2016

Laboratory Exercises in Microbiology: Discovering the Unseen World Through Hands-On Investigation

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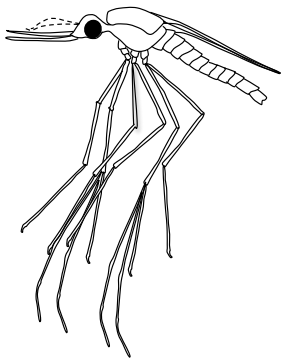
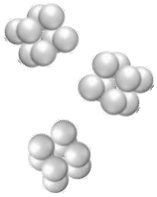
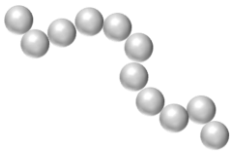

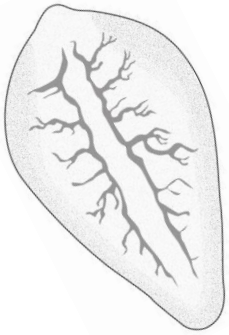

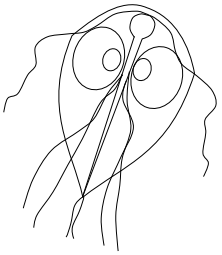
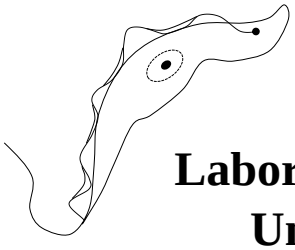

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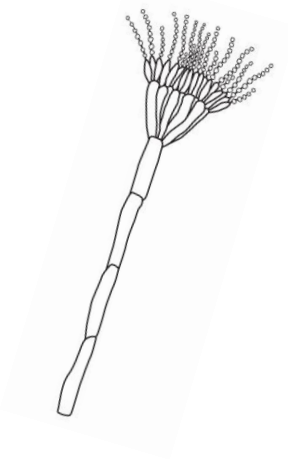
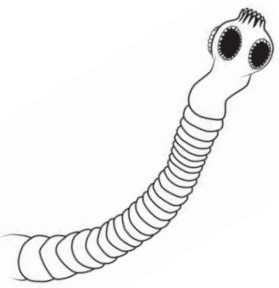

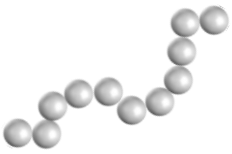


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Preface

Dear Student:

Welcome to the world of Microbiology! Although invisible to the unaided eye, microbes play many important and beneficial roles in nature as well as within the human body. For example, they are critical for processes of decomposition and nutrient cycling, produce natural antimicrobial compounds, and help protect our bodies from dangerous pathogens (disease-causing microorganisms). However, not all microbes are "good guys"-pathogenic bacteria, viruses, and parasites continue to challenge health care providers in numerous ways. The evolution of antibiotic resistance among bacteria, as well as emerging and re-emerging infectious diseases, are just two examples of the significant challenges that we are currently facing. In recent years we have become increasingly aware of the critical roles of the human microbiome (all the microbes we carry within and on our bodies) on human health and disease, yet there is still so much that remains unknown. Health care providers need to have a full understanding of the microbial world to properly detect, diagnose, treat and prevent infectious diseases, as well as to know how to protect themselves and others from harm.

We have designed the laboratory exercises in this book around a few major concepts-proper use of aseptic techniques, bacterial staining and microscopy, bacterial metabolism, and control of microbial growth. As you read through each exercise, perform the experiments, and interpret your results, try to always remember the "big picture"—how your knowledge of the microbial world will help you in your future career.

Each laboratory exercise begins with objectives and key terms, and review questions are found at the end of each chapter. Be sure to familiarize yourself with the key terms—they are bolded within the text of each exercise. Review questions will help ensure that you understand the "big picture" as well. In addition, there are appendices that provide supplementary information to help you understand the material found in some of the exercises.

The creation of this manual involved several semesters of testing out new laboratory exercises, modifying experiments and revising write-ups based on feedback from other instructors and students. We welcome additional comments and suggestions as to how we can improve the manual in the future.

Best of luck with your studies—we hope that you will enjoy learning about and exploring the wonderful world of microbes as much as we have. Always remember- you are never alone because your microbes are always with you!

A note to instructors: At Queensborough Community College, Lab 13 (Case studies in Microbiology) is not included in the manual but provided to our students as a handout in the last class—this is done so that the students can answer the questions based on what they observe in the lab rather than on prior preparation. A copy of this exercise, as well as answers to review questions can be requested by emailing either of the authors.

Please feel free to contact us with questions or comments on the manual.

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Acknowledgments

The authors would like to thank the following people who contributed to the preparation of this manual:

- Our College Laboratory Technician, Ms. Laura Rachiele, for her tireless dedication and willingness to try out numerous experiments with us, and for providing the photographs used in the manual
- Our two student readers, Bharti Kumari and Stephanie Solomon, for the great effort they put into reading, evaluating, and providing the student perspective on the exercises
- Our colleague Dr. Monica Trujillo for providing the *Streptomyces* cultures and for her expertise in helping to develop this exercise
- Mr. Adam Morgenstern & Antonios Tsimounis for providing many of the illustrations
- Our fellow Microbiology instructors at QCC for their helpful comments and suggestions on previous versions of the manual
- QCC's library for providing funding for us to complete this project and for their helpful guidance about publishing open educational resources
- And finally, to all Microbiology students who have used this manual in its earlier versions for providing us with their comments and suggestions during the revision process

Microbiology Lab Safety Instructions

The instructions below are designed to keep you safe in the laboratory. Please read them **carefully**. If you have any questions about safe laboratory practices, ask your instructor.

1. No eating, drinking or smoking at any time. Do not bring food or drink items into the lab. Avoid all finger/hand-to-mouth contact.

2. A lab coat must be worn at all times in the microbiology laboratory. You will not be allowed to participate in the lab without one.

3. Follow all directions given by the instructor. Bring any safety concerns to the attention of the instructor.

4. Come to lab on time and prepared for that day's experiments.

5. Wash hands, and wipe down bench area with disinfectant prior to working. Before you leave the lab for the day wipe down your bench area with disinfectant and then wash your hands. Wash your hands at any time during the lab if you think you may have contaminated them. Wipe any surfaces or equipment with disinfectant immediately if you suspect contamination with living cultures.

6. Loose clothing and long hair must be tied back while working to avoid burning with open flames or inadvertent contamination.

7. Open-toed shoes (sandals, flip-flops etc.) cannot be worn in lab.

8. Use care with the Bunsen burners. Keep paper, alcohol and other flammable items away from the open flame.

9. Treat all living cultures of microorganisms (bacteria, yeast, etc.) as potential pathogens. Avoid spilling or spreading the microorganisms. Place all used materials in the appropriate waste containers designated for cultures (to be autoclaved). Use the techniques specified by the instructor for handling microorganisms. If there is a spill notify your instructor **immediately**.

10. Know where fire extinguishers and safety equipment are located in the lab.

11. To prevent contamination of these articles, books, coats, backpacks, etc. (anything you do not need for the Micro lab) must be placed in the designated area and should not be kept at the laboratory table.

12. Make sure to carefully read through the entire procedure before beginning an experiment in the lab. This will help prevent you from making mistakes that could compromise your safety.

Notes:

- Since many laboratory procedures are carried over to the next week, make sure you bring previous lab write-ups with you to the following lab.
- For supplementary color pictures of media, microorganisms, videos, etc., see the Blackboard Review site. Instructions for signing up for this site are provided in the syllabus.

Instructions for Good Laboratory Practice and Care of Laboratory Equipment

Correct use and care of the laboratory equipment is considered a fundamental part of good laboratory technique. All students working in the microbiology laboratory are responsible for maintaining equipment and materials in proper working condition.

Please read over and follow the instructions listed below:

Microscopes
The most critical (and most expensive) piece of equipment in the microbiology laboratory is the microscope. If you expect to see specimens through the microscope, it must be kept clean and in good condition. You must use the microscope assigned to your seat. Instructions for the use and care of the microscope can be found in Lab 1 of the lab manual. Report any problems with your microscope to your instructor.

Inoculating loops and inoculating needles
Inoculating loops and needles are used to transfer bacteria into and from culture media. Inoculating loops have a loop at the end, while inoculating needles end in a point. Inoculating loops are the most common method of transferring bacteria. Inoculating needles are used when stabbing into a medium during specific inoculation procedures, or when it is necessary to pick up a small amount of bacteria from one colony on an agar plate without contacting bacteria in other colonies.

Bunsen burners
A Bunsen burner is a source of open flame that is used to sterilize loops and needles, as well as flaming the lips of test tubes during inoculations. You must always take great care when operating a Bunsen burner!

To light the Bunsen burner, turn the handle of the valve so it is in line with the tubing connecting the Bunsen burner to the gas. Using a striker or a BBQ lighter, light the Bunsen burner. If the Bunsen burner does not immediately light, turn off the gas and determine the cause of the problem. NEVER leave the gas on if the Bunsen burner is not lit. DO NOT lean over the Bunsen burner while lighting it.

Once the Bunsen burner is lit, be careful to keep all flammable items, including lab coats, hair, shirt sleeves, scarves, tissues, alcohol, etc. away from the flame. When the Bunsen burner is not actively in use it should be kept in the pilot setting for safety. If you smell gas at any time, check to make sure that the Bunsen burner is still lit. If the flame goes out at any time, TURN OFF THE GAS. When you are finished using the Bunsen burner, be sure to return it to the pilot setting before turning off the gas.

Before leaving the lab, make sure that the gas is off (handle of the valve perpendicular to the tubing connected to the Bunsen burner).

Microscope slides
Any disposable glass slides should be discarded in the sharps container. Do not discard glass slides in the waste cans.

Petri dishes and test tubes
All materials used for handling or culturing microorganisms are to be disposed of as follows: test tubes placed in racks in a bin for autoclaving; petri dishes in the other bin for autoclaving and disposal.

Spillage
Any living culture material that is spilled, either on tables or on the floor, is to be treated immediately with disinfectant and cleaned up with paper towels. Notify the instructor of any spills. The paper towels that you use to clean up the spill should be placed in the bin with the petri dishes for autoclaving.

Prepared slides
Prepared slides that are used during the semester must be returned clean to the trays from which they were taken.

Cleanliness of the room
Any papers on the floor at the end of the laboratory period are to be picked up and discarded in the wastebasket. The same is true for your laboratory bench area.

VERY IMPORTANT!

DO NOT throw plates, tubes, swabs, slides, pipets, pipet tips, broken glass, etc. into the regular garbage. These items need to be disposed of properly. Throwing potentially contaminated items into the regular garbage is a safety issue for students, instructors, lab techs and the cleaning staff. If these items are found in the regular garbage the ENTIRE BAG OF GARBAGE must be autoclaved before disposal. If you are unsure about where an item should go, always ask your instructor.

Laboratory Exercise 1: Introduction to Microscopy and Diversity of Cell Types

Objectives

1. Review the principles of light microscopy and identify the major parts of the microscope.
2. Learn how to use the microscope to view slides of several different cell types, including the use of the oil immersion lens to view bacterial cells.
3. Learn about the shapes and arrangements of some common types of bacteria.
4. Review the taxonomic classification system used in scientific nomenclature.

Key Terms: microorganism, magnification, resolution, working distance, parfocal, parcentric, prokaryotic, eukaryotic, bacillus, coccus, spirillum, spirochete, morphology, bacterial arrangements, depth of field, field of view, taxonomic classification

Introduction

The first microscope was developed in 1590 by Dutch lens grinders Hans and Zacharias Jansen. In 1667, Robert Hooke described the microscopic appearance of cork and used the term cell to describe the compartments he observed. Anton van Leeuwenhoek was the first person to observe living cells under the microscope in 1675—he described many types of cells, including bacteria. Since then more sophisticated and powerful scopes have been developed that allow for higher magnification and clearer images.

Microscopy is used by scientists and health care professionals for many purposes, including diagnosis of infectious diseases, identification of **microorganisms** (microscopic organisms) in environmental samples (including food and water), and determination of the effect of pathogenic (disease-causing) microbes on human cells. This exercise will familiarize you with the microscopes we will be using to look at various types of microorganisms throughout the semester.

The Light microscope

What does it mean to be microscopic? Objects are said to be microscopic when they are too small to be seen with the unaided eye—they need to be magnified (enlarged) for the human eye to be able to see them. This includes human cells and many other types of cells that you will be studying in this class.

The microscope you will be using uses visible light and two sets of lenses to produce a magnified image. The total magnification will depend on which objective lens you are using—the highest magnification possible on these microscopes is 1000X—meaning that objects appear 1000X larger than they actually are.

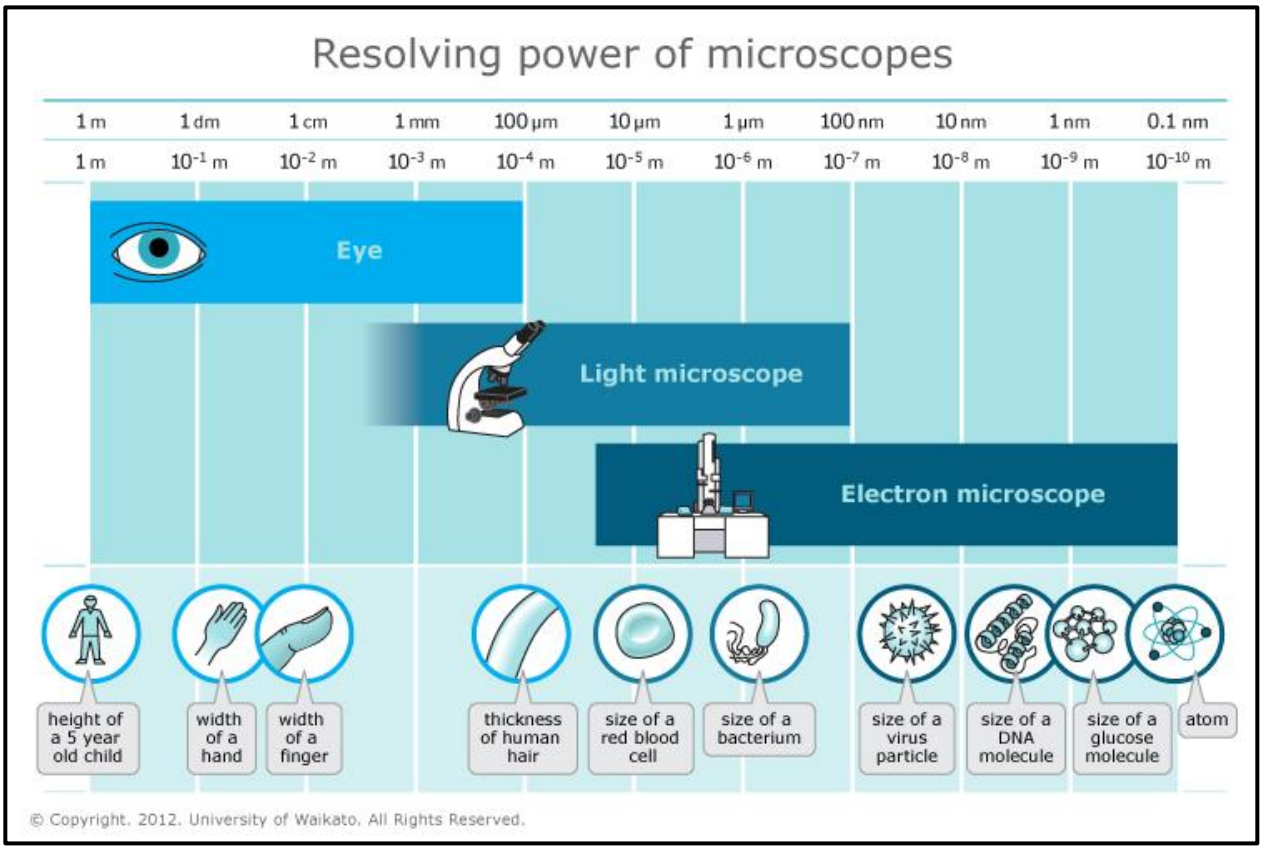
Resolution vs. magnification: **Magnification** refers to the process of making an object appear larger than it is; whereas **resolution** is the ability to see objects clearly enough to tell two distinct objects apart. Although it is possible to magnify above 1000X, a higher magnification would result in a blurry image. (Think about magnifying a digital photograph beyond the point where you can see the image clearly). This is due to the limitations of visible light (details that are smaller than the wavelength of light used cannot be resolved).

The limit of resolution of the human eye is about 0.1 mm, or 100 microns (see Table 1 for metric review). Objects that are smaller than this cannot be seen clearly without magnification. Since most cells are much smaller than 100 microns, we need to use microscopes to see them.

The limit of resolution of the light microscope you will be using today is about 0.1 μm, or 100 nm. This means that we can view objects that are 1000X smaller than what we can see with our eyes alone. Biologists typically use microscopes to view all types of cells, including plant cells, animal cells, protozoa, algae, fungi and bacteria. The nucleus and chloroplasts of eukaryotic cells can also be seen—however smaller organelles and viruses are beyond the limit of resolution of the light microscope (see Figure 1).

Table 1: Metric units commonly used in Microbiology
The basic unit of measurement of length in the metric system is the meter.
There are 1000 millimeters (mm) in one meter. 1 mm = 10^{-3} meter
There are 1000 micrometers (microns, or μm) in one millimeter. 1 μm = 10^{-6} meter
There are 1000 nanometers in one micrometer. 1 nm = 10^{-9} meter

Figure 1.



Procedures

A. Use and Care of the Microscope

When instructed to do so, obtain the microscope that corresponds to your seat number from the cabinet (power cords are in a plastic box in the drawer near your seat). Familiarize yourself with the major parts and their functions—you may use the information found at the end of this chapter to guide you.

Basic Guidelines for Using the Microscope

1. Always carry the microscope with **two hands**.
2. Always use the microscope that is assigned to your seat number.

3. Clean the lenses with lens cleaner (Windex) and lens tissue before and after use.
4. Report any problems with the microscope to your instructor immediately.
5. Oil **must** be cleaned off completely before returning the microscope to the cabinet. If you accidentally get oil on the 40X objective, clean it immediately. Microscopes must always be returned to the cabinet clean.
6. Microscopes should always be put away with a low power objective (4X or 10X) over the stage.
7. Always **lift** the microscope to reposition it—do not drag it across the surface of the table!

Total Magnification: The microscope you are using has two sets of lenses that both contribute to the total magnification of the image. The ocular lenses magnify your image 10X. There are 4 different objective lenses—each with a different magnification. The total magnification is calculated as follows:

Total magnification= ocular magnification x objective magnification

Since the ocular magnification of our microscope is 10X, determining the total magnification of an object with this microscope simply requires multiplying the objective magnification by 10. (Note: other microscopes may have ocular lenses with a different magnification, for example 12X.)

Fill in the chart below

Name of lens	**Objective magnification**	**Total magnification**
Scanning		
Low		
High-dry		
Oil immersion		

Diversity of Cells: There are many types of cells found among the diverse forms of life on the planet. All cells have certain features in common, such as a plasma membrane surrounding the cell, cytoplasm within the plasma membrane, and DNA as the molecule that stores genetic material. However, there is a great deal of diversity among the cells that make up living organisms.

Some living organisms are composed of one cell (unicellular); others are composed of many cells (multicellular).

Cells that have a nucleus and other organelles are **eukaryotic**; bacterial cells do not have these intracellular structures—they are **prokaryotic**.

Some types of cells (plants, algae and some bacteria) are photosynthetic (capable of using light energy to make organic compounds from inorganic materials.) Others take in organic molecules as a source of energy.

There are several other similarities and differences between cell types that you will learn about throughout the semester. In today's lab you will use the microscope to look at various types of cells that are often studied in a Microbiology course.

B. Examination of different cell types under the microscope

In this activity you will look at some prepared slides, as well as make a few of your own.

Materials:

- Prepared slides: Blue-green algae**,** mixed protozoa, bacterial types
- Slides and cover slips
- Pond water sample (if available)
- Live *Saccharomyces* culture
- Methylene blue stain
- Prepared slides (demos): may include Pinworm (*Enterobius),* pinworm eggs, mosquito, yeast cells (*Saccharomyces*), bacterial slides showing varying morphologies and arrangements

Instructions: Use the spaces provided at the end of this exercise to draw pictures as you are viewing the slides.

1. Place your first slide (**blue-green algae**) on the mechanical stage and make sure that it is level and held firmly in place. Your sample /smear should be facing upwards.
2. Use the knobs located below the stage to move the slide left and right, and up and down until the stained area of the slide is centered over the light source.
3. Position the scanning power objective (4X) in place over the slide. (Note: objective will "click" into place).

4. Use the course adjustment knob to bring the stage up as far as it will go.
    - When using the scanning or low power objectives the **working distance** (the distance between the lens and the slide) is large enough so that the slide will never make contact with the lens. This is not the case when using the high-dry and oil immersion lenses, where the working distance is significantly less. This is why the coarse adjustment knob can **only** be used with the two low-power lenses.

5. Rotate the coarse adjustment knob away from you until the image comes into focus.
    - You will not be able to make out much detail at this power—the purpose is to find where your specimen is on the slide so that it is easier to locate when you switch to high power. Low power objectives have a large **field of view** (the circular area seen when looking through the microscope) and a large **depth of field** (the thickness of a specimen that is in sharp focus). As magnification increases, both the field of view and depth of field decrease, which is why it is easier to locate your specimen using a low power objective.

6. If needed, use the fine adjustment knob to improve the clarity of the image.

7. After viewing the slide at scanning power, move the slide so that the area you want to focus on is in the center of the field of view.
    - Since your microscope is **parcentric**, when you increase magnification you will be zooming in at the center of the field of view. Objects that are not centered at low power may be out of the field of view at high power.

8. Rotate the objective lens nosepiece so that the 10X objective is in place over the slide. Re-focus and adjust the light (if needed) under the 10X objective.
    - The microscope you are using is **parfocal**—this means that when it is in focus with one lens in place the same stage position will be in focus with all other lenses. Therefore when switching objectives, DO NOT change the position of the stage—just click the objective you wish to use into place.

Note the differences in the appearance of the cells from when you were using the 4X objective.

9. After focusing at 10X, rotate the objective lens nosepiece so that the 40X objective is positioned over the slide. Re-focus using the fine adjustment knob and adjust the light if needed.
    - Note: Remember that you CANNOT use the coarse adjustment knob at high power (40X or 100X objectives). When you are using the high-power lenses the lens is very close to the slide (small working distance) therefore using the coarse adjustment knob at high power could result in damage to the lens, damage to the slide, or both.

Remember to sketch what you are looking at in the spaces provided.

10. When you are finished looking at the blue-green algae slide, return it to the slide box and proceed to the next slide—**Mixed protozoa**. Follow the instructions above to view the slide with the 4X, 10X and 40X objectives in place. Draw diagrams of each.
    - When viewing the mixed protozoa slide, you should scan different areas of the slide, as there are many types of protozoans and algae present on this slide. Some organisms you might see include *Volvox, Amoeba,* and *Paramecium.* Note that in most of these cells the nucleus is clearly visible.

11. Sketch a few areas of this slide with the 10X or 40X objective in place. Use the charts in the lab to identify some of the organisms you see.

12. Return the mixed protozoa slide to the slide box and use a clean slide and cover slip to prepare a wet mount of a live pond sample (see instructions below). View this slide at 4X, 10X and 40X magnification.

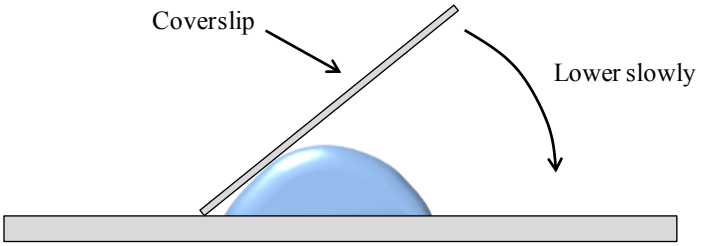
C. Preparation of Wet Mounts

These slides will be prepared with live microbes on them. Since the liquid is left on the slide, cover slips are needed to prevent this liquid from touching the lenses.

a. Live protozoa: use a dropper to place 1-2 drops of a pond water sample onto a clean microscope slide. Take a cover slip and place it down over the water as shown in the diagram below (try to avoid air bubbles).

b. Preparation of a live yeast culture: place one drop of the yeast (*Saccharomyces*) suspension and one drop of methylene blue onto a clean microscope slide, and prepare a wet mount as shown.

Figure 2. Preparation of a wet mount



Observe these slides under the microscope (use the magnifications suggested by your instructor).
As time permits, your instructor may have you examine other types of cells that are set up on demonstration scopes.

D. Observing bacterial cells under the microscope.

In this exercise you will learn how to use the oil immersion lens, and observe some common morphologies and arrangements of bacteria.

1. Obtain a **bacterial types** slide from the slide box and place it on your microscope. Since bacterial cells are very small, you will need to use the highest magnification (100X objective, or 1000X total magnification) to see them clearly—however, as with all slides, you should use a low-power objective lens to focus on the slide before moving to high power.

   The bacterial types slide may be viewed with all 4 objectives—alternatively, your instructor may ask you to skip some (for example, just use the 10X and 100X objectives). Follow the instructions you receive in class.

- There are three areas of bacteria on the slide. The area closest to the slide label is the darkest and therefore the easiest to find: the area on the rightmost side of the slide is very faint and you may not be able to detect any stain with your eyes.

2. Focus with a low-power objective on the first area of the slide.
   - The cells will still be very small at this magnification and you will not see any detail.

3. Once in focus, switch the objectives to 10X and then to 40X (this step may be skipped). Adjust light and focus as needed.

4. You are now ready to use the oil immersion lens. This lens requires the use of immersion oil, which has the same index of refraction as glass, to prevent light from scattering and focus it on your specimen (we need a lot of light to see clearly at this high magnification).
   - Immersion oil MUST be used with the 100X lens.
   - Immersion oil should NEVER be used with the high dry lens.

5. Rotate the nosepiece so that there is no objective over the stage. Add 1-2 drops of immersion oil to the slide right above where your sample is.

6. Click the 100X objective into place. If done correctly, you should only need to fine focus a little bit to bring the cells into view.
   - Remember to NEVER use the coarse adjustment knob when focusing under 40X or 100X objectives.
   - If you "get lost," it's better (and faster) to go back to low power and refocus, then switch back to 100X.
   - Once there is oil on the slide you CANNOT use the 40X lens—rotate the nosepiece the other way to use 4X or 10X to refocus.

7. Use the spaces provided to draw the cells. At this point, your instructor will discuss the morphology (shape) and arrangements of the cells you are looking at. (See Figure 3. at the end of the exercise for more information.)

8. When you are finished, move the slide to the right to find the second area of cells (middle of the slide), and then to the third area of cells (right side of the slide).

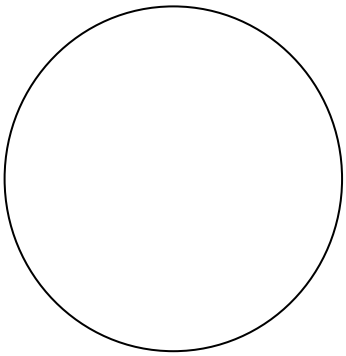
    - The third area of cells is very faint and the cells are more spread out. If you "get lost"—try going back to find the second area and then carefully move the slide across to the third area while keeping the slide in focus.

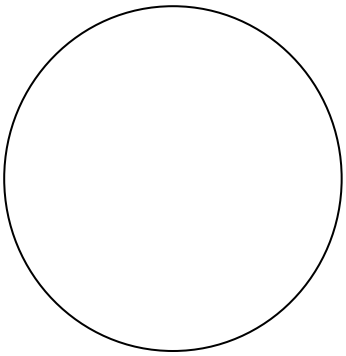
9. When you are finished, clean all oil off of the slide and return it to the slide box.

Results

Blue Green Algae

Low power **High power**

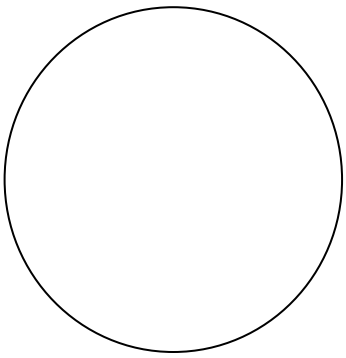


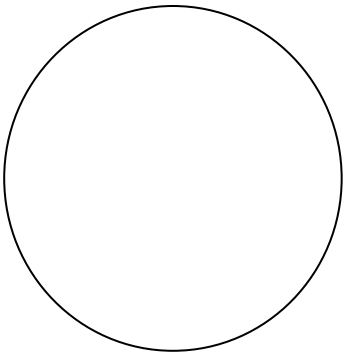


_____ **Total Magnification** _____

Mixed Protozoa

Low power **High power**

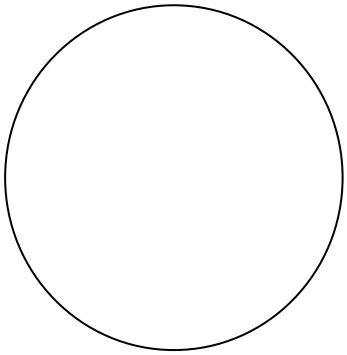


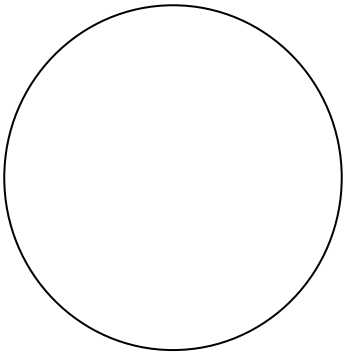


_____ **Total Magnification** _____

Bacterial Types

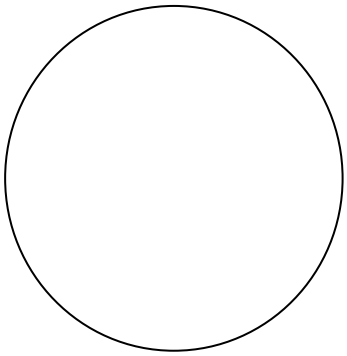
Bacilli **Low power** **1000X (oil immersion)**

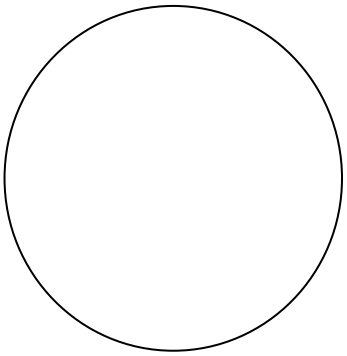




_____ **Total Magnification** _____

Cocci **Low power** **1000X**





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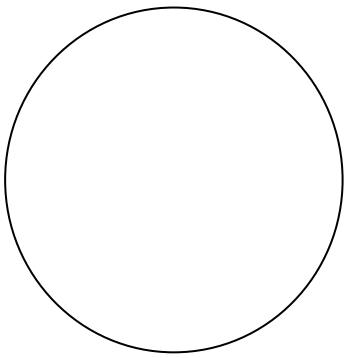
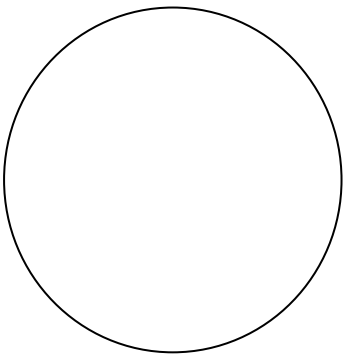
Spirilla **Low power** **1000X**

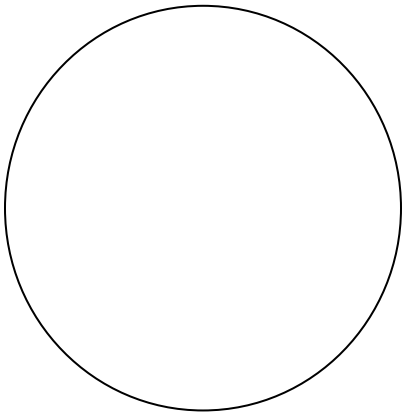




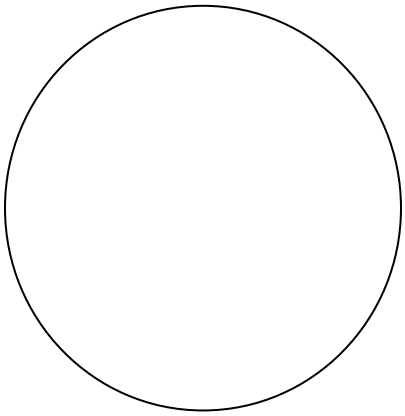
_____ **Total Magnification** _____

Use the page below to draw additional sketches of the organisms you observe.

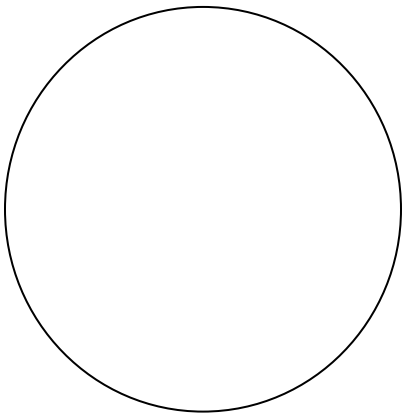
Name of organism _____
Magnification _____



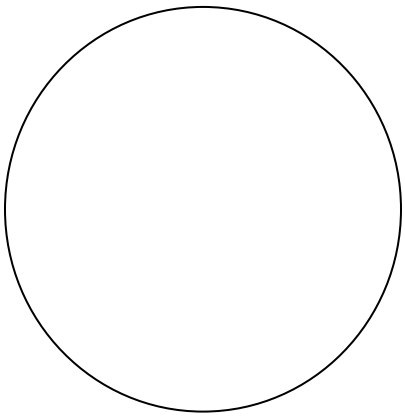
Name of organism _____
Magnification _____



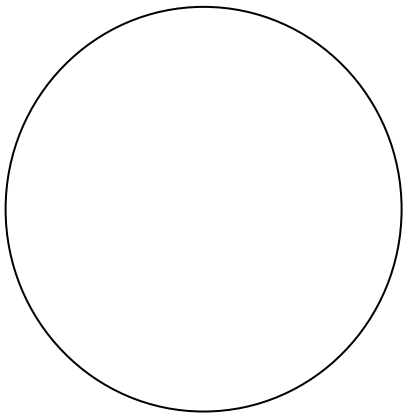
Name of organism _____
Magnification _____



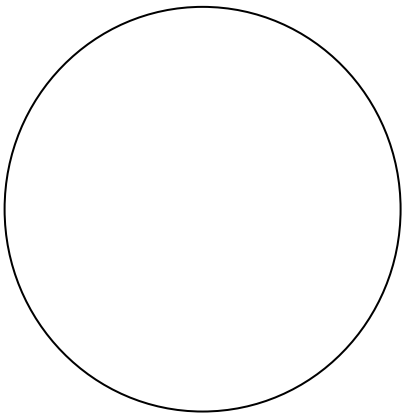
Name of organism _____
Magnification _____



Name of organism _____
Magnification _____



Name of organism _____
Magnification _____



Review Questions

1. Why is it important that health care professionals know about microorganisms?

2. What is the main difference between prokaryotic and eukaryotic cells?

3. Why is it important to use immersion oil when using the 100X objective?

4. Are there things that are too small to be seen with a light microscope? Explain.

5. Of the organisms you have looked at today, which are unicellular and which are multicellular?
   Unicellular:
   Multicellular:

6. Fill in the blanks: As magnification increases, the area of the field of view _____, the depth of the field of view _____, the working distance _____, and the amount of light required _____.

7. Distinguish between morphology and arrangement.

8. What do you think would happen if you tried to view a slide using the oil immersion lens but forgot to add the oil?

9. Use the information below to label the parts of the microscope on the figure provided.

Parts of the Microscope

a) Ocular (eyepiece): what you look through to view your slide. Our microscopes are **binocular**, which means they have two eyepiece tubes and both eyes are used (monocular microscopes have only one tube). Typically ocular lenses magnify an image 10X, although some have other magnifications (ex: 12X). Binocular microscopes allow for adjustment of the distance between pupils so that both eyes can be used to observe one image.

b) Objective lenses are the lenses close to the stage. Usually microscopes will have 3-5 lenses of different magnifications (ours have 4—4X, 10X, 40X and 100X magnifications). Objective lenses are located on a rotating turret to allow for changes in magnification.

c) Coarse adjustment knob: the larger (and outermost) of the two focusing knobs—moves the stage toward or away from the objectives to bring the image into focus at low power.

d) Fine adjustment knob: smaller knob within the coarse adjustment knob—used for "fine-tuning" an image. Only fine focus can be used when the 40X and 100X objectives are in place.

e) **Stage**: the platform where the slide to be viewed is placed. A **mechanical stage** holds the slide in place and allows for the movement of the slide to view different areas.

f) **Illuminator** (light source): found at the base (bottom) of the microscope below the stage.

g) **Condensor/iris diaphragm assembly:** found directly beneath the stage. This assembly can be raised and lowered using a knob at the side of the microscope. For our purposes, the assembly should be positioned very close to the bottom of the stage. The condenser is a lens that focuses the light from the illuminator onto the specimen. The iris diaphragm controls the amount of light that passes through the specimen. The iris diaphragm can be opened and closed by twisting the ridged ring of the assembly.

h) **Base:** the bottommost part of the microscope that contains the illuminator.

i) **Arm:** positions the objective lenses and the oculars above the stage. When moving the microscope, the base should be supported with one hand, while the arm is grasped with the other hand.

Figure 2. Label the Microscope Diagram

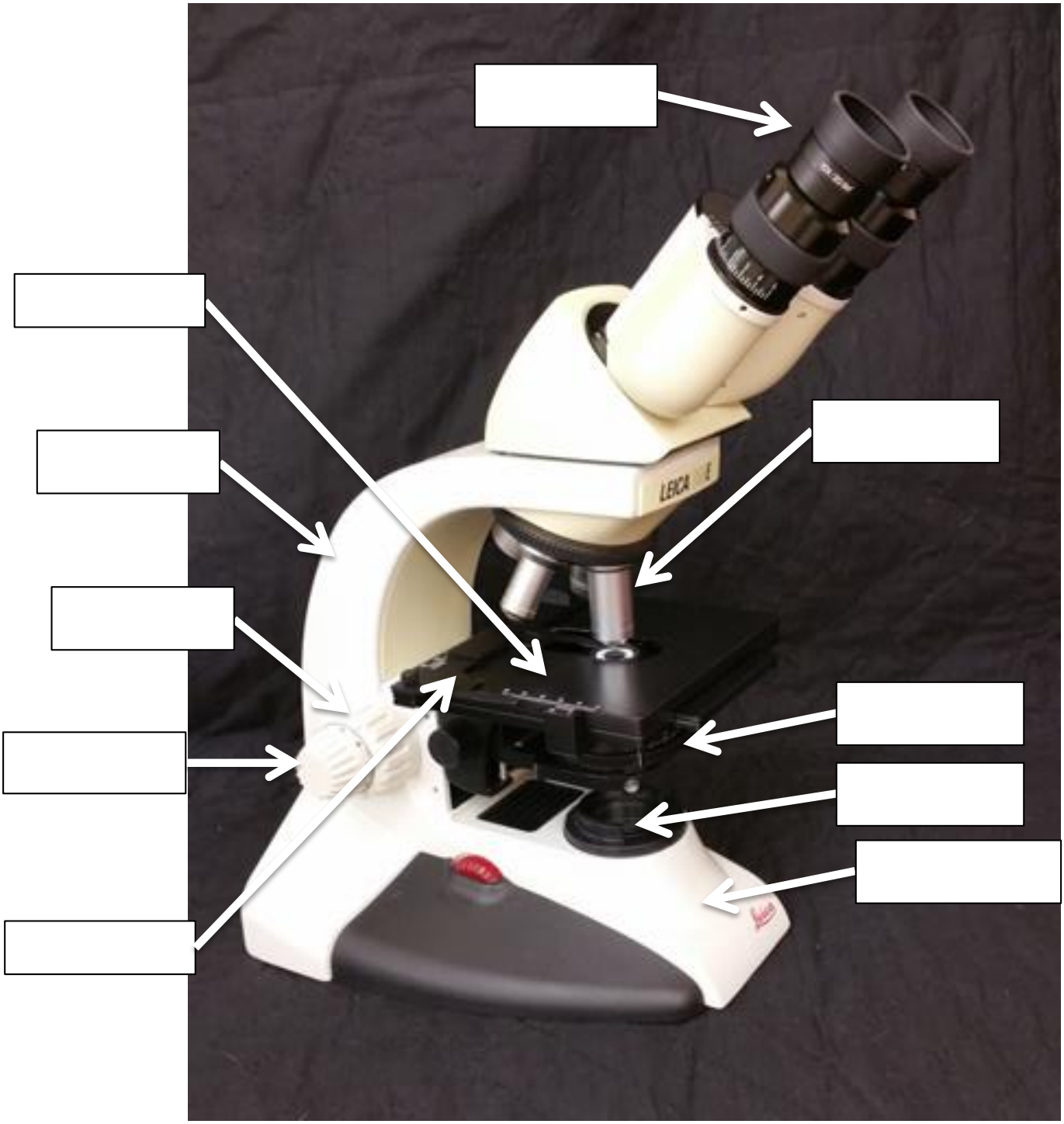
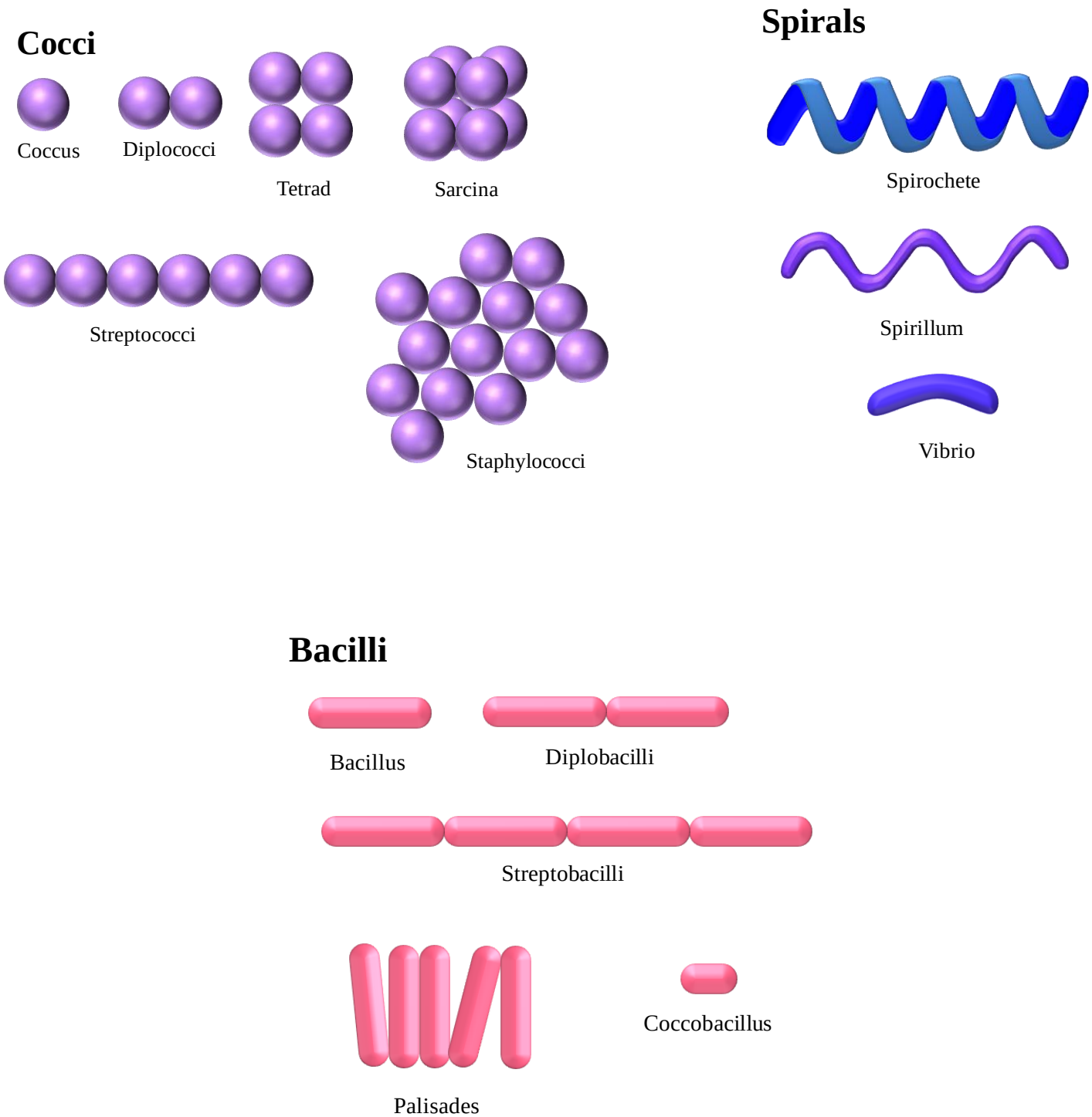


Figure 3. Common shapes and arrangements of bacteria

Note: There are other less common bacterial morphologies (e.g., filamentous, squares, etc.) that are not shown here.



Scientific Nomenclature

Scientific nomenclature is based on a taxonomic classification system. **Taxonomic classification** is a hierarchical system used to classify and compare organisms. There are 8 ranks in this system, listed below in the order of the most general (broadest) to the most specific:

1. Domain
2. Kingdom
3. Phylum
4. Class
5. Order
6. Family
7. Genus
8. Species

Prokaryotic organisms like bacteria may have classifications below the species level, including strain, subspecies, serotype, morphotype or variety.

Taxonomic classification indicates how closely organisms are related. For example, two organisms sharing the same Class are more closely related than two organisms sharing the same Phylum.

Binomial nomenclature: The scientific name of an organism consists of two words: the genus name and the specific epithet. The genus name comes first and is always capitalized; once identified it can be abbreviated to a single letter. The second word is known as the specific epithet and is not capitalized. The two words together make up the scientific name or species name. The genus can be used alone (you can refer to the genus *Staphylococcus* or the genus *Bacillus*) but the specific epithet without the genus name has no scientific significance. Scientific names in print should always be either italicized or underlined and should always be underlined when written. For example, the scientific name for human beings is *Homo sapiens* or *H. sapiens*. The scientific name of a bacterium is *Staphylococcus aureus* or *S. aureus*.

The scientific name often includes a description of the characteristics of an organism. The scientific name *Staphylococcus aureus* tells you the morphology and arrangement of the individual cells belonging to this bacterial genus (staphylococcus = spheres in clusters) and also tells you that *S. aureus* often grows in colonies with a golden color ("aureus"). Although scientific names are often descriptive occasionally these descriptions can be deceiving. For example, *Haemophilus influenza* is a bacterium (not a virus), and does not cause influenza.

Laboratory Exercise 2: Introduction to Aseptic Techniques and Growth Media

Objectives

1. Learn how to inoculate growth media using proper aseptic procedures
2. Learn how to streak for single colonies
3. Understand the uses of selective and differential growth media
4. Determine the properties of some common bacterial types when grown on selective and differential growth media

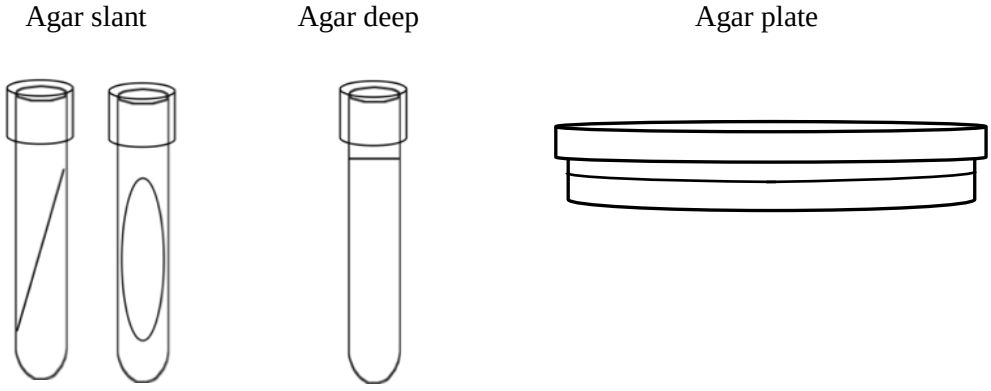
Key Terms: agar, broth, general purpose medium, selective medium, differential medium, colony, aseptic techniques, inoculation, streak-plate technique, contamination, sterile, pure culture, mixed culture

Introduction

Growth Media

To study bacteria and other microorganisms, it is necessary to grow them in controlled conditions in the laboratory. Growth media contain a variety of nutrients necessary to sustain the growth of microorganisms. There are two commonly used physical forms of growth media: liquid media and solid growth media. A liquid medium is called a **broth**. Solid growth media usually contains **agar**, which is a mixture of polysaccharides derived from red algae. It is used as a solidification agent because it (1) is not broken down by bacteria, (2) contains no nutrients that can be used by bacteria and (3) melts at high temperatures, and yet is solid at temperatures used for most bacterial growth. Solid growth media is used in the following forms: agar plates, agar slants and agar deeps. To make agar deeps or agar slants, melted agar is poured into a test tube and then allowed to solidify vertically (agar deep), or at a slant (agar slant). Agar plates are made by pouring melted agar into a petri dish.

Figure 1.



Broths can be used to determine growth patterns in a liquid medium, and for certain types of inoculations and metabolic tests that you will be doing later in the semester. They are also the method of choice for growing large quantities of bacteria. Agar slants are commonly used to generate stocks of bacteria. Agar plates can be used to separate mixtures of bacteria and to observe colony characteristics of different species of bacteria (you will perform an experiment in this lab to illustrate this). Deeps are used for several different types of differential metabolic tests (e.g., the gelatinase test, which you will perform in Lab 5).

Growth media can be categorized based on their chemical constituents, or the purpose for which they are used.

- **Complex growth media** contain ingredients whose exact chemical composition is unknown (e.g. blood, yeast extract, etc.).
- **Synthetic** (also called **chemically defined**) **growth media** are formulated to an exactly defined chemical composition.
- A **general purpose growth medium** (e.g. tryptic soy agar (TSA) or Luria broth (LB) is used to grow a wide variety of non-fastidious bacteria. This type of medium is often a complex growth medium.
- A **selective growth medium** contains chemicals that allow some types of bacteria to grow, while inhibiting the growth of other types. An example of a purely selective growth medium is PEA, phenylethyl alcohol agar, which allows Gram positive bacteria to grow while inhibiting the growth of Gram negative bacteria.
- A **differential growth medium** is formulated such that different types of bacteria will grow with different characteristics (e.g. colony color). An example of a differential growth medium is blood agar, which differentiates among bacteria based on their ability to break down red blood cells and hemoglobin. Blood agar is also a complex growth medium because it contains blood.

A growth medium can be both selective and differential. For example, EMB (eosin methylene blue agar) inhibits the growth of Gram positive bacteria. Gram negative bacteria that grow on this medium are differentiated based on their ability to ferment the sugars lactose and sucrose. (Note: the Gram staining procedure divides bacteria into 2 main groups: Gram-positive bacteria and Gram-negative bacteria, based on their cell wall structure. You will be doing Gram staining in the next lab period.)

Characteristics of Bacterial Growth

Even on general purpose growth media, bacteria can exhibit characteristic growth patterns. On agar plates, bacteria grow in collections of cells called **colonies**. Each colony arises from a single bacterium or a few bacteria. Although individual cells are too small to be viewed, masses of cells can be observed. Colonies can have different forms, margins, elevations and colors. Observing colony characteristics is one piece of information that microbiologists can use to identify unknown bacteria. Some examples of growth characteristics on different forms of growth media are shown at the end of the lab.

Aseptic Technique and Inoculation

Inoculation is the purposeful introduction of bacteria into a **sterile** growth medium. A material is sterile when it has no living organisms present; **contamination** is the presence of unwanted microorganisms. **Aseptic techniques** are practices that prevent the contamination of growth media.

When working in a microbiology laboratory, you must always remember that bacteria are present on all surfaces in the lab, as well as on your own hands and clothing. Aseptic techniques are designed to prevent the transfer of bacteria from the surrounding environment into a culture medium. These techniques require care and concentration. Pay attention to what you are doing at all times!

Aseptic techniques include the following practices:

1. Minimize the time that cultures and growth media are open to the environment.
2. Disinfect the work area before and after use.
3. Do not touch or breathe into the sterile culture media or the stock cultures.
4. Loops, needles, pipets, etc. should be sterilized before they are used.
5. **When working with tubes, the tube caps should not be placed on the table top; they should be held in your hand while inoculating.**
6. When removing the caps from test tubes, flame the lip of the test tube after the cap is removed. This heats the air inside the tube, so the air moves out of the tube, preventing contaminants from entering the tube.
7. Information about the use of the Bunsen burner can be found in the General Introduction in the Lab Manual.

General Procedure for inoculating media

Note: See figure on next page

1. Sterilize an inoculating loop or needle in the flame of a Bunsen burner. The portion of the loop or needle that will contact the stock culture or the growth medium must turn bright orange for effective sterilization. For the most rapid sterilization, place the loop at the top of the inner blue cone of flame—this is where the temperature of the Bunsen burner is the hottest. Remove the loop from the flame after it is properly heated- keeping the loops in the flame for too long will eventually cause them to crack.

2. If you are picking a colony from a plate, cool the inoculating loop on agar that does not contain any bacterial colonies.

3. Pick a small amount of bacteria (you do not need much). If you are inoculating a tube of broth or an agar slant, remove the cap of the tube (do not set the cap down on the table) and flame the lip of the tube. Throughout the procedure, hold the tube at an angle to reduce the probability of particles entering the opening. Insert the loop into the tube and transfer bacteria to the growth medium. Be careful that only the sterilized part of the loop touches the tube or enters the growth medium.

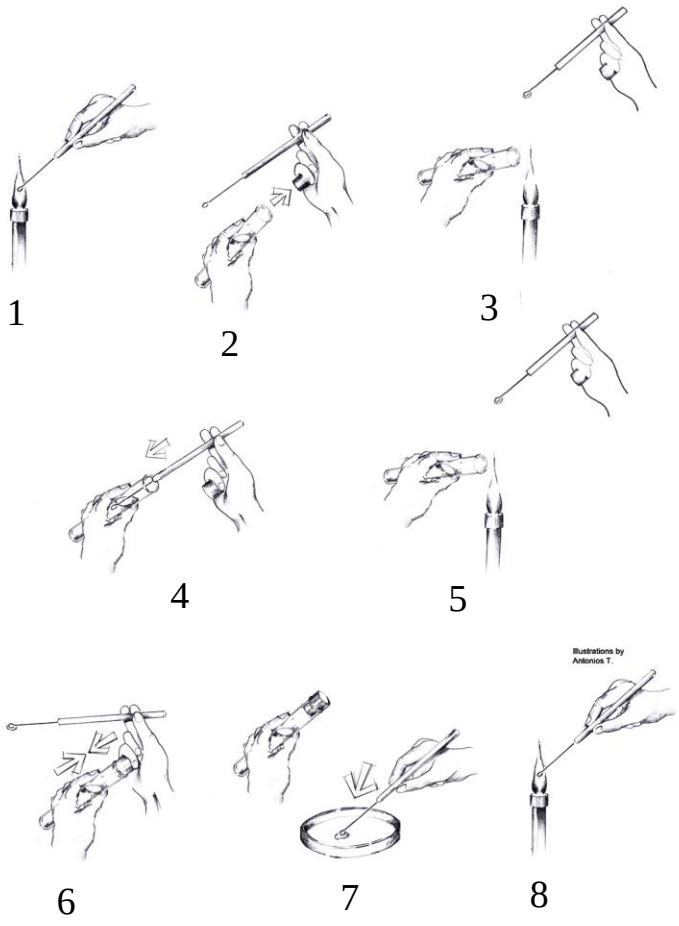
4. Flame the lip of the test tube before replacing the cap.

5. Sterilize the inoculating loop again.

Streaking for single colonies

In the real world outside the laboratory, bacteria grow in communities made of many bacterial species. If you need to identify the types of bacteria present in environmental or medical samples, you must have a way to separate out the different types and produce pure cultures. A **pure culture** contains a single bacterial species, whereas a **mixed culture** may contain many different types of bacteria. The process described in Procedure B (the streak plate method) describes the method that you will use to separate different types of bacteria in a mixture (see pg. 29).

Inoculating a Plate from a Broth Culture



1. Sterilize the inoculating loop.
2. Remove the cap from tube. Do NOT put the cap of the tube down on the lab bench—hold it in your hand.
3. Flame the lip of the tube.
4. Place sterile portion of inoculating loop into broth, then remove.
5. Flame the lip of the tube
6. Replace the cap.
7. Gently streak the surface of an agar plate with the inoculating loop.
8. Sterilize the inoculating loop.

Notes about Labeling and Incubating Plates

1. Always label your plates/tubes BEFORE you do your inoculations. You can use Sharpies on the plates, but wax markers ONLY on tubes. When labeling tubes, label the tube itself—don't label the cap!

2. Make sure you label the **bottom** of the plates (the part of the plate that holds the agar).

3. Place plates inverted (upside down) for incubation. This prevents condensation from falling on the surface of the agar and disrupting the streaking pattern.

Media Used in This Lab Exercise

Note: See the Microbiology Review site for color pictures of media.

Tryptic soy agar (TSA): General purpose complex growth medium.

Mannitol-salt agar (MSA): Differential and selective growth medium.

This medium contains 7.5% NaCl, the carbohydrate mannitol and the pH indicator phenol red (yellow at pH <6.8; red at pH 7.4 – 8.4; pink at pH >8.4). It is selective for staphylococci due to the high concentration of NaCl, and differentiates based on the ability to ferment mannitol. Staphylococci that ferment mannitol produce acidic byproducts that cause the phenol red to turn yellow. This produces a yellow halo in the medium around the bacterial growth.

MS Agar		
Selectivity	**Interpretation**	**Identification**
Growth	Organism not inhibited by NaCl	*E.g., Staphylococcus*, *Micrococcus*
No growth	Organism inhibited by NaCl	Not *Staphylococcus*
Differentiation		
Yellow halo	Organism ferments mannitol	Probable *S. aureus*
No yellow halo	Organism does not ferment mannitol	*Staphylococcus* species (other than *S. aureus*); *Micrococcus* (yellow colonies)

Eosin-methylene blue agar (EMB): Differential and selective growth medium.

This medium contains peptone, lactose, sucrose and the dyes eosin Y and methylene blue. Gram positive organisms are inhibited by the dyes, so this medium is selective for Gram negative bacteria. The medium differentiates based on the ability to ferment lactose (and/or sucrose.) Organisms that cannot ferment either of the sugars produce colorless colonies. Organisms that ferment the sugars with some acid production produce pink or purple colonies; organisms that ferment the sugars and produce large amounts of acid form colonies with a green metallic sheen. This medium is commonly used to detect the presence of fecal coliforms (like *E. coli*)—bacteria that grow in the intestines of warm-blooded animals. Fecal coliforms produce large amounts of acid when fermenting lactose and/or sucrose; non-fecal coliforms will produce less acid and appear as pink or purple colonies.

EMB Agar		
Result	**Interpretation**	**Identification**
No or poor growth	Organism inhibited by dyes	Organism is Gram-positive
Good growth	Organism not inhibited by dyes	Organism is Gram-negative
Colorless growth	Organism does not ferment sucrose or lactose	Non-coliform
Growth is pink and mucoid	Organism ferments lactose and/or sucrose with some acid production	Coliform bacteria
Growth is dark (purple to black with or without green metallic sheen)	Organism ferments lactose and/or sucrose, with large amounts of acid production	Possible fecal coliform (*E. coli*)

Procedures

Make sure you follow aseptic procedures and label everything carefully!

A. Observation of bacterial growth characteristics on selective and differential media

Each pair of students: 1 MSA and 1 EMB plate

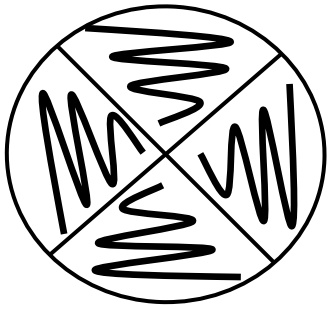
Bacteria used: *Staphylococcus aureus*, *Escherichia coli*, *Micrococcus luteus*, and *Pseudomonas aeruginosa*

Inoculation of selective and differential media

1. Divide the MS and EMB agar plates into 4 areas. Label each area with the type of bacteria that will be streaked there.

2. Use the inoculation loop to make a single streak of *S. aureus, E. coli*, *M. luteus,* and *P. aeruginosa* in each of the 4 areas (see below).

3. Incubate the plate until the next lab period.

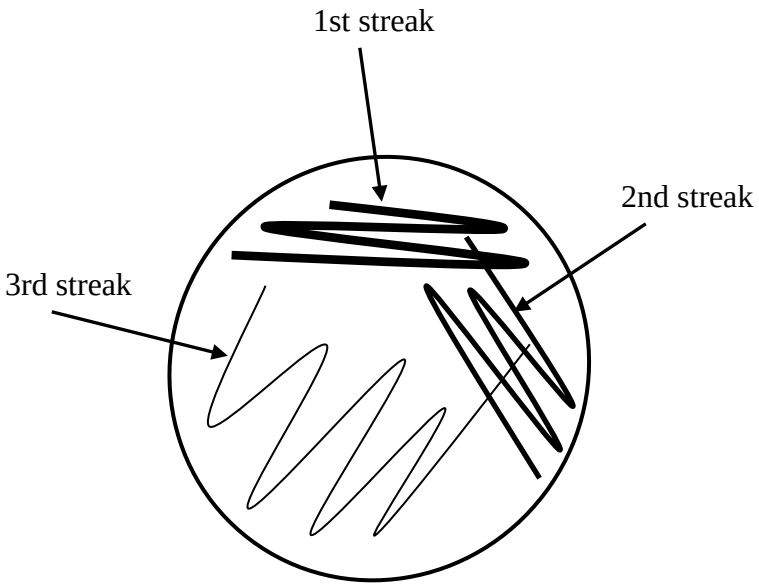


B. Inoculation for single colony isolation

Each pair of students: 4 TSA plates

1. Sterilize your inoculating loop.
2. Use your loop obtain some bacteria from your sample.
3. Streak the bacteria on a small portion of the plate.
4. Sterilize your loop.
5. Cool the loop on an uninoculated part of the agar surface, and then pass it once through the already-streaked region of the plate. Streak another small portion of the plate.
6. Sterilize your loop. Cool the loop, then pass it once through the second streak, and make a third streak.

Depending on the density of the original sample, single colonies will usually appear by the second or third streak.



Use the procedure outlined above to streak *S. aureus*, *E. coli*, *M. luteus*, and *P. aeruginosa* for single colonies onto individual TSA plates. Remember that you are trying to isolate single cells or a cluster of cells to form individual colonies. Do not pick up too much bacteria or spread it evenly all throughout the plate! Incubate until the next lab period.

C. Isolation of single colonies from mixed cultures using the streak plate method

Each pair of students: 1 MSA and 1 EMB plate

Mixed cultures: Unknown A and Unknown B

1. Streak Unknown A for single colonies on an MSA plate.
2. Streak Unknown B for single colonies on an EMB plate.
3. Incubate until the next lab period.

D. Culturing Microorganisms from Environmental Samples

Each pair of students: 1 MSA plate, 1 EMB plate, 1 TSA plate; 1 tube of sterile water; 1 sterile swab.

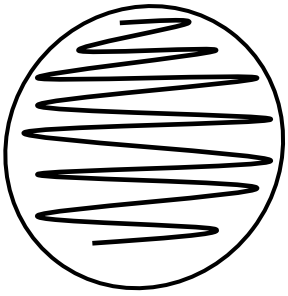
1. Pick one part of the environment that you would like to sample. Possibilities include: bottom of shoe, floor, cell phone, sponge on table, or the bottom of your handbag or backpack. Be inventive—pick an area you would find interesting!

Communicate with your lab partners so that different environments are represented at your table.

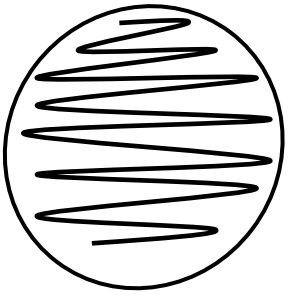
My environmental sample is from: _____

2. Moisten a swab in the sterile water; before the removing the swab, press it against the side of the tube to remove excess moisture.
3. Firmly swab the region you would like to sample.
4. Use the swab to inoculate the TSA plate, streaking the entire surface of the plate (see below). Use the same swab to streak the MS plate and the EMB plate.
5. Dispose of the swab in designated plastic container at your lab table. (DO NOT throw swabs in the regular garbage!)
6. Incubate the plates until the next lab period.

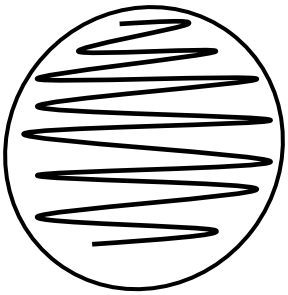
TSA Plate



EMB Plate



MS Plate



Results

Record the results of your experiments in the tables below.

A. Observation of growth characteristics on selective and differential media

Mannitol salt Agar			
Bacteria	**Growth (+/-)**	**Describe growth characteristics**	**Interpretation**
S. aureus			
E. coli			
P. aeruginosa			
M. luteus			

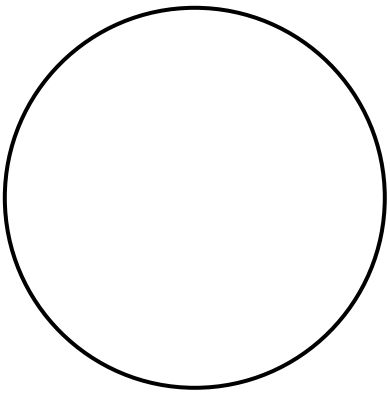
EMB Agar			
Bacteria	**Growth (+/-)**	**Describe growth characteristics**	**Interpretation**
S. aureus			
E. coli			
P. aeruginosa			
M. luteus			

B. Inoculation for single colony isolation

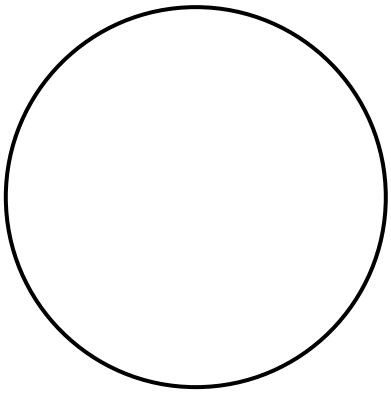
Tryptic soy agar		
Bacteria	**Growth (+/-)**	**Describe growth characteristics**
S. aureus		
E. coli		
P. aeruginosa		
M. luteus		

C. Isolation of single colonies from mixed cultures

Unknown A: Describe growth characteristics on MSA and make a sketch in the circle below.



Unknown B: Describe growth characteristics on EMB and make a sketch in the circle below.



Based on your results, answer the following questions:

1. How effective was the streaking procedure in separating the different types of bacteria?

2. What conclusions do you have regarding the types of bacteria in the unknowns?

Unknown A

Unknown B

D. Environmental Sample

Record the results of your own environmental sample and the other sample at your bench in the two tables below

Your environmental sample from _____

	Growth (+/-)	Describe growth characteristics
TSA		
EMB		
MSA		

Lab partner environmental sample from _____

	Growth (+/-)	Describe growth characteristics
TSA		
EMB		
MSA		

Answer the following questions and provide evidence to support your answers.

1. Did you detect any *Staphylococcus* species in your environmental sample?

2. Were Gram-negative bacteria present?

3. Were there any coliforms present? Was *E. coli* present?

4. Was *S. aureus* present?

5. Was there evidence for organisms other than bacteria? (Ask your instructor.)

Your instructor may ask you to save some of your environmental samples for further analysis.

Review Questions

1. Based on your results and the information in the lab manual, fill in the table below.

	Predicted Gram Reaction	**Ability to ferment lactose/sucrose**	**Ability to ferment mannitol**	**Coliform or Non-coliform**
S. aureus				
E. coli				
P. aeruginosa				
M. luteus				

2. What general type of growth medium would you use to:

(a) grow one type of bacteria but inhibit the growth of another type?

(b) discriminate between different types of bacteria?

3. Why is it necessary to sterilize the loop between streaks when streaking for single colonies?

4. Define and/or explain the use of the following:

(a) synthetic medium

(b) agar

(c) broth

5. A bacterial species is inoculated on EMB agar.

(a) The bacteria do not grow. Why?

(b) If the bacteria ferment lactose, what would you expect to see?

(c) The bacteria produce clear colonies. Why?

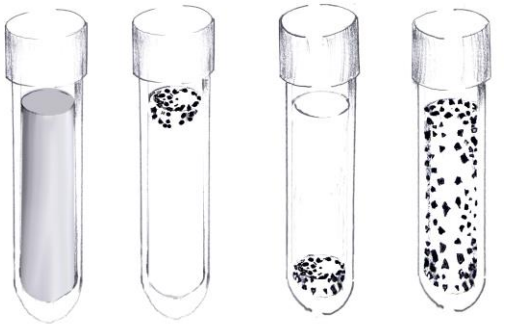
6. What medium would you use (TSA, EMB, MS) if you wanted to determine if a *Staphylococcus* isolate could ferment mannitol? Describe what you would see on this medium.

7. If you were testing water for the presence of fecal coliforms, what sort of medium would you use: TSA, EMB agar or MS agar? If fecal coliforms were present, what would their growth characteristics be on this medium?

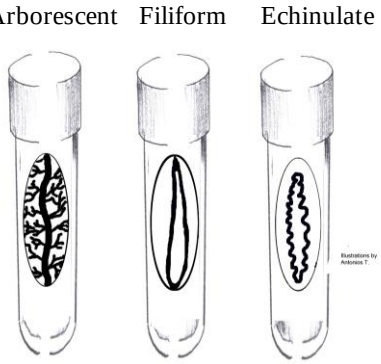
Examples of Bacterial Growth Characteristics in Broths, Slants and Plates

Even on general purpose growth media, bacteria can exhibit characteristic patterns of growth. Some examples are shown below. While these growth patterns are an important piece of information when identifying a bacterial species, they are not sufficient for a positive identification. Staining procedures and metabolic tests must be used for a definitive identification.

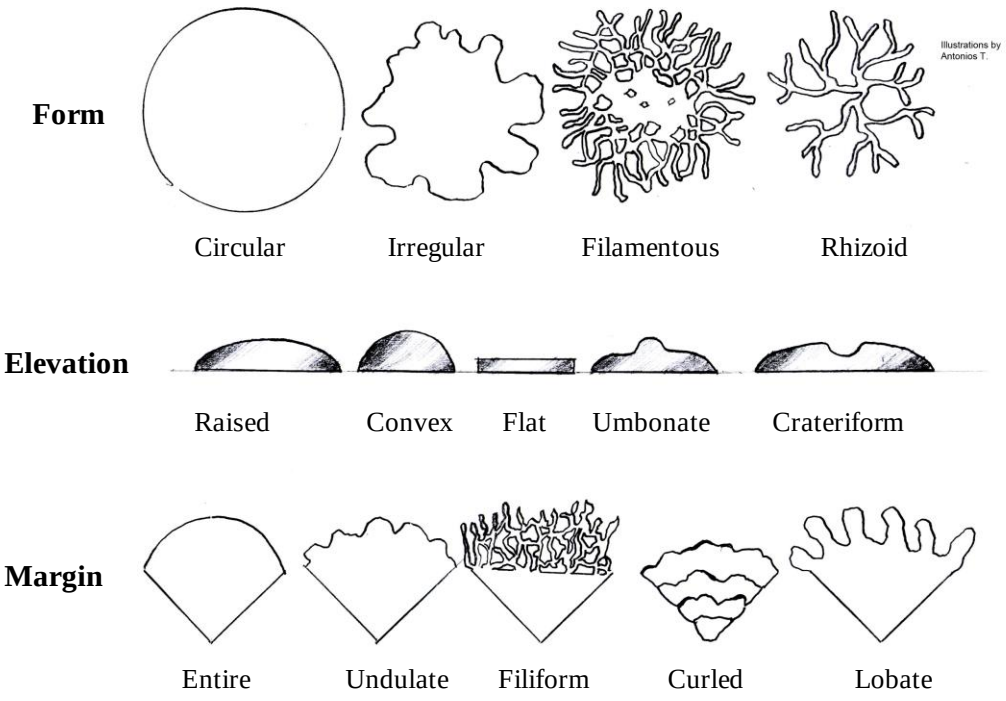
Growth Characteristics in Broths



Growth Characteristics on Slants



Growth Characteristics on Plates



Laboratory Exercise 3: Preparation of Bacterial Smears and Introduction to Staining

Objectives

1. Learn the differences between simple staining and differential staining techniques.
2. Learn how to prepare a bacterial smear from cultured organisms.
3. Learn the differences between gram positive and gram negative bacteria.
4. Learn how to perform the gram stain procedure.
5. Use microscopy to examine gram stained cells.
6. Learn about some special staining procedures, and view examples of these under oil immersion.

Key Terms: Gram stain, bacterial smear, simple stain, differential stain, Gram positive, Gram negative, Gram variable, capsule, spirochete, flagella, negative staining, silver stain

Introduction

Most types of cells do not have much natural pigment and are therefore difficult to see under the light microscope unless they are stained. Several types of stains are used to make bacterial cells more visible. In addition, specific staining techniques can be used to determine the cells' biochemical or structural properties, such as cell wall type and presence or absence of endospores. This type of information can help scientists identify and classify microorganisms, and can be used by health care providers to diagnose the cause of a bacterial infection.

One type of staining procedure that can be used is the **simple stain**, in which only one stain is used, and all types of bacteria appear as the color of that stain when viewed under the microscope. Some stains commonly used for simple staining include crystal violet, safranin, and methylene blue. Simple stains can be used to determine a bacterial species' morphology and arrangement, but they do not give any additional information.

Scientists will often choose to perform a **differential stain**, as this allows them to gather additional information about the bacteria they are working with. Differential stains use more than one stain, and cells will have a different appearance based on their chemical or structural properties. Some examples of differential stains are the Gram stain, acid-fast stain, and endospore stain. In this lab you will learn how to prepare bacterial cells for staining, and learn about the gram staining technique.

The Gram Stain

This very commonly used staining procedure was first developed by the Danish bacteriologist Hans Christian Gram in 1882 (published in 1884) while working with tissue samples from the lungs of patients who had died from pneumonia. Since then, the Gram stain procedure has been widely used by microbiologists everywhere to obtain important information about the bacterial species they are working with. Knowing the Gram reaction of a clinical isolate can help the health care professional make a diagnosis and choose the appropriate antibiotic for treatment.

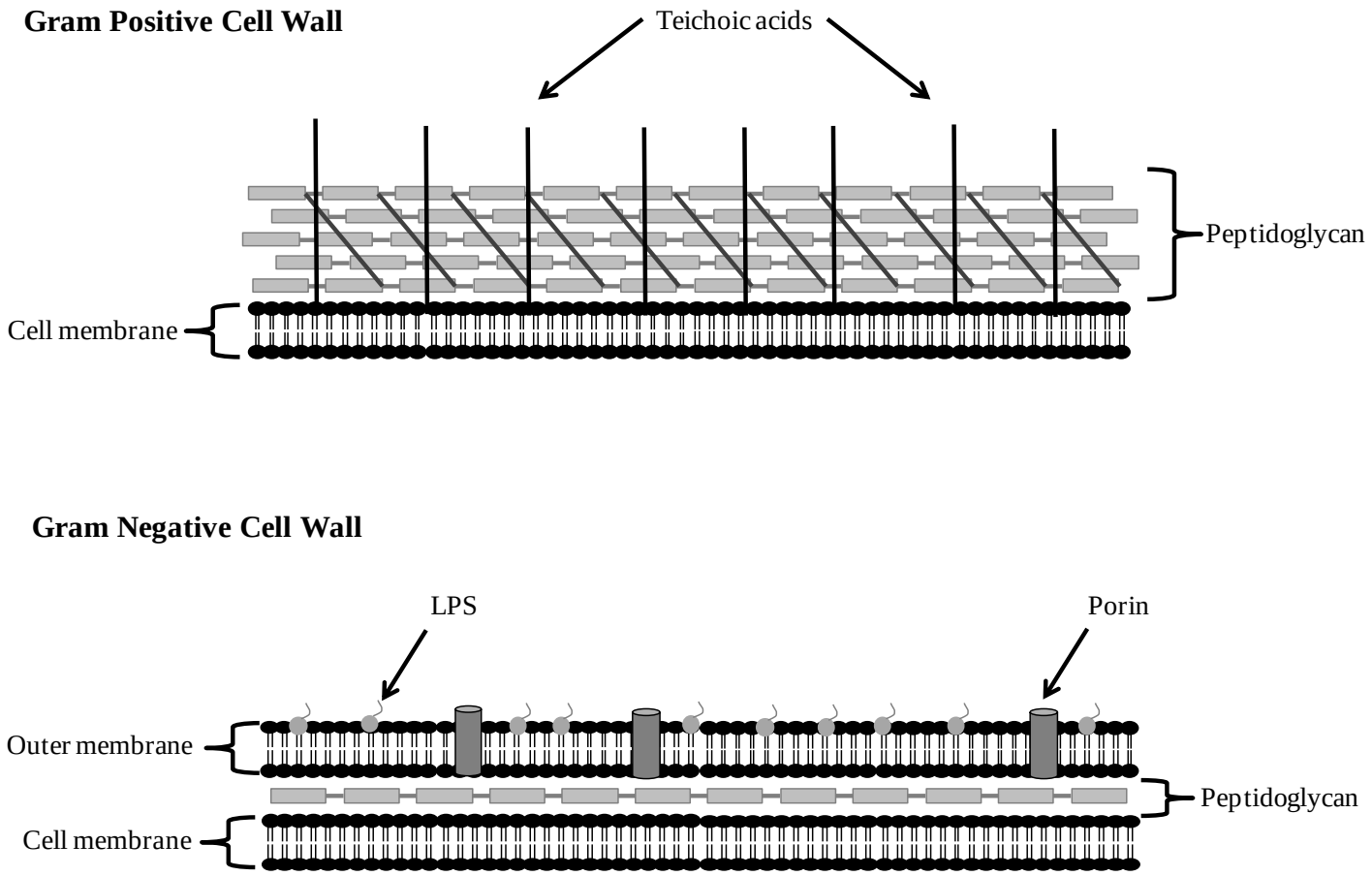
Gram stain results reflect differences in cell wall composition. **Gram positive** cells have thick layers of a peptidoglycan (a carbohydrate) in their cell walls; **Gram negative** bacteria have very little. Gram positive bacteria also have teichoic acids, whereas Gram negatives do not. Gram negative cells have an outer membrane that resembles the phospholipid bilayer of the cell membrane. The outer membrane contains lipopolysaccharides (LPS), which are released as endotoxins when Gram negative cells die. This can be of concern to a person with an infection caused by a gram negative organism.

Figure 1. shows the major differences between the Gram positive and Gram negative cell walls (also refer to your textbook for additional information). The differences in the cell wall composition are reflected in the way the cells react with the stains used in the Gram stain procedure.

Gram stains are best performed on fresh cultures—older cells may have damaged cell walls and not give the proper Gram reaction. Also, some species are known as **Gram-variable**, and so both Gram positive and Gram negative reactions may be visible on your slide.

Although the vast majority of bacteria are either Gram positive or Gram negative, it is important to remember that not all bacteria can be stained with this procedure (for example, Mycoplasmas, which have no cell wall, stain poorly with the Gram stain).

Figure 1.



Special Stains

There are a variety of staining procedures used to identify specific external or internal structures that are not found in all bacterial species (see table at the end of this exercise for a comparison of staining procedures). You will do some of these staining procedures in the next lab (acid-fast staining and endospore staining). In today's lab, you will observe prepared slides of special stains: a capsule stain (*Klebsiella pneumoniae*), flagella stain (*Proteus vulgaris*) and spirochete stain (*Treponema pallidum*).

Capsule Stain

Some bacteria secrete a polysaccharide-rich structure external to the cell wall called a glycocalyx. If the glycocalyx is thin and loosely attached, it is called a slime layer; if it is thick and tightly bound to the cell, it is

called a **capsule**. The glycocalyx can protect the cell from desiccation and can allow the cell to stick to surfaces like tissues in the body. They may also provide cells with protection against detection and phagocytosis by immune cells and contribute to the formation of a biofilm: in this way a glycocalyx can act as a virulence factor; (contributes to the ability of an organism to cause disease).

Capsules can be detected using a **negative staining** procedure in which the background (the slide) and the bacteria are stained, but the capsule is not stained. The capsule appears as a clear unstained zone around the bacterial cell. Since capsules are destroyed by heat, the capsule staining procedure is done without heat-fixing the bacteria.

Silver Stain

Flagella (long whip-like structures used for bacterial motility) and some bacteria (e.g. **spirochetes**) are too thin to be observed with regular staining procedures. In these cases, a **silver stain** is used. Silver nitrate is applied to the bacteria along with a special mordant; the silver nitrate precipitates around the flagella or the thin bacteria, thus thickening them so they can be observed under the light microscope.

Procedures

A. Preparation of a Bacterial Smear and Gram Staining

This semester you will be performing three staining procedures: Gram stain, acid-fast stain, and endospore stain. All three of these staining procedures begin with the preparation of a bacterial smear.

Materials

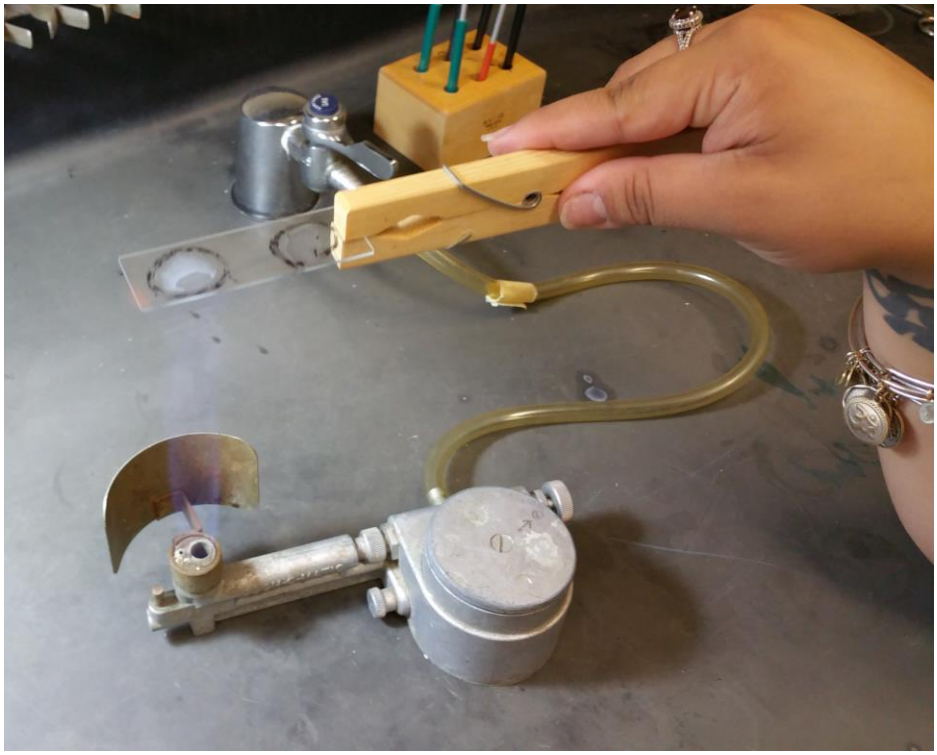
- Clean microscope slides
- Staining trays and newspaper
- Gram stain reagents: crystal violet, Gram's iodine, safranin, 95% ethanol
- Water bottle (for rinsing)
- Bacterial cultures: *Escherichia coli, Staphylococcus aureus, Micrococcus luteus, Pseudomonas aeruginosa, Corynebacterium xerosis,* and *Neisseria sicca*

How to make a bacterial smear

1. Label a clean glass slide as demonstrated by your instructor.
2. Add a small drop of saline to the slide (you will usually put two bacteria on one microscope slide- Follow your instructor's specific instructions). This can be done by placing a drop of saline onto your inoculation loop and then transferring it to the slide. If you use the saline dropper directly on the slide, do not release a full drop.
3. With an inoculation loop or needle, pick up a small amount of bacteria. Mix it well with the saline and spread the mixture over a wider area of the slide.
    - Be careful not to have the two smears run into each other.
4. Air dry the bacterial specimen on the slide (slide warmers may also be used).
5. When slides are completely air-dry, heat fix the bacterial specimen by passing the slide slowly over the flame twice (your instructor will demonstrate this).
    - Heat fixing kills cells, and adheres them to the slide.
    - Cells will be rinsed off the slides if they are not heat fixed properly.
    - Be careful not to overheat the slides in this procedure

After heat-fixing is complete, you are ready to gram stain your slide.

Figure 2. Heat fixation



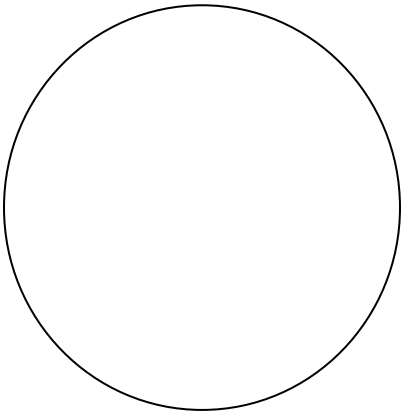
GRAM STAINING PROCEDURE

- For all steps in the gram staining procedure, add enough of the solution to cover the areas of the slide that have bacteria on them. You do not need to flood the entire slide.
- All staining should be done over a staining tray. Be sure to put newspaper under the tray in case of spillage.
- Gloves should be worn while staining and removed before working with the microscope.

STEPS	EXPLANATION
1. Add a few drops of **crystal violet (primary stain)** to the smear and let it sit for 60 seconds.	
2. Rinse the slide with water.	All cells are **purple** after this step. Stopping here would be a simple stain.
3. Add a few drops of **Gram's iodine (mordant)** to the smear and let it sit for 60 seconds.	Gram's iodine forms a complex with crystal violet
4. Rinse the slide with water.	All cells are **purple** after this step.
5. **Decolorize** with **95% ethanol**: let the alcohol run over surface of slide until no more crystal violet color comes out of the smear (time varies—no more than 5-10 seconds).	Gram positive cells retain crystal violet and remain **purple** Gram negative cells lose crystal violet and are now **colorless**
6. Rinse with water.	Water rinse stops the decolorization process
7. Add a few drops of **safranin (counterstain)** and let it sit for 60 seconds.	Safranin is a pink/red dye
8. Rinse with water. Blot dry on bibulous paper. Be careful not to wipe off the bacteria.	Gram positive cells remain **purple**; gram negative cells are now **pink/red**
9. Observe your slide under the microscope.	For each organism, determine morphology, arrangement and Gram reaction

Gram Stain Results

Draw sketches for each type of bacteria that you observe. Identify its morphology, arrangement, and Gram reaction.

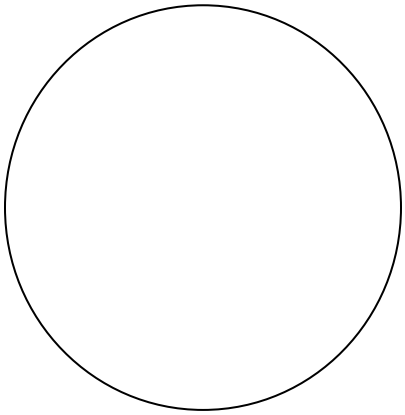


Species _____

Morphology _____

Arrangement _____

Gram reaction _____

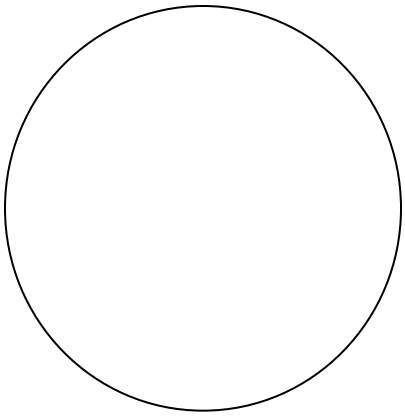


Species _____

Morphology _____

Arrangement _____

Gram reaction _____

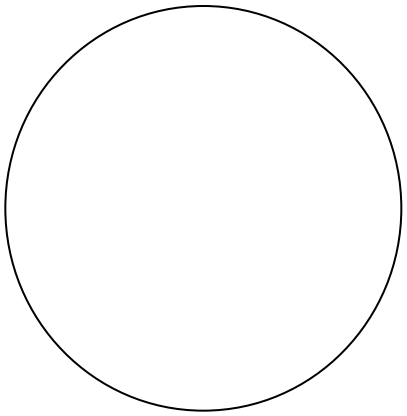


Species _____

Morphology _____

Arrangement _____

Gram reaction _____

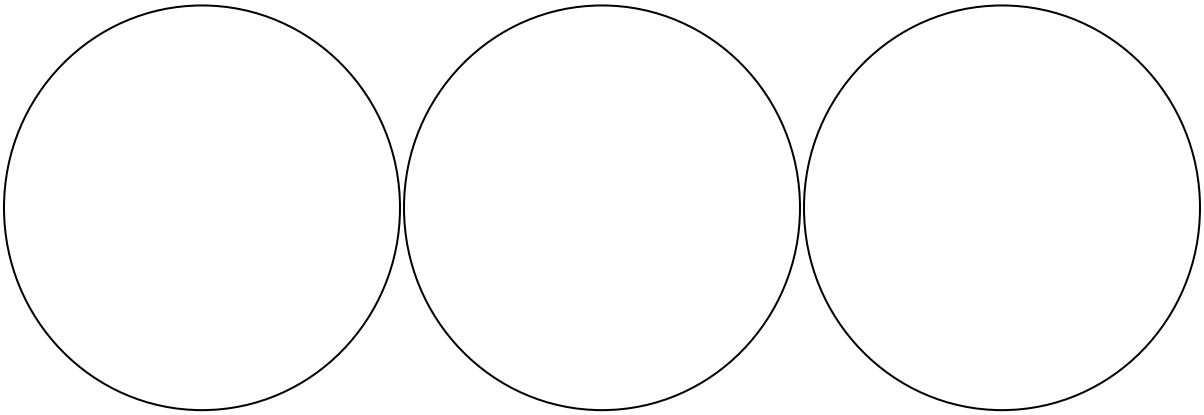


Species _____

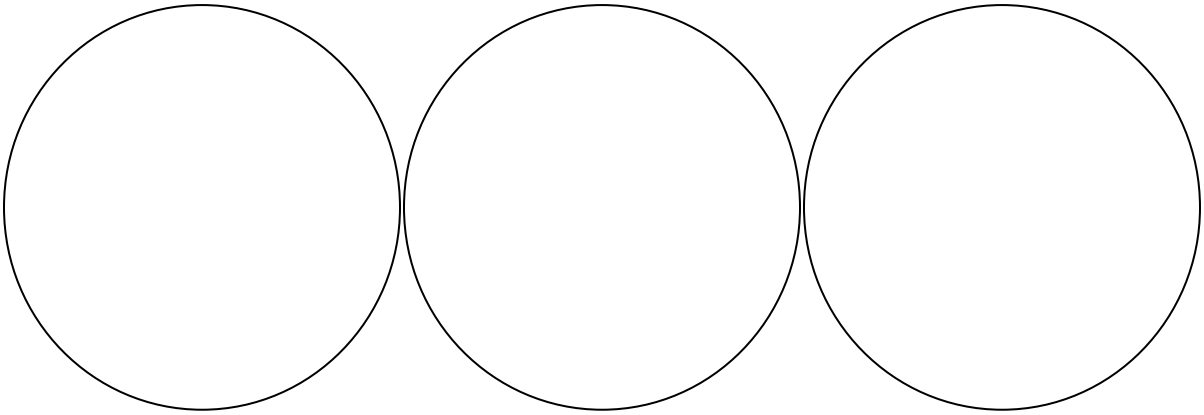
Morphology _____

Arrangement _____

Gram reaction _____



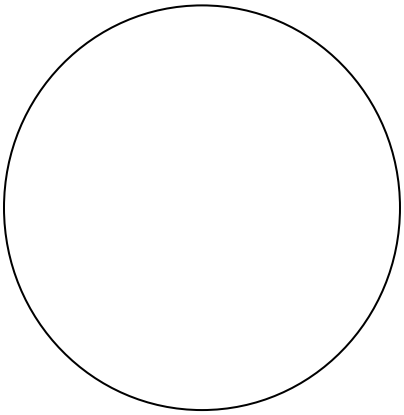
Species _____	Species _____	Species _____
Morphology _____	Morphology _____	Morphology _____
Arrangement _____	Arrangement _____	Arrangement _____
Gram reaction _____	Gram reaction _____	Gram reaction _____



Species _____	Species _____	Species _____
Morphology _____	Morphology _____	Morphology _____
Arrangement _____	Arrangement _____	Arrangement _____
Gram reaction _____	Gram reaction _____	Gram reaction _____

Special Stain Results

Observe the special stains set up at the demo microscopes at the back of the lab. Make sketches below.



Species _____

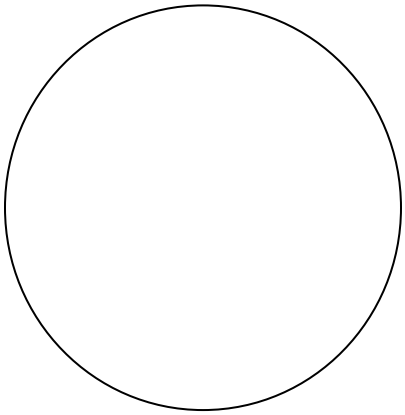
Staining technique

_____

Comments:

_____

_____



Species _____

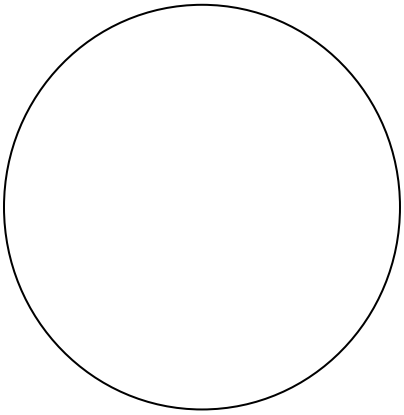
Staining technique

_____

Comments:

_____

_____



Species _____

Staining technique

_____

Comments:

_____

_____

Summary of Common Bacterial Staining Techniques

Simple Stains Crystal Violet, Methylene Blue, Safranin	• Used to provide color to otherwise transparent bacterial cells • Can be used to determine cell size, morphology and arrangement
Gram Stain Primary stain – crystal violet Mordant – iodine; decolorizer- 95% Ethanol Counterstain – Safranin	• Common differential stain • Gram reaction (positive or negative) reflects cell wall properties • Also used to determine cell size, morphology and arrangement
Acid-Fast Stain Primary stain – Carbol fuchsin Decolorizer – acid alcohol Counterstain – Methylene blue	• A differential stain used to detect bacteria with mycolic acid cell walls (genera *Mycobacterium* and *Nocardia*) • Developed to detect the bacterial species that causes tuberculosis • Acid-fast organisms resist decolorization with acid-alcohol
Endospore Stain Primary stain: Malachite green Counterstain: safranin	• Endospores resist staining with basic stains • Endospores stain with malachite green; vegetative cells stain with safranin
Capsule Stain (Negative staining) Uses an acidic stain: (Congo red or Nigrosin) and a basic stain: (crystal violet or safranin)	• Negative stains are neither heat-fixed nor rinsed • The background of the slide is stained by acidic stains (capsule remains unstained) • The cells within the capsule are stained with Basic stains • Examples of encapsulated cells: *Bacillus anthracis*, *Streptococcus pneumoniae*, and *Klebsiella pneumonia*
Flagella Stain Silver nitrate	• Used to see bacterial flagella that are too slender to be seen with other staining techniques • Silver nitrate makes flagella appear larger than they are • Can be used to determine arrangement of flagella for identification. ○ Ex: *Proteus vulgaris* has peritrichous flagella
Spirochete stain Silver nitrate	• Used to visualize slender spirochetes like *Treponema pallidum*

Review Questions

1. Explain the major differences between the gram positive and gram negative cell wall.

2. *Salmonella typhi* is a gram negative organism.
    a. What color will it appear when simple stained with crystal violet?

    b. What color would it be if it was gram stained correctly?

3. Explain how bacterial cells would look in the gram staining procedure if the following mistakes were made:
    a. Decolorizer left on too long

    b. Decolorizer not left on long enough

    c. Slide not heat-fixed before staining

4. What is the difference between a simple stain and a differential stain?

5. Explain why it is important to use only a small amount of bacteria when preparing a smear.

6. What are the two things that are stained in a capsule stain?

   What is NOT stained in a capsule stain?

7. Why would a health care provider be interested in knowing the gram reaction of a pathogenic bacterium?

Laboratory Exercise 4: Acid fast and Endospore Staining

Objectives

1. Learn about microorganisms that have acid-fast cell walls.
2. Perform the acid-fast staining procedure and view cells under oil immersion.
3. Learn about some of the microorganisms that are endospore formers.
4. Perform the endospore staining procedure and view cells under oil immersion.

Key Terms: mycolic acid, endospore, carbol fuchsin, methylene blue, malachite green, Ziehl-Neelson method, Kinyoun method, Schaeffer-Fulton method

Acid-Fast Staining

Introduction

Most bacterial species are either Gram positive or Gram negative, however some organisms have different cell wall properties that make them difficult to stain with this method. For example, some species of bacteria have a waxy lipid (**mycolic acid**) in their cell walls. These organisms generally do not Gram stain very well (those that do would usually appear gram positive) and are more clearly visible with the acid-fast staining technique.

Acid-fast staining was developed by Robert Koch in 1882 and later modified by other scientists. Koch used the method to observe the "tubercle bacillus"—what we now call *Mycobacterium tuberculosis*, in sputum samples. While acid-fast and gram staining are both differential stains, the acid-fast stain is much more specific. Many bacteria are either gram positive or gram negative, but very few are acid-fast. Two acid-fast genera that are important as human pathogens are *Mycobacterium* and *Nocardia:* Pathogenic species include *M. tuberculosis, M leprae, M. bovis, M. avium, and N. asteroides.* The protozoan parasite *Cryptosporidium* can also be stained using this procedure.

There are 2 different methods of acid-fast staining—both involve techniques that make the cell wall more permeable to the primary stain. The **Ziehl-Neelson method** uses steam heat to allow stain to penetrate, whereas the **Kinyoun** (cold method) uses a wetting agent mixed in with the primary stain. In this lab we will be using the Kinyoun method.

Kinyoun Staining Procedure

1. Prepare a slide with *Mycobacterium smegmatis* on one side and *Micrococcus luteus* on the other side. (Alternatively, both bacteria may be mixed into one smear).
    - Be sure to break up clumps of *M. smegmatis* before staining.
2. Air dry and heat fix as usual.
3. Add carbol fuchsin (primary stain): leave on for 5-7 minutes.
4. Rinse with water.
    - Note: not all of the primary stain will be removed by water in this step
5. Decolorize with Acid-alcohol: 1-2 quick rinses
6. Rinse with water.
7. Add **methylene blue** (counterstain) and leave on for 2-3 minutes.
8. Rinse with water, blot dry, and look at beautiful bacteria.

Acid-fast organisms retain the primary stain and will appear bright red: non acid-fast organisms are decolorized with acid-alcohol and pick up the methylene blue counterstain. Epithelial cells that may be present in a clinical sample will also appear blue.

Endospore staining

Introduction

Endospores are the most resistant forms of life. They can resist desiccation (drying), boiling and radiation—in addition, disinfectants and antibiotics cannot penetrate an intact spore coat. For this reason they are difficult to eliminate from the environment with standard methods of disinfection, and they are difficult to treat in the case of an infection.

Endospores are a survival mechanism for the bacterial species that produce them. When conditions are favorable, vegetative bacterial cells will continue to grow and divide; however when nutrients are depleted, cells will begin to form endospores. Endospores are not metabolically active, but contain all the materials needed by cells to survive. When conditions for growth are again favorable, the spore will germinate and form a cell that is identical to the cell that produced it. Endospores are produced by certain types of Gram positive

bacilli, like *Clostridium* and *Bacillus*, as well as other species. Endospore-forming pathogens include *C. tetani*, *C. botulinum, C. difficile, and B. anthracis*.

In today's lab we will use the **Schaeffer-Fulton** method (without heat) to view endospores. Since we are not using heat, it is important to leave the stain on for a long time to allow it to penetrate the spore coat.

Schaeffer-Fulton Staining Procedure

1. Prepare a smear with *Bacillus subtilis* or *Clostridium sporogenes* on one side of the slide, any other bacteria on the other side
2. Air dry and heat fix as usual.
3. Add **malachite green** (stains spores) and leave on for at least 10 minutes.
4. Rinse briefly with water.
5. Stain cells with safranin (stains vegetative cells) for 1 minute.
6. Rinse with water, blot dry and look at beautiful bacteria

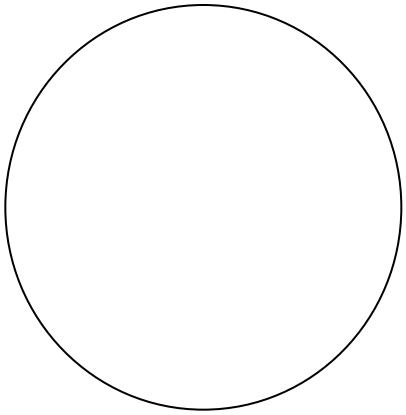
If the bacterial species is an endospore former, you will see pink vegetative cells as well as green oval-shaped endospores: non-spore formers will appear only as pink vegetative cells.

Note: Color pictures of organisms stained with the Ziehl-Neelson procedure and the Schaeffer-Fulton procedure can be found on the Blackboard Microbiology Review site.

Additional Gram staining: if time allows, your instructor may have you prepare some additional slides with the Gram stain procedure.

Results

Acid Fast Stain

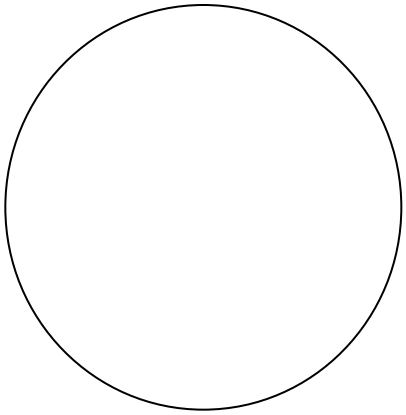


Species _____

Morphology _____

Arrangement _____

Acid fast result _____



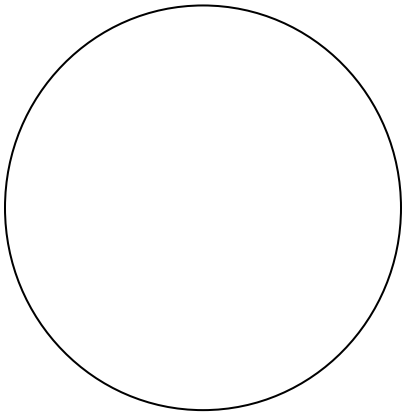
Species _____

Morphology _____

Arrangement _____

Acid fast result _____

Endospore Stain

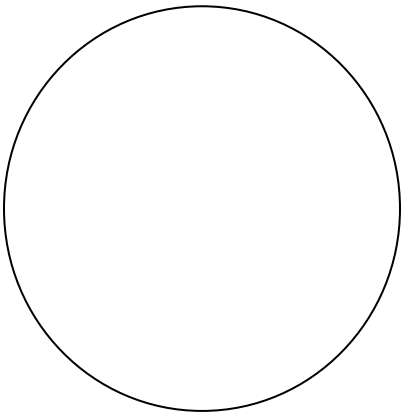


Species _____

Morphology _____

Arrangement _____

Endospore result _____



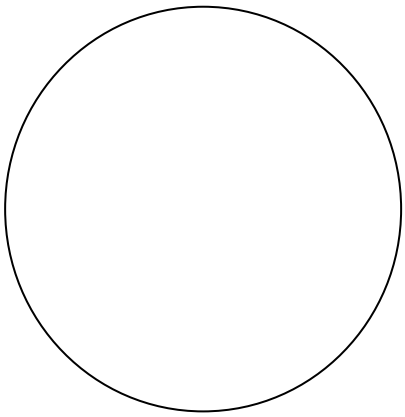
Species _____

Morphology _____

Arrangement _____

Endospore result _____

Additional Gram Stains

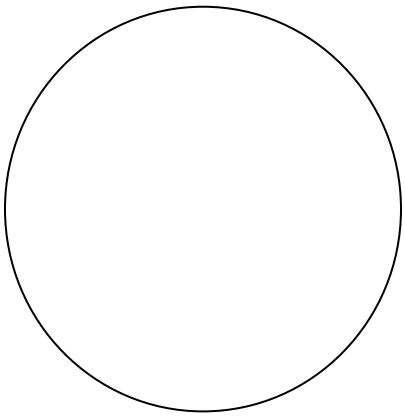


Species _____

Morphology _____

Arrangement _____

Gram reaction _____

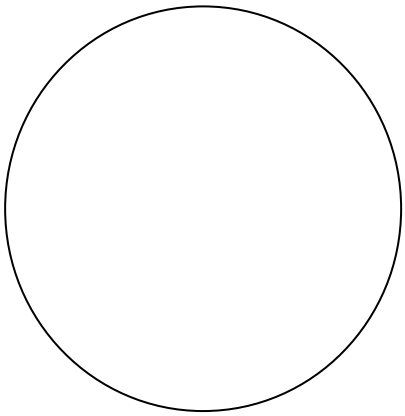


Species _____

Morphology _____

Arrangement _____

Gram reaction _____

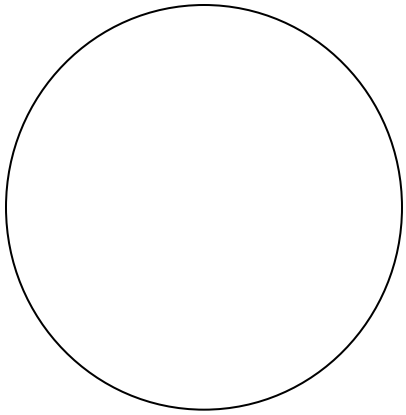


Species _____

Morphology _____

Arrangement _____

Gram reaction _____

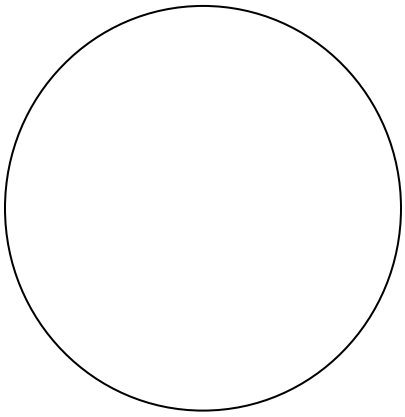


Species _____

Morphology _____

Arrangement _____

Gram reaction _____

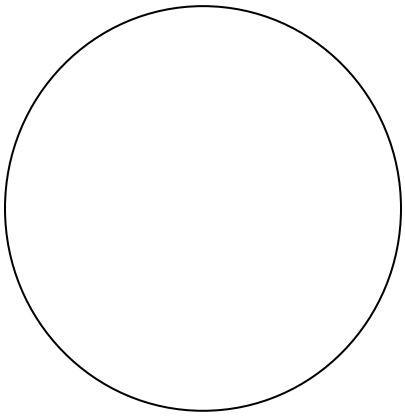


Species _____

Morphology _____

Arrangement _____

Gram reaction _____

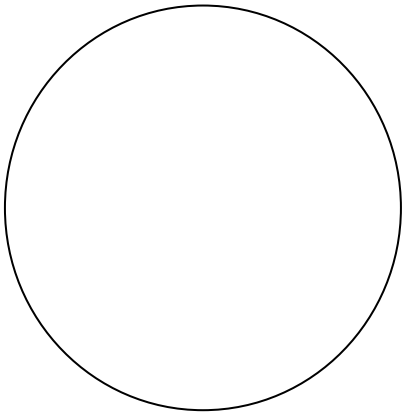


Species _____

Morphology _____

Arrangement _____

Gram reaction _____

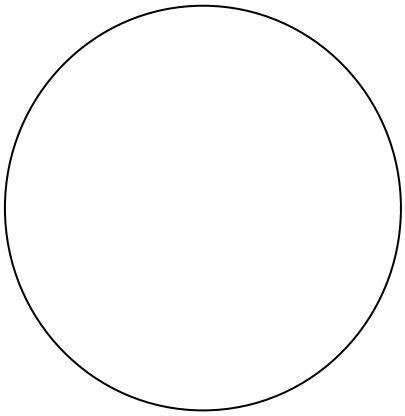


Species _____

Morphology _____

Arrangement _____

Gram reaction _____



Species _____

Morphology _____

Arrangement _____

Gram reaction _____

Review Questions

1. Distinguish between the Ziehl-Neelson and Kinyoun methods of acid-fast staining.

2. Why is it important to leave the malachite green on the slide for at least 10 minutes in the endospore-staining procedure?

3. Do you think you would find more endospores in a freshly prepared culture or in an older culture of *Bacillus subtilis*? Explain.

4. Why do you think an infection caused by an endospore former might be harder to treat than one caused by a non-spore former?

Review of Staining Procedures

To help you review the staining procedures in Labs 3 and 4, fill out the table below with information about these staining procedures. This information should include (but not be limited to) the following:

- What does the staining procedure tell you about bacterial cell structure, or the types of structures produced by bacteria?
- Is the staining procedure used to detect specific types of cells? If so, what are they?
- What do the positive and negative results look like at the end of the procedure?
- Is there any clinical relevance to the results of the staining procedure?
- Any other information that would help you to understand what the staining procedure detects and how it works.

Staining Procedure	Information
Gram	
Ziehl-Neelson	
Schaeffer-Fulton	

Silver Nitrate	
Negative Staining	

Laboratory Exercise 5: Metabolic Activities of Bacteria

Objectives

1. Learn how to use differential and selective media to identify bacterial species.
2. Be able to perform the following biochemical tests and understand how they work: carbohydrate fermentation, gelatin hydrolysis, starch hydrolysis, casein hydrolysis, urea hydrolysis, citrate utilization, catalase activity, tryptophan hydrolysis (indole test).
3. Be able to relate the results of the tests performed to the different types of metabolic activities carried out by individual bacterial species.

Key Terms: fermentation, amylase, gelatinase, caseinase, urease, citrate permease, catalase, indole, tryptophanase, Kovac's reagent, BBL Dry Slide, agar slant (locate butt and slant), Durham tube, Simmon's citrate

Introduction

As you observed in Labs 3 and 4, staining procedures can be used to discriminate between bacteria based on morphology, arrangement, cell wall structure, and their possession of other structures associated with bacterial cells such as flagella, capsules and spores. However, staining procedures are not sufficient to identify bacteria at the species level. For example, *E. coli*, *P. aeruginosa*, *P. vulgaris*, *E. aerogenes*, and many other bacteria are all Gram negative rods and are indistinguishable by Gram staining. Therefore, additional methods are required to identify individual species.

Even though different bacterial species may appear identical under the microscope, they all have a different genetic makeup (different DNA), and therefore produce different types of enzymes that allow them to carry out a characteristic set of biochemical reactions. In other words, each bacterial species has a characteristic metabolism that can be used to distinguish them from other species, and therefore unknown bacteria are often identified based on both their appearance under the microscope as well as their metabolic properties.

Examples of differences in metabolic properties were observed in Lab 2, when you inoculated *E. coli* and *P. aeruginosa* onto EMB agar. Each of these bacteria produced different colored colonies on this media, depending on their ability to ferment lactose. In addition to EMB and MS, there are many other types of differential media that can be used in the identification of bacteria.

When using differential media, it is always important to remember that reactions in these media can vary based on the incubation time, the incubation temperature, the number of bacteria in the initial inoculum and how the medium is inoculated, among other variables. For example, an inoculum containing large amounts of bacteria might give a strong positive reaction, while one containing a very small number of bacteria might only produce a weakly positive, or perhaps a negative reaction. It is also important to remember that not all bacterial species will give definitive positive or negative results on these media. Since not all differential media will provide meaningful results for all bacterial species, it is important to choose the media carefully when identifying unknowns.

In many metabolic tests, end products are produced that change the pH of the medium. To measure this pH change, pH indicators (chemicals that change color depending on pH) are included in the medium. Some common pH indicators are phenol red, bromocresol purple and bromothymol blue. Each pH indicator has a range of pH values over which it changes color (see below).

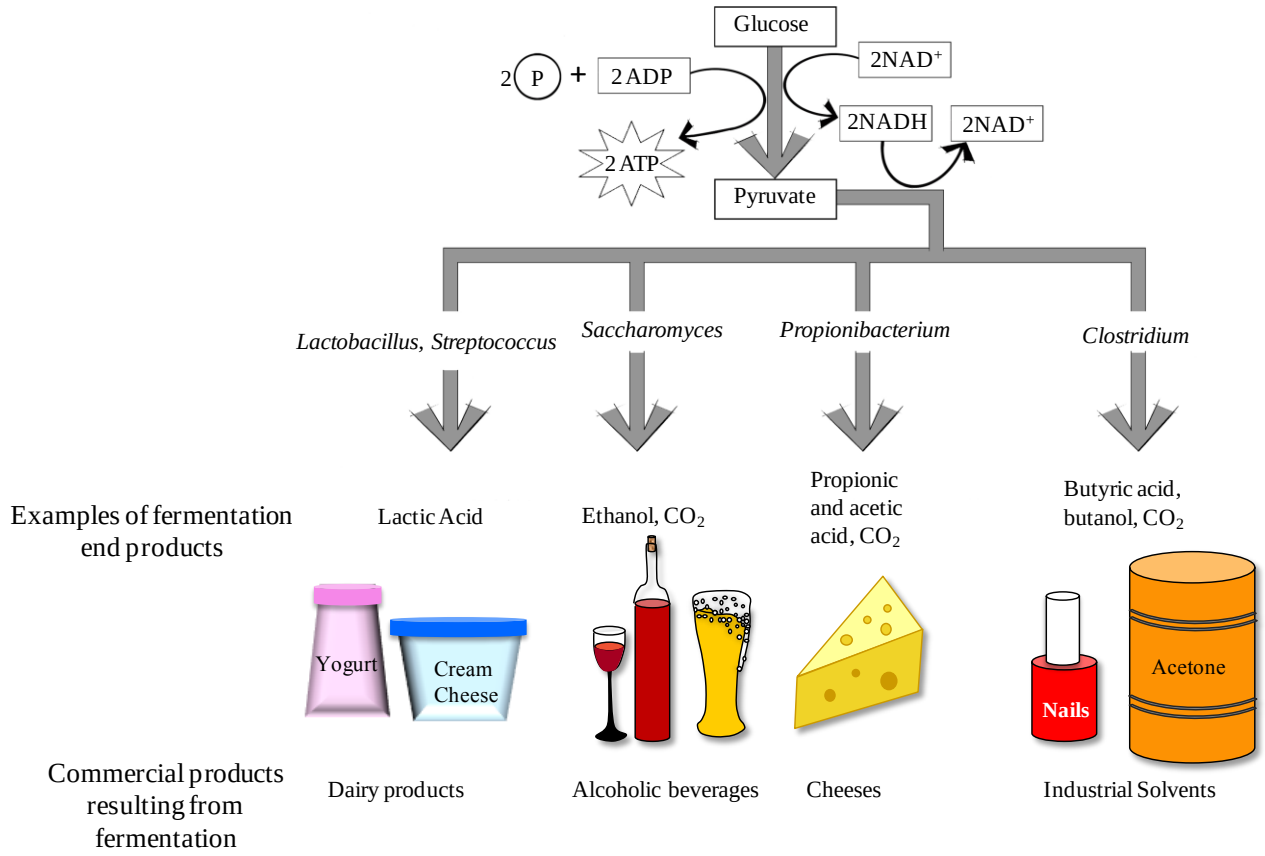
pH Indicator			
phenol red	< pH 6.8 = yellow	pH 6.8 – 7.4 = red	pH >7.4 = pink/magenta
bromocresol purple	< pH 6.8 = yellow	> 6.8 pH = purple	
bromothymol blue	< pH 6.0 = yellow	pH 6.1 – 7.5 = green	pH >7.5 = blue

Note: color pictures showing the results for all metabolic tests can be found on the Microbiology Review site on Blackboard.

A. Carbohydrate Fermentation

Fermentation is a metabolic process that some bacteria use to break down glucose when O_2 is not available. Fermentation includes the reactions of glycolysis (where a single molecule of glucose is broken down into 2 molecules of pyruvate), as well as additional reactions that produce a variety of end products (acids, alcohols, gases). The end products are characteristic of individual bacterial species.

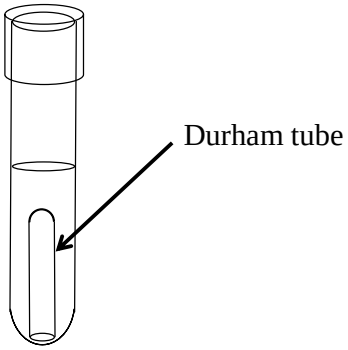
Figure 1. Carbohydrate Fermentation



Although the ultimate substrate molecule for fermentation is always glucose, some bacteria use additional chemical reactions to convert other monosaccharides as well as disaccharides into glucose. Therefore bacteria can be differentiated both based on their ability to ferment various carbohydrates, as well as the end products that result from the fermentation process.

The medium used to test carbohydrate fermentation is a nutrient broth that contains a fermentable carbohydrate (usually a monosaccharide or a disaccharide), peptone (amino acids) as well as a pH indicator. The pH of the medium is adjusted to approximately 7.5, so it appears orange/red when using phenol red. If the carbohydrate in the medium is fermented and acidic end products are formed, a color change to yellow will result. Occasionally, bacteria will not ferment the carbohydrate, but instead will break down proteins producing ammonia (NH_3) in the growth medium. In this case, the medium will become more alkaline.

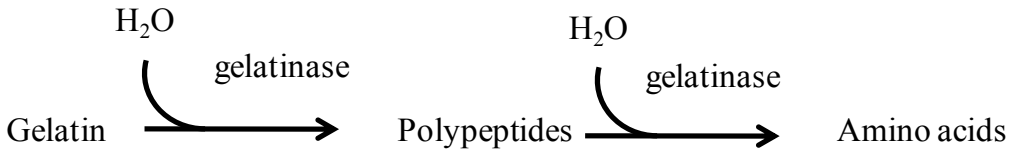
Some bacteria will produce gases when fermenting a carbohydrate. To detect these gases, a **Durham tube** is used. This is a small inverted tube that is placed within the larger glass tube containing the fermentation medium (see below). If gases (typically CO_2) are produced during the fermentation process, a bubble will form at the top of the Durham tube. If you see a bubble in the Durham tube, the medium will also be acidic.



Carbohydrate fermentation media are often used to differentiate members of the family Enterobacteriaceae (e.g., *Escherichia coli, Enterobacter aerogenes*) from each other.

B. Gelatin Hydrolysis

Gelatin is a protein derived from the collagen found in connective tissue of bones, skin and tendons. Robert Koch first used gelatin as a solidifying agent for growth media, but switched to agar when it was realized that gelatin does not stay solid at temperatures above 28°C and that some bacteria have the ability to chemically break it down. Some species of bacteria secrete an extracellular enzyme (exoenzyme) called **gelatinase** (a type of proteinase), that breaks gelatin down into its constituent polypeptides and amino acids. The small amino acids can then be brought into the bacterial cell. This is why agar is now used as the solidification agent in bacterial growth media. Since gelatinase breaks down connective tissue, it is sometimes called "spreading factor" and contributes to the virulence of a pathogen.



The presence of gelatinase can be detected by using nutrient gelatin deeps. This medium contains gelatin and nutrients for bacterial growth. A positive result is indicated by the gelatin becoming liquefied due to the enzyme activity. It is important to note that if the nutrient gelatin deeps are incubated at 37°C, then the medium **must be cooled** to below 25°C before a result can be recorded. A negative control is therefore very important in this experiment.

C. Starch Hydrolysis

Starch is a polymer of glucose that is too large to be transported into bacterial cells. For bacteria to be able to use the glucose in starch as an energy source, the macromolecule must be broken down extracellularly into smaller monosaccharide and disaccharide subunits. Some bacteria secrete the extracellular enzyme (exoenzyme) **amylase** that breaks down starch into monosaccharides and disaccharides (glucose and maltose). These smaller molecules can then be transported inside the bacterial cell.

Starch agar is used to determine if bacteria can produce the amylase enzyme. Bacteria that secrete amylase will break down the starch in the medium surrounding the bacterial colony. After the plate is inoculated and incubated, iodine is poured on the plate. The combination of iodine and starch results in the formation of a dark blue-black color. Regions of the agar where no starch is present (i.e., where starch has been broken down by amylase) will remain the color of the iodine (light yellow or gold).

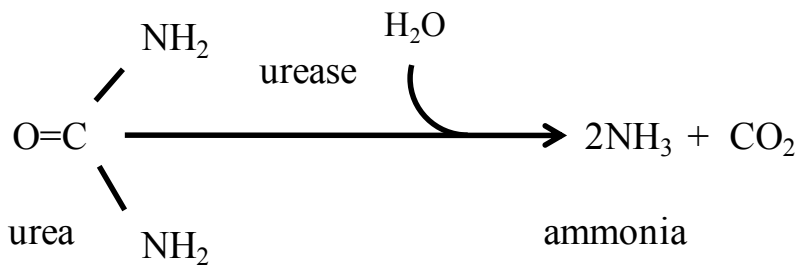
D. Casein Hydrolysis

Some bacteria secrete enzymes called proteinases that break down proteins. You have already read about one kind of proteinase—gelatinase. Milk contains large proteins called **caseins**. Some bacteria secrete **caseinases** that break down casein outside of the bacterial cell so the smaller products (e.g., amino acids) can be transported inside the cell and further metabolized.

Milk agar (which contains powdered milk) is used to detect the presence of bacterial caseinases. This medium is cloudy because when milk is mixed with agar, the casein forms a colloid through which light cannot pass. The presence of caseinases can be detected by observing a clearing in the agar around the bacterial growth, which indicates that the caseins have been broken down into transparent end products (amino acids and peptides), which are then taken up by the cells.

E. Urea Hydrolysis

Urea is a common waste product that results from the breakdown of proteins. Urea agar slants are used to test for the presence of a urease enzyme that is produced by some bacteria. Bacteria that produce **ureases** can break urea down into ammonia and carbon dioxide (see below). The ammonia that results from the breakdown of urea causes the medium of the slant to become more alkaline; this pH change is detected by the phenol red, which changes to a hot pink color. Organisms that do not break down urea may grow on the slant, but the slant will remain its original color, or will become more yellow (due to the production of acids).

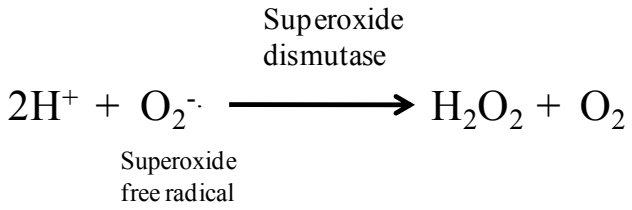


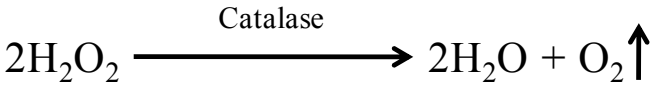
F. Citrate Utilization

Organisms that can survive using citrate as the sole source of carbon have a **citrate permease** enzyme that can transport citrate molecules into the cell. The citrate is then made into pyruvate, which can be converted into a variety of different products. **Simmon's citrate** is a chemically defined medium that contains sodium citrate as the sole carbon source as well as the pH indicator bromothymol blue. Bacteria that can grow on this medium (i.e., that can survive on citrate as the sole source of carbon) produce alkaline byproducts that will change the medium from green (neutral pH) to blue (alkaline pH).

G. Catalase Activity

Byproducts of aerobic metabolism include two toxic compounds: superoxide free radicals ($2O_2^-$) and hydrogen peroxide (H_2O_2). To remove these compounds, cells produce enzymes to break them down. Cells can convert superoxide free radicals to hydrogen peroxide by using the enzyme superoxide dismutase (SOD); **catalase** breaks down hydrogen peroxide into water and oxygen.

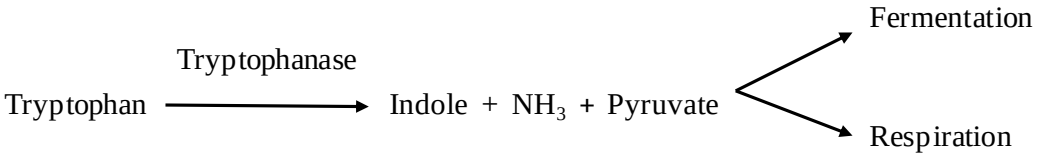




A simple test to determine if bacteria produce catalase is to add hydrogen peroxide to bacteria on an agar slant or to bacteria spread on a slide. If catalase is present, the hydrogen peroxide will be broken down into water and oxygen gas, resulting in the production of bubbles. This test does not require any special type of medium, however it should never be performed on organisms that have been grown on blood agar (a medium that contains blood). This is because there is a catalase activity in blood that would produce a false positive result. Most aerobic and facultatively anaerobic organisms produce SOD and catalase (note: some species use peroxidase rather than catalase to break down hydrogen peroxide). Obligate anaerobes lack these enzymes, which is why they cannot survive in an atmosphere containing oxygen.

H. Tryptophan Hydrolysis: The Indole Test

Tryptophan is an amino acid found in most proteins. Some bacteria produce **tryptophanase**, an enzyme that breaks tryptophan down into **indole**, ammonia and pyruvate (see below). The pyruvate and ammonia are converted into other molecules, but the indole accumulates, and thus can be detected in the media.



The presence of indole indicates that an organism produces the enzyme tryptophanase. Indole can be detected using a chemical known as **Kovac's reagent**. We will be using a **BBL dry slide** to detect indole. This is a disposable slide that employs a dry reagent slide impregnated with Kovac's reagent. The slide is divided into four large segments to enable multiple tests on one slide. The presence of indole will cause a pink color to develop on the dry-slide; no color change will occur if no indole is detected.

Note: for the indole test to be performed, the organism must be grown using medium containing tryptophan.

Procedures

Make sure you follow aseptic procedures and label everything carefully! Use the inoculation method indicated for each type of medium—these methods may differ. Make sure to thoroughly sterilize your loop or needle between inoculations to ensure that you are only introducing one bacterial species into your medium. If a medium is inoculated with more than one kind of bacterium, a positive result cannot be attributed to a single bacterial species. Be sure to use the correct bacterial species for each test. Follow directions carefully so that you do not waste media.

For comparison purposes, you will be provided with negative controls (media that have not been inoculated) in the next lab when you are analyzing your results.

Note: all media that you inoculate today will be incubated until the next lab, when you will analyze your results.

A. Carbohydrate Fermentation

Each student: 1 tube each of the following broths: Lactose + phenol red (green cap), Sucrose + phenol red (yellow cap), Glucose + phenol red (red cap)

Instructions: Choose 1 of the following bacteria: *Proteus vulgaris*, *Escherichia coli*, *Bacillis subtilis*, or *Streptococcus faecalis* (each person at the table should choose a different species)

Inoculate the 3 types of fermentation broth with your chosen bacteria. Prior to inoculating the broths, make note of any small bubbles that might be present in the Durham tubes, so these are not read as evidence of gas formation during fermentation.

B. Gelatin Hydrolysis

Each student: 1 nutrient gelatin deep

Instructions: Choose 1 of the following bacteria: *Staphylococcus aureus*, *Serratia marcescens*, *Streptococcus faecalis,* or *Bacillis subtilis*

Use an **inoculation needle (not a loop)** to obtain some bacteria from your stock culture. Inoculate the gelatin deep by using the inoculating needle to stab ~3/4 of the way down into the nutrient gelatin and then drawing the needle straight back up.

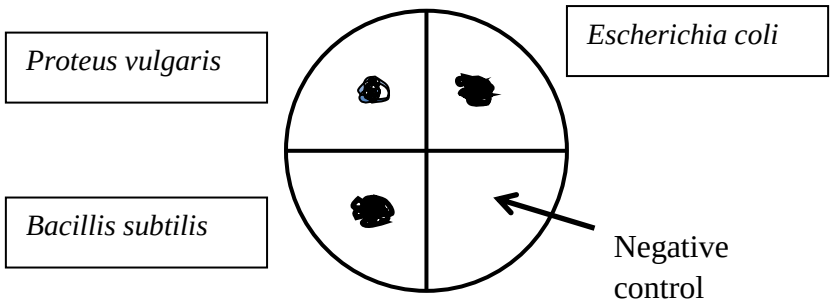
C. Starch Hydrolysis

Each student: 1 starch agar plate

Instructions: Use all of the following bacteria: *Proteus vulgaris, Escherichia coli, Bacillus subtilis*

Divide your starch agar plate into 4 areas using a wax pencil or a sharpie marker. Make sure you draw on the <u>bottom</u> of the petri dish (the part that contains the agar) rather than on the lid. Label the plate to indicate which bacterium will be inoculated into each area. One area is left as a negative control.

Using a loop, you will do spot inoculations of each bacterial species in the areas. A spot inoculation is used to ensure that a large amount of bacteria will grow at a single location and produce a concentrated amount of amylase. The spot inoculation technique is shown below.



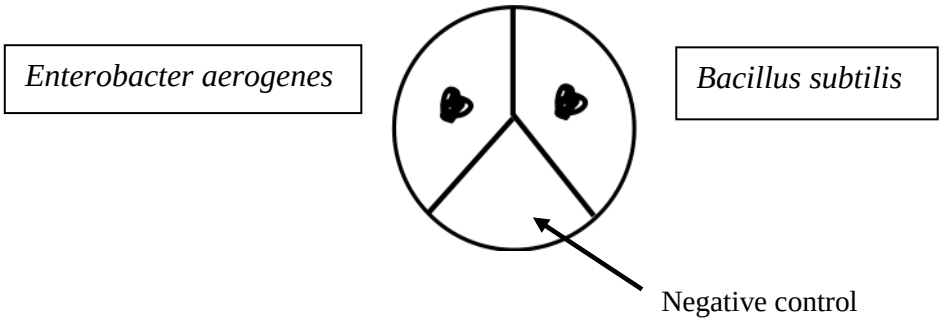
D. Casein Hydrolysis

Each student: 1 milk agar plate

Instructions: Use both these bacteria: *Bacillus subtilis* and *Enterobacter aerogenes*

Divide your milk agar plate into 3 areas using a wax pencil or a sharpie marker. Label the plate to indicate which bacterium will be inoculated into each area. One area is left as a negative control.

Using a loop, you will do spot inoculations of each bacterial species in the areas, just as you did on the starch agar plate.



E. Urea Hydrolysis

Each pair of students: 1 urea slant

Instructions: Choose one of these bacteria: *Escherichia coli* or *Proteus vulgaris*

To inoculate the urea slant, use a loop to obtain some bacteria from the culture, and then carefully streak the surface of the slant. Do not stab down into the slant's interior. Replace the cap on the slant, but leave the cap somewhat loose. Wrap in the tube in aluminum foil.

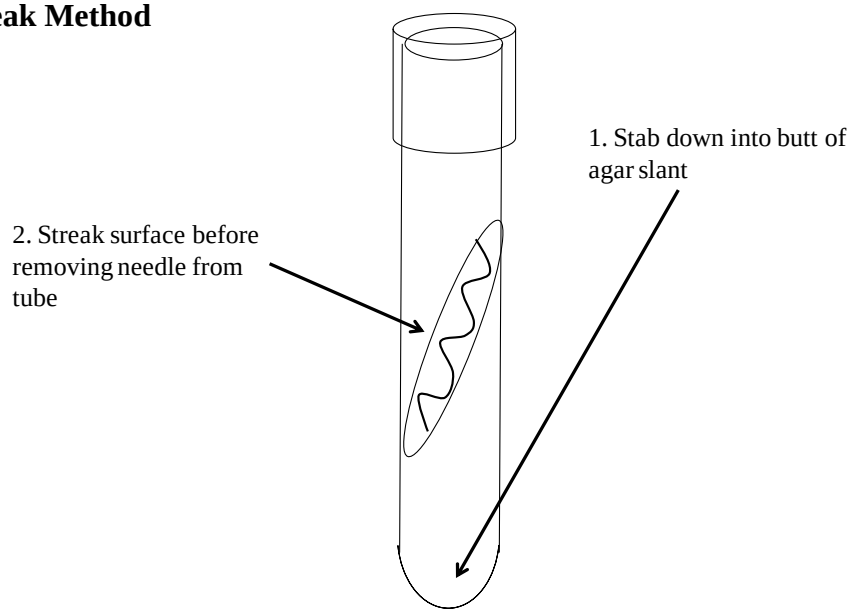
F. Citrate Utilization

Each student: 1 Simmon's citrate slant

Instructions: Choose one of these bacteria: *Escherichia coli* or *Serratia marcescens*

Using an inoculation needle (not a loop), inoculate the Simmon's citrate by first stabbing the needle into the **butt of the agar slant** (see below) and then streaking the surface of the **slant** before you pull the needle out of the tube.

Stab and Streak Method



G. Catalase Activity

Each student: 1 TSA slant

Instructions: Choose one of these bacteria: *Staphylococcus aureus* or *Streptococcus faecalis*

Using a loop, inoculate the surface of the TSA slant. Make sure to use a heavy inoculum.

H. Tryptophan Hydrolysis

Each student: 1 tryptone broth (**Note**: this medium contains tryptophan)

Instructions: Choose one of these bacteria: *Escherichia coli* or *Enterobacter aerogenes*

Inoculate the medium with your chosen bacteria. Make sure to use a heavy inoculum.

Results

Record your results for the metabolic tests in the tables on the following pages. At the end of this lab there is also a table where you can record test results for all of the organisms that will be on your first practical exam. It is important to obtain results all of the organisms in the table, as you will need this information to identify your unknown organisms for your midterm practical.

A. Carbohydrate Fermentation

Observe the results of your own carbohydrate fermentation test, as well as the tests done by your table partners. You can compare your inoculated tubes with the negative controls in the front of the class at the instructor's table. Record the results in the table below.

The following convention is used when noting the results of fermentation experiments.

A = acid

G = gas

AG means that both acid and gas are present

If neither acid nor gas is present, you can write "negative"

Bacteria	**Glucose**	**Lactose**	**Sucrose**
Proteus vulgaris			
Escherichia coli			
Bacillus subtilis			
Streptococcus faecalis			

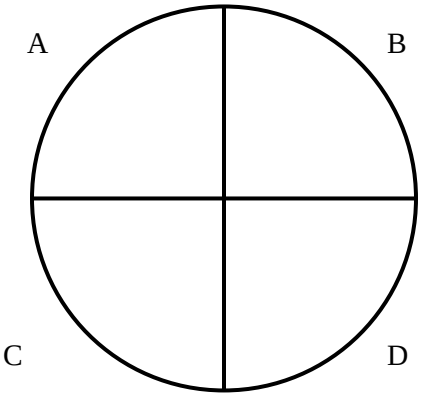
B. Gelatin Hydrolysis

Observe your gelatin deeps and those of your table partners. Record your results in the table below.

Bacteria	**Liquid or solid**	**Result (+/-)**
Staphylococcus aureus		
Serratia marcescens		
Streptococcus faecalis		
Bacillus subtilis		

C. Starch Hydrolysis

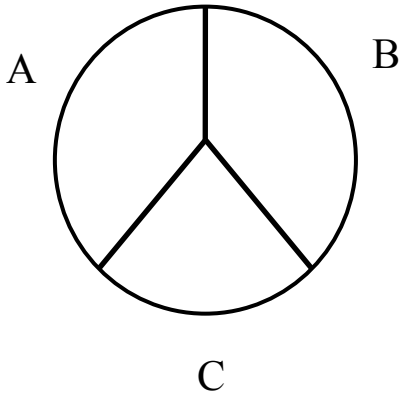
Add enough Gram's iodine to the plate to ensure that the entire areas of growth and the agar surrounding it are covered (you should also add iodine to the negative control area). Once you have added the iodine, make sure you do not tip the plate too much! Observe the appearance of your plate. Record your results in the table below.



Bacteria	Color change? (Y/N)	Starch present or absent?	Presence (+) or absence (-) of amylase
A. *Proteus vulgaris*			
B. *Escherichia coli*			
C. *Bacillus subtilis*			
D. Negative control			

D. Casein Hydrolysis

Observe your casein plate. It is helpful to hold it up to the light so you can detect the clear zones. Make a drawing of your results the circle below. Indicate the location of the bacterial growth and draw any clear zones that are present. Record your results in the table below.



Bacteria	Clear zone?	Result (+/-)
A. *Enterobacter aerogenes*		
B. *Bacillus subtilis*		
C. Negative control		

E. Urea Hydrolysis

Observe your urea slants. Record the results in the table below.

Bacteria	Color	Result (+/-)
Escherichia coli		
Proteus vulgaris		

If the urea slant is pink, what can you say about the pH of the medium?_____ This pH change is due to what molecule? _____

F. Citrate Utilization

Observe your Simmon's citrate slants. Record the results in the table below.

Bacteria	Color	Growth?	Result (+/-)
Escherichia coli			
Serratia marcescens			

If the color of the slant is blue, what does this tell you about the pH of the medium? _____

G. Catalase Activity

Add a dropper full of H_2O_2 to the surface of the slant. Record your results below.

Bacteria	Presence of bubbles	Result (+/-)
Staphylococcus aureus		
Streptococcus faecalis		

H. Tryptophan Hydrolysis

Flick your inoculated culture to stir up the bacteria. Use a sterile swab to obtain some bacteria. Make sure you swirl the swab around in the broth so you pick up a large quantity of bacteria. Rub the swab on one area of the BBL Dry Slide. Record any color changes that occur between 30 – 60 seconds. Do not wait too long before you observe your result, as false positives can occur if results are not read right away.

Record your results in the table below.

Bacteria	**Color**	**Result (+/-)**
Escherichia coli		
Enterobacter aerogenes		

Review Questions

1. Why do you think it is important to do spot-inoculations for the amylase test and the caseinase test?

2. What happens if you drop H_2O_2 into a cut on your arm?

What does this tell you about the type of enzymes your cells might produce?

3. (a) You inoculate an organism into phenol red lactose broth. The organism ferments lactose. What color is the medium? _____ What does this tell you about the pH of the medium? _____

(b) The organism ferments lactose and produces gases. What do you use to detect gas production?

_____? What do you see that indicates that gases are produced? _____

4. In what test is Kovac's reagent used? _____ What molecule does Kovac's reagent detect?

_____ To do this test, why do you have to grow the organism in

medium containing tryptophan? _____

_____

_____

5. In the urea test, what happens to the pH of the medium when an organism breaks down urea?

What molecule causes this pH change? _____

6. After inoculating a tube of nutrient gelatin, you incubate it overnight at 37^{o}C. When you remove the tube from the incubator, you notice that the gelatin has liquefied. Does this indicate that the organism produces gelatinase? Why or why not?

7. Fill in the following table to summarize the metabolic tests that you learned about.

Test	Biochemical reaction/ Enzyme involved	Medium used/Inoculation Procedure	Appearance of Result		What causes the change in the medium's appearance? (e.g. pH change, enzyme activity, etc.)
			Positive Result	Negative Result	
Lactose Fermentation					
Gelatinase					
Amylase					
Urease					
Citrate Permease (Simmon's citrate test)					
Catalase					
Tryptophanase (Indole test)					

Results for Metabolic Tests

Bacteria	Gram stain	Morphology	Arrangement	Lactose Fermentation	Gelatinase	Amylase	Urease	Citrate permease (Simmon's citrate)	Catalase	Tryptophanase (Indole test)
Bacillus subtilis										
Corynebacterium xerosis										
Enterobacter aerogenes										
Escherichia coli										
Micrococcus luteus										
Pseudomonas aeruginosa										
Proteus vulgaris										
Staphylococcus aureus										
Streptococcus faecalis										
Serratia marcescens										

The tests in the table above will be available for your practical exam. Use your results from Lab 5 to fill out this table. Results for the bacteria that were not tested in Lab 5 can be found on the Microbiology Review site on Blackboard.

Lab 6: DICHOTOMOUS KEYS

Objectives

1. Understand the uses of dichotomous keys.
2. Using the metabolic test results obtained in Lab 5, construct a dichotomous key for the identification of bacterial unknowns.

Introduction

Keys that are based upon successive choices between two alternatives are known as **dichotomous keys** (dichotomous means to "fork into two equal parts"). One use of a dichotomous key is to organize large amounts of information so identifications can be made more easily. This method is used to characterize and identify living organisms, including bacteria.

The first step in the identification of an unknown bacterium is often to determine its morphology and Gram stain reaction. This allows you to eliminate many possibilities (for example, a Gram negative bacillus is not *Staphylococcus aureus* or *Bacillus subtilis*). After determining the Gram reaction and morphology of an unknown organism, you can eliminate many possibilities, but will likely still need additional tests to determine which species you have (remember that there are lots of bacteria that look the same under the microscope). Sometimes, additional staining (like acid-fast or endospore staining, for example) can be used to further characterize bacteria. In this exercise, you will be using the metabolic tests you have learned about in Lab 5 to design a dichotomous key that will allow you determine which tests can be used to distinguish the 10 bacterial species we are using in the lab from each other. You will then be using those tests to identify your two unknowns for your midterm practical.

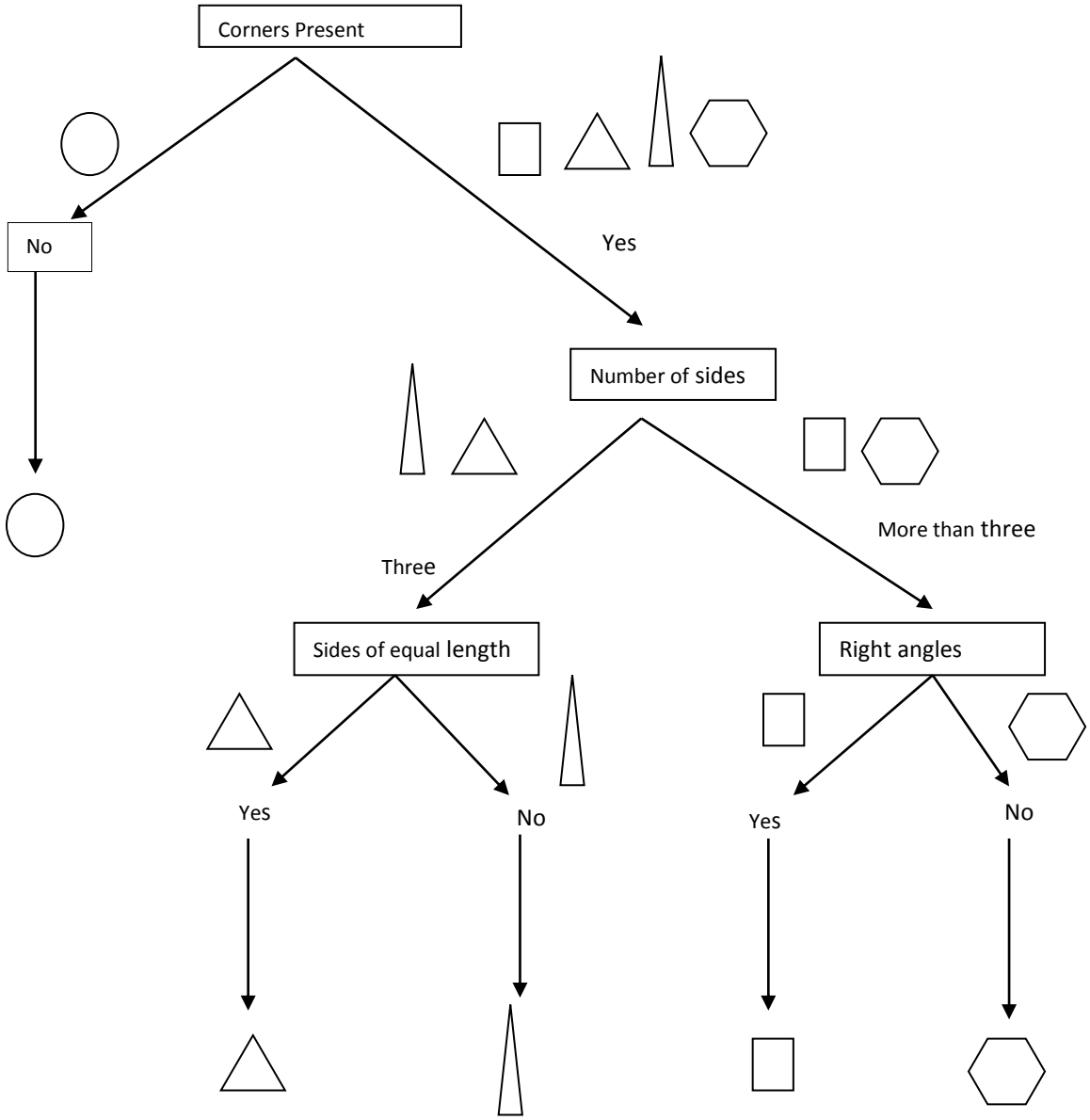
Procedure

Your instructor will show you an example of a simple dichotomous key using geometric shapes (see below). The key is based on looking for features that distinguish one shape from another. Similarly, unknown bacteria can be identified by looking for tests that distinguish one type of bacteria from another based on their different properties. Dichotomous keys for bacterial unknowns are based on Gram reactions, morphologies and metabolic assays like the ones you have learned in lab. Once you understand the concept of developing a dichotomous key, you will design your own keys that will allow you to identify your particular unknowns based on these characteristics.

There are many ways to make a useful key, so your key may not resemble those made by other students. **Note**: although additional staining can sometimes be used as part of a dichotomous key, you will be only using the metabolic tests for this exercise.

Once you have completed your dichotomous key, review the gram reaction and morphologies of your two unknowns. Based on these results, decide which media should be used to identify your unknowns, and write them down in the space provided on the back of your dichotomous key. When you are finished, turn it in to your instructor. You can do this during the lab period (please make sure it is legible), or you can take it home to work on it, provided you return it to your instructor **at least 3 days before** your next scheduled lab. Your instructor will check it to make sure it is correct and that you have identified the correct media to use to identify your unknowns. Your key will be graded and returned to you by the next lab period when you will inoculate your chosen media with your unknowns. **Note**: Points will be deducted for late dichotomous keys.

Sample Dichotomous Key for Geometric Shapes



Optional Class Exercise: Construct a dichotomous key using your classmates as objects.

Divide into two groups, each containing 8 students. Fill out the characteristics of the individuals in your group in the provided table. You can start with the characteristics listed in the table, but you may have to use different or additional characteristics for a complete identification (e.g., if all of the members of your group have brown hair, then hair color cannot be used as an identifier).

Using the information in your table, design (draw) a dichotomous key (a branch diagram) that can be used to identify the members of your group. Each person in the group should have a copy of the key. Design the key so that each choice has only 2 alternatives. For example, if members of your group range in height from 5'4" to 6' 2", your key could include two branch points: < 6' tall and > 6'tall.

Once each of you have completed a dichotomous key for your group, exchange keys with an individual from the other group. Using the key, try to determine their name without speaking to them (unless you need to ask their height or their shoe size). Ask that individual if your identification is correct. If not, review the key to see if you made a mistake, or if there was a point where the key was misleading.

Student Characteristics

Student Name	Gender	Height	Eye color	Hair color	Shoe size	Additional characteristics

Construct Classmate Dichotomous Key Below

SAMPLE DICHOTOMOUS KEY DATA FOR CLASSMATE IDENTIFICATION

Table of Characteristics for Sample Dichotomous Key

Name	Gender	Hair color	Eye color	Age	Shoe Size
Jim	M	Blonde	Grey	19	10
Tom	M	Brown	Green	55	9
George	M	Blonde	Brown	23	11
Fred	M	Black	Blue	35	12
Dan	M	Brown	Brown	22	10
Martha	F	Black	Brown	43	7
Amy	F	Brown	Brown	32	7
Connie	F	Brown	Brown	21	6
Carol	F	Blonde	Brown	23	8
Fran	F	Red	Blue	23	6

Construct Sample Dichotomous Key Below

Dichotomous Key for Bacterial Unknowns

Use the space below to draw your dichotomous key based on the staining and metabolic activity results you have obtained for the 10 organisms (see table at end of Lab 5). You will be using this key to identify your own two unknowns.

Based on your dichotomous key, list the metabolic tests you will need to identify each of your unknowns, and the media that you will used for each test.

My unknown number: _____

Unknown A		**Unknown B**	
Metabolic test required	**Media**	**Metabolic test required**	**Media**

Laboratory Exercise 7: The Effect of Physical Factors on Microbial Growth

Objectives:

1. Learn how temperature, moisture and UV light affect microbial growth.
2. Be able to determine the thermal death point (TDP) and thermal death time (TDT).
3. Understand the uses of dry heat and moist heat and the advantages and disadvantages of each.

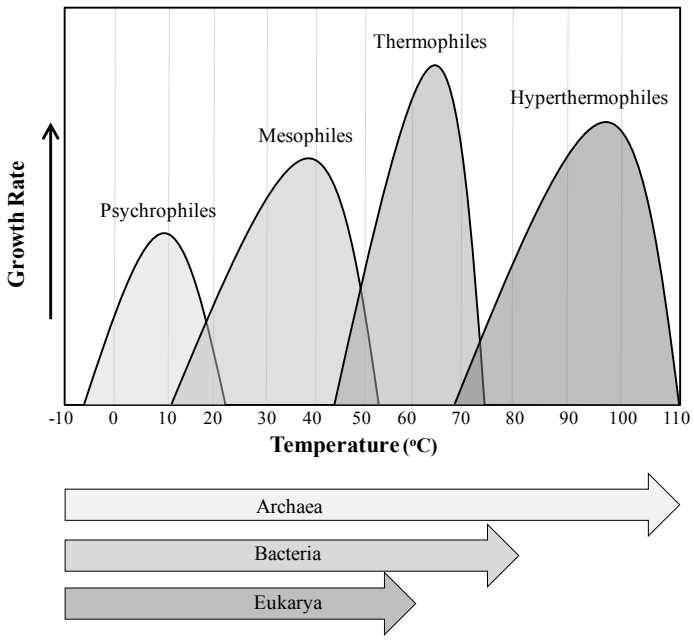
Key Terms: psychrophile, mesophile, thermophile, hyperthermophile, thermoduric, UV radiation, pyrimidine dimers, bacteriostatic, bactericidal, dry heat, moist heat, thermal death point, thermal death time, autoclave, pasteurization

Introduction

Just as there is a great deal of diversity in the metabolic properties of bacteria, there is also a great deal of diversity in the types of environments in which different species of bacteria can survive. Microbes are affected in different ways by their physical environment. In today's lab you will perform experiments that will determine the effects of some of these physical factors on microbial growth.

Effect of temperature: Prokaryotes are found in all types of environments. Some microbes are adapted to live in very cold temperatures, whereas others can survive only in very hot temperatures. Each species has a minimum temperature (lowest temperature for growth), maximum temperature (highest temperature for growth) and optimal temperature (temperature at which it grows best). The ranges of temperatures that different types of microbes grow at are as follows:

Figure 1. Growth Rates for Different Microorganisms in Response to Temperature.



Use the chart to determine the range of growth temperatures for the following:

Psychrophiles _____

Mesophiles _____

Thermophiles _____

Hyperthermophiles _____

Note: In addition to the categories listed here, some bacteria are classified as **thermoduric**—these species can survive short bursts of heat but will only grow at lower temperatures.

Temperature Control Methods: Because microbial growth can be affected by temperature, both high and low temperatures can be used to control the growth of microorganisms.

Low Temperatures: One of the most important means of controlling bacterial growth is through the use of temperature. Low temperatures are primarily **bacteriostatic**—they inhibit bacterial growth and/or reduce the total number of bacteria. Low temperatures inhibit enzyme activity, so biochemical reactions are slowed or cease, thus reducing the rate at which the bacteria can metabolize and reproduce. This inhibition of enzymatic activity is usually not permanent—if the temperature increases, the enzymes can function at their normal rate, and the bacteria will resume metabolizing and reproducing. Low temperatures are commonly used to prevent food from "spoiling"—i.e., to inhibit bacterial growth, but low temperatures cannot be used to sterilize materials.

High Temperatures: High temperatures can be **bactericidal**—they can kill bacteria. When you heat your inoculating loop in the Bunsen burner flame this results in sterilization, since the bacteria are incinerated. Of course not all materials can be sterilized by incineration. Both dry heat methods and moist heat methods can be used to kill bacteria. **Dry heat** kills by causing oxidation of cellular molecules and by desiccating (drying) the bacteria. Dry heat is commonly used to sterilize materials that could be damaged by moisture (corrosive metal surgical instruments, dry powders). Dry heat methods generally require longer times and higher temperatures, and are less penetrating than moist heat methods. For example the hot air oven requires 2 hours at 160 – 180^{o}C to sterilize materials.

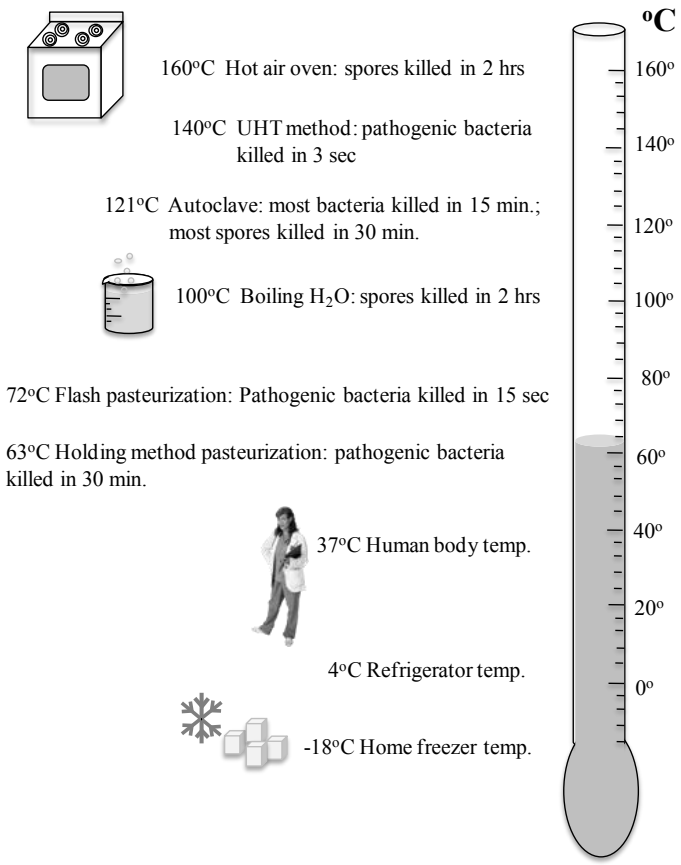
Moist heat works by denaturing nucleic acids and enzymes in the bacteria; once these molecules are denatured they are no longer capable of functioning, so even if the temperatures are reduced, the bacteria are incapable of metabolizing or reproducing. Moist heat methods include boiling, pasteurization and autoclaving.

1. Boiling: The temperature of boiling water at sea level is 100^{o}C. This temperature is high enough to kill many vegetative cells, but the exact time required varies depending on the bacterial species. Boiling does not guarantee sterilization, because some bacteria can produce spores that are resistant to high temperatures.

2. Pasteurization: **Pasteurization** is a heating method that is used to control the growth of microbes in food materials such as milk and fruit juices. Regular pasteurization (the holding or batch method) is a low heat treatment (63^{o}C for 30 min.) that is used to reduce the number of bacteria to what are considered to be acceptable levels. It is primarily focused on eliminating *Mycobacterium tuberculosis*, *Escherichia coli* and *Salmonella* sp. from milk. More recently two other pasteurization methods have been developed: flash pasteurization (71.6^{o}C for 15 sec) and the ultra-high temperature (UHT) method (140^{o}C for 3 sec.) The UHT method can sterilize if it is done under proper aseptic conditions.

3. Autoclave The **autoclave** is an instrument that uses steam under pressure to destroy microbes. An autoclave is generally set to apply 15 lbs/in^2 of pressure, which allows liquids inside the autoclave to reach a temperature of 121.5^{o}C, a higher temperature than can be achieved by a liquid at normal atmospheric pressures. Autoclaves are capable of achieving sterilization—killing all forms of life, including viruses and spores. They are commonly used to in the laboratory to sterilize growth media, glassware and other solutions. They are used in hospital settings to sterilize bedding, IV solutions, instruments and other heat-resistant objects.

Figure 2. Effect of Temperature on Bacterial Growth



Determining the Effectiveness of Heating Methods

Since heating methods are commonly used to control microbial growth, it is important to be able to define the effectiveness of a heating method for a particular bacterial species. One way to do this is to determine the thermal death point (TDP) and the TDT (thermal death time).

Thermal Death Time (TDT)

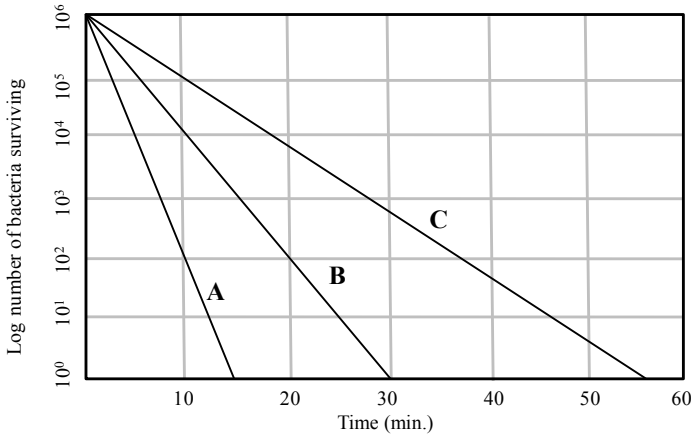
The TDT is the minimum time it takes to kill a population of microbes at a specific temperature.

Figure 3. shows death curves for 3 bacterial species (A, B, C), each treated at 70ºC for the given time period. Based on the definition of TDT, provide the following information:

What is the TDT for Bacterial species A at 70ºC? _____

Which bacterial species is more likely to be a thermophile? _____

Figure 3.



Thermal Death Point (TDP)

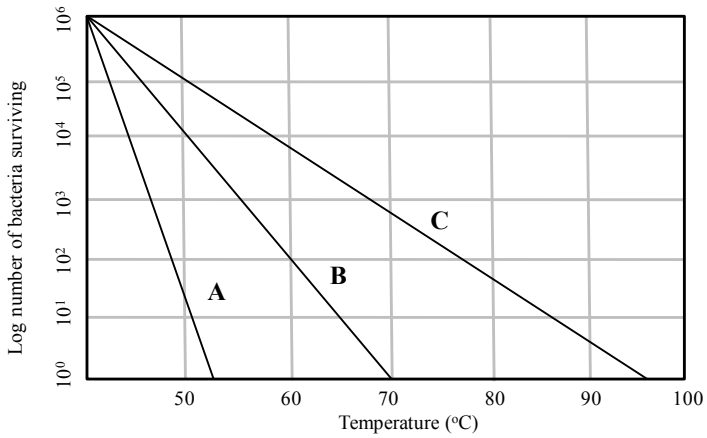
The TDP is the lowest temperature that is required to kill a population of microbes when applied for a specific time.

Figure 4. below shows death curves for bacterial species A, B and C, each treated for 10 min. over a range of temperatures. Based on the definition of TDP, provide the following information:

What is the TDP for Bacterial species C at 10 min.? _____

Which bacterial species is more likely to be a psychrophile? _____

Figure 4.

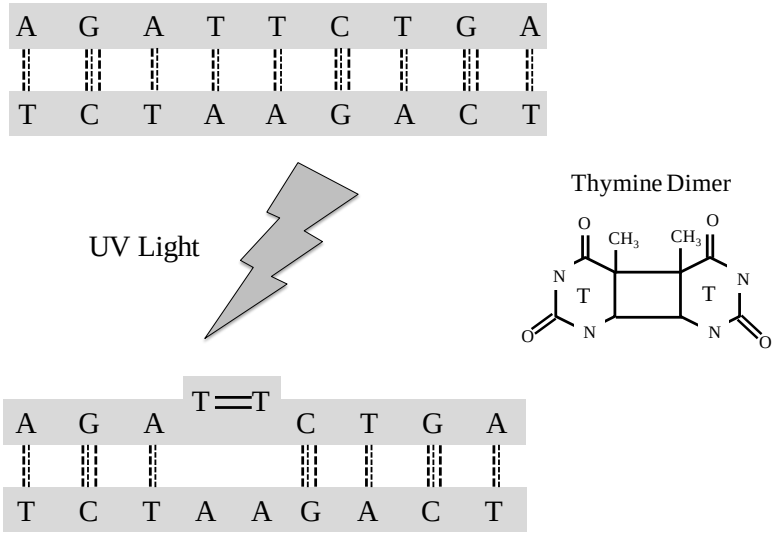


As you learned in above, different bacterial species have different temperature requirements for growth. It is therefore not surprising that it would require higher temperatures or longer heating times to kill some bacteria than others.

Effect of moisture: all living organisms, including bacteria, require water to survive. However, bacterial species vary in their ability to survive in a dry environment. Although some species will die very quickly under dry conditions, others can persist for varying amounts of time. The bacterial endospore is not affected by dry conditions, and can germinate to form vegetative cells when it finds itself in a moist environment. Other non-spore formers can also persist in the environment for varying amounts of time based on their cell wall properties and amount of glycocalyx they produce.

Effect of UV radiation: **Ultraviolet (UV) radiation** is electromagnetic radiation or light having a wavelength longer than that of x-rays but shorter than that of visible light (between 100 nm - 400 nm). UV radiation damages the DNA molecule by causing the formation of **pyrimidine dimers**, which are unnatural bonds between adjacent thymine or cytosine nucleotides. These bonds distort the DNA molecule (see Figure 5.), which can interfere with the processes of DNA replication and transcription. Although cells do have repair mechanisms to fix the damage, DNA damage caused by UV radiation exposure (particularly in the range of 240-260 nm) can result in mutations, and long-term exposure can overwhelm the repair mechanisms and cause cell death. UV radiation is used to control microbial growth in such locations as sterile hoods, operating rooms, and other areas when they are not in use. Humans need to use these methods with caution, as the UV radiation can damage skin cells as well.

Figure 5. Effect of UV light on DNA



Procedures

A. The Autoclave: (Optional Field Trip)

Each table: 1 broth culture of *Geobacillus stearothermophilus*
Each table: 1 TSA plate

1. Divide TSA plate into 2 areas, labeled "before" and "after".
2. Make a streak of *Geobacillus stearothermophilus* on the area labeled "before".
3. Place the culture tube in a wire rack for autoclaving.

Your instructor will take you to the Micro Prep room, where our technician will discuss the use of the autoclave.

4. After the culture has been autoclaved, make another streak on the side of the plate labeled "after".
5. Incubate the plate at 55°C until the next lab period.

B. Effect of Temperature

Each student: broth cultures of *Micrococcus roseus, Geobacillus stearothermophilus, Escherichia coli,* and *Serratia marcescens*
Each student: 1 TSA plate

1. Divide your agar plate into 4 areas, and inoculate with each of the following bacteria (use a small streak or spot inoculation): *Micrococcus roseus, Geobacillus stearothermophilus, Escherichia coli,* and *Serratia marcescens*

Your instructor will collect all 4 plates from your table and place them at 4 different incubation temperatures: 4˚C (refrigerator), room temperature (about 25˚C), 37˚C, and 55˚C. After incubation, you will determine which temperatures are appropriate for the growth of these species.

C. Effect of Moisture

Each student: 1 empty sterile tube, sterile cotton swab and liquid culture of one of the following: *Proteus vulgaris, Staphylococcus aureus, Mycobacterium phlei,* or *Bacillus subtilus*

Instructions

Week 1: Each student at the table should choose <u>one</u> of the bacteria listed above.

1. Make sure your bacteria are suspended in the broth by gently flicking the tube.
2. Dip a sterile cotton swab into your liquid culture to pick up some bacteria.

3. Press the swab against the inside of the culture tube to make sure you are not transferring too much moisture.
4. Use the swab to introduce bacteria into the bottom of your empty sterile tube.
5. Discard the swab in the disinfectant beaker, and incubate the tube until the following week.

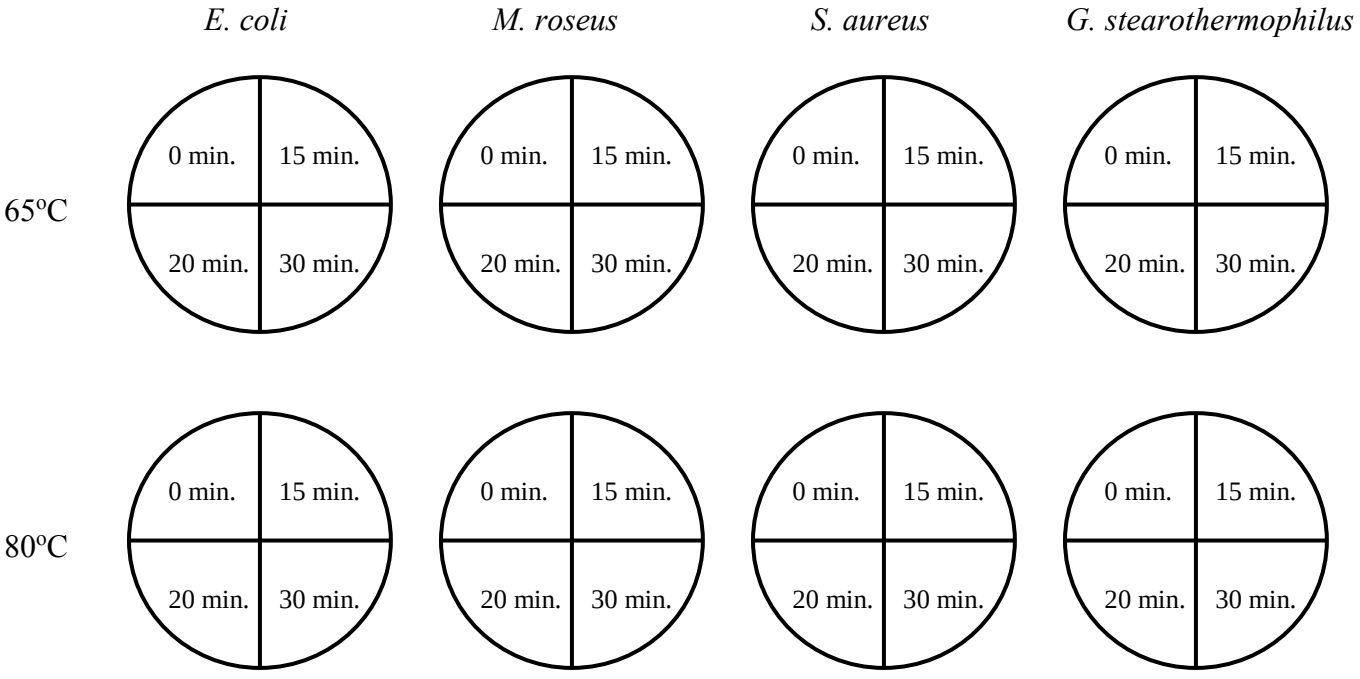
Week 2: tubes of nutrient broth, 5 ml pipets, pipet pumps

1. Observe any changes in the appearance of your tube.
2. Use a sterile 5 ml pipet and pipet pump to add 2 ml of nutrient broth to your tube.
3. Incubate this tube until the following lab period.

Week 3: record growth (a turbid culture indicates that the bacteria survived dry conditions for one week and were able to grow after nutrient broth was added).

D. Demo Experiment: Determination of the TDT

There will be 8 plates at the instructor's table which you will use to determine the TDT at 65˚C and at 80˚C for the following organisms: *M. roseus, E. coli, S. aureus* and *G. stearothermophilus*. Each of these organisms was heated at 65˚C and at 80ºC for 15 min., 20 min. and 30 min. 0 min. is the control. They were then streaked on the plates. Record the results as instructed, and then calculate the TDT for each organism at each temperature.



E. Effect of UV radiation

Per table: 12 TSA plates (6/pair of students), liquid cultures of *Serratia marcescens* and *Bacillus subtilis*, sterile cotton swabs, UV lamp, visible light source

Note: *Serratia marcescens* produces a red pigment known as prodigiosin especially when it is grown at room temperature. However, cells that have mutations in the genes responsible for producing this pigment may lose their ability to make it.

1. Each pair of students will work with one type of bacteria *(Serratia marcescens or Bacillus subtilis).*

Label your plates with the name of the organism and the experimental conditions as follows:

1. UV lid off 5 sec
2. UV lid off 10 sec
3. UV lid off 1 min
4. UV lid off 10 min
5. UV lid on 10 min (control)
6. Visible light (lid off) 10 min (control)

2. Use the sterile cotton swab to inoculate a lawn of bacteria (cover the whole surface of the plate) for all 6 plates.

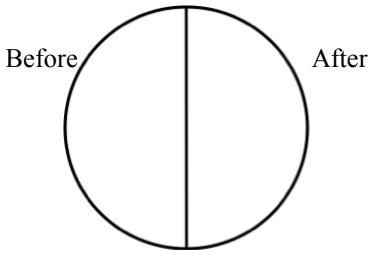
3. Your instructor will demonstrate how to use the UV lamp (be extremely careful to minimize your exposure). Expose each plate to the UV light source with the lid off for the specified amount of time. Be sure to time the exposure carefully!
    a. Visible light control: this plate should be placed under the small lamp with the lid off for the maximum exposure time.
    b. UV lid on control: this plate should be placed under the UV lamp for the maximum exposure time used but with the lid in place.

4. Incubate all plates until the next lab period. Note: if you are working with *Serratia*, your plates will need to be wrapped in foil before incubating them. This is to present any photoreactivation (a DNA repair mechanism) from occurring.

Results

A. The Autoclave

Describe and draw the results of the autoclave experiment.



What can you conclude about the effectiveness of the autoclave as an instrument for sterilization?

_____

_____

B. Effect of Temperature

For each incubation temperature, record growth pattern as follows:

0 no growth

\+ light growth

++ medium growth

+++ heavy growth

	Week 2 Results			
Bacterial species	**4˚C**	**Room temperature**	**37˚ C**	**55˚ C**
Micrococcus roseus				
Escherichia coli				
Geobacillus stearothermophilus				
Serratia marcescens				

Based on these results, how would you classify:

Micrococcus roseus _____

Escherichia coli _____

Geobacillus stearothermophilus _____

Serratia marcescens _____

Do you notice any differences in the *appearance* of the growth of *Serratia* at different temperatures? If so, describe what you observe.

_____

_____

_____

Your instructor will again collect the plates that had been incubated at 4˚C and incubate them for another week at 4 ˚C, room temperature, 37˚C and 55˚C (one plate at each temperature).

Week 3: Record the results for plates that were initially incubated at 4˚C and then placed in warmer temperatures.

		Week 2 incubation temperature		
Bacterial species	**Initial incubation temperature (week 1)**	**Room temperature**	**37˚ C**	**55˚ C**
Micrococcus roseus	4˚C			
Escherichia coli	4˚C			
Geobacillus stearothermophilus	4˚C			
Serratia marcescens	4˚C			

Based on these results, what can you conclude about the effect of cold temperatures on these organisms?

_____

_____

C. Effect of Moisture

Week 2: describe any changes in appearance of the tubes.

_____

_____

Week 3: Record + or – growth for each bacteria tested

Bacteria	**Growth**
Staphylococcus aureus	
Proteus vulgaris	
Bacillus subtilis	
Mycobacterium phlei	

Based on these results, which bacteria can survive drying conditions?

_____

_____

Can you think of any reasons why these species would be able to survive in a dry environment?

_____

_____

D. Demo: Determination of the TDT

Observe the plates at the instructor's table, and record the growth of the bacteria at each incubation time in the table below. Record growth as 0, +, ++, or +++.

Temperature	**65°C**				**80°C**			
Incubation Time (min.)	**0**	**15**	**20**	**30**	**0**	**15**	**20**	**30**
Escherichia coli								
Micrococcus roseus								
Staphylococcus aureus								
Geobacillus stearothermophilus								

Based on the information in the data table, determine the TDT for each organism at the given temperature.

Bacterial Species	**65ºC**	**80ºC**
Escherichia coli		
Micrococcus roseus		
Staphylococcus aureus		
Geobacillus stearothermophilus		

E. Effect of UV Radiation

Record the results for each plate at follows:

0 no growth

\+ light growth

++ medium growth

+++ heavy growth

Also note any differences in appearance of growth on each of the plates.

			UV lid off			
Bacteria	**Visible light**	**UV lid on**	**5 sec**	**10 sec**	**1 min**	**10 min**
Serratia marcescens						
Bacillus subtilis						

Based on these results, answer the following questions:

1. Did visible light have any effect on microbial growth? _____
2. Did UV exposure with the lid on have any effect on microbial growth?_____
3. Explain any differences you observe between the two species tested.

   _____
4. Describe any other differences in the appearance of colonies on the irradiated plates.

   _____

   _____

   _____

Review Questions

1. Classify the following types of organisms by their temperature requirements
    a. A bacteria that is spoiling food in your refrigerator _____
    b. A bacteria that live in the hot springs of Yellowstone _____
    c. A bacterial species that can cause human disease _____

2. Some hospital operating rooms have UV light bulbs that are switched on when the room is not in use
    a. What is the purpose of these lights?

    b. Why are they only used when the room is not in use?

3. Would you expect an endospore-former to be thermoduric? Why or why not?

4. In the UV radiation experiment, what was the purpose of exposing one plate to visible light?

5. What did you conclude from observing the plate that was exposed to UV radiation with the lid on?

6. Why would we need to expose the *Serratia* and the *Bacillus* to UV radiation for different amounts of time to achieve sterilization?

Lab 8: Chemical Control of Microbial Growth--Disinfectants and Antibiotics

Objectives: In this lab you will:
1. Learn about disinfectants and the various factors that need to be considered when choosing a disinfectant.
2. Set up an experiment to determine the effectiveness of five different common disinfectants.
3. Learn how to determine antibiotic susceptibility using the Kirby-Bauer method.
4. Set up an experiment to test for antibiotic production by three different strains of *Streptomyces.*

Key Terms: **disinfectant, antiseptic, filter paper disk method (agar disk diffusion assay), zone of inhibition, Kirby-Bauer method, broad-spectrum antimicrobial drugs, narrow-spectrum antimicrobial drugs, susceptibility, resistance, intrinsic resistance, acquired resistance, *Streptomyces*, saprophytic, secondary metabolites**

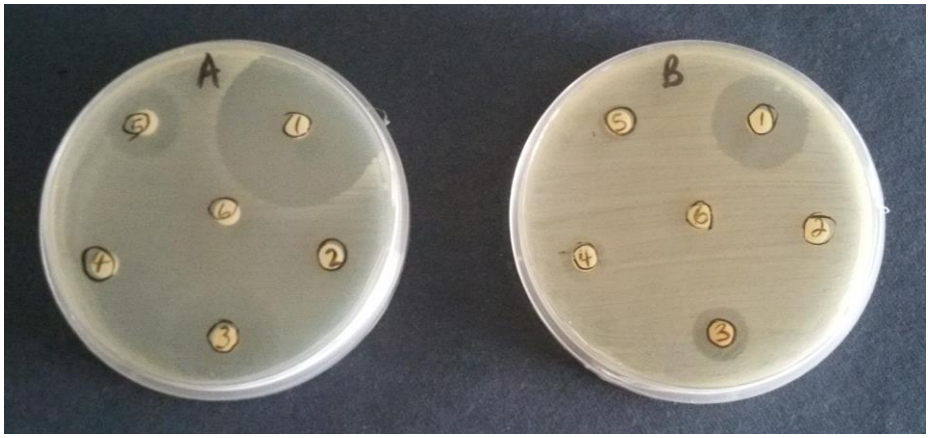
Introduction

The use of chemicals to control microbial growth dates back at least as far as the 1800's. Tincture of iodine was used as antiseptic during the Civil War, and Joseph Lister established the practice of aseptic surgery using a disinfectant known as carbolic acid (phenol) in the 1860's. Since that time, many types of **disinfectants** (agents that are used to eliminate or kill vegetative cells on surfaces) and **antiseptics** (agents that are used to eliminate or reduce vegetative cells on living tissue) have been used. Although disinfectants and antiseptics may be effective at killing vegetative cells, they do not usually achieve sterilization.

Various factors need to be considered when choosing a disinfectant or antiseptic. It is very important to know which microbes are present to determine what type of disinfectant would work best. It is also important to realize that the effectiveness of a particular disinfectant may be affected by pH, temperature, concentration**,** and exposure time. Ideal disinfectants should be effective against the particular contaminants present, usable at a low concentration, require a relatively short exposure time, and have a long shelf life. It should also be water soluble, non-toxic to humans and animals, and cost-effective.

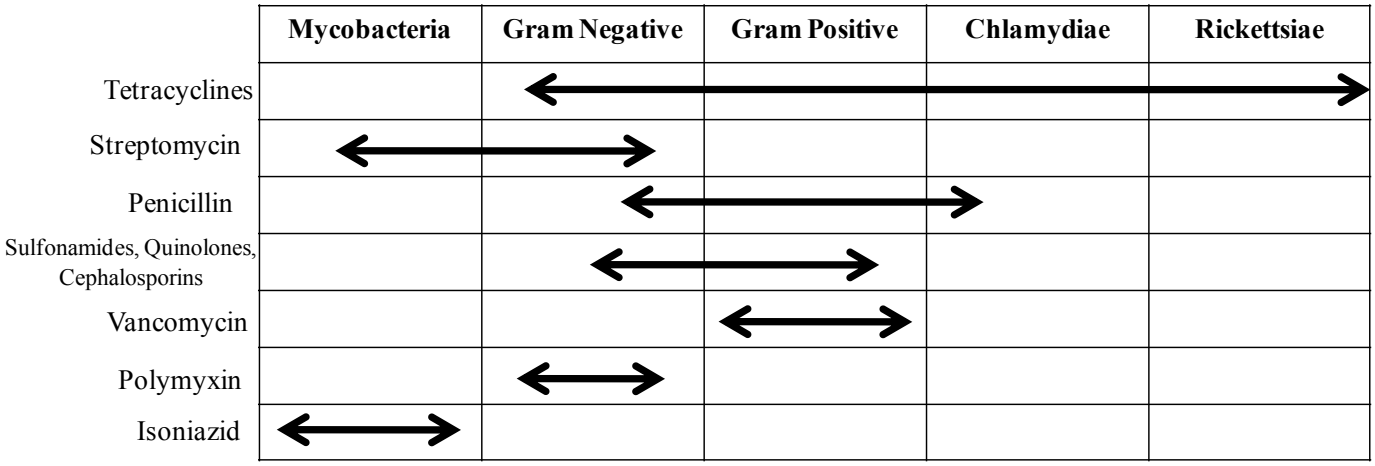
The efficacy of a disinfectant or antiseptic can be tested in several ways. One way is to inoculate an agar plate with a lawn of bacteria and add filter paper disks that have been moistened with the disinfectant being tested. This is known as the **filter paper disk method**, or **agar disk diffusion assay**. After incubation, plates are observed for the presence of a **zone of inhibition** (area around a disk where no microbial growth is detected). Generally speaking, the larger the zone, the more effective the disinfectant is against that particular microbe. However, other factors such as the solubility of the test agent and the molecular weight of the disinfectant molecules (which determines the diffusion rate of the disinfectant through the agar) can also affect results.

Figure 1. Filter paper disk method experiment showing zones of inhibition



The use of antimicrobial agents to treat infections began in the early 1900's, when Paul Ehrlich developed Salvarsan to treat individuals infected with *Treponema pallidum*, the spirochete that causes syphilis. In 1928, Alexander Fleming later observed that the *Penicillium* mold growing on his agar plates could inhibit the growth of bacteria: years later penicillin was purified and used to treat many types of infections. Since this time, many other antimicrobial agents have been used to treat a wide variety of bacterial infections. Antibiotic producers include many types of fungi (*Penicillium, Cephalosporium*) and bacteria (*Bacillus, Streptomyces*). In addition, many antimicrobial agents currently used to treat infections are either synthetic (made in a laboratory) or semi-synthetic (a modification of a naturally-produced antibiotic). Today there are over 100 different antimicrobials that are used to treat infectious diseases. These include **broad-spectrum** and **narrow-spectrum antimicrobial drugs** (see chart below). Narrow-spectrum drugs are more desirable to use whenever possible because they target the pathogen more specifically and do less damage to the normal microbiota; broad-spectrum drugs are used when the cause of the infection is unknown or when other antibiotics are not effective.

Figure 2. Activity spectra of the major classes of antibiotics



It is important to remember that not all antibiotics are effective at killing all types of bacteria. Bacteria may have **intrinsic resistance** to a particular antibiotic. For example, gram negative bacteria are intrinsically resistant to vancomycin because the drug cannot penetrate the outer membrane of the gram negative cell wall. Also, the misuse and overuse of antibiotics has led to the evolution of **resistance** among bacteria by selecting for individual cells within a population that are not affected by the drug. This **acquired resistance** can occur in several ways, including through transformation, conjugation and mutation. Antibiotic-resistant bacteria have become a major problem of growing concern in health care, as it is often difficult (or impossible) to treat bacterial infections caused by these microbes (for example, multidrug-resistant *Staphylococcus aureus*, or MRSA). Therefore clinical isolates are often tested for their antibiotic susceptibility in a laboratory setting so that health care providers can choose an appropriate drug to treat a particular infection.

There are several ways to determine antibiotic **susceptibility** in a laboratory setting—one common test is called the **Kirby-Bauer method**. This method is similar to the filter paper disk method used to test disinfectants, except that it uses filter paper disks impregnated with a known concentration of an antimicrobial compound. It also uses Mueller-Hinton agar, and is often performed with larger (150 mm) petri dishes that allow for the testing of several antibiotics simultaneously. When performing the Kirby-Bauer method, it is important to measure the size of the zones of inhibition and compare them to a set of standardized values established by the Clinical Laboratory Standards Institute (CLSI).

Antibiotic Production by Soil Bacteria

Streptomyces is a bacterial genus of the order *Actinomycetales*. These Gram-positive spore-formers closely resemble fungi because of their branched filamentous structure. They are commonly found in soils and are primarily **saprophytic**, which means that they feed off of decaying matter. *Streptomyces* are characterized by having complex and abundant gene clusters that code for bioactive **secondary metabolites**. From a human health perspective they are very valuable, as they produce 2/3 of all the antibiotics of natural origin. Secondary metabolites from *Streptomyces* have antifungal, antiviral, antitumor, and immunosuppressant activities.

In this lab you will set up experiments to evaluate the effectiveness of several disinfectants and antibiotics. In addition, you will test three strains of *Streptomyces* for their ability to produce antimicrobial compounds, and determine the effect of these compounds on different types of bacteria.

Procedures

A. Disinfectants: For this exercise you will perform a filter paper disk diffusion assay to determine the effectiveness of 5 different types of disinfectants.

Materials: 1 TSA plate/student, liquid cultures of *Staphylococcus aureus* and *Escherichia coli*, sterile cotton swabs, filter paper disks, 5 different disinfectants, forceps, and beakers with 70% ethanol

Go up to the instructor's table and write down the names of the 5 disinfectants you will test in the chart below.

Disinfectant #	**Name**	**Concentration**	**Active ingredient/class of disinfectant**
6	Sterile distilled water (control)		

1. Choose one of the bacterial species listed above. Use a cotton swab to inoculate a lawn of bacteria on your TSA plate. Discard swabs in the beaker provided at your table.

2. Label your TSA plate with the name of the microbe you are using, and numbers 1-6 evenly spread out on the plate (see demo plate done by your instructor). The numbers (and corresponding disks) should not be too close to the edges of the plate.

3. Bring your inoculated plate up to the front table, where you will be adding filter paper disks soaked in disinfectants.

4. Before each use, forceps should be flame sterilized as follows:
    a) Remove forceps from beaker of alcohol. **Keep the tips angled down at all times**.
    b) Put the forceps in the Bunsen burner flame **just to ignite the alcohol.**

    NOTE: you are not heating the forceps, just igniting the alcohol.

c) Keep a careful eye on the forceps (hold them steady in one location) until all the alcohol has burned off (this will be very quick). To avoid fires, do not hold them over any beakers of alcohol!

d) Once the alcohol has burned off, use the forceps to pick up one filter paper disk from the glass Petri dish.

5. Dip the disk into disinfectant #1.

NOTE: just touch the surface of the liquid—the disinfectant will soak into the disk by capillary action. It is important to not have the disks too wet when you place them on your TSA plate.

6. Place disinfectant #1 on the appropriate area of the agar plate. Tap it down gently with the forceps to ensure that it adheres to the surface of the agar.

7. **Repeat this step for all disinfectants.**

NOTE: if you want to test some other product, just omit one of the disinfectants in the front of the room.

8. Add a filter paper disk that has been dipped in sterile water to the area of the plate labeled "#6"- this will serve as your negative control.
9. After each use, place the forceps back into the beaker with 70% ethanol. DO NOT heat them after use before returning them to the beakers.

10. Incubate plates (inverted, as usual) until the next lab period.

B. Antibiotic susceptibility testing

Each table: 3 large (150 mm) Mueller-Hinton agar plates, liquid cultures of *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*, sterile cotton swabs, antibiotic disk dispensers

1. Work as a table for this exercise. Use a sterile cotton swab to inoculate the Mueller-Hinton agar plates with a lawn of bacteria for each species listed (one species/plate). Be sure to cover the entire area of the plate. This can best be accomplished by swabbing the entire surface of the plate three times, rotating the plate approximately 60° each time, and finishing the swabbing by going around the entire outer surface of the plate.

2. After all plates are inoculated, your instructor will show you how to use the disk dispenser to introduce antibiotic disks onto the plates. Be careful to always keep the dispensers in the upright position.
    a. You will notice that each disk that is introduced to the plate is stamped with a letter and a number—these indicate the type of antibiotic as well as the concentration. For example, P10 is an abbreviation for 10 units of penicillin; PIP-100 is an abbreviation for 100 mcg (micrograms) of piperacillin.

3. Return to your lab table, and use your inoculation loop or needle to gently push down on the disks to ensure that they are adhered to the agar surface.

NOTE: flame your loop in between plates to avoid cross-contamination.

4. Incubate the Mueller-Hinton agar plates (inverted) until the next lab period.

C. Demonstrating antibiotic production with *Streptomyces*

Materials: Soy Flour Mannitol (SFM) plates containing cultures of *Streptomyces coelicolor* and two additional Streptomyces cultures, liquid cultures of *Escherichia coli, Staphylococcus aureus,* and *Mycobacterium smegmatis,* TSA agar plates (1/pair of students), sterile cotton swabs, sterile 1000 µl pipet tips.

Before beginning this experiment, write down the names of the organisms you will be testing here, and provide a brief description of their appearance.

Strain #	Strain name	Description	Additional information

1. Divide your plate into 4 areas (labeled 1, 2, 3 and – for negative control).

2. Choose one bacterial liquid culture from the list above, and use a sterile cotton swab to inoculate a lawn of bacteria. Discard swab in the beaker at your table.

3. Using the large round end of a sterile pipet tip, punch a hole out of the center of each of the 4 quadrants you created on your TSA Plate. Discard the tip in the disinfectant beaker.

4. Use another sterile tip to cut out a plug of agar from the lawn of the corresponding SFM plate (your instructor may provide these for you).

5. Transfer this plug of agar into the well you created in your agar plate.

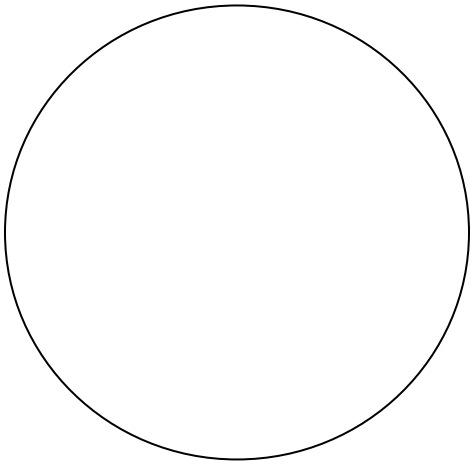
6. Repeat for the other two *Streptomyces* strains, using a fresh sterile tip each time.

7. Place a plug of uninoculated SFM agar on your plate in the negative control quadrant.

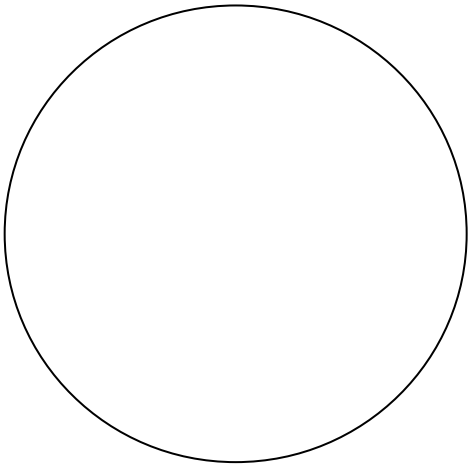
8. Incubate the plates at 28˚C until the next lab period.

Results

A. Disinfectant experiment: Use the spaces below to sketch the appearance of your own TSA plate and your lab partner's plate. Record your observations in the chart below.



Escherichia coli



Staphylococcus aureus

Disk #	Name	Observations
1		
2		
3		
4		
5		
6		

Based on your results, which disinfectant(s) worked best against the bacteria you tested?

_____

Which disinfectant(s) were least effective?

_____

Compare your plate with that of your lab partner. Did you see the same pattern of zones of inhibition for both bacteria tested? Explain.

_____

_____

_____

B. Antibiotic susceptibility testing

Observe the three Mueller-Hinton plates, and note any differences in their overall appearance.

Which plate seems to have the most zones of inhibition? _____

Which has the least? _____

Do you see any small colonies within the zones of inhibition on any of your plates? _____

What do these colonies represent?

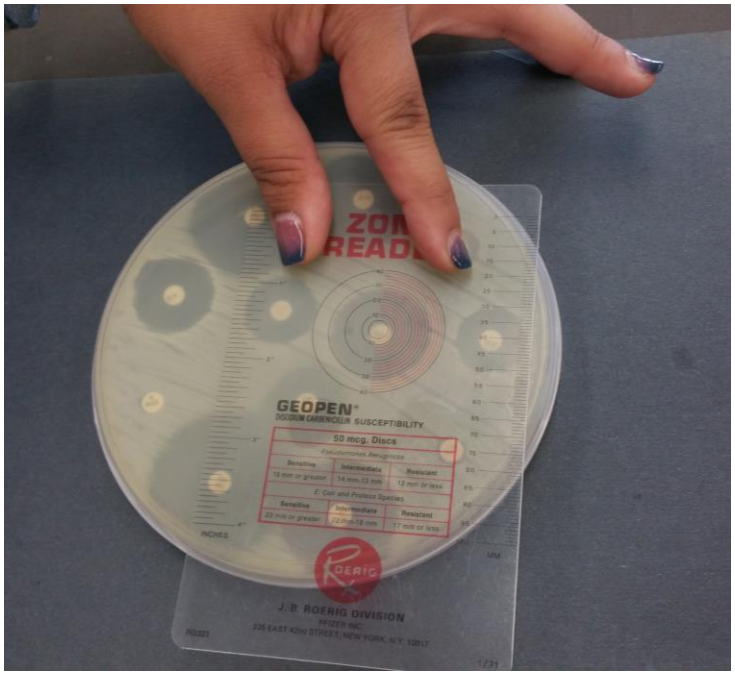
_____

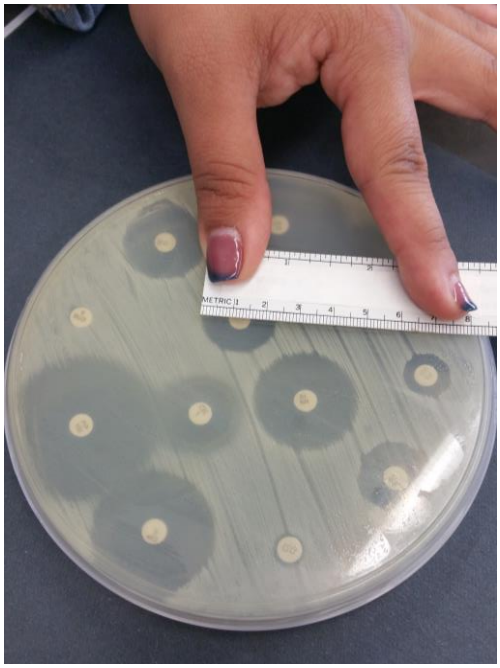
For each plate, measure zones of inhibition for 3-4 antibiotics as follows:

1. With a metric ruler, measure the diameter of the zone of inhibition. Express the values **in millimeters** (mm). It is best to choose some small, medium-sized, and large zones for comparison.

2. Use the charts provided by your instructor to look up the interpretation for each of the zones you measured. Record measurements and interpretations in the chart below.

Figure 2. Measuring zones of inhibition





Bacterial species	**Disk label**	**Antibiotic/concentration**	**Zone of inhibition (mm)**	**Interpretation (R, I, S)**
E. coli				
S. aureus				
P. aeruginosa				

Based on your results, do all antibiotics work equally as well against all types of bacteria? Explain.

_____

_____

Why do we need to look up values in the charts for each antibiotic?

_____

_____

C. Antibiotic production in *Streptomyces*

Examine the plates for evidence of antibiotic activity. Measure the zones of inhibition and record your observations in the table below.

	E. coli	*S. aureus*	*M. smegmatis*
MTE4a			
Streptomyces coelicolor			
Streptomyces violaceusniger			

Do you notice any differences in the ability of the *Streptomyces* strains to inhibit gram positive and gram negative bacteria?

_____

_____

Review Questions

1. Why would bacteria such as *Streptomyces* species evolve the ability to produce molecules that are toxic to other bacteria?

2. Do you think that a disinfectant or antiseptic that works well on an agar plate always works well in a real-world setting? Why or why not?

3. You are asked by your place of employment to order a disinfectant that will be used for daily cleaning. What are some of the factors that you will consider when choosing which one to order?

4. Give an example of when a health-care provider might choose a broad-spectrum antibiotic over a narrow-spectrum antibiotic.

5. What are some of the ways that antibiotics are misused or overused?

6. Explain the connection between the misuse/overuse of antibiotics and the evolution of antibiotic resistant bacteria.

Lab 9: The Microbiology of Milk and Food

Objectives

1. Learn how to use serial dilutions.
2. Learn how to do a standard plate count.
3. Understand the importance of standard microbiological tests for food safety.

Key Terms: CFU, indicator bacteria, serial dilution, coliform bacteria, fecal coliform bacteria, standard plate count, UHT pasteurization

Introduction

Pathogens can be introduced into foods at any stage: during growth/production at the farm, during processing (grinding, chopping, milling, etc.), during handling and packaging, and when the food is prepared in the kitchen. In many cases, small numbers of pathogenic bacteria are not dangerous, but improper storage and/or cooking conditions can allow these bacteria to multiply to dangerous levels.

Fecal contamination of water (and through water, contamination of food materials) is another one of the ways in which pathogens can be introduced. **Coliform bacteria** are Gram-negative non-spore forming bacteria that are capable of fermenting lactose to produce acid and gas. A subset of these bacteria are the **fecal coliforms**, which are found at high levels in human and animal intestines. Fecal coliform bacteria such as *E. coli*, are often used as **indicator species**, as they are not commonly found growing in nature in the absence of fecal contamination. The presence of *E. coli* suggests feces are present, indicating that serious pathogens, such as *Salmonella* species and *Campylobacter* species, could also be present.

In this lab, you will examine bacteria found in milk, chicken, and in other assorted food materials.

Milk

Milk contains carbohydrates, minerals, fats, vitamins and proteins, and is therefore susceptible to breakdown by a wide variety of microorganisms. Several different kinds of bacteria may be present in milk, most commonly the genera *Lactobacillus*, *Micrococcus* and *Streptococcus*. As discussed in Lab 7, regular pasteurization is process used to reduce microbial loads to acceptable levels in foods like milk and fruit juices. Milk that has undergone a regular pasteurization procedure can still contain bacteria. If this milk is stored at 4^{o}C (refrigerator temperature) the bacteria are prevented from multiplying, but if the milk is left out at room temperature, the bacteria will reproduce and the milk will spoil.

If done aseptically, **UHT (ultra high temperature) pasteurization** can sterilize foods. This is why UHT milk can be left unopened at room temperature for long periods of time.

Microorganisms of concern in milk include *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Mycobacterium bovis*, *Mycobacterium tuberculosis*, *Salmonella* species (most common: *S. enteritidis* and *S. typhimirium*), and *Yersinia enterocolitica*.

Chicken

Raw poultry products are frequently contaminated by pathogens (e.g. *Salmonella*, *Staphylococcus aureus*, *Clostridium perfringens*, *Campylobacter jejuni*, etc.). Salmonellosis is one of the most common serious foodborne infection caused by contaminated poultry. The prevalence of *Salmonella* in raw fresh and frozen poultry approaches 80% in some countries.

Fruits and Vegetables

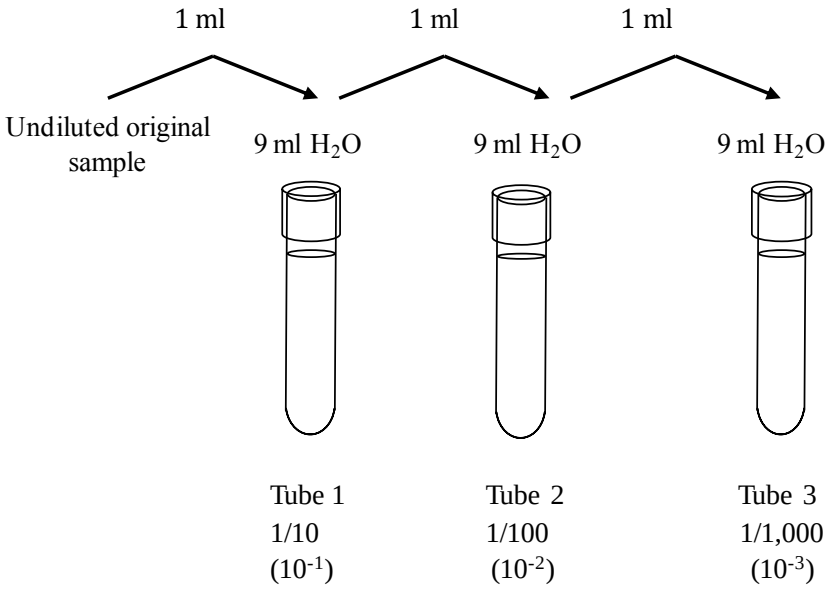
Fruits and vegetables may become contaminated with potential pathogens from the soil or water they come in contact with while growing. They may also pick up harmful bacteria during processing, storage and preparation. While the use of potable (uncontaminated, drinkable) water to wash and freshen harvestable fruits and vegetables is useful both commercially and in the home, residual water may support extensive microbial growth during further storage.

Standard Plate Count and Serial Dilution Techniques

One of the most common methods of determining the amount of bacteria in a food product is a **standard plate count**. In this method, serial dilutions of the food are plated on general purpose and/or differential/selective growth media. Bacterial colonies are then counted, and the number of **CFUs** (colony forming units) in the original undiluted sample is calculated. (CFUs are as a measure of the number of bacteria to take into account that one colony might be the product of more than a single bacterium).

Serial dilution is a technique that is used to produce very dilute solutions without the necessity of measuring very small quantities of liquids. It is a series of stepwise dilutions, in which one first dilutes a solution, then dilutes the dilution, then dilutes the dilution of the dilution and so forth. The dilution factor at each step is usually constant, resulting in a geometric progression of concentration. An example of a serial dilution is seen below. In this example, each dilution is a 10-fold dilution (transferring 1 ml into 9 ml of H_2O results in a 1/10 dilution; i.e., 1 ml in a total volume of 10 ml).

Serial dilutions are often used in standard plate counts because the number of bacteria in a sample (water, food, or a medical sample such as a urine or a fecal sample) is unknown. The sample is diluted to obtain a number of CFUs that supplies statistically significant results, yet is still easily countable. The general recommendation for a countable plate is between 30 – 300 CFUs/plate.



After dilutions are prepared, a set amount of liquid (typically between 0.1- 1 ml) is spread out over the surface of an agar plate, and then incubated to allow for bacterial growth.

CFU counts from these diluted plates are used to calculate the number of bacterial cells/ml in your original (undiluted) sample.

If you plate a full milliliter (ml) of your dilution, you would simply multiply the number of CFU's counted by the dilution factor of the plate you counted.

- Example: I count 55 CFU's on a plate diluted 1:1000 (10^{-3}) in which I added a full ml of my dilution. My calculation is 55 x 1000 = 55,000 (5.5×10^4) cells/ml.

However, it is important to note that we do not always plate a full ml (it can be difficult to get that much liquid to be absorbed into an agar plate). Therefore an additional calculation is often necessary to be able to express your results as cells/ml.

If you plate 0.1 ml of sample, you will need to multiply the number of CFU's by 10 to determine # CFU's/ml.

If you plate 0.5 ml of a sample, you will need to multiply by 2 to determine the number of CFU's/ml.

- Here's another example: you set up serial dilutions for a milk sample and plate out 0.5 ml of your dilutions. The countable plate that came from your 1:100 (10^{-2}) dilution has 114 colonies.

Your calculation:

1. Multiply: #CFU's x dilution factor = 114 x 100 = 11400 cells/0.5 ml.
2. Multiply by 2 to express your result as cells/ml 11400 x 2 = 22800 cells/ml (or 2.28 x 10^4)

If dilutions are done properly, we should expect to see a geometric progression in the number of cells in each dilution as shown below.

	Dilution	Number of bacteria/ml in each tube after dilution	Number of CFU's (1 ml plated)	Number of CFU's (0.1 ml plated)
Undiluted sample		1,000,000/ml = 10^6/ml	1,000,000 (10^6)	100,000 (10^5)
Tube 1	1/10 =10^{-1}	100,000/ml = 10^5/ml	100,000 (10^5)	10,000 (10^4)
Tube 2	1/100 = 10^{-2}	10,000/ml = 10^4/ml	10,000 (10^4)	1,000 (10^3)
Tube 3	1/1000 = 10^{-3}	1,000/ml = 10^3/ml	1,000 (10^3)	100 (10^2)

Of course your results would not turn out as perfectly as those shown above. Slight inaccuracies in pipetting would cause variations in the final results. So in the real world the results of multiple dilutions are averaged together when determining the final number of bacteria/ml in a sample.

For a review of scientific notation and additional practice problems to help you understand serial dilution, see Appendix II. For additional information about the use of pipettors for measuring small volumes, see Appendix III.

Procedures

Note: You will be using sterile spreaders when you inoculate plates in Procedures A and B of this lab. Each table will have a tinfoil packet of sterile spreaders. Please be careful when you open the packet (unfold the narrow end first) so you do not contaminate the spreaders. There will be a beaker of vesphene specifically for these spreaders at each table. Place the spreaders in this beaker. DO NOT throw the spreaders in the garbage—they will be autoclaved and reused for later lab sections. DO NOT use spreaders from the disinfectant beakers in your experiment.

A. The Microbiology of Milk

In this exercise, you will compare the amount of bacteria found in regular pasteurized milk with the amount found in UHT pasteurized milk. You will also examine the effects of leaving the two different types of milk at room temperature.

Per pair of students: 2 TSA plates
Per pair of students:

Either: 2 tubes of pasteurized milk: one refrigerated, one left out at room temperature for 1 week, **or**
2 tubes of UHT pasteurized milk: one refrigerated, one left out at room temperature for 1 week

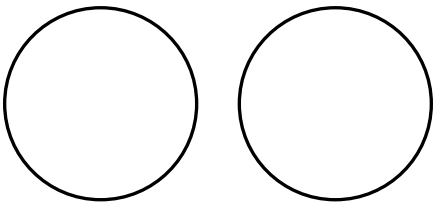
1. Each pair of students will choose either the regular pasteurized milk or the UHT pasteurized milk.
2. Using the 20-200 µl pipettor (also called the P200), transfer 200 µl of the refrigerated milk on a TSA plate. (Appendix III provides instructions on how to use micropipettors.)
3. Use a sterile spreader to spread the 200 µl of the milk across the plate.
4. Using a **different** sterile spreader, repeat Steps 2-3 using the room temperature milk.
5. Incubate the plates until the next lab.

Type of milk chosen: _____

Per Table, you should have the following plates:

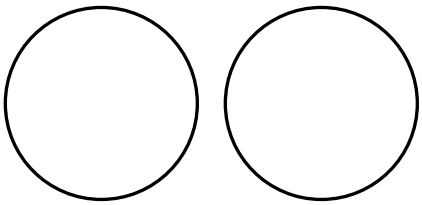
Regular Pasteurized

Refrigerated Room temp.



UHT Pasteurized

Refrigerated Room temp.



What type of bacteria will grow on the TSA plates? _____

Predict which type of milk will have the most bacteria. Explain the rationale for your prediction.

_____

_____

B. The Microbiology of Poultry

In this exercise you will determine the number of bacteria found on chicken wings using serial dilutions followed by a standard plate count.

There will be 1 resealable plastic bag containing 9 ml of sterile water and a chicken wing at the instructor's table.

Per table: 3 TSA plates
4 tubes, each containing 9 ml of sterile H_2O
1 tube of chicken wing wash fluid (given to you by your instructor)

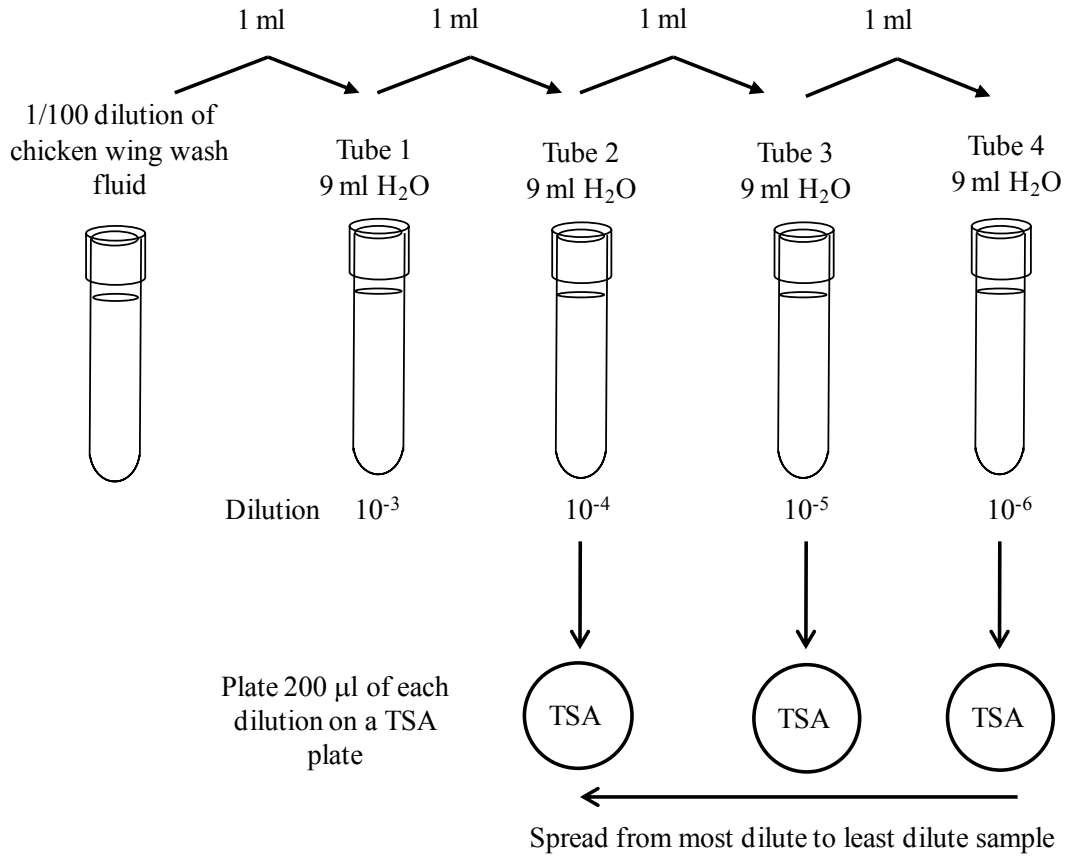
1. The instructor will rinse the chicken wing in the water, massaging the chicken wing to remove any bacteria that are adhering to the skin. Using a 200 - 1000 µl pipettor (also called the P1000), the instructor will transfer 1 ml of the wash fluid into 99 ml of H_2O, and will mix thoroughly. This will be a dilution of 1/100 (10^{-2}). The instructor will transfer 2-3 ml of this 1/100 dilution into 4 sterile tubes (1/table).

2. Label one of the tubes of sterile water 10^{-3}(1/1,000)—this will be Tube 1. Label one of the tubes of sterile water 10^{-4} (1/10,000)—this will be Tube 2. Label the next tube of sterile water 10^{-5} (1/100,000)—this will be Tube 3. Label the last tube of sterile water 10^{-6} (1/1,000,000)—this will be Tube 4.

3. Using the P1000, remove 1 ml of the 1/100 wash fluid from the tube given to you by the instructor, and transfer it to Tube 1. Mix the contents of the tube by carefully by flicking the tube. Using the same pipettor, transfer 1 ml from Tube 1 into Tube 2; transfer 1 ml from Tube 2 to Tube 3, and transfer 1 ml from Tube 3 to Tube 4. After each transfer, flick to mix thoroughly. The figure below shows you how to make the transfers.

Instructions for plating

Using the P200, transfer 100 µl from Tube 2, Tube 3 and Tube 4 to 3 different TSA plates. Make sure to label your plates. The figure below indicates how the transfers should be done.



You will be using 1 sterile spreader for all of your plates, so you must be careful to work from the most dilute sample to the least dilute sample. Make sure that all of the fluid has been absorbed into the plate before you invert the plate for incubation.

C. Microbiology of Food

Per table: 1 TSA plate

Per table: Choose 1 of the following four food materials: ground beef, mushroom, bread or lettuce.

Chosen food material: _____

1. Flame sterilize forceps. (Note: follow the recommendations for forcep sterilization described in Lab 9.)

2. Pick up a portion of your chosen food product, gently rub it over the surface of the TSA plate, then discard the food.

3. Incubate the plate until the next lab.

Of the four food materials, which do you think will contain the most bacteria? _____

Results

A. The Microbiology of Milk

Record the results of your experiment in the table below. Record growth as 0, +, ++, or +++. Describe any notable growth characteristics.

TSA Plates

	Amount of growth	Growth Characteristics
Regular pasteurized (refrigerated)		
Regular pasteurized (room temperature)		
UHT pasteurized (refrigerated)		
UHT pasteurized (room temperature)		

Which type of milk had the largest number of bacteria? _____

Was your earlier prediction correct? _____

B. The Microbiology of Poultry

Count the CFUs on your plates. Record your numbers in the table below. If there are too many CFUs to count, just write "TMTC" (too many to count). Remember—a countable plate that is considered statistically significant should have between 30 -300 CFUs.

Record your plate counts below. Make note of any notable growth characteristics (e.g., fungal-like growth, colony colors, etc.) Note: not all of your plates may be in the countable plate range (they may have many more or many fewer colonies). You need only record the numbers for the countable plates.

Based on the results for each countable dilution, determine the total number of bacteria in the original sample. Then average these numbers to obtain a more significant result.

Dilution plated	Amount spread on plate	Growth characteristics	Number of CFUs/plate	Number of bacteria/ml in original sample (No. CFUs x Dilution factor x 10)
Tube 2 =10^{-4}	0.1 ml			
Tube 3 = 10^{-5}	0.1 ml			
Tube 4 = 10^{-6}	0.1 ml			
Average no. bacteria/ml in original sample				

If you also did this experiment using EMB plates, would you expect more colonies on the TSA or the EMB plates? Explain your answer.

_____

_____

What type of bacteria could you identify by using the EMB plates, and why would these bacteria be of concern for human health?

_____

_____

Class Data

For the results of the experiment to be more statistically significant, data from the entire class will be collected and written in the following table

	Number of bacteria/ml in original sample
Table 1	
Table 2	
Table 3	
Table 4	
Average	

C. Microbiology of Food

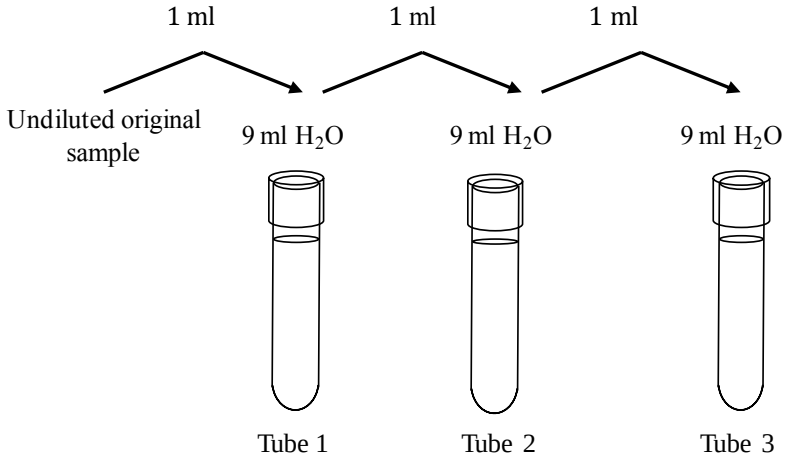
Observe the plates of all of the food materials. Rate the food material based on the relative number of bacterial colonies seen on each plate:

Least Bacteria Most bacteria

_____ < _____ < _____ < _____

Review Questions

1. You are testing ground beef to determine how many bacteria are present per gram of meat. You place 1 gram of meat in sterile water, mix vigorously, and then do serial dilutions as shown below. You inoculate a plate with 0.1 ml of the sample from the last tube. The next day you count 122 colonies. How many bacteria/ml were in the undiluted sample?



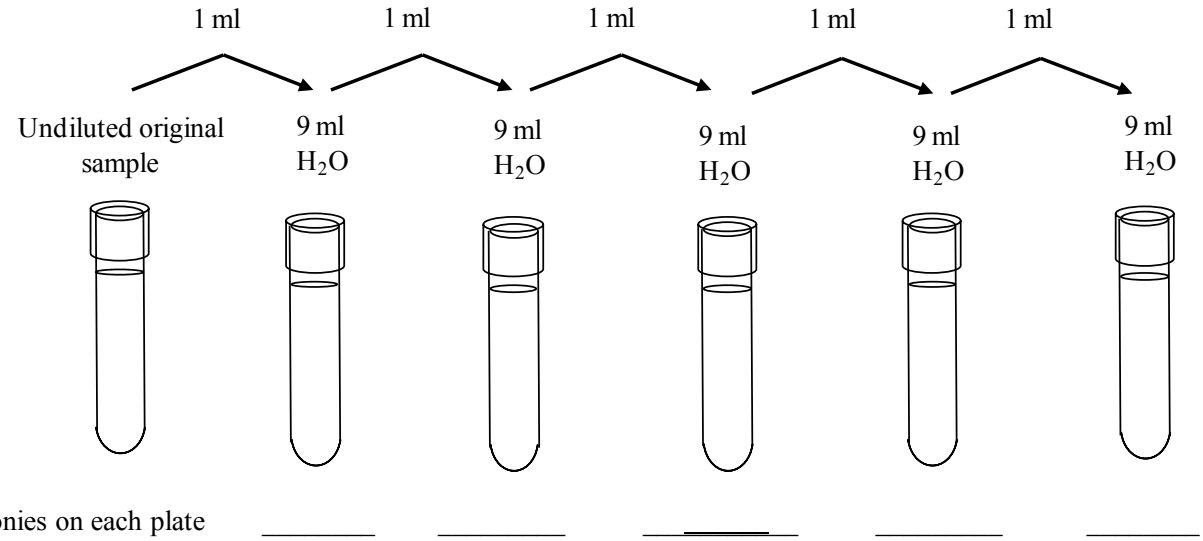
2. When doing serial dilutions, why is it necessary to plate more than one dilution?

3. If you observe *Escherichia coli* in a food sample, what could this indicate?

4. You left a carton of orange juice on your counter for 3 days. When you taste it, it is very bubbly (as though it was carbonated), and it tastes more bitter than usual. (Note: acids taste bitter). What do you think could have happened? (Note: you can't just say that bacteria grew—you must explain how the growth of the bacteria resulted in the changes in the orange juice.)

5. Based on what you now know about the presence of bacteria on chicken and beef, why is it a good idea to use separate chopping boards for meat and for vegetables?

6. You are testing unpasteurized milk for the presence of bacterial contamination. Starting from the undiluted milk, you do serial dilutions as shown below, and plate 1.0 ml of each dilution on agar. If the undiluted milk contains 5 x 10^6 bacteria/ml, how many colonies would you expect to see on each plate?



Number of colonies on each plate _____ _____ _____ _____ _____

7. Do you think standard plate counts are very accurate? Why or why not?

LAB 10: The Eukaryotes

Objectives

In this lab, you will:
1. Learn about the characteristics of fungi, protozoa and helminthes, including pathogenic species.
2. Observe prepared slides and agar plates with several types of fungi including yeasts and molds.
3. Observe prepared slides of several protozoan pathogens, and live protozoans in a lake water sample.
4. Observe prepared slides and preserved specimens of parasitic worms.
5. Prepare a collective forehead swab for microbial identification in Lab 12.

Key Terms: Fungi, heterotroph, saprotroph, mold, hyphae, mycelium, aerial (reproductive) hyphae, vegetative hyphae, thermal dimorphism, yeast, budding, opportunistic infections, Zygomycete, Ascomycete, Basidiomycete, ascospore, conidiospore, sporangiospore, zygospore, basidiospore, protozoa, vector, Amoebozoans, ciliates, flagellates, apicomplexans, cyst, trophozoite, helminthes, Platyhelminthes, cestodes, trematodes, nematodes, mode of transmission.

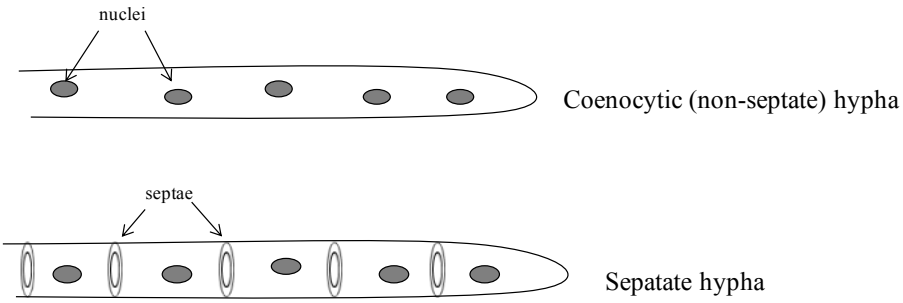
Introduction

So far our laboratory exercises have been primarily focused on prokaryotic organisms—those that lack a nucleus and other membrane-bound organelles. However, there are other types of eukaryotic microorganisms that exist in nature, some of which cause human disease. In this exercise we will examine representative types of eukaryotic microorganisms: fungi, protozoa, and parasitic worms (helminthes), and learn about the diseases that they cause.

The Fungi

Fungi are **heterotrophs** (organisms that require organic carbon). In nature they are important **saprotrophs**-organisms that decompose dead organic matter. Many fungi produce enzymes that decompose woody plant material—thus making them of critical importance for nutrient recycling in forests. Some types of fungi live in a symbiotic relationship with a photosynthetic algae or bacterium (lichens)—others live in symbiosis with plant roots (mycorrhizae). Fungi are also an important food source for humans and other organisms, and are used in food production as well. Fungi have a cell wall composed primarily of chitin (a polymer of glucose). Members of the Kingdom Fungi exist as multinucleate filaments (**molds**) or unicellular **yeasts**. Molds have long branching cellular structures called **hyphae** that grow continuously without complete division of cytoplasm. Several hyphae may form a visible mat called **mycelium**. Most hyphae grow along the substrate (**vegetative hyphae**) but those that produce spores extend upwards to disperse them (**aerial or reproductive hyphae**). Hyphae may or may not have septa that partially separate the cytoplasm (Figure 1).

Figure 1. Fungal Hyphae



Yeasts are unicellular fungi with an oval or spherical shape that replicate either by uneven or even cell division (uneven cell division is called **budding**). Some fungi exhibit **thermal dimorphism**: they grow as filamentous molds at room temperature, but grow as yeasts at 37ºC.

Among the human pathogens, many can cause **opportunistic infections** by taking advantage of a weakened or immunocompromised host. Other species of fungi produce toxins that can affect humans when consumed: some affect humans indirectly causing disease in crop plants and animals that humans rely on for food.

Fungi are often grouped based on the types of spores produced during sexual reproduction.

- **Zygomycetes** form **zygospores** in sexual reproduction and **sporangiospores** (encased in a sac known as a sporangium) in asexual reproduction.
    - *Rhizopus stolonifer* (black bread mold)
- **Ascomycetes** form **ascospores** (encased in a sac known as an ascus) in sexual reproduction and unprotected **conidiospores** in asexual reproduction.
    - *Penicillium notatum*: produces penicillin
    - *Aspergillis*—includes *A. fumigatus* (causes aspergillosis) *and A. flavus* (produces carcinogenic aflatoxins)
    - *Saccharomyces* (Baker's yeast): used in baking and alcohol production
    - *Candida albicans*: causes yeast infections and thrush
    - *Pneumocystis jiroveci*: leading cause of pneumonia among AIDS patients

- **Basidiomycetes** form **basidiospores** in sexual reproduction but do not have a well-defined asexual mode of reproduction. The mushroom is a macroscopic fruiting body of this type of fungus-basidiospores are formed on the underside of the mushroom cap (basidiocarp). Although mushrooms are clearly visible without a microscope, most of the living biomass of these fungi exists as microscopic hyphae.
    - *Agaricus* (edible)
    - *Amanita* (poisonous)
    - *Cryptococcus neoformans*: found in pigeon droppings; can cause severe infections in immunocompromised patients

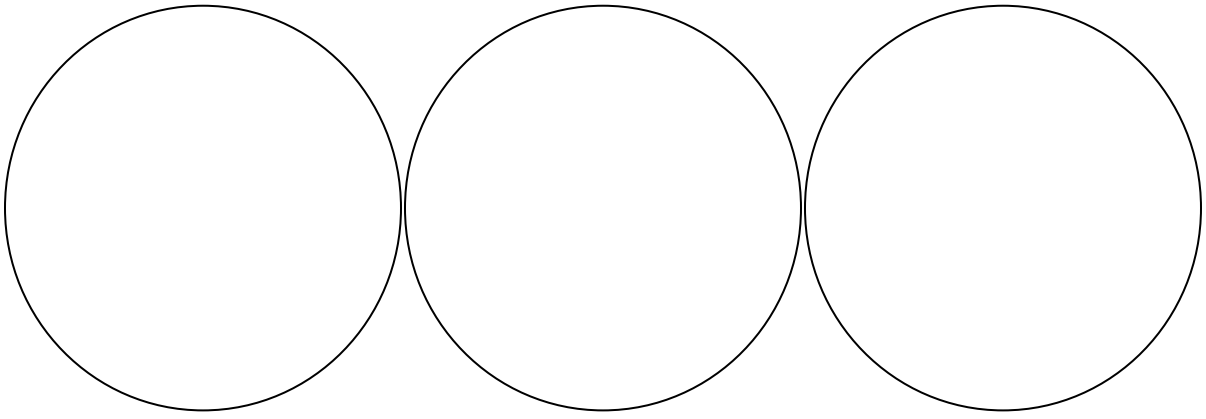
Procedures

Procedure A. The Fungi

Observe the following slides under the microscope. Use the space provided to draw diagrams of the slides to help you remember them.

1. *Rhizopus stolonifor*- zygospore
2. *Rhizopus stolonifor*- sporangiospore
3. *Penicillium notatum*- conidiospore
4. *Aspergillus niger*- conidiospore
5. *Candida albicans*
6. *Saccharomyces cereviseae* (budding yeast)
7. Assorted live molds (set up on demo scopes)

FUNGI



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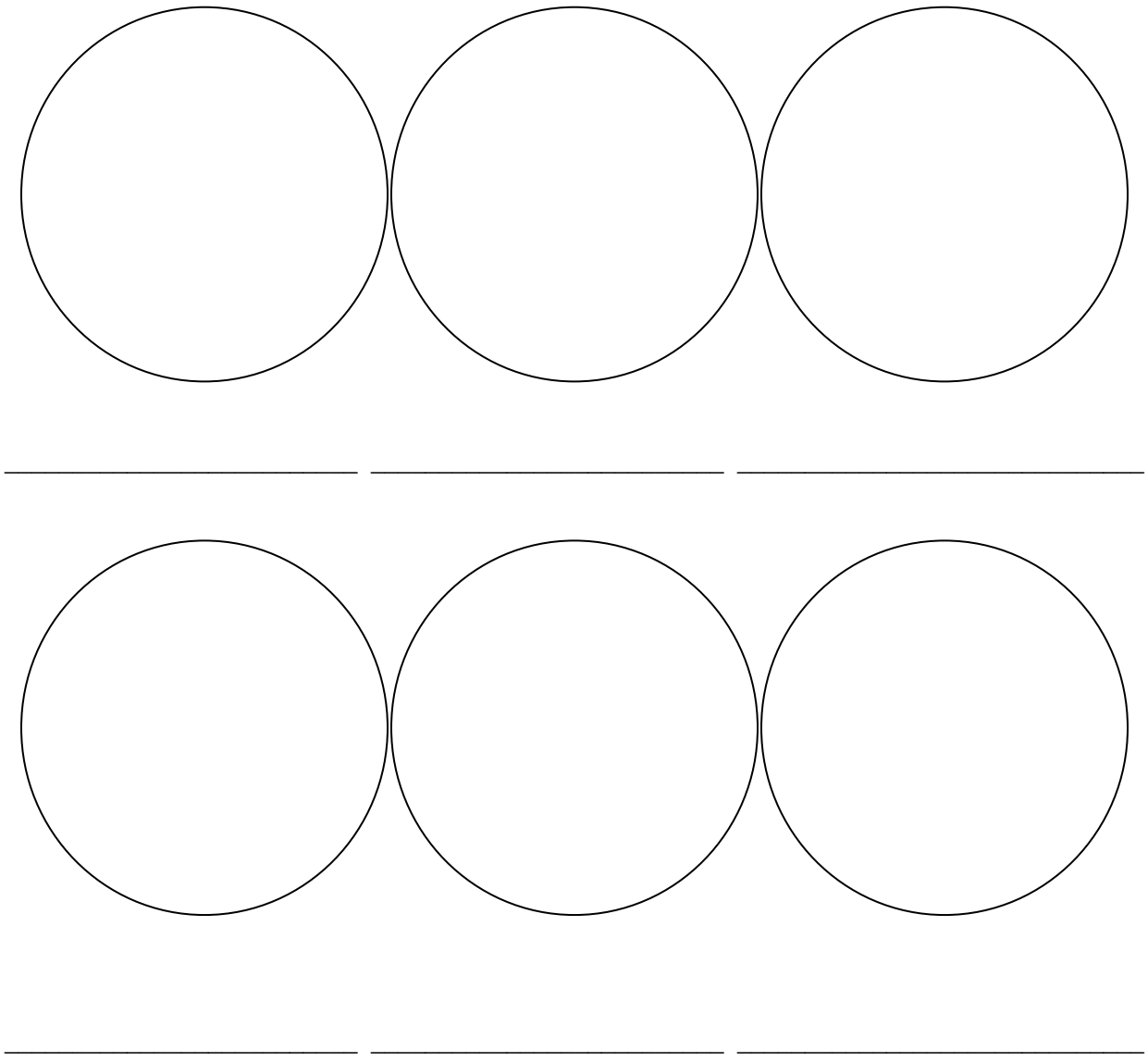


The Protozoa

Protozoa are a very diverse group of unicellular, heterotrophic eukaryotes that are members of the kingdom Protista (photosynthetic algae are also in this kingdom). They are found in all types of habitats, including soil, freshwater and saltwater. Some live in symbiosis with other organisms, others are free-living consumers, and a few are parasitic to humans. Protozoans do not have cell walls, but many are surrounded by a proteinaceous outer covering called a pellicle. Both sexual and asexual modes of reproduction occur in this group.

Protozoan pathogens vary in their **mode of transmission**, or method of gaining access to a new host. Some are transmitted by **vector** (an insect or arthropod that transmits a microbial pathogen), by ingestion of contaminated food or water, or even by sexual contact.

Many species form a dormant stage called a **cyst** that is resistant to adverse environmental conditions. This allows them to exist outside of a host cell for some time and is often the stage that is transmitted to a new host. The feeding (metabolically active) form of these organisms is known as a **trophozoite**.

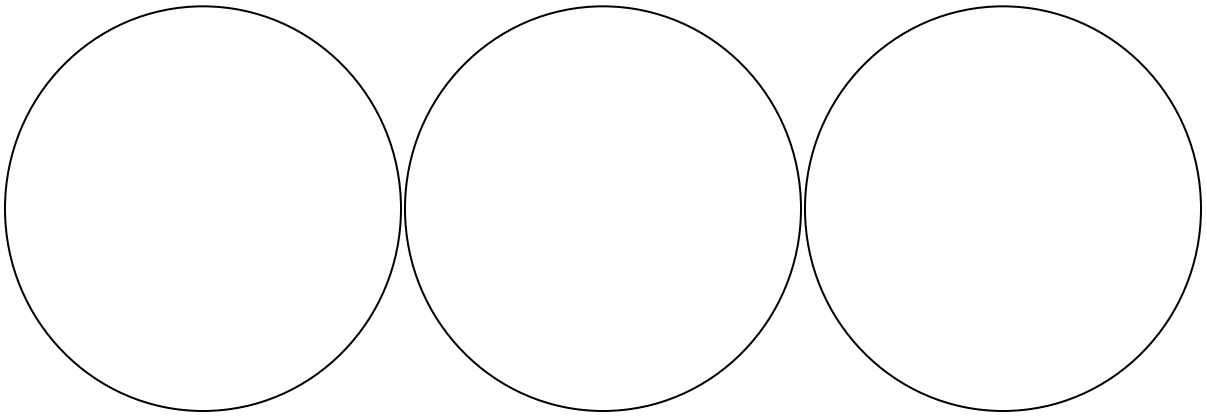
Protozoa are often grouped based on the type of structures they use for locomotion (motility).

- **Amoebozoans** use cytoplasmic projections called pseudopodia
  - ex: *Entamoeba histolytica*
- **Flagellates** use flagella
  - *Trypanosoma gambiense*
  - *Trypanosoma cruzi*
  - *Giardia lamblia (intestinalis)*
  - *Trichomonas vaginalis*
- **Ciliates** use cilia.
  - *Balantidium coli*
  - *Paramecium* spp (non-pathogenic)
- **Apicomplexans** have no means of locomotion in their mature form
  - *Plasmodium vivax*
  - *Toxoplasma gondii*

Procedure B. The Protozoa

Observe the slides listed above. Draw diagrams to help you remember the organisms. Then use the reference materials available in the lab to fill in the information below for each organism in Table 1 (your instructor may assign you to research a particular species or group).

PROTOZOA



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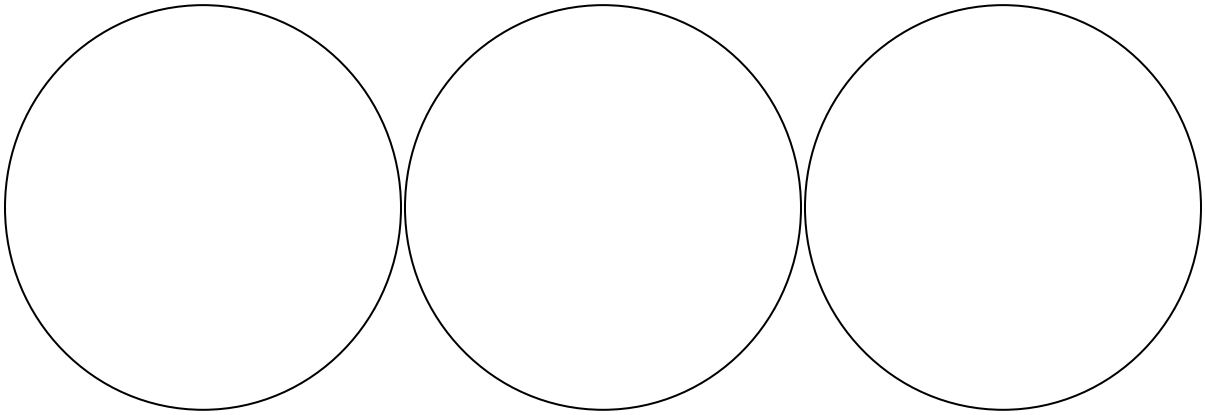

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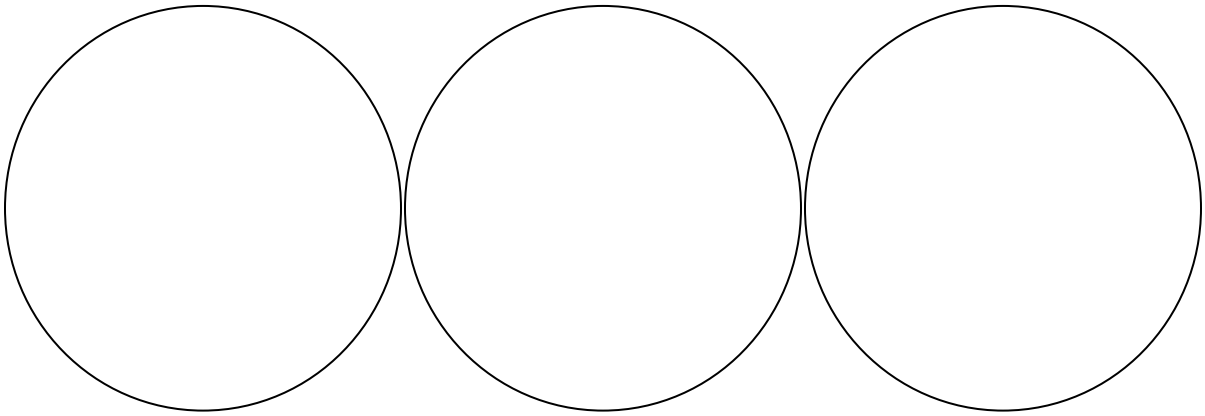


_____ _____ _____

Table 1: Protozoan pathogens

Organism (species name)	Taxonomic grouping (based on mode of locomotion)	Disease	How transmitted	Part of body affected	Other info

Organism	Taxonomic grouping (based on mode of locomotion)	Mode of locomotion	Disease	How transmitted	Part of body affected	Other info

The Helminthes (parasitic worms)

Helminthes are worms that live off of other living organisms. They are multicellular heterotrophs in the kingdom Animalia. There are two major phyla (a taxonomic grouping below the kingdom) that contain parasitic worms that are important to humans.

1. Phylum **Platyhelminthes**: these worms are commonly called flatworms because of their flat body structure. They do not have respiratory or circulatory structures, or a digestive tract, and thus rely on diffusion of nutrients and other chemicals. Two distinct types of Platyhelminthes exist: (a) **trematodes**, or flukes, and (b) **cestodes,** or tapeworms.

2. Phylum **Nematoda** (roundworms): these worms have more complex organ systems and are found in many types of habitats on Earth; a few species are parasitic to humans and other organisms.

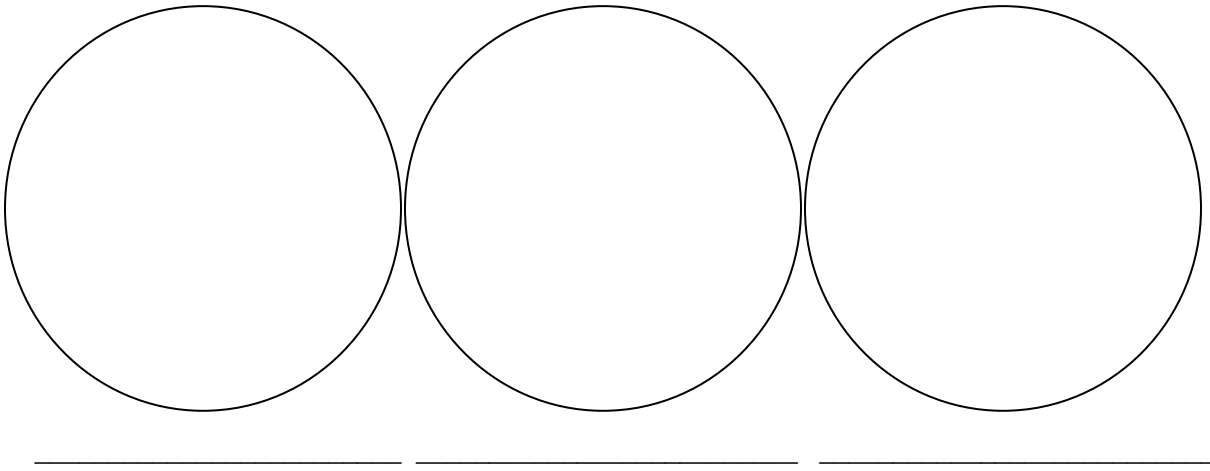
Helminthes vary in their mode of transmission and the part of the body infected. Some are transmitted by cysts, eggs, or larval stages of development. Many parasitic worms infect the digestive tract but may be disseminated to other body parts as well. The severity of a parasitic worm infection varies with type of worm, number of individual organisms present, and whether or not they spread to other organs.

Procedure C. The Helminthes

Observe the following slides and preserved specimens. Use the spaces provided to draw diagrams that will help you remember them. Then use the sources of information provided to fill in Table 2 (your instructor may assign you to research a particular species or group of species)

1. *Enterobius vermicularis*
2. *Necator americanus*
3. *Schistosoma mansoni*
4. *Fasciola hepatica*
5. *Trichinella spiralis*
6. *Wuchereria bancrofti*
7. *Taenia solium*
8. Preserved samples of tapeworms
9. Preserved samples of *Ascaris lumbricoides*

HELMINTHES



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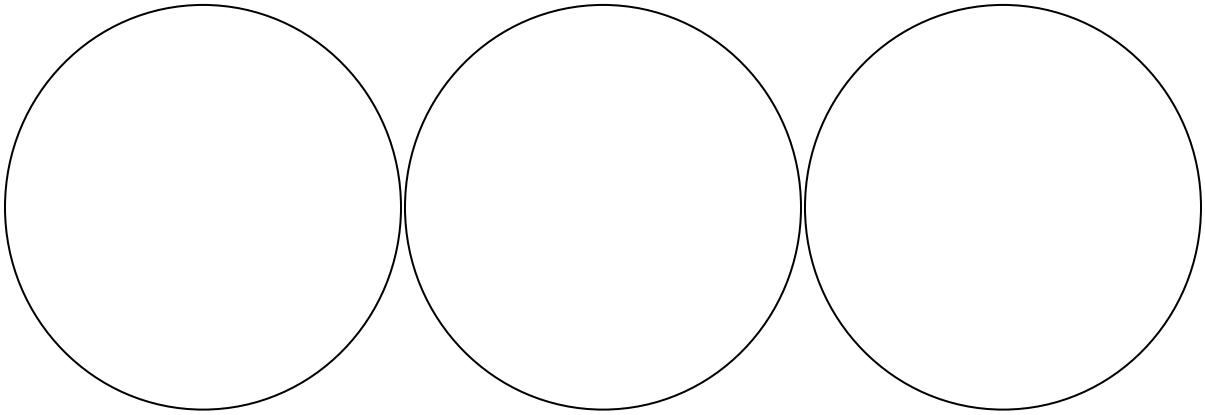

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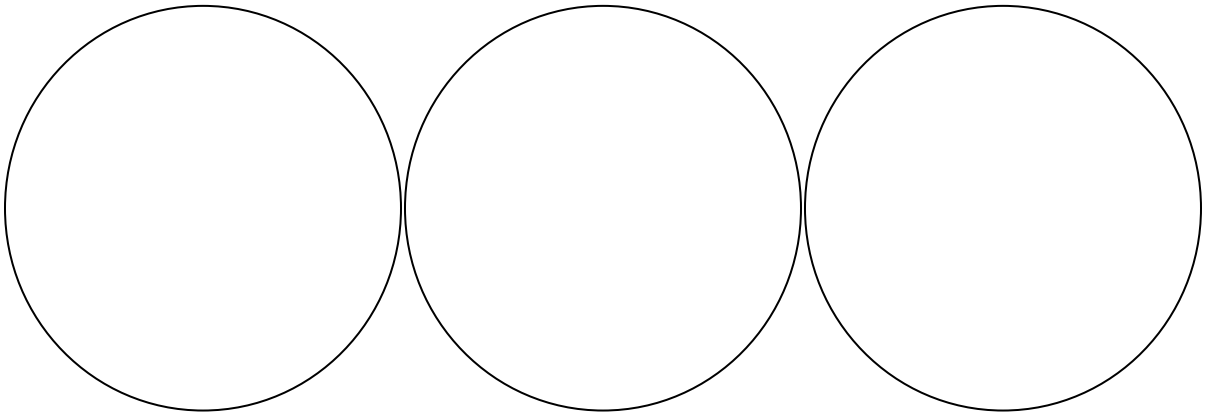


_____ _____ _____

Table 2: Helminthes

Species name	Common name	Taxonomic grouping(s)	How transmitted	Part of body affected	Other information

Species name	Common name	Taxonomic grouping(s)	How transmitted	Part of body affected	Other information

Procedure D. Forehead cultures

In this lab you will culture bacterial inhabitants of a human forehead, and in Lab 12 you will use the Biolog bacterial identification system to identify the isolate.

Per pair of students: 1 TSA plate

1. Each student will swab their forehead with a sterile cotton swab, and then swab the TSA plate. A composite culture will be used so a specific bacterial species cannot be linked to a particular student.
2. Plates will be incubated until the next lab period.

Results

Forehead Cultures

1. Examine the 2 forehead culture plates from your bench. Do you observe different colony types?

_____

2. Select a single colony (if possible, a well-isolated colony) for subculturing from one of the two plates inoculated at your bench. What are the characteristics of this specific colony (colors, elevation, margins, etc.)?

_____

_____

_____

3. Using the bacteria from your chosen colony, streak for single colonies on a second TSA plate. (If you need to review this procedure, see Lab 2.)

4. Incubate the plate until next week (Lab 12).

Why do you think it is important to streak this colony out for a second time before proceeding with the identification?

_____

_____

_____

Review Questions

1. Summarize the major differences between prokaryotes and eukaryotes.

2. What type of organism alternates between a trophozoite and a cyst form? What is the difference between a trophozoite and a cyst?

3. List some differences between fungi, protists and helminthes.

4. Why is it important to learn about parasitic infections that don't normally occur in the United States?

5. Would you expect an antibiotic like penicillin to be an effective treatment for a fungal or protist infection? Why or why not?

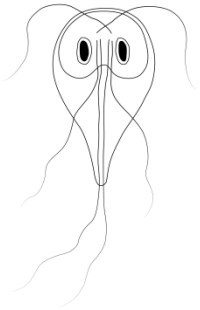
Identify the Organism!

Give the scientific name and identify the type of organism for each of the pictures below.

Scientific name: _____

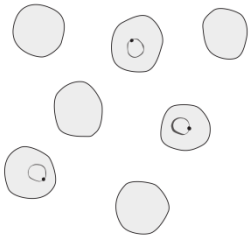
Type: protist helminth fungus

Hint: Is a common infection in children



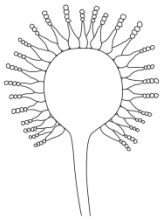
Scientific name: _____

Type: protist helminth fungus



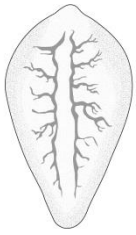
Scientific name: _____

Type: protist helminth fungus



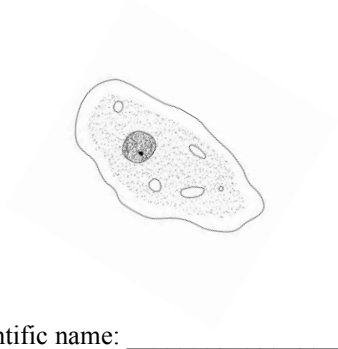
Scientific name: _____

Type: protist helminth fungus



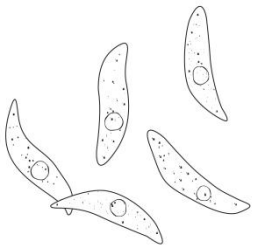
Scientific name: _____

Type: protist helminth fungus



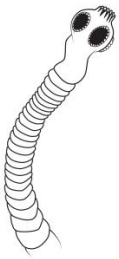
Scientific name: _____

Type: protist helminth fungus



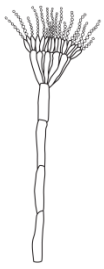
Scientific name: _____

Type: protist helminth fungus



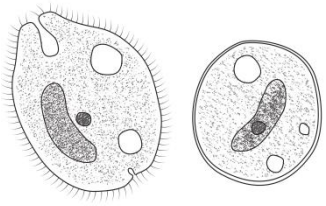
Scientific name: _____

Type: protist helminth fungus



Scientific name: _____

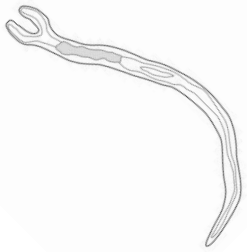
Type: protist helminth fungus



Form: _____ _____

Scientific name: _____

Type: protist helminth fungus

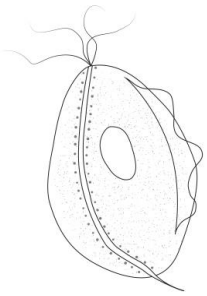


Scientific name: _____

Type: protist helminth fungus

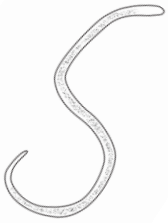
Scientific name: _____

Type: protist helminth fungus



Scientific name: _____

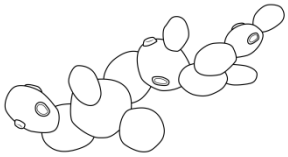
Type: protist helminth fungus



Scientific name: _____

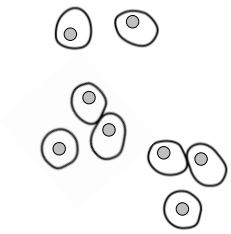
Type: protist helminth fungus

Hint: is found in tropical regions



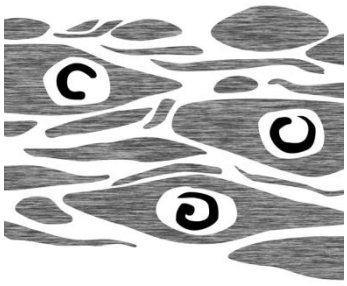
Scientific name: _____

Type: protist helminth fungus



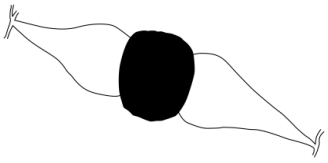
Scientific name: _____

Type: protist helminth fungus



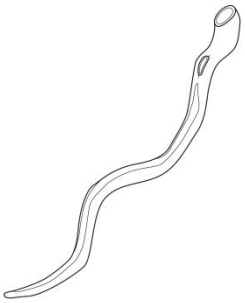
Scientific name: _____

Type: protist helminth fungus



Scientific name: _____

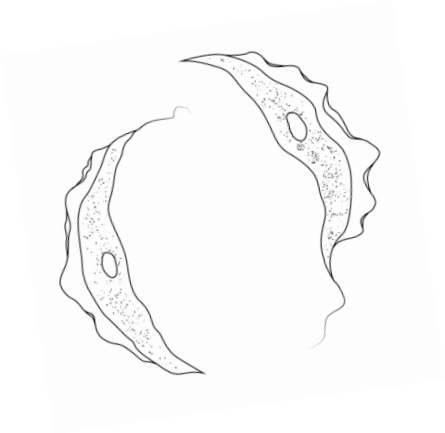
Type: protist helminth fungus



Scientific name: _____

Type: protist helminth fungus

Hint: Commonly known as a blood fluke



Scientific name (genus only): _____

Type: protist helminth fungus

Laboratory Exercise 11: Clinical Microbiology I; Anaerobic pathogens; Vectors of Infectious Disease

Objectives

1. Learn how to do some clinical tests (throat cultures and urine cultures) using selective and differential media.
2. Learn about some significant anaerobic pathogens, and about how anaerobic bacteria are grown.
3. Examine prepared slides of some vectors and the diseases that they transmit.

Key Terms: obligate anaerobes, GasPak system, nosocomial infection, biological vector, mechanical vector, bacteriuria, α, β and γ hemolysis

Introduction

Health care providers will often be expected to obtain samples from patients to diagnose a particular infectious disease. When doing this, it is important to use aseptic technique and proper precautions to protect the person handling the sample, avoid contamination of the surrounding area, and preserve the integrity of the sample. Following proper aseptic technique guarantees that what grows on the Petri dish came from the patient's sample and is not an environmental contaminant.

There are numerous clinical tests that can be used to identify specific types of infections—you will use two of these in this laboratory activity.

Throat Cultures: health care providers will often take a throat culture from a patient complaining of a sore throat. This is most commonly used to diagnose strep throat, which is caused by *Streptococcus pyogenes*. Although there are now "rapid-strep" tests that may be used, a more traditional way of identifying this organism in a throat culture is to use Blood agar. This media contains 5% sheep's blood, and allows for the distinction between types of hemolysis. Many bacterial species are **α- hemolytic**, which means they can partially break down red blood cells. Bacteria that are **β- hemolytic** can completely break down red blood cells; those that are γ-hemolytic do not break down red blood cells at all. *Streptococcus pyogenes* is a β-hemolytic organism—colonies of this species will completely lyse red blood cells, and so clear zones will be seen around the colonies on a blood agar plate (BAP). Other species, such as *Streptococcus pneumoniae*, are α- hemolytic, meaning that they can partially break down red blood cells—these colonies will have a greenish pigment around them on a BAP. Species that are non-hemolytic (**γ-hemolytic**) will grow on Blood agar, but no color change will be observed (e.g. *Enterococcus faecalis, Streptococcus salivarius*).

Note: For a review of hemolysis (including color images), see the Microbiology review site on Blackboard.
The diagnosis of strep throat is important because if left untreated, it may progress to scarlet fever and/or a more serious infection that can result in cardiac tissue damage. A positive strep test indicates the need for antibiotic therapy.
Streptococcus pyogenes infections are not confined to the respiratory tract. This organism can also cause skin infections such as erysipelas, cellulitis and impetigo. In rare cases, some strains of *S. pyogenes* can invade and multiply in the fascia, causing necrotizing fasciitis, resulting in rapid tissue destruction with mortality rates that can exceed 40%.

Urine cultures: urine cultures are taken to diagnose urinary tract infections (UTIs), a very common type of **nosocomial infection** (an infection acquired during a stay at a hospital or chronic care facility). Many types of bacteria can cause UTIs but *E. coli* is the most common culprit. Although urine within the body is generally microbe-free, some bacteria are picked up as urine leaves the body. Therefore UTIs are generally diagnosed by determining the amount of bacteria present—low numbers are not usually indicative of an infection unless the organism detected is a very serious pathogen that would not be part of the normal microbiota.
To minimize the amount of bacteria from the external genitalia that are present in a urine sample, patients are usually asked to wipe the area before urinating, and a "clean catch" is taken by letting some urine go before collecting—this flushes away the external normal microbiota. A urine sample will often appear cloudy if an infection is present. Selective and differential media, like EMB and BA, can be used to determine the types of bacteria that are present in a urine samples. *E. coli* can be identified on EMB by the green metallic sheen of the colonies. Generally, more than 100,000/ml of one type of organism reflects significant **bacteriuria** (bacteria in the urine), but lower numbers of certain pathogens may also indicate infection. Types of UTIs include urethritis (infection of the urethra), cystitis (bladder infection) and pyelonephritis (kidney infection).

Anaerobic pathogens: Many bacterial species are aerobic or facultative (can grow with or without oxygen). A few pathogenic species are **obligate anaerobes**, which means they cannot grow in the presence of free oxygen. These pathogens would not cause superficial or lung infections, but rather may be responsible for infections within the body in environments where free oxygen is scarce. Infections occur by way of puncture wounds, ingestion, or when a body part is cut off from its blood supply via injury or disease.

One genus of pathogenic anaerobes is *Clostridium*. Members of this genus are gram positive, bacillus-shaped endospore formers. Pathogenic members of this genus include *C. tetani*, *C. botulinum*, *C. difficile*, and *C. perfringens.*

Note: not all endospore formers are anaerobic—*Bacillus anthracis*, which causes anthrax, is an example of an aerobic pathogen that may cause infections in the lungs and on the skin.

Growing obligate anaerobes requires creating an environment that is free of oxygen. One way to do this is using the **GasPak system**. Agar plates are placed into a special jar and sealed tightly. A sachet is placed in the jar along with the plates—this sachet contains chemicals that bind up the free oxygen in the jar; an indicator strip is used to ensure that no free oxygen remains. In this lab you will be using the GasPak system to determine the oxygen requirements of two types of bacteria.

Vectors of pathogenic microbes

Disease-causing organisms can be transmitted in many different ways. Some are transmitted by contaminated food or water, some by respiratory droplet, and some by sexual contact. Some pathogens are also transmitted to humans by vectors including insects (flies, mosquitoes) and arthropods (ticks, mites).

Vectors themselves are not microscopic organisms, but transmit microbes in one of two ways:

1. **Mechanical vectors** carry microbes on their body (e.g., a fly landing on your food).
2. **Biological vectors** carry microbes within their body, and will transmit to a new host via bite or sting (e.g., mosquito bite transmitting malaria; tick bite transmitting Lyme disease).

Diseases caused by vector-borne pathogens are difficult to control or eradicate because it would be necessary to kill all of the individual organisms that carry them. Disease prevention for vector-borne pathogens include the use of insecticides and insect repellents (which pose their own health risks), avoiding contact with the vector by using mosquito netting, or avoiding being outside in areas when/where the vector is most active.

In this lab you will observe slides of a few common vectors, as well as the pathogens that some of them transmit.

Procedures

NOTE: materials that come in contact with our bodily fluids are treated as biohazardous waste and must be autoclaved before discarded. Place these items (swabs, tongue depressors, and empty urine cups into the orange biohazard bag in the front of the room. Wrappers may be placed in regular garbage unless they also come in contact with body fluids.

A. Throat cultures: **one blood agar plate/student**

1. Obtain a sterile tongue depressor and cotton swab.
2. Take a throat culture from your lab partner by swabbing the back of their throat in the region of the uvula (be careful not to touch the sides of the mouth or the tongue).
3. Use the cotton swab to inoculate the entire surface of a Blood agar plate.
4. Discard the swab and tongue depressor in the biohazard waste container provided.
5. Plates will be incubated at 37˚C for 48 hours and then refrigerated until the next lab period.

B. Urine cultures: **one blood agar (BA) plate and one Eosin Methylene Blue (EMB) plate/ student**

1. Divide your plates into 2 areas- one labeled "simulated" and one labeled "mine".
2. Obtain a sterile container and cap, and bring it with you to the bathroom to collect a urine sample (do a "clean-catch"). **Note**: you do not need a large amount of urine- just enough to moisten a cotton swab!
3. Bring your sample back to the lab, and use it to inoculate the side of the EMB and BA plates labeled "mine."
4. Discard the cotton swab in the biohazard waste container provided.
5. Take another trip to the bathroom to discard the remaining urine from the cup, and then discard the **EMPTY** urine cup in the biohazard waste container provided.
6. Use one of the four simulated urine samples at your table to inoculate the other side of the BA and EMB plates (you may use your inoculation loop for this).
7. Plates will be incubated at 37˚C for 48 hours and then refrigerated until the next lab period.

C. Anaerobic cultures

Per pair of students: 1 TSA plate

1. Divide your plate into two areas—one labeled BS (for *Bacillus subtilis*) and the other CS (for *Clostridium sporogenes*).
2. Inoculate your plate with these two bacteria (use inoculation loop).
3. Your instructor will divide the plates from your class—half will be incubated aerobically, the other half will be placed into an anaerobic jar. Your instructor will also demonstrate the proper use of the anaerobic jar.
4. Plates will be incubated until the next lab period.

E. Examination of prepared slides of aerobic and anaerobic pathogens

When you have finished setting up your cultures, examine the following prepared slides under the microscope. Sketch what you see in the spaces provided, and try to look for distinguishing characteristics to help you remember what these organisms look like.

Note: not all slides have been gram stained, and you should not assume anything from the color of the organism unless you know how the slide was stained (Remember: *E. coli* would be purple if simple stained with crystal violet!)

Slides to view:

1. Streptococcus pyogenes

2. Streptococcus pneumoniae

3. Clostridium perfringens

4. Clostridium tetani

F. Vectors of infectious disease: Demonstration of a mechanical vector-the sowbug

Sowbugs (also called pillbugs) belong to Phylum Arthropoda and Class Crustacea (and are therefore related to crabs, lobsters, etc.) There are 4000 described species of pillbugs. They live humid areas in leaf litter, in rock piles or at the base of buildings. They primarily act as decomposers. THEY DO NOT BITE AND THEY DO NOT CAUSE DISEASES. We are using them today as an example of a mechanical vector.

One TSA plate/table

TSA Plate Number: _____

1. Number the 4 TSA plates (1/table) 1 – 4. Bring all of the TSA plates to the front of the room at the same time.

2. Obtain a sowbug from the container at the instructor's table. (Please treat them gently—they will be released into the wild when we are done with them.)

2. Allow it to walk across the surface of TSA # 1 plate for 2 minutes.

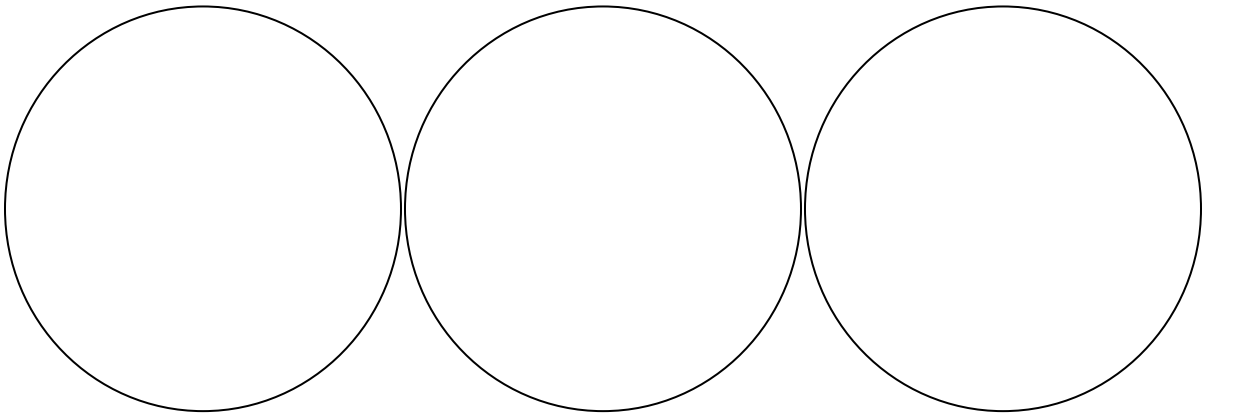
3. Pass the sowbug on to TSA plate #2, and repeat the above process. Continue until the sowbug has walked across each of the four plates.

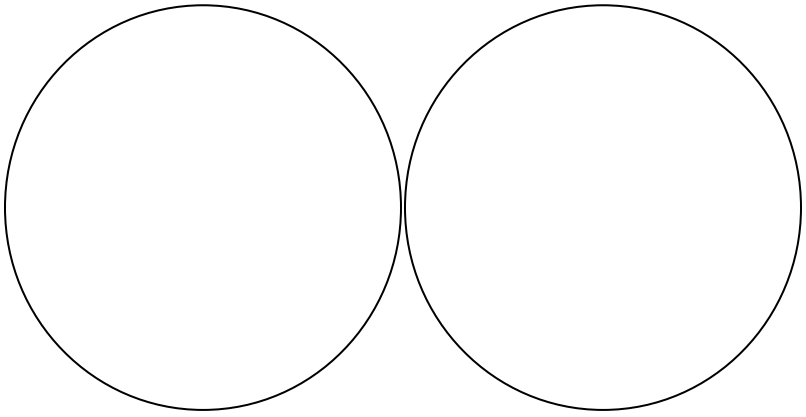
4. Incubate plates until the next lab period.

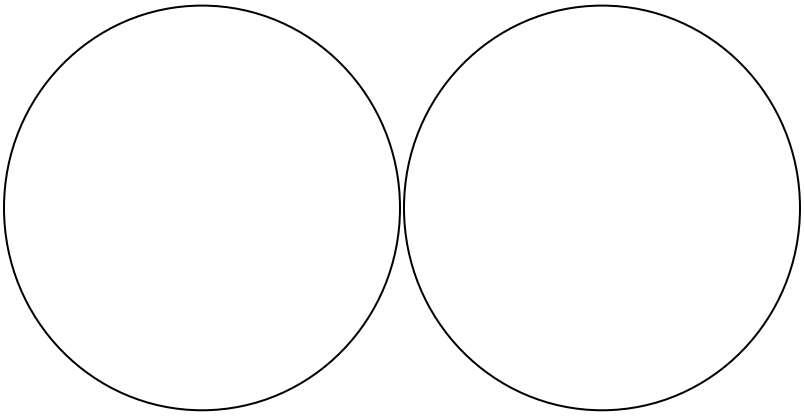
G. Vectors and Pathogens

The following slides have been set up on demonstration scopes. Where possible, the vector slide is set up next to the slide of the pathogen that it transmits (in bold). Observe these slides and sketch the organisms in the spaces provided. Then fill out the table included in the results section.

1. ***Xenopsylla cheopis*** (rat flea): transmits 2. ***Yersinia pestis*** (bubonic plague)
3. ***Ixodes scapularis*** (tick): transmits 4. ***Borrelia burgdorferi*** (Lyme disease)
5. ***Anopheles gambiae*** (mosquito): transmits 6. ***Plasmodium vivax*** (malaria)
7. ***Pediculus corporis*** (body louse): transmits 8. ***Borrelia recurrentis*** (relapsing fever)





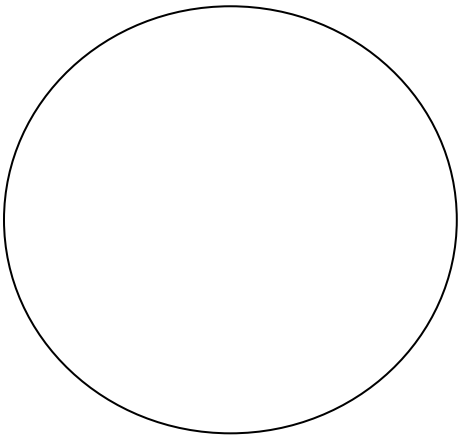


_____ _____

Results

A. Throat cultures

1. Examine your blood agar plates and sketch your plate in the space provided, noting any specific characteristics of the colonies. In particular you should note the type of hemolysis you observe.



Did you observe colonies that showed α, β, and γ hemolysis? _____

Note: your instructor has demo plates of all types of hemolysis—be sure to view them if you do not see all types on your own plate.

B. Urine culture

1. Examine your blood agar plate, and record the type of hemolysis observed.

What type(s) of hemolysis were observed on your BA plate?

Your sample: _____

Simulated sample: _____

2. Examine your EMB plate, and note the color and appearance of the colonies you observe from your own urine sample as well as from the simulated sample.

Describe the growth observed on the EMB plate for:

Your sample:

_____

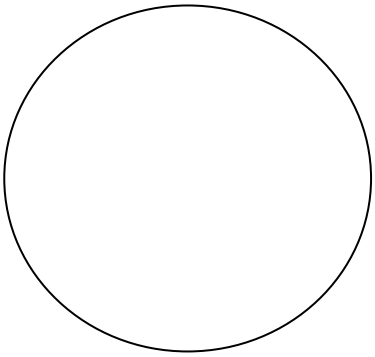
_____

Simulated sample:

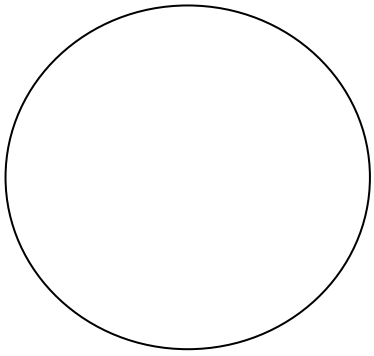
_____

_____

Sketch both plates (BA and EMB) in the space provided below.



Blood Agar



EMB agar

Based on the appearance of the growth you observed, what can you conclude about the bacteria in your samples?

_____

_____

_____

D. Anaerobic cultures

Your TSA plates were either incubated aerobically (on the tray) or anaerobically (within the anaerobic jar). Note: before opening the jar, observe the indicator strip that was added when the jar was set up. Recall that the methylene blue strip appeared blue when it was first added to the jar. If anaerobic conditions were achieved within the jar, the indicator should have turned white.

What color is the indicator strip? _____

Based on this, can you say proper conditions were achieved within the jar?

_____

For plates incubated aerobically: which bacteria shows growth on these plates? _____

For plates incubated anaerobically: which bacteria shows growth on these plates? _____

Based on your results, what can you conclude about these two organisms?

_____

_____

Vector experiment: Describe the type/amount of growth you observe on the plates on which the sowbug walked.

_____

_____

_____

Did the amount of microbes decrease from the first plate to the 4th?

_____

_____

Slides:

Fill in the table on the next page. Use the reference materials available in the lab to fill in the information for each organism (your instructor may assign you to research a particular species or group).

Vector name	Common name of vector	Name of pathogen transmitted by vector	Characteristics of pathogen (type of organism, morphology)	Name of disease	Body part affected	Additional information

Review Questions

1. What additional information can you get by using selective and differential media that you cannot get by using a general media (like TSA)?

2. Explain how the type of hemolysis can be used to tell apart two species that would look the same under the microscope.

3. You set up an anaerobic jar and after incubation the indicator strip is blue. What can you conclude from this? Are the organisms growing on your plate anaerobes? Explain.

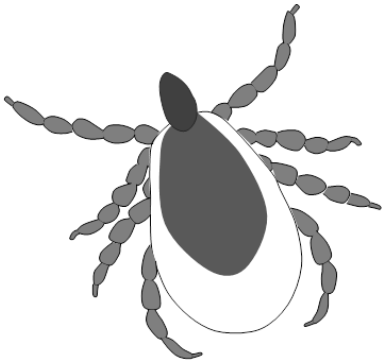
4. Why do you think *E. coli* and other enteric organisms are common causes of UTIs?

5. Explain why it is important to follow proper precautions and use aseptic technique when working with clinical samples.

6. Explain why vector-borne pathogens are difficult to control.

7. Why do you think that samples from urine should be tested within 2 hours of collection, or if not possible, that the sample should be refrigerated?

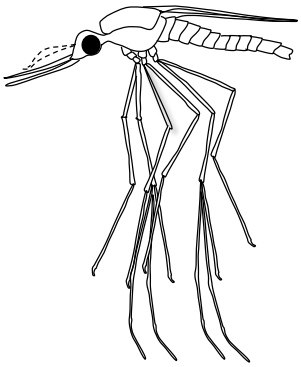
A Review of the Vectors



This vector is: _____

It transmits _____(organism)

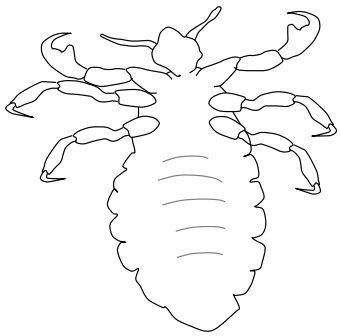
that causes _____(disease)



This vector is: _____

It transmits _____(organism)

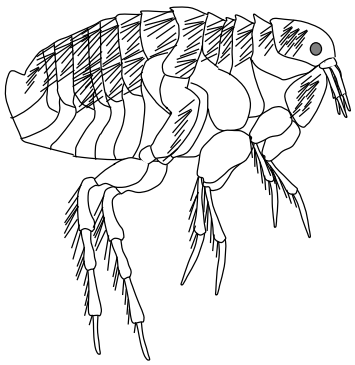
that causes _____(disease)



This vector is: _____

It transmits _____(organism)

that causes _____(disease)



This vector is: _____

It transmits _____(organism)

that causes _____(disease)

Lab 12: Clinical Microbiology Part II- Immunology and the Biolog System

Objectives

1. Learn about the principles behind the ELISA test, and perform an experiment that simulates an ELISA test for infectious mononucleosis
2. Learn another way bacteria can be identified using the Biolog microplate system (using the forehead isolate subcultured in Lab 11)

Key Terms: **Enzyme-linked immunosorbent assay (ELISA), antigen, antibody, colorimetric detection, secondary antibody, Epstein-Barr virus, infectious mononucleosis, Biolog, turbidimeter, tetrazolium, transmittance**

Introduction

The rapid and accurate diagnosis of infectious disease is often crucial to determining patient treatment, supportive care, and precautions for the health care worker. However, some infectious diseases are more easily diagnosed than others. For example, although many types of bacteria may be easily identified by appearance on differential media, gram staining, metabolic testing, etc.… these identification methods may be time-consuming and are not always practical. In addition, not all pathogens (e.g., viruses) can be cultured in the laboratory, and so other types of diagnostic tests are necessary.

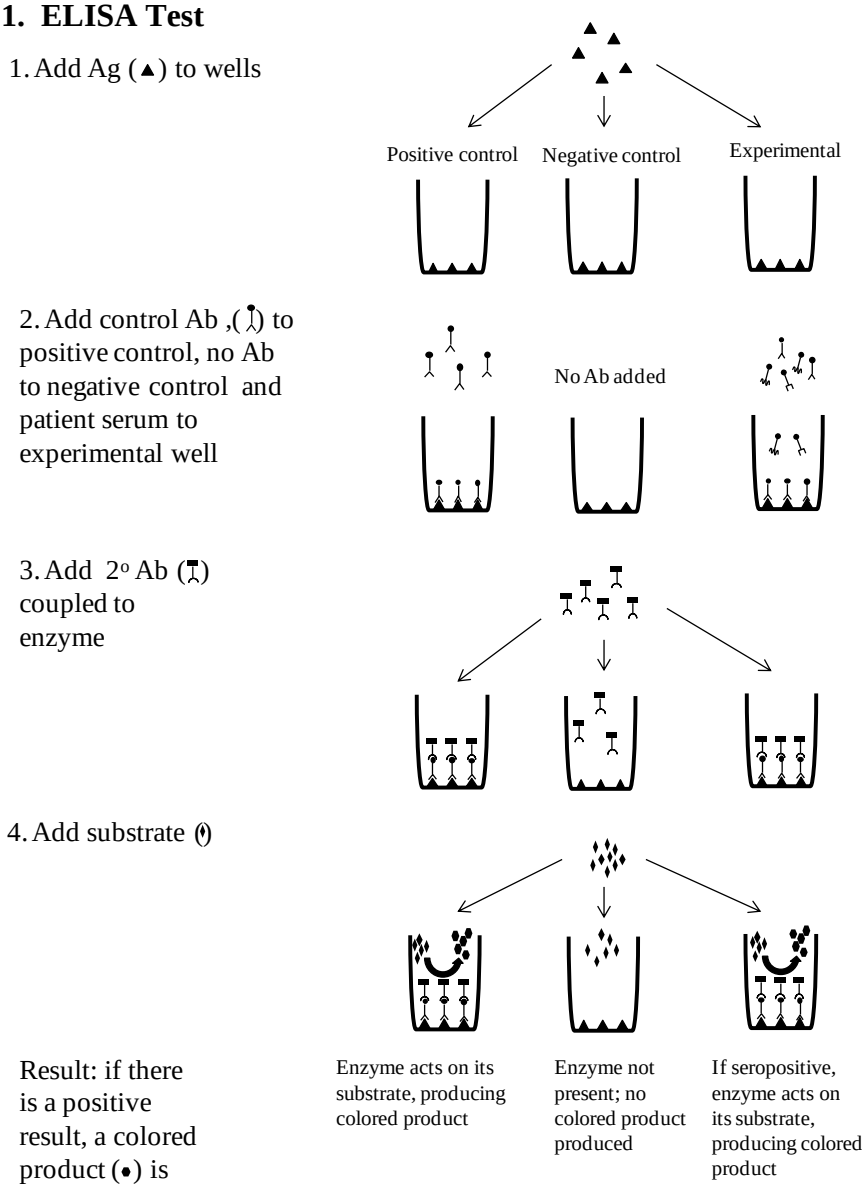
Our immune systems respond to the presence of **antigens** (a foreign substance that stimulates an immune response) by producing **antibodies** (globular proteins produced and secreted by B lymphocytes) that bind specifically to them. Many diagnostic tests take advantage of the specificity of antigen-antibody binding to detect exposure to a particular pathogen. For example, some diagnostic tests detect antibodies in a patient's serum- their presence indicates exposure to a specific pathogen, as these antibodies will only be present if the patient has been exposed to that pathogen. Other diagnostic tests may look for specific antigens in serum (or in a cultured clinical sample) by reacting them with known antibodies in clinical laboratory tests.

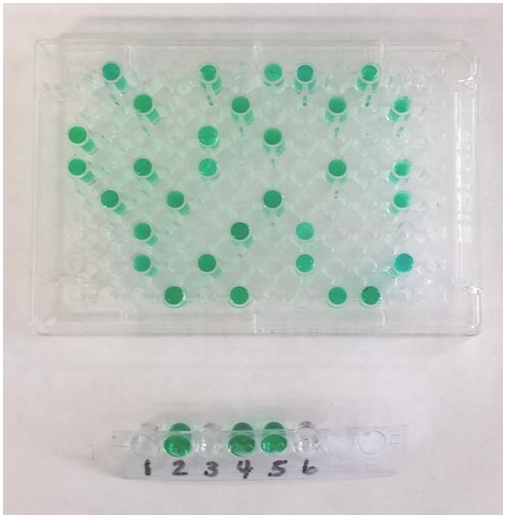
In this lab, you will learn how to use a standard immunological test—an ELISA that detects serum antibodies to diagnose infectious disease. In addition, you will use the Biolog microbial identification system to identify an isolate from human skin.

ELISA TEST: **The Enzyme-linked Immunosorbent Assay (ELISA)** is used to detect several types of pathogens and is particularly useful for the diagnosis of viral infections. Although there are many variations of

this test, it is most commonly used to detect serum antibodies to a particular viral pathogen. ELISAs are used to diagnose exposure to human immunodeficiency virus (HIV), **Epstein-Barr virus** (EBV) and several other pathogens. The test typically uses 96-well plates that have viral antigens bound to the wells. When serum is added, only antibodies that are specific to the antigen in the well will bind to it—all other serum components (including other types of antibodies) will be removed during washing steps. A **secondary antibody** (one that recognizes the first antibody) is then added. This secondary antibody is conjugated (attached) to an enzyme (horseradish peroxide is one commonly used enzyme). After washing away unbound secondary antibodies, the enzyme's substrate is added, and produces a color reaction in the well (this is known as **colorimetric detection**). A color change is an indication of serum antibody, which means that the patient has been exposed to that particular pathogen. Further testing may be done to confirm a positive ELISA test.

Figure 1. ELISA Test





A typical ELISA plate

What your ELISA experiment will look like

In this lab, you will be using a kit that simulates the ELISA test using non-pathogenic materials. Our simulation will show how ELISAs are used to detect **infectious mononucleosis**, commonly called "kissing disease" because it is transmitted by saliva. Although young children are the largest population of people affected by this viral pathogen, they generally exhibit very few symptoms. Adolescents and young adults are more likely to have symptoms of infection, including fatigue, sore throat, fever, swollen lymph nodes and, in severe cases, an enlarged spleen. The disease is caused by the **Epstein-Barr virus** (EBV) that is also associated with Chronic Fatigue Syndrome, and in some geographic locations, with certain types of cancers (ex: Burkitt Lymphoma)

B. Biolog System

The **Biolog** system is designed to identify bacteria based on their metabolic properties. This identification system uses a 96-well plate—each well contains: (a) a redox dye (**tetrazolium**), and (b) a substrate or chemical sensitivity test. For example, some wells contain sugars or amino acids; others test the bacteria's ability to survive in the presence of acid, high salt concentrations, and antibiotics. There are also positive and negative control wells to ensure that the test is working correctly.

To perform the test, a suspension of bacteria is added to a 96-well microplate and incubated (usually at 33˚C). If the bacteria are able to use the substrate in a particular well, a tetrazolium dye is reduced (has electrons transferred to it), and as a result the dye in that well will turn purple. Individual species will give unique

patterns of substrate utilization. These patterns can be compared to a database of known species for identification. In this experiment you will: (1) inoculate your unknown (forehead) bacterial isolate onto a Biolog microplate and next week you will (2) record the results for your unknown, and (3) use the software and database to identify your unknown bacterial species based on its pattern of substrate utilization (Steps 2 and 3 will be done in Lab 13).

Procedures

A. ELISA

Per Table: 1 "strip" of test wells; viral antigen solution (EBV), positive and negative control solutions, anti- IgG peroxidase conjugate (second antibody solution), Phosphate buffered saline (PBS, for washing steps), substrate solution, 4 "unknown" donor serum samples.

Note: follow all directions carefully. Be particularly careful to avoid cross-contamination of the solutions.

1. Label the first 6 wells of your strip 1-6.
2. Add 50 µl of EBV (viral antigens) to each of the 6 wells using the P200 pipettor.
3. Incubate for 5 minutes at room temperature.
4. Remove the liquid using a transfer pipet. Discard the liquid in the beaker labeled "liquid waste."
5. Using a transfer pipet, add PBS to each of the 6 wells until almost full (do not allow them to spill over). Be careful not to touch the transfer pipet to the well.
6. Remove the liquid from the well as demonstrated by your instructor (Important: do **NOT** use the PBS transfer pipet to remove the liquid- this will result in contamination of the PBS with antigen.
7. Add 50 µl of the appropriate test reagent to each well as outlined in the table below. Use a new pipet tip for each solution!

Well #	**Test Reagent**	**Description**
1	(-) PBS	Negative control
2	(+)	Positive control
3	DS 1	Donor serum 1
4	DS 2	Donor serum 2
5	DS 3	Donor serum 3
6	DS 4	Donor serum 4

8. Incubate at 37˚C for 15 minutes.
9. Remove all the liquid from each well with a new pipet tip for each.
10. Wash each well once with PBS. (You can use the same tip for all of the wells provided you don't contaminate the tip.)
11. Add 50 µl of anti-IgG peroxidase conjugate (secondary antibody) to each of the 6 wells (see above).
12. Incubate at 37˚C for 15 minutes.
13. Remove the liquid from each well.
14. Wash each well twice with PBS.
15. Add 50 µl of the substrate to each of the 6 wells.
16. Incubate at 37˚C for 5 minutes.
17. Remove the test strip from the incubator and examine each well. Development of a color reaction is an indication of a positive result.

Note: wells may be incubated for a longer period of time if the color reaction does not develop after 5 minutes.

B. Biolog

Before you begin: make note of the appearance of growth on your agar plate. Record appearance of growth as you have before. More information about the contents of the wells and how the test is set up can be found by watching the videos found at this link:

http://www.biolog.com/videos.php

Per table: one Biolog microplate, 1 swab for picking up bacterial growth, 1 tube of inoculation fluid, 1 reservoir, 1 multichannel pipettor (octapet), pipet tips, and an empty pipet tip box

1. Unwrap your Biolog microplate and label it on the side of the plate with your table number. Place the plate over a white sheet of paper to make the wells more visible.
2. Use a swab to pick up a **small amount** of bacterial growth from your agar plate (your instructor will guide you as to how much to pick up).
   NOTE: it's very important not to add too many cells to the tube. It is easier to correct the mistake of not adding enough than it is to correct adding too much, so go slowly!
3. Transfer the cells you have picked up into a tube of inoculating fluid to make a suspension.

4. Mix thoroughly and break up any clumps (vortex gently if needed).
5. Use the **turbidimeter** (a machine that estimates the concentration of cells in a suspension based on its turbidity) to determine the % **transmittance** (how much light passes through) for your sample. Ideally this should be 90-98%. Your instructor will assist you with this part.
6. When your sample is at the proper % transmittance, add the contents of the entire tube into a clean reservoir.
7. Secure 8 pipet tips onto the octapet. Make sure all the tips are firmly attached.
8. Use the pipet tips to transfer 100 µl to each of the 98 wells (8 at a time)

   NOTE: it is very important to do this step carefully.
   a) Be sure you are pipetting the correct amount of fluid. (If you pipet too much you will run out of your suspension before inoculating all wells).
   b) After drawing up the suspension, check the pipet tips—they should all have the same amount and should be free of bubbles. If they do not, release the suspension back into the reservoir and start again.
   c) Always start on the left side of the plate (so A1, the negative control, gets inoculated first).
   d) Make sure your tips (all 8) are directly over the wells before beginning to release the bacterial suspension from the pipet tip.
   e) **DO NOT** let the tips touch the bottom of the wells. If you do this you will be transferring small amounts of substrate/tetrazolium dye, which could lead to false positive results.
   f) After each 2-3 transfers, use the empty pipet tip holder to press the tips back into place (they may become loose after a few uses).
9. Replace the lid on the plate, and incubate your sample at 33˚C.
10. Your plate will be incubated until color development is complete (3-36 hours) and then refrigerated until you can read the results.
11. Discard all contaminated materials (inoculation fluid tube, inoculator, reservoir and pipet tips as instructed. DO NOT put any of these items in the regular garbage.

Results

A. ELISA test

Record the appearance of each of the wells here.

Well	Contents	Color appearance	Interpretation
1			
2			
3			
4			
5			
6			

B. Biolog

Record your Biolog results below.

Describe the colony that you picked for Biolog (color, etc.): _____

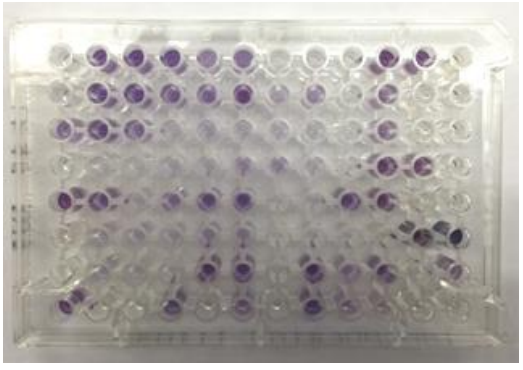
_____

_____

Use the sheet on the next page to record your results. In each box, write "+" if the well appears positive (purple color), "-" if there is no color change, and "+/-" if the result is borderline (light purple).

Your instructor will show you how to enter your results into the database to achieve an identification.

An example of what a Biolog plate looks like is shown below.



Identification: _____

% match: _____

A1 Negative Control	A2 Dextrin	A3 D-Maltose	A4 D-Trehalose	A5 D-Cellobiose	A6 Gentiobiose	A7 Sucrose	A8 D-Turanose	A9 Stachyose	A10 Positive Control	A11 pH 6	A12 pH 5
B1 D-Raffinose	B2 α-D-Lactose	B3 D-Melibiose	B4 β-Methyl-D-Glucoside	B5 D-Salicin	B6 N-Acetyl-D-Glucosamine	B7 N-Acetyl-β-D-Mannosamine	B8 N-Acetyl-D-Galactosamine	B9 N-Acetyl Neuraminic Acid	B10 1% NaCl	B11 4% NaCl	B12 8% NaCl
C1 α-D-Glucose	C2 D-Mannose	C3 D-Fructose	C4 D-Galactose	C5 3-Methyl Glucose	C6 D-Fucose	C7 L-Fucose	C8 L-Rhamnose	C9 Inosine	C10 1% Sodium Lactate	C11 Fusidic Acid	C12 D-Serine
D1 D-Sorbitol	D2 D-Mannitol	D3 D-Arabitol	D4 myo-Inositol	D5 Glycerol	D6 D-Glucose- 6-PO4	D7 D-Fructose- 6-PO4	D8 D-Aspartic Acid	D9 D-Serine	D10 Troleandomycin	D11 Rifamycin SV	D12 Minocycline
E1 Gelatin	E2 Glycyl-L-Proline	E3 L-Alanine	E4 L-Arginine	E5 L-Aspartic Acid	E6 L-Glutamic Acid	E7 L-Histidine	E8 L-Pyroglutamic Acid	E9 L-Serine	E10 Lincomycin	E11 Guanidine HCl	E12 Niaproof 4
F1 Pectin Acid	F2 D-Galacturonic Acid	F3 L-Galactonic Acid Lactone	F4 D-Gluconic Acid	F5 D-Glucuronic Acid	F6 Glucuronamide	F7 Mucic Acid	F8 Quinic Acid	F9 D-Saccharic Acid	F10 Vancomycin	F11 Tetrazolium Violet	F12 Tetrazolium Blue
G1 p-Hydroxy Phenylacetic Acid	G2 Methyl Pyruvate	G3 D-Lactic Acid Methyl Ester	G4 L-Lactic Acid	G5 Citric Acid	G6 α-Keto-Glutaric Acid	G7 D-Malic Acid	G8 L-Malic Acid	G9 Bromo-Succinic Acid	G10 Nalidixic Acid	G11 Lithium Chloride	G12 Potassium Tellurite
H1 Tween 40	H2 γ-Amino-Butryric Acid	H3 α-HydroxyButyric Acid	H4 β-Hydroxy-D,LButyric Acid	H5 α-Keto-Butyric Acid	H6 Acetoacetic Acid	H7 Propionic Acid	H8 Acetic Acid	H9 Formic Acid	H10 Aztreonam	H11 Sodium Butyrate	H12 Sodium Bromate

Review Questions

1. Why are the washing steps necessary in the ELISA test?

2. Why are positive and negative controls always required for immunological tests? (be specific)

3. What is the purpose of the enzyme conjugated to the secondary antibody in the ELISA test?

4. Why is it important to include a negative control well on the Biolog plate?

5. What does it mean if an environmental sample does not match 100% to a species in the database?

6. What possible error do you think could occur by inoculating too much bacteria into the Biolog wells?

Lab 13: Putting it all Together—Case Studies in Microbiology

In this lab, a series of stations will be set up around the room, each of which will present a microbiology case study. Some background information will be given to you, along with organisms on slides, growth media, etc.

This is your opportunity to put together everything that you have learned about microbiology this semester. You will be expected to integrate the various pieces of information that are provided to answer some questions about the situation and come up with a diagnosis.

The case studies will not be graded, and you will have an opportunity to work together to solve these problems.

All of these activities will be good practice for your lab practical—try to enjoy the process!

Appendix I: Information about Lab Practical Exams

During the course of the semester, there will be two lab practical exams: the Midterm Practical and the Final Practical. The midterm practical will occur in several steps beginning in week 5 and extending through week 10. The final practical will occur on the last day of class.
The practical exams are each worth 20% of your lab grade. Each practical exam has a different format.

The Midterm Practical is an open book exam in which you will use Gram staining and metabolic tests to determine the identity of two bacterial unknowns, and write a lab report based on your results. A more complete description of what is required for the midterm and lab report can be found below.

The Final Practical is a cumulative exam that is **not open book**. Twenty five stations will be set up around the lab, and you will be expected to answer questions about the material at these stations. You may be asked to identify microorganisms, analyze the results of a metabolic test or interpret the results of an experiment. Since the exam is cumulative, it can cover material from the first lab through the last lab of the class. The Microbiology Review site on Blackboard contains material that will help you review the material that you have covered throughout the semester.

Midterm Practical

This first lab practical involves the identification of two unknown bacteria using both Gram staining and metabolic assays. There are 3 parts to this lab practical—Part I: Gram staining (Lab 5); Part II: Preparation of dichotomous key (Lab 6) and metabolic testing (Lab 7); Part III: a written lab report (due on Lab 10). Each part of the lab practical is graded separately (see below). This first lab practical is an open book exam. You are being tested on your ability to correctly perform procedures and interpret results. Although you can use your books and your notes, you **cannot** seek the advice or assistance of other students or the instructor!

Schedule for Midterm Practical

Lab 5: You will be given two unknown bacteria and asked to Gram stain them following the procedures you have learned in previous labs. After Gram staining, you will observe the cells under the microscope, and determine their morphology and Gram reaction. At the end of the lab period you will submit your results (see sample sheet below) to your instructor for grading. Your slide will be saved and may also be photographed for use in your lab report.
Note: You will be required to show the results of your Gram staining procedure to your instructor. Make sure you don't wait until the last minute to call the instructor over to view your slides!

Lab 6: You will learn how to construct a dichotomous key for bacterial identification. Using what you know about the Gram reactions of your unknown bacteria as well as information about the metabolic reactions of bacterial species obtained in Lab 5, you will construct a dichotomous key that can be used to identify your 2 unknown bacteria. You will also choose the media that you will need to use for the inoculations you will do in the next lab. You will turn in the dichotomous key for grading and the instructor will return it to you on Lab 7 (corrected if necessary).

Lab 7: You will inoculate your unknown bacteria in the media that you chose in Lab 6.

Lab 8: You will analyze the results of your metabolic tests. Using these results and your dichotomous key, you will identify your two unknown bacteria.

Lab 10: You will turn in the Lab Report for the Midterm Practical.

Midterm Practical Grading

Point Distribution—out of 100%:

Gram reactions: 25%

Metabolic assays: 25%

Written lab report: 50% (see Lab Report Rubric)

Grading Breakdown:

Part I: Gram Reactions (25/100)

Morphology for unknown A correct – 5 points

Morphology for unknown B correct- 5 points

Gram reaction for unknown B: color and interpretation is correct -5 points

Gram reaction for unknown B: color and interpretation is correct- 5 points

Unknown A slide shown to professor -2.5 points

Unknown B slide shown to professor - 2.5 points

Part II: Metabolic Assays (25/100)

Dichotomous key- complete and correct - 5 points

Media required based on student's key complete and correct - 5 points

Metabolic test results recorded and interpreted correctly - 15 points

Part III: Lab Report (50/100)

Lab Report

General Guidelines

1. Lab reports must be typewritten—no handwritten reports will be accepted! Font size should be no larger than 11 or 12. The lab report format is described below.

2. While writing your lab report, please remember that everything must be in your own words. There is a no tolerance policy on plagiarism. Your instructors can Google along with the best of you. If they find that segments of your lab report are copied word-for-word from another source (e.g., from the web, your lab handouts or text book), you will receive a 0 for that lab report. If they find that lab reports from different people are word-for-word copies, all involved will receive a 0 for their lab reports.

3. Approximate length. The lab report should be at least 2 pages of text (not including figures, tables, pictures and references)

4. Make sure to include the **original, graded** sheets of the following items:

a. Gram stain results

b. Dichotomous key/required media

c. Metabolic test results

(These can be placed at the end of the lab report if you want to make neater, corrected copies for your lab report.)

Lab Report Format

TITLE

You should have an appropriate title for your lab report. Include on your title page: your name, your instructor's name, and your class section.

Your lab report should be divided into the sections listed below. Pay careful attention to what material belongs in each section. **Each of the sections should be labeled** in the lab report.

INTRODUCTION

In this section you should discuss the importance of bacterial identification procedures. This section should also include general background information about the use of the Gram stain and metabolic tests to identify bacteria and you should discuss why staining procedures alone are not sufficient for bacterial identification. (2-3 paragraphs)

METHODS

In this section, you should explain the steps followed to identify your unknowns. For the Gram stain procedure, you may simply reference the lab manual, unless you have deviated from the protocol (if you do anything different from the instructions, you should explain that here). This section should also include the dichotomous key that you used to set up your experiments, and you should describe the rationale that led you to the formation

of this key. You should also describe each metabolic test you used (name the medium, describe the inoculation procedure, describe any procedures necessary post-inoculation and explain what metabolic process you are testing for). (3-4 paragraphs)

RESULTS

This section will be used to present your results for the Gram stain and the metabolic tests. Divide this section into 2 parts: 1 part for each of your unknowns.

Example:

Unknown 1A

Gram stain results. Describe color, Gram reaction, morphology and arrangement. Include either a photograph or a color drawing here. Students can use the microscope at the instructor's desk to take a picture, which can then be saved to a flash drive and printed out to be added to the lab report. The photograph added to the lab report should be in color. If you make a drawing, it must also be in color, and make sure that the bacteria's morphology and characteristic arrangement (if any) can be seen. Drawings/photographs should be labeled. You should include the table that you filled out in Lab 5, but you must also describe your Gram staining results in the body of your lab report.

Metabolic Test results. You should include the table you filled out in Lab 8, but you must also summarize your results in the body of the lab report. You need to describe the results (colors, bubbles, etc.), make a determination as to whether the result is a positive or a negative result, and then interpret the result. For example: "After inoculation my urea slant turned pink. This was a positive result, indicating that this bacteria produces a urease."

Unknown 1B (as above for Unknown 1A)

(1-2 paragraphs for each unknown, not including the tables.)

CONCLUSIONS

It is here where you will make your identification of your unknowns based on the results of the Gram stain and the metabolic tests. Divide it into 2 sections, 1 for each unknown. Describe in words and sentences your rationale for making your final identifications. If you have any problems or inconsistencies with your experiments (ex: an incorrect result for your gram stain or a metabolic test result that is not consistent with your bacterial identification), you should discuss them here. Try to explain what might have happened to give you an incorrect result.

Also include in this section an additional test that would confirm your results for each unknown. This is a test that you did not perform; it could be a staining procedure or an additional metabolic assay. **Make sure you mention the expected result for these additional tests.**

After you have discussed the identification of your unknowns, include 1-2 paragraphs about each of your identified bacteria. This section should include what your test results tell you about the bacteria's structure, morphology, and metabolism. Also add some general information about the bacteria, including any diseases that this species has been shown to cause. You can use your textbook to obtain this information, or use the following websites:

- CENTERS FOR DISEASE CONTROL AND PREVENTION: Contains information about infectious diseases, their causes, treatments, epidemiology and prevention.
- http://www.cdc.gov/

AMERICAN SOCIETY FOR MICROBIOLOGY: allows for searches of their journals; also contains links to current information about Microbiology and Microbiology education.http://www.asm.org/

- NATIONAL LIBRARY OF MEDICINE, part of the National Institutes of Health; includes information on infectious diseases; search engine can be used to find current information on a particular topic

http://www.nlm.nih.gov/

- CELLS ALIVE: links to information about bacteria, viruses and eukaryotic pathogens
- http://www.cellsalive.com/inex.htm
- OTHER RESOURCES MAY BE USED BUT PLEASE MAKE SURE THEY ARE RELIABLE (WHEN IN DOUBT ASK YOUR INSTRUCTOR!)

DO NOT RELY ON WIKIPEDIA!

The Lab Report will be due on Lab 10. Lab reports that are turned in late will have **5 points** deducted for each day they are late. No reports will be accepted after lab 11—no exceptions!

Suggestions for writing a good lab report:

1. Have someone proofread your lab report before you turn it in.

2. Use the grading rubric for the lab report (see below) as a checklist to make sure that you formatted the lab report correctly, and that you have all the required information.

2. The Campus Writing Center is located in the Library (L-118). You can visit them for additional help with your writing.

Sample Forms you will fill out for Midterm Practical

Gram Stain Results

Name: _____ **Unknown Number _____**			
Unknown	**Color after Gram stain**	**Gram reaction (positive or ne*g*ative)**	**Morphology**
A			
B			

Results from Metabolic Tests

Name: _____

Unknown number: _____

	A		B	
Test	**Describe result**	**Result (+ or -)**	**Describe result**	**Result (+ or -)**
Catalase				
Simmon's citrate (Citrate permease)				
Indole (Tryptophanase)				
Urease				
Amylase				
Gelatinase				
Lactose fermentation				

Fill in only for the tests you have performed. Result descriptions could include colors, presence/absence of bubbles, colored or colorless zones around inoculation regions, etc., depending on the test used. Based on the appearance of your test, determine if the result was positive (+) or negative (-)

Microbiology Lab Report Grading Rubric

Introduction (max 8 points)	Student properly explains the importance of bacterial identification and why both staining and metabolic tests are necessary **7-8 points**	Student offers some explanation of the importance of bacterial identification and why both staining and metabolic tests are necessary **5-6 points**	Student offers little explanation of the importance of bacterial identification and why both staining and metabolic tests are necessary **3-4 points**	Student offers very little or no explanation of the importance of bacterial identification and why both staining and metabolic tests are necessary **0-2 points**
Methods (max 10 points)	• Student clearly explains how dichotomous key was generated and used to choose appropriate metabolic tests • dichotomous key is correct and neatly organized • all of the expected information about the basic chemistry behind each metabolic test chosen is provided • Student identifies Gram staining method used and mentions any changes to procedure **8-10 points**	• Student provides some explanation about how dichotomous key was generated and used to choose appropriate metabolic tests • dichotomous key contains one error and/or is somewhat disorganized • most of the expected information about the basic chemistry behind each metabolic test chosen is provided **6-7 points**	• Student provides inadequate information about how dichotomous key was generated and used to choose appropriate metabolic tests • dichotomous key contains several errors and/or is difficult to interpret • some information about the basic chemistry behind each metabolic test chosen is provided **3-5 points**	• Student provides little to no explanation about how dichotomous key was generated and used to choose appropriate metabolic tests • dichotomous key contains multiple errors and/or is impossible to interpret • little to no information/inaccurate information about the basic chemistry behind each metabolic test chosen is included • Student does not identify Gram stain method used **0-2 points**
Results (max 10 points)	• all gram stain results (morphology, arrangement, and gram reaction) are explained correctly • all metabolic test results (appearance of media and interpretation) are explained completely and correctly • Both tables (gram stain and metabolic test results) with all correct information are included • Labeled drawings or photographs of slides (A and B) are included **8-10 points**	• most gram stain results (morphology, arrangement, and gram reaction) are explained correctly • most metabolic test results (appearance of media and interpretation) are explained completely and correctly • Both tables (gram stain and metabolic test results) with mostly correct information are included • Drawing or photographs of slides (A and B) are included **6-7 points**	• some gram stain results (morphology, arrangement, and gram reaction) are explained correctly • some metabolic test results (appearance of media and interpretation) are explained completely and correctly • Both tables (gram stain and metabolic test results) with some correct information are included • Drawings or photographs of slides are included but inadequate (A and B) **3-5 points**	• gram stain results (morphology, arrangement, and gram reaction) is either missing or incorrect • little or none of the metabolic test results (appearance of media and interpretation) are explained correctly • Tables (gram stain and metabolic test results) are missing or incorrect • Drawings or photographs of slides (A and B) are missing **0-2 points**

Conclusion (max 10 points)	• both unknowns A and B are correctly identified, and student fully explains rationale for their identification • an appropriate additional test for each organism that would confirm its identity is explained • an appropriate explanation for any incorrect or inconsistent results is provided • an appropriate summary of the bacterial characteristics determined by their experiments is provided • student provides adequate background information about both species from outside sources (text, web resources) **8-10 points**	• both unknowns A and B are correctly identified, and student mostly explains rationale for their identification • an additional test for each organism that would confirm its identity is explained • some explanation for any incorrect or inconsistent results is provided • some summary information about the bacterial characteristics determined by their experiments is provided • Student provides some background information about both species from outside sources (text, web resources) **6-7 points**	• one unknown is identified correctly and/or student inadequately explains rationale for their identification • an additional test for one organism that would confirm its identity is explained • little explanation for any incorrect or inconsistent results is provided • little and/or inaccurate summary information about the bacterial characteristics determined by their experiments is provided • student provides little and/or inaccurate background information about both species from outside sources (text, web resources) **3-5 points**	• neither unknown is correctly identified and/or student does not explain rationale for their identification • no additional tests for either organism that would confirm their identity is explained • no explanation for any incorrect or inconsistent results is provided • no summary information about the bacterial characteristics determined by their experiments is provided • student provides no background information about either species from outside sources (text, web resources) **0-2 points**
References (max 6 points)	• References and citations are used correctly • All references used are appropriate • Report is clearly written in the student's own words **5-6 points**	• References and citations are sometimes used correctly • most references are appropriate **3-4 points**	• Either references or citations are missing or not used correctly • Some references are appropriate **1-2 points**	• Both references and citations are missing or not used correctly • References are not appropriate **0 points**
General overview and format (max 6 points)	• All topics are covered in the appropriate sections • Document is free of spelling and grammar errors • Text follows a logical order and is easy to understand • An appropriate font and page layout is used • An appropriate title is provided **5-6 points**	• Most topics are covered in the appropriate sections • document contains few spelling and/or grammar errors • text is fairly easy to follow and understand • Font and page layout are acceptable **3-4 points**	• Some topics are covered in the appropriate sections • Document contains some spelling and/or grammar errors • Some text does not follow a logical order and is difficult to understand • Font and page layout generally acceptable **1-2 points**	• Many topics are covered in the wrong sections • Document contains many spelling and grammar • Text does not follow a logical order and is extremely difficult to understand • Font and page layout is not acceptable • Title is missing **0 points**

How to Create a Proper Works Cited Page

A works cited page is required for any assignment that asks you obtain information from sources written by someone else. Ask your teacher how to include a type of source that is not listed below.

If you are using a ….

A Book with one author:
Last Name, First Name. *Title of Book*. Place of Publication: Publisher, Year of Publication. Print.

A Book with two authors:
Last Name, First Name and First Name Last Name. *Title of Book*. Place of Publication: Publisher, Year of Publication. Print.

A Book with three authors:
Last Name, First Name, First Name Last Name, and First Name Last Name. *Title of Book*. Place of Publication: Publisher, Year of Publication. Print.

A Book with more than three authors:
Last Name, First Name, et al. *Title of Book*. Place of Publication: Publisher, Year of Publication. Print.

An Encyclopedia Entry:
Last Name, First Name (if available). "Name of Entry." *Title of Encyclopedia*. edition. Copyright year. Print.

A Website:
Last Name, First Name (if available). "Title of webpage." *Title of Entire Website*. Date of posting/revision. Date of publication. Web. Date of access.

A Newspaper or Magazine Article:
Last Name, First Name. "Title of Article." *Title of Magazine*. Date of Article: Page(s). Print.

An Online Newspaper or Magazine Article:
Last Name, First Name. "Title of Article." *Title of Newspaper*. Date of Article. Web. Date you viewed the webpage.

An Online Database:
Last name, First name. "Title of article." *Title of journal or magazine:* Pages (if available). *Title of Database*. Web. Date of access.

A DVD Video:
Title of film or video. Film studio or distributor, Release year. DVD.

An Online Video:
"Title of Video." *Website*, Year Posted. Web. Date Viewed.

This is what your works cited page should look like....

Works Cited

Batchelor, John and James Davidson. *The American Nation*. New Jersey: Prentice Hall, 1994. Print.

"Biography of Franklin D. Roosevelt." *The White House*. Web. 26 Sept. 2009.

"George Washington." *World Book Encyclopedia*. 3rd ed. 1996. Print.

Glory. TriStar Pictures, 1989. DVD.

O'Brien, Steven. "Bill Clinton." *American Government*. ABC-CLIO, 2012. Web. 18 Aug. 2012.

"President Barack Obama's Inaugural Address." *Youtube*, 2009. Web. 18 Aug. 2012.

Rubel, David. *Scholastic Encyclopedia of the President*. New York: Scholastic, 1994. Print.

Sisk, Richard. "President Obama blasts Iran for secret nuclear facility." *New York Daily News*. 12 July. 2009. Web. 26 Sept. 2012.

Note:

- All entries are in **alphabetical** order.
- Books, websites, newspaper and magazine articles, etc. are ***NOT separated by type.***
- **All punctuation marks must be in the proper place!!**
- All entries that go onto a second line are **indented**, are lined up, and single-spaced.
- The entries ***are not*** numbered or bulleted.
- There is a line skipped between entries. All entries are **lined up**.
- All titles are *in italics*.
- All entries in your work cited must indicate what type of source it is (e.g. "Print", "Web", etc.)
- The online database ABC CLIO gives the proper citation at the end of each article.
- All dates are listed : Day Month (abbreviated) Year e.g.: 26 Sept. 2012
- A "Works Cited" page is always a SEPARATE page.
- It is not necessary to include the URL Address for webpages.

Appendix II: Scientific Notation and Serial Dilution

Scientific notation is a concise method of expressing numbers that are too large or too small to be conveniently represented as decimals. Since science is often concerned with very large things (e.g., galaxies, light years, planets) and very small things (atoms, bacteria, viruses), scientific notation is commonly used in science and engineering.

In scientific notation, all numbers are written in the following form:

$A \times 10^n$ (A times 10 raised to the power of n)

"A" represents the digits in the number and "n" is the exponent (or power) to which ten is raised.

Numbers both larger and smaller than one can be represented in scientific notation. If the number is larger than one, the exponent will have a positive value, and if the number is smaller than one, the exponent will have a negative value. For example:

(a) The number 100 can also be written as 1×10^2 (or: 1 times 100 = 100). Scientific notation tells you that you take the number 1, and move the decimal point 2 places to the right of the number.

(b) The number 0.01 can also be written as 1×10^{-2}. Scientific notation tells you that you take the number 1 and move the decimal 2 places to the left of the number.

Some examples:

a. $223 = 2.23 \times 10^2$

b. $0.000156 = 1.56 \times 10^{-4}$

c. $7{,}650{,}000 = 7.65 \times 10^6$

d. $0.00382 = 3.82 \times 10^{-3}$

Exercises

Below are a series of exercises that will help familiarize you with scientific notation.

1. Convert the following fractions to their decimal form and their scientific notation form.

Fraction	Decimal	Scientific notation
1/10		
1/100		
1/1,000		
1/10,000		
1/100,000		

2. Write the following numbers in scientific notation:

a. 4,500 _____

b. 2,220,000 _____

c. 0.0035 _____

d. 0.7 _____

e. 858.67 _____

3. The following numbers are written using scientific notation. Convert them to their decimal form.

a. 5.65×10^{-3} _____

b. 9.25×10^{-4} _____

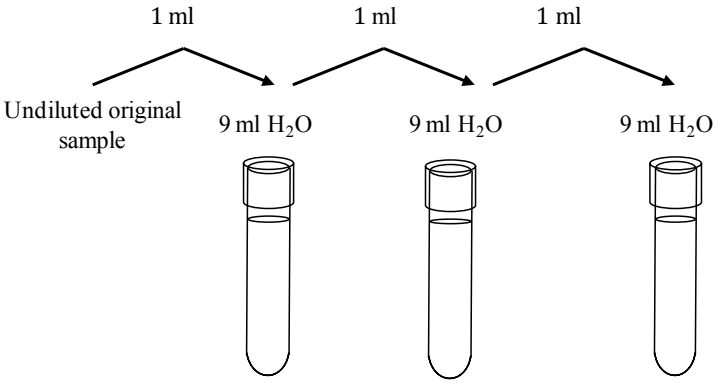
c. 5.68×10^{5} _____

d. 1.632×10^{3} _____

e. 4.8932×10^{-2} _____

Serial Dilution Problems

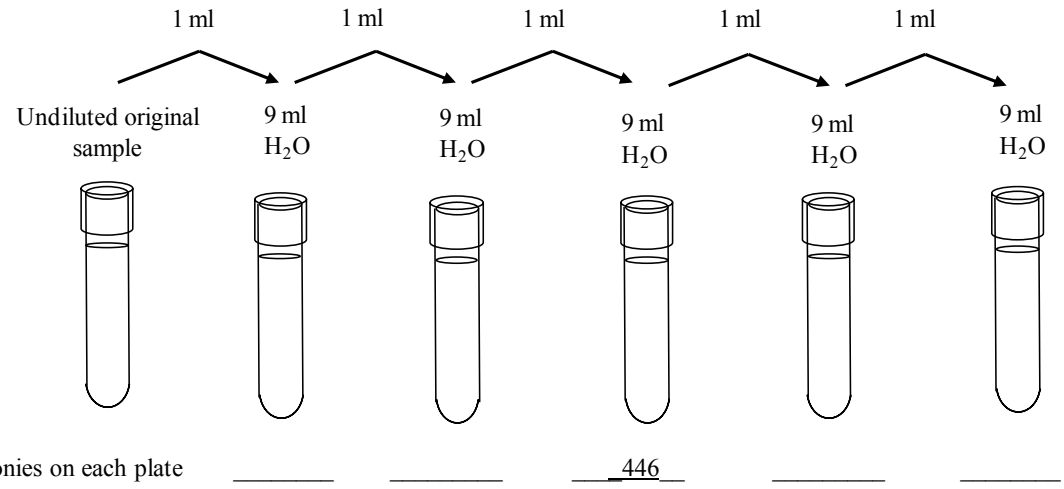
1. You have a urine sample from a patient that you suspect has a urinary tract infection. You make ten-fold dilutions of this sample as shown below, and then plate 0.5 ml (500 μl) of the last dilution on a TSA plate. There are 45 colonies on the plate. How many CFUs/ml were in the original urine sample?



2. You have received a sample from a sewage treatment plant, and have been asked to determine how many CFUs/ml are in this sample. You want to make a 1/100,000 fold dilution, but the smallest volume you can measure is 1.0 ml, and the tubes available to you only hold 10 ml. Explain/draw how you would do this!

3. You have a bacterial culture that you know has 650,000 bacteria/ml. You do serial dilutions to achieve a 1/10,000 dilution, and then plate 0.1 ml of each of these dilutions. How many colonies will you see at each dilution?

4. You do a series of dilutions as shown below, and you plate 1.0 ml of each dilution. Given the information below, fill in the number of colonies you would expect on each of the plates.



5. You do serial dilutions on a water sample, and plate the dilutions on TSA plates. You count the colonies on each of the plates as follows: (Note: TMTC = too many to count)

Dilution	Number of CFUs
10^{-2}	TMTC
10^{-3}	TMTC
10^{-4}	352
10^{-5}	45
10^{-6}	6
10^{-6}	0

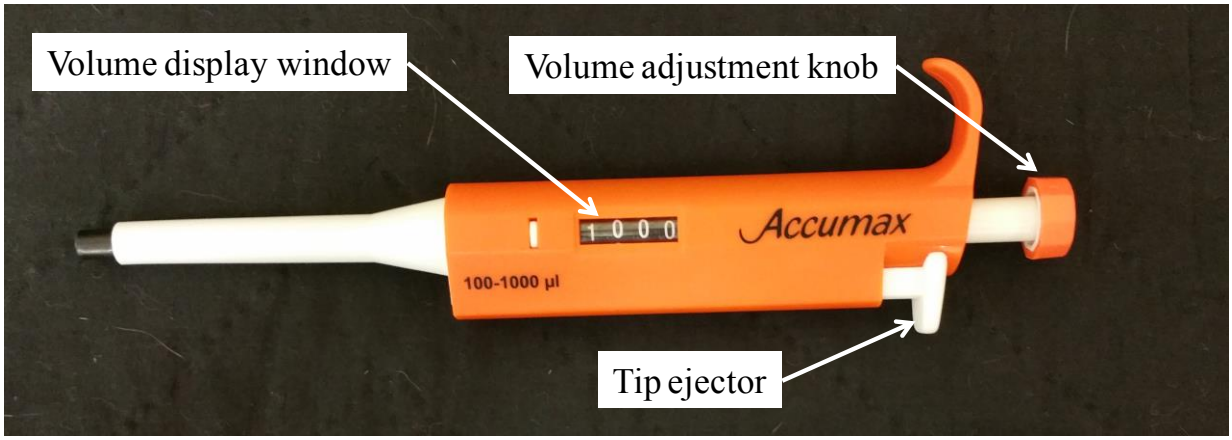
Based on these results, what is your estimate for the total number of CFUs/ml in the original sample?

Appendix III: Introduction to Micropipetting

When scientists need to transfer small volumes of liquid, they use a piece of equipment known as a micropipettor (or pipettor). There are a few different types of micropipettors based on the minimum and maximum volumes of liquid they are designed to transfer. Today you will learn how to use a micropipettor to a) transfer 1 ml (1000 µl) volumes of liquid in the preparation of serial dilutions, and 2) transfer 0.1 ml (100 µl) of these dilutions onto agar plates.

A. How to set the micropipettor for the appropriate volume:

The picture of the micropipettor shown below is similar to the one you will be using. As you can see, this micropipettor is designed to transfer volumes between 100 µl - 1000 µl (this type of micropipettor is sometimes called a **P1000**). The display window reads "1000" when the pipettor is set to its maximum volume (1000 µl or 1 ml). If we were using this same pipettor to transfer 500 µl (or 0.5 ml) the window would read 500.



Similarly, the pipettor shown below (a P200) is designed to transfer volumes between 20- 200 µl (0.02- 0.2 ml) It is set at 50 µl —to pipet 100 µl you would set the display to read '100'.

- The volume is set by turning the knob at the top of the pipettor until the correct volume is reached.

***Note:** never try to turn the knob above the minimum or maximum volumes or you may damage the micropipettor!

B. How to attach a pipet tip:

After setting the appropriate volume, you will be attaching a pipet tip to the narrow end of the micropipettor. The P1000 uses blue tips; P200's use clear or yellow tips. The tips have been sterilized in the autoclave so as not to introduce any contaminating microbes into your sample. To pick up a tip:

1. Open the box of tips and insert the end of the pipettor into the end of a pipet tip.
2. Press down gently to ensure that the tip stays attached to the micropipettor.
3. Close the box to prevent the other tips from getting contaminated.

C. To transfer liquid using the pipettor: Note: follow these steps very carefully!

1. With your thumb, depress the plunger on the top of the pipettor until you **first** feel resistance.
   Note: it is important not to go past the first stop. If you do, you will picking up too much liquid!
2. While holding the plunger in the depressed position, place the pipet tip into the liquid you will be transferring.
   Note: if you depress the liquid after placing the tip in the liquid you will be blowing air into the liquid!
3. Slowly release the plunger to allow the tip to pick up the liquid.
   Note: Be sure to keep the tip in the liquid the entire time so that you don't get air bubbles.
4. Place the tip into the tube (or plate) where you will be transferring the liquid to.
5. Slowly press the plunger down again until all the liquid leaves the pipet tip.
6. Withdraw the tip from the tube before releasing your thumb from the plunger.
7. Use the tip ejector to discard the tip in the container as instructed by your professor.
   Note: pipet tips **NEVER** get disposed of in the regular garbage.

Other important things to remember:

1. It is important not to let the pipet tip touch anything other than the liquid you are transferring.
2. Always keep the pipettor in the upright position when in use. If you tilt it too much the liquid in the tip will make contact with the pipettor and will no longer be sterile (this could also damage the pipettor).

Practice:

Set your P200 pipettor to 100 µl and practice pipetting by transferring the liquid onto a piece of parafilm. Let your lab partners try as well. All droplets should be the same size (and the same size as your instructor's). Try transferring different volumes as instructed until you feel comfortable using the pipettor.