Laboratory Manual for General Microbiology (MCB 2010C)

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Lab Manual Overall Objectives

The objectives for each exercise of this Microbiology laboratory manual are simple: You are expected to know and understand all of the material for each exercise as well as any lab procedures used. In addition, you must be able to identify the different types of organisms covered in this manual (plus the characteristics of each organism) when viewed under the microscope or if a PowerPoint presentation, on the computer's screen.. The development of good, safe microbiology techniques are essential and expected in this course. You are responsible for understanding and recognizing, therefore, all material and microbes covered in this manual. You are also responsible for correctly answering all exercise quiz questions that are found towards the end of each exercise before the next week's exercise. All students are expected to have thoroughly studied the lab exercise before attending the lab that covers that particular exercise's material.

Your Name_____

LAB SAFETY

General Lab Safety

1. **Do not eat, drink, store food or drink, bring food or drink or apply cosmetics while in the lab. (Water in bottles included.)**
2. **Always wear shoes at all times.**
3. **Never place any personal items on the floor.**
4. **Never place any personal items on the countertops.**
5. **If you have long hair, tie it back.**
6. **If you wear long sleeves, roll them back up your arm.**
7. **Disinfect your work area before lab starts and at lab's end.**
8. **Disinfect your work area if you make a spill.**
9. **Wash your hands before and at the end of the lab period.**
10. **Wash your hands if they become contaminated with microbes.**
11. **Wash your hands before you leave the room for whatever reason.**
12. **Wash your hands when you remove your gloves.**
13. **For whatever reason, always scrub your hands for a minimum of 24 seconds.**
14. **Keep hands, pencils, pens etc. out of your mouth.**
15. **Keep your work area uncluttered.**
16. **Do not insert contact lenses in the lab.**
17. **Wear eye protection when heating chemicals of if you wear contact lenses.**
18. **Turn off Bunsen burners when not in use. They are a hazard and also a source of heat in the room.**
19. **No horseplay ever in any lab anywhere.**
20. **Do not experiment on your own.**
21. **Follow lab manual directions carefully and correctly.**
22. **Never pipette by mouth.**
23. **Call your instructor if there is microbial spillage.**
24. **Don't touch broken glassware. Use a broom and dust pan to remove.**
25. **Wear disposable gloves while staining or handling microbes.**

Student Conduct

1. **Again, do not eat, drink, store food or drink, bring food or drink or apply cosmetics while in the lab. (Water in bottles included.)**
2. **Come prepared for the day's exercise. Study the exercise before you come to lab.**
3. **Don't remove anything from the lab.**
4. **It is not permitted to apply cosmetics or insert contact lenses in lab.**
5. **Do not talk while the instructor is talking to the lab class.**
6. **Always bring your lab manual to lab.**
7. **Wash hands thoroughly after handling microbes and anytime you leave the lab.**
8. **At all times, practice safety—be mentally alert at all times!!!**
9. **When the instructor is talking, pay attention--you may learn something.**

Fire Safety

1. Learn all exits from the lab the first day of lab.
2. Know the locations of Bunsen burners at all times.
3. Turn off Bunsen burners when not in use.
4. Tie long hair back; roll up long sleeves.
5. Find the fire extinguisher and fire blanket.
6. In case of fire, calmly leave the lab via exit doors.
7. In the case of fire in the laboratory, there are four exit doors through which you can exit the lab. Locate, during your first lab meeting, these four doors. All of these doors are located in the front of the lab (left and right sides). The other two doors up front lead into the chemistry lab and can be used for exit.
8. After leaving the building calmly, go immediately to the formation area described by your instructor on the first day of lab. Do not leave this area.
9. Your instructor will handle the fire emergency.
10. Locate all fire alarm boxes in the lab and the hallway during the first lab period and know their locations.
11. Advise the instructor immediately if a fire starts in lab.
12. Always be extra careful when using Bunsen burners.

Contaminated Materials Disposal:

1. Place all plate cultures in an autoclavable bag for disposal when finished with them.
2. Place all microscope slides in the container marked “slides”. They will be immersed in a disinfectant solution in this container.
3. Remove all labels from plates or tubes before disposal.
4. Place all gloves in their respective autoclavable bag.

Table of Contents

Exercise 1:
The Microscope

Materials

1. Microscope PowerPoint presentation on lab computer and on WebCT.
2. Binocular microscope.
3. The following prepared microscope slides.
   a. Computer Chip b. Human Helminth Eggs
   c. Drosophila Chromosome d. Cyanobacteria Water Bloom

Objectives

1. Learn the location and function of the listed microscope's parts.
2. Learn how to place a specimen onto a microscope and then properly use the microscope for specimen examination.
3. Learn how to properly use the different objective lenses to bring a specimen into perfect focus.
4. Learn how to properly clean the oil immersion lens.
5. Learn the specified metric measurements.
6. Be able to define resolution and N.A. .
7. Be able to compute the total magnification of a specimen.
8. To always follow proper hand washing and countertop disinfection procedures.
9. Learn all terms in bold print.

INTRODUCTION

The Microscope

. You are expected to access WebCT and learn the Lab Exercise 1 material before you attend the first lab (using the microscope). The online exercise presentation has sound. Be sure to adjust your computer's speakers using the second slide in the online exercise. The first slide has no sound but advises you to turn on your computer's speakers. It is required that you go over this online material several times to ensure that you are well familiar with the microscope and its parts as well as any measurements and defined terms. You will be tested on these during the first lab quiz as well as the lab mid-term. The online material is also found on the hard drive of the lab's computers but with no sound.

Your lab microscope is known as a **brightfield compound microscope** since it contains more than one lens (thus compound and it has three separate lens systems) and the illumination source for your specimen is a light bulb in the base of the microscope. The lab's microscopes are **binocular** which means there is not one but two **eyepieces (or ocular lenses)** through which you can view the specimen. The magnification of each ocular lens is **10X**. The capital "X" after the number always indicates, in science, the magnifying power of a lens. Within one of the ocular lenses (or eyepieces) is a **"pointer"** that allows one to "point" to a particular area or object on a slide's specimen that they are viewing. Then someone else can look into the eyepieces and see what they were referring to. One can adjust the distance between eyepieces (their **intraocular distance**) by gently pulling or pushing on them both (at the same time). At least one eyepiece has

INTRODUCTION CONTINUED:

a **focus adjustment dial**, the other may or may not. If not both, then you should first bring your specimen into focus using the eyepiece that **does not** have a focus dial. Then, you would next adjust the eyepiece that has the adjustment dial (thus bringing the specimen into perfect focus with it). If both have a dial, then choose one to initially focus the specimen; then adjust the other.

A second group of lenses on your binocular microscope is the **objective lenses**. They are found on the **rotating nosepiece** and can be moved around in a circle. When moving objective lenses, one can tell when they are in their proper position for viewing a specimen because one can hear or feel them click into place. Try it a few times to make sure you understand and can do this. Each objective lens has its own name and magnification. In their order from the smallest in magnification and proceeding counterclockwise on the nosepiece to the highest in magnification would be the **scanning lens (also called the low power lens)**, the **medium power lens**, the **high dry (hi dry) lens or high power lens** and then the most powerful one called the **oil immersion lens**. **The oil immersion lens is the only lens you immerse in oil. Immersing the high dry lens in oil can and will ruin it. PLEASE DON'T DO THAT! PAY ATTENTION!**

The following are the magnifications for each of the objective lenses:

Low Power lens - 4X
Medium Power lens – 10X
High Power lens - 40X
Oil Immmersion lens – 100X

How does one determine the **total magnification** of the specimen they are viewing? After all, one is looking through the eyepieces as well as one of the objective lenses. The circular area you see with your eyes when looking through the eyepieces is called your "**microscopic field**" or just your "**field**". We call your microscope a "**brightfield**" microscope since the bulb makes your field bright and the specimen dark. The following formula allows you to determine the total magnification of the specimen you are viewing in your field:

Total magnification = ocular lens magnification x objective lens magnification

As an example, let's say you're looking at a specimen using the high dry lens. What is the total magnification of your specimen once in focus with this objective lens?

Substituting in the formula:

Total magnification = 10X x 40X = 400X
Your specimen has been magnified 400 times. Be sure you can figure the total magnification of a specimen when using any of the four objective lenses.

INTRODUCTION CONTINUED:

Where is the third lens system in your microscope? It is in the **condenser**. The condenser lies beneath the stage. The condenser grabs as many light rays as possible coming from you illumination source (light bulb in our microscopes) and directs then straight upwards (in the form of a cone) into the specimen on your slide. Light rays coming from an illumination source such as a light bulb try to wander off in another direction besides "straight". However, the condenser grabs as many of these wayward light rays as possible and directs them straight upwards in the form of a cone thus trying to assure that the specimen is uniformly illuminated. Once you have focused on a specimen, play with the condenser and see how it can "**contrast**" the specimen you're looking at. It is an important structure on the microscope. I expect you to know what it does and how to use it properly. **Before you start for the day, make sure the condenser is lowered slightly downwards from the stage (just a little bit) before you start the day's exercise**. Inside the condenser is an **iris diaphragm**. By moving the **iris diaphragm lever** on the outside of the condenser, one can affect the diameter and angle of the cone of light hitting the specimen and hence the illumination of the specimen. Be sure to move the iris diaphragm lever back and forth slowly to see what it does. **When starting for the day, make sure the iris diaphragm is full open.**

What is the "**resolution**" of a microscope or its "**resolving power**"? One way to look at it is to say that it is the measurement of how far two points must be in order for the microscope to tell that they are separate points. The best compound microscopes have a resolution of down to about 0.2 micrometers. In other words, at the highest magnification you can get, you cannot see objects less than 0.2 microns apart.

What is the "**N. A**." or "**Numerical aperture**" of an objective lens? It is a measurement of a lens's ability to capture light coming from the specimen. The higher the N. A. number, the greater the ability of that lens to capture light. Thus, if you use a lens with a higher N. A. than the preceding lens, you must add more light from your illumination source in order to properly illuminate your specimen. If you use a lens with a lower N. A. than the previous lens, then you need to lower your **rheostat** since you don't need as much light in this new lens (as you did in the previous one).

a. Which objective lens on your microscope has the least ability to capture light?

b. Which objective lens has the greatest ability to capture light?

INTRODUCTION CONTINUED:

Measurements

The metric system of measurements is important thorough out all of science. An ant is about 5 **millimeters** (5 **mm**) long. A dust mite is somewhere near 200 **micrometers** (200 **microns or** 200 **μm)** long. The head of a pin is about 1 to 2 millimeters (1 – 2 mm) in diameter. An erythrocyte is approximately 8.5 to 11.5 microns (micrometers or μm) in diameter. A leukocyte is about 10 μm (micrometers) in diameter. Bacteria vary in length, depending on who they are, from 0.2 to 100 microns (micrometers) in length. A typical human hair is about 60,000 **nanometers** (60,000 **nm**) wide. DNA is about 2.5 nanometers (2.5 nm) wide. An atom of hydrogen is only 0.1 nanometers (1 **angstrom**) in diameter. Did you know that there are nanobacteria? It is essential that you understand and can use these measurements in science, especially mm, nm and μm. One **meter** is approximately 39.36 inches in length if you refer to the English measurement system.

1 centimeter = 1/100 meter (abbreviated: 1 cm)
1 millimeter = 1/1000 meter (abbreviated: 1 mm)
1 micrometer (1 micron or 1 μm) = 1/1,000,000 meter
1 nanometer = 1/1,000,000,000 meter (abbreviated: 1 nm)
1 angstrom = 1/10 nanometer

Measurement Practice Problems:

1. **1 meter = ? cm**
2. **1 meter = ? mm**
3. **1 meter = ? μm**
4. **1 meter = ? nm**
5. **? micrometers (? μm) = 1 mm**
6. **20 mm = ? micrometers (μm)**
7. **100 micrometers (100 μm) = ? mm**
8. **10 micrometers (10 μm) = ? mm**
9. **1 micrometer (1 μm) = ? nm**
10. **100 nm = ? micrometers (? μm)**
11. **Which is the larger number? 1/1,000,000 or 1/1,000,000,000**
12. **4 nm = ? angstroms**
13. **.5 nm = ? angstroms**
14. **100 angstroms = ? nm**
15. **3 angstroms = ? nm**
16. **400 mm = ? cm**
17. **3.5 cm = ? mm**
18. **1 micron = ? angstroms**
19. **1 nm = ? angstroms**
20. **? nm = 2 micrometer (2 μm)**
21. **? angstroms = 1 micrometer**

Using the Microscope

It is essential that you understand and use the microscope properly in lab**.** For an assignment, you are to study and learn the microscope's WebCT presentation before your first lab meeting. Click on the following item in WebCT's NAV bar or else the icon in "Course Home" ----- **"Lab Exercise #1 Material"**. Here, I will lecture to you online about the microscope as well as other exercise #1 items during this presentation.

You will be responsible for learning and understanding all of the following items including their location on the microscope as well as their function or meaning (as appropriate):

Ocular lens or eyepiece
Magnification of each eyepiece
Interpupillary distance
Eyepiece focus adjustment
Pointer
What the "X" after a number means
Arm
Revolving nosepiece
Objective lenses and their appropriate names
The magnification of each objective lens
Why the 40X lens can't be placed in oil
N. A. or numerical aperture
N. A. of each objective lens
The resolution (the limit of resolution) of a microscope
Stage
Stage Aperture
Slide Holder
Mechanical stage
Mechanical stage control (adjustment) knobs
Condenser
Condenser control knob
Iris diaphragm lever
Iris diaphragm
Base
Rheostat
On/Off switch
Illumination source

Setting Up and focusing on the specimen:

Get Out the Microchip Slide. Use these steps as a guide:

1. Make sure the microscope is plugged firmly into an electrical outlet.
2. Make sure the microscope's rheostat is on zero (0).
3. Make sure the condenser is lowered a little way from the stage.
4. Make sure the iris diaphragm is full open (use its lever).
5. Using the on/off switch, turn the microscope on.
6. Lower your stage all full down using coarse adjustment knob.
7. Firmly grasp your slide (labeled microchip) and place it gently into the slide holder. Please do not drop the slides------they cost money and are hard to replace. Center this slide over your stage aperture.
8. Ascertain that the medium power objective lens is properly positioned for use on the revolving nosepiece.
9. Look through your ocular lenses with **both eyes**. Make any adjustments to your interpupillary distance now. You are now looking at your **microscopic field**. Notice the **pointer**.
10. Note which eyepiece you cannot adjust with a focus dial (if there is only one). Use it first to bring your specimen into focus. Then adjust the other.
11. Using the coarse focus knob and while looking through your ocular lenses, slowly bring the specimen slide (stage) towards you or upwards. You cannot break the slide with this objective lens. It is true that some others state that for the initial focusing of your specimen, once all is ready, bring the stage all the way upwards and then slowly lower the stage away from you (while looking in both eyepieces). It doesn't matter to me which way you do it. Just get the specimen in focus with medium power.
12. Once you have the specimen in focus, next, using the fine focus knob, "fine tune" your specimen's focus.
13. From here on, when you go to higher magnifications, only use the fine focus knob.
14. Go to high power next and bring the chip into focus.
15. We will not use oil immersion today.
16. Next, your instructor will "walk" you through the other specimen slides. Please do not go ahead of the instructor.

When finished for the day

1. First, make sure to turn your microscope's rheostat all the way to zero (0).
2. If you have used oil immersion, thoroughly clean the oil immersion lens with **lens paper** and **xylene**.
3. Wrap the microscope's electrical cord gently around the arm.
4. Carefully and properly, carry the microscope and return it to its storage area.
5. If we have used any live microorganisms in the lab, disinfect your countertop work area. Then wash your hands a minimum of 24 seconds.
6. Place all paper towels etc. into the waste can. Leave your work area clean.
7. Place all prepared slides back into their storage box. **Do not break them!**
8. Wash your hands a minimum of 24 seconds again before leaving the lab.

Exercise Questions:

1. List 5 things that could affect the illumination of your specimen.
2. Where would you find the "pointer"? What does it allow you to do?
3. Which ocular lens should one use when first focusing on a specimen in our lab?
4. How do you properly carry a microscope?
5. What is the magnification of your ocular lens or eyepiece in lab?
6. What is the magnification of your high dry lens? What type of lens is it?
7. What is the magnification of your oil immersion lens? Your medium power lens?
8. Which lens does not require as much light going through it (in order to properly illuminate the specimen) when compared to all of the others? Which one requires the most light going through it in order to see the specimen?
9. What is the purpose of the revolving nosepiece?
10. Why do they call your microscope a brightfield, compound microscope?
11. Millie is looking at her slide specimen. It is magnified 400X. Which objective lens is she using?
12. Alamander is using the oil immersion lens. What will be the total magnification of his specimen?
13. On your microscope, what part is used to give you greater or lesser contrast of your specimen?
14. Where should your rheostat setting be if you're using the oil immersion lens?
15. Where should the rheostat setting be if using the medium power lens?
16. Using mathematics (show your work here), would our microscopes resolve a specimen that is 1000 nm wide?
17. True or False: You can use the coarse focus knob on high power or oil immersion.

Exercise Measurement Questions

1. This is a virus. Notice its length and width given in "μm':

.8 μm x 0.015 μm

What would these dimensions be in nanometers?

2. This is a protein molecule called hemoglobin. Its length is:

length = 15 nm

What would be its length in μm?

3. This is a bacterial cell. Its shape is called a "bacillus". Its length and width are:

2.0 nm x 10 nm

What would its length and width be in μm?

4. This is a type of leukocyte called a neutrophil. Neutrophils average 10 – 14 μm in diameter.

How would you write this average diameter range in nm?

Exercise No. 2 STREAK PLATE PROTOCOL

History

The modern streak plate procedure has evolved from attempts by Robert Koch and other early microbiologists to obtain pure bacterial cultures in order to study them, as detailed in an 1881 paper authored by Koch (5). Slices of sterilized potatoes became the first solid media employed on which to grow bacteria. This process was a procedure that worked only for a few organisms and only until the bacteria decomposed the potato surface. A search for other materials led to experimentation with the suitability of gelatin and agar-agar as solidifying agents. Gelatin was difficult to prepare and difficult to use at room temperature, let alone at the higher temperature of an incubator, and many bacteria digest the protein. Agar, because of its characteristics of melting only when boiled, rarely being digested by bacteria, and providing a substance in which other nutrients could be dissolved, proved to be a suitable material on which to grow bacteria. Agar was originally called agar-agar and is derived from seaweed. The agar that we use today is the same substance as agar-agar, but it has been processed by the manufacturer. Agar, as purchased 100 years ago, required filtering before it was clear enough to use in media (12). In the early eras of microbiology, making media was an extensive process of preparing the extracts of meat or other nutrient sources, as well as purifying and filtering the gelatin or agar. Before the invention of the autoclave, sterilizing the media properly was also time consuming. The 1939 edition of An introduction to Laboratory Technique in Bacteriology, an early microbiology lab manual, contains extensive instruction for students to prepare their own media from "scratch" (7) for use in the lab. Before R. J. Petri invented the petri dish, flat plates of glass covered by glass lids were most commonly used to culture organisms in gelatin.

Even after agar was adopted and solid media were available, the streak plate was not commonly used. Historically, microbiologists most frequently used pour plates to isolate organisms for pure cultures. A pure culture was made from an isolated colony, represented only one species or strain, and traditionally arose through the growth of a single cell. Colonies are considered isolated if they are not touching any other colony. Isolated colonies were identified and transferred by streaking onto a new agar or gelatin plate using a sterile needle, a process called "picking colonies." More rarely, a researcher would try to isolate organisms directly on the surface of a gelatin or agar plate. A typical description of the streaking process was given by Huber Williams, revised by Meade Bolton in *A Manual of Bacteriology* published in 1908 (11). "...the isolation of bacteria may sometimes be effected by drawing a platinum wire containing material to be examined rapidly over the surface of a petri dish containing solid gelatin or agar; or over the surface of the slanted culture medium in a test tube; or by drawing it over the surface of the medium in one test tube, then without

sterilizing, over the surface of another, perhaps over several in succession."

Bacteriology textbooks and lab manuals from the early and mid 20th century did not mention the streak plate nor did they have our typical "isolation streak" exercise. For instance, isolation by streaking is absent from Buchanan and Buchanan, 1938 (2) and from Sherwood, Billings and Clawson's manual published in 1952 (10). During a literature search to pinpoint the first appearance of our modern streak plate, several papers published in the 1940s were found to mention streak plates. However, these did not describe the process or illustrate the results, and from the context, most probably referred to the process of picking colonies and creating a pure culture in fresh media.

An early version of our modern isolation streak is found in Levine's An Introduction to Laboratory Technique in Bacteriology published in 1939 (7) and a similar version from 1954, in Salle's Laboratory Manual on Fundamental Principles of Bacteriology, 4th ed. (9). In that process, the student picked up organisms on a needle or loop and then either stabbed into the agar or spread the loopful of the culture at the upper end of the petri dish to thin it out. Then a series of strokes 1/4-inch apart was made over the rest of the plate. Dr. Salle noted that the first streaks would contain too many organisms but that the last streaks should give isolated colonies. He suggested that a second plate be inoculated without flaming the wire loop first, to give a better chance of obtaining isolated colonies. This process dilutes the bacteria as the plate is streaked, similar to the dilution observed in a modern streak plate.

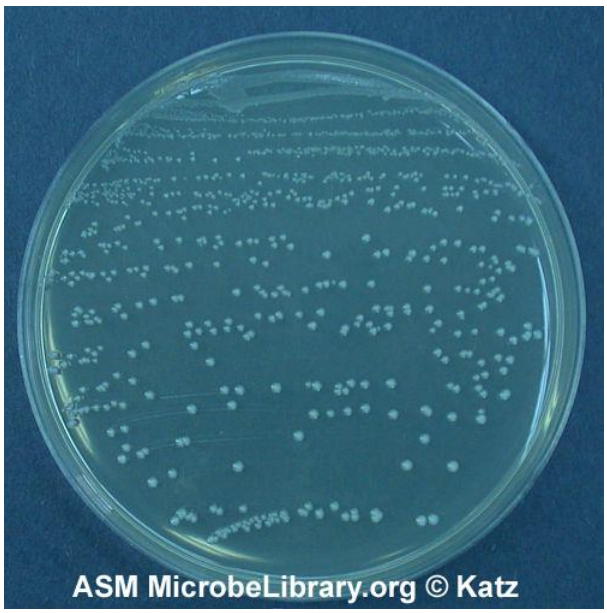


FIG. 1. An example of the one-directional streak pattern as described in the lab manuals by Levine and Salle (7, 9). The plate illustrated is a 100-mm petri dish.

In 1958, in the first edition of Laboratory Exercises in Microbiology, Pelczar and Reid (8) presented a streak plate exercise. It utilized a 4-quadrant streak pattern, and the procedure described using both a loop and a needle in the streak and all streaks were in the same direction, rather than both back and forth.

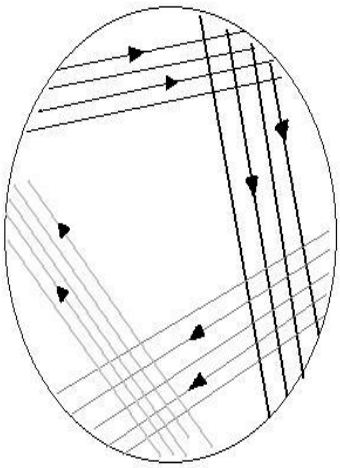


FIG. 2. A drawing representing the streak pattern recommended by Pelczar and Reid (8). All strokes of the loop or needle are done in a single forward direction, rather than in a back-and-forth pattern, as indicated by the arrowhead directions. The initial sector is at the top of the plate, followed clockwise by sectors 2, 3, and 4.

The earliest appearance of the three sector streak pattern (called the T streak) commonly used today may be the 1961 photos published in Finegold and Sweeney (4). An illustration detailing how to perform this streak is in the 1968 edition of the Manual of BBL Products and Laboratory Procedures (1). In addition to the T streak, the BBL Manual illustrates two other streak patterns, neither of which is the simple monodirectional streak pattern used earlier in the century.

Today, there are two most commonly used streak patterns, a three sector T streak and a four quadrant streak. Microbiology lab manuals since the 1970s have presented an isolation streak exercise. Lab manual editions published between 1970 and 2000 illustrated and described several streak pattern variations. However, today, almost all published microbiology lab manuals illustrate at least the T streak.

FIG. 3. A three sector T streak of *Serratia marcescens* grown on trypticase soy agar. This illustrates a streak plate which has many isolated colonies.

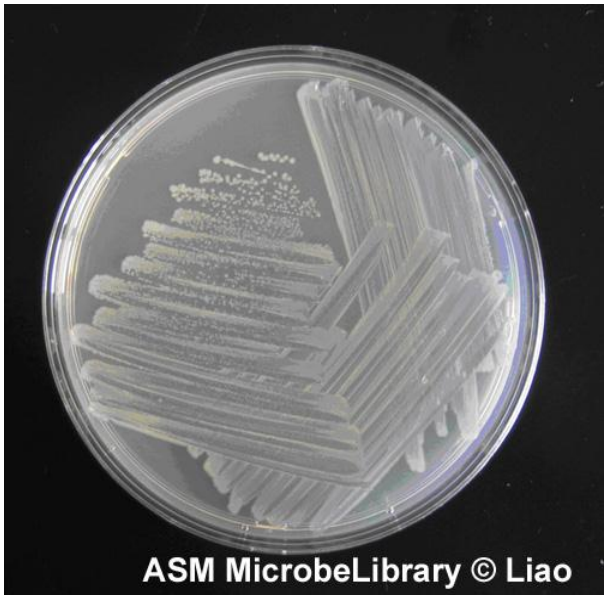


FIG. 4. This plate illustrates a streak plate which did not achieve isolation, and which would not be considered a good streak plate example. This photograph is by Dr. Min-Ken Liao, Furman University.

Purpose

The purpose of the streak plate is to obtain isolated colonies from an inoculum by creating areas of increasing dilution on a single plate. Isolated colonies represent a clone of cells, being derived from a single precursor cell. When culture media is inoculated using a single isolated colony, the resulting culture grows from that single clone. Historically, most microbiology research and microbial characterization has been done with pure cultures.

Theory

One bacterial cell will create a colony as it multiplies. The streak process is intended to create a region where the bacteria are so dilute that when each bacterium touches the surface of the agar, it is far enough away from other cells so that an isolated colony can develop. In this manner, spreading an inoculum with multiple organisms will result in isolation of the different organisms.

PROTOCOL

Mesophilic bacteria are generally streaked onto media solidified with 1.5% agar or agarose. Gelatin can be used if a high enough concentration of gelatin protein or a low enough incubation temperature is used. Thermophiles and hyperthermophiles can also be streaked onto growth media solidified with agar substitutes, such as Gelrite and guar gum.

One-hundred-mm-diameter petri dishes are the most commonly used size of plate for streaking. The agar surface of the plate should be dry without visible moisture such as condensation drops. Traditionally, inoculated petri dishes are incubated with the agar side up to prevent condensed moisture from falling onto the agar surface, which would ruin the isolation by allowing bacteria to move across the moist surface creating areas of confluent growth instead.

The inoculum for a streak plate could come from any type of source, for example clinical specimen, sedimented urine, environmental swab, broth, or solid culture. The two most common streak patterns are the three sector T streak and the four sector quadrant streak.

In a streak plate, dilution is achieved by first spreading the specimen over the agar surface of one sector. If a cotton swab or disposable loop or needle was used to inoculate the first sector, it is now discarded into an appropriate container, while reusable loops, usually with nichrome or platinum wire (24 gauge), are flamed to incinerate any organisms on the loop. When cooled, the sterile loop is streaked through the initial sector and organisms are carried into the second sector where they are spread using a zig-zag movement. In a similar manner, the organisms present on the loop are incinerated after the second sector is streaked, and the third sector is streaked. For a four quadrant plate, the process is carried an extra step.

Detailed procedure for a Three Sector Streak, the T Streak:
Reference J. Lammert, Techniques in Microbiology, A Student Handbook (6)

Materials:

- Specimen to be streaked; this protocol is written for a test tube culture
- Transfer loop (usually nichrome, a nickel-chromium alloy, or platinum; it may also be a single-use disposable plastic loop, which would be discarded between sectors rather than resterilized)
- Bunsen burner
- Sterile petri dish with appropriate bacterial media, such as trypticase soy agar
- Labeling pen
- Sterile cotton swabs (if necessary to remove condensation from the agar surface and from around the inner rim of the petri dish)

A. Label a petri dish.

Petri dishes are labeled on the bottom rather than on the lid. In order to preserve area to observe the plate after it has incubated, write close to the edge of the bottom of the plate. Labels usually include the organism name, type of agar, date, and the plater's name or initials. Using sterile cotton swabs, remove any visible water on the agar in the plate or around the inner rim of the petri plate. Observe the plate and mentally divide it into three sectors, a "T." The area above the "T" will become the first sector streaked. The plate will then be turned clockwise (if you are right handed) with the agar side up. The second sector will then be at the top for streaking and then the plate is turned again so that the third sector can be streaked.

B. Sterilize the transfer loop before obtaining a specimen.

In order to streak a specimen from a culture tube, metal transfer loops are first flamed so that the entire wire is red-hot. The incineration and flaming

process is described below in the Tips section. When flaming, the wire loop is held in the light blue area of a bunsen burner just above the tip of inner flame of the flame until it is red-hot. If a hot incinerator is available, the loop may be sterilized by holding it inside the incinerator for 5 to 7 seconds. Once sterile, the loop is allowed to cool by holding it still. Do not wave it around to cool it or blow on it. When manipulating bacteria, transfer loops are usually held like a pencil. If plastic disposable loops are being utilized, they are removed from the packaging to avoid contamination and after being used, are discarded into an appropriate container. A new loop is recommended for each sector of an isolation streak plate.

C. Open the culture and collect a sample of specimen using the sterile loop.

Isolation can be obtained from any of a variety of specimens. This protocol describes the use of a mixed broth culture, where the culture contains several different bacterial species or strains. The specimen streaked on a plate could come in a variety of forms, such as solid samples, liquid samples, and cotton or foam swabs. Material containing possibly infectious agents should be handled appropriately in the lab (see biosafety references below), only by students with appropriate levels of skill and expertise.

Remove the test tube cap. It is recommended that the cap be kept in your right hand (the hand holding the sterile loop). Curl the little finger of your right hand around the cap to hold it or hold it between the little finger and third finger from the back. See the illustration. Modern test tube caps extend over the top of the test tube, keeping the rim of the test tube sterile while the rim of the cap has not been exposed to the bacteria. The cap can also be placed on the disinfected table, if the test tube is held at an angle so that air contamination does not fall down into the tube and the test tube cap is set with the sterile rim on the table.

Insert the loop into the culture tube and remove a loopful of broth.

Replace the cap of the test tube and put it back into the test tube rack.

D. Streak the plate.

Inoculating the agar means that the lid will have to be opened. Minimize the amount of agar and the length of time the agar is exposed to the environment during the streak process.

1) Streak the first sector.

Raise the petri dish lid to insert the loop. Touch the loop to the agar area on the opposite side of the dish, the first sector. Bacteria on the loop will be transferred to the agar. Spread the bacteria in the first sector of the petri dish by moving the loop in a back and forth manner across the dish, a zig-zag motion. Make the loop movements close together and cover the entire

first region. The loop should glide over the surface of the agar; take care not to dig into the agar.

2) Between sectors.

Remove the loop from the petri dish and obtain a sterile loop before continuing to the second sector. Either incinerate the material on the loop or obtain a sterile loop if using plastic disposable loops. The loop must be cool before streaking can continue. Metal loops can be touched to an uninoculated area of agar to test whether they are adequately cooled. If the loop is cool, there will be no sizzling or hissing and the agar will not be melted to form a brand. If a brand is formed, avoid that area when continuing with the streaking process.

3) Streak the second sector.

Open the petri dish and insert the loop. Touch the cooled loop to the first sector once, invisibly drawing a few of the bacteria from the first sector into the second sector. The second sector is streaked less heavily than the first sector, again using a zig-zag motion.

4) Obtain a sterile loop for the third sector (see 2, above).

5) Streak the third sector.

Open the petri dish and insert the loop. Touch the cooled loop (if the loop has been flamed) once into the second sector and draw bacteria from the second sector into the third sector. Streak the third sector with a zig-zag motion. This last sector has the widest gap between the rows of streaking, placing the bacteria a little further apart than in the previous two sectors. Watch closely to avoid touching the first sector as the streak is completed.

6) Final step.

Flame the loop to incinerate any bacteria that are left on the loop. Allow the loop to cool before placing it near anything that is flammable. Invert the petri dish so that the agar side is up and incubate the plates.

SAFETY

The ASM advocates that students must successfully demonstrate ability to explain and practice safe laboratory techniques. For more information, visit the ASM Curriculum Recommendations: Introductory Course in Microbiology and read the section on laboratory safety.

Three additional articles provide important information:

Biosafety Levels-What We Need to Know About Them in Teaching Labs by

Christina Thompson (2004)

Update of Biosafety Level Designations by Erica Suchman (2004)

Safety Recommendations from the Concurrent Sessions on Safety in the Microbiology Teaching Laboratory at the Undergraduate Microbiology Education Conference 2003 by Jackie Laxon (2003)

COMMENTS AND TIPS

A. Alternate streak patterns and different culture media

A variety of alternate streak patterns exist. Some are used for specific inocula, such as a urine specimen. The patterns also differ in the number of sectors as well as in the number of times the loop is sterilized.

The four quadrant streak pattern would be recommended for use when large amounts of bacteria are expected in the inoculum. The extra sector will provide additional dilution and increase the probability of isolated colonies on the plate. The four quadrant streak plate is described in a variety of references, e.g., in Cappuccino and Sherman's Microbiology, A Laboratory Manual, 8th ed. (3).

Sometimes, cultures will be streaked on enrichment media or various selective and differential media. For instance, a culture which is expected to have a gram-negative pathogen will be streaked on a MacConkey agar plate, which inhibits the growth of gram-positive organisms.

B. Incinerating material on transfer loops—flaming

Reusable microbiological loops and needles are sterilized by flaming. A Bunsen burner is traditionally used for this process. Most microbiology manuals show the microbiologist positioned with his/her hand above the burner, with the loop placed into the flame. To avoid possible contact with the flame, the microbiologist might consider placing his/her hand below the flame with the loop/needle above the hand in the flame. The flame of the Bunsen burner should be adjusted to blue, with the darker blue cone of cooler air visible in the center of the flame. The loop or needle should be placed into the hotter part of the flame and kept there until it glows red. There is a possible aerosolization hazard if the loop or needle contains liquid or a bacterial clump. These loops and needles should be placed into the heat slowly so that the moisture evaporates rather than sputters.

If an incinerator such as a Bacti-Cinerator is used to sterilize the loop, the loop is to remain inside the incinerator for 5 to 7 seconds. When warmed up (which will take 5 minutes), the temperature inside the incinerator is 815°C.

The incinerator will take 5 to 10 minutes to warm up to working temperature.

C. Several techniques decontaminate transfer loops between sectors of a streak plate: flame, dig into agar, flame once and rotate loop

A variety of methods exist for removing organisms from the loop between sectors. Beginning students are generally taught to sterilize the loop between each sector by incinerating and then cooling the loop. Clinical microbiologists practice a variety of methods. Some flame once after the initial sector and then rotate the loop so that the next sectors can be streaked with an unused side of the loop. Other laboratorians (as clinical microbiologists name themselves) stab the loop several times into the agar to clear the loop between sectors.

D. Isolated colony appearances

Isolated colonies can be described using the traditional colony descriptions. The Colony Morphology Atlas-Protocol project provides information about bacterial colony appearance and characteristic photographs. The appearance of an organism can vary. For instance, a colony of an organism growing in a crowded sector of the plate will not grow as large as the identical organism growing in isolation. The media composition, pH, and moistness, as well as the length of time and temperature can all affect the organism's appearance. Colonies selected for subculturing should be colonies which are isolated, i.e., there is no other colony visibly touching the colony.

Agar with a surface layer of water is not suitable for obtaining isolated colonies. Obvious water drops should be removed from the surface of the plate and from the rim of the plate by using sterile cotton swabs. Plates should be incubated agar side up, to avoid condensation that would drop onto the growing colonies on the agar surface.

E. Flaming tube mouths

Many protocols suggest flaming the tube mouth before and after removing organisms from a tube. Flaming was important when test tubes were capped with a cotton plug. Flaming would still be appropriate if a foam plug were being used. If a screw cap, KimKap, or similar test tube cap is used, the open end of the tube remains sterile since the cap normally covers that area.

F. Rehearsing the streak procedure

Some instructors have students practice the streaking procedure on a piece of paper. The process helps the student visualize the completed product and practice the fine muscle movements that are required in successful streaking for isolation.

Students may also find that they can visualize the pattern better if they mark the back of the petri dish (for instance, a T streak divide the plate into three sectors).

Before learning to streak, students should have had the opportunity to work with 1.5% agar media. Ideally they will have also previously had the opportunity to practice using a loop on a plate to determine the best angle of approach and the amount of force required to glide the loop over the surface of the agar without gouging the surface.

G. Holding the plate while streaking

If possible, adequate lighting should be available to help the microbiologist follow the tracings of the loop on the agar. For most labs, this means that the petri dish should be held in one's hand while being streaked in order to reflect the light properly. Additionally, the length of time the petri dish lid is removed should be minimized in order to limit contamination. There are several ways to hold the petri dish. Beginning students may find that they obtain the best results leaving the plate on the lab bench and lifting the lid to work. Other students may find that they can place the plate upside down on the workbench and lift the agar containing bottom, hold it to streak and then quickly replace it into the lid. Yet other students may have the manual dexterity to manipulate the entire dish in their hand, raising the lid with thumb and two fingers while balancing the plate in the rest of their hand.

REFERENCES

1. BBL. 1973. BBL manual of products and laboratory procedures. Becton Dickson Microbiology Systems, Cockeysville, MD.

2. Buchanan, E. D., and R. E. Buchanan. 1938. Bacteriology for students in general and household science, 4th ed. Macmillan Company, New York, NY.

3. Cappuccino, J. G., and N. Sherman. 2008. Microbiology a laboratory manual, 8th ed. Pearson/Benjamin Cummings, San Francisco, CA.

4. Finegold, S. M., and E. E. Sweeney. 1960. New selective and differential medium for coagulase-positive staphylococci allowing rapid growth and strain differentiation. J. Bacteriol. 81(4):636–641.

6. Lammert, J. M. 2007. Techniques in microbiology. A student handbook. Pearson/Prentice Hall, Upper Saddle River, NJ.

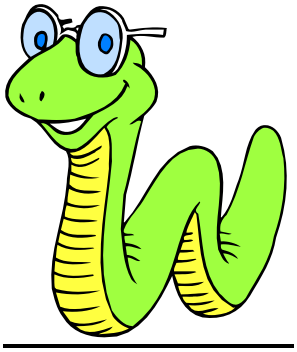
7. Levine, M. 1939. An introduction to laboratory technique in bacteriology, revised ed. The Macmillan Company, New York, NY.

8. Pelczar, M. J., Jr., and R. D. Reid. 1958. Laboratory exercises in microbiology, p. 45–47. McGraw-Hill Book Company, Inc., New York, NY.

9. Salle, A. J. 1954. Laboratory manual on fundamental principles of bacteriology, 4th ed., p. 39. McGraw-Hill Book Company, Inc., New York, NY.

10. Sherwood, N. P., F. H. Billings, and B. J. Clawson. 1992. Laboratory exercises in bacteriology and diagnostic methods, 7th ed. The World Co., Lawrence, KS.
11. Williams, H. U. 1908. A manual of bacteriology, p. 100. Revised by B. M. Bolton. P. Blakiston's Son & Co., PA.
12. Williams, C. L., and H. P. Letton. 1916. A note on the preparation of agar agar culture media. J. Bacteriol. 1:547-548. http://jb.asm.org/cgi/reprint/1/5/547?maxtoshow=&HITS=10&hits=10&RESULTFORMAT=
&author1=williams&author2=letton&titleabstract=agar-agar&searchid=1&FIRSTINDEX=0&tdate=3/31/1931&resourcetype=HWCIT.

Exercise No. 3: Endoparasites: Helminthes



Materials

1. **Helminthology PowerPoint presentation on lab computers' hard drive.**
2. **The Helminthology PowerPoint presentation is also found online in WebCt under the "Image Database".**
3. **Lab Manual (always bring your lab manual to lab)**
4. **Microbiology textbook by Talaro. Read the following pages:**

Objectives

1. **Have the student learn and understand basic Helminthology/Parasitology terms.**

2. **Learn the basic facts, as presented in this exercise, for the following helminths:**

    a. **Ascaris lumbricoides**
    b. **Enterobius vermicularis**
    c. **Genus Ancyclostoma**
    d. **Genus Trichuris**
    e. **Trichinella spiralis**

3. **Be able to answer general questions about each picture on the Helminthology PowerPoint slides .**

INTRODUCTION

Your travels throughout life will more than likely expose you to persons who have one or more helminth infestations. A general knowledge of some of the frequently encountered worms (helminths) is therefore necessary, not only for your personal and family's health, but also for having the ability to intelligently discuss with your patients (or family members) the important details concerning the particular endoparasite they have been unfortunate enough to contract. People and patients ask questions. Hopefully, you will be able to answer most of their basic questions (as well as your own) if you study and learn the material presented on the following pages. The helminths we will study are **endoparasites**---they live inside the body. The lab's "Helminthology" PowerPoint is also on WebCT under the "Image Database".

I. Terms You Need To Learn

A. Parasitism
A relationship (with two organisms living together) wherein one is harmed while the other benefits (benefits by extracting food from its living host). Examples of parasites: certain bacteria, fungi, protozoans, parasitic worms, etc. Viruses are considered nonliving parasites but do not obtain food – they only replicate in living cells and have no characteristics of life.

B. Parasitology
A branch of biology dealing with parasites.

C. Helminthology
The study of parasitic worms.

D. Oviparous Parasite
A Parasite that lays eggs.

E. Viviparous Parasite
A Parasite that gives birth to living young.

I. Terms You Need To Learn Continued:

F. Larva (plural: larvae)
A microscopic, immature (baby) worm that hatched from an egg. Once in the body and depending on who the parasite is, larvae can be found in sputum, feces or tissue samples.

G. Rhabditiform Larvae
Larvae that must live part of their life cycle in the soil. Ancyclostoma has rhabditiform larvae.

H. Adult Helminth
The Helminth stage that can reproduce.

I. Antihelminthic Agent
A drug that kills the adult stage. Most available drugs (worm medicine).cannot kill immature, migrating larvae in the body The drugs can only kill the adult stage.

J. Ectoparasites
Parasites who live on superficial surfaces of the body. Examples are fleas, ticks, and lice. They are facultative parasites – they can leave their host without harm to themselves.

K. Endoparasites
Parasites who must live within the body. Examples are tapeworms, flukes, roundworms, hookworms, and heartworms. They are obligate parasites – they are unable to leave their host without harm to themselves.

L. Host
A parasite lives at the expense of another organism which is called its host.

M. Definitive Host
The host which eventually harbors the adult parasite.

N. Intermediate Host
The host which harbors the immature larval stage or stages only. Some parasites have only one intermediate host (such as a snail) whereas others can have two intermediate hosts (such as a snail and then a fish). A few have the same organism as both its intermediate host and definitive host (an example of this being

Enterobius vermicularis nicknamed "roundworms"). We humans are both hosts for the helminths in this exercise.

I. Terms You Need To Learn Continued:

O. Aberrant Host
A host that is accidental and not normally in the natural parasite cycle. Larvae do not continue normal cycle development in this type of host. Example: "Creeping Eruption" due to hookworm larvae.

P. Monecious
Both male and female reproductive organs are contained within one body.

Q. Dioecious
Separate sexes (male and female parasites who eventually mate). In this exercise, all of the helminthes are dioecious.

R. Cycle
The stages in the development of a parasite. Includes the required environment and hosts.

S. Pathology
The study of the macroscopic (autopsy) and microscopic (biopsy) damage done to the body by an invading parasite or disease process (ex. Disease process such as cancer).

II. General Information

A. Two helminths account for about 1 billion infestations yearly throughout the world: the genera **Ascaris** (nicknamed "**roundworms**") and **Enterobius** (nicknamed "**pinworms**"). It is possible to be infested with both at the same time (as well as other helminths besides these).

B. Some pathology of the helminths we will study: Depending on the helminth or parasitic infestation:

1. Adults cause damage to the intestinal mucosa (lining) thereby disrupting digestion and absorption.
2. All produce toxic waste products and other substances that damage the body (since you absorb them).
3. Migrating larvae produce bodily tissue damage throughout their migration phase. Both adults and migrating larvae cause damage.
4. If there are enough adults or migrating larvae, (the worm burden) they could and do cause death.

5. Some can obstruct or perforate intestines.
6. One helminth we'll study causes anemia.

II. General Information Continued:

C. **Diagnosis (Dx)** – How does one diagnose (Dx) that a patient has a helminth infestation? **What do we look for?** Generally, depending on the particular parasitic worm infecting a patient, the diagnosis depends on **looking for** and identifying:

1. **Ova (eggs)** – depending on the genus and species involved, one would begin by performing a lab procedure on the feces of a patient. Most helminthes that we will study live as adults in the intestinal tract. Thus, eggs from females will be deposited in one's feces. Feces is the most common substance examined for ova in a lab.

2. **Larvae (singular: larva)**– immature forms that have hatched from ova. They are most often found in feces since many adult helminths target the digestive tract as their final home.

3. **Adults** – the full-grown parasite. One doesn't find these often.

D. Classification: Most important helminth pathogens are found in the:

1. Class Trematoda (flukes)

2. Class Cestoda (tapeworms)

3. Phylum Nematoda (roundworms)—we will study some of these.

III. General Features of Nematodes:

A. According to production:

1. Oviparous – egg-bearing and hatch larvae.

2. Viviparous – bear living young (filarial or microfilaria); these roundworms are called the filarial worms. We will not study them.

B. According to pathology:

1. Intestinal – pathology occurs in the gut… destruction of lining affects digestion/

absorption.

2. Tissue – pathology occurs in the tissue

III. General Features of Nematodes Continued:

C. Groupings

1. Plant nematodes – at least 1,000 nematode species parasitize plants; estimated that they consume approximately 10% of all plant crops annually. None infest humans. They are economically important.

2. Human parasitic nematodes – nearly 50 species parasitize humans. Depending on the genus / species, they enter by food, contaminated water, simply swallowing contaminated soil or other object, or by skin penetration. Insects are vectors for some (the filarial worms). Poor hygiene often plays a tremendous role in obtaining nematode infestations (oral ovas ingestion). Children are notorious for lack of good hygiene.

   Remember: Feces, fingers, flies, fomites and filth.

3. There are several reasons why we can't digest nematodes that exist in our digestive tracts. The first is that they have a tough outer layer called a cuticle which our digestive enzymes cannot penetrate. Also, many helminthes secrete enzymes that inactivate our digestive enzymes.

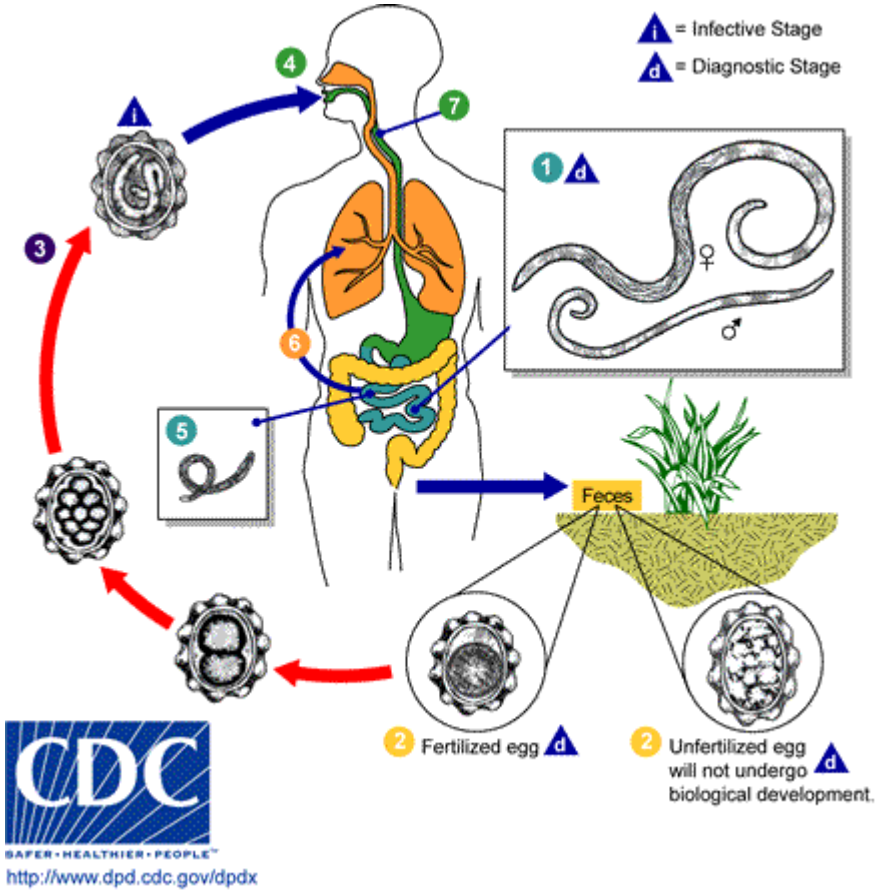
EXERCISE #2 CONTINUED

IV. *THE PHYLUM NEMATODA*

All members of this phylum are generally referred to as the ”roundworms”. The members we will discuss are oviparous worms: “roundworms” (genus Ascaris), “pinworms” (genus Enterobius), “hookworms” (genus Ancyclostoma) and “whipworms” (genus Trichuria) In a different nematode grouping is the genus Trichinella (nickname “pork roundworm”) which is unusually considered to be both oviparous and viviparous. The quotation marks in this paragraph indicate nicknames or common names for these worms. Here are facts about the nematodes you need to know:

Helminth #1: <u>Ascaris</u> <u>lumbricoides (nickname: “roundworms”:</u>

Cycle:



Ascaris lumbricoides Facts:

1. a roundworm (nematode) nicknamed (or common name) "roundworms".

2. dioecious (separate sexes).

3. adult female – approximately 6 inches; male smaller.

4. definitive host also the intermediate host – **autoinfection** (autoinfestation) possible with your own eggs (the result of poor hygiene). We humans are considered both the intermediate and definitive host.

5. Ascaris infections often occur when children or adults eat dirt contaminated with ascarid eggs or by consuming food or drink contaminated with eggs (ova). Dogs and cats can transmit their roundworms to humans. (Get these animals checked for worms and dewormed if needed by 2 – 6 weeks of age---don't wait!!).

6. Roundworm eggs can remain viable for months in the soil. They do not hatch in the soil.

7. Once the eggs are swallowed, they hatch in the intestinal tract. The larvae then penetrate the intestinal wall and over the next few weeks, migrate through the abdomen to the lungs. In the lungs the larvae molt (mature a little) and are eventually coughed up and swallowed. These larvae are microscopic but cause damage during their migration. I have seen death due to pneumonia when the worm burden was very, very large. Once these larvae have arrived again in the intestinal tract, they will mature into large adults who will mate. Females pass thousands of eggs in the person's or animal's feces each day. If you get these eggs on your hands or fomites (candy bars, cold drinks etc..) or swallow dirt (soil) containing the eggs, then infestation can occur. If a person has roundworms and does not wash their hands after using the bathroom, it is very possible for that person to have an **autoinfestation** with that worm. Poor hygiene, whether it be dirt or feces, is a common causative factor for obtaining this parasite.

Ascaris lumbricoides Facts Continued:

8. Remember, the migrating roundworm larvae cause tissue damage as well as intestinal adults. If there are enough larvae and damage, death can result. We have no drugs to kill larval stages.

9. Adults damage the intestinal wall thus disrupting digestion and absorption. They can perforate the intestinal wall yielding peritonitis. Sufficient numbers of adults leads to malnutrition, diarrhea, fluid electrolyte loss and death. They can partially obstruct or totally obstruct the intestines which in either case is an emergency situation.

Other facts about Ascaris Lumbricoides

Distribution: Although the highest frequency of ascariasis is found in the tropical areas, it is also common in many temperate regions of the world.

Pathology: The maturing and adult worms live in the small intestine deriving their nourishment from semi-digested food. The detrimental effect on the host's nutrition is approximately proportional to the number of worms. The survival time of mature A. lumbricoides in the human intestine is relatively short, generally not exceeding a year.

Epidemiology: Ascariasis is a disease due to fecal contamination of the soil. In most hyper-endemic areas infected small children in and around the home provide the major source for the infection by their promiscuous defecation. Infective-stage eggs remain viable for weeks or months; only desiccation, freezing, heat, and direct sunlight are detrimental to them.

Other facts about <u>Ascaris Lumbricoides</u> Continued:

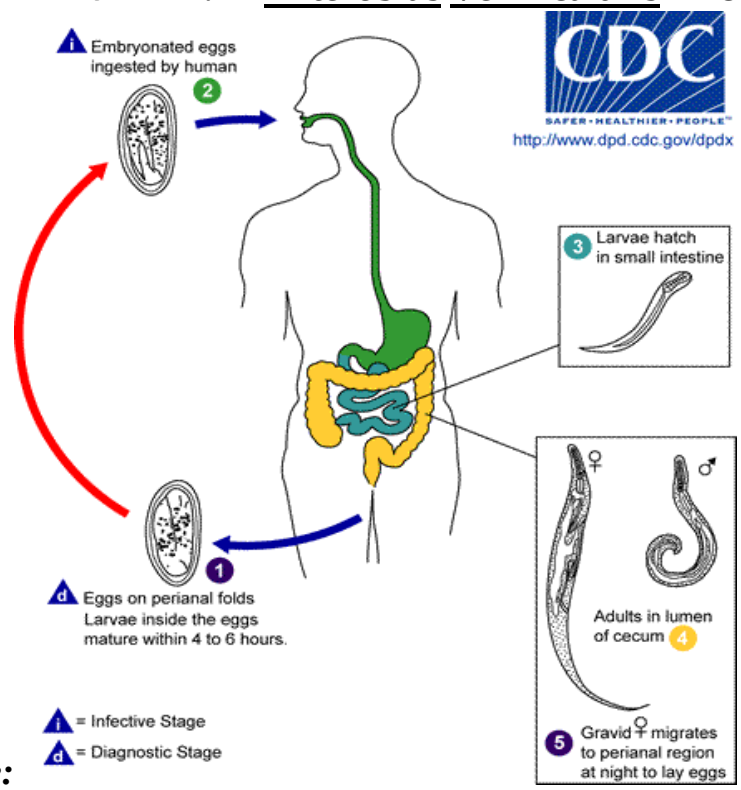
Life Cycle: The adult Ascaris is the largest roundworm parasitizing the human intestinal tract. When ingested, the fertile eggs hatch in the duodenum and the emerging robust larvae penetrate into the nearby intestinal wall, enter the mesenteric venules and via the liver eventually make their way into the alveoli of the lungs. The period from exposure to maturity requires 8 to 12 weeks. They are eventually coughed up, swallowed, and mature in the intestinal tract.

Therapeusis: There is no conclusive evidence that any antihelminthic presently employed in intestinal ascariasis is lethal to the larval worms migrating through the lungs. Obstructive jaundice in a child with intestinal ascariasis indicates the need for surgical intervention to remove the worm from the common bile duct. Prognosis is usually excellent following appropriate treatment, but at times it is grave when there is massive larval invasion of the lungs, in case of hypersensitization, or when surgical complications develop. Some larvae can wander and cause blindness in an eye. Adults have been known to come out of the nose of the individual.

Prophylaxis: The problem of control is concerned directly with home and community sanitation.

Helmint #2: <u>Enterobius Vermicularis</u>– nickname: pinworms

Cycle:



a. Facts:

1. as adults are approximately ¼ inch long

2. dioecious; spread via fomites or autoinfestation. Dust particles can spread eggs/larvae.

3. mate in lower colon causing colitis.

4. can cause appendicitis. The appendix is part of the immune system.

5. can enter female vaginal tract and migrate up to fallopian tubes causing PID.

6. nocturnal – female migrates at night to lay eggs outside anal opening. Eggs mature in a few hours.

7. major symptoms: vaginal and/or anal pruritis

Detailed facts for <u>Enterobius</u> <u>Vermicularis</u> – The pinworm, seatworm, or threadworm causing enterobiasis or oxyuriasis.

Distribution: More common in persons living in cool or temperate zones than in strictly tropical areas. Although it is almost exclusively a parasite of man, it has been found, on a few occasions, in the chimpanzee**. It is not found in dogs or cats.**

Pathology: First recognizable symptom is pruritus as the worms emerge from the rectum and crawl over the perianal and perineal skin. As worms in various stages of development frequently are seen in the appendix, and occasionally are found deep in the inflamed mucosa of the colon, pinworms often are suspected of causing appendicitis. At times worms enter the female genital tract and become encapsulated within the uterus or fallopian tubes dragging bacteria with them and causing PID.

Epidemiology: Pinworm infestation is more prevalent in large family groups, in schools and mental institutions than it is in the population at large. Cool, moist atmosphere is optimal for survival of the eggs; dry heat and good ventilation produce rapid death of the enclosed larvae.

Life Cycle: The eggs discharged on the skin are essentially mature and within a few hours contain a fully developed infective-stage larva. They are swallowed either on fomites (even dust particles in the air) or by autoinfestation (anus-to-mouth). Chlorine in swimming pools does not kill them. In all cases one becomes both a definitive and intermediate host. On reaching maturity in the cecal area of the intestine, the worms mate and complete the life cycle in 15 to 28 days.

Therapeusis: Two groups of drugs are useful in infections of this nature – the piperazine compounds and cyanine dyes.

Detailed facts for <u>Enterobius</u> <u>Vermicularis</u> Continued:

Prophylaxis: The infection can be controlled by personal and group hygiene and mass chemotherapy. Infection in a family group can be reduced by developing habits of personal hygiene in the children; by providing small children with closed sleeping garments and by keeping the fingernails short. Eggs of pinworms are not killed by chlorination of water in swimming and wading pools. Dust particles can spread.

Dx: Scotch tape analysis. Your instructor will explain this.

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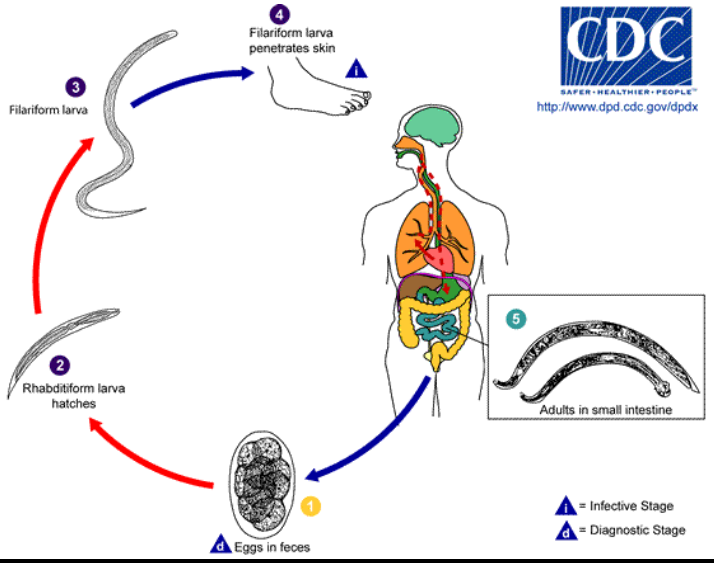
Helminth #3: The genera Ancyclostoma and Necator: Nicknamed "Hookworms":



A. <u>A few examples of hookworm genera and species:</u>

1. <u>Ancyclostoma duodenale</u> – "old world hookworm" in man

2. <u>Ancyclostoma caninum</u> – a dog hookworm

3. <u>Ancyclostoma braziliense</u> – a dog hookworm

4. <u>Necator Americana</u> – "New world hookworm" in man

The genera Ancyclostoma and Necator: Nicknamed "Hookworms" Continued:

Discussion

1. Especially prevalent in the South.

2. Symptoms: There are two possibilities that can occur when hookworm larvae penetrate the skin. What will occur depends on the genus/species we are referring to:

    a. Skin penetration and eventual migration through the body until the larvae reach the intestinal tract where they mature into adults who mate. Skin penetration sites can cause eruptions and itching; however, the larvae will begin a migration to the GI tract.

    b. Skin penetration which will not usually migrate beyond the skin and subcutaneous tissues. This is due to the fact that we are not a true host for this particular hookworm (aberrant host). This is usually referred to as the **creeping eruption** (or cutaneous larval migrans). This occurs because we are not the normal host of these particular genera and species. The immune system and the inflammatory response usually keep these larvae from migrating further into the body. Thiobendazole and ethylene chloride are treatments for this.

Pathology

1. If we are a true definitive host, the penetrating larvae migrate from the skin and eventually reach the intestinal tract where they mature into male and female adults. In the intestinal tract adults feed on blood and other tissues. They attach to the wall of the gut with sharp ridges or teeth. When they are finished with that site of attachment, they move over to a new site and reattach (called grazing). The old site continues

to hemorrhage since the hookworms secrete an anticoagulant. One adult hookworm can cause a

The genera Ancyclostoma and Necator: Nicknamed "Hookworms" Continued:

Pathology Continued:

blood loss of up to **.4cc of blood per day**. They can live for several years. Disruption of nutrition can also occur – deficiencies of iron and protein results in weight loss. Anemia often occurs with these parasites and is the number one cause of death and pathology. In humans and animals death from anemia and malnutrition can occur. These worms have teeth.

2. Digested blood in the stool is called melena. Black, tarry stools often indicate digested blood. Frank blood (red in color) in the feces can also occur. Weight loss, anemia, low albumin, diarrhea, and abdominal pains are a few of the symptoms that can be seen.

Life Cycle

1. Eggs from the adult female pass in the feces and contaminate the soil.

2. Eggs hatch in moist soil releasing free-living larvae that feed on bacteria and organic debris (rhabditiform larvae).

3. The rhabditiform larvae grow, molt (mature some), and become Infective larvae that will penetrate the skin and cause ground itch Or creeping eruption. Sometimes the larvae bring bacteria into the skin with them creating an infection as well. When it does, it is referred to as "ground itch".

4. In aberrant hosts creeping eruption occurs when the infective larvae in the soil penetrate the skin.

5. In a normal definitive host, after skin penetration, blood vessels carry the larvae to the heart and lungs. They penetrate the lung tissue and eventually some are coughed up and swallowed. Upon reaching the intestinal tract, they will mature into adults who mate.

6. Migrating larvae do cause damage to the host. Of course,

the adults cause damage as previously described. Enough of either can cause death.

Other facts for Hookworms:

Ancyclostoma duodenale – The "Old World Hookworm"

Distribution:	Europe, Asia, Africa, North and South America.
Pathology:	Man is probably the only normal host of A. duodenale. Papules and vesicles accompanied by intense itching and burning, edema, and erythema, and known as "ground itch" or "dew itch", mark the site of entry of the larvae.
Epidemiology:	Local hemorrhages, a pneumonitis, or bronchitis may be produced by larvae reaching the lungs. In chronic infections cardiac symptoms may be evident, and infected children may show retardation.
Life Cycle:	Embryonation to the first larval stage takes place in 24 to 48 hours on moist, sandy loam in about 25 degrees C. Free-living larval stages on the soil are similar to those of Necator americanus.
Therapeusis:	There is no consistently successful treatment for hookworm infection.

Ancyclostoma duodenale – The "Old World Hookworm" continued:

Prophylaxis:	Two major lines of attack are indicated, namely (1) antihelmenthic treatment of all infected individuals to reduce to a minimum the sources of soil infestation, and (2) selective treatment of individuals showing evidence of hookworm disease. Equally important is the sanitary disposal or sterilization of human feces to prevent infestation of the soil (and of animals also).

Other facts for Hookworms Continued:

Ancyclostoma caninum – The Common Hookworm of Dogs and Cats

Distribution: Common in temperate climates.

Pathology: It has been reported on four occasions as an incidental intestinal parasite of man. Occasionally, creeping eruption of the human skin is caused by A. caninum.

Epidemiology: In light infections, the blood loss can be completely compensated and in moderately heavy infections compensated by an adequate, well-balanced diet containing iron, other minerals, quality animal proteins and vitamin A. In severe hookworm disease even with a highly fortified diet, the hematopoietic mechanism is unable to produce new supplies of normal red blood cells as rapidly as they are lost. Moreover, underlying protein deficiency in the diet, even with adequately absorbed iron intake, may contribute measurably to the anemia of hookworm patients, a majority of whom subsist essentially on carbohydrates.

In hookworm areas exposure begins fairly early in childhood and is repeated again and again throughout life.

Life Cycle: The life cycle is similar to that of A. duodenale but prenatal infection is common in the dog.

Therapeusis: There is no consistently successful treatment for hookworm infection. To date, best results have been obtained with tetrachlorethylene. In animals, pryantel pamoate and others are efficient.

Ancyclostoma duodenale – The "Old World Hookworm" continued:

Prophylaxis: Sanitary improvements, health education, nutrition and improved agricultural practices.

Other facts for Hookworms Continued:

Ancyclostoma braziliense – Dog Hookworm

Distribution:	This species was first described from the intestines of cats and dogs in Brazil and it is now found in many warm areas of the world.
Pathology:	Clinical interest in A. braziliense is concerned primarily with human skin exposure to larvae of this species derived from feline and canine hosts, causing a dermatitis referred to as "**creeping eruption**".
Epidemiology:	Human intestinal infection with A. braziliense does not occur or is relatively uncommon.
Life Cycle:	Similar to A. duodenale.
Therapeusis:	Same drugs as others for hookworms.
Prophylaxis:	Sanitary improvements, health education, and removal of infected animals.

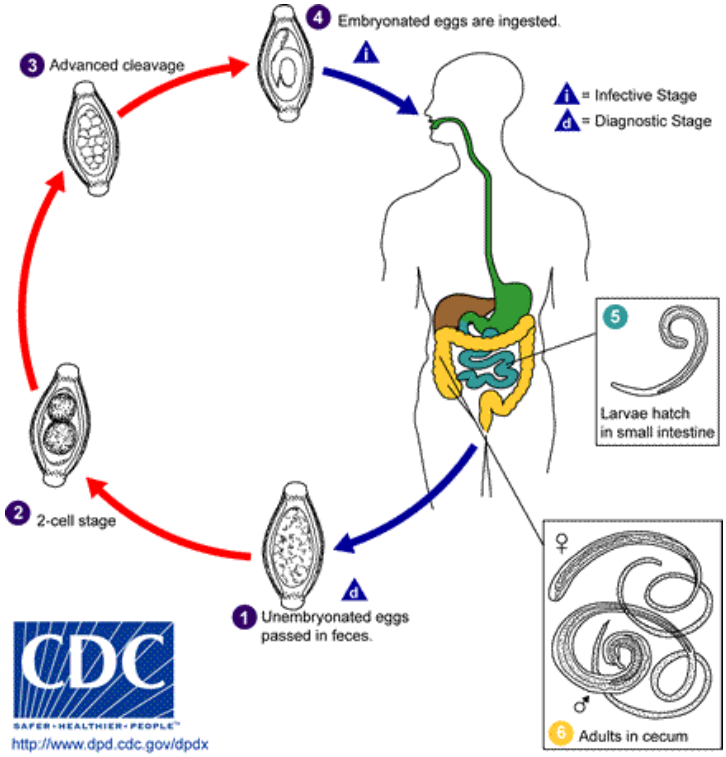
Necator americanus – "New World" hookworm, causing human hookworm infection of warm climates.

Distribution:	Southern United States, Mexico, Central America, the West Indies and South America east of the Andes. This hookworm was introduced into the Western hemisphere with the importation of African slaves.
Pathology:	Intense itching and burning, edema and erythema mark the site of entry into the skin of the filariform larvae.

Other facts for Hookworms Continued:

Epidemiology:	Identification of eggs in fecals is definitive, but larvae from older stool samples do hatch from the eggs and must then be distinguished from larvae of Stroniloides and Trichostrongylus. N. americanus is attached to the upper levels of the small intestine onto the mucosa from mid duodenum through the jejunum. Heavy infections may extend far down into the ileum and occasionally into the cecum.
Life Cycle:	When the host's feces are deposited on moist sandy loam, in a warm, shaded location, hatching takes place in 24 to 48 hours. Optimal conditions include good aeration of the top soil, which must be moist but not saturated with water, and be protected from the direct sun and at 31 to 34.5 degrees C. On contact with exposed human skin, the hatched larvae penetrate under epidermal scales or into hair follicles. The most common area for invasion is the tender skin between the toes. From here they migrate through the tissues eventually reaching the lungs. From here they are eventually swallowed and mature in the intestinal tract.
Therapeusis:	Iron must be administered to replace that lost through intestinal hemorrhage caused by the grazing worms. Occasionally, in humans, whole blood transfusion may be needed. Liver is not indicated for the patient with hookworm disease.
Prophylaxis:	Similar to A. duodenale.

Helminth #4: The Genus Trichuris – Nickname "Whipworms"



Life Cycle: Similar to hookworms.

Pathology: Similar to hookworms: severe anemia, mucosal damage with diarrhea and hemorrhage.
Severe infection can lead to rectal prolapse (since adults inhabit the colon) and intussusception of the intestines. These are due to severe smooth muscle spasms in the intestinal wall caused by these worms.

Examples: Trichuris vulpis - dogs
Trichuris trichiura - humans

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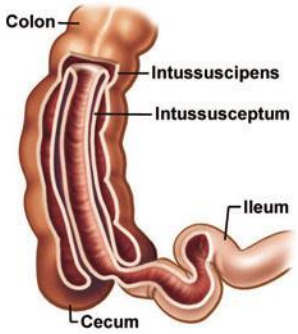
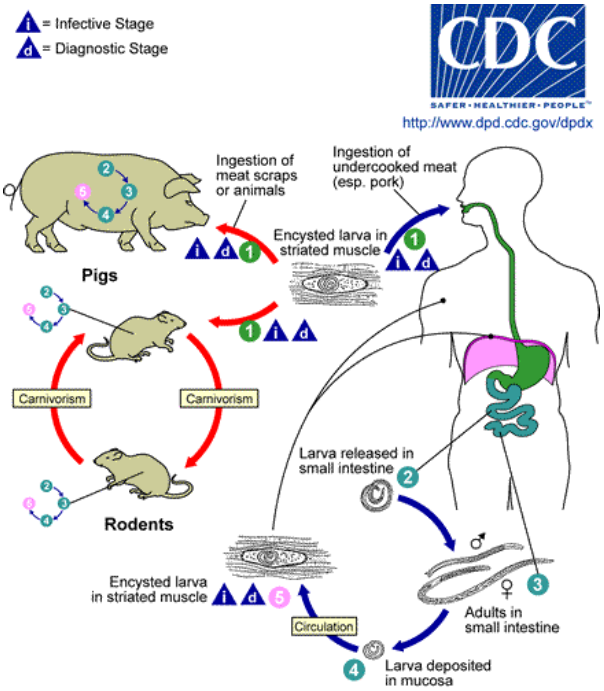


Helminth #5: A Nematode in a class all by itself

<u>Trichinella</u> <u>spiralis</u> – The "pork roundworm" also called Trichinella.



Discussion:

1. One ingests encysted larvae by eating undercooked pork.

2. Most Americans have a few worms in their body if they have eaten pork meat. Incidence on the rise in the U.S.

3. The usual infection is by ingestion of encysted larvae in undercooked pork meat; venison, other game meat (bear). Horse meat has also rendered infections.

4. Once in our intestines, cysts release larvae that mature into male and female adults in about 2 days. The males

die after mating. Before the females also die in the intestinal tract, they produce eggs which hatch inside of them (oviparous) and then living larvae are released (viviparous) who then penetrate the intestinal wall. The larvae migrate through blood and lymph vessels to the liver, heart, lungs, and skeletal muscles as well as other tissues. Upon reaching the skeletal muscles, especially tongue, eye, and chewing muscles, they form a cyst. Cysts last for years (10-12). We do not term the baby worms "filarial" since no vector is involved.

Trichinella Discussion Continued:

5. The cycle is the same in pigs.

    a. Pig eats undercooked garbage containing infested pork (slop).

    b. Cycle same as in humans – larvae penetrate intestinal wall and migrate to target tissues where they encyst.

6. This parasite causes damage as adults. Females penetrate the intestinal wall to some degree releasing toxins which mimic food poisoning. Wandering larvae damage blood vessels and tissues. Death can result from kidney or heart failure, respiratory disorders, and reactions to toxins. Encysted larvae cause muscle pain. Most damage occurs if larvae burrow into brain or heart muscle.

7. Just because one has Trichinosis doesn't mean they will die. It depends on the worm burden. COOK MEAT THOROUGHLY.
8. The cycle suggests that Trichinella is both oviparous and viviparous, unusual to say the least. (Note: Trichinella is a roundworm or nematode that is not a filarial worm.)

Facts for Trichinella spiralis – Trichina worm, causing trichinosis.

Distribution: Germany, Poland, Spain, Hungary, and the lower Danube countries. It has come to be recognized as a widely disseminated, clinically important disease in the United States and parts of Latin America, with epidemic outbreaks in small or moderate-size groups of the population.

Pathology: Only larvae reaching the striated muscle survive;

they cause a myositis and eventually calcification of the cysts. The diaphragm, tongue, pectoral and intercostals muscles are especially involved. Sudden onset of edema of the upper eyelids is one of the earliest signs, appearing about the 11th day of infection. If myocardial failure develops, it occurs between the 4th and 8th week. By about the 3rd month, most symptoms disappear; vague muscular pains and fatigue may persist for months.

Trichinella spiralis – The "pork roundworm" Trichinella facts continued:

Epidemiology: Infection with the roundworm T. spiralis results from ingesting raw or inadequately cooked or processed pork or pork products containing encysted larvae.

Life Cycle: See previous discussion.

Thereapeusis: There is no specific therapy for trichinosis. Hence, palliative and supportive treatment must be relied upon to carry the patient through the critical phase. In patients critically ill due to allergic reactions to, or intoxication with substances derived from the worms, administration of ACTH may ameliorate the crisis.

Prognosis: In heavy infections this is poor to grave; in lighter infections it is fair to good. In severe epidemics 0.5 to 30% of the patients succumb, with an average of about 3%.

Prophylaxis: It has been demonstrated that refrigeration at minus 18 degrees C. for 24 hours provides essentially safe pork. However, one can't buy a freezer going that low nor do grocery companies have freezers going that low. Thorough cooking of pork at 55 degrees C. also will kill the larvae. There is a possibility that irradiation may eventually be used to kill Trichinella cysts in carcasses.

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When Finished with Lab:

Properly shut down your computer. Make sure the CPU is OFF and the monitor is OFF.

Lab Exercise Questions:

You are to correctly answer the exercise questions
That begin on the next page, You are responsible for
having completed then before the next lab exercise.

MICROBIOLOGY LAB QUIZ FOR EXERCISE #2

1. In the Lab what do laboratory technologists **look for** in order to diagnose a helminth infestation in a patient?

   a.
   b.
   c.

2. In a Lab what do lab technologists **look in** in order to diagnose a helminth infestation in a patient?

   a.
   b.
   c.

3. Helminths that produce eggs are termed _____.

4. Helminths that give birth to living young are termed _____.

5. Antihelminthic drugs usually kill which form or stage of the parasite?

6. What is a host?

7. What is a definitive host?

8. What is an intermediate host?

9. Why can't we digest parasitic helminths?
   a.
   b.

10. What is an aberrant host?

11. What are larvae?

12. What two genera of helminths account for the most infestations throughout the world? What are their nicknames or common names?
    a.
    b.

13. Define pathology.

Lab Exercise Questions Continued:

14. List four (4) examples of pathology (that we discussed) which parasitic helminths could cause to the body.

    a.

    b.

    c.

    d.

15. How and from where does one obtain an Ascaris infection?

16. How long can Ascaris eggs remain viable in the soil?

17. What happens after a person swallows an Ascaris egg?
    (Be specific, descriptive and complete).

18. How do maturing Ascaris larvae in the lungs get to the intestinal tract where they will mature into mating adults?

19. What drugs can be used against migrating larvae such as Ascaris larvae?

20. List the common name for <u>Enterobius vermicularis</u>.

21. List the ONLY two definitive hosts for Enterobius.

    a.
    b.

22. List the ONLY two intermediate hosts for Enterobius.

    a.
    b.

23. Where do the adult Enterobius males and females mate?

24. List two different areas inside the female body where one could find Enterobius. They must be from different systems. (By the way, they don't live in the stomach, esophagus, or small intestine and anal opening isn't an answer.)

    a.
    b.

Lab Exercise Questions Continued:

25. List two different anatomical areas inside a male where Enterobius could be found. One belongs to the immune system, the other to the digestive system.
    a.
    b.

26. What do the female pinworms do when they come out of the anal opening usually at night? (Note: The anal opening is, for our purposes, to be considered outside the body and not inside).

27. List two possible, different, system signs (symptoms) of a pinworm infestation in a female.

    a.
    b.

28. What are the usual signs (symptoms) in a male?

29. List the two MAJOR (general) ways or means one obtains a pinworm infestation.

    a.
    b.

30. Can dust particles in the air carry pinworm eggs?

31. Can swimming and wading pools be infested with pinworm eggs?

32. What is the scotch tape analysis and what are we looking for?

33. Discuss the procedure for a scotch tape analysis.

34. What is the MAJOR method by which hookworms enter the body?

35. In hookworm infestations, where we are a true definitive host, what is the major consequence or pathology to us?

36. In hookworm infestations, where we are not a true definitive host, what is the disease it causes in us?

37. A hookworm larvae, migrating under the skin, and for which we are not a true definitive host, cause the condition known as the _____ _____.

Lab Exercise Questions Continued:

38. Do hookworms have rhabditiform larvae?

39. In general, for hookworms in which we are a true definitive host, where in the body do the adults reside and mate?

40. What damage do the hookworm adults cause and list two pathological results to the body of this damage.

41. Are hookworm females oviparous, viviparous, monoecious or dioecious?

42. Give the common name or nickname for the following genera:

    a. Ascaris =
    b. Ancyclostoma =
    c. Trichuris =
    d. Necator =
    e. Enterobius =
    f. Trichinella =

43. What is a rhabditiform larva?

44. Which roundworm has rhabditiform larvae?

45. How do hookworms attach to the intestinal wall?

46. What is grazing?

47. What happens to the old "site" in grazing and why?

48. How much blood loss could possibly result from 10 hookworm adults in one day's time?

49. What is melena?

50. What does melena look like?

51. Which helminth causes melena?

52. What do rhabditiform larvae feed upon?

53. One hookworms larvae enter the body and reach the intestinal tract, they undergo a body migration phase. How do hookworm larvae eventually wind up again in the intestinal tract?

Lab Exercise Questions Continued:

54. How does mankind obtain Trichinosis?

55. How do pigs obtain Trichinosis?

56. How does the cycle differ between pigs and humans?

57. What happens to the encysted larvae in the undercooked meat that you just ate?

58. What happens, and where does it happen, to the male adult Trichinella worm?

59. What happens, and where does it happen, to the female adult Trichinella worm?

60. What does the female do, before she dies, to continue the cycle in Trichinella?

61. Where are the eggs hatched in the Trichinella cycle?

62. What happens to the female's larvae after birth?

63. List four (4) different meats from which one can obtain Trichinosis.

    a.
    b.
    c.
    d.

64. How many Americans, who have eaten pork, have Trichinosis?

65. Where, in general, do the larvae from the female Trichinella worm like to go in the body?

66. How does one try to avoid obtaining Trichinosis?

67. Does freezing meat kill the Trichinella larvae? What about microwaving?

68. Do migrating larvae, in all the roundworms we've studied, cause damage to the host?
69. Do Ascaris worms have rhabditiform larvae?

Lab Exercise Questions Continued:

70. Do the adult worms, in all the roundworms we've studied, cause damage to the host?

71. Is it possible to die from the damage caused by migrating larvae in the roundworms we've studied?

72. In life, what two possible outcomes could happen to Trichinella encysted larvae?

73. Trichuris has a life cycle similar to which type of roundworm?

74. Where do Trichuris adults live and mate?

75. What can Trichuris adults cause to happen to the smooth muscle sheets in the intestinal wall?

76. What could be two results of this problem in question #75?

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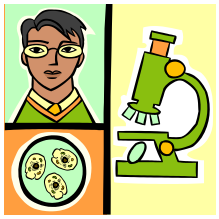
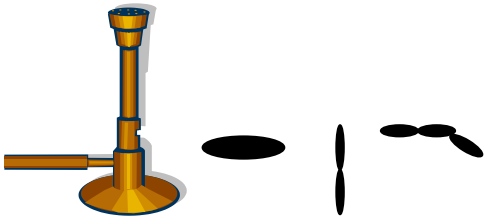
EXERCISE No. 4: THE SIMPLE STAIN PROCEDURE





Materials

1. Microscope
2. Immersion oil, lens paper, xylene, Chinese marker
3. Microscope slides
4. New Methylene Blue stain
5. Microbiology loop and Bunsen burner
6. Water in glass beakers
7. Soap/paper towels for washing hands (At sink in first row)
8. Lysol disinfectant and paper towels for disinfecting your counter top
9. Bacterial plate cultures of Escherichia coli and Bacillus cereus or other species (I may substitute, at times, Bacillus megaterium for Bacillus cereus.)
10. Patience, organization and time management
11. Disposable, sterile nitrile gloves and protective eyewear.
12. Slide disposal beaker and glove disposal bag in the back of the room

Objectives

1. To understand why we stain specimens.
2. To describe what the ideal stain is.
3. To learn how to properly place bacteria onto a glass slide (and keep them there).
4. To learn how to accurately stain the bacteria.
5. To define aseptic technique.
6. To describe at least 5 aseptic techniques we will have employed in lab during this exercise.
7. **To practice lab safety at all times**.
8. To be able to give two reasons why we heat-fix a slide.
9. To properly light and use Bunsen burners.
10. To properly "flame" the microbiology loop.
11. To properly disinfect your work area before you start the exercise and at its conclusion.
12. To learn how and when to properly wash your hands.
13. To learn how to properly dispose of used specimen slides and used gloves.

EXERCISE #4 CONTINUED:

DISCUSSION

A rod-shaped or cylindrical bacterium is termed a **bacillus** (plural = **bacilli** pronounced bas-il'e). Notice the little "b". If I use the word Bacillus (which has a capitalized "B"), then I am now referring to the genus Bacillus whose shape is a bacillus. A lower case "b" refers to a shape.

Bacilli are found everywhere in nature – soil, water, intestines, mouth, on skin, etc. and many are essential due to their role in ecology – decomposers and nitrifyers. Some species can cause disease – Clostridium tetani (tetanus), Bacillus anthracis (anthrax), Escherichia coli strain 0157 (not all strains out there of Escherichia coli are harmful; many Escherichia coli strains are beneficial – you have huge numbers of them in your gut). The H7: 0157 strain secretes a toxin that can destroy your kidneys. Dairy cattle were first detected as having this strain. Cook raw hamburger meat or beef extremely well to avoid getting this strain of Escherichia coli.

Some bacilli (notably the **genera Clostridium** & **Bacillus**) are capable of producing a survival structure called an **endospore**. Many genera of bacilli do not. Be sure to identify endospores in your stain today if you can see any. Their thick, calcium/dipicolinic acid cell wall and very low metabolism allow them to endure a long time as a survival structure. Upon reactivation in better environmental conditions, each one germinates into a **vegetative cell** that can reproduce by binary fission. Endospores themselves do not reproduce. They are simply a survival structure.

This exercise is designed to allow you to observe stained bacilli and observe/determine their arrangement (be sure you understand the difference between shape and arrangement) as well as possibly find endospores. Observe the slides closely for the presence of endospores. Bacillus cereus and Bacillus megaterium produce endospores; they are soil microbes. Escherichia, a fecal bacterium, does not. **Shape and arrangement are always given in the singular. Do not abbreviate in my course.**

Arrangement is simply how bacteria are placed together in nature or "arranged". If they are always singular or separated from one another, then their shape is a bacillus, their arrangement is a **single bacillus**. If two bacilli are arranged together in pairs, then their shape is a bacillus and their arrangement is a **diplobacillus**. If three or more are attached to one another, then their shape is a bacillus, their arrangement is a **streptobacillus**. There is one organism that belongs to the "bacillus" family that has different shapes: Corynebacterium diptheriae which causes diphtheria. We call its shape **pleomorphic** since it has different shapes. Corynebacterium has no arrangement. A curved or comma-shaped bacillus is described as being a "**vibrio**" in shape.

Asceptic Techniques

NOW IS THE TIME TO BEGIN PRACTICING ASEPTIC TECHNIQUES.
These are techniques that you employ to prevent contaminating the pure culture (contains only one genus and species), you and your lab environment with the organisms you are working with.

Some examples of aseptic techniques we will use in lab are:

1. Flaming your loop
2. Disinfecting your countertop
3. Washing your hands
4. Getting in and out of the culture quickly and only opening its lid 45 degrees (or using vertical retrieval).
5. Heat-fixing the slide

Procedures and Tips for Exercise #4:

1. Each student will make a bacterial, stained smear from each culture plate: one containing Escherichia coli, the other containing Bacillus cereus (or other species of Bacillus).

2. Preparing the slide for the Simple Stain:

    a. In order to stain a slide containing organisms, you can, if you want to, first draw a half dollar-sized circle, using a Chinese marker, on the BOTTOM of your slide. Always label the top of the slide with the bacterium's initials. You will eventually place your bacteria onto the top of your slide, working in the circle (work area) you have drawn with the Chinese marker.

    b. **All lab work is always performed while sitting down. Also, wear nitrile gloves when working with the bacteria.**

    c. When first using your microbiology loop, thoroughly **flame it before entering the culture plate**. Then, after allowing it to cool, (approximately 10 seconds), gently touch the surface of the plate culture and obtain a very small amount of the bacteria within your loop. **CAUTION**: Do not get a lot of the organisms onto your microbiology loop. If you do

Procedures and Tips for Exercise #4 Continued:

c. so, your smear will be so thick with organisms that you will not be able to see individual cells under the microscope nor determine their shape and arrangement. Instead, you will have a dark, massive, dark purple mess. The loop can contain billions of bacteria. **You want to see individual cells intensely stained and separated (the ideal smear).**

d. **Heat-fixing the slide**: Heat-fixing a slide accomplishes two important tasks: **1) It causes the organisms to adhere to the glass surface**. If you didn't do this, then they would "wash off" during the staining procedure. **2) It usually kills them.**

WARNING!!

DO NOT slowly pass the slide through the flame when heat-fixing. If you do, you will "cook" the organisms. They will not stain properly if cooked and you will not be able to determine their shape or arrangement. This is a common, MAJOR, mistake students make. You have fried them.

e. Using the microscope: Start off on medium power and eventually work your way up to oil immersion. You EVENTUALLY observe ALL bacteria under oil immersion in order to make your decision about their shape and arrangement. No cover slip is needed. **Do not place the high dry lens into oil. Your instructor will discuss how to use the oil immersion lens. Remember, it has already been shown to you on WebCT's Lab Exercise 1 presentation—so, there's no excuse.**

To usually determine arrangement on a slide, scan the entire slide and look for the largest, orderly arrangement when under oil immersion. Make no decisions concerning the bacteria until under oil immersion.

The Simple Stain Procedure

Now you are ready to stain your bacteria. New Methylene Blue stains the cell wall of bacteria. It does not enter the cell. Follow these steps carefully. All work is done sitting down. Watch out for Bunsen burners!!!!! Pay attention to what you are doing:

THE SIMPLE STAIN PROCEDURE STEPS

1. Label the top of your slide with the bacterium's initials. If you want to draw a circular work area on the bottom of the slide then do so. Do not stain the bottom of the slide-----only the top of the slide with the bacteria gets stained.

2. Using a dropper, place a small drop of water towards the opposite end of the slide (middle is ok). A microbiology loop can also be used instead of the dropper method. If you use the loop, then I advise you to use at least two loops of water.

2. Using your microbiology loop, obtain some bacteria from the surface of the pure culture plate. **Remember aseptic technique here. Also, have you flamed your loop before entering the plate?** Place the bacteria on your loop into the drop(s) of water on your slide and then, using a rapid stirring motion, spread them outwards into a thin film of bacteria over your slide's surface (spread them out to the size of between a quarter and a half dollar).

4. Let this film air dry.

5. Once air-dried, heat-fix the film by **quickly** passing the slide through the Bunsen flame (upper part of flame).

6. Place several drops of new Methylene Blue stain onto the slide. You don't use many drops of stain; only enough to cover most of the bacterial smear. You can gently rock the slide to help cover most of the bacteria.

7. Let stand for several minutes and then gently rise off the stain by dipping the slide in beaker water.

8. Gently blot the slide dry using bilbous paper **(not lens paper!!!).**

9. Once the slide is dry, eventually work your microscope up to oil immersion for your final decision on shape and arrangement.

When Finished With The Lab:

A. **When you are finished for the Lab period, place your slides in the glass disposal beaker in the back of the room. Remove your gloves and place them in the glove disposal container (bag).**

B. **Clean your microscope oil immersion lens and high power lens using xylene and lens paper before you leave the lab. Then place your microscope back in its original location.**

C. **THE LAST THING YOU MUST ACCOMPLISH IS TO THOROUGHLY DISINFECT YOUR COUNTERTOP WITH LYSOL AND THEN THOROUGHLY WASH YOUR HANDS BEFORE LEAVING THE LAB.**

Shapes and Arrangements:

Do not abbreviate in my course. For credit, All shapes and arrangements are to be given in the singular and properly spelled. No credit for abbreviations.

The following shapes and arrangements are listed under the "bacillus" family of bacteria:

1. Shape: Bacillus

Arrangements:

a. single rod

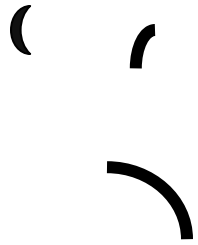
b. chain of rods.

c. paired rods

Shapes and Arrangements Continued:

2. Shape: vibrio

Arrangement: single vibrio



3. Shape: pleomorphic

Arrangement: None

Exercise Questions

1. What is a bacillus?

2. What is meant when we write: Bacillus?

3. List three (3) genera of bacilli that can cause disease.
(note: genera is the plural of genus)

   a.
   b.
   c.

4. Which genus has some strains that are not harmful to us (or to animals), lives in our colon but has some strains that cause disease?

5. What strain in question #4 above causes severe and sometimes fatal disease in humans?

6. List two (2) genera of bacilli that produce endospores.

   a.
   b.

7. Do all bacilli produce endospores?

8. Which bacillus is a major inhabitant of the flora of your gut?

Exercise Questions Continued:

9. What is the purpose of an endospore?

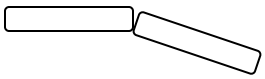
10. Cell-wise, where does an endospore form?

11. List two (2) reasons why an endospore can survive for a long time.

12. Is an endospore considered to be alive?

13. What happens to an endospore when it "comes back to life"?

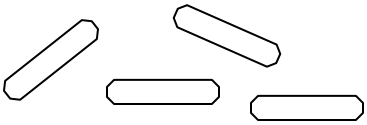
14. What is the a) shape and b) arrangement of the following bacilli?

a. 
a)
b)

b. 
a)
b)

c. 
a)
b)

d. 
a)
b)

e. 
a)
b)

15 Define aseptic technique.

16. List five things you do in Lab to practice aseptic technique.
    a.
    b.
    c.
    d.
    e.

17. What two things does "heat-fixing" bacteria accomplish?
    a.
    b.

Exercise Questions Continued:

18. What is the major problem that could occur when students heat-fix a slide?

19. What happens if you get too many organisms on your microbiology loop and then make your smear?

20. Describe the ideal smear.

21. New Methylene Blue is used for what type of staining procedure?

22. Why do we stain cells or specimens?

23. List two specific roles that bacilli perform in nature (that we discussed).

24. Name the genus and species we mentioned that is pleomorphic.

EXERCISE No. 5: Gram Stain (A Differential staining Procedure)

Materials

1. Microscope
2. Immersion oil, lens paper, xylene, Chinese marker
3. Microscope slides, bilbous paper
4. Crystal Violet, Gram Iodine, Acetone Alcohol, Safrinin
5. Microbiology loop and Bunsen burners
6. Glass beaker for slide disposal (in back of room)
7. Soap/paper towels for washing hands (at 1st row's sink)
8. Lysol Disinfectant and paper towels for disinfecting your counter top
9. Bacterial plate culture of Micrococcus luteus,
10. Water in beakers
11. Patience, organization and time management
12. Disposable nitrile gloves and disposal bag in back of room
13. Protective eyewear

Objectives

1. To be able to identify different coccal arrangements under the microscope.
2. To become more proficient at using oil immersion and staining.
3. To learn the name and roll of each chemical used in the gram stain procedure and how each affects gram positive and gram negative bacteria being stained.
4. To list the basic components of a gram positive cell wall and a gram negative cell wall.
5. To be able to describe the final color of Gm+ and Gm- bacteria and why they ended up these colors.
6. To be able to list a genus that doesn't gram stain, why it doesn't do so and what staining procedure is used for them.
7. To be able to list 4 things a gram stain could tell you about the bacteria being stained.
8. To learn to perform the gram stain procedure.

Discussion

The purpose of this lab exercise is to familiarize you with the shape and arrangement of cocci and how to perform the gram stain procedure. You will be performing the gram stain on Micrococcus luteus.

Like the bacilli, the cocci are ubiquitous in nature. Cocci are spherical bacteria. Depending on the genus and species, they may be arranged singularly (single coccus), in pairs (diplococcus), in chains of three or more (streptococcus), in square groups of four (tetrad or gaffkya such as seen in the genera Micrococcus and Gaffkya), cuboidal packets of 8 or sarcina (as seen in the genus Sarcina or Sporosarcina) or in grape-like clusters (staphylococcus). Sporosarcina is the only genus of coccus to produce endospores.

Many cocci are free-living saprophytes in nature. Some are pathogens in man and animals with examples being Staphylococcus aureus (abscess formation – boils, etc.), Streptococcus pyogenes (tonsillitis, the flesh-eating strep), and Neisseria gonorrheae (causes gonorrhea---gonorrhea is a gonococcus). Cocci usually have no flagella.

A **pure culture** is growth, on a culture medium or agar medium, of only one genus and species of organism. No other genus and species of organism is present. If another is present, then it is a "mixed" culture. In laboratories, the clinician needs pure cultures to work with. Since some infections are often mixed, techniques, such as the **streak plate** technique, are employed in the beginning to separate the different bacteria when placing them onto the plate. Then, each isolate can be worked with separately and each genus and species eventually identified.

Small, individual (isolated) growth areas on a culture medium are referred to as **colonies**. The entire plate and all of its growth is termed a **culture**. Some of the colonies may "run" together and make a large, confluent growth area. In the laboratories they are interested in **INDIVIDUAL, ISOLATED PURE colonies**; not those that are confluent or running together (This is because you may have a "mixed" culture and confluent growth areas may contain more than one genus and species). Bacteria of different genera and species usually have different appearing colonies on the isolation plate. Sometimes the color of each colony is different, or its texture (softness, roughness), size, etc. is different. Sometimes it is difficult to tell that there are different genera and species present.

Laboratory medical technologists are well trained in microbiology and can easily tell different appearing colonies from one another on a plate. Pus, sputum, urine, tissues, etc. can contain more than one bacterial genus and species. Therefore, what you isolate on your culture plate may be a "mixed" culture. However, it may contain only one genus and species. Occasionally, you isolate nothing and have to

Discussion Continued:

re-culture again from pus, sputum, etc., until you isolate something. It is possible to never isolate anything. Then where are you?

Gram staining is the essential, first-step procedure in any laboratory for BEGINNING TO IDENTIFY the genus and species of bacterium which as been isolated. **IT DOES NOT IDENTIFY THE GENUS AND SPECIES**. It merely starts one off in the right direction for the detective's journey in identifying the genus and species isolated. **Biochemical tests must be performed on each isolate in order to identify who they are (the genus and species).** We'll perform biochemical tests later on in the lab manual. These biochemical tests will be performed by you in the last four laboratory exercises.

One can learn four things from a gram stain:

1. The shape of the bacterium
2. The arrangement of the bacterium
3. Whether the bacterium is gram+ or gram-. (Differential)
4. That the bacterium is neither gram+ or gram-; it could be an acid-fast organism (Ex: Mycobacterium tuberculosis which is one bacterium that doesn't gram stain at all). The genus **Mycobacterium** has a wax capsule surrounding each bacterium. This prevents the gram stain from working on them. We stain them with a procedure called the "Acid-Fast Staining Procedure". Hence, they are known as **acid-fast organisms**. Most bacterial capsules are composed of polysaccharide. There are other bacteria out there who do not gram stain. Some gram stain poorly. Some are gram variable.

Many things can affect the outcome of your gram stain procedure. If you don't gram stain "true", then the following are possible causes:

1. Your technique has a mistake in it (improper technique)
2. The culture is too old – you need to gram stain young, freshly-prepared cultures 24 – 36 hours old. Bacteria in old cultures, such as 3 – 4 days old or older, can gram stain not true.
3. When you heat-fix the bacteria to the slide, you "cooked" them. This is the most common mistake students make.
4. The stains are too old.

Discussion Continued:

Antibiotic Selection and The C & S

We usually select an antibiotic to use on a patient in part on whether we are dealing with gram+ or gram- bacteria. However, this is not the sole criteria for selecting an antibiotic to use. Antibiotics can be "cidal" (kill the bacteria or bacteriocidal) or "static" (inhibit binary fission or bacteriostatic). There are three groups of antibiotics: One group affects only certain gram positive bacteria (the gram + antibiotics), some antibiotics only affect certain gram negative bacteria (gram negative antibiotics) and some antibiotics can kill or inhibit certain gram+ and gram- bacteria (broad spectrum antibiotics). In addition to gram staining our unknown(s) infecting a patient, we additionally perform what is called a C & S (culture and sensitivity). It is here that we determine what antibiotics are effective against our isolate(s). The C & S, the patient's condition, the physician's experience, the patient's sex and age and their type illness plus the gram stain result will determine the choice of antibiotic to use.

Gram Stain Background

The Danish bacteriologist Christian Gram (1884) developed a staining technique which separated many (not all) bacteria into two colored groups: bacteria that were gram-positive and those that were gram-negative. His staining procedure was based on the fact that some organisms retained the purple color of crystal violet and iodine when decolorized with alcohol. Gram-negative bacteria were decolorized (crystal violet and the iodine were removed) by the alcohol and became colorless again. Gram-positive bacteria were not decolorized by alcohol and remained dark purple. After the gram-negative bacteria were decolorized by the alcohol, they were counterstained with the red stain safranin which imparted a pink or reddish color to the decolorized gram-negative bacteria.

The chemicals used in the gram staining procedure - crystal violet, Gram iodine, 95% ethyl alcohol (or acetone) and safrinin - each have a specific effect on gram-positive and gram-negative bacteria. Most of these chemicals go inside the bacterial cell. The **crystal violet** is called **the primary stain** and it causes BOTH gram-positive and gram-negative bacteria to become purple after about 20 second of staining. When the **Gram iodine**, called **the mordant**, is applied to both types of bacteria for about one minute, it enters inside BOTH cells and the color of BOTH gram-positive and gram-negative bacteria becomes a darker purple. In gram-positive bacteria the mordant, Gram iodine, combines with the crystal violet to form a relatively insoluble crystal violet-iodine complex. The complex also forms in gram-negative bacteria but is rather soluble.

Discussion Continued:

Gram Stain Background Continued:

When the **95% ethanol (decolorizing agent or decolorizer***)* is added to each type cell for about 10-20 seconds, it removes the crystal violet-iodine complex from within the gram-negative cells rendering them colorless, but does not remove the complex from within gram-positive cells who remain dark purple. Do you know why? As a final step, **the counterstain safrinin** enters the colorless gram-negative bacteria rendering them a pink or reddish color; it has no effect on the color of the gram-positive bacteria who remain dark purple.

The gram staining procedure is the most important procedure needed to be understood and correctly performed by you, the microbiology student. The technique may appear to be simple to you, but performing it with a high degree of reliability requires much practice and experience. Make sure that your smear is not too thick – a common mistake made by students. Also, pay particular attention to the decolorizing step in the staining procedure since it is here that the most critical step lies. Don't cook them, even slightly. **Watch your timing very closely in each step.**

Remember:

a. Gram positive bacteria stain blue, purple, dark blue, dark purple, etc.
b. Gram negative bacteria stain red, pink, reddish-pink, or orange

Procedure

The purpose of this exercise is to allow you to gram stain some representative cocci and determine their arrangements and gram reaction. Bacteria are very small. You must patiently study, with your eyes, the bacteria in your oil immersion, microscopic field. Move around the entire slide. Not all bacteria will be in chains or all diplococci, tetrads, streptococci or staphylococci. This is because the smearing technique breaks them apart and also bunches many together. Use your mechanical stage to move the slide around so that you can observe many different microscopic fields. By studying different fields, you should be able to decide on the shape, arrangement and gram reaction of the bacteria being observed. As a general rule, look for the largest, orderly arrangement you can find. Is it possible for a bacterial genus and species to have more than one arrangement? What would you call a bacterium that has different arrangements?

BE SURE AND FOLLOW ASEPTIC TECHNIQUE THROUGHOUT THE EXERCISE

CAUTION:

Do not get a lot of the organisms onto your microbiology loop. If you do so, your smear will be so thick with organisms, that you will not be able to see individual cells or determine shape and arrangement. Instead, you will have a dark, massive mess. The loop can contain billions of bacteria. **You want to see individual cells intensely stained and separated (the ideal smear). Also, do not get a large drop of water onto your slide. Now you know how long it takes one water drop to air dry---- the bigger the drop, the longer it takes.**

The Gram Stain Procedure

1. Label your slide.

2. Using a dropper, place a small drop of water on the slide

3. Using your flamed microbiology loop, spread a thin film of bacteria from the culture into this drop of water on your microscope slide. Spread the mix outwards until it reaches the size of a quarter to a half dollar.

4. Let the film dry.

5. Heat-fix the film by quickly passing the slide through the Bunsen flame (upper part of flame).

6. Add several drops of Crystal Violet stain (primary stain).

7. Let stand for one minute, then rise by dipping in water in the water beaker.

8. Next, add several drops of Gram Iodine (mordant) solution to "set" the stain.

9. Let this stand for one minute, then rise by dipping in the water beaker.

10. Next, tilt the slide over the sink and then slowly add 95% alcohol as the counterstain. Add the alcohol **drop by drop** (slowly) letting the alcohol flow over the stained smear. Do this until no more color runs off (**do for a maximum of 20 seconds).** This step will remove the color (CV-I) from the gram-negative bacteria. The gram-positive bacteria will still retain their color (CV-I). The alcohol is known as the decolorizer.

11. **Immediately**, **rinse with water in the water beaker**.

12. Next, counter-stain with Gram's Safrinin stain. Allow this to stand for approximately 20 seconds. Then rinse by dipping in the water beaker.

13. Gently blot away excess moisture (gently blot the stained bacteria) and allow the slide to totally air dry. **Bilbous paper is used for the blotting but be gentle.**

14. Once the slide is dry, start on medium power and eventually go to oil immersion for your final decision on gram reaction, shape and arrangement. **Never make a decision under high dry and don't put it in oil!.**

The Gram Stain Procedure Continued:

Remember:

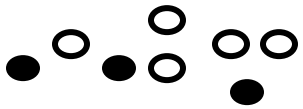
a. All decisions about your bacterium must be made under oil immersion.

Upon Completion of Lab

1. When you are through, place your slides in the glass disposal beaker in the back of the room.
2. Place gloves in disposal container in the back of the lab.
2. Clean your microscope oil immersion lens and high power lens using xylene and lens paper. Then place your microscope back in its original location.
3. WHEN FINISHED WITH THE LAB, THOROUGHLY DISINFECT THE COUNTERTOP WITH LYSOL AND THEN PROPERLY WASH YOUR HANDS.
4. **Make sure you have seen all of the arrangements. They will be on the mid-term under the microscope for you to identify.**

Bacterial Arrangements

The following arrangements (the shape is: coccus) are listed under the "coccus" family of bacteria:

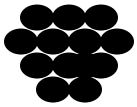
1. Single Coccus: 

2. Diplococcus: 

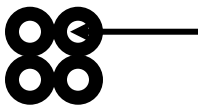
3. Diplococcus:  <u>Neisseria gonorrheae</u> has flattened, medial adjacent sides.

4. chain of cocci: 

5. Tetrad or gaffkya: 

6. Cluster: 

7. Sarcina: The genus Sporosarcina appears as tetrads that are fully large. However, it is a cuboidal packet of 8 bacteria – 4 on the top, 4 on the bottom. Sporosarcina is the only genus of coccus that produces endospores:



Exercise #5 Questions

1. List 4 things a gram stain will tell you.
2. Which stain is the counterstain? Why is it called that?
3. Which stain is the mordant?
4. What is a mordant?
5. Why do we call the gram stain a "differential stain"?

6. What is the purpose of the alcohol?
7. Which chemical is the decolorizer?
8. Which chemical is the primary stain?
9. At the conclusion of the gram staining procedure, what color would a Gm- bacterium be?
10. At the end of the gram staining procedure, what color would a Gm+ bacterium be?
11. What is a pure culture?
12. What is a bacterial colony?
13. How many bacteria do you think are in one bacterial colony (give me a number)?
14. What would a Gm**-** bacterium look like, under the microscope, just before you added the alcohol?
15. What would a Gm**+** bacterium look like, under the microscope, after you added the alcohol?
16. Does the alcohol affect a Gm+ or Gm- bacterium or does it affect both or neither? If it affects one of them, then how does it affect it?
17. Does the alcohol affect you?
18. Give the singular for the following:

    a. cocci
    b. staphylococci
    c. streptococci
    d. tetrads
    e. diplococci

19. Give the plural for the following:

    a. staphylococcus
    b. tetrad
    c. diplococcus
    d. streptococcus
    e. coccus

20. List two genera that have a tetrad arrangement.
    a.
    b.

Exercise #5 Questions Continued:

21. Cocci in cuboidal, groups of eight (one group of four on top of the other group of four cocci below) have an arrangement called

    _____

22. List two genera that have the arrangement of #21.

a.

b.

23. Give the singular (first) and plural (second) of cocci that live in ORDERLY clusters:
    a.

    b.

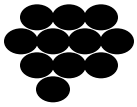
24. What name do we give to a bacterium that has different shapes in a microscopic field?

25. What do we call a bacterium that has different arrangements?

26. Give the shape and arrangement of the following:

    a.  Shape: _____
    Arrangement: _____

    b.  Shape: _____
    Arrangement: _____

    c.  Shape: _____
    Arrangement: _____

    d.  Shape: _____
    Arrangement: _____

27. Give the genus name of the only coccus that can produce endospores.

28. What is the unusual about Neisseria gonorrhoeae's arrangement?

29. Most bacterial capsules are composed of what type of material?

30. List two genera of cocci whose arrangement is in orderly clusters.
    a.
    b.

Exercise #5 Questions Continued:

31. Draw and label a Petri dish showing colonies.

32. As far as we are concerned, in the Laboratory, what type of colony growth are we interested in on our growth plate?

33. When picking out colonies on our culture medium, which colonies do we want to work with?

34. On a growth plate, how can one tell the difference between different genera and species?

35. What is the first step, in a Laboratory, for BEGINNING TO IDENTIFY a genus and species of bacterium?

36. Does gram-staining tell one the genus or species?

37. Give the genus and species name of a bacterium that is acid-fast. (Write it properly).

38. Give an example of an acid-fast genus.

39 List the four (4) chemicals used in the gram-staining procedure.

a.
b.
c.
d.

40. Which stain is the mordant in the gram-staining procedure?

41. What is a decolorizer?

42. Which chemical is the decolorizer in the gram-staining procedure?

43. What is a counterstain?

44. Which stain is the counterstain in the gram-staining procedure?

43. Which stain was the primary stain?

Exercise #5 Questions Continued:

44. Fill in the chart below. You have both a gram-positive and gram-negative bacterium on the same slide next to each other. Starting with crystal violet, what color would each bacterium be as you proceeded step-by-step in gram-staining?

	Gram-Positive	Gram-Negative

Crystal violet

Gram iodine

Acetone alcohol

Safrinin

45. Why won't bacteria, such as Mycobacterium tuberculosis, gram stain?

46. What type of staining procedure is used on bacteria such as the tuberculosis organism?

EXERCISE No. 6 WINOGRADSKY COLUMN

Objectives

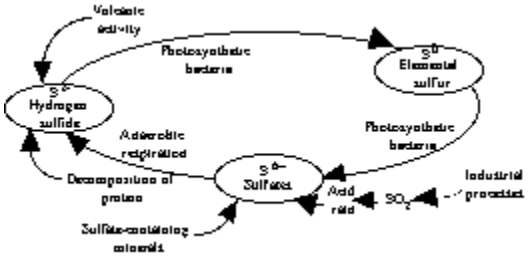
1. Define the following terms: photosynthesis, light reaction, and dark reaction.
2. Differentiate between oxygen-producing photosynthesis and bacterial photosynthesis.
3. Diagram the carbon and sulfur cycles as they occur in a Winogradsky column.
4. Determine the rate of decomposition of various substrates.

Background

Photoautotrophs use light as a source of energy, and carbon dioxide as their chief source of carbon. The process by which photoautotrophs transform carbon dioxide into carbohydrates for use in catabolic processes is called **photosynthe**sis. Photosynthesis can be divided into two parts: the light reaction and the dark reaction. In the **light** reaction, light energy is converted into chemical energy (ATP) using light-trapping pigments. Chlorophyll molecules trap light energy and provide electrons that are used to *generate* ATP. Carbon dioxide is reduced to a carbohydrate by the **dark** reaction. Carbon dioxide reduction or **carbon dioxide fixation** requires an electron donor and energy.

The two types of photosynthesis are classified according to the way ATP is generated and the source of electrons. Cyanobacteria, algal protists, and green plants use *chlorophyll* a to generate ATPs. The resulting oxygen is produced by hydrolysis (splitting water) of the electron donor, *water*. This photosynthetic reaction is summarized as follows:

Figure 1. Anaerobic sulfur cycle

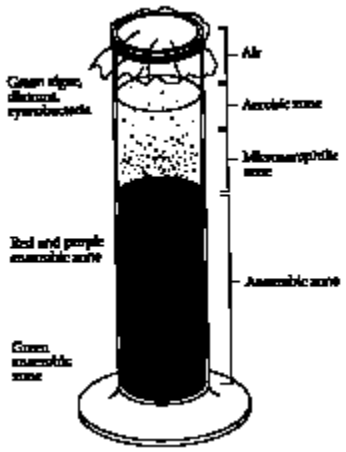


In addition to cyanobacteria, there are several other photosynthetic prokaryotes. These are classified in *Bergey's* Manual Part I, "Phototrophic Bacteria." Most photosynthetic bacteria use **bacteriochlorophylls** to generate electrons for ATP

synthesis and use *sulfur,* sulfur-containing *compounds,* hydrogen *gas,* or *organic* molecules as electron donors. The generalized equation for bacterial photosynthesis is:

$CO_2 + H_2 = C_6H_{12}O_6 + S$

Figure 2. WInogradsky column.



Some photosynthetic bacteria store sulfur granules in or on their cells as a result of the production of sulfide ions. The stored sulfur can be used as an electron donor in photosynthesis, resulting in the production of sulfates.

Bacterial photosynthesis differs from green plant photosynthesis in that **bacterial photosynthesis:**

1. Occurs in an anaerobic environment

2. Does not produce oxygen

Green photosynthetic **bacteria** are colored by bacteriochlorophylls although they may appear brown due to the presence of red accessory photosynthetic pigments called **carotenoids. Purple photosynthetic bacteria** appear purple or red because of large amounts of carotenoids. Purple bacteria also have bacteriochlorophylls.

Purple and green bacteria are involved in another biogeochemical cycle, the **sulfur** cycle (Figure 1). In this exercise we will enrich for bacteria involved in an *anaerobic* sulfur cycle.

In nature, hydrogen sulfide is produced from the reduction of sulfates in anaerobic respiration and the degradation of sulfur-containing amino acids. Sulfates can be reduced to hydrogen sulfide by five genera of *sulfate-reducing* bacteria (the best known of which is *Desulfovibrio*). Carbon dioxide used by photosynthetic bacteria is provided by the fermentation of carbohydrates in an anaerobic environment.

An enrichment culture technique involving a habitat-simulating device called a Winogradsky column (Figure 2) will be utilized in this exercise. A variety of organisms will be cultured depending on their exposure to light and the availability of oxygen.

Materials

Mud from fresh or marine water
Paper
Plant fertilizer (nitrogen and phosphorous), 1 tsp.
Iron nail or screw
Fresh or marine water
Large test tube, graduated cylinder, or clear 2-liter soda bottle
Plastic wrap
Light source

Procedure

1. Stuff one handful of shredded paper into the bottom of the column.
2. Mix mud with an equal part of water and pack a large test tube two-thirds full with the mud mixture. Pack it to eliminate air bubbles. Why?
3. Add fertilizer and iron source to the column.
4. Cover the column with the plastic wrap and secure with a rubber band.
5. Place the column in front of a light source.
6. Observe the column at weekly intervals for 4 to 6 weeks. Record the appearance of colored areas. Aerobic mud will be brownish and anaerobic mud will be black.
7. After 4 weeks, prepare wet mounts from the purple or green patches. Record whether bacteria grew in aerobic areas; whether algae grew in anaerobic areas.
8. Examine the paper for signs of decomposition.

Questions

1. What was the purpose of each of the following in the Winogradsky enrichment? Paper; Nitrogen; Phosphorous; Nail.
2. Indicate the aerobic and anaerobic regions on the diagrams of your enrichment column. How can you tell?
3. Is there evidence of any nonphotosynthetic growth in the Winogradsky column? Explain.

Additional activities

1. Hay and paper are the traditional starting points, however anything (e.g., styrofoam, orange peels) can be buried in the mud and examined later for the presence of bacterial colonies or other evidence of decomposition.
2. Use different light sources (e.g., red, green, fluorescent, incandescent) and compare the colors of photosynthesizers that grow.

References

C. L. Case. and T. R. Johnson. *Laboratory Experiments in Microbiology.* San Francisco, CA: Benjamin/Cummings, 2004

King-Thom Chung and Christine L. Case. "Sergei Winogradsky: Founder of Soil Microbiology." *SIM News* 51(3):133-135, MayJune 200. View article in pdf.

Why do different bacteria grow in different colored light?

Green photosynthetic bacteria did not grow in the column illuminated with blue or green light because bacteriochlorophyll cannot absorb these colors. The green bacteria did grow in the red and yellow lights. Accessory pigments allow the purple bacteria to absorb green and blue light and grow in these columns.

Exercise No. 7: WINE MAKING

Wine Making

Wine was first made 8,000 years ago. Learning about the history of wine making is helpful and at least interesting when considering this rewarding hobby. Most people have the opinion that you can only get fine wines by buying it from well know wineries, however this is not so, you can learn *how to make homemade wine* by learning the basic fundamentals of winemaking used thousands of years ago.

You may also think that how to make homemade wine is only about using fermented juices that are made by grapes, another misconception, because there are many fine and popular wines that are made from many fruits, flowers and vegetables. You will also learn that there are some major differences in the pricing between your homemade wine and the commercial wine that you buy on a regular basis at the store. Using these fundamentals you will be able to make six bottles of quality wine for the price you will pay for one bottle of great wine at the store or a winery. Making wine will be an enjoyable and interesting hobby that will showcase you skill at being a great winemaker.

Many of the great homemade wines (called “country wines) are the best wines in the country. These wines use many great ingredients like grapes, elderberries, blackberries, apples, peaches, and many other wonderful cultivated fruits that you may be able to find in any of the many winemaking books available online and in stores. How to grow grapes is a science of its own. You may even find recipes for vegetables and flowers and some other interesting ingredients.

The great thing about learning wine making is that there are many opportunities for information. There are many places that not only have detailed recipe books and free advice, they also sell a wide range of the much needed equipment and may even carry all of the ingredients that you will need to get started.

Learning **how to make homemade wine** is a fascinating and enjoyable hobby that allows you to explore your creative side. As a novice winemaker you should start with the easiest and most recognized wines in the world, red and white wines. After learning these basics you may want to learn more advanced and involved methods of making wine at home.

The Basic Fundamental Stages

In the effort to learn *how to make homemade wine* you will need to know the basic fundamental stages of making wine, such as flavor extraction, fermentation, bottling and aging. Besides learning these concepts you will also need to understand the types of equipment you need for making wine at home, and the ingredients needed to make the wine you make at home, great.

Stage One: Flavor Extraction

Some wines can be made frm pure undiluted juice from the grape and may have little to no sugar added to them. But many times homemade wines may use fruits that has stronger flavors and may need to be diluted with water so that it will have a more agreeable concentration. In this process the juice (or flavor) is extracted and the liquid that is obtained is watered down or diluted, this is called

the “must”. Sugar may be dissolved in the must and the yeast will need to be added so that the fermentation process can begin. You will have to decide if you will use real fruit or juice concentrate. If you choose to purchase juice concentrate you will avoid all the squeezing.

Stage Two: Fermentation

When you are learning how to make wine from home, one of the most important concepts you must learn regarding the making of wine is the fermentation process. Fermentation is a natural occurrence that happens through the use of yeasts as well as fungi, bacteria and moulds. However, for the wine making concern, fermentation usually refers to the use of yeast and its action on the sugar that is dissolved in your must.

In wine fermentation, the yeast uses the sugar as food, it consumes the sugar that you have added to the must and then it produces alcohol. The actual fermentation process involves to stages, the first stage is the aerobic stage in which the yeast builds up a colony and the second stage is the production of alcohol in the much. Many times the first stage occurs in your primary fermentation bucket which contains the must and the pulp of the ingredients. This is the first step in the extraction of the juice or flavor, so you may notice that the extraction and the fermentation work together. After waiting the allotted time as directed by your recipe, you will need to strain the liquid (raking) and then pour the liquid into your demijohn or a vessel with a fermentation lock to block the air out. While the yeast is using the remaining oxygen you may see some bubbling, this will stop when the oxygen is gone and a slower fermentation will occur. When the yeast has completed its work the yeast will drop to the bottom of your vessel and you will notice that your wine will start to clear. You will want to rake it again at this point (siphoning) and put it into a clean jar to age. The siphoning and cleaning process is known as wine clarification.

Bottling and Aging

Another important aspect of learning how to make wine at home is bottling and aging wine, this is the easiest part of the process but it is very important that it is done properly. When your fermentation process is done and you have allowed it to age some, it will be time to bottle your wine, and allow it to continue to age. A lot of times new wines will not taste good when you first finish the fermentation process, so you will want to store it for a while just to allow the flavor to mellow and to allow all of the ingredients to continue interacting with each other so that they will produce a wonderful bouquet and flavor you would usually find in the finer wines.

The Equipment You Need

Another important part of learning how to make homemade wine is what equipment you will need to complete all of the three stages of the winemaking process. If you are new to winemaking you will need only the basic equipment, but later as you have more experience you may want to buy more sophisticated equipment. The following is equipment needed to make your wine:

- A plastic bucket of about 2 gallons with a lid that fits securely on top.
- A plastic or wooden spoon

- A large enamel or aluminum pan that can be used if you must boil the ingredients
- A mesh sheet or a pair of ladies nylons for straining the must
- A demijohn or a fermenting vessel with a rubber bung and airlock
- A funnel
- A storage demijohn (optional)
- Bottles with corks or stoppers
- Plastic tube for siphoning
- A bottle brush for cleaning your equipment
- Other useful equipment includes an electric heater, a hydrometer, corking machine, a juice extractor, and glass carboys for storage. Each of these is optional items that you may buy to make the process easier but are not necessary.

The equipment found on this list can be found in any specialty winemaking store, some wine retailers and online vendors. Wine equipment can be bought inexpensively on-line at Wine Making Equipment Store.

It is important to avoid different kinds of equipment, which includes metal pans, stainless steel, or colored plastics. This applies to vessels for long term storage and fermentation as well. The best equipment should be bought from specialty dealers who only sell the winemaking equipment that is acceptable for those learning how to make homemade wine.

Sterilization and Hygiene - How to Make Homemade Wine

Another thing that is very important when learning how to make homemade wine is hygiene. You will want to know how bacteria can infect your wine and turn it bad during any stage of the production, if your equipment or bottles are dirty when you are making wine the wine will be no good. It is a fact that many wines are lost to bacteria infection more than through any other variable that can occur during the making of the wine. The most common infection is the Acetobacter, which will turn your wine into vinegar by a process of acetification. The only good thing is that you can use it fro cooking when this occurs.

Another reason you will need good hygiene is that wild yeasts live on fruit and in the air, they can produce small amounts of alcohol, but they also taint the wine and produce unpleasant flavors in your wine. This means that you will need to wash and sterilize all utensils, equipment and ingredients. If you are using small things that can be overlooked double check to make sure that you have indeed sterilized them. This is one of the biggest reasons for failure!

Methods of Sterilization

- Boiling water is the most effective method of method of sterilization
- House hold bleach solution, while it is ideal for sterilizing plastic and glass equipment, it may corrode the metals. You must also remember to rinse thoroughly because bleach will spoil the wine and is extremely poisonous so you want to remember to clean it off of your hands.
- Products used for sterilizing baby bottles are good for wine making equipment

- Chempro SDP is a cleaner and sterilizer and is very effective. The direction for its use is on the package.

The Ingredients

You will need to learn the basic ingredients needed for making your wine product. The main ingredients for making great wine are as follows:

1. Fruit: You will need fresh fruit, canned fruit or frozen fruit concentrate
2. Water: for dilution of the fruit
3. Yeast: turns sugar into alcohol, used for fermentation
4. Pectin Enzyme: breaks down pectin in your wine, makes more juice and adds more color to the wine/
5. Grape Tannin
6. Sugar: Yeast eat this and turns it into alcohol
7. Potassium Sorbate: inhibits yeast production and fermentation at bottling
8. Yeast nutrient, the energy for the yeast
9. Campden tablet are needed before fermentation and before bottling

This is just a short list of ingredients that will be needed, be sure to consult your recipes to make sure you are buying what is needed. More ingredients needed are discussed here. It will help you stay prepared if you know what the ingredients are that you will need. Please continue to read our free articles on winemaking or you may wish to look at another expert's perspective in his book, How to Make Wine From Home or check out the other E-Books we have for sale.

Additional Resources:

Please realize there is considerable **free** content on the how to make homemade wine website in addition to the articles. Our FAQ includes details not mentioned elsewhere and we also have 60 tips & tricks catagorized into wine making starter tips, fermentation & process tips, understanding wine yeast and racking & clarification tips. We also offer a free E-course and for specific questions you can always go to our wine making forum.

Exercise No. 8 SOIL PRODUCTIVITY – PLATE COUNT METHOD

SOIL PRODUCTIVITY – PLATE COUNT METHOD

Objectives

1. Estimate the number of microorganisms in soil using the plate count method.
2. Describe the general activity of microorganisms in the soil.
3. Distinguish between bacterial, actinomycete, and fungal colonies.

Background

The soil is one of the main reservoirs of microbial life. Typical garden soil has millions of bacteria in each gram. The most numerous microbes in soil are bacteria (Table 1). Although actinomycetes are bacteria, they are listed separately because conidiospores make their dry, powdery colonies easily recognizable. Soil bacteria include aerobes and anaerobes with a wide range of nutritional requirements, from photoautotrophs to chemoheterotrophs. As usable nutrients and suitable environmental conditions (such as light, aeration, temperature) become available, the microbial populations and their metabolic activity rapidly increase until the nutrients are depleted or physical conditions change, and they then return to lower levels.

Human pathogens, with the exception of endospore-formed bacteria, are uncommon in the soil. Soil microorganisms are responsible for recycling elements so they can be used over and over again. The numbers of bacteria and fungi in soil are usually estimated by the plate count method. The actual number of organisms is probably much higher than the estimate, however, because a plate count only detects microbes that will grow under the conditions provided (such as nutrients and temperature).

In a plate count, the number of **colony-forming units** (c.f.u.) are determined. Each colony may arise from a group of cells rather than from one individual cell. The initial soil sample is diluted through serial dilutions in order to obtain a small number of colonies on each plate. A known volume of the diluted sample is plated on sterile nutrient agar. After incubation, the number of colonies is counted. Plates with between 25 and 250 colonies are suitable for counting. A plate with fewer than 25 colonies is inaccurate because a single contaminant could influence the results. A plate with greater than 250 colonies is extremely difficult to count. The microbial population in the original soil sample can then be calculated. For example, if 232 bacterial colonies were present on the plate containing 0.01 mL of the 1:1,000,000 dilution, the calculation would be

$$\text{bacteria (c.f.u.)/g} = \frac{232 \text{ colonies}}{0.01 \text{ mL} \times 10^{-6}} = 2.32 \times 10^{10}$$

Distribution of microorganisms in numbers per gram of typical garden soil at various depths

Depth	Bacteria	Actinomycetes	Fungi	Algae
3-8 cm	9,750,000	2,080,000	119,000	25,000
20-25 cm	2,179,000	245,000	50,000	5,000
35-40 cm	570,000	49,000	14,000	500
65-75 cm	11,000	5,000	6,000	100
135-145 cm	1,400	-	3,000	-
350-450 m	100			

Materials

Balance

99 mL sterile water in a bottle (3)

Pipettes or calibrated one-piece plastic droppers

Sterile nutrient agar plates (3)

Spreading rod (L-shaped glass rod or place of piece of No. 16 shrinkable Teflon tubing over a large, straightened (L-shaped) paper clip or metal spreaders can be purchased from Carolina Biological Supply)

Alcohol (rubbing alcohol, 70% isopropyl alcohol)

Procedure

This procedure can also be used with food, milk, and water.

Figure 2. Use of a spreading rod.

9. Each group should start with a soil sample collected from a different environment, e.g., leaf litter, sand, stream bottom, clayey soil, etc.

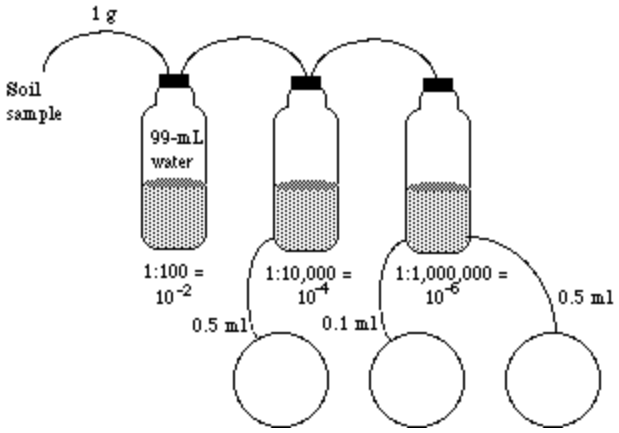
10. Prepare a 1:100 (or 10^-2) dilution of the soil. Weigh out 1 g of soil and add it to 99 mL sterile water. Mix the soil and water. Be sure to keep all tubes and bottles capped to prevent addition of bacteria and fungi from the air.
11. Prepare a 1:10,000 (or 10^-4) dilution by pipetting 1 mL of the 10^-2 dilution to another 99mL bottle of sterile water. Mix thoroughly by pipetting up and down.
12. Prepare a 1:1,000,000 (10^-6) dilution by pipetting 1 mL of the 10^-4 dilution to another 99mL bottle of sterile water. Mix thoroughly by pipetting up and down.
13. Label one nutrient agar plate "0.5." Label a second plate "0.1" and a third plate, "10^-40.5."
14. Aseptically pipette 0.5 mL of the 1:1,000,000 dilution onto the surface of the nutrient agar. See Figure 1.
15. Disinfect a spreading rod by dipping in alcohol, quickly igniting the alcohol in a Bunsen burner flame, and letting the alcohol burn off. Let the spreading rod cool.
16. Spread the liquid over the surface of the agar. (See Figure 2.) Disinfect the spreading rod and return it.
17. Repeat inoculating the second plate with 0.1 mL of the 1:1,000,000 and the third plate with 0.1 mL of the 1:10,000 dilution.
18. Incubate the plates inverted for 24 to 48 hours.
19. After incubation select a plate with a countable number of colonies (between 25 and 250). Count the number of colonies and calculate the number of bacteria in 1 g of that soil

$$\frac{\#\text{ colonies}}{\text{amount plated} \times \text{dilution}} = \text{number of bacteria/g soil}$$

20. Record class data in a table:

	Soil type
Number of bacterial c.f.u./g.	
Number of actinomycete c.f.u./g.	
Number of fungal c.f.u./g.	

Figure 1. Serial dilutions



Questions

Questions

4. Why do these numbers represent the minimum number of microorganisms in the soil?
5. Which soil type has the most microorganisms? Why?
6. Which type of microorganism (fungi, bacteria, or actinomycetes) predominates in each soil type?

EXERCISE 9: SERIAL DILUTIONS

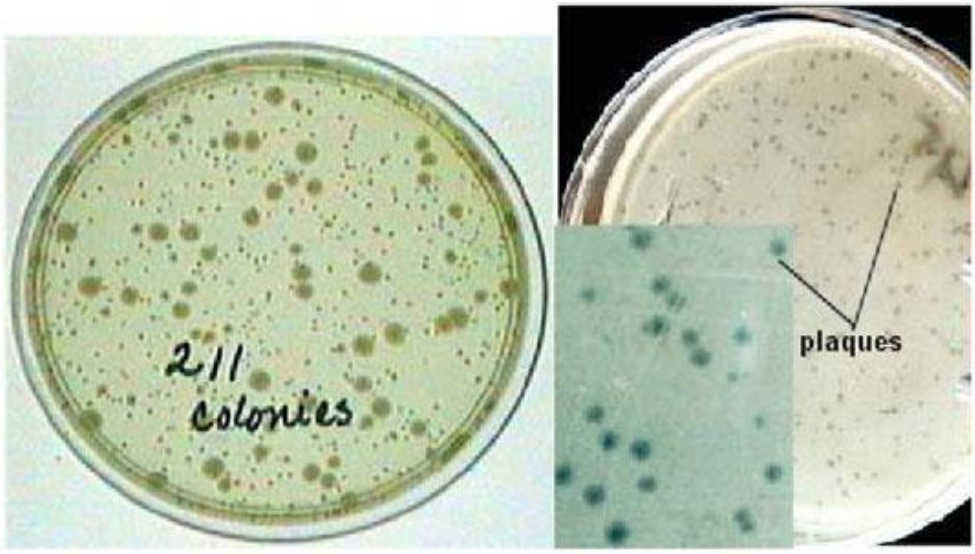
It is a common practice to determine microbial counts for both liquid and solid specimens---suspensions of *E. coli* in nutrient broth all the way to soil samples and hamburger meat. Most specimens have high enough numbers of microorganisms that the specimen has to be serially diluted to quantitate effectively.The following is a step-by-step procedure to working dilution problems, and includes some practice problems at the end.

The purpose can be determination of bacterial, fungal, or viral counts. This protocol is specific for bacterial counts (colony-forming units, CFUs), but can be modified for fungi (CFUs) and viruses (plaque-forming units, PFUs for viral counts).

History

Robert Koch is credited with identifying a method for bacterial enumeration, used first for the study of water quality. His article, *About Detection Methods for Microorganisms in Water*. was published in 1883.

The standard plate count is a reliable method for enumerating bacteria and fungi. A set of serial dilutions is made, a sample of each is placed into a liquefied agar medium, and the medium poured into a petri dish. The agar solidifies, with the bacterial cells locked inside of the agar. Colonies grow within the agar, as well as on top of the agar and below the agar (between the agar and the lower dish). The procedure described above produces a set of pour plates from many dilutions, but spread plates (sample spread on top of solidified agar) can be used also. The agar plate allows accurate counting of the microorganisms, resulting from the equal distribution across the agar plate. This cannot be done with a fluid solution since 1) one cannot identify purity of the specimen, and 2) there is no way to enumerate the cells in a liquid.



Principles

THE STANDARD FORMULA

$$\text{total dilution of tube (used to make plate for colony count)} \times \text{volume plated} = \frac{\text{colony count (CFUs) on an agar plate}}{}$$

To work the problem, you need 3 values---a colony count from the pour or spread plates, a dilution factor for the dilution tube from which the countable agar plate comes, and the volume of the dilution that was plated on the agar plate.

PROTOCOL

STEP 1: Determine the appropriate plate for counting:

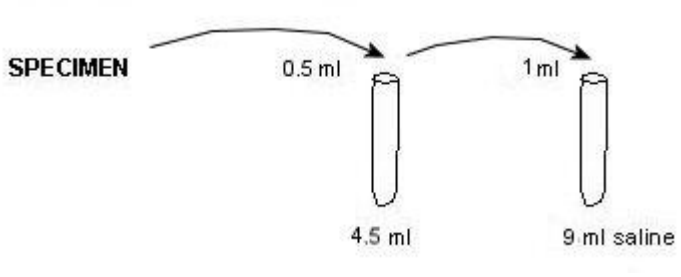
Look at all plates and find the one with 30-300 colonies (see COMMENTS & TIPS section at end for explanation).
Use the total dilution for the tube from where the plate count was obtained.
If duplicate plates (with same amount plated) have been made from one dilution, average the counts together.

STEP 2: Determine the total dilution for the dilution tubes:

Dilution factor = amount of specimen transferred divided by the total volume after transfer[amount of specimen transferred + amount of diluent already in tube].

Determine the dilution factor for each tube in the dilution series.
Multiply the individual dilution factor for the tube and all previous tubes.

To calculate this dilution series:



Determine the dilution factor of each tube in the set.

$$\text{dilution factor for a tube} = \frac{\text{amount of sample}}{\text{volume of specimen transferred + volume of diluent in tube}}$$

But after the first tube, each tube is a dilution of the previous dilution tube.

SO…..

total dilution factor = previous dilution factor of tube **X** dilution of next tube

FOR THE ABOVE DILUTION SERIES:

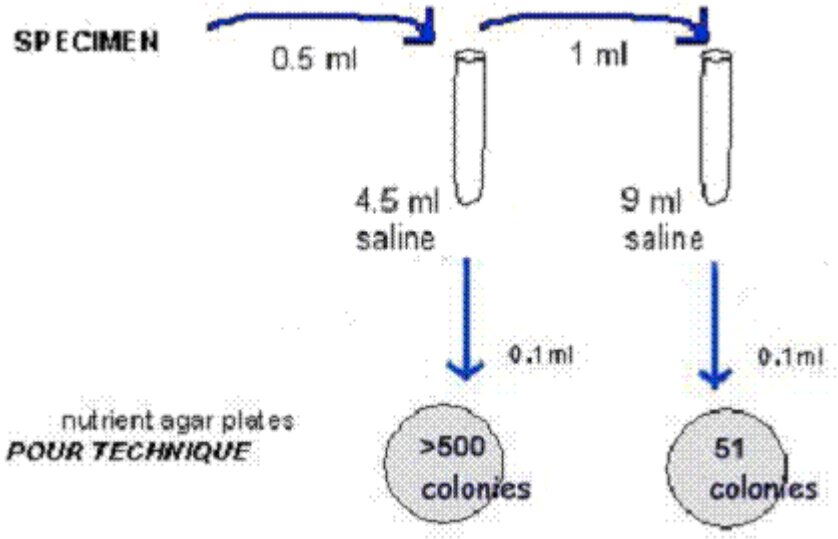
0.5 ml added to 4.5ml = 0.5/5.0 = 5/50 = 1/10 for 1st tube

1ml added to 9ml = 1/10 (2nd tube) **X** previous dilution of 1/10 (1st tube) = total dilution of 1/100 for 2nd tube.

STEP 3: Determine the amount plated (the amount of dilution used to make the particular pour plate or spread plate).

There is nothing to calculate here: the value will be stated in the procedure, or it will be given in the problem.

STEP 4: Solve the problem



1. The countable plate is the one with **51** colonies.

2. The total dilution of the 2nd tube from which that pour plate was made = $1/10^2$

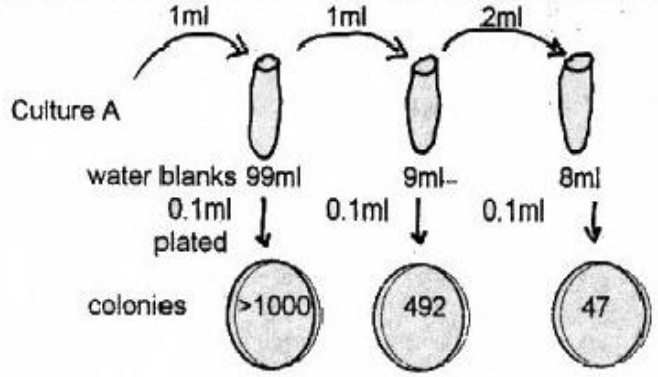
3. The amount used to make that pour plate = **0.1ml** (convert to 1/10 - it is easier to multiply fractions and decimals together).

51 colonies = 51 X 10^3 = **5.1 X 10^4** (scientific notation) OR **51,000 CFUs/ml**
$1/10^2$ X 1/10

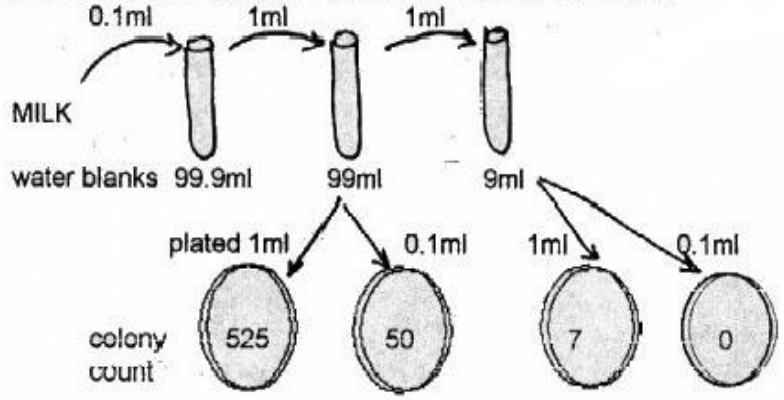
45 colonies = 45 X 10^4 = **4.5 X 10^5** (scientific notation) OR **450,000/ml**
$1/10^3$ X 1/10

SAMPLE PROBLEMS:

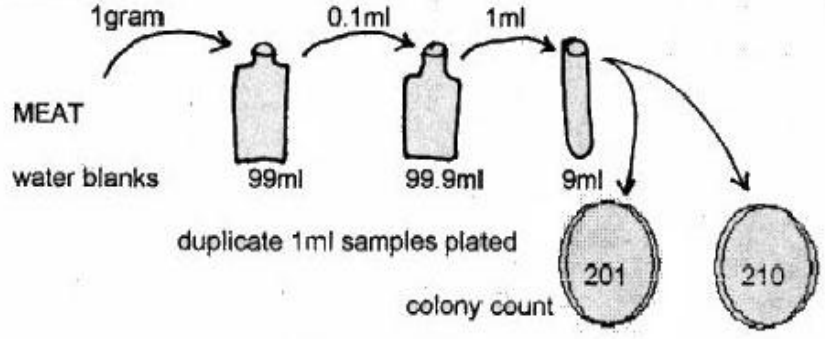
CALCULATE THE NUMBER OF BACTERIA PER MILLILITER OF CULTURE A.



CALCULATE THE NUMBER OF BACTERIA PER ML OF MILK.



CALCULATE THE NUMBER OF BACTERIA PER GRAM OF HAMBURGER MEAT.
(1 gram = 1ml)



SAFETY

Tubes and agar plates should be discarded properly in a biohazard container for proper sterilization. The pipettes will also be sterilized (washed first if using reusable glass pipettes.

Do not pipette by mouth.

Use sterile technique in the transfer of microorganisms from tube to tube, as well as in the production of the pour plates.

The ASM advocates that students must successfully demonstrate ability to explain and practice safe laboratory techniques. For more information, visit the ASM Curriculum Recommendations: Introductory Course in Microbiology and read the section on laboratory safety.

Three additional articles provide important information:

Biosafety Levels-What We Need to Know About Them in Teaching Labs by Christina Thompson (2004)

Update of Biosafety Level Designations by Erica Suchman (2004)

Safety Recommendations from the Concurrent Sessions on Safety in the Microbiology Teaching Laboratory at the Undergraduate Microbiology Education Conference 2003 by Jackie Laxon (2003)

COMMENTS AND TIPS

Greater than 300 colonies on the agar plate and less than 30 leads to a high degree of error. Air contaminants can contribute significantly to a really low count. A high count can be confounded by error in counting too many small colonies, or difficulty in counting overlapping colonies.

Use sterile pipettes for the dilutions, and use different ones in between the different dilutions. To do otherwise will increase the chances of inaccuracy because of carry-over of cells.

Accuracy in quantitation is determined by accurate pipette use and adequate agitation of dilution tubes.

Exercise 10: Swabs

Objectives

1. **Perform a throat culture on your classmate.**
2. **When the plate is ready, determine the type or types of hemolysis present.**
3. **Be able to define, describe or discuss the following terms and be able to use them correctly within sentences:**

Enriched medium	**Lysis**
Fastidious organism	**Hemolysis**
Steptococcus pyogenes	**Alpha hemolysis**
Antibody	**Beta hemolysis**
Colony	**Nonhemolytic**
Zone of hemolysis	**Gamma hemolytic**
Radial diffusion	**Heme**
Hemolyzed	***Hemoglobin***
Lancefield Group	**RBC or erythrocyte**
Streptococcal	**Streptococcus (streptococci)**
Chocolate agar	**Facultative anaerobe**

Candle jar or CO2 incubator

4. **Be able to discuss the use of a candle jar and its environment within**

Materials

a. **Blood agar plate (Tryptic Soy agar with Sheep Red Blood Cells)**
b. **Sterile, cotton-tipped applicator**
c. **Your posterior pharynx**
d. **Chinese marker**
e. **Candle jar**
f. **Beaker with disinfectant**
g. **Bunsen burner and Microbiology loop**
h. **Mannitol Salt Agar**
i. **MacConkey Agar**
j. **Hektoen Agar**
k. **Brilliant Green Agar**
l. **Bile Esculin Agar**
m. **Eosin Methylene Blue Agar**

Discussion

Blood agar is an **enriched**, bacterial growth **medium**. **Fastidious** organisms, such as streptococci, do not grow well on ordinary growth media. Blood agar is a type of growth medium that encourages the growth of bacteria, such as streptococci, that otherwise wouldn't grow well at all on other types of media. This bacterial culture medium is used by physicians (and laboratories) when performing a "throat culture" on an individual that has come to the doctor complaining of a sore throat. In particular, the physician is interest in determining if the sore throat is being caused by the oral presence

of the bacterium ***Streptococcus pyogenes.*** This microbe is the cause of tonsillitis (strep throat), scarlet fever and is also known as the flesh-eating strep. These diseases can kill the patient. The physician uses blood agar in an attempt to isolate *Streptococcus pyogenes*, if it is present, from the patient with the sore throat complaint. There is also an **antibody** test that can be performed on the isolated material from the patient's mouth. This antibody procedure is quicker than growing organisms on a blood agar plate.

Discussion Continued

In everyday medicine, blood agar has two major uses:

1. To grow streptococci.
2. To determine the type of hemolysis, if any.

In addition to encouraging the growth of streptococci , this medium is also used in determining the type(s) of **hemolysis**, if present, on the plate. There are two types of hemolysis possible depending on the organism: **Alpha hemolysis** and **beta hemolysis.** If the **colony** you're observing on the plate's surface indicates there is no type of hemolysis present, then we state that the microbe creating that colony is **nonhemolytic**. They have caused no change in the erythrocytes.Your **Identibacter interactus** CD from Purdue will use the term **gamma hemolytic** (gamma streptococci) in lieu of the term **nonhemolytic**. Nonhemolytic, however, is the preferred description.

How does one know if the colonies they are observing on a plate have caused **alpha hemolysis** or **beta hemolysis**? If either type of **hemolysis** is present, then one will observe a **zone of hemolysis** surrounding a growing colony. **Alpha hemolytic** bacteria produce an olive green zone of hemolysis around their colony. The bacteria have fragmented the **erythrocyte**s in the medium and have altered the hemoglobin content within the RBCs. This causes the medium to turn an **olive green color**. All agar media contain a lot of water. **Alpha hemolytic bacteria** produce and secrete an **exotoxin** called **alpha hemolysin** that **radially diffuses** away from their colonies in the medium's water and causes a partial destruction of the red blood cells within the blood agar as it progresses outwards (it is a partial or incomplete **lysis** of the cells). In addition, the hemoglobin within the erythrocytes are partially denatured to various **heme** products. Can you describe the basic structure of a hemoglobin molecule? What is the purpose of hemoglobin? Thus, these bacteria cause **alpha hemolysis** on the plate. **Beta hemolytic bacteria** produce a **clear zone of hemolysis** surrounding their colonies. They produce an extotoxin that radially diffuses outwards from the colony (or colonies) causing complete destruction of the red cells in the medium and complete denaturation of hemoglobin within the cells to colorless products. This causes a clear zone of hemolysis to form around these growing colonies. No cellular debris would be found around the **hemolyzed** cells.Their exotoxin is called **beta hemolysin** which causes **beta hemolysis** to occur on the plate.

Why are we so concerned with the type of hemolysis present on a blood agar plate? ***Streptococcus pyogenes*** is a beta hemolytic, **Lancefield Group A streptococcal** bacterium. If one isolates this bacterium from the throat of a patient, then aggressive antibiotic therapy is indicated. This bacterium can kill the patient, sometimes very

quickly. If we isolate a beta hemolytic, streptococcal bacterium from our throat culture, then we are dealing with *Streptococcus pyogenes*.

Examples of alpha hemolytic, beta hemolytic and nonhemolytic streptococci:

While the beta hemolytic streptococci are the most dangerous of the streptococci, the alpha hemolytic streptococci, if given the opportunity (**opportunistic bacteria**), can cause disease that could be fatal. They are not outright pathogens like the beta hemolytic streptococci, but are opportunistic if given the chance. Many of the alpha hemolytic streptococci are part of the normal body flora. One of these, ***Streptococcus pneumoniae***, is the causative agent of a bacterial pneumonia and can be very serious, opportunistic pathogen. It is presently the only infectious disease listed among the top 10 causes of death in the United States. It is spread primarily by carriers who harbor it in their nasopharynx. Virtually all of the alpha hemolytic streptococci, except *Streptococcus pneumoniae*, are resistant to lysis by bile-salt solutions and will grow in the presence of such salts. This characteristic is used to determine if any of the alpha hemolytic streptococci that are cultured from the throat are *Streptococcus pneumoniae*. The sensitivity of this bacterium to bile salts can be determined by either placing one drop of bile-salt solution over a colony or by using prepared disks. Sensitive organisms will dissolve in the bile solution or will not grow adjacent to the disk.

The beta hemolytic streptococci, such as *Streptococcus pyogenes*, are all pathogenic and must be carefully identified. One of the most important differential tests is the sensitivity of Lancefield Group A streptococci to the antibiotic Bacitracin. This sensitivity is measured by placing a disk with the antibiotic on blood agar that has been streaked with the beta hemolytic isolate. Further differentiation of the other Lancefield groups (B, C D) requires the determination of several biochemical characteristics. It is interesting to note that it is not just streptococci that could be beta hemolytic. *Staphylococcus aureus*, who is not a streptococcus, is also beta hemolytic.

The **nonhemolytic streptococci** (**or gamma hemolytic**) and other types of bacteria produce **no zones of hemolysis** when growing on blood agar. ***Streptococcus lactis***, found in milk, is a nonhemolytic streptococcus. ***Neisseria gonorrheae***, which is a diplococcus, can grow on blood agar but produces no hemolytic zone (it is therefore nonhemolytic also). The medium of choice for growing *Neisseria gonorrhea* is **chocolate agar**.

Even though we will not be using chocolate agar, a brief discussion of its use and its composition is advantageous to those who will be working within a medical environment. **Chocolate agar** is blood agar that has been heated to denature the proteins of the sheep red blood cells. This causes the cells to lyse and to turn a

light brown color, not unlike that of milk chocolate (hence the name). Most formulas for chocolate agar include certain enrichment factors (such as adding hemoglobin and other factors) that encourage the growth of the more **fastidious** pathogens, including Neisseria and Haemophilus genera. It is the choice medium for growing ***Neisseria gonorrheae*.**

The Candle Jar

Streptococci are **facultative anaerobes**. They grow best in a facultative anaerobic environment and also in a CO2- rich atmosphere. Remember, they are **fastidious** organisms. By placing throat cultures into a large jar, lighting a candle within the jar and then screwing on the lid, the candle's flame will eventually become extinguished. Products of combustion are heat, light, water and carbon dioxide. The amount of CO2 within the jar will increase up to about 4%. Normal atmospheric CO2 content is approximately **.**3%. In addition, normal O2 content of air is approximately 21%. After the flame goes out, is all the O2 gone from the jar? No. It has decreased to about 16% and at that percentage will not support combustion. Well, what have we done here? **We have created a CO2-rich environment and a facultative anaerobic environment for streptococci.** We are doing all we can to encourage the growth of any possible streptococcal isolates from a sick persons oral cavity. Of course, a hospital (or lab) doesn't place blood agar plates (throat cultures) into a candle jar. They have CO2 incubators that create this environment for growing streptococci. Besides streptococci, other organism that are grown in CO2 incubators are *Campylobacter fetus*, a common cause of diarrhea, and *Haemophilus influenza*, frequently the etiological agent of meningitis as well as otitis media in infants. Streptococci primarily spread by aerosol; occasionally by fomites.

Procedure

1. Clearly label the bottom of your blood agar plate with your full name.
2. Have a lab companion obtain your throat swab with a sterile cotton applicator. Then they will hand it to you.
3. Gently, using the spread plate technique your instructor explained to you on the blackboard and with the plate's lid no more than 45 degrees open, place your organisms onto the blood agar plate's surface. Spread an area the size of a half dollar and be gentle. Rotate your swab as you spread your organisms onto the plate's surface.
4. When finished with the spread, close the lid and place the applicator stick into the disposal container in the back of the room.
5. Next, flame your loop, let it cool, and then gently streak into and out of your spread using your loop. Remember to keep the lid only 45 degrees open. Streak the entire plate with **many streaks** into and out of your spread. Your instructor has explained to you, using the blackboard, how to perform a streak plate.
6. Then flame your loop before placing it onto the countertop.
7. Bring your finished plate to the instructor. Do not put it into the jar.

8. When all plates are in, the instructor will light the candle, place it in the jar, and place the lid onto the jar.
9. During the next lab, we will interpret your plates.

When Finished With the Lab

1. Disinfect your countertop**.**
2. Wash your hands.

Exercise #7 Questions

1. Fill in the blanks with the following words:

hemolytic
hemolysis
hemolysis

Upon observing our blood agar plate, one of the colonies had a clear zone of _____ around it. This indicated that the bacterium was a beta _____ bacterium. In order to get this zone of _____, the bacterium had to produce the toxin beta _____. Therefore, this beta _____ bacterium, who caused beta _____ on the blood agar plate, can produce the toxin beta _____.

2. Give the genus and species name of a streptococcus that is beta hemolytic.

3. Give the genus and species name of a bacterium that is beta hemolytic but is not a streptococcus. (In lecture note).

4. What is the principle use of chocolate agar?

5. In a sick patient with a sore throat, what genus and species of bacterium are we looking for when we perform a throat culture using blood agar?

6. Is <u>Neisseria goorrheae</u> alpha, beta, or non-hemolytic?

7. How can one tell they have a beta-hemolytic streptococcus on their blood agar plate?

8. In #7 what did the bacteria specifically produce in order to cause the beta hemolysis?

9. How does one tell they have an alpha-hemolytic streptococcus on their blood agar plate?

10. What did the streptococci produce to give the alpha-hemolytic effect in #9?

11. How does one know they have a streptococcus that is non-hemolytic on their blood agar plate?

12. Give a genus and species of streptococcus that is alpha-hemolytic and causes pneumonia.

13. Give the genus and species name of a streptococcus that is beta-hemolytic.

Exercise #7 Questions Continued:

14. Which type of hemolytic streptococcus is always pathogenic and the most dangerous?

15. Name the streptococcus that is the flesh-eating streptococcus.

16. What two (2) environmental conditions occur in a candle jar for growing streptococci? (Moisture is not one).

    a.
    b.

17. Is a candle jar anaerobic? If not, what is it?

18. Fill in the table below for a candle jar:

    % $C0^2$ in atmospheric air =
    % $C0^2$ in candle car =

    % 0^2 in atmospheric air =
    % 0^2 in candle jar =

19. Why did the candle eventually go out in the candle jar?

20. Is it normal to have alpha-hemolytic bacteria in your mouth?

21. How do streptococci spread from one person to another?
    (Give two ways).

Exercise #11Bacterial DNA Extraction of *Escherichia coli*

Materials:

1. **Spooling tube of freeze-died *Escherichia coli***
2. **Vial of Sodium Dodecyl Sulfate (SDS)**
3. **Bottle of bacterial suspension solution**
4. **Glass spooling rod**
5. **Medicine cup**
6. **2 graduated 1-mL dropping pipets**
7. **95% ethanol**
8. **Tube of 95% ethanol**
9. **Disinfectant (Lysol)**
10. **Test tubes and test tube rack**
11. **Hot water bath**
12. **Thermometer**
13. **Ice bath**

Objectives:

1. **Perform a DNA extraction procedure on Escherichia coli.**
2. **Understand the structural features of the DNA molecule that make the extraction work.**
3. **Explain the functions of DNA.**
4. **Understand the molecular structure of DNA, its nucleotides and how these nucleotides bond together to form DNA.**
5. **Understand what a gene is.**

Time Requirements:

1. **Preparation of the lysate takes about 40 minutes and can be stored up to one week at 4 degrees C.**
2. **The extraction of the DNA from the lysate and completion of the procedure will take about 35 to 45 minutes.**

Warnings and Notes:

1. **Ethanol is flammable. Keep it away from Bunsen burners.**
2. **Wash your hands before performing the experiment and immediately afterwards.**
3. **Disinfect your work area immediately upon exercise's completion.**
4. **Rinse out your test tubes thoroughly in the sink when finished.**

Procedure:

1. **Measure out 5 mL of the bacterial suspension solution using a medicine cup and add the freeze-dried *Escherichia coli* (in the spooling tube). Cap the tube very tightly and shake it gently, but thoroughly until the bacterial cells go into suspension (this will require about 5 minutes).**

2. **Measure out 1.0 mL sodium dodecyl sulfate (SDS) with a pipet and add it to the *Escherichia coli* bacterial suspension. Discard the pipet in proper bag in back of lab. Cap the tube tightly and rotate or invert the tube gently so as to avoid excessive bubbling. Repeat this rotation several times over a 5 minute period. *(You may notice that the suspension becomes more viscous as the bacteria are lysed).***

3. **Stand the tube for 30 minutes in a hot water bath preheated to 60 to 65 degrees C.**

4. **Remove the lysate from the water bath. Allow the lysate to cool to room temperature. *(At this point the lysate may be stored up to 1 week at 4 degrees C. If you do refrigerate the lysate, allow it to warm to room temperature before you proceed. Your instructor will tell you if we are going to do this or not).***

5. **As the tubes are cooling back down to room temperature, place the 95% alcohol on ice until needed for Step 7.**

6. **Carefully lower the spooling rod into the lysate in the spooling tube. With a pipet add approximately 3 – 4 mL of the cold 95% alcohol solution to the spooling tube. To prevent aqueous and ethanol layers from mixing, tip the tube at a 45 degree angle, hold the pipet against the side of the tube, and allow the ethanol to flow very slowly down the side of the tube onto the aqueous layer. Discard the pipet in the back of the lab.**

7. **Continue to hold the tube at an angle of 45 degrees so that maximum interface surface is exposed (see fig. 1). Let the spooling rod rest on the bottom of the tube. Slowly rotate the spooling rod in a continuous clockwise direction. Avoid touching the sides of the tube. Fibers of DNA will come out of solution and attach to the glass rod at the interface of the two layers. Continue twirling the rod for several minutes until a visible mass of DNA has attached to the rod.**

Procedure continued:

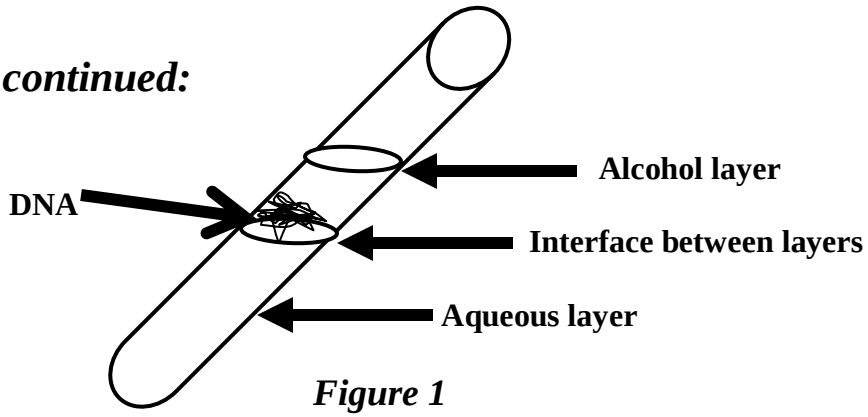


Figure 1

8. **Remove the spooling rod and gently immerse it in a tube of 95% ethanol for 2 minutes.**

9. **Remove the rod from the ethanol and allow the extracted DNA to air dry for 5 minutes.**

Extraction Discussion:

Each cell of Escherichia coli contains a single chromosome of DNA. Sodium dodecyl sulfate (SDS), a detergent used in laundry products, removes lipids from bacterial cell walls (is *Escherichia coli* Gm + or Gm - ?). When the cell walls are damaged, the cell lyses, releasing the contents into the bacterial suspension solution. Along with DNA, enzymes and many other cellular proteins are present in the **lysate**. Enzymes harmful to DNA must be inactivated at this point by heating the suspension to 60 – 65 degrees C, a temperature that degrades proteins (**denatures**) but not DNA. DNA must be heated to about 80 degrees C before it **denatures**. In addition, DNA is protected by sodium citrate, which is incorporated into both the bacterial and DNA suspensions solutions in this exercise. The citrate **ion** is a **chelating agent** with a strong affinity for magnesium ions. These ions are essential to the activity of **DNAase,** the enzyme that degrades DNA and is present in the cell.

The spooling rod is lowered into the lysate. DNA is soluble in water but insoluble in ethanol. Ethanol is mixed with an alkaline **pH indicator**, which is yellow at pH 8.0 and pink at pH 6.5. As the ethanol-indicator solution is added to the lysate, a pink layer develops at the interface, indicating that the lysate has an acid pH. As the rod is rotated, the DNA fibers come out of solution and attach to the rod. The extracted DNA is then soaked in ethanol to stabilize it and is air dried to allow the 95% ethanol to evaporate.

Discussion of DNA:

Deoxyribonucleic acid (DNA) is contained in all living cells. Not only does DNA pass along the genetic information of the parent organism to the offspring, it also directs the synthesis of proteins in the cells. In bacterial cells, DNA is contained in the form of a circular chromosome.

The saying "like begets like" sums up the central principle of genetics: living things breed true in the sense that the offspring of a given creature share so many features with their parents that their kinship cannot be doubted. What is the chemical basis of this ability to keep a biological image constant from one generation to another? **Genes**, the units of heredity, are packets of DNA, the molecule that contains information passed from one generation to the next, as well as instructions for synthesizing and regulating the synthesis of proteins in the cells. A bacterium contains about 3000 genes. Humans have many more genes as bacteria (appears we have about 30,000 genes). There are about 75 trillion cells in the human body, most of which contain DNA (do you know of a mature human cell that doesn't contain DNA?). The average length of the DNA molecule in a human cell is 3 **meters**. If all the DNA in the cells of a human were laid end to end, it would form a molecular ladder stretching for 100 billion kilometers, the equivalent of going to the sun and back over 300 times.

The composition of DNA

The chemical structure of DNA was unlocked in the early 1950s when an Englishman, Francis Crick, and an American, James Watson, developed a three-dimensional model of the DNA molecule by combining what was known about the chemical content of DNA with the X-ray diffraction studies of Rosalind Franklin and Maurice Wilkins. The result was a model for DNA that has since become the symbol for modern biology----the double helix.

DNA (a **macromolecule**) is composed of building blocks (**micromolecules**) called nucleotides, each made up of a **five-carbon sugar (deoxyribose), a phosphate group, and a nitrogenous base** (Fig. 2). The four kinds of nucleotides in DNA differ only in their nitrogenous bases. Two of these bases, **adenine (A)** and **guanine (G),** are **double-ringed structures** or **purines**; the other two bases, **cytosine (C)** and **thymine (T)**, are **single-ringed structures**, or **pyrimidines** (Fig. 3). The sugar of one nucleotide is bonded to the phosphate group of the next nucleotide (Fig. 4). The function of the sugar-phosphate groups is structural, while the nitrogenous bases carry the genetic information.

The composition of DNA continued:

The DNA molecule does not ordinarily exist in a single chain (polymer or strand) but as two chains (strands) oriented in opposite directions. The whole DNA molecule may be compared to a rope ladder twisted on itself (Fig. 5). The sides of the ladder are composed of alternating sugar and phosphate groups; the rungs of the ladder are composed of **complemetary nitrogenous bases, or complementary base pairs.** (A typical gene contains about 1000 base pairs). Each rung of the ladder is composed of one purine and one pyrimidine. The space between the sugar-phosphate sides of the ladder will accommodate a three-ring structure. Therefore, each rung is composed of one pyrimidine and one purine **hydrogen bonded** together. Only in this way can the rungs of the ladder be the same distance apart down the length of the DNA molecule. In addition, adenine can only bond to thymine by two hydrogen bonds and guanine only to cytosine by three hydrogen bonds.

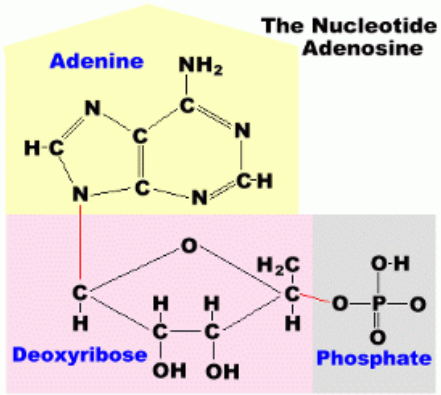


Figure 2

Regardless of the source of DNA, whether microorganisms or man, the amount of adenine equals the amount of thymine, and the amount of guanine equals the amount of cytosine. However, there is wide variation in the ratios of adenine and thymine to guanine and cytosine in different organisms. Among viruses, bacteria and lower plants there is great variation; some species have a preponderance of adenine-thymine bases, while others have a preponderance of cytosine-guanine bases. Taxonomically related organisms exhibit similar ratios of adenine and thymine to guanine and cytosine.

The phosphate and sugar components of DNA are both readily soluble in water. The phosphate groups on the outside of the DNA ladder carry a negative charge. When DNA is in solution **in vitro**, the charges are

The composition of DNA continued:

neutralized by binding to metal ions such as sodium. **In vivo**, positively charged proteins neutralize the negative charges. The chromosomes of plant and animal cells are normally complexed with basic compounds such as **histone**.

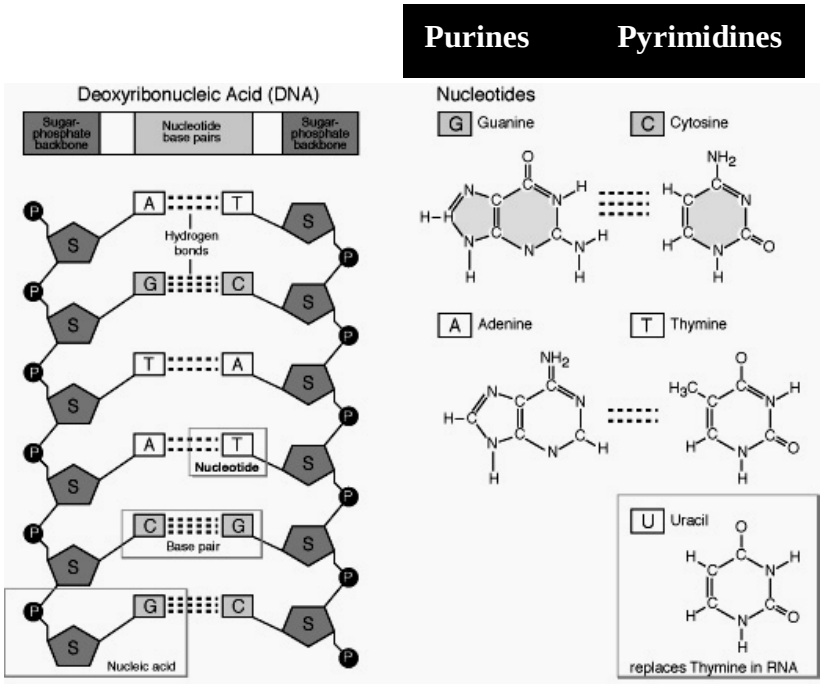


Figure 3

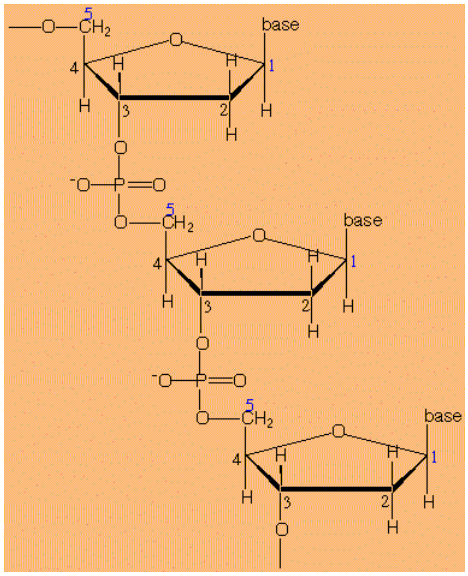


Figure 4

The composition of DNA continued:

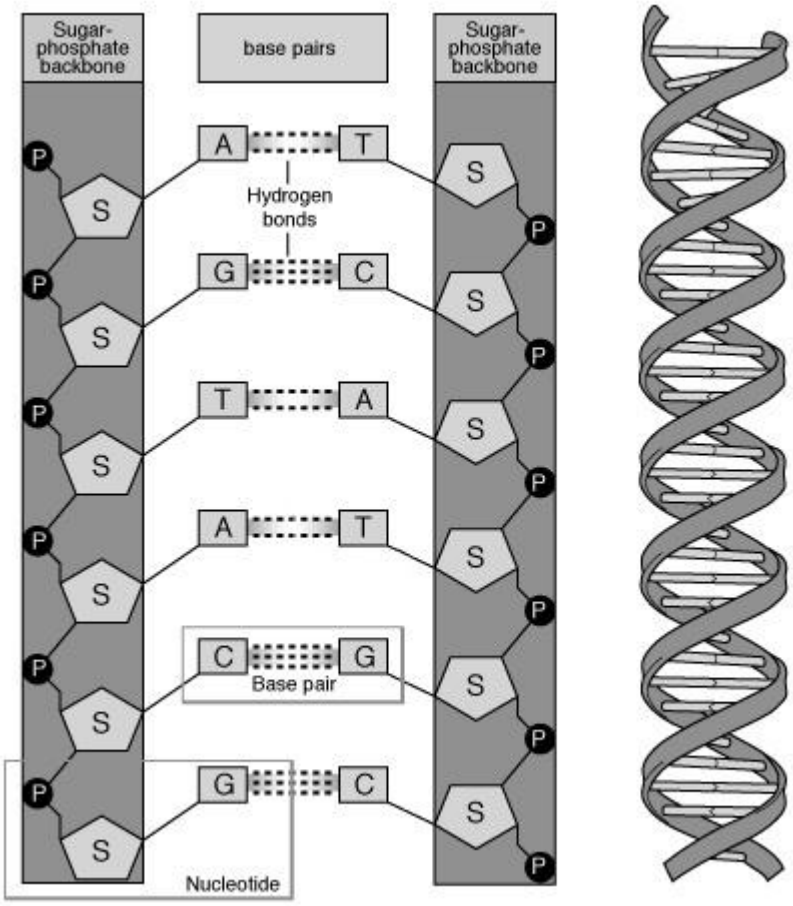


Figure 5

The double helix configuration is chemically very stable because energy is released by hydrogen bonding between two base pairs. Also, the base pairs are stacked one above the other in the double helix like a stack of plates. Energy is released by the hydrophobic bases from the interactions between electron systems of stacked bases. The double helical configuration of DNA is a consequence of the tendency of hydrophobic bases to minimize their contact with water and the tendency of the phosphate-sugar end of the molecule to maximize contact. In addition, the double helix is the most energy-stable configuration possible for the molecule.

One characteristic of DNA is its asymmetry: its length is tremendous in comparison to its width of about only 2**nm**. The length of the DNA molecule makes it very susceptible to cleavage. The DNA fibers can be fractured by processes as simple as pipetting. Because of its length, solutions of DNA are noticeably **viscous**.

The composition of DNA continued:

Functions of DNA

The cell copies DNA to provide instructions for constructing proteins and regulating their synthesis (**transcription or making mRNA from DNA**) and to perpetuate the species (**replication or making a copy of the chromosome**). Replication involves chain (strands) separation and formation of complementary molecules of DNA on each free single strand (fig. 6). Each free strand attracts to itself the very sequence of nucleotides needed to rebuild its partner. Each single strand then serves as a template for the formation of its **complement**. This assures that each daughter molecule is identical to the parent. Each daughter double strand is composed of one strand from the parent and one newly synthesized complementary strand. This characteristic is called **semiconservative replication**.

The sequence of bases on the DNA molecule indicates the sequence of amino acids in the proteins synthesized in the cell. DNA transcribes the instructions for synthesizing amino acid sequencing in a protein in the cell to **ribonucleic acid (RNA),** another type of nucleic acid. RNA differs from DNA in that it is single rather than double stranded, the pentose sugar is **ribose** instead of deoxyribose, and the **pyrimidine uracil** is substituted for thymine, RNA is synthesized along one strand of separated DNA (we say that the double stranded DNA has "unzipped") in a manner similar to replication. This **messenger RNA (mRNA**) then migrates from the nucleus to the **ribosomes** in the cytoplasm where the sequence of nucleotides is translated into an amino acid sequence (and hence a protein). This process is called "**translation**". The amino acids are gathered from throughout the cytoplasm and delivered to the ribosomes by **transfer RNA (tRNA).** Still a third type of RNA, **ribosomal RNA (rRNA),** is a structural part of the ribosomes.

The coding unit of mRNA, or **codon**, must be at least three nucleotides long, allowing for 64 possible combinations of the four nucleotides in the codons. Since there are 20 amino acids, the DNA code is described as being degenerate, meaning that there are synonyms in the genetic code. For example, ACU, ACC, ACA, and ACG all code for the amino acid threonine. In fact, all but two amino acids, methionine and tryptophan, are coded for by more than one codon. There are also start codons to begin protein synthesis on the ribosme's surface and stop codons that signal the end of **translation**.

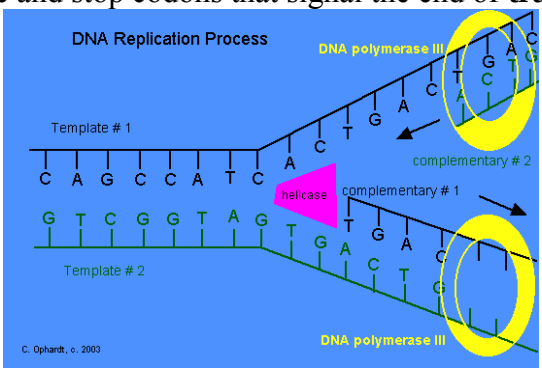


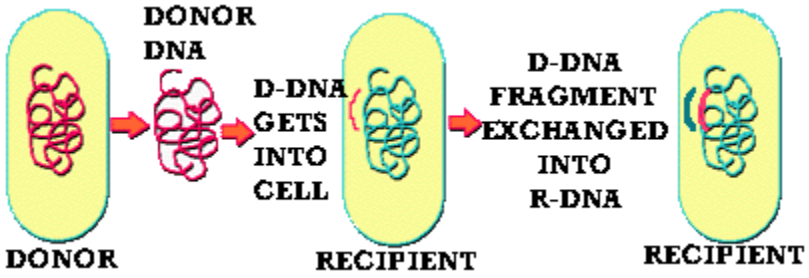
Figure 6

Exercise #11 Questions

1. Draw the general structure of any nucleotide. You can use either circles or squares for the molecular parts.
2. What type of molecular bonding holds the parts of a nucleotide together?
3. Using circles and squares, draw the 4 basic nucleotides for DNA. Be specific when labeling their molecular parts.
4. Which nitrogenous bases in DNA nucleotides are pyrimidines? Are they single-ringed or double ringed molecules?
5. Which nitrogenous bases in DNA nucleotides are purines? Are they single-ringed or double-ringed molecules?
6. What do we call a polymer or chain of nucleotides?
7. Draw a section of one of the chains referred to in question #6. It must be at least 4 nucleotides long.
8. Draw a section of the double helix of DNA. It must be at least 5 nucleotides long. Be specific in labeling its parts and properly show the bonding between all molecules. Make sure all possible bases are used.
9. What is a gene?
10. Draw a gene on a chromosome. Use your own method here.
11. What do genes usually code for in the cell?
12. The average bacterial cell has how many genes?
13. Approximately how many genes does a human have?
14. Describe a typical bacterial chromosome.
15. What holds the chains of a DNA molecule together?
16. What is meant by "complementary base pairing" in DNA?
17. What is the term used when chains of DNA break apart?
18. What is transcription?
19. What is replication?
20. What is translation?
21. List the three types of RNA.
22. Is RNA single or double chained?
23. What is the purpose of each of the three types of RNA?
24. What does semiconservative replication mean?

Exercise #12

Bacterial Transformation



Objectives

1. To be able to define transformation.
2. To understand present day uses of genetic engineering and what organisms are involved (and examples of how they are used).
3. To understand the steps involved in the transformation process and the purpose of the chemicals and other materials used.
4. To precisely follow all steps in the transformation experiment.

Materials

1. Escherichia coli culture
2. Viral plasmid DNA of pGREEN at a concentration of 0.005 ug/ul
3. vial of sterile calcium chloride
4. vial of sterile LB broth, 3 mL
5. 2 15-mL sterile tranformation tubes
6. 6 sterile 1-mL pipets, individually sealed in plastic
7. 1 bottle of glass beads
8. 4 sterile transfer loops
9. wire inoculating loop
10. 3 LB agar plates
11. 2 LB agar plates with ampicillin (LB/Amp)
12. Masking tape

Material Storage

The Eshcerichia coli culture, plasmid DNA, ready-made LB plates and the vial of ampicillin can be stored in the refrigerator for no more than 8 weeks. Other materials can be stored at room temperature (about 25 degrees C).

Biotechnology Discussion

Transformation occurs when organisms receive foreign DNA thereby acquiring one or more new genes or characteristics. Once they receive this new, foreign DNA, they are called **transgenic organisms**. The technology used to accomplish this is known as **recombinant DNA technology**. We also refer to this process as **genetic engineering**. Transferring DNA from one organism to another has already improved our daily lives. In the human biotechnology industry, insulin and growth hormone genes have been transferred into bacteria that now produce as much of these hormones as we need. These genetically engineered bacteria are grown in giant vats known as bioreactors. Another use is the removal of genes from deadly, pathogenic organisms and then by dividing these genes into many pieces, inserting these pieces into the DNA of nonpathogenic bacteria. This allows the pathogenic genes to be safely studied by scientists. Examples of such DNA study are the Ebola virus, HIV and anthrax.

Biotechnology Discussion Continued

Many different organisms have been already used for recombinant DNA technology and we are now seeing the fruits of our genetic engineering labor. Bacteria have been created that can remove sulfur from coal before it is burned; some can help clean up toxic waste dumps. One strain cleans up toxins that normally would kill other bacteria. Interestingly, these toxic-waste, genetically transformed bacteria have "death genes" that terminate their lives once their jobs have been fulfilled. Why do you think it was wise to give them these death genes? Bacteria have been genetically manipulated to produce certain enzymes that give us a product we want such as phenylalanine (which is used in the making of the dipepetide aspartine which is "NutraSweet"). Other bacteria have been manipulated to produce products such as t-PA (tissue plasminogen activator) and Hepatitis B vaccine. Plants have been used to produce hormones, clotting factors (factor VIII) and antibodies. Animals are being engineered to produce pharmaceuticals and therapeutic proteins for things such as cancer, cystic fibrosis and blood diseases. The animal, genetic products appear in and are isolated from the animal's milk. Genetic engineering and the cloning of animals are being used to produce human organs for transplant purposes.

Some bacteria can undergo transformation naturally. *Streptococcus pneumoniae, Neisseria gonnorheae and Haemophilus influenzae* are examples. Each has surface proteins that bind to the DNA and transport it into the cell. Bacterial recombination occurs if the base sequence of the extracellular DNA is similar to a base sequence in a particular region of the circular chromosome. If not similar, it is not incorporated and is broken

down by intracellular enzymes. The DNA-binding surface proteins only bind to particular base sequences in the case of Haemophilus and Neisseria. Haemophilus has about 600 copies of this sequence on its chromosome. Hence, incorporation is likely to occur in it. This assures DNA comes from members of the same species of Haemophilus (the same holds true for Neisseria). *Neisseria gonorrheae* incorporates new genes naturally from other strains of gonorrhea. In gonorrhea organisms, some genes are for surface, stalk-like proteins called pilins that stick out and help the bacterium evade the immune system. We make antibodies against these pilins. However, if a gonorrhea organism undergoes transformation and receives a new gene for a new surface, pilin protein, the immune system has not seen this particular protein pilin before and this fact will allow the newly transformed gonorrhea bacterium to enter the body and evade the immune system. Transformation in nature, however, is not that common for bacteria.

Biotechnology Discussion Continued

What if scientists want to recombine genes into a bacterial cell but the base sequence for this DNA fragment is not similar to any sequence on the bacterial chromosome? Scientists avoid this problem by transforming host cells with **plasmids**. A host cell can be made "**competent**" (making it easier for DNA to enter into the cell). Instead of having the DNA insert at a specific **locus** on the chromosome, entire plasmids can enter the cell, not have to insert on the chromosome, and then can express their gene(s). Competency of cells can be made to occur by heating cells in the presence of increased positive ions in the medium (such as calcium). Plasmids then can more easily enter the cell and then can express their genes in this newly transformed cell.

Plasmids are **extrachromosomal DNA**. They occur in yeast and bacteria. They are small and are double-stranded DNA. Plasmids have an **origin of replication**. An origin of replication is a sequence of bases at which DNA replication begins. By having this origin, a plasmid will be copied during cell replication by the cell's replication enzymes and the new daughter cells will have an exact copy of the plasmid. This is important because it means that plasmids do not need to be recombined into the genome (circular chromosome) to be expressed. Also, since plasmid DNA does not have to be similar to genome DNA, DNA from other organisms can simply be maintained in the cell as a plasmid. Now we don't have to worry about trying to insert foreign genes (recombine) onto the circular chromosome at the location of a similar base sequence, especially since many genes we want to deal with have no sequence of bases similar to the genome's base sequences. It is very easy to introduce plasmids into a cell. Plasmids are

commonly used today to introduce foreign genes into a bacterial or yeast cell. To date, however, we have no analogs of plasmids for higher plants or animals (thus making genetic engineering more difficult in them).

Selecting for Transformed Bacteria

Biotechnologists must overcome two problems in order to transform bacteria:

1. Plasmid-containing cells have a disadvantage over cells not containing the plasmid. Cell resources are diverted from daily cell activities to replicate plasmids for new daughter cells and resources are used to synthesize plasmid-encoded proteins. Because of this, cells lacking the plasmid grow faster than cells that contain the plasmid. This causes pressure on plasmid-containing cells to get rid of their plasmids.

Selecting for Transformed Bacteria Continued

1. To overcome this pressure, the plasmid must contain genes that are an advantage to the cell.

2. Biotechnologists must be able to determine which bacteria received our plasmid. In other words, we need a "**marker**" that informs us that certain bacterial cells (a colony on a growth plate for instance) successfully received the transferred plasmid.

To accomplish both goals (making it an advantage for cells to retain plasmids and also having a marker so we can recognize cells having the new DNA), we will use a system that involves antibiotics and genes for resistance to antibiotics. This is a most powerful tool in biotechnology.

Antibiotics and **antibiotic resistance** should be terms already familiar to you, the student. We will use an antibiotic that is basically the same one for humans. This antibiotic will be incorporated throughout our agar growth medium. We will use, in this experiment, a plasmid that contains a gene for **ampicillin** resistance. This plasmid gene allows a bacterium to produce the protein "**beta-lactamase**" which inhibits the activity of the antibiotic ampicillin. We will transform cells using a plasmid cotaining the beta lactamase gene. Colonies that grow on our plate (that contains the ampicillin) will indicate that we successfully transferred the plasmid into them. Hence, this result will be our marker for success.

During a biotechnology procedure such as the one we are about to perform in this exercise, billions of bacteria are treated and then exposed to plasmid DNA. Usually, fewer than 1 in 1000 will acquire the plasmid. By using our antibiotic

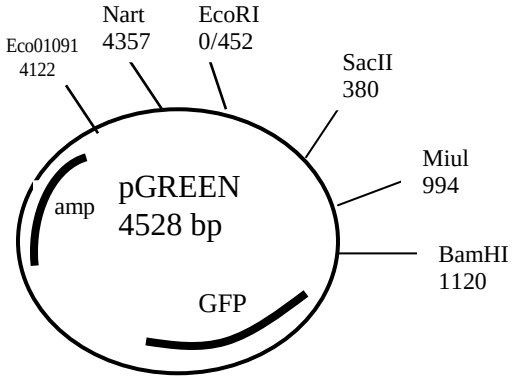
resistance gene, this will allow us to find the bacteria that acquired our plasmid amongst all of the other bacteria who did not. By plating our hopefully-transformed bacteria onto a plate that contains this antibiotic, then the ones that grow will have acquired the plasmid that contained the antibiotic resistance gene. Our plasmid may have other genes on it that we are concerned with (which is usually the case). However, by plating them on a plate containing the antibiotic, we can assure ourselves that the bacteria that do grow on the plate contain the plasmid we wanted to transform them with (along with genes that we were concerned with or wanted to work with). Thus, the antibiotic resistance gene becomes a **marker gene**.

Our plasmid in this experiment contains the gene for ampicillin resistance. Our procedures therefore will use ampicillin to select transformed cells. In addition, our plasmids will also contain a gene called the **GFP gene**. This gene codes for a protein that glows yellow-green, especially under fluorescent light. The letters stand for **Green Fluorescent Protein**. This gene will also act as a marker, even though it is the gene we want to transform our bacteria with in this experiment.

Selecting for Transformed Bacteria Continued

The name of our plasmid that contains both the ampicillin resistance gene and the GFP gene is "pGREEN". So, the pGREEN plasmid contains the GFP gene we want to transfer. The green fluoresent protein glows all by itself (unlike other fluorescent genes in fish, jellyfish, etc.). We say it is "**autoflourescent**". The GFP gene is widely used throughout research.

The GFP gene originates in the aquatic environment from a bioluminescent jellyfish named *Aequorea victoria*. In the ocean, these jellyfish emit an easily seen green glow in the water around their belly-like areas. We don't know why they do this. We are going to place the GFP jellyfish gene into bacteria in this exercise. We will use plasmids containing this gene to do so. The pGREEN plasmid contains the gene for both ampicillin resistance as well as the GFP gene. The colonies of our transformed bacteria will become bright yellow-green (normally they are light yellow-green) and will fluoresce under UV light. Below is a drawing of the pGREEN plasmid and the approximate locus of these two genes on this plasmid:



Exercise 13: AGAROSE GEL ELECTROPHORESIS

Gel electrophoresis is a widely used technique for the analysis of nucleic acids and proteins. Most every molecular biology research laboratory routinely uses agarose gel electrophoresis for the preparation and analysis of DNA. We will be using agarose gel electrophoresis to determine the presence and size of PCR products.

Background:
Electrophoresis is a method of separating substances based on the rate of movement while under the influence of an electric field. Agarose is a polysaccharide purified from seaweed. An agarose gel is created by suspending dry agarose in a buffer solution, boiling until the solution becomes clear, and then pouring it into a casting tray and allowing it to cool. The result is a flexible gelatin-like slab. During electrophoresis, the gel is submersed in a chamber containing a buffer solution and a positive and negative electrode. The DNA to be analyzed is forced through the pores of the gel by the electrical current. Under an electrical field, DNA will move to the positive electrode (red) and away from the negative electrode (black). Several factors influence how fast the DNA moves, including; the strength of the electrical field, the concentration of agarose in the gel and most importantly, the size of the DNA molecules. Smaller DNA molecules move through the agarose faster than larger molecules. DNA itself is not visible within an agarose gel. The DNA will be visualized by the use of a dye that binds to DNA.

Purpose: To determine the presence or absence of PCR products and quantify the size (length of the DNA molecule) of the product.

Materials needed:
- Agarose
- TAE Buffer
- 6X Sample Loading Buffer
- DNA ladder standard
- Electrophoresis chamber
- Power supply
- Gel casting tray and combs
- DNA stain
- Staining tray
- Gloves
- Pipette and tips

Recipes:

TAE Buffer
- 4.84 g Tris Base
- 1.14 ml Glacial Acetic Acid

2 ml 0.5M EDTA (pH 8.0)
- bring the total volume up to 1L with water

Add Tris base to ~900 ml H_2O. Add acetic acid and EDTA to solution and mix. Pour mixture into 1 L graduated cylinder and add H_2O to a total volume of 1 L. Note – for convenience a concentrated stock of TAE buffer (either 10X or 50X) is often made ahead of time and diluted with water to 1X concentration prior to use.

6X Sample Loading Buffer
1 ml sterile H_2O
1 ml Glycerol
enough bromophenol blue to make the buffer deep blue (~ 0.05 mg)

-for long term storage, keep sample loading buffer frozen.

QUIKView DNA Stain

25 ml WARDS QUIKView DNA Stain
475 ml warm water (50-55° C)

Agarose Gel Electrophoresis Protocol

Preparing the agarose gel

- Measure 1.25 g Agarose powder and add it to a 500 ml flask
- Add 125 ml TAE Buffer to the flask. (the total gel volume well vary depending on the size of the casting tray)
- Melt the agarose in a microwave or hot water bath until the solution becomes clear. (if using a microwave, heat the solution for several short intervals - do not let the solution boil for long periods as it may boil out of the flask).
- Let the solution cool to about 50-55°C, swirling the flask occasionally to cool evenly.
- Seal the ends of the casting tray with two layers of tape.
- Place the combs in the gel casting tray.
- Pour the melted agarose solution into the casting tray and let cool until it is solid (it should appear milky white).
- Carefully pull out the combs and remove the tape.
- Place the gel in the electrophoresis chamber.
- Add enough TAE Buffer so that there is about 2-3 mm of buffer over the gel.

Note – gels can be made several days prior to use and sealed in plastic wrap (without combs). If the gel becomes excessively dry, allow it to rehydrate in the buffer within the gel box for a few minutes prior to loading samples.

Loading the gel

- Add 6 μl of 6X Sample Loading Buffer to each 25 μl PCR reaction
- Record the order each sample will be loaded on the gel, including who prepared the sample, the DNA template - what organism the DNA came from, controls and ladder.
- Carefully pipette 20 μl of each sample/Sample Loading Buffer mixture into separate wells in the gel.
- Pipette 10 μl of the DNA ladder standard into at least one well of each row on the gel.

Note – if you are running multiple gels, avoid later confusion by loading the DNA ladder in different lanes on each gel

Running the gel

- Place the lid on the gel box, connecting the electrodes.
- Connect the electrode wires to the power supply, making sure the positive (red) and negative (black) are correctly connected. (Remember – "Run to Red")
- Turn on the power supply to about 100 volts. Maximum allowed voltage will vary depending on the size of the electrophoresis chamber – **it should not exceed 5 volts/ cm between electrodes!** .
- Check to make sure the current is running through the buffer by looking for bubbles forming on each electrode.
- Check to make sure that the current is running in the correct direction by observing the movement of the blue loading dye – this will take a couple of minutes (it will run in the same direction as the DNA).
- Let the power run until the blue dye approaches the end of the gel.
- Turn off the power.
- Disconnect the wires from the power supply.
- Remove the lid of the electrophoresis chamber.
- Using gloves, carefully remove the tray and gel.

Gel Staining

- Using gloves, remove the gel from the casting tray and place into the staining dish.
- Add warmed (50-55°) staining mix.
- Allow gel to stain for at least 25-30 minutes (the entire gel will become dark blue).
- Pour off the stain (the stain can be saved for future use).

• Rinse the gel and staining tray with water to remove residual stain.
• Fill the tray with warm tap water (50-55°). Change the water several times as it turns blue. Gradually the gel will become lighter, leaving only dark blue DNA bands. Destain completely overnight for best results.
• View the gel against a white light box or bright surface.
• Record the data while the gel is fresh, very light bands may be difficult to see with time.

Note – Gels stained with blue stains are stable for long periods. When destaining is complete, remove gel from water and allow the gel to dehydrate. Dark bands can be seen for in a dried gel for weeks or months.

Date _____

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