A Laboratory Manual
Diagnostic Microbiology
(BIOM 422)

Pre-requist
(BIOM 322)

Organized by

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Microbiological analysis of Urine specimens

Purpose : To acquaint students with:

1. Organisms commonly responsible for infections of the Urinary tract.
2. Laboratory methods for detection of bacteriuria and identifications of microorganisms associated with the urinary tract.

Principle:

The anatomical structure of the mammalian urinary system is such that the external genitalia and the lower aspects of the urethra are normally contaminated with a diverse population of microorganisms, the tissues and organs that comprise the remainder of the urinary system, the bladder, ureters, and kidneys, are sterile, and therefore urine that passes through these structures is also sterile. When pathogens gain access to this system, they can establish infection. Some etiological agents of urinary-tract diseases are illustrated in figure 1.1.

Method:

1. Mid-stream specimens of urine following adequate cleansing of external genitalia should be sent to the laboratory in sterile containers with the minimum of delay.
2. A film of the specimen without centrifugation should be made and stained with simple stain, if organisms are seen, this indicates that organisms are present in large numbers enough to cause infection.

3. Note the general appearance of urine:
    a. Color, opacity, consistency
    b. Presence of blood, mucous or pus.
    c. Presence of macroscopic bodies such as parasites.

4. Using a standard loop (0.001 ml of water) insert vertically into the urine and inoculate in: a. MacConkey or CLED agar plate.
    b. Blood agar plate.

Incubate at 37^{o} c for 24 hrs.

5. Transfer the urine to a centrifuge tube and centrifuge at 3000 rpm for 15 min. Pour off the supernatant into another tube for biochemical analysis:

    a. Sugar content
    b. Albumin
    c. pH.
    d. Specific gravity refer to Box 1

6. Make a wet preparation (wet mount) from the sediment to examine for cells, casts, crystals and organisms.

7. Report your results in the given chart.

BOX 1

Biochemical analysis.

a. Sugar test: Benedict
Mix equal amounts of the urine and Benedict's solution, heat on the flame. If the color changes to brown it is a Positive test indicating the presence of glucose.

b. Albumin:
Pour some of the urine in a test tube and Heat at 1/3 of the tube if protein is present turbidity will be formed at the middle. Upon addition of Conc. Hcl. The turbidity will be increased indicating a positive result for albumin. If the turbidity disappeared, this indicates the presence of salts.

c. pH.
Check the pH of the urine using a pH paper.

d. Specific gravity:
Using the refractometer.

Next day:

- <u>Quantitative cultures:</u>

- Colony count of $10^{5/}$ml. or greater is indicative of infection.

- Colony count between 10^3 and 10^4/ml. indicates probable contamination

- Colony count above 10^4/ml. indicates probable infection.

- Bacterial colonies observed should be isolated and identified as shown in figure 1.2.

- Antibiotic sensitivity testing should be performed.

- Provided the specimen has been taken with adequate aseptic precautions, the organisms in figure 1.1 may be considered.

Urinary Tract Pathogens

A. Quantitative Evaluation

After counting the colonies on the plate, record the count as follows.

Number of colonies: _____

Dilutions: _____

No. of organisms/ml. of urine:_____

Gram- stained slide. If organisms are seen on a gram-stained slide of an uncentrifuged sample, sketch in color in the circle below

Conclusion: Do the plate count and gram-stained side of the uncentrifuged sample provide presumptive evidence of a urinary infection?

B. Microscopic Study (Centrifuged Sample)

Illustrate in the circle below the microscopic appearance of a centrifuged sample.

Conclusion: Describe here the morphological appearance of the predominant organism seen:

_____
_____
_____
_____

C. Culture Analyses

After studying the organisms on the three plates;
what organism do you believe is causing the infection?

Organism: _____ _____

What further testing should be performed for confirmation?

_____
_____
_____

This course deals with :

1- Systems rather than individual groups of bacteria.
2- Obtaining, handling and processing clinical specimens.
3- Logical steps which lead to isolation and identification of causative organisms in a systematic manner.
4- Various methods of antibiotic sensitivity testing are also considered.

Course Outline of Diagnostic Microbiology Practical

Week No.	Subject
1.	Sample collection and Transport
2.	Normal Flora of the Body Skin, Throat and Mouth
3.	Urinary Tract Infection
4.	Meningitis – CSF Examination and Culture / Body Fluids. Body Fluids - Examinations (Synovial)
5.	Bacteremia, Septicemia and P.U.O. Blood culture
6.	Ear, Eye, and Throat Infections
7.	Respiratory Tract Infections (Sputum)
8.& 9 10	Gastro Intestinal / Stool Exam. Genital Tract Infection / Urethral, Vaginal and Cervical Swabs
11&12	Wounds, Abscesses and Skin Infections. Various, Culture Techniques Anaerobic Techniques
13	Sensitivity Testing, MIC.

SAMPLE COLLECTION AND TRANSPORT

Purpose:

To familiarize students with:

1. Specific functions and procedures that are carried out in diagnostic microbiology laboratories.

2. Types of specimens, specimen collection, transport and processing

INTRODUCTION:

The chief function of the diagnostic microbiology laboratory is to assist physicians in the diagnosis and treatment of patients with infectious disease. Excellence in patient care must remain the prime focus, and the work performed by the staff microbiologists should be directed toward the production of clinically useful results in as short a time as possible. The delay of microbiology reports beyond a time when the results can be useful in directing patient care is one of the major criticisms voiced by physicians on the performances of clinical laboratories.

The delivery of diagnostic laboratory service has become quite complex and requires the constant attention of the laboratory director, supervisors, and qualified personnel. **Figure 1-1** is a schematic representation of the sequence of steps necessary in deriving a clinical and laboratory diagnosis of infectious disease. Note that the cycle begins with the patient who presents with signs or symptoms of an infectious disease.

Each step in the above cycle must be completed with accuracy and precision in as short a time as possible. Note that the laboratory is directly involved in only a portion of the cycle, and it is the obligation of the laboratory director and laboratory personnel to also be involved in decisions that will improve the efficiency of functions external to the laboratory. Thus, although transcription of orders, proper collection of specimens, specimen transport to the laboratory, and posting and interpretation of final results are not under the direct control of laboratory personnel, they must assume some responsibility for seeing that these functions are properly carried out. Each step is equally important if optimal patient care is to be provided.

Patient consults
Physician because of
Signs or symptoms
of an infectious disease

Physician examines patient and makes tentative diagnosis

Physician writes Laboratory orders

Written orders are transcribed to a requisition form

Appropriate specimen is collected for culture

Specimen and request form are transported to lab

Date on request
Form is entered into
Laboratory log book.

Specimen is directly examined, processed, and incubated.

Cultures are interpreted bacterial identification is made

Final culture report is prepared

Report is sent to hospital ward or physician

Physician interprets Report and institutes Appropriate therapy

Fig. 1-1. The clinical and laboratory diagnosis of infectious disease. A schematic overview of the diagnostic cycle.

DIAGNOSIS OF INFECTIOUS DISEASE

It is the physician's responsibility to suspect an infectious disease in patients with suggestive signs and symptoms and confirm or reject this suspicion by ordering appropriate cultures or serologic tests on biologic fluids or tissues.

An infection is said to occur when an invading microorganism demonstrates pathogenicity by eliciting a local or systemic inflammatory response in the host.

Table 1-1 summarizes the common sites of infections, clinical signs and symptoms, types of specimens to culture, and a list of organisms associated with infections at these sites.

The laboratory should be forwarded by the physician that certain microorganisms are suspected, particularly if a culture medium other than that commonly used is required for their recovery. The physician should always indicate on the laboratory request slip if an infection with some microorganisms such as (fungi, Mycobacteria and some others) are suspected because special media re required for their isolation.

SPECIMEN COLLECTION

The proper collection of a specimen for culture is possibly the most important step in the recovery of microorganisms responsible for infectious disease. A poorly collected specimen may be responsible for failure to isolate the causative microorganism, and recovery of contaminants can lead to an incorrect or even harmful course of therapy.

BASIC CONCEPTS

1. The clinical specimen must be material from the actual infection site and must be collected with a minimum of contamination from adjacent tissues, organs, or secretions.

"There is always a problem with salivary contamination of sputum samples or lower respiratory secretions. Other examples of problems encountered in specimen collection include failure to culture the depths of a wound or draining sinus without touching the adjacent skin, inadequate cleansings of the Para urethral tissue and perineum prior to collecting a clean-catch urine samples from a woman, contamination of an endometrial sample with vaginal secretions, and failure to reach deep abscesses with aspirating needles or cannulas.

2. Optimal times for specimen collection must be established for the best chance of recovery of causative microorganisms.

Knowledge of the natural history and pathophysiology of the infectious disease process is important in determining the optimal time for specimen collection. The causative microorganism can be recovered optimally from the blood during the first week of illness. Culture of the feces or urine is usually positive during the second and third weeks of illness.

Because of the high risk of contamination or overgrowth with more rapidly growing commensal bacterial, 24 hour collections of clinical materials for culture, particularly of sputum and urine, should be discouraged. On the other hand, Kaye has shown that urine from normal persons may be inhibitory or bactericidal for some

microorganisms, particularly if the urine pH is 5.5 or lower (acidic), if the osmolality is high, or if the urea concentration is increased. The ability of bacterial to grow in urine may represent a failure in a host defense mechanism.

Historically, 24 hour collections of sputum or urine were necessary at a time when laboratory methods were inadequate to allow for a high recovery rate of some types of infectious bacterial. Early morning specimens are also recommended.

3. A sufficient quantity of specimen must be obtained to perform the culture techniques requested.

The low volume is insufficient to enable the carrying out of all the procedures requested. Again, a repeat specimen should be requested if clinically indicated.

4. Appropriate collection devices, specimen containers, and culture media must be used to ensure optimal recovery of microorganisms.

Sterile containers should be used for collection of all specimens. It is also important that containers be constructed for ease of collection, particularly if the patients are required to obtain their own specimens. Narrow-mouthed bottles are poorly designed for collection of sputum or urine samples. The containers should also be provided with tightly fitting caps or lids to prevent leakage or contamination during transport.

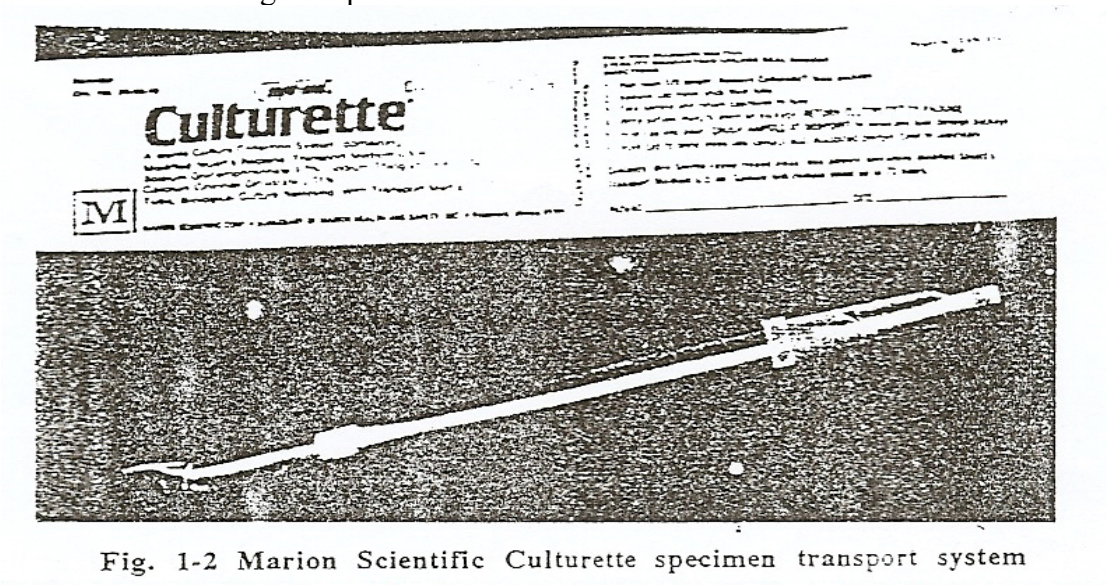


Fig. 1-2 Marion Scientific Culturette specimen transport system

Swabs are commonly used for collection of specimens for culture. These are acceptable in most instances if certain precautions are taken. Because of residual fatty acids on the cotton fibers that may inhibit some strains of fastidious bacteria, it is recommended that swabs tippes with calcium alginate, Dacron, or polyester be used. Specimens should not be allowed to remain in contact with the swab any longer that is necessary. Except for throat swabs where drying does not seem to affect the recovery of streptococci, it is recommended that swabs be placed into a transport medium or moist container to prevent drying and death of bacteria. One commonly used tube, Culturette, illustrated in Figure 1-2, includes a glass vial containing Stuarts transport medium that can be broken when the inoculated swab is reinserted to provide moisture during transport. Good recovery of most

bacterial species from these tubes has been demonstrated for up to 48 hours or longer. The use of culture tubes containing semisolid Stuart or Amies transport medium also serves as an adequate means for holding swab cultures during transport.

Use of swabs for collection of specimens for recovery of anaerobic bacteria is discouraged; rather, aspiration with needle and syringe is recommended. In either event, specimens once collected must be protected from exposure to ambient oxygen and kept from drying until they can be processed in the laboratory.
A number of transport containers suitable for anaerobic specimens are listed in Table 1-2, some of which are commercially available.

Regardless of the transport system used, the major principle is to keep the time delay between collection of specimens and inoculation of media to a minimum .

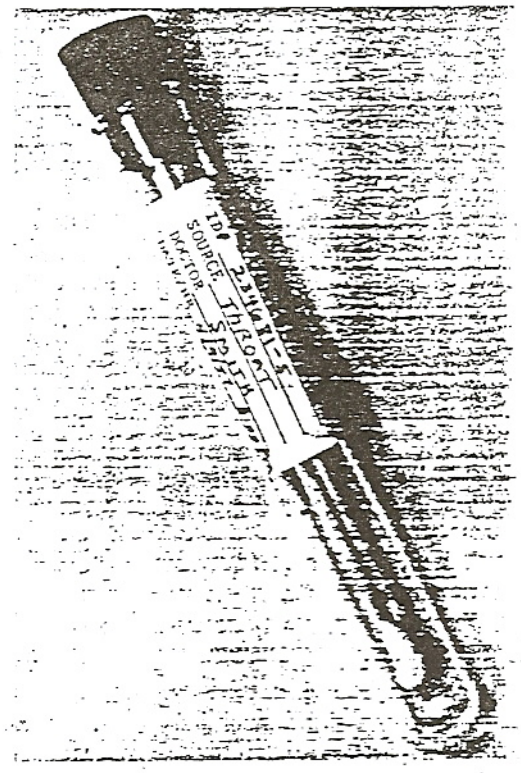
5. Whenever possible, obtain cultures prior to the administration of antibiotics.
It is recommended that culture be obtained before administration of antibiotics. However, administration of antibiotics does not necessarily preclude recovery of other species of microorganisms from clinical specimens, and therefore specimens should not be rejected on the basis of this criterion alone.
The action of many antibiotics may be bacteriostatic, not bactericidal, and often microorganisms can be recovered when they are transferred to an environment devoid of the antibiotic (a fresh culture medium) Also, the concentration of antibiotic may be below the minimal inhibitory concentration for the organism in question at the site of infection, and recovery in culture is no problem. Thus, one should always make an attempt to culture these sites, although the results must be interpreted accordingly or qualified in the written report.

6. The culture container must be properly labeled.
In order for the microbiologist to use proper culture techniques and provide the physician with accurate and complete information, each culture container must have a legible label, with the following minimum information:

Name_____
ID#_____
Source_____
Doctor_____
Date/ Hour_____

Figure 1-3 illustrates a culture tube with a label that has been properly filled out. The patient's full name should be used and initials should be avoided. The identification number may be the hospital number, clinic or office number, home address, or social security number, depending upon the circumstances. The physicians, name or office title is necessary in the event that consultation or early reporting is required. The specimen source should be identified in the event that special culture media are required. The date and time of collection should appear on the label to ensure that the specimen is cultured within a n acceptable length of time after it has been collected. Other potentially useful information includes the clinical diagnosis and the antibiotic treatment history of the patient.

FIG. 1-3 A culture transport tube with a properly written identification label.



COLLECTION FROM VARIOUS ANATOMIC SITES

Throat and Nasopharynx

The proper method for obtaining a throat sample is illustrated in Figure 1-4. A bright light from over the shoulder of the person obtaining the specimen should be focused into the opened oral cavity so that the swab can be guided into the posterior pharynx. The patient is instructed to breathe deeply and the tongue is gently depressed with a tongue blade. The swab is then extended between the tonsillar pillars and behind the uvula. Care should be taken not to touch the lateral walls of the buccal cavity. Having the patient phonate an ah serves to lift the uvula and aids in reducing the gag reflex. The swab should be swept back and forth across the posterior pharynx to obtain and adequate sample. After the sample is collected the swab should be placed immediately into
a sterile tube or other suitable container for transport to the laboratory.

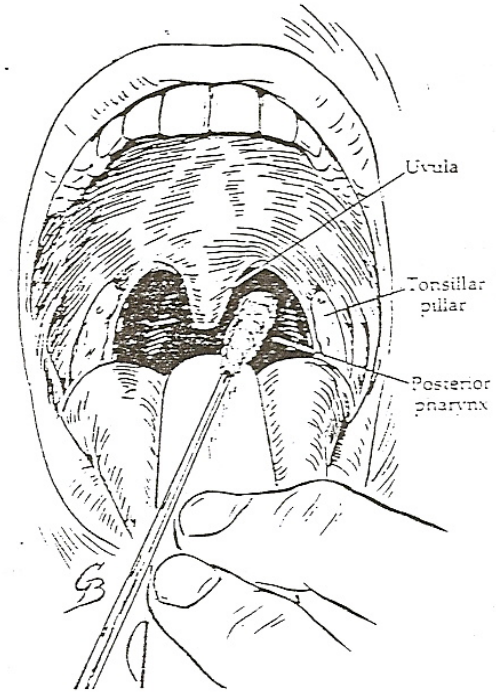


Fig. 1-4 Throat Culture Technique

Sputum and Lower Respiratory Tract

It is difficult to prevent contamination of sputum samples with upper respiratory secretions. Having the patient gargle with water immediately prior to obtaining the specimen reduces the number of contaminating oropharyngeal bacteria. In cases in which sputum production is scant, induction with nebulized saline (avoid saline for injection because it contains antibacterial substances) through a positive pressure respirator apparatus may be effective in producing a sample more representative of the lower respiratory tract.

Translaryngeal (transtracheal) aspiration may be indicated when the patient is debilitated and cannot spontaneously expectorate sputum sample.

Urine

For optimal recovery of bacteria from the urinary tract, and to reduce potential contamination, it is imperative that careful attention be paid to the proper collection of urine samples. For best results, a nurse or a trained aide should personally supervise the collection of clean-catch samples from women.

For proper collection of clean-catch urine samples from women the periurenthral area and perineum should be cleansed with soapy water and thoroughly rinsed with sterile saline or water. The labia should be held apart during voiding, and the first few milliliters of urine passed into a bedpan or toilet bowl to flush out bacteria from the urethra. The midstream portion of urine is then collected in a sterile container (Fig.1-5)

How well this procedure is being carried out can be monitored by noting the frequency with which urine colony counts in the intermediate range of 10,000 to 100,000 organisms per ml of urine are reported. Patients without urinary tract infection should have no bacterial or very few colonies at most; those with infection most commonly have more than 100,000 organisms per ml. Intermediate counts should be uncommon if the urine collection procedure is properly carried out by the patients. The recovery of three or more bacterial species also generally indicates that the specimen has been contaminated through faulty collection or delay in transport.

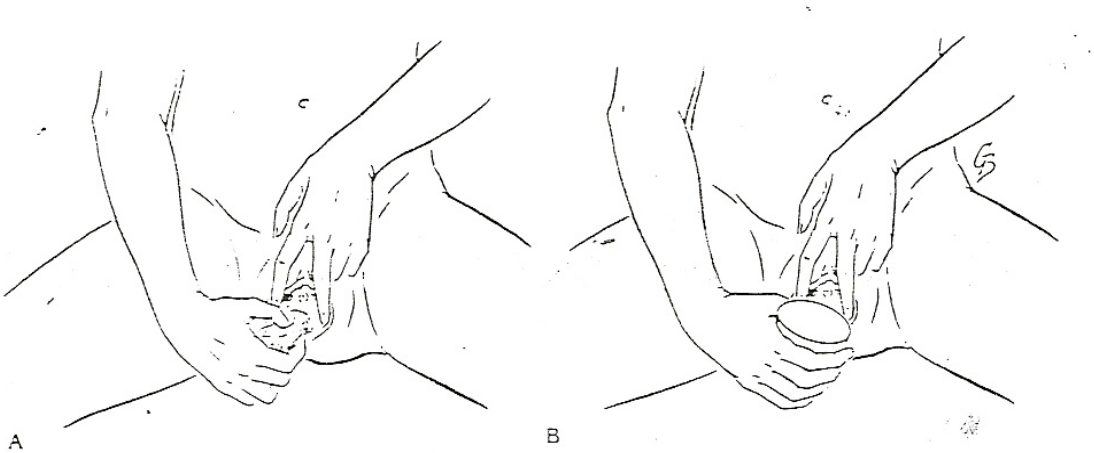


FIG. 1-5 Midstream clean-catch urine collection. (*A*) The labia are separated with the fingers and cleansed with a 4 × 4-inch gauze pad saturated with green soap. The midstream portion of the urine is collected into a sterile container (*B*).

INSTRUCTION FOR OBTAINING CLEAN-CATCH URINE SPECIMENS (FEMALE)

1. Remove underclothing completely and sit comfortably on the seat, swinging one knee to the side as far as you can.

2. Spread yourself with one hand, and continue to hold yourself spread while you clean and collect the specimen.

3. Wash, be sure to wash well and rinse well before you collect the urine sample. Using each four separate 4" X 4" sterile sponges soaked in 10% green soap, wipe from the front of your body towards the back. Wash between the folds of the skin as carefully as you can.

4. Rinse, after you have washed with each soap pad, rinse with a moistened pad with the same front-to-back motion. Do not use any pad more than once.

5. Hold cup outside and pass your urine into the cup. If you touch the inside of the cup or drop it on the floor, ask the nurse to give you a new one.

6. Place the lid on the container or ask the nurse to do so for you.

On occasion suprapubic aspiration of the urinary bladder is required to obtain a valid urine specimen for culture, particularly from your children. The technique is illustrated in Figure-1-6.

> Catheterized urine specimens may be used for culture. The free flow from the month of the catheter should be obtained. Urine from catheter bags is generally unsuitable for culture except from infants when special precautions have been taken.

Wound

The surfaces of cutaneous wounds decubitus ulcers are frequently colonized with environmental bacteria, and swab samples often do not reflect the true cause of the infectious process. For this reason, the most desirable method of collecting cutaneous specimens is aspirating loculated purulent material from the depths of the wound with a sterile needle and syringe. The wound margins should be decontaminated as much as possible with surgical soap and application of 70% ethyl or isopropyl alcohol. If material is obtained in the syringe, the needle cap can be replaced and the syringe sent to the laboratory for culture. If it is anticipated the processing will be delayed beyond 20 to 30 minutes, the specimen should be transferred to an anaerobic container.

If material cannot be obtained with a needle and syringe and a swab must be used to collect the specimen, the wound margins should be gently separated with the thumb and forefinger of one hand (wearing a sterile glove) while extending the tip of the swab deep into the wound with the other hand, taking care not to touch the adjacent skin margins. The swab should be transported in an anaerobic container.

Stool

Laboratory confirmation of an intestinal infection caused by microorganisms is usually made by detecting ova and parasites in direct saline or iodine mounts of fecal material or by recovering pathogenic bacteria from stool specimens. Samples obtained directly in sterile wide-mounted containers should be covered with a tightly fitting lid.

Rectal swabs may be necessary in some instances. The swab should be inserted just beyond the anal sphincter, avoiding direct contact with fecal material within the rectum.

Stool samples should be examined and cultured as soon as possible after collection. As the stool specimen cools, the drop in pH soon becomes sufficient to inhibit the growth of most Shigella species and some Salmonella species. If a delay in transporting the specimen to the laboratory is anticipated, stool specimens should be placed in an appropriate stool preservative. If the recovery of pathogenic bacteria is desired, a preservative consisting of equal quantities of 0.033 molar sodium or potassium phosphate buffer and glycerol is suggested; for recovery of parasites, polyvinyl alcohol (PVA) fixative is recommended. A small amount of feces can be added to an enrichment broth such as GN or Selenite broth if shigellosis or salmonellosis is suspected. Stool specimens are not suitable for recovery of ova and parasites for 10 days after a barium enema; however, recovery of enteric bacterial pathogens is not compromised.

Cerebrospinal Fluid

Lumbar spinal puncture is the procedure used by physicians to obtain cerebrospinal fluid (CSF) for culture and other laboratory studies. After properly disinfecting the skin of the lower back, the patient is asked to lie on his side with the torso bent forward to separate the spinous processes of the lumbar vertebrae. Under local anesthesia, a long spinal needle is inserted into the spinal canal between the third and fourth lumbar vertebrae (fig .1-7). Cerebrospinal fluid need not be aspirated; since it flows from the mouth of the needle under a pressure of approximately 90 mm to 150 mm of CSF in normal persons.

CSF is commonly collected into three tubes, the third of which is selected for culture, presumably because the chance for the recovery of skin contaminants, which tend to wash into the first two tubes, is reduced. A total of 10ml is usually collected, since this volume is required if cultures for the recovery of bacteria, fungi, and acid-fast organisms are to be set up. If there is to be a delay in processing specimens, the fluid **should be left at room temperature or paced in the incubator**. **Refrigeration is contraindicated because of the killing effect that chilling has on *Neisseria meningitide*s and *Haemophilus influenczae*, the two most common bacterial species causing meningitis.**

Female Genital Tract

Vaginal cultures do not often produce meaningful results. In cases of suppurative vaginitis, direct wet mounts should be prepared at the bedside and examined microscopically shortly thereafter for the presence of *Trichomonas vaginalis* or the budding yeast form of *Candida albicans. Gardnerella vaginalis*, probably in combination with anaerobic bacteria, causes a non-suppurative superficial infection of the vaginal mucosa. The observation of dense aggregates of bacilli on desquamated epithelial cells (**so-called clue cells**) in stained smears of the vaginal secretion or the production of vaginal secretions more alkaline than pH 5.5 should lead the clinician to suspect this etiology. Other bacterial species, particularly members of the family Enterobacteriaceae, are only rarely incriminated. The significance of anaerobic bacteria recovered from vaginal secretions is difficult to interpret because some are present as normal flora.

Eye, Ear, and Sinus

Suppurative material from an infected eye should be collected from the lower cul-de-sac or from the inner canthus. A direct Gram's stain of the material obtained should always be prepared to determine the presence and type of bacteria. If infection with *Clalmydia trachomatis* (Trachoma) is suspected, corneal scraping should be smeared on a glass microscope slide, air dried, and fixed in absolute methanol. This preparation can be stained with Lugol's solution or 5% iodine in 10% potassium iodide and examined as a wet mount for the presence of red-brown staining intracytoplamic inclusion. This procedure lacks sensitivity, and culture for recovery of the causative agent is recommended for a definitive diagnosis. Cultures of the external auditory canal generally do not reflect the bacterial cause of otitis media unless there has been recent rupture of the tympanic membrane. Tympanic membrane aspiration is rarely performed. In some cases of acute otitis media, the causative micro-organism can be cultured from the posterior nasopharynx.

Cultures from the maxillary, frontal or other sinuses should be collected by the syringe aspiration technique and cultures set up for recovery of both aerobic and anaerobic species of bacteria that are commonly found in cases of chronic sinusitis.

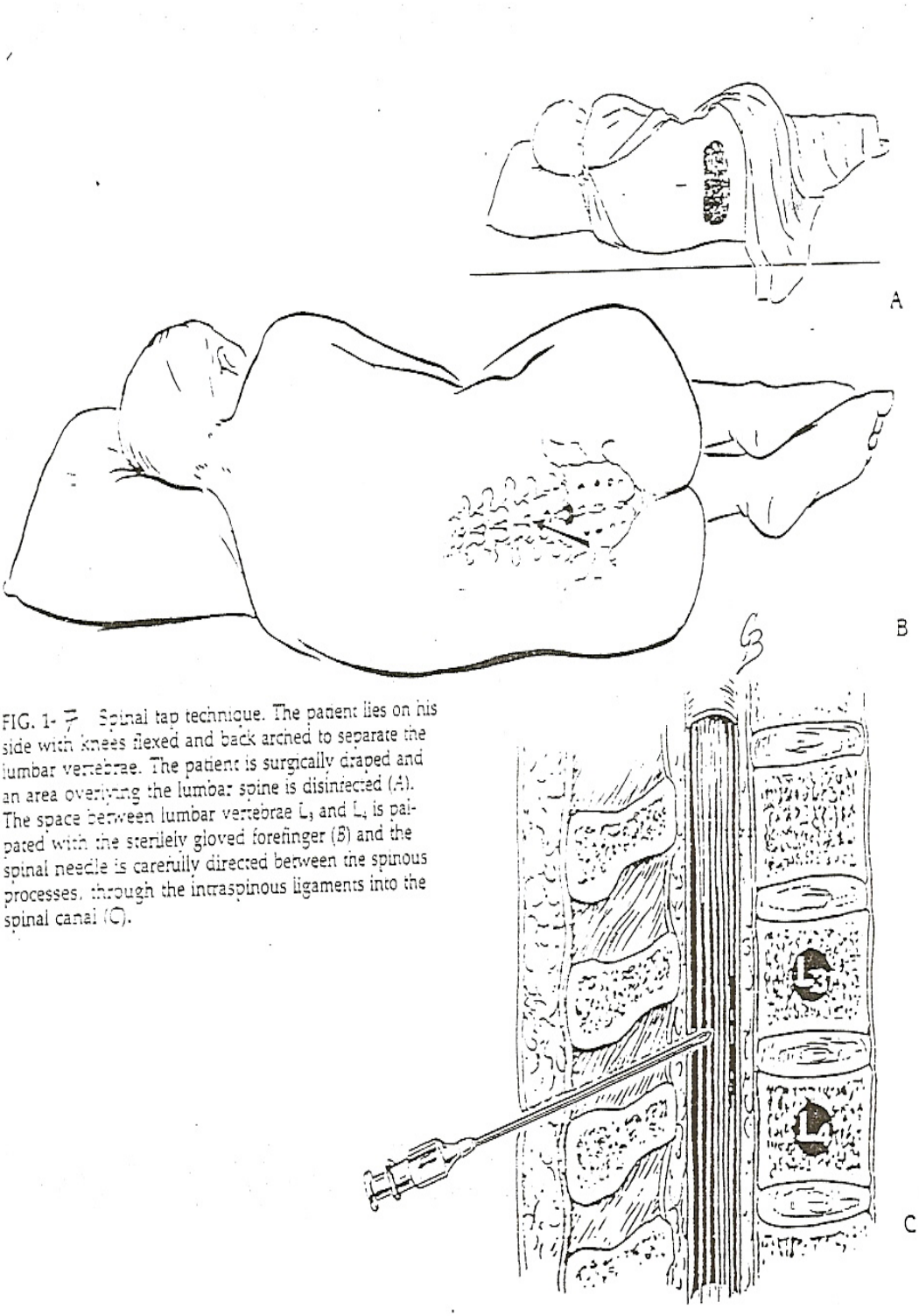


FIG. 1- 7 Spinal tap technique. The patient lies on his side with knees flexed and back arched to separate the lumbar vertebrae. The patient is surgically draped and an area overlying the lumbar spine is disinfected (*A*). The space between lumbar vertebrae L_3 and L_4 is palpated with the sterilely gloved forefinger (*B*) and the spinal needle is carefully directed between the spinous processes, through the intraspinous ligaments into the spinal canal (*C*).

Blood

Because the mortality from septicemia may reach as high as 40%, or more in some populations of hospitalized patients, it is urgent that the laboratory perform blood culture correctly and report accurate results as soon as possible. The critical factors that must be decided by laboratory supervisors include the collection, number, and timing of blood cultures; the volume of blood cultured; the amount and composition of the culture medium: when and how frequently to subculture; and the interpretation of results. Space here allows only a brief summary of these factors: however, reviews by Bartlett et al, Reller et al. and Washington can be consulted for details.

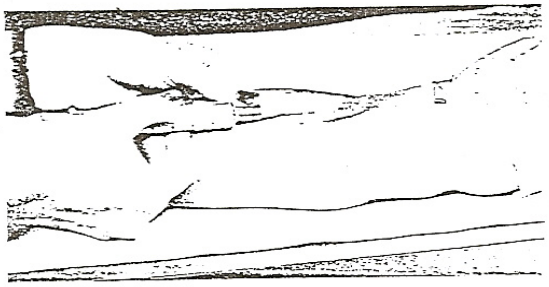


FIG. 1-8 Venipuncture technique for blood culture using a sterile needle and syringe. A tourniquet is applied to the upper arm above the venipuncture site to distend the antecubital veins. The site has previously been prepared with tincture of iodine and alcohol. The blood is removed with the syringe and needle and injected into an appropriate blood culture bottle. To reduce the chance of skin contamination, it is recommended that a second syringe be used to draw the blood to be cultured, with the first syringe theorencally containing any organisms that were washed from the needle.

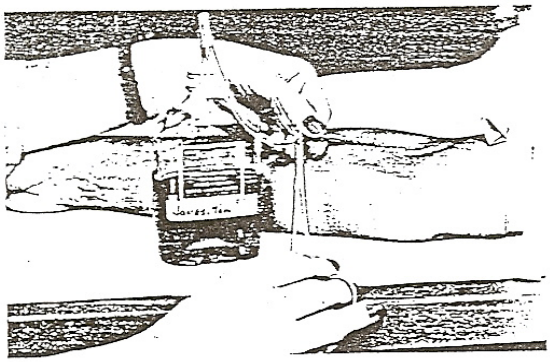


FIG. 1-9 The closed system of blood culture collection. The closed blood culture system consists of a vacuum blood culture bottle and a double-needle collection tube. The tube is first clamped with a hemostat and one needle is placed into the stopper of the blood culture bottle. The opposite needle is used for the venipuncture. Again, note the tourniquet above the venipuncture site. When the needle enters the vein, the hemostat is released and blood is aspirated directly into the bottle. The vacuum is regulated so that exactly 10 ml of blood is delivered into the bottle.

Collection: Blood cultures can be obtained either by using a needle and syringe (Fig.1 -8) or by the closed system using a vacuum bottle and the double-needle collection tube (Fig. 1-9). In either instance, the venipuncture site should be properly disinfected to minimize skin contamination. The incidence of contamination of blood cultures during collection should not exceed 3 %. Optimal skin preparation includes (1), a 30-second wash with green soap, (2) a rinse with sterile water, (3) an application of tincture of iodine or an iodophor preparation that is allowed to dry, and (4) and alcohol wash to remove the iodine (do not use alcohol to remove the iodophor).

Number and Timing: Most investigators agree that obtaining more than three blood cultures within a 24 hour period does not result in a significant increase in positive results. In case of proven septicemia, the cumulative rates of organism recovery from three blood cultures are 99% or greater. Obtaining the culture immediately before temperature, spike is ideal because this is the time of the highest concentration of circulating organisms. Since a temperature spike usually cannot be predicted, it is generally recommended that routine blood cultures be obtained from different veni-puncture sites at least 1 hour apart. It takes about 30 minutes for the normal defenses to clear bacteria from the circulation: therefore, positive cultures on successive venipuncture separated by at least 1 hour tend to indicate a true septicemia a rather than an isolated bactermic episode.

It is generally accepted that two blood culture bottles should be inoculated from each venipuncture: an aerobic vented bottle and a closed anaerobic bottle.

Volume of Blood Cultured: At least 10 ml of blood should be obtained from adults for each venipuncture. The percentage yield of positive blood cultures drops significantly if this amount is reduced by half; the yield is not substantially increased if each draw contains more than 10 ml. In infants and children, 1 ml. to 5 ml. suffices an amount that in relationship to the total blood volume is comparable to the 10-ml volume drawn in adults. It is generally recommended that the blood be added to culture broth in a ratio of 1:10 to protect the organisms from the bactericidal activity of human serum; however, a 1:5 ration of blood to broth is also effective in patients not receiving high doses of effective antibiotics. The yield of positive blood cultures is greater with 10ml of blood cultured at a 1:5 ratio of blood to broth than with 5 ml blood at a 1: 10 ratio.

Culture Medium: Tryptic or Trypticase soy, supplemented peptone, brain-heart infusion, Columbia, brucella, and 16B medium (for laboratories using the BACTEC system) are the blood culture media most commonly used. All are commercially available; however one should produced by different manufacturers. Variables other than the type of media have not been controlled is most studies; therefore, conclusions on the percentage yield for these various media are difficult to reach. A variation in the composition of the same medium by different manufacturers also makes comparisons difficult. Most commercial vacuum bottles have a 5% to 10% concentration of CO2 in the gas phase of the culture bottle to affect a maximal yield of fastidious organisms. The recovery of bacteria from blood is highest when cultures are incubated at 35^{o} C

Most commercially available blood culture media contain the anticoagulant sodium poly-anetholsulfonate (SPS) in concentrations of 0.025% to 0.05%. In addition to preventing clotting (an affect that is desirable because small numbers of bacteria within clots may not survive), SPS also inhibits the activity of complement and lysozymes, deters phagocytosis and inactivates therapeutic concentrations of aminoglycosides. However, SPS may inhibit and some strains, therefore, blood from patients suspected of having septicemia caused by one of these organisms should also be inoculated into anticoagulant free broth.

Blood culture media supplemented with 20% sucrose is available; however, investigators do not agree about whether the incidence of recovery of bacteria is improved. The turbidity of the medium resulting from lysis of the red cells makes visual assessment of growth difficult. The addition of penicillinase to blood culture medium is also generally discouraged because risk of contaminating the broth is increased.

Blood culture bottles are also available that contain a treated plastic resin called the antibiotic removal device (ARD-Marion Laboratories, Kansas City, MO 64114), designed to be used with blood specimens collected from septic patients who are receiving antibiotics. Studies by Wallis and associates and by Lindsey and Riely indicate that the lowered concentrations of antibiotics effected by this device have resulted in a significant improvement in recovery of some species of bacteria. These findings, however, have not been corroborated by Wright and associates; therefore, recommendations on the use of ARD must await further parallel studies between resin-containing and nontreated blood culture media.

The BACTEC radiometric and the Dupont Isolator systems are being used with increasing frequency in clinical microbiology laboratories. Each laboratory director must determine whether the implementation of these systems into any given laboratory is cost effective and in keeping with the needs of the community being served.

Tissues and Biopsies

Tissue samples for culture should be delivered promptly to the laboratory in sterile gauze of in a suitably capped, sterile container. Formalinized specimens are not suitable for culture unless the exposure time has been short and the culture is obtained from a portion of the tissue not exposed to formalin.

SPECIMEN TRANSPORT

The primary objective in the transport of diagnostic specimens, whether within the hospital or clinic or externally by mail to a distant reference laboratory, is to maintain the sample as near its original state as possible with minimum deterioration. Adverse environmental conditions, such as exposure to extremes of heat and cold, rapid changes in pressure (during air transport), or excessive drying, should be avoided.

Specimens should be transported to the laboratory as quickly as possible. In a hospital setting, a 2 hour time limit between collection and delivery of a specimen to the laboratory is recommended. This time limit poses a problem for specimens collected in physicians offices, and a transport medium is often required. Stuart, Amies, and Carey-Blaier transport media are most frequently used. For Stuart transport medium the medium is essentially a solution of buffers with carbohydrates, peptones, and other nutrient, however growth factors are excluded. This medium is designed to preserve the viability of bacteria during transport without significant multiplication of the microorganisms. Sodium thioglycollate is added as a reducing agent to improve a recovery of anaerobic bacteria, and the small amount o f agar provides a semisolid consistency to prevent oxygenation and spillage during transport.

Table 1-1 The Diagnosis of Bacterial Infections at Different Body sites

Site of Infection	Presenting Signs and Symptoms	Specimens to Culture	Bacterial Species Potentially Associated with Infections
Urinary tract	**Urinary bladder infection** Pyuria Dysuria Hematuria **Pain and tenderness :supra public or lower abdomen** Kindly infection Back pain Tenderness : costovertabral angie (CVA)	Clean – catch midstream urine Catheterized urine Catheter base : newborns and infants only Suprapubic aspiration of urine	**Enterobacteriaceae** *Escherichia coli* *Klebsiella spp.* *Proteus spp.* Group D strepotococci (enterococci) *Pseudomonas aeruginosa* *Staph.aureus* *Staph.epidermidis* and *S. saprophiticus*
Respiratory tract	**Upper tract – nose and sinuses** Headache Pain and redness over maiar area **Rhinitis** X – ray sinus consolidation, fluid leveis , or membrane thickening **Upper tract throat and pharynx** Redness an edema of mucosa Exudanon of tonsils Pseudomembrane Edema of uvula Gray coating of tongue : straw berry tongue Enlargement of cervical nodes	Acute Naseoopharyngeal swab Sinus washings Chronic Sinus washings Surgical biopsy specimen Swab of posterior pharynx Swab of tonsils abscess Nasopharyngeal swab	*Streptococcus pneumonia* *Streptococcus*, beta-hemolytic Group – A *Staph.aureus* *Haemophilus influenzae* *Klebsieilla spp.* and other Enterobacteriaceae Bacteroides species and other anaerobes (sinus) *Streptocouccus* , beta-hemolyac Group – A *Haemophilus influenzae* *Corynebacterium diphtheriae* *Neisseria menigitidis* *Bordetella pertussis*
Respiratory tract	**Lower tract- lungs and bronchi** Cough : bloody or profuse Chest pain Dyspnea Consolidation to lung Rales and bronchi Diminished breath sounds Dullness to percussion X-ray infiltrates Cavitary lesion Empyema	Sputum (poor-return) Blood Brochoscopy secretions Transtrachaeal aspirate Lung aspirate or biopsy	*Streptococcus pneumonia* *Haemophilus influenzae* *Staph.aureus* *Klebsiella pneumonia and other members of Entetobacteriaceae* *Lagionella* spp. *Mycobacterium* species *Fusobacterium nucleatum* , *Bacteroides melaninogenicus* and other anaerobic species

Gastrointestinal tract	Diarrhea Dysentery Purulent Mucous Bloody Cramping abdominal pain	Stool specimen Rectal swab or rectal mucous Blood culture (typhoid fever)	Campylobacter jejuni Salmonella species Shigella species Escherichta coli (entrotoxigenic) Vibrio species Yersina species Clostridium difficile (demonstration of toxin)
Wounds	Discharge : serous or purulent Abscess : subcutaneous or sub mucous Redness and edema Crepitation (gas formation) Pain Ulceration or sinus formation	Aspirate of drainage Deep swab of purulent drainage Swab from wound margins or depths of ulcer Tissue biopsy	*Staphylocouccus aureus* *Streptococcus pyogenes* *Clostridium* species, and other anaerobic bacteria Enterobacteriaceae *Pseudomonas aeruginosa* Enterococci
Meningitis	Headache Pain in neck and back Stiff neck Straight leg raising (positive kernig's sign) Nausea and vomiting Stupor to coma Petechial skin rash	Spinal fluid Subdural aspirate Blood culture Throat or sputum culture	*Neisseria menintidis* *Haemophilus influenzae* *Streptococcus pneumonia* Streptococcus , beta Groups A and B (Group B in infants) Enterobactenaceae : debilitated patients , infants post craniotomy *Listeria monocytogenes*
Genital tract	**Males** Urethral discharge : serous or puruient Burning on urination Terminal hematuria **Females** Purulent vaginal discharge Burning on urination Lower abdominal pain spasm and tenderness Mucous membrane chancre of chancroid	Urethral discharge Prostatic secretions Uterine cervix Rectum (anal sphincter swab) Urethral swab Dark – field examination	Neisseria gonorrinoese (N Meningudis) Haemophilus aucreyi Trponema pallidum syphilis) Gardneella vaginalls Nonbacterial : Trichomonal vainalls Candida albieens Mycopiasma species Chiamyida species Herpes simplex mrus
Bacteriemia	Spiking fewer Chills Cardiac murmur (endocarditis) Petechiae: skin and mucous membranes "Spunter hemorrhages" of nails Malaise	Blood 3 or 4 cultures per day at 1 hour intervals or greater. Urine Wounds Any suspected primary site of infection Cerebrospinal fluid Respiratory tract Skin -umbilicus Skin -ear	Streptococcus species Group – A all ages Alpha – hemolytic endocardts Group – A B D- newborns *Staphylocouccus aureus* Streptococcus pneumoniae Escherichia coli *Bacteroides fragilis* and other anaerobic bacteria *Pseudomonas aerugnisa* *Listeria monocyrogenes* *Haemophilus influenzae* Salmonella typhi typhoid fever

Eye	Coniuncnval discharge : serous or purulent Conjuvential redness (hyperemuaj: pinkeye) Ocular pain and tenderness	Purulent discharge Lower cui-de-sac Inner canthus	*Haemophilus spp.* *Moraxella spp*. *Neisseria gonorrhoeae*: neonates *Staphylccoccus aureus* *Streptococcus pneumonia* Streptococcus pyogenes]
Middle ear	Serous or purulent drainage Deep pain in ear and jaw Throbbing headache Red or bulging tympanic membrane	Acute No culture Nasopharyngeal swab Tympanic membrane aspirate Chronic drainage of external meatus	Acute *Streptococcus pneumoniae* and other Streptococcus *Haemophilus influenzae* *Proteus* spp. Anaerobic bacteria
Bones and joints	Joint swelling Redness and heat Pain on motion Tenderness on palpation X-rays synovities or osteomyelitis	Joint aspirate Synovial biopsy Bone spicules or bone marrow aspirate	*Staphylococcus aureus* *Haemophilus influenzae* *Streptococcus pyogenes* *Neisseria gonorrhoeae* *Streptococcus pneumoniae* Entrobacteriaceae Mycobacteria

Table 1-2 Transport Containers for anaerobic Specimens

Container	Rational or Description	Reference
Rubber stoppered tubes or bottles for aspirates	Container filled with oxygen-free CO_2 Dry bottles for fluid specimens Container containing nonnutritive agar or liquid medium with a reducing agent and resazurin indicator	Holdeman . Cato and Moore Sutter , Cinon and Finegold
Syringe and needle for aspirates	Fresh exudates or liquid specimen can be transported to laboratory after bubbles are carefully expelled and the needle is inserted in to sterile rubber stopper. These should be used only if specimen can be taken to laboratory without delay	Sutter , Cinon and Finegoid
Tissue transport containers	Tissue can be placed into a Petri dish on moist gauze or into a loosely screw capped vial and transported within a type A biobag (anaerobic culture set) a plastic bag containing an anaerobic gas generator catalyze and redox indicator.	
Two tube system for swabs	One tube contains sterile swab in oxygen free CO: or N2. Second tube contains either a few drops of a prereduced salts solution and oxygen free gas or semisolid agar containing a reducing agent and redox indicator. A deep tube or Stuart , Amies or modified Cary- Blair medium can be used because the oxidation- reduction potential in the deeper portions is sufficiently low to preserve the viability of most clinically encountered anaerobes	Holdeman . Cato and Moore Sutter , Cinon and Finegold
Reduced medium system	The BBL Port A Cul tube and vialt (Fig 1-5) is an example of this type. Even if the superficial portion of the medium is oxygenated as indicated by the indicator dye. This reverse back to an anaerobic condition soon after the cap is replaced because of the action of the reducing agent in the medium.	

- Scott laboratories, Inc. Fiskeville , R. I 02823
- Marion Scientific Corp,, Kansas City, Mo.
- BBL , Division of Becton – Dickinson and Co. Cockeysville , Md . 21030

Normal Flora of the body
Skin, Ear and Mouth

Purposes:

To identify microorganisms that normally reside in the ear, skin and mouth

Principle:

The SO-called normal flora are regularly found in a specific areas of the body. This specificity is far from arbitrary and depends on environmental factors such as pH. Oxygen concentration, amount of moisture present, and types of secretions associated with each anatomical site. Native microbial flora are broadly located as follows:

1- **Skin:** Staphylococci (predominantly *S. epidermidis*), streptococci (α-hemolytic, non-hemolytic and enterococci, diphtheriod bacilli, yeasts and fungi)

2- **Eye conjunctive**: Staphylococci, Streptococci, diphtheroids and neisseriae.

3- **Upper respiratory tract**: Staphylococci, streptococci (a- hemolytic non hemolytic, Enterococci, and *S. pneumoniae*) diphtheroids, spirochetes, and members of the genera Branhamella, Neisseria, and Hemophillus.

4- **Mouth and teeth**: Anaerobic spirochetes and vibrios, fusiform bacteria staphylococci, anaerobic levan-producing and dextran producing streptococci responsible for dental caries.

5- **Intestinal tract**: In the upper intestine, predominantly lactobacilli and enterococci, In the lower intestine and colon 96% to 99% anaerobes such as members of the genera Bacteroides, Lactobacillus, Clostridium and Streptococcus: 1% to 4% aerobes including coliforms, enterococci, and a small number of Proteus, Pseudomonas and Candida species.

6- **Genitourinary tract**: Staphylococci, Streptococci, Lactobacilli, gram-negative enteric bacilli, Clostridia, Spirochetes, Yeasts and Protozoa such as *Trichomonas* spp.

In this exercise the resident flora of the ear, mouth and skin will be studied. As these sites represent sources of mixed microbial populations, Steak plate inoculations will be used to effect their separation. The isolated colonies formed can then be studied morphologically, biochemically, and microscopically to identify the individual genera of these mixed flora.

Method:

A. Ear

1- Swab the auditory canal of your ear using a sterile swab.

2- Using the swab inoculate a: -Sabouraud agar
-Nutrient agar plate.
-Blood agar plate

3- Using the same swab make a gram stain.

4- Incubate the Sabouraud's plate at room temperature for one week.
Incubate the nutrient agar plate at 37^{o} overnight.

5- Identify organisms isolated. Report your results.

B. Skin

6- Press your fingers slightly on top of a
- Blood agar plate
-Sabouraud agar plate

and incubate as in step 4.

7- Wash your hands with soap and disinfect with 75% ethyl alcohol, wait until dry. Repeat step 6.

C. Mouth

8- Using a clean tooth pick scratch between your teeth
9- Inoculate the following plates : Blood agar
MacConkey

10- Prepare a smear and do gram stain.

11- Identify your organisms and report your results.

Report Form

Sample	Identification tests	Results	Organism

Microbiological Analysis of Urine Specimens

Purposes: To acquaint students with:

1- Organisms commonly responsible for infections of the Urinary tract.
2- Laboratory methods for detection of bacteriuria and identification of microorganisms associated with the urinary tract.

Principle:

The anatomical structure of the mammalian urinary system is such that the external genitalia and the lower parts of the urethra are normally contaminated with a diverse population of microorganisms. The issues and organs that comprise the remainder of the urine that passes through these structures are also sterile. When pathogens gain access to this system, they can establish infection. Some etiological agents of urinary-tract diseases are illustrated in figure – 1.1.

Method:

1- Mid-stream specimens of urine following adequate cleansing of external genitalia should be sent to the laboratory in sterile containers with the minimum of delay.
2- A film of the specimen without centrifugation should be made and stained with simple stain, if organisms are seen; this indicates that organisms are present in large numbers enough to cause infection.
3- Note the general appearance of urine:
   a- Color, opacity, consistency.
   b- Presence of blood, mucous or pus.
   c- Presence of macroscopic bodies such as parasites.

4- Using a standard loop (.001 ml of water) insert vertically into the urine and inoculate on:

   a. MacConkey or CLED agar plate.
   b. Blood agar plate.
   Incubate at 37^{o} C for 24 hrs.

5- Transfer the urine to a centrifuge tube and centrifuge at 3000 rpm for 15min. Pour off the supernatant into another tube for biochemical analysis:

a. Sugar content.
b. Albumin
c. pH
d. Specific gravity.

6- Make a wet preparation (wet mount) from the sediment to examine for cells, casts, crystals and organisms.

7- Report your results in the given chart.

Box 1

Biochemical analysis

a. Sugar test: Benedict.
Mix equal amount of the urine and Benedict solution, heat on the flame. The color change to brown; it is a positive test indicating the presence of glucose.

b. Albumin:
Pour some of the urine in a test tube. Heat at 1/3 of the tube if protein present turbidity will be formed at the middle. Upon addition of conc. HCI. The turbidity will be increased indicating a positive result for albumin. If turbidity disappeared, this indicates the presence of salts.

c. pH
Check the pHof the urine using a pH paper.

d. Specific Gravity:
Using the refractometer

Next day:

Quantitative Cultures:

- Colony count of 10^5/ml or greater is indicative of infection.
- Colony count between 10^3 and 10^4 / ml indicates probable contamination.
- Colony count above 10^4 / ml. indicates probable infection.
- Bacterial colonies observed should be isolated and identified as shown in figure 1.2.
- Antibiotic sensitivity testing should be performed.
- Provided the specimen has been taken with adequate aseptic precautions, the organisms in figure 1.1 may be considered.

Fig 1.1

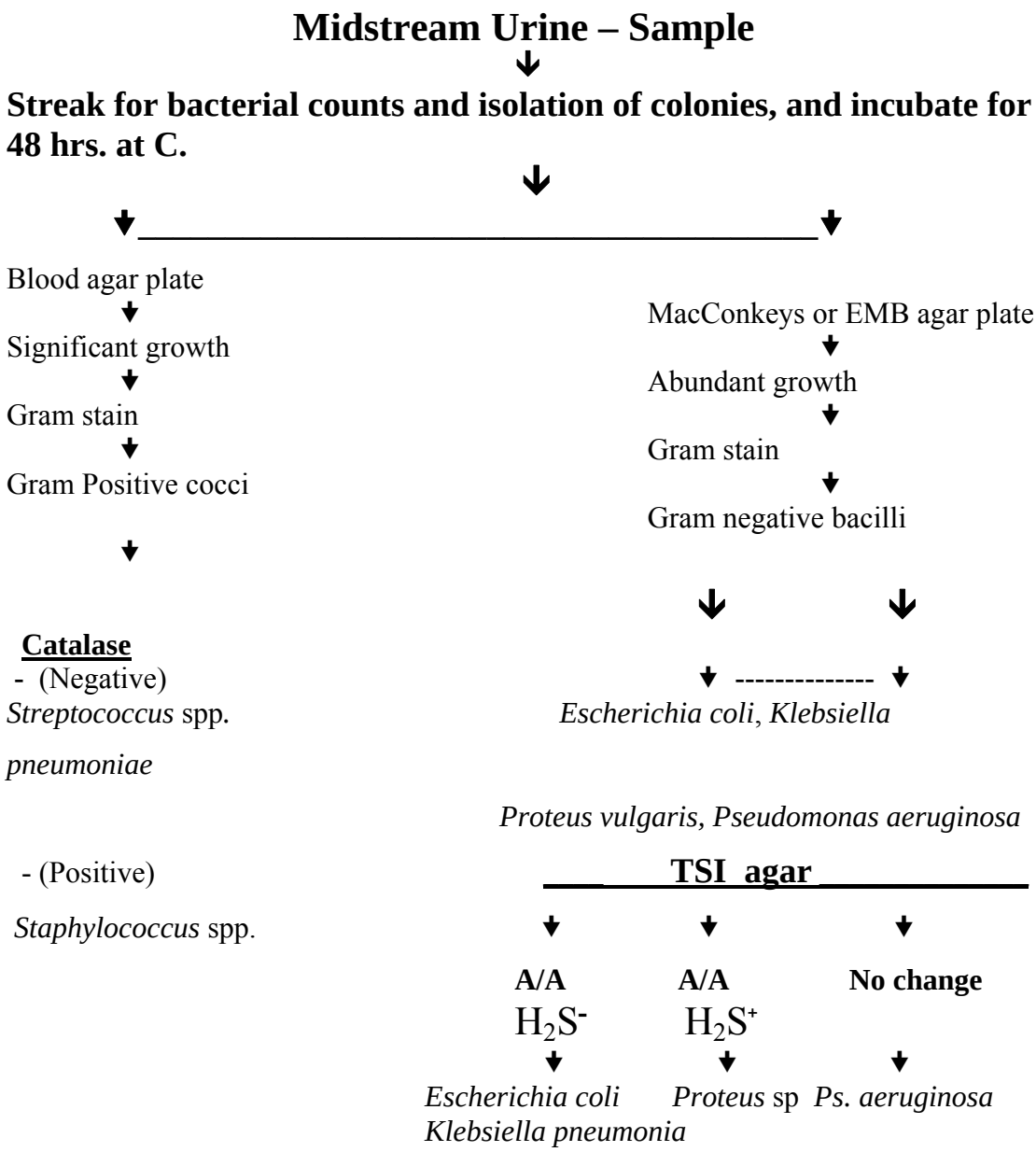
<table>
<tr><td></td><td></td><td colspan="2">Stapylococcus aureus
Streptococcus pyogenes</td></tr>
<tr><td rowspan="2">Bacteria</td><td>Gram positive</td><td>Enterococci</td><td>Streptococcus facalis

Streptococcus faecium</td></tr>
<tr><td>Gram negative</td><td colspan="2">Escherichia coli
Pseudomonas aeruginosa
Proteus vulgaris
Klebsiella pneumoniae</td></tr>
<tr><td>Viruses_____</td><td>Venereal Disease

_____</td><td colspan="2">Treponema pallidum
Neisseria gonorrhoeae
Hemophillus ducreyi
Calymnatobacterium granulomatis

_Herpes hominus (type 11)</td></tr>
<tr><td>Fungi</td><td colspan="3">Candida albicans
Blastomyces dermatitidis
Coccidioides bancrofti</td></tr>
<tr><td>Protozoa</td><td colspan="3">Trichomonas vaginalis
Entameoba histolytica</td></tr>
</table>

Fig 1.2


Midstream Urine – Sample
Streak for bacterial counts and isolation of colonies, and incubate for 48 hrs. at C.
Blood agar plate
Significant growth
Gram stain
Gram Positive cocci
Catalase
- (Negative)
Streptococcus spp.
pneumoniae
- (Positive)
Staphylococcus spp.
MacConkeys or EMB agar plate
Abundant growth
Gram stain
Gram negative bacilli
Escherichia coli, Klebsiella
Proteus vulgaris, Pseudomonas aeruginosa
TSI agar
A/A
H2S-
A/A
H2S+
No change
Escherichia coli
Klebsiella pneumonia
Proteus sp
Ps. aeruginosa

Examination of cerebrospinal And other body fluids

Principle:

Sterile fluids of the body are Pleural, peritoneal, cerebrospinal, synovial, and ascetic fluids.

Fluids are sent to the lab in a sterile narrow – mouthed I oz bottle. In case of CSF the bottles are new, sterile empty but in case of other fluids the bottle contains 3 ml of 3.8% sodium citrate of prevent clotting of the fluid.

N.B If delay is expected, Samples should always be refrigerated except CSF sample, which should be inoculated immediately.

Method:

1. General appearance:

Note the appearance & the color of the fluid whether clear or cloudy and the quantity of the fluid.

2. Transfer part of the fluid to a clean sterile centrifuge tube, and centrifuge at 3000r pm for 5 min.

3. Pour the supernatant fluid into a jar of disinfectant (Unless required for biochemical testing) Make two thick smears from the deposit.
4. Stain one smear by gram stain and stain the other for acid-fast organisms.
5. Inoculate the following media from the deposit:

a. Blood agar plate; incubate aerobically 37^{o} C for 24 hrs.
b. Chocolate agar plate incubate in 10% CO2 at 37^{o} C
c. Direct sensitivity test may be made inoculating with a swab a nutrient or MH agar plates and seeding it with the antibiotics.
d. In fungal meningitis is suspected, India ink preparation from the centrifuged sediment is often done to detect *Cryptococcus neoformans*.

* If tuberculous meningitis is suspected, inoculate two Lowenstein- Jensen slants in addition to the plates and incubate at 37oC for 8 weeks examining these slants weekly.

Next day:

Culture should be identified and sensitivity tests are made.

The following organisms are associated with meningitis:

Neisseria spp. *S. pneumonia* *H. influenza.*

S. Pyogenes *S. aureus* *Listeria* spp. *Cryptococcus* spp.

M. tuberculosis

Microbiological analysis of Blood Specimens

Purposes

To acquaint students with:

1. Microorganisms most frequently associated with septicemia
2. Laboratory methods for the isolation and presumptive identification of the etiological agents of septicemia.

Principle:

Blood is normally a sterile body fluid. This sterility may be lost, however, when microorganisms gain access into the blood stream during the coarse of an inflectious process.

Bacteremia:

A condition characterized by rapid multiplication of microorganisms with the possible release of their toxins into the blood stream.

Microbial population implicated in case of Septicemia:

1. Gram-negative bacteria:

H. influenzae. Neisseria meningitides, Serratia marcescens, E.coli, Pseudomonas aeruginosa and *Salmonella sp.* Less frequently implicated are *Francisella tublarensis* and members of the general *Campylobacter* and *Brucella*.

2. Gram Positive bacteria:

Primarly include members of the genera *Streptococcus* and *Staphylococcus.*

3. *Candida albicans* is the major fungal invader of the blood stream.

Procedure:

In the clinical setting, to facilitate the rapid initiation of effective chemotherapy. The following steps are followed:

1. Blood sample withdrawn from the patient is inoculated at the bedside. Directly into bottles containing Brain heart infusion broth (BHI). The bottles are incubated aerobically and anaerobically.

2. Bottles are checked for turbidity over a period of 3,5, and 7 days

3. Upon observing turbidity, using a sterile syringe withdraw 1 ml from the Sample and apply one drop on a slide and make a gram stain.

4. According to your gram stain inoculate the suitable plates as follows:

Gram negative bacilli
Blood, MacConkey 37^{o}C

Gram negative cocci
Chocolate agar 10% Co2 at 37^{o} C

Gram positive cocci
Blood agar

Yeast
Sabaroud
Incubate the plate. Identify your organisms.

Prepare a pure culture plate from your isolated organism for the next lab period.

Examination of
Ear, Throat, Eye Swabs

Purpose:

- Familiarize the student with the routine procedure for examination of ear, throat and eye swabs.
- Acquaint the Student with pathogenic organism more likely to be in these areas.

Introduction:

Throat and nasal swabs: (Nasopharyngeal swabs)

Nasal cultures are frequently performed to detect carrier states of *Staph, aureus, Streptococcus pyogenes, Corynebacterium diphtheriae, Bordetella pertussis and Neisseria meningitides*.

Bacteria that produce pharyngitis are: *Streptococcus pyogenes* (group A) *Neisseria gonorrhoeae, Corynebacterium diphtheriae and Bordetella pertussis.*

Upon receiving a nasopharyngeal swab the lab should be notified with the suspected diagnosis, so that to use the appropriate selective media or else it might be unnoticed, since some of these organisms require special requirements for their cultivation.

In case of:

Corynebacterium diphtheriae:

A swab from the membrane itself should be collected.

Smears are made for staining with loeffler alkaline methylene blue or Alberts stain to look for Corynbacterium with metachromatic granules.

Pertussis:

May be rapidly diagnosed by staining smears directly with fluorescin conjugated anti-Bordetella pertussis antiserum

Meningococcal carrier or gonococcal pharyngitis:

Directly inoculated onto modified Thayer-Martin agar that has been brought to room temperature.

Group A streptococcal pharyngitis:

Certain precautions should be observed to ensure accurate diagnosis of this disease.

1- Throat culture should be made, diagnosis should not relay on symptoms alone.
2- Areas of purulence, ulceration and inflammation should be cultured.
3- The swab is used to inoculate media that is enriched with 5% sheep blood.
4- The area of primary inoculation must be streaked out for isolation with a sterile wire loop using the streak-stab method, for better hemolysis to be observed.
5- Bacitracin disk and penicillin G disk should be placed on the pure culture and not from the primary isolation.

6- Culture should be incubated at $35^{O}C$ for 18-24 hrs.

Examination of Sputum Samples

Purposes:

1. To Acquaint the students with the pathogens of the lower respiratory tract
2. Students would be aware of the procedure to follow in sputum samples examination.

Introduction:

Sputum is a secretion from the trachebronchial area that is made of plasma, water, electrolytes and mucin. These secretions when passes through lower and upper respiratory tract gets contaminated with nasal and salivary gland secretions. This mixture is referred to as sputum.

Specimen Collection:

Sputum samples are often contaminated with food particles, mouth microbial flora: thus the following steps should be requested from the patient:

1. First morning specimen should be taken.
2. Rinsing of the mouth before sample collection.

Problems in sputum collection always arise with children thus one of the following three methods could be used:

1- Nasopharyngeal swab is obtained, which is believed to represent the pathogen in LRT.

2- Cough plate: A plate is placed in front of the child's mouth and the child is urged to cough.

3- Cough –swab technique: Most appropriate gives non contaminated sputum sample: the mouth of the child is kept open by pressing the tongue with a tongue depressor and the epiglottis is touched by a swab to induce the cough. The sputum will be collected on the swab and cultured on the appropriate plates.

Problems can also arise in non cooperative patients and this can be overcome by using inducers.

- Induction increases the flow of bronchial secretions and stimulates a cough e.g. of inducers are:

- 10% sodium chloride and sterile distilled water aerosols + 10% propylene glycol.

- Acetylcysteine together with a bronchodilator

Specimens should be collected in a sterile disposable screw cap container. The sample should be examined as soon it reaches the lab.

Specimen examination:

A) Samples should be first examined macroscopically. The sputum sample be transferred to a sterile empty Petri dish which is placed against a dark background, sterile wooden applicator is used to spread the sample and then the sample is examined by :

Eye Swabs

Swabs taken from eye infections should be inoculated immediately for several reasons:

1- Scarcity of microorganisms.
2- To avoid enzymatic activity of enzymes found in tears.

Certain devises should be available in the lab for the ophthalmologist to use:

1- Sterile tipped with calcium alginate Dacron or cotton.
2- Platinum spatula
3- Glass slides
4- Media should be ready for inoculation

Which are: fluid thioglycollate
Sabouraud dextrose agar
Lowenstein Jensen
Blood agar
Chocolate agar.

Corneal scrapings are spot inoculated on chocolate blood agar and Sabouraud dextrose agar and into fluid thioglycollate medium.

Lowenstein- Jensen agar can be inoculated in suspected mycobacterial disease.

Possible Pathogens are *Staph. aureus*, pneumococci, *Moraxella, Haemophilus*, *N. gonorrhoeae*, haemolytic streptococci, pathogenic fungi, and diphtheroids.

Ear Swabs:

Culture is usually indicated in patients with sever pain, pulging tympanic membranes, impaired host deafness or failure to respond to therapy.

In most cases the organisms encountered as pathogens are *Streptococcus pneumoniae, Streptococcus pyogenes, or H. influenzae*.

In neonates most propably *Staphylococcus aureus, E. coli* or *Klebsiella peneumonia.*

In chronic infections *Staph. aureus* and *Pseudomonas aeruginosa* are predominant.

General Procedure:

- All media should be inoculated immediately, with the least delay upon receiving the samples. Smears for gram stain or other stains should be prepared later.
- Streak the swab primarily on two blood agar plates and incubate one aerobically and the other one anaerobically at 37^{o} C.
- Unless stated suspicion of gonococcal infection or heamopilus, plates should be incubated at 10% CO2 containing jars.
- Inoculate other plates suitable for the suspected diagnosis stated on the request form
- Make a smear for gram stain and for any other recommended stain as stated before.
- Identify the organism and report your results.

Naked eye or using a magnifying lenses. The following macroscopic features are checked:

1- Consistency and appearance:
Liquid (serous), mucoid, purulent, bloody or combinations.
2- Color
3- Odor
4- Cheesy masses: necrotic pulmonary tissue.
5- Bronchial Casts: tree like casts of bronchi with various sizes
6- Broncholitis or lung stones: calcifications of necrotic or infected tissue.
7- Dittrich's plugs: yellowish or gray bodies that vary in size, usually, contain cellular debris, fatty acid crystals, fat globules and bacteria.
8- Parasites.
9- Foreign bodies.

B) Suspicious particles are examined <u>microscopically</u>. Stained or Unstained.
Stains used:-
Gram stain
Wright's stain for RBC's
- Ziehl- Neelsen stain for *M. tuberculosis*

Papanicolau stain for malignant cells.
Buffered crystal violet for epithelial cell.

<u>Types of cells seen</u>:

- The presence of alveolar Macrophages is the best assurance that the specimen has been correctly collected.
- Bronchial epithelia
Ciliated columnar bronchial epithelia.
- Blood cells.

C) Sputum Culture:
Microscopic examination would determine if the sputum sample is acceptable for culture or not.
Generally speaking gram stain preparation of mucus samples that would contain less than 25 squamous epithelial cells (mouth) and at least 10 leukocytes per field (x100) are acceptable for culture.
Each specimen is routinely cultured on:
Blood agar, MacConkeys chocolate agar and thioglycollate broth by the streak plate technique incubated at 37^{o} for 24 hrs. and at 5% CO2 for blood and Chocolate.

- The culture results should correlate with the gram stain results.

Suspected organisms are:

1- Mycobacterium tuberculosis stained with acid fast stain or Auramin- Rodamin stain.
2- -Gram-positive cocci – *Streptococcus pneumonia* gram positive diplococci lancet shape alpha-hemolytic can survive for long period in sputum and if refrigerated at 4^{o} c it can survive for weeks.
- Staphylococci
3- Gram negative bacilli:
Almost any gram - ve bacilli have the potential to cause disease of the lower respiratory tract. The more common one are *Klebsiella*, *Hemophilus, Enterobacter, Pseudomonas* and *E Coli.*

The Ziehl – Neelsen Stain

Ziehl – Neelsens Method:
Principle:
The waxy material of the capsule of the acid fast bacilli resists staining with ordinary stains penetration of the stain occurs only upon using a strong mordant dye. e.g. strong carbol fuchsin in phenol in the presence of heat. The bacteria resist decolorization with 20% sulphuric acid and alcohol. Methylene blue or malachite green are counter stains.

Procedure:

1- Prepare an air dried heat fixed smear from your culture.
2- Food the slide with strong carbol fuchsin and heat until steam rises but do not let it boil.
3- After 3-4 min. apply heat again until steam rises. Do not let the stain dry on the slide.
4- After 5 – 7 min. from the initial application of heat, wash the slide with running water.
5- Decolorize with acid alcohol until red color disappears from the film.
6- Wash and reapply the decolorizer:
7- When decolorization is complete wash well.
8- Counter stain with Loffler's methylene blue or 5% malachite green for 1 min.
9- Wash and air dry do not blot.

Results:

Acid fast organisms are red.
Other organisms are blue or green

Bile Solubility test

Principle:

This test is used to differentiate between pneumococci and viridans types of streptococci (This test is not specific for pneumococci).

Pneumococci differ from other streptococci species in having an autolytic enzyme which is activated by the presence of bile

Procedure:

1- Prepare two sterile test tubes containing 5ml. of 18 hrs culture of the heamolytic streptococci species.
2- Add to tube No.1 5ml of 10% sodium deoxycolate (bile salt).
3- Add to tube No.2 5ml of sterile normal saline.
4- Incubate at 37^{o} for 2 hours and examine the rubes every 15 min.

Results:

Positive: tube no.1 becomes clear after 15 min.
Negative: tube no. 1 remains turbid. after 2 hrs.

Examination of Stool Samples for Gastro Intestinal Pathogens

Purpose:

1) To acquaint the student with the proper way of examining stool sample for routine and culture.

2) Identification of gastrointestinal pathogens.

Introduction:

In this lab, we are going to deal with infection of the gastrointestinal system, which is either food poisoning or enteritis.

Bacterial Enteritis:

Inflammatory diseases of the small intestine and colon as a consequence of bacterial infections, Bacteria that causes enteritis are numerous:-

Shigellosis or bacillary *Shigella spp*
Salmonellosis – *Salmonella spp.*
Campylobacter enteritis, Campylobacter fetus.
Clostridium perfringens enteritis
Cholera *Vibrio cholerae*
Yersinia enteritis *Y. entercolitica*.
Infantile diarrhea Enterotoxogenic *E. Coli* (ETEC)

The enteric pathogens of prime medical concern is salmonella and *Shigella* Routing testing and isolation of these pathogens from the faces is complicated by the fact that colon contains a diverse population of coliform bacteria. Proteus Pseudomonas and clostridium exist in large numbers Hence its necessary to use enrichment media as well as selective and differential to favor the growth of these pathogens.

The enrichment media widely used in the selenite F broth, this media enrich for the growth of Salmonella and some of the *Shigella* Second Party. Differential and selective media often used in laboratories are MacConkey, S.S., or DCA (deoxycholate – citrate agar).

Food poisoning:

Symptoms caused from bacterial toxins preformed and ingested or bacteria that are ingested and release their toxins in the intestine. So it's useful to detect the presence of the toxins as well as the bacterial and the results should be correlated.

The causes of food poisoning are shown it table 1.

Examination of stool samples:

Stool sample submitted to the lab in wide mouth containers with tightly fitted lid. Rectal swabs may be necessary in some instance. Samples should be examined as soon as they reach the lab, PH drop in the sample is harmful for *Shigella* and salmonella species.

1- Microscopic examination

* Consistency
Odor
Color
Presence of mucous

Microscopic examination

Routine stool analysis involves preparing a wet mount of the sample to check the presence of parasites as the cause of enteritis. Adult parasites, parasite fragments (taenia) ova or protozoa may be seen.

Culture:

In case f suspecting cholera a small amount of the stool is placed in alkaline peptone water as enrichment for *V. cholerae.* Incubated at 35^{o} c for 6-8 hours which is then streaked on TCBS agar (thiosulfate citrate bile salt sucrose media). Sucrose fomenter or large-sized yellow colonies are subcultered, biochemical and serological tests for identification is carried out.

Vibrio Cholera:

Gram negative comma shaped bacteria, motile, catalase, oxidase positive, ferment sugars without gas.

To test for Salmonella and Shigella.

Stool Sample is inoculated in selenite F broth for enrichment as well as directly plated on any of the differential selective media mentioned before. In the following day characteristic colony are identified by biochemical and serological tests.

Examination of Genital tract infection

Purposes:

1- Familiarize the students with the methods of examining specimens for genitourinary tract infection.

2- Training the student of ELISA technique used for detection of chamydial antigen in urethral exudates.

Introduction:

Infections of the genital tract garies between venereal diseases (sexually transmitted) and non venereal diseases. The following table shows the disease and its causative agent:

Disease	Causative agent
Venereal Disease:	
Gonococcal Urethritis	*Niesseria gonorrhoeae*
Nongonococcal urethritis	Chlamydia trachomatis Trichomonas vaginalis
Chancroid	*Haemophilus ducreyi*
Lymphoganuloma Vanerevm LGV	*Chlamydia trachomatis*
Syphilis of the genital tract	*Treponema palladium*
Genital Herpes	Herpes simplex virus type 2
Non Venereal Disease:	
Septic abortion	Anaerobes *C. perfernigens*
Pureperal spsis	Streptococcus pyogenes, *S.aureus*, *St. pneumoniae*
Chorio amnionitis	"
Post operative infection	"
Non specific pelvic inflammatory disease	"
Vulvovaginitis	*T. vaginalis* *C. albicans* *Gardenella vaginalis*
Toxic shock syndrome TSS	*S. aureus*

Sample Collection and transport:

Sample usually sent to the lab are urethral discharge, scrapings, swabs, lymph node aspirate

In case of suspected gonococcal urethritis the swab should be non toxic (calcium alginate) and should be cultured as soon as it reaches the lab. If delay is expected swabs should be placed in sterile transport media, Stuart media is widely used.

Processing of Samples:

In cases of Gonococcal urethritis, urethral discharge is cultured on Thayer-Martin media or on chocolate agar, the isolation of *Niesseria* requires the use of selective and enriched media.

Thayer martin is a selective media it contains the following antibiotics:

Vancomycin ------- >Gram positive bacteria.
Trimethoprim ------- > Gram positive bacteria
Nystatin -------- > Commensal fungi.
Colstin --------> Gram negative bacteria

Cultures are often made in cases of female gonorrhaea, but in cases of males a gram stained smear of the exudates in sensitive and specific in establishing the diagnosis positive gram stains show typical gram negative diplococci within polymorphonuclear leukoeytes, this result correlates well with the cultural findings.

Diagnosis of NGV can be made clinically if there is a negative gonococcal culture. The presence of >5 polymorphonuclear leukocyte per oil immersion field or in the sediment from the first 10ml of overnight urine (>15/high-dry field) supports the diagnosis of Urethritis.

If the causative agent is to be investigated the following test should be carried out:

1. Chlamydia:
   -Immunoflourescence antigen detection from discharge
   1gM antibody detection from serum samples.
   -MacCoy Cells – cycloheximide culture system.
   -Antigen detection of Elisa.
2. Candida albicans

- Direct smear and simple stain will show the oval shape body of yeast.
- Germ tube:

Yeast grown on peptone containing media is inoculated in a tube containing human serum. Incubated at 37 and in 30 min intervals the serum is checked for the presence of a curved germ tube developing from the pole of some of the yeast cell.

This test is specific for Candida albicans.

3. *Trichomonas vaginalis*:

Wet preparation from the swab and examine under the microscope for the presence of motile trichomonas and / or dense aggregations of bacilli around desquamated epithelial cells.

Examination of Wounds, Burns, Skins Ulcers And Abscesses

Purposes:

1- Training the student on the examination of samples from burns, contaminated wounds, closed and opened abscesses.

2- Acquaint the students with methods of cultivating and identifying anaerobic bacteria.

Introduction:

1- **Sample Collection:**

a) Tissues, Biopsies:

Biopsies come from ulcers, lesions, Tissues obtained surgically for culture should be placed into a sterile, wide mouthed screw-capped bottle. The surgeon should take an adequate amount of material for both histologic and microbiologic examination. Specimens should be kept away from fixatives and disinfectants.

b) Pus from abscesses; wounds or burns:

Pus should be injected into an anaerobic vial for transport to the laboratory or it can be sent in the syringe from which it can be aspirated by plugging the needle with a sterile rubber stopper. When an abscess cavity is opened and drained a portion of its wall should also be submitted for culture. Pus or samples from wounds and burns can also be supplied in swabs which is unsuitable for anaerobes.

Processing:

* Specimens of tissues:

Tissue should be bisected aseptically by the surgeon in the operating room and material representative of the pathological and microbiologic examination.
Good communication between histopathologist and microbiologist is important especially in cases of fever of unknown origin.

1- Tissues received by the lab. Should be examined and its characteristics described on a work card before processing.
2- It should then be finely minced with sterile scissors into a mortar where its minced with sterile abrasive in broth and ground with a pistol to render 20% suspension.
3- This material is then transferred to a sterile dropper bottle that can be used later to inoculate the different types of media. The suspension is then stored for 2 weeks until discarded. Histopathological results are an aid for determining which media to be used and which stains can be applied.

In closed abscesses the pus contains one organism only e.g. of the most common is staphylococci, streptococci anaerobes or coliforms.
In open wounds number of organisms is usually encountered which make it difficult to decided which is significant. When deep suppurating lesion drain onto exterior surface, normal flora of that surface must not be mistaken for that of the deep lesion.

Bacteriologic examination of pus from closed or deep lesions must always include anaerobic methods. Anaerobic bacteria play an important etiologic role always while aerobes are mostly contaminants. Typical wound infection due to clostridia is readily suspected in gas gangrene, pseudomonas in wounds give rise to blue green pus.
The methods employed must be for both common bacteria and specialized microorganisms such as anaerobes, mycobacteria and fungi. Eroded skin and mucous membranes are usually the sites of fungal and yeast infection like in case of burns and wounds. Candida. & Asperigellus can be seen microscopically in smears of scrapings from suspicious areas and can be grown in cultures.

Anaerobic infections:

Characteristics suggestive of anaerobic infections:

1- They tend to be found in a mixture of anaerobic organisms.
2- Always found in closed areas infection e.g. abscesses or burrowing through tissue layers.
3- Foul odor from pus.
4- Most of the pathogenic anaerobes are highly sensitive to penicillin except for *Bacteroids fragilis*.
5- Anaerobic infections are favored by low O2 tension → low blood supply.

It is necessary to use special methods to obtain organisms in culture or else it will be missed.
Figure I shows a flow chart for processing anaerobic specimens.
Samples arrive as a swab or aspirate, it should be inoculated on:
-fluid thioglycollate
-Blood agar
-EMB, CLED or MacConkey:

Media for anaerobes:

To achieve media for anaerobes cultivation the following principles should be met.

1- Freshly steamed media that has been oiled rapidly.

2- Reducing agents should be added e.g. 5% glucose
1% Sodium thioglycolate
Particles of meat in cooked meat both

3- Oxidation-reduction potential indicator that would show that the media is anaerobic e.g. methylene blue or resazurin, that would changes its color in the presence of oxygen.

These media are usually liquid if semisolid media is used a layer of liquid petroleum is added at the top of the media.
Growth of aerobes is mostly at the lower layer of the tube. In case of solid media the plates should be incubated under anaerobic conditions and this is achieved by inculcating the plates in the anaerobic jar.

Principle of Anaerobic Jars.

Gas Pack System:

- A jar with a tightly fitted lid.
- Plates are placed in the jar

- Water is added to Aluminum foil packet
- The packet is placed in the jar and the lid is screwed on.

The packet contains pellets of sodium hydrochloride, cobalt chloride, citric acid and sodium bicarbonate. Upon the addition of water the reaction between these components takes place with the release of H2 and CO2.

This lid of the jar contains Palladium which serves as a catalyst for the combustion of hydrogen and removal of O2 and the final formation H2O.

Procedure:

- The student will receive a sample from an infected wound.
- Primary inoculation is on two B.A. plates should be inoculated aerobically and anaerobically.

- Gram stain from the sample.

- Proceed with your samples until final identification.

- Student will be presented with B.A. plates, thioglycolate broth and cooked meat with media clostridium sp.

- Note its cultural characteristics on B.A. perform spore stain. Is the spore terminal, sub- terminal or central.

- Note the growth of the bacteria in the liquid media and note the formation of gas bubbles.

Methods for antibiotic sensitivity Testing and drug monitoring in Body fluids

Purposes:

- Acquaint the student with various methods of antibiotic sensitivity testing.

- Measurement of antimicrobial agent level in body fluids.

Introduction:-

Isolating the infectious agent from a patient is not often sufficient for determining proper therapy. The extensive use of antibiotics for therapeutic purposes have created the Antibiotic Resistant Strains, thus, testing individual pathogens against appropriate antibiotics is necessary.

There are certain characteristics for the antibiotic of choice. The most important are:-

a. To show the most activity against the pathogen.
b. To show the least toxicity to the host.
c. To be the most economical.

One point should be stressed is that *invitro* susceptibility tests cannot be performed on mixed cultures. Only pure cultures will yield valid results.

The student should be familiar with the following vocabulary.

Antibiotic: A chemical produced by a microorganism that inhibit the growth of other microorganisms.

A Susceptible Organism: - An organism is inhibited or killed by the concentration of the antimicrobial usually achieved in the Serum, other body fluids and tissues of the antimicrobial by the usual rout of administration.

Resistant Organism: An Organism is resistant to antimicrobial when its not killed or inhibited by the dose of that is normally achieved in the human body by the usual dose given by the usual rout of administration.

Bacteriostatic antibiotic: - Antimicrobial that has an inhibitory effect on the microorganism. Thus the organism can re-grow upon the antibiotic removal.

Bactericidal antibiotic: Antibiotic that can kill the microorganism.

Minimal inhibitory Concentration: The lowest concentration of the antibiotic that inhibit the growth of the organism as detected by lack of Visual turbidity.

Minimal Bactericidal concentration: The lowest concentration of an antimicrobial that would allow less than 1% of the original inoculum to survive.

Experiment No. 1

Disk diffusion method
Stoke method

Principle:
The susceptibility of an Organism against a Variety of antibiotics a is tested. A standard strain with known sensitivity to these antibiotics is inoculated. The antibiotic disks are placed in the border lines between the lest and the standard organism to achieve comparable results.

Standard organisms are as follows:-

- 24 hrs. culture of *E. coli* a standard for g-ve bacteria ATCC 25922
- 24 hrs. culture of Staph aureus a standard for g + ve bacteria ATCC 29213
- 24 hrs. culture of *Pseudomonas aeruginosa*. A standard for Pseudomonas species. ATCC 27853

Materials:

- a 24 hrs. MH both cultures of Pseudomonas, E Coil, Staph aureus
- a 24 hrs. MH both cultures of the test organism.
- Sterile swabs
- Mueller Hinton agar plate
- Antibiotic sensitivity disks.

Methods:-

- Divide the bottom of the MH plate into 3 parts as shown in fig. 1.1
- Swab the two sides of the plate with the suitable standard culture.
- Swab the test organism in the middle sector of the plate.
- Place the antibiotic disks at the two lines using a sterile needle.
- Incubate the plates overnight at 35^{o}c
- Measure the zone of inhibition at the test organism side and at the standard side.
- Compare the results of the test and the standard organism and report your results as:-
  Susceptible (S)
  Resistant (R)
  Write your antibiotic of choice.

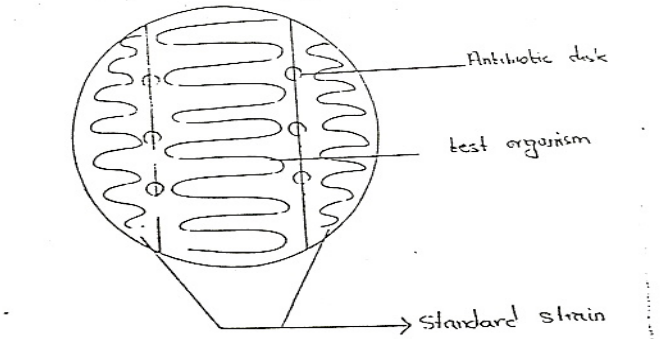


Figure: 1.1

Broth Dilution method

Principle:-

Decreasing concentrations of antimicrobial agent to be tested, are prepared in two fold dilutions and placed in tubes of Muller Hinton broth that would support the growth of the test microorganism. After overnight incubation the tubes are checked for turbidity, that is an indication of the growth of the microorganism. The-organism will grow in the control tube that doesn't contain antibiotic to inhibit the growth of the microorganism. The lowest concentration of the antibiotic that will inhibit the growth of microorganism. The lowest concentration of the antibiotic that will inhibit the growth of microorganism is considered as the minimal inhibitory concentration MIC. And is measured by the lack of turbidity. The minimal bactericidal concentration is then calculated by growing a loopful of tube and the two tubes following it and calculating the number of colony forming units CFU per plate, finding which tube showed growth less than. 1% than the original inoculum plated from the control tube. MBC can be calculated.

Materials:-

- Sterile Screw capped tubes
- Calibrated loop
- Mueller Hinton broth.
- Gentamicin in 200 ug/ml concentration
- 24 hrs. culture of *E. coli* containing 1 x 10^6 CFU /ml
- Mueller Hinton plate
- Sterile pipettes

Procedure:-

As shown in figure II

Results:-

- Examine the tube for turbidity

- Record your MIC result
- Calculate your MBC.

Measurement of antibiotic levels in body fluids (serum)

Principle:-

Known Concentrations of the antibiotic being used are placed in wells made in a previously seeded plate with the microorganism.

The test serum which contains an Unknown amount of that antibiotic is placed in a well that is made on another seeded plate with the test organism. The size of the zone of inhibition around the Serum well is compared with the one of inhibition in the patient serum is estimated.

The results of tests for levels of antimicrobial agents are called. Therapeutic drug monitoring TDM

Materials:-

1- Mueller Hinton plates.
2- Sterile Pasteur pipettes
3- Serum of a patient under antibiotic treatment
4- Micropipettes to deliver 50 ul.
5- Standard plates of E. Coli with various dilutions of gentamicin. 12-6-1.5-0 ug /ml. *
6- A 24 hrs broth culture of E Coli
7- Sterile swabs.

Method:-

1- Using a sterile swab. Swab the surface of MH agar plate with the *E. coli* culture.
2- Using the back of your Pasteur pipette make a well by pressing it is the middle of your plate.
3- Using a sterile needle remove the piece of agar and place it in a disinfectant.
4- Fit the sterile tip to the micropipettes and add 0.05 ml of the patient serum to the well.
5- Be careful not to over flood it or make any splashes around the well.
6- Leave the plate for 1 hour on the bench for the drug to diffuse.
7- Incubate over nigh at 35^{o}c

*** Draw a standard curve with the drug concentration on the axis and the zone diameter on the other**

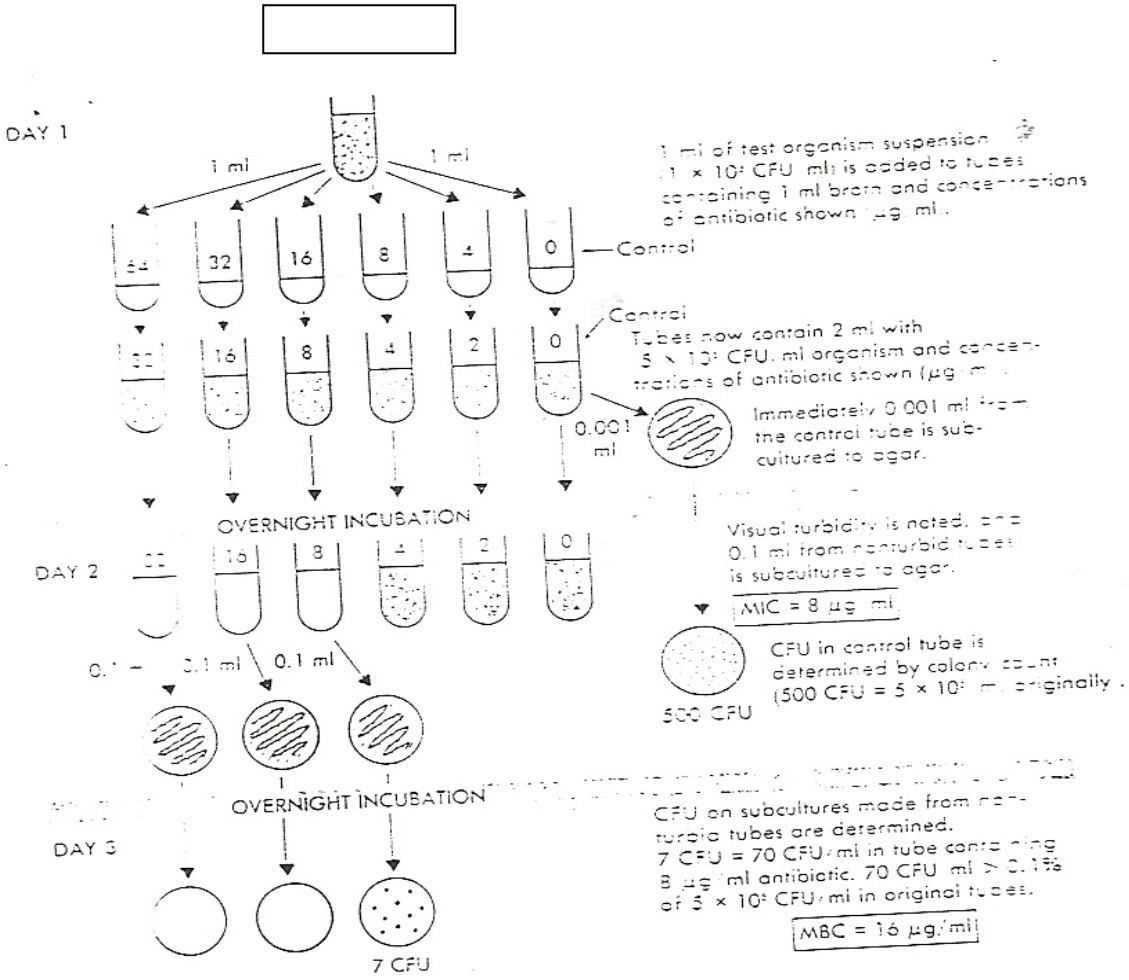


Figure: 2

Determining MIC and MBC for one organism and one antibiotic.