LABORATORY EXERCISES

to accompany

MICROBIOLOGY LABORATORY

BIO 225

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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 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 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.

12. 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 pathogenic 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- distinguishes 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** = trypsin 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 distinguish 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 *Escherichia 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 O 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 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, peritoneal/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 posted on Blackboard.

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
(5) appropriate storage temperatures

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 (CO₂) jar for incubation

Procedure:

1. Obtain a throat specimen from your assigned partner's throat or your own with a sterile swab. Place the sterile swab against the back wall of the throat gently and move it up and down.
2. Discard the swab in the biohazard box on the student bench top. Place wrappers in regular trash can.
3. Inoculate a blood agar plate with the throat specimen. Streak it out using the streak plate method.
4. Incubate in a candle jar for increased CO₂ at 37°C for 24-48 hours.
5. Take the previously inoculated, dried out throat swab and inoculate the second BAP. Streak for isolation and incubate in the candle jar at 37°C for 24-48 hours.
6. Record the amount of growth on each plate in the Results and Observations.
THROAT CULTURE RESULTS and OBSERVATIONS

Estimated amount of growth*

<table>
<thead>
<tr>
<th></th>
<th>Fresh culture</th>
<th>Dried culture</th>
</tr>
</thead>
</table>

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

STUDY QUESTIONS: THROAT SWAB

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 amounts 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

8. (1) sterile transfer pipette

Procedure:

1. Disinfect your bench top with the disinfectant provided. Place paper towels in the regular trash can. If a bacteria-related spill is disinfected, place paper towels in the regular trash can.

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. After all transfers are completed, discard pipettes in the biohazard box on the student bench top and place urine sample in the designated area in the back of the lab.

7. 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.

8. Invert the agar plates and incubate the streak plates at 37°C 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:________________________________________________________________________

Once you have observed and described your results, all petri plates may be placed into the large biohazard trashcan at the designated discard area in the back of the room.
## Colony Morphology

<table>
<thead>
<tr>
<th><strong>Shape</strong></th>
<th><strong>Surface</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Circular</td>
<td>- Smooth, Shiny</td>
</tr>
<tr>
<td>Irregular</td>
<td>- Rough</td>
</tr>
<tr>
<td>Punctiform</td>
<td>- Wrinkled</td>
</tr>
<tr>
<td>Rhizoid (branched</td>
<td>- Dry, Powdery</td>
</tr>
<tr>
<td>like roots)</td>
<td>- Mucoid</td>
</tr>
<tr>
<td>Filamentous</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Margin</strong></th>
<th><strong>Elevation</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Entire (smooth)</td>
<td>Flat</td>
</tr>
<tr>
<td>Curled</td>
<td>Raised</td>
</tr>
<tr>
<td>Undulate (wavy)</td>
<td>Convex</td>
</tr>
<tr>
<td>Lobate (lobed)</td>
<td>Umbonate</td>
</tr>
<tr>
<td>Fillamentous</td>
<td>Pulvinate (very convex)</td>
</tr>
</tbody>
</table>
STUDY QUESTIONS: MEDIA AND SPECIMEN HANDLING

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 OF AN ISOLATED COLONY ON A STREAKED PLATE (e.g., TSA) FROM YOUR STREAK PLATES

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 (note that a gram stain can be used on a pure culture or mixed culture from a specimen). 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 is used to kill the bacteria and keep your bacterial smear from washing off the slide during the staining procedure. These smears will be used to perform the Gram Stain.
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 also 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 -) made during previous lab
2. unknown bacteria smear provided by instructor
3. wash bottle (with tap water)
4. rinse bucket
5. clothespins (slide holders)
6. absorbent mat
7. glass marker
8. reagents used in the Gram stain:
   - crystal violet
   - Gram's iodine
   - 95% ethyl alcohol
   - safranin
The Gram Stain Procedure

1. Ensure that the rough-frosted side of the slide is facing up.

2. Secure the slide with clothes pin over the staining bucket and under pad.

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

4. 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.

5. 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.

6. 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.)

7. 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.

8. 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.

9. Rinse with water, remove slide from clothes pin, 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
Revolving nosepiece = used to rotate objective lenses
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 four objectives:

(1) scanning (4X magnification)
(2) low power (10X)
(3) high power (40-45X)
(4) 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 (4X, 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 AND show your instructor before putting your microscope away.
4. After each use, the following steps should be followed:
   a. clean off all lenses and the stage
   b. make sure the light is turned off
   c. lower the condenser and the stage
   d. rotate the 4X or 10X objective into place
   e. wrap the cord around the base
   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 frosted-rough side of the smear is up before placing it on the microscope stage.
3. Use the stage control knobs to move the stained smear into the center of the viewing area over the light source.
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. Since the objectives are parfocal, 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. After viewing the smear on 40X, practice focusing with the oil-immersion objective (100X). Place a drop of immersion oil on the slide, over the area of the smear. Slowly rotate the nose piece to the oil-immersion objective; it should touch the oil.
8. Next, bring the specimen into a fuzzy focus very slowly with the fine focus adjustment knob. The field will come in and out of view quickly.
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 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.

Sketch of A.

Morphology of A: ______________________________________________________
Gram reaction A: _________________________________
Sketch of B.

Morphology of B: ________________________________
Gram reaction of B: ________________________________

Sketch of C.

Morphology of C: ________________________________
Gram reaction of C: ________________________________

Sketch of D.

Morphology of D: ________________________________
Gram reaction of D: ________________________________
STUDY QUESTIONS: MICROSCOPY AND GRAM STAIN

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:

<table>
<thead>
<tr>
<th>Steps</th>
<th>Appearance of bacterial cell after each step (color)</th>
</tr>
</thead>
<tbody>
<tr>
<td>crystal violet</td>
<td>Gram positive cell</td>
</tr>
<tr>
<td>Gram's iodine</td>
<td></td>
</tr>
<tr>
<td>95% alcohol</td>
<td></td>
</tr>
<tr>
<td>safranin</td>
<td></td>
</tr>
</tbody>
</table>

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 is 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 instructor. 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
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, and coagulase.
4. Describe the actions of the enzymes catalase and coagulase as they relate to microbial metabolism and pathogenicity.
5. Define hemolysis and hemolysin.
6. List the three types of hemolysis produced by Gram-positive streptococci 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, numerous bacterial species 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 without first performing the Gram stain.)

**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. It is important to be able to distinguish **Staphylococcus aureus** from other staphylococcus species since **S. aureus** is responsible for most infections and deaths. Other species of staphylococci, such as **S. epidermidis** and **S. saprophyticus**, are also part of the normal flora and are important pathogens. **Staphylococcus saprophyticus** causes the second most UTI infections among women behind **E.coli**. **Staphylococcus epidermidis** is the most common cause of hospital-acquired bacteremia and the principle organism responsible for infections associated with implanted prosthetics and intravascular catheters.
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**. The catalase test is useful in differentiating between the Gram-positive genera Staphylococci (catalase +) and Streptococci (catalase -). If catalase is being produced, the following chemical reaction will occur:

\[
\text{Catalase} \\
2\text{H}_2\text{O}_2 + \text{bacterium} \rightarrow 2\text{H}_2\text{O} + \text{O}_2
\]

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°C) 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.**

Most 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. *Staphylocoeci* 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" then 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°C for 6-24 hours.

6. Observe each tube for coagulation of the plasma by tilting the tube slightly. DO NOT INVERT test tubes, as uncoagulated tubes will spill! 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

<table>
<thead>
<tr>
<th>UNKNOWN ORGANISM:</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>growth on mannitol salt agar (yes or no)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>color of mannitol salt agar (yellow or red)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slide catalase test: bubbles(+) or little/no bubbling (-)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tube coagulase test: plasma coagulated(+) or liquid (-)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDENTIFICATION OF UNKNOWN ORGANISM:</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
STUDY QUESTIONS: STAPHYLOCOCCI

1. Why would you perform a coagulase test on a bacterial isolate that tested positive for mannitol salt agar fermentation?
______________________________________________________________________________________
______________________________________________________________________________________

2. Would a catalase positive test always signify the presence of Staphylococci spp.?
______________________________________________________________________________________
______________________________________________________________________________________

3. What is the medical significance of a coagulase positive test result?
______________________________________________________________________________________
______________________________________________________________________________________
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* is responsible for the majority of community-acquired pneumonia; it also causes ear infections (otitis media), and meningitis. Other alpha-hemolytic *streptococci* are primarily normal flora, such as *Streptococcus salivarius* and *Streptococcus mutans*, each found in the mouth. Collectively, these streptococci are called "viridans" strep. *Streptococcus salivarius* rarely causes disease, whereas *Strep mutans* causes dental caries. Other members of the viridans group can cause endocarditis.

**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:

<table>
<thead>
<tr>
<th>Beta-hemolytic Strep</th>
<th>Alpha-hemolytic Strep</th>
<th>Gamma-hemolytic Strep</th>
</tr>
</thead>
<tbody>
<tr>
<td>bacitracin sensitivity</td>
<td>optochin sensitivity</td>
<td>bile esculin hydrolysis</td>
</tr>
<tr>
<td>hippurate hydrolysis</td>
<td></td>
<td>growth in 6.5% salt</td>
</tr>
</tbody>
</table>

**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.

<table>
<thead>
<tr>
<th>Group</th>
<th>Major Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td><em>S. pyogenes</em></td>
</tr>
<tr>
<td>B</td>
<td><em>S. agalactiae</em></td>
</tr>
</tbody>
</table>
| D     | *E. faecalis*  
|       | *E. faecium*  
|       | *E. durans*  
|       | *E. 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. Three to four drops of distilled water is added to the sodium hippurate tube. From an 18-24 hour culture, a heavy suspension of the organism is placed in the sodium hippurate tube with a standard inoculating loop. Tubes are incubated for 2 hours at 37 degrees C. After the 2-hour incubation period, 2 drops of the Ninhydrin Indicator solution is added to the Hippurate Reagent/organism mixture. Ninydrin acts as an indicator to detect glycine, a byproduct of hippurate Hydrolysis. Tubes are reincubated at 37 degrees C for 30 minutes. Tubes are observed at 10-minute intervals for the appearance of a deep blue/violet color, which is a positive test for *S. agalactiae*. The color change will usually appear in 10-15 minutes after the Ninhydrin Indicator solution has been added. A negative reaction is indicated by a faint blue color or no color change and signifies that 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 \textit{E. faecalis}, \textit{E. faecium}, \textit{E. durans}, and \textit{E. 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 any 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 \textit{enterococci}.

However, some streptococci that are BE + are not \textit{enterococci} species. Therefore, another test must be done to differentiate these strep species from the true \textit{enterococci}. The test used for this purpose is the 6.5\% NaCl 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. \textit{If growth occurs, the organism is an enterococcus.}

In summary: \textbf{bile esculin hydrolysis positive, growth in 6.5\% salt = enterococcus group Group D} \newline \textbf{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 \textit{Enterococcus} \newline 2. non-hemolytic, BE (+), salt (-) non-\textit{enterococcus}
## Unknown Streptococci RESULTS SHEET

<table>
<thead>
<tr>
<th>Unknown #</th>
<th>Bacitracin Sensitivity</th>
<th>Hippurate Hydrolysis</th>
<th>Optochin Sensitivity</th>
<th>Bile Esculin Hydrolysis</th>
<th>Growth in 6.5% salt</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>2</td>
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<td>3</td>
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<td>4</td>
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<tr>
<td>5</td>
<td></td>
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</tr>
</tbody>
</table>

**IDENTIFICATION:**

#1 __________________________ __________________________

#2 _________________________________________________

#3 _________________________________________________

#4 _________________________________________________

#5 _________________________________________________
STUDY QUESTIONS: STREPTOCOCCI

1. Give two examples of Streptococcus spp. that are inhibited in the presence of bacitracin.

______________________________________________________________________________________

2. During prenatal care, a pregnant woman is likely to be tested for what Streptococcus sp.? What test(s) would be used to identify this organism?

______________________________________________________________________________________

______________________________________________________________________________________

3. Gamma hemolytic organisms lack hemolysins. Does this make them less pathogenic compared to organisms that possess hemolysins?

______________________________________________________________________________________

______________________________________________________________________________________

______________________________________________________________________________________
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”. **Neisseria gonorrhoeae** is the etiological agent for the sexually transmitted disease gonorrhea. Over 500,000 new cases of gonorrhea are reported each year in the U.S. **Neisseria meningitidis** is rare in the U.S. (0.5-1.5 per 100,000); however, it is a leading cause of bacterial meningitis in the U.S. **Hemophilus influenzae** and **Moraxella catarrhalis** cause a number of diseases including pneumonia, bacteremia, meningitis, epiglottitis, conjunctivitis, and otitis media.

These bacteria grow best on enrichment media such as **chocolate agar** in an increased 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 (performed by instructor):**

1. Grow a culture of the suspected bacteria on chocolate agar (instructor demo, DO NOT open the lid).

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.

**STUDY QUESTIONS GRAM-NEGATIVE COCCI and COCCOBACILLI:**

1. If the Gram stain shows gram-negative cocci or coccobacilli, and the **oxidase test is positive**, what genera might 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 (Steps 1-7 performed by instructor):**

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-CO₂ incubator.

**Reading the Strip:**

1. You will be provided with two test strips labeled “before reagents” and “after reagents”

2. Record the “before reagents” strip results first for the entire strip using the API NH Key provided to you. This will allow you to record the results for tests “PEN” through “BGAL”.

3. Read “after reagents” test strip only for the last three cupules of “ProA”, “GGT”, and “IND”. These are read after the addition of one drop of **ZYM B** reagent to microcupules 8 and 9 (LIP/ProA and PAL/GGT) and one drop of **JAMES** reagent to microcupule 10 (BGAL/IND), and waiting for 3 minutes. Note that these procedures have already been performed for you.
### READING TABLE

<table>
<thead>
<tr>
<th>Test</th>
<th>Color for positive test result</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEN</td>
<td>yellow, yellow-green, yellow-blue</td>
</tr>
<tr>
<td>GLU</td>
<td>yellow or orange</td>
</tr>
<tr>
<td>FRU</td>
<td>“</td>
</tr>
<tr>
<td>MAL</td>
<td>“</td>
</tr>
<tr>
<td>SAC</td>
<td>“</td>
</tr>
<tr>
<td>ODC</td>
<td>blue</td>
</tr>
<tr>
<td>URE</td>
<td>pink-violet</td>
</tr>
<tr>
<td>LIP</td>
<td>blue</td>
</tr>
<tr>
<td>PAL</td>
<td>yellow</td>
</tr>
<tr>
<td>BGAL</td>
<td>yellow</td>
</tr>
<tr>
<td>ProA</td>
<td>orange</td>
</tr>
<tr>
<td>GGT</td>
<td>dark orange</td>
</tr>
<tr>
<td>IND</td>
<td>pink</td>
</tr>
</tbody>
</table>

**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](https://apiweb.biomerieux.com) (user name and password required) will give the identification of the organism.

**RESULTS SHEET:**

<table>
<thead>
<tr>
<th>Unknown #</th>
<th>Numerical profile</th>
<th>Identification of organism (Genus and Specie)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**STUDY QUESTIONS API NH:**

1. Choose one organism from your results and answer the following questions: Is this organism normal microbiota or a pathogen? If a pathogen, what type of infectious diseases does it cause?

   ____________________________________________

   ____________________________________________
<table>
<thead>
<tr>
<th>API Profile</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>001</td>
<td>Neisseria cinerea/gonorrhoeae</td>
</tr>
<tr>
<td>002</td>
<td>Neisseria meningitidis</td>
</tr>
<tr>
<td>0010</td>
<td>Branhamella catarrhalis</td>
</tr>
<tr>
<td>1001</td>
<td>Neisseria gonorrhoeae</td>
</tr>
<tr>
<td>1002</td>
<td>Neisseria meningitidis</td>
</tr>
<tr>
<td>1003</td>
<td>Neisseria meningitidis</td>
</tr>
<tr>
<td>1020</td>
<td>Haemophilus influenzae</td>
</tr>
<tr>
<td>1024</td>
<td>Haemophilus influenzae</td>
</tr>
<tr>
<td>1103</td>
<td>Neisseria spp.</td>
</tr>
<tr>
<td>1224</td>
<td>Haemophilus influenzae</td>
</tr>
<tr>
<td>1420</td>
<td>Haemophilus influenzae</td>
</tr>
<tr>
<td>1424</td>
<td>Haemophilus influenzae</td>
</tr>
<tr>
<td>1426</td>
<td>Haemophilus influenzae</td>
</tr>
<tr>
<td>1620</td>
<td>Haemophilus influenzae</td>
</tr>
<tr>
<td>1624</td>
<td>Haemophilus influenzae</td>
</tr>
<tr>
<td>1720</td>
<td>Haemophilus parainfluenzae/influenzae</td>
</tr>
<tr>
<td>3001</td>
<td>Neisseria spp.</td>
</tr>
<tr>
<td>3003</td>
<td>Neisseria spp.</td>
</tr>
<tr>
<td>3020</td>
<td>Haemophilus influenzae</td>
</tr>
<tr>
<td>3024</td>
<td>Haemophilus influenzae</td>
</tr>
<tr>
<td>3026</td>
<td>Haemophilus influenzae</td>
</tr>
<tr>
<td>3100</td>
<td>Neisseria spp/Haemophilus parainfluenzae</td>
</tr>
<tr>
<td>3101</td>
<td>Neisseria spp.</td>
</tr>
<tr>
<td>3103</td>
<td>Neisseria spp.</td>
</tr>
<tr>
<td>3120</td>
<td>Haemophilus parainfluenzae</td>
</tr>
<tr>
<td>3200</td>
<td>Haemophilus somnus</td>
</tr>
<tr>
<td>3204</td>
<td>Haemophilus somnus</td>
</tr>
<tr>
<td>3220</td>
<td>Haemophilus influenzae</td>
</tr>
<tr>
<td>3224</td>
<td>Haemophilus influenzae</td>
</tr>
<tr>
<td>3320</td>
<td>Haemophilus parainfluenzae</td>
</tr>
<tr>
<td>3324</td>
<td>Haemophilus parainfluenzae/influenzae</td>
</tr>
<tr>
<td>3360</td>
<td>Haemophilus parainfluenzae</td>
</tr>
<tr>
<td>3420</td>
<td>Haemophilus influenzae</td>
</tr>
<tr>
<td>3422</td>
<td>Haemophilus influenzae</td>
</tr>
<tr>
<td>3424</td>
<td>Haemophilus influenzae</td>
</tr>
<tr>
<td>3426</td>
<td>Haemophilus influenzae</td>
</tr>
<tr>
<td>3520</td>
<td>Haemophilus parainfluenzae/influenzae</td>
</tr>
<tr>
<td>3524</td>
<td>Haemophilus parainfluenzae</td>
</tr>
<tr>
<td>3560</td>
<td>Haemophilus parainfluenzae</td>
</tr>
<tr>
<td>3620</td>
<td>Haemophilus influenzae</td>
</tr>
<tr>
<td>3622</td>
<td>Haemophilus influenzae</td>
</tr>
<tr>
<td>3624</td>
<td>Haemophilus influenzae</td>
</tr>
<tr>
<td>3626</td>
<td>Haemophilus influenzae</td>
</tr>
<tr>
<td>3720</td>
<td>Haemophilus parainfluenzae/influenzae</td>
</tr>
<tr>
<td>3724</td>
<td>Haemophilus parainfluenzae/influenzae</td>
</tr>
<tr>
<td>3760</td>
<td>Haemophilus parainfluenzae</td>
</tr>
<tr>
<td>4002</td>
<td>Neisseria meningitidis</td>
</tr>
<tr>
<td>4003</td>
<td>Neisseria meningitidis</td>
</tr>
<tr>
<td>5001</td>
<td>Neisseria polysaccharea/spp</td>
</tr>
<tr>
<td>5002</td>
<td>Neisseria meningitidis</td>
</tr>
<tr>
<td>5003</td>
<td>Neisseria meningitidis</td>
</tr>
<tr>
<td>5041</td>
<td>Neisseria lactamica</td>
</tr>
<tr>
<td>5060</td>
<td>Haemophilus aphrophilus/paraphrophilus</td>
</tr>
<tr>
<td>5103</td>
<td>Neisseria spp.</td>
</tr>
<tr>
<td>5120</td>
<td>Haemophilus influenzae/aphrophilus/paraphrophilus</td>
</tr>
<tr>
<td>5122</td>
<td>Haemophilus influenzae/aphrophilus/paraphrophilus</td>
</tr>
<tr>
<td>5160</td>
<td>Haemophilus influenzae/aphrophilus/paraphrophilus</td>
</tr>
<tr>
<td>5162</td>
<td>Haemophilus aphrophilus/paraphrophilus</td>
</tr>
<tr>
<td>5320</td>
<td>Haemophilus parainfluenzae</td>
</tr>
<tr>
<td>5324</td>
<td>Haemophilus parainfluenzae</td>
</tr>
<tr>
<td>5360</td>
<td>Haemophilus parainfluenzae</td>
</tr>
<tr>
<td>5420</td>
<td>Haemophilus influenzae/parainfluenzae</td>
</tr>
</tbody>
</table>
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 loopful 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, H₂S, and URE with mineral oil.

7. Place the plastic lid on the tray and incubate the strip in aerobic conditions at 37øC 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

2. 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)

RESULTS SHEET:

<table>
<thead>
<tr>
<th>Unknown #</th>
<th>Numerical profile</th>
<th>Identification of organism (Genus and Specie)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

STUDY QUESTIONS API 20E:

1. Choose one organism from your results and answer the following questions: Are these organisms normal enteric microbiota or enteric pathogens? Under what circumstances can they become pathogenic?

________________________________________________________________________
________________________________________________________________________
## API 20E SYSTEM READING TABLE
(Interpretation of reactions)

<table>
<thead>
<tr>
<th>TUBE</th>
<th>POSITIVE</th>
<th>NEGATIVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ONPG</td>
<td>yellow</td>
<td>clear/colorless</td>
</tr>
<tr>
<td>ADH</td>
<td>red or orange-red</td>
<td>yellow/yellow-orange</td>
</tr>
<tr>
<td>LDC</td>
<td>red or orange-red</td>
<td>yellow/yellow-orange</td>
</tr>
<tr>
<td>ODC</td>
<td>red or orange-red</td>
<td>yellow/yellow-orange</td>
</tr>
<tr>
<td>CIT</td>
<td>turquoise or dark blue</td>
<td>light green or yellow</td>
</tr>
<tr>
<td>H2S</td>
<td>blackening of the media</td>
<td>no blackening present</td>
</tr>
<tr>
<td>URE</td>
<td>pink or coral (red-orange)</td>
<td>yellow/no pink or coral</td>
</tr>
<tr>
<td>TDA</td>
<td>reddish-dark brown</td>
<td>light red-brown or yellow</td>
</tr>
<tr>
<td>IND</td>
<td>red</td>
<td>yellow/no red color</td>
</tr>
<tr>
<td>VP</td>
<td>pink-red</td>
<td>pale pink or no pink-red color</td>
</tr>
<tr>
<td>GEL</td>
<td>diffusion of the black granules throughout the cupule</td>
<td>no diffusion/black granules remain clumped together at the bottom</td>
</tr>
</tbody>
</table>

### GLU
### MAN
### INO
### SOR
### RHA
### SAC
### MEL
### AMY
### ARA

*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.
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°C 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: ______________________________________________
**Procedure #4:** Preparation of a microculture/slide culture for viewing fungi.

Sometimes, to be able to view the type of sporulation of a mold properly, it is necessary to view the mold being studied in its intact, undisturbed state. This is achieved by setting up a microculture or slide culture. A slide culture is a miniature version of a culture, using a small piece of agar placed on a microscope slide. After the slide culture has grown, the slide can be placed directly onto a microscope and observed for growth. The mold may then be seen in its living, undisturbed, growing state.

1. Assemble the materials necessary to prepare a slide culture of one of the molds supplied by your instructor.
   - a. teasing needle
   - b. Sabouraud dextrose agar plate
   - c. small test tube ("cookie cutter")
   - d. slide culture package
   - e. sterile water
   - f. culture plate of a mold

2. Unwrap the slide culture package, but **DO NOT** remove the lid of the Petri dish until you ready. The contents are sterile.

3. Flame the mouth of the small test tube, let it cool, and then use it to cut out a small circle of “Sab” agar from the agar plate.

4. Remove the circle aseptically and transfer it to the center of the microscope slide inside the slide culture dish. The microscope slide should be resting on the wooden sticks above the filter paper in the bottom of the dish. Close the lid when you are done.

5. Using the teasing needle and aseptic technique, cut a very tiny piece of the mold culture and touch it to the top edge on the side of the agar circle. Repeat the procedure on the opposite side of the circle. Replace the lid immediately afterwards.

6. With sterile forceps, place a cover slip on top of the agar circle. Pour a small amount of sterile water into the bottom of the dish so that the filter paper is well soaked, but not swimming. This will make the chamber stay moist for the fungi to grow properly.

7. Incubate the slide culture dish at **room temperature** in your drawer until the next lab period.

8. After the incubation period, remove the microscope slide with its agar circle and cover slip, and place the entire set-up directly onto a microscope. Using **low power** (10X), focus on the edge of the agar circle where the fungus is growing; you should be able to see hyphae and spores. Carefully switch to high power (40X) to see more details. (NOTE: the fungal structures will appear black, since this is not a stained preparation.)

9. After viewing the slide culture, remove it from the microscope.

10. Obtain a clean microscope slide and place 1-2 drops of **lactophenol cotton blue** (LCB) stain in the center.
11. Remove the cover slip from the slide culture with forceps. The underside of the cover slip will have a circular imprint of fungal growth. Place this side down onto the LCB stain.

12. Take the microscope slide with the agar “cookie” left on it, and hold it over the container of disinfectant. Using the forceps, remove the agar circle and drop it into the disinfectant. Remaining will be the microscope slide with a circular imprint of fungal growth.

13. Place 1-2 drops of lactophenol cotton blue stain onto this imprint and cover it with a cover slip.

14. You should now have two LCB-stained preparations of the fungus. Observe both slides under low and high power. Sketch the fungal structures that you see, label the hyphae and spores using proper terminology.

15. Write a description of the morphology of this mold.
**Study Questions Fungi:**

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 sterile pipette to dilute it until it is the same turbidity as the standard. **DO NOT DISCARD the MacFarland 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°C 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.)
<table>
<thead>
<tr>
<th>ANTIMICROBIAL DRUG OR ANTIBIOTIC TESTED</th>
<th>INHIBITION ZONE DIAMETER IN MM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RESISTANT</td>
</tr>
<tr>
<td></td>
<td>mm or less</td>
</tr>
<tr>
<td>Ampicillin (AM)</td>
<td></td>
</tr>
<tr>
<td>gram negatives</td>
<td>13</td>
</tr>
<tr>
<td>Staphylococci</td>
<td>28</td>
</tr>
<tr>
<td>Carbenicillin (CB)</td>
<td></td>
</tr>
<tr>
<td>gram negatives</td>
<td>19</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>13</td>
</tr>
<tr>
<td>Cephalothin (CR)</td>
<td></td>
</tr>
<tr>
<td>Ceftiofur (XNL)</td>
<td></td>
</tr>
<tr>
<td>Ceftazidime (CAZ)</td>
<td></td>
</tr>
<tr>
<td>Cefmetazole (CMZ)</td>
<td></td>
</tr>
<tr>
<td>Clindamycin/Lincomycin (CC/L)</td>
<td>14</td>
</tr>
<tr>
<td>Erythromycin (E)</td>
<td>13</td>
</tr>
<tr>
<td>Gentamycin (GM)</td>
<td>12</td>
</tr>
<tr>
<td>Kanamycin (K)</td>
<td>13</td>
</tr>
<tr>
<td>Neomycin (N)</td>
<td>12</td>
</tr>
<tr>
<td>Nitrofurantoin (FD or F/M)</td>
<td>14</td>
</tr>
<tr>
<td>Penicillin (P)</td>
<td></td>
</tr>
<tr>
<td>Staphylococci only</td>
<td>28</td>
</tr>
<tr>
<td>Tetracycline (Te)</td>
<td>14</td>
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<tr>
<td>Ticarcillin (TIC)</td>
<td></td>
</tr>
<tr>
<td>gram negatives only</td>
<td>14</td>
</tr>
<tr>
<td>Tobramycin (NN)</td>
<td>12</td>
</tr>
<tr>
<td>Trimethoprim/</td>
<td></td>
</tr>
<tr>
<td>Sulfamethoxazole (SXT)</td>
<td>10</td>
</tr>
</tbody>
</table>
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 "5 sec.", "15 sec.", "30 sec." and “90 sec.” 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 (5 sec., 15 sec., 30 sec. and 90 sec.)

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)

<table>
<thead>
<tr>
<th>Lamp #</th>
<th>5 seconds covered</th>
<th>5 seconds exposed</th>
<th>15 seconds covered</th>
<th>15 seconds exposed</th>
<th>30 seconds covered</th>
<th>30 seconds exposed</th>
<th>90 seconds covered</th>
<th>90 seconds exposed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
</tbody>
</table>
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: ______________________________

<table>
<thead>
<tr>
<th>Antiseptic</th>
<th>Zone of inhibition (mm)</th>
<th>Satellite colonies (yes/no)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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
**IMMUNODIFFUSION**

In this exercise you will study a technique of immunology called **immunodiffusion**. Each team should obtain a dropper and a petri dish containing agar. Become familiar with the following procedures before beginning the exercises.

**Making the Wells in the Agar**

Set the petri dish right side up over the pattern below. Remove the dish cover and hold the dropper vertically over one of the circles on the pattern. Squeeze the dropper bulb and gently touch the tip to the surface of the agar. While releasing the bulb, push the pipette tip down through the agar to the bottom of the dish. Lift the pipette vertically; this should leave a straight-walled well in the agar.

**CAUTION:** As the bulb is released, a vacuum pull is exerted on the agar. If this vacuum is not maintained while pushing the pipette in to the agar, hairline fractures can develop in the well which will interfere with the results.

**Filling the Wells**

Each vial of antigen or antibody has a dropper tip. Draw a small amount of solution into the dropper, avoiding air bubbles. Wipe the dropper tip on the inside edge of the vial to remove any excess solution from the tip. Insert the dropper tip to the bottom of the appropriate well and slowly eject solution until the well is filled to, but not above, the surface of the agar. Either underfilling or overfilling a well may cause poor results. Immediately return the dropper to its vial.

**CAUTION:** Do no exchange the droppers or use the dropper of one vial for solution in another vial.

**EXERCISE 1: ANTIBODY-ANTIGEN REACTION IN AGAR**

Place petri dish section over template. Make the four wells indicated and then fill them as listed below. Replace the cover and set the dish at room temperature. Observe your results 16 to 48 hours later. The results are best viewed by holding the dish (without its cover) vertically between the fact and light source and then moving the dish to the side until all glare vanishes. Diagram the results on template below.

<table>
<thead>
<tr>
<th>Well</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bovine Albumin (antigen)</td>
</tr>
<tr>
<td>2</td>
<td>Anti-horse Albumin (antibody)</td>
</tr>
<tr>
<td>3</td>
<td>Anti-bovine Albumin (antibody)</td>
</tr>
<tr>
<td>4</td>
<td>Anti-swine Albumin (antibody)</td>
</tr>
</tbody>
</table>
EXERCISE 2: IDENTIFICATION OF AN UNKNOWN

Set petri dish section over template and make the indicated four wells. Your instructor will prepare an extract for testing. Fill the wells as listed below. Replace the cover and set the dish at room temperature. Observe and record your results 16 to 48 hours later. Diagram the results on template below.

<table>
<thead>
<tr>
<th>Well</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>“Mystery Meat” Extract (antigen)</td>
</tr>
<tr>
<td>2</td>
<td>Anti-horse Albumin (antibody)</td>
</tr>
<tr>
<td>3</td>
<td>Anti-bovine Albumin (antibody)</td>
</tr>
<tr>
<td>4</td>
<td>Anti-swine Albumin (antibody)</td>
</tr>
</tbody>
</table>

QUESTIONS

1. Which serum functions as the antigen in Exercise 1? ______________________
   Exercise 2? ______________________

2. Which antiserum reacted with the antigen in Exercise 1? __________________
   Exercise 2? ______________________

3. According to your test results from Exercise 2, what did the unknown extract contain?
   ______________________
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 **antiserum**. 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.

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

4. 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.

5. 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.

6. 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.

7. 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.

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**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?

____________________________________________________________________________________

____________________________________________________________________________________

____________________________________________________________________________________
<table>
<thead>
<tr>
<th>Scientific name of vector</th>
<th>Type of pathogenic microbe transmitted</th>
<th>Scientific name of pathogen</th>
<th>Disease Process</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Anopheles</em> mosquito</td>
<td>protozoan</td>
<td><em>Plasmodium</em></td>
<td>malaria</td>
</tr>
<tr>
<td><em>Aedes</em> mosquito</td>
<td>viruses</td>
<td>arboviruses</td>
<td>dengue fever, yellow fever</td>
</tr>
<tr>
<td><em>Culex</em> mosquito</td>
<td>viruses</td>
<td>arboviruses</td>
<td>encephalitis</td>
</tr>
<tr>
<td><em>Dermacentor</em> tick</td>
<td>bacteria</td>
<td><em>Rickettsia</em></td>
<td>Rocky Mountain spotted fever</td>
</tr>
<tr>
<td><em>Ixodes</em> tick</td>
<td>bacteria</td>
<td><em>Borrelia</em></td>
<td>Lyme disease</td>
</tr>
<tr>
<td><em>Glossinia</em> tsetse fly</td>
<td>protozoan</td>
<td><em>Trypanosoma</em></td>
<td>African trypanosomiasis (sleeping sickness)</td>
</tr>
<tr>
<td><em>Triatoma</em> kissing bug</td>
<td>protozoan</td>
<td><em>Trypanosoma</em></td>
<td>Chagas’ disease</td>
</tr>
<tr>
<td><em>Pediculus</em> louse (lice)</td>
<td>bacteria</td>
<td><em>Rickettsia</em></td>
<td>epidemic typhus</td>
</tr>
<tr>
<td><em>Xenopsylla</em> rat flea</td>
<td>bacteria</td>
<td><em>Yersinia pestis</em></td>
<td>plague</td>
</tr>
<tr>
<td>Classification</td>
<td>Scientific or Common Name</td>
<td>Type of Sporulation</td>
<td>Portal of Entry or Mode of Transmission</td>
</tr>
<tr>
<td>------------------------</td>
<td>-----------------------------</td>
<td>-------------------------------------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td><strong>Systemic mycoses:</strong></td>
<td>*Histoplasma capsulatum</td>
<td>dimorphic fungus</td>
<td>respiratory inhalation of spores</td>
</tr>
<tr>
<td></td>
<td>*Cryptococcus neoformans</td>
<td>budding yeast with capsule</td>
<td>respiratory inhalation of spores</td>
</tr>
<tr>
<td><strong>Subcutaneous mycoses:</strong></td>
<td>*Sporothrix schenckii</td>
<td>subcutaneous implantation of spores</td>
<td>sporotrichosis</td>
</tr>
<tr>
<td><strong>Opportunistic Mycoses:</strong></td>
<td>*Pneumocystis carinii</td>
<td>cysts</td>
<td>respiratory opportunist pneumonia</td>
</tr>
<tr>
<td></td>
<td>*Aspergillus</td>
<td>conidiospores</td>
<td>respiratory, brain aspergilosis</td>
</tr>
<tr>
<td></td>
<td>*Candida albicans</td>
<td>budding yeast (blastoconidia)</td>
<td>normal microbiota vaginitis, thrush</td>
</tr>
</tbody>
</table>
**SUMMARY OF SIGNIFICANT CHARACTERISTICS OF PARASITIC PROTOZOA/ALGAE**
*slides available in lab*

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

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Disease</th>
<th>Infective or Diagnostic Stage</th>
<th>Infective Stage/Mode of Transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Platyhelminths (Flatworms)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Trematoda (Flukes)</strong></td>
<td>schistosomiasis: liver damage, dysentery</td>
<td>ova in feces; elongated, with a single, lateral spine cercaria (larvae) with forked tail</td>
<td>free-swimming cercaria in fecally contaminated water penetrate skin</td>
</tr>
<tr>
<td><em>Schistosoma mansoni</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cestoda (Tapeworms)</strong></td>
<td>intestinal involvement</td>
<td>ova or proglottids in feces</td>
<td>ingestion of cysticerus or ova in undercooked beef, pork, or fish</td>
</tr>
<tr>
<td><em>Taenia</em> species</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Nematoda (Roundworms):</strong></td>
<td>intestinal or lung involvement</td>
<td>ova in feces; (oval with thick, course, bumpy outer shell)</td>
<td>ingestion of ova; often in fecally contaminated water or food</td>
</tr>
<tr>
<td><em>Ascaris lumbricoides</em> (Roundworm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Trichuris trichiura</em> (Whipworm)</td>
<td></td>
<td>ova in feces; (lemon-shaped with bipolar knobs)</td>
<td>same as <em>Ascaris</em></td>
</tr>
<tr>
<td><em>Enterobius vermicularis</em> (Pinworm)</td>
<td></td>
<td>ova from perianal region by Scotch tape method (asymmetrical oval shape with well-formed larva)</td>
<td>ingestion or inhalation of ova</td>
</tr>
<tr>
<td><em>Necator americanus</em> (Hookworm)</td>
<td></td>
<td>ova in feces; (rounded with single, thin, transparent shell; larvae not usually seen in feces)</td>
<td>larvae in fecally contaminated soil burrow through skin of bare feet OR ingestion of ova for <em>Ancylostoma duodenale</em></td>
</tr>
<tr>
<td><em>Strongyloides stercoralis</em></td>
<td>similar to hookworm</td>
<td>microscopic larva in feces; ova not found in feces</td>
<td>larvae in fecally contaminated soil burrow through skin</td>
</tr>
<tr>
<td><em>Trichinella spiralis</em></td>
<td>trichinosis</td>
<td>muscle biopsy for encysted larvae; serologic tests</td>
<td>ingestion of larvae in undercooked pork or other meat</td>
</tr>
<tr>
<td></td>
<td>muscle involvement</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>