Lab # 1: Safety First!

Welcome to the laboratory section for Biology 2500 –Microbiology for Nursing and Allied Health. The purpose of this laboratory is provide students with exposure to real-world situations, techniques, and practices that they will encounter as health professionals. To this end, the labs have been designed based on the skills guidelines established by the American Society for Microbiology for such a class:

Skills

I. Discipline specific

A. Practicing laboratory safety
B. Collecting and handling specimens
C. Isolating and identifying microorganism (differentiation)
D. Using a microscope
E. Pipetting and micropipetting
F. Using aseptic technique
G. Growing and controlling microorganisms
H. Utilizing basic antigen-antibody interactions
I. Making dilutions

II. General

A. Effectively communicating scientific information
B. Finding and using appropriate resources
C. Critically evaluating information, results, and incompatibilities
D. Demonstrating ethical behavior and scientific integrity

The objective of the laboratories of this course is to give students training in the above skill sets so that they may proficiently perform these skills once they enter the workforce. Additionally, the labs will closely model real-world situations, so students will have some (albeit artificial) exposure to their future professions. In other words, get ready to handle some pathogens and body fluids!

But before I can feel safe about turning you loose with infectious agents, you need to demonstrate that you can correctly handle and decontaminate such agents. Please bear in mind that special permission has been granted to us to work with such bacteria, and that some of these pathogens are on the government's list of bioterrorism agents (Figure 1). So, dealing with such organisms (which, by the way, you may encounter in a future job!) demands your attention and respect!

Now, while we will only work with a few organisms that are known pathogens, always remember that all microbes are potential opportunistic pathogens, so treat them with respect! Use common sense and good judgment when handling them, and if you ever have a question about what you are doing, ask me!

Before we begin learning a few techniques, let's go over some of the safety rules for the lab.

Safety rules for Biology 2500:

1. Eye protection and closed-toed shoes must be worn at all times! Failure to comply with this rule will prevent you from attending lab!
2. No eating or drinking is permitted in the lab. This should be obvious, but needs to be stated. Additionally, I highly recommend not going outside to grab a quick bite during lab, nor chewing gum. Bacteria have a nasty habit of aerosolizing, and if it should happen while you have your mouth open – let's just say no gum. *E. coli*, *Salmonella*, and *Campylobacter* can cause you to spend your week on a toilet.
3. **NO SMOKING.**
4. At the beginning of the lab, scrub down your work area with alcohol. This will reduce the possibility of contaminating your cultures with other bacteria left from the previous lab or contaminating yourself. Wipe

the table with alcohol at the end of the lab.

5. Books, purses, etc. should be placed at the front of the lab in the areas assigned by the professor. The only things on your lab table should be your handouts, supplies and pen.
6. Don't put the pen in your mouth.
7. Tie back long hair that would be any hair touching the shoulder.
8. Do not wear loose fitting clothing in lab. It is advisable to bring an old shirt or a lab coat to lab. Do not wear clinical uniforms to lab if you plan to return to the clinical setting after the lab.
9. Wash your hands **APPROPRIATELY** before starting and after finishing the lab.
10. Remove all labels from the broth cultures and dump the contents into the bacterial morgue immediately after use. Place tubes with their caps in the racks provided by the bacterial morgue. **DO NOT RINSE THESE TUBES IN THE SINK** instead rinse with water and pour it into the morgue. Then fill the tube half full of water and place in the rack.
11. Do not keep any of your cultures longer than necessary. Be sure to clean out the incubator at the end of every experiment. Failure to do this will result in points being deducted from your total points earned.
12. Do not remove cultures, reagents, plates and other items from the lab.
13. Keep tops on alcohol bottles at all times unless using them. Replace top immediately after dipping spreading tool in the alcohol.
14. Keep all supplies tools, etc in the middle drawer. **DO NOT MOVE EQUIPMENT FROM ONE TABLE TO ANOTHER.** Do not place equipment except microscopes in the cabinets.
15. **NEVER LEAVE THE BUNSEN BURNER UNATTENDED WHEN IT IS LIT**. Turn it off when not in use or if you need to leave your table.
16. Bacterial spills should be cleaned by applying alcohol to the spill and carefully collecting the liquid with paper towels. All used towels are to be placed in the biohazardous waste bin. **IN CASE OF A SPILL, NOTIFY ME IMMEDIATELY, SO I MAY ASSIST WITH THE CLEANUP**.
17. Always carry pipette canisters horizontally.
18. Do not horde media – spare media can be obtained if available by asking the professor. Please do not waste media since it is costly to provide. Always be sure you know what you need to do before opening the media.
19. Try not to touch your face or clothes with your hands during the lab.
20. **NEVER MOUTH PIPET**.
21. Always clean up your lab station – your momma ain't here!

REMEMBER – if you injure yourself, let me know immediately!

Rule 21 is an important one. However, in an effort to get to lunch or class more quickly, students have been known to skip out or not fully perform rule 21. Lack of cleaning includes not removing old Petri dishes from an incubator, forgetting to take the tape off of used tubes, and not wiping down your lab station. As such, I provide a maid service that can be used for a price:

Prices for Maid Services: Removed from the incubator	
Test tubes	5 points each
Petri dishes	5 points each
Bottle	1 point each
Remove tape from test tube	1 point each
Add tape to Petri dishes in disposal bin	1point each
Dump contents from tube into bacterial morgue	1point each
Borrowing goggles	
First time	Free
Each time thereafter	5 points each use
Removal of items from lab table	1 point each
Wipe down lab station	5 points each

Other services can be provided at a fee of my discretion!

Bacterial species that we may be working with in this lab are starred.

Bacillus anthracis	Anthrax
Brucella species	Brucellosis
Burkholderia mallei	Glanders
Burkholderia pseudomallei	Melioidosis
*Campylobacter jejuni**	Campylobacterosis (food poisoning)
Chlamydia psittaci	Psittacosis (Chlamydia, or "The Clap")
Clostridium botulinum	Botulism
Clostridium perfringens	
Coxiella burnetii	Q fever
Cryptosporidium parvum	
Ebola virus	Hemorrhagic fever
E. coli O157:H7	*Escherichia coli* food poisoning
Francisella tularensis	Tularemia ("Rabbit Fever")
Hanta virus	
Marburg virus	Hemorrhagic fever
Rickettsia prowazekii	Typhus fever
Salmonella species	Salmonellosis (Food poisoning)
*Salmonella typhi**	Typhoid fever
Shigella species	Shigellosis (dysentery)
Staphylococcal enterotoxin B	
Variola major (smallpox)	
Viral encephalitis	Alphaviruses [e.g., Venezuelan equine encephalitis, eastern equine encephalitis, western equine encephalitis)
Viral hemorrhagic fevers	Filoviruses [e.g., Ebola, Marburg] Arenaviruses [e.g., Lassa, Machupo])
Vibrio cholerae	Cholera
Yersinia pestis	Plague

Figure 1. A list of Bioterrorism Agents (taken from the Center for Disease Control's website, www.cdc.gov).

Lab #1: Hand washing and Safety

Exercise 1.1 Hand washing Lab

Hand washing is one of the simplest tools we can use to keep ourselves healthy and prevent the spread of disease. Anyone working with pathogenic microbes, food, or toxic chemicals should always wash his or her hands thoroughly prior to leaving the workplace.

1. Squirt 1-2 strokes of lotion from the bottle. .
2. Thoroughly rub the solution into your hands, taking care not to get any on your clothing. Scrape your nails gently on the palms of your hands
3. Examine your hands under the UV light. Note: anywhere your hands are glowing represents a typical covering of bacteria on the surface of your hands.
4. Wash your hands with soap and water as you normally would.
5. Re-examine your hands under the UV light. If you still have glo-germs visible try washing your hands again.

Unfortunately, most individuals **DO NOT** know the proper way to wash their hands. The general rule of thumb is to use moderately warm water and soap (antibacterial soap is not necessary!), scrubbing to create a good lather. Bubbles are vital, as the surface tension created by the bubbles helps to denature proteins. How long should you do it? Experts think that the time it takes to recite the alphabet song (about 30 seconds) is sufficient.

Now think about the signs in the restrooms in restaurants. Why are they there? Do you think the staff follows through on this?

Exercise 1.2: Spread of Germs.
What are your impressions of how quickly and easily germs are spread?

Often when you go to a restaurant, you will find the members of wait staff will keep their billfolds or order pads tucked in the back of their pants. Why is this a health code violation?

Explain why chocolate does not cause acne. What is the most likely cause of occasional (not chronic) acne?

Exercise 1/3: Safety Quiz (True or False) If you answer False then rewrite the statement to make it True.

1. Open-toed shoes are acceptable for lab during heat waves.
2. Antibacterial soap containing triclosan should be used when available.
3. Long hair should be worn pulled back and fastened.
4. *Salmonella* is on the list of potential bioterrorism agents.
5. Although eating is prohibited, chewing gum is acceptable.
6. If you are injured during lab, the first thing you should do is run around screaming until you pass out.
7. Mouth pipetting is acceptable and encouraged, as it is a better way to control the volume.
8. Bunsen burners should always be attended.
9. You are to only wipe down your lab workstation with 95% ethanol after each lab exercise.
10. You are to use only 95% ethanol to wipe down your lab workstation after each lab exercise.

11. The eyewash station doubles as an in-lab water fountain.

12. All biohazardous waste goes into the biohazard waste bin, including Petri dishes you are not sure have cultures on them.

13. Proper hand washing includes using warm water, soap, a good lather, and a minimum of 10 seconds.

14. If you have not lit the Bunsen burner after 7 seconds, keep trying, as it is only a matter of time!

15.. Not only are lab goggles stylish, but essential for lab safety!

Lab #2: The Ubiquity of Microorganisms

Microorganisms are everywhere. From your skin to your clothes to the food you eat, microorganisms cover everything. In fact, of the 10^{13} cells that make up your body, 90% of those are bacterial in nature! Although this may sound disturbing, the simple fact is that humans and microorganisms have had a (relatively) peaceful co-existence for thousands of years. Without microbes, we would not be able to digest food properly, be more susceptible to harmful pathogens, and have an atmosphere unsuitable to life as we currently know it.

In a setting where biological control of microbes is paramount (such as a clinical lab or site of health care), the constant presence of life poses problems by causing continual contamination. As such, care must be taken to prevent accidental **contamination** – the carryover of unwanted microbes into undesired places. This can be done be utilizing **aseptic technique** – the laboratory methods used to prevent unwanted contaminants.

In this lab, you will get a feel for how omnipresent microbes are in our world. We will be using a variety of media (singular, medium) – the material we use to grow the bacteria. In addition, you will learn how to transfer bacteria and media using aseptic techniques.

Exercise 2.1 – Flaming glassware.

The objective of this exercise is to learn how to use the Bunsen burner in the process of aseptic technique.

Whenever you are using the Bunsen burner, remember the rules! Never leave a burner in use unattended; hair is to be pulled back, and remember to light within 7 seconds after turning on the gas or turn it off and start over!

The Bunsen burner, of course, uses the heat from the flame to kill microorganisms off the surface of an instrument or glassware you are working with. As a side benefit, the burner causes an up-draft in the local air currents (remember that heat rises!) helping to prevent microbes from dropping out of the air, usually from air vents and into the bottle or tube of sterile media. Bunsen burners can also be used in conjunction with alcohol for sterile technique. When using this method, the alcohol (usually 95% ethanol) does the sterilizing. The burner is used to rid the instrument of any excess alcohol that might kill the sample you are working with.

1. Carefully light the Bunsen burner by turning on the gas and depressing the pressure plate. You then strike the flint to create a spark.
2. Take the bottle of Tryptic Soy broth (TSB), and unscrew the cap.
3. Quickly pass the lip of the bottle through the flame, taking care **NOT** to leave the bottle in the flame for more than a few seconds.
4. Return the cap back to the bottle.
5. Now practice the same procedure using an empty capped sterile test tube.
6. Once you are proficient at this, master the next step by doing it with **ONE HAND** and **NO ASSISTANCE** from your lab partner.

Why one hand? Because in the next few exercises, you will be transferring media from one container into another. If you use two hands (one to hold the cap, the other the bottle), your hands are tied up to get the pipette. The natural reaction is to place the cap on the bench top, leaving it susceptible to contamination. Learning how to do it now will allow you to do future lab techniques more quickly and more correctly.

Exercise 2.2: Handling solid media.

One of the most common forms of media is solid media. It is basically the same as the broths you will use to grow bacteria, with one exception: it contains 1.7% weight/volume agar, a solidifying agent derived from seaweed. This mixture is poured into **Petri plates** while it is still molten from the autoclave used to sterilize it, and allowed to cool and harden in the Petri plate.

1. Examine the Tryptic Soy agar (TSA) plate in front of you. Whenever you need to gain access to the plate, lift the lid off of the plate, keeping it in front of your face. This is to prevent your breath from getting onto the plate, and will help prevent anything from the plate getting into your lungs!
2. Lift the lid and touch the surface of the plate with your finger tip or the side of your hand. Note how the agar feels.
3. Return the lid to the plate. Turn the plate upside down. All plates are to be stored upside down – this will prevent any condensation from the lid from falling onto the surface of the plate.
4. On the bottom of the plate, write the date, your initials, and lab section. Also, label the plate with what is on it. (Example – finger, or hand). Secure the plate with the masking tape you will find in your middle drawer. These will go in the 37°C incubator at the end of the lab.

Exercise 2.3: Sterilizing the inoculating loop.

The metal wire loop on your lab table is an excellent tool for transferring bacteria from one source to another. The process of transferring bacteria from one source to a new one is called **inoculation**. The bacteria transferred are termed the **inoculum** (plural **inocula**).

1. Hold the sterilizing loop like you would a pencil.
2. To sterilize the loop, pass the loop at an angle through the blue flame, until the entire wire is glowing orange. DO NOT leave the loop in too long – the wire can melt off!
3. Before transferring an inoculum, make sure the loop has had time to cool. A few seconds will suffice. If transferring to a new media plate, you can cool the loop by touching to the STERILE surface of the plate – just take care not to damage or contaminate the plate
4. Remember not to set the loop down again, as it will no longer be sterile!

Exercise 2.4: Aseptic transfer of liquid media.

1. Label the tube using Masking tape with your initials, date, and lab section and "-".
2. Carefully light your Bunsen burner.
3. Remove a pipette from the canister. Do not touch the tip, as you will contaminate it!
4. Attach the pipette aide to the pipette. Briefly sterilize the pipette tip by passing through the flame.
5. USING ASEPTIC TECHNIQUE, transfer 5 ml of the TSB from the bottle to an empty sterile test tube. After everyone at the table has finished using the bottle label it with everyone's initials, lab section and Table number.
6. We will place this in the 37°C incubator after class. IF ANYTHING GROWS, YOU MUST ARRANGE WITH THE PROFESSOR TO REPEAT THIS TEST PRIOR TO THE NEXT LAB! This will demonstrate your ability to perform sterile techniques.

Exercise 2.5: Inoculation of an *E. coli* liquid culture.

This is your first go at inoculating a culture. Take care not to kill your bacteria!

1. Use the tube of 5ml of TSB provided for each person at the table and label it with your initials, lab section and "+".
2. Sterilize your inoculating loop.
3. Pick a colony from the plate labeled *E. coli* on your bench top. Note any smells you detect when you open the plate. Take care not to contaminate the plate! To do this, all you need is to touch the individual colony on the plate. Each colony has millions of bacteria, so you do not need to see anything on the loop to ensure a successful inoculation.
4. Using aseptic technique, place your inoculated loop into you test tube containing the media and briefly swirl around.
5. Remove the loop, pass the tube through the flame and recap the tube.
6. Re-sterilize the loop. TAKE CARE WHEN DOING THIS! A wet loop will splatter (and aerosolize any bacteria) if placed directly into the flame. Slowly move the loop to the flame. This allows the loop to dry before sterilizing.

   6. Label the tube with Masking Tape and put aside. We will place this in the 37°C incubator.

   This serves as a positive control for contamination. *E. COLI* MUST BE PRESENT IN THIS TUBE, OR ELSE YOU MUST ARRANGE WITH THE PROFESSOR TO REPEAT THIS TEST PRIOR TO THE NEXT LAB! This will demonstrate your ability to sterile inoculate bacterial cultures. ADDITIONALLY, THE *E. COLI* PLATE WILL BE PLACED INTO THE INCUBATOR TO DETERMINE HOW STERILE YOU AND YOUR LABMATES WERE. If anything grows on this plate, YOU MUST ARRANGE WITH THE PROFESSOR TO REPEAT THIS TEST!

Exercise 2.6: Starting your unknown body organism.

To better demonstrate the ubiquity of microorganisms on our bodies, I am inviting you to test some external area of your body for the presence of microbes. Through the course of the semester, you will determine certain characteristics of your microbe, which will be stored and used for future microbiology labs!

1. Each person should take one fresh TSA plate. Also on your bench are sterile cotton swabs DO NOT OPEN THE SWAB UNTIL YOU ARE READY TO USE IT!
2. When you have decided what body part you want to swab, carefully open and remove the swab. Take care not to touch the cotton end.
3. Dip the swab into the sterile water and swab the body part using 3-5 strokes with the cotton swab.
4. Immediately and quickly swab the plate using aseptic techniques.
5. Cover the plate, label it, and place it in the **37°C** incubator.
6. Dispose of the used swab in the beaker with alcohol on top of the flammables cabinet.

Use this space to record any observations or to take any notes you would like. This for your benefit, and will help you in studying or repeating any labs.

Laboratory #3: Tools of the Trade

Modern-day microbiology involves cutting edge technology and relies heavily on advances in molecular biology. However, basic techniques are still used today and are essential for any microbiologist. In this lab, students will be exposed to these basic techniques: isolation of bacterium from a mixed culture, basic microscopy, and differential staining (the Gram stain). You are expected to become comfortable and proficient in all of these techniques during the lab period. Once you have acquired these skills, you will employ them numerous times throughout the semester, and on your identification of your bacterial unknown.

PART A
Exercise 3.1: Isolation of bacteria from an established culture.

Arguably the most essential technique for any microbiologist to master is the T-streak isolation. This process involves taking cells from an established culture and performing a dilution in order to obtain a single isolated cell. When this is done on solid media (an agar plate), this single cell should, given the proper growing conditions, begin dividing to establish a bacterial colony. This colony, in theory, should be essentially genetic clones of the original cell. This allows microbiologists to work with clonal populations of bacteria, essential for obtaining consistent results from experiments.

Refer to Figure 3.1 for assistance.

1. Draw a large "T" on the underside of your tryptic soy agar (TSA) plate, with the lines touching the edge of the plate.
2. Sterilize your inoculating loop.
3. Once cooled, dip the loop into the tube labeled *E. coli*. The loop should have a film of culture across its opening. This small volume contains millions of cells. You will dilute these cells to a single bacterium.
4. Starting at the edge of the TSA plate, swab the loop back and forth in the top third of the plate, located above your "T".
5. Flame your loop again, taking care not to aerosolize the bacteria still on the loop.
6. Cool the loop by touching a sterile area on the edge of the TSA plate. Once the loop cools, carefully touch the third on which you previously spread the bacteria.
7. Without picking up the loop, drag the loop to the third of the TSA plate to the right of the stem of the "T". Without picking up the loop, swab the plate by moving the loop back and forth to the stem of the "T", taking care not to cross over into another third OR going back over the previous streaks.
8. Flame your loop again, taking care not to aerosolize the bacteria still on the loop.
9. Cool the loop as before and once the loop cools, carefully touch the third on which you previously spread the bacteria.
10. Without picking up the loop, drag the loop to the third of the TSA plate to the left of the stem of the "T". Without picking up the loop, swab the plate by moving the loop back and forth to the stem of the "T" taking care not to cross over into another third OR going back over the previous streaks.

11. Place the lid on the plate, label the bottom of the plate with your name, lab section, contents and date. Tape the plate shut and place in the 37°C incubator on your lab sections shelf.

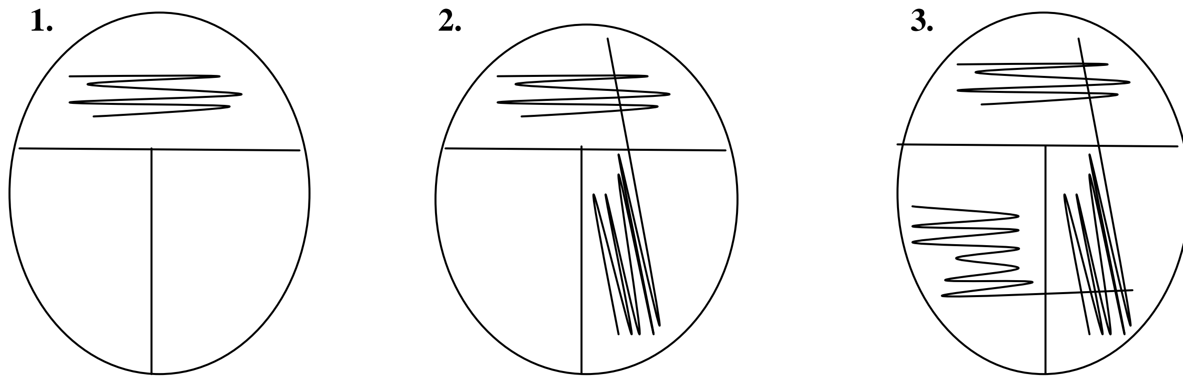


Figure 3.1 "T streak" method of isolation.

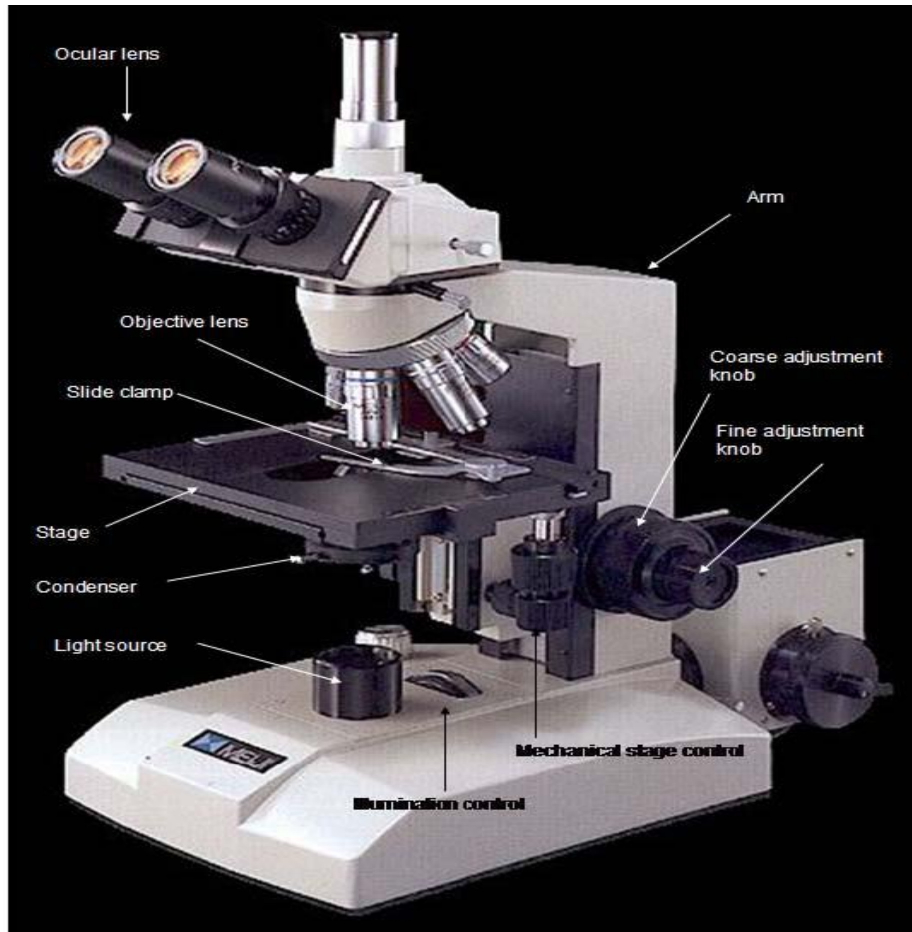
Exercise 3.2: Isolation of *Serratia marcerans* and *Eschericia coli* from a mixed culture.

The real power of the "T" streak can be seen when one is trying to isolate a bacterium from a mixed culture. A mixed culture is a bacterial culture that consists of 2 or more different types of bacteria. In this exercise, you will separate the bacterium *Serratia marcerans* from *Eschericia coli*. *Serratia marcerans* is unique in that when grown at lower temperatures (30°C), it produces a red pigment that makes cells and colonies red. At higher temperatures (37°C), it fails to produce this pigment and appears white, the same color as *E. coli* colonies (which do not produce any pigment regardless of temperature).

1. Using the T-streak isolation method, isolate single colonies of *S. marcerans* and *E. coli* from the tube labeled "Mix 1".
2. Label this plate, tape it shut, and place in the 30°C incubator.
3. Pick a colony from your unknown body organism plate and inoculate the 3 mL of sterile TSB provided at your table. Label tube with initials, lab section and date and place it in the 37°C incubator. Get a TSB slant from the rack on the handicapped station label it and put it back on the handicapped station. Tomorrow take a loopful of bacteria from your body organism tube and streak it up the slant. Then put the slant in the 37°C incubator.

Exercise 3.3: Using the microscope.

Perhaps no tool is more recognizable in microbiology than the microscope. Before we examine specimens under the scope, students must be comfortable with handling and using the microscope. TAKE CARE – microscopes are high precision instruments and are thus expensive. Identify the different object lenses located on the scope.



The concept of magnification and use of oil immersion.

Magnification is the apparent increase in size of an object. This is different from resolution, which is the clarity of an image. On a compound microscope, the magnification is calculated by multiplying the ocular lens power by the objective lens power. Thus, **Ocular Lens x Objective Lens = Total Magnification**. So if you are using the 40X objective and an ocular lens of 10X, the total magnification is 10X x 40X = 400X.

However, most microbes are too small to be seen clearly enough to distinguish Gram stain and shape with any objective lens other than the 100X lens. This lens requires the use of immersion oil. This is due to the fact that air has the capability to bend and scatter light (refraction), which is required for viewing. At lower objectives, the lens is large enough to allow sufficient light into the lens despite some refraction. The size of the 100X lens is smaller and less forgiving – air scatters enough light to prevent a clear image. By using immersion oil, the light will not be refracted as much, allowing for more light to enter the lens, resulting in the best possible image. Whenever you are observing bacteria, you will start with 10X objective focus, progress to 40X objective focus and finally use the 100X objective and immersion oil.

Exercise 3.4 Calibration of the Binocular Compound Microscope

1. Obtain a slide of a prepared specimen from the box of slides on the handicapped station.
2. Place the slide on the stage and find the specimen with the 10x objective.
3. Pull the eyepieces all the way apart and then push them together until you see one image. Record the micrometers on the scale between the eyepieces here:_______.
4. Adjust the diopter on the right eyepiece until the scale on its left side is set to the number recorded in step 3.
5. Close the left eye and look through the right eyepiece with the right eye. Focus the specimen using the fine focus.
6. Now cover the right eye and look through the left eyepiece. **DO NOT ATTEMPT TO FOCUS WITH THE FINE FOCUS.**
7. Focus the specimen by turning the left diopter. Record settings for both eyepieces here: Right:__________ Left:__________.
8. The microscope should now be calibrated for your eyes. If you now look through both eyepieces, the specimen should be in focus. You may have to use the fine focus slightly.
9. Always set the microscope for your eyes before you look through the scope.

Exercise 3.5: Preparing and viewing a wet-mount slide.

When viewing a live specimen is required, a **wet-mount slide** is required. This involves taking a specimen and adding it to liquid on the surface of the glass slide. A glass coverslip is placed over the sample, and a drop of immersion oil is added on top of the glass coverslip. The sample is then viewed using the microscope.

1. Obtain a sample of the *Rhodospirillum* culture from the tube labeled "*Rhodospirillum*" using a sterile inoculating loop.
2. Place it on a glass microscope slide.
3. Place a glass coverslip over the sample as demonstrated. *Hint: try to prevent the formation of air bubbles!*
4. Focus on the 10 X and then 40 X next move the 40X objective out of the way and add a drop of immersion oil on top of the coverslip and view using the 100X lens
5. In the space below, draw what you see.

PART B

Exercise 3.6: Heat fixation.

Often, viewing microorganisms using light microscopes can prove difficult; specimens appear more like small rod-like bubbles. This is due to the amount of light that is passing through them – lots of light washes out the image. To offset this problem, microbiologists will stain the samples with a variety of dyes.

Some dyes will stain a variety of features common among many different cells, turning the entire specimen a particular color. Such a stain is referred to as a **simple stain**, since it stains the sample indiscriminately. More involved stains may stain a particular structure or organelle. Still other stains are used to differentiate one microorganism from another. These procedures are referred to as **differential staining**. The most useful differential staining technique is the **Gram stain**.

Prior to staining, specimens usually require a **heat fixation** step. Heat fixation involves preparing a sample similar to a wet mount, but then you let it air dry and add an additional heating step and does not require a coverslip. After the sample is air dried on the glass microscope slide, the slide is passed through the flame of the Bunsen burner several times. This step has three desired effects: 1) the sample is affixed to the slide, making it less likely to be washed off; 2) the sample is killed, protecting you from any potential hazard; and 3) it makes the cell more permeable to the dye, allowing for optimal staining.

1. Draw a circle under the slide using the wax pencil in the middle drawer.
2. Obtain a sample of the culture from the tube labeled "Mix 1" using a sterile inoculating loop and aseptic technique. Place it on a glass microscope slide.
3. Allow the sample to air dry. Then clip the slide into a clothes pin.
4. Pass the sample slowly through the flame, taking care not to allow it to stay too long in the flame.
5. *Do not place slide on the bench – it is cold and will cause the hot slide to crack!* Instead place it on the staining rack.

Exercise 3.7: The Gram Stain.

The Gram Stain is a differential staining technique developed by Danish microbiologist Hans Christian Gram. The stain differentiates bacteria as either Gram negative or Gram positive. As the following diagram shows, Gram positive cells have their cell membranes surrounded by a thick peptidoglycan layer. By contrast, Gram negative cell membranes are surrounded by a thin layer of peptidoglycan, which itself is surrounded by an additional lipopolysaccharide layer. After heat fixation, the slide is flooded with the primary stain, crystal violet. The dye binds to the peptidoglycan layer of the cells. After the excess dye is rinsed off with water, a mordant, Gram iodine, is added. This serves to enhance the binding of the crystal violet. Following this, a decolorizing step using ethanol is carried out. The ethanol works to remove the crystal violet. Since the Gram positive cells have a thick peptidoglycan layer, the dye does not rinse out easily.

Gram negative cells, with their much thinner peptidoglycan layers, easily lose the dye. The cells are subsequently counterstained with safranin, a pink dye. Both cells acquire the counterstain dye; however, since the Gram positive cells maintain the purple dye, they appear purple, while Gram negative cells appear pink. (See Figure 3.3)

1. Take a glass slide and place 3 circles the size of the end of your pinkie under the slide in a row using the Wax pencil in the middle drawer. Label the first one "-" the middle one "u" and the last one "+". Using a sterile loop and aseptic technique smear the "-" with the negative control, the "u" with the tube labeled mix 2 and the "+" with the positive control. Let all air dry.
2. Heat-fix the slide as before.
3. After the slide has sufficiently cooled, flood the slide with crystal violet for 60 seconds.
4. Using the squirt water bottle carefully rinse the slide with water. Be careful to let the water run down over the samples. NEVER squirt the water directly onto the sample as this may cause it to rinse off. Shake off excess water. DO NOT BLOT OR WIPE SLIDE!!
5. Flood the slide with iodine for 60 seconds.
6. Rinse the slide with water as described in step 4.
7. Rinse the slide with the decolorizer (ethanol) by adding it for approximately 15 seconds.
8. Rinse the slide with water as described in step 4.
9. Flood the slide with safranin for 60 seconds.
10. Rinse the slide with water as described in step 4.
11. Use the Bibulous paper to blot the slide to dry, and then view the slide.

NOTE: Whenever you do a Gram stain in lab from this point on, you must ALWAYS do a known Gram positive and known Gram negative controls on the same slide EVERY time!

E. coli are the bacilli, and *Staphylococcus aureus* are the cocci.

Which is Gram positive?

Gram negative?

Next week, determine whether your unknown body sample is Gram positive or Gram negative. This result is due when we do antibiotic sensitivity testing of your body organism.

Use the space below to take any notes you wish.

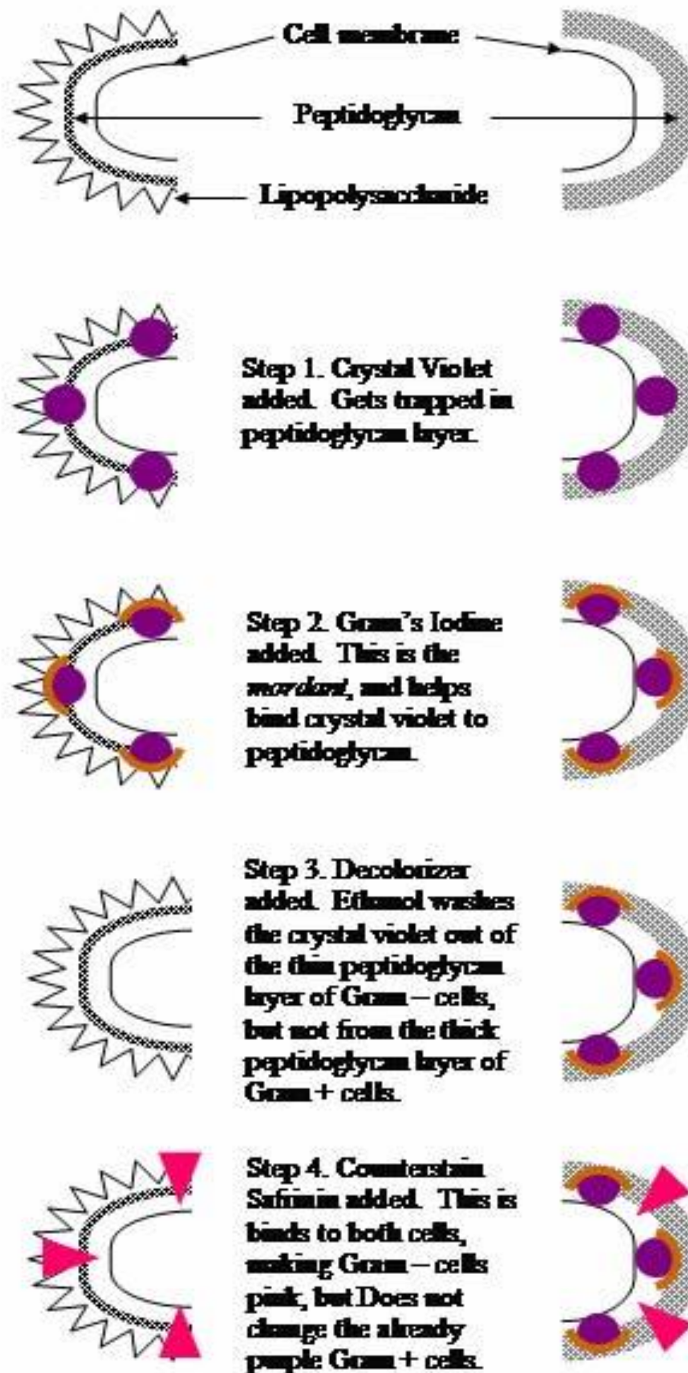


Figure 3.3 This indicates the presence or absence of Crystal Violet dye from the cell wall after the various steps of the Gram's stain.

Laboratory #4: Monitoring Bacterial Growth

Much of the new species of bacteria that have been identified over the last ten years have been detected via DNA sequence alone. Why? Many of the bacteria cannot yet be cultured in the laboratory because the conditions for growing these bacteria have yet to be established. Factors such as nutrients, atmospheric conditions, pH and temperature all have an impact on growth, and researchers must determine suitable conditions for each species of bacteria.

Once these conditions have been established, researchers can begin to study bacteria in controlled laboratory environments. Bacterial growth is one of the most fundamental characteristics to study, as it is easy to monitor and manipulate. In this lab, you will be exposed to two fundamental ways to monitor growth: **optical density** and **viable counts**.

Exercise 4.1: Monitoring growth of a liquid culture.

The most fundamental way to measure bacterial growth in a liquid culture is to measure the optical density. The optical density is a measure of turbidity, or cloudiness, of a liquid culture. As the number of cells increase, the cloudier the solution becomes. To measure the turbidity, researchers employ the use of a spectrophotometer. A spectrophotometer can measure the amount of light emitted from a light source- transmittance or the amount of light absorbed by the culture - absorbance. (See Figure 4.1)

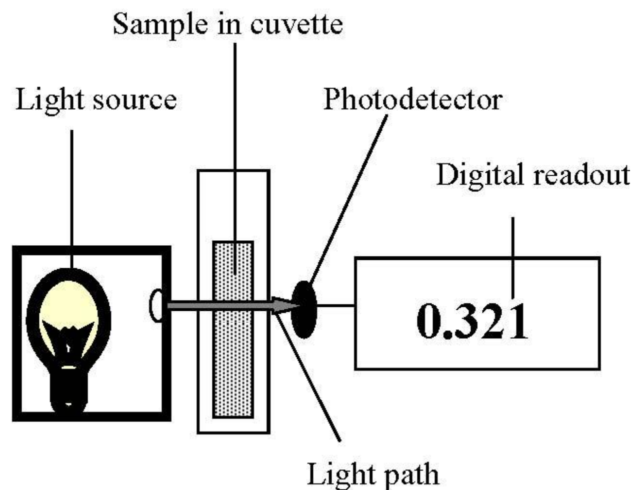


Figure 4.1. Basic setup of a spectrophotometer

The typical wavelength of light used to measure bacterial growth is 600 nm. Once the wavelength is set, a cuvette filled with sterile TSB is placed into the cuvette and the machine zeroed (that is, the spectrophotometer is calibrated to the turbidity of sterile TSB(no turbidity). This is the reference. A small volume from the growing culture is removed, placed into the cuvette, which is then analyzed in the spectrophotometer.
As the number of cells increase, the ability of the spectrophotometer to detect the correct

absorbance becomes compromised. This limit is usually around the optical density (OD) reading of 0.700. At this point, light can be scattered to the point where not all bacteria are allowed to light (See Figure 4.2).

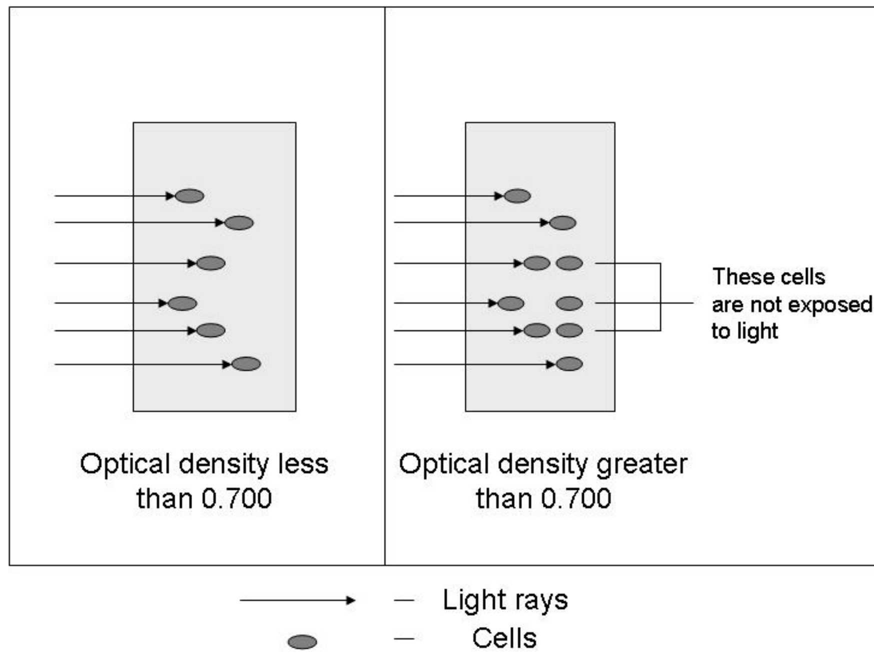


Figure 4.2: Diagram representing the limitations of a spectrophotometer when the optical density is greater than 0.700

Figure 4.2. Limitations of the spectrophotometer.

When this occurs, the user must dilute the culture with the media the culture was grown in. For instance, if the OD reading was 0.888 for a 3 ml sample, the limit of the spectrophotometer has been met. The user would than take 1.5 ml of the culture and add 1.5 ml of fresh media (a 1:1 mix, or a 1:2 dilution). The user would then take the reading, and multiply by the dilution factor (in this instance, 2). So if the reading for the diluted culture was 0.451, the actual OD would be 0.902.

1. Watch the demonstration on how to use the spectrophotometer.
2. Zero the machine. Take the starting culture and measure the absorbance. Record the time as 0 minutes.
3. Take a reading every 10 minutes throughout the lab as directed by the instructor. Dump contents of cuvette in bacterial morgue then clean cuvette with ethanol dump in morgue and drain the cuvette on a paper towel.
4. Record the values below.

Exercise 4.3: The logarithmic scale.

Since binary fission is the form of cell division for bacteria, bacterial numbers will double with each round of division. If we start with a single bacterium, one round of cell division results in 2 cells, another, 4, then 8, 16, 32, 64, 128, 256, 512, 1024, 2048, 4096, 8192, 16384, and so on. The numbers increase dramatically. If you were to plot this as a function of divisions, your graph would be larger than the building! However, by plotting this as a logarithmic function (by powers of 10), one can easily graph and visualize any trends in growth. This will produce a straight line if the bacteria are in the log phase of growth. This allows us to see the doubling of the bacteria. If we were to plot the number of bacteria versus time, we could calculate the doubling time of the bacteria and we could see the various phases of bacterial growth. Note at the first few points of the graph in Figure 4.3, there is no increase. This is called the **lag phase** of growth, and is the point where the cells are adapting to the environment and preparing for maximal growth. The curve then rapidly increases in almost a linear fashion; this is the **log** or **exponential phase** of growth, where the bacteria are dividing at a maximal rate. As they continue to grow, the readings stabilize, and the curve flattens. This represents **stationary phase,** the period where the cells have used most of the nutrients in the media, and begin to prepare to exist in an environment less suitable for growth.

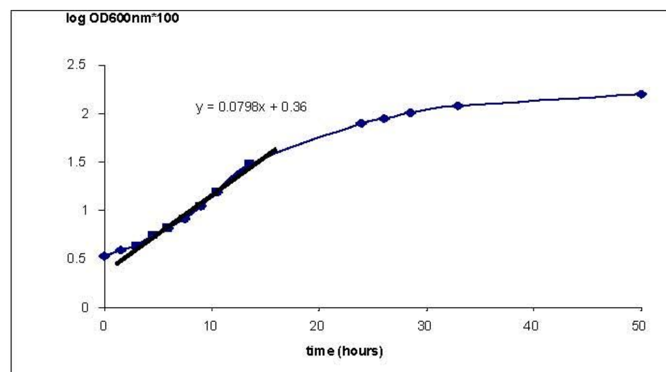


Figure 4.3. Growth curve of *Campylobacter jejuni.* (*Courtesy of J. Andrus*)

WORK IN GROUPS OF FOUR

1. Pick up the pennies on the table (50). Count them to be sure you have 50 pennies.
2. Carefully drop the pennies onto the bench.
3. Count the number of tails showing and record.
4. Remove all the pennies showing heads.
5. Pick up those displaying tails and repeat steps 2 through 4 until there are no pennies displaying tails.
6. Repeat the entire experiment one more time.
7. **Average the results** and plot the numbers on a graph using Microsoft Excel. **Note, if Trial #1 had 7 throws and Trial #2 had 11 throws, extend the number of throws for Trial #1 to 11 and use 0 for the number of tails observed for throws 8-11..*

Your Y axis will be the *dependant variable* (your # of Tails) and the X – axis will be your

independent variable (Throw). If you are having trouble graphing, take the tutorial at http://www.mistupid.com/viewlets/excel/xlgraphs.htm. (HINT: The best graph type will be the X-Y scatter plat with connected data points!) Be sure to put a title on each axis and a title on the graph that is not the axis titles but gives a description of what the graph shows. **Your individual Excel graph is due at the beginning of the next lab period.**

Exercise 4.4. Number of viable bacteria in a culture of *E. coli.*
While measuring the turbidity of the culture is effective to determine growth rates of bacteria, it is not without drawbacks. Of greatest concern is the fact that it does not discern between living and dead bacteria, which for all practical purposes, absorb light at 600nm equally. Likewise, examining bacteria under a microscope does not distinguish between living and dead cells. To determine the number of live cells in a culture, researchers must use **viable plate counts**, which roughly determine the number of living cells. This technique involves plating dilutions of the culture to determine the number of viable cells that can produce a colony on an agar plate.

To effectively carry this out, we are going to perform dilutions in an effort to obtain one plate containing somewhere between 30 – 300 colonies on it. This range has been determined to most accurately reflect the number of viable cells readable on a plate.
When performing this technique, remember to always use sterile technique, and to always vortex dilutions! (**NOTE: no extra tubes will be provided!**)

WORK IN GROUPS OF FOUR

1. Fill 2 test tubes with 9.9mL of sterile water. Label them 10^{-2} and 10^{-4}.
2. Fill 3 test tubes with 9.0mL of sterile water. Label them 10^{-5}, 10^{-6}, and 10^{-7}.
3. Finger vortex or mix the tube labeled "*E. coli*". Using the 1.0 mL pipette remove 0.1mL of the culture and transfer to the 10^{-2} tube.
4. Finger vortex or mix the 10^{-2} tube. Remove 0.1mL of the dilution and transfer to the 10^{-4} tube.
5. Finger vortex or mix the 10^{-4} tube. Remove 1.0mL of the dilution and transfer to the 10^{-5} tube.
6. Finger vortex or mix the 10^{-5} tube. Remove 1.0mL of the dilution and transfer to the 10^{-6} tube.
7. Finger vortex or mix the 10^{-6} tube. Remove 1.0mL of the dilution and transfer to the 10^{-7} tube.
8. Label four plates 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}.
9. Remove 0.1mL of the dilution from 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} tubes and add it to the 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} plates, respectively. (**REMEMBER TO VORTEX EACH SAMPLE PRIOR TO REMOVING!**)
10. Sterilize a metal spreader by dipping in alcohol. Light the alcohol on fire and remove the spreader from the flame and when the flame goes out spread the added culture around on each plate. **EVERYONE SHOULD DO AT LEAST ONE DILUTION AND ONE PLATING**.
11. Tape plates together and place in the 37°C incubator. Check samples after one day, and record the number of colonies on the plate that has 30-300 colonies. Wrap up the plate

used in parafilm, and place on bench – I will collect and store them. Dispose of the other plates in the biohazardous waste bin.

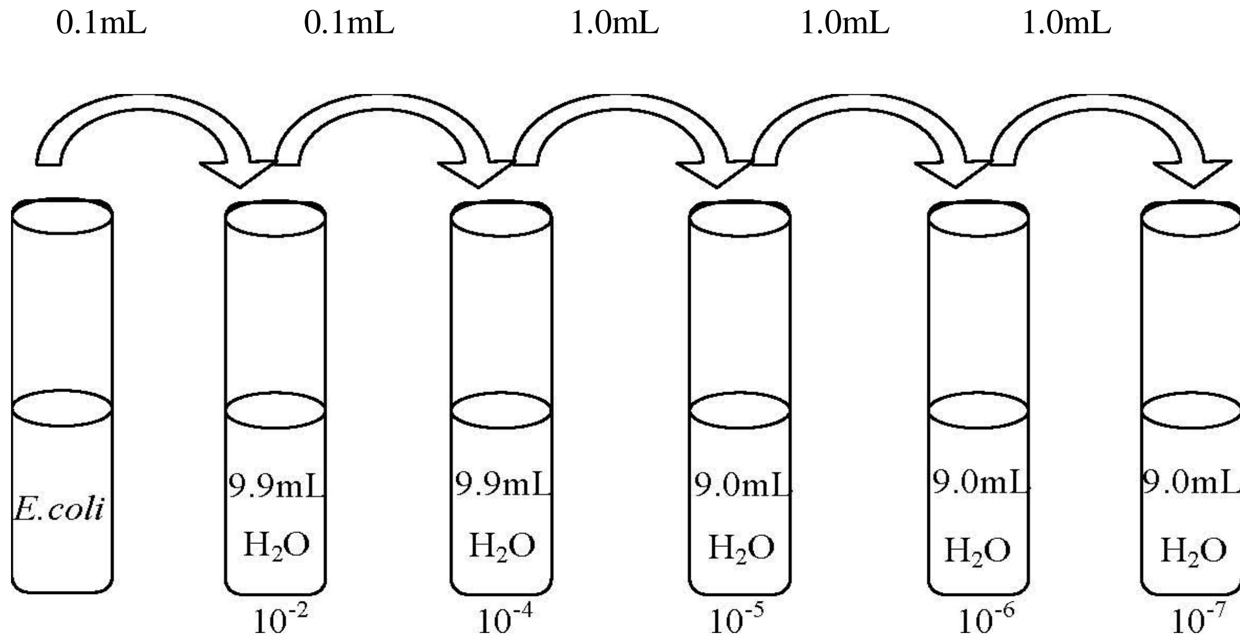


Figure 4.4. Dilution strategies for viable counts.

Exercise 4.5: Graphing of growth curve from Exercise 4.1.

Using the class data from Exercise 4.1, produce a growth curve. To accomplish this, you must transform the data into the logarithmic scale. To do this, multiply each of your values by the constant 100. (This is done to prevent log values that are negative). Take the log (base 10) for each of your transformed data point. For example, if the OD for time point 20 minutes was 0.252, multiply this value by 100. The new value will be 25.2. Take the log (base 10) of this number (the answer is 1.40). **These mathematical transformations are easily and quickly done in Excel.** Plot this number versus time on the graph. Your Y axis will be the *dependant variable* (your log(OD600*100) values) and the X – axis will be you *independent variable* (time, recorded in minutes). If you are having trouble graphing, take the tutorial at http://www.mistupid.com/viewlets/excel/xlgraphs.htm. (HINT: The best graph type will be the X-Y scatter plat with connected data points!)

Your individual Excel graph is due by next lab period.

Lab Assignments Summary:

1. Graph the penny toss results and the results of the optical density of growth on separate graphs INDIVIDUALLY. (Use of Microsoft Excel is required). Assignment is due at the beginning of lab... IF it is determined you have Xeroxed the graph of a lab partner both you and the lab partner will receive zeros.
2. Monitor growth of your unknown and record below.

3. Clean up used tubes and plates.

Use this space to take notes.

Laboratory #5: Control of Bacterial Growth

In the past few labs, we have identified ways to grow bacterial cells and monitor growth. However, in many instances, stopping microbial growth is critical. Obviously, pre-empting growth in a clinical setting is paramount to maintain good health, but it is also important in areas of food preparation and storage, laboratory settings, even in such unusual arenas as space travel (to prevent accidental contamination of other planets and moons!) In this lab, we will apply several microbial growth control techniques and evaluate their effectiveness.

Exercise 5.1: Calculation of viable counts from Laboratory #4.

Now that you have living cells on your plate, you can determine the approximate number of viable cells in the culture. The values are reported as colony forming units (CFU) per volume or bacteria/mL

The volume is often 1 mL. The formula used to calculate the number of viable cells in the culture is:

$$\text{CFU/mL or Bacteria/mL} = \frac{\text{Number of colonies}}{\text{Volume plated}} \times \frac{1}{\text{Dilution factor}}$$

EXAMPLE:

$$\text{CFU mL} = \frac{40}{0.1 \text{ mL}} \times \frac{1}{1 \times 10^{-5}} = 4 \times 10^{7} \text{CFU/mL}$$

1. Count the number of colonies on your plate you saved from last week.
2. Calculate the number of viable cells in the culture.
3. What is the CFU/ml (colony forming units per milliliter) of the culture?

Exercise 5.2: Controlling growth with temperature.

As we have discussed in class, temperature is a physical property that can be used to modulate microbial growth. In this lab, we will take more extreme temperatures and put them to the tests.

WORK IN GROUPS OF FOUR –Use Assigned Bacteria

1. Prepare 4 test tubes for inoculation (everyone is to do at least one). You will inoculate a 1% volume of the overnight culture (the one on your bench)
into the new tube. That is, for a 10 ml sample, you will use 0.1 ml of the overnight culture and 9.9 ml of sterile TSB.

2. After inoculation, record the OD600nm using the spectrophotometer of each tube. This reading will be time 0.

3. Place one tube in the 37°C water bath in the prep room, one in the refrigerator (14°C) in the prep room, and one in the 55°C water bath in the prep room, and one at room temperature (25°C) on the table in the prep room. This is so you can access your tubes and a spectrophotometer even if there is a class in the lab. Please be quiet and respectful of any class in the lab while you are in the prep room.

4. Take an additional 3 readings over the next 48 hours for each tube (Remember the lab is inaccessible after 9 PM until 8AM M-R and after 5 PM on Friday) and record the data

and the time from time 0.

5. Using the formula used to plot your growth curve form last week (log (OD600*100)), plot out new growth curves for each tube, all on the same graph using Microsoft Excel.

Graphs (1 Graph per Group) are due at the beginning of lab in one week

5.3: Growth temperatures of unknown.

1. The day before lab each person needs to prepare a T-streak plate of their unknown body organism.
2. Using aseptic technique, transfer 3 mL of sterile TSB into 4 test tubes be sure to clearly label the tubes.
3. Pick a single colony and inoculate each tube with your unknown body organism.
4. Place one tube in the 25°C, 30°C, 37°C, and 45°C incubator (location indicated at beginning of lab.
4. Determine if growth is present after 24 hours and after 48 hours.
6. Record your results on the chart at the end of this lab. Use "-" for no growth, "+" for light growth, "++" for moderate growth, "+++" for heavy growth
7. Upon completion, clean up you tubes as previously described.

Temperature	**24 hours**	**48 hours**
25°C		
30°C		
37°C		
45°C		

Exercise 5.4: Kirby-Bauer assessment of over-the-counter antimicrobial solutions.
There are many topical antimicrobials solutions available at stores. But which ones are most effective against inhibiting bacterial growth? In this lab, we will investigate the efficacy of classic antimicrobials: hydrogen peroxide, 70% isopropyl alcohol or Genetian Violet, witch hazel (an "all natural" astringent), and iodine. We will determine the effectiveness by measuring the zones of inhibitions created by the diffusion of the substances from a paper disk. The diffusion disk method is a common technique used to assess how effective certain compounds are at inhibiting growth. The larger the zone, the more effective the substance (the rate of diffusion for each compound is assumed to be identical). This experiment will be performed on your unknown body organisms.

WORK INDIVIDUALLY

1. The day before lab each person needs to inoculate a tube of sterile TSB with their unknown body organism, put the appropriate label on the tube and place it in the 37°C.

2. Using a sterile cotton-tipped swab, inoculate a fresh TSA plate with your unknown body organism ensuring that the culture has been spread over the ENTIRE plate. This will require you to dip the swap several times into the broth and spreading in multiple directions.
3. Add 5 sterile paper disks using sterile tweezers in the conformation shown below.
4. Add a drop of each antimicrobial solution to each paper disk, and a drop of sterile water to the fifth disk as a control. Mark the side of the plate to designate which product is on which disk.
5. Place in the 37°C incubator, and allow the cells grow overnight.
6. The following day, measure the diameter of the zone of inhibition, if any, and record the results in millimeters.
7. Next week, average you groups results. We will compare the data and discuss the results in class.

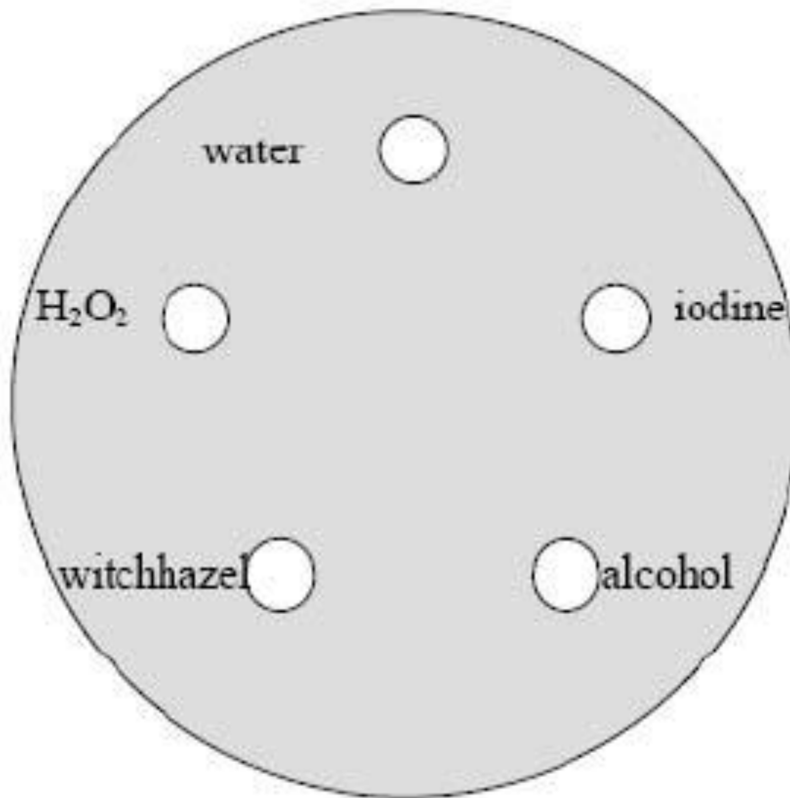


Figure 5.2 Diagram indicating the positioning of the disks on the Petri dish. The labels were made on the back of the Petri plate.

Exercise 5.3: Gram stain of unknowns. Repeat the Gram stain technique of your unknown body organism. Use *E. coli* and *S. aureus* as you Gram negative and Gram positive controls, respectively. Also, make note of the shape of the cells of your unknown.

WORK INDIVIDUALLY

1. Prepare a microscope slide with your unknown, as well as the *E. coli* and *S. aureus* from the plates on you bench. When preparing a smear from a colony you need to first put a drop of tap water in the circle on the slide. Then just touch the colony on the plate and smear that in the drop of water on your slide. Let the slide air dry.
2. Heat-fix the slide as before.
3. After the slide has sufficiently cooled, flood the slide with crystal violet for 60 seconds.
4. Rinse the slide with water using the indirect method.
5. Flood the slide with iodine for 60 seconds.
6. Rinse the slide with water using the indirect method.
7. Rinse the slide with the decolorizer (ethanol) by adding it for approximately 15 seconds.
8. Rinse the slide with water using the indirect method.
9. Flood the slide with safranin for 60 seconds.
10. Rinse the slide with water using the indirect method.
11. Dry the slide using the Bibulous paper in your drawer, and then view the slide. View the controls prior to viewing your sample. Use the rest of this space for notes and data.

Table 1: Class Data for the Over –the-Counter Antimicrobial Solutions effects on Body Organisms.

Last Name	Water (mm)	H_2O_2 (mm)	Witch Hazel(mm)	Genetian Violet(mm)	Iodine(mm)	Gram Stain

Laboratory #6: Use of Selective and Differential Media

Often one wishes to select for or eliminate certain types of organisms. This activity can be accomplished by the use of selective media. Some selective media are also differential, they allow one to distinguish between or among various organisms based on some biochemical property. The Table below indicates selective and differential media that may be used in this and other labs.

Table 1: Characteristics of Various Selective and Differential Media

Media	Use	Selective Agent	Differential Reagent	Color of different colonies
Eosin-methylene Blue (EMB)	Selection of Gram neg. enteric, Differentiates lactose fermentation	Eosin, Methylene blue	Methylene blue	*E.coli* -green sheen Enterbacter –pink with black edges Lac+ pink Lac- cream
Phenyl ethyl Alcohol (PEA)	Selection of Gram pos., especially cocci	Phenyl ethyl alcohol	none	N/A
MacConkeys (MAC)	Selection of Gram neg., differentiates lactose fermentation	Bile salts	Neutral red	Lac + pink to purple Lac - cream
Mannitol salt Agar (MSA)	Selection of salt tolerance, especially staphylococci	7.5% NaCl	Phenol red	Man+ yellow halo Around colony Man- no halo

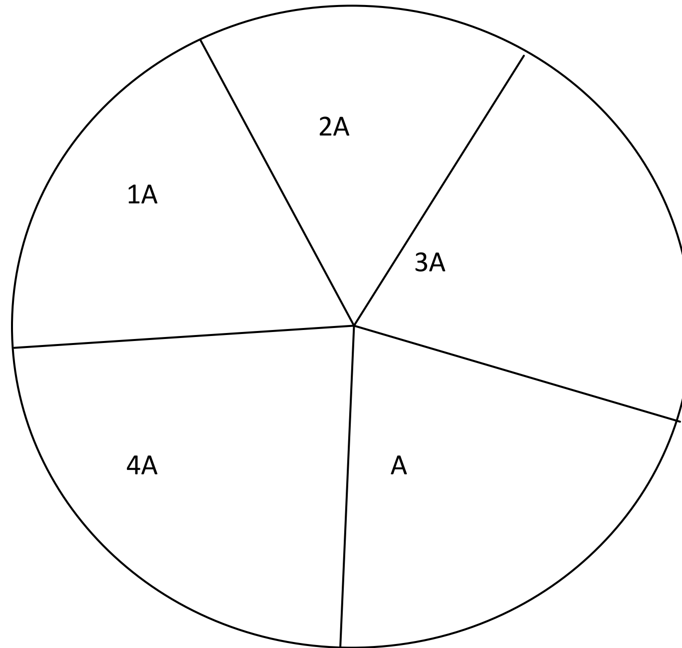
In this lab will be given a number of different cultures chosen from this group: *E. coli*, *Enterobacter aerogenes*, *Bacillus cereus* (or *Bacillus megaterium*), *Staphylococcus aureus*, *Serratia marcescens*, and an unknown mixture of two of the bacteria listed here. The cultures will be numbered rather than named. Your job will be to determine which number corresponds to which culture. To solve this puzzle, you will need to use information about the various media, as well as information that can be found in various parts of the textbook and lab manual.

<u>MATERIALS PER GROUP</u>

1 TSA plate
1 PEA plate
1 EMB plate
1 MAC plate
1 MSA plate
4 cultures labeled 1-4 and a letter and 1 culture with a letter but no number (this is a mixture of 2 of the others)

PROCEDURE

1. Divide the plate into five sections. Label each section 1-4 and the letter for your set of cultures plus one for your letter only culture



2. Using a loop and sterile technique make a straight streak of each culture across the appropriate section on each plate.
3. Incubate plates not more than 24 hours at 37°C.
4. Record the results of relative growth on the Table found on the next page using +++, ++, +, - Compared to the growth on the control plate (TSA) and indicate the color of the growth.
5. Then using this information complete the section below the Table naming what bacteria is in each numbered culture and giving your reason for this determination. Where it says unknown you need to name both bacteria that are present and give a reason for each.

UNIT 13 RESULTS

Name ____ Section_____________

Part A

CULTURE	TSA	PEA	MAC	EMB	MSA

Identity of each culture. Be sure to include your reasoning for each culture. Be sure to give the letter of your unknown!!! **Failure to do so will result in a loss of points.**

CULTURE ORGANISM REASON

1___

2___

3___

4___

Unknown___

CHARACTERIZATION OF AN UNKNOWN

As you recall the first step on characterizing an organism is to obtain a pure culture. Since the characterization and identification will take a couple of weeks we will ensure that the organism remains visible throughout this time by storing it on an agar slant. Labs 7 and 8 are designed to help you attempt to identify the unknown you have randomly chosen. If you were performing this identification in a research lab you would run tests that would then tell you what other tests you should run but that takes far more than 2 weeks so we will run them all and then you can sort out the results afterwards to identify your unknown. You will work in pairs.

You will select a culture that may contain 1-2 different organisms. By streaking, you will isolate one organism to be identified. You do not need to identify all the organisms present in the mix. In fact you will pick one from the streak plate to work with for identification purposes. Proper and immediate recording of the results is imperative; you should not trust your memory. In most cases, all that needs to be recorded is a + or – in the Table that follows the summary Table of the media. The culture that you chose is the only source of your unknown. If your culture dies or becomes contaminated, then you grade will reflect this. Therefore, exercise caution and proper technique when performing all the tests.

In order to identify the organism, you will need to consult Bergey's Manual; we have both the multi-volume version in the reserve section of the library and the short version at the reserve desk in the library. **DO NOT WRITE IN EITHER OF THESE MANUALS**. The multi-volume manual is divided into sections that depend on Gram stain reactions, cellular morphology, oxygen requirements and spore forming ability. Within each section are written descriptions of the cellular and colonial morphology of each organism as well as many Tables of the results of various biochemical tests.

Since much of these manuals are based on the Gram stain reactions, one of the first things you should do once you have isolated the unknown is to perform a Gram stain. Remember, cultures must be less than 48 hours old to obtain accurate results from the Gram stain. It is not possible to perform all the definitive tests described in the manual, and some tests that you do perform may not be relevant to your organism. Space is provided on the unknown sheet to list the results of other tests you may have run. Feel free to use any of the left over media that is available in the lab. This is done by visiting Bergey's manual and determining which leftover media would help then ask the professor if there is any of that media left. If you request is valid based on your justification from Bergey's and there is media available you can then run that test. A caveat: if your gram stain suggests that you have a Gram negative diplococcus, you don't. This genus represented by this shape is Neisseria, a genus that contains either extremely pathogenic organisms or organisms that are difficult to grow under the conditions of our lab. I don't have Neisseria in stock. You either have a Gram positive diplococcus or a rod that is either Gram positive or negative.

The final exam will cover this material and will include questions that require you to know the positive and negative results for various media. A summary Table of the media is provided for your use as a study aid on the next page.

SUMMARY OF DIFFERENTIAL MEDIA

Medium	**Test**	**Differential reagent**	**+ result**	**-result**
Phenol Red sugar				
MR-VP	MR			
MR-VP	VP			
Simmons citrate				
Malonate broth				
Tyrptone broth				
Urea broth				
DNA agar				
Catalase Test				
Skim Milk Agar				
Starch agar				
TSI	Butt: Slant: H_2S			
Indol Test				
Oxidase				

UNKNOWN ANALYSIS

Name______________________________________Section__________Unknown number______

Colony morphology_______________

Gram Stain______________________

Cellular morphology______________

Phenol Glucose acid_______gas_______

Phenol Lactose acid_______gas_______

Phenol sucrose____________

Phenol mannose____________

Phenol Malate_____________

MR__________

VP___________

Citrate_________

Malonate_______

Catalase________

Indole________

Urea__________

DNase_________

Amylase________

Casein hydrolysis_______

Oxidase____________

TSI butt________

slant_________

H_2S__________

Other tests run, results

1.__________________________

2.__________________________

3.__________________________

Possible organisms

1.__________________________

2.__________________________

3.__________________________

UNKNOWN CONTINUED

If you listed more than one genus and species for your unknown, provide three other tests that if they could have been run to distinguish between the organisms you listed. Your choice of tests must be such that the results will unambiguously identify the organism. If you are almost certain that you have a particular organism, provide three more tests that could have been run to confirm your suspicions.

Organism (from the list on the previous page)	TEST 1 – must be the same for all three organisms Name: List results below	TEST 2 – must be the same for all three organisms Name: List results below	TEST 2 – must be the same for all three organisms Name: List results below

Laboratory #7: Biochemical Test of Unknowns I: Enzymes

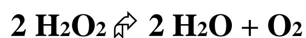
Up to this point, you have been learning various skills that make up the basis for microbiological research. Now that you have become technically capable of handling and growing microorganisms, you next step is to ply these skills towards identifying your unknown microorganism. Over the next two weeks, we will perform a variety of biochemical tests that will enable us to identify the microorganisms using Bergey's Manual and The Prokaryotes. All of these tests exploit the metabolic processes (the total chemical reactions of a cell) that are unique to you unknowns, whether they check for the presence or absence of an enzyme, utilize certain compounds, or produce a particular compound as a by-product of metabolism. The results of your tests will give you a composite of your microbial unknown. You will be given opportunities during the semester to repeat any test that results in ambiguous results. In this lab, we will be checking for the presence of enzymes used by the microorganisms.

Exercise 7.1: T-streak of your unknowns.

1. Choose a partner to work with on your unknown.
2. You and your partner select a numbered unknown and print each of your names on the line next to the number you have chosen.
3. Each person needs to take one TSA plate and streak the unknown to isolation.
4. Label each plate, tape them shut and put one in the 37°C incubator and the other plate in the 30°C
5. Pick up two TSA slants and label each one with both partners initials, the date and the number of the unknown. Then put the slants back in to the rack on the handicap bench.
5. The next day pick a single colony and streak half on the working slant and the other half on the reserve slant. Place both slants at the temperature the bacteria grew best and incubate overnight.

Exercise 7.2: The catalase test.

Catalase is an enzyme that breaks down the potentially toxic compound hydrogen peroxide, H_2O_2 into water and oxygen:



$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

Normally, hydrogen peroxide will breakdown to these components over time. However, this reaction occurs very slow and would allow the hydrogen peroxide ample time to react with cellular components such as DNA and proteins to damages cells. The presence of catalase speeds up this reaction, detoxifying hydrogen peroxide before it has a chance to damage the cell. In this assay, you will use live cells to determine the presence or absence of this enzyme. Presence of catalase is confirmed if the addition of hydrogen peroxide results in the generation of bubbles, which is the oxygen evolving.

1. The day before lab prepare a T-streak of your unknown.
2. Aseptically transfer a small amount of cells from a single isolated colony of your unknown

to a glass microscope slide. Also transfer to a different region of the slide *E. coli* (positive control).

3. Place the slide on the bench top. The black background will provide excellent contrast to visualize the bubbles better.
4. Add 2-3 drops of hydrogen peroxide to the cells. Bubbles should form in 2-10 seconds.
5. Vigorous bubbling that occurs immediately is indicative of strong catalase activity, while tiny bubbles produced after longer periods of time signal weak activity. No bubble indicates a catalase negative reaction.

Exercise 7.3: DNase test.

Many microorganisms are capable of secreting the enzyme deoxyribonuclease, or DNase. This enzyme is responsible for breaking down DNA, and may be used by the bacteria to provide an advantage over other bacteria growing nearby. In this test, we are going to determine whether your unknowns are capable of producing this enzyme using DNase agar. The test agar contains an emulsion of DNA, peptides as a nutrient source, and methyl green dye. The dye and polymerized DNA form a complex that gives the agar a blue-green color at pH 7.5 Bacterial colonies that secrete DNase will hydrolyze (breakdown using water!) the DNA in the medium into smaller fragments unbound from the methyl green dye. This results in clearing around the bacterial growth. Another type of DNase agar does not contain the Methyl Green dye. This media is inoculated in the same manner but after the bacteria have grown 1N HCl is added to the plate to precipitate the undigested bacteria. A positive is still a clear halo around the bacteria.

http://www.austin.cc.tx.us/microbugz/37dnase.html (results from a DNase test)

1. Using aseptic technique, transfer cells of *S. marcerans* (DNase positive) to the DNase plate, smearing a broad line (or lines) across the top of the plate.
2. Below this line (in the center of the plate) transfer cells of *E. coli* (DNase negative) to the DNase plate, smearing a broad line (or lines). Leave enough space so not to confuse the results between samples!

3. Below this line transfer cells of your unknown to the DNase plate, smearing a broad line (or lines) at the bottom of the plate.
4. Place your plate in the 37˚C incubator and check tomorrow for results. If you have the plate without methyl green don't forget to add the 1n HCl.

Exercise 7.4: Urease test.

http://www.austin.cc.tx.us/microbugz/35urease.html (results from a urease test).

Some microorganisms have the ability to hydrolyze the nitrogen containing compound urea. When urea is broken down, ammonia is released. Ammonia is basic, and can help to neutralize acidic environments, making them more suitable for supporting microbial life. Urea test media (either broth or slant) contains urea and the pH indicator phenol red. This indicator turns to a dark pink (magenta) color when the pH is greater than 8.4 When urea is hydrolyzed, ammonia will be produced, raising the pH of the medium above 8.4 A positive reaction is magenta, and a negative reaction will be orange or peach color**.**

1. Inoculate a urease tube with your unknown using aseptic technique.
2. Incubate the tubes overnight at 37˚C. Compare your results with that of the controls provided.

Exercise 7.5: Casein hydrolysis (casease test).

Casein is the protein in milk that gives milk its white color. When milk sours, the curd that is left is coagulated casein (due to the acid produced by the microbes in the milk). Casein also contains all 20 naturally occurring amino acids (making hydrolyzed casein an excellent nutritional additive in microbiological media!). Casein is also the cause of many dairy allergies. The enzyme that is responsible for the hydrolysis of casein is casease. Some bacteria secrete the casease, which breaks down the large casein molecules into smaller polypeptides which pass

through the cell membrane. This hydrolytic reaction, called proteolytic action, will clear the area surrounding the colony as it attacks the molecules.

1. Using aseptic technique, transfer your unknown to a milk plate,
2. Place your plate in the 37˚C incubator and check tomorrow for results. If your plate looks like B. subtilis below it is positive. If your plate looks like *S. aureus* or *E. coli* it is negative.

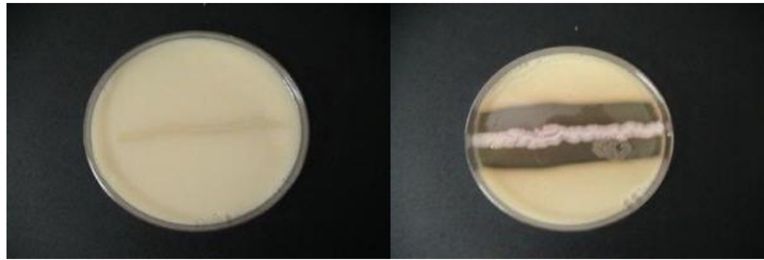


Figure 7.5 The results of the casein test. The plate on the left is a bacteria that does not produce caseinase. The plate on the right shows the clear zone produced when the bacteria does produce caseinase.

Exercise 7.6: Gram stain of unknowns.

Gram stain your unknown. Use *E. coli* and *St. aureus* as your Gram negative and Gram positive controls, respectively. Also, make note of the shape of the cells of your unknown.

WORK INDIVIDUALY

1. Prepare a microscope slide with your unknown, as well as the *E. coli* and *St. aureus* from the plates on you bench.
2. Air dry and then Heat-fix the slide as before.
3. After the slide has sufficiently cooled, flood the slide with crystal violet for 60 seconds.
4. Rinse the slide with water.
5. Flood the slide with iodine for 60 seconds.
6. Rinse the slide with water.
7. Rinse the slide with the decolorizer (ethanol) by adding it for approximately 15 seconds.
8. Rinse the slide with water.
9. Flood the slide with safranin for 60 seconds.
10. Rinse the slide with water.
11. Allow the slide to dry, and then view the slide. View the controls prior to viewing your sample.

Use the space below to record the results of your tests. Keep for future reference, as you will need this data!!

Laboratory #8: Biochemical tests: metabolism

Continuing our examination of biochemical tests, we will now turn our attention to microbial metabolism. One of the reasons that microbes can be found virtually anywhere is because they can have such a varied metabolism. Many microbes utilize fermentation, either aerobic or anaerobic, to generate ATP. Fermentation is another alternative for microbes to generate energy. Some bacteria, such as the facultative anaerobe *Escherichia coli*, have the ability to employ all three of these forms of metabolism, depending on the environmental conditions they are in.

Exercise 8.1: Oxidase test.

The oxidase test is used to determine if the bacterial species in question contains the respiratory enzyme cytochrome oxidase. The cytochrome oxidase enzyme catalyzes the transport of electrons from a donor compound (e.g., NADH) to the final electron acceptor, oxygen. In this test, the electron donor phenylenediamine is a redox dye. In its reduced form, the dye is clear. If the enzyme is present, it will oxidize the dye, turning it blue-purple in color.

1. Hold the reagent dropper upright and point the tip away from yourself. Grasp the middle with your thumb and forefinger and squeeze gently to crush the glass ampule inside the dropper.
2. Tap the bottom on the bench top a few times, then invert for drop-dispensing.
3. Add a few drops of the reagent dye to a strip of Whatman #1 filter paper.
4. Aseptically transfer a loopful of bacteria onto the reagent saturated paper.
5. Reaction should occur within 10 – 30 seconds. Do not add more reagent to the strip, as this could result in a false positive. Any results longer than 30 seconds should be interpreted as a negative result.

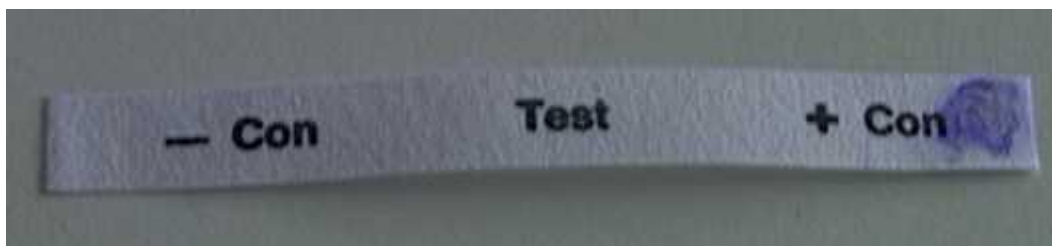


Figure 8.1: An oxidase test. Note the blue-purple color of the positive reaction. (From http://www.staffs.ac.uk/schools/sciences/biology/dr/oxidase.jpg)

Exercise 8.2: Carbohydrate fermentation.

Many bacteria can ferment various sugars in an effort to generate energy and regenerate NAD+ and other electron carriers. The final electron acceptor in fermentation is an organic compound, such as lactic acid. In this test, you will examine your unknown's ability to ferment a number of different carbohydrate sources. Each medium has a single fermentable carbohydrate added to a peptone medium. Phenol red is also added as a pH indicator. A small tube (Durham tube) is inverted and placed in each larger test tube of liquid medium. The inverted tube is able to trap any gas products. The indicator, phenol red will turn yellow below pH 6.8 and a darker pinkish-red above pH 7.4. If the organism metabolizes the carbohydrate, subsequent acid production will

result in lowered pH. If the organism does not ferment the carbohydrate, the pH may remain neutral. If the organism does not ferment the carbohydrate and also utilizes the peptone, accumulation of the ammonia as a degradation product will raise the pH.

You will examine the ability of your unknown to ferment the sugars glucose, lactose, sucrose, and mannitol. The results of the test will be one of the following:

Acid Fermentation: If your tube changes from red to yellow, the pH has dropped, indicating that you bacterium has fermented the carbohydrate source in the tube. An organism that produces only one end product from fermentation is considered to be a ***homofermentor.***

Acid and Gas Fermentation: If your tube changes from red to yellow, the pH has dropped, indicating that you bacterium has fermented the carbohydrate source in the tube. Additionally, a bubble appearing in the Durham tube indicates that the microorganism in question also generates gas (usually CO2) as a by-product of fermentation. This type of organism is considered to be a ***heterofermentor***, an organism that ferments sugar to two or more end products.

Negative Reaction: Negative fermentation can be indicated two ways:
1. No color change in the tube means that the sugar was not utilized by the organism. 2. Color change to a dark, pinkish-red: this darker color indicates alkaline or basic metabolic products which are *due to the utilization of the peptone, rather than the sugar*. If the tube is read within 48 hours, the darker red color would be an indication of negative fermentation; although the result is usually recorded as alkaline.

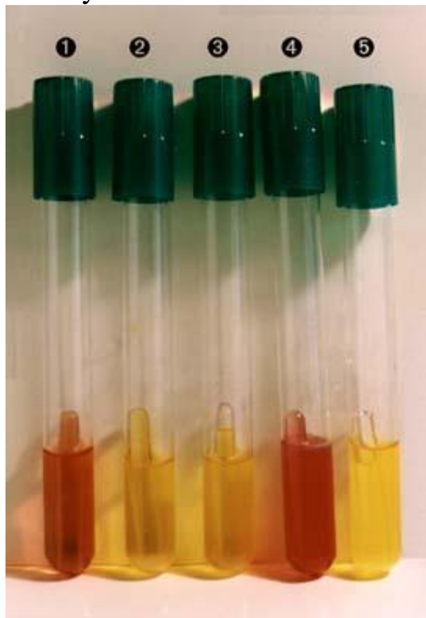


Figure 8.2: A result of a glucose fermentation test. Tube 1, negative control; tube 2, acid positive; tube 3, acid and gas positive; tube 4, negative reaction; tube 5, acid gas positive. Note the bubbles in the Durham tube in tubes # 3 and 5. (From http://www.austin.cc.tx.us/microbugz/26fermentation.html)

1. Inoculate your unknown into a tube of glucose phenol red, lactose phenol red, sucrose phenol red, and mannitol phenol red.
2. Place in the 37˚C incubator.
3. Check and record results within 24 hours.

Exercise 8.3. Oxygen requirements of your unknowns.

We have learned from lecture that the oxygen requirements of microorganisms can be quite varied. One way to determine what requirements a microorganisms has is the use of thioglycollate broth tubes. Along with nutrients to support bacterial growth, it contains sodium thioglycollate, thioglycollic acid, L-cystine, and 0.05% agar. The sodium thioglycollate, thioglycollic acid, and L-cystine reduce the oxygen to water. The agar helps retard oxygen diffusion and helps maintain the stratification of organisms growing in different layers of the broth. Additionally, a chemical dye (methylene blue) may be added to the media to show the presence of oxygen, which can be seen a bluish or greenish color.

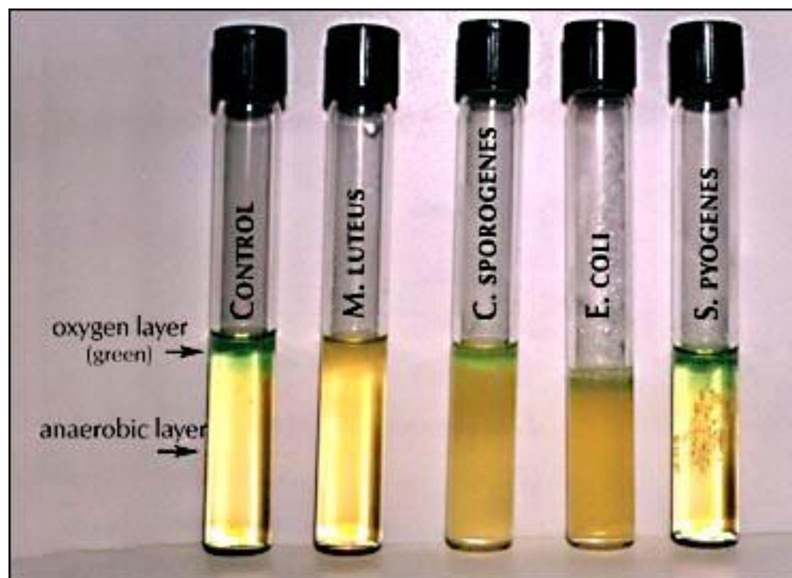


Figure 8.3: results of a thioglycollate test. From left to right: tube 1, negative control; tube 2, strict aerobe; tube 3, strict anaerobe; tube 4, facultative anaerobe; tube 5, microaerophile. (from http://www.austin.cc.tx.us/microbugz/05thio.html)

1. Grow a 5ml overnight culture of your known in tryptic soy broth.
2. Get a molten thioglycollate tube from the 55˚C water bath.
3. Cool to 44 C. in water bath (cooling to avoid killing bacteria).
4. Add 0.5 mL of a 24-hour liquid culture of your unknown.
5. Mix tube by rolling between hands. *Don't get air into medium!*
6. Cool tubes in an upright position until agar is solid. If using screw cap tubes, loosen caps
7. Incubate at 30˚C or 37˚C
8. After incubation (24 hours or as needed), determine location of bacterial growth by holding the tube to light and trying to view through the tube. Record your results

Exercise 8.4 Citrate Utilization
Some organisms such as Enterobacter and Serratia can use citrate as a sole C source. Simmons citrate, an agar slant, contains brom-thymol blue, a dye that turns a deep blue as the pH rises as a result of the use and elimination of citrate.

Figure 8.4: Simmons Citrate agar. The tube on the left is a positive result and the tube on the right is a negative result.

Materials per pair
1 tube of Simmons citrate agar slant

Procedure
1. Take your loop and straighten it out to form a needle for this test. Sterilize it as in previous experiments. Extract a small amount of bacteria from your slant and then stab the butt of the tube and streak the slant (drag the needle across the surface of the slant).
2. Incubate for 2-3 days
3. Examine the tube for the appearance of a deep blue color for a positive result.

Exercise 8.5 Malonate Utilization
This reaction is similar in principle to the citrate utilization test, except the medium is a liquid and malonate is the sole C source.

Figure 8.5: Malonate Broth: the tube on the left is a negative reaction and the tube on the right is a positive reaction

Materials per pair
1 tube of malonate broth

Procedure
1. Inoculate the tube with a loop of your unknown.

2. Incubate the tube for 2-3 days
3. Look for the appearance of a blue color which indicates a positive result

Exercise 8.5 Triple Sugar Iron Agar

This medium allows one to test for the fermentation of three different sugars (glucose, lactose, and sucrose), gas production and hydrogen sulfide (H_2S) production. Phenol red is used as the indicator for acid production, and hence an acid result is indicated by a yellow color. If gas is produced, it will be trapped between the side of the tube and the agar and appears as bubbles. If H_2S is produced, it will react with the iron compound to form a black precipitate that covers much of the butt of the tube.

In order to determine which sugar's is/are fermented, both the butt and the slant must be examined as indicated below:

Butt	Slant	Interpretation
Red	Red	No sugars are fermented
Yellow	Red	Only glucose is fermented
Yellow	Yellow	Glucose and at least one of the other two sugars are fermented

NOTE: You cannot have a yellow slant and a red butt. This would indicate that at least one of the disaccharides was fermented but not the monosaccharide, glucose. All organisms will preferentially ferment glucose. In order to ferment the other sugars, the organisms must first break them down into glucose and some other sugar.

If an organism produces H_2S, the black precipitate will often obscure the color of the butt. Since H_2S is produced after the fermentation of a sugar to acid the butt must also be assumed to be yellow.

TSI tubes must be scored within 24 hours. If you allow it to go for longer periods of time the amino acids will be deaminated, the pH will rise and the medium will revert to red.

Figure 8.5 Triple Sugar Iron Agar. From the left: The first tube is yellow butt, yellow slant and gas (glucose and one other sugar fermented and gas produced), the second tube is a yellow butt and a red slant (glucose fermented), the third tube is yellow butt, black butt and red slant (glucose fermented and H_2S produced), and the last tube is red butt and red slant (no sugars fermented).

Materials per pair
1 TSI slant

Procedure
1. Inoculate the TSI with your unknown by stabbing the butt and streaking the slant. Just as you did for the Simmons citrate.
2. Incubate the tube for no more than 24 hours.
3. Score the tube for acid, gas and H_2S production.

Exercise 8.6 Amylase Test
Amylase is an enzyme that hydrolyzes starch into maltose, glucose, and dextrins. Iodine reacts with starch to give a blue black color, whereas the hydrolytic products do not react with Iodine. An organism that produces amylase will have a yellow or amber halo against a black background

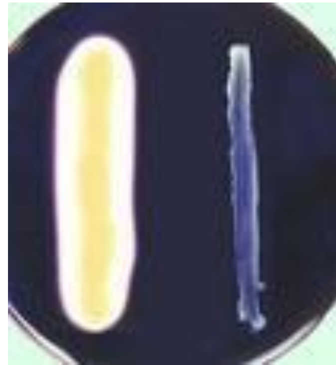


Figure 8.6: Amylase agar after the addition of Gram's iodine. The bacteria on the left produced amylase and broke down the starch in the plate. The bacteria on the left did not secrete amylase.

Material per pair
1 Starch agar plate

Procedure
1. Make a straight streak of your unknown across the starch plate.
2. Incubate 2-4 days (until you have a streak of bacteria growing on the plate).
3. Flood the plate around the organism with Grams Iodine and look for a yellow halo.

Exercise 8.7 Tryptophan Hydrolysis (Indole Test)
The hydrolysis of the amino acid tryptophan by the enzyme tryptophanase produces indole and pyruvic acid. Kovac's reagent reacts with indole to produce a red ring at the top of the tube. If no indole is present, the ring will be colorless, brown, amber or sometimes green.

Material per pair
1 tube of tryptone broth or 1 tube of MR-VP broth

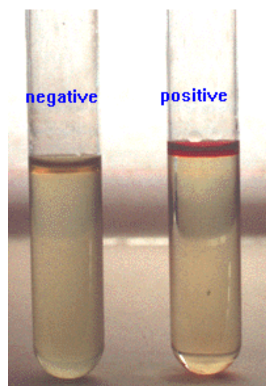


Figure 8.7 The tube on the left is a negative for the Indole test and the tube on the right is a positive result for the Indole test. The image is from: http://biology.fullerton.edu/biol302/302labf99/biochem.html

Procedure
1. Inoculate the tube of broth with your unknown.
2. Incubate the tube for 2-3 days
3. Add about 15 drops of Kovac's reagent to the tube. **DO NOT MIX THE TUBE**. Look for the presence of a red ring at the top of the tube after 1-2 minutes.

Exercise 8.8 Mixed Acid (methyl red) and butanediol (Voges-Proskauer) tests

Some organisms ferment sugars first to lactic acid then to other acids such as formic and acetic acid. These acids have a pH lower than 5.0 and cannot be detected by phenol red. Methyl red is used instead. In a positive result, the tube will turn red after the addition of Methyl red (any hint of red color should be considered positive); a yellow tube is indicative of a negative result.

Other organisms convert lactic acid into alcohols, especially butanediol. Acetyl carbinol, or acetoin, the precursor to butanediol, is readily detected by using Barritt's reagents. A red color is a positive result; a negative result is sometimes a dark green, sometimes brown, and sometimes greys.

Materials per pair
2 tubes of MR-VP broth

Procedure
1. Inoculate both tubes of MR-VP broth with your unknown. Label one tube MR and the other tube VP. Incubate both tubes at 37°C.
2. After two days take out the one labeled VP, remove 1.0 ml of culture from tube and add this volume to a sterile tube.
3. Add 18 drops of Barritt's A (alpha-naphthol) to the tube containing the 1.0 mL
4. Add 18 drops of Barritt's B (KOH) to the tube containing the 1.0 mL
5. Shake the tube vigorously every 15 seconds for 5-10 minutes. The reaction is dependent upon the presence of ample oxygen. Allow the tube to sit for 5 more minutes. A red,

usually blood red or brick red is appositive result for VP.

6. After 7 days remove the tube labeled MR and add 8 drops of methyl red. Mix the tube. Any hint of red (orange, peach, pink) is a positive result.

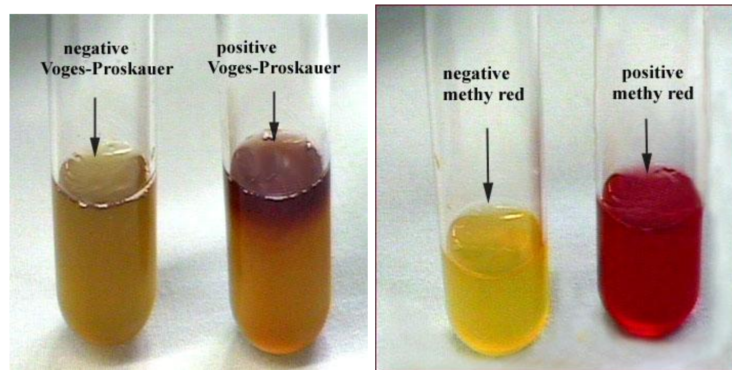


Figure 8.8 The image on the left is the negative and positive for the Voges-Proskauer test. The image on the right is negative and positive results for the Methyl Red Test. Images are from: http://www.mc.maricopa.edu/~johnson/labtools/Dbiochem/imvic.html

Lab #9: Epidemiology

When a new disease enters a population, several different outcomes: (1) the disease could quickly die out, (2) the disease could remain in the population at more or less stable levels, perhaps "settling down" after a major outbreak (i.e., become *endemic*), (3) the disease could cycle in incidence, causing periodic epidemics (the cycles could increase or decrease in amplitude, or remain about the same), (4) epidemics could come and go at more or less random intervals, perhaps exhibiting "chaotic" behavior, or (5) the disease could cause the population to go extinct. Being able to predict how the disease is spreading will allow health care professionals to correctly prescribe the course of action to stem the flow of the disease. Epidemiology is the study of where and when diseases occur, and how they are transmitted within populations. Modern-day epidemiologists have a broader scope than just infectious diseases; they track injuries, accidental deaths, poisonings, and a variety of other human plights.

The job of the epidemiologist is to track the incidence and prevalence of a disease, and classify disease outbreaks as **endemic** (usually present in the population), **sporadic** (occasionally found in the population), **epidemic** (more cases than usual in the population), or **pandemic** (disease present on more than one continent. The incidence of disease is the number of new cases of a disease in a specific area or population over time, while the prevalence of a disease is the total number of cases in a specific area or population over a given time. These are often expressed as ratios of a given population:

Incidence = #of new cases/# of people at risk

Prevalence = # of old and new cases/ # of people at risk

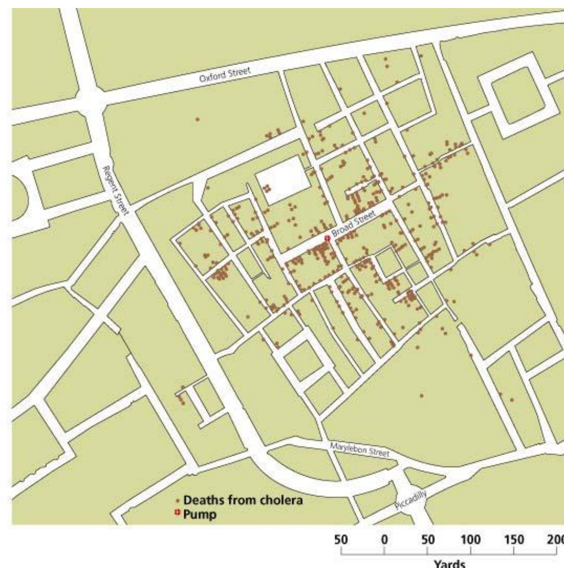
For example, in 2002 the number of new TB cases in the Unites States was 11,878 and the population at risk was 289,939,000. Thus the incidence was roughly 4.1 persons for every 100,000 people (i.e., a city like Augusta with a population around 200,000 would likely see about 8 new cases of TB in 2002). But the prevalence of the disease was 6.87/100,000. Why the difference? Because prevalence factors in new *and* old cases into the calculation. However, one must consider other factors, such as age, gender, race, and geography. These factors can all influence the spread of disease.

How all of these factors play roles in disease can be understood by looking at the history of AIDS. Initially, doctors noted an increase in incidence of the usually benign Kaposi's sarcoma and rare lung infection *Pneumocystis carinii* pneumonia (PCP) in young gay men in California and New York. Since no disease had presented in women or non-homosexuals, it did not appear to be caused by a contagion. In fact, doctors thought that there was no apparent danger to non-homosexuals and women. However, over time, the cause of the disease (the etiological agent) was discovered to be HIV, and the risks to these other populations (heterosexuals and women) were better understood. Knowing who is at risk changes the threat of the disease, which is now known globally as a primarily heterosexual disease.

Epidemiology can be approach from three different ways: descriptive epidemiology, analytical

epidemiology, and experimental epidemiology. A descriptive approach involves the collection and tabulation of data concerning disease, as well as trying to identify the index patient (sometimes called patient zero). The index case is the first case of the disease in a given population. Analytical approaches are used to determine the probable cause, mode of transmission, and possible means of prevention. This approach is often retrospective in nature, looking at the data after the fact. The experimental approach involves testing a hypothesis concerning the cause of disease. Application of Koch's postulates to a disease would fall under this classification.

In summation, epidemiology is much like detective work. In a classic example, Dr. John Snow tracked down the source of a cholera outbreak in London in 1854 by applying the location of cholera deaths to a map and identifying the water pump that was the cause (Figure 10.1). His work saved the lives of hundreds of Londoners.



Copyright © 2006 Pearson Education, Inc., publishing as Benjamin Cummings.

Figure 10.1.

In this lab, we will stage a mock outbreak of the sexually transmitted disease chlamydia. Chlamydia is the most prevalent STD in the United States caused by *Chlamydia trachomatis,* obligate intracellular bacteria. Approximately 3 - 4 million Americans suffer from infections every year costing nearly 1 billion dollars. No one appears to develop immunity after contracting the disease. Often infected women show no obvious symptoms, while approximately 75% of men are symptomatic. In men the disease produces urethritis resulting in painful urination and a discharge from the urethra, sometimes being confused with gonorrhea. Two serious complications occur as a result of this disease:

1) Chlamydia may spread to the fallopian tubes resulting in PID (pelvic inflammatory disease). Approximately 500,000 cases of PID due to Chlamydia occur every year.
2) Infants, born to infected mothers, are at high risk for developing eye infections leading to blindness. Other than trauma related injury, Trachoma is the leading cause of blindness in the world.

Chlamydia can be treated with tetracycline and erythromycin. There are no vaccines available. It is your job to trace the spread of the disease and work to identify the index case, as well as determine the incidence and prevalence of the disease, as well as the possible portals of entry and modes of transmission.

Exercise 10.1: Mock Chlamydia outbreak.

PLEASE FOLLOW AL INSTRUCTIONS CAREFULLY AND USE CAUTION. We are spreading bacteria via contact in this lab. Although the strains used are not normal human pathogens (*E. coli* and *S. marcerans*), it is important to remember that ALL bacteria can be opportunistic pathogens.

1. Everyone has 4 TSA plates. Please label them with your assigned number. Additionally please label you section letter on each plate. Lastly, number the plates 1-4 to represent the round of transmission.

2. Each person has a rubber glove to put on one hand (either right or left). Place the glove on one hand.

3. Each person has a Petri dish with a paper towels that has been saturated with a particular culture.

4. Prior to your "night out", you are to handle the paper towels with your gloved hand only! Your goal is to coat the glove so that it is damp but not be dripping with fluid!

5. When the music starts, randomly pick one person not from your table to "socialize" with.

6. Shake and rub your gloved hand with your partner's gloved hand. Try to be careful and not drip.

7. When the music is over, swab your gloved hand and spread onto plate #1. Label the plate with the number of the person you mingled with and circle it.

8. Again, handle the paper towels. Go out and party again with a different partner. Again make sure this person is not from your Table.

9. Repeat steps 5-7. Continue until all plates have been swabbed. During the last round you can party with someone from your Table.

10. Remove your glove and place in the Petri dish with the paper towels. The professor will pick up both and dispose of them for you.

11. Place your plates in the 30˚C incubator.

12. Check the plates within 2 days. You are looking for plates that have the red *Serratia* culture on it (positive for *Chlamydia*).

Compile you information to determine the flow of the disease. Identify the possible modes of transmission for the real *Chlamydia* (airborne? Contact? Fluid exchange? Water borne?) and portals of entry for the real *Chlamydia* (site of the body where the bacteria could have entered). You are to turn in a flow chart showing the spread of the disease and the 2 possible index cases (I will demo in class), along with your possible modes of transmission and portals of entry for the disease (image this is Chlamydia). Also, state the incidence of mock Chlamydia in the class. **<u>It is due AT THE BEGINNING OF NEXT CLASS - LATE REPORTS WILL NOT BE ACCEPTED.</u>**

Put example of flow chart given in class here:

Lab 11: Antimicrobials

Antimicrobial chemotherapy is the use of chemicals to inhibit or kill microorganisms in or on the host. The process of chemotherapy is based on **selective toxicity**, the ability of the chemical to kill or inhibit the pathogen without causing harm to the host. To accomplish this, the chemicals being used target specific structures or processes unique to the microorganism.

There are two general classes of antimicrobial chemicals: **antibiotics** and **antimicrobial** chemotherapeutic chemicals. Antibiotics are substances produced as metabolic products of one microorganism which inhibit or kill other microorganisms. Antimicrobial chemotherapeutic chemicals are chemicals synthesized in the laboratory which can be used therapeutically on microorganisms. Antimicrobials have revolutionized medicine. Alexander Fleming's serendipitous discovery of penicillin is wonderfully recounted by Dr. Andrew J. Schuman in "A concise history of antimicrobial therapy (serendipity and all)", *Contemporary Pediatrics*:

> "The initial focus of antibiotic research was on synthetic chemicals with antimicrobial properties, but microbiologists next began to search for "natural" antibiotics. It had long been observed that one bacterial species inhibited the growth of others when introduced into the same culture medium. Researchers assumed that one species produced antibiotic substances that assured its survival at the expense of potential invaders."

The British physician and surgeon Alexander Fleming had gained limited notoriety in 1922 by discovering that tears and nasal secretions could inhibit the growth of bacteria. He subsequently identified and isolated the enzyme lysozyme from these secretions as well as from saliva, hair, and skin, and eggs, flowers, and vegetables. He speculated that lysozyme was a part of a universal defense mechanism that all living creatures possessed to prevent invasion by bacteria. Unfortunately, lysozyme had no effect on pathologic bacteria, and Fleming eventually abandoned research on the enzyme to study staphylococci.

Fortunately, Fleming was somewhat untidy in his laboratory. One day in 1928, he noticed that a mold (*Penicillium notatum*) that had contaminated old culture dishes in which staphylococci were growing produced a zone of inhibition where it grew. He later isolated a substance, which he called penicillin, from the mold and found that it effectively eradicated many different types of bacteria. He published his findings in 1929 but never attempted to administer penicillin to lab animals inoculated with bacteria.

Had it not been for other investigators at Oxford University in England who chanced upon Fleming's original paper, penicillin might never have been introduced as an antibiotic. The investigators—Howard Florey and Ernst Chain, a German Jew who had fled from Germany to England as Hitler rose to power—began to attempt to produce enough penicillin to determine its potential utility as an antibiotic. With the aid of a $5,000 grant from the Rockefeller Institute, Florey and Chain increased the yield of penicillin by growing *P notatum* in porcelain bedpans, and Chain produced small quantities of purified penicillin for testing. The penicillin he prepared was 1,000 times more potent than Fleming's original "mold juice extract" and appeared to have at least 10 times the antibiotic activity of sulfa drugs.

In a now-famous study published in the *Lancet* in 1940, Florey and Chain injected 50 mice with streptococci and treated half of them with penicillin. At the end of the experiment, all the untreated mice were dead; 24 of the 25 penicillin-treated mice survived.

One year later, Florey and Chain produced enough penicillin for a clinical test. The antibiotic was first administered to a policeman with streptococcal septicemia, who improved while receiving penicillin but eventually died once the penicillin supply was exhausted after five days of treatment. The next beneficiaries were children—a 15-year-old with hemolytic septicemia and a 4-year-old with cavernous-sinus thrombosis and sepsis. The 15-year-old survived; the 4-year-old died from a ruptured aneurysm after being cured of infection by the penicillin."

From:
http://www.contemporarypediatrics.com/contpeds/article/articleDetail.jsp?id=111791

Interestingly, Fleming was one of the first to predict that the careless use of antimicrobials would lead to the development of bacterial resistance to the compounds. Indeed, by 1946, just three years after the widespread use of penicillin to combat disease, hospitals began to report a rise in the numbers of penicillin resistant staphylococci they were seeing.

For some microorganisms, susceptibility to chemotherapeutic agents is predictable. However, for many microorganisms (*Pseudomonas, Staphylococcus aureus,* and gram-negative enteric bacilli such as *Escherichia coli, Serratia, Proteus*, etc.) there is no reliable way of predicting which antimicrobial agent will be effective in a given case. This is especially true with the emergence of many antibiotic-resistant strains of bacteria. Because of this, antibiotic susceptibility testing is often essential in order to determine which antimicrobial agent to use against a specific strain of bacterium. Several tests may be used to tell a physician which antimicrobial agent is most likely to combat a specific pathogen. In this laboratory, you will use one of the most common clinical tests of antimicrobial resistance, the Kirby-Bauer method) to determine the antimicrobial resistance of your body organism that you isolated at the beginning of the semester.

Exercise 11.1: Kirby-Bauer Method

The Kirby Bauer method employs the use of an assay we have previously seen before – the disk diffusion method. In this method, bacteria are spread evenly across the plate using a sterile swab. After the swab, paper disks containing antibiotics are added to the top of the plate. The plates are incubated overnight, and the zones of inhibition created are measured the next day (see example). Bear in mind, the size of the zone of clearing determines whether the microorganism is resistant (R), intermediate (I), moderately susceptible (ms), susceptible (s), based on the National Committee for Clinical Laboratory Standards.

Work Individually

1. The day before lab you will need to go into lab and inoculate your body organism into a tube of TSB.

2. Spread a culture of your body organism on a TSA plates.

3. The professor will dispense the disks containing the antimicrobial onto each plate. The automatic dispersal will ensure that the disks have sufficient space to make a zone of clearing. Each disk has a label indicating the concentration and the antibiotic that the disk contains.

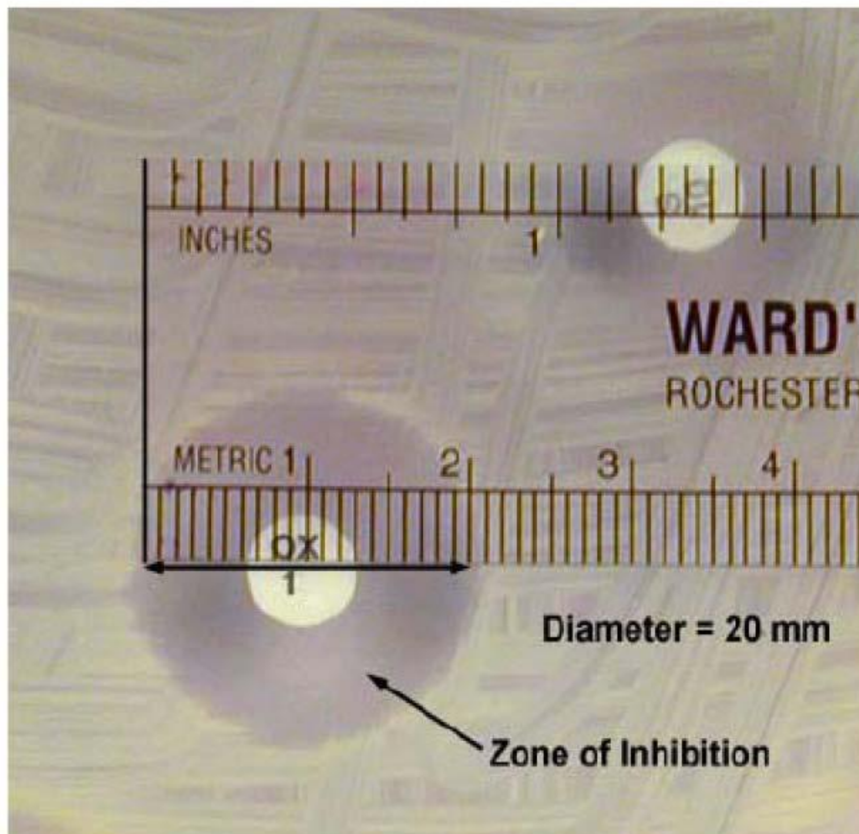
4. Incubate the TSA plate overnight at 37°C.

5. Measure the zones of inhibition that result using a ruler, and using millimeters.

6. Record these averages on the chart provided.

7. Turn in a brief write-up, stating if the bacteria are resistant, intermediate, moderately susceptible, or susceptible, based on the NCCLS charts provided.

Measuring the diameter of zones of inhibition in mm.



Zone Size Interpretive Chart for Bauer-Kirby Test

Antimicrobial agent	Disc code	R= mm or less	I= mm	MS= mm	S= mm or more
Amikacin	AN-30	≤15	15-16	-	≥16
Amoxicillin/ Clavulanic Acid - *Staphylococcus* -other organisms	AmC-30	 ≤19 ≤13	 - 14-17	 - -	 ≥20 ≥18
Ampicillin - *Staphylococcus* -G- enterics	AM-10	 ≤28 ≤11	 - 12-13	 - -	 ≥29 ≥14
Azlocillin	AZ-75	≤14	15-17	-	≥13
Aztreonam	ATM-30	≤15	-	16-21	≥22
Carbenicillin -*Enterobacteriaceae* *Pseudomonas*	CB-100	 ≤17 ≤13	 18-22 14-16	 - -	 ≥23 ≥17
Cefamandole	MA-30	≤14	15-17	-	≥18
Cefazolin	CZ-30	≤14	15-17	-	≥18
Cefonicid	CID-30	≤14	15-17	-	≥18
Cefoperazone	CFP-75	≤15	-	16-20	≥21
Cefotaxime	CTX-30	≤14	-	15-22	≥23
Cefotetan	CTT-30	≤12	-	13-15	≥16
Cefoxitin	FOX-30	≤13	-	15-17	≥18
Ceftazidime	CAZ-30	≤14	15-17	-	≥18
Ceftizoxime -*Pseudomonas* -other organisms	ZOX-30	 ≤10 ≤14	 - -	 ≥11 15-19	 - ≥20
Ceftriaxone	CRO-30	≤13	-	14-20	≥21
Cefuroxime	CXM-30	≤14	15-17	-	≥18
Cephalothin	CF-30	≤14	15-17	-	≥18
Chloramphenicol	C-30	≤12	13-17	-	≥18
Cinoxacin	CIN-100	≤14	15-18	-	≥19
Ciprofloxacin	CIP-5	≤15	16-20	-	≥21
Clindamycin	CC-2	≤14	15-20	-	≥21

Antimicrobial agent	Disc code	R= mm or less	I= mm	MS= mm	S= mm or more
Doxycycline	D-30	≤12	13-15	-	≥16
Erythromycin	E-15	≤13	14-22	-	≥23
Gentamicin	GM-10	≤12	13-14	-	≥15
Imipenem	IPM-10	≤13	14-15	-	≥16
Kanamycin	K-30	≤13	14-17	-	≥18
Methicillin - *Staphylococcus*	DP-5	≤9	10-13	-	≥14
Mezlocillin	MZ-75	≤12	13-15	-	≥16
Minocycline	MI-30	≤14	15-18	-	≥19
Moxalactam	MOX-30	≤14	-	15-22	≥23
Nafcillin - *Staphylococcus*	NF-1	≤10	11-12	-	≥13
Nalidixic Acid	NA-30	≤13	14-18	-	≥19
Netilmicin	NET-30	≤12	13-14	-	≥17
Nitrofurantoin	F/M-300	≤14	15-16	-	≥17
Norfloxacin	NOR-10	≤12	13-16	-	≥17
Oxacillin - *Staphylococcus*	OX-1	≤10	11-12	-	≥13
Penicillin - *Staphylococcus*	P-10	≤28	-	-	≥29
Piperacillin/Tazobactum -*Enterobactereaceae* - *Staphylococcus* or *P. aeruginosa*	TZP-110	≤17 ≤17	18-20 -	-	≥21 ≥18
Sulfamethoxazole + Trimethoprim	SXT	≤10	11-15	-	≥16
Tetracycline	Te-30	≤14	15-18	-	≥19
Ticarcillin	TIC-75	≤11	12-14	-	≥15
Ticarcillin/ Clavulanic Acid	TIM-85	≤11	12-14	-	≥15
Tobramycin	NN-10	≤12	13-14	-	≥15
Vancomycin	Va-30	≤9	10-11	-	≥12

Name of Organism used: Gram + or Gram -?		
Name of Antibiotic	Clearing in mm	Classification (R, I, MS, S)

Look up the antibiotics used against your organism and write an explanation below of how your organism should have responded to each antibiotic used. Then conclude with an indication of whether or not your organism responded as expected and if not why not?