LABORATORY EXERCISES

to accompany

MICROBIOLOGY LABORATORY

BSL 214

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INTRODUCTION TO CULTURING, MEDIA, AND ASEPTIC TECHNIQUES

Even though you may be starting a career in a health-related field, you may be unaware of the number and variety of microorganisms (microbes) found everywhere in our environment, including the human body. In this laboratory you will learn new techniques and observations which relate to the concepts of microbiology and to your future health career. Most of the microorganisms that you will use in these laboratories are normal inhabitants of our environment. These microbes are called **normal microbiota** for the environment in which they normally reside. Health professionals need this knowledge in order to be able to distinguish normal flora from a possible infectious agent when interpreting microbiological reports. They also need to understand how normal flora can occasionally cause an infection when they invade a different area of the body, or when the patient's immune responses have been compromised.

Microorganisms are found almost everywhere. In these first laboratory exercises you will be introduced to **aseptic techniques**, the procedures followed by microbiologists and healthcare workers to prevent contamination of cultures from outside sources and to prevent introduction of potentially disease-causing microbes (pathogens) into the human body. The methods for handling previously sterilized materials, for taking samples, for handling cultures, and for disposing contaminated materials are all designed to prevent the spread of microbes from one area to another. Pay close attention to the details in the written procedures and to the instructor's demonstrations to prevent contamination of your cultures, yourself, your environment, and the other people in your laboratory as well as prevention of infecting people outside of the laboratory, such as your friends and family. These techniques can be applied not only here in the microbiology laboratory, but also throughout your career, and in your daily life.

Most of the laboratory exercises performed in this course will involve a two-step process. During the lab session you will set up the cultures and then after these cultures have incubated for the appropriate length of time (usually 24 to 48 hours) you will need to observe the growth and record your observations and results.

Wait for the instructor to demonstrate the procedures described and to make the specific assignments.

In the rest of the exercises in the course, you are dealing with living bacteria, so it is very important to follow the procedures exactly to avoid contamination or infection. **The following precautions are especially important:**

1. Always **wash your hands** with the antiseptic soap provided before you begin and after you have finished each procedure.

2. Always **wipe off your work area** with the disinfectant provided before you begin and after you have finished each procedure.

3. Always **wear gloves** when handling cultures or specimens.

4. Discard all used materials in the appropriate designated place after you are done. Put all used materials and cultures into the special containers for contaminated material. Never put any used materials back into the supply area.

5. Do not lay liquid broth cultures, test tubes, swabs, or pipettes on the tabletop or touch anyone with them.

6. The lid of the culture (Petri plate) is held over the surface while you are inoculating the surface and then is immediately replaced.

7. The cultures you will observe after the 24-48 hour incubation period will have a high concentration of bacteria on them. Even though they are "normal inhabitants" of the environment or human body, they can cause an infection if they get into an open cut or sore or transmitted to the mouth, hair, or eyes from your hands because of the large number of bacteria present. Thus, it is extremely critical that the Petri dishes be examined when the covers are in place. Never hand one to someone else with the lid removed.

8. A **disposable, fluid-resistant**, **full-length, long-sleeved lab coat** must be worn at all times in the lab. The coat must be removed before leaving the room for any reason**.** If the lab coat becomes contaminated, it must be removed, put into a biohazard bag, and autoclaved before disposal into the trash.

9. If a spill occurs, notify the instructor immediately and decontaminate the area right away.

10. Long hair must be pulled back.

11. **Closed-toed shoes** must be worn in the lab at all times. No sandals are permitted.

10. If you have any doubts or questions about what you are doing; **ASK THE INSTRUCTOR FOR HELP!**

BACTERIAL MEDIA

Objectives: *After completion of this laboratory experiment, the student will be able to:*

1. Perform a commonly used method of isolating bacteria in pure culture - the streak plate method.
2. Perform essential aseptic techniques.
3. Use selective media to isolate an organism from a mixture of organisms.
4. Transfer microorganisms from liquid nutrient broth to an agar plate using a pipette or an inoculating loop.

In this exercise you will use different types of **culture media** to grow various species of bacteria from a mixed culture.
To study microorganisms properly, we have to be able to grow them. To accomplish this, it is necessary to transfer the specimens to an environment that will simulate the same conditions under which they occur in nature. Nutritional requirements vary widely from one species of bacteria to another and in many cases are not clearly known. Much has been accomplished concerning the duplication of conditions necessary for the cultivation of microorganisms, and most microbes can now be cultivated on or in artificial media. Ingredients in media are intended to supply the nutritional and growth requirements of microorganisms so that the cultures studied will present characteristics comparable to those that exist in nature.

1. **Primary or Isolation Media:** Media used for primary inoculation of specimen; usually prepared in Petri dishes so they can be streaked to obtain isolated colonies of any organisms present.
   Media used routinely in most laboratories are:
   Trypticase soy agar (TSA) and Nutrient agar

2. **Enrichment Media:** Media that has been enriched by the addition of extra ingredients to enhance the growth of fastidious microbes.

   Examples: blood agar
   chocolate agar

3. **Selective Media:** Media used to grow one particular type of bacteria from a mixed culture by inhibiting the growth of the other bacterial species.

   Examples: Phenylethyl alcohol (PEA) agar-selects for gram-positive bacteria
   Mannitol salt agar-selects for staphylococci
   MacConkey agar-selects for gram-negative bacteria
   Eosin methylene blue agar-selects for gram-negative bacteria

4. **Differential Media:** Media used to distinguish between species of bacteria which may look exactly alike or very similar by other methods, such as the Gram stain, or on TSA.

   Examples: MacConkey agar – distinguishes between lactose fermenters and non-lactose fermenters
   Mannitol salt agar- distinquishes between *Staphylococcus aureus* and other *Staphylococcus* species
   Eosin methylene blue – distinguishes between *E.coli* and other enteric bacilli

The media that you will be using in this experiment are:

TSA = **trypticase soy agar**; nutrient primary isolation media; will grow many types of bacteria (both gram-positive and gram-negative bacteria)

PEA = **phenylethyl alcohol agar**; selective media; grows only gram-positive bacteria. The phenylethylalcohol is inhibitory to gram-negative bacteria.

MAC = **MacConkey agar**; selective media; grows only gram-negative bacteria; gram-positive bacteria are inhibited by the crystal violet dye in the agar. MacConkey agar is also used as differential media to distinquish between lactose-fermenting and non-lactose fermenting bacteria. Incorporation of lactose, bile salts, and phenol red indicator causes lactose-fermenters to appear red, whereas non-lactose fermenters will appear colorless or transparent.

MSA = **Mannitol salt agar;** selective media; grows only *Staphylococcus* bacteria. 7.5% salt is inhibitory to most other bacteria. Mannitol salt is also differential media used to distinguish between *Staphylococcus aureus* and other *Staphylococcus* species. Mannitol fermentation with subsequent acid production by *S. aureus* is indicated by a change in the color of the phenol red indicator to yellow.

EMB = **Eosin methylene blue;** selective media; grows only gram-negative bacilli. Eosin methylene blue is also differential media used to distinguish *E.coli* from other gram-negative enteric bacilli. *E.coli* ferments the lactose in the agar, causing acid production, which precipitates the eosin and methylene blue dyes. This results in a metallic blue-black color with a greenish sheen. Other gram-negative enteric bacilli will appear pink or transparent.

BAP = **Blood agar** plate; enrichment media used to grow a variety of fastidious microorganisms such as *Streptococcus*. Blood agar is also used to demonstrate different types of hemolysis:

- **beta hemolysis** = complete lysis of the red blood cells by streptolysin 0 and streptolysin S enzymes
- **alpha hemolysis** = incomplete lysis of red blood cells resulting in the breakdown of hemoglobin, which produces a greenish halo around the bacterial colonies
- **gamma hemolysis** = no lysis of the red blood cells; no significant change in in the color of the agar surrounding the colonies

Specimens submitted to the laboratory for microbiological examination often contain a mixture of microorganisms. In order to study the characteristics of a microorganism, it is first necessary to separate it from other microorganisms present in the mixture; we must isolate the suspected organism in **pure culture**. A pure culture is one in which all of the cells present in the culture originated from a single cell type. The **streak plate method** is the method classically used for isolating a pure culture from a mixed culture.

With this method you will attempt to purify a mixed broth culture containing several different species of bacteria. Once isolated, the bacterial colonies can be differentiated from each other.

An essential component for isolating a pure culture is **aseptic technique**, which involves the transfer of microorganisms from one environment to another in such a way that neither you nor the environment around you is contaminated with the specimen that you are transferring and that the pure culture you are making is not contaminated with other organisms from the environment. In the aseptic preparation of pure cultures, the transfers are usually made with sterile **inoculating loops** or **needles** or with sterile pipettes. Your instructor will first demonstrate the aseptic techniques to be used.

SPECIMEN HANDLING

Objectives: *After completing this exercise the student should be able to:*

1. obtain a throat swab specimen
2. explain the effect of drying on swab specimens prior to their inoculation onto bacteriological media
3. describe correct collection and handling procedure for the following specimens: throat swabs, wound swabs, CSF, peritonal/pleural/synovial fluids, blood cultures, sputum, sputum for AFB, cultures for gonorrhea, stools, urines.

****Assignment**: Read the article entitled "Know your Specimen Collection Techniques to avoid Errors" by Mahesh C. Goel, D.V.M., Ph.D. You will be held responsible for the material in this article. The article is on reserve in the LCC Library and is also available on-line through the LCC Library's homepage. Here is how to access this item:
*start from the library's homepage at http://www.bluegrass.kctcs.edu/lrc/ereserves
*click on BSL 214 (instructors name)
*Username: Will be announced at the first lab meeting (type exactly as shown; case sensitive)
*Password: Will be announced at the first lab meeting (type exactly as shown; case sensitive)
*click on the article you want: "Know Your Specimen Collection Techniques"

The proper handling of specimens for microbiological analysis requires:

(1) aseptic collection techniques
(2) the use of appropriate containers
(3) suitable means for preservation
(4) suitable means of transporting specimens to the laboratory.

All specimens should be handled aseptically and treated as potentially infectious. In cases of spillage or contamination of the outside of a container, some form of disinfection should be carried out immediately.

SPECIMEN HANDLING: Throat swabs

Materials:

1. Two blood agar plates (BAP).
2. Sterile cotton swabs.
3. Tongue depressors to hold the tongue down during specimen-taking.
4. Sterile test tube with a previously inoculated throat swab that has been left to dry out.
5. candle (CO_2) jar for incubation

Procedure:

1. Obtain a throat specimen from your assigned partner's throat with a sterile swab. Place the sterile swab against the back wall of the throat gently and move it up and down.
2. Inoculate a blood agar plate with the throat specimen. Streak it out using the streak plate method.
3. Incubate in a candle jar for increased CO_2 at 37^0C for 24-48 hours.
4. Take the previously inoculated, dried out throat swab and inoculate the second BAP. Streak for isolation and incubate in the candle jar at 37^0C for 24-48 hours.
5. Record the amount of growth on each plate in the Results and Observations.

THROAT CULTURE RESULTS and OBSERVATIONS

	Estimated amount of growth*
Fresh culture	
Dried culture	

*0 = no growth
1+ = a few colonies
2+ = a moderate # of colonies
3+ = heavy growth (almost solid – no distinct colonies)

Study Questions:

1. What difference did you notice between the culture grown from the fresh throat swab and the one grown from the dried-up throat swab?

2. What explains the difference between the amount of growth on the two cultures?

3. Give two methods that would be used to prevent the loss of microbes after collection of the specimen.

4. What type of hemolysis did you observe?

SPECIMEN HANDLING: Urine Samples

Materials:

1. Urine sample containing ***Staphylococcus epidermidis***, a gram-positive coccus in clusters and ***Escherichia coli***, a gram-negative bacillus.

2. One plate of **trypticase soy agar** (**TSA**) (primary isolation media).

3. One **phenylethylalcohol agar (PEA)** plate (selective media for the growth of gram positive bacteria).

4. One **MacConkey** (**MAC**) agar plate (selective/differential media for the growth of gram negative bacilli).

5. One **eosin methylene blue** (**EMB**) agar plate (selective/differential media for the growth of gram negative bacilli; growth of *Escherichia coli* has a green metallic sheen)

6. One **mannitol salt agar** (**MSA**) plate (selective/differential media for the growth of staphylococcus species)

7. (1) inoculating loop

6. (1) sterile transfer pipette

Procedure:

1. Disinfect your bench top with the disinfectant provided.

2. Using a marker, label the **bottom** (contains the agar) of each Petri dish with (a) your name, (b) date, (c) class and section number and (d) description of the specimen.

3. Obtain a sample of urine. Be sure the urine is well mixed beforehand. This can be done by gently swirling the cup.

4. Remove a drop of urine from the cup using a pipette or an inoculating loop using proper aseptic technique.

5. Lift the lid of the Petri dish just enough to get the pipette tip or loop inside. Place a drop of urine in the top half section.

6. Using your inoculating loop, streak back and forth in the pattern demonstrated by your instructor, using proper aseptic techniques. Do this for each of the 5 agar plates.

7. Invert the agar plates and incubate the streak plates at **37° Centigrade** (body temperature) for **24 - 48 hours.**

URINE CULTURE RESULTS and OBSERVATIONS

Record your observations on each type of culture media:

Trypticase soy agar:

Phenylethylalcohol agar:

MacConkey agar:

Eosin methylene blue agar:

Mannitol salt agar:

STUDY QUESTIONS

1. Explain the difference between normal microbiota and pathogenic microbes. Is *Staphylococcus epidermidis* normal microbiota or a pathogen? *E.coli*?

_____

_____

_____

2. Under what circumstances can normal microbiota become pathogenic?

_____

_____

_____

3. Explain the importance of the aseptic techniques used in microbiology as they relate to your career as a health care practitioner.

_____

_____

_____

_____

4. Describe five aseptic techniques that you used during this laboratory exercise.

a. _____

_____

b. _____

_____

c. _____

_____

d. _____

_____

e. _____

5. What is the purpose of trypticase soy agar? What type of bacteria will grow on TSA?_____

_____

Phenylethylalcohol agar? _____

_____

MacConkey agar? _____

_____

Eosin methylene blue agar? _____

_____

Mannitol salt agar?_____

_____

8. What is the purpose of the streak plate technique?

_____

_____

PREPARATION OF A BACTERIAL SMEAR

As you use this procedure throughout this course, remember these precautions for achieving the best results:

1. Use fresh cultures between 24-48 hours old, whenever possible.

2. When making smears, use a medium-sized drop of water and a small amount of bacteria. Mix the bacteria in the drop quite well with an inoculating needle, and spread it out thinly. A smear that is too thick will not only be difficult to stain properly but it will also be very difficult to observe individual bacterial cells under the microscope.

Materials:

glass slide	Bunsen burner
tube of sterile water	slide warmer
gloves	pencil
inoculating needle	sterile transfer pipette ("transpette")
inoculating loop	
culture of *Staphylococcus epidermidis* and *Escherichia coli*	

a. Take your streak plates from the last lab period and examine them for the two different colony types. The TSA plate should have well-isolated ***Staphylococcus epidermidis*** (Gram-positive) and ***Escherichia coli*** (Gram-negative) colonies. The PEA and MSA should only have one colony type (*S.epidermidis*), and the MAC and EMB should only have one colony type (*E.coli*).

b. Assemble the materials necessary for making the smears.

c. With a pencil, label two glass slides on the frosted end with the names of the respective test organisms: *Staphylococcus epidermidis* and *Escherichia coli*.

d. Using the aseptic techniques demonstrated by the instructor put a medium-sized drop of water on the slide in the center, using a sterile pipette or an inoculating loop. Transfer a small amount from a single, well-isolated colony from the Petri plate to the drop of sterile water on the slide**.** When transferring an isolated colony from the streak plate, an **inoculating needle** rather than a loop is used.

e. Touch the inoculating needle to the center of a well-isolated colony. You may use any one of your plates. However, if you use a selective agar, remember that the bacterial type that did not appear to grow is only inhibited. Therefore, you should touch the needle to the very top or edge of the colony without going too deep. **DO NOT TOUCH THE AGAR SURFACE**! Transfer the colony aseptically to the appropriately labeled glass slide and thoroughly mix the bacteria with the drop of sterile water on the slide.

f. Repeat the procedure for the other colony type.

g. Let each smear air-dry thoroughly and then heat-fix gently using either the flame of a Bunsen burner or a slide warmer. **Heat-fix** the bacteria onto the slide by passing the slide, smear side up, quickly through the flame of the bunsen burner 4-5 times. Avoid getting the slide too hot; this will cause distortion of the morphology of the cells. This step will keep your smear from washing off of the slide during the staining procedure.

These smears will be used to perform the **Gram Stain** procedure.

THE GRAM STAIN

Objectives: *After completion of this laboratory exercise, the student will be able to:*

1. Explain the technique and theory of the Gram Stain.
2. Describe bacterial cell morphology.
3. Explain the importance of the Gram stain as an important step in the identification of a bacterial species.
4. Properly perform a Gram stain.

Individual bacterial cells exhibit **morphology** typical of their species: **size, shape, and arrangement** of cells. These can be demonstrated by making a smear on a glass slide, then staining the smear with a suitable dye. The use of a stained smear permits microscopic examination of the smear with the oil immersion lens, which gives the greatest magnification, revealing the size, shape, and arrangement. The study of individual bacterial cells is thus frequently one of the first steps in the identification of bacteria.

In this exercise you will use the **Gram stain.** This is called a **differential stain**, because it not only shows bacterial morphology but allows differentiation of different bacterial types since different species react differently to the stain. The differential Gram stain gives information about the bacterial cell wall, which may be **gram-positive** or **gram-negative**. Gram-positive bacteria will appear purple, the color of the primary stain, crystal violet. Gram-negative bacteria will appear pink-red, the color of the counterstain, safranin. The Gram stain is especially useful as one of the first steps in the identification of a bacterial species, since it reveals both the morphology and the Gram reaction of the bacteria.

The bacteria may show the following shapes: **coccus/cocci**(spherical), **bacillus/bacilli**(rod-shaped), or **spirillum/spirilli**(curved or spiral). The cells may assume a characteristic arrangement: some occur singly, others appear in pairs **(diplo-),** chains **(strepto-),** or clusters **(staphylo-).**

Materials:

1. slides of ***Staphylococcus epidermidis*** (Gram +)and ***Escherichia coli*** (Gram -)
2. wash bottle (with tap water)
3. rinse bucket
4. clothespins (slide holders)
5. absorbent mat
6. glass marker
7. reagents used in the Gram stain:
   - crystal violet
   - Gram's iodine
   - 95% ethyl alcohol
   - safranin

The Gram Stain Procedure

1. Add crystal violet stain until the slide is completely covered. Stain for one minute.

2. Do not drain the stain off of the slide before rinsing, because the crystal violet will form dye crystals on the slide. Dilute the crystal violet stain on the slide with a gentle stream of water from a wash bottle. Then tip the slide and drain off the stain, and continue rinsing until all the purple color has washed off of the slide. Drain off excess rinse water. If viewed under the microscope at this point, all bacterial cells will appear purple.

3. Cover the slide with Gram's iodine solution and let it stand for one minute. This step will not change the color of the cells; the iodine forms a complex with the crystal violet in the cell wall. Rinse with water, using the wash bottle.

4. Decolorize the smear by letting 95% ethyl alcohol run down over the slide, which should be held at an angle with the clothespin until the purple stain no longer is being visibly removed from the slide. This step should only take a few seconds. (NOTE: a thick smear will take longer to decolorize than a thin one.)

5. Quickly rinse the slide with water. At this stage, if viewed under the microscope, gram-positive bacteria will still appear purple and gram-negative bacteria will appear colorless.

6. Add safranin, the counterstain, to cover the slide. Stain for two minutes. At this stage, if viewed under the microscope, gram-positive bacteria will still appear purple, and gram-negative bacteria will appear the color of the counterstain, pink-red.

7. Rinse with water, and let the slide air-dry or blot gently (DO NOT RUB) with bibulous paper. The slide must be completely dry before adding oil for observation under the oil-immersion lens.

USE OF THE MICROSCOPE

Objectives: *After completion of this laboratory, the student will be:*

1. acquainted with the basic principles of compound light microscopy.
2. able to properly use the low power, high power, and oil immersion objectives.
3. able to exercise the steps necessary for proper care of a microscope.

In microbiology, the small size of the microorganisms requires that you become a microscopist. Development of this skill requires practice and experience. The purpose of this exercise is to allow you to become familiar with the use of the microscope. At first you are all thumbs, but with patience and practice, you will become better as time progresses.

First, familiarize yourself with the parts of the microscope and their functions. Refer to your textbook for complete descriptions. Starting at the **base** of the microscope and following the path of light upward:
Illuminator = lamp or light source
Substage condenser = a lens system located below the microscope stage that directs ("condenses") the light rays through the specimen
Iris diaphragm = controls the amount of light that can pass through the condenser; integrated into the condenser itself and is usually controlled by a rotating ring or a lever
Mechanical stage = platform with clips that hold the specimen (microscope slide) in place; the slide can be moved up/down and side to side using **stage knobs**
Objective lenses = primary lenses that magnify the specimen
Body tube = transmits the image from the objective lens to the ocular lens
Ocular lens (eyepiece) = remagnifies the image received from the objective lens
Coarse adjustment/focusing knob = used initially to bring the desired image into view
Fine adjustment/focusing knob = used to make final focus adjustments to the image

There are two sets of lenses that make up the magnification system in a **compound light microscope**. The **objective lenses** provide the initial magnification of the specimen. This "real image" is then projected up through the **body tube** to the **ocular lens**, which magnifies the real image 10X. This is the image that is seen by your eyes.

Microscopes for bacteriological use are usually equipped with at least three objectives:

(1) **low power** (10X magnification)
(2) **high power** (40-45X)
(3) **oil immersion** (100X)

The desired objective is rotated into place by a **revolving nosepiece.**

To calculate the total magnification, the power of the ocular lens (10X) is multiplied by the power of the objective being used (10X, 40X, or 100X).

Proper illumination is a major part of compound light microscopy. The amount of light entering the objective lens is regulated in three ways:

(1) raising or lowering the amount of light coming from the **lamp** or light source,
(2) opening or closing the **iris diaphragm**
(3) focusing the light up through the objective is controlled by raising or lowering the **condenser**.

With increasing magnification, the objective lens requires more light. For example, when the oil immersion objective is used, the maximum amount of light possible is necessary. To achieve this, the lamp must be turned up all the way, the condenser is raised up to stage level, and the iris diaphragm is opened completely.

The lamp, condenser, stage, objective, and ocular lenses must be kept clean to achieve optimal results. The lenses are highly susceptible to scratching, so they must be cleaned carefully. This can be done by moistening a piece of lens paper with special lens cleaner, wiping off the lens, and then drying it off with a piece of dry lens paper. To clean oil from the lenses on stage, use the same procedure until no oil is seen on the lens paper.

Precautions:

1. Do not touch the lenses with your fingers. Always use special lens cleaning paper.
2. Do not force the adjustments. If you have problems making adjustments, consult the instructor before proceeding.
3. Always clean off the lenses and stage with special cleaner and lens paper before putting your microscope away.
4. After each use, the following steps should be followed:

a.	clean off all lenses and the stage	d.	rotate the 4X or 10X objective into place
b.	make sure the light is turned off	e.	wrap the cord around the base
c.	lower the condenser and the stage	f.	cover the microscope with a plastic cover

Procedure:

1. Place the microscope on your desk and identify the different parts of the microscope and their function. Refer to your textbook for a diagram and description of each microscope part, and the path of light through the microscope.

2. Obtain a stained bacterial smear from your instructor, or use one of the smears that you have prepared yourself. Make sure that the smear side is up before placing it on the microscope stage.

3. Place the slide on the stage with the smear centered over the opening.

4. Rotate the **low-power** (10X) objective into position. For initial coarse focusing, first use the large **coarse adjustment** knob. The **fine adjustment** knob, the smaller knob, can then be used to complete your focusing.

5. After examining the smear under low power, rotate the nosepiece until the **high-dry objective** (40X) snaps into place. You should only have to refocus slightly, using the fine adjustment knob.

6. Note the increased size of the bacterial cells and the decreased number of cells present per microscopic field.

7. For practice focusing with the oil-immersion objective (100X), place a drop of **immersion oil** on the slide, over the area of the smear. Lower the **oil-immersion objective** slowly until it just touches the oil.

8. Next bring the specimen into a fuzzy focus very slowly with the coarse adjustment knob, and then into sharp focus with the fine adjustment knob. The field will come in and out of view quickly.

9. If the microscope is **parfocal**, an alternate method is to find the smear with the low power objective (10X) or high power objective (40X) and then carefully switch over to the oil immersion lens.

10. Sketch and describe the appearance of the cells on the Results Sheet.

11. Remove the slide when finished and put it into one of the cans labeled "for glassware only."

RESULTS

1. After performing the Gram Stain procedure on your bacterial smear, use the oil-immersion objective to examine the bacteria. You should see a mixture of two different species of bacteria: one gram-positive, and one gram-negative. Sketch the appearance of each type of cell. Describe the morphology and give the gram reaction. (Be sure to use the correct terminology in describing the morphology.*)

   (*morphology means size, shape, and arrangement)

   Morphology: _____
   Gram reaction: _____

   Morphology:_____
   Gram reaction:_____

2. Examine the unknown pre-prepared Gram stains provided by the instructor. Sketch a few cells of each, describe the morphology, and give the gram reaction.

   A.
   Morphology: _____
   Gram reaction: _____

B.
Morphology: _____
Gram reaction: _____

C.
Morphology: _____
Gram reaction: _____

D.
Morphology: _____
Gram reaction: _____

STUDY QUESTIONS

1. What conclusion can you make about the relationship between the size of the microscopic field (average number of organisms per field) and the magnification used?

_____

_____

_____

2. How do you determine the actual total magnification of the specimen you are looking at? (Show your calculations for each of the three objectives.)

_____

_____

a. low power objective:

b. high-dry objective:

c. oil-immersion objective:

3. Why do you have to use the oil-immersion objective to view bacteria?

_____

_____

4. Describe the type of information the Gram stain can give:

the microbiologist

the physician.

5. Fill in the following table:

Steps	Appearance of bacterial cell after each step (color) Gram positive cell	Gram negative cell
crystal violet		
Gram's iodine		
95% alcohol		
safranin		

6. What is the function of the Gram's iodine (the mordant) in the Gram stain?

7. What is the function of the safranin counterstain?

8. What is the function of the 95% alcohol decolorizer?

9. Explain the chemistry behind how the Gram stain distinguishes between gram-positive and gram-negative bacteria.

Special Staining Techniques: Acid-fast Bacilli Stain, Capsule Stain, Endospore Stain, and Flagella Stain

Objectives: *After completion of this laboratory exercise, the student will be able to:*

1. Identify special bacterial structures: capsules, flagella, and endospores.
2. Explain the significance of these bacterial structures in diagnosis and identification of disease.
3. Perform or describe the techniques that identify these special structures.
4. Identify acid-fast bacilli on a stained preparation.
5. Explain the significance of acid-fast bacilli in a specimen.

Some bacteria possess cell walls and other structures that are best demonstrated by methods other than the Gram Stain. This exercise deals with a differential stain for the special type of waxy cell walls possessed by *Mycobacterium* and with methods used to demonstrate endospores, capsules, and flagella. In addition to their value in identification of certain bacteria, demonstration of these structures is important for your understanding of the basic structure and function of bacterial cells in disease processes.

Bacterial Capsules

The **capsule** is a gelatinous, slimy material surrounding the bacterial cell. In many cases the capsule helps protect the cell against phagocytosis. Thus potential pathogens are protected from the body's natural defenses and are more likely to cause disease than non-capsulated strains. The capsule also allows bacteria to adhere to surfaces, such as mucous membranes and teeth. Other functions of a capsule include protection from dehydration and loss of nutrients. In this exercise, capsules are demonstrated by the **negative stain,** in which the capsule shows up as a clear area or halo surrounding the cell against the dark background of **nigrosin** stain.

Bacterial Flagella

Flagella are structures that enable bacteria to be motile. They may occur singly at one end, in tufts at one or both ends, or arranged all around the cell.

monotrichous = a single flagellum
amphitrichous = a single flagellum at both ends of the cell
lophotrichous = two or more flagella at one or both ends of the cell
peritrichous = flagella distributed over the entire cell

The number and arrangement of flagella can be used to help identify bacteria.

Flagella are demonstrated by special stains using **mordants** that increase the width of the flagella and are then stained with **carbol-fuchsin** so that they may be seen with the microscope. NOTE: The pink color of the microbes is due to the color of the primary carbol-fuchsin stain, and is NOT an indication of a gram reaction, as in the Gram stain procedure.

Bacterial Endospores

Endospores are very resistant structures that are formed by certain bacteria under adverse conditions. Two genera of gram-positive bacilli (rods) are endospore-formers: ***Bacillus*** and ***Clostridium.*** Endospores enable the organism to survive drying and lack of nutrients, so they can exist in dust and soil for many years. Endospores are the most resistant form of life known. Their presence in dust accounts for much of the laboratory contaminants. The very thick spore wall does not stain easily, so the endospores will appear in Gram stains as unstained areas inside the cell. To stain the spores themselves, **carbol-fuchsin** stain is heated so that it will be absorbed by the wall of the endospore so that they appear red. The vegetative part of the cell will decolorize upon rinsing with 95% ethanol and can then be counterstained with methylene blue or brilliant green for contrast.

Acid-Fast Bacilli

The cell walls of the genus ***Mycobacterium***, which includes the pathogens of tuberculosis and leprosy, are different from most other types of bacterial cell walls because they are waxy and stain poorly, if at all. However, they will take up the **acid-fast stain**. This stain uses **carbol-fuchsin** to which phenol has been added. The cell wall then resists decolorization with acid-alcohol. (alcohol plus hydrochloric acid; thus the name "acid-fast") The end result is an organism that retains the carbol-fuchsin color. Other organisms will decolorize with the acid-alcohol and will take up the counterstain brilliant green or methylene blue. *Mycobacterium* species are therefore often called **"acid-fast bacilli" (AFB).**

Materials:
Prepared demonstration slides of capsules, flagella, endospores, and acid-fast bacilli

Procedures:

I. **Capsule stain by the negative method**
Examine the demonstration slides with oil immersion for the presence of capsules. They should appear as tiny, unstained, “halos” around the bacteria cells. The bacteria may be seen inside the capsule as tiny blue bacilli.

II. **Flagella stain:** Examine the demonstration slides under oil-immersion for the presence of flagella. They should appear as thin, whip-like "tails". Remember, this in not a gram stain, and the color does not designate a gram reaction.

III. **Endospores stain**
Examine the demonstration slides under oil-immersion for bacterial endospores. They will appear as small pink or colorless circles or ovals inside the streptobacilli.

IV. **Acid-fast stain**
Examine the demonstration slides with oil-immersion for the presence of the acid-fast organisms (“AFB” = acid-fast bacilli). They should appear as clumps (“cords”) of tiny, fuschia-colored bacilli. Other, non-acid fast bacteria will appear blue.

RESULTS SHEET
SPECIAL STAINS

Examine the **special stains** provided by the instuctor. Draw the appearance of the structures. Describe the appearance of the structure and the bacterial cell. Label your diagrams.

I. Capsules

II. Endospores

III. Acid-fast bacilli

IV. monotrichous flagellum

amphitrichous flagella

lophotrichous flagella

peritrichous flagella

STUDY QUESTIONS
SPECIAL STAINS

I. What is the importance of performing these special stains? What information do they give you?

_____

_____

_____

_____

II. a. Is a bacterium that possesses a capsule always considered a pathogen?

_____

_____

b. What are the functions of a capsule?

_____

_____

III. a. Why are endospores important to a bacterial cell? Under what conditions are they formed?

_____

_____

b. What genera of bacteria can produce endospores?

_____

c. Give an example of the genus and species of four(4) pathogenic bacteria that produce bacterial endospores.

_____

_____

_____

IV. a. What are the genera that the acid-fast stain is used to identify?

_____

b. Name two diseases that can be diagnosed with the aid of the acid-fast stain.

_____

_____

V. What is the function of flagella?

_____

_____

VI. Write a brief explanation of why each one of the following bacterial structures requires a "special" staining technique in order to be observed. (Explain why they cannot be demonstrated using a Gram Stain.)

a. capsule

b. endospore

c. acid-fast bacilli

d. flagella

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IDENTIFICATION OF GRAM-POSITIVE COCCI

Objectives: *After completion of these laboratory exercises, the student will be able to:*

1. Name the medically significant Gram-positive cocci.
2. List the media and biochemical tests that are commonly used to identify Gram-positive staphylococci and Gram-positive streptococci.
3. Explain the theory behind the following tests for the identification of Gram-positive staphylococci: mannitol salt agar, catalase, coagulase.
4. Describe the actions of the enzymes catalase and coagulase as they relate to microbial metabolism and pathogenicity.
5. Define hemolysis, hemolysin.
6. List the three types of hemolysis produced by Gram-positive streptocci on blood agar media and describe the appearance of each type.
7. List the medically significant streptococci that produce each of the three types of hemolysis.
8. Explain how the production of hemolysis relates to pathogenicity.
9. Identify the type of hemolysis produced by various species of streptococci on blood agar.

BIOCHEMICAL TESTING FOR THE IDENTIFICATION OF GRAM-POSITIVE COCCI

In a clinical microbiology, specimens from infected patients are cultured, and then pathogens must be distinguished from normal and transient microbiota. Normally, the first step in this identification process is to perform a microscopic examination of the morphology and staining characteristics of the suspected pathogen by performing stains such as the Gram stain. However, the problem is that through a microscope, there is often too much similarity between organisms to rely on microscopic descriptions alone. For example, there are numerous bacterial species that are gram-positive cocci.

Therefore, further testing must be done to identify bacteria. These include the use of selective and differential media, and biochemical tests.

IDENTIFICATION OF STAPHYLOCOCCI

Staphylococci are gram-positive cocci in clusters. After a Gram stain has determined that the organism to be identified is a **gram-positive coccus in clusters**, the tests for identification of ***staphylococci*** can be performed. (Note: other species of bacteria can also have biochemical activity similar to that of the staphylococci, such as production of the enzymes catalase and coagulase; therefore, a test is meaningless with first performing the Gram stain.)

It is important to be able to distinguish *Staphylococcus. aureus* from other staphylococcus species. *Staphylococcus aureus* can be part of the normal flora of the skin and upper respiratory tract, but it is also a potential pathogen. *S. aureus* is one of the most common causes of nosocomial (hospital-acquired) infections. Other species of staphylococci, such as *S. epidermidis* and *S.saprophyticus*, are also part of the normal flora, but are not normally pathogenic.

Biochemical tests used to identify Staphylococci:

Three biochemical tests that are commonly used to isolate, differentiate, and identify *Staphylococci* are:

1. mannitol salt
2. catalase
3. coagulase

1. **Mannitol salt agar (MSA)** is a type of selective and differential medium that can be used to isolate staphylococcus species from a specimen. MSA is **selective** for *staphylococci* because of the high salt content; only *staphylococci* will grow on mannitol salt agar. All other organisms are inhibited. MSA is also **differential** for *staphylococci*: *S. aureus* will cause the agar to turn yellow because of the fermentation of the carbohydrate mannitol in the agar; other species of staphylococci (such as *S. epidermidis*) will not change the color of the agar because they do not ferment mannitol, and it will remain red.

2. *Staphylococci* are capable of producing the enzyme **catalase.** This enzyme can be tested for by mixing the bacteria in question with a drop of **hydrogen peroxide**. If catalase is being produced, the following chemical reaction will occur:

$$2H_2O_2 + \text{bacterium} \xrightarrow{\text{catalase}} 2H_2O + O_2$$

The oxygen that is liberated will produce a bubbling effect.

3. As a potentially pathogenic organism, *S. aureus* produces an invasive enzyme, **coagulase**. This enzyme is capable of coagulating plasma. This clot may protect the bacteria from phagocytosis and isolate them from the body's defenses. Coagulase production can be tested for by mixing the bacteria in question with sterile plasma. This mixture is allowed to incubate at body temperature (37°) for several hours. If the mixture coagulates, the test is positive for coagulase.

** **In summary, *S. aureus* is catalase positive and coagulase positive, with yellow growth on mannitol salt agar.**

Other species of staphylococci, such as *S. epidermidis*, are catalase positive and coagulase negative, with red growth on mannitol salt agar.

MANNITOL SALT AGAR
for the selection and differentiation of *Staphylococcus* species

1. Obtain a mannitol salt agar plate that has been divided into three sections. Label the bottom of the plate with your name, date, course, and section number.

2. Label one section "**A**", the second section "**B**", and the third section "**C**".

3. Aseptically streak out the unknown organism "A" on that third of the plate. Repeat the procedure for unknown organism "B" and "C".

4. Invert the plate and incubate for **24-48 hours at 37°C**.

5. After the incubation period, observe each section of the agar for bacterial growth. *Staphylococci* can tolerate high concentrations of salt and will grow on MSA; other organisms will not grow well, if at all..

6. Also observe each section of the plate for a change in the color of the agar. The presence of a distinct yellow color indicates fermentation of the mannitol sugar by ***S. aureus***. Other staphylococci species will not change the color of the agar.

7. Record your results on the Results Sheet.

8. Discard the used culture plates into the buckets marked "For Plastic Petri Dishes Only".

SLIDE CATALASE TEST
for the detection of *Staphylococcus* species

1. Obtain three clean, glass microscopic slides.

2. Label the first slide "**A**", the second "**B**", and the third "**C**".

3. Aseptically place a drop of **hydrogen peroxide** onto each slide.

4. Using a sterile inoculating needle, aseptically transfer a visible amount of unknown organism "A" to the hydrogen peroxide on slide "A" and mix. Observe for the immediate production of vigorous **oxygen bubbling**, which indicates a **positive catalase test**. Little or no bubbling is a negative catalase test. Record your observation on the Results Sheet.

5. Sterilize your transfer needle and repeat Step #4 for organism "B" and "C" and record your results.

6. Discard the slides in disinfectant.

7. The presence of vigorous oxygen bubbling indicates that the hydrogen peroxide has been broken down by the enzyme catalase. Little or no oxygen bubbling is a negative for catalase activity. **All *Staphylococci* produce strong catalase activity.**

TUBE COAGULASE TEST
for the detection of pathogenic *Staphylococcus aureus*

1. Obtain three (3) small test tubes containing sterile rabbit plasma.

2. Label each tube with a piece of tape with your name, date, course, and section number. Label one tube "**A**", the second tube "**B**", and the third tube "**C**".

3. Using a sterile inoculating loop, transfer a loop-full of unknown organism "A" into tube A.

4. Repeat Step #3 with unknown organisms "B" and "C".

5. Incubate the inoculated plasmas at **37^0C for 6-24 hours**.

6. Observe each tube for coagulation of the plasma by tilting the tube slightly. If the plasma is still liquid, the test is negative for coagulase activity. If the plasma has **coagulated**, it will be semi-solid, and the test is considered **positive for coagulase activity.**

7. Record your results on the Results Sheet.

8. Place the culture tubes into a rack in the corner for "Items to be Autoclaved".

RESULTS SHEET

UNKNOWN ORGANISM:	A	B	C
growth on mannitol salt agar (yes or no)			
color of mannitol salt agar (yellow or red)			
Slide catalase test: bubbles(+) or little/no bubbling (-)			
Tube coagulase test: plasma coagulated(+) or liquid (-)			
IDENTIFICATION OF UNKNOWN ORGANISM:			

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IDENTIFICATION OF STREPTOCOCCI

If a Gram stain performed on a patient's specimen or from a culture shows the presence of **gram-positive cocci in pairs or chains**, this morphology is typical of ***streptococci*.** *Streptococci* are responsible for more infectious disease processes than any other type of bacteria. Therefore, differentiation and identification of *streptococci* is an important step in diagnosis.

There are many different species of *streptococci*, which makes them more difficult to identify. One method for the differentiation of *streptococci* is to divide them into groups based on their action on blood agar. This action is called **"hemolysis",** which means "breakdown of red blood cells". *Streptococci* produce enzymes called **"hemolysins"** that cause this breakdown. The type of hemolysis on blood agar is the most important test in the identification of the different groups of *streptococci*. **The three groups of *streptococci* are:**

1. **beta-hemolytic** *streptococci*
2. **alpha-hemolytic** *streptococci*
3. **non-hemolytic (gamma)** *streptococci*

Beta-hemolytic *streptococci* produce colonies on blood agar that are surrounded by a relatively clear zone of hemolysis in which the red blood cells in the agar are completely lysed. Many serious infections such as pharyngitis, scarlet fever, impetigo, rheumatic fever, and glomerulonephritis are caused by the beta-hemolytic species ***Streptococcus pyogenes*.** Another beta-hemolytic *streptococcus* species, ***Streptococcus agalactiae***, is often the cause of bacterial meningitis in newborns, and can also cause childbirth sepsis. (This is due to the fact that ***S. agalactiae*** is present in the vaginal normal flora of up to 25% of all women.)

Alpha-hemolytic *streptococci* produce colonies on blood agar that are surrounded by a greenish zone of hemolysis, due to the incomplete breakdown of the hemoglobin in the red blood cells. ***Streptococcus pneumoniae*** is an example of a pathogenic alpha-hemolytic *streptococcus*. ***S. pneumoniae*** causes pneumonia, ear infections (otitis media), and meningitis. Other alpha-hemolytic *streptococci* are primarily normal flora, such as ***Streptococcus salivarius*** and ***Streptococcus mutans***, found in the mouth. Collectively, these non-pathogenic streptococci are called **"viridans" strep**.

Gamma or non-hemolytic *streptococci* do not produce any hemolysis on blood agar. ***Enterococcus faecalis*** is an example of a non-hemolytic *streptococcus* that is normally found in the intestinal tract, and is therefore included in a group of streptococci called the **"enterococci".** These enterococci can migrate to other areas of the body to cause conditions such as urinary tract infections or peritonitis.

After determination of the type of hemolysis produced by a *streptococcus* colony on blood agar, further biochemical tests should be performed to identify the species of *streptococcus*. For example, the tests used to identify the various species of beta-hemolytic *streptococci* are different from those used to identify the alpha-hemolytic *streptococci*. The following is a summary of some of the biochemical tests commonly used to identify *streptococcus* species:

Beta-hemolytic Strep	Alpha-hemolytic Strep	Gamma-hemolytic Strep
bacitracin sensitivity	optochin sensitivity	bile esculin hydrolysis
hippurate hydrolysis		growth in 6.5% salt

LANCEFIELD ANTIGENIC GROUP (SEROLOGICAL) TYPING

Beta-hemolytic *streptococci* and enterococci possess chemicals called **CH (carbohydrate) antigens**. The presence and type of CH antigen can be demonstrated by extraction of the antigen from the cell, and reacting it with antibodies specific to each antigen. Lancefield found thirteen different antigenic groups, A-O. Of these, Groups A, B, and D are most commonly implicated in human infections. Groups C, F, and G are also occasionally cultured from patients.

Group	Major Species
A	*S. pyogenes*
B	*S. agalactiae*
D	*E. faecalis** *S. faecium** *S. durans** *S. avium**

(*enterococci)

IDENTIFICATION OF BETA-HEMOLYTIC STREPTOCOCCI

The two most common beta-hemolytic *streptococcal* pathogens are ***Streptococcus pyogenes*** and ***Streptococcus agalactiae***. It is important to differentiate these two beta-hemolytic strep species from other beta-hemolytic strep and from each other for a correct diagnosis.

TESTING FOR BACITRACIN SENSITIVITY

S. pyogenes is sensitive to the antibiotic bacitracin, whereas other beta-hemolytic strep are not.
When a paper disk impregnated with bacitracin is placed on a blood agar plate upon which ***S. pyogenes*** is growing, there will be a **zone of inhibition** around the bacitracin disk where the ***S. pyogenes*** cannot grow. This is a positive test for ***S. pyogenes***.

Observe the demonstration blood agar plates of:

1) beta-hemolytic ***S. pyogenes*** (also known as "Group A" strep by Lancefield typing), sensitive to bacitracin
2) beta-hemolytic strep species that is resistant to bacitracin. (further I.D. required)

In summary: ***S. pyogenes* = beta-hemolytic, sensitive to bacitracin**
resistant to bacitracin = other species of beta-hemolytic *streptococci;* (*further ID required)

If the organism is a beta-hemolytic streptococcus that is resistant to bacitracin, the next step in the identification process is to perform further testing to determine whether it is a Group B strep such as *S. agalactiae* or some other beta-hemolytic strep such as Groups C, F, or G.

TESTING FOR HIPPURATE HYDROLYSIS

To confirm the I.D. of ***S. agalactiae***, the **sodium hippurate hydrolysis** test is often used. Sodium hippurate broth is inoculated with the organism and incubated overnight. The tube is then centrifuged and the supernatant fluid removed. A reagent called **ferric chloride** is added to the supernate and observed for the development of a heavy precipitate, which is a positive test for ***S. agalactiae***. (If little or no precipitate results, the test is negative, and the organism is not ***S. agalactiae***.)

Observe the demonstration of the hippurate hydrolysis test:

1) hippurate (+) ***S. agalactiae*** (Group B strep)
2) hippurate (-) (*further I.D. req.)

In summary:

***S. agalactiae* = beta-hemolytic, bacitracin (R), hippurate hydrolysis (+)**

beta-hemolytic, bacitracin (R), hippurate hydrolysis (-) = other beta-hemolytic streptococcus species (*further I.D. required*.)

IDENTIFICATION OF ALPHA-HEMOLYTIC STREPTOCOCCI

The most common human pathogen in the alpha-hemolytic streptococci group is ***Streptococcus pneumoniae*** (also called the **pneumococcus**). Most other species of alpha-hemolytic strep are usually normal flora of the oral cavity or upper respiratory tract. As a group, these streptococci are called **"viridans" strep**. This group consists of at least ten different known species, including ***S. mutans***, the oral bacteria implicated in the formation of dental caries. To differentiate ***S. pneumoniae*** from the viridans streptococci, one of the biochemical tests often used is the **optochin sensitivity test.**

TESTING FOR OPTOCHIN SENSITIVITY

The optochin sensitivity test is similar to the bacitracin sensitivity test, except that the disk used is impregnated with the chemical optochin. The presence of a **zone of inhibition** around the optochin disk is a presumptive identification of ***S. pneumoniae***.

In summary: **optochin sensitive = *S. pneumoniae***
optochin resistant = possible viridans *streptococci* (*further I.D. required)

Observe the demonstration of the optochin sensitivity tests:

1. alpha-hemolytic, optochin sensitive ***S. pneumoniae***
1. alpha-hemolytic, optochin resistant strep (*further I.D. required.)

IDENTIFICATION OF NON-HEMOLYTIC STREPTOCOCCI

The major pathogens in the non-hemolytic (gamma) *streptococcus* group are the **Group D *enterococci,*** such as ***E. faecalis***, ***S. faecium***, ***S. durans***, and ***S. avium***. The most accurate tests for identification of *enterococci* are the **bile esculin (BE) hydrolysis test and growth in 6.5% salt.**

TESTING FOR BILE ESCULIN HYDROLYSIS

BE media can be made into agar plates or slants. The surface is then inoculated with the suspected organism and incubated for 24-48 hours. If **blackening of the media** occurs, the test is **positive for bile esculin hydrolysis,** and the organism can be identified as part of the group of streptococci called the *enterococci.*

However, some streptococci that are BE + are not *enterococci* species. Therefore, another test must be done to differentiate these strep species from the true *enterococci.* The test used for this purpose is the 6.5% NaC1 tolerance test.

TESTING FOR GROWTH IN 6.5% SALT

The salt can be incorporated into an agar plate or a tube of broth. The media is then inoculated with the strep, incubated for 24-48 hours, and checked for growth. **If growth occurs, the organism is an *enterococcus*.**

In summary: **bile esculin hydrolysis positive, growth in 6.5% salt = *enterococcus* group Group D**
bile esculin hydrolysis positive, no growth in 6.5% salt = non-enterococcus group

Observe the demonstration of the bile esculin hydrolysis and growth in 6.5% salt tests
:

1. non-hemolytic, BE (+), salt (+) Group D *Enterococcus*
2. non-hemolytic, BE (+), salt (-) non-*enterococcus*

Unknown Streptococci RESULTS SHEET

Unknown #	Bacitracin Sensitivity	Hippurate Hydrolysis	Optochin Sensitivity	Bile Esculin Hydrolysis	Growth in 6.5% salt
1					
2					
3					
4					
5					

IDENTIFICATION:

#1 _____

#2 _____

#3 _____

#4 _____

#5 _____

IDENTIFICATION OF GRAM-NEGATIVE COCCI and COCCOBACILLI

If a gram stain performed from a specimen or culture shows the presence of **gram-negative cocci** or **coccobacilli,** this morphology is typical of several genera, including **Neisseriae, Hemophilus, and Moraxella (**formerly **Branhamella)** Most of these bacteria are normal microbiota of the respiratory, digestive, and genitourinary tracts of humans. However, several species are pathogenic, including ***Neisseria gonorrhoeae* (the "gonococcus") and *Neisseria meningitidis* (the "meningococcus"), *Hemophilus influenzae*, and *Moraxella catarrhalis***. These microbes often appear as small, kidney-bean shaped diplococci, often seen inside phagocytes on a smear from a clinical specimen, or as small bacteria that have a typical "in between" morphology called "coccobacilli".

These bacteria grow best on enrichment media such as **chocolate agar** in an increased $\mathbf{CO^2}$ atmosphere. Some are also extremely sensitive to cold; clinical specimens sent to the lab for possible isolation of *Neisseriae* must not be refrigerated. Specimens typically collected for detection of *Neisseriae* include cerebrospinal fluid (CSF), cervical or urethral swabs. *Hemophilus* and *Moraxella* specimens are most often respiratory or eye samples.

Preliminary identification can be done after 24-48 hours of incubation by gram-staining and testing suspicious colonies for **oxidase** activity.

THE OXIDASE TEST

Procedure

1. Grow a culture of the suspected bacteria on chocolate agar.

2. Put a drop of **oxidase reagent** directly onto an area of the plate where there are isolated colonies.

3. Wait up to 60 seconds and observe for a color change to dark purple-black.

Results:

Study Questions:

1. If the Gram stain shows gram negative cocci, and the oxidase test is positive, what genus does this bacterium belong to?

   _____

2. If the Gram stain shows gram negative cocci or coccobacilli, and the oxidase test is negative, what genera might this bacterium belong to?

   _____

IDENTIFICATION OF NEISSERIA SPECIES
USING THE API NH system

The API NH system for identification of ***Neisseria***, ***Hemophilus***, and ***Moraxella*** species consists of microcupules containing dehydrated test medium. The media are rehydrated by filling them with a heavy saline suspension of bacteria. The strip is then incubated and observed for color changes, which indicates the metabolism of the medium.

Procedure:

1. Set up an incubation tray and lid. Dispense tap water into the bottom of the tray using a squeeze bottle, to provide a humid atmosphere. Record the specimen number on the end flap.

2. Open a pouch and remove an API strip. Place the strip into the incubation tray. The strip should be at room temperature before using.

3. Open an ampule of NaCl 0.85% medium. Using a sterile swab, inoculate the sterile saline with bacteria taken from a culture of the suspected bacteria. This inoculum should be taken from a fresh (18-24 hr) culture on recommended media. Transfer enough inoculum into the saline so that a heavy suspension is achieved. The turbidity should be equivalent to or greater than a **No. 4** McFarland standard. Suspensions should be used immediately after preparation.

4. Use a sterile pipette to fill the first seven cupules about 2/3 full with the bacterial suspension. For the last three cups with a box around them, fill the cup all the way up.

5. Cover the first seven cups (those that are underlined) with mineral oil.

6. Place a plastic lid on the tray.

7. Incubate the test strip at **37°C for 2 hours** in aerobic conditions in a non-CO_2 incubator.

Reading the Strip:

1. First, on the result sheet provided, record all reactions as **positive** (+) or **negative** (-) before the addition of reagents. (Refer to the Reading Table provided)

2. Add one drop of **ZYM B** reagent to microcupules **8** and **9** (LIP/ProA and PAL/GGT).

3. Add one drop of **JAMES** reagent to microcupule **10** (BGAL/IND).

4. Wait **three minutes**, and then read these reactions according to the Reading Table, and record on the results sheet.
   Note: If the LIP reaction is blue (+), interpret the ProA reaction as negative, whether the ZYM B reagent has been added or not.

READING TABLE

Test		Color for positive test result
PEN	Penicillinase	yellow, yellow-green, yellow-blue
GLU	Glucose	yellow or orange
FRU	Fructose	"
MAL	Maltose	"
SAC	Saccharose/Sucrose	"
ODC	Ornithine decarboxylase	blue
URE	Urease	pink-violet
LIP	Lipase	blue
PAL	alkaline phosphatase	yellow
BGAL	beta galactosidase	yellow
ProA	proline arylamidase	orange
GGT	gamma glutamyl transferase	dark orange
IND	indole	pink

HINT: If the test reads any color other than that clearly defined as "positive", call it negative.

Interpretation of Test Results:

Identification is obtained with a **numerical profile**. To determine the numerical profile, the test results are divided into groups of three on the results sheet. A value of 1, 2 or 3 is assigned to each of the three tests in the group. By adding the three values together for each group, a 4-digit number is obtained.

Note: do not code the first test (penicillinase)

Example: the first group consists of the tests GLU – FRU – MAL.

Looking up this 4-digit number in the profile list provided or on https://apiweb.biomerieux.com (user name and password required) will give the identification of the organism.

Results:

1. The number of the unknown organism you were assigned:

2. The API NH numerical profile obtained for your organism:

Study Questions:

1. What is the identification of your organism according to the API NH profile index?

   _____

2. Is this organism normal microbiota or a pathogen? If a pathogen, what type of infectious diseases does it cause?

   _____

   _____

RESULTS SHEET

Unknown #	Identification
1	
2	
3	
4	

LIST OF API NH NUMERICAL PROFILES

0001 Neisseria cinerea/gonorrhoeae
0002 Neisseria meningitidis
0010 Branhamella catarrhalis
1001 Neisseria gonorrhoeae
1002 Neisseria meningitidis
1003 Neisseria meningitidis
1020 Haemophilus influenzae
1024 Haemophilus influenzae
1103 Neisseria spp.
1224 Haemophilus influenzae
1420 Haemophilus influenzae
1424 Haemophilus influenzae
1426 Haemophilus influenzae
1620 Haemophilus influenzae
1624 Haemophilus influenzae
1626 Haemophilus influenzae
1720 Haemophilus parainfluenzae/influenzae
3001 Neisseria spp.
3003 Neisseria spp.
3020 Haemophilus influenzae
3024 Haemophilus influenzae
3026 Haemophilus influenzae
3100 Neisseria spp/Haemophilus parainfluenzae
3101 Neisseria spp.
3103 Neisseria spp.
3120 Haemophilus parainfluenzae
3200 Haemophilus somnus
3204 Haemophilus somnus
3220 Haemophilus influenzae
3224 Haemophilus influenzae
3320 Haemophilus parainfluenzae
3324 Haemophilus parainfluenzae/influenzae
3360 Haemophilus parainfluenzae
3420 Haemophilus influenzae
3422 Haemophilus influenzae
3424 Haemophilus influenzae
3426 Haemophilus influenzae
3520 Haemophilus parainfluenzae/influenzae
3524 Haemophilus influenzae/parainfluenzae
3560 Haemophilus parainfluenzae
3620 Haemophilus influenzae
3622 Haemophilus influenzae
3624 Haemophilus influenzae
3626 Haemophilus influenzae
3720 Haemophilus parainfluenzae/influenzae
3724 Haemophilus influenzae/parainfluenzae
3760 Haemophilus parainfluenzae
4002 Neisseria meningitidis
4003 Neisseria meningitidis
5001 Neisseria polysaccharea/spp
5002 Neisseria meningitidis
5003 Neisseria meningitidis
5041 Neisseria lactamica
5060 Haemophilus aphrophilus/paraphrophilus
5103 Neisseria spp.
5120 H.parainfluenzae/aphrophilus/paraphrophilus
5122 H.aphrophilus/paraprophilus/parainfluenzae
5160 H.aphrophilus/paraprophilus/parainfluenzae
5162 Haemophilus aphrophilus/paraphrophilus
5320 Haemophilus parainfluenzae
5324 Haemophilus parainfluenzae
5360 Haemophilus parainfluenzae
5424 Haemophilus influenzae
5520 Haemophilus parainfluenzae
5560 Haemophilus parainfluenzae
5620 Haemophilus influenzae/parainfluenzae
5624 Haemophilus influenzae
5720 Haemophilus parainfluenzae
5724 Haemophilus parainfluenzae
5760 Haemophilus parainfluenzae
7000 Neisseria spp.
7001 Neisseria spp.
7003 Neisseria spp.
7020 Haemophilus spp.
7022 Haemophilus spp.
7024 Haemophilus influenzae/parainfluenzae.
7060 H. aprophilus/paraphrophilus/parainfluenzae
7062 Haemophilus aphrophilus/paraphrophilus
7100 Neisseria spp/Haemophilus parainfluenzae
7101 Neisseria spp.
7103 Neisseria spp.
7120 H.aphrophilus/paraprophilus/parainfluenzae
7122 H.aphrophilus/paraprophilus/parainfluenzae
7124 Haemophilus parainfluenzae
7160 H.aphrophilus/paraprophilus/parainfluenzae
7162 Haemophilus aphrophilus/paraphrophilus
7164 Haemophilus parainfluenzae
7220 Haemophilus parainfleunzae/influenzae
7224 Haemophilus influenzae/parainfluenzae
7260 Haemophilus parainfluenzae
7300 Haemophilus parainfluenzae
7320 Haemophilus parainfluenzae
7322 Haemophilus parainfluenzae
7324 Haemophilus parainfluenzae
7326 Haemophilus parainfluenzae
7340 Haemophilus parainfluenzae
7360 Haemophilus parainfluenzae
7362 Haemophilus parainfluenzae
7364 Haemophilus parainfluenzae
7420 Haemophilus influenzae/parainfluenzae
7424 Haemophilus influenzae
7426 Haemophilus influenzae
7460 Haemophilus parainfluenzae
7500 Haemophilus parainfluenzae
7520 Haemophilus parainfluenzae
7522 Haemophilus parainfluenzae
7524 Haemophilus parainfluenzae/influenzae
7540 Haemophilus parainfluenzae
7560 Haemophilus parainfluenzae
7562 Haemophilus parainfluenzae
7564 Haemophilus parainfluenzae
7620 Haemophilus influenzae/parainfluenzae
7624 Haemophilus influenzae/parainfluenzae
7626 Haemophilus influenzae/parainfluenzae
7660 Haemophilus parainfluenzae
7700 Haemophilus parainfluenzae
7720 Haemophilus parainfluenzae
7722 Haemophilus parainfluenzae
7724 Haemophilus parainfluenzae
7726 Haemophilus parainfluenzae
7740 Haemophilus parainfluenzae
7760 Haemophilus parainfluenzae
7762 Haemophilus parainfluenzae
7764 Haemophilus parainfluenzae

IDENTIFICATION OF ENTEROBACTERIACEAE

Enteric bacteria are gram-negative bacilli (**the Enterobacteriaceae**). They are microbes whose normal habitat is the intestinal tract of humans and other animals, birds, and reptiles. Examples of some of the more common enteric bacilli are ***Escherichia coli, Enterobacter***, ***Salmonella***, and ***Shigella***. Whereas ***E. coli*** and ***Enterobacter*** are usually normal flora, ***Salmonella*** and ***Shigella*** are enteric pathogens. These various genera of enteric bacilli can be differentiated and identified by using selective and differential media and biochemical tests.

Identification of Enteric Bacteria

The API 20E system is a miniaturized version of the conventional test tube procedures for identifying enteric bacteria. The system contains 20 or more different biochemical tests. Each microcupule consists of dehydrated media that is reconstituted by adding several drops of a bacterial suspension. The strip is then incubated at 37ø C for 18-24 hours and read.

Procedure:

PREPARATION OF STRIPS

1. Using aseptic technique, inoculate a tube of sterile water with a loopfull of the organism provided by the instructor.

2. Set up an incubation tray and lid. Dispense tap water into the bottom of the tray using a squeeze bottle, to provide a humid atmosphere.

3. Remove one API strip from the sealed packet and place the strip into the incubation tray. Label the end of the strip.

4. Using a sterile pipette, fill each microtube with the bacterial suspension prepared in step #1.

5. Fill both the microtube and the cupule of the |CIT|, |VP|, and |GEL|.

6. Upon completion of all the inoculations, completely cover the cupule of the ADH, LDC, ODC, H_2S, and URE with mineral oil.

7. Place the plastic lid on the tray and incubate the strip in aerobic conditions at **37^0C for 18-24 hours**.

READING THE STRIPS

1. Record all reactions not requiring the addition of reagents. This will be all tubules **except TDA, VP,** and **IND.** Interpretation of reactions are given in the reading table provided.

2. After recording the above reactions, add **one drop of 10% ferric chloride to the TDA tubule**. The reaction should be immediate.

3. Next, add **one drop of solution A (40% potassium hydroxide) to the VP tubule**. Then add **one drop of solution B (6% alpha-naphthol**). This reaction may take up to 10 minutes.

4. Last, add **one drop of Kovac's reagent to the IND tubule**. This reaction should occur within two minutes.

INTERPRETATION OF RESULTS (IDENTIFICATION)

1. Using a marker, mark the strip off in groups of three tubules.

2. Within each group of three tubules, assign the following numbers:

   tubule #1 = 1
   tubule #2 = 2
   tubule #3 = 4

3. To obtain the identification number for your organism, add up the numbers within each separate group of tubules that corresponds to a positive reaction. For example: API 20E identification number = 5146572

   3. Once the identification number has been obtained, you can look it up in the API 20E Analytical Profile Index or on https://apiweb.biomerieux.com. (user name and password required)

STUDY QUESTIONS:

1. What is the identification of each organism according to the API 20E Analytical Profile Index? (Fill in the chart below)

2. Are these organisms normal enteric microbiota or enteric pathogens? Under what circumstances can they become pathogenic?

   _____

   _____

RESULTS SHEET:

Unknown #	API #	Identification (Genus and species)

API 20E SYSTEM READING TABLE
(Interpretation of reactions)

TUBE	POSITIVE	NEGATIVE
ONPG	yellow	clear/colorless
ADH	red or orange-red	yellow/yellow-orange
LDC	red or orange-red	yellow/yellow-orange
ODC	red or orange-red	yellow/yellow-orange
CIT	turquoise or dark blue	light green or yellow
H2S	blackening of the media	no blackening present
URE	pink or coral (red-orange)	yellow/no pink or coral
TDA	reddish-dark brown	light red-brown or yellow
IND	red	yellow/no red color
VP	pink-red	pale pink or no pink-red color
GEL	diffusion of the black granules throughout the cupule	no diffusion/black granules remain clumped together at the bottom
GLU, MAN, INO, SOR, RHA, SAC, MEL, AMY, ARA	→ yellow or yellow-green (<u>any</u> yellow color is +)	blue or blue green

***NOTE**: The other tests listed after ARA with a dotted line around the cupule (OX, NO2, N2, MOB, McC, OF-O, OF-F) are **optional** and can be used for further differentiations. You will leave these blank.

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IDENTIFICATION OF FUNGI

Objectives: *After completion of this part of the laboratory exercise, you should be able to:*

1. Describe and compare **colony morphology** of yeasts vs. molds vs. bacteria.

2. Describe and compare the **microscopic appearance** of yeasts vs. molds vs. bacteria.

3. Describe and diagram the microscopic features and sporulation of *Aspergillus, Penicillium*, *Rhizopus, Saccharomyces*, *Candida*, *Histoplasma capsulatum*, *Cryptococcus neoformans*, and *Sporothrix schenckii*.

Before beginning this laboratory exercise, read the sections on Fungi in your textbook. Also, refer to the table of Medically Important Fungi at the back of this lab manual.

Materials:

1. Sabouraud's agar culture ***Saccharomyces*** yeast
2. Sabouraud's agar cultures of ***Rhizopus***, ***Aspergillus***, and ***Penicillium*** molds
3. culture of bacteria for comparison purposes
4. sterile water for preparation of wet mount
5. sterile transpettes
6. microscope slides
7. cover slips
8. lactophenol cotton blue stain
9. prepared demonstration slides of various molds and yeasts
10. microscope
11. lens paper and cleaner

Procedure #1: Comparisons of colony (macroscopic) characteristics of molds and yeasts and bacteria

1. Examine the culture plates of ***Aspergillus, Penicillium***, and ***Rhizopus*** molds provided by the instructor. Describe the colonies of these molds. Observe the aerial mycelium and the vegetative mycelium.

2. Examine the culture plates of ***Saccharomyces*** and ***Candida*** (yeasts). Describe the colonies of the yeasts.

3. Examine the culture of bacterial colonies and compare them to the colonies of the molds and yeasts.

4. Write a comparison of the colonies of molds, yeasts, and bacteria.

A. ***Rhizopus*** (mold)

B. ***Aspergillus*** (mold)

C. ***Penicillium*** (mold)

D. ***Candida*** (yeast)

E. ***Saccharomyces*** (yeast)

F. Bacterial Colonies

Procedure #2: Comparison of microscopic characteristics of molds and yeasts

1. Using the ***Saccharomyces*** ("bakers" or "brewers" yeast) cultures provided, prepare a wet mount of yeast cells stained with lactophenol cotton blue. Examine the wet mount under low power and high power magnification. Draw a few cells and describe their morphology. Look for the budding (blastoconidia) (NOTE: the descriptive terms used to describe bacterial morphology, such as streptococcus, do not apply to fungi.)

2. Examine the prepared demonstration slides of the various molds and yeasts provided by the instructor. Draw the structures that you see for each fungus and describe their morphology. Look for hyphae and sporulation. Write a description of the disease processes caused by each.

   For the medically important mycoses, refer to the chart in the back of your lab manual entitled "Summary of Significant Characteristics of Medically Important Fungi."

   You will be required to:
   1. Identify the fungus and specific structures microscopically
   2. Describe the mode of infection and disease process caused by each fungus.
   3. Identify the type of specimen required for identification

3. Write a comparison of the microscopic appearance of yeasts vs. molds vs. bacteria.

Saprophytic fungi:

Saccharomyces under low and high power magnification (10X and 40X)

Rhizopus (10X and 40X)

Penicillium (10X and 40X)

Systemic mycoses:

Histoplasma capsulatum

Mold form

Yeast form

Cryptococcus neoformans

Subcutaneous mycoses:

Sporothrix schenckii

Opportunistic mycoses:

Candida albicans (100X)

Aspergillus (40X)

Pneumocystis carinii cysts in lung tissue (100X)

Procedure #3: Identification of yeast species using the API C AUX system

API 20C AUX is a system for precise identification of frequently encountered yeasts. The system consists of 20 cupules containing dehydrated substrates. The yeast will grow only if it is capable of utilizing that substrate as its sole carbon source.

The reactions are read by comparing them to a control cupule. Identification is obtained by looking up the resulting profile number in the API Index.

1. Set up an incubation tray. Dispense distilled water into the bottom of the tray to provide humid atmosphere.
2. Record the specimen number on the end flap.
3. Open a pouch and remove an API strip from the pouch. Place the strip in to the tray on top of the water-filled wells.
4. Open an ampule of API Suspension Medium of API 8.5 % NaCl medium. Using a sterile pipette, pick up a yeast colony either by suction or by repeated touching. Transfer aseptically to the suspension, creating a turbidity equal to a #2 McFarland standard.
5. Open an ampule of API 20 Medium. Transfer 100 µl of the suspension prepared in step #4.
6. Using a pipette, fill the cupules with the suspension from step #5. Avoid bubbles.
7. Place the lid on the tray and incubate at 30^0C for 48-72 hours.

Reading the Strip:

1. After 48-72 hours, compare growth in each cupule to the "0" cupule which is used as a negative control.
2. A cupule more turbid than the control indicates a positive reaction.

Morphology Test:

1. Determine the presence or absence of hyphae or pseudohyphae using Rice Agar Tween (RAT) medium.
2. This is considered to be the 21st test of the strip. It is recorded as positive if either hyphae or pseudohyphae are present.

Interpretation:

Identification is obtained with a numerical profile. To determine the profile number, the test results are divided into groups of three on the results sheet (see next page). A value of 1, 2, or 4 is assigned to each of the three test in the group. By adding the numbers corresponding to the positive results within each group, a 7 digit numerical profile is obtained.

Look up the numerical profile in the API C AUX Index or on https://apiweb.biomerieux.com. (user ID and password required)

RESULTS

Unknown yeast #1

API profile # = _____

Identification: _____

Unknown yeast #2

API profile # = _____

Identification: _____

Unknown yeast #3

API profile # = _____

Identification: _____

Study Questions:

1. Are these yeast species normal microbiota or are they pathogenic? Under what circumstances could they become pathogenic?

   _____

   _____

2. What is the difference between hyphae and pseudohyphae? Which are usually seen with yeasts?

   _____

   _____

3. How do yeasts reproduce? _____

   _____

4. What type of tests are those used in the API C AUX for identifying yeast species?

   _____

IDENTIFICATION OF PROTOZOA

Before beginning this laboratory exercise, read the sections on Protozoa in your textbook. Also, refer to the table of entitled "Summary of Significant Characteristics of Parasitic Protozoa" at the back of this lab manual.

Draw and describe the microscopic appearance of the following protozoans. Write a description of the disease process caused by each.

You will be required to:

1. Identify the protozoan microscopically
2. Describe the mode of transmission and disease process caused by each protozoan.
3. Identify the type of specimen required for identification

A. ***Entamoeba histolytica*** cysts and trophozoites in feces (oil-immersion)

B. ***Giardia lamblia*** cysts and trophozoites in feces (oil-immersion)

C. ***Trichomonas vaginalis*** trophozoites from vaginal exudate (oil-immersion)

D. ***Trypanosoma*** species hemoflagellate (blood smear on oil-immersion)

E. ***Plasmodium*** species trophozoites (merozoites) (blood smear on oil-immersion)

F. ***Toxoplasma gondii*** trophozoites in tissue (oil immersion)

ARTHROPOD VECTORS

Before beginning this laboratory exercise, read the section in your textbook on arthropod vectors. Also refer to the table on Medically Important Arthropod Vectors at the end of this lab manual.

Examine each vector under the dissecting microscope. You will be required to:

1. recognize the vector
2. name the microorganism the insect vector transmits
3. name and describe the disease process the microbe causes in humans

Anopheles (mosquito)

Xenopsylla (rat flea)

Aedes (mosquito)

Pediculus (louse)

Culex (mosquito)

Triatoma (kissing bug)

Dermacentor (tick)

Glossina (tsetse fly)

Ixodes (tick)

IDENTIFICATION OF HELMINTHS

Before beginning this laboratory exercise, read the section on helminths in your textbook. Also, refer to the table of Medically Important Helminths at the end of this lab manual.

Draw and describe the microscopic appearance of the following helminths.

You will be required to:

1. Identify the helminth microscopically
2. Describe the mode of transmission and disease process caused by each helminth.
3. Identify the type of specimen required for identification

A. ***Trichinella spiralis*** cysts in muscle tissue (10X-40X)

B. ***Schistosoma*** cercaria (10X-40X) and ova (10X-40X)

C. ***Strongyloides*** larva (10X-40X)

D. ***Ascaris lumbricoides*** ova (10X-40X)

E. ***Trichuris trichiura*** ova (10X-40X)

F. ***Enterobius vermicularis*** ova (10X-40X)

G. ***Necator americanus*** ova (10X-40X)

H. ***Taenia*** sp. ova (10X-40X) and proglottid

HELMINTH UNKNOWNS

Using the prepared fecal specimens supplied by the instructor, make a wet mount of each, and examine under low (10X) and high (40X) power for the presence of ova or larvae. Write the scientific name of the parasite on the results sheet, and have it checked by the lab instructor before you leave.

A.

B.

C.

D.

E.

F.

G.

Antibiotic Susceptibility Testing

Objectives: *After completion of this laboratory exercise, the student will be able to:*

1. Demonstrate the activity of certain antibiotics against certain microbes.
2. Show the antibiotic susceptibility patterns of microorganisms that cause human infections.
3. Explain the importance of susceptibility testing in clinical microbiology.

As antibiotics have been used to treat infections over the years, resistant strains of bacteria have developed. The development of **resistance** to an antibiotic involves these processes:

1. **Genetic mutation**: natural selection operates to promote "survival of the fittest": survival of new mutant strains that are resistant to the effects of a particular drug with the old, sensitive bacteria being killed off by the antibiotic.

2. Transfer of a **plasmid** (**the R factor**) to the bacterial cell. The plasmid contains a gene or group of genes causing resistance to an antibiotic. This transfer occurs when resistant bacteria (carrying an R factor) come in contact with sensitive bacteria (do not have a R factor).

In order to choose the proper antibiotic for therapy it is important not only to identify the causative bacterium but to test it for its susceptibility to a variety of antibiotics. The variety of antibiotics to which a given organism is susceptible or resistant is called its **antibiotic susceptibility pattern.** This susceptibility is based on the genetic characteristics of each individual species of microorganism.

Among the variety of tests that are available, the disk-diffusion method (**Kirby-Bauer test**) is probably the simplest to perform and interpret. Discs of filter paper are impregnated with antibiotic solutions in the same range of concentrations obtainable in the human body. These are placed on an agar plate that has been uniformly inoculated with the organism to be tested. The test organisms grow in a smooth "lawn" of growth on the plate except in a clear round zone around each antibiotic disc which inhibits the growth of the organism. This zone indicates the susceptibility of the organism. Bacteria resistant to an antibiotic show little or no inhibition.

You will perform antibiotic susceptibility tests on different bacterial species to a variety of antibiotics. The microorganisms used in this exercise are common (such as *S. aureus*) and the antibiotics used here have been selected because they are widely used. They are not necessarily the most appropriate therapeutic choice.

Materials:

1. 24-hour broth cultures of ***Staphylococcus aureus*** (gram-positive coccus)
   Escherichia coli (gram-negative bacillus)

2. antibiotic discs
3. forceps
4. Mueller-Hinton agar plates
5. sterile cotton swabs
6. MacFarland Standard or spectrophotometer
7. sterile pipettes
8. sterile TSB

Procedure:

1. Using the 24-hour broth culture you were assigned, **standardize** the inoculum by either comparing the turbidity (cloudiness) of your culture to the **MacFarland Standard** provided, or use a spectrophotometer. If the culture is too turbid ,use sterile TSB and a sterile pipette to dilute it until it is the same turbidity as the standard.

2. Using a sterile swab, dip the swab into the standardized bacterial suspension. Spread the bacteria out over the surface of a **Mueller-Hinton agar** plate to create a solid "lawn" of bacteria.

3. Using a lab marker, divide the bottom of the Mueller-Hinton agar plate into four quadrants.

4. Choose four antibiotic discs to test. Make sure that you are using the appropriate type antibiotics for the microorganism you were assigned. For example, penicillin is used to test with ***S.aureus***, a gram-positive bacterium. It should not be used to test with ***E.coli***, a gram-negative bacterium.

5. Label the bottom of each quadrant of the petri dish with the abbreviation (code) of the antibiotic being tested. Also put your name, date, and class section.

6. Dip the forceps into a bottle of alcohol and then hold the forceps in the flame of your Bunsen burner until the alcohol has burned off. This will sterilize the forceps. Allow them to cool before using.

7. With the sterile forceps, remove an antibiotic disc aseptically from its container and place it gently on the surface of the agar in the center of the section labeled for that disc.

8. Tap the disc gently onto the surface of the agar so that it will not fall off when the plate is inverted in the incubator.

9. Reflame the forceps.

10. Place the other disc onto the agar in the same manner. Flame the forceps in between each use. **DO NOT CONTAMINATE THE ANTIBIOTIC VIALS!**

11. Invert the plate and incubate at **37^0C for 24 hours**.

12. After incubation, examine the plate for a **zone of inhibition**. Using a ruler marked in millimeters (mm), measure the diameter of each zone. Be sure to make a note of any colonies growing inside the zone of inhibition. These are called "**satellite colonies**" and indicate the development of a resistant mutation.

Look up each zone measurement in the interpretation chart provided. Record the measurement in the appropriate box on the sample report sheet. (**R= resistant, I=intermediate, S=sensitive**). Zones containing satellite colonies should be recorded as "R" (resistant.)

ZONE SIZE INTERPRETATION CHART

ANTIMICROBIAL DRUG OR ANTIBIOTIC TESTED	INHIBITION ZONE DIAMETER IN MM		
	RESISTANT	INTERMEDIATE	SENSITIVE
	mm or less	mm	mm or more
Ampicillin (AM)			
gram negatives	13	14-16	17
Staphylococci	28		29
Carbenicillin (CB)			
gram negatives	19	20-22	23
Pseudomonas	13	14-16	17
Cephalothin (CR)	14	15-17	18
Ceftiofur (XNL)			
Ceftazidime (CAZ)			
Cefmetazole (CMZ)			
Clindamycin/Lincomycin (CC/L)	14	15-20	21
Erythromycin (E)	13	14-22	23
Gentamycin (GM)	12	13-14	15
Kanamycin (K)	13	14-17	18
Neomycin (N)	12	13-16	17
Nitrofurantoin (FD or F/M)	14	15-16	17
Penicillin (P)			
Staphylococci only	28		29
Tetracycline (Te)	14	15-18	19
Ticarcillin (TIC)			
gram negatives only	14	15-19	20
Tobramycin (NN)	12	13-14	15
Trimethoprim/ Sulfamethoxazole (SXT)	10	11-15	16

Physical Control of Microbial Growth
Ultraviolet Light

Objectives: *After completing this laboratory exercise, the student will be able to:*

1. observe the effects of exposure to ultraviolet radiation as a mutagenic agent.
2. describe the variables that must be controlled in order to use UV irradiation as an effective disinfecting agent

Ultraviolet light is often toxic to bacteria. The DNA in the bacterial cell is distorted by the formation of **thymine dimers**. The mutation that results can take several forms. A different protein may be formed, such as a change in an enzyme, which can produce a different trait. A protein or enzyme may be destroyed, so that it no longer functions and the trait is lost. The most serious result is death of the organism due to a lethal mutation. When attempting to achieve disinfection or sterilization, obviously a lethal mutation is desirable.

The advantages of using UV light for sterilization are its ease of application, and its rapid effect. Therefore, it is widely used in clinical applications such as in hospital operating rooms over instrument trays. However, UV light also has some disadvantages. UV light has very little penetrating power, so that unless the microorganisms are directly exposed to the UV light, they will not be killed. Glass, plastic, and dust can block the penetration of UV light. In addition, UV light can burn the skin and eyes, and must be carefully used around human contact areas.

UV light is also not as effective against bacterial endospores, and some species of bacteria can recover from the damage imposed by UV light. This process is called **photoreactivation**, and can take place if the bacteria are reexposed to light.

Sunlight, since it contains UV light, is harmful to bacteria. That is why drying clothes on a line outside in the sun is more beneficial than using a clothes dryer, and the clothes smell fresher.

Other types of radiation, such as gamma rays, have more energy and greater penetrating power. For example, gamma radiation is used in sterilizing medical supplies. However, they are more dangerous to use than UV light because normal tissue can be damaged.

Materials:

1. 24-hour TSB culture of ***Serratia marcescens***
2. four (4) TSA plates
3. small, sterile tube of TSB
4. sterile pipettes
5. 3 x 5" cards and tape
6. safety glasses
7. ultraviolet lamp
8. sterile cotton swabs

Procedure:

1. Transfer two (2) drops of a 24-hour broth culture of ***Serratia marcescens*** with a sterile pipette to a small tube of sterile TSB. Mix by tapping the tube gently with your fingers.

2. Spread this diluted broth culture over the entire surface of a TSA plate, using streaks back and forth across the entire plate with a cotton swab. Repeat with 3 more TSA plates.

3. Label the lids and bottoms **"30 sec.", "1 min.", "5 min."** and **"10 min."** This indicates the exposure time to be used.

4. Put on safety glasses. If you already wear glasses, use the safety glasses designed to go over your own. These glasses have special UV protective coatings to protect your eyes.

5. Remove the lids of the Petri dishes. Tape an index card over one-half of the agar plate.

6. Have a partner time the exposure. Expose each plate for the time specified (30 sec., 1 min., 5 min. and 10 min.)

7. After exposure, remove the cards, replace the lids, invert the plates, and incubate **in the dark at 25°C** (in your lab drawer) to prevent the possibility of photoreactivation until the next lab period.

8. During the next lab period, examine the plates and record your results.

Results:

0 = no growth
1+ = a few colonies
2+ = moderate # of colonies
3+ = heavy growth (solid)

	30 seconds		**1 minute**		**5 minutes**		**10 minutes**	
Lamp #	covered	exposed	covered	exposed	covered	exposed	covered	exposed

Chemical Control of Microbial Growth
Filter Paper Disc Method for Evaluating Antiseptics

The filter paper disc method is a simple method for evaluating the effectiveness of an antiseptic. In this method, a disc of filter paper is soaked with the antiseptic and placed on a nutrient agar plate that has been streaked with a particular type of organism. The plate is then incubated for 24 hours. If the antiseptic is inhibitory, a clear **zone of inhibition** will surround the disc. The size of the zone is related to the effectiveness of the antiseptic, and therefore can be measured and compared to other substances. In this exercise we will measure the relative effectiveness of various antiseptics against a common inhabitant of the skin and respiratory tract, and a potential pathogen, ***Staphylococcus aureus***.

Materials:

1. four (4) antiseptics
2. sterile filter paper discs
3. TSA plate
4. forceps
5. paper towel
6. a 24-hour broth culture of ***Staphylococcus aureus***
7. sterile cotton swabs

Procedure:

1. With a marker, divide the bottom of the TSA plate into four quadrants and label them with the names of the antiseptics to be used.

2. Label the plate with the name of the organism tested, your initials and section number, and the date.

3. Take a sterile cotton swab and carefully insert the swab using aseptic technique into the 24-hour broth culture that you are assigned. Press the swab against the walls of the tube to remove excess liquid. Streak this swab thoroughly across the entire surface of the TSA plate, making sure that there are no uncovered areas.

4. With sterile forceps, remove one of the paper discs provided and dip it into the antiseptic solution.

5. Blot off any excess liquid on the paper towel. Then place the disc in the center of the quadrant labeled for that particular antiseptic. Tap it gently into place so that it will stick to the surface of the agar. DO NOT PRESS IT INTO THE AGAR.

6. Repeat the procedure for the other three antiseptics.

7. Invert the plate and incubate it at **37°C for 24 hours**.

8. After incubation, measure the zones of inhibition surrounding each disc. Use the ruler marked off in millimeters, and record the zone sizes in **millimeters**, not centimeters or inches. Measure the complete diameter of the zone, from one side of the circle to the other. (This will include the paper disc.)

9. Also note in your results if there are any colonies growing inside the zone of inhibition. These are called **"satellite colonies"** and indicate a resistant mutation has occurred.

RESULTS:

Organism tested:_____

Antiseptic	Zone of inhibition (mm)	Satellite colonies (yes/no)

Study Questions:

1. Which antiseptics were the most effective against this strain of *Staphylococcus aureus*? How do you know this?

   _____

   _____

2. Which antiseptics were the least effective against this strain of *Staphylococcus aureus*? How do you know this?

   _____

   _____

3. Explain "satellite" growth. What does this mean in terms of the effectiveness of the antiseptic?

   _____

   _____

STUDY QUESTIONS

Physical Methods of Microbial Control

1. Give an example of a medical or laboratory use of each of the following to control microbial growth:

   incineration _____

   _____

   pasteurization _____

   _____

   autoclaving _____

   _____

   filtration _____

   _____

   osmotic pressure _____

   _____

   desiccation _____

   _____

2. Is UV light effective in controlling microbial growth? Why or why not, according to your results?

   _____

   _____

3. What length of time gave the most killing, using UV light?

   _____

4. What factors could have affected the outcome of UV treatment? (What variables do you have to control in order for UV light to be an effective killing agent?)

   _____

   _____

   _____

5. What mechanism is responsible for the killing action of UV light?

_____

_____

_____

6. Give a practical application for the use of UV light.

_____

_____

7. For each of the following items, choose the best or most practical method of controlling microbes:

plastic Petri dishes, test tubes, or pipettes packaged inside a plastic wrapping

canned fruits or vegetables

inoculating loop or needle

milk

water inside of a glass container with a screw-cap

beef jerky

a used, soiled paper lab coat

bacteria to be sent through the mail

IMMUNOLOGICAL TESTS FOR IDENTIFICATION OF MICROORGANISMS AND INFECTIOUS DISEASES

An often used and alternative method for identification of microbes and the diseases they cause is to identify them by their antigenic structure, or by the antibodies that are produced against them. **Antigens** are molecular markers that are part of the structure of the microbes themselves. When the body is exposed to these antigens, serum proteins called **antibodies** ("immunoglobulins") are usually produced that will specifically react with these microbial antigens in an attempt to eliminate them. Serum or solutions containing antibodies are called **antisera**. These antigen-antibody reactions are very specific; that is to say, for example, that antibodies produced against *S.aureus* will only react with *S. aureus* and not with other microbial species.

LATEX AGGLUTINATION TEST FOR IDENTIFICATION OF ***STAPHYLOCOCCUS AUREUS***

INTRODUCTION

The latex agglutination procedure is used for the rapid identification of ***S. aureus*** utilizing the detection of protein A in the cell wall. The coagulase test has long been recognized as the principle aid in the identification of ***S. aureus***. This test takes a minimum of four hours to perform, and sometimes as long as 24 hours to become positive. ***S. aureus*** can be differentiated by a rapid slide agglutination procedure using latex particles coated with antibody. When bacteria resembling ***S. aureus*** are mixed with this *S.aureus* antiserum, agglutination of the cells (clumping) that is visible to the naked eye will occur.

MATERIALS

latex reagent (antiserum) (latex particles coated with antibody)
disposable reaction cards
disposable stirring sticks
culture of suspected ***S.aureus***

PROCEDURE

Step 1. Add one-two drops of latex antiserum to a circle on the test card.

Step 2. Using a plastic or wood stirring stick, mix at least 3-5 colonies of suspected ***S. aureus*** in the latex antiserum to achieve an even, **heavy** suspension. Discard the stick in disinfectant.

Step 3. Continue stirring with the stick for 30 seconds and observe for clumping. Discard the used stick and card in disinfectant.

Results:

unknown A _____

unknown B _____

unknown C _____

INDIRECT ELISA TEST FOR IDENTIFICATION OF HIV ANTIBODIES

The **ELISA** test ("**enzyme-linked immunosorbent assay**") is a screening test that is currently used to detect the presence of antibodies to HIV. The procedure is done by placing a drop of blood on a piece of clean filter paper. The sample is placed in a microtiter well that has previously been coated with HIV antigens and allowed to incubate. Any HIV antibodies present in the blood sample will then bind to the antigens on the surface of the well. The well is then rinsed to wash away any unbound antibodies.

The next step is to "visualize" the presence of antigen-antibody complexes attached to the well. This is done by adding a solution of antibodies designed to attach to human immunoglobulins. These "**anti-human IgG antibodies**" will attach to the HIV antibodies that are already bound to the HIV antigens on the well surface. These secondary antibodies have been tagged with an enzyme and are therefore called "**conjugated antibodies**". If there are any HIV antigen-antibody complexes present in the well, the conjugated antibodies will attach to them, creating a "sandwich" with the HIV antibodies in the middle (HIV antigen - HIV antibody - conjugated antibody). The well is then rinsed again; if there are no HIV antibodies in the patient's sample, the conjugated antibodies will be washed away.

The last step is to add a "**substrate-chromagen**" to the well. This substrate will undergo a chemical reaction when it comes in contact with its enzyme, and will change color. If the patient has HIV antibodies, the HIV antigen-HIV antibody complex will be detected when this substrate is added.

NOTE: This test kit that you will be using is a simulation. This kit contains no blood or blood products or HIV. However, as with any chemicals, care should be taken when handling any of the reagents.

PROCEDURE:

1. Obtain a plastic microtiter plate. You will use only the rows labeled with the letter of the serum samples you are to test.

2. Obtain one microtiter pipette for each serum sample. Label each pipette with the letter of the sample.

1. Place **six (6) drops** of serum in the first two wells of the row labeled for that sample.

2. Obtain another pipette and label it for distilled water. Skip the first well and add **six (6) drops** of distilled water to wells #2 thru 7 in each row.
   Since there is undiluted serum in well #1, this is commonly referred to as the **undiluted sample**.
   Since there is an equal amount of water and serum in well #2, this is commonly called a **1:2 dilution.**

3. Using the appropriate serum sample pipette labeled for each row, mix the sample in the second well by gently sucking the solution up and down into the pipette. Then suck the contents of well #2 into the pipette and transfer only **six (6) drops** to well #3. Squirt the remaining solution in the pipette back into well #2.

4. Using the same pipette, mix the contents of well #3 and then transfer **six (6) drops** to well #4. Return the remaining solution back into well #3.

5. Continue this **serial dilution** process until you reach the eighth well. The dilution of antibody in well #7 is **1:64**.

8. Let the plate sit undisturbed for **10 minutes** to allow any antibodies in the serum to react with the antigen in the wells.

9. Label a clean pipette "**conjugate**". Add two drops of conjugate to wells #1-7**.** This simulates the addition of the conjugated antibody-enzyme in the actual ELISA test

10. Let the plate sit undisturbed for **5 minutes** to allow the conjugate to adhere to any antigen-antibody complexes in the well.

11. Label a clean pipette "**chromogen**". Add three drops of **chromogen** to each well. This simulates the addition of the substrate-chromogen in the actual ELISA test.

12. Observe the color change that occurs in each well. A light yellow or clear color is a negative test result. A reddish color is a positive test for HIV antibodies.

CLINICAL LAB REPORT

Date: _____ Technologist: _____

Test: Enzyme Linked Immunosorbent Assay (ELISA) for detection of antibodies to the Human Immunodeficiency Virus (HIV)

Patient A _____ (positive or negative?)

Patient B _____

Patient C _____

Patient D _____

Patient E _____

Patient F _____

Patient G _____

Patient H _____

Study Questions:

After reading the biographical sketches for each of the above patients, which ones did you predict would be positive? Why or why not? Which behaviors are considered to be of the highest risk for HIV infection? Which are the lowest risk?

_____

_____

_____

_____

SUMMARY OF MEDICALLY IMPORTANT ARTHROPOD VECTORS

Scientific name of vector	Type of pathogenic microbe transmitted	Scientific name of pathogen	Disease Process
Anopheles mosquito	protozoan	*Plasmodium*	malaria
Aedes mosquito	viruses	arboviruses	dengue fever, yellow fever
Culex mosquito	viruses	arboviruses	encephalitis
Dermacentor tick	bacteria	*Rickettsia*	Rocky Mountain spotted fever
Ixodes tick	bacteria	*Borrelia*	Lyme disease
Glossinia tsetse fly	protozoan	*Trypanosoma*	African trypanosomiasis (sleeping sickness)
Triatoma kissing bug	protozoan	*Trypanosoma*	Chagas' disease
Pediculus louse (lice)	bacteria	*Rickettsia*	epidemic typhus
Xenopsylla rat flea	bacteria	*Yersinia pestis*	plague

SUMMARY OF SIGNIFICANT CHARACTERISTICS
OF MEDICALLY IMPORTANT FUNGI

*slides available in lab

Classification	Scientific or Common Name	Type of Sporulation	Portal of Entry or Mode of Transmission	Disease or Condition in Humans	Specimen of Choice for Identification
Systemic mycoses:	**Histoplasma capsulatum*	dimorphic fungus 25°C=mold (tuberculated macroconidia) 37^0C=yeast	respiratory inhalation of spores	histoplasmosis	sputum/lung tissue
	**Cryptococcus neoformans*	budding yeast with capsule	respiratory inhalation of spores	cryptococcal meningitis	CSF
Subcutaneous mycoses:	**Sporothrix schenckii*		subcutaneous implantation of spores	sporotrichosis	exudate from draining lesion
Opportunistic Mycoses:	**Pneumocystis carinii*	cysts	respiratory opportunist	pneumonia	sputum, lung tissue
	**Aspergillus*	conidiospores	respiratory, brain	aspergillosis	sputum, tissue
	**Candida albicans*	budding yeast (blastoconidia)	normal microbiota	vaginitis, thrush	throat or vaginal swab

SUMMARY OF SIGNIFICANT CHARACTERISTICS
OF PARASITIC PROTOZOANS/ALGAE
*slides available in lab

CLASSIFICATION by means of locomotion	**PARASITIC REPRESENTATIVE**	**PORTAL OF ENTRY OR MODE OF ENTRY**	**DISEASE OR CONDITION IN HUMANS**	**SPECIMEN OF CHOICE FOR IDENTIFICATION**
Amoebas (pseudopods)	**Entamoeba histolytica*	ingestion of cysts	amoebic dysentery	fresh stool
Flagellates	**Trichomonas vaginalis*	sexual contact	vulvovaginitis	vaginal or urethral discharge
	**Giardia lamblia*	ingestion of cysts	enteritis and diarrhea "backpacker's disease"	fresh stool
Hemoflagellate	**Trypanosoma* species	bite of insect vector (tsetse fly or kissing bug)	African sleeping sickness/S. American Chagas' disease	blood smear
Nonmotile obligate Intracellular parasite	**Plasmodium* species	bite of insect vector (*Anopheles* mosquito)	malaria	blood smear
	**Toxoplasma gondii*	ingestion or inhalation of oocysts (cat feces)	toxoplasmosis	tissue culture serologic tests

A SUMMARY OF THE PARASITIC HELMINTHS

PARASITE	DISEASE	INFECTIVE OR DIAGNOSTIC STAGE	INFECTIVE STAGE/MODE OF TRANSMISSION
PLATYHELMINTHS (flatworms)			
TREMATODA (flukes) *Schistosoma mansoni*	schistosomiasis: liver damage, dysentery	ova in feces; elongated, with a single, lateral spine cercaria (larvae) with forked tail	free-swimming cercaria in fecally contaminated water penetrate skin
CESTODA (tapeworms) *Taenia* species	intestinal involvement	ova or proglottids in feces	ingestion of cysticerus or ova in undercooked beef, pork, or fish
NEMATODA (roundworms): *Ascaris lumbricoides* (roundworm)	intestinal or lung involvement	ova in feces; (oval with thick, course, bumpy outer shell)	ingestion of ova; often in fecally contaminated water or food
Trichuris trichiura (whipworm)	intestinal	ova in feces; (lemon-shaped with bipolar knobs)	same as *Ascaris*
Enterobius vermicularis (pinworm)	intestinal	ova from perianal region by Scotch tape method (asymmetrical oval shape with well-formed larva)	ingestion or inhalation of ova
Necator americanus (hookworm)	intestinal	ova in feces; (rounded with single, thin, transparent shell; larvae not usually seen in feces)	larvae in fecally contaminated soil burrow through skin of bare feet OR ingestion of ova
Strongyloides stercoralis	similar to hookworm	microscopic larva in feces; ova not found in feces	larvae in fecally contaminated soil burrow through skin
Trichinella spiralis	trichinosis muscle involvement	muscle biopsy for encysted larvae; serologic tests	ingestion of larvae in undercooked pork or other meat