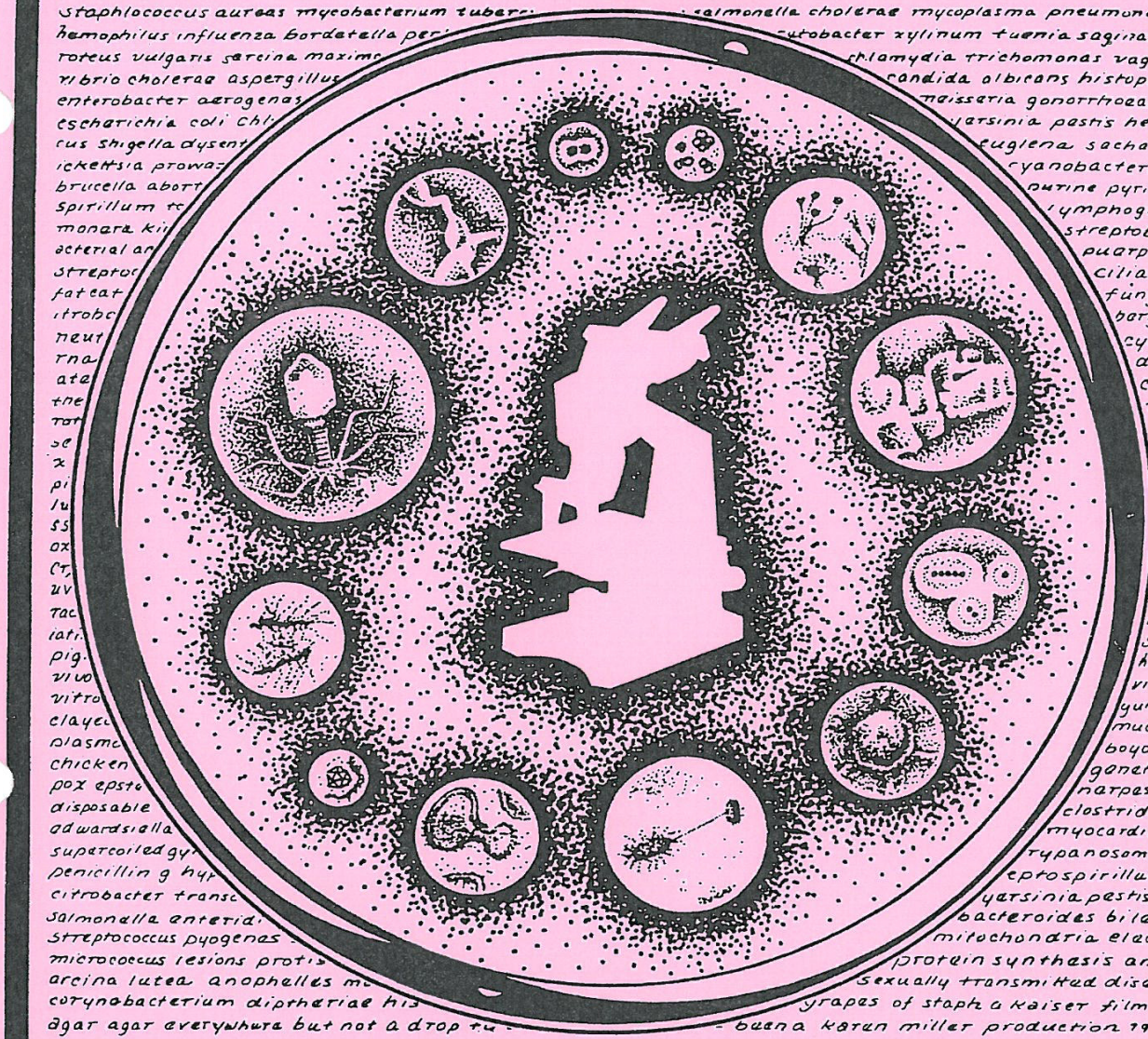

THE GRAPES OF STAPH

a microbiology laboratory manual

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PROFESSOR of BIOLOGY

THE CATONSVILLE CAMPUS OF
THE COMMUNITY COLLEGE OF BALTIMORE COUNTY

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PREFACE

This laboratory manual has evolved over the years to meet the needs of students taking microbiology at the Catonsville Campus of The Community College of Baltimore County. The intention is to provide the student with an organized, user-friendly tool to better enable him or her to understand laboratory aspects of microbiology as well as to hopefully make learning laboratory material and preparing for lab quizzes a bit easier.

Each lab exercise is set up as a complete module that demonstrates some microbiological principle or technique. Each exercise begins with a detailed **Discussion** that provides all the information needed to understand that lab and has key words and phrases presented in **boldface**. The discussion section is followed by a detailed step-by-step **Procedure**, again with key elements presented in boldface. The **Results** section of each exercise is where the student records important results and conclusions that will enable him or her to prepare for the practical portion of each lab quiz. There is also an **online version of this manual** which has the added advantage of providing students with a **complete set of color photographs and photomicrographs of the results for each laboratory exercise** to use in reviewing and studying for quizzes, as well as **concept maps and self quizzes** for each laboratory exercise. The URL for the online lab manual is

http://faculty.ccbcmd.edu/~gkaiser/index.html.

Finally, each lab exercise ends with a set of **Performance Objectives** that tells the student exactly what he or she is responsible for on lab quizzes.

The lab exercises are designed to give the student "hands-on" laboratory experience to better reinforce certain topics discussed in lecture as well as to present a number of selected microbiological principles not covered in lecture. The first several labs enable the student to master techniques essential in working with and studying microorganisms. The next series covers microbial morphology. A number of labs deal with the laboratory isolation and identification of common opportunistic and pathogenic microorganisms as well as other techniques used in the diagnosis of infectious diseases; several of these labs revolve around a problem-solving case study. Two of the labs pertain to the control of microorganisms. Near the end of the course there is a final problem-solving group project that revolves around a case study and applies knowledge and lab procedures learned during the course.

I hope you enjoy this laboratory manual and hope it makes your study of microbiology a bit easier. Keep in mind that the labs are meant to be informal and your instructor is more than willing to answer any questions on either lab or lecture topics.

Gary Kaiser
2018

ACKNOWLEDGEMENTS

I would like to extend my deepest thanks to my co-worker and treasured friend Dr. David R. Jeffrey, for his many comments, suggestions, and support during the various editions of this manual, and for the many hours he has spent in editing. Also, special thanks for encouraging me over these many years I have known him to maintain some degree of sanity in this sometimes crazy world.

Continuous thanks to another friend and co-worker Ms. Emily Plasterer whose efforts and ability assure that the microbiology labs always run smoothly.

A special thanks to Ms. Karen Miller for her great cover to this lab manual.

I would like to thank my past, current, and future students in my microbiology classes. You always seem to make coming to work each day a pleasure.

Also, my complete love and heartfelt thanks is given to my wonderful wife and colleague Dr. Sonja Schmitz. You are my dream-maker!

Finally, thanks to microorganisms everywhere --- for without their existence, I'd be out of a job and forced to join the "real world."

Gary E. Kaiser

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INTRODUCTION

A. Using Performance Objectives

This manual contains **performance objectives** for each of the 20 lab exercises covered in BIOL 230. The objectives tell exactly what you are expected to perform after the completion of each lab exercise.

When verbs such as "define," "state," "discuss," "describe," or "differentiate" are used in the objective, you will be expected to "perform" that objective by way of short answer, multiple choice, or matching questions on a pre-announced quiz. When verbs such as "demonstrate" or "perform" are used, you will be expected to demonstrate a particular technique or procedure to the instructor during the course of the laboratory exercise. When verbs such as "recognize" or "interpret" are used, you will be expected to give a written interpretation of the results of an experiment when given these results in either a written form or a practical form.

As a general rule, when the objective falls under the discussion sections of a lab exercise, it will be tested by means of short answer, multiple choice, or matching questions. When an objective falls under the procedure section of a lab exercise, it represents a procedure or technique that must be mastered during the course of the lab period. Finally, when an objective is found under the results section of an exercise, it will most likely be tested for by a practical question.

B. Laboratory Rules

For the safety and convenience of everyone working in the laboratory, it is important that the following laboratory rules are observed **at all times**:

1. **Anyone working with bacterial cultures, chemicals, and/or projectile and sharps hazards must wear:**
    a. **Approved (ANSI Z87.1) safety goggles;**
    b. **Gloves; and**
    c. **A lab coat buttoned all the way to the bottom**.

Goggles and gloves will be provided in the lab but you must purchase your own lab coat at the college bookstore. **Lab coats cannot be removed from a microbiology lab. They will be stored in the lab and must be discarded at the end of the semester**.

2. **Place only those materials needed for the day's laboratory exercise on the bench tops.** Purses, coats, extra books, etc., should be placed in the lab bench storage areas or under the lab benches in order to avoid damage or contamination.

3. Since some of the microorganisms used in this class are pathogenic or potentially pathogenic (opportunistic), it is essential to **always follow proper aseptic technique in handling and transferring all organisms**. Aseptic technique will be learned in Laboratory 2.

4. **No smoking, eating, drinking, or any other hand to mouth activity while in the lab.** If you need a short break, wash or sanitize your hands and leave the room.

5. If you should **spill a culture**, observe the following procedures:

   a. **Immediately place the culture tube in the plastic baskets found in the hood in the back of the room so no one else touches the contaminated tube**.

   b. **Have your partner place paper towels over the spill and spray liberally with lysol. After 10 minutes, place the paper towels in the biohazard container**.

   c. **Both you and your partner wash your hands with disinfectant soap and sanitize your hands**.

   d. **Notify your instructor** of the spill.

6. Report any cuts, burns, or other injuries to your instructor.

7. **Use only the provided pens or pencils in the lab**. Any pen or pencil you use during the lab should be wiped down with lysoll before returning them.

8. Using a **wax marker**, properly **label all inoculated culture tubes or Petri plates** with the name or the **initials of the microorganism** you are growing, your initials or a group symbol, and any other pertinent information. It is important to know what microorganisms are growing in each tube or on each plate.

9. **Place all inoculated material only on your assigned incubator shelf**, the shelf **corresponding to your lab section**. **Culture tubes** should be stored upright in **your dedicated test tube rack**, while **Petri plates should be stacked and incubated upside-down** (lid on the bottom) in the Petri plate hohder.

10. Tie back long hair.

11. Do not touch the face, apply cosmetics, adjust contact lenses, or bite nails.

12. Handle all glassware carefully. **Notify your instructor of any broken glassware** (culture tubes, flasks, beakers, etc.) or microscope slides. **DO NOT PICK UP BROKEN GLASSWARE WITH YOUR HANDS! Use the dust pan and brush**. **All broken glassware must be disposed of in one of the two biohazard disposal containers** located in the front of the room and under the hood.

13. **Use caution around the Bunsen burners.** In a crowded lab it is easy to lean over a burner and ignite your hair or clothing. Keep the burner well away from the edge of the lab bench to avoid hair or clothing from contacting the flame.

14. Always **clean the oil from of the oil immersion lens of the microscope with a piece of lens paper** at the completion of each microscopy lab.

15. **You must wear shoes that cover the tops of your feet** to prevent injury from broken glass, spilled chemicals, and dropped objects. **Sandals are not permitted in the lab!**

16. When doing any lab where microbes are being stained for viewing with a microscope, **make sure the used dye in the staining tray is poured into the waste dye collection container, not down the sink**.

17. Return all equipment, reagents, and other supplies to their proper places at the end of each lab period.

18. **Disinfect the bench top** with **lysol before and after** each lab period.

19. **Always wash your hands with disinfectant soap and/or sanitize them with hand sanitizer before leaving the laboratory.**

20. Do not run in the laboratory. Avoid horseplay.

21. To avoid contamination and damage, **do not use cell phones or other personal media devices in the laboratory**.

22. Please read the laboratory exercises and **follow the laboratory directions carefully**.

23. **Do not place regular trash such as Kim wipes and paper towels in the biohazard containers**.

IN CASE OF EMERGENCY, CONTACT CAMPUS SECURITY AT (443) 840-1111 and describe the situation and your location. **In a medical emergency, first call 911** and then campus security.

Students who engage in any actions that may damage college property, create an unsafe condition, injure another person, or result in a disruption that interferes with learning may have any, or a combination of the following sanctions imposed as determined by the instructor:

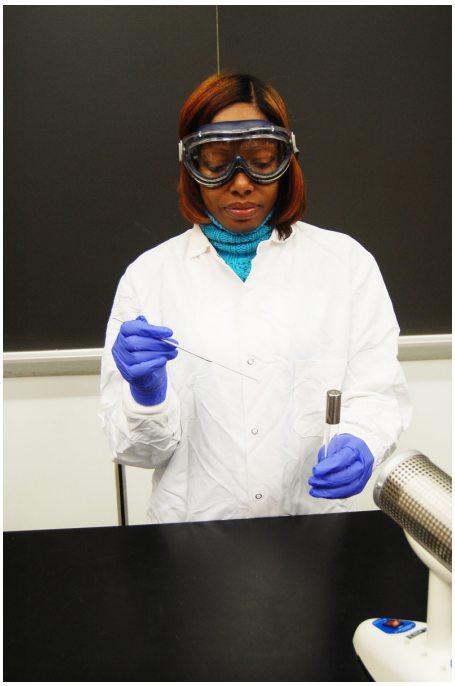
a. A verbal or written warning;
b. Being directed to leave the class for the remainder of the period;
c. A referral to either the Campus Ombudsman or the Department Chairperson;
d. Suspension from the class or the college.

Please see Code of Conduct in the most recent Student Handbook.

C. Personal Protection Equipment (PPE)

The following personal protection equipment must be worn in all microbiology labs where chemicals, stains, microbiological cultures, and glassware or microscope slides are used:

1. A full length lab coat that is fully buttoned to avoid contamination of clothing and skin.



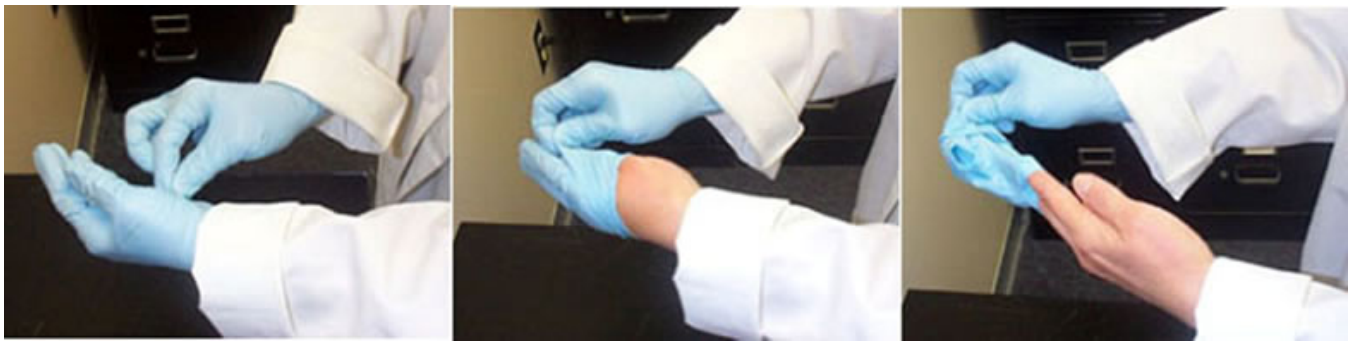
2. OSHA recommended safety goggles ANSI Z87.1 splash goggles. These will be provided in lab.

3. Gloves. These will be provided in lab.

4. At the end of lab, gloves must be removed safely and disposed of only in the designated biohazard containers. Do NOT dispose of gloves in the trash can!

Safe removal of gloves:

a. Pinch the palm of the first glove and remove by pulling the glove inside out as shown below.



b. Hold the first glove with other hand, slide your fingers inside the second glove and turn it inside out as it is removed.

c. **Dispose of the gloves in the correct biohazard container, NOT in the trash can**.

D. General Directions

1. **Anyone working with bacterial cultures, chemicals, and/or projectile and sharps hazards must wear:**
   a. **Approved (ANSI Z87.1) safety goggles;**
   b. **Gloves; and**
   c. **A lab coat buttoned all the way to the bottom**.

Goggles and gloves will be provided in the lab but you must purchase your own lab coat at the college bookstore. **Lab coats cannot be removed from a microbiology lab. They will be stored in the lab and must be discarded at the end of the semester**.

2. Always familiarize yourself in advance with the exercises to be performed.

3. Disinfect the bench tops with lysol before and after each lab.

4. The first part of each lab period will be used to complete and record the results of prior experiments. When you come into the lab, always pull out and organize any culture tubes or petri plates you have in the incubator from previous labs. We will always go over these results as a class.

5. The latter part of each lab period will be used to begin new experiments. Preliminary instructions, demonstrations, and any changes in procedure will be given by your instructor prior to starting each new lab exercise.

6. After completing an experiment, dispose of all laboratory media and contaminated materials in the designated areas as described above.

7. Sanitize your hands or wash them with soap and water for 20 seconds before leaving the lab.

E. Binomial Nomenclature

Microorganisms are given specific scientific names based on the **binomial** (two names) system of nomenclature. The first name is referred to as the **genus** and the second name is termed the **species**. The names usually come from Latin or Greek and describe some characteristic of the organism.

To correctly write the scientific name of a microorganism, the first letter of the genus should be capitalized while the species name should be in lower case letters. Both the genus and species names are **italicized or underlined**. Several examples are given below.

Bacillus subtilus

Bacillus: L. dim. noun *Bacillum*, a small rod
subtilus: L. adj. *subtilus*, slender

Escherichia coli

Escherichia: after discoverer, Prof. Escherich
coli: L. gen. noun *coli*, of the colon

Staphylococcus aureus

Staphylococcus: Gr. noun *Staphyle*, a bunch of grapes; Gr. noun *coccus*, berry
aureus: L. adj. *aureus*, golden

F. Metric Length and Fluid Volume

The study of microorganisms necessitates an understanding of the metric system of length. The basic unit of length is the **meter** (m), which is approximately 39.37 inches. The basic unit for fluid volume is the **liter** (l), which is approximately 1.06 quarts. The prefix placed in front of the basic unit indicates a certain fraction or multiple of that unit.

The most common prefixes we will be using are:

centi (c) = 10^{-2} or 1/100
centimeter (cm) = 10^{-2} m or 1/100 m

milli (m) = 10^{-3} or 1/1000
millimeter (mm) = 10^{-3} m or 1/1000 m
milliliter (ml) = 10^{-3} l or 1/1000 l

Introduction

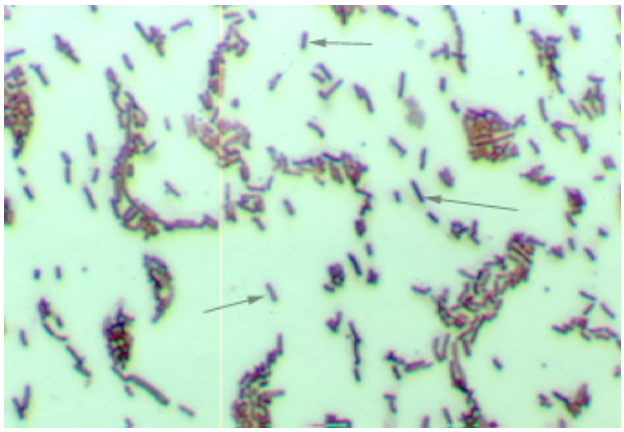
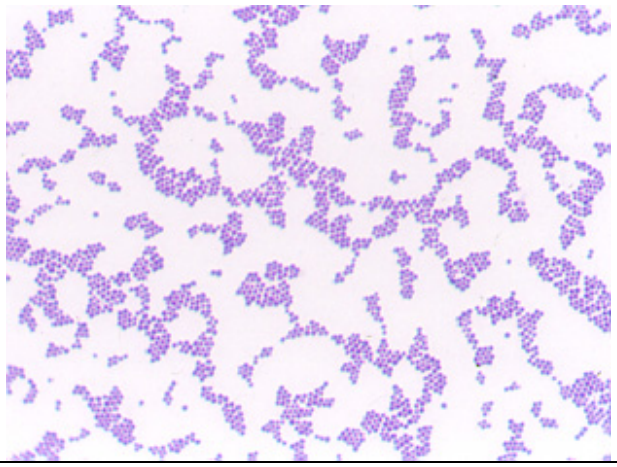
micro (μ) = 10^{-6} or 1/1,000,000
micrometer (μm) = 10^{-6} m or 1/1,000,000 m
microliter (μl) = 10^{-6} l or 1/1,000,000 l

nano (n) = 10^{-9} or 1/1,000,000,000
nanometer (nm) = 10^{-9} m or 1/1,000,000,000 m

In microbiology, we deal with extremely small units of metric length (micrometer, nanometer). The main unit of length is the micrometer (μm) which is 10^{-6} (1/1,000,000) of a meter or approximately 1/25,400 of an inch.

The average size of a bacillus-shaped (cylindrical) bacterium (see Fig. 1) is 0.5-1.0 μm wide by 1.0-4.0 μm long. An average coccus-shaped (spherical) bacterium (see Fig. 2) is about 0.5-1.0 μm in diameter. A volume of one cubic inch is sufficient to contain approximately nine trillion average-sized bacteria. It would take over 18,000,000 average-sized cocci lined up edge to edge to span the diameter of a dime!

In several labs we will be using pipettes to measure fluid volume in ml.

Fig. 1: A Bacillus or Rod-Shaped Bacterium	**Fig. 2:** A Coccus-Shaped Bacterium
	
Escherichia coli, a rod-shaped bacterium.	*Staphylococcus aureus*, a coccus-shaped bacterium.

G. Using the Microscope (Olympus Model CX31 Microscope)

1. **Moving and transporting the microscope**

Grasp the arm of the microscope with one hand and support the base of the microscope with the other. Handle the microscope gently!

2. Before you plug in the microscope, turn the **light intensity control dial** on the right side of the microscope **to 1** (see Fig. 3). Now plug in the microscope and use the on/off switch on the right-hand side of the microscope to turn it on. Make sure the entire cord is on the bench top and not hanging down where it could be caught by a leg. **Adjust the light intensity control dial to 6** (see Fig. 3).

3. **Adjusting the eyepieces**

These microscopes are binocular, that is, they have 2 ocular lenses or eyepieces (see Fig. 4). To adjust them, first find the proper distance between your eyes and the eyepieces by closing one eye and slowly moving your head toward that eyepiece until you see the complete field of view - about 1 inch away. Keep your head steady and both eyes in the same plane. Now open the other eye and gradually raise or lower the other eyepiece until it matches the distance between your eyes. At the correct distance you will see one circular field of view with both eyes.

4. **Positioning the slide**

Place the slide specimen-side-up on the stage so that the specimen lies over the opening for the light in the middle of the stage. Secure the slide **between** - not under- the slide holder arms of the mechanical stage (see Fig. 3). The slide can now be moved from place to place using the 2 control knobs located under the stage on the right of the microscope (see Fig. 3).

5. **Adjusting the illumination**

a. Adjust the **light intensity** by turning the **light intensity control dial** located on the right-hand side of the microscope (see Fig. 3). For oil immersion microscopy (1000X) set the light on 6. At lower magnifications less light may be needed.

b. Adjust the **amount of light coming through the condenser** using the **iris diaphragm lever** located under the stage in the front of the microscope (see Fig. 3A). Light adjustment using the iris diaphragm lever is critical to obtaining proper contrast. For oil immersion microscopy (1000X), the iris diaphragm lever should be set at approximately 0.9 (most of the way to the left.). For low powers such as 100X the iris diaphragm lever should be set mostly closed (to your right for minimum light).

c. The **condenser height control** (the single knob under the stage on the left-hand side of the microscope) should be set so the condenser is all the way up.

6. **Obtaining different magnifications**

The final magnification is a product of the 2 lenses being used. The **eyepiece or ocular lens** magnifies **10X**. The **objective lenses** (see Fig. 4) are mounted on a turret near the stage. The small **yellow-striped lens** magnifies **10X**; the **blue-striped lens** magnifies **40X**, and the **white-striped oil immersion lens** magnifies **100X**.

Final magnifications are as follows:

ocular lens	X	objective lens	=	total magnification
10X	X	4X (red)	=	40X
10X	X	10X (yellow)	=	100X
10X	X	40X (blue)	=	400X
10X	X	100X (white)	=	1000X

Fig. 3A: Olympus CX31 Microscope Iris Diaphragm

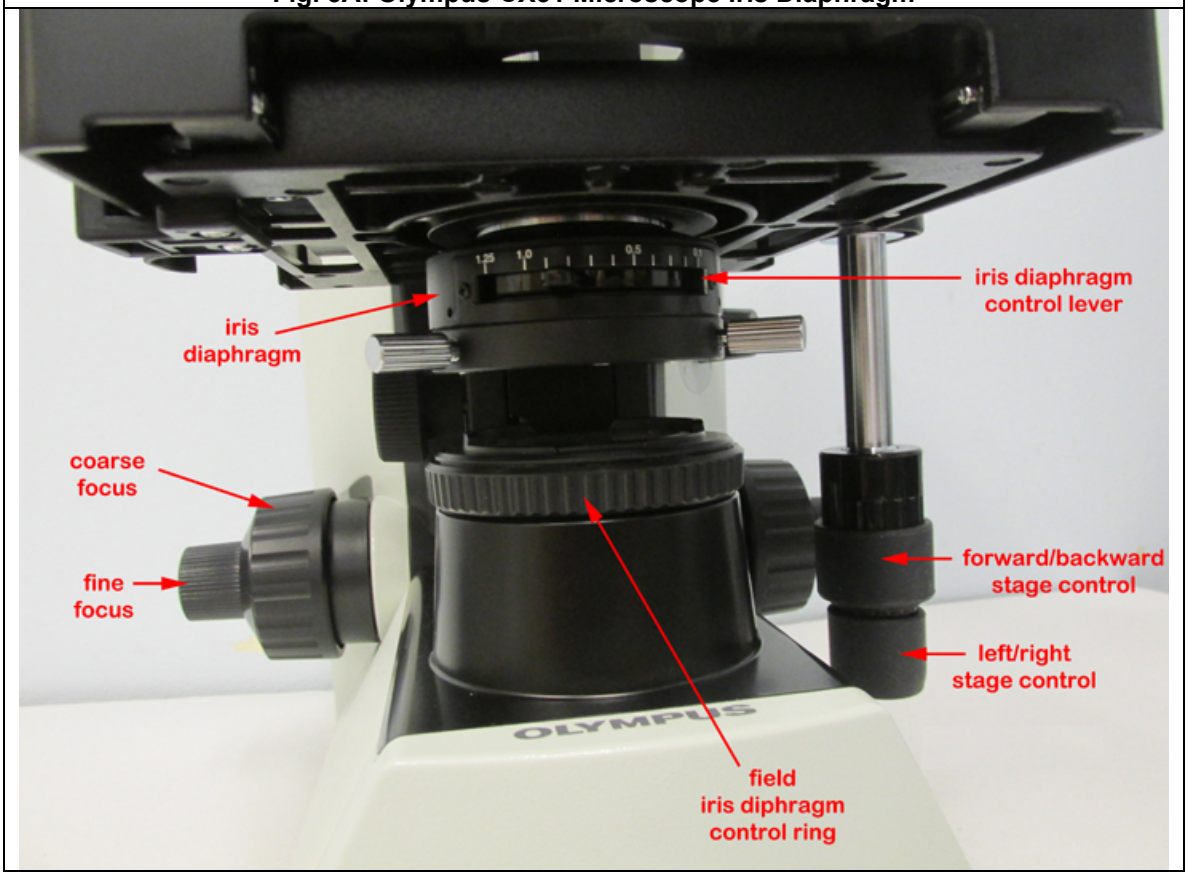
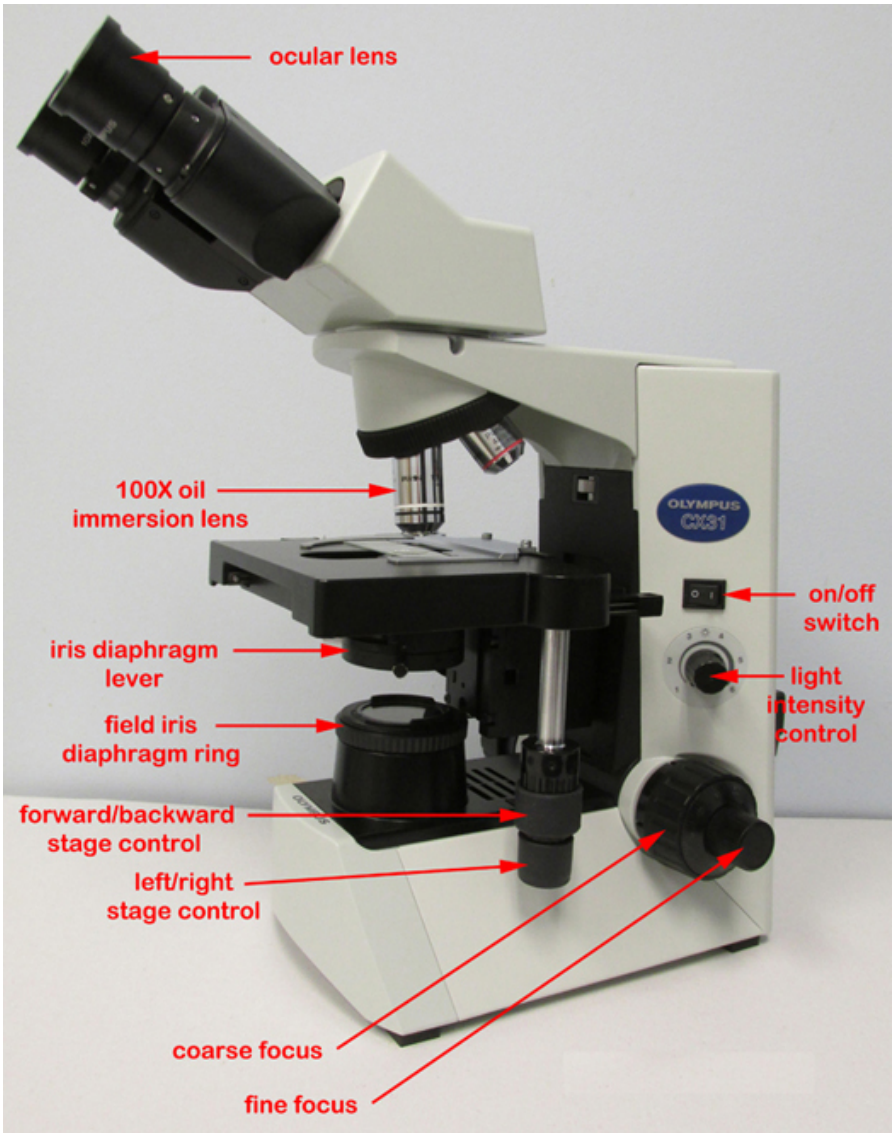
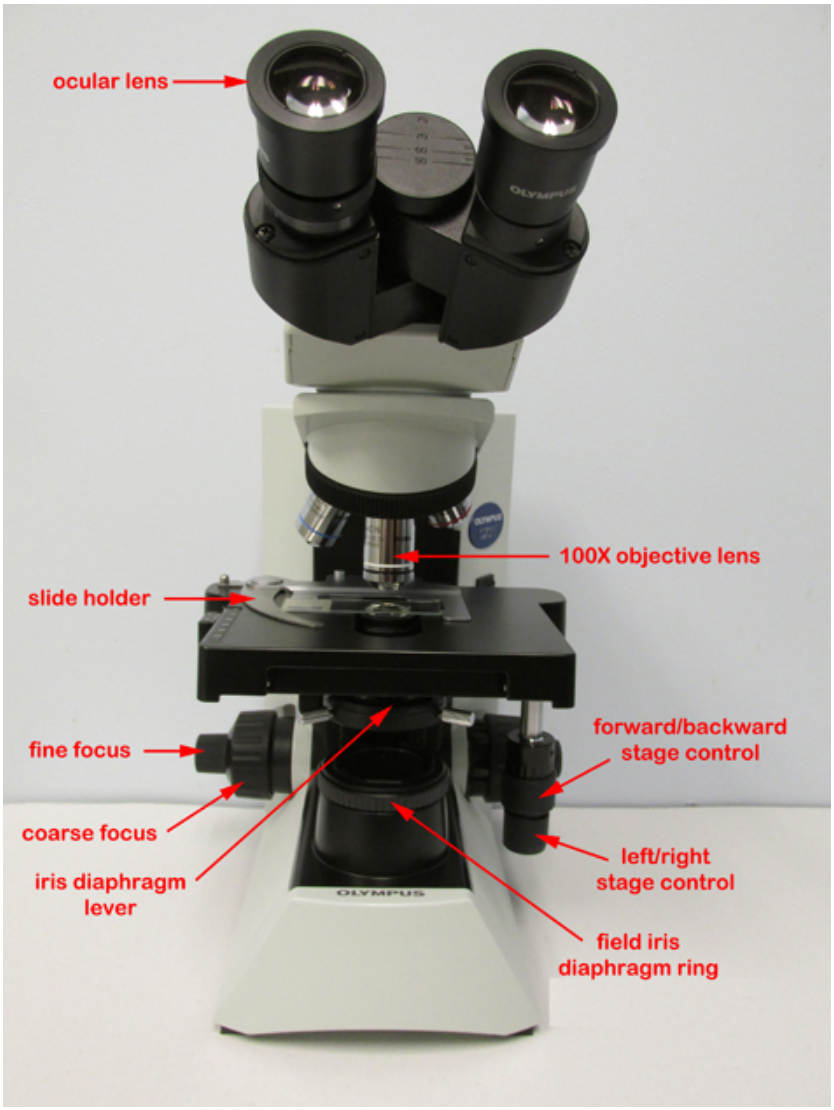


Fig. 3: An Olympus CX31 Microscope



Iris diaphragm lever: moving the lever to the left increases the light; moving the lever to the right decreases the light.
Forward/backward stage control: moves the mechanical stage holding the slide forward and backward.
Left/right stage control: moves the mechanical stage holding the slide left and right.
Coarse focus: turning the knob away from you raises the stage; turning the knob towards you lowers the stage.
Fine focus: turning the knob away from you raises the stage; turning the knob towards you lowers the stage.

Fig. 4: An Olympus CX312 Microscope



7. **Focusing from lower power to higher power**

a. Rotate the **yellow-striped 10X objective** until it locks into place (total magnification of 100X).

b. Turn the **coarse focus control** (larger knob; see Fig. 4) all the way **away from you** until it stops.

c. Look through the eyepieces and turn the **coarse focus control** (larger knob) **towards you** slowly until the specimen comes into focus.

d. Get the specimen into sharp focus using the **fine focus control** (smaller knob; see Fig. 4) and adjust the light for optimum contrast using the iris diaphragm lever.

e. If higher magnification is desired, simply rotate the **blue-striped 40X objective** into place (total magnification of 400X) and the specimen should still be in focus. (Minor adjustments in fine focus and light contrast may be needed.)

f. For maximum magnification (**1000X or oil immersion**), rotate the blue-striped 40X objective slightly out of position and place a **drop of immersion oil** on the slide. Now rotate the **white-striped 100X oil immersion objective** into place. Again, the specimen should remain in focus, although minor adjustments in fine focus and light contrast may be needed.

Directions for focusing directly with oil immersion (1000X) without first focusing using lower powers will be given in Laboratory 1.

8. **Cleaning the microscope**

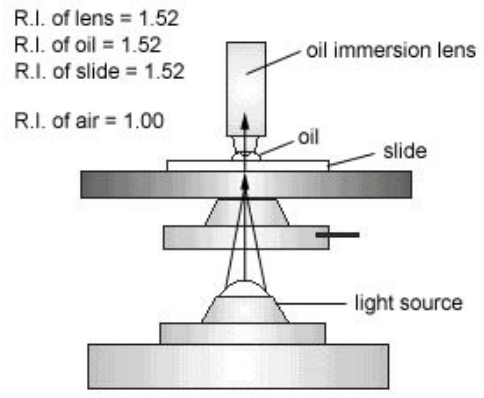
Clean the exterior lenses of the eyepiece and objective before and after each lab using **lens paper** only. (Paper towel or kim-wipes may scratch the lens.) **Remove any immersion oil from the oil immersion lens before putting the microscope away.**

9. **Reason for using immersion oil**

Normally, when light waves travel from one medium into another, they bend. Therefore, as the light travels from the glass slide to the air, the light waves bend and are scattered similar to the "bent pencil" effect when a pencil is placed in a glass of water. The microscope magnifies this distortion effect. Also, if high magnification is to be used, more light is needed.

Immersion oil has the same refractive index as glass and, therefore, provides an optically homogeneous path between the slide and the lens of the objective. Light waves thus travel from the glass slide, into glass-like oil, into the glass lens without being scattered or distorting the image (Fig. 5). In other words, **the immersion oil "traps" the light and prevents the distortion effect that is seen as a result of the bending of the light waves.**

Fig. 5: The Oil Immersion Lens



The immersion oil has the same refractive index as the glass lens and the glass slide. Because the light waves follow a homogeneous path, there is no distortion.

PERFORMANCE OBJECTIVES
INTRODUCTION

After completing this introduction, the student will be able to perform the following objectives:

A. USING PERFORMANCE OBJECTIVES

1. Answer all performance objectives as soon as possible after completing each laboratory exercise.

B. LABORATORY RULES

1. Follow all laboratory rules stated in the Introduction.

C. GENERAL DIRECTIONS

1. Follow all general directions stated in the Introduction.

D. BINOMIAL NOMENCLATURE

1. Define genus and species and state how to correctly write the scientific name of a microorganism.
2. Be able to correctly write the scientific names of microorganisms.

E. METRIC LENGTH

1. Define and give the commonly used abbreviations for the following units of metric length and fluid volume: centimeter, millimeter, micrometer, nanometer, milliliter, and microliter.
2. State the length and width of an average bacillus-shaped bacterium and the diameter of an average coccus-shaped bacterium in micrometers.

F. USING THE MICROSCOPE

1. Correctly clean the eyepiece and the objective lenses before and after each lab.
2. Define ocular lens and objective lens.
3. Place a slide in the slide holder of a mechanical stage correctly.
4. Focus on a specimen using 10X, 40X, and 100X objectives.
5. Adjust the light using the iris diaphragm lever for optimum contrast after focusing.
6. State the reason for using immersion oil at 1000X.
7. Calculate the total magnification of a lens system when using a 10X, 40X, or 100X objective in conjunction with a 10X eyepiece.

LABORATORY 1
INTRODUCTION TO THE MICROSCOPE AND COMPARISON OF SIZES AND SHAPES OF MICROORGANISMS

A. Bacterial Shapes and Arrangements
B. Yeasts
C. Measurement of Microorganisms
D. Focusing Using Oil Immersion Microscopy

DISCUSSION

In this lab, you will become familiar with the use of the microscope (particularly oil immersion microscopy) and will compare the relative size and shape of various microorganisms.

A. BACTERIAL SHAPES, ARRANGEMENTS, AND FORMS

Bacteria are **unicellular prokaryotic microorganisms** that **divide by binary fission**, a process by which one bacterium splits into two. For a review of prokaryotic versus eukaryotic cellular characteristics, see Unit 1, section IB in your Lecture Guide.

There are three common **shapes of bacteria**:

- **coccus**
- **bacillus**
- **spiral**

The cocci come in 5 different arrangements; the bacilli in 3 different arrangements; and the spirals in 3 different forms.

1. Coccus

A coccus-shaped bacterium is usually spherical, although some appear oval, elongated, or flattened on one side. Most cocci are approximately **0.5 – 1.0 micrometer (μm)** in diameter and may be seen, based on their **planes of division** and **tendency to remain attached** after replication, in one of the following **arrangements** (see Fig. 1A):

a. Division in **one plane** produces either a **diplococcus** arrangement (cocci in pairs; see Fig. 1B) or a **streptococcus** arrangement (cocci in chains; see Fig. 1C), as shown in Figs. 1A.

b. Division in **two planes** produces a **tetrad** arrangement (cocci forming a square of four; see Fig. 1D), as shown in Fig. 1A.

c. Division in **three planes** produces a **sarcina** arrangement (cocci forming a cube of eight), as shown in Figs. 1A. It is difficult with a conventional light microscope to tell a tetrad arrangement (square of four cocci) from a sarcina arrangement (cube of eight) so in our lab, anytime you see a square of four cocci, say it is either a tetrad or a sarcina arrangement.

d. Division in **random planes** produces a **staphylococcus** arrangement (cocci in irregular, often grape-like clusters; see Fig. 1E), as shown in Figs. 1A.

As you observe these different cocci, keep in mind that **the procedures used in slide preparation may cause some arrangements to break apart or clump together** (see Figs. 1D and 1E). The correct form, however, should **predominate. Also remember that each coccus in an arrangement represents a complete, individual, one-celled organism**.

Fig. 1: Arrangement of Cocci

Fig. 1A: Arrangements of Cocci

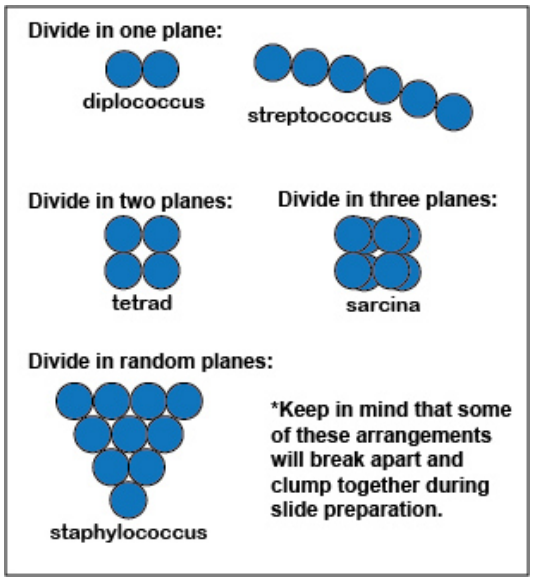


Fig. 1B: Diplococcus Arrangement

Fig. 1C: Streptococcus Arrangement

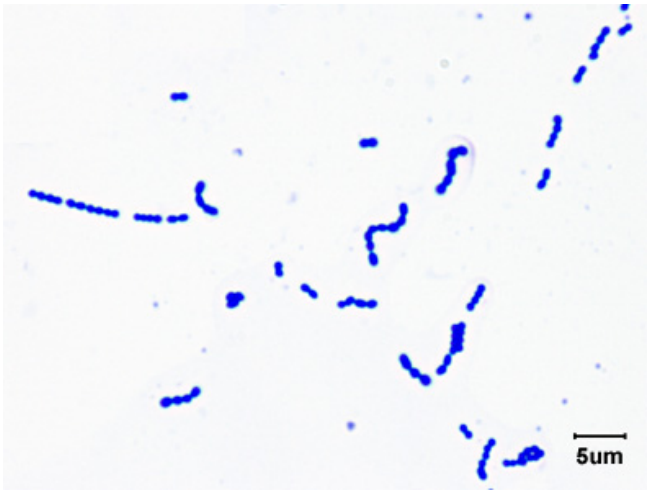


Fig. 1D: Tetrad Arrangement

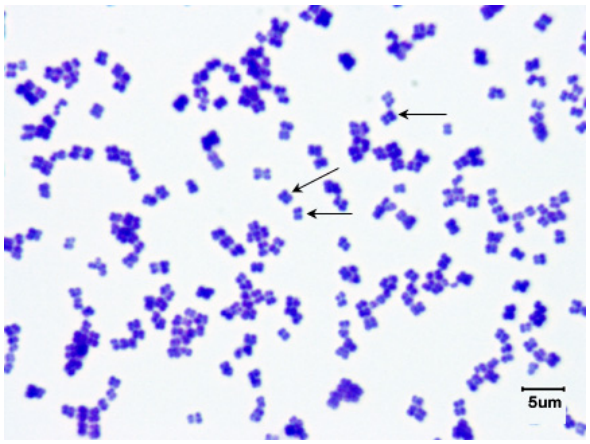
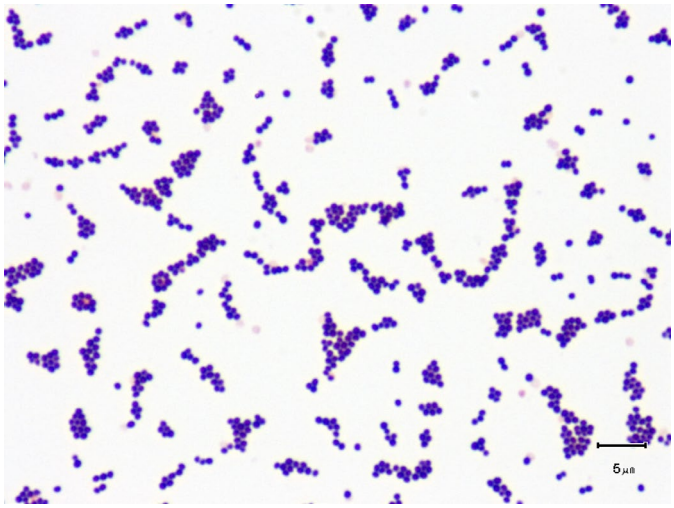


Fig. 1E: Staphylococcus Arrangement



2. Bacillus (rod)

A bacillus or rod is a hotdog-shaped bacterium having one of the following **arrangements** (see Fig. 2A):

a. **bacillus**: a single bacillus (see Fig. 2B).

b. **streptobacillus**: bacilli in chains (see Fig. 2C).

c. **coccobacillus**: oval and similar to a coccus.

A single bacillus is typically **0.5-1.0 µm wide** and from **1-4 µm long**. **Small bacilli or bacilli that are dividing or have just divided by binary fission may at first glance be confused for diplococcic or cocci** (see Fig. 2A) so they must be **observed carefully**. You will, however, be able to see bacilli that have not divided and are definitely rod-shaped as well as bacilli in the process of dividing.

3. Spiral

Spiral-shaped bacteria occur in one of three **forms** (see Fig. 3A):

a. **vibrio**: an incomplete spiral or comma-shaped (see Fig. 3B).

b. **spirillum**: a thick, rigid spiral (see Fig. 3C).

c. **spirochete**: a thin, flexible spiral (see Fig. 3D).

While some spirochetes are over 100 µm in length, the spirals you will observe today range from **5 µm to 40 µm long**.

Fig. 2: Arrangements of Bacilli

Fig. 2A: Arrangements of Bacilli

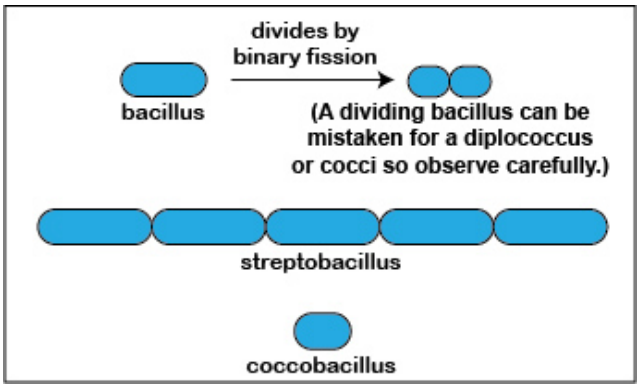


Fig. 1B: Single Bacillus Arrangement

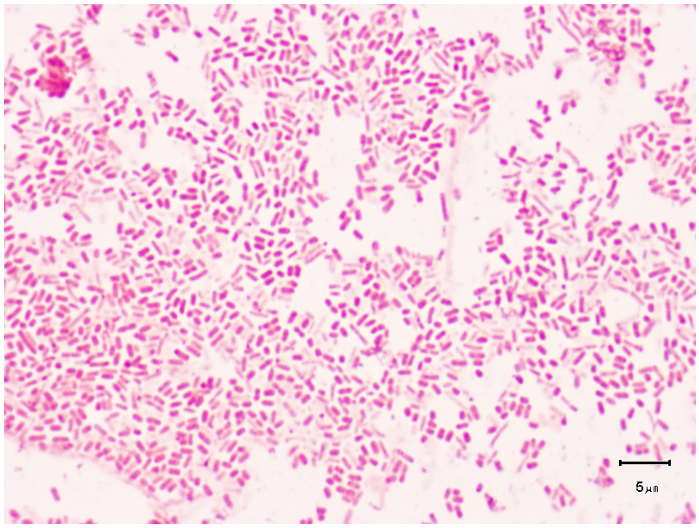
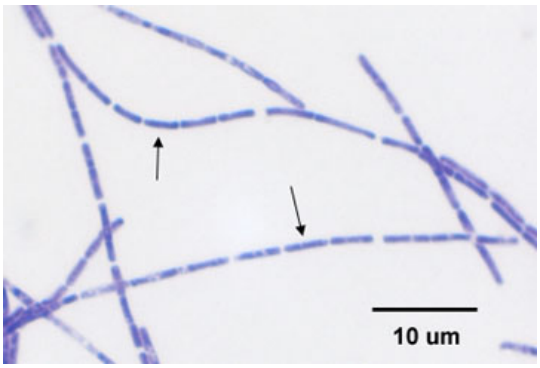


Fig. 1C: Streptobacillus Arrangement



LABORATORY 1:
INTRODUCTION TO THE MICROSCOPE AND COMPARISON OF SIZES AND SHAPES OF MICROORGANISMS

Fig. 3: Spiral Forms

Fig. 3A: Forms of Spirals

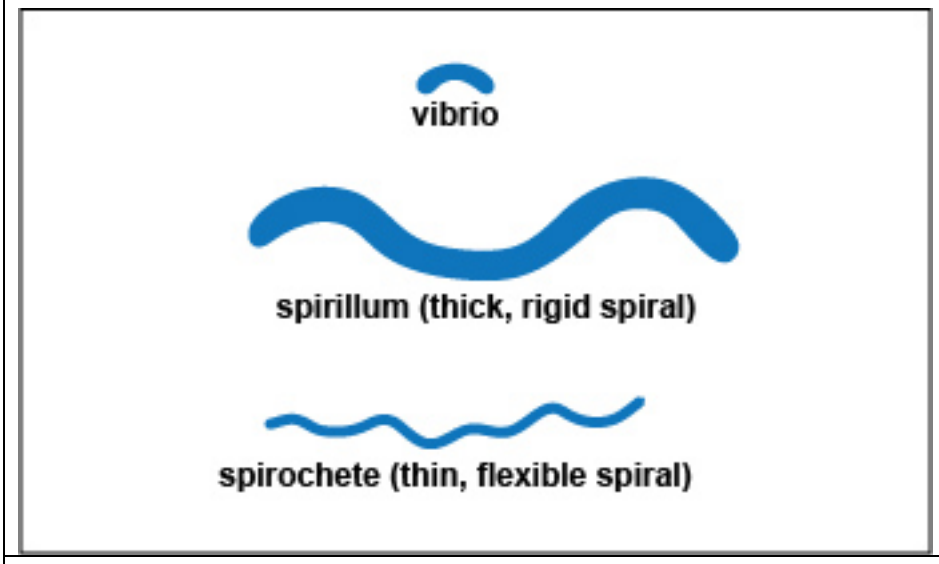


Fig. 3B: Vibrio

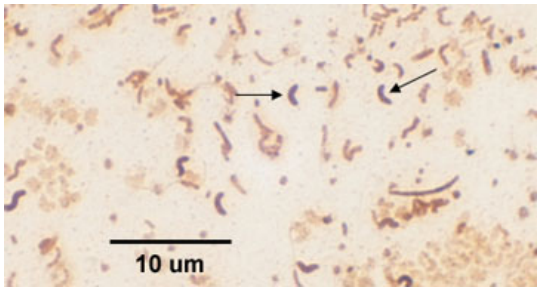


Fig. 3C: Spirillum

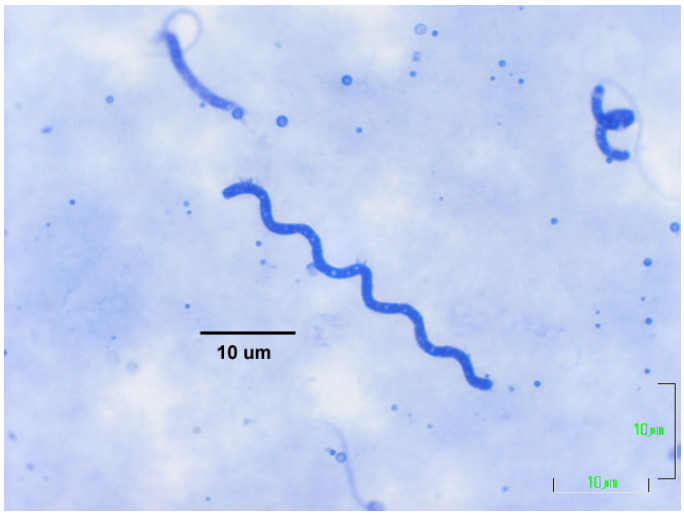
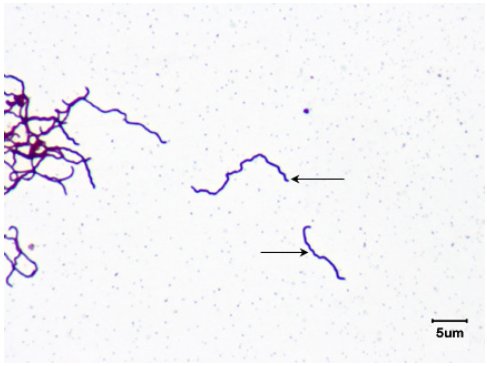


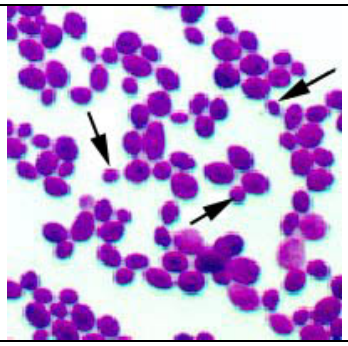
Fig. 3D: Spirochete



B. YEASTS

Yeasts, such as the common baker's yeast *Saccharomyces cerevisiae*, are **unicellular fungi**. They usually appear spherical and have a diameter of **3 - 5 µm**. Yeasts commonly **reproduce asexually by a process called budding** (see Fig. 4). Unlike bacteria, which are prokaryotic, yeasts are **eukaryotic**. For a review of prokaryotic versus eukaryotic cellular characteristics, see Unit 1, section IB in your Lecture Guide.

Fig. 4:
A Budding Yeast



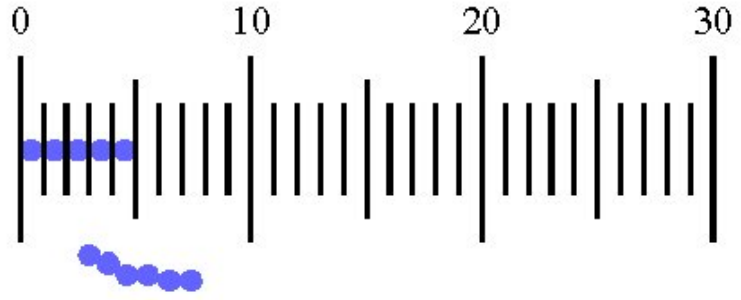
Note budding (arrows).

C. MEASUREMENT OF MICROORGANISMS

The approximate size of a microorganism can be determined using an **ocular micrometer,** an eyepiece that contains a scale that will appear superimposed upon the focused specimen.

The ocular micrometers provided are calibrated so that when using 1000X oil immersion microscopy, the distance between any two lines on the scale represents a length of approximately one micrometer (see Fig. 5). Remember this does not hold true when using other magnifications.

Fig. 5: Ocular Micrometer



When using oil immersion microscopy, **the distance between any two lines is one micrometer**. The streptococcus seen here is five micrometers long.

D. FOCUSING THE MICROSCOPE

Focusing With the 1000X Oil Immersion Objective - Olympus CX31 Microscope (see Fig. 7)

1. Before you plug in the microscope, **turn the light intensity control dial** on the right-hand side of the microscope **to 1** (see Fig. 6). Now plug in the microscope and **turn it on using the on/off switch** (see Fig. 6).

2. Place a **rounded drop of immersion oil** on the area of the slide that is to be observed under the microscope, typically an area that shows some visible stain. Place the slide in the slide holder and center the slide using the two mechanical stage-control knobs under the right-hand side of the stage (see Fig. 6).

3. Rotate the **white-striped 100X oil immersion objective** until it is locked into place. This will give a total magnification of **1000X**.

4. Turn the **light intensity control dial** on the right-hand side of the microscope to **6** (see Fig. 6). Make sure the **iris diaphragm lever** in front under the stage is **set approximately at 0.9**, (toward the left side of the stage; see Fig. 6A). Do not close the field iris diaphragm ring on the light source; that should remain fully open. The knob under the stage on the left-hand side of the stage controlling the height of the condenser should be turned so that the **condenser is all the way up**.

5. Watching the slide and objective lens carefully from the front of the microscope, lower the oil immersion objective into the oil by **raising the stage until the white-striped, 100X, oil immersion lens just touches the immersion oil on the slide** (see Fig. 8). Do this by turning the **coarse focus** (larger knob; see Fig. 7) **away from you until the 100X oil immersion objective lens just touches the oil**. When the lens enters the immersion oil you will see the light scatter away from the lens. **Do not push the spring-loaded portion of the lens up into the barrel of the oil immersion lens**.

6. While looking through the eyepieces, turn the **fine focus** (smaller focus knob; see Fig. 7) **away from you at a slow steady speed until the specimen comes into focus**. (If the specimen does not come into focus within a few complete turns of the fine focus control, reverse direction and start turning the fine focus toward you - or go back and repeat step 5.

7. Using the **iris diaphragm lever, adjust the light** to obtain optimum contrast (see Fig. 6A).

8. When finished, **wipe the oil off of the oil immersion objective** with lens paper, **turn the light intensity control dial back to 1**, **turn off the microscop**e, unplug the power cord, and wrap the cord around the cord holder on the back of the microscope.

An **alternate focusing technique** is to first focus on the slide with the yellow-striped 10X objective by using only the coarse focus control. Then without moving the stage, add immersion oil, rotate the white-striped 100X oil immersion objective into place, and adjust the fine focus and the light as needed. This procedure is discussed in the Introduction to the lab manual.

Focusing With the 10X Objective - Olympus CX31 Microscope (see Fig. 7)

1. Rotate the **yellow-striped 10X objective** until it locks into place. This will give a total magnification of **100X**.

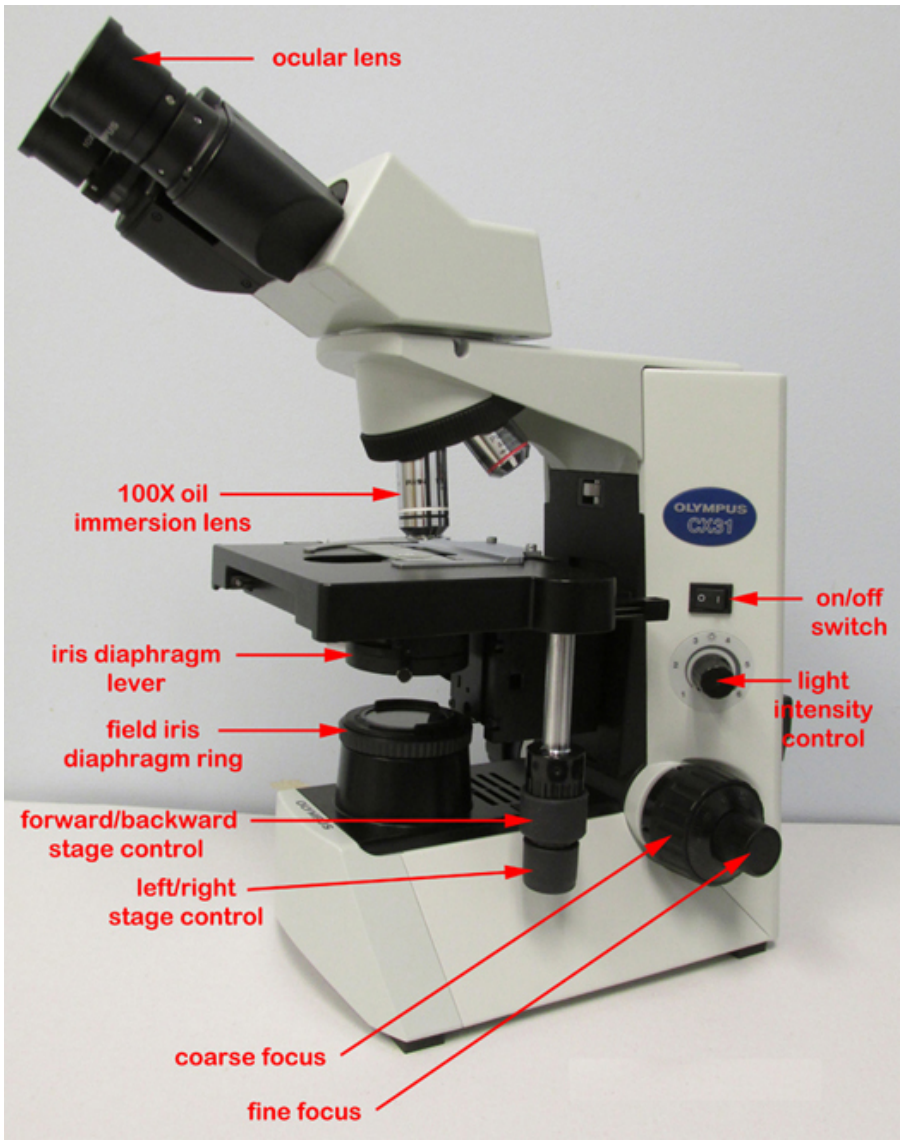
2. Using the **iris diaphragm lever** under the stage (see Fig. 6A), **reduce the light** by sliding the lever most of the way to the right.

3. Turn the **coarse focus control** (larger knob; see Fig. 7) all the way **away from you** until it stops.

4. Look through the eyepieces and turn the **coarse focus control** (larger knob) **towards you** slowly until the specimen comes into focus.

5. Get the specimen into sharp focus using the **fine focus control** (smaller knob; see Fig.7) and adjust the light for optimum contrast using the iris diaphragm lever (see Fig. 6A) and/or the light intensity control (see Fig. 6).

Fig. 6: An Olympus CX31 Microscope



Iris diaphragm lever: moving the lever to the left increases the light; moving the lever to the right decreases the light.
Forward/backward stage control: moves the mechanical stage holding the slide forward and backward.
Left/right stage control: moves the mechanical stage holding the slide left and right.
Coarse focus: turning the knob away from you raises the stage; turning the knob towards you lowers the stage.
Fine focus: turning the knob away from you raises the stage; turning the knob towards you lowers the stage.

Fig. 6A: Olympus CX31 Microscope Iris Diaphragm

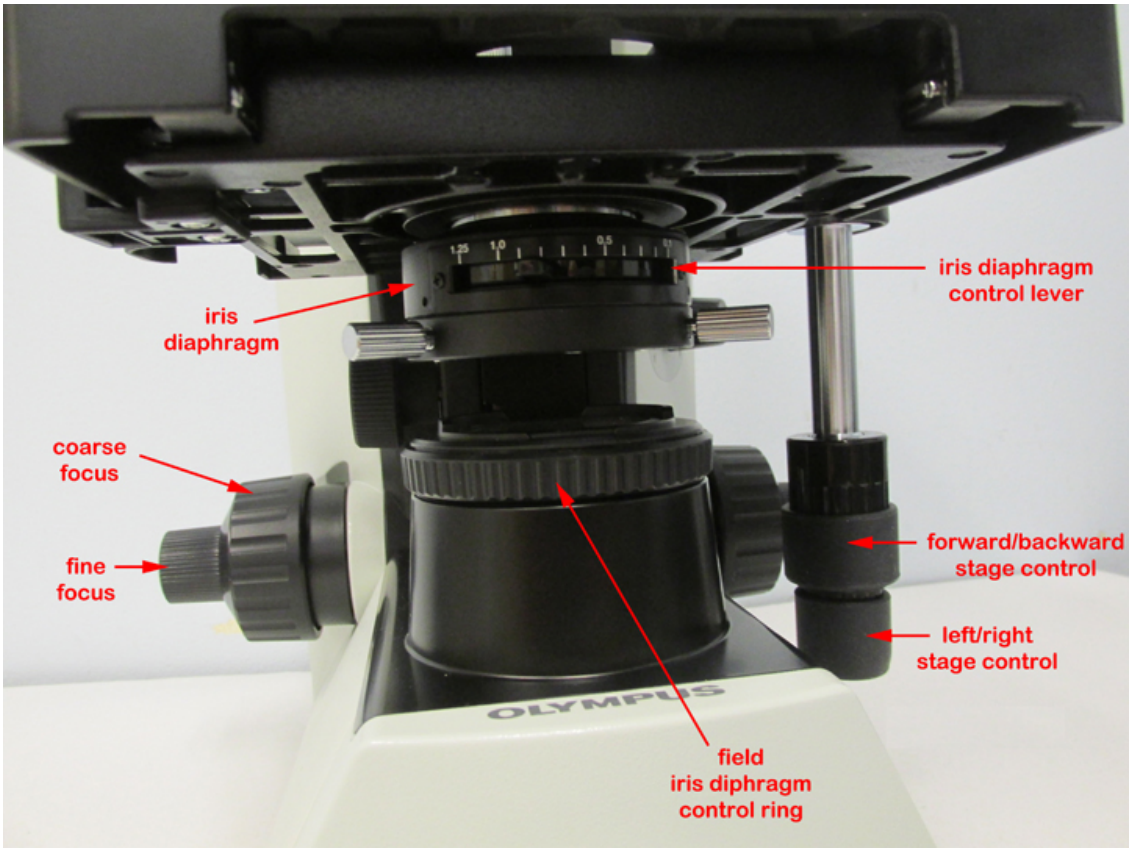


Fig. 7: Olympus CX31 Microscope

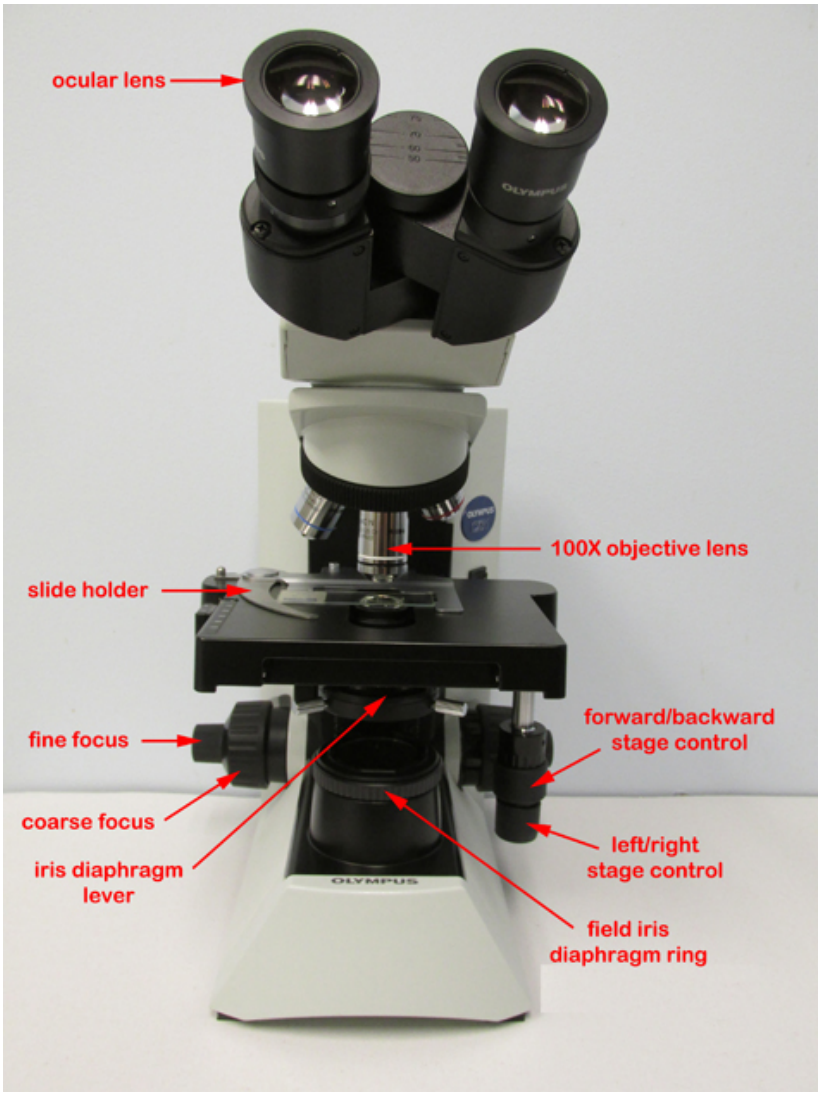
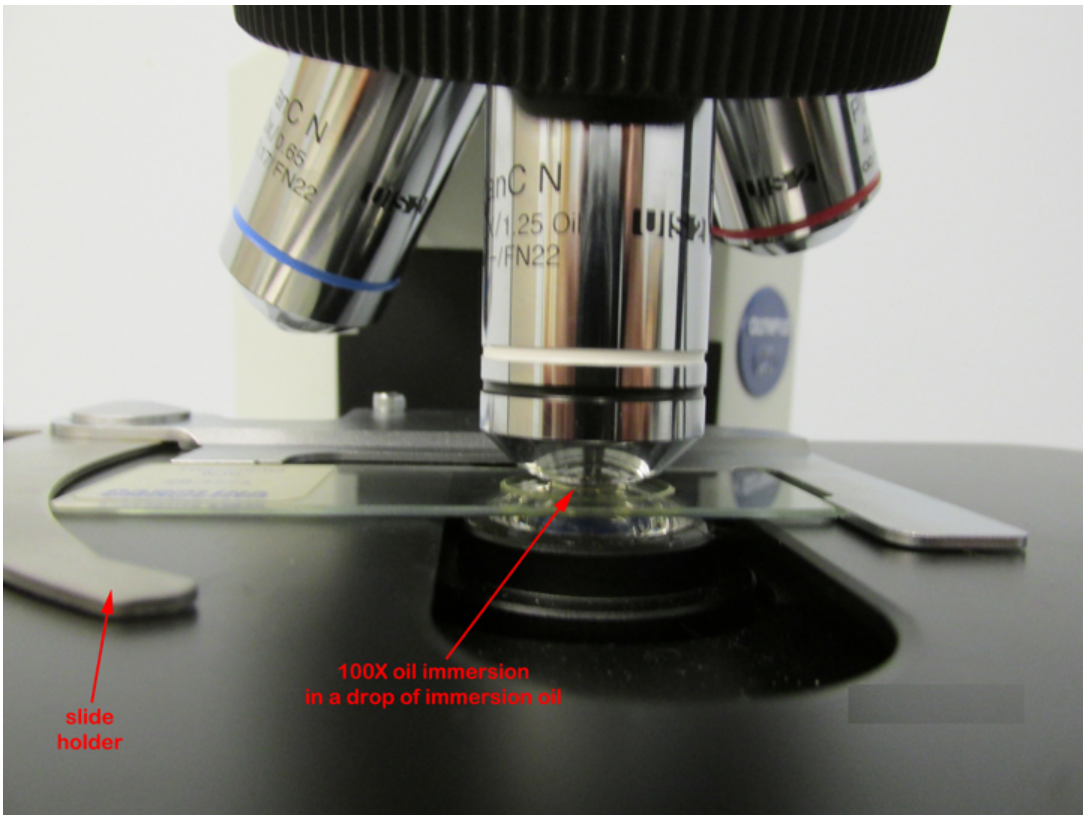


Fig. 8: Olympus CX31 Microscope: Oil Immersion



SPECIMENS

Prepared slides of the following bacteria:

- *Staphylococcus aureus*
- *Escherichia coli*
- *Treponema pallidum*
- *Spirillum* species

On-line demonstration slides of the following bacteria:

- *Micrococcus luteus*
- *Neisseria gonorrhoeae*
- *Streptococcus pyogenes*
- *Bacillus megaterium*

Broth culture of *Saccharomyces cerevisiae*
Human hair

LABORATORY 1:
INTRODUCTION TO THE MICROSCOPE AND COMPARISON OF SIZES AND SHAPES OF MICROORGANISMS

PROCEDURE

TIPS FOR MICROSCOPIC OBSERVATIONS

Move the slide around until you see an area representing the true arrangement of each organism. Also remember:

- In the process of making the slide**, some of the coccal arrangements will clump together and others will break apart. Look carefully to determine the true arrangement**.
- **Small bacilli (such as *Escherichia coli*) that are dividing or have just divided by binary fission will look similar to cocci**. **Look carefully for bacilli that are not dividing and are definitely rod-shaped as well as bacilli in the process of dividing to confirm the true shape**.
- **Bacilli do not divide so as to form clusters. Any such clusters you see are artifacts from preparing the slide**.
- Look carefully to see the spirochetes as they are the thinnest of the bacteria. When seen microscopically, **spirochetes resemble extremely thin, wavy pencil lines**.

1. Using **oil immersion microscopy** (1000X), observe and measure the bacteria listed below.

    a. *Staphylococcus aureus: Staphylococcus* species, as the genus name implies, are cocci possessing a staphylococcus arrangement (cocci in irregular, often grape-like clusters). Measure the diameter of a single coccus.

    b. *Escherichia coli: Escherichia coli* is a small bacillus. Measure the length and width of a typical rod.

    c. *Treponema pallidum*: *Treponema pallidum* is a spirochete, a thin, flexible spiral. On this slide you are examining a direct stain of *Treponema pallidum*, the bacterium that causes syphilis. Measure the length and width of a typical spirochete.

    d. *Spirillum* species: Spirillum species appear as thick, rigid spirals. Measure the length and width of a typical spirillum.

When finished, remove the oil from the prepared slides using either a paper towel or a Kim wipe and return them to their proper tray.

2. Observe the **on-line demonstration slides** of the following bacteria:

    a. *Micrococcus luteus: Micrococcus luteus* can appear in tetrads or cubes of 8. This strain is a coccus usually exhibiting a tetrad or a sarcina arrangement. Note the shape and arrangement.

    b. *Neisseria gonorrhoeae: Neisseria* species are cocci usually having a diplococcus arrangement. Note the shape and arrangement.

    c. *Streptococcus pyogenes: Streptococcus* species, as the genus name implies, are cocci that usually possess a streptococcus arrangement (cocci in chains). Note the shape and arrangement.

    d. *Bacillus megaterium: Bacillus megaterium* appears as large bacilli in chains (a streptobacillus). Note the shape and arrangement.

3. Prepare a wet mount of baker's yeast (*Saccharomyces cerevisiae.*

a. Using a pipette, put a **small drop** of the yeast culture on a microscope slide and place a cover slip over the drop.

b. Using your iris diaphragm lever, **reduce the light for improved contrast** by moving the lever almost all the way to the right.

c. Observe using **oil immersion microscopy**. Measure the diameter of a typical yeast cell.

d. When finished, **discard the coverslip in the broken glass disposal container**, wash the slide with water and dry with a paper towel, and use the same slide again for step 4.

4. Prepare a wet mount of your hair.

a. Remove a small piece of a hair from your head and place it in a **small drop of water** on a slide.

b. Place a cover slip over the drop and observe using **oil immersion microscopy**.

c. Measure the diameter of your hair and compare this with the size of each of the bacteria and the yeast observed in steps 1-3.

d. **Discard the slide and coverslip in the broken glass disposal container**.

5. At the completion of the lab, **remove the oil** from the oil immersion objective using **lens paper** and put your microscope away.

LABORATORY 1:
INTRODUCTION TO THE MICROSCOPE AND COMPARISON OF SIZES AND SHAPES OF MICROORGANISMS

RESULTS

1. Make drawings of several of the bacteria from each of the four prepared slides and indicate their approximate size in micrometers.

Staphylococcus aureus	*Escherichia coli*
Shape =	Shape =
Arrangement =	Length = μm
Diameter = μm	Width = μm

Treponema pallidum	*Spirillum* species
Shape =	Shape =
Form =	Form =
Length = μm	Length = μm
Width = μm	Width = μm

2. Make drawings of several of the bacteria from each of the four demonstration slides and indicate their approximate size in micrometers.

Micrococcus luteus	*Neisseria gonorrhoeae*
Shape =	Shape =
Arrangement =	Arrangement =
Streptococcus pyogenes	*Bacillus megaterium*
Shape =	Shape =
Arrangement =	Arrangement =

3. Make a drawing of several yeast cells and indicate their size in micrometers.

Saccharomyces cerevisiae
Diameter = μm

4. Make a drawing indicating the size of the bacteria and yeast observed above relative to the diameter of your hair.

Hair
Diameter = μm

LABORATORY 1:
INTRODUCTION TO THE MICROSCOPE AND COMPARISON OF SIZES AND SHAPES OF MICROORGANISMS

PERFORMANCE OBJECTIVES
LABORATORY 1

After completing this lab, the student will be able to perform the following objectives:

DISCUSSION

1. State three basic shapes of bacteria.
2. State and describe five different arrangements of cocci.
3. State and describe three different arrangements of bacilli.
4. State and describe three different spiral forms.
5. Describe the appearance of a typical yeast cell.

RESULTS

1. When given an oil immersion microscope, a prepared slide of a microorganism, and an ocular micrometer, determine the size of that organism in micrometers.
2. Using a microscope, identify different bacterial shapes, arrangements, and forms.
3. Differentiate a yeast cell from a coccus-shaped bacterium by its size.
4. Compare the size of the microorganisms observed in this lab with the diameter of a hair when using oil immersion microscopy.

LABORATORY 2

ASEPTIC TECHNIQUE AND TRANSFER OF MICROORGANISMS

A. Aseptic Technique
B. Forms of Culture Media
C. Oxygen Requirements for Microbial Growth
D. Temperature Requirements
E. Colony Morphology and Pigmentation

DISCUSSION

In natural environments, microorganisms usually exist as mixed populations. However, if we are to study, characterize, and identify microorganisms, we must have the organisms in the form of a pure culture. A **pure culture** is one in which all organisms are descendants of the same organism. Techniques for obtaining pure cultures from a mixed population will be described in Lab 3.

In working with microorganisms we must also have a sterile nutrient-containing medium in which to grow the organisms. Anything in or on which we grow a microorganism is termed a **medium**. A **sterile** medium is one that is free of all life forms. It is usually sterilized by heating it to a temperature at which all contaminating microorganisms are destroyed.

Finally, in working with microorganisms, we must have a method of transferring growing organisms (called the **inoculum**) from a pure culture to a sterile medium without introducing any unwanted outside contaminants. This method of preventing unwanted microorganisms from gaining access is termed **aseptic technique**.

A. ASEPTIC TECHNIQUE (Figs. 1 & 2)

The procedure for aseptically transferring microorganisms is as follows:

1. **Using a microincinerator to sterilize the inoculating loop.**

Microincinerators enable the sterilization of inoculating loops without having to use an open flame of a Bunsen burner. The microincinerator uses infrared heat at a temperature of 816°C to sterilize the wire portion of the inoculating loop. **Be careful not to touch the top portion of the microincinerator. It becomes very hot!**

a. **At the start of class, turn on the microincinerator and wait 10 minutes for it to heat up**.

b. **Place the entire wire portion of the inoculating loop into the opening of the microincinerator and hold it there for 10 seconds** (see Fig. 1A). In this way all contaminants on the wire are incinerated. **To avoid burning your hand on the handle of an overheated inoculating loop, never lay the loop down in the microincinerator**

and then attempt to pick it up. Never lay the loop down once it is sterilized or it may again become contaminated.

c. **Allow the loop to cool 10-20 seconds** before removing the inoculum.

2. **Remove the inoculum.**

a. **Removing inoculum from a broth culture** (organisms growing in a liquid medium):

1. Hold the culture tube in one hand and in your other hand, hold the sterilized inoculating loop as if it were a pencil (see Fig. 1).

2. **Remove the cap of the pure culture tube with the little finger of your loop hand** (Fig. 1B). **Never lay the cap down** or it may become contaminated.

3**. Place the lip of the culture tube at the opening of the microincinerator for 2-3 seconds** (Fig. 1C). This heats the glass and creates a convection current which forces air out of the tube and prevents airborne contaminants from entering the tube.

4. **Insert the inoculating loop and remove a loopful of inoculum** (Fig. 1D).

5. Again **place the lip of the culture tube at the opening of the microincinerator for 2-3 seconds** (Fig. 1E).

6. **Immediately replace the cap** (Fig. 1F).

b. **Removing inoculum from a plate culture** (organisms growing on an agar surface in a petri plate):

1) **Sterilize the inoculating loop by placing it in the microincinerator for 10 seconds** (Fig. 3A).

2) Lift the lid of the petri plate slightly and **stab the loop into the agar away from the growth** to cool the loop.

3) Scrape off a **small amount** of the organisms and immediately close the lid (Fig. 3B).

3. **Transfer the Inoculum to the Sterile Medium.**

a. **Transferring the inoculum into a broth tube:**

1) Pick up the sterile broth tube and remove the cap with the **little finger** of your loop hand. **Do not set the cap down** (Fig. 2A).

2) **Place the lip of the culture tube at the opening of the microincinerator for 2-3 seconds** (Fig. 2B).

3) **Place the loopful of inoculum into the broth**, and withdraw the loop (Fig. 2C). Do not lay the loop down!

4) **Place the lip of the culture tube at the opening of the microincinerator for 2-3 seconds** (Fig. 2D).

5) **Replace the cap** (Fig. 2E).

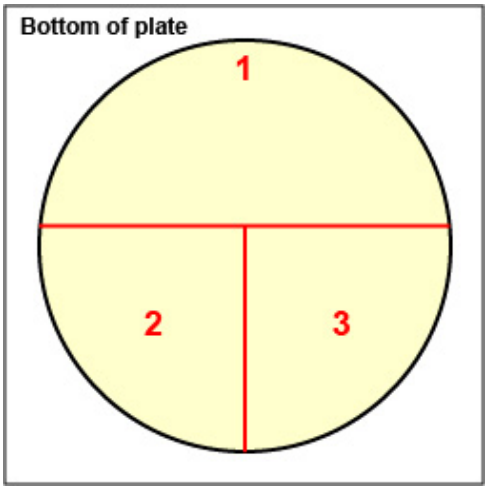
6) **Resterilize the inoculating loop by placing it in the microincinerator for 10 seconds** (Fig. 2F). Now you may lay the loop down until it is needed again.

This procedure is summarized in Figures 1A-1F and 2A-2F.

b. **Transferring the inoculum onto a petri plate:**

1) If the agar surface of the plate is visibly wet, use a sterile swab to gently remove the water.

2) On the bottom of the petri plate, divide the plate into thirds with your wax marker and label as shown below. This will guide your streaking.



3) **Lift the edge of the lid just enough to insert the loop**.

4) **Streak the loop across the surface of the agar medium using the either the pattern shown in Fig. 4 or the pattern shown in Fig. 5**. These streaking patterns allow you to obtain single isolated bacterial colonies originating from a single bacterium or arrangement of bacteria (see Fig. 6).

In order to avoid digging into the agar as you streak the loop over the top of the agar you must **keep the loop parallel to the agar surface**. Always start streaking at the **"12:00 position" of the plate (see Fig. 3C)** and streak side-to-side as you pull the loop toward you. As you follow either Fig. 4 or Fig. 5, each time you flame and cool the loop between sectors, rotate the plate counterclockwise so you are **always working in the "12:00 position" of the plate**. This keeps the inoculating loop parallel with the agar surface and helps prevent the loop from digging into the agar.

5) Remove the loop and immediately close the lid.

6) **Resterilize the inoculating loop by placing it in the microincinerator for 10 seconds**.

See “Flash animation showing how to streak an agar plate for isolation: 3 sector method” in the on-line lab manual.

In the future, every procedure in the lab will be done using similar aseptic technique.

B. FORMS OF CULTURE MEDIA

1. **Broth tubes** are tubes containing a liquid medium. A typical nutrient containing broth medium such as Trypticase Soy broth contains substrates for microbial growth such as pancreatic digest of casein, papaic digest of soybean meal, sodium chloride, and water. After incubation, **growth** (development of many cells from a few cells) may be observed as one or a combination of three forms (**Figure 7**):

    a. **Pellicle:** A mass of organisms is floating on top of the broth.

    b. **Turbidity:** The organisms appear as a general cloudiness throughout the broth.

    c. **Sediment:** A mass of organisms appears as a deposit at the bottom of the tube.

2. **Slant tubes (Figure 8A)** are tubes containing a nutrient medium plus a solidifying agent, agar-agar. The medium has been allowed to solidify at an angle in order to get a flat inoculating surface (**Figure 8B**). Growth on agar slants can be described by means of the terms found in **Appendix A.**

3. **Stab tubes (deeps)** are tubes of hardened agar medium that are inoculated by "stabbing" the inoculum into the agar (**Figure 9**).

4. **Agar plates** are sterile petri plates that are aseptically filled with a melted sterile agar medium and allowed to solidify. Plates are much less confining than slants and stabs and are commonly used in the culturing, separating, and counting of microorganisms. Single colonies of microorganisms on agar plates can be described using the terms found in **Appendix A.**

LABORATORY 2:
ASEPTIC TECHNIQUE AND TRANSFER OF MICROORGANISMS

FIG. 1:
Aseptic Removal of Microorganisms from a Broth Culture

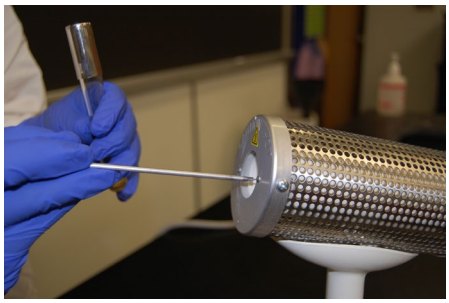
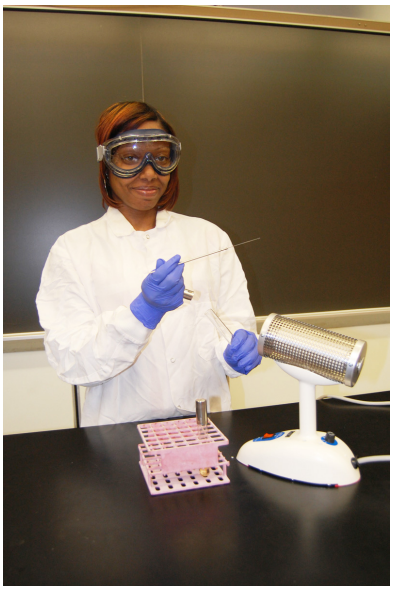
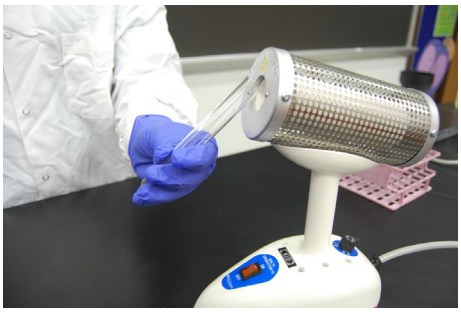
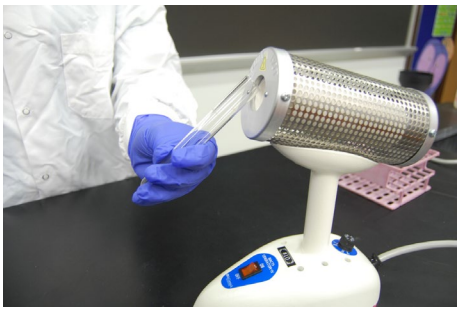
		
A. Sterilize the inoculating loop by placing it in the microincinerator for 10 seconds.	B. Remove the cap of the broth culture with the little finger of the loop hand.	C. Place the lip of the culture tube at the opening of the microincinerator for 2-3 seconds.
		
D. Remove the inoculum.	E. Place the lip of the culture tube at the opening of the microincinerator for 2-3 seconds.	F. Replace the cap.

FIG. 2:
Transferring Microorganisms into a Broth Tube

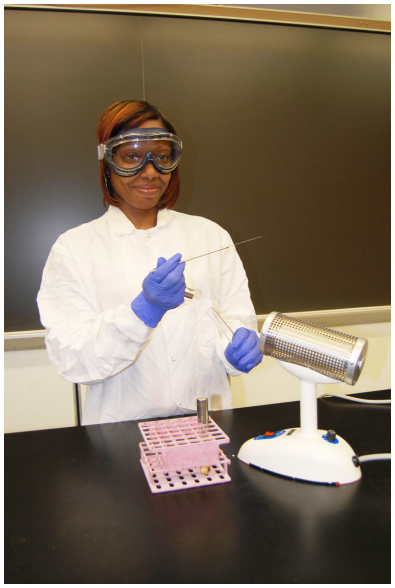
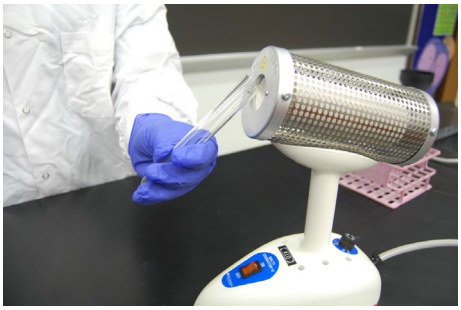
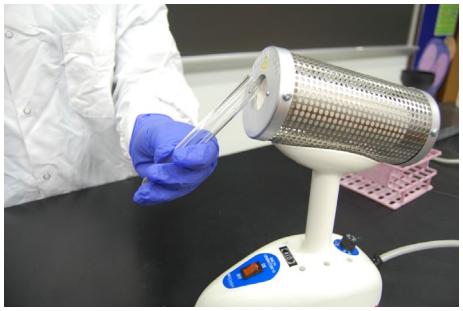
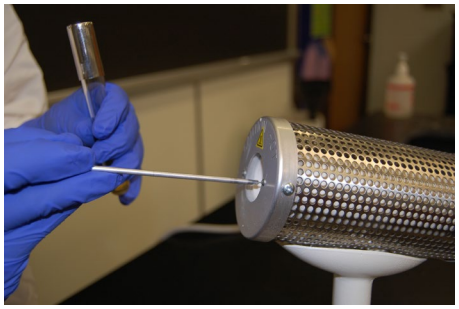
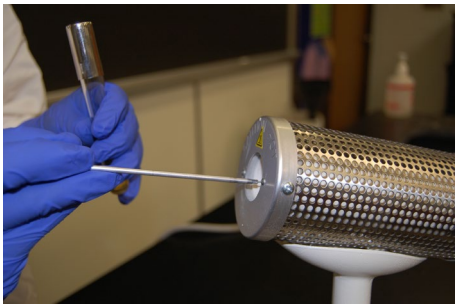
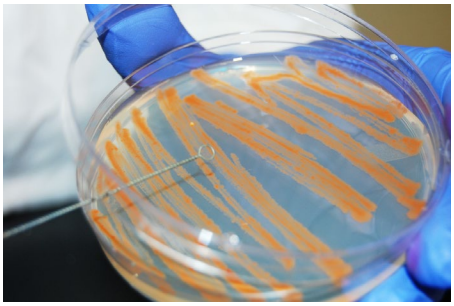
		
A. Remove the cap of the broth tube with the little finger of the loop hand.	B. Place the lip of the culture tube at the opening of the microincinerator for 2-3 seconds.	C. Inoculate the tube.
		
D. Place the lip of the culture tube at the opening of the microincinerator for 2-3 seconds.	E. Replace the cap.	F. Resterilize the inoculating loop by placing it in the microincinerator for 10 seconds.

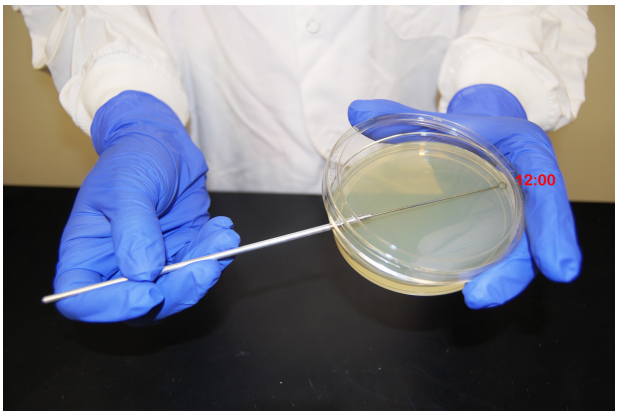
Fig. 3:
Aseptic Removal of Microorganisms from a Plate Culture



A. Sterilize the inoculating loop by placing it in the microincinerator for 10 seconds.

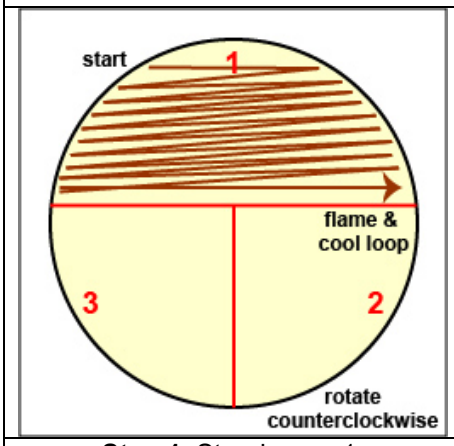


B. Scrape off a small amount of the organisms and close the lid.

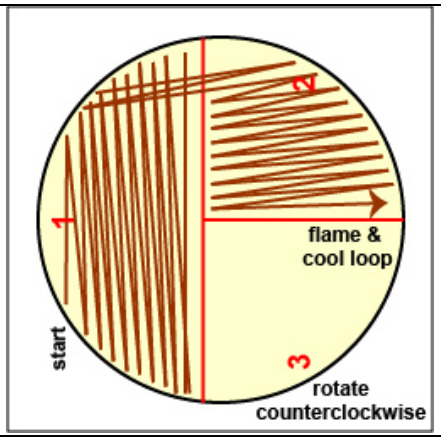


C. Keep the inoculating loop parallel with the agar and rotate the plate so you are always streaking the agar in the "12:00" position.

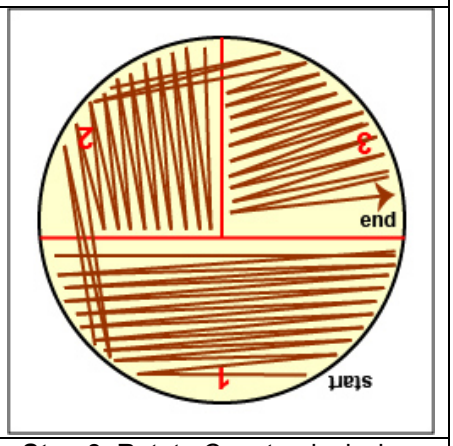
Fig. 4:
Inoculating a Petri Plate - Method 1



Step 1: Streak area 1. Sterilize the loop.

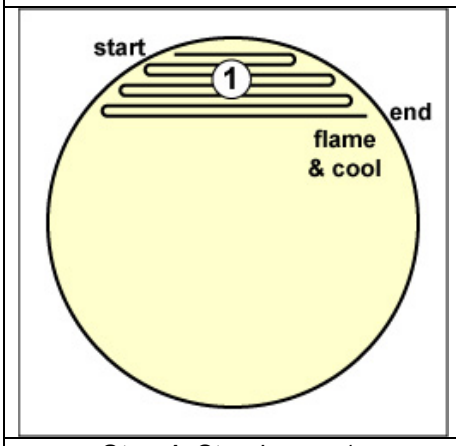


Step 2: Rotate counterclockwise. Spread area 1 over area 2. Sterilize the loop.

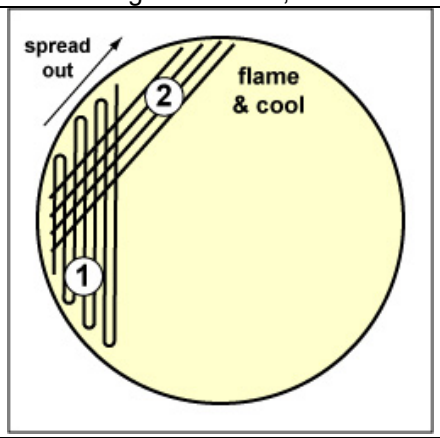


Step 3: Rotate Counterclockwise. Spread area 2 over area 3. Sterilize the loop.

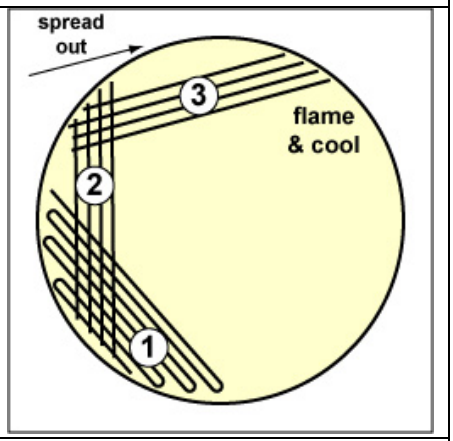
Fig. 5:
Streaking for Isolation, Method 2



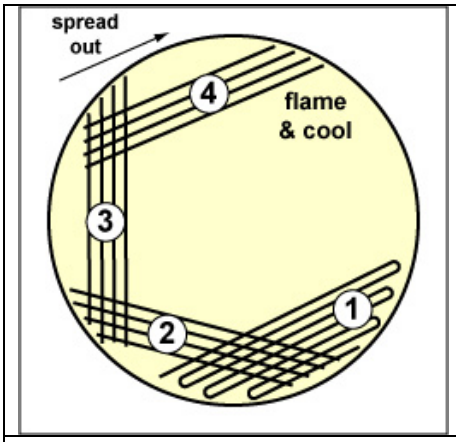
Step 1: Streak area 1.
Sterilize the loop.



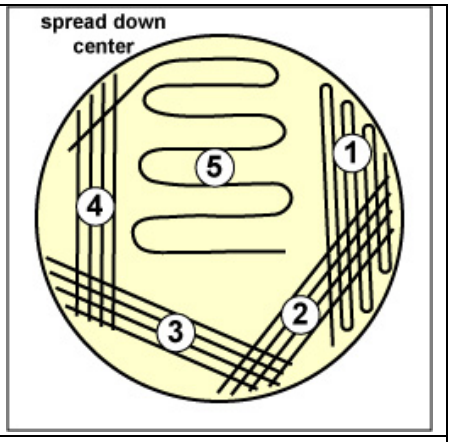
Step 2: Rotate counterclockwise.
Spread area 1 over area 2.
Sterilize the loop.



Step 3: Rotate counterclockwise.
Spread area 2 over area 3.
Sterilize the loop.



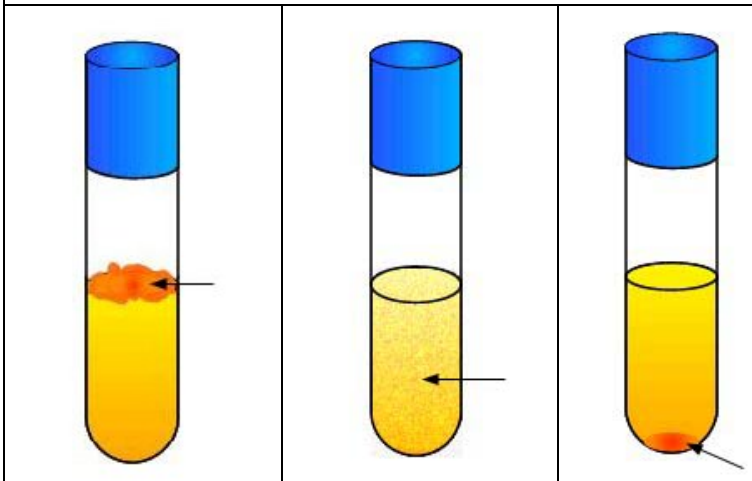
Step 4: Rotate counterclockwise.
Spread area 3 over area 4.
Sterilize the loop



Step 5:Draw your loop through area "4" and spread it down the center of the plate without touching any of the areas already streaked.

Fig. 6:
Single Colonies of a Bacterium on an Agar Plate

Fig. 7:
Bacterial Growth in Broth Tubes



Pellicle	Turbidity	Sediment

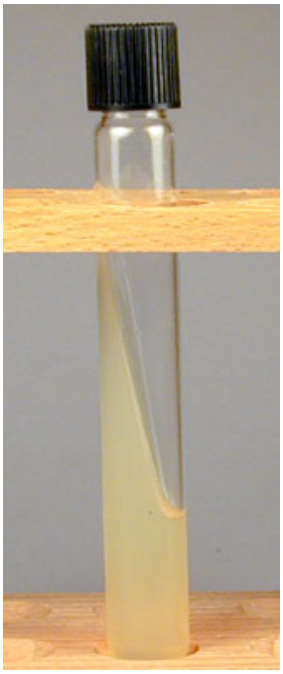
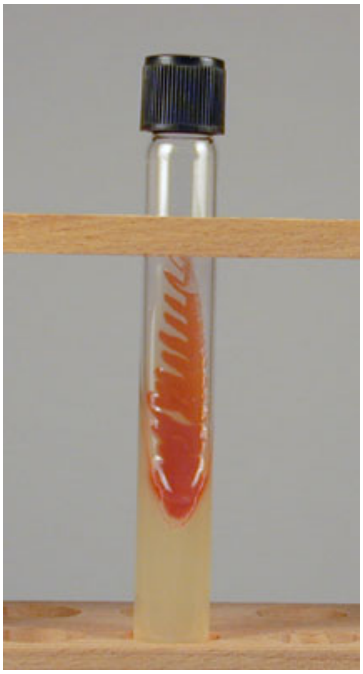
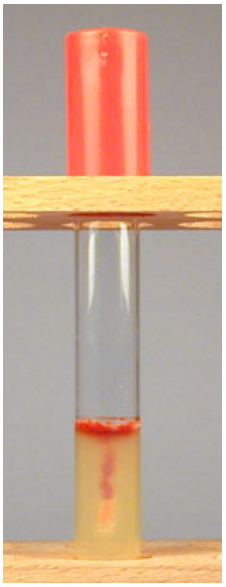
Fig. 8A: Slant Tube	**Fig. 8B:** Slant Culture
	
Uninoculated slant tube (side view)	Bacterial Growth on a Slant tube

Fig. 9:
Bacterial Growth in a Stab Tube



C. OXYGEN REQUIREMENTS FOR MICROBIAL GROWTH

Microorganisms show a great deal of variation in their requirements for gaseous oxygen. Most can be placed in one of the following groups:

1. **Obligate aerobes** are organisms that grow **only** in the presence of oxygen. They obtain energy from aerobic respiration.

2. **Microaerophiles** are organisms that require a low concentration of oxygen for growth. They obtain energy from aerobic respiration.

3. **Obligate anaerobes** are organisms that grow **only** without oxygen and, in fact, are inhibited or killed by oxygen. They obtain energy from anaerobic respiration or fermentation.

4. **Aerotolerant anaerobes**, like obligate anaerobes, cannot use oxygen for growth but they tolerate it fairly well. They obtain energy from fermentation.

5. **Facultative anaerobes** are organisms that grow with or without oxygen, but generally better with oxygen. They obtain energy from aerobic respiration, anaerobic respiration, or fermentation. Most bacteria are facultative anaerobes.

D. TEMPERATURE REQUIREMENTS

Microorganisms have a minimum and maximum temperature at which they can grow, as well as an **optimum temperature where they grow best**. Microorganisms can be divided into groups on the basis of their preferred range of temperature:

1. **Psychrophiles** are cold-loving organisms. Their optimum growth temperature is between -5C and 15C. They are usually found in the Arctic and Antarctic regions and in streams fed by glaciers.

2. **Mesophiles** are organisms that grow best at moderate temperatures. Their optimum growth temperature is between 25C and 45C. Most bacteria are mesophilic and include common soil bacteria and bacteria that live in and on the body.

3. **Thermophiles** are heat-loving organisms. Their optimum growth temperature is between 45C and 70C and are comonly found in hot springs and in compost heaps.

4. **Hyperthermophiles** are bacteria that grow at very high temperatures. Their optimum growth temperature is between 70C and 110C. They are usually members of the *Archae* and are found growing near hydrothermal vents at great depths in the ocean.

E. COLONY MORPHOLOGY AND PIGMENTATION

A **colony** is a visible mass of microorganisms growing on an agar surface and usually originating from a single organism or arrangement of organisms. Different microorganisms will frequently produce colonies that differ in their morphological appearance (form, elevation, margin, surface, optical characteristics, and pigmentation). Single colonies can be described using standard terms, as listed in **Appendix A**.

Probably the most visual characteristic is **pigmentation** (color). Some microorganisms produce pigment during growth and are said to be **chromogenic**. Often, however, formation of pigment depends on

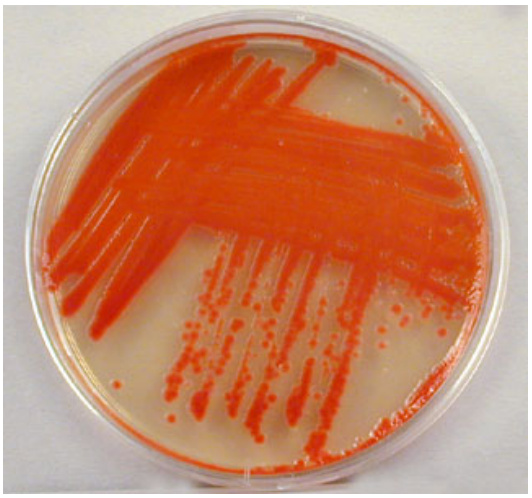
environmental factors such as temperature, nutrients, pH and moisture. For example, *Serratia marcescens* produces a deep red pigment at 25°C, but does not produce pigment at 37°C.

Pigments can be divided into two basic types: water-insoluble and water-soluble. If the pigment is **water-insoluble** (see Fig. 10A), as is the case with most chromogenic bacteria, it does not diffuse out of the organism. As a result, **the colonies are pigmented but the agar remains the normal color**. If the pigment is **water-soluble (Fig. 10B)** (as in the case of *Pseudomonas aeruginosa*) it will diffuse out of the organism into the surrounding medium **causing the agar to appear pigmented**. Note that pigment can generally be detected only on a medium that was **originally colorless**.

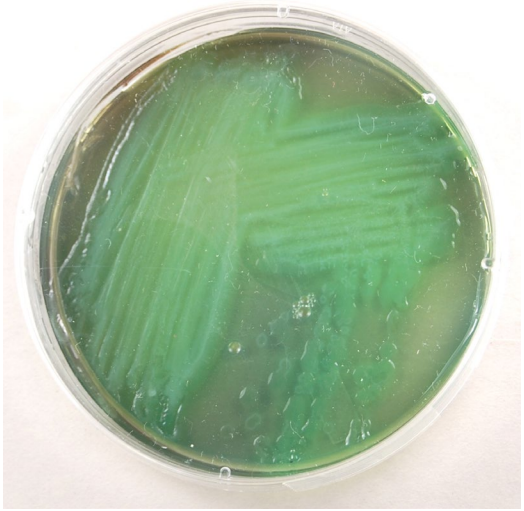
Below is a list of several common chromogenic bacteria:

Staphylococcus aureus - gold; water-insoluble
Micrococcus luteus - yellow; water-insoluble
Micrococcus roseus - pink; water-insoluble
Mycobacterium phlei - orange; water-insoluble
Serratia marcescens - orange/red; water-insoluble
Pseudomonas aeruginosa - green/blue; water-soluble

Fig. 10:
Water Insoluble and Water-Soluble Pigment
in Chromogenic Bacteria



A. Water-insoluble pigment



B. Water-soluble pigment

MEDIA

Trypticase Soy Broth tubes (4), Trypticase Soy Agar slant tubes (4), Trypticase Soy Agar stab tubes (4), and Trypticase Soy Agar plates (7).

LABORATORY 2:
ASEPTIC TECHNIQUE AND TRANSFER OF MICROORGANISMS

ORGANISMS

Trypticase Soy Broth cultures of *Bacillus subtilis, Escherichia coli* and *Micrococcus luteus*, and Trypticase Soy Agar plate cultures of *Mycobacterium phlei*.

PROCEDURE: to be done in pairs

1. Aseptically inoculate one Trypticase Soy Broth tube, one Trypticase Soy Agar slant tube, one Trypticase Soy Agar stab tube, and one Trypticase Soy Agar plate with B. subtilis. (See Fig. 11)

Remember to label all tubes with a wax marker. When streaking the agar plates, use either of the patterns shown in Figure 2A or 2B. This procedure is termed **streaking for isolation** and has a diluting effect. The friction of the loop against the agar causes organisms to fall off the loop. Near the end of the streaking pattern, individual organisms become separated far enough apart on the agar surface to give rise to **isolated single colonies** after incubation. (See Fig. 6)

2. Aseptically inoculate one Trypticase Soy Broth tube, one Trypticase Soy Agar slant tube, one Trypticase Soy Agar stab tube, and one Trypticase Soy Agar plate with E. coli. (See Fig. 11)

3. Aseptically inoculate one Trypticase Soy Broth tube, one Trypticase Soy Agar slant tube, one Trypticase Soy Agar stab tube, and one Trypticase Soy Agar plate with M. luteus. (See Fig. 11)

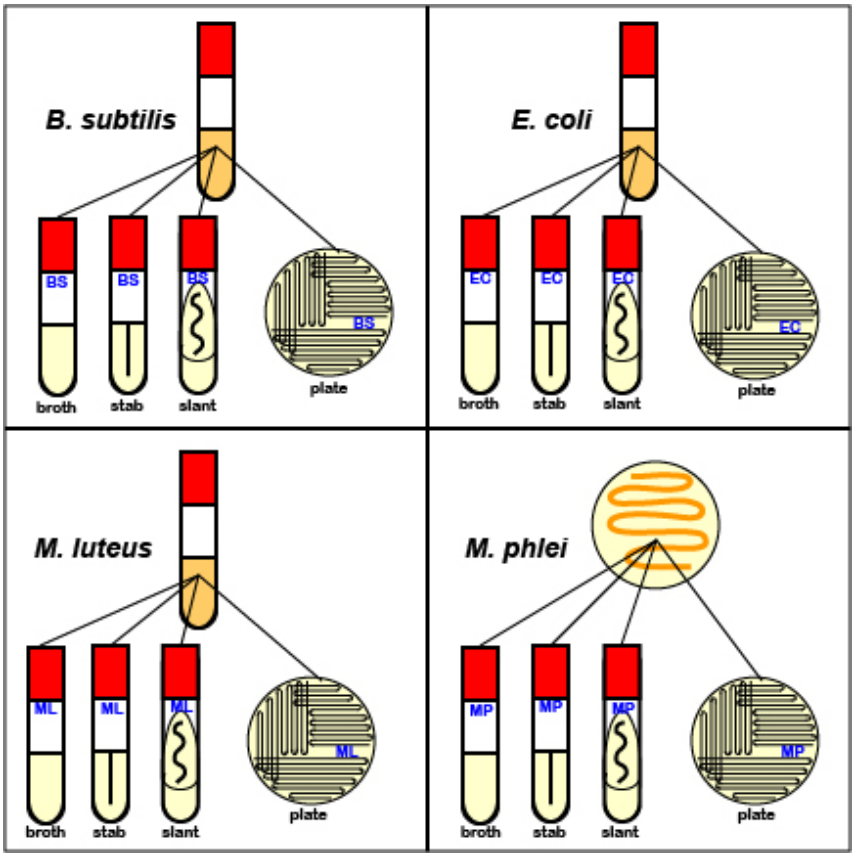
4. Aseptically inoculate one Trypticase Soy Broth tube, one Trypticase Soy Agar slant tube, one Trypticase Soy Agar stab tube, and one Trypticase Soy Agar plate with M. phlei. (See Fig. 11)

5. Incubate all the tubes and plates inoculated with *B. subtilis, E. coli, M. luteus*, and *M. phlei* at **37°C**. **Place the tubes in your dedidated test tube rack**. **Incubate the petri plates upside down** (lid on the bottom) **and stacked in the petri plate holder on the shelf of the 37°C incubator corresponding to your lab section**. Incubating the plates upside down prevents condensing water from falling down on the growing colonies and causing them to run together. (Store your test tube rack on your incubator shelf when not in use.)

6. In order to illustrate that microorganisms are all around us and to demonstrate the necessity for proper aseptic technique, **contaminate** three Trypticase Soy Agar plates as follows:

> a. Remove the lid from the first agar plate and place the exposed agar portion in or out of the building for the duration of today's lab. Replace the lid, label, and incubate it **upside-down at room temperature. Do this plate first.**
>
> b. Using a wax marker, divide a second petri plate in half. You and your partner both moisten a sterile cotton swab in sterile water. Rub your swab over some surface in the building or on yourself. Use this swab to inoculate your half of the second agar plate. Label the plate and incubate **upside-down at room temperature**.
>
> c. With a wax marker, divide a third petri plate into quarters and label as shown in Fig. 12. On your half of the plate, first rub the fingers of one of your gloved hands over your "glove" quadrant. Remove that glove and rub your fingers over your "fingers" quadrant. Your partner will do the same on his or her half of the plate. Label the plate and incubate **upside-down in your petri plate holder at 37°C. Do this plate last.**

Fig. 11:
Procedure for Lab 2



Inoculate 1 broth tube, 1 stab tube, 1 slant tube, and 1 petri plate with each bacterium.

Fig. 12:
Inoculation of Your Fingers Plate

On your half of the plate, first rub the fingers of one of your gloved hands over your "glove" quadrant. Remove that glove and rub your fingers over your "fingers" quadrant. Your partner will do the same on his or her half of the plate.

RESULTS

1. Draw and describe the growth seen in each of the four broth cultures.

			
B. subtilis Type(s) of growth:	***E. coli*** Type(s) of growth:	***M. luteus*** Type(s) of growth:	***M. phlei*** Type(s) of growth:

2. Observe the growth in the slant cultures and stab cultures for pigmentation and purity.

3. Using the terms in the Appendix A, compare a single colony of *B. subtilis* with a single colony of *M. luteus*. Use a hand lens or a dissecting microscope to magnify the colony.

Characteristics	***B. subtilis***	***M. luteus***
Form of colony		
Elevation		
Margin (edge)		
surface		
Optical characteristics		
pPgmentation		

4. Observe the results of the three "contamination" plates and note the differences in colony appearances.

5. Observe the demonstration plates of chromogenic bacteria and state the color and water-solubility of each pigment.

Organism	Color	Solubility
Micrococcus luteus		
Micrococcus roseus		
Mycobacterium phlei		
Serratia marcescens		
Pseudomonas aeruginosa		

PERFORMANCE OBJECTIVES
LABORATORY 2

After completing this lab, the student will be able to perform the following objectives:

DISCUSSION

1. Define the following terms: pure culture, sterile medium, inoculum, aseptic technique, and colony.
2. State and define the three types of growth that may be seen in a broth culture.
3. Define the following terms: obligate aerobe, microaerophile, obligate anaerobe, aerotolerant anaerobe, and facultative anaerobe.
4. Define the following terms: psychrophile, mesophile, thermophile, and hyperthermophile.
5. Define the following terms: chromogenic, water-soluble pigment, and water-insoluble pigment.

PROCEDURE

1. Using an inoculating loop, demonstrate how to aseptically remove some inoculum from either a broth tube, slant tube, stab tube, or petri plate, and inoculate a sterile broth tube, slant tube, stab tube, or petri plate without introducing outside contamination.
2. Label all tubes and plates and place them on the proper shelf in the incubator corresponding to your lab section.
3. Return all class pure cultures to the instructor's lab bench.
4. Dispose of all materials when the experiment is completed, being sure to remove all markings from the glassware. Place all culture tubes in the plastic baskets in the biohazard hood and all petri plates in the buckets in the biohazard hood.

RESULTS

1. Recognize and identify the following types of growth in a broth culture: pellicle, turbidity, sediment, and any combination of these.
2. State the color and water-solubility of pigment seen on a plate culture of a chromogenic bacterium.

LABORATORY 3
OBTAINING PURE CULTURES FROM A MIXED POPULATION

A. Streak Plate Method of Isolation
B. The Pour Plate and Spin Plate Methods of
C. Use of Specialized Media

DISCUSSION

As stated in Lab 2, microorganisms exist in nature as mixed populations. However, to study microorganisms in the laboratory we must have them in the form of a pure culture, that is, one in which all organisms are descendants of the same organism.

Two major steps are involved in obtaining pure cultures from a mixed population:

1. First, the mixture must be diluted until the various **individual microorganisms become separated** far enough apart on an agar surface that after incubation they form visible **colonies isolated from the colonies of other microorganisms**. This plate is called an **isolation plate**.

2. Then, an isolated colony can be aseptically **"picked off"** the isolation plate (see Fig. 1) and **transferred** to new sterile medium (see Fig. 3). After incubation, all organisms in the new culture will be descendants of the same organism, that is, a **pure culture**.

A. STREAK PLATE METHOD OF ISOLATION

The most common way of separating bacterial cells on the agar surface to obtain isolated colonies is the streak plate method we used in Lab 2 to inoculate a petri plate. It provides a simple and rapid method of diluting the sample by mechanical means. As the loop is streaked across the agar surface, more and more bacteria are rubbed off until individual separated organisms are deposited on the agar. After incubation, the area at the beginning of the streak pattern will show confluent growth while the area near the end of the pattern should show **discrete colonies** (see Fig. 2).

B. THE POUR PLATE AND SPIN PLATE METHODS OF ISOLATION

Another method of separating bacteria is the pour plate method. With the **pour plate method**, the bacteria are mixed with melted agar until evenly distributed and separated throughout the liquid. The melted agar is then poured into an empty plate and allowed to solidify. After incubation, discrete bacterial colonies can then be found growing both on the agar and in the agar.

The **spin plate method** involves diluting the bacterial sample in tubes of sterile water, saline, or broth. Small samples of the diluted bacteria are then pipetted onto the surface of agar plates. A sterile, bent-glass rod is then used to spread the bacteria evenly over the entire agar surface. In Lab 4 we will use this technique as part of the plate count method of enumerating bacteria.

LABORATORY 3:
OBTAINING PURE CULTURES FROM A MIXED POPULATION

Fig. 1:
Picking off an isolated colony from an isolation plate

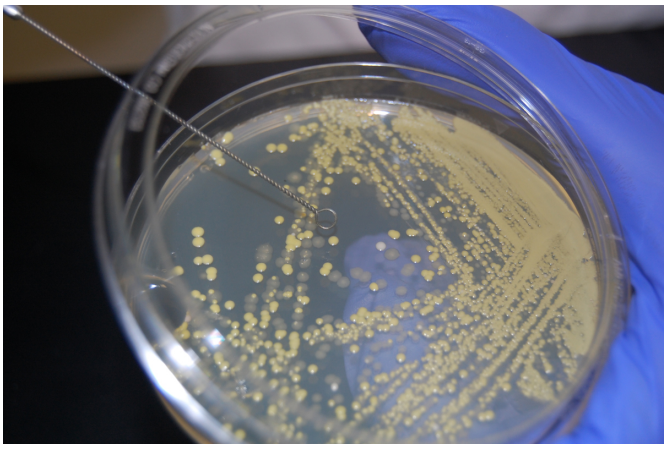


Fig. 2:
Isolated colonies of two bacteria seen on an isolation plate

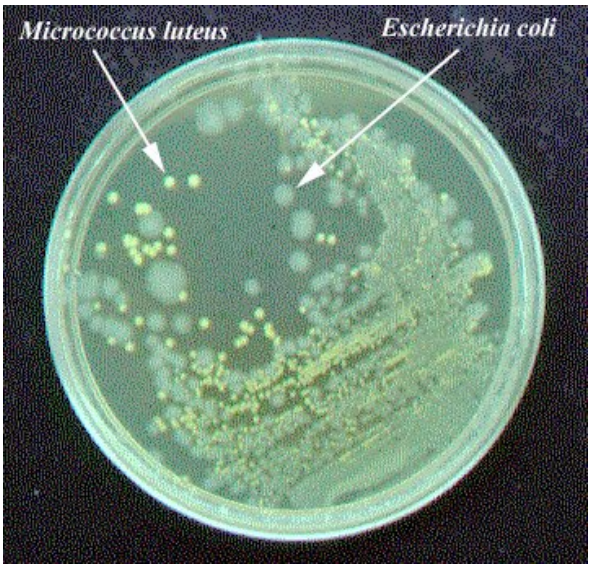
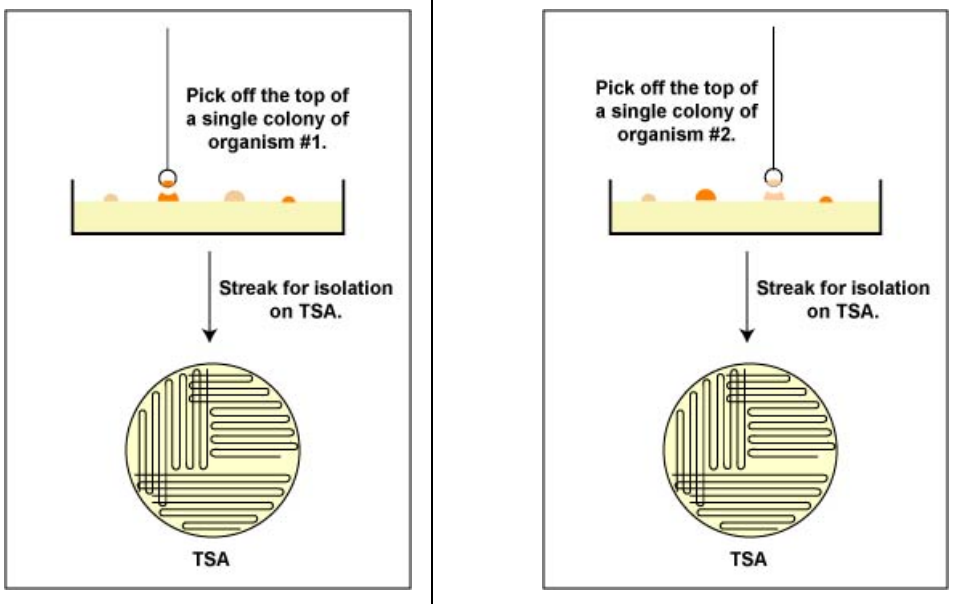


Fig. 3:
Obtaining Pure Cultures from an Isolation Plate



C. USE OF SPECIALIZED MEDIA

To supplement mechanical techniques of isolation such as the streak plate method, many **special-purpose media** are available to the microbiologist to aid in the isolation and identification of specific microorganisms. These special purpose media fall into four groups: selective media, differential media, enrichment media, and combination selective and differential media.

1. **Selective media**

A selective medium has agents added which will **inhibit the growth of one group of organisms while permitting the growth of another**. For example, **Columbia CNA agar** has the antibiotics colistin and nalidixic acid added which inhibit the growth of Gram-negative bacteria but not the growth of Gram-positives. It is, therefore, said to be **selective for Gram-positive organisms**, and would be useful in separating a mixture of Gram-negative and Gram-positive bacteria.

Remember that even if you can only see the the Gram-positive bacterium growing on a plate of Columbia CNA agar that was streaked with a mixture of a Gram-positive and a Gram-negative bacterium, this is NOT a pure culture. Tha Gram-negative bacteria are **inhibited, not killed**, and they are still alive on the agar surface. They have just not grown sufficiently that colonies are visible to the naked eye. **Colonies still have to be picked off of the Columbia CNA agar and streaked on a new agar plate before you have obtained a pure culture**.

2. **Differential media**

A differential medium contains additives that **cause an observable color change in the medium when a particular chemical reaction occurs**. They are useful in differentiating bacteria according to some biochemical characteristic. In other words, **they indicate whether or not a certain organism can carry out a specific biochemical reaction** during its normal metabolism. Many such media will be used in future labs to aid in the identification of microorganisms.

3. **Enrichment media**

An enrichment medium contains additives that **enhance the growth of certain organisms**. This is useful when the organism you wish to culture is present in relatively small numbers compared to the other organisms growing in the mixture.

4. **Combination selective and differential media**

A combination selective and differential medium **permits the growth of one group of organisms while inhibiting the growth of another**. In addition, it **differentiates those organisms that grow based on whether they can carry out particular chemical reactions**.

For example, **MacConkey agar** is a selective medium used for the **isolation of non-fastidious Gram-negative rods**, particularly members of the family *Enterobacteriaceae* and the genus *Pseudomonas*, and the **differentiation of lactose fermenting from lactose non-fermenting Gram-negative bacilli**. MacConkey agar **contains the dye crystal violet well as bile salts that inhibit the growth of most Gram-positive bacteria but do not affect the growth of most Gram-negatives**. If the Gram-negative bacterium ferments the sugar lactose in the medium, the acid end products lower the pH of the medium. The neutral red in the agar turns red in color once

the pH drops below 6.8. As the pH drops, the neutral red is absorbed by the bacteria, causing the colonies to appear bright pink to red.

Results are interpreted as follows:

- **Strong fementation of lactose** with high levels of acid production by the bacteria causes the colonies and confluent growth to appear **bright pink to red**. The resulting acid, at high enough concentrations, can also causes the bile salts in the medium to precipitate out of solution causing **a pink halo to appear around the the growth**.
- **Weak fermentation of lactose** by the bacteria causes the colonies and confluent growth to appear **pink to red,** but without the precipitation of bile salts there is **no pink halo around the growth**.
- If the bacteria **do not ferment lactose**, the colonies and confluent growth appear **colorless** and the agar surrounding the bacteria remains relatively transparent.

Escherichia coli: colonies and confluent growth appear bright pink to red and surrounded by a pink halo

Enterobacter and *Klebsiella*: colonies and confluent growth appear bright pink to red but are not surrounded by a pink halo

Pseudomonas. Salmonella and *Shigella* and *Proteus*: colorless colonies

Remember that even if you can only see the the Gram-negative bacterium growing on a plate of MacConkey agar that was streaked with a mixture of a Gram-positive and a Gram-negative bacterium, this is NOT a pure culture. Tha Gram-positive bacteria are **inhibited, not killed**, and they are still alive on the agar surface. They have just not grown sufficiently that colonies are visible to the naked eye. **Colonies still have to be picked off of the MacConkey agar and streaked on a new agar plate before you have obtained a pure culture**.

There are literally hundreds of special-purpose media available to the microbiologist. Today we will combine both a mechanical isolation technique (the streak plate) with selective and selective-differential media to obtain pure cultures from a mixture of bacteria. In future labs, such as 12 - 16, which deal with the isolation and identification of pathogenic bacteria, we will use many additional special-purpose media.

MEDIA

One plate of each of the following media: Trypticase Soy agar, Columbia CNA agar, and MacConkey agar.

ORGANISMS

A broth culture containing a mixture of one of the following Gram-positive bacteria and one of the following Gram- negative bacteria:

Possible Gram-positive bacteria:

Micrococcus luteus. A **Gram-positive coccus with a tetrad or a sarcina arrangement**; produces circular, convex colonies with a **yellow, water-insoluble pigment** on Trypticase Soy agar and Columbia CNA agar.

Staphylococcus epidermidis. A **Gram-positive coccus with a staphylococcus arrangement**; produces circular, convex, **non-pigmented colonies** on Trypticase Soy agar and Columbia CNA agar.

Possible Gram-negative bacteria:

Escherichia coli. A **Gram-negative bacillus**; produces irregular, raised, **non-pigmented colonies** on Trypticase Soy agar.

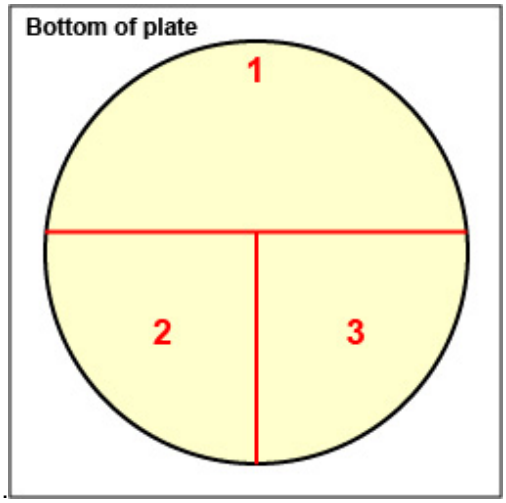
Klebsiella aerogenes (formerly called *Enterobacter aerogenes*). A **Gram-negative bacillus**; produces irregular raised, **non-pigmented colonies** on Trypticase Soy agar.

Pseudomonas aeruginosa. A **Gram-negative bacillus**; produces irregular raised colonies and a **green to blue water-soluble pigment** on Trypticase soy agar.

During the next two labs you will attempt to obtain pure cultures of each organism in your mixture and determine which two bacteria you have. **Today** you will try to separate the bacteria in the mixture in order to obtain isolated colonies; **next lab** you will identify the two bacteria in your mixture and pick off single isolated colonies of each of the two bacteria in order to get a pure culture of each. The **following lab** you will prepare microscopy slides of each of the two pure cultures to determine if they are indeed pure.

PROCEDURE: to be done in pairs

1. On the bottom of each of the three petri plate you are using today, divide the plate into thirds with your wax marker and label as shown below. This will guide your streaking.



2. Although Trypticase Soy agar (TSA), which grows both Gram-positive and Gram-negative bacteria, is not normally used as an isolation medium, we will attempt to obtain isolated colonies of the two organisms in your mixture by using strictly mechanical methods. Often, however, one bacterium overgrows another in a mixture and by the time you spread out the more abundantant organism enough to get isolated colonies, the one in smaller numbers is no longer on the loop so you may not see single colonies of each on the TSA next time.

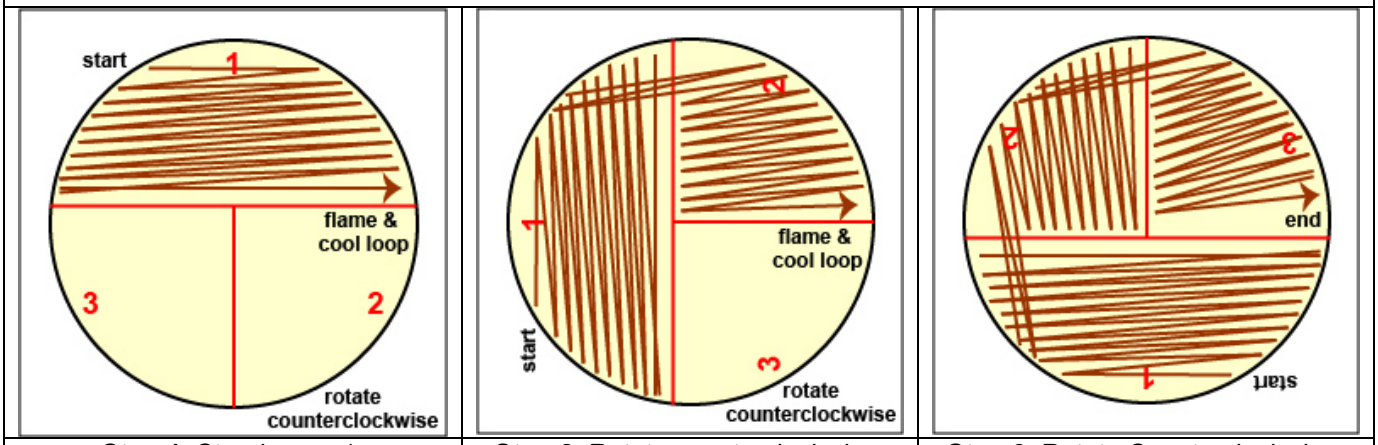
Streak the mixture on a plate of Trypticase Soy agar using one of the two streaking patterns **illustrated in Lab 2, Fig. 4 or Fig. 5**.

3. Streak the same mixture for isolation on a plate of Columbia CNA agar (selective for Gram-positive bacteria).

3. Streak the same mixture for isolation on a plate of MacConkey agar (selective for Gram- negative bacteria and differential for certain members of the bacterial family *Enterobacteriaceae* as well as *Pseudomonas*.

4. Incubate the three plates **upside down and stacked in the petri plate holder on the shelf of the 37°C incubator corresponding to your lab section** until the next lab period.

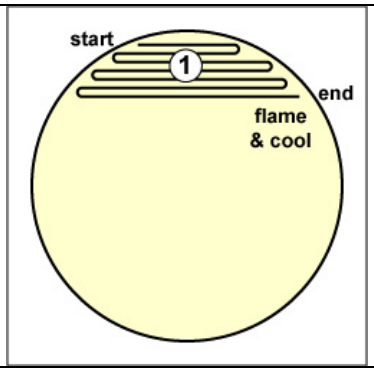
Lab 2, Fig. 4:
Inoculating a Petri Plate - Method 1



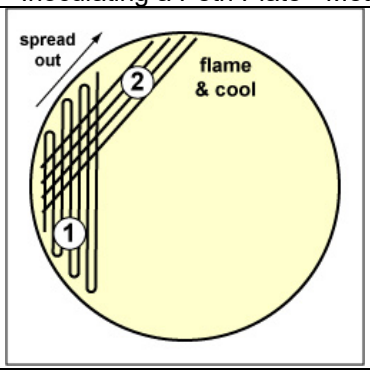
Step 1: Streak area 1. Sterilize the loop.	**Step 2**: Rotate counterclockwise. Spread area 1 over area 2. Sterilize the loop.	**Step 3**: Rotate Counterclockwise. Spread area 2 over area 3. Sterilize the loop.

LABORATORY 3:
OBTAINING PURE CULTURES FROM A MIXED POPULATION

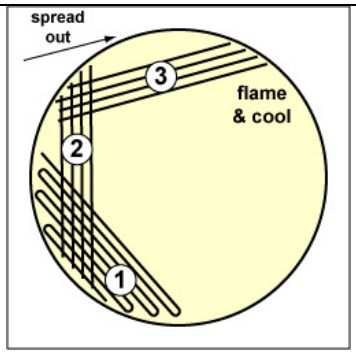
Lab 2, Fig. 5:
Inoculating a Petri Plate - Method 2



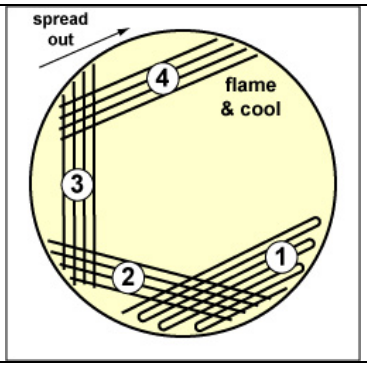
Step 1: Streak area 1.
Sterilize the loop.



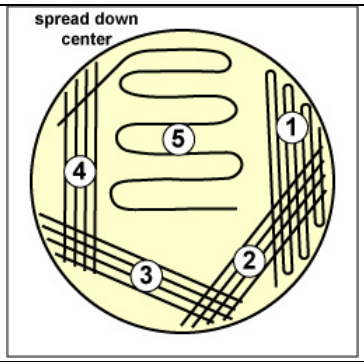
Step 2: Rotate counterclockwise.
Spread area 1 over area 2.
Sterilize the loop.



Step 3: Rotate counterclockwise.
Spread area 2 over area 3.
Sterilize the loop.



Step 4: Rotate counterclockwise.
Spread area 3 over area 4.
Sterilize the loop



Step 5: Draw your loop through area "4" and spread it down the center of the plate without touching any of the areas already streaked.

RESULTS

1. Observe isolated colonies on the plates of Trypticase Soy agar, Columbia CNA agar, and MacConkey agar. Record your observations and conclusions.

Trypticase Soy agar	
Observations	
Conclusions	
Columbia CNA agar	
Observations	
Conclusions	
MacConkey agar	
Observations	
Conclusions	

2. Using any of the three plates on which they are growing:

a. **Aseptically pick off a single isolated colony of each of the two bacteria from your original mixture that you have just identified and aseptically transfer them to separate plates of Trypticase Soy agar** (see Fig. 3). Remember to **streak the plate for isolation** as you learned in labs 2 and 3.

b. When picking off single colonies, **remove the top portion of the colony without touching the agar surface itself** to avoid picking up any inhibited bacteria from the surface of the agar. **Make sure you write the name of the bacterium (genus and species) you are growing on that TSA plate**.

c. Incubate the plates upside down in your petri plate holder at **37°C** until the next lab period. These will be your pure cultures for Lab 5 (Direct and Indirect stains).

PERFORMANCE OBJECTIVES
LABORATORY 3

After completing this lab, the student will be able to complete the following objectives:

DISCUSSION

1. Given a mixture of a Gram-positive and a Gram- negative bacterium and plates of Columbia CNA, MacConkey, and Trypticase Soy agar, describe the steps you would take to eventually obtain pure cultures of each organism.
2. Define the following: selective medium, differential medium, enrichment medium, and combination selective-differential medium.
3. State the usefulness of Columbia CNA agar and MacConkey agar.
4. Describe how each of the following would appear when grown on MacConkey agar:
    a. *Escherichia coli*
    b. *Klebsiella aerogenes*
    c. *Pseudomonas aeruginosa*

PROCEDURE

1. Using the streak plate method of isolation, obtain isolated colonies from a mixture of microorganisms.
2. Pick off isolated colonies of microorganisms growing on a streak plate and aseptically transfer them to sterile media to obtain pure cultures.

RESULTS

1. When given a plate of Columbia CNA agar or MacConkey agar showing discrete colonies, correctly interpret the results.

LABORATORY 4
ENUMERATION OF MICROORGANISMS

A. Plate Count
B. Direct Microscopic Method
C. Turbidity

DISCUSSION

As part of daily routine, the laboratory microbiologist often has to determine the number of bacteria in a given sample as well as having to compare the amount of bacterial growth under various conditions. Enumeration of microorganisms is especially important in dairy microbiology, food microbiology, and water microbiology.

Since the enumeration of microorganisms involves the use of extremely small dilutions and extremely large numbers of cells, scientific notation is routinely used in calculations. **A review of exponential numbers, scientific notation, and dilutions is found in Appendix B.**

A. THE PLATE COUNT (VIABLE COUNT)

The number of bacteria in a given sample is usually too great to be counted directly. However, if the sample is serially diluted (see Fig. 7) and then plated out on an agar surface in such a manner that **single isolated bacteria form visible isolated colonies** (see Fig. 1), the number of colonies can be used as a measure of the number of viable (living) cells in that known dilution. However, keep in mind that if the organism normally forms multiple cell arrangements, such as chains, the colony-forming unit may consist of a chain of bacteria rather than a single bacterium. In addition, some of the bacteria may be clumped together. Therefore, when doing the plate count technique, we generally say we are determining the number of **Colony-Forming Units (CFUs)** in that known dilution. By extrapolation, this number can in turn be used to calculate the number of CFUs in the original sample.

Normally, the bacterial sample is diluted by factors of 10 and plated on agar. After incubation, the number of colonies on a dilution plate showing **between 30 and 300 colonies** is determined (see Fig. 1). A plate having 30-300 colonies is chosen because this range is considered statistically significant. If there are less than 30 colonies on the plate, small errors in dilution technique or the presence of a few contaminants will have a drastic effect on the final count. Likewise, if there are more than 300 colonies on the plate, there will be poor isolation and colonies will have grown together.

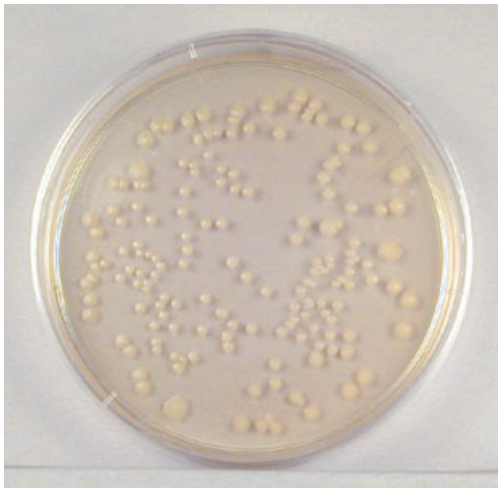
Generally, one wants to determine the number of CFUs **per milliliter (ml) of sample**. To find this, the number of colonies (on a plate having 30-300 colonies) is multiplied by the number of times the original ml of bacteria was diluted (the **dilution factor** of the plate counted). For example, if a plate containing a 1/1,000,000 dilution of the original ml of sample shows 150 colonies, then 150 represents 1/1,000,000 the number of CFUs present in the original ml. Therefore, the number of CFUs per ml in the original sample is found by multiplying 150 x 1,000,000 as shown in the formula below:

The number of CFUs per ml of sample =

The number of colonies (30-300 plate)
X
The dilution factor of the plate counted

In the case of the example above, 150 x 1,000,000 = 150,000,000 CFUs per ml. ($1.5 \times 10^2 \times 10^6 - 1.5 \times 10^8$ in scientific notation.)

Fig. 1:
A petri plate showing between 30 and 300 colonies



For a more accurate count it is advisable to plate each dilution in duplicate or triplicate and then find an average count.

There are two practice plate count problems with answers found in the Performance Objectives in the online version of your lab manual. Make sure you practice these for Lab Quiz 1.

B. DIRECT MICROSCOPIC METHOD (TOTAL CELL COUNT)

In the direct microscopic count, a counting chamber consisting of a ruled slide and a coverslip is employed. It is constructed in such a manner that the coverslip, slide, and ruled lines delimit a known volume. **The number of bacteria in a small known volume is directly counted microscopically and the number of bacteria in the larger original sample is determined by extrapolation.**

The Petroff-Hausser counting chamber (Fig. 2), for example, has small etched squares 1/20 of a millimeter (mm) by 1/20 of a mm and is 1/50 of a mm deep. The volume of one small square therefore is 1/20,000 of a cubic mm or 1/20,000,000 of a cubic centimeter (cc). There are 16 small squares in the large double-lined squares that are counted, making the volume of a large double-lined square 1/1,250,000 cc. The normal

procedure is to count the number of bacteria in **five large double-lined squares and divide by five to get the average number of bacteria per large square**. This number is then **multiplied by 1,250,000,** since the square holds a volume of 1/1,250,000 cc, to find the total number of organisms per cc in the original sample.

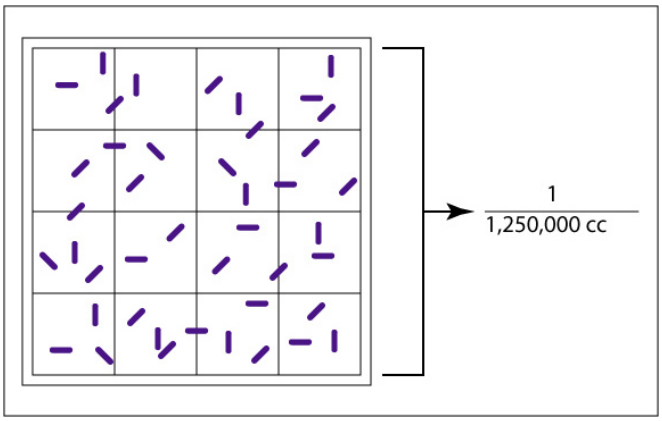
If the bacteria are diluted, such as by mixing the bacteria with dye before being placed in the counting chamber, then this dilution must also be considered in the final calculations.

The formula used for the direct microscopic count is:

The number of bacteria per cc =

The average number of bacteria per large double-lined square
X
The dilution factor of the large square (1,250,000 or 1.25 X 10^6)
X
The dilution factor of any dilutions made prior to placing the sample in the counting chamber

Fig. 2:
Large double-lined square of a Petroff-Hausser counting chamber



There are two direct microscopic count problems with answers found in the Performance Objectives in the online version of your lab manual. Make sure you practice these for Lab Quiz 1.

C. TURBIDITY

As seen in Lab 2, when you mix the bacteria growing in a liquid medium, the culture appears **turbid**. This is because a bacterial culture acts as a colloidal suspension that blocks and reflects light passing through the culture. Within limits, the light absorbed by the bacterial suspension will be directly proportional to the concentration of cells in the culture. By measuring the amount of light absorbed by a bacterial suspension, one can estimate and compare the number of bacteria present.

The instrument used to measure turbidity is a **spectrophotometer**. It consists of a light source, a filter that allows only a single wavelength of light to pass through, the sample tube containing the bacterial suspension,

and a photocell that compares the amount of light coming through the tube with the total light entering the tube.

The ability of the culture to block the light can be expressed as either percent of light transmitted through the tube or the amount of light absorbed in the tube (see Fig. 4). The **percent of light transmitted** is inversely proportional to the bacterial concentration. (The greater the number of bacteria, the lower the percent light transmitted.) The **absorbance**, or optical density, is directly proportional to the cell concentration. (The greater the number of bacteria, the higher the absorbance.)

Turbidimetric measurement is often correlated with some other method of cell count, such as the direct microscopic method or the plate count. In this way, turbidity can be used as an indirect measurement of the cell count. For example:

1. Several dilutions can be made of a bacterial stock.
2. A Petroff-Hausser counter can then be used to perform a direct microscopic count on each dilution.
3. Then a spectrophotometer can be used to measure the absorbance of each dilution tube.
4. A standard curve comparing absorbance to the number of bacteria can be made by plotting absorbance versus the number of bacteria per cc (see Fig. 5)
5. Once the standard curve is completed, any dilution tube of that organism can be placed in a spectrophotometer and its absorbance read. Once the absorbance is determined, the standard curve can be used to determine the corresponding number of bacteria per cc (see Fig. 6)

Fig. 4:
Absorbance and % Light Transmitted

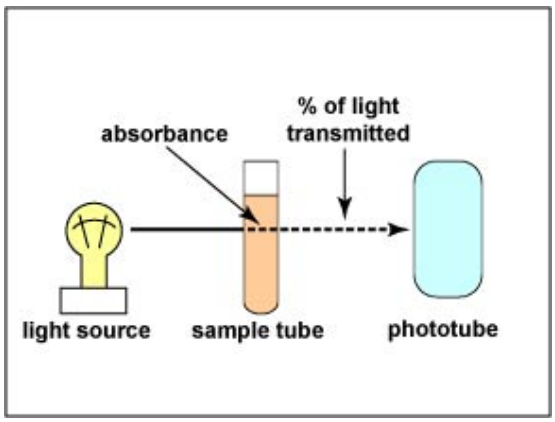
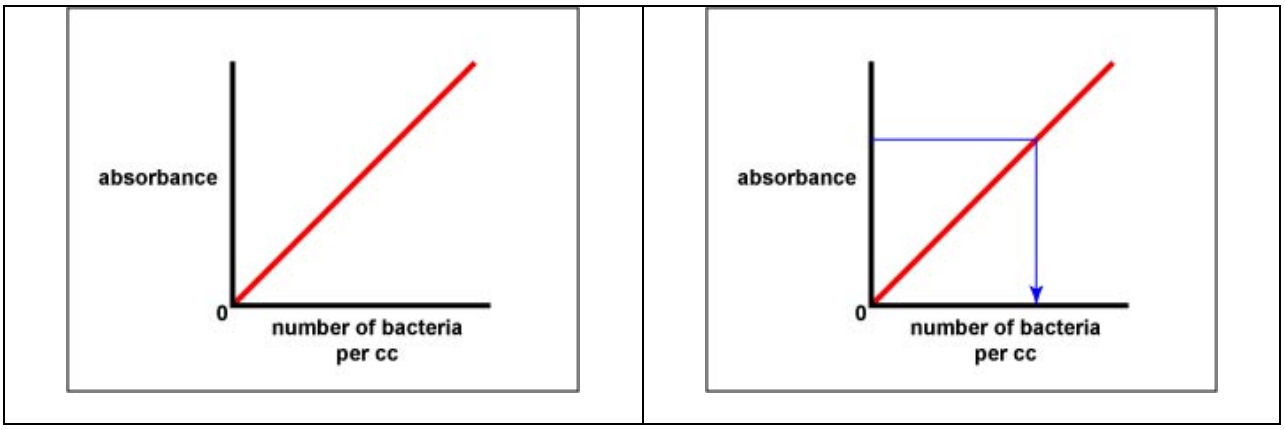


Fig. 5: A Standard Curve Plotting the Number of Bacteria per cc versus Absorbance	**Fig. 6:** Using a Standard Curve to Determine the Number of Bacteria per cc in a Sample by Measuring the Sample's Absorbance



MATERIALS

6 tubes each containing 9.0 ml sterile saline, 3 plates of Trypticase Soy agar, 2 sterile 1.0 ml pipettes, pipette filler, turntable, bent glass rod, dish of alcohol

ORGANISM

Trypticase Soy broth culture of *Escherichia coli*

PROCEDURE

A. Plate Count: to be done in pairs

1. Take 6 dilution tubes, each containing 9.0 ml of sterile saline. Aseptically dilute **1.0 ml** of a sample of *E. coli* **as shown in Fig. 7** and described below.

a. Remove a sterile 1.0 ml pipette from the bag. **Do not touch the portion of the pipette that will go into the tubes and do not lay the pipette down**. From the tip of the pipette to the "0" line is **1 ml**; each numbered division (0.1, 0.2, etc.) represents **0.1 ml** 1.0 ml (see Fig. 8).

b. Insert the cotton-tipped end of the pipette into a **blue** 2 ml pipette filler.

c. Place the lip of the culture tube at the opening of the microincinerator for 2-3 seconds, insert the pipette to the bottom of the flask, and withdraw **1.0 ml** of the sample (up to the "0" line; see Fig. 8) by turning the filler knob **towards** you. Draw the sample up **slowly** so that it isn't accidentally drawn into the filler itself (see Fig. 9). Re-flame and cap the sample.

d. Place the lip of the first dilution tube at the opening of the microincinerator for 2-3 seconds and dispense the 1.0 ml of sample into the tube by turning the filler knob **away** from you.

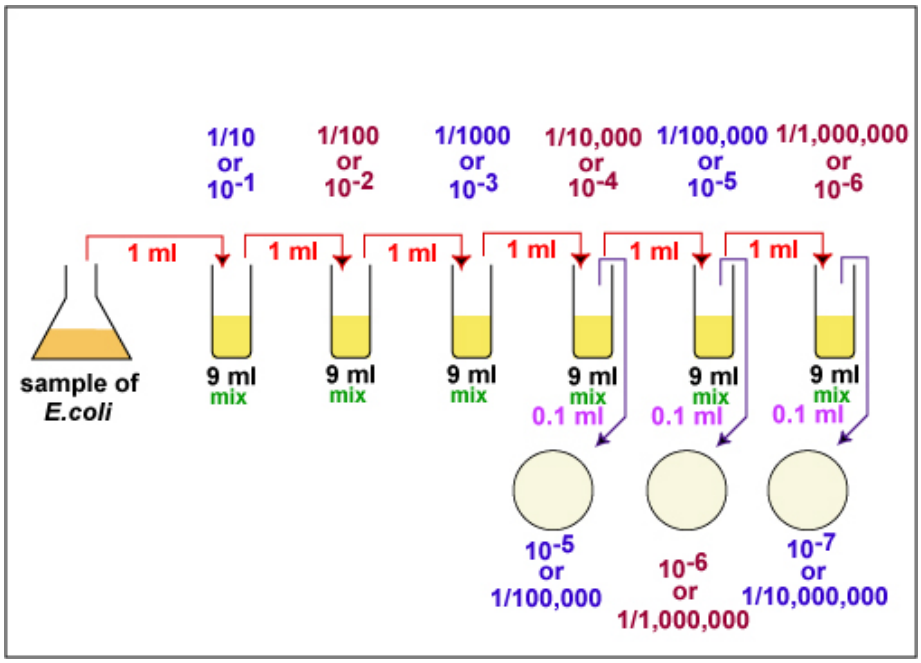
Draw the liquid up and down in the pipette several times to rinse the pipette and help mix. Re-flame and cap the tube.

e. **Mix the tube thoroughly** by either holding the tube in one hand while vigorously tapping the bottom with the other hand, or by using a vortex mixer. This is to assure an even distribution of the bacteria throughout the liquid.

f. Using the same procedure, aseptically withdraw 1.0 ml of the sample (see Fig. 8) from the first dilution tube and dispense into the second dilution tube. Continue doing this from tube to tube as shown in **Fig. 7** until the dilution is completed. Discard the pipette in the used pipette container.

Your instructor will demonstrate these pipetting and mixing techniques.

Fig. 7:
Plate count dilution procedure



2. Using a new 1.0 ml pipette, aseptically transfer **0.1 ml** (see Fig. 8) **from each of the last three dilution tubes** onto the surface of the corresponding plates of trypticase soy agar as shown in Figure 7 and Figure 10. **Note** that since only 0.1 ml of the bacterial dilution (rather than the desired 1.0 ml) is placed on the plate, the bacterial dilution **on the plate** is 1/10 the dilution of the tube from which it came.

3. Using a turntable and sterile bent glass rod, immediately spread the solution over the surface of the plates as follows:

a. Place the plate containing the 0.1 ml of dilution on a turntable.

b. Sterilize the glass rod by dipping the bent portion in a dish of alcohol and igniting the alcohol with the flame from your burner. Let the flame burn out.

c. Place the bent portion of the glass rod on the agar surface and spin the turntable for about 30 seconds to distribute the 0.1 ml of dilution evenly over the entire agar surface (see Figure 12).

d. Replace the lid and re-sterilize the glass rod with alcohol and flaming.

e. Repeat for each plate.

f. Discard the pipette in the used pipette container.

4. Incubate the 3 agar plates **upside down and stacked in the petri plate holder on the shelf of the 37°C incubator corresponding to your lab section** until the next lab period. Place the used dilution tubes in the disposal baskets in the hood.

Fig. 8:
A 1.0 ml pipette

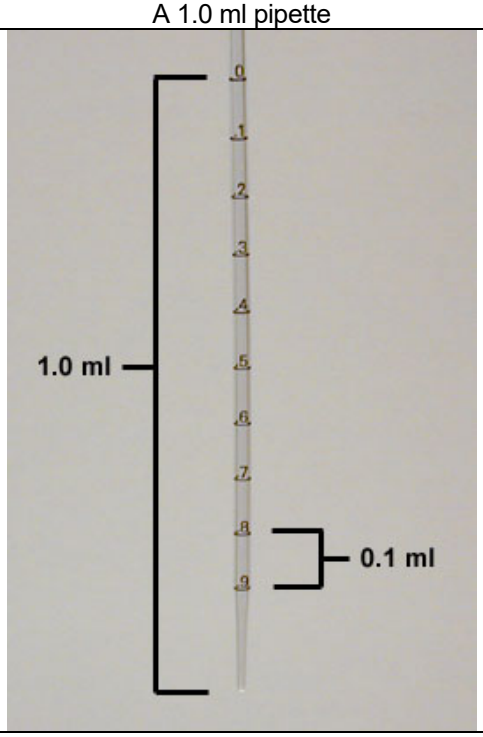


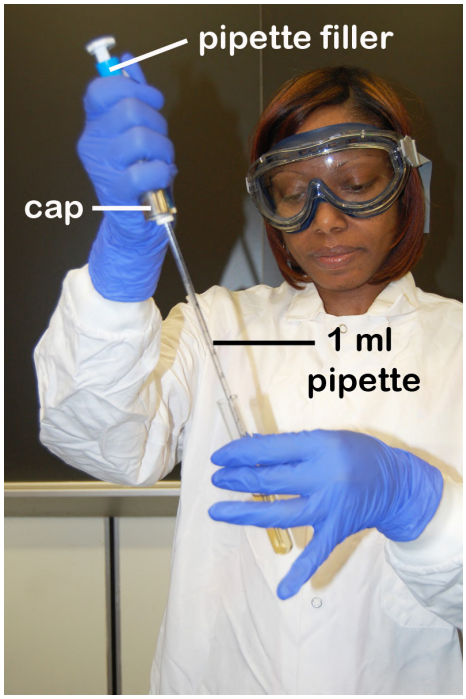
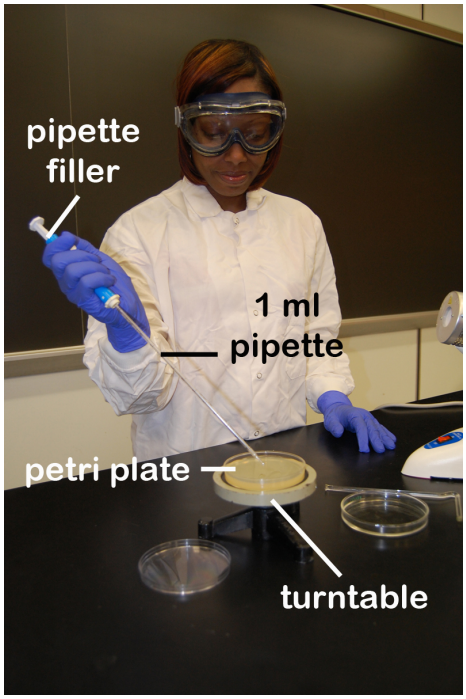
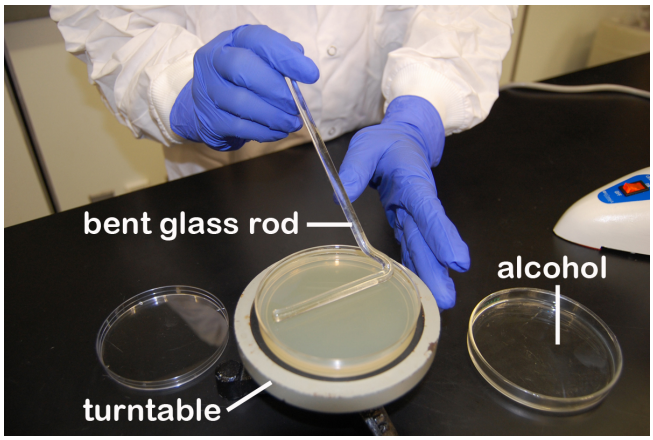
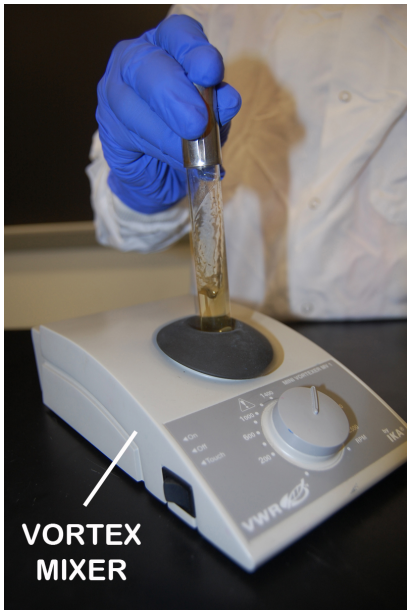
Fig. 9: Removing bacteria from a tube with a pipette.	**Fig. 10:** Using a pipette to transfer bacteria to an agar plate.
	

Fig. 11: Using a turntable and bent glass rod to spread bacteria evenly over the surface of an agar plate.	**Fig. 12:** Using a vortex mixer to distribute bacteria evenly throughout the tube.
	

B. Direct Microscopic Method: demonstration

1. Pipette 1.0 ml (see Fig. 8) of the sample of *E. coli* into a tube containing 1.0 ml of the dye methylene blue. This gives a 1/2 dilution of the sample.

2. Using a Pasteur pipette, fill the chamber of a Petroff-Hausser counting chamber with this 1/2 dilution.

3. Place a coverslip over the chamber and focus on the squares using 400X (40X objective).

4. Count the number of bacteria in 5 large double-lined squares. For those organisms on the lines, count those on the left and upper lines but not those on the right and lower lines. Divide this total number by 5 to find the average number of bacteria per large square.

5. Calculate the number of bacteria per cc as follows:

The number of bacteria per cc =

The average number of bacteria per large square
X
The dilution factor of the large square (1, 250,000)
X
The dilution factor of any dilutions made prior to placing the sample in the counting chamber (2 in this case)

C. Turbidity

Your instructor will set up a spectrophotometer demonstration illustrating that as the number of bacteria in a broth culture increases, the absorbance increases (or the percent light transmitted decreases).

RESULTS

A. Plate Count: demonstration

1. Choose a plate that appears to have between 30 and 300 colonies. Count the exact number of colonies on that plate using the colony counter (as demonstrated by your instructor).

2. Calculate the number of CFUs per ml of original sample as follows:

The number of CFUs per ml of sample =

The number of colonies (30-300 plate)
X
The dilution factor of the plate counted

_____ = The number of colonies

_____ = The dilution **factor** of plate counted

_____ = The number of CFUs per ml

3. Record your results on the blackboard.

B. Direct Microscopic Method

Observe the demonstration of the Petroff - Hausser counting chamber.

C. Turbidity

Observe your instructor's demonstration of the spectrophotometer.

PERFORMANCE OBJECTIVES
LABORATORY 4

After completing this lab, the student will be able to perform the following objectives:

DISCUSSION

1. State the formula for determining the number of CFUs per ml of sample when using the plate count technique.
2. When given a diaGram of a plate count dilution and the number of colonies on the resulting plates, choose the correct plate for counting, determine the dilution factor of that plate, and calculate the number of CFUs per ml in the original sample. **(See this objective in the on-line Lab Manual for practice problems.)**
3. State the principle behind the direct microscopic method of enumeration.
4. State the formula for determining the number of bacteria per cc of sample when using the direct microscopic method of enumeration.
5. When given the total number of bacteria counted in a Petroff-Hausser chamber, the total number of large squares counted, and the dilution of the bacteria placed in the chamber, calculate the total number of bacteria per cc in the original sample. **(See this objective in the on-line Lab Manual for practice problems.)**
6. State the function of a spectrophotometer.
7. State the relationship between absorbance (optical density) and the number of bacteria in a broth sample.
8. State the relationship between percent light transmitted and the number of bacteria in a broth sample.

PROCEDURE

1. Perform a serial dilution of a bacterial sample according to instructions in the lab manual and plate out samples of each dilution using the spin-plate technique.

RESULTS

1. Count the number of colonies on a plate showing between 30 and 300 colonies and, by knowing the dilution of this plate, calculate the number of CFUs per ml in the original sample.

LABORATORY 5
DIRECT STAINING and INDIRECT STAINING

A. Introduction to Staining
B. Direct Staining Using a Basic Dye
C. Indirect Staining Using an Acidic Dye

A. INTRODUCTION TO STAINING

DISCUSSION

In our laboratory, bacterial morphology (form and structure) may be examined in two ways:
- Observing living unstained organisms (wet mount), or
- Observing killed stained organisms.

Since bacteria are almost colorless and therefore show little contrast with the broth in which they are suspended, they are difficult to observe when unstained. Staining microorganisms enables one to:

- See greater contrast between the organism and the background,

- Differentiate various morphological types (by shape, arrangement, Gram reaction, etc.),

- Observe certain structures (flagella, capsules, endospores, etc.).

Before staining bacteria, you must first understand how to "fix" the organisms to the glass slide. If the preparation is not fixed, the organisms will be washed off the slide during staining. There are two common tecniques for doing this: heat fixation and chemical fixation.

1. **Heat fixation**

The bacteria are heat fixed by holding the bottom of the slide against the opening of a microincinerator for 10 seconds. The heat coagulates the organisms' proteins causing the bacteria to stick to the slide.

2. **Chemical fixation**

An air-dried smear of the organism is covered with several drops of 95% methanol and allowed to sit for 2 minutes, after which the methanol is poured off and the slide is again allowed to air dry. Chemical fixation has been shown to be better at enabling bacteria to adhere to the slide while causing less damage and distortion to the bacteria.

The **procedure for slide preparation prior to staining** is as follows:

1. If the culture is taken from an **agar medium**:

a. First place a small piece of **tape** at one end of the slide and **label it with the name of the bacterium** you will be placing on that slide.

b. Using the dropper bottle of **deionized water found in the staining rack**, place **1/2 of a normal sized drop of water on a clean slide** by touching the dropper to the slide. Altenately, use your sterilized inoculating loop to place a drop of deionized water on the slide.

c. Using your sterile inoculating loop, aseptically remove a **small amount** of the culture from the agar surface and **gently touch it several times to the drop of water** until the water **becomes visibly cloudy**.

d. **Incinerate the remaining bacteria on the inoculating loop**. If too much culture is added to the water, you will not see stained individual bacteria.

e. After the inoculating loop cools, **spread the suspension over the slide to form a thin film**.

f. Allow this thin suspension to **completely air dry**. The smear must be completely dry before the slide is fixed!

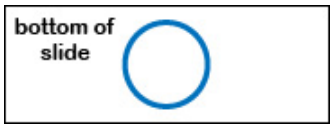
g. **If your professor instructs you to heat-fix the bacteria to the slide**, pick up the air-dried slide with provided closepin and **hold the bottom of the slide opposite the smear against the opening of the microincinerator for 10 seconds** as demonstrated by your instructor. If the slide is not heated enough, the bacteria will be washed off the slide. If it is overheated, the bacteria structural integrity can be damaged.

h. **If your professor instructs you to fix the bacteria to the slide using methanol**, add several drops of **95% methanol** to the air-dried smear of bacteria and let sit for **2 minutes**. Pick up the slide and **allow the excess methanol to drain off** as demonstrated by your instructor. Let the slide again **air dry** before staining.

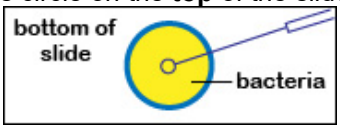
2. If the organism is taken from a **broth culture:**

a. First place a small piece of **tape** at one end of the slide and **label it with the name of the bacterium** you will be placing on that slide.

b. Using a sharpie, **draw a circle about the size if a nickel** on the **<u>bottom</u>** of your microscope slide.



c. Turn the slide over. **Using your sterile inoculating loop, aseptically place 1 -2 loops of the culture** within this circle on the **top** of the slide. **Do not use water**.



d. Using your inoculating loop, **spread the suspension over the area delineated by the circle** to form a thin film.

e. Allow this thin suspension to **completely air dry**.

f. **If your professor instructs you to heat-fix the bacteria to the slide**, pick up the air-dried slide with provided closepin and **hold the bottom of the slide opposite the smear against the opening of the microincinerator for 10 seconds** as demonstrated by your

instructor. If the slide is not heated enough, the bacteria will wash off the slide. If it is overheated, the bacteria structural integrity can be damaged.

g. **If your professor instructs you to fix the bacteria to the slide using methanol**, add several drops of **95% methanol** to the air-dried smear of bacteria and let sit for **2 minutes**. Pick up the slide and **allow the excess methanol to drain off** as demonstrated by your instructor. Let the slide again **air dry** before staining.

In order to understand how staining works, it will be helpful to know a little about the physical and chemical nature of stains. Stains are generally salts in which one of the ions is colored. (A salt is a compound composed of a positively charged ion and a negatively charged ion.) For example, the dye methylene blue is actually the salt methylene blue chloride that will dissociate in water into a positively charged methylene blue ion that is blue in color and a negatively charged chloride ion that is colorless.

Dyes or stains may be divided into two groups: basic and acidic. If the color portion of the dye resides in the **positive ion**, as in the above case, it is called a **basic dye** (examples: methylene blue, crystal violet, safranin). If the color portion is in the **negatively charged ion**, it is called an **acidic dye** (examples: nigrosin, congo red).

Because of its chemical nature, **the cytoplasm of all bacterial cells has a slight negative charge** when growing in a medium of near neutral pH. Therefore, when using a **basic dye**, the positively charged color portion of the stain combines with the negatively charged bacterial cytoplasm (opposite charges attract) and the organism becomes **directly stained** (see Fig. 1 and Fig. 2). An **acidic dye**, due to its chemical nature, reacts differently. Since the color portion of the dye is on the negative ion, it will not readily combine with the negatively charged bacterial cytoplasm (like charges repel). Instead, it forms a **deposit around the organism**, leaving the organism itself colorless (see Fig. 1 and Fig. 3). Since the organism is seen indirectly, this type of staining is called **indirect** or **negative,** and is used to get a more accurate view of bacterial size, shapes, and arrangements.

LABORATORY 5:
DIRECT STAINING AND INDIRECT STAINING

Fig. 1:
Direct Staining and Indirect Staining

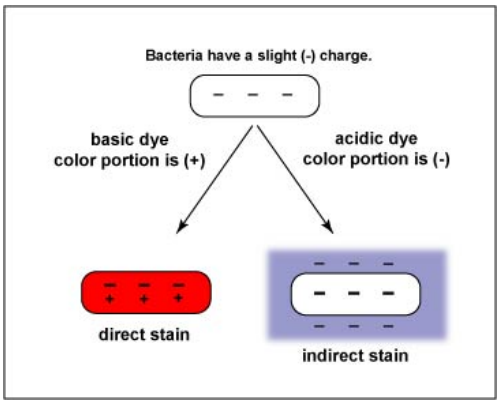


Fig. 2:
Direct stain of *Escherichia coli*

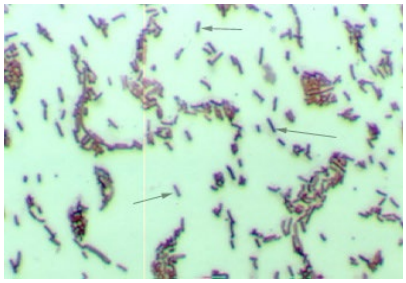
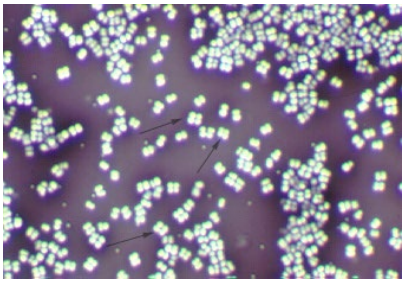


Fig. 3:
Indirect stain of *Micrococcus luteus*



In today's lab, we will make both direct and indirect stains of several microorganisms.

B. DIRECT STAIN USING A BASIC DYE

In direct staining the positively charged color portion of the basic dye combines with the negatively charged bacterium and the organism becomes directly stained (Fig. 1 and Fig. 2).

ORGANISMS

Your pure cultures of *Staphylococcus epidermidis* (coccus with staphylococcus arrangement) or *Micrococcus luteus* (coccus with tetrad or sarcina arrangement) and *Escherichia coli* (small bacillus), *Pseudomonas aeruginosa* (small bacillus), or *Enterobacter aerogenes* (small bacillus) from Lab 3.

PROCEDURE: to be done individually

1. ***Escherichia coli, Pseudomonas aeruginosa*, or *Enterobacter aerogenes***

a. Fix a smear of either *Escherichia coli, Pseudomonas aeruginosa*, or *Enterobacter aerogenes* to the slide as follows:

1. First place a small piece of **tape** at one end of the slide and **label it with the name of the bacterium** you will be placing on that slide

2. Using the dropper bottle of **deionized water found in the staining rack**, place **1/2 of a normal sized drop of water on a clean slide** by touching the dropper to the slide. Altenately, use your sterilized inoculating loop to place a drop of deionized water on the slide.

3. Using your sterile inoculating loop, aseptically remove a **small amount** of the culture from the agar surface and **gently touch it several times to the drop of water** until the water **becomes visibly cloudy**.

4. **Incinerate the remaining bacteria on the inoculating loop**. If too much culture is added to the water, you will not see stained individual bacteria.

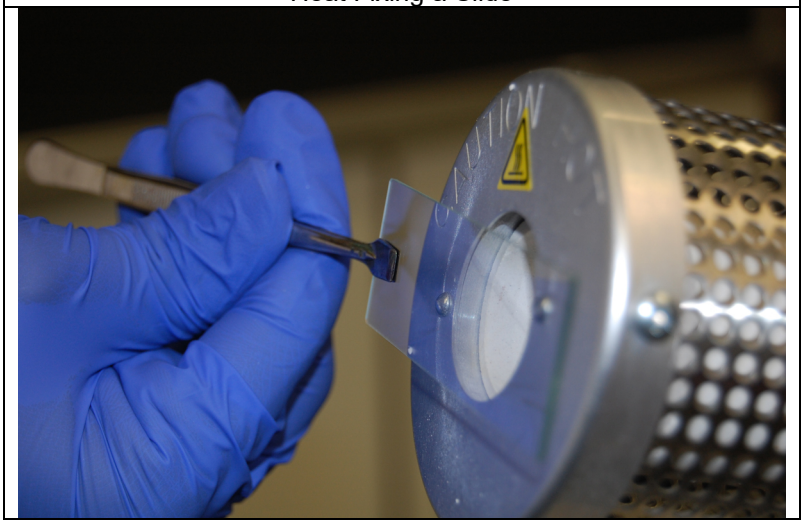
5. After the inoculating loop cools, **spread the suspension over the slide to form a thin film**.

6. Allow this thin suspension to **completely air dry**. The smear must be completely dry before the slide is fixed!

7. **If your professor instructs you to heat-fix the bacteria to the slide**, pick up the air-dried slide with provided closepin and **hold the bottom of the slide opposite the smear against the opening of the microincinerator for 10 seconds** as shown in **Fig. 3**. If the slide is not heated enough, the bacteria will be washed off the slide. If it is overheated, the bacteria structural integrity can be damaged.

8. **If your professor instructs you to fix the bacteria to the slide using methanol**, add several drops of **95% methanol** to the air-dried smear of bacteria and let sit for **2 minutes**. Pick up the slide and **allow the excess methanol to drain off** as demonstrated by your instructor. Let the slide again **air dry** before staining.

Fig. 4:
Heat Fixing a Slide



b. Place the slide on a staining tray and cover the entire film with **safranin**. Stain for **one minute**.

c. Pick up the slide by one end and hold it at an angle over the staining tray. Using the wash bottle on the bench top, gently **wash off the excess safranin** from the slide. Also wash off any stain that got on the **bottom** of the slide as well.

d. Use a book of blotting paper to blot the slide dry. Observe using oil immersion microscopy.

(Review the focusing steps from Lab 1, section D before you start.)

2. ***Micrococcus luteus* or *Staphylococcus epidermidis***

a. Fix a smear of either *Micrococcus luteus* or *Staphylococcus epidermidis* to a slide as follows:

1. First place a small piece of **tape** at one end of the slide and **label it with the name of the bacterium** you will be placing on that slide

2. Using the dropper bottle of **deionized water found in the staining rack**, place **1/2 of a normal sized drop of water on a clean slide** by touching the dropper to the slide. Altenately, use your sterilized inoculating loop to place a drop of deionized water on the slide.

3. Using your sterile inoculating loop, aseptically remove a **small amount** of the culture from the agar surface and **gently touch it several times to the drop of water** until the water **becomes visibly cloudy**.

4. **Incinerate the remaining bacteria off of the inoculating loop**. If too much culture is added to the water, you will not see stained individual bacteria.

5. After the inoculating loop cools, **spread the suspension over the slide to form a thin film**.

6. Allow this thin suspension to **completely air dry**. The smear must be completely dry before the slide is fixed!

7. **If your professor instructs you to heat-fix the bacteria to the slide**, pick up the air-dried slide with provided closepin and **hold the bottom of the slide opposite the smear against the opening of the microincinerator for 10 seconds** as shown in **Fig. 3**. If the slide is not heated enough, the bacteria will be washed off the slide. If it is overheated, the bacteria structural integrity can be damaged.

8. **If your professor instructs you to fix the bacteria to the slide using methanol**, add several drops of **95% methanol** to the air-dried smear of bacteria and let sit for **2 minutes**. Pick up the slide and **allow the excess methanol to drain off** as demonstrated by your instructor. Let the slide again **air dry** before staining.

b. Stain with **crystal violet** for **one minute.**

c. Wash off the excess crystal violet with water.

d. Blot dry and observe using oil immersion microscopy.

3. Prepare a third slide of the normal microbiota and cells of your mouth as follows:

a. Use a sterile cotton swab to vigorously scrape the inside of your mouth and gums.

b. Rub the swab over the slide (**do not use water**), **air dry**, and **heat-fix**.

c. Stain with **crystal violet** for **30 seconds.**

d. Wash off the excess crystal violet with water.

e. Blot dry and observe. Find epithelial cells using your 10X objective, center them in the field, and switch to oil immersion to observe your normal flora bacteria on and around your epithelial cells.

4. **Make sure you carefully pour the used dye in your staining tray into the designated drains located in the three lab benches and <u>not</u> down the sinks used for hand washing**.

C. INDIRECT STAIN USING AN ACIDIC DYE

In negative staining, the negatively charged color portion of the acidic dye is repelled by the negatively charged bacterial cell. Therefore, the background will be stained and the cell will remain colorless (Fig. 1 and Fig. 3).

ORGANISM

Use the pure culture of *Micrococcus luteus* provided.

PROCEDURE: to be done individually

1. Place a **small drop of nigrosin** towards one end of a clean microscope slide.

2. Aseptically add a small amount of *Micrococcus luteus* to the dye and mix gently with the loop.

3. Using the edge of another slide, spread the mixture with varying pressure across the slide so that there are **alternating light and dark areas**. **Make sure the dye is not too thick or you will not see the bacteria**!

4. Let the slide **air dry completely** on the slide. **Do not heat fix and do not wash off the dye.**

5. Observe using oil immersion microscopy. Find an area that has neither too much nor too little dye (an area that appears **light purple** where the light comes through the slide). If the dye is too thick, not enough light will pass through; if the dye is too thin, the background will be too light for sufficient contrast.

6. **Make sure you carefully pour the used dye in your staining tray into the designated drains located in the three lab benches and <u>not</u> down the sinks used for hand washing**.

RESULTS

Make drawings of your three direct stain preparations and your indirect stain preparation.

<table>
<tr><td></td><td></td></tr>
<tr><td>Direct stain of
Escherichia coli,
Pseudomonas aeruginosa,
or Enterobacter aerogenes</td><td>Direct stain of
Staphylococcus epidermidis
or Micrococcus luteus</td></tr>
<tr><td rowspan="2">Shape =</td><td>Shape =</td></tr>
<tr><td>Arrangement =</td></tr>
</table>

<table>
<tr><td></td><td></td></tr>
<tr><td rowspan="3">Direct stain of
epithelial cells and microbiota
from your mouth</td><td>Indirect stain of
Micrococcus luteus</td></tr>
<tr><td>Shape =</td></tr>
<tr><td>Arrangement =</td></tr>
</table>

PERFORMANCE OBJECTIVES
LABORATORY 5

After completing this lab, the student will be able to perform the following objectives:

INTRODUCTION TO STAINING

DISCUSSION

1. Describe the procedure for heat fixation and methanol fixation.
2. Define the following: acidic dye, basic dye, direct stain, and indirect stain.
3. State in chemical and physical terms the principle behind direct staining and the principle behind indirect staining.

DIRECT STAINING

PROCEDURE

1. Transfer a small number of bacteria from an agar surface or a broth culture to a glass slide and fix the preparation to the slide.
2. Prepare a direct stain when given all the necessary materials.

RESULTS

1. Recognize a direct stain preparation when it is observed through a microscope and state the shape and arrangement of the organism.

INDIRECT STAINING

PROCEDURE

1. Perform an indirect stain when given all the necessary materials.
2. State why the dye is not washed off during an indirect stain.

RESULTS

1. Recognize an indirect stain preparation when it is observed through a microscope and state the shape and arrangement of the organism.

LABORATORY 6
GRAM STAIN AND CAPSULE STAIN

A. The Gram Stain
B. The Capsule Stain

A. THE GRAM STAIN

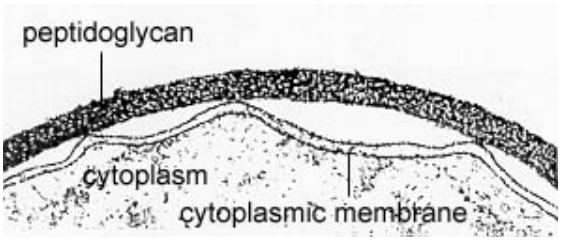
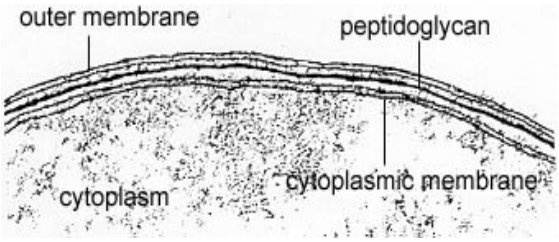
DISCUSSION

The Gram stain is the most widely used staining procedure in bacteriology. It is called a **differential stain** since it differentiates between Gram-positive and Gram-negative bacteria. Bacteria that stain **purple** with the Gram staining procedure are termed **Gram-positive;** those that stain **pink** are said to be **Gram- negative**. The terms positive and negative have nothing to do with electrical charge, but simply designate two distinct morphological groups of bacteria.

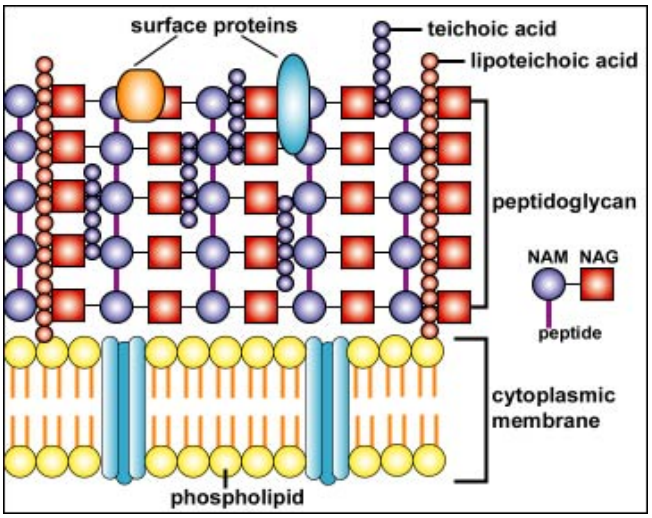
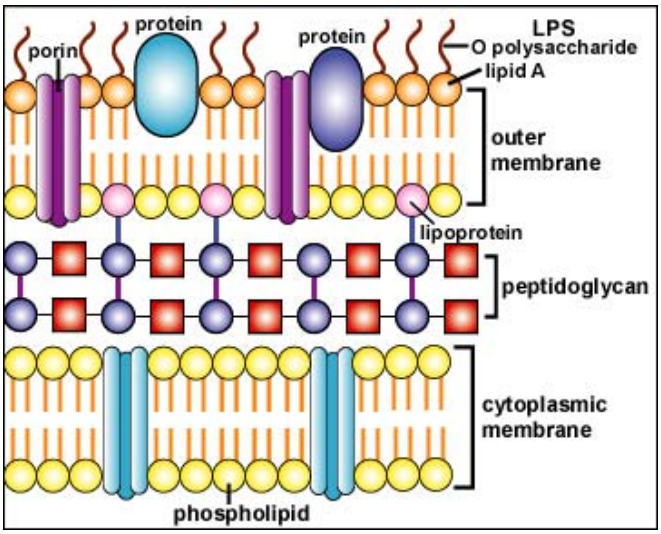
Gram-positive and Gram- negative bacteria stain differently because of fundamental differences in the structure of their cell walls. The bacterial cell wall serves to give the organism its size and shape as well as to prevent osmotic lysis.

In electron micrographs, the **Gram-positive cell wall** appears as a broad, dense wall 20-80 nm thick and consisting of numerous interconnecting layers of peptidoglycan (see Figs. 1A and 1B). Chemically, 60 to 90% of the Gram-positive cell wall is peptidoglycan. Interwoven in the cell wall of Gram-positive are teichoic acids. Teichoic acids, which extend through and beyond the rest of the cell wall, are composed of polymers of glycerol, phosphates, and the sugar alcohol ribitol. Some have a lipid attached (lipoteichoic acid). The outer surface of the peptidoglycan is studded with proteins that differ with the strain and species of the bacterium.

The **Gram- negative cell wall**, on the other hand, contains only 2-3 layers of peptidoglycan and is surrounded by an outer membrane composed of phospholipids, lipopolysaccharide, lipoprotein, and proteins (see Figs. 2A and 2B). Only 10% - 20% of the Gram- negative cell wall is peptidoglycan. The phospholipids are located mainly in the inner layer of the outer membrane, as are the lipoproteins that connect the outer membrane to the peptidoglycan. The lipopolysaccharides, located in the outer layer of the outer membrane, consist of a lipid portion called lipid A embedded in the membrane and a polysaccharide portion extending outward from the bacterial surface. The outer membrane also contains a number of proteins that differ with the strain and species of the bacterium.

Fig. 1A: Electron Micrograph of a Gram-Positive Cell Wall	**Fig. 2A:** Electron Micrograph of a Gram- negative Cell Wall
	

LAB 6:
GRAM STAIN AND CAPSULE STAIN

Fig. 1B: Illustration of a Gram-Positive Cell Wall	**Fig. 2B:** Illustration of a Gram- negative Cell Wall
	

The Gram staining procedure involves four basic steps:

1. The bacteria are first stained with the basic dye **crystal violet**. Both Gram-positive and Gram-negative bacteria become directly stained and appear purple after this step.

2. The bacteria are then treated with **Gram's iodine solution**. This allows the stain to be retained better by forming an insoluble crystal violet-iodine complex. Both Gram-positive and Gram- negative bacteria remain purple after this step.

3. **Gram's decolorizer**, a mixture of **ethyl alcohol and acetone**, is then added. This is the differential step. Gram-positive bacteria retain the crystal violet-iodine complex while Gram- negative are decolorized.

4. Finally, the counterstain **safranin** (also a basic dye) is applied. Since the Gram-positive bacteria are already stained purple, they are not affected by the counterstain. Gram- negative bacteria, which are now colorless, become directly stained by the safranin. Thus, Gram-positive appear purple, and Gram- negative appear pink.

With the current theory behind Gram staining, it is thought that in Gram-positive bacteria, the crystal violet and iodine combine to form a larger molecule that precipitates out within the cell. The alcohol/acetone mixture then causes dehydration of the multilayered peptidoglycan, thus decreasing the space between the molecules and causing the cell wall to trap the crystal violet-iodine complex within the cell. In the case of Gram- negative bacteria, the alcohol/acetone mixture, being a lipid solvent, dissolves the outer membrane of the cell wall and may also damage the cytoplasmic membrane to which the peptidoglycan is attached. The single thin layer of peptidoglycan is unable to retain the crystal violet-iodine complex and the cell is decolorized.

It is important to note that Gram-positivity (the ability to retain the purple crystal violet-iodine complex) is not an all-or-nothing phenomenon but a matter of degree. There are **several factors that could result in a Gram-positive organism staining Gram- negatively:**

1. **The method and techniques used.** Overheating during heat fixation, over decolorization with alcohol, and even too much washing with water between steps may result in Gram-positive bacteria losing the crystal violet-iodine complex.

2. **The age of the culture.** Cultures more than 24 hours old may lose their ability to retain the crystal violet-iodine complex.

3. **The organism itself.** Some Gram-positive bacteria are more able to retain the crystal violet-iodine complex than others.

Therefore, one must use very precise techniques in Gram staining and interpret the results with discretion.

ORGANISMS

Trypticase Soy agar plate cultures of *Escherichia coli* (a small, Gram- negative bacillus) and *Staphylococcus epidermidis* (a Gram-positive coccus with a staphylococcus arrangement).

PROCEDURE (to be done individually)

1. ***Escherichia coli***

a. Fix a smear of ***Escherichia coli*** to the slide as follows:

1. First place a small piece of **tape** at one end of the slide and **label it with the name of the bacterium** you will be placing on that slide

2. Using the dropper bottle of **deionized water found in your staining rack**, place **1/2 of a normal sized drop of water** on a clean slide by touching the dropper to the slide. Altenately, use your sterilized inoculating loop to place a drop of deionized water on the slide.

3. Using your sterile inoculating loop, aseptically remove a **small amount** of the culture from the agar surface and **gently touch it several times to the drop of water** until the water **becomes visibly cloudy**.

- A good smear with the correct amount of bacteria is essential to Gram staining. **Too many bacteria on the slide could result in under-decolorization; too few could lead to over-decolorization**.

4. **Incinerate the remaining bacteria on the inoculating loop.** If too much culture is added to the water, you will not see stained individual bacteria and you may not have a reliable Gram stain.

5. After the inoculating loop cools, **spread the suspension over the slide** to form a thin film.

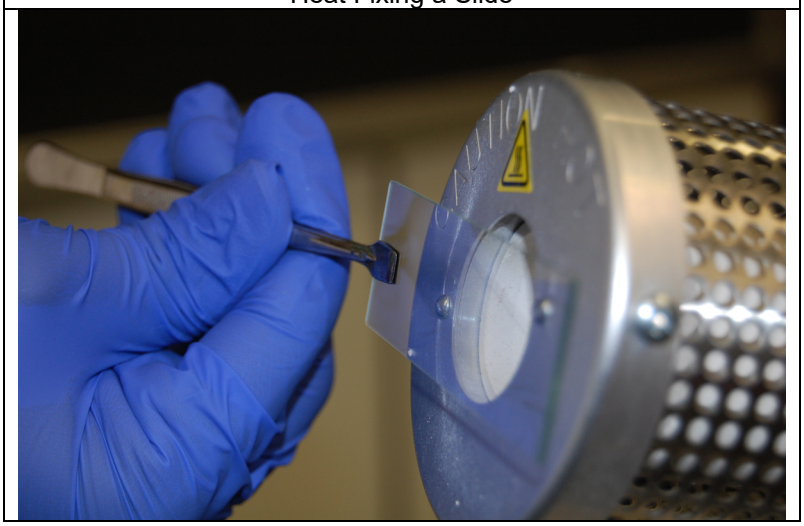
6. Allow this thin suspension to **completely air dry**. **The smear must be completely dry before the slide is fixed!**

7. **If your professor instructs you to heat-fix the bacteria to the slide**, pick up the air-dried slide with provided closepin and **hold the bottom of the slide opposite the smear against the opening of the microincinerator for 10**

seconds) as shown in **Fig. 3**. If the slide is not heated enough, all of the bacteria will wash off. If it is overheated, the bacteria structural integrity can be damaged.

8. **If your professor instructs you to fix the bacteria to the slide using methanol**, add several drops of **95% methanol** to the air-dried smear of bacteria and let sit for **2 minutes**. Pick up the slide and **allow the excess methanol to drain off** as demonstrated by your instructor. Let the slide again **air dry** before staining.

Fig. 5:
Heat Fixing a Slide



b. Stain with Hucker's **crystal violet** for **one minute**. **Gently wash with water**. Shake off the excess water but **do not blot dry between steps**.

c. Stain with **Gram's iodine solution** for **one minute** and **gently wash with water**.

d. **Decolorize by picking up the slide and letting the Gram's decolorizer run down the slide until the purple just stops flowing at the bottom of the slide**.

- **Make sure the entire smear is evenly decolorized and that you are not under-decolorizing or over-decolorizing**.
- **Wash immediately with water**.

e. Stain with **safranin** for **one minute**. **When you wash off the excess safranin, be very careful to wash gently and briefly** as it is possible to wash out some of the sarfanin in the bacterium.

f. **Blot dry** and observe using oil immersion microscopy.

(**First review the focusing steps from Lab 1, section D**.)

2. ***Staphylococcus epidermidis*** and repeating steps 1-6.

a. Fix a smear of ***Staphylococcus epidermidis*** to the slide as follows:

1. First place a small piece of **tape** at one end of the slide and **label it with the name of the bacterium** you will be placing on that slide

2. Using the dropper bottle of **deionized water found in your staining rack**, place **1/2 of a normal sized drop of water** on a clean slide by touching the dropper to the slide. Altenately, use your sterilized inoculating loop to place a drop of deionized water on the slide.

3. Using your sterile inoculating loop, aseptically remove a **small amount** of the culture from the agar surface and **gently touch it several times to the drop of water** until the water **becomes visibly cloudy**.

- A good smear with the correct amount of bacteria is essential to Gram staining. **Too many bacteria on the slide could result in under-decolorization; too few could lead to over-decolorization**.

4. **Incinerate the remaining bacteria on the inoculating loop.** If too much culture is added to the water, you will not see stained individual bacteria and you may not have a reliable Gram stain.

5. After the inoculating loop cools, **spread the suspension over the slide** to form a thin film.

6. Allow this thin suspension to **completely air dry**. **The smear must be completely dry before the slide is fixed!**

7. **If your professor instructs you to heat-fix the bacteria to the slide**, pick up the air-dried slide with provided closepin and **hold the bottom of the slide opposite the smear against the opening of the microincinerator for 10 seconds**) as shown in **Fig. 3**. If the slide is not heated enough, all of the bacteria will wash off. If it is overheated, the bacteria structural integrity can be damaged.

8. **If your professor instructs you to fix the bacteria to the slide using methanol**, add several drops of **95% methanol** to the air-dried smear of bacteria and let sit for **2 minutes**. Pick up the slide and **allow the excess methanol to drain off** as demonstrated by your instructor. Let the slide again **air dry** before staining.

b. Stain with Hucker's **crystal violet** for **one minute**. **Gently wash with water**. Shake off the excess water but **do not blot dry between steps**.

c. Stain with **Gram's iodine solution** for **one minute** and **gently wash with water**.

d. **Decolorize by picking up the slide and letting the Gram's decolorizer run down the slide <u>until the purple just stops flowing at the bottom of the slide</u>**.

- **Make sure the entire smear is evenly decolorized and that you are not under-decolorizing or over-decolorizing**.
- **Wash immediately with water**.

e. Stain with **safranin** for **one minute**. **When you wash off the excess safranin, be very careful to wash gently and briefly** as it is possible to wash out some of the sarfanin in the bacterium.

f. **Blot dry** and observe using oil immersion microscopy.

3. **Make sure you carefully pour the used dye down the designated drains at the three lab benches, <u>not</u> down the sink**.

B. THE CAPSULE STAIN

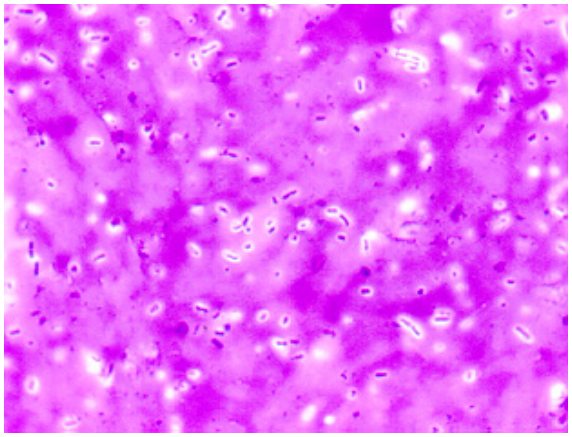
DISCUSSION

Many bacteria secrete a slimy, viscous covering called a **capsule or glycocalyx** (see Fig. 3). This is usually composed of polysaccharide, polypeptide, or both.

The ability to produce a capsule is an inherited property of the organism, but the capsule is not an absolutely essential cellular component. Capsules are often produced only under specific growth conditions.

Even though not essential for life, capsules probably help bacteria to survive in nature. Capsules help many pathogenic and normal flora bacteria to initially **resist phagocytosis** by the host's phagocytic cells. In soil and water, capsules help prevent bacteria from being engulfed by protozoans. Capsules also help many bacteria to **adhere to surfaces** and thus resist flushing.

Fig.3:
Capsule stain of Klebsiella aerogenes, formerly called *Enterobacter aerogenes*



ORGANISM

Skim Milk broth culture of *Klebsiella aerogenes*. The skim milk supplies essential nutrients for capsule production and also provides a slightly stainable background.

PROCEDURE (to be done individually)

1. **Stir up the Skim Milk broth culture with your loop** and place **2-3 loops** of *Klebsiella aerogenes* on a microscope slide.

2. Using your inoculating loop, **spread the sample out to cover about one inch of the slide**.

3. Let it completely air dry. **Do not heat fix**. Capsules stick well to glass, and heat may destroy the capsule.

4. Stain with **crystal violet** for **one minute**.

5. **Wash off the excess dye with 20% copper sulfate solution**.

6. **Shake off the excess copper sulfate solution** and immediately **blot dry**.

7. **Observe using oil immersion microscopy**. The organism and the milk dried on the slide will pick up the purple dye while the capsule will remain colorless. (Fig. 3)

8. Observe the demonstration capsule stain of *Streptococcus lactis*, an encapsulated bacterium that is normal flora in milk.

9. **Make sure you carefully pour the used dye in your staining tray into the waste dye collection container, not down the sink**.

RESULTS

A. The Gram Stain

Make drawings of each bacterium on your Gram stain preparation.

Gram stain of *Escherichia coli*	Gram stain of *Staphylococcus epidermidis*
Color =	Color =
Gram reaction =	Gram reaction =
Shape =	Arrangement =

B. The Capsule Stain

Make a drawing of your capsule stain preparation of *Klebsiella aerogenes* and the demonstration capsule stain of *Streptococcus lactis*.

Capsule stain of *Klebsiellar aerogenes*	Capsule stain of *Streptococcus lactis*

PERFORMANCE OBJECTIVES
LABORATORY 6

After completing this lab, the student will be able to perform the following objectives:

THE GRAM STAIN

DISCUSSION

1. State why the Gram stain is said to be a differential stain.
2. Describe the differences between a Gram-positive and a Gram- negative cell wall.
3. Describe a theory as to why Gram-positive bacteria retain the crystal violet-iodine complex while Gram- negatives become decolorized.
4. Describe three conditions that may result in a Gram-positive organism staining Gram- negatively.

PROCEDURE

1. State the procedure for the Gram stain.
2. Perform a Gram stain when given all the necessary materials.

RESULTS

1. Determine if a bacterium is Gram-positive or Gram- negative when microscopically viewing a Gram stain preparation and state the shape and arrangement of the organism.

THE CAPSULE STAIN

DISCUSSION

1. State the chemical nature and major functions of bacterial capsules.

RESULTS

1. Recognize capsules as the structures observed when microscopically viewing a capsule stain preparation.

LABORATORY 7
ENDOSPORE STAIN AND BACTERIAL MOTILITY

A. Endospore Stain
B. Bacterial Motility

A. ENDOSPORE STAIN

DISCUSSION

A few genera of bacteria, such as *Bacillus* and *Clostridium* have the ability to produce **resistant survival forms** termed **endospores**. Unlike the reproductive spores of fungi or plants, these endospores are resistant to heat, drying, radiation, and various chemical disinfectants (see Labs 19 & 20).

Endospore-producing bacteria need an environment that is physiologically favorable for biosynthesis in order to produce endospores. Once produced, however, these endospores can survive various adverse conditions. Endospore formation (sporulation) occurs through a complex series of events. One endospore is produced within each vegetative bacterium. Once the endospore is formed, the vegetative portion of the bacterium is degraded and the dormant endospore is released.

First the DNA replicates and a cytoplasmic membrane septum forms at one end of the cell. A second layer of cytoplasmic membrane then forms around one of the DNA molecules (the one that will become part of the endospore) to form a forespore. Both of these membrane layers then synthesize peptidoglycan in the space between them to form the first protective coat, the cortex. Calcium dipocolinate is also incorporated into the forming endospore. A spore coat composed of a keratin-like protein then forms around the cortex. Sometimes an outer membrane composed of lipid and protein and called an exosporium is also seen (see Fig. 1).

Finally, the remainder of the bacterium is degraded and the endospore is released. Sporulation generally takes around 15 hours.

The endospore is able to survive for long periods of time until environmental conditions again become favorable for growth. The endospore then germinates, producing a single vegetative bacterium.

Bacterial endospores are resistant to antibiotics, most disinfectants, and physical agents such as radiation, boiling, and drying. The impermeability of the spore coat is thought to be responsible for the endospore's resistance to chemicals. The heat resistance of endospores is due to a variety of factors:

- Calcium-dipicolinate, abundant within the endospore, may stabilize and protect the endospore's DNA.Specialized DNA-binding proteins saturate the endospore's DNA and protect it from heat, drying, chemicals, and radiation.
- The cortex may osmotically remove water from the interior of the endospore and the dehydration that results is thought to be very important in the endospore's resistance to heat and radiation.
- Finally, DNA repair enzymes contained within the endospore are able to repair damaged DNA during germination.

LAB 7:
ENDOSPORE STAIN AND BACTERIAL MOTILITY

Although harmless themselves until they germinate, bacterial **endospores are involved in the transmission of some diseases to humans**. Infections transmitted to humans by endospores include:

a. **Anthrax**, caused by *Bacillus anthracis*.

Endospores can be inhaled, ingested, or enter wounds where they germinate and the vegetative bacteria subsequently replicate and produde exotoxins. In the case of the two anthrax exotoxins, two different A-components known as lethal factor (LF) and edema factor (EF) share a common B-component known as protective antigen (PA). Protective antigen, the B-component, first binds to receptors on host cells and is cleaved by a protease creating a binding site for either lethal factor or edema factor. At low levels, LF inhibits the release of proinflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor-alpha, (TNF-alpha), and NO. This may initially reduce immune responses against the organism and its toxins. But at high levels, LF is cytolytic for macrophages, causing release of high levels of interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-alpha), and NO. Excessive release of these cytokines can lead to a massive inflammatory response and the shock cascade, similar to septic shock. Edema factor impairs phagocytosis, and inhibits production of TNF and interleukin-6 (IL-6) by monocytes. This most likely impairs host defenses.

b. **Tetanus**, caused by *Clostridium tetani*.

Endospores enter anaerobic wounds where they germinate and the vegetative bacteria subsequently replicate and release exotoxin. Tetanus exotoxin (tetanospasmin), produced by *Clostridium tetani* is a neurotoxin that binds to inhibitory interneurons of the spinal cord and blocks their release of inhibitor molecules. It is these inhibitor molecules from the inhibitory interneurons that eventually allow contracted muscles to relax by stopping excitatory neurons from releasing the acetylcholine that is responsible for muscle contraction. The toxin, by blocking the release of inhibitors, keeps the involved muscles in a state of contraction and leads to spastic paralysis, a condition where opposing flexor and extensor muscles simultaneously contract. Death is usually from respiratory failure.

c. **Botulism**, caused by *Clostridium botulinum*.

Endospores enter the anaerobic environment of improperly canned food where they germinate and subsequently replicate and at a neutral pH, secrete botulinal exotoxin. This is a neurotoxin that acts peripherally on the autonomic nervous system. For muscle stimulation, acetylcholine must be released from the neural motor end plate of the neuron at the synapse between the neuron and the muscle to be stimulated. The acetylcholine then induces contraction of the muscle fibers. The botulism exotoxin binds to and enters the presynaptic neuron and blocks its release of acetylcholine. This causes a flaccid paralysis, a weakening of the involved muscles. Death is usually from respiratory failure.

d. **Gas gangrene**, caused *by Clostridium perfringens*.

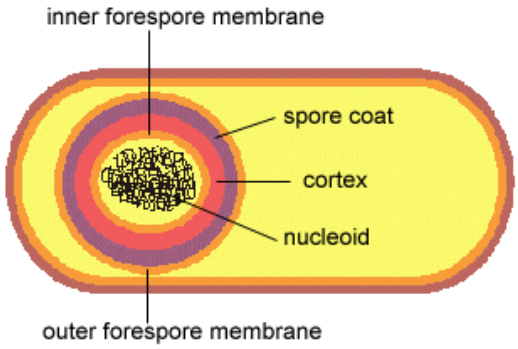
Endospores enter anaerobic wounds where they germinate and the vegetative bacteria subsequently replicate and produce a variety of exotoxins. This bacterium produces at least 20 exotoxins that play a role in the pathogenesis of gas gangrene and producing expanding zones of dead tissue (necrosis) surrounding the bacteria. Toxins include: Alpha toxin (lecithinase) that increases the permeability of capillaries and muscle cells by breaking down lecithin in cytoplasmic membranes resulting in the gross edema associated with gas gangrene as well as being necrotizing, hemolytic, and cardiotoxic; Kappa toxin (collagenase) breaks down supportive connective tissue resulting in the mushy lesions of gas gangrene and is also necrotizing; Mu toxin (hyaluronidase) breaks down the tissue cement that holds cells together in tissue; and epsilon toxin Increases vascular permeability and causes edema and congestion in various organs including lungs and kidneys. Additional necrotizing toxins include beta toxin, iota toxin, and nu toxin. A major characteristic of gas gangrene is the ability of *C. perfringens* to very rapidly spread from the initial wound site leaving behind an expanding zone of dead tissue. This organism spreads as a result of the pressure from fluid accumulation (due to increased capillary permeability from alpha toxin) and gas production (anaerobic fermentation of glucose by the organisms produces hydrogen and carbon dioxide), coupled with the breakdown of surrounding connective tissue (kappa toxin) and tissue cement (mu toxin).

e. **Antibiotic-associated pseudomembranous colitis**, caused by *Clostridium difficile*.

Clostridium difficile causes severe antibiotic-associated colitis and is an opportunistic Gram-positive, endospore-producing bacillus transmitted by the fecal-oral route. *C. difficile* is a common healthcare-associated infection (HAIs) and is the most frequent cause of health-care-associated diarrhea. *C. difficile* infection often recurs and can progress to sepsis and death. CDC has estimated that there are about 500,000 *C. difficile* infections (CDI) in health-care associated patients each year and is linked to 15,000 American deaths each year. Antibiotic-associated colitis is especially common in older adults. It is thought that *C. difficile* survives the exposure to the antibiotic by sporulation. After the antibiotic is no longer in the body, the endospores germinate and *C. difficile* overgrows the intestinal tract and secretes toxin A and toxin B that have a cytotoxic effect on the epithelial cells of the colon. *C.difficile* has become increasingly resistant to antibiotics in recent years making treatment often difficult. There has been a great deal of success in treating the infection with fecal transplants.

Due to the resistant nature of the endospore coats, endospores are difficult to stain. Strong dyes and vigorous staining conditions such as heat are needed. Once stained, however, endospores are equally hard to decolorize. Since few bacterial genera produce endospores, the endospore stain is a good diagnostic test for species of *Bacillus* and *Clostridium*.

Fig. 1:
Endospore formation within a vegetative bacterium



ORGANISMS

Trypticase Soy agar plate cultures of *Bacillus megaterium.*

PROCEDURE (to be done individually)

1. Heat-fix a smear of *Bacillus megaterium* as follows:

    a. Using the **dropper bottle of deionized water found in your staining rack**, place **1/2 of a normal sized drop of water** on a clean slide by touching the dropper to the slide. Altenately, use your sterilized inoculating loop to place a drop of deionized water on the slide.

b. Using your sterile inoculating loop, aseptically remove a small amount of the culture **from the edge of the growth** on the agar surface and generously mix it with the drop of water until the water becomes **visibly cloudy**.

c. **Incinerate the remaining bacteria on the inoculating loop**.

d. After the inoculating loop cools, **spread the suspension over approximately half of the slide** to form a thin film.

e. Allow this thin suspension to **completely air dry**.

f. To heat-fix the bacteria to the slide, **pick up the air-dried slide with coverslip forceps and hold the bottom of the slide opposite the smear against the opening of the microincinerator for 10 seconds** as demonstrated by your instructor.If the slide is not heated enough, all of the bacteria will wash off. If it is overheated, the bacteria structural integrity can be damaged.

2. Place a piece of blotting paper over the smear and saturate with **malachite green**.

3. Let the malachite green sit on the slide for **one minute** and proceed to the next step.

4. Fill a glass beaker approximately one-fourth full with tap water, place it on a hot plate, and bring the water to a boil. Reduce the heat so the water simmers and **place your slide on top of the beaker**. Your slide will get hot so be sure to **handle the slide with a test tube holder**. **Steam the slide for 5 minutes**. **As the malachite green evaporates, continually add more. Do not let the paper dry out!**

5. After five minutes of steaming, **wash** the excess stain and blotting paper off the slide **with water**. Don't forget to wash of any dye that got onto the **bottom** of the slide.

6. Blot the slide dry.

7. Now flood the smear with **safranin** and stain for **one minute**.

8. Wash off the excess safranin with water, blot dry, and observe using oil immersion microscopy. With this endospore staining procedure, endospores will stain **green** while vegetative bacteria will stain **red** (see Fig. 2**)**.

9. **Make sure you carefully pour the used dye in your staining tray into the waste dye collection container, <u>not</u> down the sink**.

10. Observe the demonstration slide of *Bacillus anthracis*. With this staining procedure, the vegetative bacteria stain blue and the endospores are colorless. Note the long chains of rod-shaped, endospore-containing bacteria (Fig. 3).

11. Observe the demonstration slide of *Clostridium tetani*. With this staining procedure, the vegetative bacteria stain blue and the endospores are colorless. Note the "tennis racquet" appearance of the endospore-containing *Clostridium* (Fig. 4).

Fig. 2:
Endospores of *Bacillus megaterium*

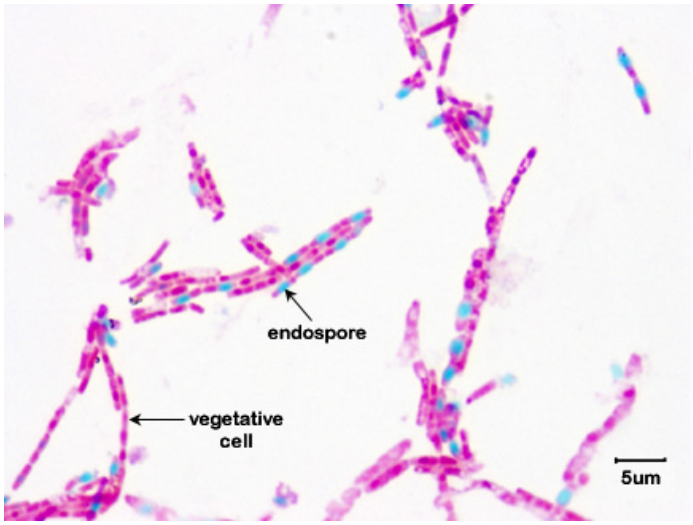


Fig. 3:
Endospores of *Bacillus anthracis*

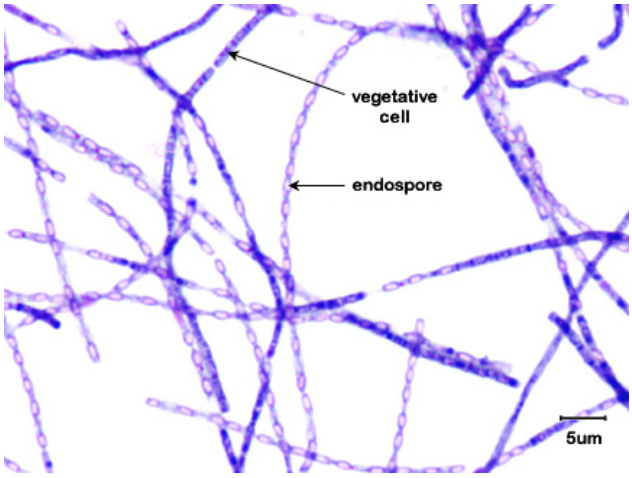
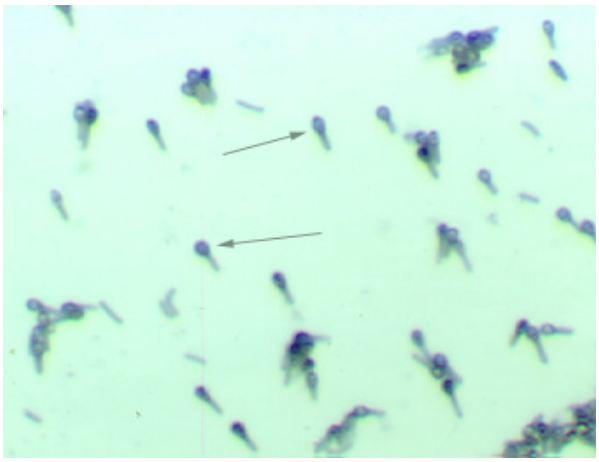


Fig. 4:
Endospores of *Clostridium tetani*



B. BACTERIAL MOTILITY

DISCUSSION

Many bacteria are capable of motility (the ability to move under their own power). Most motile bacteria propel themselves by special organelles termed **flagella** (Fig. 5**)**.

A bacterial flagellum has 3 basic parts: a filament, a hook, and a basal body.

1) The filament is the rigid, helical structure that extends from the cell surface. It is composed of the protein flagellin arranged in helical chains so as to form a hollow core. During synthesis of the flagellar filament, flagellin molecules coming off of the ribosomes are transported through the hollow core of the filament where they attach to the growing tip of the filament causing it to lengthen. With the exception of a few bacteria, such as *Bdellovibrio* and *Vibrio cholerae*, the flagellar filament is not surrounded by a sheath (see Fig. 5).

2) The hook is a flexible coupling between the filament and the basal body (see Fig. 5).

3) The basal body consists of a rod and a series of rings that anchor the flagellum to the cell wall and the cytoplasmic membrane (see Fig. 5). Unlike eukaryotic flagella, the bacterial flagellum has no internal fibrils and does not flex. Instead, the basal body acts as a rotary molecular motor, enabling the flagellum to rotate and propell the bacterium through the surrounding fluid. In fact, the flagellar motor rotates very rapidly. The Mot proteins surround the MS and C rings of the motor and function to generate torque for rotation of the flagellum. Energy for rotation comes from the proton motive force provided by protons moving through the Mot proteins.

Bacterial motility constitutes unicellular behavior. In other words, motile bacteria are capable of a behavior called **taxis.** Taxis is a motile response to an environmental stimulus and functions to keep bacteria in an optimum environment.

The arrangement of the flagella about the bacterium is of use in classification and identification. The following flagellar arrangements may be found:

1. **monotrichous** - a single flagellum at one pole (see Fig. 7).

2. **amphitrichous** - single flagella at both poles (Fig. 8A).

3. **lophotrichous** - two or more flagella at one or both poles of the cell (Fig. 8).

4. **peritrichous** - completely surrounded by flagella (Fig. 9).

These arrangements are shown in Figure 6.

One group of bacteria, the **spirochetes**, has internally-located **axial filaments** or endoflagella. Axial filaments wrap around the spirochete towards the middle from both ends. They are located above the peptidoglycan cell wall but underneath the outer membrane or sheath (Fig. 10).

Some bacteria use motility to contact host cells and disseminate within a host. The mucosal surfaces of the respiratory tract, the intestinal tract, and the genitourinary tract constantly flush bacteria away in order to prevent colonization of host mucous membranes. Motile bacteria can use their motility and chemotaxis to swim through mucus towards mucosal epithelial cells. Many bacteria that can colonize the mucous membranes of the bladder and the intestines, in fact, are motile. Motility probably helps these bacteria move through the mucus between the mucin strands or in places where the mucus is less viscous.

In addition, because of their thinness, their internal flagella (axial filaments), their corkscrew shape, and their motility, certain spirochetes are more readily able to penetrate host mucous membranes, skin abrasions, etc., and enter the body. Motility and penetration may also enable the spirochetes to penetrate deeper in tissue and enter the lymphatics and bloodstream and disseminate to other body sites. Spirochetes that infect humans include *Treponema pallidum* that causes syphilis, *Leptospira* that causes leptospirosis, and *Borrelia burgdorferi* that causes Lyme disease.

Fig. 5:
Insertion of a bacterial flagellum

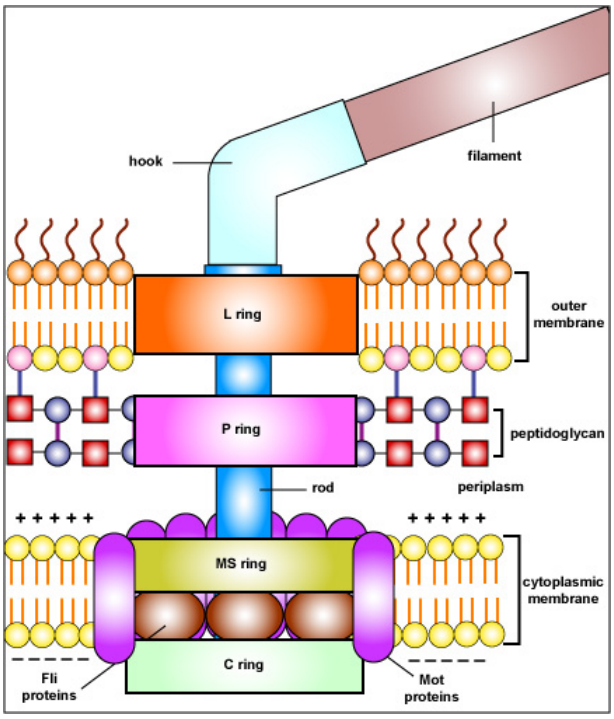


Fig. 6:
Arrangements of bacterial flagella

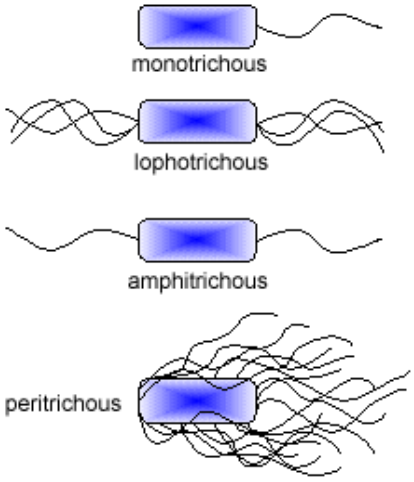


Fig. 7:
Monotrichous arrangement of flagella

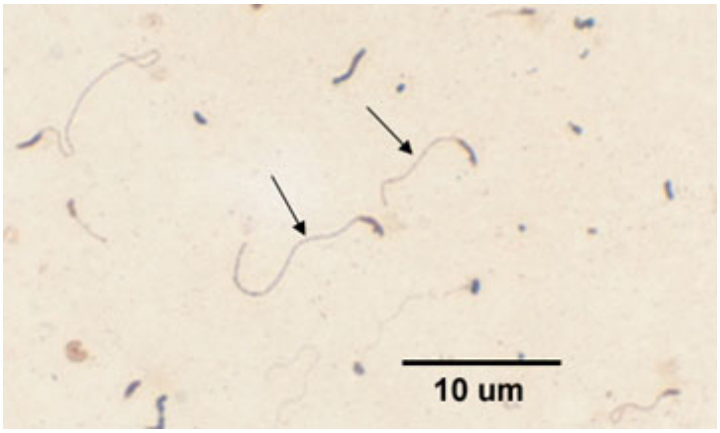
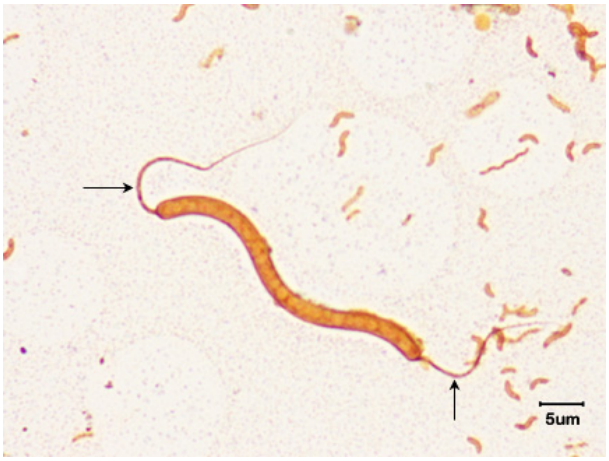


Fig. 8A:
Amphitrichous arrangement of flagella



LAB 7:
ENDOSPORE STAIN AND BACTERIAL MOTILITY

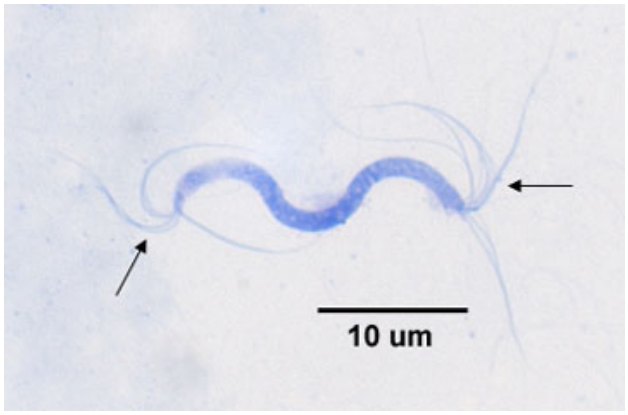
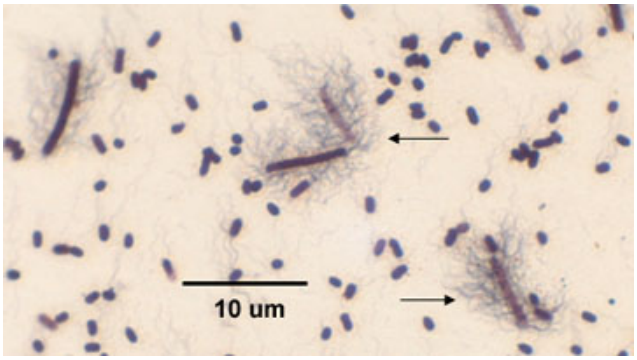
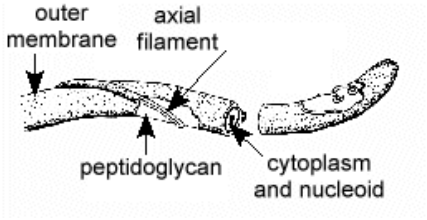
Fig. 8: Lophotrichous arrangement of flagella	**Fig. 9:** Peritrichous arrangement of flagella
	

Fig. 10 Axial filaments of a spirochete	**Fig. 11** *Leptospira* seen under dark-field microscopy
	

To detect bacterial motility, we can use any of the following three methods: 1) direct observation by means of special-purpose microscopes (phase-contrast and dark-field), 2) motility media, and, indirectly, 3) flagella staining.

1. **Direct observation of motility using special-purpose microscopes.**

a. **Phase-contrast microscopy**
A phase-contrast microscope uses special phase-contrast objectives and a condenser assembly to control illumination and give an optical effect of direct staining. The special optics will convert slight variations in specimen thickness into corresponding visible variation in brightness. Thus, the bacterium and its structures appear **darker than the background**.

b. **Dark-field microscopy**
A dark-field microscope uses a special condenser to direct light away from the objective lens. However, bacteria (or other objects) lying in the transparent medium will scatter light so that it enters the objective. This gives the optical effect of an indirect stain. The organism will appear **bright against the dark background**. Dark field microscopy is especially valuable in observing the very thin spirochetes (see Fig. 11).

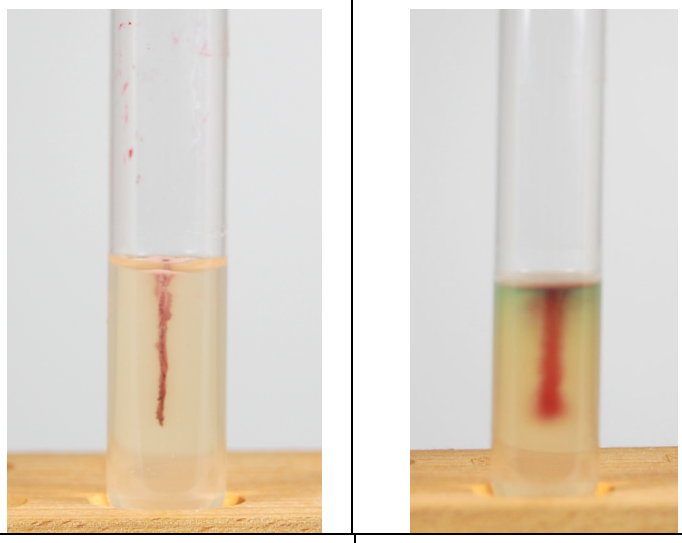
2. **Motility Test medium**

Semi-solid Motility Test medium may also be used to detect motility. The agar concentration (0.3%) is sufficient to form a soft gel without hindering motility. When a **non-motile organism** is stabbed into Motility Test medium, growth occurs **only along the line of inoculation**. Growth along the stab line is very **sharp and defined**. When **motile organisms** are stabbed into the soft agar, they swim away from the stab line. Growth occurs **throughout the tube** rather than being concentrated along the line of inoculation (Fig. 12). Growth along the stab line appears much more **cloud-like as it moves away from the stab**. A dye incorporated in the medium turns the growth red as a result of bacterial metabolism. **The more bacteria present at any location, the darker red the growth appears.**

3. **Flagella staining**

If we assume that bacterial flagella confer motility, flagella staining can then be used indirectly to denote bacterial motility. Since flagella are very thin (20-28 nm in diameter), they are below the resolution limits of a normal light microscope. They cannot be seen unless one first treats them with special dyes and mordants that build up as layers of precipitate along the length of the flagella, making them microscopically visible. This is a delicate staining procedure and will not be attempted here. We will, however look at several demonstration flagella stains.

Fig. 12:
Motility Test Medium



Non-motile | Motile

ORGANISMS

Trypticase Soy broth cultures of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. **Caution: handle these organisms as pathogens.**

MEDIUM

Motility Test medium (2 tubes)

PROCEDURE: (to be done individually and in pairs)

1. Observe the phase-contrast microscopy demonstration of motile *Pseudomonas aeruginosa*.

2. Observe the dark-field microscopy demonstration of motile *Pseudomonas aeruginosa*.

3. Take 2 tubes of Motility Test medium per pair. Stab one with *Pseudomonas aeruginosa* and the other with *Staphylococcus aureus*. **Stab the bacterium about 1/2 - 3/4 of an inch into the agar, taking care not to tilt or twist the loop so that the loop comes up through the same cut as it went down**. Incubate the tubes in your test tube rack **at 37°C** until the next lab period.

4. Observe the flagella stain demonstrations of a *Vibrio* species (monotrichous), *Proteus vulgaris* (peritrichous) and *Spirillum undula* (amphitrichous) as well as the dark-field photomicrograph of the spirochete *Leptospira*. When observing flagella stain slides, keep in mind that **flagella often break off during the staining procedure** so you have to look carefully to observe the true flagellar arrangement.

RESULTS

A. Endospore Stain

Make drawings of the various endospore stain preparations.

Endospore stain of *Bacillus megaterium*	Endospore stain of *Bacillus anthracis*	Endospore stain of *Clostridium tetani*

B. Bacterial Motility

1. Observe the phase contrast and dark-field microscopy demonstrations of bacterial motility.

2. Observe the two tubes of Motility Test medium.

	
Pseudomonas aeruginosa	*Staphylococcus aureus*
Conclusion:	Conclusion:

3. Make drawings of the flagella stain demonstrations.

Vibrio	*Proteus*
Arrangement:	Arrangement:

Spirillum
Arrangement:

PERFORMANCE OBJECTIVES
LABORATORY 7

After completing this lab, the student will be able to perform the following objectives:

ENDOSPORE STAIN

DISCUSSION

1. Name two endospore-producing genera of bacteria.
2. State the function of bacterial endospores.

RESULTS

1. Recognize endospores as the “structures” observed in an endospore stain preparation.
2. Identify a bacterium as an endospore-containing *Clostridium* by its "tennis racquet" appearance.

BACTERIAL MOTILITY

DISCUSSION

1. Define the following flagellar arrangements: monotrichous, lophotrichous, amphitrichous, peritrichous, and axial filaments.
2. State the function of bacterial flagella.
3. Describe three methods of testing for bacterial motility and indicate how to interpret the results.

RESULTS

1. Recognize bacterial motility when using phase-contrast or dark-field microscopy.
2. Interpret the results of Motility Test Medium.
3. Recognize monotrichous, lophotrichous, amphitrichous, and peritrichous flagellar arrangements.

LABORATORY 8
IDENTIFICATION OF BACTERIA THROUGH BIOCHEMICAL TESTING

A. INTRODUCTION

In the three previous labs we examined bacteria microscopically. Staining provides valuable information as to bacterial morphology, Gram reaction, and presence of such structures as capsules and endospores. Beyond that, however, microscopic observation gives little additional information as to the genus and species of a particular bacterium.

To identify bacteria, we must rely heavily on biochemical testing. The types of biochemical reactions each organism undergoes act as a "thumbprint" for its identification. This is based on the following chain of logic:

> Each different species of bacterium has **a different molecule of DNA** (i.e., DNA with a unique series of nucleotide bases).
>
> Since DNA codes for protein synthesis, then different species of bacteria must, by way of their unique DNA, be able to synthesize **different protein enzymes**.
>
> Enzymes catalyze all the various chemical reactions of which the organism is capable. This in turn means that different species of bacteria must carry out **different and unique sets of biochemical reactions**.

When identifying a suspected organism, you inoculate a series of differential media (see Lab 3). After incubation, you then observe each medium to see if **specific end products** of metabolism are present. This can be done by adding **indicators** to the medium which react specifically with the end product being tested, giving **some form of visible reaction such as a color change**. The results of these tests on the suspected microorganism are then compared to known results for that organism to confirm its identification.

Lab 7 will demonstrate that different bacteria, because of their unique enzymes, are capable of different biochemical reactions. It will also show the results of the activity of those enzymes. In later labs we will use a wide variety of special purpose differential media frequently used in the clinical laboratory to identify specific pathogenic and opportunistic bacteria.

In general, we can classify enzymes as being either exoenzymes or endoenzymes. **Exoenzymes** are secreted by bacteria into the surrounding environment in order to break down larger nutrient molecules so they may enter the bacterium (**see Fig. 1**). Once inside the organism, some of the nutrients are further broken down to yield energy for driving various cellular functions, while others are used to form building blocks for the synthesis of cellular components. These later reactions are catalyzed by **endoenzymes** located within the bacterium.

Fig. 1: *B. subtilis* producing an exoenzyme to hydrolize the milk protein casein.

B. STARCH HYDROLYSIS

DISCUSSION

Starch is a polysaccharide that appears as a branched polymer of the simple sugar glucose. This means that starch is really a series of glucose molecules hooked together to form a long chain. Additional glucose molecules then branch off of this chain as shown below.

GLU
|
(---GLU-GLU-GLU-GLU-GLU-GLU-GLU---)$_n$

Some bacteria are capable of using starch as a source of carbohydrate but in order to do this they must first **hydrolyze or break down** the starch so it may enter the cell. The bacterium secretes an exoenzyme that hydrolyzes the starch by breaking the bonds between the glucose molecules. This enzyme is called a **diastase**.

(---GLU GLU GLU GLU GLU GLU GLU---)$_n$

The glucose can then enter the bacterium and be used for metabolism.

MEDIUM

Starch agar (one plate)

LAB 8:
IDENTIFICATION OF BACTERIA THROUGH BIOCHEMICAL TESTING

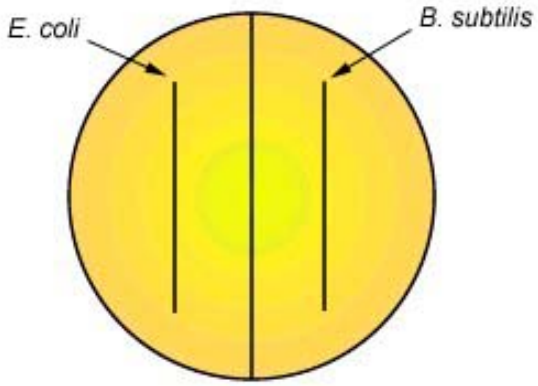
ORGANISMS

Trypticase Soy **broth cultures** of *Bacillus subtilis* and *Escherichia coli*.

PROCEDURE: to be done in pairs

1. Using a wax marker, draw a line on the **bottom** of a Starch agar plate so as to divide the plate in half. Label one half *B. subtilis* and the other half *E. coli*.

2. Make a **single streak line** with the appropriate organism on the corresponding half of the plate as shown below.



3. Incubate **upside down and stacked in the petri plate holder on the shelf of the 37°C incubator corresponding to your lab section** until the next lab period.

4. Next period, **iodine will be added** to see if the starch remains in the agar or has been hydrolyzed by the exoenzyme diastase. Iodine reacts with starch to produce a dark brown or blue/black color. **If starch has been hydrolyzed there will be a clear zone around the bacterial growth** because the starch is no longer in the agar to react with the iodine. **If starch has not been hydrolyzed, the agar will be a dark brown or blue/black color**.

C. PROTEIN HYDROLYSIS

DISCUSSION

Proteins are made up of various amino acids linked together in long chains by means of peptide bonds. Many bacteria can hydrolyze a variety of proteins into peptides (short chains of amino acids) and eventually into individual amino acids. They can then use these amino acids to synthesize their own proteins and other cellular molecules or to obtain energy. The hydrolysis of protein is termed **proteolysis** and the enzyme involved is called a **protease**. In this exercise we will test for bacterial hydrolysis of the protein **casein,** the protein that gives milk its **white, opaque appearance (see Fig. 1)**.

MEDIUM

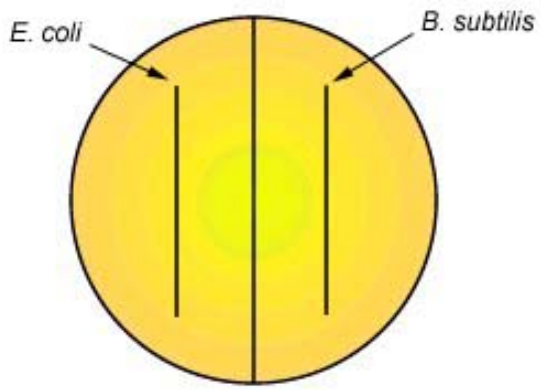
Skim Milk agar (one plate)

ORGANISMS

Trypticase Soy **broth cultures** of *Bacillus subtilis* and *Escherichia coli*.

PROCEDURE: to be done in pairs

1. Divide the Skim Milk agar plate in half and inoculate one half with *Bacillus subtilis* and the other half with *Escherichia coli* as done above with the above starch agar plate.



2. Incubate **upside down and stacked in the petri plate holder on the shelf of the 37°C incubator corresponding to your lab section** until the next lab period. **If casein is hydrolyzed, there will be a clear zone around the bacterial growth** (see Fig. 1). **If casein is not hydrolyzed, the agar will remain white and opaque**.

D. FERMENTATION OF CARBOHYDRATES

DISCUSSION

Carbohydrates are complex chemical substrates that serve as energy sources when broken down by bacteria and other cells. They are composed of carbon, hydrogen, and oxygen (with hydrogen and oxygen being in the same ratio as water; [CH_2O]) and are usually classed as either sugars or starches.

Facultative anaerobic and anaerobic bacteria are capable of **fermentation**, **an anaerobic process during which carbohydrates are broken down for energy production**. A wide variety of carbohydrates can be fermented by different bacteria in order to obtain energy and the types of carbohydrates which are fermented by a specific organism can serve as a diagnostic tool for the identification of that organism.

We can detect whether a specific carbohydrate has been fermented by **looking for common end products of fermentation**. When carbohydrates are fermented as a result of bacterial enzymes, the following fermentation end products may be produced:

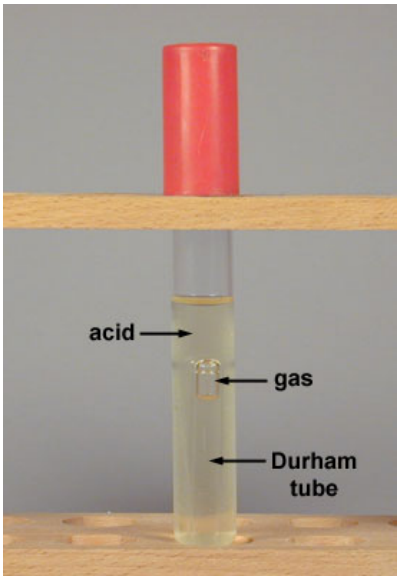
1. **acid end products**, or

2. **acid and gas end products**.

In order to test for these fermentation products, you inoculate and incubate tubes of media containing a **single carbohydrate** (such as lactose or maltose), a **pH indicator** (such as phenol red) and a **Durham tube** (a small inverted tube to detect gas production).

- **If the bacterium ferments that particular carbohydrate producing acid end products alone**, **the acid will lower the pH**, **causing the pH indicator phenol red to change form its original red color at a neutral pH to a yellow or clear color**.
- **If the bacterium ferments that particular carbohydrate producing both acid and gas end products**, **the pH indicator phenol red to change form its original red color at a neutral pH to a yellow or clear color and the gas will collect in the Durham tube as a substantial gas bubble appearing at the top of the Durham tube** (Fig. 2).
- **If the carbohydrate is not fermented by the bacterium**, **no acid or gas will be produced and the phenol red will remain red**.

Fig. 2:
Gas bubble (arrow) seen in a Durham tube.



MEDIA

3 tubes of Phenol Red Lactose broth and 3 tubes of Phenol Red Maltose broth

ORGANISMS

Trypticase Soy **agar cultures** of *Bacillus subtilis*, *Escherichia coli*, and *Staphylococcus aureus*.

PROCEDURE: to be done in pairs

1. **Label each tube with the name of the sugar in the tube and the name of the bacterium you are growing**.

2. Inoculate one Phenol Red Lactose broth tube and one Phenol Red Maltose broth tube with *Bacillus subtilis.*

3. Inoculate a second Phenol Red Lactose broth tube and a second Phenol Red Maltose broth tube with *Escherichia coli*.

4. Inoculate a third Phenol Red Lactose broth tube and a third Phenol Red Maltose broth tube with *Staphylococcus aureus*.

5. **Incubate the tubes in your test tube rack on your shelf of the 37°C incubator corresponding to your lab section** until the next lab period.

E. INDOLE AND HYDROGEN SULFIDE PRODUCTION

DISCUSSION

Sometimes we look for the production of products produced by only a few bacteria. As an example, **Indole** is a compound formed by the breakdown of the amino acid tryptophan by the enzyme tryptophanase. Since only a few bacteria contain this enzyme, the formation of indole from a tryptophan substrate can be another useful diagnostic tool for the identification of an organism. Indole production is a key test for the identification of *Escherichia coli.* The pathway for the production of indole is shown below:

$$\text{tryptophan} \xrightarrow{\text{tryptophanase}} \text{indole} + \text{pyruvic acid} + \text{ammonia}$$

By adding **Kovac's reagent** to the medium after incubation we can determine if indole was produced. Kovac's reagent will react with the indole and turn **red**.

Likewise, some bacteria are capable of breaking down sulfur containing amino acids (cystine, methionine) or reducing inorganic sulfur-containing compounds (such as sulfite, sulfate, or thiosulfate) to produce **hydrogen sulfide** (H_2S). This reduced sulfur may then be incorporated into other cellular amino acids, or perhaps into coenzymes. The ability of an organism to reduce sulfur-containing compounds to hydrogen sulfide can be another test for identifying unknown organisms such as certain *Proteus* and *Salmonella*. To test for hydrogen sulfide production, a medium with a sulfur-containing compound and iron salts is inoculated and incubated. If the sulfur is reduced and hydrogen sulfide is produced, it will combine with the iron salt to form a visible **black** ferric sulfide (FeS) in the tube. The pathway for hydrogen sulfide production from sulfur reduction is shown below:

$$S^{2-} \xrightarrow{2H^+} H_2S \xrightarrow{Fe^{2+}} FeS \text{ (black)}$$

MEDIUM

Three tubes of SIM (Sulfide, Indole, Motility) medium. This medium contains a sulfur source, an iron salt, the amino acid tryptophan, and is semi-solid in agar content (0.3%). It can be used to detect hydrogen sulfide production, indole production, and motility.

ORGANISMS

Trypticase Soy **agar cultures** of *Proteus mirabilis, Escherichia coli*, and *Enterobacter cloacae*.

PROCEDURE: to be done in pairs

1. Stab one SIM medium tube with *Proteus mirabilis.*

2. Stab a second SIM medium tube with *Escherichia coli.*

3. Stab a third SIM medium tube with *Enterobacter cloacae.*

4. **Incubate the tubes in your test tube rack on your shelf of the 37°C incubator corresponding to your lab section** until the next lab period

5. Next lab period add Kovac's reagent to each tube to detect indole production.

F. CATALASE ACTIVITY: demonstration

DISCUSSION

Catalase is the name of an enzyme found in most bacteria which initiates the breakdown of **hydrogen peroxide** (H_2O_2) into water and free oxygen:

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

During the normal process of aerobic respiration, hydrogen ions (H^+)are given off and must be removed by the cell. The electron transport chain takes these hydrogen ions and combines them with half a molecule of oxygen (an oxygen atom) to form water (H_2O). During the process, energy is given off and is trapped and stored in ATP. Water is then a harmless end product. Some cytochromes in the electron transport system, however, form toxic hydrogen peroxide (H_2O_2) instead of water and this must be removed. This is done by the enzyme catalase breaking the hydrogen peroxide into water and oxygen as shown above. Most bacteria are catalase-positive; however, certain genera that don't carry out aerobic respiration, such as the genera *Streptococcus, Enterococcus, Lactobacillus*, and *Clostridium*, are catalase-negative.

MATERIALS

Trypticase Soy agar cultures of *Staphylococcus aureus* and *Streptococcus lactis*, 3% hydrogen peroxide.

PROCEDURE: demonstration

Add a few drops of 3% hydrogen peroxide to each culture and look for the release of oxygen as a result of hydrogen peroxide breakdown. This appears as **foaming**.

RESULTS

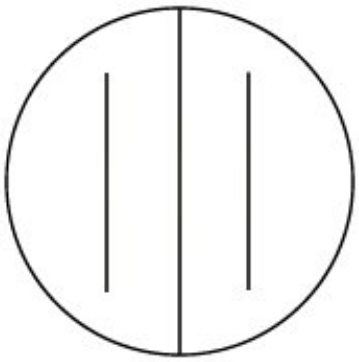
A. Starch Hydrolysis

When **iodine is added to starch, the iodine-starch complex that forms gives a characteristic dark brown or deep purple color reaction**. If the starch has been hydrolyzed into glucose molecules by the diastase exoenzyme, it no longer gives this reaction.

Flood the surface of the Starch agar plate with Gram's iodine.

- **If the bacterium produced an exoenzyme that hydrolized the starch in the agar**, a **clear zone** will surround the bacterial growth because **the starch is no longer there to react with the iodine**.
- **If the bacterium lacks the exoenzyme to hydrolize the starch**, the agar around the growth should turn **dark brown or deep purple due to the iodine-starch complex**.

Record your results and indicate which organism was capable of hydrolyzing the starch
(+ = hydrolysis; - = no hydrolysis).



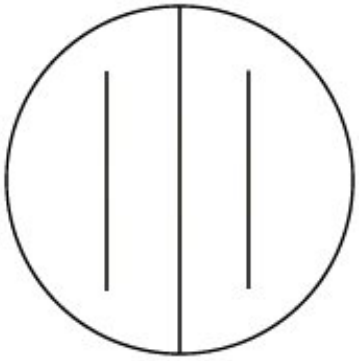
Escherichia coli	*Bacillus subtilis*
Starch hydrolysis =	Starch hydrolysis =

B. Protein Hydrolysis

The protein casein exists as a colloidal suspension in milk and **gives milk its characteristic white, opaque appearance**. If the **casein in the agar is hydrolyzed** into peptides and amino acids **it will lose its opaqueness**.

- **If the bacterium produced an exoenzyme capable of hydrolyzing the casein, there will be a clear zone around the bacterial growth** (**Fig. 1**).
- **If the bacterium lacks the exoenzyme to break down casein**, **the Skim Milk agar will remain white and opaque** (**Fig. 1**).

Record your results and indicate which organism was capable of hydrolyzing casein
(+ = hydrolysis; - = no hydrolysis).

	
Escherichia coli Casein hydrolysis =	*Bacillus subtilis* Casein hydrolysis =

C. Fermentation of Carbohydrates

As mentioned above, we can detect whether a specific carbohydrate is fermented **by looking for common end products of fermentation**. When carbohydrates are fermented as a result of bacterial enzymes**, the following fermentation end products may be produced**:

1. **acid end products**, or
2. **acid and gas end products**.

The results of fermentation may be acid alone or acid plus gas, but never gas alone.

Phenol red pH indicator appears **red or orange at neutral pH** but appears **yellow or clear at an acidic pH**.

- A change in color in the tube **from red or orange to yellow or clear** indicates that **the organism has fermented that particular carbohydrate, producing acid end products**.
- **A substantial gas bubble** at the top of the Durham tube**,** the inverted test tube within the broth, **indicates gas was also produced from the fermentation of the carbohydrate**.
- If the **phenol red remains red, no acid was produced** and **the carbohydrate was not fermented**.

Possible results are as follows:

- **Carbohydrate fermentation** producing **acid but no gas**: acidic (yellow or clear); no substantial gas bubble in the Durham tube.
- **Carbohydrate fermentation** producing **acid and gas**: acidic (yellow or clear); a substantial gas bubble in the Durham tube.
- **No carbohydrate fermentation**. **No acid or gas** (neutral pH (red or orange); no substantial gas bubble in the Durham tube.

Carbohydrate Fermentation

Record your results below (+ = positive; - = negative).

Organism	Phenol Red Maltose	Phenol Red Lactose
Bacillus subtilis		
Acid		
Gas		
Fermentation		
Escherichia coli		
Acid		
Gas		
Fermentation		
Staphylococcus aureus		
Acid		
Gas		
Fermentation		

D. Production of Indole and Hydrogen Sulfide

Carefully add about ¼ inch of Kovac's reagent to each of the 3 SIM agar tubes and observe.

1. **Production of hydrogen sulfide (H2S)**

   - If **the bacterium produces the enzyme to reduce sulfur to hydrogen sulfide** (H2S), **the agar will turn black** indicating that the organism has **produced hydrogen sulfide**.
   - If **the bacterium lacks the enzyme, the agar does not turn black, indicating that hydrogen sulfide was not produced**.

2. **Production of indole**

- If the bacterium **produces the enzyme to break down tryptophan into molecules of indole**, pyruvic acid, and ammonia, **the Kovac's reagent will turn red**, indicating the organism is **indole-positive**.

- If the Kovac's **reagent remains yellow, no indole was produced** and the organism is **indole-negative**.

SIM Medium

Record your results below (+ = positive; - = negative).

Organism	Indole	Hydrogen sulfide
Escherichia coli		
Enterobacter cloacae		
Proteus mirabilis		

E. Catalase Activity

Catalase is the name of an enzyme found in most bacteria which initiates the breakdown of **hydrogen peroxide** (H_2O_2) into water and free oxygen.

- If the **bacterium produces the enzyme catalase, then the hydrogen peroxide** added to the culture will be **broken down into water and free oxygen**. The **oxygen will bubble through the water** causing a **surface froth** to form. This is a **catalase-positive** bacterium.

- A **catalase-negative bacterium will not produce catalase to break down the hydrogen peroxide**, and **no frothing** will occur.

Catalase Test

Record your results below (foaming = positive; no foaming = negative).

Organism	Catalase reaction
Staphylococcus aureus	
Streptococcus lactis	

PERFORMANCE OBJECTIVES
LABORATORY 8

After the completion of this lab, the student will be able to complete the following objectives:

A. INTRODUCTION

DISCUSSION

1. State the chemical nature and function of enzymes.
2. Define endoenzyme and exoenzyme.

B. STARCH HYDROLYSIS

DISCUSSION

1. Describe a method of testing for starch hydrolysis and state how to interpret the results.

RESULTS

1. Interpret the results of starch hydrolysis on a Starch agar plate that has been inoculated, incubated, and flooded with iodine.

C. PROTEIN HYDROLYSIS

DISCUSSION

1. Describe a method of testing for casein hydrolysis and state how to interpret the results.

RESULTS

1. Interpret the results of casein hydrolysis on a Skim Milk agar plate after it has been inoculated and incubated.

D. FERMENTATION OF CARBOHYDRATES

DISCUSSION

1. Name the general end products which may be formed as a result of the bacterial fermentation of sugars and describe how these end products change the appearance of a broth tube containing a sugar, the pH indicator phenol red, and a Durham tube.

RESULTS

1. Interpret the carbohydrate fermentation results in tubes of Phenol Red Carbohydrate broth containing a Durham tube after it has been inoculated and incubated.

E. INDOLE AND HYDROGEN SULFIDE PRODUCTION

DISCUSSION

1. State the pathway for the breakdown of tryptophan to indole.
2. State the pathway for the detection of sulfur reduction in SIM medium.
3. State three reactions that may be tested for in SIM medium and describe how to interpret the results.

RESULTS

1. Interpret the hydrogen sulfide and indole results in a SIM medium tube after inoculation, incubation, and addition of Kovac's reagent.

F. CATALASE ACTIVITY

DISCUSSION

1. State the function of the enzyme catalase and describe a method of testing for catalase activity.

RESULTS

1. Interpret the results of a catalase test after adding hydrogen peroxide to a plate culture of bacteria.

LABORATORY 9
FUNGI PART 1: THE YEASTS

INTRODUCTION

Fungi are eukaryotic organisms (see **Fig. 3**) and include the yeasts, molds, and fleshy fungi. Yeasts are microscopic, unicellular fungi; molds are multinucleated, filamentous fungi (such as mildews, rusts, and common household molds); the fleshy fungi include mushrooms and puffballs.

All fungi are **chemoheterotrophs,** requiring organic compounds for both an energy and carbon source, which obtain nutrients by absorbing them from their environment. Most live off of decaying organic material and are termed **saprophytes**. Some are **parasitic**, getting their nutrients from living plants or animals.

The study of fungi is termed **mycology** and the diseases caused by fungi are called mycotic infections or **mycoses**.

In general, fungi are **beneficial** to humans. They are involved in the decay of dead plants and animals (resulting in the recycling of nutrients in nature), the manufacturing of various industrial and food products, the production of many common antibiotics, and may be eaten themselves for food. Some fungi, however, damage wood and fabrics, spoil foods, and cause a variety of plant and animal diseases, including human infections.

YEASTS

DISCUSSION

Yeasts are unicellular, oval or spherical fungi which increase in number asexually by a process termed **budding** (see Fig. 1). A bud forms on the outer surface of a parent cell, the nucleus divides with one nucleus entering the forming bud, and cell wall material is laid down between the parent cell and the bud. Usually the bud breaks away to become a new daughter cell but sometimes, as in the case of the yeast *Candida*, the buds remain attached forming fragile branching filaments called hyphae (see Fig. 10). Because of their unicellular and microscopic nature, **yeast colonies appear similar to bacterial colonies** on solid media. It should be noted that certain dimorphic fungi (see Lab 10) are able to grow as a yeast or as a mold, depending on growth conditions.

Yeasts are facultative anaerobes and can therefore obtain energy by both aerobic respiration and anaerobic fermentation. The vast majority of yeasts are nonpathogenic and some are of great value in industrial fermentations. For example, *Saccharomyces* species are used for both baking and brewing.

The yeast ***Candida*** is **normal flora** of the gastrointestinal tract and is also frequently found on the skin and on the mucous membranes of the mouth and vagina. *Candida* is normally held in check in the body by:

1) The body's normal immune defenses; and

2) The body's normal bacterial microbiota.

However, *Candida* may become an opportunistic pathogen and overgrow an area of colonization if the host becomes immunosuppressed or is given broad-spectrum antibiotics that destroy the normal bacterial flora. (Since *Candida* is eukaryotic, antibiotics used against prokaryotic bacteria do not affect it.)

LABORATORY 9:
YEASTS

Fig. 1:
A typical budding yeast.

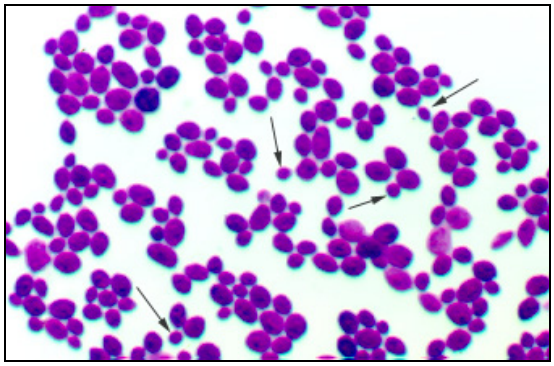


Fig. 2:
Candida albicans on Rice Extract agar. Note hyphae, blastoconidia (blastospores), and chlamydoconidia (chlamydospores).

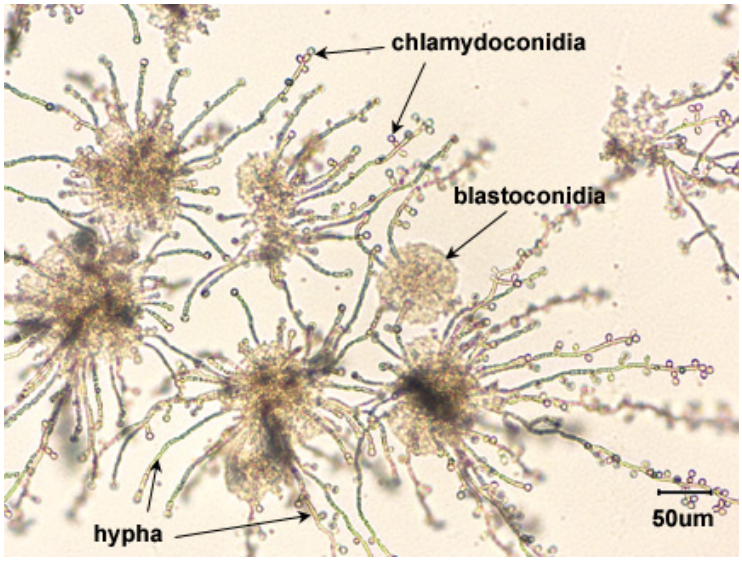


Fig. 3:
Candida albicans (Eukaryotic Cell)

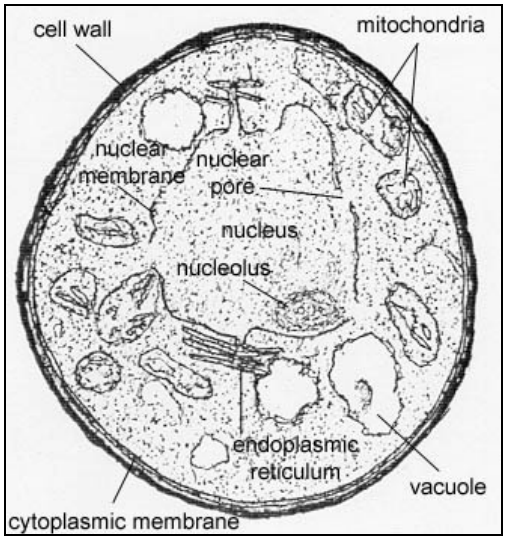
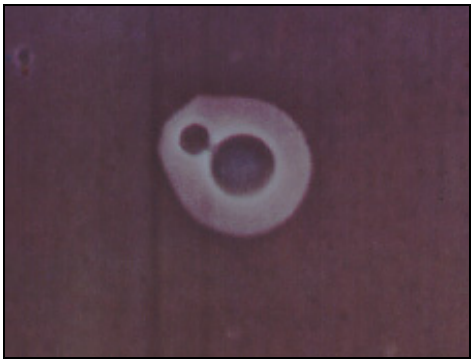


Fig. 4:
India ink preparation of *Cryptococcus neoformans* in spinal fluid.



Any infection caused by the yeast *Candida* is termed **candidiasis**. The most common forms of candidiases are oral mucocutaneous candidiasis (**thrush**; see Fig. 7A), **vaginitis** (see Fig. 7B), **balantitis** (infection of the penis),

onychomycosis (infection of the nails), and **dermatitis** (diaper rash and other infections of moist skin). However, antibiotic therapy, cytotoxic and immunosuppressive drugs, and immunosuppressive diseases such as diabetes, leukemias, and AIDS can enable *Candida* to cause severe opportunistic systemic infections involving the skin, lungs, heart, and other organs. In fact, *Candida* now accounts for 10% of the cases of septicemia. Candidiasis of the esophagus, trachea, bronchi, or lungs, in conjunction with a positive HIV antibody test, is one of the indicator diseases for AIDS.

The most common *Candida* species causing human infections is *C. albicans*, causing 50-60% of all *Candida* infections. *Candida glabrata* is second, causing 15-20% of *Candida* infections; *Candida parapsilosis* is third, responsible for 10-20%. More recently, *Candida auris* has been causing relatively rare but severe infections in patients that have been hospitalized for long periods of time, sometimes entering the bloodstream causing invasive infections throuout the body. *C. auris* is often resistant to common antifungal drugs and kills as many as 1 out of 3 patients with invasive infections.

Candida is said to be **dimorphic**, that is it **has two different growth forms**. It can grow as an oval, budding yeast, but under certain culture conditions, **the budding yeast may elongate and remain attached producing filament-like structures called pseudohyphae**. *C. albicans* may also **produce true hyphae** similar to molds. In this case long, branching filaments lacking complete septa form. The hyphae help the yeast to invade deeper tissues after it colonizes the epithelium. Asexual spores called **blastoconidia** (blastospores) develop in clusters along the hyphae, often at the points of branching. Under certain growth conditions, thick-walled survival spores called **chlamydoconidia** (chlamydospores) may also form at the tips or as a part of the hyphae (see Fig. 2).

A lesser known but often more serious pathogenic yeast is *Cryptococcus neoformans*. Like many fungi, this yeast can also reproduce sexually and the name given to the sexual form of the yeast is *Filobasidiella neoformans*. It appears as an oval yeast 5-6 µm in diameter, forms buds with a thin neck, and is surrounded by a **thick capsule** (see Fig. 4). It does not produce pseudohyphae and chlamydospores. The capsule enables the yeast to **resist phagocytic engulfment**. The yeast is dimorphic. In its sexual form, as well as in its asexual form under certain conditions, it can produce a hyphal form.

Cryptococcus infections are usually mild or subclinical but, when symptomatic, usually begin in the lungs after inhalation of the yeast in dried bird feces. It is typically associated with pigeon and chicken droppings and soil contaminated with these droppings. *Cryptococcus*, found in soil, actively grows in the bird feces but does not grow in the bird itself. Usually the infection does not proceed beyond this pulmonary stage. However, in an immunosuppressed host it may spread through the blood to the meninges and other body areas, often causing cryptococcal meningoencephalitis. Any disease by this yeast is usually called **cryptococcosis**.

Dissemination of the pulmonary infection can result in severe and often fatal **cryptococcal meningoencephalitis**. Cutaneous and visceral infections are also found. Although exposure to the organism is probably common, large outbreaks are rare, indicating that an immunosuppressed host is usually required for the development of severe disease. Extrapulmonary cryptococcosis, in conjunction with a positive HIV antibody test, is another indicator disease for AIDS. People with AIDS-associated cryptococcal infections account for 80%-90% of all patients with cryptococcosis.

Cryptococcus can be identified by preparing an India ink or nigrosin negative stain of suspected sputum or cerebral spinal fluid in which the encapsulated, budding, oval yeast cells may be seen (**Fig. 4**). It can be isolated on Saboraud Dextrose agar and identified by biochemical testing. Direct and indirect serological tests (discussed in Lab 16) may also be used in diagnosis.

Pneumocystis jiroveci (formerly called *Pneumocystis carinii*) causes ***Pneumocystis*** **pneumonia (PCP)**. It is seen a**lmost exclusively in highly immunosuppressed individuals** such as those with AIDS, late stage malignancies, or leukemias. PCP is one of the more common infection associated with AIDS.

LABORATORY 9:
YEASTS

P. jiroveci can be found in **3 distinct morphologic stages**:

- The **trophozoite (trophic form)**, a haploid amoeboid form 1-4 µm in diameter that replicates by mitosis and binary fission. The trophic forms are irregular shaped and often appears in clusters.

- A **precystic form** or early cyst. Haploid trophic forms conjugate and produce a diploid precyst form or sporocyte.

- The precyst form matures into a **cyst form**, which contains several intracystic bodies or spores are 5-8 µm in diameter. It has been postulated that in formation of the cyst form (late phase cyst), the zygote undergoes meiosis and subsequent mitosis to typically produce eight haploid ascospores (sporozoites). As the haploid ascospores are released the cysts often collapse forming crescent-shaped bodies. *P. jiroveci* is usually transmitted by inhalation of the cyst form. Released ascospores then develop into replicating trophic forms that attach to the wall of the alveoli and replicate to fill the alveoli.

In biopsies from lung tissue or in tracheobronchial aspirates, both a trophic form about 1-4 µm in diameter with a distinct nucleus and a cyst form between 5-8 µm in diameter with 6-8 intracystic bodies (ascospores) can be seen.

When viewing cysts of *P. jiroveci* in lung tissue after utilizing the Gomori methenamine silver stain method, the walls of the cysts are stained black and often appear crescent shaped or like crushed ping-pong balls (see Fig. 5). The intracystic bodies are not visible with this stain.

- *P. jiroveci* cysts from bronchoalveolar lavage (see Fig. 5)

- *P. jiroveci* cysts from the lungs (see Fig. 9)

Malassezia globosa

Malassezia globosa is a **dimorphic yeast** that is the most frequent cause of a superficial skin infection called **tinea versicolor** that commonly appears as a hypopigmentation or hyperpigmentation of the infected skin. *M. globosa* is also the most common cause of **dandruff** and **seborrheic dermatitis**. The yeast is naturally found on the skin.

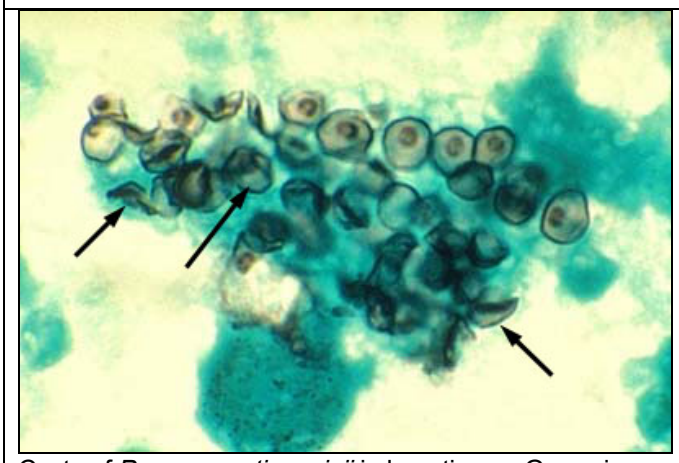
For a decription of **antifungal agents used to treat fungal infections**, see section IIE: Chemotherapeutic Control of Fungi in you lecture E-text.

Today we will use three agars to grow our yeast: Saboraud Dextrose agar (SDA), Mycosel agar, and Rice Extract agar. **Saboraud Dextrose agar (SDA)** is an agar similar to trypticase soy agar but with a higher sugar concentration and a lower pH, both of which **inhibit bacterial growth but promote fungal growth**. SDA, therefore, is said to be **selective for fungi**. Another medium, **Mycosel agar,** contains chloramphenicol to **inhibit bacteria** and cycloheximide to **inhibit most saprophytic fungi**. Mycosel agar, therefore, is said to be **selective for pathogenic fungi**. **Rice Extract agar** with polysorbate 80 **stimulates the formation of hyphae, blastoconidia** (blastospores), and **chlamydoconidia** (chlamydospores), structures unique to *C. albicans*, and may be used in its identification. The speciation of *Candida* is based on sugar fermentation patterns.

For a decription of antifungal agents used to treat fungal infections, see Chemotherapeutic Control of Fungi in my Softchalk Lecture Lessions.

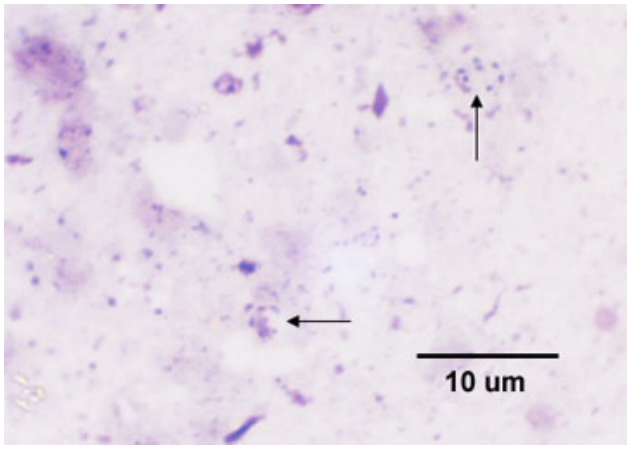
LABORATORY 9:
YEASTS

Fig. 5:
Cysts of *Pneumocystis jiroveci* in Smear from Bronchoalveolar Lavage



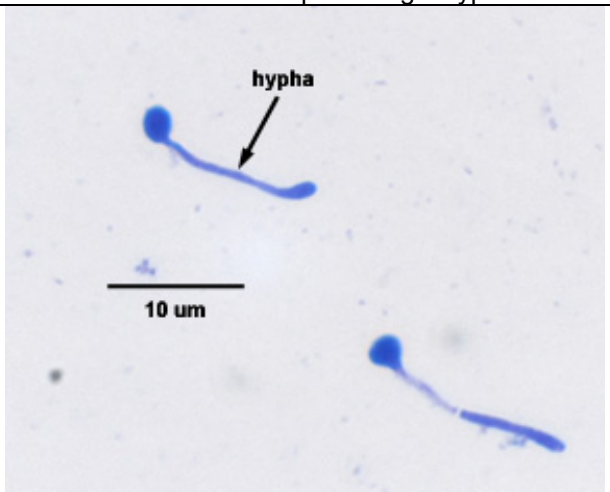
Cysts of *Pneumocystis carinii* in lung tissue, Gomori methenamine silver stain method. The walls of the cysts are stained black and often appear crescent shaped or like crushed ping-pong balls. The intracystic bodies are not visible with this stain. (Courtesy of the Centers for Disease Control and Prevention.)

Fig. 9:
Cysts of*Pneumocystis jiroveci* from the Lungs of a Person with PCP



Cysts of *Pneumocystis jiroveci* in bronchoalveolar material, Giemsa stain method.The rounded cysts (arrows) are 4 to 7 µm in diameter and contain 6 to 8 intracystic bodies, whose nuclei are stained by the dye. The walls of the cysts are not stained

Fig. 10:
Candida albicans producing a hypha



Dimorphic *Candida albicans* switching from a yeast form to a filamentous form.

LABORATORY 9:
YEASTS

MATERIALS

Coverslips, alcohol, forceps, and one plate each of Saboraud Dextrose agar, Mycosel agar, and Rice Extract agar.

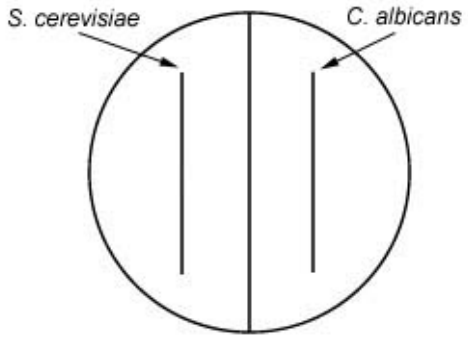
ORGANISMS

Trypticase Soy broth cultures of *Candida albicans* and *Saccharomyces cerevisiae*.

PROCEDURE (to be done in pairs)

1. With a wax marker, divide a **Saboraud Dextrose agar** and a **Mycosel agar** plate in half. Using a sterile **swab**, inoculate one half of each plate with *C. albicans* and the other half with *S. cerevisiae* as shown below. Incubate the two plates **upside down and stacked in the petri plate holder on the shelf of the 37°C incubator corresponding to your lab section** until the next lab period.

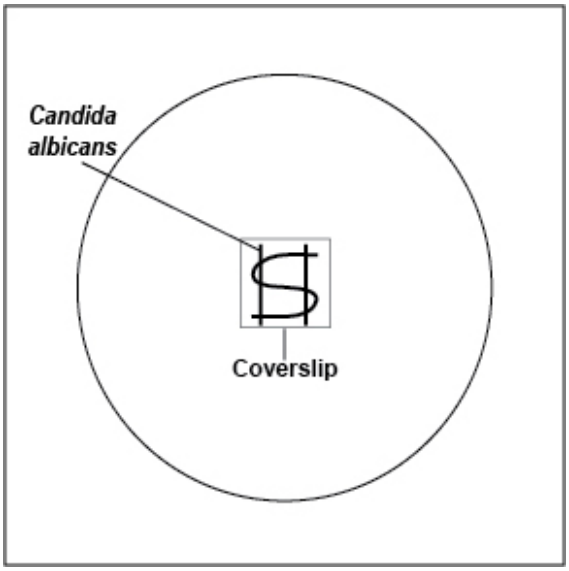
Inoculation of SDA and Mycosel Agars



2. Using your **inoculating loop**, **streak two parallel lines of *Candida albicans* approximately 1.5 cm long and 1.0 cm apart onto the surface of a plate of Rice Extract agar**.Sterilize your inoculating loop and let it cool. Using your sterile loop, **make an S-shaped streak lightly back and forth across the two parallel streak lines** as shown in the figure below. Pick up a **glass coverslip** with forceps, dip the coverslip in alcohol, and ignite with the flame of your butane lighter. Let the coverslip cool for a few seconds and place it over a portion of the streak line so that the plate can be observed directly under the microscope after incubation. **Incubate upside down at room temperature** for 3-5 days and examine microscopically.

LABORATORY 9:
YEASTS

Inoculation of Rice Extract Agar



3. Observe the following demonstrations:
   a. Direct stain of *Saccharomyces cerevisiae*
   b. Direct stain of *Candida albicans*
   c. Oral smear from a person with thrush
   d. Lung tissue infected with *Candida albicans*
   e. India ink preparation of *Cryptococcus neoformans*
   f. Cyst form of *Pneumocystis jiroveci* from lung tissue.

RESULTS

1. Describe the appearance of *Candida albicans* and *Saccharomyces cerevisiae* on Saboraud Dextrose agar and on Mycosel agar.

Yeast	SDA	Mycosel agar
Candida albicans Description:		
Saccharomyces cerevisiae Description:		

2. Remove the lid of the Rice Extract agar plate and put the plate on the stage of the microscope. Using your **yellow-striped 10X objective**, observe an area under the coverslip that appears "fuzzy" to the naked eye.

LABORATORY 9:
YEASTS

Reduce the light by moving the iris diaphragm lever almost all the way to the right. Raise the stage all the way up using the **coarse focus** (large knob) and then lower the stage using the coarse focus until the yeast comes into focus. Draw the hyphae, blastoconidia (blastospores), and chlamydoconidia (chlamydospores.) **See lab 1 for focusing instructions using the 10X objective.**

Candida albicans producing hyphae,blastoconidia, and chlamydoconidia on Rice Extract agar

3. Observe and make drawings of the demonstration yeast slides.

Direct stain of *Saccharomyces cerevisiae*	Direct stain of *Candida albicans*

Mouth smear showing thrush *(Candida albicans)*	Mouse lung infected with *Candida albicans*

LABORATORY 9:
YEASTS

India ink preparation of *Cryptococcus neoformans*	Lung tissue showing cysts of *Pneumocystis jiroveci*

PERFORMANCE OBJECTIVES
LABORATORY 9

After completing this lab, the student will be able to perform the following objectives:

INTRODUCTION

1. Define mycology and mycosis.
2. State three ways fungi may be beneficial to humans and three ways they may be harmful.

THE YEAST

DISCUSSION

1. Describe the typical appearance of a yeast cell and its usual mode of reproduction.
2. Describe yeasts in terms of their oxygen requirements.
3. State two ways the yeast *Saccharomyces* is beneficial to humans.
4. Name three yeasts that commonly infect humans.
5. Name four common forms of candidiasis.
6. Describe two conditions that may enable *Candida* to cause severe opportunistic systemic infections.
7. Describe pseudohyphae, hyphae, blastoconidia (blastospores), and chlamydoconidia (chlamydospores).
8. State the usefulness of Saboraud Dextrose agar, Mycosel agar, and Rice Extract agar.
9. State how *Cryptococcus neoformans* is transmitted to humans, where in the body it normally infects, and possible complications.
10. State the primary method of identifying *Cryptococcus neoformans* when causing cryptococcal meningoencephalitis.
11. State what disease is caused by *Pneumocystis jiroveci* and indicate several predisposing conditions a person is normally seen to have before they contract the disease.
12. Name an infection caused by *Malassezia globosa*.

RESULTS

1. Describe the appearance of *Saccharomyces cerevisiae* and *Candida albicans* on Saboraud Dextrose agar and on Mycosel agar.
2. When given a plate of Mycosel agar showing yeast-like growth and a plate of Rice Extract agar showing hyphae, (blastospores), and chlamydoconidia (chlamydospores), identify the organism as *Candida albicans*.
3. Recognize the following observed microscopically:
    a. *Saccharomyces cerevisiae* and *Candida albicans* as yeasts in a Gram stain preparation
    b. A positive specimen for thrush by the presence of budding *Candida albicans*
    c. *Cryptococcus neoformans* in an India ink preparation
    d. *Pneumocystis jiroveci* in lung tissue

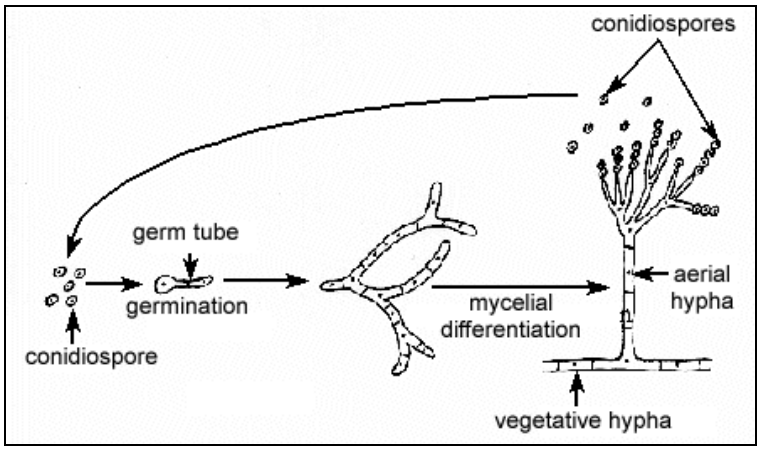
LABORATORY 10
FUNGI PART 2: THE MOLDS

A. Non-pathogenic Molds
B. Dermatophytes
C. Dimorphic Fungi

DISCUSSION

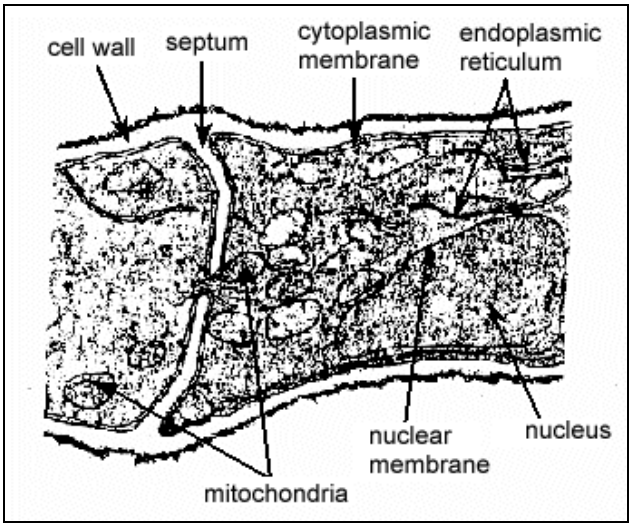
Molds are multinucleated, filamentous fungi composed of **hyphae**. A hypha is a branching, tubular structure from 2-10 µm in diameter and is usually divided into eukaryotic cell-like units by crosswalls called **septa**. The total mass of hyphae is termed a **mycelium**. The portion of the mycelium that anchors the mold and absorbs nutrients is called the **vegetative mycelium;** the portion that produces asexual reproductive spores is termed the **aerial mycelium** (see Fig. 1).

Fig. 1:
Asexual reproduction in molds.



Molds possess a rigid polysaccharide cell wall composed mostly of chitin and, like all fungi, are eukaryotic (see Fig. 2). Molds reproduce primarily by means of **asexual reproductive spores** such as conidiospores, sporangiospores, and arthrospores. These spores are disseminated by air, water, animals or objects and upon landing on a suitable environment, germinate and produce new hyphae (see Fig. 1). Molds may also reproduce by means of sexual spores such as ascospores and zygospores, but this is not common. The form and manner in which the spores are produced, along with the appearance of the hyphae and mycelium, provide the main criteria for identifying and classifying molds.

Fig. 2:
Segment of a Mold Hypha Showing Eukaryotic Nature



A. COMMON MOLDS

To illustrate how morphological characteristics such as the type and form of asexual reproductive spores and the appearance of the mycelium may be used in identification, we will look at three common non-pathogenic molds.

The two most common types of asexual reproductive spores produced by molds are conidiospores and sporangiospores. **Conidiospores** are borne externally in chains on an aerial hypha called a conidiophore (see Fig. 3); **sporangiospores** are produced within a sac or **sporangium** on an aerial hypha called a sporangiophore (see Fig. 4).

Penicillium and *Aspergillus* are examples of molds that produce **conidiospores**. ***Penicillium*** is one of the most common household molds and is a frequent food contaminant. The **conidiospores** usually appear grey, green, or blue and are produced in chains on **finger-like projections called phialides coming off the conidiophore** (see Fig. 5).

Aspergillus is another common contaminant. Although usually nonpathogenic, it may become opportunistic in the respiratory tract of a compromised host and, in certain foods, can produce mycotoxins. The conidiophore terminates in a ball-like structure called a vesicle. Its **conidiospores**, which typically appear brown to black, are produced in chains on phialides **coming off of the vesicle** (see Fig 6).

Although generally harmless in most healthy individuals, *Aspergillus* species do cause allergic bronchopulmonary aspergillosis (ABPA), chronic necrotizing *Aspergillus* pneumonia (or chronic necrotizing pulmonary aspergillosis [CNPA]), aspergilloma (a mycetoma or fungus ball in a body cavity such as the lung), and invasive aspergillosis. In highly immunosuppressed individuals, however, *Aspergillus* may disseminate beyond the lung via the blood.

Rhizopus is an example of a mold that produces **sporangiospores**. Although usually nonpathogenic, it sometimes causes opportunistic wound and respiratory infections in the compromised host. At the end of its sporangiophore is dome-shaped end called a columella that extends into a **sac-like structure called a sporangium**. Its **sporangiospores**, typically brown or black, are produced within the **sporangium** (see Fig 7). Anchoring structures called **rhizoids** are also produced on the vegetative hyphae.

LABORATORY 10:
MOLDS

Mucormycoses are infestions caused by fungi belonging to the order of Mucorales. *Rhizopus* species are the most common causative organisms. The most common infection is a severe infection of the facial sinuses, which may extend into the brain. Other mycoses include pulmonary, cutaneous, and gastrointestinal.

Rhizopus can also reproduce sexually. During sexual reproduction (see Fig 8), hyphal tips of (+) and (-) mating type join together and their nuclei fuse to form a sexual spore called a **zygospore** (see Fig. 8). This gives rise to a new sporangium producing sporangiospores having DNA that is a recombination of the two parent strain's DNA.

Molds are commonly cultured on fungal-selective or enriched media such as Saboraud Dextrose agar (SDA), Corn Meal agar, and Potato Dextrose agar.

Fig. 3:
Fungal Conidiospores

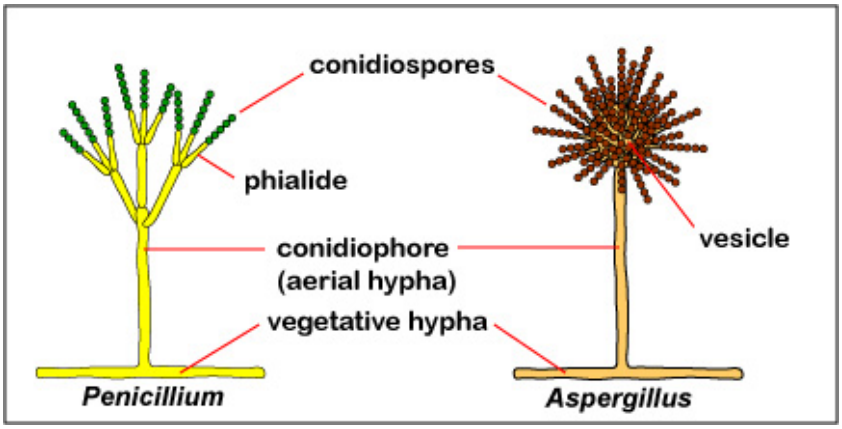
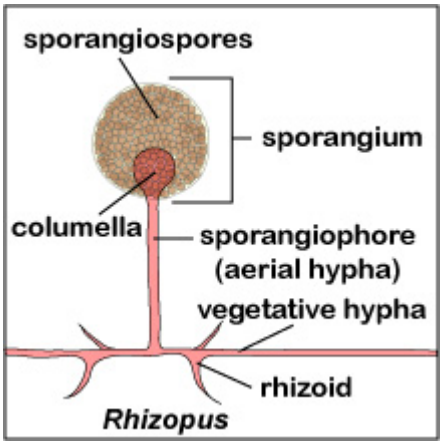


Fig. 4
Fungal Sporangiospores within a Sporangium



LABORATORY 10:
MOLDS

Fig. 5:
Penicillium
Note chains of conidiospores on phialides.

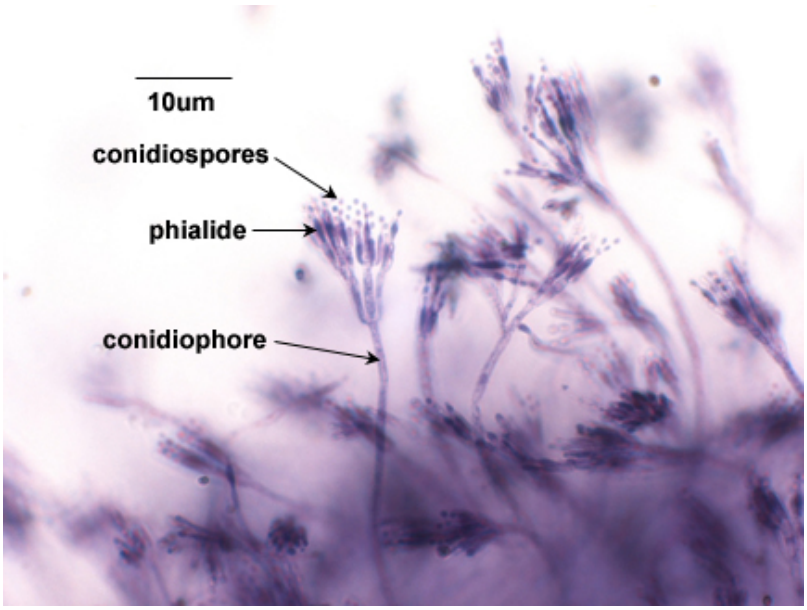


Fig. 6:
Aspergillus
Note chains of conidiospores on a vesicle.

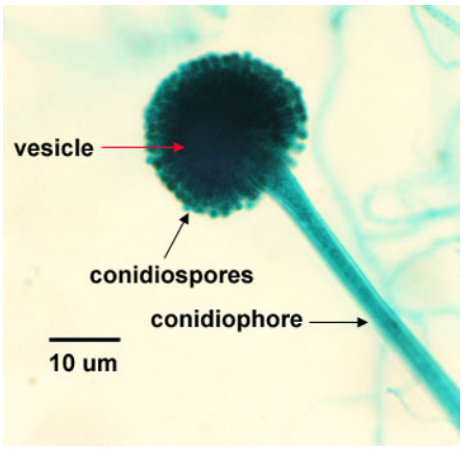


Fig. 7:
Rhizopus:
Note sporangium filled with sporangiospores.

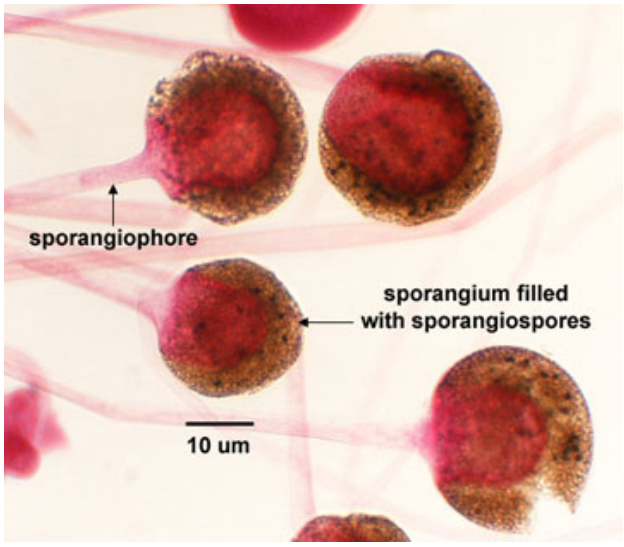
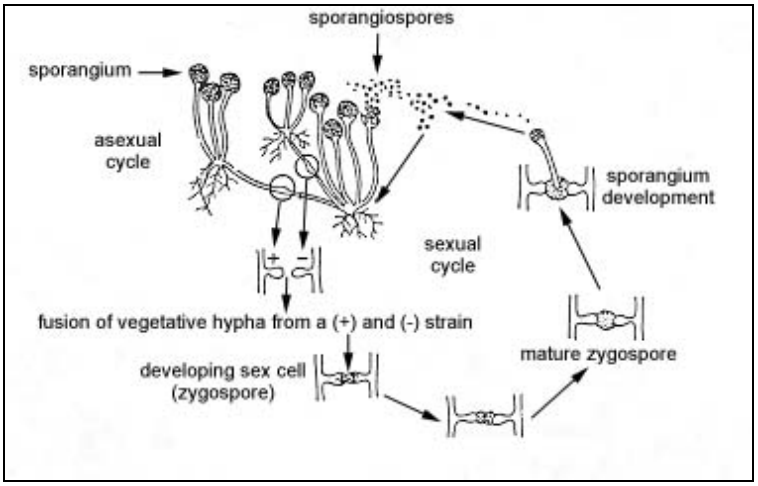


Fig. 8:
Sexual reproduction of *Rhizopus*



B. DERMATOPHYTES

The **dermatophytes** are a group of molds that cause **superficial mycoses of the hair, skin, and nails** and **utilize the protein keratin**, found in hair, skin, and nails, **as a nitrogen and energy source**. Infections are commonly referred to as ringworm or tinea infections and include:

- tinea capitis (infection of the skin of the scalp, eyebrows, and eyelashes)
- tinea barbae (infection of the bearded areas of the face and neck)
- tinea faciei (infection of the skin of the face)
- tinea corporis (infection of the skin regions other than the scalp, groin, palms, and soles)
- tinea cruris (infection of the groin; jock itch)
- tinea unguium (onchomycosis; infection of the fingernails and toenails)
- tinea pedis (athlete's foot; infection of the soles of the feet and between the toes).

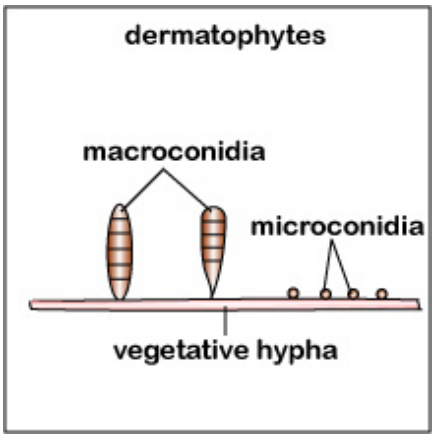
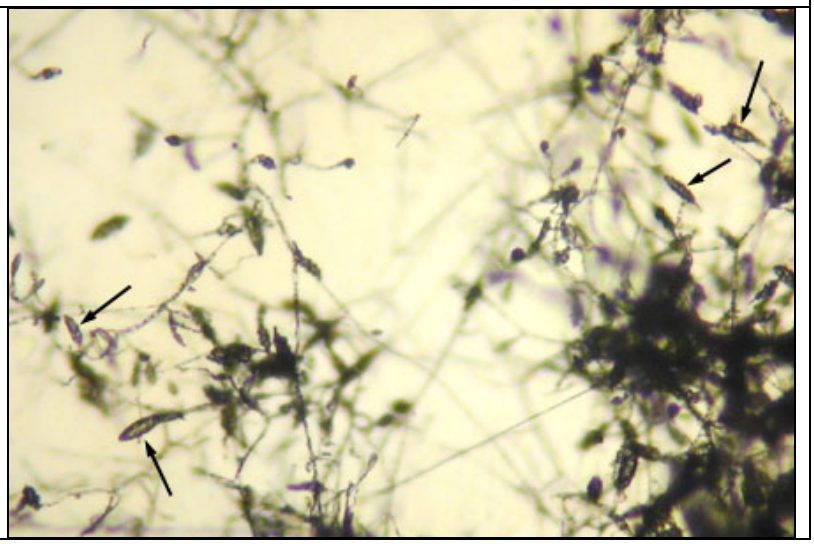
The three common dermatophytes are ***Microsporum, Trichophyton,*** and ***Epidermophyton.*** These organisms grow well at 25°C. They may produce large leaf or club-shaped asexual spores called **macroconidia** as well as small spherical asexual spores called **microconidia**, both from vegetative hyphae (see **Fig. 10A** and **Fig. 10B**).

Microsporum commonly infects the skin and hair, *Epidermophyton*, the skin and nails, and *Trichophyton*, the hair, skin, and nails. Dermatophytic infections are acquired by contact with fungal spores from infected humans, animals, or objects. On the skin, the dermatophytes typically cause reddening, itching, edema, and necrosis of tissue. This is a result of fungal growth and a hypersensitivity of the host to the fungus and its products. Frequently there is secondary bacterial or *Candida* invasion of the traumatized tissue.

To diagnose dermatophytic infections, **tissue scrapings** can be digested with 10% potassium hydroxide (which causes lysis of the human cells but not the fungus) and examined microscopically for the presence of **fungal hyphae and spores**. To establish the specific cause of the infection, fungi from the affected tissue can be cultured on Dermatophyte Test Medium (DTM) and Saboraud Dextrose agar (SDA).

Dermatophyte Test Medium (DTM) has phenol red as a pH indicator with the medium yellow (acid) prior to inoculation. **As the dermatophytes utilize the keratin in the medium, they produce alkaline end products** that raise the pH, thus **turning the phenol red** in the medium **from yellow (acid) to red (alkaline)**.

The types of **macroconidia and microconidia** can be observed by growing the mold on **SDA** and observing under a microscope (see Fig. 10B). Many dermatophyte species produce yellow to red-pigmented colonies on SDA and the most common species of *Microsporum* fluoresce under ultraviolet light.

Fig. 10A: Macroconidia and macroconidia of dermatophytes	**Fig. 10B:** Macroconidia of the dermatophyte *Microsporum* (arrows)
	

C. DIMORPHIC FUNGI

Dimorphic fungi may exhibit two different growth forms. Outside **the body they grow as a mold**, producing hyphae and asexual reproductive spores, but **inside the body they grow as a yeast-like form**. Dimorphic fungi may cause **systemic mycoses** that usually begin by **inhaling spores from the mold form**. After germination in the lungs, the fungus grows as a yeast. Factors such as body temperature, osmotic stress, oxidative stress, and certain human hormones activate a dimorphism-regulating histidine kinase enzyme in dimorphic molds, causing them to switch from their avirulent mold form to their more virulent yeast form.

The infection usually remains localized in the lungs and characteristic lesions called **granuloma** may be formed in order to wall-off and localize the organism. In rare cases, usually in an immunosuppressed host, the organism may disseminate to other areas of the body and be life threatening. Examples of dimorphic fungi include *Coccidioides immitis*, *Histoplasma capsulatum*, and *Blastomyces dermatitidis*.

1. ***Coccidioides immitis***

Coccidioides immitis (See Fig. 11) and *Coccidioides posadasii* are dimorphic fungithat causes **coccidioidomycosis**, a disease endemic to areas of the southwestern United States where there is a semiarid climate, an alkaline soil, and hot summers. An estimated 150,000 infections occur annually in the United States, but two thirds of these cases are subclinical. The mold form of the fungus grows in arid soil and produces thick-walled, barrel-shaped asexual spores called **arthroconidia** by a fragmentation of its vegetative hyphae.

After inhalation, the arthroconidia sheds its outer coating, swells, and becomes an **endosporulating spherule** in the terminal bronchioles of the lungs. The spherule contains hundreds to thousands of yeast-like endospores. The spherule subsequently ruptures and releases endospores that develop into new spherules.

Coccidioidomycosis can be diagnosed by culture, by a coccidioidin skin test, and by indirect serologic tests (discussed in Lab 16).

2. ***Histoplasma capsulatum***

Histoplasma capsulatum (see Fig. 12) is a dimorphic fungus that causes **histoplasmosis**, a disease that is endemic to the Ohio, Missouri, and Mississippi River valleys in the United States. Approximately 250,000 people are thought to be infected annually in the US, but clinical symptoms of histoplasmosis occur in less than 5% of the population. Most individuals with histoplasmosis are asymptomatic. Those who develop clinical symptoms are typically either immunocompromised or are exposed to a large quantity of fungal spores.

The mold form of the fungus often grows in acidic, damp soil with high organic content, especially near areas inhabited by bats and birds and produces large **tuberculate macroconidia** and small **microconidia**. Although birds cannot be infected by the fungus and do not transmit the disease, bird excretions contaminate the soil and enrich it for mycelial growth. Bats, however, can become infected and transmit histoplasmosis through their droppings. After inhalation of the fungal spores and their germination in the lungs, the fungus grows as a **budding, encapsulated yeast**.

Histoplasmosis can be diagnosed by culture, by a histoplasmin skin test, and by indirect serologic tests (discussed in Lab 16).

3. ***Blastomyces dermatitidis***

Blastomycosis (see Fig. 13), caused by *Blastomyces dermatitidis*, is most commonly found in the Mississippi and Ohio River valley states and Canada provinces bordering the Great Lakes. Infection can range from an asymptomatic, self-healing pulmonary infection to widely disseminated and potentially fatal disease. Pulmonary infection may be asymptomatic in nearly 50% of patients. *Blastomyces dermatitidis* can also sometimes infect the skin.

Blastomyces dermatitidis produces a mycelium with small **conidiospores** and grows actively in wet soil that has been enriched with animal droppings, decaying vegetable matter, and rotting wood. When spores are inhaled or enter breaks in the skin, they germinate and the fungus grows as a **yeast** having a characteristic thick cell wall. It is diagnosed by culture and by biopsy examination.

For a decription of **antifungal agents used to treat fungal infections**, see Chemotherapeutic Control of Fungi in you lecture Softchalk lessons.

Fig. 11:
Dimorphic cycle of *Coccidioides immitis*.

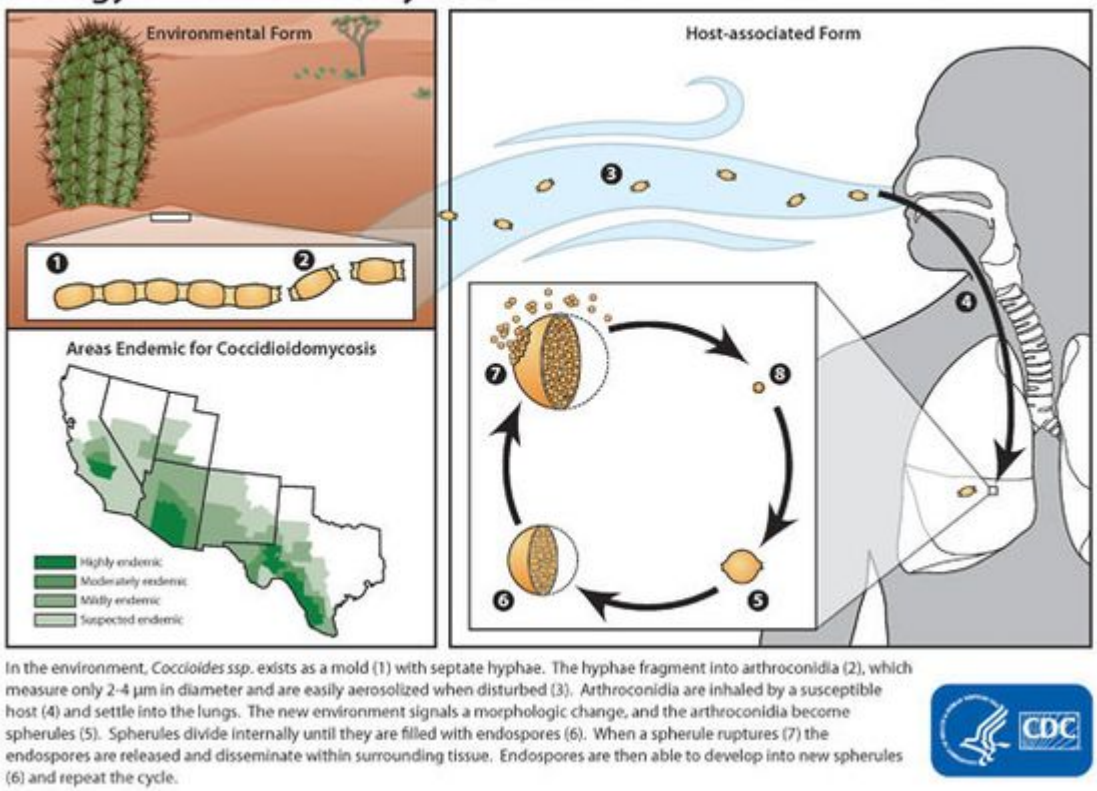


Fig. 12:
Dimorphic cycle of *Histoplasma capsulatum*.

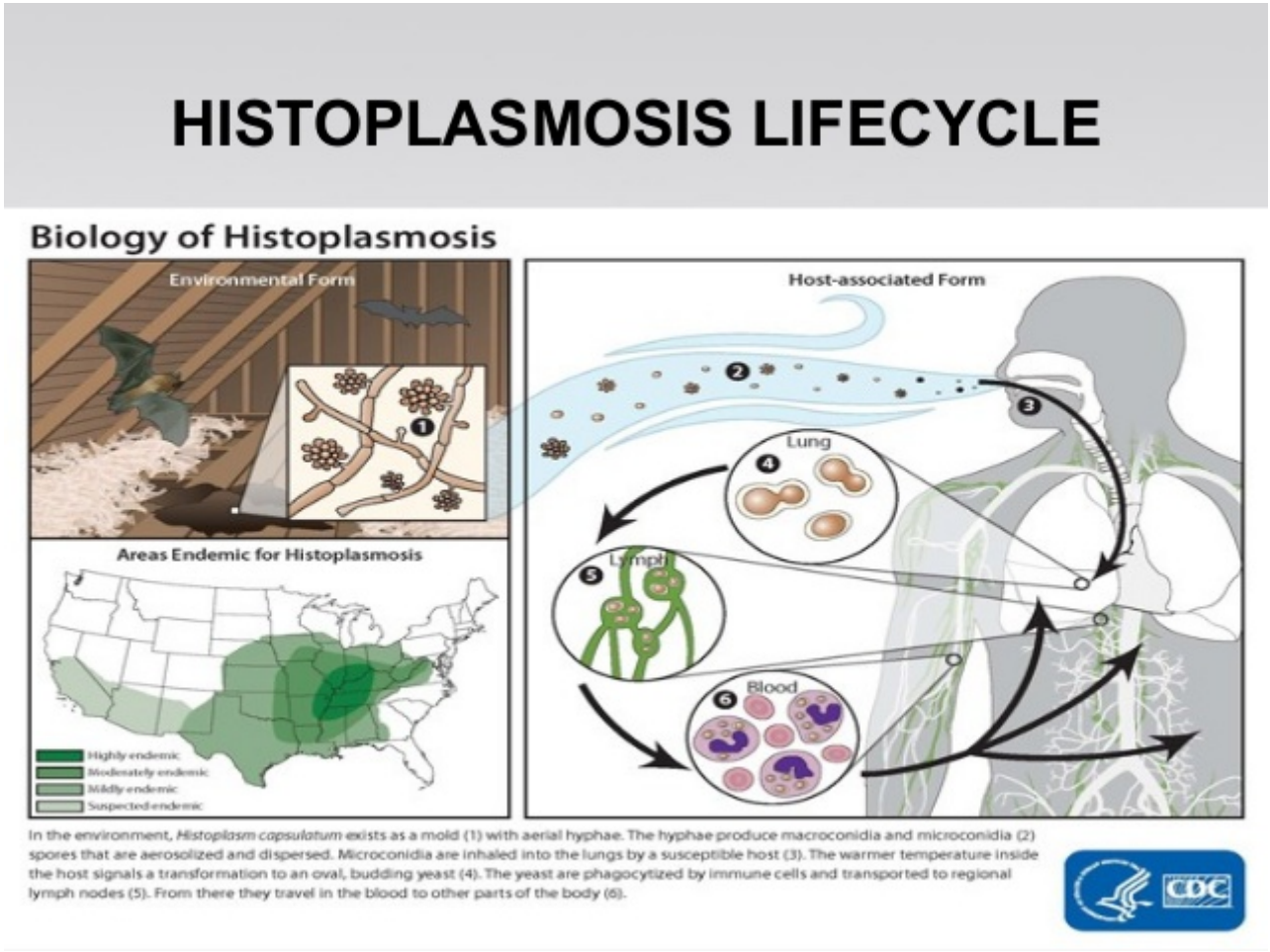
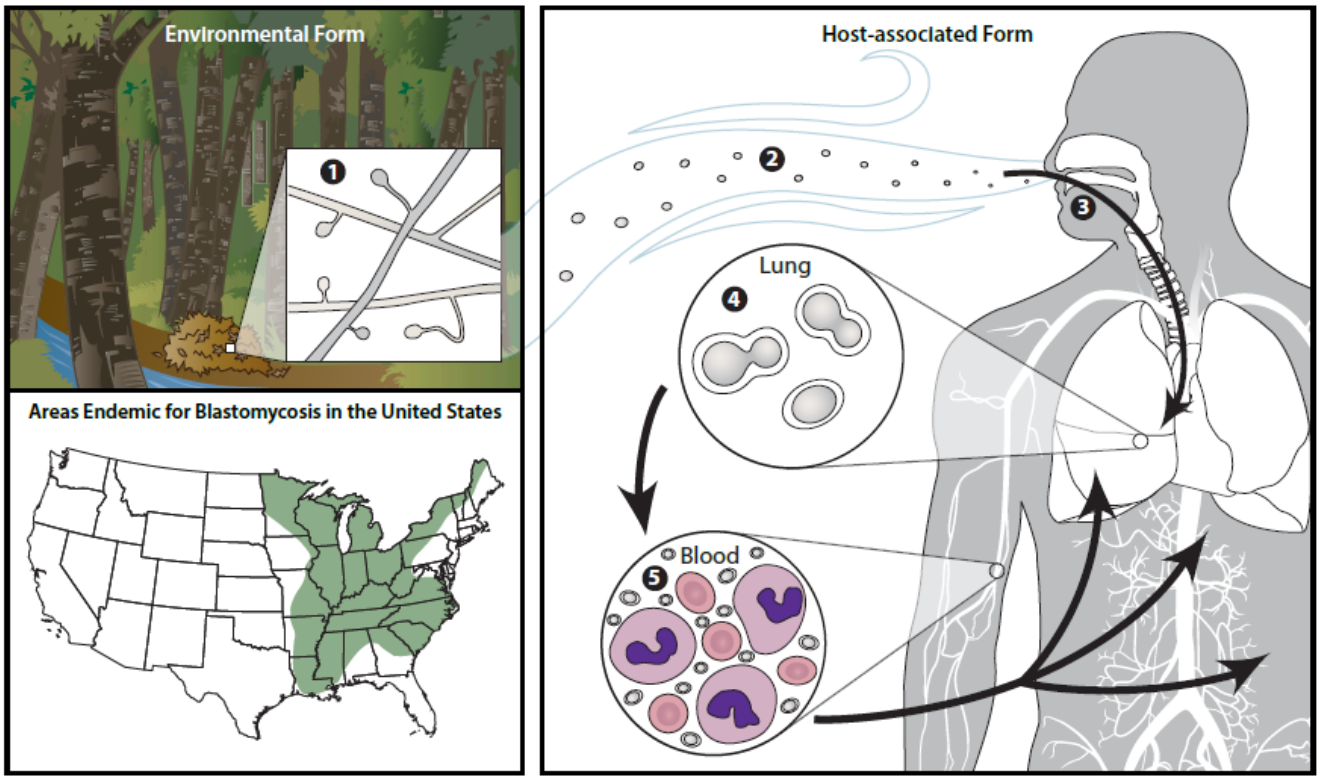


Fig. 13:
Dimorphic cycle of *Blastomyces dermatitidis*.



PROCEDURE (to be done individually)

A. COMMON MOLDS

1. Using your microscope, observe a prepared slides of Penicillium. **Focus first using the yellow-striped 10X objective (100X magnification) and then rotate to the 40X objective (400X magnification)**. Note the type of asexual spores produced and on what they are borne. **Focusing instructions when using the 10X objective can be found in Lab 1**.

2. Using your microscope and **using the yellow-striped 10X objective** (100X magnification), observe a prepared slides of Aspergillus. Note the type of asexual spores produced and on what they are borne.

3. Using your microscope and **using the yellow-striped 10X objective** (100X magnification), observe a prepared slides of Rhizopus. Note the type of asexual spores produced and on what they are borne.

4. Observe the prepared slide showing the zygospore of *Rhizopus* produced during sexual reproduction.

B. DERMATOPHYTES

1. Observe the dermatophyte *Microsporum* growing on DTM. Note the red color from production of alkaline end products indicating that it is breaking down the keratin in the agar. This indicates that the organism is a dermatophyte.

2. Microscopically observe the the macroconidia and microconidia of *Microsporum*.

C. DIMORPHIC FUNGI

1. Observe the prepared slide of *Coccidioides immitis* arthroconidia.

2. Observe the pictures of *Coccidioides immitis* showing the mold form with arthroconidia seen in the soil as well as the endosporulating spherule form seen in the lungs.

3. Observe the pictures of *Histoplasma capsulatum* showing the mold form with tuberculate macroconidia seen in the soil as well as yeast form seen in the lungs.

RESULTS

A. COMMON MOLDS

Make drawings of the molds as they appear microscopically under high magnification and indicate the type of asexual spore they produce. Also note their color and appearance on SDA.

Penicillium	*Aspergillus*
Type of asexual spore: Spores are borne on:	Type of asexual spore: Spores are borne on:

Rhizopus	zygospores of *Rhizopus*
Type of asexual spore: Spores are found in:	

B. DERMATOPHYTES

1. Describe the results of *Microsporum* growing on **Dermatophyte Test Medium (DTM)**:

   Original color of DTM =

   Color following growth of *Microsporum* =

   Reason for Color Change =

2. Draw the **macroconidia and microconidia** of *Microsporum*.

Macroconidia and microconidia of *Microsporum*

C. DIMORPHIC FUNGI

1. Draw the two forms of *Coccidioides immitis*.

Mold form of *Coccidioides* Type of spores seen:	Endosporulating spherules of *Coccidioides* in the lungs.

2. Draw the mold form and yeast form of *Histoplasma capsulatum*.

Mold form of *Histoplasma* Type of spores seen:	Yeast form of *Histoplasma* in the lungs.

PERFORMANCE OBJECTIVES
LABORATORY 10

After completing this lab, the student will be able to perform the following objectives:

DISCUSSION

1. Define the following: hypha, mycelium, vegetative mycelium, and aerial mycelium.
2. Describe the principle way molds reproduce asexually.
3. State the main criteria used in identifying molds.

COMMON MOLDS

1. Describe conidiospores and sporangiospores and name a mold that produces each of these.

2. Recognize the following genera of molds when observing a prepared slide under high magnification and state the type of asexual spore seen:
    a. *Penicillium*
    b. *Aspergillus*
    c. *Rhizopus*
3. Recognize *Rhizopus* zygospores.

DERMATOPHYTES

1. Define dermatophyte and list three common genera of dermatophytes.
2. Name four dermatophytic infections and state how they are contracted by humans.
3. Describe macroconidia and microconidia.
4. Describe how the following may be used to identify dermatophytes: potassium hydroxide preparations of tissue scrapings, DTM, and SDA.
5. Recognize a mold as a dermatophyte and state how you can tell when given the following:
    a. A flask of DTM showing alkaline products
    b. An SDA culture (under a microscope) or picture showing macroconidia.
6. Recognize macroconidia and microconidia.

DIMORPHIC FUNGI

1. Define dimorphic fungi and state how humans usually contract them.
2. Name three common dimorphic fungal infections found in the United States, state how they are transmitted to humans, and indicate where they are found geographically.
3. Describe the mold form and the yeast-like form of the following:
    a. *Coccidioides immitis*
    b. *Histoplasma capsulatum*
    c. *Blastomyces dermatitidis*
4. Recognize *Coccidioides immitis* and its arthroconidia when given a prepared slide and a microscope.

LABORATORY 11
VIRUSES: THE BACTERIOPHAGE

DISCUSSION

Viruses are infectious agents with both living and non-living characteristics.

1. **Living characteristics** of viruses

    a. They reproduce at a fantastic rate, but only in living host cells.

    b. They can mutate.

2. **Non-living characteristics** of viruses

    a. They are acellular, that is, they contain no cytoplasm or cellular organelles.

    b. They carry out no metabolism on their own and must replicate using the host cell's metabolic machinery. In other words, viruses don't grow and divide. Instead, new viral components are synthesized and assembled within the infected host cell.

    c. With few exceptions, they possess DNA or RNA but never both.

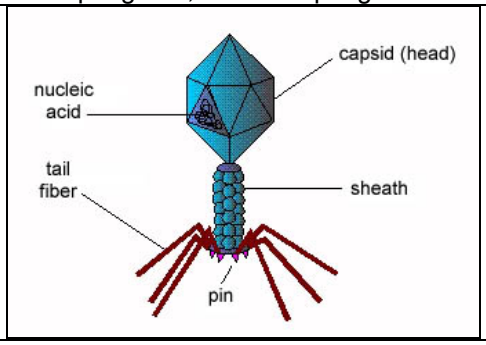
Viruses are usually much smaller than bacteria. Most are submicroscopic, ranging in size from **10-250 nanometers**.

Structurally, viruses are much more simple than bacteria. Every virus contains a **genome** of single-stranded or double-stranded DNA or RNA that functions as its genetic material. This is surrounded by a protein shell, called a **capsid** or core, composed of protein subunits called capsomeres. Many viruses consist of no more than nucleic acid and a capsid, in which case they are referred to as **nucleocapsid or naked viruses**.

Most animal viruses have an **envelope** surrounding the nucleocapsid and are called **enveloped viruses**. The envelope usually comes from the host cell's membranes by a process called budding, although the virus does incorporate glycoprotein of its own into the envelope.

Bacteriophages are viruses that infect only bacteria. In addition to the nucleocapsid or head, some have a rather complex tail structure used in adsorption to the cell wall of the host bacterium (**Fig. 1**).

Fig. 1:
Structure of Coliphage T4, a bacteriophage that infects *E. coli*



LABORATORY 11:
VIRUSES: THE BACTERIOPHAGES

Since viruses lack organelles and are totally dependent on the host cell's metabolic machinery for replication, they cannot be grown in synthetic media. In the laboratory, animal viruses are grown in animals, in embryonated eggs, or in cell culture. (In cell culture, the host animal cells are grown in synthetic medium and then infected with viruses.) Plant viruses are grown in plants or in plant cell culture. Bacteriophages are grown in susceptible bacteria.

Today we will be working with bacteriophages since they are the easiest viruses to study in the lab. Most bacteriophages, such as Coliphage T4 that we are using today, replicate by the **lytic life cycle** and are called **lytic bacteriophages**.

The **lytic life cycle of Coliphage T4 (see Figs. 2-7)** consists of the following steps:

1. **Adsorption**
   Attachment sites on the bacteriophage tail adsorb to receptor sites on the cell wall of a susceptible host bacterium (**Fig. 2**).

2. **Penetration**
   A bacteriophage enzyme "drills" a hole in the bacterial cell wall and the bacteriophage injects its genome into the bacterium (**Fig. 3**). This begins the eclipse period, the period in which no intact bacteriophages are seen within the bacterium.

3. **Replication**
   Enzymes coded by the bacteriophage genome shut down the bacterium's macromolecular (protein, RNA, DNA) synthesis. The bacteriophage genome replicates and the bacterium's metabolic machinery is used to synthesize bacteriophage enzymes and bacteriophage structural components (**Fig. 4 and 5**).

4. **Maturation**
   The bacteriophage parts assemble around the genome (**Fig. 6**).

5. **Release**
   A bacteriophage-coded lysozyme breaks down the bacterial peptidoglycan causing osmotic lysis of the bacterium and release of the intact bacteriophages (**Fig. 7**).

6. **Reinfection**
   From 50-200 bacteriophages may be produced per infected bacterium and they now infect surrounding bacteria.

Some bacteriophages replicate by the **lysogenic life cycle** and are called **temperate bacteriophages**. When a temperate bacteriophage infects a bacterium, it can either: 1) replicate by the lytic life cycle and cause lysis of the host bacterium, or it can 2) incorporate its DNA into the bacterium's DNA and assume a noninfectious state. In the latter case, the cycle begins by the bacteriophage adsorbing to the host bacterium and injecting its genome, as in the lytic cycle. However, the bacteriophage does not shut down the host bacterium. Instead, the bacteriophage DNA inserts or integrates into the host bacterium's DNA. At this stage, the virus is called a **prophage**. Expression of the bacteriophage genes controlling bacteriophage replication is repressed by a repressor protein and the bacteriophage DNA replicates as a part of the bacterial nucleoid. However, in approximately one in every million to one in every billion bacteria containing a prophage, **spontaneous induction** occurs. The bacteriophage genes are activated and bacteriophages are produced as in the lytic life cycle.

Figs. 2-7
The Lytic Life Cycle of Coliphage T4.

Fig. 2: Adsorption
Tail fibers of Coliphage T4 adsorb to receptors on the cell wall of *E. coli* B.

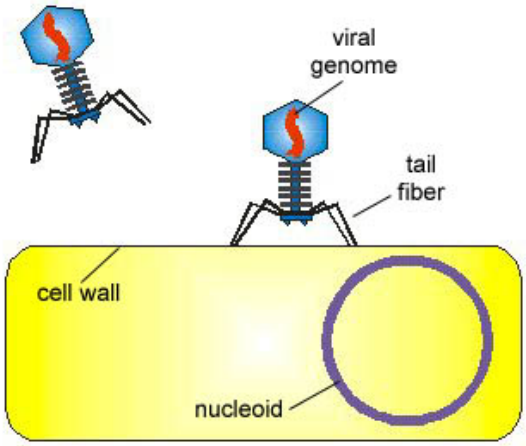


Fig. 3: Penetration
The bacteriophage injects its DNA genome into the cytoplasm of the bacterium.

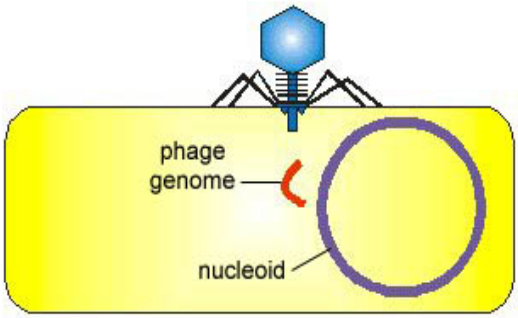


Fig. 4: Early Replication
The genome of the bacteriophage replicates and begins to direct the metabolic machinery of the bacterium to produce phage structural components and enzymes.

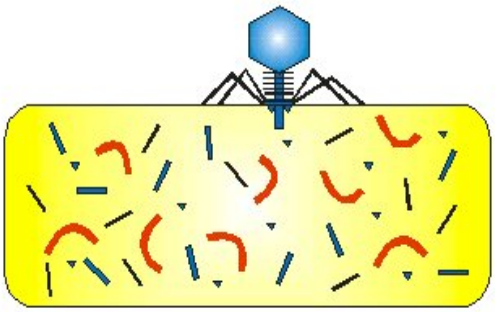


Fig. 5: Late Replication, Early Maturation
Bacteriophage parts continue to be produced and begin to assemble.

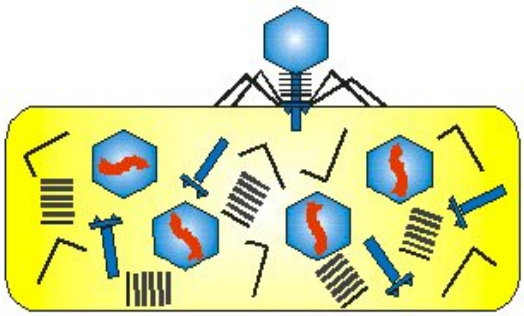
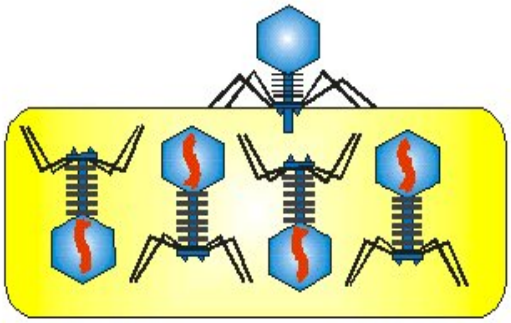
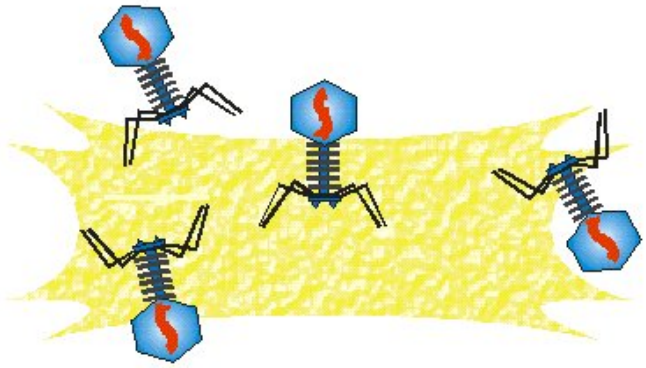
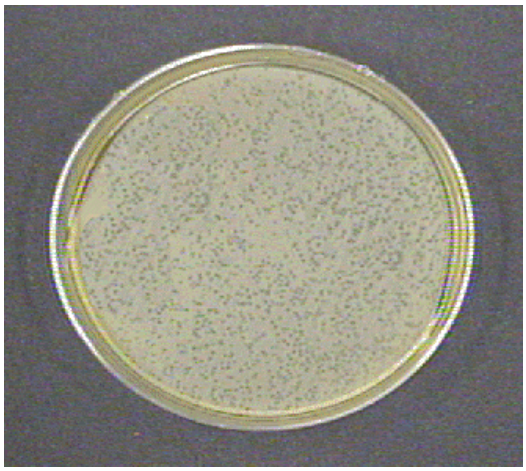


Fig. 6: Maturation The phage parts assemble producing intact bacteriophages.	**Fig. 7: Release** Phage-coded lysozyme breaks down the bacterium's cell wall causing lysis. The bacteriophages are released.
	

Today you will infect the bacterium *Escherichia coli* B with its specific bacteriophage, Coliphage T4.

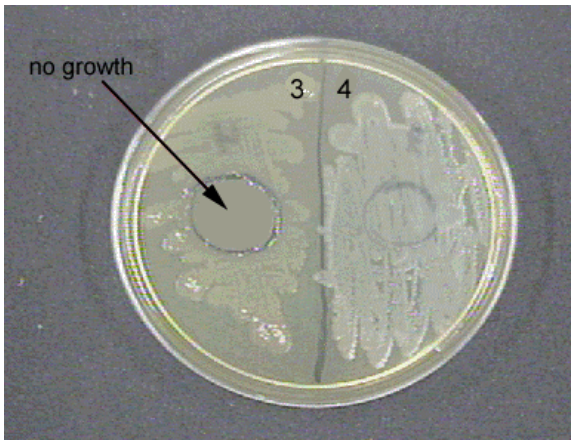
In the first part of the lab you will perform a plaque count. A **plaque** is a small, clear area on an agar plate where the host bacteria have been lysed as a result of the lytic life cycle of the infecting bacteriophages (**see Fig. 14**). As the bacteria replicate on the plate they form a "lawn" of confluent growth. Meanwhile, each bacteriophage that adsorbs to a bacterium will reproduce and cause lysis of that bacterium. The released bacteriophages then infect neighboring bacteria, causing their lysis. Eventually a visible self-limiting area of lysis, a plaque, is observed on the plate.

Fig. 14:
Plaques



The second part of the lab will demonstrate viral specificity. **Viral specificity** means that a specific strain of bacteriophage will only adsorb to a specific strain of susceptible host bacterium. In fact, viral specificity is just as specific as an enzyme-substrate reaction or an antigen-antibody reaction. Therefore, viral specificity can be used sometimes as a tool for identifying unknown bacteria. Known bacteriophages are used to identify unknown bacteria by observing whether or not the bacteria are lysed (**see Fig. 15**). This is called **phage typing**.

Fig. 15:
Lysis of an unknown bacterium by a known bacteriphage.



Phage typing is useful in identifying strains of such bacteria as *Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella* species. For example, by using a series of known staphylococcal bacteriophages against the *Staphylococcus aureus* isolated from a given environment, one can determine if it is identical to or different from the strain of *Staphylococcus aureus* isolated from a lesion or from a food. This can be useful in tracing the route of transmission.

A. PLAQUE COUNT

MATERIALS

1 tube containing 9.9 ml of sterile saline; 3 tubes containing 9.0 ml of sterile saline; 3 sterile empty dilution tubes; 3 Trypticase Soy agar plates; 3 sterile 1.0 ml pipettes; 1 sterile 10.0 ml pipette; bottle of melted Motility Test Medium from a water bath held at 47C.

CULTURES

Trypticase Soy broth culture of *Escherichia coli* B, suspension of Coliphage T4

PROCEDURE: to be done in groups of three

1. Take 1 tube containing 9.9 ml of sterile saline, 3 tubes containing 9.0 ml of sterile saline, and 2 sterile empty dilution tubes and label the tubes as shown in **Fig. 8**. Label the 3 TSA plates 10^{-4}, 10^{-5}, and 10^{-6}.

2. Dilute the **Coliphage T4** stock as described below and shown in **Fig. 8**.

   a. Remove a sterile **1.0 ml pipette** from the bag. **Do not touch the portion of the pipette that will go into the tubes and do not lay the pipette down**. From the tip of the pipette to the "0" line is **1 ml**; each numbered division (0.1, 0.2, etc.) represents **0.1 ml** (see Fig. 11).

   b. Insert the cotton-tipped end of the pipette into a **blue** 2 ml pipette filler.

c. Uncap the sample of Coliphage T4, insert the pipette to the bottom of the tube, and withdraw **0.1 ml** (see Fig. 11) of the sample by turning the filler knob **towards** you. Re-cap the tube.

d. Place the lip of the 10^{-2} dilution tube at the opening of the microincinerator for 2-3 seconds and dispense the 0.1 ml of sample into the tube by turning the filler knob **away** from you. Draw the liquid up and down in the pipette several times to rinse the pipette and help mix. Flame and cap the tube. This will give a 1/100 or 10^{-2} dilution of the bacteriophage.

e. Using a vortex mixer, **mix the tube thoroughly**. This is to assure an even distribution of the bacteriophage throughout the liquid.

f. Using the same pipette and procedure, aseptically withdraw **1.0** ml (see Fig. 11H) from the 10^{-2} dilution tube and dispense into the 10^{-3} dilution tube and mix. This will give a 1/1000 or 10^{-3} dilution of the bacteriophage. Using a vortex mixer, **mix the tube thoroughly**.

g. Using the same pipette and procedure, aseptically withdraw **1.0 ml** (up to the "0" line; see Fig. 11H) from the 10^{-3} dilution tube and dispense into the 10^{-4} dilution tube. This will give a 1/10,000 or 10^{-4} dilution of the bacteriophage. Using a vortex mixer, **mix the tube thoroughly**.

h. Using the same pipette and procedure, aseptically withdraw **1.0 ml** (see Fig. 11H) from 10^{-4} dilution tube and dispense into the 10^{-5} dilution tube. This will give a 1/100,000 or 10^{-5} dilution of the bacteriophage. Using a vortex mixer, **mix the tube thoroughly**.

i. Discard the pipette in the used pipette container.

3. Take 3 empty, sterile tubes and treat as described below and shown in **Fig. 8**.

a. Using a new sterile **1.0 ml** pipette and the procedure described above, aseptically remove **0.1 ml** (see Fig. 11H) of the 10^{-5} bacteriophage dilution and dispense into the 10^{-6} empty tube (**Fig. 8**).

b. Using the same pipette and procedure, aseptically remove **0.1 ml** of the 10^{-4} bacteriophage dilution and dispense into the 10^{-5} empty tube (**Fig. 8**).

c. Using the same pipette and procedure, aseptically remove **0.1 ml** of the 10^{-3} bacteriophage dilution and dispense into the 10^{-4} empty tube (**Fig. 8**).

d. Discard the pipette in the used pipette container.

4. Using a new sterile **1.0 ml pipette**, add **0.5 ml** (see Fig. 11H) of ***E. coli* B** to the 0.1 ml of diluted bacteriophage in each of the 3 tubes from step 3 and mix (**Fig. 9**). Discard the pipette in the used pipette container.

5. **Let the 3 tubes of *E. coli* / coliphage T4 mixture sit for 10-15** minutes to promote adsorption.

6. Using a sterile **10.0 ml pipette**, add **2.5 ml** (see Fig. 11I) of sterile, **melted Motility Test Medium** to the *E. coli* / bacteriophage mixture in each of the 3 tubes from step 4 and mix (**Fig. 10**).

7. Quickly pour the melted Motility Medium - *E. coli* - bacteriophage mixtures onto separate **plates of Trypticase Soy agar** and swirl to distribute the contents over the entire agar surface.

8. Incubate the 3 TSA plates **right-side up** and stacked in the petri plate holder on the shelf of the 37°C incubator corresponding to your lab section until the next lab period.

Fig. 8:
Diluting Coliphage T4 for the plaque count experiment.

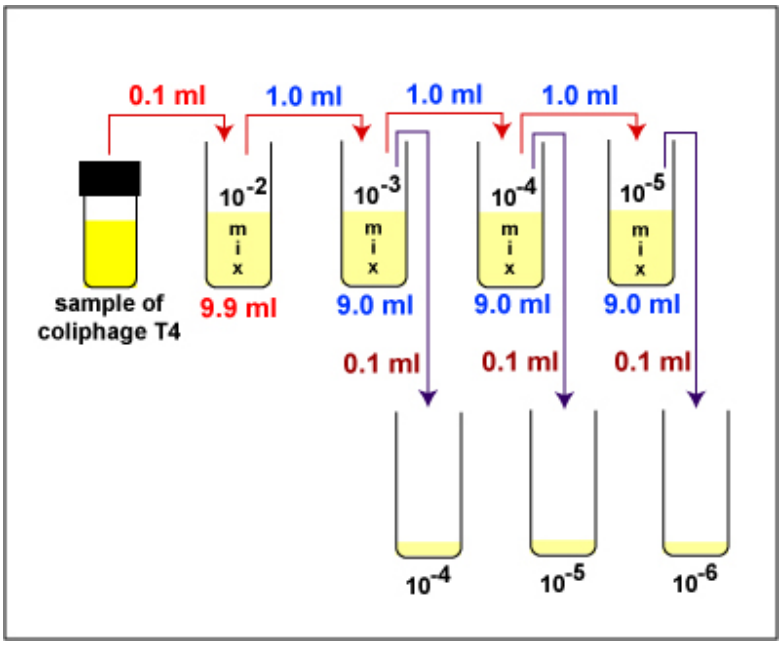


Fig. 9:
Adding the *E. coli* to the diluted bacteriophages

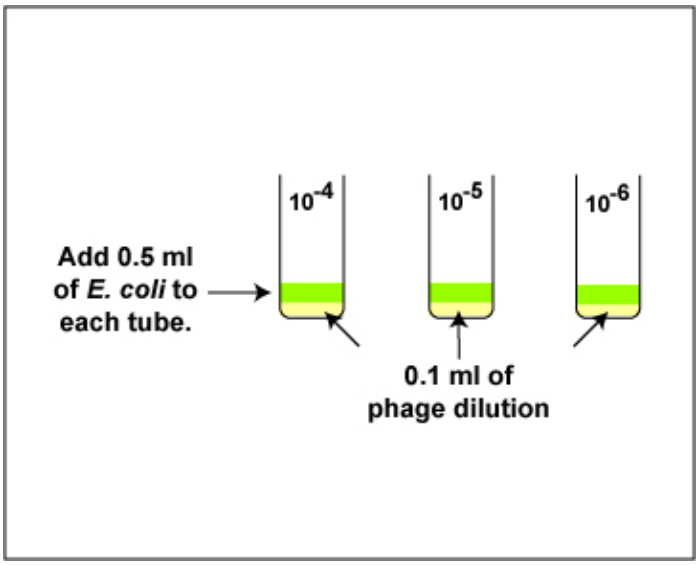


Fig. 10:
Adding the melted Trypticase Soy Agar (TSA) to the *E. coli* and bacteriophages

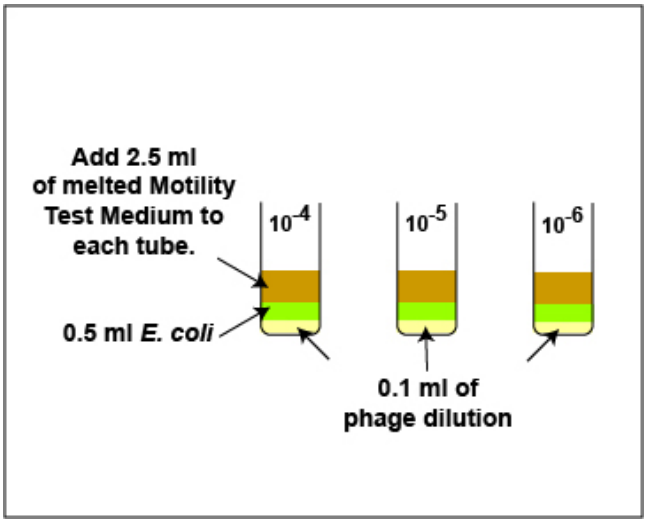
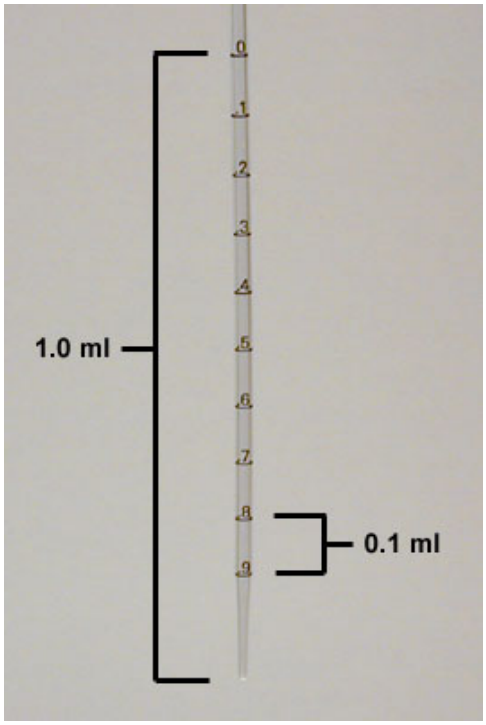
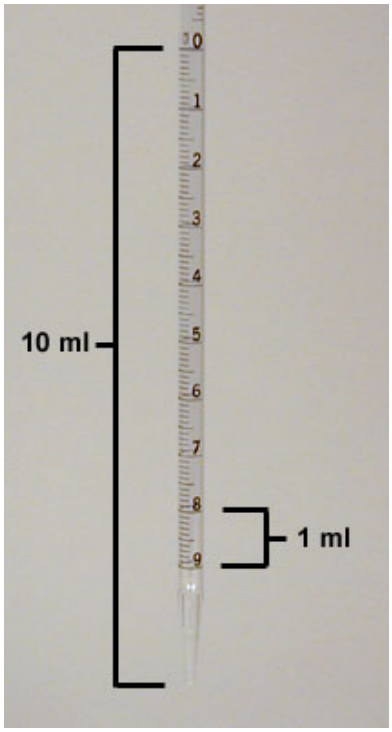


Fig. 11: A 1.0 ml pipette. Use the blue filler.	**Fig. 12:** A 10.0 ml pipette. Use the green filler.
	

B. VIRAL SPECIFICITY

MATERIALS

Trypticase Soy agar plates (2)

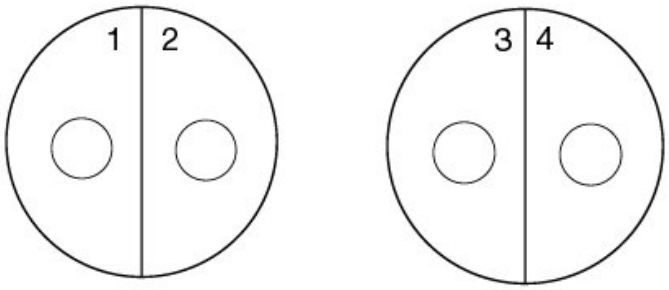
CULTURES

Trypticase Soy broth cultures of 4 unknown bacteria labelled #1, #2, #3, and #4; suspension of Coliphage T4.

PROCEDURE: (To be done in groups of three)

1. Using a wax marker, draw a line on the bottom of both Trypticase Soy agar plates dividing them in half. Number the 4 sectors 1, 2, 3, and 4, to correspond to the 4 unknown bacteria.

2. Draw a circle about the size of a dime in the center of each of the 4 sectors.



3. Using a sterile inoculating loop, streak unknown bacterium #1 on sector 1 of the first Trypticase Soy agar plate by streaking the loop **through the circle** you drew. **Be careful not to streak into the other half of the plate.**

4. Streak unknown bacterium #2 on sector 2 of the first Trypticase Soy agar plate.

5. Streak unknown bacterium #3 on sector 3 of the second Trypticase Soy agar plate.

6. Streak unknown bacterium #4 on sector 4 of the second Trypticase Soy agar plate.

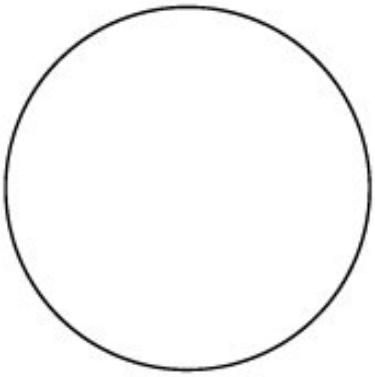
7. Your instructor will add **1 drop** of concentrated Coliphage T4 to each sector in the area outlined by the circle.

8. Incubate the 2 TSA plates **right-side up** and stacked in the petri plate holder on the shelf of the 37°C incubator corresponding to your lab section until the next lab period.

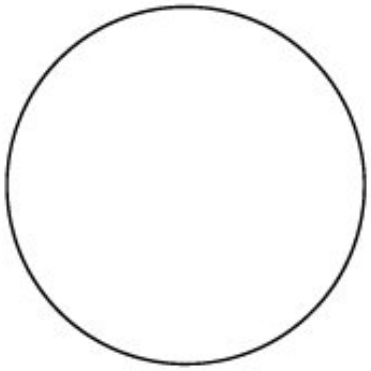
RESULTS

A. Plaque Count

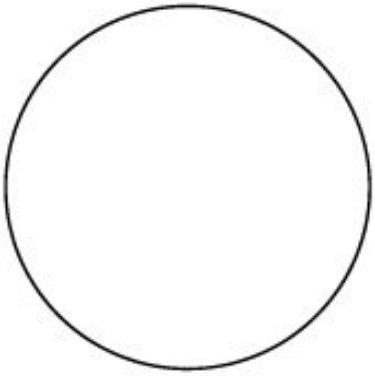
Observe the 3 plates for plaque formation and make a drawing.



10^{-4} dilution



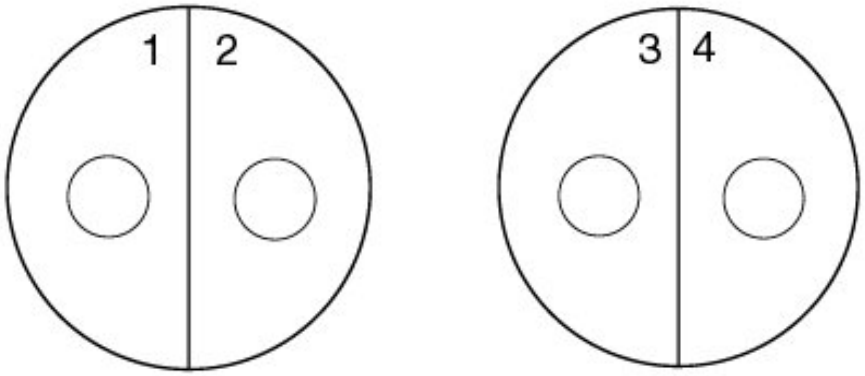
10^{-5} dilution



10^{-6} dilution

B. Viral Specificity

Make a drawing of your results and state which of the unknowns (#1, #2, #3, or #4) was *E. coli*.



Which unknown bacterium is *E. coli*? _____

How can you tell?

PERFORMANCE OBJECTIVES LABORATORY

After completing this lab, the student will be able to perform the following objectives:

DISCUSSION

1. Define the following: bacteriophage, plaque, and phage typing.
2. Describe the structure of the bacteriophage coliphage T4.
3. Describe the lytic life cycle of bacteriophages.
4. Define viral specificity.

RESULTS

1. Recognize plaques and state their cause.
2. Interpret the results of a viral specificity test using Coliphage T4.

LABORATORY 12
ISOLATION AND IDENTIFICATION OF *ENTEROBACTERIACEAE* AND *PSEUDOMONAS:* PART 1

A. *Enterobacteriaceae*: Glucose Fermenting Gram- negative Enteric Bacilli
B. *Pseudomonas* and Other Glucose non-fermenting Gram- negative Bacilli
C. Isolation of *Enterobacteriaceae* and *Pseudomonas*
D. Differentiating Between the *Enterobacteriaceae* and *Pseudomonas*
E. Identifying the *Enterobacteriaceae* Using the Enterotube®II

DISCUSSION

Labs 12 and 13 deal with **opportunistic and pathogenic glucose fermenting Gram-negative bacilli that are members of the bacterial family Enterobactereaceae, as well as glucose non-fermenting Gram- negative bacilli such as *Pseudomonas* and *Acinetobacter*.**

A. *ENTEROBACTERIACEAE*: THE GLUCOSE FERMENTING, GRAM-NEGATIVE, ENTERIC BACILLI

Bacteria belonging to the family ***Enterobacteriaceae*** are the most commonly encountered organisms isolated from clinical specimens. The *Enterobacteriaceae* is a large diverse family of bacteria belonging to the order *Enterobacteriales* in the class *Gammaproteobacter* of the phylum *Proteobacter*. **Medically important members of this family are commonly referred to as glucose fermenting, Gram- negative, enteric bacilli**, because they are Gram- negative bacilli that can **ferment the sugar glucose**. Many are normal flora of the intestinal tract of humans and animals while others infect the intestinal tract. Members of this family have the following characteristics in common:

1. They are **Gram-negative rods.**
2. If motile, they possess a **peritrichous arrangement of flagella.**
3. They are **facultative anaerobes.**
4. With few exceptions, they are **oxidase negative.**
5. All species **ferment the sugar glucose** but otherwise vary widely in their biochemical characteristics.
6. Most **reduce nitrates to nitrites**.

The *Enterobacteriaceae* represents a very large and diverse family of bacteria. Some of the more common clinically important genera of the family *Enterobacteriaceae* include:

Salmonella	*Citrobacter*	*Morganella*
Shigella	*Enterobacter*	*Yersinia*
Proteus	*Serratia*	*Edwardsiella*
Escherichia	*Klebsiella*	*Providencia*

LABORATORY 12:
ENTEROBACTERIACEAE AND *PSEUDOMONAS*: PART 1

Several genera of *Enterobacteriaceae* are associated with **gastroenteritis** and food-borne disease. These include:

- ***Salmonella***.
- ***Shigella***.
- **Certain strains of *Escherichia coli.***
- **Certain species of *Yersinia***.

All intestinal tract infections are **transmitted by the fecal-oral route**.

There are two species of *Salmonella*, *Salmonella enterica* and *Salmonella bongori*. Any infection caused by *Salmonella* is called a **salmonellosis**. Non-typhoidal *Salmonella* accounts for an estimated 520 cases per 100,000 population. CDC estimates that there are approximately 1,200,000 cases of non-typhoidal salmonellosis each year in the U.S resulting in 450 deaths. Since many different animals carry *Salmonella* in their intestinal tract, people usually become infected from ingesting improperly refrigerated, uncooked or undercooked poultry, eggs, meat, dairy products, vegetables, or fruit **contaminated with animal feces**.

Enteritis is the most common form of salmonellosis. Symptoms generally appear 6-48 hours after ingestion of the bacteria and include **vomiting, nausea, non-bloody diarrhea, fever, abdominal cramps, myalgias, and headache**. Symptoms generally last from 2 days to 1 week followed by spontaneous recovery. All species of *Salmonella* can cause **bacteremia** but *S. enterica* serotype Typhi, isolated only from humans, frequently disseminates into the blood causing a severe form of salmonellosis called typhoid fever. About 400 cases of typhoid fever occur each year in the U.S. but approximately 75% of these are acquired while traveling internationally.

Salmonella serotyping is a subtyping method of identification based on the identification of distinct cell wall, flagellar, and capsular antigens with known antiserum, as will be discussed in Lab 17. *Salmonella* serotypes Enteritidis and Typhimurium are the two most common serotypes in the United States, accounting for approximately 35 to 40% of all infections confirmed by laboratory culture. As mentioned above, *S. enterica* serotype Typhi is responsible for typhoid fever.

Any ***Shigella*** infection is called a **shigellosis.** Unlike *Salmonella*, which can infect many different animals, *Shigella* only infects humans and other higher primates. There are approximately 14,000 laboratory cases of shigellosis a year reported in the US with an estimated 450,000 total cases and 70 deaths.

Shigellosis frequently **starts with a watery diarrhea, fever, and abdominal cramps** but may **progress to dysentery with scant stools containing blood, pus, and mucus**. The incubation period is 1-3 days. Initial profuse watery diarrhea typically appears first as a result of enterotoxin. Within 1-2 days this progresses to abdominal cramps, with or without bloody stool. Classic shigellosis presents itself as lower abdominal cramps and stool abundant with blood and pus develops as the *Shigella* invade the mucosa of the colon.

Escherichia coli is one of the **dominant normal flora in the intestinal tract of humans and animals**. **Some strains, however, can cause infections of the intestines while others are capable of causing infections outside the intestines**. Extraintestinal pathogenic *E. coli* cause such opportunistic infections as urinary tract infections, wound infections, and septicemia and will be discussed in greater detail below. Intestinal or diarrheagenic *E. coli* cause infections of the intestinal tract. **Diarrheagenic *E. coli*** include the following:

a. Enterotoxigenc *E. coli* (ETEC) produce enterotoxins that cause the loss of sodium ions and water from the small intestines resulting in a watery diarrhea. It is an important cause of diarrhea in impoverished countries. ETEC accounts for between 11-15% of cases of traveler's diarrhea in persons visiting developing countries and 30-45% of cases among those visiting Mexico. There are approximately 80,000 cases of ETEC a year in the U.S.

b. Enteropathogenic *E. coli* (EPEC) causes an endemic diarrhea in in impoverished countries, especially in infants younger than 6 months of age. The bacterium disrupts the normal microvilli on the epithelial cells of

the small intestines resulting in maladsorbtion and diarrhea. They do not produce enterotoxin or shiga toxin and are not invasive. It is rare in industrialized countries.

c. Enteroaggregative *E. coli* (EAEC) is a cause of endemic diarrhea in children in impoverished countries and industrialized countries. EAEC causes around 30% of cases of traveler's diarrhea. It is also responsible for a persistant diarrhea in people infected with HIV. It probably causes diarrhea by adhering to mucosal epithelial cells of the small intestines and interfering with their function.

d. Enteroinvasive *E. coli* (EIEC) invade and kill epithelial cells of the colon usually causing a watery diarrhea but sometimes progressing to a dysentery-type syndrome with blood in the stool. It occurs mostly in impoverished countries and is rare in industrialized countries.

e. Shiga toxin-producing *E. coli* (STEC), such as *E. coli* 0157:H7, produce a shiga toxin that kills epithelial cells of the colon causing hemorrhagic colitis, a bloody diarrhea. In rare cases, the shiga toxin enters the blood and is carried to the kidneys where, usually in children, it damages vascular cells and causes hemolytic uremic syndrome. *E. coli* 0157:H7 is thought to cause more than 20,000 infections and up to 250 deaths per year in the U.S.

Several species of ***Yersinia***, such as *Y. enterocolitica* and *Y. pseudotuberculosis* are also causes of **diarrheal disease**.

Many other genera of the family *Enterobacteriaceae* are **normal microbiota of the intestinal tract** and are considered **opportunistic pathogens**. The **most common genera of *Enterobacteriaceae* causing opportunistic infections in humans** include the following:

- ***Escherichia coli*.**
- ***Proteus*.**
- ***Enterobacter*.**
- ***Klebsiella*.**
- ***Citrobacter*.**
- ***Serratia*.**

They act as opportunistic pathogens when they are **introduced into body locations where they are not normally found**, especially if the host is debilitated or immunosuppressed. They all cause the same types of **opportunistic infections**, namely:

- **Urinary tract infections**.
- **Wound infections**.
- **Pneumonia**.
- **Septicemia**.

These normal flora Gram- negative bacilli, along with Gram-positive bacteria such as *Enterococcus* species (see Lab 14) and *Staphylococcus* species (see Lab 15), are among the most common causes of **healthcare-associated infections** (formerly called **nosocomial infections**).

According to the Centers for Disease Control and Prevention (CDC) Healthcare-associated infection's website, "In American hospitals alone, **healthcare-associated infections account for an estimated 1.7 million infections and 99,000 associated deaths each year**. Of these infections:

- 32 percent of all healthcare-associated infection are urinary tract infections (UTIs.)
- 22 percent are surgical site infections.
- 15 percent are pneumonia (lung infections.)

- 14 percent are bloodstream infections.

Most patients who have healthcare-associated infections are predisposed to infection because of invasive supportive measures such as urinary catheters, intravascular lines, and endotracheal intubation.

By far, the most common Gram-negative bacterium causing nosocomial infections is *Escherichia coli*. *E. coli* causes between 70 and 90% of both upper and lower urinary tract infections (UTIs). It is also a frequent cause of **abdominal wound infections and septicemia**. Depending on the facility, *E. coli* is responsible for between 12% and 50% of all healthcare-associated infections.

However, according to a 2008 study, ***Enterobacteriaceae* other than *E. coli* were responsible for 7 of the 10 most common Gram-negative organisms isolated from urinary tract, respiratory tract, and bloodstream infections from intensive care unit patients** between 2002 and 2008 in the United States. These include *Klebsiella pneumoniae* (15%), *Enterobacter cloacae* (9%), *Serratia marcescens* (6%), *Enterobacter aerogenes* (4%), *Proteus mirabilis* (4%), *Klebsiella oxytoca* (3%), and *Citrobacter freundii* (2%). Furthermore, the National Healthcare Safety Network reported *K. pneumoniae* (6%), *Enterobacter* spp. (5%), and *K. oxytoca* (2%) among the top 10 most frequently isolated health care-associated infections between the years between 2006 and 2007.

1. Urinary Tract Infections

The most common infection caused by opportunistic *Enterobacteriaceae* is a **urinary tract infection (UTI)**. UTIs account for more than 8, 000,000 physician office visits per year in the U.S and as many as 100,000 hospitalizations. Among the non-hospitalized and non-debilitated population, UTIs are more common in females because of their shorter urethra and the closer proximity between their anus and the urethral opening. (Over 20 percent of women have recurrent UTIs.) However, anyone can become susceptible to urinary infections in the presence of predisposing factors that cause functional and structural abnormalities of the urinary tract. These abnormalities increase the volume of residual urine and interfere with the normal clearance of bacteria by urination. Such factors include prostate enlargement, sagging uterus, expansion of the uterus during pregnancy, paraplegia, spina bifida, scar tissue formation, and catheterization. Between 35 and 40 percent of all nosocomial infections, about 900,000 per year in the U.S., are UTIs and are usually associated with catheterization.

E. coli* and *Staphylococcus saprophyticus (a Gram-positive staphylococcus that will be discussed in Lab 15) cause **around 90 percent of all uncomplicated UTIs**. Most of the remaining uncomplicated UTIs are caused by other Gram- negative enterics such as *Proteus mirabilis* and *Klebsiella pneumoniae* or by *Enterococcus faecalis* (a Gram-positive streptococcus that will be discussed in Lab 14). ***E. coli* is responsible for more than 50 percent of healthcare-associated UTIs**. Other causes of hospital-acquired UTIs include other species of *Enterobacteriaceae* (such as *Proteus*, *Enterobacter*, and *Klebsiella*), *Pseudomonas aeruginosa* (discussed below), *Enterococcus* species (discussed in lab 14), *Staphylococcus saprophyticus* (discussed in Lab 15), and the yeast *Candida* (discussed in lab 9).

The traditional laboratory culture standard for a UTI has been the presence of more than 100,000 CFUs (colony-forming units; see Lab 4) per milliliter (ml) of midstream urine, or any CFUs from a catheter-obtained urine sample. More recently, this has been modified and counts of as few as 1000 colonies of a single type per ml or as little as 100 coliforms per ml are now considered as indicating a UTI.

2. Wound Infections

Wound infections are due to fecal contamination of external wounds or a result of wounds that cause **trauma to the intestinal tract**, such as surgical wounds, gunshot wounds, and knife wounds. In the latter case, fecal bacteria get out of the intestinal tract and into the peritoneal cavity causing peritonitis and formation of abcesses on the organs found in the peritoneal cavity.

3. Pneumonia

Although they sometimes cause **pneumonia**, the *Enterobacteriaceae* account for less than 5% of the bacterial pneumonias requiring hospitalization.

4. Bloodstream Infections

Gram-negative septicemia is a result of these opportunistic Gram- negative bacteria getting into the blood. They are **usually introduced into the blood from some other infection site, such as an infected kidney, wound, or lung**. Looking at patients that develop septic shock:

- Lower respiratory tract infections are the source in about 25% of patients.
- Urinary tract infections are the source in about 25% of patients.
- Soft tissue infections are the source in about 15% of patients.
- Gastrointestinal infections are the source in about 15% of patients.
- Reproductive tract infections are the source in about 10% of patients.
- Foreign bodies (intravascular lines, implanted surgical devices, etc.) are the source in about 5% of patients.

There are approximately 750,000 cases of septicemia per year in the U.S. and 200,000 cases of septic shock. Septic shock results in approximately 100,000 deaths per year in the U.S. Approximately 45 percent of the cases of septicemia are due to Gram- negative bacteria. *Klebsiella, Proteus, Enterobacter, Serratia*, and *E. coli*, are all common *Enterobacteriaceae* causing septicemia. (Another 45 percent are a result of Gram-positive bacteria (see Labs 14 and 15) and 10 percent are due to fungi, mainly the yeast *Candida* (see Lab 9).

- Diagnosis and symptoms for SIRS and septicemia can be found in Appendix F.

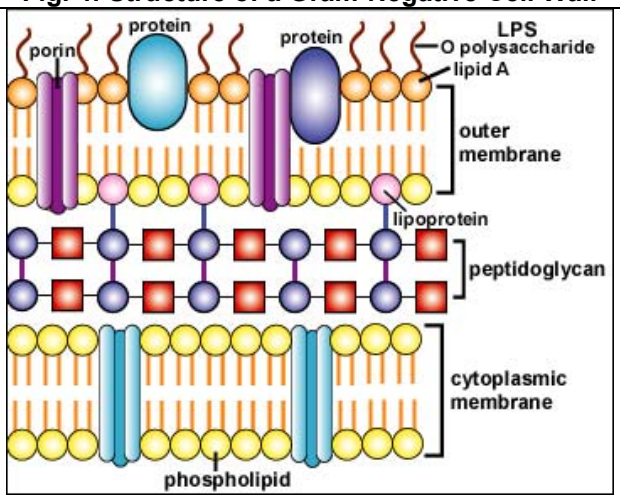
In the outer membrane of the Gram-negative cell wall, the lipid A moiety of the lipopolysaccharide functions as an **endotoxin** (see Fig 4). Endotoxin indirectly harms the body when massive amounts are released during severe Gram- negative infections. This, in turn, causes an excessive cytokine response.

1. The LPS released from the outer membrane of the Gram- negative cell wall first binds to a LPS-binding protein circulating in the blood and this complex, in turn, binds to a receptor molecule (CD_{14}) found on the surface of body defense cells called macrophages (see Fig. 1) located in most tissues and organs of the body.

2. This is thought to promote the ability of the toll-like receptor TLR-4 to respond to the LPS, triggering the macrophages to release various defense regulatory chemicals called cytokines, including tumor necrosis factor-alpha (TNF-alpha), interleukin-1 (IL-1), interleukin-6 (IL-6), and interleukin-8 (IL-8), and platelet-activating factor (PAF). The cytokines then bind to cytokine receptors on target cells and initiate inflammation and activate both the complement pathways and the coagulation pathway (see Fig. 5).

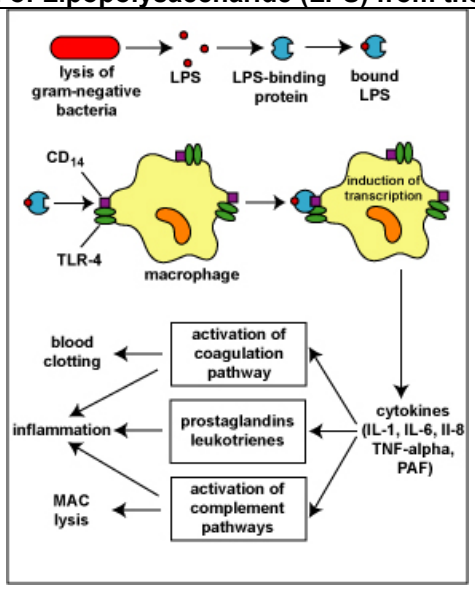
3. The complex of LPS and LPS binding protein can also attach to molecules called CD14 on the surfaces of phagocytic white blood cells called neutrophils causing them to release proteases and toxic oxygen radicals for extracellular killing. Chemokines such as interleukin-8 (IL-8) also stimulate extracellular killing. In addition, LPS and cytokines stimulate the synthesis of a vasodilator called nitric oxide.

Fig. 4: Structure of a Gram-Negative Cell Wall



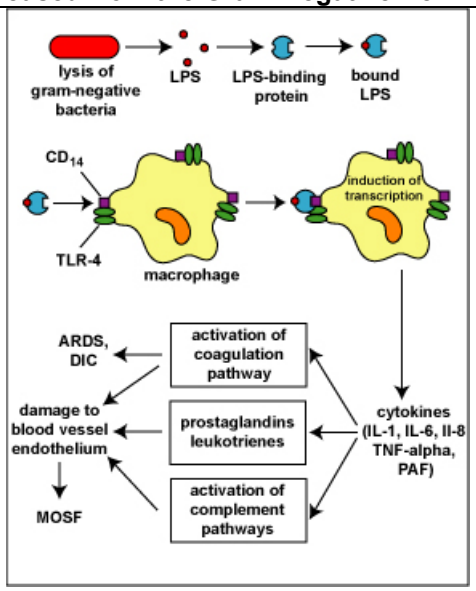
The Gram- negative cell wall is composed of a thin, inner layer of peptidoglycan and an outer membrane consisting of molecules of phospholipids, lipopolysaccharides (LPS), lipoproteins and surface proteins. The lipopolysaccharide consists of lipid A and O polysaccharide.

Fig. 5: Physiologic Action of Lipopolysaccharide (LPS) from the Gram-Negative Cell Wall



The lysis of Gram- negative bacteria causes them to release lipopolysaccharide (LPS; endotoxin) from the outer membrane of their cell wall. The LPS binds to a LPS-binding protein circulating in the blood and this complex, in turn, binds to a receptor molecule (CD14) found on the surface of body defense cells called macrophages. This is thought to promote the ability of the toll-like receptor TLR-4 to respond to the LPS, triggering the macrophage to release various defense regulatory chemicals called cytokines, including IL-1, IL-6, IL-8, TNF-alpha, and PAF. The cytokines then bind to cytokine receptors on target cells and initiate inflammation and activate both the complement pathways and the coagulation pathway. LPS can also bind directly to TLR-4 molecules. (LPS, lipopolysaccharide; TLR, toll-like receptor; IL-1, interleukin-1; IL-6, interleukin-6; IL-8, interleukin-8, TNF-alpha, tumor necrosis factor-alpha; PAF, platelet-activating factor.)

Fig. 6: Harmful Effects of Lipopolysaccharide (LPS; Endotoxin) Released from the Gram-Negative Cell Wall



Excessive production of inflammatory cytokines and clotting factors may lead to damage to the vascular endothelium, ARDS, DIC, shock and MOSF. (LPS, lipopolysaccharide; IL-1, interleukin-1; IL-6, interleukin-6; IL-8, interleukin-8, TNF-alpha, tumor necrosis factor-alpha; PAF, platelet-activating factor; ARDS, acute respiratory distress syndrome; DIC, disseminated intravascular coagulation; MOSF, multiple organ system failure.)

During minor local infections with few bacteria present, low levels of LPS are released leading to moderate cytokine production by the monocytes and macrophages and in general, promoting body defense by stimulating inflammation and moderate fever, breaking down energy reserves to supply energy for defense, activating the complement pathway and the coagulation pathway, and generally stimulating immune responses. Also as a result of these cytokines, circulating phagocytic white blood cells such as neutrophils and monocytes stick to the walls of capillaries, squeeze out and enter the tissue, a process termed diapedesis. The phagocytic white blood cells such as neutrophils then kill the invading microbes with their proteases and toxic oxygen radicals.

However, during severe systemic infections with large numbers of bacteria present, high levels of LPS are released resulting in excessive cytokine production by the monocytes and macrophages and this can harm the body. In addition, neutrophils start releasing their proteases and toxic oxygen radicals that kill not only the bacteria, but the surrounding tissue as well. Harmful effects include high fever, hypotension, tissue destruction, wasting, acute respiratory distress syndrome (ARDS), disseminated intravascular coagulation (DIC), and damage to the vascular endothelium resulting in shock, multiple system organ failure (MOSF), and often death (see Fig. 6).

This excessive inflammatory response is referred to as Systemic Inflammatory Response Syndrome or SIRS. Death is a result of what is called the shock cascade.

The release of excessive levels of inflammatory cytokines in response to PAMPs binding to PRRs during a systemic infection results in:

1. A drop in blood volume or hypovolemia. This is caused by the following events:

    a. Extracellular killing by neutrophils damages the capillary walls results in blood and plasma leaving the bloodstream and entering the surrounding tissue.

b. Depletion of clotting factors during disseminated intravascular coagulation (DIC) can lead to hemorrhaging as the capillaries are damaged.

c. Prolonged vasodilation results in plasma leaving the bloodstream and entering the surrounding tissue.

2. A drop in blood pressure or hypotension. This is a result of the following events:

a. Prolonged vasodilation causes decreased vascular resistance within blood vessels decreases blood pressure.

b. High levels of TNF, inhibit vascular smooth muscle tone and myocardial contractility decreasing the ability of the heart to pump blood throughout the body.

c. Hypovolemia from capillary damage, plasma leakage, and hemorrhaging.

3. The inability to deliver nutrients and oxygen to body cells or hypoperfusion. This is a result of the following events:

a. Activation of the blood coagulation pathway can cause clots called microthrombi to form within the blood vessels throughout the body causing disseminated intravascular coagulation (DIC) which blocks the flow of blood through the capillaries and, as mentioned above, depletion of clotting factors can lead to hemorrhaging in many parts of the body.

b. Increased capillary permeability, as a result of vasodilation in the lungs as well as neutrophil-induced injury to capillaries in the alveoli leads to acute inflammation, pulmonary edema, and loss of gas exchange in the lungs (acute respiratory distress syndrome or ARDS). As a result, the blood does not become oxygenated.

c. Hypovolemia decreases the volume of circulating **blood** and leads to hypotension.

d. Hypotension decreases the pressure needed to deliver blood throughout the body.

6. Hypoperfusion in the liver can result in a drop in blood glucose level from liver dysfunction. Glucose is needed for ATP production during glycolysis and aerobic respiration. A drop in glucose levels can result in decreased ATP production and insufficient energy for cellular metabolism.

7. The lack of oxygen delivery, as a result of hypoperfusion, causes cells to switch to fermentation for energy production. The increased lactic acid end products (hyperlactemia) of fermentation may then lead to lactic acidosis and a blood pH to low for the functioning of the enzymes involved in cellular metabolism. This can result in irreversible cell death.

Collectively, this can result in:

- End-organ ischemia: a restriction in blood supply that results in damage or dysfunction of tissues or organs
- Multiple system organ failure (MSOF)
- Death

Both pili and surface proteins in the Gram- negative cell wall function as adhesins, allowing the bacterium to adhere intimately to host cells and other surfaces in order to colonize and resist flushing. Some Gram- negative bacteria also produce invasins, allowing some bacteria to penetrate host cells. Pili, flagella, capsules, and exotoxins also play a role in the virulence of some *Enterobacteriaceae*

Many of the *Enterobacteriaceae* also carry **R (resistance) plasmids** (see Lab 21). These plasmids are small pieces of circular non-chromosomal DNA that may code for **multiple antibiotic resistance.** In addition, the plasmid may code for a **sex pilus**, enabling the bacterium to pass R plasmids to other bacteria by **conjugation**. As mentioned earlier, there are over 2,000,000 nosocomial infections per year in the U.S. Between 50 and 60 percent of the bacteria causing these infections are antibiotic resistant.

The identification of lactose-fermenting Gram- negative rods belonging to the bacterial family *Enterobacteriaceae* (bacteria commonly referred to as **coliforms**) in water is often used to determine if water has been fecally contaminated and, therefore, may contain disease-causing pathogens transmitted by the fecal-oral route. The procedure for this is given in **Appendix E**.

B. *PSEUDOMONAS* AND OTHER GLUCOSE NON-FERMENTING GRAM-NEGATIVE BACILLI

Glucose non-fermenting Gram- negative bacilli refer to Gram-negative bacilli or coccobacilli that **cannot ferment the sugar glucose**. The glucose non-fermenting Gram- negative bacilli are often normal inhabitants of soil and water. They may cause human infections when they colonize immunosuppressed individuals or gain access to the body through trauma. However, less than one-fifth of the Gram- negative bacilli isolated from clinical specimens are glucose non-fermenting bacilli. By far, the **most common** Gram- negative, glucose non-fermenting rod that causes human infections is *Pseudomonas aeruginosa*. *Pseudomonas* belongs to the family *Pseudomonadaceae* in the order *Pseudomonadales* in the class *Gammaproteobacter* of the phylum *Proteobacter*.

Pseudomonas aeruginosa is also an **opportunistic pathogen**. It is a common cause of nosocomial infections and can be found growing in a large variety of environmental locations. In the hospital environment, for example, it has been isolated from drains, sinks, faucets, water from cut flowers, cleaning solutions, medicines, and even disinfectant soap solutions. It is especially dangerous to the debilitated or immunocompromised patient.

Like the opportunistic *Enterobacteriaceae*, *Pseudomonas* is a Gram- negative rod, it is frequently found in small amounts in the feces, and it causes similar **opportunistic infections**: **urinary tract infections, wound infections, pneumonia, and septicemia**. *P aeruginosa* is the fourth most commonly isolated nosocomial pathogen, accounting for 10% of all hospital-acquired infections. *P. aeruginosa* is responsible for 12 percent of hospital-acquired pneumonia cases, 10 percent of the cases of septicemia, and 16 percent of nosocomial pneumonia cases. In addition, *P. aeruginosa* is a significant cause of **burn infections** with a 60 percent mortality rate. It also colonizes and chronically infects the lungs of people with cystic fibrosis. Like other opportunistic Gram- negative bacilli, *Pseudomonas aeruginosa* also releases endotoxin and frequently possesses R-plasmids. A number of other species of *Pseudomonas* have also been found to cause human infections.

Other glucose non-fermenting Gram-negative bacilli that are sometimes opportunistic pathogens in humans include *Acinetobacter, Aeromonas, Alcaligenes, Eikenella, Flavobacterium,* and *Moraxella*.

***Acinetobacter* has become a frequent cause of nosocomial wound infections, pneumonia, and septicemia.** The bacterium has become well known as a cause of infections among veterans of the wars in Iraq and Afghanistan and is becoming a growing cause of nosocomial infections in the U.S. *Acinetobacter* is thought to have been contracted in field hospitals in Iraq and Afghanistan and subsequently carried to veteran's hospitals in the U.S. Because most species are multiple antibiotic-resistant, it is often difficult to treat. *Acinetobacter* is commonly found in soil and water, as well as on the skin of healthy people, especially healthcare personnel. Although there are numerous species of *Acinetobacter* that can cause human disease, *Acinetobacter baumannii* accounts for about 80% of reported infections.

SCENERIOS FOR TODAY'S LAB

Students will be assigned **either Case Study 1A or 1B** to do today. **All students will do Case Study 2** as part of the results next time.

Case Study #1A

A 26 year old female presents to her doctor complaining of 2 days of increased urinary frequency, dysuria, and sensation of incomplete voiding. Her abdominal exam indicates mild suprapubic tenderness. Her blood pressure is normal and she does not have fever, chills, costovertebral angle (CVA) tenderness, or vaginal discharge. She reports that she became sexually active with her new boyfriend one month ago. She and her boyfriend have sexual intercourse 3-4 times a week. She is using a combination of a diaphragm and spermicide for contraception. She is otherwise healthy. A microscopic examination of her centrifuged urine shows 9 white blood cells and 15 bacteria per high-power microscopic field. A urine dipstick shows a positive leukocyte esterase test and a positive nitrite test.

Assume that your unknown is from the urine of this patient.

Case Study #1B

A 90 year old woman resides at an area nursing home. She shows signs of mild dementia, and because of severe arthritis and requiring a walker for ambulation, sits in a chair most of the day. She has not used any form of estrogen in at least 30 years. She also has a history of 4-5 confirmed urinary tract infections per year.
This morning, her caregiver is unable to coax the patient out of her bed. She seems confused and disoriented. Vital signs reveal tachycardia in the 120's, respirations at 24/min, and a blood pressure of 78/49. She is immediately taken to an ER for evaluation. A CT of the abdomen and a chest x-ray appear normal. She has a WBC count of 2300/µL. She continues to exhibits marked confusion compared to her baseline and is exhibiting anxiety. Urine and blood samples are taken and sent for culture and sensitivity.

Assume your unknown is from both a urine sample and a blood sample.

Case Study #1C

A 79 year old man living in a nursing home has COPD, a lifetime history of heavy smoking, and hypertension. His caregivers note that he is exhibiting rigor, has a temperature of 103°F, and lacks his normal alertness. Vital signs include a blood pressure is 165/90, a pulse of 128 beats per minute, a respiratory rate of 32 breaths per minute, and a pulse oximetry on room air of 80%. He is transferred to an acute care facility where a chest X-ray reveals a right lower lobe infiltrate and his white blood cell count is 18,000/µL with a marked left shift. He has thick, foul-smelling yellow-green sputum.

Assume you unknown is from the sputum sample.

CAUTION: TREAT EACH UNKNOWN AS A PATHOGEN! Inform your instructor of any spills or accidents. **WASH AND SANITIZE YOUR HANDS WELL before leaving the lab.**

LABORATORY 12:
***ENTEROBACTERIACEAE* AND *PSEUDOMONAS*: PART 1**

MATERIALS

Gibson oxidase swab and **either** a plate of MacConkey agar and an EnteroPluri-*Test* **or** a plate of Cetrimide agar and an EnteroPluri-*Test*

PROCEDURE: to be done in groups of 3

[Keep in mind that organisms other than the *Enterobacteriaceae* and *Pseudomonas* can cause these infections, so in a real clinical situation other lab tests and cultures for bacteria other than those upon which this lab is based would also be done.]

1. Perform a **Gram stain** on your unknown.

Record the results of your Gram stain in the Gram stain section of Lab 13.

2. If you have a Gram-negative bacillus, determine if it is a **glucose fermenting** Gram-negative bacillus like most *Enterobacteriaceae* or a **glucose non-fermenting** Gram-negative bacillus such as *Pseudomonas* by performing an **oxidase test** as follows:

To perform the oxidase test, touch the Gibson oxidase test swab to a colony on your onknown plate. An **oxidase-positive** reaction will appear **purple to black within 10 seconds**. An **oxidase-negative** will **result in no color change.** Ignore any color development toccurring after 60 seconds.

Record your oxidase test results in the Oxidase test section of Lab 13.

3. If your unknown is **oxidase-negative**, **usually indicating a glucose fermenting Gram-negative bacillus**, do the following inoculations:

a. Streak your unknown for isolation on a plate of **MacConkey agar, a selective medium used for the isolation of non-fastidious Gram-negative rods** and particularly members of the **family *Enterobacteriaceae*** (see Fig. 4 and Fig. 5). Incubate **upside down and stacked in the petri plate holder on the shelf of the 37°C incubator corresponding to your lab section**.

See "Flash animation showing how to streak an agar plate for isolation: 3 sector method" in the on-line lab manual.

b. Inoculate an **EnteroPluri-*Test*** as follows:

1. Remove both caps of the EnteroPluri-*Test* and with the **straight end of the inoculating wire**, pick off the equivalent of a colony from your unknown plate. **A visible inoculum should be seen on the tip and side of the wire**.

2. **Inoculate** the EnteroPluri-*Test* by grasping the **bent-end of the inoculating wire**, twisting it, and withdrawing the wire through all 12 compartments using a turning motion.

3. **Reinsert the wire** into the tube (use a turning motion) **through all 12 compartments** until the notch on the wire is aligned with the opening of the tube. (The tip of the wire should be seen in the citrate compartment.) **Break the wire at the notch** by bending. Do not discard the wire yet.

4. Using the broken off part of the wire, **punch holes through the cellophane which covers the air inlets located on the rounded side of the last 8 compartments**. Your instructor will show you their correct location. Discard the broken off wire in the disinfectant container.

5. **Replace both caps** and incubate the EnteroPluri-Test **on its flat surface at 37°C**.

4. If your unknown is **oxidase-positive**, **usually indicating a glucose non-fermenting Gram-negative bacillus**, do the following inoculation:

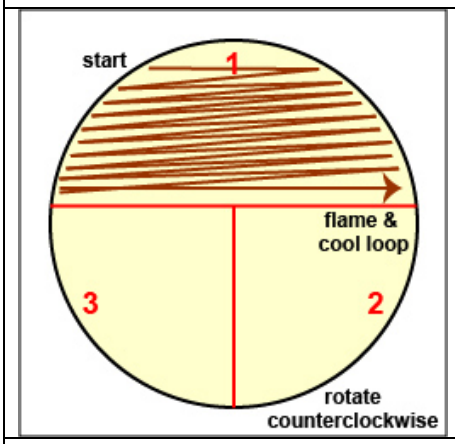
a. Streak your unknown for isolation on a plate of **Cetrimide agar, a selective and differential medium for *Pseudomonas*** (see Fig 4 and Fig. 5). Incubate at **upside down and stacked in the petri plate holder on the shelf of the 37°C incubator corresponding to your lab section**.

See “Flash animation showing how to streak an agar plate for isolation: 3 sector method” in the on-line lab manual.

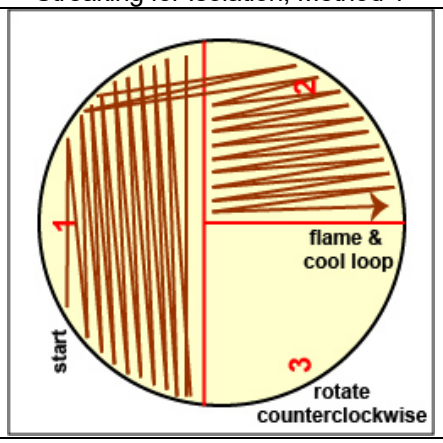
Note that **MacConkey agar can also be used to isolate *Pseudomonas*** but we are using the Cetrimide agar today because it enables us to detect the production of the blue to green water-soluble pigment by *Pseudomonas aeruginosa*, as well as the production of fluorescein.

You will also inoculate an EnteroPluri-*Test* **for practice only, but keep in mind that the EnteroPluri-*Test* is used to identify *Enterobacteriaceae,* not *Pseudomonas***.

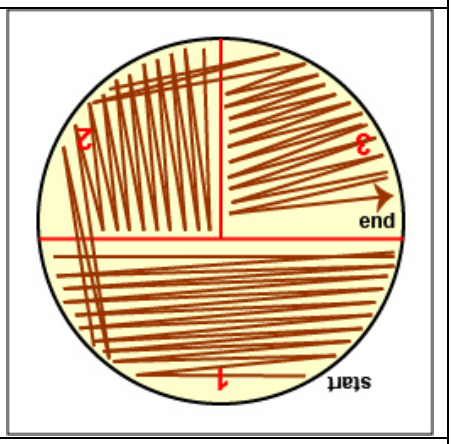
Fig. 4:
Streaking for Isolation, Method 1



Step 1: Streak area 1. Sterilize the loop.

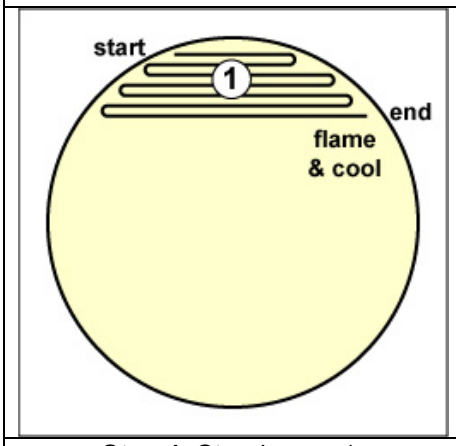


Step 2: Rotate counterclockwise. Spread area 1 over area 2. Sterilize the loop.

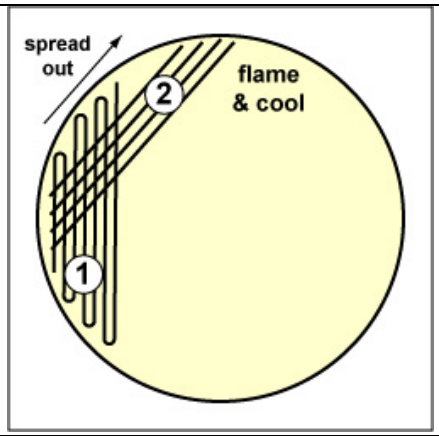


Step 3: Rotate Counterclockwise. Spread area 2 over area 3. Sterilize the loop.

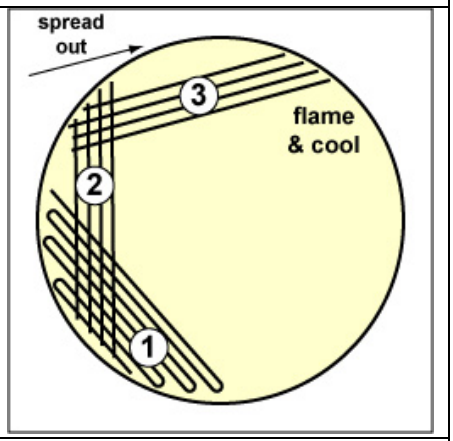
Fig. 5:
Streaking for Isolation, Method 2



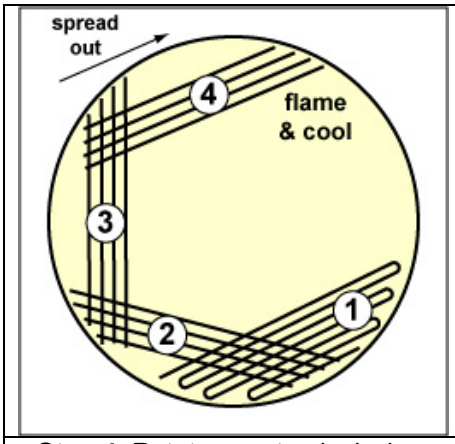
Step 1: Streak area 1.
Sterilize the loop.



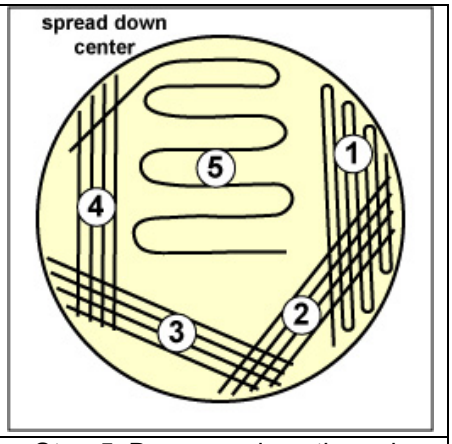
Step 2: Rotate counterclockwise.
Spread area 1 over area 2.
Sterilize the loop.



Step 3: Rotate counterclockwise.
Spread area 2 over area 3.
Sterilize the loop.



Step 4: Rotate counterclockwise.
Spread area 3 over area 4.
Sterilize the loop



Step 5: Draw your loop through area "4" and spread it down the center of the plate without touching any of the areas already streaked.

CASE STUDY #2

After receiving a baby chicken for Easter, a 7 year old boy is taken to the emergency room with symptoms of vomiting, nausea, non-bloody diarrhea, abdominal cramps, and a temperature of 100°F. A complete blood count (CBC) shows the WBC count to be within the reference range.

This XLD agar plate and this EnteroPluri-*Test* are from a stool culture from this patient.

CAUTION: TREAT THE UNKNOWN AS A PATHOGEN! Inform your instructor of any spills or accidents. WASH AND SANITIZE YOUR HANDS WELL before leaving the lab.

MATERIALS

Demonstration XLD agar plate and EnteroPluri-*Test*

PROCEDURE: to be done in groups of 3

1. Observe the following demonstrations **shown in the links of your online lab manual** and identify the causative bacterium:

    a. An **XLD agar plate**, a selective medium used for isolating and differentiating Gram-negative enteric bacteria, especially intestinal pathogens such as *Salmonella* and *Shigella*.

    b. The **EnteroPluri-*Test***.

2. Record your results in the Results section of Lab 13.

C. Lab Tests Used as Part of Today's Lab

To isolate *Enterobacteriaceae* and *Pseudomonas*, specimens from the infected site are plated out on any one of a large number of **selective and differential media** such as EMB agar, Endo agar, Deoxycholate agar, MacConkey agar, Hektoen Enteric agar, and XLD agar. We will look at three of these.

1. MacConkey Agar

MacConkey agar is a **selective medium used for the isolation of non-fastidious Gram-negative rods**, particularly members of the family *Enterobacteriaceae* and the genus *Pseudomonas*, and the **differentiation of lactose fermenting from lactose non-fermenting Gram-negative bacilli**. MacConkey agar **contains the dye crystal violet well as bile salts that inhibit the growth of most Gram-positive bacteria but do not affect the growth of most Gram-negatives**.

If the Gram- negative bacterium ferments the sugar lactose in the medium, the **acid end products lower the pH of the medium. The neutral red in the agar turns red in color once the pH drops below 6.8**. As the pH drops, the neutral red is absorbed by the bacteria, causing the **colonies to appear bright pink to red**.

- **Strong fementation of lactose with high levels of acid production** by the bacteria causes the **colonies and confluent growth to appear bright pink to red**. The resulting acid, at high enough concentrations, can also causes the bile salts in the medium to precipitate out of solution causing **a pink halo to appear around the the growth**.

- **Weak fermentation of lactose** by the bacteria causes the **colonies and confluent growth to appear pink to red**, but without the precipitation of bile salts there **is no pink halo around the growth**.

- If the bacteria **do not ferment lactose**, the **colonies and confluent growth appear colorless** and the **agar surrounding the bacteria remains relatively transparent**.

Typical results for our strains of *Enterobacteriaceae* and *Pseudomonas aeruginosa* on MacConkey agar is as follows:

A. Strong fermentation of lactose

Escherichia coli

B. Weak fermentation of lactose

Klebsiella pneumoniae, Enterobacter aerogenes, Enterobacter cloacae.

C. No fermentation of lactose

Proteus mirabilis, Proteus vulgaris, Serratia marcescens, Salmonella enterica, Pseudomonas aeruginosa.

2. XLD Agar

Xylose Lysine Desoxycholate (XLD) agar is used for **isolating and differentiating Gram-negative enteric bacteria, especially intestinal pathogens such as *Salmonella* and *Shigella***. XLD agar contains sodium desoxycholate, which **inhibits the growth of Gram-positive bacteria but permits the growth of Gram-negatives**. It also contains the sugars lactose and sucrose, the amino acid L-lysine, sodium thiosulfate, and the pH indicator phenol red. Results can be interpreted as follows:

- If the Gram- negative bacterium **ferments lactose and/or sucrose**, **acid end products will be produced** and cause the colonies and the phenol red in the agar around the colonies to turn **yellow**.

- If **lactose and sucrose are not fermented** by the bacterium but the **amino acid lysine is decarboxylated**, ammonia, an **alkaline end product** will cause the phenol red in the agar around the colonies to turn a **deeper red**.

- Sometimes the bacterium **ferments the sugars producing acid end products and breaks down lysine producing alkaline end products**. In this case **some of the colonies and part of the agar turns yellow and some of the colonies and part of the agar turns a deeper red**.

- If **hydrogen sulfide is produced** by the bacterium as a result of thiosulfate reduction, **part or all of the colony will appear black**. Well-isolated colonies are usually needed for good results.

Typical colony morphology on XLD agar is as follows:

1. *Escherichia coli*: flat yellow colonies; some strains may be inhibited.

2. *Enterobacter* and *Klebsiella*: mucoid yellow colonies.

3. *Proteus*: red to yellow colonies; may have black centers.

4. *Salmonella*: usually red colonies with black centers.

5. *Shigella, Serratia,* and *Pseudomonas*: red colonies without black centers

3. Cetrimide Agar (*Pseudomonas* P agar)

Cetrimide agar contains the chemical cetrimide (cetyl timethylammonium bromide) for **the selective inhibition of most bacteria other than *Pseudomonas***. The medium also stimulates *Pseudomonas* aeruginosa to produce a number of pigmented compounds, including pyoverdin and pyocyanin. **The green water soluble pigment characteristic of *Pseudomonas aeruginosa* is due to production of a green to blue water-soluble toxin called pyocyanin**. A fluorescent siderophore called **pyoverdin**, often produced by *Pseudomonas*, **will typically fluoresce when the plate is placed under a short wavelength ultraviolet light**. After a few minutes at room temperature, the plate loses its fluoresence. The fluoresence, however, can be restored by placing the plate back at 37°C for several minutes.

4. Oxidase Test

In this lab a Gibson Oxidase Swab is used to perform the oxidase test. The oxidase test is based on the bacterial production of an oxidase enzyme. **Cytochrome oxidase**, in the presence of oxygen, oxidizes the N-N-N-tetramethyl-p-phenylenediamine oxidase test reagent in a Gibson oxidase swab.

To perform the oxidase test, ouch the oxidase test swab to a colony on a culture medium. An **oxidase-positive** reactions will appear **purple to black within 10 seconds**. An **oxidase-negative** will **result in no color change.** Ignore any color development toccurring after 60 seconds.

***Pseudomonas aeruginosa* and most other glucose non-fermenting, Gram-negative bacilli are oxidase-positive.** With the exception of the genus *Plesiomonas*, the **glucose fermenting *Enterobacteriaceae* are oxidase-negative**.

5. Pigment production in *Pseudomonas aeruginosa*

The green water-soluble pigment characteristic of *Pseudomonas aeruginosa* is due to production of a green to blue water-soluble toxin called pyocyanin. A fluorescent siderophore called pyoverdin, often produced by *Pseudomonas*, will typically fluoresce when the plate is placed under a short wavelength ultraviolet light. After a few minutes at room temperature, the plate loses its fluoresence. The fluoresence, however, can be restored by placing the plate back at 37°C for several minutes. None of the *Enterobacteriaceae* produces pigment at 37°C.

6. Odor

Most of the *Enterobacteriaceae* have a rather foul smell; ***Pseudomonas aeruginosa* produces a characteristic fruity or grape juice-like aroma** due to production of an aromatic compound called aminoacetophenone.

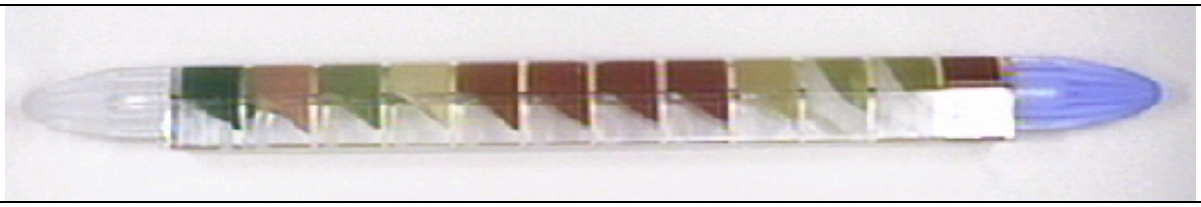
7. The EnteroPluri-*Test*

A number of techniques can be used for the identification of specific species and subspecies of *Enterobacteriaceae*. Speciation is important because it provides data regarding patterns of susceptibility to antimicrobial agents and changes that occur over a period of time. It is also essential for epidemiological studies such as determination of nosocomial infections and their spread.

In an effort to simplify the speciation of the *Enterobacteriaceae* and reduce the amount of prepared media and incubation space needed by the clinical lab, a number of self-contained **multi-test systems** have been commercially marketed. Some of these multi-test systems have been combined with a computer-prepared manual to provide identification based on the **overall probability of occurrence** for each of the biochemical reactions. In this way, a large number of biochemical tests can economically be performed in a short period of time, and the results can be accurately interpreted with relative ease and assurance.

The EnteroPluri-*Test* is a self-contained, compartmented plastic tube containing 12 different agars (enabling the performance of a total of 15 standard biochemical tests) and an enclosed inoculating wire (see Fig. 7). After inoculation and incubation, the resulting combination of reactions, together with a Computer Coding and Identification System (CCIS), allows for easy identification. The various biochemical reactions of the Enterotube® II and their correct interpretation are discussed below. Although it is designed to identify members of the bacterial family *Enterobacteriaceae*, it will sometimes also identify common biotypes of *Pseudomonas* and other non-fermentative Gram-positive bacilli. It does not identify *Pseudomonas aeruginosa*.

Fig. 7:
The EnteroPluri-*Test*



IDENTIFYING MEMBERS OF THE *ENTEROBACTERIACEAE* WITH THE ENTEROPLURI-*TEST*

The EnteroPluri-*Test* contains 12 different agars that can be used to carry out 15 standard biochemical tests. Interpret the results of your EnteroPluri-*Test* using the instructions below and record them on the EnteroPluri-*Test* table on your Results page. For more detail on the 15 biochemical tests in the EnteroPluri-*Test*, see **Table 12-A**.

1. Interpret the results of **glucose** fermentation in **compartment 1**.
   Any yellow = +; red = -
   If positive, circle the number 4 under glucose on your Results page.

2. Interpret the results of **gas** production also in **compartment 1**.
   White layer of wax is lifted from the yellow agar = +; wax not lifted from agar = -
   If positive, circle the number 2 under gas on your Results page.

3. Interpret the results of **lysine** decarboxylase in **compartment 2**.
   Any violet = +; yellow = -
   If positive, circle the number 1 under lysine on your Results page.

4. Interpret the results of **ornithine** decarboxylase in **compartment 3**.
   Any violet = +; yellow = -
   If positive, circle the number 4 under ornithine on your Results page.

5. Interpret the results of **H_2S** production in **compartment 4**.
   Black/brown = +; beige = - (The black may fade or revert back to negative if the EnteroPluri *Test* is read after 24 hours of incubation.)
   If positive, circle the number 2 under H_2S on your Results page.

6. **Indole** production also in **compartment 4**. Do not interpret the indole test at this time. Add Kovac's reagent only after all other tests have been read (see **step 16** below).

7. Interpret the results of **adonitol** fermentation in **compartment 5**.
   Any yellow = +; red = -
   If positive, circle the number 4 under adonitol on your Results page.

8. Interpret the results of **lactose** fermentation in **compartment 6**.
   Yellow or orange = +; red = -
   If positive, circle the number 2 under lactose on your Results page.

9. Interpret the results of **arabinose** fermentation in **compartment 7**.
   Any yellow = +; red = -
   If positive, circle the number 1 under arabinose on your Results page.

10. Interpret the results of **sorbitol** fermentation in **compartment 8**.
    Any yellow = +; red = -
    If positive, circle the number 4 under sorbitol on your Results page.

11. **Voges-Praskauer** test in **compartment 9**. Do not interpret the VP test at this time. Add alpha-naphtol and potassium hydroxide (KOH) reagents only after all other tests have been read (see **step 17** below).

12. Interpret the results of **dulcitol** fermentation in **compartment 10**.
    Yellow or pale yellow = +; green or dark brown = -
    If positive, circle the number 1 under dulcitol on your Results page.

13. Interpret the results of **PA** deaminase also in **compartment 10**.
    Dark brown = +; green or yellow = -
    If positive, circle the number 4 under PA on your Results page.

14. Interpret the results of **urea** hydrolysis in **compartment 11**.
    Pink, red or purple = +; beige = -
    If positive, circle the number 2 under urea on your Results page.

15. Interpret the results of **citrate** utilization in **compartment 12**.
    Any blue = +; green = -
    If positive, circle the number 1 under citrate on your Results page.

16. **Your instructor will add 2-3 drops of Kovac's reagent to the indole test compartment**.
    Pink or red = +; yellow = -
    If positive, circle the number 1 under indole on your Results page.

17. **Your instructor will add 3 drops of alpha-naphtol reagent and 2 drops of potassium hydroxide (KOH) to the VP test compartment**.
    Red = +; colorless = -
    If positive, circle the number 2 under indole on your Results page.

18. **Add all the positive test number values in each bracketed section** and **enter each sum in its code box** on the EnteroPluri-*Test* chart on your Results page

19. Locate the 5-digit number in the **Computer Coding and Identification System (CCIS)** booklet and find the best identification in the column entitled "ID Value." (Should more than one organism be listed, the confirmatory tests indicated in the CCIS would normally then have to be performed. In addition, an identification of *Salmonella* or *Shigella* would usually be confirmed by direct serologic testing as will be described in Lab 17.)

If there are any problems, consult your instructor.

Here is a description of the individual biochemical tests in the EnteroPluri *Test*.

18. **Add all the circled numbers in each bracketed section** and enter each sum in its box provided below the arrows on the EnteroPluri *Test*.

TABLE 12-A
Interpretation of the **EnteroPluri-*Test***

Compartment	Reaction	Negative	Positive
1	glucose fermentation	red	yellow
1	gas production	wax not lifted	wax lifted

Remarks: Glucose - Any degree of yellow is positive. Acid end products from glucose fermentation turns the pH indicator from red (alkaline) to yellow (acid).

Remarks: Gas - Positive is a definite and complete separation of the white wax overlay from the surface of the glucose medium. Detects gas from glucose fermentation.

Compartment	Reaction	Negative	Positive
2	lysine decarboxylase activity	yellow	violet

Remarks: Any degree of violet is positive. Alkaline end products from the decarboxylation of lysine changes the pH indicator from pale yellow (acid) to violet (alkaline).

TABLE 12-A, continued
Interpretation of the **EnteroPluri-*Test***

Compartment	Reaction	Negative	Positive
3	ornithine decarboxylase activity	yellow	violet

Remarks: Any degree of violet is positive. Alkaline end products from the decarboxylation of ornithine changes the pH indicator from pale yellow (acid) to violet (alkaline).

Compartment	Reaction	Negative	Positive
4	H_2S production	beige	Black/brown
4	indole production (**done last**)	yellow	Pink/red

Remarks: H_2S - Reduction of thiosulfate produces H_2S which reacts with iron salts to produce black/brown FeS.

Remarks: Indole - **This test is not interpreted until all other compartments have been read**. Kovac's Reagent must be added before reading. Indole, produced from the breakdown of tryptophan, reacts with Kovac's reagent turning it red.

Compartment	Reaction	Negative	Positive
5	adonitol fermentation	red	yellow

Remarks: Any degree of yellow is positive. Acid end products from adonitol fermentation turn the pH indicator from red (alkaline) to yellow (acid).

Compartment	Reaction	Negative	Positive
6	lactose fermentation	red	yellow/orange

Remarks: Any degree of yellow or orange is positive. Acid end products from lactose fermentation turn the pH indicator from red (alkaline) to yellow (acid).

TABLE 12-A, continued
Interpretation of the **EnteroPluri-*Test***

Compartment	Reaction	Negative	Positive
7	arabinose fermentation	red	yellow

Remarks: Any degree of yellow is positive. Acid end products from arabinose fermentation turn the pH indicator from red (alkaline) to yellow (acid).

Compartment	Reaction	Negative	Positive
8	sorbitol fermentation	red	yellow

Remarks: Any degree of yellow is positive. Acid end products from sorbitol fermentation turn the pH indicator from red (alkaline) to yellow (acid).

Compartment	Reaction	Negative	Positive
9	Voges-Proskauer	colorless	red

Remarks: **This test is not interpreted until all other compartments have been read**. Acetoin produced during the production of butylene glycol from glucose fermentation reacts with the added reagents KOH and alpha-naphtol and turns red.

Compartment	Reaction	Negative	Positive
10	dulcitol fermentation	green or dark brown	yellow
10	phenylalanine deaminase activity	green or yellow	dark brown

Remarks: Dulcitol - Yellow or pale yellow is positive. Green or dark brown is negative. Acid from dulcitol fermentation turns the pH indicator from green (alkaline) to yellow (acid).

Remarks: PA - Pyruvic acid produced from deamination of phenylalanine reacts with ferric salts in the medium turning it dark brown. Green or yellow is negative.

TABLE 12-A, continued
Interpretation of the **EnteroPluri-*Test***

Compartment	Reaction	Negative	Positive
11	urea hydrolysis	beige	pink/red/ purple

Remarks: Hydrolysis of urea forms ammonia which causes the pH indicator to turn from yellow (acid) to pink/red/purple (alkaline).

Compartment	Reaction	Negative	Positive
12	citrate utilization	green	blue

Remarks: Any degree of blue is positive. Utilization of citrate produces alkaline products turning the pH indicator from green (acid) to blue (alkaline).

	Group 1			Group 2			Group 3			Group 4			Group 5		
Test	**Glucose**	**Gas**	**Lysine**	**Ornithine**	**H_2S**	**Indole**	**Adonitol**	**Lactose**	**Arabinose**	**Sorbitol**	**VP**	**Dulcitol**	**PA**	**Urea**	**Citrate**
Value	**4**	**2**	**1**	**4**	**2**	**1**	**4**	**2**	**1**	**4**	**2**	**1**	**4**	**2**	**1**
Result															
Code															
CODICE NUMBER:				**Identification:**											

8. The Complete Blood Count (CBC) Test

9. Urinalysis (The Dipstick Tests)

See Appendix D.

10. SIRS and Sepsis

See Appendix F

PERFORMANCE OBJECTIVES
LABORATORY 12

After completing this lab, the student will be able to perform the following objectives:

A. ENTEROBACTERIACEAE: GLUCOSE FERMENTING, GRAM-NEGATIVE, ENTERIC BACILLI

1. Name the bacterial family to which the most commonly encountered organisms isolated from clinical specimens belong.
2. List five characteristics used to place bacteria into the family *Enterobacteriaceae*.
3. State what infections are caused by *Salmonella* and by *Shigella* and how they are transmitted to humans.
4. Name four strains of *Escherichia coli* that may infect the gastrointestinal tract.
5. Name five genera of *Enterobacteriaceae* considered as common **opportunistic** pathogens, state their normal habitat, and list four common types of opportunistic infections that they all may cause.
6. Name several predisposing factors that make one more susceptible to urinary tract infections.
7. In terms of CFUs, state the laboratory culture standard for a urinary tract infection.
8. Define nosocomial infection.
9. State the significance of endotoxins in infections caused by many of the *Enterobacteriaceae*.
10. Discuss the significance of R-plasmids in our attempts to treat infections caused by the *Enterobacteriaceae*.

B. *PSEUDOMONAS* AND OTHER GLUCOSE NON-FERMENTING, GRAM-NEGATIVE BACILLI

1. Name the most common glucose non-fermenting Gram-negative rod that infect humans and list five types of opportunistic infections it may cause.

2, State 3 infections being caused with increased frequency by *Acinetobacter*.

C. ISOLATION OF *ENTEROBACTERIACEAE* AND *PSEUDOMONAS*

1. State the usefulness of MacConkey agar and Cetrimide agar for the isolation of *Enterobacteriaceae* and *Pseudomonas*.

D. DIFFERENTIATING BETWEEN THE *ENTEROBACTERIACEAE* AND *PSEUDOMONAS*

1. State how to differentiate *Pseudomonas aeruginosa* from the *Enterobacteriaceae* using the following tests:
    a. oxidase test
    b. production of pigment and fluorescent products
    c. odor

E. IDENTIFYING THE *ENTEROBACTERIACEAE* USING THE ENTEROPLURI-*TEST*

1. Briefly describe the EnteroPluri-*Test*.

LABORATORY 13
GRAM-Negative Bacilli: ISOLATION AND IDENTIFICATION OF *ENTEROBACTERIACEAE* AND *PSEUDOMONAS*
PART 2: RESULTS OF LAB 12

A. Case Study #1
B. Case Study #2

The concept behind the case studies presented in Lab 12 used to illustrate the *Enterobacteriaceae* and *Pseudomonas* is for you and your lab partners as a group to:

1. **First come up with a valid diagnosis of the type of infectious disease seen in your case study and then identify the bacterium causing that infection**, and

2. **Support your group diagnose** based on:

   a. Any relevant facts in the **patient's history**. (A reliable on-line source will be used to support this.)
   b. The patient's **signs and symptoms.** (A reliable on-line source will be used to support this.)
   c. **Each of the individual lab tests** given in your case study.
   d. All **microbiological lab tests you performed** as part of the project.

The due date for this report can be found on the class calendar. Your grade for this lab is based on the **completeness of your report and written evidence of the critical thinking process that went into making and supporting your diagnosis.** Remember, you are trying to convince your instructor that you understand how the diagnosis was made by **supporting that diagnosis with data.**

Grading:

The lab 12 Lab Report is worth 32 points.

These case studies are based in part on your in-class participation as part of your group. Therefore:

a. **If you were not in lab when the inoculations with your unknown were performed, 3 points will be deducted from your Lab Report score for labs 12, 14, and 15; 6 points from your Lab Report score for the Final Project.**

b. **If you were not in lab when the results of your lab tests were observed, 3 points will be deducted from your Lab Report score for labs 12, 14, and 15; 6 points from your Lab Report score for the Final Project**.

c. **For each day your Lab Report is late, 2 points will be deducted from your Lab Report score for labs 12, 14, and 15; 4 points from your Lab Report score for the Final Project.**

Each person in the group must write and submit their own individual report and it cannot be the same as that of other members of that group.

The rubric that will be used to grade this lab report can be found under this Assignment on Blackboard.

LABORATORY 13:
***ENTEROBACTERIACEAE* AND *PSEUDOMONAS*: PART 2**

Be sure to handle all the bacterial cultures you are using in lab today as if they are pathogens! Be sure to wash and sanitize your hands well at the completion of today's lab.

Also, make sure you **observe several MacConkey agar plates and the Cetrimide agar plate** used by others in your lab so that you can answer practical questions from Lab 12 and Lab 13. The Performance Objectives for Lab 13 tell you what you are expected to be able to do on the practical.

Case Study Lab Report for Labs 12 and 13: The *Enterobacteriaceae* and *Pseudomonas*

Your Name:

Others in your group:

Unknown number (1-6):

Lab section:

Date:

Case Study #1A from Lab 12

A 26-year old female presents to her doctor complaining of 2 days of increased urinary frequency, dysuria, and sensation of incomplete voiding. Her abdominal exam indicates mild suprapubic tenderness. Her blood pressure is normal and she does not have fever, chills, costovertebral angle (CVA) tenderness, or vaginal discharge. She reports that she became sexually active with her new boyfriend one month ago. She and her boyfriend have sexual intercourse 3-4 times a week. She is using a combination of a diaphragm and spermicide for contraception. She is otherwise healthy. A microscopic examination of her centrifuged urine shows 9 white blood cells and 15 bacteria per high-power microscopic field. A urine dipstick shows a positive leukocyte esterase test and a positive nitrite test.

Assume that your unknown is from the urine of this patient.

Case Study #1B from Lab 12

A 90-year old woman resides at an area nursing home. She shows signs of mild dementia, and because of severe arthritis and requiring a walker for ambulation, sits in a chair most of the day. She has not used any form of estrogen in at least 30 years. She also has a history of 4-5 confirmed urinary tract infections per year. This morning, her caregiver is unable to coax the patient out of her bed. She seems confused and disoriented. Vital signs reveal tachycardia in the 120's, respirations at 24/min, and a blood pressure of 78/49. She is immediately taken to an ER for evaluation. A CT of the abdomen and a chest x-ray appear normal. She has a WBC count of 2300/μL. She continues to exhibit marked confusion compared to her baseline and is exhibiting anxiety. Urine and blood samples are taken and sent for culture and sensitivity.

Assume your unknown is from both a urine sample and a blood sample.

Case Study #1C from Lab 12

A 79-year old man living in a nursing home has COPD, a lifetime history of heavy smoking, and hypertension. His caregivers note that he is exhibiting rigor, has a temperature of 103°F, and lacks his normal alertness. Vital signs include a blood pressure is 165/90, a pulse of 128 beats per minute, a respiratory rate of 32 breaths per minute, and a pulse oximetry on room air of 80%. He is transferred to an acute care facility where a chest X-ray reveals a right lower lobe infiltrate and his white blood cell count is 18,000/μL with a marked left shift. He has thick, foul-smelling yellow-green sputum.

Assume you unknown is from the sputum sample

Did you have Case Study 1A, 1B, or 1C? _____

LABORATORY 13:
ENTEROBACTERIACEAE AND *PSEUDOMONAS*: PART 2

1. Patient's history and predisposing factors

Read the case study. Explain how any relevant parts of the patient's history contributed to your diagnosis of the type of infectious disease that is present here. You are urged to use the computers in lab to search reliable medically oriented Internet sources to support this. Reliable sources you might consider are Medscape (http://emedicine.medscape.com/infectious_diseases) and The Centers for Disease Control and Prevention (CDC) at http://www.cdc.gov/. Cite any sources you use at the end of this Patient's History section in APA style (http://www.apastyle.org/).

The **patient's history should suggest a general type of infectious disease** that is present, such as a urinary tract infection, a wound infection, gastroenteritis, pharyngitis, pneumonia, septicemia, etc. **Do not look up the bacterium you eventually identify** as the cause of this infectious disease. You do not know the causative bacterium at this point. **You need to determine the general type of infection to determine what microbiological tests to perform in order to identify the bacterium causing the infection**. Search at least one medically oriented reference article from a reliable site such as *Medscape* and **use this article to support your diagnosis the type of infectious disease seen here**. Don't forget to **cite any sources you used in APA style directly under this Patient's History and Patient's Symptoms sections** of this Lab Report.

2. Patient's signs and symptoms

Read the case study. Explain how the patient's signs and symptoms contributed to your diagnosis of the type of infectious disease that is present here. You are urged to use the computers in lab to search reliable Internet sources to support this. Reliable sources you might consider are *Medscape* (http://emedicine.medscape.com/infectious_diseases) and the Centers for Disease Control and Prevention (CDC) at http://www.cdc.gov/. Also see appendix F (SIRS and Sepsis) in your lab manual for an indication of whether or not the patient has SIRS. Cite any sources you use at the end of this Patient's History section in APA style (http://www.apastyle.org/).

The **patient's signs and symptoms should suggest a general type of infectious disease** that is present, such as a urinary tract infection, a wound infection, gastroenteritis, strep throat, pneumonia, septicemia, etc. **Do not look up the bacterium you eventually identify** as the cause of this infectious disease. You do not know the causative bacterium at this point. **You need to determine the general type of infectious disease present in order to determine what microbiological tests to perform to identify the bacterium causing the infection**. Search at least one medically oriented reference article from a reliable site such as *Medscape* and **use this article to support your diagnosis the type of infectious disease seen here**. Don't forget to **cite any sources you used in APA style under this Patient's History and Patient's Symptoms sections** of this Lab Report.

3. Vocabulary list for medical terms used in the case study under signs and symptoms

List and define any medical terms used in your case study that describe the patients's signs and symptoms that the average person not in the medical profession might not know.

4. Results of laboratory test given in the case study

List each lab test given and explain how the results of that test helps to contribute to your l diagnosis. **Refer to appendix C (Complete Blood Count), appendix D (Urinalysis), and appendix F (SIRS and Sepsis) in your lab manual.**

5. Microbiological lab tests you performed in Lab 12

a. Gram stain

Give the Gram reaction (Gram-positive or Gram negative and how you reached this conclusion) and the shape and arrangement of the unknown you were given. **State how these Gram stain results contributed to your decision of what microbiological test to perform next**. The Gram stain is discussed in Lab 6.

b. Oxidase test

Give the results of the oxidase test (positive or negative) you performed on the unknown you were given, and how you reached this conclusion. **State how these oxidase results contributed to your decision as to what microbiological media to use next**. The oxidase test is discussed in Lab 12 under C. Lab Tests Used in Today's Lab.

c. Maconkey agar (if used)

Describe the results of the MacConkey agar plate you inoculated with the unknown you were given. State how this contributed to your identification of the bacterium causing the infectious disease. MacConkey agar is discussed in Lab 12 under C. Lab Tests Used in Today's Lab.

c. Cetrimide agar (if used)

Describe the results of the Cetrimide agar plate you inoculated with the unknown you were given. State how this contributed to your identification of the bacterium causing the infectious disease. Cetrimide agar is discussed in Lab 12 under C. Lab Tests Used in Today's Lab.

d. EnteroPluri-Test.
Using your EnterPluri-*Test*, identify the unknown you were given. The EnteroPluri-*Test* and its use are described in Lab 12 under C. Lab Tests Used in Today's Lab.

1. In the table below, **put a (+) or a (-) in the Result row for each test**.
2. **Add up the value of each positive test** in a group and put that number in the code for each group.
3. The 5-digit number is the CODICE number. Look that number up in the **Codebook** and identify your unknown.

	Group 1			Group 2			Group 3			Group 4			Group 5		
Test	**Glucose**	**Gas**	**Lysine**	**Ornithine**	**H_2S**	**Indole**	**Adonitol**	**Lactose**	**Arabinose**	**Sorbitol**	**VP**	**Dulcitol**	**PA**	**Urea**	**Citrate**
Value	**4**	**2**	**1**	**4**	**2**	**1**	**4**	**2**	**1**	**4**	**2**	**1**	**4**	**2**	**1**
Result															
Code															
CODICE NUMBER:							**Identification:**								

Genus and species of bacterium: _____

Final diagnosis:

What infectious disease does the patient have?

What is the genus and species of the bacterium causing this infectious disease?

Case Study #2

Each member of the group must:

1. Print a copy of the rubric from the link found in Lab 13 in the online Lab Manual.
2. Print and fill out a copy of the Team Member Evaluation Form from the link found in Lab 13 in the online Lab Manual.
3. Staple them together and hand them in to me the day your Lab 13 Case Study Lab Report is due.

LABORATORY 13:
ENTEROBACTERIACEAE AND *PSEUDOMONAS*: PART 2

After receiving a baby chicken for Easter, a 7 year old boy is taken to the emergency room with symptoms of vomiting, nausea, non-bloody diarrhea, abdominal cramps, and a temperature of 100°F. A complete blood count (CBC) shows the WBC count to be within the normal reference range.

1. Patient's history

Read the case study. Explain how any relevant parts of the patient's history contributed to your diagnosis of the type of infectious disease that is present here. You are urged to use the computers in lab to search reliable medically oriented Internet sources to support this. Reliable sources you might consider are Medscape (http://emedicine.medscape.com/infectious_diseases) and The Centers for Disease Control and Prevention (CDC) at http://www.cdc.gov/. Cite any sources you use at the end of this Patient's History section in APA style (http://www.apastyle.org/).

The **patient's history should suggest a general type of infectious disease** that is present, such as a urinary tract infection, a wound infection, gastroenteritis, pharyngitis, pneumonia, septicemia, etc. **You need to determine the general type of infection to determine what microbiological tests to perform to identify the bacterium causing the infection**. Search at least one medically oriented reference article from a reliable site such as *Medscape* and **use this article to support your diagnosis the type of infectious disease seen here**. Don't forget to **cite any sources you used in APA style directly under this Patient's History and Patient's Symptoms sections** of this Lab Report.

2. Patient's symptoms

Read the case study. Explain how the patient's signs and symptoms contributed to your diagnosis of the type of infectious disease that is present here. You are urged to use the computers in lab to search reliable Internet sources to support this. Reliable sources you might consider are *Medscape* (http://emedicine.medscape.com/infectious_diseases) and the Centers for Disease Control and Prevention (CDC) at http://www.cdc.gov/. Cite any sources you use at the end of this Patient's History section in APA style (http://www.apastyle.org/).

The **patient's signs and symptoms should suggest a general type of infectious disease** that is present, such as a urinary tract infection, a wound infection, gastroenteritis, strep throat, pneumonia, septicemia, etc. **You need to determine the general type of infectious disease present to determine what microbiological tests to perform to identify the bacterium causing the infection**. Search at least one medically oriented reference article from a reliable site such as *Medscape* and **use this article to support your diagnosis the type of infectious disease seen here**. Don't forget to **cite any sources you used in APA style under this Patient's History and Patient's Symptoms sections** of this Lab Report.

3. Vocabulary list for medical terms used in the case study under signs and symptoms

List and define any medical terms used in your case study that describe the patients's signs and symptoms that the average person not in the medical profession might not know.

4. Results of laboratory test given in the case study

List each lab test given and explain how the results of that test helps to contribute to your diagnosis. Refer to appendix C (Complete Blood Count) in your lab manual as well as your source paper used above.

LABORATORY 13:
ENTEROBACTERIACEAE AND *PSEUDOMONAS*: PART 2

5. Microbiological lab tests you performed in Lab 12

a. XLD

Describe the results of the XLD agar plate you inoculated with the sample from the patient. State how this contributed to your to your identification of the bacterium causing this infectious disease. XLD agar is discussed in Lab 12 under C. Lab Tests Used in Today's Lab.

b. EnteroPluri-*Test*.

Using the EnteroPluri-*Test* inoculated with a colony from the MacConkey agar described above, identify the bacterium causing the infection. The EnteroPluri-*Test* and its use are described in Lab 12 under C. Lab Tests Used in Today's Lab.

1. In the table below, **put a (+) or a (-) in the Result row for each test**.
2. **Add up the value of each positive test** in a group and put that number in the code for each group.
3. The 5-digit number is the CODICE number. Look that number up in the **Codebook** and identify your unknown.

LABORATORY 13:
***ENTEROBACTERIACEAE* AND *PSEUDOMONAS*: PART 2**

	Group 1			Group 2			Group 3			Group 4			Group 5		
Test	**Glucose**	**Gas**	**Lysine**	**Ornithine**	**H_2S**	**Indole**	**Adonitol**	**Lactose**	**Arabinose**	**Sorbitol**	**VP**	**Dulcitol**	**PA**	**Urea**	**Citrate**
Value	**4**	**2**	**1**	**4**	**2**	**1**	**4**	**2**	**1**	**4**	**2**	**1**	**4**	**2**	**1**
Result															
Code															
CODICE NUMBER:							**Identification:**								

Genus of the bacterium: _____

Final diagnosis:

What infectious disease does the patient have?

What is the genus of the bacterium causing this infectious disease?

PERFORMANCE OBJECTIVES
LABORATORY 13

After completing this lab, the student will be able to perform the following objectives:

ISOLATION OF *ENTEROBACTERIACEAE* AND *PSEUDOMONAS*

1. Interpret the results of MacConkey agar and Cetrimide agar.

IDENTIFICATION OF *PSEUDOMONAS*

1. Interpret the results of the following tests:
    a. growth on Cetrimide agar
    b. oxidase test (Gibson Oxidase Test Swab)
    c. pigment production on Cetrimide agar
    d. odor

2. Recognize an organism as *Pseudomonas aeruginosa* and state the reasons why based on the results of the above tests.

IDENTIFICATION OF *ENTEROBACTERIACEAE* USING AN ENTEROTUBE® II

1. Interpret the results of an EnteroPluri-*Test*.

LABORATORY 14
ISOLATION AND IDENTIFICATION OF STREPTOCOCCI AND ENTEROCOCCI

A. The Genus Streptococcus
- **1. The Beta Streptococci**
- **2. The Pneumococcus (*Streptococcus pneumoniae*)**
- **3. The Viridans Streptococci**

B. The Enterococci

There are two genera of bacteria that we will take up in the lab that can appear as a streptococcus arrangement: **the genus *Streptococcus* and the genus *Enterococcus***. Both are Gram-positive cocci 0.5-1.0 µm in diameter, typically **occurring in pairs and chains of varying length when grown in a liquid medium**, and often **occurring singly, in pairs, short chains, and clusters when taken from an agar culture**. As learned in Lab 8, they are both catalase-negative.

A. The Genus *Streptococcus*

Streptococcus species are usually classified clinically based on their **hemolytic properties on blood agar** and according to their **serologic groups**.

The streptococci are usually isolated on Blood agar. **Blood agar** is one of the most commonly used media in a clinical lab. It consists of an enriched agar base (Tryptic Soy agar) to which **5% sheep red blood cells** have been added. Blood agar is commonly used to isolate not only streptococci, but also staphylococci and many other pathogens. Besides providing enrichments for the growth of fastidious pathogens, Blood agar can be used to detect hemolytic properties.

Hemolysis refers to is the lysis of the red blood cells in the agar surrounding bacterial colonies and is a result of bacterial enzymes called **hemolysins**. Although hemolysis can often be observed with the naked eye, ideally it should be examined microscopically using low power magnification, especially in cases of doubtful hemolysis. Reactions on blood agar are said to be beta, alpha, gamma, or double-zone:

1. **Beta hemolysis** (see Fig. 1) refers to a **clear, red blood cell-free zone** surrounding the colony, where a **complete lysis of the red blood cells** by the bacterial hemolysins has occurred. This is **best seen in subsurface colonies where the agar has been stabbed** since some bacterial hemolysins, like streptolysin O, are inactivated by oxygen.

2. **Alpha hemolysis** appears as a zone of **partial hemolysis** surrounding the colony, often accompanied by a **greenish discoloration of the agar**. This is also best seen in subsurface colonies where the agar has been stabbed.

3. **Gamma reaction** refers to **no hemolysis or discoloration of the agar** surrounding the colony.

4. **Double-zone hemolysis** refers to **both a beta and an alpha zone of hemolysis** surrounding the colony.

Many of the streptococci can also be classified under the **Lancefield system**. In this case, they are divided into 19 different **serologic groups** on the basis of carbohydrate antigens in their cell wall. These antigenic groups are

designated by the letters A to H, K to M, and O to V. Lancefield serologic groups A, B, C, D, F, and G are the ones that normally infect humans, however, not all pathogenic streptococci can be identified by Lancefield typing (e.g., *Streptococcus pneumoniae*). **Serologic typing** to identify microorganisms will be discussed in more detail later in **Lab 16**. Single-stranded DNA probes complementary to species-specific r-RNA sequences of streptococci and enterococci are also being used now to identify these organisms.

1. The Beta Streptococci

a. Discussion

Lancefield serologic groups A, B, C, D, F, and G are all streptococci that may show beta hemolysis on Blood agar. However, some group B streptococci are non-hemolytic and group D streptococci (discussed below) usually show alpha hemolysis or are non-hemolytic.

Streptococcus pyogenes, often referred to as **group A beta streptococci** or **GAS** because they belong to Lancefield serologic group A and show beta hemolysis on blood agar, are responsible for most acute human streptococcal infections. *S. pyogenes* isolates are Gram-positive cocci 0.5-1.0 µm in diameter that typically form short chains in clinical specimens and longer chains in laboratory media. The most common infection is **pharyngitis** (streptococcal sore throat) with the organism usually being limited to the mucous membranes and lymphatic tissue of the upper respiratory tract. *S. pyogenes* is responsible for 15-30% of cases of acute pharyngitis in children and 5-10% of cases in adults. Between 5% and 20% of children are asymptomatic carriers. Pharyngitis is pread person to person primarily by respiratory droplets; skin infections are spread by direct contact with an infected person or through fomites. *S. pyogenes* produces a hyaluronic acid capsule which is chemically similar to host connective tissue and masks the bacteria from immune recognition as well as enabling the bacteria to resist phagocytosis. Another characteristic of *S. pyogenes* is the organism's ability to invade epithelial cells. This may play a role in some people becoming carriers of *S. pyogenes* and the bacteria not being erradicated by antibiotics.

From the pharynx, however, the streptococci sometimes **spread to other areas of the respiratory tract** resulting in **laryngitis, bronchitis, pneumonia, and otitis media (ear infection)**. Occasionally, it may enter the **lymphatic vessels or the blood** and disseminate to other areas of the body, causing **septicemia, osteomyelitis, endocarditis, septic arthritis, and meningitis**. It may also **infect** the **skin**, causing **erysipelas**, **impetigo**, or **cellulitis.**

Group A beta streptococcus infections can result in **two autoimmune diseases, rheumatic fever and acute glomerulonephritis**, where antibodies made against streptococcal antigens cross react with joint membranes and heart valve tissue in the case of rheumatic fever, or glomerular cells and basement membranes of the kidneys in the case of acute glomerulonephritis.

Streptococcal pyrogenic exotoxin (Spe), produced by rare invasive strains and scarlet fever strains of *Streptococcus pyogenes* (the group A beta streptococci). .*S pyogenes* produces a number of SPEs that are cytotoxic, pyrogenic, enhance the lethal effects of endotoxins, and contribute to cytokine-induced inflammatory damage. **SPEs are responsible for causing streptococcal toxic shock syndrome (STSS)** whereby excessive cytokine production leads to fever, rash, and triggering the shock cascade. The **SPEs also appear to be responsible for inducing necrotizing fasciitis, a disease that can destroy the skin, fat, and tissue covering the muscle (the fascia).** SPE B is also a precursor for a cysteine protease that can destroy muscles tissue.

CDC reports that approximately 9,000-11,500 cases of invasive GAS disease occur each year in the U.S., with STSS and necrotizing fasciitis each accounted for approximately 6-7% of the cases. STSS has a mortality rate of around 35%. The mortality rate for necrotizing fasciitis is approximately 25%.

The **group B streptococci (GBS)** (*Streptococcus agalactiae*) usually show a small zone of beta hemolysis on Blood agar, although some strains are non-hemolytic. *S. agalactiae* isolates are Gram-

positive cocci 0.6-1.2 µm in diameter that typically form short chains in clinical specimens and longer chains in laboratory media. They are **found in the gastrointestinal tract and genitourinary tract of 15%-40% healthy woman**. This reservoir, along with nosocomial transmission, provides the inoculum by which many infants are colonized at birth. The transmission rate from a mother colonized with GBS to her baby is thought to be around 50%. Most colonized infants (and adults) remain asymptomatic, however, an estimated **1-2% of neonates colonized will develop invasive GBS diseases, including pneumonia, septicemia, and/or meningitis**. Pregnant women should be tested to determine if they are GBS carriers and be given IV antibiotics if they are a carrier Other infections associated with group B streptococci include **urinary tract infections, skin and soft tissue infections, osteomyelitis, endometritis, and infected ulcers (decubitus ulcers and ulcers associated with diabetes)**. In the immunocompromised patient it sometimes causes **pneumonia and meningitis**.

The **group C streptococci** (mainly *S..equi, S. equisimilis* and *S. zooepidemicus*) are beta hemolytic. They sometimes cause pharyngitis and, occasionally, bacteremia, endocarditis, meningitis, pneumonia, septic arthritis, and cellulitis. Group C streptococci are a common cause of infections in animals.

The **group F streptococci** (mainly *S. anginosus*) have been isolated from abscesses of the brain, mouth, and jaw. They also sometimes cause endocarditis.

The **group G streptococci** also show beta hemolysis. They sometimes cause pharyngitis and can also cause serious infections of the skin and soft tissues (mainly in the compromised host) as well as endocarditis, bacteremia, and peritonitis.

All of these beta hemolytic streptococci can be identified by biochemical testing and/or by serologic testing. Today you will look at the isolation and identification of group A beta streptococci (*Streptococcus pyogenes*) by biochemical testing. Serological identification will be performed in Lab 16.

b. Isolation and Identification of Group A Beta Streptococci (*Streptococcus pyogenes*)

Group A beta streptococci are usually isolated on **Blood agar**. *Streptococcus pyogenes* produces:

1. Very **small, white to grey colonies** approximately 1mm in diameter.

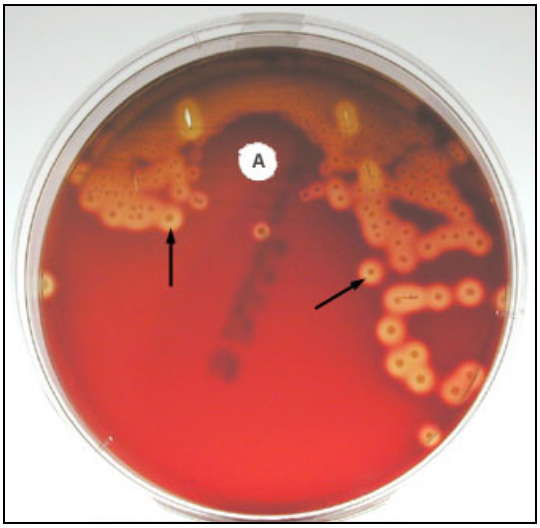
2. A **zone of beta hemolysis** around 2-3mm in diameter surrounding each colony.

There are two streptococcal hemolysins, streptolysin S and streptolysin O. Streptolysin O can be **inactivated by oxygen** so more distinct hemolysis can be seen by **stabbing the agar several times**. In this way, some of the organisms form subsurface colonies growing away from oxygen. Since both streptolysin S and streptolysin O are active in the stabbed area, a clearer zone of beta hemolysis can be seen.

3. Sensitivity to the antibiotic bacitracin.

Only the group A beta streptococci (*Streptococcus pyogenes*) are sensitive to bacitracin, as shown by a **zone of inhibition around a Taxo A® disc**, a paper disc containing low levels of bacitracin (**Fig 1A**). Other serologic groups of streptococci are resistant to bacitracin and show no inhibition around the disc. (The Lancefield group of a group A beta streptococcus can also be determined by direct serologic testing as will be demonstrated in Lab 17.)

Fig. 1A:
Blood agar plate with a Taxo A disc inoculated with *Streptococcus pyogenes*.



2. The Pneumococcus (*Streptococcus pneumoniae*)

a. Discussion

Streptococcus pneumoniae, or the pneumococcus, is a Gram-positive lancet-shaped (pointed like a lance) coccus 0.5-1.2 µm in diameter. They typically appear as a **diplococcus**, but occasionally appear singularly or in short chains. Pneumococci are frequently found as **normal flora of the nasopharynx of healthy carriers**. Pharyngeal colonization occurs in 40%-50% of healthy children and 20%-30% of healthy adults.

Worldwide, as well as in the U.S., *S. pneumoniae* remains the most common cause of community-acquired pneumonia, otitis media, bacteremia, and bacterial meningitis. In the U.S pneumococci are **the most common cause of community-acquired pneumonia requiring hospitalization**, causing around 500,000 cases per year and usually occuring as a secondary infection in the debilitated or immunocompromised host. The pneumococci also cause between 6 and 7 million cases of **otitis media** per year, are the leading cause of **sinusitis** in people of all ages, are responsible for 55,000 cases of **bacteremia**, and 3000 cases of **meningitis**, being the most common cause of meningitis in adults and children over 4 years of age.

The capsule serves as the major virulence factor, enabling the pneumococcus to resist phagocytic engulfment, and glycopeptides from its Gram-positive cell wall can lead to excessive cytokine production and a massive inflammatory response.

Pneumococci show **alpha hemolysis** on Blood agar.

b. Isolation and Identification Pneumococci (*Streptococcus pneumoniae)*

1. **Isolation on Blood agar**

Pneumococci frequently require enriched media and increased CO_2 tension for initial isolation. They are usually isolated on **Blood agar** and incubated in a candle jar (a closed container in which a lit candle is placed to remove O_2 and increase CO_2) at 37°C. On Blood agar, colonies appear small, shiny, and translucent. They are surrounded by a zone of **alpha hemolysis.** Due to autolysis with age, the colonies may show a depressed center with an elevated rim.

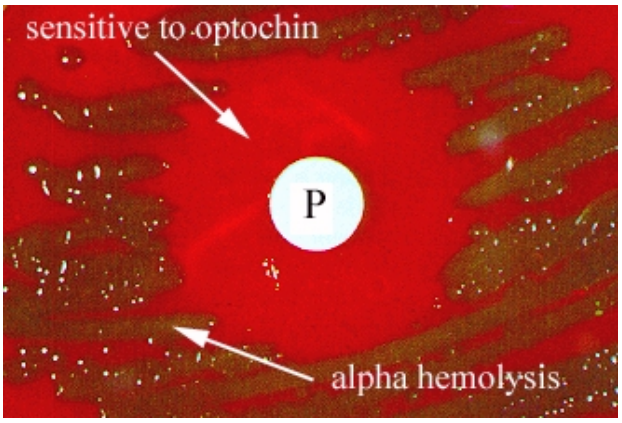
2. **Optochin sensitivity**

Pneumococci are the only streptococci that are **sensitive to the drug optochin** (ethylhydrocupreine hydrochloride). This can be detected by a **zone of inhibition around a Taxo P® disc,** a paper disc containing the drug optochin, which is placed on the Blood agar plate prior to incubation (**Fig. 1B**).

3. **Bile solubility test**

Most colonies of S. pneumoniae will dissolve within a few minutes when a drop of bile is placed upon them. (This test will not be done in lab today.)

Fig. 1B:
A blood agar plate with a Taxo P disc inoculated with *Streptococcus pneumoniae*.



4. **Gram stain of sputum**

Streptococcus pneumoniae will usually appear as encapsulated, **Gram-positive, lancet-shaped diplococci**.

3. The Viridans Streptococci

a. Discussion

Ten species of streptococci are known as the **viridans streptococci**. They are the **dominant normal flora in the upper respiratory tract**. Species include *S. mutans, S. sanguis, S. mitis*, and *S. salivarius*. *S. mutans* is the primary cause of **dental caries**. Viridans streptococci are responsible for between 50% and 70% of the cases of **bacterial endocarditis**, especially in people with previously damaged heart valves. They are also frequently associated with **bacteremia, deep wound infections, dental abscesses, and abscesses of internal organs**. The viridans streptococci show **alpha hemolysis or no hemolysis** on Blood agar, do not possess Lancefield group antigens, and can be differentiated from other alpha streptococci by biochemical testing.

B. The Genus *Enterococcus*

a. Discussion

Enterococci are **Gram-positive streptococci that are normal flora of the intestinal tract**. **They typically occur singly, in pairs, short chains, and clusters**, especially when taken off an agar culture for staining. Like the genus *Streptococcus*, the genus *Enterococcus* is **catalase-negative**. Enterococci **responsible for a variety of opportunistic infections** in humans, and serologically belong to **Lancefield group D streptococci**.

Enterococcus faecalis is the most common enterococcus causing human infections, representing 80-90% of human enterococcal clinical isolates. *E. faecalis* is **normal flora of the intestinal tract** in humans and is regularly isolated from **infections within the peritoneal cavity** (especially following penetrating trauma), **urinary tract infections, kidney infections, prostate infections, and infections of damaged or compromised skin** such as **diabetic or decubitus ulcers, burns, and surgical wounds**. **Trauma to the intestines can lead to intra abdominal and pelvic infections**. Other opportunistic enterococcal species include *E. faecium* and *E. durans*. **The enterococci have become the second most common bacterium isolated from nosocomial urinary and wound infections, and the third most common cause of nosocomial bacteremia**. Each year in the U.S enterococci account for approximately 110,000 urinary tract infections, 40,000 wound infections, 25,000 cases of nosocomial bacteremia, and 1100 cases of endocarditis. Furthermore, the enterococci are **among the most antibiotic resistant** of all bacteria, with some isolates resistant to all known antibiotics. The ability of enterococci to produce biofilms protects the organism from the body's defenses as well as promotes exchange of genetic material with other pathogens

b. Isolation and Identification of Enterococci

The enterococci may be isolated and identified using a selective and differential media called **Bile Esculin Azide Agar**. Unlike most bacteria, the enterococci will grow in the presence of the bile salts in the medium. They **hydrolyze the esculin**, producing esculetin which reacts with the iron salts in the medium turning the agar **black**. The sodium azide in the medium inhibits Gram-negative bacteria.

On blood agar, most strains of *Enterococcus faecalis* show gamma reaction on sheep blood agar, however some strains exhibit beta hemolysis. Colonies are usually 1-2 millimeters in diameter. Enterococci are also being identified using chemiluminescent labelled DNA probes complementary to species-specific bacterial ribosomal RNA (rRNA) sequences.

SCENERIOS FOR TODAY'S LAB

CASE STUDY #1

Choose either unknown #1 or unknown #2 as your unknown for Case Study #1.

A 21 year old male complains of a sore throat and painful swallowing. A physical exam of the throat shows tonsillopharyngeal edema and erythema, a patchy exudate, petechiae on the soft palate, and a red, swollen uvula. He has a temperature of 101.6 °F. He doesn't have a cough or a noticeably runny nose.

Assume that your unknown is a transport medium from a swab of this person's throat.

CAUTION: TREAT EACH UNKNOWN AS A PATHOGEN! Inform your instructor of any spills or accidents. WASH AND SANITIZE YOUR HANDS WELL before leaving the lab.

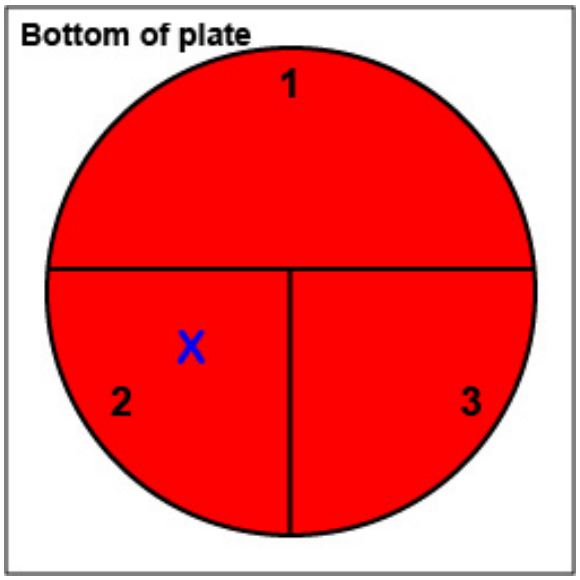
LABORATORY 14:
STREPTOCOCCI AND ENTEROCOCCI

MATERIALS

1 plate of blood agar, 1 Taxo A ® disc, 1 sterile swab, inoculating loop

PROCEDURE (to be done in groups of 3)

1. On the bottom of the petri plate, **divide the plate into thirds with your wax marker and label as shown below**. Before you streak your plate **draw an "X" on the bottom of the blood agar plate in sector 2** to indicate where you will eventually place the Taxo A disk as indicated in the illustration below.



2. Using a sterile inoculating loop, **streak your unknown for isolation** on a blood agar plate so as to get **single, isolated colonies** (see Fig. 2, step 1, Fig. 2, step 2, and Fig. 2, step 3).

3. Using your inoculating loop, **stab** the agar 2-3 times in each of the growth areas in order to detect oxygen-sensitive hemolysins (Fig. 2, step 4).

4. **Place a Taxo A ® disc containing bacitracin where you drew the "X" in sector 2). Tap it lightly with your loop so that the disk sticks to the agar.** (Fig. 2, step 5).

5. Incubate the blood agar plate **upside down and stacked in the petri plate holder on the shelf of the 37°C incubator corresponding to your lab section** until the next lab period.

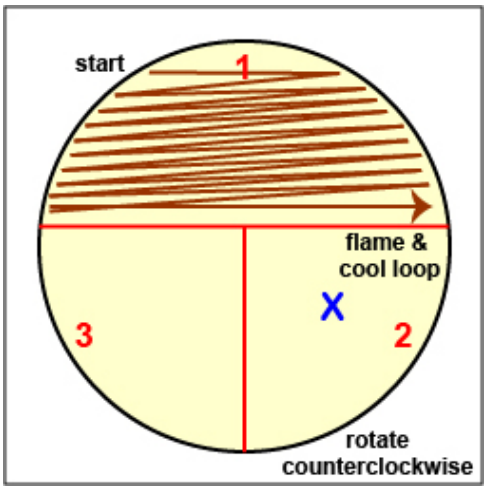
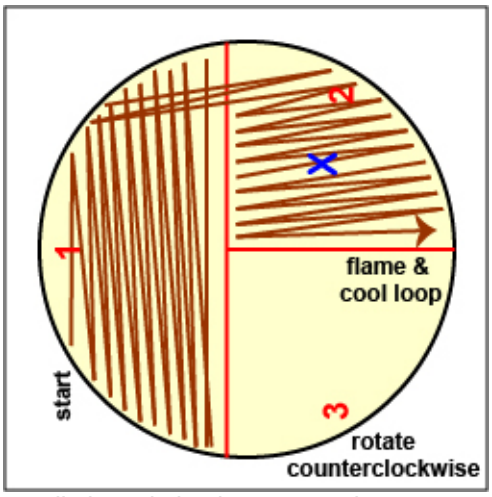
Fig. 2, step 1 Inoculating a Blood Agar Plate with your unknown	Fig. 2, step 2 Inoculating a Blood Agar Plate with your unknown
 Using a sterile inoculating loop, streak one-third of the blood agar plate with your unknown. Flame the loop and let it cool.	 Using a sterile inoculating loop, spread out some of the bacteria in area 1 over area 2. Flame the loop and let it cool.

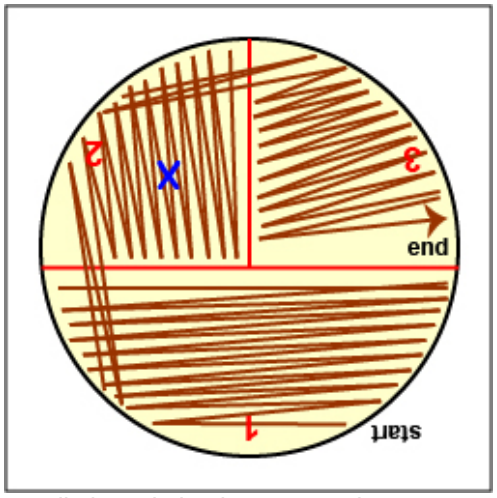
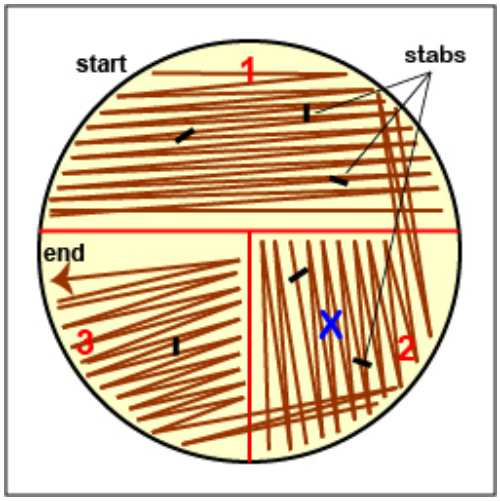
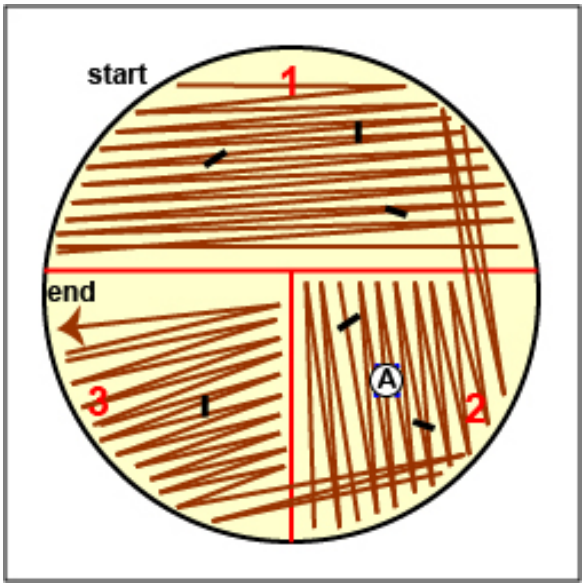
Fig. 2, step 3 Inoculating a Blood Agar Plate with your Unknown	Fig. 2, step 4 Inoculating a Blood Agar Plate with your Unknown
 Using a sterile inoculating loop, spread out some of the bacteria in area 2 over area 3.	 Stab the agar 2-3 times in each if the growth areas.

Fig. 2, step 5
Inoculating a Blood Agar Plate with your Unknown



Place a Taxo A disc over the "X" in sector 2 and tap it lightly with your loop so that it sticks to the agar.

CASE STUDY #2

Choose unknown #3 as your unknown for Case Study #2.

A 57 year old diabetic male hospitalized following hip replacement surgery has had an indwelling urinary catheter inserted for 8 days. He presents with suprapubic discomfort. His blood pressure is normal and he does not have fever, chills, or flank pain. There is no costovertebral angle (CVA) tenderness. A complete blood count (CBC) shows leukocytosis with a left shift. A urine dipstick shows a positive leukocyte esterase test, a negative nitrite test, 30mg of protein per deciliter, and red blood cells in the urine. Microscopic examination of centrifuged urine shows 50 white blood cells, as well as 20 bacteria and 5 red blood cells per high-power field.

CAUTION: TREAT EACH UNKNOWN AS A PATHOGEN! Inform your instructor of any spills or accidents. WASH AND SANITIZE YOUR HANDS WELL before leaving the lab.

MATERIALS

1 bile esculin azide agar plate, materials to perform a Gram stain, inoculating loop

PROCEDURE (to be done in groups of 3)

[Keep in mind that in a real clinical situation other lab tests and cultures for bacteria other than those upon which this lab is based would also be done.]

1. Do a **Gram stain** on the unknown (see Lab 6). Make sure you **review the instructions before you do the Gram stain.** Because Enterococci and Staphylococci can sometimes look similar in Gram stains done from a plate culture, **perform a catalase test on your unknown** to help differentiate an Enterococcus from a *Staphylococcus* (see **Lab 8**).

2. Inoculate a **Bile Esculin Azide agar plate** with your unknown. Incubate in **your petri plate holder on the shelf of the 37°C incubator corresponding to your lab section** until the next lab period.

RESULTS

1. Pneumococci (*Streptococcus pneumoniae*)

Blood agar with Taxo P® (optochin) disc

Description of colony	
Type of hemolysis (alpha, beta, or gamma)	
Taxo P®disc (optochin) results (inhibition or no inhibition)	

Case Study Lab Report for Lab 14: *Streptococcus* and *Enterococcus*

The concept behind the case studies presented in Lab 14 used to illustrate the genus *Streptococcus* and the genus *Enterococcus* is for you and your lab partners as a group to:

1. **Determine whether or not the patient in case study #1 has streptococcal pharyngitis**.

2. **Come up with a valid diagnosis of the infectious disease in case study #2 and identify the bacterium causing that infection**.

3. **Support your group diagnose** based on:

    a. Any relevant facts in the **patient's history**. (A reliable on-line source will be used to support this.)
    b. The patient's **signs and symptoms.** (A reliable on-line source will be used to support this.)
    c. **Each of the individual lab tests** given in your case study.
    d. All **microbiological lab tests you performed** as part of the project.

The due date for this report can be found on the class calendar. Your grade for this lab is based on the **completeness of your report and written evidence of the critical thinking process that went into making and supporting your diagnosis.** Remember, you are trying to convince your instructor that you understand how the diagnosis was made by **supporting that diagnosis with data.**

Grading:

The lab 14 Lab Report is worth 21 points.

These case studies are based in part on your in-class participation as part of your group. Therefore:

a. **If you were not in lab when the inoculations with your unknown were performed, 3 points will be deducted from your Lab Report score for labs 12, 14, and 15; 6 points from your Lab Report score for the Final Project.**

b. **If you were not in lab when the results of your lab tests were observed, 3 points will be deducted from your Lab Report score for labs 12, 14, and 15; 6 points from your Lab Report score for the Final Project**.

c. **For each day your Lab Report is late, 2 points will be deducted from your Lab Report score for labs 12, 14, and 15; 4 points from your Lab Report score for the Final Project.**

Each person in the group must write and submit their own individual report and it cannot be the same as that of other members of that group.

The rubric that will be used to grade this lab report can be found under this Assignment on Blackboard.

Be sure to handle all the bacterial cultures you are using in lab today as if they are pathogens! Be sure to wash and sanitize your hands well at the completion of today's lab.

Also, make sure you **observe the results of of someone in your lab who had an unknown different from yours in case study #1**. The Performance Objectives for Lab 14 tell you what you are expected to be able to do on the practical.

A. Case Study #1 from Lab 14: Unknown #1

Your Name:

Others in your group:

Unknown number (1 or 2):

Lab section:

Date:

Case Study #1, Unknown #1

A 21-year old male complains of a sore throat and painful swallowing. A physical exam of the throat shows tonsillopharyngeal edema and erythema, a patchy exudate, petechiae on the soft palate, and a red, swollen uvula. He has a temperature of 101.6 °F. He doesn't have a cough or a noticeably runny nose.

LABORATORY 14:
STREPTOCOCCI AND ENTEROCOCCI

1. Patient's signs and symptoms

Read the case study. Explain how the patient's signs and symptoms contributed to your diagnosis of the type of infectious disease seen here. You are urged to use the computers in lab to search reliable Internet sources to support this. Reliable sources you might consider are *Medscape* (http://emedicine.medscape.com/infectious_diseases) and the Centers for Disease Control and Prevention (CDC) at http://www.cdc.gov/. Cite any sources you use at the end of this Patient's History section in APA style (http://www.apastyle.org/).

The patient's signs and symptoms should suggest a general type of infectious disease that is present, such as a urinary tract infection, a wound infection, gastroenteritis, pharyngitis, pneumonia, septicemia, etc. **You need to determine the general type of infection to determine what microbiological tests to perform to identify the bacterium causing the infection**. Search at least one medically oriented reference article from a reliable site such as Medscape and **use this article to support your diagnosis of the type of infectious disease seen here**. Don't forget to **cite any sources you used in APA style** directly under this Patient's Signs and Symptoms sections of this Lab Report.

2. Vocabulary list for medical terms used in the case study under signs and symptoms

List and define any medical terms used in your case study that describe the patients's signs and symptoms that the average person not in the medical profession might not know.

3. Microbiological lab tests you performed in Lab 14

a. **Blood agar with Taxo A® (bacitracin) disc: Unknown #1**

Give the results of the Blood agar with Taxo A® (bacitracin) disc you performed on the unknown you were given, and how you reached this conclusion. **State how this contributed to your final diagnosis as to whether or not the person has streptococcal pharyngitis**. The possible results for blood agar and Taxo A® disc were discussed in the beginning pages of this lab.

4. If he has streptococcal pharyngitis, state the genus and species of this bacterium.

B. Case Study #1 from Lab 14: Unknown #2

Case Study #1, Unknown #2

A 21-year old male complains of a sore throat and painful swallowing. A physical exam of the throat shows tonsillopharyngeal edema and erythema, a patchy exudate, petechiae on the soft palate, and a red, swollen uvula. He has a temperature of 101.6 °F. He doesn't have a cough or a noticeably runny nose.

1. Patient's signs and symptoms

Read the case study. Explain how the patient's signs and symptoms contributed to your diagnosis of the type of infectious disease seen here. You are urged to use the computers in lab to search reliable Internet sources to support this. Reliable sources you might consider are *Medscape* (http://emedicine.medscape.com/infectious_diseases) and the Centers for Disease Control and Prevention (CDC) at http://www.cdc.gov/. Cite any sources you use at the end of this Patient's History section in APA style (http://www.apastyle.org/).

The **patient's history and patient's symptoms should suggest a general type of infection**, such as a urinary tract infection, a wound infection, gastroenteritis, strep throat, pneumonia, septicemia, etc. Search at least one medically oriented reference article from a reliable site such as *Medscape* and **use this article to support your diagnosis of the type of infection**. Don't forget to **cite any sources you used in APA style under the Patient's History and Patient's Symptoms sections** of your Lab Report.

2. Vocabulary list for medical terms used in the case study under signs and symptoms

List and define any medical terms used in your case study that describe the patients's signs and symptoms that the average person not in the medical profession might not know.

3. Microbiological lab tests you performed in Lab 14

a. **Blood agar with Taxo A® (bacitracin) disc: Unknown #1**

Give the results of the Blood agar with Taxo A® (bacitracin) disc you performed on the unknown you were given, and how you reached this conclusion. **State how this contributed to your final diagnosis as to whether or not the person has streptococcal pharyngitis**. The possible results for blood agar and Taxo A® disc were discussed in the beginning pages of this lab.

4. If he has streptococcal pharyngitis, state the genus and species of this bacterium.

C. Case Study #2

Your Name:

Others in your group:

Lab section:

Date:

Case Study #2, Unknown #3

A 57-year old diabetic male hospitalized following hip replacement surgery has had an indwelling urinary catheter inserted for 8 days. He presents with suprapubic discomfort. His blood pressure is normal and he does not have fever, chills, or flank pain. There is no costovertebral angle (CVA) tenderness. A complete blood count (CBC) shows leukocytosis with a left shift. A urine dipstick shows a positive leukocyte esterase test, a negative nitrite test, 30mg of protein per deciliter, and red blood cells in the urine. Microscopic examination of centrifuged urine shows 50 white blood cells, as well as 20 bacteria and 5 red blood cells per high-power field.

1. Patient's history and predisposing factors

Read the case study. Explain how any relevant parts of the patient's history contributed to your diagnosis of the type of infectious disease seen here. You are urged to use the computers in lab to search reliable medically oriented Internet sources to support this. Reliable sources you might consider are Medscape (http://emedicine.medscape.com/infectious_diseases) and The Centers for Disease Control and Prevention (CDC) at http://www.cdc.gov/. Cite any sources you use at the end of this Patient's History section in APA style (http://www.apastyle.org/).

The **patient's history should suggest a general type of infectious disease** that is present, such as a urinary tract infection, a wound infection, gastroenteritis, pharyngitis, pneumonia, septicemia, etc. **Do not look up the bacterium you eventually identify** as the cause of this infectious disease. You do not know the causative bacterium at this point. **You need to determine the general type of infection to determine what microbiological tests to perform to identify the bacterium causing the infection**. Search at least one medically oriented reference article from a reliable site such as *Medscape* and **use this article to support your diagnosis the type of infectious disease seen here**. Don't forget to **cite any sources you used in APA style directly under this Patient's History and Patient's Symptoms sections** of this Lab Report.

2. Patient's signs and symptoms

Read the case study. Explain how the patient's signs and symptoms contributed to your diagnosis of the type of infectious disease seen here. You are urged to use the computers in lab to search reliable Internet sources to support this. Reliable sources you might consider are *Medscape* (http://emedicine.medscape.com/infectious_diseases) and the Centers for Disease Control and Prevention (CDC) at http://www.cdc.gov/. Cite any sources you use at the end of this Patient's History section in APA style (http://www.apastyle.org/).

The **patient's signs and symptoms should suggest a general type of infectious disease** that is present, such as a urinary tract infection, a wound infection, gastroenteritis, strep throat, pneumonia, septicemia, etc. **Do not look up the bacterium you eventually identify** as the cause of this infectious disease. You do not know the causative bacterium at this point. **You need to determine the general type of infectious disease present to determine what microbiological tests to perform to identify the bacterium causing the infection**. Search at least one medically oriented reference article from a reliable site such as *Medscape* and **use this article to support your diagnosis the type of infectious disease seen here**. Don't forget to **cite any sources you used in APA style under this Patient's History and Patient's Symptoms sections** of this Lab Report.

3. Vocabulary list for medical terms used in the case study under signs and symptoms

List and define any medical terms used in your case study that describe the patients's signs and symptoms that the average person not in the medical profession might not know.

4. Results of laboratory test given in the case study

List each lab test given and explain how the results of that test helps to contribute to your diagnosis. **The CBC and urinalysis tests are described in** Appendix C and Appendix D of this lab manual.

5. Microbiological lab tests you performed in Lab 14

a. **Gram stain and catalase test results**

Give the Gram reaction (Gram-positive or Gram-positive and how you reached this conclusion) and the shape and arrangement of the unknown you were given. **Because Enterococci and Staphylococci can sometimes look similar in Gram stains done from a plate culture, perform a catalase test on your unknown to help differentiate an *Enterococcus* from a *Staphylococcus*. State how this contributed to your decision as to which microbiological tests and/or media to use next**. The Gram stain is discussed in Lab 6; the catalase test in Lab 8.

b. **Bile Esculin Azide Agar**

Give the results of the Bile Esculin Azide Agar plate you inoculated with the unknown you were given, and how you reached those conclusions. **State how this contributed to your final diagnosis of the bacterium causing this infection**. The possible results for Bile Esculin Azide Agar were discussed earlier in this lab.

Genus of the bacterium: _____

Infection: _____

PERFORMANCE OBJECTIVES
LABORATORY 14

After completing this lab, the student will be able to perform the following objectives:

A THE GENUS *STREPTOCOCCUS*

1. State the Gram reaction and morphology of the streptococci.
2. State two ways the streptococci are classified.
3. Describe alpha hemolysis, beta hemolysis, and gamma reaction on Blood agar plates.
4. State what is meant by the Lancefield system.
5. State the Lancefield group of streptococcus that is the most common cause of acute streptococcal infections in humans and name five other Lancefield groups that frequently cause human infections.

1. THE BETA STREPTOCOCCI

DISCUSSION

1. State what the term "group A beta" means when referring to streptococci.
2. State the genus and species of the group A beta streptococci.
3. State the most common infection caused by *Streptococcus pyogenes* and name six other infections it may cause.
4. Name two autoimmune diseases associated with the group A beta streptococci.
5. State the genus and species of the group B streptococci.
6. State the normal habitat of the group B streptococci, name three infections they may cause in newborns, and describe how the infants become colonized.
7. Name three infections the group B streptococci may cause in adults.

ISOLATION AND IDENTIFICATION OF GROUP A BETA STREPTOCOCCI

1. Describe the appearance of group A beta streptococci on Blood agar.
2. State why Blood agar is usually stabbed during streaking when isolating beta streptococci.
3. Describe the reaction of group A beta streptococci to a Taxo A® disc containing bacitracin.

RESULTS OF GROUP A BETA STREPTOCOCCI

1. Identify an organism as a group A beta streptococcus (or *Streptococcus pyogenes*) and state the reasons why when it is seen growing on a Blood agar plate with a Taxo A® disc containing bacitracin.
2. Recognize beta hemolysis on Blood agar.

2. THE PNEUMOCOCCUS

DISCUSSION

1. State the genus and species of the pneumococcus.
2. State the Gram reaction and morphology of *Streptococcus pneumoniae*.
3. State the natural habitat of *Streptococcus pneumoniae* and name four infections it may cause in humans.

ISOLATION AND IDENTIFICATION OF PNEUMOCOCCI

1. Describe the appearance of *Streptococcus pneumoniae* on Blood agar with a Taxo P® disc containing the drug optochin.

RESULTS OF PNEUMOCOCCI

1. Identify an organism as *Streptococcus pneumoniae* and state the reasons why when it is seen growing on a Blood agar plate with a Taxo P® disc containing optochin.
2. Recognize alpha hemolysis on Blood agar.

3. THE VIRIDANS STREPTOCOCCI

1. State the normal habitat of the viridans streptococci and name three infections they may cause in humans.
2. State the hemolytic reactions of the viridans streptococci on Blood agar.

B. THE GENUS *ENTEROCOCCUS*

DISCUSSION

1. Name the most common enterococcus that infects humans and state its normal habitat.
2. State the Lancefield group of the enterococci.
3. Name four infections commonly caused by *Enterococcus faecalis*.

ISOLATION AND IDENTIFICATION OF ENTEROCOCCI

1. Describe the reactions of enterococci on Bile Esculin Azide Agar.
2. State the Gram reaction and morphology of the enterococci.

RESULTS OF THE ENTEROCOCCI

1. Identify an organism as an *Enterococcus* and state the reasons why when it is seen growing on Bile Esculin Azide Agar.

LABORATORY 15
ISOLATION AND IDENTIFICATION OF STAPHYLOCOCCI

DISCUSSION

The Staphylococci

Staphylococci are often found in the human nasal cavity (and on other mucous membranes) as well as on the skin. They are **Gram-positive cocci** 0.5-1.0 µm in diameter and occur singly, in pairs, in short chains, and most commonly, in **irregular grape-like clusters**. The staphylococci are strongly catalase positive and generally tolerate relatively high concentrations of sodium chloride (7.5-10%). This ability is often employed in preparing media selective for staphylococci.

Staphylococcal **capsules** play a major role in the ability of the bacteria to **adhere to and colonize biomaterials**.

There are five species of staphylococci **commonly associated with clinical infections:** *Staphylococcus aureus, S. epidermidis, S. haemolyticus, S. hominis* and *S. saprophyticus*.

A. *Staphylococcus aureus* (coagulase-positive staphylococci)

Staphylococcus aureus is the most pathogenic species and is implicated in a variety of infections. *S. aureus* is with some frequency found as normal human flora in the anterior nares (nostrils). It can also be found in the throat, axillae, and the inguinal and perineal areas. Approximately 30% of adults and most children are healthy periodic **nasopharyngeal carriers** of *S. aureus*. Around 15% of healthy adults are persistent nasopharyngeal carriers. The colonization rates among health care workers, patients on dialysis, and people with diabetes are higher than in the general population.

In the majority of *S. aureus* infections, the **source of the organism** is either:

- A **healthy nasal carrier**, or
- **Contact with an abscess** from an infected individual.

The portal of entry is usually the skin. *S. aureus* causes pus-filled inflammatory lesions known as **abscesses**. Depending on the location and extent of tissue involvement, there are three categories of abscesses:

1. **Pustules**
A pustule is an **infected hair follicle** where the base of the hair follicle appears red and raised with an accumulation of pus just under the epidermis. Infected hair follicles are also referred to as **folliculitis**.

2. **Furuncles** or **boils**
Furuncles appear as large, **raised, pus-filled, painful nodules** having an accumulation of dead, necrotic tissue at the base. The bacteria **spread from the hair follicle to adjacent subcutaneous tissue**.

3. **Carbuncles**
Carbuncles occur when **furuncles coalesce and spread into surrounding subcutaneous and deeper connective tissue**. **Superficial skin perforates**, **sloughs off**, and **discharges pus**.

S. aureus also causes **impetigo**, a **superficial blister-like infection of the skin** usually occuring on the face and limbs and seen mostly in young children. *S. aureus* may also cause **cellulitis**, a diffuse inflammation of connective tissue with severe inflammation of dermal and subcutaneous layers of the skin. *S. aureus* is also a frequent cause of **accidental wound and postoperative wound infections**.

Less commonly, *S. aureus* may escape from the local lesion and spread through the blood to other body areas, causing a variety of **systemic infections** that may involve every system and organ. Such systemic infections include **septicemia, septic arthritis, endocarditis, meningitis, and osteomyelitis**, as well as **abscesses in the lungs, spleen, liver, and kidneys.** *S. aureus* pneumonia may also be a secondary respiratory complication of viral infections such as measles and influenza, and is a frequent cause of nosocomial pneumonia in patients who are debilitated. Finally, *S. aureus* is frequently introduced into food by way of abscesses or the nasal cavity of food handlers. If it is allowed to grow and produces an **enterotoxin,** it can cause **staphylococcal food poisoning**.

In a 1990-1992 National Nosocomial Infections survey, CDC found *S. aureus* to be the **most common cause of nosocomial pneumonia and operative wound infections**, as well as the **second most common cause of nosocomial bloodstream infections**. Antibiotic resistant *S. aureus* is a common problem. For example, a survey conducted by CDC reported the proportion of methicillin-resistant isolates *S. aureus* (MRSA) with sensitivity only to vancomycin increased from 22.8% in 1987 to 56.2% in 1997.

Virulence factors for *S. aureus* include exotoxins such as leukocidin (kills leukocytes), alpha and delta toxins (damage tissue membranes), microcapsules (resist phagocytic engulfment and destruction), coagulase and protein A (both help resist phagocytic engulfment). Some strains also produce **TSST-1** (toxic shock syndrome toxin-1) and cause **toxic shock syndrome,** usually associated with wounds. Approximately 25% of all *S. aureus* strains are toxigenic and approximately 6000 gases of toxic shock syndrome occur each year in the U.S. Some strains also produce **exfoliatin**, an exotoxin that causes **scalded skin syndrome**, an infection usually seen in infants and young children.

In the past 20 years, both community-associated and hospital-acquired infections with *Staphylococcus aureus* have increased. This infection rate has been accompanied **by a rise in antibiotic-resistant strains - most significantly, methicillin-resistant *S. aureus* (MRSA) and, more recently, vancomycin-resistant *S. aureus***.

Since most *S. aureus* strains produce the enzyme coagulase (see the coagulase test described below), they are often referred to as **coagulase-positive staphylococci**.

B. Coagulase-Negative Staphylococci

Clinically common species of staphylococci other than *S. aureus* are often referred to as **coagulase-negative staphylococci**. These staphylococci are normal flora of the skin and, as such, frequently act as **opportunistic pathogens**, especially in the compromised host. *S. saprophyticus* is a relatively common cause of **urinary tract infections**, especially in young, sexually active women, but is seldom isolated from other sources. The great **majority of infections caused by other coagulase-negative staphylococci**, including *S. epidermidis, S. haemolyticus, and S. hominis,* are **associated with intravascular devices** (prosthetic heart valves and intra-arterial or intravenous lines) **and shunts**. Also quite common are **infections of prosthetic joints, wound infections, osteomyelitis** associated with foreign bodies, and **endocarditis**.

Although certain reactions may vary from strain to strain, a series of biochemical tests will usually differentiate the most common clinically encountered species of staphylococci. Today we will use a number of tests to determine if an unknown is *S. aureus, S. epidermidis,* or *S. saprophyticus*.

LABORATORY 15:
STAPHYLOCOCCI

ISOLATION AND IDENTIFICATION OF STAPHYLOCOCCI

1. Blood agar with a novobiocin (NB) disc

To isolate staphylococci, clinical specimens are usually grown on **Blood agar** (described in Lab 14). Staphylococci produce round, raised, opaque colonies 1-2mm in diameter. The novobiocin disc is used to detect sensitivity or resistance to the antibiotic novobiocin.

Test	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Staphylococcus saprophyticus*
Hemolysis*	Usually beta[1]	Usually gamma[2]	Usually gamma[2]
Pigment	Often creamy gold[1]	Usually white[2]	Usually white[2]
Novobiocin disc	Sensitive	Sensitive	Resistant

* see Lab 14 for descriptions of hemolysis
1. Some strains do not show hemolysis and/or pigment
2. Some strains do show hemolysis and/or pigment
Sensitive = zone of inhibition around disc
Resistant = no zone of inhibition around disc

2. Gram stain

All staphylococci appear as Gram-positive cocci, usually in irregular, often grape-like clusters.

3. Mannitol fermentation on Mannitol Salt agar (MSA)

Staphylococci are able to tolerate the high salt concentration found in Mannitol Salt agar and thus grow readily. If mannitol is fermented, the acid produced turns the phenol red pH indicator from red (alkaline) to yellow (acid).

Test	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Staphylococcus saprophyticus*
Mannitol fermentation	Positive	Negative	Usually positive

Positive = acid end products turn the phenol red pH indicator from red to yellow
Negative = phenol red remains red

4. Production of coagulase

The staphylococcal enzyme coagulase will cause inoculated citrated rabbit plasma to gel or coagulate. The coagulase converts soluble fibrinogen in the plasma into insoluble fibrin.

Test	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Staphylococcus saprophyticus*
Coagulase production	Positive	Negative	Negative

Positive = plasma will gel or coagulate
Negative = plasma will not gel

5. The Staphyloslide® Latex Test for cell-bound coagulase (clumping factor) and/or Protein A

The Staphyloslide® Latex Test is an agglutination test that detects **cell-bound coagulase (clumping factor) and/or Protein A.** Approximately 97% of human strains of *S. aureus* possess both bound coagulase and extracellular coagulase. Approximately 95% of human strains of *S. aureus* possess Protein A on their cell surface. This test uses blue latex particles coated with human fibrinogen and the human antibody IgG. Mixing of the latex reagent with colonies of the suspected *S. aureus* having coagulase and/or Protein A bound to their surface causes agglutination of the latex particles.

Test	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Staphylococcus saprophyticus*
Cell-bound coagulase (clumping factor) and/or Protein A	Positive	Negative	Negative

Positive = clumping of latex particles
Negative = no clumping of latex particles

Staphylococci are also being identified using chemiluminescent labelled DNA probes complementary to species-specific bacterial ribosomal RNA (rRNA) sequences as well as by other direct DNA techniques.

SCENERIO FOR TODAY'S LAB

CASE STUDY

Choose either unknown #1 or unknown #2 as your unknown for this Case Study.

A 57-year old female who is diabetic, a long-time smoker, and who 28 days ago had hip replacement surgery presents with swelling, pain, inflammation, and erythema at the surgical site. Examination shows she has a fever of 101°F, is exhibiting malaise, and has an increased total white blood cell count with a left shift. Ultrasonography examination indicates a deep abscess. A culture from an aspiration of the infected surgical site was taken.

LABORATORY 15:
STAPHYLOCOCCI

Assume that unknown you are given is the culture from this patient.

CAUTION: TREAT EACH UNKNOWN AS A PATHOGEN! Inform your instructor of any spills or accidents. WASH AND SANITIZE YOUR HANDS WELL before leaving the lab.

MATERIALS (per group of 3)

1 Blood agar plate, 1 Mannitol Salt agar plates, 1 DNase agar plate, 1 tube of citrated rabbit plasma (coagulase test), 1 novobiocin disc, inoculating loop

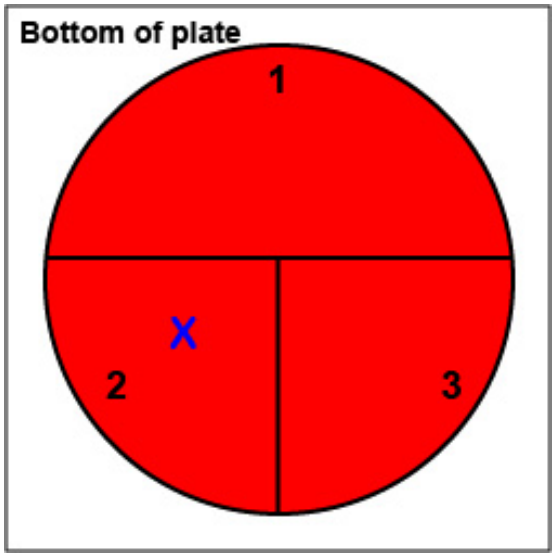
LABORATORY 15:
STAPHYLOCOCCI

PROCEDURE: to be done in groups of 3

[Keep in mind that in a real clinical situation other lab tests and cultures for bacteria other than those upon which this lab is based would also be done.]

1. Do a **Gram stain** on the unknown (see Lab 6). Make sure you **review the instructions before you do the Gram stain**. Because Enterococci and Staphylococci can sometimes look similar in Gram stains done from a plate culture, **perform a catalase test on your unknown** to help differentiate an *Enterococcus* from a *Staphylococcus* (see **Lab 8**).

2. On the bottom of the petri plate, **divide the plate into thirds with your wax marker and label as shown below**. Before you streak your plate **draw an "X" on the bottom of the blood agar plate in sector 2** to indicate where you will eventually place the Taxo NB disk as indicated in the illustration below.



3. Using your inoculating loop, streak your unknown **for isolation** on a plate of **Blood agar** as described below and shown in **Fig. 2**.

   a. Using a **sterile inoculating loop**, streak your unknown for isolation on a blood agar plate so as to get **single, isolated colonies** as shown in **Fig. 2, step 1**, **Fig. 2, step 2**, and **Fig. 2, step 3**.

   b. Using your inoculating loop, **stab** the agar several times in each of the 3 growth as shown in **Fig. 2, step 4**. This detects oxygen sensitive hemolysins.

   c. Place a **novobiocin antibiotic disc in the center of the area of the plate where you drew the "X")** as shown in **Fig. 2, step 5**. Tap it lightly with your loop so that the disk sticks to the agar.

   d. Incubate **upside down and stacked in the petri plate holder on the shelf of the 37°C incubator corresponding to your lab section** until the next lab period.

4. Streak your unknown **for isolation** on a plate of **Mannitol Salt agar (MSA)** as shown in **Fig. 3**. Incubate **upside down and stacked in the petri plate holder on the shelf of the 37°C incubator corresponding to your lab section**.

5. Inoculate a tube of **citrated rabbit plasma** with your unknown and incubate in your test tube rack **at 37°C**.

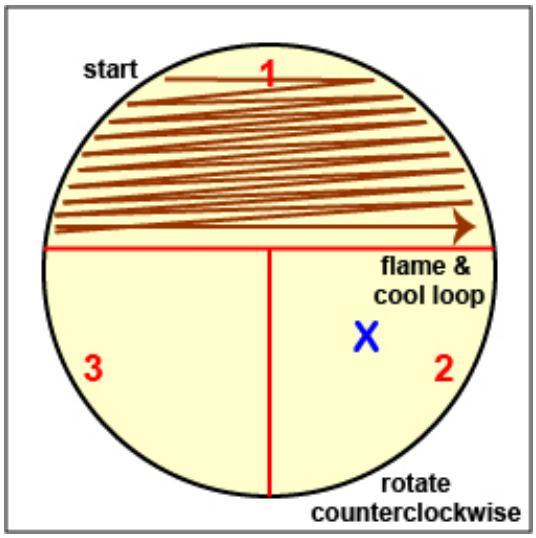
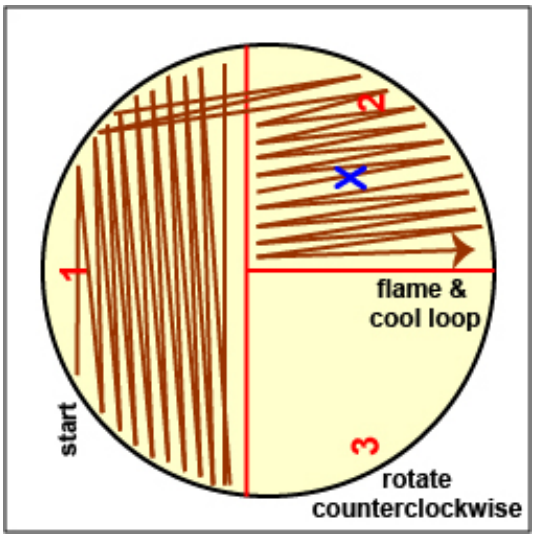
Fig. 2, step 1 Inoculating a Blood Agar Plate with your unknown	Fig. 2, step 2 Inoculating a Blood Agar Plate with your unknown
 Using a sterile inoculating loop, streak one-third of the blood agar plate with your unknown. Flame the loop and let it cool.	 Using a sterile inoculating loop, spread out some of the bacteria in area 1 over area 2. Flame the loop and let it cool.

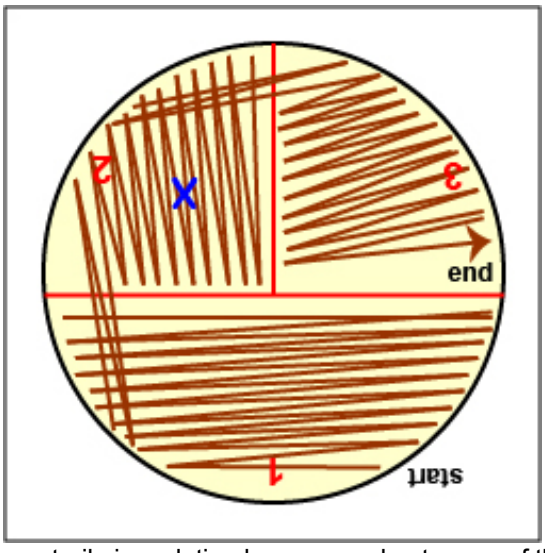
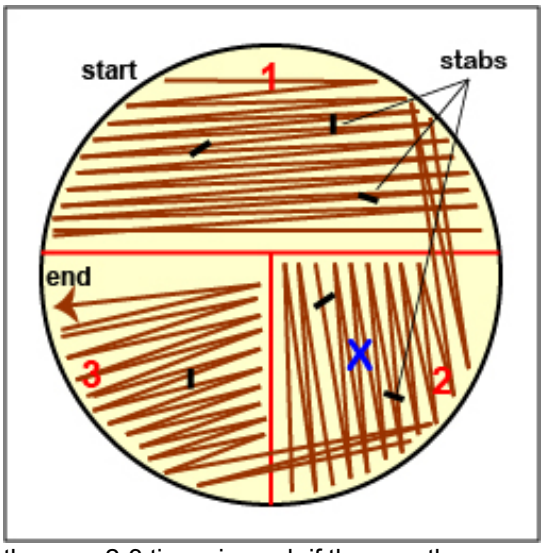
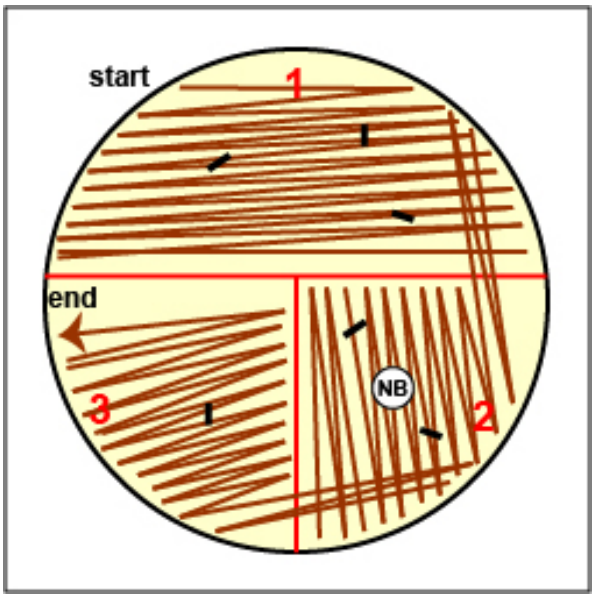
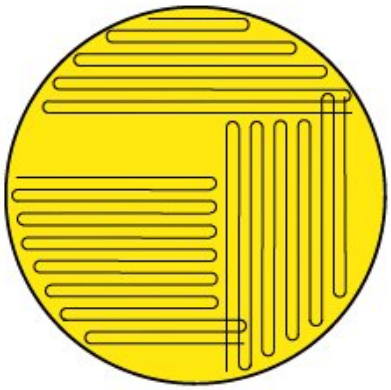
Fig. 2, step 3 Inoculating a Blood Agar Plate with your Unknown	Fig. 2, step 4 Inoculating a Blood Agar Plate with your Unknown
 Using a sterile inoculating loop, spread out some of the bacteria in area 2 over area 3.	 Stab the agar 2-3 times in each if the growth areas.

Fig. 2, step 5
Inoculating a Blood Agar Plate with your Unknown



Place a Taxo NB disc in the area that you first streaked with the loop.

Fig. 3:
Inoculation of Mannitol Salt agar.



RESULTS

Case Study Lab Report for Lab 15: Staphylococci

The concept behind the case studies presented in Lab 15 used to illustrate the genus *Staphylococcus* is for you and your lab partners as a group to:

1. **Come up with a valid diagnosis of the infectious disease seen in your case study and identify the bacterium causing that infection**; and

2. **Support your group diagnose** based on:

   a. Any relevant facts in the **patient's history**. (A reliable on-line source will be used to support this.)
   b. The patient's **signs and symptoms.** (A reliable on-line source will be used to support this.)
   c. **Each of the individual lab tests** given in your case study.
   d. All **microbiological lab tests you performed** as part of the project.

The due date for this report can be found on the class calendar. Your grade for this lab is based on the **completeness of your report and written evidence of the critical thinking process that went into making and supporting your diagnosis.** Remember, you are trying to convince your instructor that you understand how the diagnosis was made by **supporting that diagnosis with data.**

Grading:

The lab 15 Lab Report is worth 30 points.

These case studies are based in part on your in-class participation as part of your group. Therefore:

a. **If you were not in lab when the inoculations with your unknown were performed, 3 points will be deducted from your Lab Report score for labs 12, 14, and 15; 6 points from your Lab Report score for the Final Project.**

b. **If you were not in lab when the results of your lab tests were observed, 3 points will be deducted from your Lab Report score for labs 12, 14, and 15; 6 points from your Lab Report score for the Final Project**.

c. **For each day your Lab Report is late, 2 points will be deducted from your Lab Report score for labs 12, 14, and 15; 4 points from your Lab Report score for the Final Project.**

Each person in the group must write and submit their own individual report and it cannot be the same as that of other members of that group.

The rubric that will be used to grade this lab report can be found under this Assignment on Blackboard.

Be sure to handle all the bacterial cultures you are using in lab today as if they are pathogens! Be sure to wash and sanitize your hands well at the completion of today's lab.
Also, make sure you **observe the results of someone in your lab who had an unknown different from yours**. The Performance Objectives for Lab 15 tell you what you are expected to be able to do on the practical.

A. Case Study from Lab 15: Unknown #1

Your Name:

Others in your group:

Lab section:

Date:

Case Study

57-year old female who is diabetic, a long-time smoker, and who 28 days ago had hip replacement surgery presents with swelling, pain, inflammation, and erythema at the surgical site. Examination shows she has a fever of 101°F, is exhibiting malaise, and has an increased total white blood cell count with a left shift. Ultrasonography examination indicates a deep abscess. A culture from an aspiration of the infected surgical site was taken.

Assume that unknown you are given is the culture from this patient.

LABORATORY 15:
STAPHYLOCOCCI

1. Patient's history and predisposing factors

Read the case study. Explain how any relevant parts of the patient's history contributed to your diagnosis of the type of infectious disease seen here. You are urged to use the computers in lab to search reliable medically oriented Internet sources to support this. Reliable sources you might consider are Medscape (http://emedicine.medscape.com/infectious_diseases) and The Centers for Disease Control and Prevention (CDC) at http://www.cdc.gov/. Cite any sources you use at the end of this Patient's History section in APA style (http://www.apastyle.org/).

The **patient's history should suggest a general type of infectious disease** that is present, such as a urinary tract infection, a wound infection, gastroenteritis, pharyngitis, pneumonia, septicemia, etc. **Do not look up the bacterium you eventually identify** as the cause of this infectious disease. You do not know the causative bacterium at this point. **You need to determine the general type of infection to determine what microbiological tests to perform to identify the bacterium causing the infection**. Search at least one medically oriented reference article from a reliable site such as *Medscape* and **use this article to support your diagnosis the type of infectious disease seen here**. Don't forget to **cite any sources you used in APA style directly under this Patient's History and Patient's Symptoms sections** of this Lab Report.

2. Patient's signs and symptoms

Read the case study. Explain how the patient's signs and symptoms contributed to your diagnosis of the type of infectious disease seen here. You are urged to use the computers in lab to search reliable Internet sources to support this. Reliable sources you might consider are *Medscape* (http://emedicine.medscape.com/infectious_diseases) and the Centers for Disease Control and Prevention (CDC) at http://www.cdc.gov/. Cite any sources you use at the end of this Patient's History section in APA style (http://www.apastyle.org/).

The **patient's signs and symptoms should suggest a general type of infectious disease** that is present, such as a urinary tract infection, a wound infection, gastroenteritis, strep throat, pneumonia, septicemia, etc. **Do not look up the bacterium you eventually identify** as the cause of this infectious disease. You do not know the causative bacterium at this point. **You need to determine the general type of infectious disease present to determine what microbiological tests to perform to identify the bacterium causing the infection**. Search at least one medically oriented reference article from a reliable site such as *Medscape* and **use this article to support your diagnosis the type of infectious disease seen here**. Don't forget to **cite any sources you used in APA style under this Patient's History and Patient's Symptoms sections** of this Lab Report.

3. Vocabulary list for medical terms used in the case study under signs and symptoms

List and define any medical terms used in your case study that describe the patients's signs and symptoms that the average person not in the medical profession might not know.

4. Results of laboratory test given in the case study

List each lab test given and explain how the results of that test helps to contribute to your diagnosis. The CBC test is described in Appendix C of this lab manual.

5. Microbiological lab tests you performed in Lab 15

a. **Gram stain and catalase test results**

Give the Gram reaction (Gram-positive or Gram-positive and how you reached this conclusion) and the shape and arrangement of the unknown you were given. **Because Enterococci and Staphylococci can sometimes look similar in Gram stains done from a plate culture, perform a** catalase test **on your unknown to help differentiate an *Enterococcus* from a *Staphylococcus*. State how this contributed to your decision as to which microbiological tests and/or media to use next**.The Gram stain is discussed in Lab 6.

b. **Blood agar with novobiocin (NB) disc**

Give the results of the Blood agar with Taxo NB disc you performed on the unknown you were given, and how you reached this conclusion. **State how this contributed to your your decision as to what bacterium is causing the infection**. The possible results for Blood agar and NB disc were discussed in the beginning pages of this lab.

c. **Mannitol Salt agar**

Give the results of the Mannitol Salt agar you performed on the unknown you were given, and how you reached this conclusion. **State how this contributed to your your decision as to what bacterium is causing the infection**. The possible results for Mannitol Salt agar were discussed in the beginning pages of this lab.

d. **Coagulase test**

Give the results of the Coagulase test you performed on the unknown you were given, and how you reached this conclusion. **State how this contributed to your decision as to what bacterium is causing the infection**. The possible results for the Coagulase test were discussed in the beginning pages of this lab.

Final Diagnosis

Genus and species of unknown #1 = _____

Infection: _____

B. Case Study from Lab 15: Unknown #2

Your Name:

Others in your group:

Lab section:

Date:

Case Study

A 57-year old female who is diabetic, a long-time smoker, and who 28 days ago had hip replacement surgery presents with swelling, pain, inflammation, and erythema at the surgical site. Examination shows she has a fever of 101°F, is exhibiting malaise, and has an increased total white blood cell count with a left shift. Ultrasonography examination indicates a deep abscess. A culture from an aspiration of the infected surgical site was taken.

Assume that unknown you are given is the culture from this patient.

LABORATORY 15:
STAPHYLOCOCCI

1. Patient's history

Read the case study. Explain how any relevant parts of the patient's history contributed to your diagnosis. You are urged to use the computers in lab to search reliable medically oriented Internet sources to support this. Reliable sources you might consider are Medscape (http://emedicine.medscape.com/infectious_diseases) and The Centers for Disease Control and Prevention (CDC) at http://www.cdc.gov/. Cite any sources you use at the end of this Patient's History section in APA style (http://www.apastyle.org/). The **patient's history and patient's symptoms should suggest a general type of infection**, such as a urinary tract infection, a wound infection, gastroenteritis, strep throat, pneumonia, septicemia, etc. Search at least one medically oriented reference article from a reliable site such as *Medscape* and **use this article to support your diagnosis of the type of infection**. Don't forget to **cite any sources you used in APA style under the Patient's History and Patient's Symptoms sections** of your Lab Report.

2. Patient's symptoms

Read the case study. Explain how the patient's symptoms contributed to your diagnosis. You are urged to use the computers in lab to search reliable Internet sources to support this. Reliable sources you might consider are *Medscape* (http://emedicine.medscape.com/infectious_diseases) and the Centers for Disease Control and Prevention (CDC) at http://www.cdc.gov/. Cite any sources you use at the end of this Patient's History section in APA style (http://www.apastyle.org/). The **patient's history and patient's symptoms should suggest a general type of infection**, such as a urinary tract infection, a wound infection, gastroenteritis, strep throat, pneumonia, septicemia, etc. Search at least one medically oriented reference article from a reliable site such as *Medscape* and **use this article to support your diagnosis of the type of infection**. Don't forget to **cite any sources you used in APA style under the Patient's History and Patient's Symptoms sections** of your Lab Report.

3. Vocabulary list for medical terms used in the case study under signs and symptoms

List and define any medical terms used in your case study that describe the patients's signs and symptoms that the average person not in the medical profession might not know.

4. Results of laboratory test given in the case study

List each lab test given and explain how the results of that test helps to contribute to your final diagnosis. The CBC test is described in Appendix C of this lab manual.

5. Microbiological lab tests you performed in Lab 15

a. **Gram stain and catalase test**

Give the Gram reaction (Gram-positive or Gram-positive and how you reached this conclusion) and the shape and arrangement of the unknown you were given. **Because Enterococci and Staphylococci can sometimes look similar in Gram stains done from a plate culture, perform a** catalase test **on your unknown to help differentiate an *Enterococcus* from a *Staphylococcus*. State how this contributed to your diagnosis and choice of microbiological tests and/or media to perform next**. The Gram stain is discussed in Lab 6.

b. **Blood agar with novobiocin (NB) disc**

Give the results of the Blood agar with Taxo NB disc you performed on the unknown you were given, and how you reached this conclusion. **State how this contributed to your final diagnosis**. The possible results for Blood agar and NB disc were discussed in the beginning pages of this lab.

c. **Mannitol Salt agar**

Give the results of the Mannitol Salt agar you performed on the unknown you were given, and how you reached this conclusion. **State how this contributed to your final diagnosis**. The possible results for Mannitol Salt agar were discussed in the beginning pages of this lab.

d. **Coagulase test**

Give the results of the Coagulase test you performed on the unknown you were given, and how you reached this conclusion. **State how this contributed to your final diagnosis**. The possible results for the Coagulase test were discussed in the beginning pages of this lab.

Final Diagnosis

Genus and species of unknown #2 = _____

Infection: _____

PERFORMANCE OBJECTIVES
LABORATORY 15

After completing this lab, the student will be able to perform the following objectives:

DISCUSSION

1. Name three common clinically important species of *Staphylococcus* and state which species is most pathogenic.
2. State two sources and the portal of entry for most *Staphylococcus aureus* infections.
3. Name and describe three types of abscesses caused by *Staphylococcus aureus*.
4. Name four systemic *Staphylococcus aureus* infections.
5. State the significance of *Staphylococcus aureus* enterotoxin, the exotoxin TSST-1, and the exotoxin exfoliatin.
6. Name the infection normally caused by *Staphylococcus saprophyticus*.
7. Name the types of infections most commonly caused by coagulase-negative staphylococci other than *Staphylococcus saprophyticus*.

ISOLATION AND IDENTIFICATION OF STAPHYLOCOCCI

1. State the Gram reaction and morphology of all staphylococci.
2. Describe the typical reactions of *S. aureus, S. epidermidis,* and *S. saprophyticus* on each of the following media:
    a. Blood agar (pigment, hemolysis, novobiocin resistance)
    b. Mannitol Salt agar (for mannitol fermentation)
    c. coagulase test with citrated rabbit plasma

RESULTS

1. Recognize staphylococci in a Gram stain preparation.
2. Recognize an organism as *Staphylococcus aureus* and state the reasons why after seeing the results of the following:
    a. a Blood agar plate with a novobiocin disc
    b. a Mannitol Salt agar plate
    c. a tube of citrated rabbit plasma

LABORATORY 16
SEROLOGY:
DIRECT AND INDIRECT SEROLOGIC TESTING

A. Introduction to Serologic Testing
B. Direct Serologic Testing: Using Antigen-Antibody Reactions in the Laboratory to Identify Unknown Antigens
C. Indirect Serologic Testing: Using Antigen-Antibody Reactions in the Laboratory to Indirectly Diagnose Disease by Detecting Antibodies in a Person's Serum Produced Against a Disease Antigen

DISCUSSION

A. INTRODUCTION TO SEROLOGIC TESTING

The adaptive immune responses refer to the **ability of the body (self) to recognize specific foreign antigens (non-self)** that threaten its biological integrity. There are two major branches of the adaptive immune responses:

1. Humoral immunity: humoral immunity involves the production of antibody molecules in response to an antigen and is mediated by B-lymphocytes.

2. Cell-mediated immunity: Cell-mediated immunity involves the production of cytotoxic T-lymphocytes, activated macrophages, activated NK cells, and cytokines in response to an antigen and is mediated by T-lymphocytes.

To understand the immune responses we must first understand what is meant by the term antigen. Technically, an **antigen** is defined as **a substance that reacts with antibody molecules and antigen receptors on lymphocytes**. An **immunogen** is **an antigen that is recognized by the body as nonself and stimulates an adaptive immune response**. For simplicity, both antigens and immunogens are usually referred to as antigens.

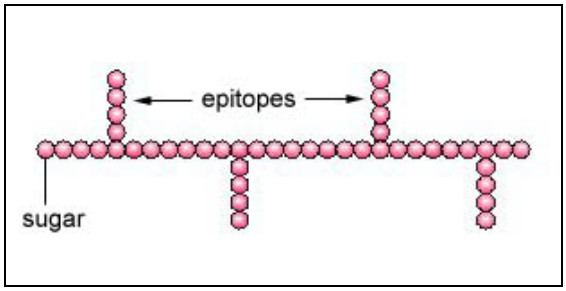
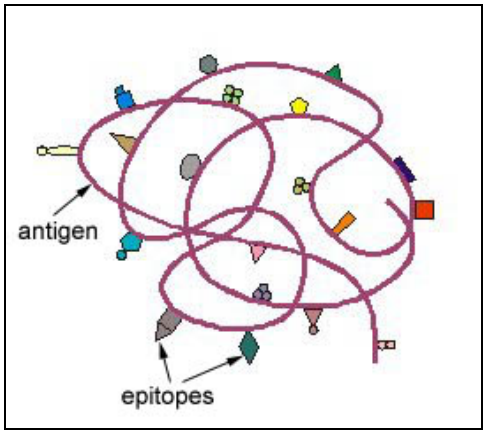
Chemically, **antigens are large molecular weight proteins** (including conjugated proteins such as glycoproteins, lipoproteins, and nucleoproteins) **and polysaccharides** (including lipopolysaccharides). These protein and polysaccharide antigens are found on the surfaces of viruses and cells, including microbial cells (bacteria, fungi, protozoans) and human cells.

As mentioned above, the B-lymphocytes and T-lymphocytes are the cells that carry out adaptive immune responses. The body recognizes an antigen as foreign when that antigen binds to the surfaces of B-lymphocytes and T-lymphocytes by way of antigen-specific receptors having a shape that corresponds to that of the antigen (similar to interlocking pieces of a puzzle). The antigen receptors on the surfaces of B-lymphocytes are antibody molecules called B-cell receptors or sIg; the receptors on the surfaces of T-lymphocytes are called T-cell receptors (TCRs).

The **actual portions or fragments of an antigen that react with receptors on B-lymphocytes and T-lymphocytes, as well as with free antibody molecules, are called epitopes**. The size of an epitope is generally thought to be equivalent to 5-15 amino acids or 3-4 sugar residues. Some antigens, such as polysaccharides, usually have many epitopes, but all of the same specificity. This is because polysaccharides may be composed of hundreds of sugars with branching sugar side chains, but usually contain only one or

two different sugars. As a result, most "shapes" along the polysaccharide are the same (see Fig. 1). Other antigens such as proteins usually have many epitopes of different specificities. This is because proteins are usually hundreds of amino acids long and are composed of 20 different amino acids. Certain amino acids are able to interact with other amino acids in the protein chain and this causes the protein to fold over upon itself and assume a complex three-dimensional shape. As a result, there are many different "shapes" on the protein (see Fig. 2). That is why proteins are more immunogenic than polysaccharides; they are chemically more complex.

A microbe, such as a single bacterium, has many different proteins (and polysaccharides) on its surface that collectively form its various structures, and each different protein may have many different epitopes. Therefore, **immune responses are directed against many different parts or epitopes of the same microbe**.

Fig. 1: Epitopes of a polysaccharide antigen.	**Fig. 2:** Epitopes of a protein antigen.
	

In terms of infectious diseases, the following may act as antigens:

1. **Microbial structures** (cell walls, capsules, flagella, pili, viral capsids, envelope-associated glycoproteins, etc.); and

2. **Microbial toxins**

Certain **non-infectious materials** may also act as antigens if they are recognized as "nonself" by the body. These include:

1. **Allergens** (dust, pollen, hair, foods, dander, bee venom, drugs, and other agents causing allergic reactions);

2. **Foreign tissues and cells** (from transplants and transfusions); and

1. **The body's own cells that the body fails to recognize as "normal self"** (cancer cells, infected cells, cells involved in autoimmune diseases).

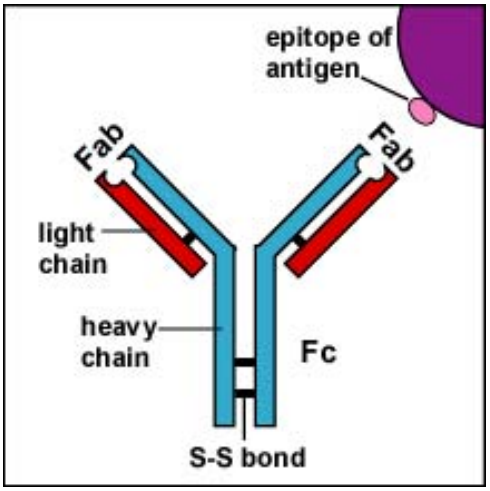
Antibodies or immunoglobulins are specific protein configurations produced by B-lymphocytes and plasma cells **in response to a specific antigen and capable of reacting with that antigen**. Antibodies are produced in the **lymphoid tissue** and once produced, are found mainly in the **plasma** portion of the blood (the liquid fraction of the blood before clotting). **Serum** is the liquid fraction of the blood after clotting.

There are 5 classes of human antibodies: IgG, IgM, IgA, IgD, and IgE. The simplest antibodies, such as IgG, IgD, and IgE, are "Y"-shaped macromolecules called monomers composed of **four glycoprotein chains**. There are two identical **heavy chains** having a high molecular weight that varies with the class of antibody. In addition, there are two identical **light chains** of one of two varieties: kappa or gamma. The light chains have a lower molecular weight. The four glycoprotein chains are connected to one another by disulfide (S-S) bonds and noncovalent bonds (see Fig. 3A). Additional S-S bonds fold the individual glycoprotein chains into a number of distinct globular domains. The area where the top of the "Y" joins the bottom is called the hinge. This area is flexible to enable the antibody to bind to pairs of epitopes various distances apart on an antigen.

The two tips of the "Y" monomer are referred to as the **Fab portions** of the antibody (see Fig. 3A). The first 110 amino acids or first domain of both the heavy and light chain of the Fab region of the antibody **provide specificity for binding an epitope on an antigen**. The Fab portions provide specificity for binding an epitope on an antigen. The bottom part of the "Y" is called the **Fc portion** and this part is responsible for the **biological activity** of the antibody (see diaGram of IgG; Fig. 3A). Depending on the class of antibody, biological activities of the Fc portion of antibodies include the ability to activate the complement pathway (IgG & IgM), bind to phagocytes (IgG, IgA), or bind to mast cells and basophils (IgE).

Two classes of antibodies are more complex. IgM is a pentamer (see Fig. 3B), consisting of 5 "Y"-like molecules connected at their Fc portions, and secretory IgA is a dimer consisting of 2 "Y"-like molecules (see Fig. 3C).

Fig. 3:
Antibody structure (IgG)



For more information on antigens, antibodies, and antibody production, see the following Learning Objects in your Lecture Guide:

Serology refers to using antigen-antibody reactions in the laboratory for **diagnostic purposes**. Its name comes from the fact that **serum, the liquid portion of the blood where antibodies are found** is used in testing. **Serologic testing** may be used in the clinical laboratory in two distinct ways:

Serologic testing may be used in the clinical laboratory in two distinct ways:

a. **To identify unknown antigens such as microorganisms**. This is called **direct serologic testing**. Direct serologic testing **uses a preparation known antibodies, called antiserum, to identify an unknown antigen** such as a microorganism.

b. **To detect antibodies being made against a specific antigen in the patient's serum**. This is called **indirect serologic testing**. Indirect serologic testing is the procedure by which **antibodies in a person's serum being made by that individual against an antigen associated with a particular disease are detected using a known antigen**.

B. DIRECT SEROLOGIC TESTING: USING ANTIGEN-ANTIBODY REACTIONS IN THE LABORATORY TO IDENTIFY UNKNOWN ANTIGENS SUCH AS MICROORGANISMS.

This type of serologic testing uses a **known antiserum (serum containing specific known antibodies)**. The preparation of known antibodies is prepared in one of two ways: in animals or by hybridoma cells.

1. Preparation of known antisera in animals.

Preparation of known antiserum in animals involves inoculating animals with specific known antigens such as a specific strain of a bacterium. After the animal's immune responses have had time to produce antibodies against that antigen, the animal is bled and the blood is allowed to clot. The resulting liquid portion of the blood is the serum and it will contain antibodies specific for the injected antigen.

However, one of the problems of using antibodies prepared in animals (by injecting the animal with a specific antigen and collecting the serum after antibodies are produced) is that up to 90% of the antibodies in the animal's serum may be antibodies the animal has made "on its own" against environmental antigens, rather than those made against the injected antigen. The development of monoclonal antibody technique has largely solved that problem.

2. Preparation of known antibodies by monoclonal antibody technique.

One of the major breakthroughs in immunology occurred when monoclonal antibody technique was developed. **Monoclonal antibodies** are antibodies of a single specific type. In this technique, an animal is injected with the specific antigen for the antibody desired. After appropriate time for antibody production, the animal's spleen is removed. The spleen is rich in plasma cells and each plasma cell produces only one specific type of antibody. However, plasma cells will not grow artificially in cell culture. Therefore, **a plasma cell producing the desired antibody is fused with a myeloma cell** (a cancer cell from bone marrow which will grow rapidly in cell culture) to produce a **hybridoma cell**. The hybridoma cell has the characteristics of both parent cells. It will **produce the specific antibodies like the plasma cell and will also grow readily in cell culture like the myeloma cell**. The hybridoma cells are grown artificially in huge vats where they produce large quantities of the specific antibody (**see Fig. 4**).

Monoclonal antibodies are now used routinely in medical research and diagnostic serology and are being used experimentally in treating certain cancers and a few other diseases.

Fig. 4:
Preparation of monoclonal antibodies.

Fig. 4, Step 1

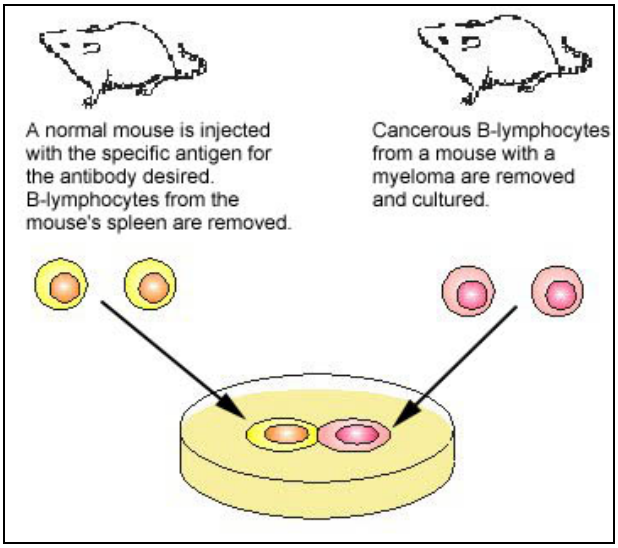


Fig. 4, Step 2

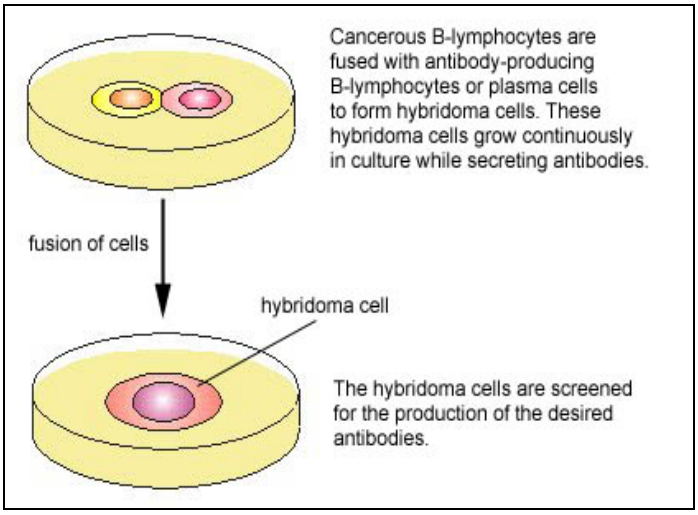
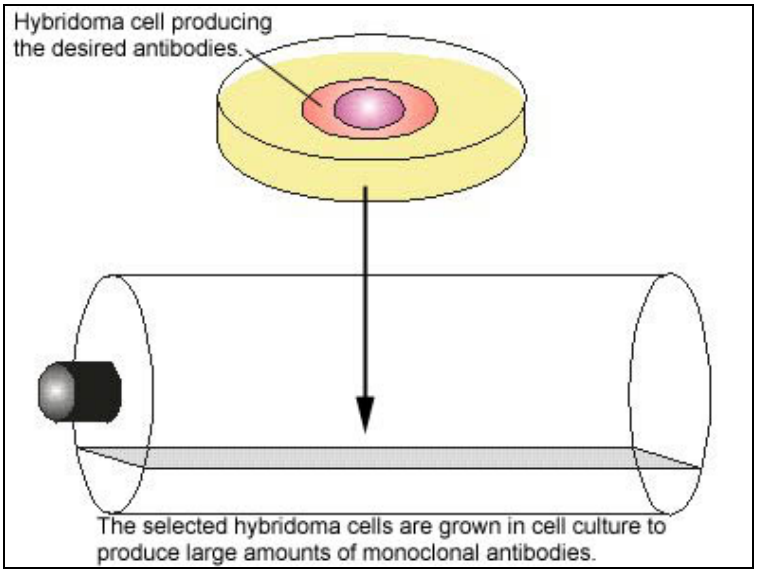


Fig. 4, Step 3



3. The concept and general procedure for direct serologic testing.

The concept and general procedure for using antigen-antibody reactions to identify unknown antigens are as follows:

Concept:

This testing is based on the fact that **antigen- antibody reactions are very specific**. Antibodies usually react **only with the antigen that stimulated their production in the first place** and are just as specific as an enzyme-substrate reaction. Because of this, one can use **known antiserum** (prepared by animal inoculation or monoclonal antibody technique as discussed above) to identify **unknown antigens such as a microorganism.**

General Procedure:

A suspension of **the unknown antigen** to be identified is mixed with **known antiserum** for that antigen. One then looks for an antigen-antibody reaction.

Examples of serologic tests used to identify unknown microorganisms include the serological typing of *Shigella* and *Salmonella*, the Lancefield typing of beta streptococci, and the serological identification of *Neisseria gonorrhoeae* and *Neisseria meningitidis*. Serological tests used to identify antigens that are not microorganisms include blood typing, tissue typing, and pregnancy testing.

4. Examples of direct serologic testing to identify unknown antigens

As stated above, this type of serologic testing uses **known antiserum (antibodies) to identify unknown antigens**. Four such tests will be looked at in lab today.

a. **Serological Typing of *Shigella***

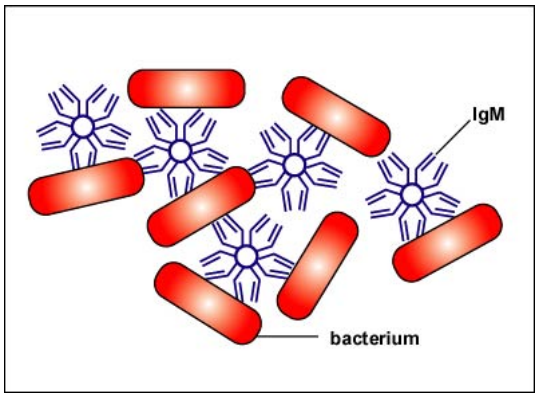
Discussion

There are **four different serological subgroups of *Shigella***, each corresponding to a different species:

subgroup A = *Shigella dysenteriae*
subgroup B = *Shigella flexneri*
subgroup C = *Shigella boydii* subgroup D = *Shigella sonnei*

Known antiserum is available for each of the 4 subgroups of *Shigella* listed above and contains antibodies against the cell wall ("O" antigens) of *Shigella.* The suspected *Shigella* (the unknown antigen) is placed in each of 4 circles on a slide and a different known antiserum (A, B, C or D) is then added to each circle. A positive antigen-antibody reaction appears as a clumping or agglutination of the *Shigella* (**Fig. 5**).

Fig. 5:
Agglutination of *Shigella* using known antiserum.



b. **Serological Typing of Streptococci**

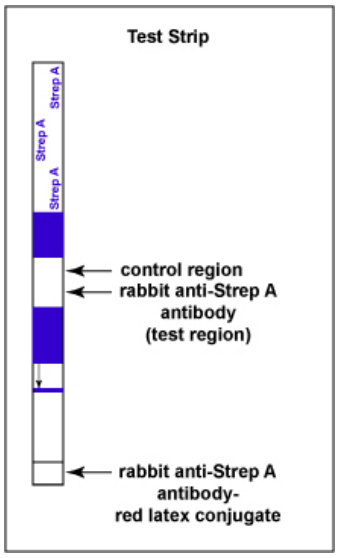
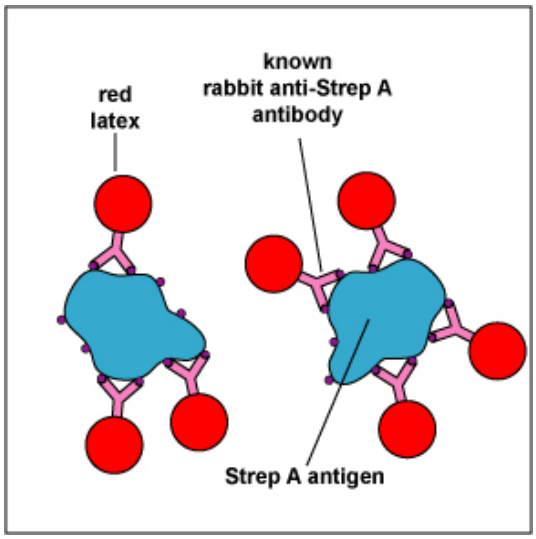
Discussion

The **Clearview® Strep A Exact II Dipstick** is a qualitative serologic test for **detecting Group A Streptococcal antigen** (the unknown antigen) directly from throat swabs and is used as an aid in **diagnosing streptococcal pharyngitis** caused by *Streptococcus pyogenes* (Group A Beta Streptococci).

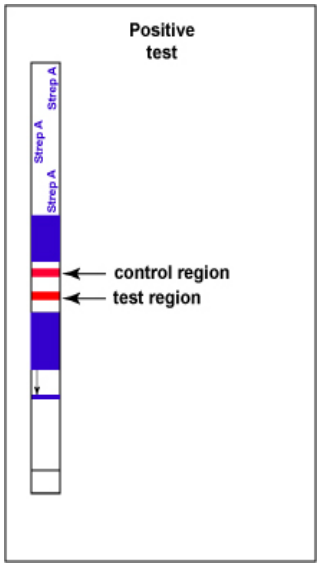
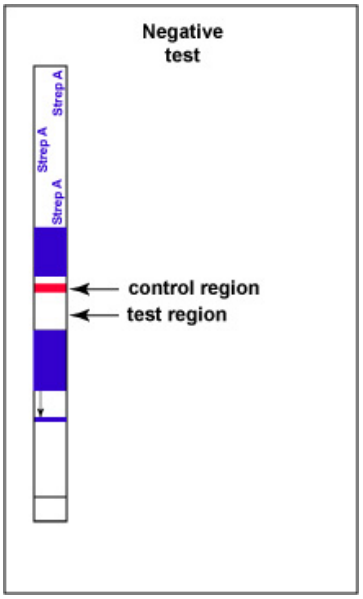
The test consists of a membrane strip that is precoated with **rabbit anti-Strep A antibody-red latex conjugate (known antibody with red latex particles attached) located in a pad** at the beginning of the strip. It is also precoated with **rabbit anti-Strep A antibody** (known antibody without attached red latex) that is immobilized at the test line where the test results are read (see Fig. 6A). The **red latex particles** attached to the rabbit anti-Strep A antibody is what ultimately causes the “positive” red band.

When the test strip is immersed in the extracted sample, the **Group A Streptococcal antigen** extracted from the *Streptococcus pyogenes* on the throat swab of a person with strep throat begins to move chromatographically up the membrane and **binds to the red-colored known antibody-latex conjugate in the pad** located at the beginning of the strip, forming a Strep A antigen-antibody complex (see Fig. 6B). This Strep A antigen-antibody complex continues to moves up the membrane to the test line region where the immobilized rabbit anti-Strep A antibodies are located.

If Group A Streptococcal antigen is present in the throat swab, a red-colored sandwich of antibody/Strep A antigen/red latex conjugate antibody forms in the test result region of the strip (see Fig. 6C). **The red color at the control line region lets you know the test is finished**. As a result, **a positive test for Group A Strep antigen appears as a red band in the test result area and a red band in the control area** (see Fig. 6C).

Fig. 6A: Strep A Test Strip	**Fig. 6B:** Strep A Antigen/Gold Conjugate Antibody Complex
	

If there is no Group A Streptococcal antigen present in the throat swab **no red band appears in the test result region** of the strip and **a single red band** appears in the control line region, **indicating a negative test for Group A Strep antigen** (see Fig. 6D).

Fig. 6C: Positive Strep A Antigen Test	**Fig. 6D:** Negative Strep A Antigen Test
	

c. **Serological Testing to Diagnose Pregnancy**

Discussion

The **Alere® hCG Dipstick** is a qualitative serologic test for detecting early pregnancy. The hormone **human chorionic gonadotropin (hCG), produced by the placenta, appears in the serum and urine of pregnant females**. The hCG is composed of two subunits - alpha and beta. The Alere® hCG Dipstick is a one step pregnancy test that detects levels of hCG as low as 25 mlU/ml. **Human chorionic gonadotropin (hCG), the unknown antigen** for which one is testing, is identified in the urine by using **known mouse monoclonal antibodies against the beta subunit of hCG bound to colloidal gold, which is red in color**. It also uses **known goat polyclonal antibodies against the alpha subunit of hCG which is bound to the test result region of the dipstick**.

Like the Strep A test mentioned above, this test uses a color immunochromatographic assay to detect the antigen-antibody reaction.The test consists of a membrane strip that is precoated with **known mouse anti- beta hCG antibody-colloidal gold conjugate (known antibody with red colloidal gold particles attached) located in a pad** at the beginning of the strip. It is also precoated with **known goat anti-alpha hCG antibody** (known antibody without attached red colloidal gold) that is immobilized at the test line where the test results are read (see Fig. 7B1). The red colloidal gold particles attached to the mouse anti-alpha hCG antibody is what ultimately causes the “positive” red band.

When the test strip is immersed in the urine sample, the **hCG** begins to move chromatographically up the membrane and **binds to the red-colored known anti-beta hCG antibody-gold conjugate in the pad** located at the beginning of the strip, forming a hCG antigen-antibody complex (see Fig. 7B2). This hCG antigen-antibody complex continues to moves up the membrane to the test line region where the immobilizedknown goat anti-beta hCG antibodies are bound.

If hCG is present in the urine, a red-colored sandwich of anti-beta antibody/hCG antigen/red gold conjugate anti-alpha antibody forms in the test line region of the strip (see Fig. 7B3). **The red color at the control line region appears when enough reagent has reached the control area and indicates that the test is finished**. As a result, **a positive test for hCG antigen appears as a red band in the test result area and a red band in the control area** (see Fig. 7B3).

If there is **no detectable hCG antigen** present in the urine **no red band appears in the test result region** of the strip and **a single red band** appears in the control line region, **indicating a negative test for hCG antigen** (see Fig. 7B4).

Fig. 7B1: Serologic Testing to Detect hCG Antigen: The hCG Test Dipstick

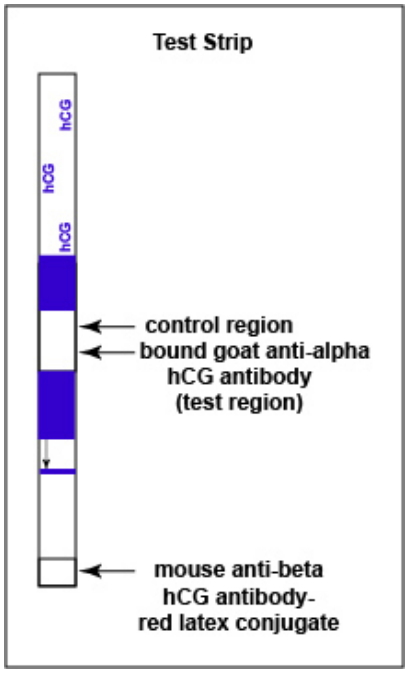
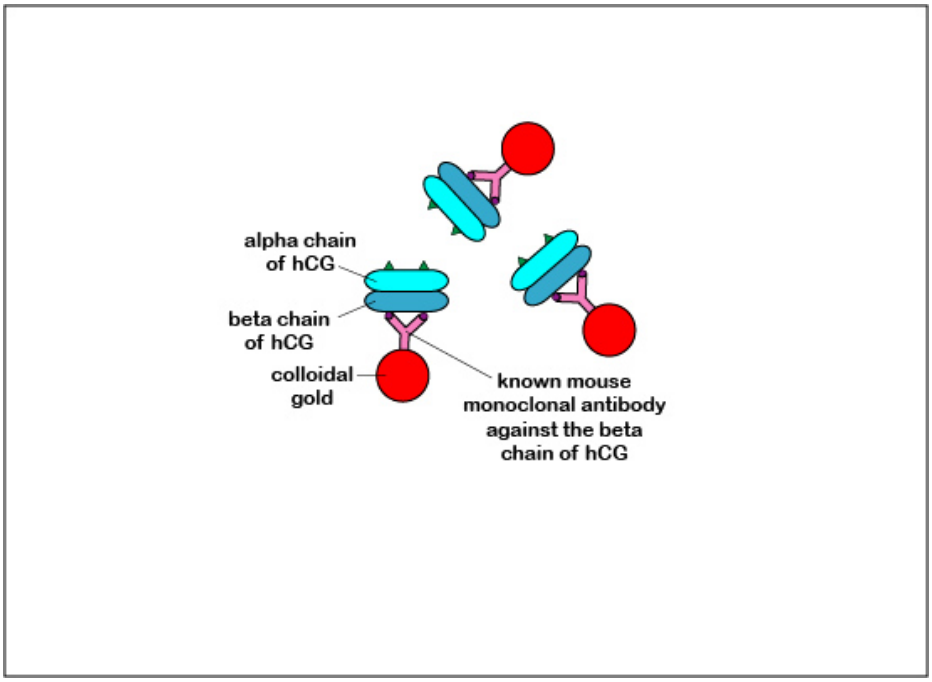
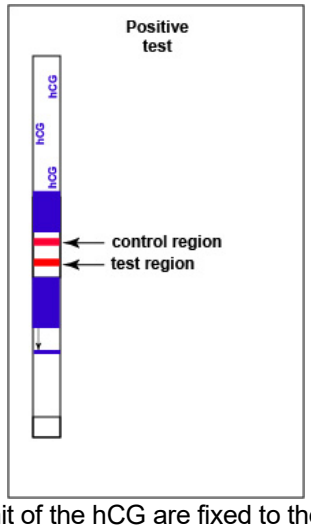


Fig. 7B2: Serologic Testing to Detect hCG Antigen



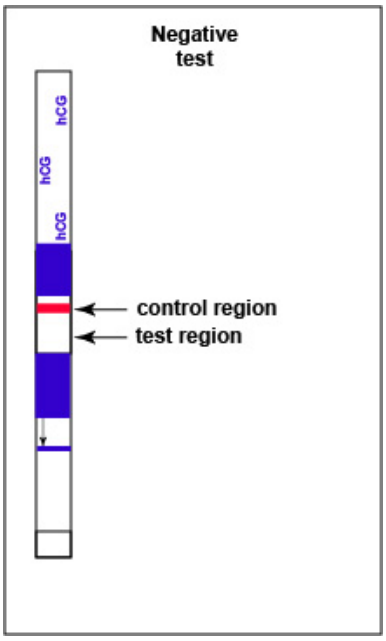
Known antibodies against the beta subunit of the hCG in the urine react with the beta chain of the hCG. Red colloidal gold particles are attached to these antibodies.

Fig. 7B3: Serologic Testing to Detect hCG Antigen: A Positive Test



Known antibodies against the alpha subunit of the hCG are fixed to the membrane on the Test region of the results window. As the hCG molecules with bound anti-beta hCG antibody and red colloidal gold move up the membrane with the urine, the alpha subunit of the hCGs bind to the fixed antibodies on the membrane creating the a pink-to-red horizontal line indicating a positive test.

Fig. 7B4: Serologic Testing to Detect hCG Antigen: A Negative Test



d. **Identification of Microorganisms Using the Direct Fluorescent Antibody Technique**

Discussion

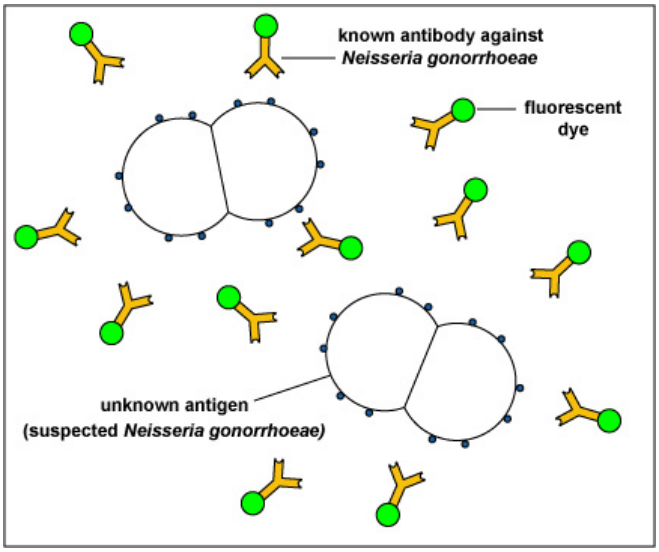
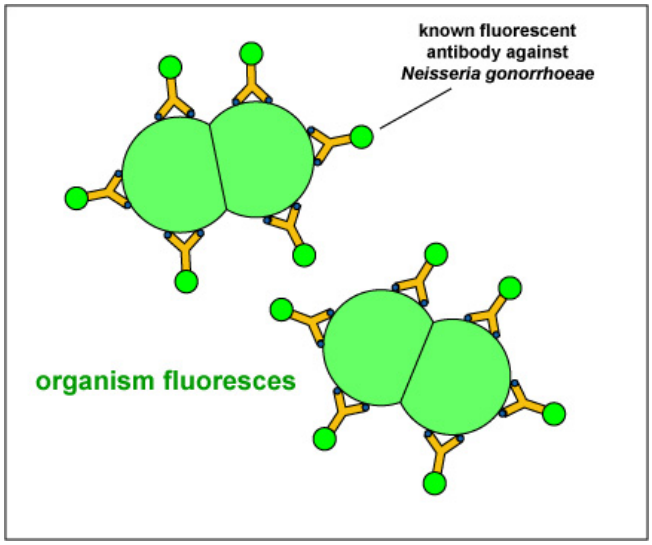
Certain **fluorescent dyes** can be chemically **attached to the known antibody molecules** in antiserum. The **known fluorescent antibody** is then mixed with the **unknown antigen** (such as a microorganism) fixed to a slide. After washing, to remove any fluorescent antibody not bound to the antigen, the slide is viewed with a **fluorescent microscope**. If the fluorescent antibody reacted with the unknown antigen, the antigen will glow or **fluoresce** under the fluorescent microscope. If the fluorescent antibody did not react with the antigen, the antibodies will be washed off the slide and the antigen will not fluoresce.

For example, in the direct fluorescent **antibody test for *Neisseria gonorrhoeae***, the unknown antigen, suspected *Neisseria gonorrhoeae*, is fixed to a microscope slide. Known fluorescent antibodies made against *N. gonorrhoeae* are then added (see Fig. 8, step 1) and the slide is then washed to remove any fluorescent antibody not bound to the antigen. The slide is then viewed under a fluorescent microscope.

If the unknown antigen is *Neisseria gonorrhoeae*, the known antibodies against *N. gonorrhoeae* with attached fluorescent dye will bind to the bacterium and will not wash off. The bacteria will fluoresce when viewed under a fluorescent microscope (see Fig. 8, step 2). If the unknown antigen is not *N. gonorrhoeae*, the known fluorescent antibodies against will wash off the slide and the bacteria will not fluoresce when viewed under a fluorescent microscope.

Many bacteria, viruses, and fungi can be identified using this technique.

Fig. 8:
Direct Fluorescent Antibody Test for *Neisseria gonorrhoeae*

Fig. 8, step 1	**Fig. 8, step 2**
	
The unknown antigen, suspected *Neisseria gonorrhoeae*, is fixed to a microscope slide. Known fluorescent antibodies made against *N. gonorrhoeae* are added. The slide is then washed and viewed under a fluorescent microscope.	If the unknown antigen is *Neisseria gonorrhoeae*, the known antibodies against *N. gonorrhoeae* with attached fluorescent dye will bind to the bacterium and will not wash off. The bacteria will fluoresce when viewed under a fluorescent microscope.

C. INDIRECT SEROLOGIC TESTING: USING ANTIGEN-ANTIBODY REACTIONS IN THE LABORATORY TO INDIRECTLY DIAGNOSE DISEASE BY DETECTING ANTIBODIES IN A PERSON'S SERUM PRODUCED AGAINST A DISEASE ANTIGEN

Indirect serologic testing is the procedure whereby **antibodies in a person's serum** being made by that individual against an antigen associated with a particular disease are detected using a **known antigen**.

1. The concept and general procedure for indirect serologic testing.

The concept and general procedure for this type of serological testing are as follows:

Concept:

:

This type of testing is based on the fact that **antibodies are only produced in response to a specific antigen**. In other words, a person will not be producing antibodies against a disease antigen unless that antigen is in the body stimulating antibody production.

General Procedure:

A sample of the **patient's serum** (the liquid portion of the blood after clotting and containing antibodies against the disease antigen if the person has or has had the disease) is mixed with the **known antigen** for that suspected disease. One then looks for an antigen-antibody reaction.

Examples of serologic tests to diagnose disease by the detection of antibodies in the patient's serum include the following: the various serological tests for syphilis or STS (such as the RPR, the VDRL, and the FTA-ABS tests), the tests for infectious mononucleosis, the tests for the Human Immunodeficiency Virus (HIV), the tests for systemic lupus erythematosus, and tests for variety of other viral infections.

2. Qualitative and quantitative serologic tests.

Indirect serologic tests may be qualitative or quantitative. A **qualitative** test only detects the presence or absence of specific antibodies in the patient's serum and is often used for screening purposes. A **quantitative** test gives the titer or amount of that antibody in the serum. **Titer indicates how far you can dilute the patient's serum and still have it contain enough antibodies to give a detectable antigen-antibody reaction**. In other words, the more antibodies being produced by the body, the more you can dilute the person's serum and still see a reaction. Quantitative serological tests are often used to follow the progress of a disease by looking for a rise and subsequent drop in antibody titer.

3. Examples of indirect serologic tests to detect antibodies in the patient's serum

a. The RPR Test for Syphilis

Discussion

Syphilis is a sexually transmitted disease caused by the spirochete *Treponema pallidum*. The **RPR** (Rapid Plasma Reagin) Card® test is a **presumptive serologic screening test** for syphilis. The serum of a person with syphilis contains a **nonspecific antilipid antibody** (traditionally termed **reagin**), which is not found in normal serum. The exact nature of the antilipid (reagin) antibody is not known but it is thought that a syphilis infection instigates the breakdown of the patient's own tissue cells. Fatty substances which are released then combine with protein from *Treponema pallidum* to form an antigen which stimulates the body to produce **antibodies against both the body's tissue lipids (nonspecific or nontreponemal) as well as the *T. pallidum* protein (specific or treponemal)**. The RPR Card® test detects the nonspecific antilipid antibody and is referred to as a **nontreponemal test for syphilis**.

It must be remembered that tests for the presence of these nonspecific anti-lipid antibodies are meant as a presumptive screening test for syphilis. Similar regain-like antibodies may also be present as a result of other diseases such as malaria, leprosy, infectious mononucleosis, systemic lupus erythematosus, viral pneumonia, measles, and collagen diseases and may give biologic false-positive results (BFP). Confirming tests should be made for the presence of specific antibodies against the *T. pallidum* itself. The confirming test for syphilis is the **FTA-ABS** test discussed below. Any serologic test for syphilis is referred to commonly as an **STS** (Serological Test for Syphilis).

The **known RPR antigen** consists of **cardiolipin, lecithin, and cholesterol bound to charcoal particles** in order to make the reaction visible to the naked eye. If the patient has

syphilis, the antilipid antibodies in his or her serum will cross-react with the known RPR lipid antigens giving a visible clumping of the charcoal particles (**see Fig. 1**).

We will do a **quantitative** RPR Card® test today in lab. Keep in mind that a quantitative test allows one to determine the **titer** or amount of a certain antibody in the serum. In this test, a constant amount of RPR antigen is added to dilutions of the patient's serum. **The most dilute sample of the patient's serum still containing enough antibodies to give a visible antigen-antibody reaction is reported as the titer**.

Fig. 1:
A positive RPR test.
Note clumping of carbon particles.

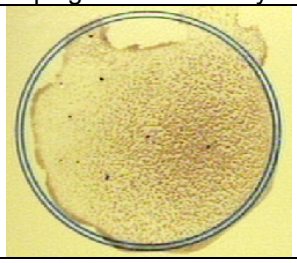
b. Serologic Tests for Infectious Mononucleosis

Discussion

During infectious mononucleosis, caused by the Epstein-Barr virus (EBV), **the body produces non-specific heterophile antibodies which are not found in normal serum**. As it turns out, these **heterophile antibodies will also cause horse, bovine, and sheep erythrocytes (red blood cells) to agglutinate** (see Fig. 2). The antigens located in the cell membrane of these erythrocytes that react with the heterophile antibodies present during infectious mononucleosis are called Paul-Bunnel antigens after their discoverers.

The infectious mononucleosis serologic test demonstrated today is a rapid **qualitative test** for infectious mononucleosis called the ASI Color Mono II Test®. The mono reagent in this test is a suspension of dyed, color-enhanced, preserved horse erythrocytes. **The Paul-Bunnel antigens in the cell membrane of these horse erythrocytes are highly specific for mononucleosis heterophile antibodies and act as the "known antigen."** Agglutination of the erythrocytes after adding the patient's serum indicates a positive test. Quantitative tests may be done to determine the titer of heterophile antibodies and follow the progress of the disease.

Fig. 2:
A positive test for mononucleosis.
Note clumping of the horse erythrocytes.

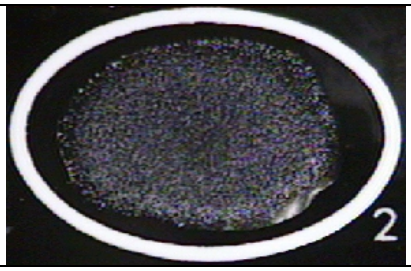


c. Serologic Tests for Systemic Lupus Erythematosus (SLE)

Discussion

Systemic lupus erythematosus or **SLE** is a systemic autoimmune disease. Immune complexes become deposited between the dermis and the epidermis, and in joints, blood vessels, glomeruli of the kidneys, and the central nervous system. It is four times more common in women than in men. In SLE, autoantibodies are made against components of DNA. This test is specific for the **serum anti-deoxyribonucleoprotein antibodies associated with SLE**. The **known antigen is deoxyribonucleoprotein adsorbed to latex particles** to make the reaction more visible to the eye (**see Fig. 3**). This is a **qualitative test** used to screen for the presence of the disease and to monitor its course.

Fig. 3:
A positive test for SLE. Note agglutination of latex particles to which the known antigen is adsorbed.



d. Detecting Antibody Using the Indirect Fluorescent Antibody Technique: The FTA-ABS test for syphilis

Discussion

The **indirect** fluorescent antibody technique involves three different reagents:

1) The **patient's serum** (containing antibodies against the disease antigen if the disease is present)

2) **Known antigen** for the suspected disease

3) **Fluorescent anti-human gamma globulin antibodies** (antibodies made in another animal against the Fc portion of human antibodies (**see Fig. 9**) by injecting an animal with

human serum. A fluorescent dye is then chemically attached to the anti-human gamma globulin (anti-HGG) antibodies.

The **FTA-ABS test** (Fluorescent Treponemal Antibody Absorption Test) **for syphilis** is an example of an indirect fluorescent antibody procedure. This is the **confirming test** for syphilis since it tests specifically for **antibodies in the patient's serum made in response to the syphilis spirochete, *Treponema pallidum***.

In this test, killed *T. pallidum*,(the known antigen), is fixed on a slide (**Fig. 4, step 1**). The patient's serum is then added. If the patient has syphilis, antibodies against the *T. pallidum* will react with the antigen on the slide (**Fig. 4, step 2**). The slide is then washed to remove any antibodies not bound to the spirochete.

To make this reaction visible, a second animal-derived antibody made against human antibodies and labelled with a fluorescent dye (fluorescent anti-human gamma globulin) is added. These fluorescent anti-HGG antibodies react with the patient's antibodies that have reacted with the *T. pallidum* on the slide (**Fig. 4, step 3**). The slide is washed to remove any unbound fluorescent anti-HGG antibodies and observed with a fluorescent microscope. If the spirochetes glow or fluoresce, the patient has made antibodies against *T. pallidum* and has syphilis **(Fig. 5)**.

Another example of the indirect fluorescent antibody test is the test for **antibodies against the measles virus**. Inactivated measles virus-infected cells (the known antigen) are fixed to a microscope slide. The patient's serum is then added. If the person has measles, antibodies of the isotype IgG will be made against the measles virus and will bind to viral epitopes on the know measles virus-infected cells. After washing the slide to remove any unbound IgG, fluorescent antihuman IgG is added. The fluoprescent antihuman IgG then binds to the patient's IgG that is bound to the infected cells. When viewed with a fluorescent microscope, the infected cells will fluoresce green.

Fig. 9:
IgG

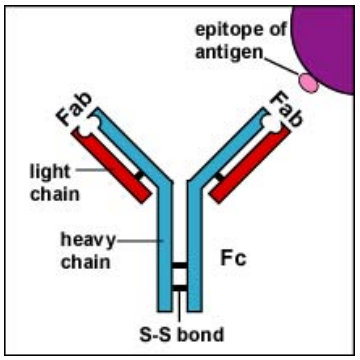


Fig. 4:
The FTA-ABS test for syphilis (Indirect fluorescent antibody test)

Fig. 4, step 1:
The known antigen, *Treponema pallidum*, is fixed to the slide.

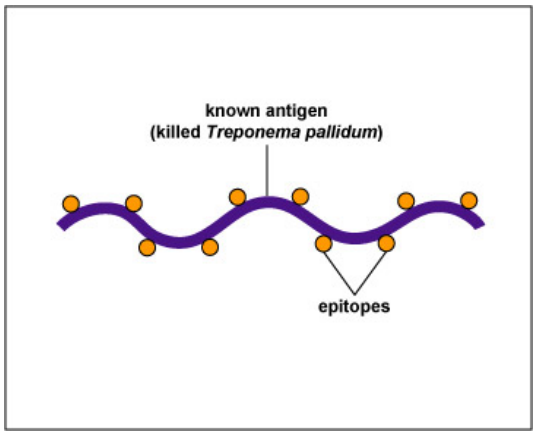


Fig. 4, step 2:
The patient's serum is added to the slide. The slide is then washed.

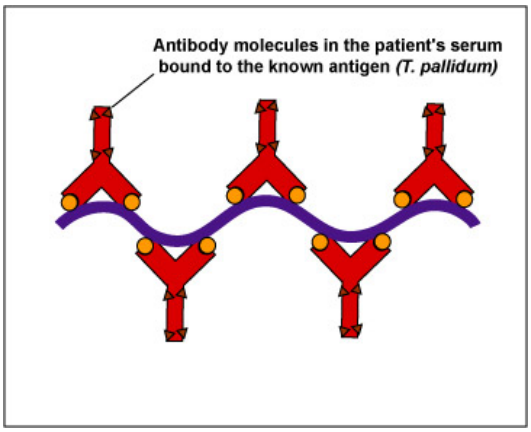


Fig. 4, step 3:
Fluorescent anti-HGG is added to the slide. The slide is then washed and viewed by means of a fluorescent microscope.

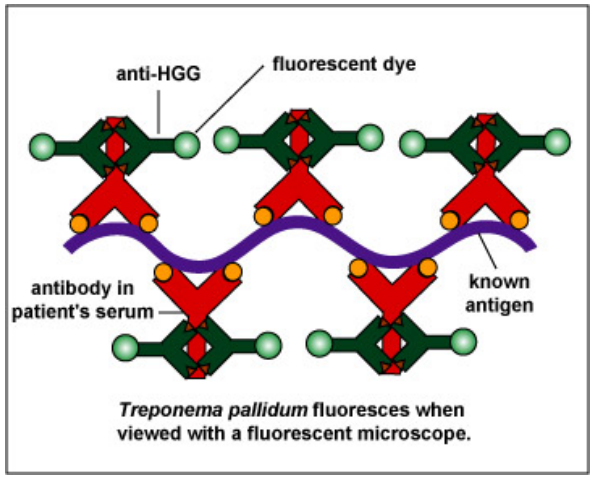
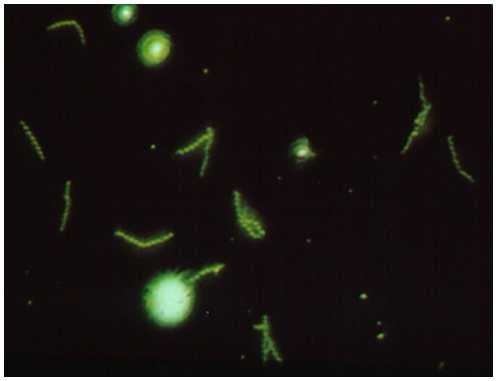


Fig. 5:
A positive FTA-ABS test for syphilis.



e. The EIA and Western Blot serologic tests for antibodies against the Human Immunodeficiency Virus (HIV)

Discussion

In the case of the current HIV antibody tests, the **patient's serum** is mixed with **various HIV antigens produced by recombinant DNA technology.** If the person is seropositive (has repeated positive antigen-antibody tests), then HIV must be in

that person's body stimulating antibody production. In other words, the person must be infected with HIV. The two most common tests currently used to detect antibodies against HIV are the **enzyme immunoassay or EIA** (also known as the enzyme-linked immunosorbant assay or ELISA) and the **Western blot or WB**. A person is considered seropositive for HIV infection only after an EIA screening test is repeatedly reactive and another test such as the WB has been performed to confirm the results.

The **EIA** is less expensive, faster, and technically less complicated than the WB and is the procedure initially done as a screening test for HIV infection. The various EIA tests give a spectrophotometric reading of the amount of antibody binding to known HIV antigens.

The EIA test kit **contains plastic wells to which various HIV antigens have been** adsorbed **(Fig. 6, step 1).** The **patient's serum** is added to the wells and any antibodies present in the serum against HIV antigens will bind to the corresponding antigens in the wells (**Fig. 6, step 2**). The wells are then washed to remove all antibodies in the serum other than those bound to HIV antigens. **Enzyme-linked anti-human gamma globulin (anti HGG) antibodies** are then added to the wells (**Fig. 6, step 3**). These antibodies, made in another animal against the Fc portion of human antibodies by injecting the animal with human serum, have an enzyme chemically attached. They react with the human antibodies bound to the known HIV antigens. The wells are then washed to remove any anti-HGG that has not bound to serum antibodies. A **substrate specific for the enzyme** is then added and the resulting enzyme-substrate reaction causes a **color change** in the wells (**Fig. 6, step 4**). If there are no antibodies in the patient's serum against HIV, there will be nothing for the enzyme-linked anti-HGG to bind to and it will be washed from the wells. When the substrate is added, there will be no enzyme present in the wells to give a color change.

Fig. 6:
Enzyme Immunoassay

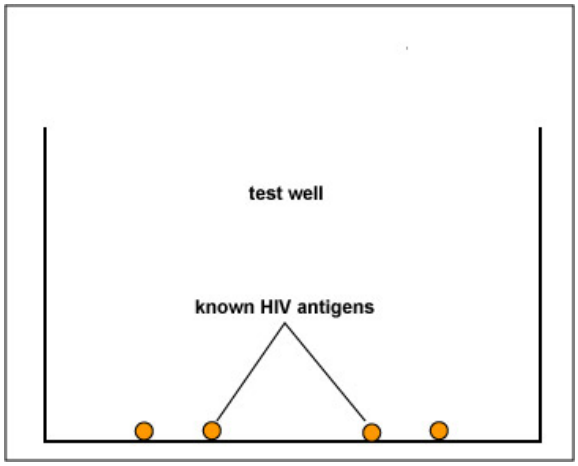
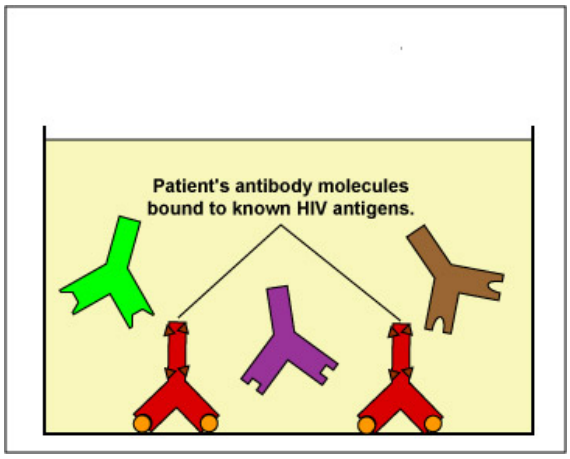
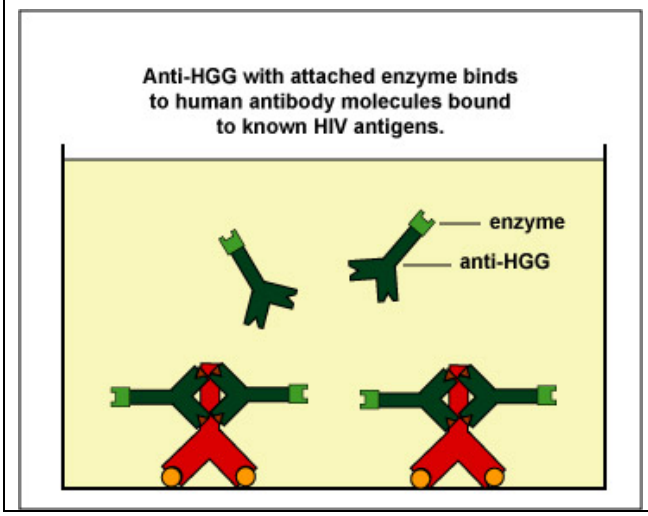
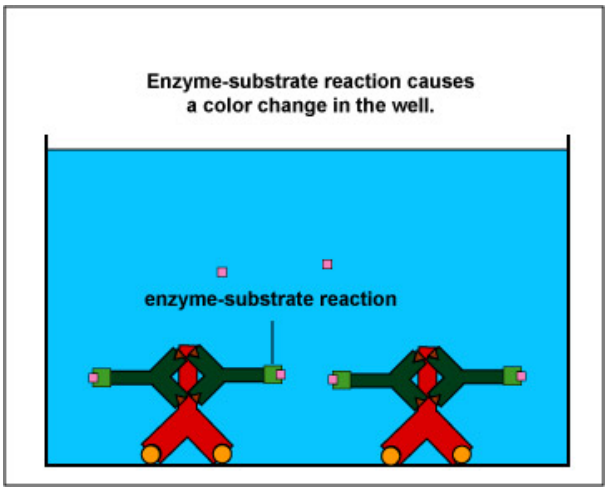
Fig. 6, step 1: The test well contains known HIV antigens.	**Fig. 6, step 2:** The patient's serum is added to the wells. The wells are then washed.
	

Fig. 6, step 3: Anti-HGG with attached enzyme is added to the wells. The wells are then washed.	**Fig. 6, step 4:** Substrate is added for the enzyme-linked anti-HGG. The enzyme-substrate reaction causes a color change in the wells.
	

If the initial EIA is reactive it is **automatically repeated** to reduce the possibility that technical laboratory error caused the reactive result. If the EIA is still reactive, it is then **confirmed by the Western blot test**.

The **Western blot WB** is the test most commonly used as a confirming test if the EIA is repeatedly positive. The WB is technically more complex to perform and interpret, is more time consuming, and is more expensive than the EIAs.

With the WB, the various protein and glycoprotein antigens from HIV are separated according to their molecular weight by gel electrophoresis (a procedure that separates charged proteins in a gel by applying an electric field). Once separated, the various HIV antigens are transferred to a nitrocellulose strip (**Fig. 7, step 1 and step 2**). The patient's serum is then incubated with the strip and any HIV antibodies that are present will bind to the corresponding known HIV antigens on the strip (**Fig. 7, step 3**). As with the EIA mentioned above, antigen-antibody reactions can then be detected using enzyme-linked anti-human gamma globulin antibodies (anti-HGG), as shown in **Fig. 7, step 4 and step 5**).

Fig. 7:
The Western Blot Test

Fig. 7, step 1

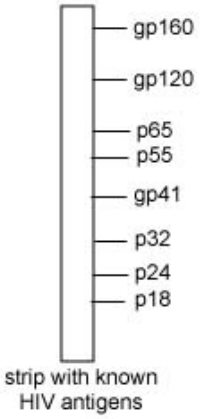


Fig. 7, step 2:
A section of the strip containing the known HIV antigen gp120.

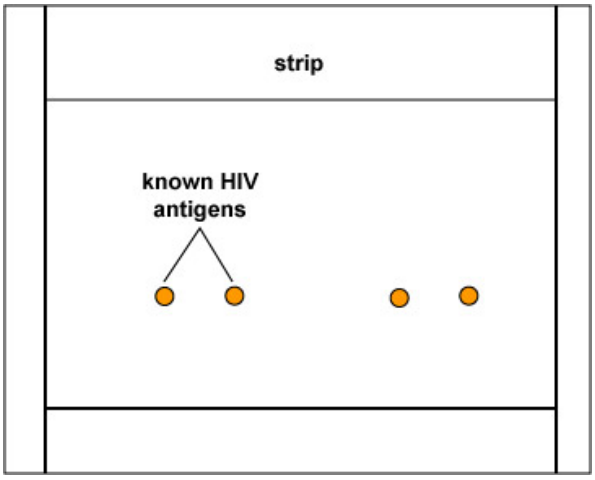


Fig. 7, step 3:
The patient's serum is added to the strip. The strip is then washed.

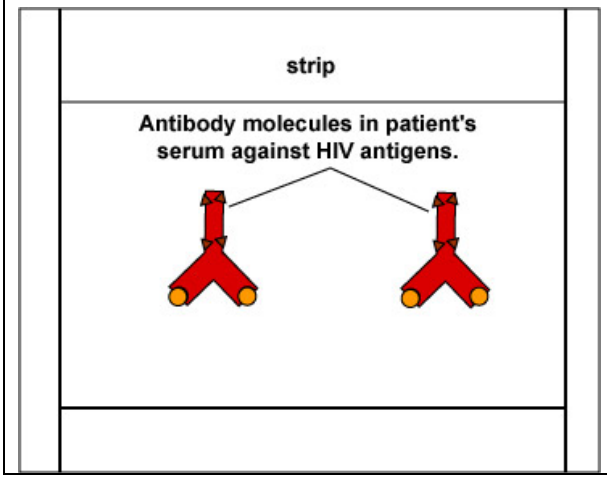


Fig. 7, step 4:
Enzyme-linked anti-HGG is added to the strip. The strip is then washed.

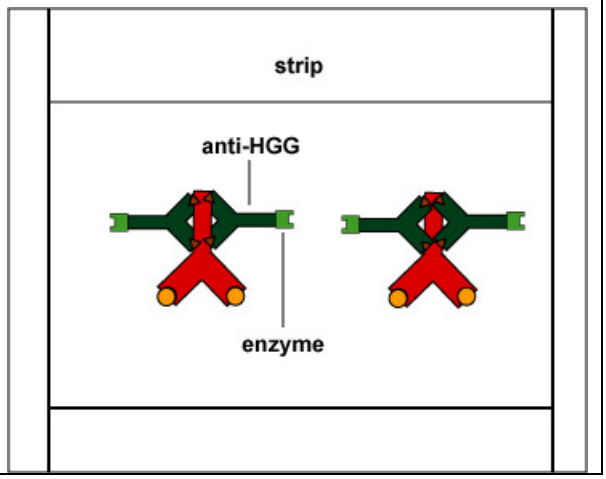
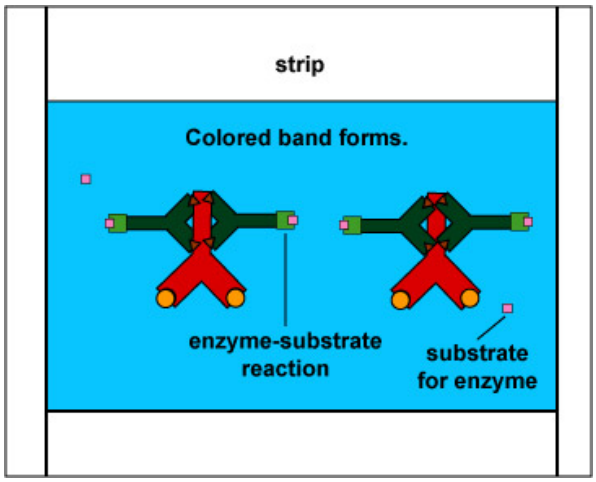


Fig. 7, step 5:
Substrate is added for the enzyme-linked anti-HGG. The enzyme-substrate reaction causes a color change on the strip.



It should be mentioned that all serologic tests give occasional **false-positive and false-negative results**. The most common cause of a false-negative HIV antibody test is when a person **has been only recently infected with HIV** and **his or her body has not yet made enough antibodies to give a visible positive serologic test**. It generally takes between 2 weeks and 3 months after a person is initially infected with HIV to convert to a positive HIV antibody test.

4. Sensitivity and Specificity in Serologic Testing

Sensitivity measures the "true-positive" rate of the serologic test, that is**, how often a test correctly generates a *positive* result for people who *have* the disease being tested.** To give an example, a test having a 95% sensitivity will correctly give a positive result for 95% of the people who have that disease, but will give a negative result, or false negative, for 5% of the people who have the disease and should have tested positive. So, **a high-sensitivity serologic test will correctly identify out almost everyone who *does* have the disease and will generate few false-negative results**.

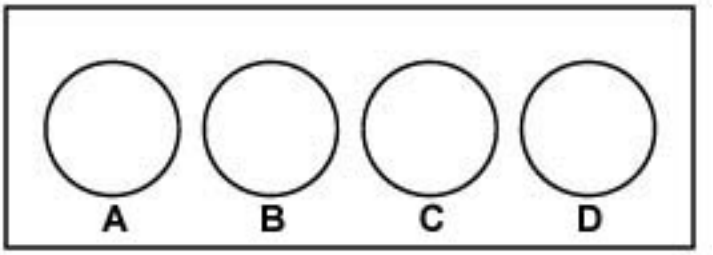
Specificity measures the "true negative" rate of the serologic test, that is, **how often a test correctly generates a *negative* result for people who *do not* have the disease being tested**. As an example, a test having a 95% specificity will correctly give a negative result for 95% of people who do not have the disease, but will return a positive result, or false-positive, for 5% of the people who don't have the disease and should have tested negative. So, a high-specificity serologic test will correctly rule out almost everyone who *does not* have the disease and will generate few false-positive results.

However**, no serologic tests are 100% accurate**. When considering test accuracy, the **rate of infection** also needs to be considered. **In a region with a low disease prevalence**, **the risk of false positive results by serologic testing is higher**, even with excellent specificity. However, **in a region with a high disease prevalence, the risk of false negative results will be higher**, even with excellent sensitivity.

PROCEDURE FOR DIRECT SEROLOGIC TESTING TO DETECT UNKNOWN ANTIGENS

A. Serologic Typing of *Shigella*

1. Using a wax marker, label four circles of a serology slide A, B, C, and D, as shown below.



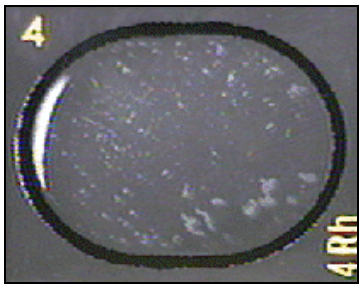
2. Add **one drop** of the **suspected *Shigella*** (unknown antigen) to each circle. (The *Shigella* has been treated with formalin to make it noninfectious but still antigenic.)

3. Now add **one drop** of known Shigella subgroup **A** antiserum to the "A" circle, **one drop** of known *Shigella* subgroup **B** antiserum to the "B" circle, **one drop** of known *Shigella* subgroup **C** antiserum to the "C" circle, and **one drop** of known *Shigella* subgroup **D** antiserum to the "D" circle.

4. **Rotate** the slide carefully for **30-60 seconds** and look for **agglutination of the bacteria**, indicating a **positive** reaction (**Fig. 9**).

5. Dispose of all pipettes and slides in the disinfectant container.

Fig. 9:
Agglutination of *Shigella*.



B. Serologic Typing of Streptococci: The Clearview® Strep A Exact II Dipstick

1. Add 4 drops of Extraction Reagent #1 to the extraction tube. This reagent contains 2M sodium nitrite and should be pink to purple in color.
2. Add 4 drops of Extraction Reagent #2 to the extraction tube. This reagent contains 0.03M citric acid. The solution must turn yellow in color.
3. Place the throat swab in the extraction tube and roll it with a circular motion inside the tube. Let stand for at least 1 minute.
4. Squeeze the swab firmly against the extraction tube to expel as much liquid as possible from the swab and discard the swab in the biowaste container.
5. Immerse the test strip into the extraction tube with the arrows pointing toward the extracted sample solution. Leave the strip in the tube and start timing.
6. Read results in 5 minutes. A red band in both the control region and the test region indicates a positive test (See Fig. 6C). A single red band in the control region only indicates a negative test (See Fig. 6D). No colored band in the control region indicates an invalid test.

C. Serologic Testing to Detect Pregnancy: The Alere hCG-Dipstick®

1. Dip the hCG dipstick into the urine **up to the maximum line on the strip for 5 seconds**.

2. Place the test dipstick on a flat, non-absorbant surface and **read the results at 3-4 minutes**. Do not interpret after the appropriate read time.

3. If hCG is present in the urine at a concentration of 25mlU/ml or greater, **a positive test, a pink-to-red Test line will appear along with a red Control line in the Result Window** (see Fig. 7B3). If hCG is absent or present at very low levels, **a negative test**, **only a red Control line appears in the Result Window** (see Fig. 7B4).

D. The Direct Fluorescent Antibody Technique

Observe the demonstration of a positive direct fluorescent antibody test for *Neisseria gonorrhoeae*.

PROCEDURE FOR INDIRECT SEROLOGIC TESTING TO DETECT ANTIBODIES IN THE PATIENT'S SERUM

A. The RPR® Card Test for Syphilis (demonstration)

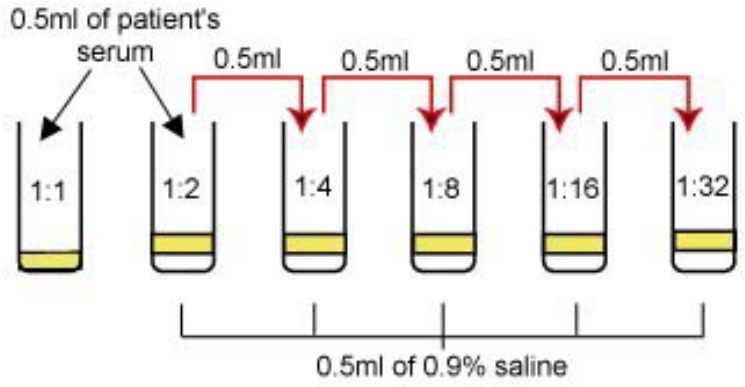
1. Label 6 test tubes as follows: 1:1, 1:2, 1:4, 1:8, 1:16, and 1:32.

2. Using a 1.0 ml pipette, add **0.5 ml of 0.9% saline solution** into tubes **1:2, 1:4, 1:8, 1:16, and 1:32**.

3. Add **0.5 ml of the patient's serum** to the **1:1** tube (undiluted serum).

4. Add another **0.5 ml of serum** to the saline in the **1:2** tube and mix. Remove **0.5 ml from the 1:2 tube** and add it to the **1:4** tube and mix. Remove **0.5 ml from the 1:4 tube**, add to the **1:8** tube and mix. Remove **0.5 ml from the 1:8 tube**, add to the **1:16** tube and mix. Remove **0.5 ml from the 1:16 tube**, add to the **1:32** tube and mix. Remove **0.5 ml from the 1:32 tube** and **discard**. The dilution of the serum is summarized in **Fig. 8**.

Fig. 8:
Diluting the patient's serum in a quantitative RPR card test.



5. Using the capillary pipettes provided with the kit, add **a drop of each serum dilution** to separate circles of the RPR card. Spread the serum over the entire inner surface of the circle with the tip of the pipette, using a new pipette for each serum dilution.

6. Using the RPR antigen dispenser, add **a drop of known RPR antigen** to each circle. Do not let the needle of the dispenser touch the serum. Using disposable stirrers, mix the known RPR antigen with the serum in each circle.

7. Place the slide on a shaker and **rotate for a maximum of 4 minutes**.

8. Read the results as follows:
- A definite **clumping** of the charcoal particles is reported as **reactive (R)** (see Fig. 1**)**.
- **No clumping** is reported as **nonreactive (N)**.

The greatest serum dilution that produces a **reactive** result is the **titer**. For example, if the dilutions turned out as follows, the titer would be reported as 1:4 or 4 dils.

1:1	1:2	1:4	1:8	1:16	1:32
R	R	R	N	N	N

B. Serologic Test for Infectious Mononucleosis (demonstration)

1. Place **0.05 ml of each of the patient's serum** in circles on the test slide.

2. Add **one drop of dyed, color-enhanced horse erythrocytes containing Paul-Bunnell antigens** (the known antigen) to each circle next to the patient's serum **and mix the two together** with disposable applicator sticks.

3. **Rock the card gently for 2 minutes and look for agglutination of the horse erythrocytes**. Agglutination indicates the presence of **heterophile antibodies** (see Fig. 2).

C. The Serologic Tests for Systemic Lupus Erythematosus (SLE) (demonstration)

1. Add **one drop of each of the patient's serum** to separate circles on the test slide.

2. Add **one drop of the Latex-Deoxyribonucleoprotein reagent** to each serum sample and mix with disposable applicator sticks. The known antigen, deoxyribonucleoprotein adsorbed to latex particles to make agglutination visible to the naked eye (**Fig. 3**).

3. **Rock the slide** gently **for 1 minute** and observe for agglutination. Agglutination indicates the presence of **antinuclear antibodies** associated with SLE.

D. The FTA-ABS Test for Syphilis (Indirect Fluorescent Antibody Technique)

Observe the 35mm slide of a positive FTA-ABS test.

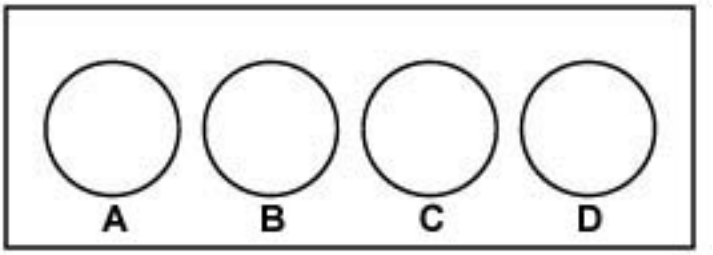
E. The EIA and WB Tests for HIV Antibodies

Observe Figures 6 and 7.

RESULTS FOR DIRECT SEROLOGIC TESTING TO DETECT UNKNOWN ANTIGENS

A. Serologic Typing of *Shigella*

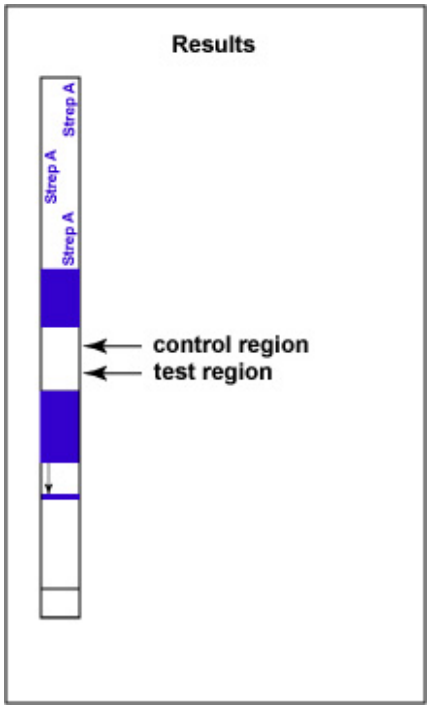
Make a drawing of your results.



Shigella typing slides

B. Serologic Identification of Group A Streptococci: The Clearview® Strep A Exact II Dipstick

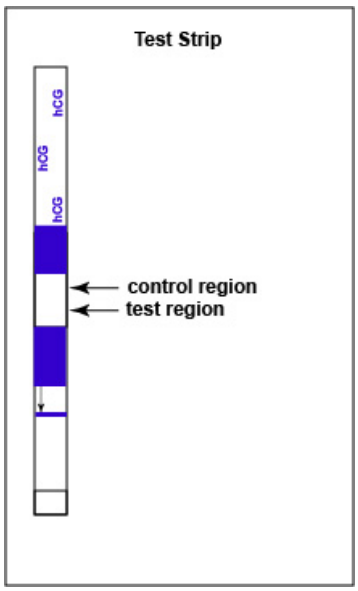
Make a drawing of your results.



Strep A strip

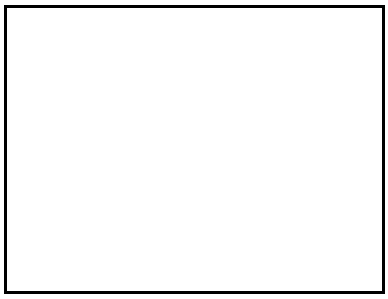
C. Serologic Testing to Diagnose Pregnancy The QuickVue+® One-Step hCG-Combo Test

Make a drawing of a positive test for pregnancy.



D. The Direct Fluorescent Antibody Technique

Make a drawing and describe a positive direct fluorescent antibody test.



A positive direct fluorescent antibody test for *Neisseria gonorrhoeae*.

RESULTS FOR INDIRECT SEROLOGIC TESTING TO DETECT ANTIBODIES IN THE PATIENT'S SERUM

A. RPR Card® Test for Syphilis (Quantitative)

Detects non-treponemal anti-lipid antibodies (reagin)

Record your results:

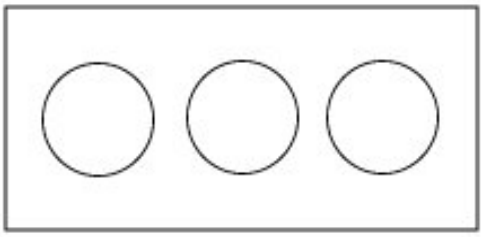
Dilution	Result
1:1	
1:2	
1:4	
1:8	
1:16	
1:32	
titer	

R = Reactive (distinct clumps)
N = Non-reactive (no clumps)

B. MONO-TEST for Infectious Mononucleosis (Qualitative)

Detects heterophile antibodies.

Draw the results of a positive and a negative test.

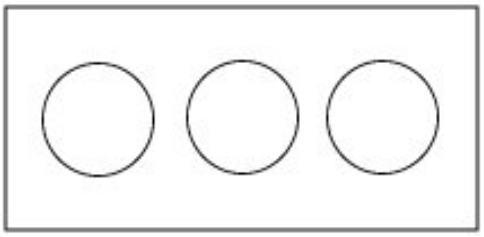


Mono-Test
(+) = Agglutination of the horse erythrocytesRBCs
(-) = No agglutination of the horse erythrocytes

C. Serologic test for SLE (Qualitative)

Detects anti-deoxyribonucleoprotein antibodies.

Draw the results of a positive and a negative test.

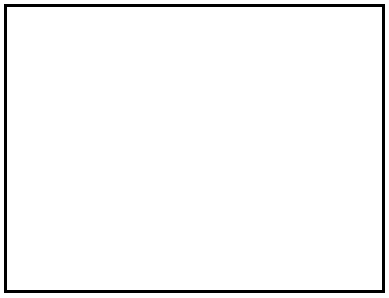


SLE Test
(+) = Agglutination
(-) = No agglutination

D. FTA-ABS Test for Syphilis (Confirming)

Detects antibodies against *Treponema pallidum*

Draw the results of a positive FTA-ABS test.



Positive FTA-ABS Test for Syphilis
(Fluorescent spirochetes)

PERFORMANCE OBJECTIVES
LABORATORY 16

After completing this lab, the student will be able to perform the following objectives:

A. INTRODUCTION TO SEROLOGICAL TESTING

1. Define serology.
2. Define antigen and state what may act as an antigen.
3. Define antibody and state where they are primarily found in the body.
4. Define direct serologic testing and indirect serologic testing.

B. DIRECT SEROLOGIC TESTING: USING ANTIGEN-ANTIBODY REACTIONS IN THE LAB TO IDENTIFY UNKNOWN ANTIGENS SUCH AS MICROORGANISMS

DISCUSSION

1. Define antiserum.
2. Describe two ways of producing known antiserum.
3. Describe the concept and general procedure for using serologic testing to identify unknown antigens (direct serologic testing).
4. Describe how to determine serologically whether an organism is a subgroup A, B, C, or D *Shigella*.
5. Describe how to serologically identify Lancefield group A *Streptococcus* causing pharyngitis using the Rapid Response Strep A Test.
6. Describe how to diagnose pregnancy serologically using the QuickVue+® One-Step hCG-Combo Test.
7. Briefly describe the direct fluorescent antibody technique.

RESULTS

1. Correctly interpret the results of the following serological tests:
    a. serological typing of *Shigella*
    b. serological identification of Group A Streptococcal antigen using the Rapid Response Strep A Test.
    c. serological testing for pregnancy using the QuickVue+® One-Step hCG-Combo Test.
    d. a direct fluorescent antibody test

C. INDIRECT SEROLOGIC TESTING: USING ANTIGEN-ANTIBODY REACTIONS IN THE LAB TO DETECT ANTIBODIES IN THE PATIENT'S SERUM

DISCUSSION

1. State the principle and the general procedure behind indirect serologic testing.
2. State the difference between a qualitative serological test and a quantitative serological test.
3. Define titer.

4. State what disease the RPR and the FTA-ABS procedures test for. Indicate which of these is a presumptive test, which is a confirming test, and why.
5. State the significance of non-treponemal anti-lipid (reagin) antibodies in serological testing.
6. State the significance of heterophile antibodies in serological testing.
7. State the significance of anti-deoxyribonucleoprotein antibodies in serological testing.
8. Briefly describe the indirect fluorescent antibody technique.
9. Briefly describe the EIA test for HIV antibodies and state the significance of a positive HIV antibody test.
10. In terms of serologic testing, define sensitivity and specificity.
11. State how the prevalence of a disease in a region effects the number of false-positive or false-negatives in the population of that region, even with tests of high sensitivity and specificity.

RESULTS

1. Interpret the results of the following serological tests:
    a. serologic test for infectious mononucleosis
    b. serologic test for SLE
    c. FTA-ABS test
2. Determine the titer of a quantitative RPR Card® test.

LABORATORY 17
USING PHYSICAL AGENTS TO CONTROL OF MICROORGANISMS

A. Introduction to the Control of Microorganisms
B. Temperature
C. Desiccation
D. Osmotic Pressure
E. Radiation
F. Filtration

A. INTRODUCTION TO THE CONTROL OF MICROORGANISMS

The next two labs deal with the inhibition, destruction, and removal of microorganisms. Control of microorganisms is essential in order to prevent the transmission of diseases and infection, stop decomposition and spoilage, and prevent unwanted microbial contamination.

Microorganisms are controlled by means of physical agents and chemical agents. Physical agents include such methods of control as high or low temperature, desiccation, osmotic pressure, radiation, and filtration. Control by chemical agents refers to the use of disinfectants, antiseptics, antibiotics, and chemotherapeutic antimicrobial chemicals.

Basic terms used in discussing the control of microorganisms include:

1. **Sterilization**
   Sterilization is the process of destroying all living organisms and viruses. A sterile object is one free of all life forms, including bacterial endospores, as well as viruses.

2. **Disinfection**
   Disinfection is the elimination of microorganisms, but not necessarily endospores, from inanimate objects or surfaces.

3. **Decontamination**
   Decontamination is the treatment of an object or inanimate surface to make it safe to handle.

4. **Disinfectant**
   A disinfectant is an agents used to disinfect inanimate objects but generally to toxic to use on human tissues.

5. **Antiseptic**
   An antiseptic is an agent that kills or inhibits growth of microbes but is safe to use on human tissue.

6. **Sanitizer**
   A sanitizer is an agent that reduces, but may not eliminate, microbial numbers to a safe level.

7. **Antibiotic**
   An antibiotic is a metabolic product produced by one microorganism that inhibits or kills other microorganisms.

8. **Chemotherapeutic antimicrobial chemical**
Chemotherapeutic antimicrobial chemicals are synthetic chemicals that can be used therapeutically.

9. **Cidal**
An agent that is cidal in action will kill microorganisms and viruses.

10. **Static**
An agent that is static in action will inhibit the growth of microorganisms.

These two labs will demonstrate the control of microorganisms with physical agents, disinfectants and antiseptics, and antimicrobial chemotherapeutic agents. Keep in mind that when evaluating or choosing a method of controlling microorganisms, you must consider the following factors that may influence antimicrobial activity:

1. The concentration and kind of a chemical agent used.
2. The intensity and nature of a physical agent used.
3. The length of exposure to the agent.
4. The temperature at which the agent is used.
5. The number of microorganisms present.
6. The organism itself.
7. The nature of the material bearing the microorganism.

B. TEMPERATURE

Microorganisms have a minimum, an optimum, and a maximum temperature for growth. Temperatures **below the minimum** usually have a **static** action on microorganisms. They inhibit microbial growth by slowing down metabolism but do not necessarily kill the organism. Temperatures **above the maximum** usually have a **cidal** action, since they denature microbial enzymes and other proteins. Temperature is a very common and effective way of controlling microorganisms.

1. **High Temperature**

Vegetative microorganisms can generally be killed at temperatures from 50°C to 70°C with moist heat. Bacterial **endospores**, however, are very resistant to heat and extended exposure to much higher temperature is necessary for their destruction. High temperature may be applied as either moist heat or dry heat.

a. **Moist heat**

Moist heat is generally more effective than dry heat for killing microorganisms because of its ability to **penetrate** microbial cells. Moist heat kills microorganisms by **denaturing their proteins** (causes proteins and enzymes to lose their three-dimensional functional shape). It also may **melt lipids** in cytoplasmic membranes.

1. **Autoclaving**

Autoclaving employs **steam under pressure**. Water normally boils at 100°C; however, when put under pressure, water boils at a higher temperature. During autoclaving, the materials to be sterilized are placed under **15 pounds per square inch of pressure** in a pressure-cooker type of apparatus. When placed under 15 pounds of pressure, the boiling point of water is raised to **121°C**, a temperature sufficient to kill bacterial endospores.

The time the material is left in the autoclave varies with the nature and amount of material being sterilized. Given sufficient time (generally 15-45 minutes), autoclaving is **cidal** for both vegetative organisms and endospores, and is the most common method of sterilization for materials not damaged by heat.

2. **Boiling water**

Boiling water (100°C) will generally kill vegetative cells after about 10 minutes of exposure. However, certain viruses, such as the hepatitis viruses, may survive exposure to boiling water for up to 30 minutes, and endospores of certain *Clostridium* and *Bacillus* species may survive even hours of boiling.

b. **Dry heat**

Dry heat kills microorganisms through a process of **protein oxidation** rather than protein coagulation. Examples of dry heat include:

1. **Hot air sterilization**

Microbiological ovens employ very high dry temperatures: 171°C for 1 hour; 160°C for 2 hours or longer; or 121°C for 16 hours or longer depending on the volume. They are generally used only for sterilizing glassware, metal instruments, and other inert materials like oils and powders that are not damaged by excessive temperature.

2. **Incineration**

Incinerators are used to destroy disposable or expendable materials by burning. We also sterilize our inoculating loops by incineration.

c. **Pasteurization**

Pasteurization is the mild heating of milk and other materials to kill **particular spoilage organisms or pathogens**. It does not, however, kill all organisms. Milk is usually pasteurized by heating to 71°C for at least 15 seconds in the flash method or 63-66°C for 30 minutes in the holding method.

2. **Low Temperature**

Low temperature **inhibits** microbial growth by **slowing down microbial metabolism**. Examples include refrigeration and freezing. Refrigeration at 5°C slows the growth of microorganisms and keeps food fresh for a few days. Freezing at -10°C stops microbial growth, but generally does not kill microorganisms, and keeps food fresh for several months.

C. DESICCATION

Desiccation, or drying, generally has a **static** effect on microorganisms. Lack of water inhibits the action of microbial enzymes. Dehydrated and freeze-dried foods, for example, do not require refrigeration because the absence of water inhibits microbial growth.

D. OSMOTIC PRESSURE

Microorganisms, in their natural environments, are constantly faced with alterations in osmotic pressure. Water tends to flow through semipermeable membranes, such as the cytoplasmic membrane of microorganisms, towards the side with a higher concentration of dissolved materials (**solute**). In other words, **water moves from greater water (lower solute) concentration to lesser water (greater solute) concentration**.

When the concentration of dissolved materials or solute is higher inside the cell than it is outside, the cell is said to be in a **hypotonic environment** and water will flow **into the cell** (**Fig. 1**). The rigid **cell walls** of bacteria and fungi, however, **prevent bursting or plasmoptysis**. If the concentration of solute is the same both inside and outside the cell, the cell is said to be in an **isotonic environment** (**Fig. 2**). Water flows equally in and out of the cell. Hypotonic and isotonic environments are not usually harmful to microorganisms. However, if the concentration of dissolved materials or solute is higher outside of the cell than inside, then the cell is in a **hypertonic environment** (**Fig. 3**). Under this condition, water flows **out of the cell**, resulting in shrinkage of the cytoplasmic membrane or **plasmolysis**. Under such conditions, the cell becomes **dehydrated** and its **growth is inhibited**.

The canning of jams or preserves with a high sugar concentration inhibits bacterial growth through hypertonicity. The same effect is obtained by salt-curing meats or placing foods in a salt brine. This **static action** of osmotic pressure thus prevents bacterial decomposition of the food. Molds, on the other hand, are more tolerant of hypertonicity. Foods, such as those mentioned above, tend to become overgrown with molds unless they are first sealed to exclude oxygen. (Molds are aerobic.)

Fig. 1:
Bacterium in an hypotonic environment.

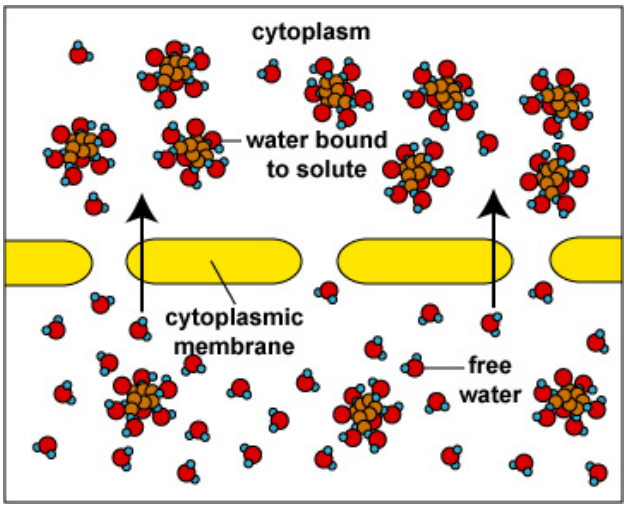


Fig. 2:
Bacterium in a isotonic environment.

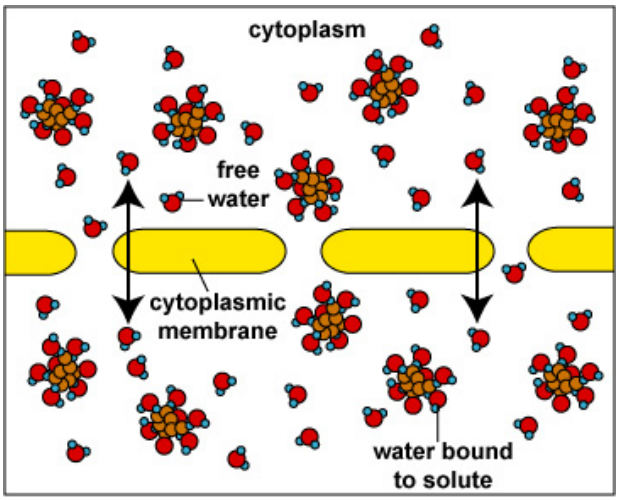
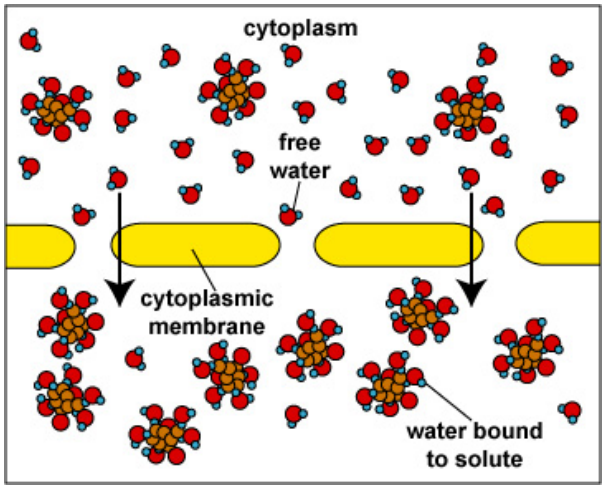


Fig. 3:
Bacterium in a hypertonic environment.



E. RADIATION

1. **Ultraviolet Radiation**

The ultraviolet portion of the light spectrum includes all radiations with wavelengths from 100 nm to 400 nm. It has low wave-length and low energy. The microbicidal activity of ultraviolet (UV) light depends on **the length of exposure**: the longer the exposure the greater the cidal activity. It also depends on the **wavelength of UV used**. The most cidal wavelengths of UV light lie in the **260 nm - 270 nm range** where it is absorbed by nucleic acid.

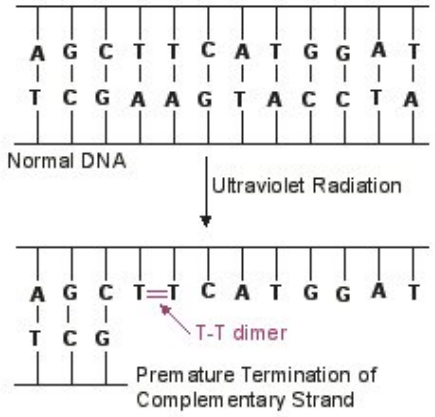
In terms of its mode of action, UV light is absorbed by microbial DNA and causes adjacent thymine bases on the same DNA strand to covalently bond together, forming what are called **thymine-thymine dimers** (**see Fig. 4**). As the DNA replicates, nucleotides do not complementary base pair with the thymine dimers and this terminates the replication of that DNA strand. However, **most of the damage from UV radiation actually comes from the cell trying to repair the damage to the DNA by a process called SOS repair**. In very heavily damaged DNA containing large numbers of thymine dimers, a process called SOS repair is activated as kind of a last ditch effort to repair the DNA. In this process, a gene product of the SOS system binds to DNA polymerase allowing it to synthesize new DNA across the damaged DNA. However, this **altered DNA polymerase loses its proofreading ability** resulting in the synthesis of DNA that itself now contains many misincorporated bases. In other words, **UV radiation causes mutation** and can lead to faulty protein synthesis. With sufficient mutation, bacterial metabolism is blocked and the organism dies. Agents such as UV radiation that cause high rates of mutation are called **mutagens**.

The effect of this incorrect base pairing may be reversed to some extent by exposing the bacteria to strong visible light immediately after exposure to the UV light. The visible light activates an enzyme that breaks the bond that joins the thymine bases, thus enabling correct complementary base pairing to again take place. This process is called **photoreactivation**.

UV lights are frequently used to reduce the microbial populations in hospital operating rooms and sinks, aseptic filling rooms of pharmaceutical companies, in microbiological hoods, and in the processing equipment used by the food and dairy industries.

An important consideration when using UV light is that it has **very poor penetrating power**. Only microorganisms on the **surface** of a material that are exposed directly to the radiation are susceptible to destruction. UV light can also damage the eyes, cause burns, and cause mutation in cells of the skin.

Fig. 4:
Thymine-thymine dimer from U.V. radiation



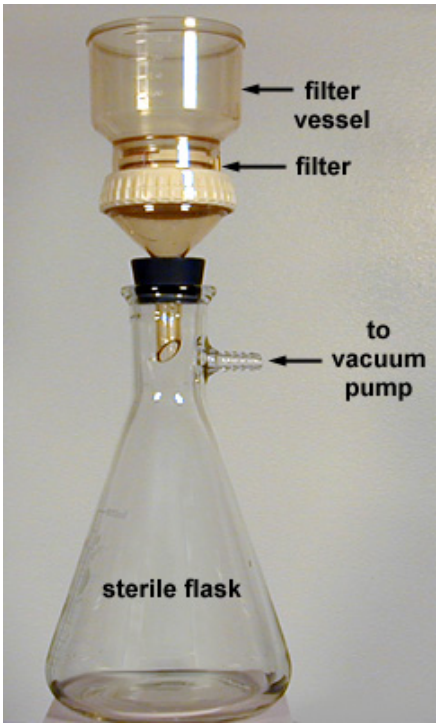
2. **Ionizing Radiation**

Ionizing radiation, such as **X-rays and gamma rays**, has much more energy and penetrating power than ultraviolet radiation. It ionizes water and other molecules to form radicals (molecular fragments with unpaired electrons) that can **disrupt DNA molecules and proteins**. It is often used to sterilize pharmaceuticals and disposable medical supplies such as syringes, surgical gloves, catheters, sutures, and petri plates. It can also be used to retard spoilage in seafoods, meats, poultry, and fruits.

F. FILTRATION

Microbiological membrane filters provide a useful way of sterilizing materials such as vaccines, antibiotic solutions, animal sera, enzyme solutions, vitamin solutions, and other solutions that may be damaged or denatured by high temperatures or chemical agents. The filters contain pores small enough to prevent the passage of microbes but large enough to allow the organism-free fluid to pass through. The liquid is then collected in a sterile flask (**see Fig. 5**). Filters with a pore diameter from 25nm to 0.45µm are usually used in this procedure. Filters can also be used to remove microorganisms from water and air for microbiological testing (see Appendix D).

Fig. 5:
Micropore filter apparatus



A. OSMOTIC PRESSURE

MEDIA

2 plates of Trypticase Soy agar, 2 plates of 5% glucose agar, 2 plates of 10% glucose agar, 2 plates of 25% glucose agar, 2 plates of 5% NaCl agar, 2 plates of 10% NaCl agar, and 2 plates of 15% NaCl agar.

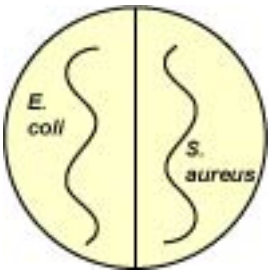
ORGANISMS

Trypticase Soy broth cultures of *Escherichia coli* and *Staphylococcus aureus*; a spore suspension of the mold *Aspergillus niger*.

PROCEDURE (to be done by tables)

1. Divide one plate of each of the following media **in half**. Using your **inoculating loop**, streak one half of each plate with *E. coli* and the other half with *S. aureus*. Incubate **upside down and stacked in the petri plate holder on the shelf of the 37°C incubator corresponding to your lab section** until the next lab period.

a. Trypticase Soy agar (control)
b. Trypticase Soy agar with 5% glucose
c. Trypticase Soy agar with 10% glucose
d. Trypticase Soy agar with 25% glucose
e. Trypticase Soy agar with 5% NaCl
f. Trypticase Soy agar with 10% NaCl
g. Trypticase Soy agar with 15% NaCl



2. Using a **sterile swab**, streak one plate of each of the following media with a spore suspension of the mold *A. niger*. Incubate the plates **upside down at room temperature** for 1 week.

a. Trypticase Soy agar (control)
b. Trypticase Soy agar with 5% glucose
c. Trypticase Soy agar with 10% glucose
d. Trypticase Soy agar with 25% glucose
e. Trypticase Soy agar with 5% NaCl
f. Trypticase Soy agar with 10% NaCl
g. Trypticase Soy agar with 15% NaCl

B. ULTRAVIOLET RADIATION

MEDIA

5 plates of Trypticase Soy agar

ORGANISM

Trypticase Soy broth culture of *Serratia marcescens*

PROCEDURE: to be done by tables

1. Using **sterile swabs**, streak all 5 Trypticase Soy agar plates with *S. marcescens* as follows:

a. Dip the swab into the culture.

b. **Remove all of the excess liquid by pressing the swab against the side of the tube.**

c. Streak the plate so as to **cover the entire agar surface with organisms**.

2. Expose **3 of the plates to UV light** as follows:

a. **Remove the lid** of each plate and place a piece of cardboard with the letter "V" cut out of it over the top of the agar.

b. Expose the first plate to UV light for **1 second**, the second plate for **3 seconds**, and the third plate for **5 seconds**.

c. Replace the lids and incubate the plates **upside down at room temperature** until the next lab period.

3. **Leaving the lid on**, lay the cardboard with the letter "V" cut out over the fourth plate and expose to UV light for **30 seconds.** Incubate the plates **upside down at room temperature** with the other plates.

4. Use the fifth plate as a **non-irradiated control** and incubate the plates **upside down at room temperature**.

NOTE: Do not look directly at the UV light as it may harm the eyes.

C. FILTRATION

MEDIUM

2 plates of Trypticase Soy agar

ORGANISM

Trypticase Soy broth cultures of *Micrococcus luteus*

PROCEDURE: demonstration

1. Using an alcohol-flamed forcep, aseptically place a sterile membrane filter into a sterile filtration device.

2. Pour the culture of *M. luteus* into the top of the filter set-up.

3. Vacuum until all the liquid passes through the filter into the sterile flask.

4. With alcohol-flamed forceps, remove the **filter** and place it organism-side-up on the surface of a Trypticase Soy agar plate.

5. Using a sterile swab, streak the surface of another Trypticase Soy agar plate with the **filtrate** from the flask.

6. Incubate the plates **upside down and stacked in the petri plate holder on the shelf of the 37°C incubator corresponding to your lab section** until the next lab period.

RESULTS

A. Osmotic Pressure

Observe the 2 sets of plates from the osmotic pressure experiment and record the results below.

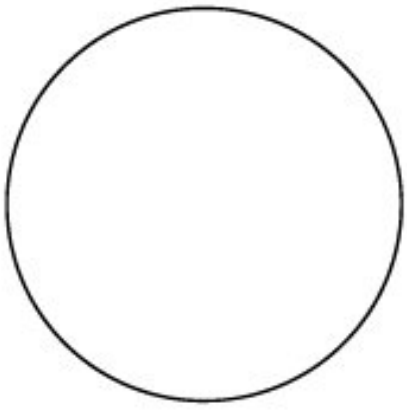
Plate	*Escherichia coli*	*Staphylococcus aureus*	*Aspergillus niger*
Control (TSA)	+++	+++	+++
5% NaCl			
10% NaCl			
15% NaCl			
5% glucose			
10% glucose			
25% glucose			

+ = Scant growth
++ = Moderate growth
+++ = Abundant growth
- = No growth

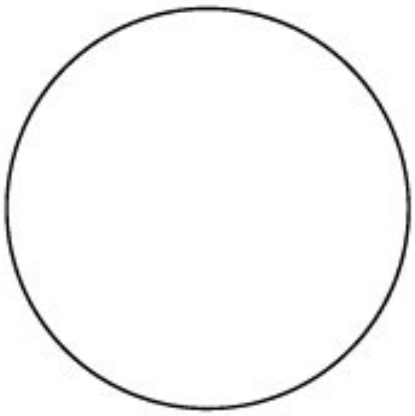
Conclusions:

B. Ultraviolet Radiation

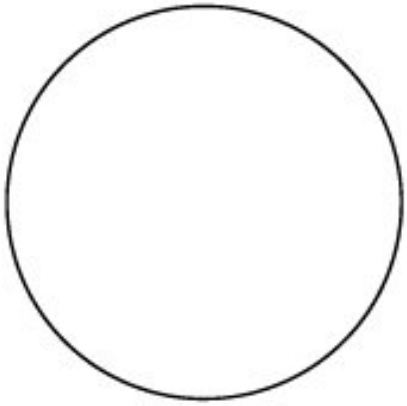
1. Make drawings of the 5 plates from the ultraviolet light experiment.



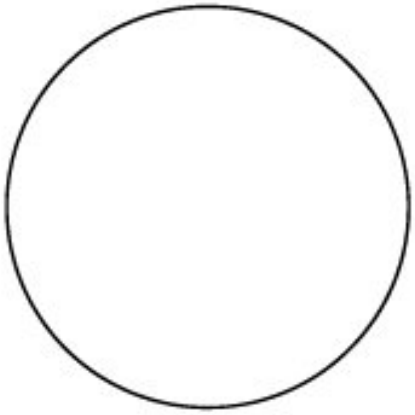
Non-irradiated control



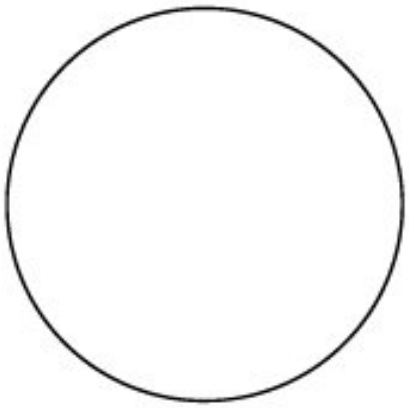
1 second; lid off



3 seconds; lid off



5 seconds; lid off



30 seconds; lid on

Conclusions:

2. Observe the plates exposed to UV light for any **non-pigmented** colonies. Aseptically **pick-off** one of these non-pigmented colonies and streak it in a plate of Trypticase Soy agar. Incubate **at room temperature** until the next lab period.

3. After incubation, observe the plate you streaked with the nonpigmented colony.

Does the organism still lack chromogenicity?

What would account for this?

C. FILTRATION

Observe the two filtration plates and describe the results below.

Plate containing the filter (growth or no growth)	
Plate streaked with filtrate (growth or no growth)	

Conclusions:

PERFORMANCE OBJECTIVES
LABORATORY 17

After completing this lab, the student will be able to perform the following objectives:

A. INTRODUCTION TO THE CONTROL OF MICROORGANISM

1. Define the following terms: sterilization, disinfection, decontamination, static, and cidal.

B. TEMPERATURE

1. State whether moist or dry heat is more effective in controlling microorganisms, and indicate why.
2. State specifically how moist heat kills microorganisms.
3. State two methods of applying moist heat.
4. Briefly describe the process of autoclaving (pressure, time, and temperature).
5. State whether or not boiling is an effective means of sterilization and state why or why not.
6. State specifically how dry heat kills microorganisms.
7. State two methods of applying dry heat.
8. Define pasteurization.
9. State whether low temperature has a static or cidal effect on microorganisms, and indicate why.

C. DESICCATION

1. State whether desiccation has static or cidal effect on microorganisms, and indicate how it affects the cell.

D. OSMOTIC PRESSURE

1. Describe osmosis in terms of water flow through a semipermeable membrane.
2. Define the following terms: hypotonic, hypertonic, isotonic, plasmoptysis, and plasmolysis.
3. State why a hypotonic environment does not normally harm bacteria.
4. Describe how bacterial growth is inhibited in jams and salt-cured meats.
5. State whether hypertonicity has a static or a cidal effect on microorganisms.

E. RADIATION

1. State how the wavelength and the length of exposure influence the bacteriocidal effect of UV light.
2. Describe specifically how UV light kills microorganisms.
3. State why UV light is only useful as a means of controlling surface contaminants and give several practical applications.
4. Describe how ionizing radiation kills microorganisms and state several common applications.

F. FILTRATION

1. State the concept behind sterilizing solutions with micropore membrane filters.
2. State why filters are preferred over autoclaving for such materials as vaccines, antibiotic solutions, sera, and enzyme solutions.

LABORATORY 18
USING CHEMICAL AGENTS TO CONTROL MICROORGANISMS

A. Disinfectants, Antiseptics, and Sanitizers
B. Evaluation of Disinfectants and Antiseptics
C. Effectiveness of Hand Washing
D. Antimicrobial Chemotherapeutic Agents
E. Microbial Resistance to Chemotherapeutic Agents
F. Antibiotic Susceptibility Testing

A. DISINFECTANTS, ANTISEPTICS, AND SANITIZERS

Disinfection is the elimination of microorganisms from inanimate objects or surfaces, whereas **decontamination** is the treatment of an object or inanimate surface to make it safe to handle.

a. The term **disinfectant** is used for an agent used to disinfect inanimate objects or surfaces but is generally too toxic to use on human tissues
b. The term **antiseptic** refers to an agent that kills or inhibits growth of microbes but is safe to use on human tissue.
c. The term **sanitizer** describes an agent that reduces, but may not eliminate, microbial numbers to a safe level.

Because disinfectants and antiseptics often work slowly on some viruses - such as the hepatitis viruses, bacteria with an **acid-fast cell wall** such as *Mycobacterium tuberculosis*, and especially bacterial **endospores**, produced by the genus *Bacillus* and the genus *Clostridium*, they are usually **unreliable for sterilization** - the destruction of **all** life forms.

There are a number of factors that influence the antimicrobial action of disinfectants, antiseptics, and sanitizers including:

1. The **concentration** of the chemical agent.

2. The **temperature** at which the agent is being used. Generally, the lower the temperature, the longer it takes to disinfect or decontaminate.

3. The **kinds of microorganisms** present. Endospore producers such as *Bacillus* species, *Clostridium* species, and acid-fast bacteria like *Mycobacterium tuberculosis* are harder to eliminate.

4. The **number of microorganisms** present. The more microorganisms present, the harder it is to disinfect or decontaminate.

5. The **nature of the material bearing the microorganisms**. Organic material such as dirt and excreta interferes with some agents.

LABORATORY 18
USING CHEMICAL AGENTS TO CONTROL MICROORGANISMS

The best results are generally obtained when the initial **microbial numbers are low** and when the **surface to be disinfected is clean** and free of possible interfering substances.

There are 2 common antimicrobial **modes of action** for disinfectants, antiseptics, and sanitizers:

1. They may **damage the lipids and/or proteins of the semipermeable cytoplasmic membrane** of microorganisms resulting in **leakage of cellular materials** needed to sustain life.

2. They may **denature microbial enzymes and other proteins,** usually by disrupting the hydrogen and disulfide bonds that give the protein its three-dimensional functional shape. This **blocks metabolism**.

A large number of such chemical agents are in common use. Some of the more common groups are listed below:

1. **Phenol and phenol derivatives**

Phenol (5-10%) was the first disinfectant commonly used. However, because of its toxicity and odor, **phenol derivatives (phenolics)** are now generally used. Th most common phenolic is orthophenylphenol, the agent found in O-syl®, Staphene®, and Amphyl®. **Bisphenols** contain two phenolic groups and typically have chlorine as a part of their structure. They include hexachlorophene and triclosan. Hexachlorophene in a 3% solution is combined with detergent and is found in PhisoHex®. Triclosan is an antiseptic very common in antimicrobial soaps and other products. **Biguanides** include chlorhexadine and alexidine. A 4% solution of chlorhexidine in isopropyl alcohol and combined with detergent (Hibiclens® and Hibitane®) is a common hand washing agent and surgical handscrub. These agents kill most bacteria, most fungi, and some viruses, but are usually ineffective against endospores. Chloroxylenol (4-chloro-3,5-dimethylphenol) is a broad spectrum antimicrobial chemical compound used to control bacteria, algae, fungi and virus and is often used in antimicrobial soaps and antiseptics. Phenol and phenolics alter membrane permeability and denature proteins. Bisphenols, biguanides, and chloroxylenol alter membrane permeability.

2. **Soaps and detergents**

Soaps are only mildly microbicidal. Their use aids in the **mechanical removal** of microorganisms by breaking up the oily film on the skin (emulsification) and reducing the surface tension of water so it spreads and penetrates more readily. Some cosmetic soaps contain added antiseptics to increase antimicrobial activity.

Detergents may be anionic or cationic. **Anionic** (negatively charged) **detergents**, such as laundry powders, mechanically remove microorganisms and other materials but are not very microbicidal. **Cationic** (positively charged) **detergents** alter membrane permeability and denature proteins. They are effective against many vegetative bacteria, some fungi, and some viruses. However, bacterial endospores and certain bacteria such as *Mycobacterium tuberculosis* and *Pseudomonas* species are usually resistant. Soaps and organic materials like excreta also inactivate them. Cationic detergents include the **quaternary ammonium compounds** such as benzalkonium chloride, zephiran, diaprene, roccal, ceepryn, and phemerol. Household Lysol® contains alkyl dimethyl benzyl ammonium chloride and alcohols.

3. **Alcohols**

70% solutions of **ethyl or isopropyl alcohol** are effective in killing vegetative bacteria, enveloped viruses, and fungi. However, they are usually ineffective against endospores and non-enveloped viruses. Once they evaporate, their cidal activity will cease. Alcohols denature membranes and proteins and are often combined with other disinfectants, such as iodine, mercurials, and cationic detergents for increased effectiveness.

4. **Acids and alkalies**

Acids and alkalies **alter membrane permeability and denature proteins and other molecules**. Salts of **organic acids**, such as calcium propionate, potassium sorbate, and methylparaben, are commonly used as food preservatives. Undecylenic acid (Desenex®) is used for dermatophyte infections of the skin. An example of an **alkali** is lye (sodium hydroxide).

5. **Heavy metals**

Heavy metals, such as mercury, silver, and copper, denature proteins. Mercury compounds (mercurochrome, metaphen, merthiolate) are only bacteriostatic and are not effective against endospores. Silver nitrate (1%) is sometimes put in the eyes of newborns to prevent gonococcal ophthalmia. Copper sulfate is used to combat fungal diseases of plants and is also a common algicide. Selinium sulfide kills fungi and their spores.

6. **Chlorine**

Chlorine gas reacts with water to form **hypochlorite ions**, which in turn denature microbial enzymes. Chlorine is used in the chlorination of drinking water, swimming pools, and sewage. Sodium hypochlorite is the active agent in household bleach. Calcium hypochlorite, sodium hypochlorite, and chloramines (chlorine plus ammonia) are used to sanitize glassware, eating utensils, dairy and food processing equipment, hemodialysis systems, and treating water supplies.

7. **Iodine and iodophores**

Iodine also denatures microbial proteins. Iodine tincture contains a 2% solution of iodine and sodium iodide in 70% alcohole. Aqueous iodine solutions containing 2% iodine and 2.4% sodium iodide are commonly used as a topical antiseptic. Iodophores are a combination of iodine and an inert polymer such as polyvinylpyrrolidone that reduces surface tension and slowly releases the iodine. **Iodophores** are less irritating than iodine and do not stain. They are generally effective against vegetative bacteria, *Mycobacterium tuberculosis*, fungi, some viruses, and some endospores. Examples include Wescodyne®, Ioprep®, Ioclide®, Betadine®, and Isodine®.

8. **Aldehydes**

Aldehydes, such as formaldehyde and glutaraldehyde, denature microbial proteins. Formalin (37% aqueous solution of formaldehyde gas) is extremely active and kills most forms of microbial life. It is used in embalming, preserving biological specimens, and in preparing vaccines. Alkaline glutaraldehyde (Cidex®), acid glutaraldehyde (Sonacide®), and glutaraldehyde phenate solutions (Sporocidin®) kill vegetative bacteria in 10-30 minutes and endospores in about 4 hours. A 10-hour exposure to a **2% glutaraldehyde** solution can be used for cold sterilization of materials. ***Ortho*-phthalaldehyde (OPA)** is dialdehyde used as a high-level disinfectant for medical instruments.

9. **Peroxygens**

Peroxygens are oxidizing agentes that include hydrogen peroxide and peracetic acid. **Hydrogen peroxide** is broken down into water and oxygen by the enzyme catalase in human cells and is not that good of an antiseptic for open wounds but is useful for disinfecting inanimate objects. The high concentrations of hydrogen peroxide overwhelm the catalase found in microbes. **Peracetic acid** is a disinfectant that kills microorganisms by oxidation and subsequent disruption of their cytoplasmic membrane. It is widely used in healthcare, food processing, and water treatment.

10. **Ethylene oxide gas**

Ethylene oxide is one of the very few chemicals that can be relied upon for **sterilization** (after 4-12 hours exposure). Since it is explosive, it is usually mixed with inert gases such as freon or carbon dioxide. **Gaseous chemosterilizers**, using ethylene oxide, are commonly used to sterilize heat-sensitive items such as plastic syringes, petri plates, textiles, sutures, artificial heart valves, heart-lung machines, and mattresses. Ethylene oxide has very high penetrating power and **denatures microbial proteins**. Vapors are toxic to the skin, eyes, and mucous membranes and are also carcinogenic. Another gas that is used as a sterilant is **chlorine dioxide** which denatures proteins in vegetative bacteria, bacterial endospores, viruses, and fungi.

B. EVALUATION OF DISINFECTANTS, ANTISEPTICS, AND SANITIZERS

It is possible to evaluate disinfectants, antiseptics, and sanitizers using either *in vitro* or *in vivo* tests. An ***in vitro*** test is one done under **artificial, controlled laboratory conditions**. An ***in vivo*** test is one done under the **actual conditions of normal use**.

A common *in vitro* test is to compare the antimicrobial activity of the agent being tested with that of phenol. The resulting value is called a phenol coefficient and has some value in comparing the strength of disinfectants under standard conditions. Phenol coefficients may be misleading, however, because as mentioned earlier, the killing rate varies greatly with the conditions under which the chemical agents are used. **The concentration of the agent, the temperature at which it is being used, the length of exposure to the agent, the number and kinds of microorganisms present, and the nature of the material bearing the microorganisms all influence the antimicrobial activity of a disinfectant**. If a disinfectant is being evaluated for possible use in a given *in vivo* situation, it must be **evaluated under the same conditions in which it will actually be used**.

C. EFFECTIVENESS OF HAND WASHING

There are 2 categories of microorganisms, or flora, normally found on the hands. **Resident flora** are the normal microbiota of the skin. **Transient flora** are the microorganisms you pick up from what you have been handling. It is routine practice to wash the hands prior to and after examining a patient and to do a complete regimented surgical scrub prior to going into the operating room. This is done in order to remove the potentially harmful transient flora, reduce the number of resident flora, and disinfect the skin.

Actual sterilization of the hands is not possible since microorganisms live not only on the surface of the skin but also in deeper skin layers, in ducts of sweat glands, and around hair follicles. These normal microbiota are mainly nonpathogenic staphylococci (Lab 15) and diphtheroid bacilli.

D. ANTIMICROBIAL CHEMOTHERAPEUTIC AGENTS

Antimicrobial chemotherapy is the use of chemicals to inhibit or kill microorganisms in or on the host. Chemotherapy is based on **selective toxicity**. This means that the agent used must **inhibit or kill the microorganism** in question **without seriously harming the host**.

In order to be selectively toxic, a chemotherapeutic agent must interact with some microbial function or microbial structure that is either not present or is substantially different from that of the host. For example, in treating infections caused by prokaryotic bacteria, the agent may inhibit peptidoglycan synthesis or alter bacterial (prokaryotic) ribosomes. Human cells do not contain peptidoglycan and possess eukaryotic ribosomes. Therefore, the drug shows little if any effect on the host (selective toxicity). Eukaryotic microorganisms, on the other hand, have structures and

functions more closely related to those of the host. As a result, the variety of agents selectively effective against eukaryotic microorganisms such as fungi and protozoans is small when compared to the number available against prokaryotes. Also keep in mind that viruses are not cells and, therefore, lack the structures and functions altered by antibiotics so antibiotics are not effective against viruses.

Based on their origin, there are 2 general classes of antimicrobial chemotherapeutic agents:

1. **Antibiotics:** substances produced as metabolic products of one microorganism which inhibit or kill other microorganisms.

2. **Antimicrobial chemotherapeutic chemicals:** chemicals synthesized in the laboratory which can be used therapeutically on microorganisms.

Today the distinction between the 2 classes is not as clear, since many antibiotics are extensively modified in the laboratory (semisynthetic) or even synthesized without the help of microorganisms.

Most of the major groups of antibiotics were discovered prior to 1955, and most antibiotic advances since then have come about by modifying the older forms. In fact, only 3 major groups of microorganisms have yielded useful antibiotics: the actinomycetes (filamentous, branching soil bacteria such as *Streptomyces*), bacteria of the genus *Bacillus*, and the saprophytic molds *Penicillium* and *Cephalosporium*.

To produce antibiotics, manufacturers inoculate large quantities of medium with carefully selected strains of the appropriate species of antibiotic-producing microorganism. After incubation, the drug is extracted from the medium and purified. Its activity is standardized and it is put into a form suitable for administration.

Some antimicrobial agents are **cidal** in action: they **kill microorganisms** (e.g., penicillins, cephalosporins, streptomycin, neomycin). Others are **static** in action: **they inhibit microbial growth** long enough for the body's own defenses to remove the organisms (e.g., tetracyclines, erythromycin, sulfonamides).

Antimicrobial agents also vary in their spectrum. Drugs that are **effective against a variety of both Gram-positive and Gram-negative bacteria are said to be broad-spectrum** (e.g., tetracycline, streptomycin, cephalosporins, ampicillin, sulfonamides). **Those effective against just Gram-positive bacteria, just Gram-negative bacteria, or only a few species are termed narrow-spectrum** (e.g., penicillin G, erythromycin, clindamycin, gentamicin).

If a choice is available, a narrow spectrum is preferable since it will cause less destruction to the body's normal flora. In fact, **indiscriminate use** of broad-spectrum antibiotics can lead to **superinfection by opportunistic microorganisms**, such as *Candida* (yeast infections) and *Clostridium difficile* (antibiotic-associated ulcerative colitis), when the body's normal flora is destroyed. Other dangers from indiscriminate use of antimicrobial chemotherapeutic agents include **drug toxicity, allergic reactions to the drug**, and **selection for resistant strains of microorganisms**.

Below are examples of commonly used antimicrobial chemotherapeutic agents arranged according to their **mode of action:**

1. **Antimicrobial agents that inhibit peptidoglycan synthesis (also see Table 1)**

Inhibition of peptidoglycan synthesis in actively dividing bacteria results in osmotic lysis.

a. **Penicillins** (produced by the mold *Penicillium*)

There are several classes of penicillins:

1. **Natural penicillins** are highly effective against Gram-positive bacteria (and a very few Gram- negative bacteria) but are inactivated by the bacterial enzyme penicillinase. Examples include **penicillin G, F, X, K, O, and V**.

2. **Semisynthetic penicillins** are effective against Gram-positive bacteria but are not inactivated by penicillinase. Examples include **methicillin, dicloxacillin, and nafcillin**.

3. **Semisynthetic broad-spectrum penicillins** are effective against a variety of Gram-positive and Gram- negative bacteria but are inactivated by penicillinase. Examples include **ampicillin, carbenicillin, and oxacillin**. Some of the newer semisynthetic penicillins include **azlocillin, mezlocillin, and piperacillin**.

4. **Semisynthetic broad-spectrum penicillins combined with beta lactamase inhibitors such as clavulanic acid and sulbactam**. Although the clavulanic acid and sulbactam have no antimicrobial action of their own, they inhibit penicillinase thus protecting the penicillin from degradation. Examples include **amoxicillin plus clavulanic acid, ticarcillin plus clavulanic acid, and ampicillin plus sulbactam**.

b. **Cephalosporins** (produced by the mold *Cephalosporium*)

Cephalosporins are effective against a variety of Gram-positive and Gram- negative bacteria and are resistant to penicillinase (although some can be inactivated by other beta-lactamase enzymes similar to penicillinase). Three "generations" of cephalosporins have been developed over the years in an attempt to counter bacterial resistance:

1. First generation cephalosporins include **cephalothin, cephapirin, and cephalexin**.

2. Second generation cephalosporins include **cefamandole, cefaclor, cefazolin, cefuroxime, and cefoxitin**.

3. Third generation cephalosporins include **cefotaxime, cefsulodin, cefetamet, cefixime, ceftriaxone, cefoperazone, ceftazidine, and moxalactam**.

4. Fourth generation cephalosporins include **cefepime and cefpirome**.

c. **Carbapenem**

Carbapenems consist of a broad-spectrum beta lactam antibiotic to inhibit peptidoglycan synthesis combined with cilastatin sodium, an agent that prevents degradation of the antibiotic in the kidneys. Examples include: **imipenem, metropenem, ertapenem, and doripenem.**

d. **Monobactems**

Monobactems are broad-spectrum beta lactam antibiotics resistant to beta lactamase. An example is **aztreonam**.

e. **Carbacephem**

A synthetic cephalosporin. An example is **loracarbef**.

f. **Glycopeptides** (produced by the bacterium *Streptomyces*)

Vancomycin, a glycopeptide, is effective against Gram-positive bacteria.

g. **Bacitracin** (produced by the bacterium *Bacillus*)

Bacitracin is used topically against Gram-positive bacteria.

h. **Fosfomycin** (Monurol)

2. A few antimicrobial chemotherapeutic agents **inhibit normal synthesis of the acid-fast cell wall** of the genus *Mycobacterium*.

a. **INH (isoniazid)** appears to **block the synthesis of mycolic acid**, a key component of the acid-fast cell wall of mycobacteria.

b. **Ethambutol interferes with the synthesis of the outer membrane of acid-fast cell walls**.

3. **Antimicrobial agents that alter the cytoplasmic membrane (also see Table 1)**

Alteration of the cytoplasmic membrane of microorganisms results in leakage of cellular materials.

a. **Polymyxins and colistins act as detergents and alter membrane permeability in Gram-negative bacteria**. They cannot effectively diffuse through the thick peptidoglycan layer in Gram-positives.

b. **Daptomycin disrupts the bacterial cytoplasmic membrane function by apparently binding to the membrane and causing rapid depolarization**. This results on a loss of membrane potential and leads to inhibition of protein, DNA and RNA synthesis, resulting in bacterial cell death.

c. **Pyrazinamide inhibits fatty acid synthesis in the membranes of *Mycobacterium tuberculosis***.

d. **Amphotericin B**, produced by the bacterium *Streptomyces*, is used for systemic fungal infections. It **interferes with membrane permeability by interacting with membrane sterols called ergosterols and forming pores in the membrane causing cellular leakage**.

e. **Nystatin**, produced by the bacterium *Streptomyces*, is used mainly for Candida yeast infections. It **interferes with membrane permeability by interacting with membrane sterols called ergosterols and forming pores in the membrane causing cellular leakage**.

f. **Imidazoles**, produced by the bacterium *Streptomyces*, are antifungal antibiotics used for yeast infections, dermatophytic infections, and systemic fungal infections. They **interfere with the synthesis of ergosterol, the sterol in fungal cytoplasmic membranes, causing cellular leakage**. Examples include **clotrimazole, miconazole, ketoconazole, itraconazole, and fluconazole**.

4. **Antimicrobial agents that inhibit protein synthesis (also see Table 1)**

These agents prevent bacteria from synthesizing structural proteins and enzymes.

a. Agents that **block transcription** (prevent the synthesis of mRNA off of DNA).

Rifampin or Rifampicin: rifadin, rifater combined with isoniazid and pyrazinamide, rimactane (produced by the bacterium *Streptomyces*). Rifaximins are effective against some Gram-positive and Gram-positive bacteria and *Mycobacterium tuberculosis.*

b. Agents that **block translation** (alter bacterial ribosomes to prevent mRNA from being translated into proteins).

1. The **aminoglycosides** (streptomycin, neomycin, netilmicin, tobramycin, gentamicin, amikacin, etc.) **bind irreversibly to the 16S rRNA in the 30S subunit of bacterial ribosomes interfering with the translation stage of protein syntheses**. Although the exact mechanism of action is still uncertain, there is evidence that some prevent the transfer of the peptidyl tRNA from the A-site to the P-site, thus preventing the elongation of the polypeptide chain. Some aminoglycosides also appear to interfere with the proofreading process that helps assure the accuracy of translation. Possibly the antibiotics reduce the rejection rate for tRNAs that are near matches for the codon. This leads to misreading of the codons or premature termination of protein synthesis. Aminoglycosides may also interfere directly or indirectly with the function of the bacterial cytoplasmic membrane. Because of their toxicity, aminoglycosides are generally used only when other first line antibiotics are not effective.

2. The **tetracyclines** (tetracycline, doxycycline, demeclocycline, minocycline, etc**.) bind reversibly to the 16S rRNA in the 30S ribosomal subunit interfering with the translation stage of protein syntheses**. They distort the ribosome in such a way that the anticodons of charged tRNAs cannot align properly with the codons of the mRNA Examples include tetracycline, minocycline, and doxycycline, produced by the bacterium Streptomyces. They are effective against a variety of Gram-positive and Gram-positive bacteria.

3. **Lincomycin and clindamycin**, produced by the bacterium *Streptomyces*, **bind reversibly to the 23S rRNA in the 50s ribosomal subunit and block peptide bond formation during the translation stage of protein synthesis**. Most are used against Gram-positive bacteria.

4. The **macrolides** (erythromycin, azithromycin, clarithromycin, dirithromycin, troleandomycin, etc.) **bind reversibly to the 23S rRNA in the 50S subunit of bacterial ribosomes interfering with the translation stage of protein syntheses**. They appear to inhibit elongation of the protein by preventing the enzyme peptidyltransferase from forming peptide bonds between the amino acids. They may also prevent the transfer of the peptidyl tRNA from the A-site to the P-siteas the beginning peptide chain on the peptidyl tRNA adheres to the ribosome, creates friction, and blocks the exit tunnel of the 50S ribosomal subunit. Macrolides are used against Gram-positive bacteria and some Gram-positive bacteria.

5. The **oxazolidinones** (linezolid), following the first cycle of protein synthesis**,** interfere with translation sometime before the initiation phase. They **appear to bind to the 50S ribosomal subunit and interfere with its binding to the initiation complex**.

6. The **streptoGramins** (synercid, a combination of quinupristin and dalfopristin) **bind to two different locations on the 23S rRNA in the 50S ribosomal subunit and work synergistically to block translation**. There are reports that the streptoGramins may inhibit the attachment of the charged tRNA to the A-site or may block the peptide exit tunnel of the 50S ribosomal subunit.

5. **Antimicrobial agents that interfere with DNA synthesis (also see Table 1)**

a. **Quinolones and Fluoroquinolones** (synthetic chemicals): The quinolones inhibit DNA replication by **inhibiting one or more of a group of enzymes called topoisomerases** that are essiential for bacterial DNA replication and transcription. Examples include

norfloxacin, ciprofloxacin, ofloxacin, enoxacin, lomefloxacin, levofloxacin, gatifloxacin, moxifloxacin, nalidixic acid, and gemifloxacin.

b. **Sulfonamides and trimethoprim** (synthetic chemicals):

Co-trimoxazole is a combination of sulfamethoxazole and trimethoprim. Both of these drugs **block the bacterial synthesis of folic acid**, a coenzyme needed to make DNA bases.

c. **Metronidazole**

Metronidazole is a drug that is activated by the microbial proteins flavodoxin and feredoxin found in microaerophilc and anaerobic bacteria and certain protozoans. Once activated, the metronidazole **puts nicks in the microbial DNA strands**.

Table 1: Common Antibacterial Antibiotics
Selected antibiotics and their modes of action.
(U.S. Brand names are in parentheses; ®.)

1. **Inhibit synthesis of peptidoglycan causing osmotic lysis.**

a. **Penicillins:** penicillin G (Pfizerpen; Bicillin; Wycillin), penicillin V (Betapen; Pen-Vee K), methicillin (Staphcillin), ampicillin (Omnipen; Polycillin), oxacillin (Bactocill), amoxicillin (Amoxil; Biomox; Polymox), ticarcillin (Ticar), carbenicillin (Geocillin), piperacillin (Pipracil), mezlocillin (Mezlin), bacampicillin (Spectrobid), dicloxacillin (Dynapen), nafcillin (Nallpen; Unipen).

b. **Penicillins plus beta lactamase inhibitors or compounds preventing antibiotic degradation on the kidneys**: amoxicillin + clavulanate (Augmentin), ticarcillin + clavulanate (Timentin), ampicillin + sulbactam (Unasyn), piperacillin + tazobactam (Zosyn), imipenem + cilastatin (Primaxin) .

c. **Cephalosporins:** cefaclor (Ceclor), cefadroxil (Duricef), cefazolin (Ancef; Kefzol), cefixime (Sulprax), cefepime (Maxipime), ceftibuten (Cedax), cefprozil (Cefzil), cefpodoxime (Vantin), cefotaxime (Claforan), cefotetan (Cefotan), cefoxitin (Cefoxitin; Mefoxin), ceftazidime (Ceptaz; Fortaz; Tazicef; Tazidime), ceftizoxime (Cefizox), ceftriaxone (Rocephin), cefuroxime (Ceftin; Kefurox; Zinacef), cephalexin (Biocef; Keflex; Keftab), cephradine (Velosef), cefdinir (Omnicef), cefditoren pivoxil (Spectracef), loracarbef (Lorabid), cefpirome (Cefrom), cefoperazone (Cefobid), cefepime (Maxipime), doripenem (Doribax).

d. **Carbapenems:** imipenem (Primaxin), meropenem (Merrem), ertapenem (Invanz) .

e. **Monobactems:** aztreonam (Azactam).

f. **Glycopeptides:** vancomycin (Lyphocin; Vancocin).

g. **Bacitracin** (AK-Tracin; Baci-IM; Baci-Rx; Ocu-Tracin; Ziba-Rx)

h. **Fosfomycin** (Monurol)

2. **Alter cytoplasmic membrane causing cellular leakage**

a. **Polymyxins:** colistimethate(Coly Mycin M), polymyxin B (Aerosporin; also mixed with other antibiotics in ointments such as Cortisporin, Neosporin, and LazerSporin).

b. **daptomycin** (Cubicin) Daptomycin disrupts the bacterial cytoplasmic membrane function by apparently binding to the membrane and causing rapid depolarization. This results on a loss of membrane potential and leads to inhibition of protein, DNA and RNA synthesis, resulting in bacterial cell death.

3. **Alter bacterial ribosomes, blocking translation and causing faulty protein synthesis**

a. Causing faulty protein synthesis by **binding to the 30S ribosomal subunit**

1. **Aminoglycosides:** amikacin (Amikin), tobramycin (Nebcin), gentamicin (Garamycin; Genoptic; Gentacidin; Gentak; Gentasol; Ocu-Mycin), kanamycin (Kantrex), neomycin (mixed with other antibiotics in antibiotic ointments such as Neosporin, Cortisporin, and LazerSporin), paromomycin (Humatin), tobramycin (AK-Tob; Tobrasol; Tobrex; Nebcin; Tobi).

2. **Tetracyclines**: tetracycline (Ala-Tet; Brodspec; Panmycin; Sumycin; Tetracon), minocycline (Arestin; Dynacin; Minocin; Vectrin), doxycycline (Adoxa; Atridox; Doryx; Monodox; Vibramycin), demeclocycline (Declomycin), tigecycline (Tygacil).

3. **Spectinomycin** (Trobicin)

b. Causing faulty protein synthesis by **binding to the 50S ribosomal subunit**

1. **Macrolides:** erythromycin (A/T/S; Akne-Mycin; E.E.S.; Emgel; E-Mycin; Eryc; Erycette; Erygel; Erymax; EryPed; Ery-Tab; Erythra-Derm; Erythrocin; Erythrocot; PCE; Romycin; Roymicin; Staticin; Theramycin; T-Stat), azithromycin (Zithromax), clarithromycin (Biaxin), dirithromycin (Dynabac), roxithromycin (Roxl-150, Roxo, Surlid, Rulide, Biaxsig, Roxar, Roximycin, Roxomycin, Tirabicin and Coroxin).

2. **Lincosamides**: lincomycin (Bactramycin; Lincocin; Lincomycin), clindamycin (Cleocin; Clinda-Derm; Clindagel; Clindamax).

3. **Chloramphenicol**: (AK-Chlor; Chloromycetin; Chloroptic; Ocu-Chlor)

4. **Oxazolidinones**: linezolid (Zyvox)

5. **StreptoGramins**: a combination of quinupristin and dalfopristin (Synercid)

6. **Telithromycin** (Ketek)

4. Inhibiting bacterial nucleic acid synthesis

a. Inhibit DNA replication by **inhibiting one or more of a group of enzymes called topoisomerases** that are essiential for bacterial DNA replication and transcription

Quinolones: norfloxacin (Noroxin, Chibroxin), ciprofloxacin (Cipro; Ciloxan; Ciprodex), ofloxacin (Floxin; Ocuflox), enoxacin (Penetrex), lomefloxacin (Maxaquin), levofloxacin (Levaquin; Quixin), gatifloxacin (Tequin), moxifloxacin (Avelox), nalidixic acid (NegGram), gemifloxacin (Factive).

b. Inhibit bacterial DNA synthesis by **blocking synthesis of tetrahydrafolate, a cofactor needed to make nucleotide bases**

1. **Sulfonamides:** sulfanilamide (AVC), sulfisoxazole (Gantrisin; Truxazole), sulfacetamide (AK-Sulf; Bleph-10; Isopto Cetamide; Klaron; Ocu-Sul; Ocusulf; Ovace; S.O.S.S.; Sebizon; Sodium Sulamyd; Sulf-10; Sulfac 10%; Sulfacet; Avar; Clenia; Nicosyn; Novacet; Plexion ; Prascion Rosac; Rosanil; Rosula; Sulfacet-R; Zetacet), Sulfadiazine (Sulfadiazine), sulfabenzamide + sulfacetamide + sulfathiazole (Gyne Sulf; Triple Sulfa), sulfisoxazole + erythromycin (Pediazole; Eryzole).

2. **Trimethoprim** (Primsol; Proloprim; Trimpex), trimethoprim + polymyxin B (Polytrim; Proloprim; Trimpex)

3. **Trimethoprim + sulfamethoxazole** (Bactrim; Bethaprim; Septra; SMX-TMP Plain; Sulfatrim; Uroplus).

4. **Trimetrexate** (NeuTrexin)

c. Drugs that are activated by the microbial proteins flavodoxin and feredoxin found in microaerophilc and anaerobic bacteria and certain protozoans. Once activated, the drug **puts nicks in the microbial DNA strands**.

Metronidazole (Flagyl; RTU; MetroCream; Metro; Metronidazole; Noritate; Protostat; Rozex)

d. Drugs that **inhibit bacterial RNA synthesis** by binding to RNA polymerase.

Rifampin or Rifampicin: rifadin, rifater combined with isoniazid and pyrazinamide , rimactane.

5. **Antituberculosis drugs**

Rifampin (Rimactane, Rifadin), rifapentine (Priftin), isoniazid (Nydrazid), ethambutol (Myambutol), capreomycin (Capastat), cycloserine (Seromycin), ethionamide (Trecator), rifabutin (Mycobutin), aminosalicylic acid (Paser D/R), clofazimine (Lamprene), rifampin + isoniazid (Rifamate), rifampin + isoniazid + pyrazinamide (Rifater).

E. MICROBIAL RESISTANCE TO ANTIMICROBIAL CHEMOTHERAPEUTIC AGENTS

A common problem in antimicrobial chemotherapy is the development of resistant strains of bacteria. Most bacteria become resistant to antimicrobial agents by one or more of the following mechanisms:

1. **Producing enzymes that inactivate the antibiotic**, e.g., penicillinase and other beta-lactamases.

2. **Altering the target site in the bacterium to reduce or block binding of the antibiotic**, e.g., producing a slightly altered ribosomal subunit that still functions but to which the drug can't bind.

3. **Altering the membranes and transport systems to prevent the entry of the antibiotic into the bacterium and/or using an efflux pump to transport the antibiotic out of the bacterium**.

4. **Developing an alternate metabolic pathway to by-pass the metabolic step being blocked by the antimicrobial agent**, e.g., overcoming drugs that resemble substrates and tie-up bacterial enzymes.

5. **Increasing the production of a certain bacterial enzyme**, e.g., overcoming drugs that resemble substrates and tie-up bacterial enzymes.

These changes in the bacterium that enable it to resist the antimicrobial agent **occur naturally** as a result of mutation or genetic recombination of the DNA in the nucleoid, or as a result of obtaining plasmids from other bacteria. Exposure to the antimicrobial agent then **selects for these resistant strains of organism**.

As an example, many Gram- negative bacteria possess **R (resistance) plasmids** that have genes coding for **multiple antibiotic resistance** through the mechanisms stated above, as well as transfer genes coding for a **sex pilus**. Such an organism can conjugate with other bacteria and transfer an R plasmid to them. *Escherichia coli, Proteus, Serratia, Salmonella, Shigella,* and *Pseudomonas* are examples of bacteria that frequently have R plasmids. Because of the problem of antibiotic resistance, **antibiotic susceptibility testing** is usually done in the clinical laboratory to determine which antimicrobial chemotherapeutic agents will most likely be effective on a particular strain of microorganism. This is discussed in the next section.

To illustrate how plasmids carrying genes coding for antibiotic resistance can be picked up by antibiotic-sensitive bacteria, in today's lab **we will use plasmid DNA to transform an *Escherichia coli* sensitive to the antibiotic ampicillin into one that is resistant to the drug**.

The *E. coli* will be rendered more "competent" to take up plasmid DNA (pAMP), which contains a gene coding for ampicillin resistance, by treating them with a solution of calcium chloride, cold incubation, and a brief heat shock. They will then be plated on 2 types of media: Lauria-Bertani agar (LB) and Lauria-Bertani agar with ampicillin (LB/amp). Only *E. coli* that have picked up a plasmid coding for ampicillin resistance will be able to form colonies on the LB/amp agar.

F. ANTIBIOTIC SUSCEPTIBILITY TESTING

For some microorganisms, susceptibility to chemotherapeutic agents is predictable. However, for many microorganisms (*Pseudomonas, Staphylococcus aureus,* and Gram- negative enteric bacilli such as *Escherichia coli, Serratia, Proteus*, etc.) there is no reliable way of predicting which antimicrobial agent will be effective in a given case. This is especially true with the emergence of many antibiotic-resistant strains of bacteria. Because of this, antibiotic susceptibility testing is often essential in order to determine which antimicrobial agent to use against a specific strain of bacterium.

Several tests may be used to tell a physician which antimicrobial agent is most likely to combat a specific pathogen:

1. **Tube dilution tests**

In this test, a series of culture tubes are prepared, each containing a liquid medium and a different concentration of a chemotherapeutic agent. The tubes are then inoculated with the test organism and incubated for 16-20 hours at 35C. After incubation, the tubes are examined for turbidity (growth). The lowest concentration of chemotherapeutic agent capable of preventing growth of the test organism is the **minimum inhibitory concentration (MIC)**.

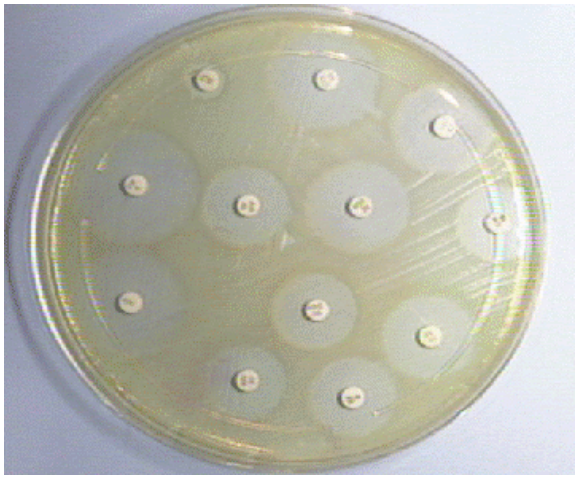
Subculturing of tubes showing no turbidity into tubes containing medium but no chemotherapeutic agent can determine the **minimum bactericidal concentration (MBC)**. MBC is the lowest concentration of the chemotherapeutic agent that results in no growth (turbidity) of the subcultures. These tests, however, are rather time consuming and expensive to perform.

2. **The agar diffusion test (Bauer-Kirby test)**

A procedure commonly used in clinical labs to determine antimicrobial susceptibility is the Bauer-Kirby disc diffusion method. In this test, the in vitro response of bacteria to a standardized antibiotic-containing disc has been correlated with the clinical response of patients given that drug.

In the development of this method, a single high-potency disc of each chosen chemotherapeutic agent was used. Zones of growth inhibition surrounding each type of disc (**Fig. 1**) were correlated with the minimum inhibitory concentrations of each antimicrobial agent (as determined by the tube dilution test). The MIC for each agent was then compared to the usually-attained blood level in the patient with adequate dosage. Categories of "Resistant," "Intermediate," and "Susceptible" were then established.

Fig. 1:
Zones of inhibition surrounding antibiotic discs.



The basic steps for the Bauer-Kirby method of antimicrobial susceptibility testing are given below. This outline of procedure is intended to be used as an adjunct to clinical laboratory instruction. The procedure is highly regulated and controlled by the Clinical and Laboratory Standards Institute (CLSI) and must be accompanied by a rigorous quality assurance proGram including performance by certified and/or licensed personnel when the results are to be reported in clinical settings.

a. Prepare a **standard turbidity inoculum** of the test bacterium so that a certain density of bacteria will be put on the plate.

- Select 3-5 isolated colonies of the bacterium that is being tested.
- If the organism is a *Staphylococcus* or is fastidious and grows unpredictably in broth like the streptococci, suspend the colonies is saline, Mueller Hinton broth or trypticase soy broth. If the organism grows rapidly in broth, place the colonies in Mueller Hinton broth or trypticase soy broth and incubate 2-8 hours.
- Match the turbidity of the test suspension or culture with a 0.5 McFarland standard. (McFarland standards are tubes containing either latex particles or barium sulfate and adjusted to a standard turbity.)
    - If the bacterial suspension is too turbid, add more saline or broth.
    - If the bacterial suspension is too light, pick off more colonies and suspend them in the broth or incubate longer.

b. **Inoculate a 150mm Mueller-Hinton agar plate** with the standardized inoculum so as to cover the entire agar surface with bacteria.

- Dip a sterile swab into the previously standardized tube of the bacterium being tested.
- Squeeze the swab against the inner wall of the tube to remove excess liquid.
- Swab the entire plate from top to bottom, edge-to-edge leaving no gaps.
- Rotate the plate approximately 60 degrees and using the same swab, again swab the entire plate from top to bottom.
- Rotate the plate approximately 60 degrees and using the same swab, and swab the entire plate from top to bottom a third time.

c. Place **standardized antibiotic-containing discs** on the plate.

d. **Incubate** the plate agar side up. For nonfastidious bacteria, incubate at 35°C for 16-18 hours. For fastidious bacteria, follow CLSI standards.

e. Measure the **diameter** of any resulting **zones of inhibition** in millimeters (mm) as shown in **Fig. 2**.

f. Determine if the bacterium is susceptible, moderately **susceptible, intermediate, or resistant** to each antimicrobial agent using a **standardized table** (**see Table 2**). (The latest interpretation tables can be found in CLSI document M100 which is updated every January.)

- If there is a double zone of inhibition, measure the diameter of the innermost zone.
- If there is a zone containing colonies, measure the diameter of the colony free zone. Measure from the colony closest to the antibiotic disc to the center of the disc (the radius) and double that number to get the diameter.
- If there is a feathered zone, measure the diameter of the point where there is an obvious demarcation between growth and no growth. Measure from the end of the feathering closest to the antibiotic disc to the center of the disc (the radius) and double that number to get the diameter.
- When testing swarming *Proteus mirabilis*, ignore the swaming.

- When testing *Staphylococcus aureus*, the haze around an oxacillin should not be ignored. Measure the diameter of the zone free of growth or haze.

The term intermediate generally means that the result is inconclusive for that drug-organism combination. The term moderately susceptible is usually applied to those situations where a drug may be used for infections in a particular body site, e.g., cystitis because the drug becomes highly concentrated in the urine.

3. **Automated tests**

Computerized automated tests have been developed for antimicrobial susceptibility testing. These tests measure the inhibitory effect of the antimicrobial agents in a liquid medium by using light scattering to determine growth of the test organism. Results can be obtained within a few hours. Labs performing very large numbers of susceptibility tests frequently use the automated methods but the equipment is quite expensive.

PROCEDURE: MICROBIAL RESISTANCE TO ANTIMICROBIAL CHEMOTHERAPEUTIC AGENTS

MATERIALS

Plasmid DNA (pAMP) on ice, calcium chloride solution on ice, 2 sterile culture tubes, 1 tube of LB broth, 2 plates of LB agar, 2 plates of LB agar with ampicillin (LB/amp), sterile 1 ml transfer pipettes, sterile plastic inoculating loops, bent glass rod, turntable, alcohol, beaker of ice, water bath at 42°C.

ORGANISM

LB agar culture of *Escherichia coli*

PROCEDURE: demonstration; see Fig. 3

1. Label one LB agar plate "Transformed Bacteria, Positive control" and the other LB agar plate "Wild-Type Bacteria, Positive Control."
Label one LB/amp agar plate "Transformed Bacteria, Experiment" and the other LB/amp agar plate "Wild-Type Bacteria, Negative Control."

2. Label one sterile culture tube "(+) AMP" and the other "(-) AMP." Using a sterile 1ml transfer pipette, add **250 µl of ice-cold calcium chloride** to each tube. Place both tubes on ice.

3. Using a sterile plastic inoculating loop, transfer **1-2 large colonies of *E. coli*** into the (+) AMP tube and vigorously tap against the wall of the tube to dislodge all the bacteria. Immediately suspend the cells by repeatedly pipetting in and out with a sterile transfer pipette until no visible clumps of bacteria remain. Return tube to the ice.

4. Repeat step 3 this time using the (-) AMP tube and return to the ice.

5. Using a sterile plastic inoculating loop, add **one loopful of pDNA (plasmid DNA) solution to the (+) AMP tube** and swish loop to mix the DNA. Return to the ice.

6. Incubate both tubes **on ice for 15 minutes**.

7. After 15 minutes, **"heat-shock"** both tube of bacteria by immersing them in a 42°C water bath for **90 seconds**. Return both tubes to the **ice for 1 minute or more**.

8. Using a sterile 1ml transfer pipette, add **250µl of LB broth** to each tube. Tap tubes with your fingers to mix. Set tubes in a test tube rack at room temperature.

9. Using a sterile 1ml transfer pipette, add **100 µl of *E. coli* suspension from the (-) AMP tube** onto the **LB/amp** agar plate labeled "Wild-Type Bacteria, Negative Control." Add another 100 µl of *E. coli* from the **(-) AMP** to the **LB** agar plate labeled "Wild-Type Bacteria, Positive Control."

10. Using a bent glass rod dipped in alcohol and flamed, spread the bacteria thoroughly over both agar plates. Make sure you re-flame the glass rod between plates.

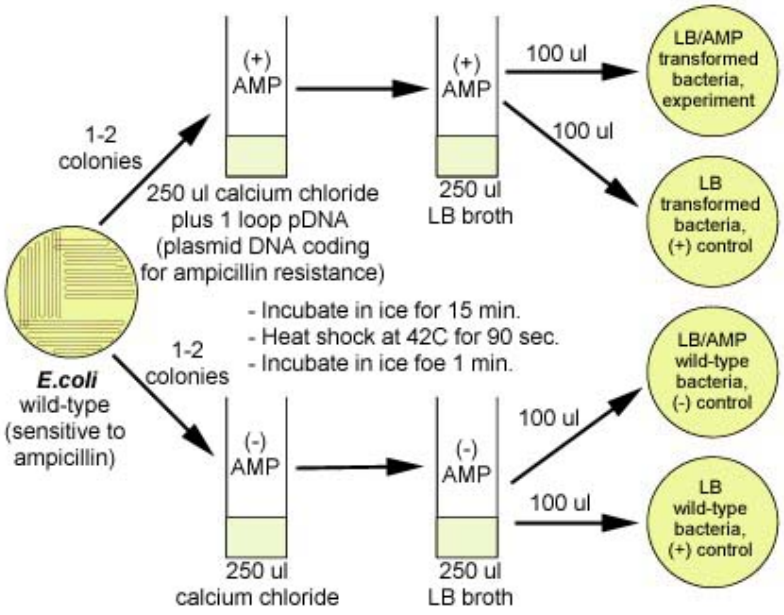
11. Using a sterile 1ml transfer pipette, add **100 µl of *E. coli* suspension from the (+) AMP tube** onto the **LB/amp** agar plate labeled "Transformed Bacteria, Experiment." Add another 100 µl of *E. coli* from the **(+) AMP** to the **LB** agar plate labeled "Transformed Bacteria, Positive Control."

12. Immediately spread as in step 10.

13. Incubate all plates **upside down and stacked in the petri plate holder on the shelf of the 37°C incubator corresponding to your lab section**.

14. The procedure is summarized in **Fig. 21C**.

Fig. 3:
Transfer of plasmid-mediated resistance to ampicillin in *E. coli*



PROCEDURE: ANTIBIOTIC SUSCEPTIBILITY TESTING

MATERIALS

150mm Mueller-Hinton agar plates (3)
Sterile swabs (3)
An antibiotic disc dispenser containing discs of antibiotics commonly effective against Gram-positive bacteria, and one containing discs of antibiotics commonly effective against Gram- negative bacteria

ORGANISMS

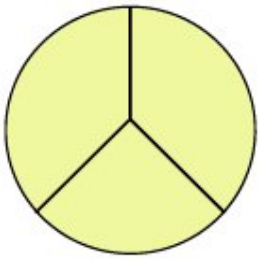
Trypticase Soy broth cultures of *Staphylococcus aureus* (Gram-positive), *Enterococcus faecalis* (Gram-positive), and *Pseudomonas aeruginosa* (Gram- negative)

PROCEDURE: to be done in groups of three

The basic steps for the Bauer-Kirby method of antimicrobial susceptibility testing are given below. This outline of procedure is intended to be used as an adjunct to general microbiology laboratory instruction. The procedure is highly regulated and controlled by the Clinical and Laboratory Standards Institute (CLSI) and must be accompanied by a rigorous quality assurance proGram including performance by certified and/or licensed personnel when the results are to be reported in clinical settings.

1. Take **3 Mueller-Hinton agar plates**. Label one *S. aureus*, one *E. faecalis*, and one *P. aeruginosa*.

2. Using your wax marker, divide each plate into **thirds** to guide your streaking.



3. Dip a **sterile swab** into the previously standardized tube of *S. aureus*. **Squeeze the swab against the inner wall of the tube** to remove excess liquid.

4.Streak the swab **perpendicular to each of the 3 lines** drawn on the plate overlapping the streaks to assure **complete coverage** of the entire agar surface with inoculum.

5. **Repeat steps 3 and 4** for the *E. faecalis* and *P. aeruginosa* plates.

6. Using the appropriate antibiotic disc dispenser, place **Gram-positive antibiotic-containing discs on the plates of *S. aureus*** and ***E. faecalis;* Gram-negative antibiotic-containing discs on the plate of *P. aeruginosa***.

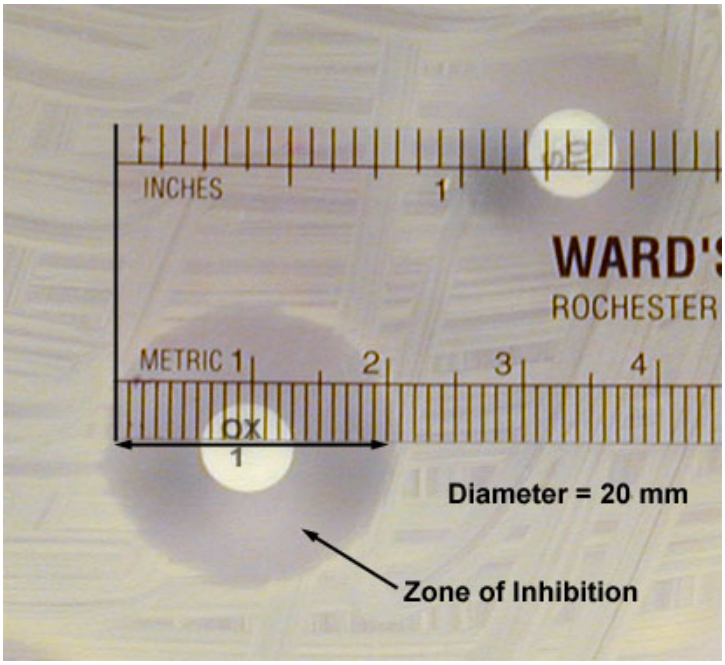
7. Incubate the 3 plates **upside down and stacked on the shelf of the 35°C incubator corresponding to your lab section** until the next lab period.

8. Using a metric ruler, measure the **diameter in mm of the zone of inhibition around each disc on each plate** by placing the ruler on the bottom of the plate (Fig. 2).

- If there is a double zone of inhibition, measure the diameter of the innermost zone (see Fig. 4).
- If there is a zone containing colonies, measure the diameter of the colony free zone. Measure from the colony closest to the antibiotic disc to the center of the disc (the radius) and double that number to get the diameter. (See Fig. 5).
- If there is a feathered zone, measure the diameter of the point where there is an obvious demarcation between growth and no growth. Measure from the end of the feathering closest to the antibiotic disc to the center of the disc (the radius) and double that number to get the diameter. (See Fig. 6).
- When testing *Staphylococcus aureus*, the haze around an oxacillin should not be ignored. Measure the diameter of the zone free of growth or haze.

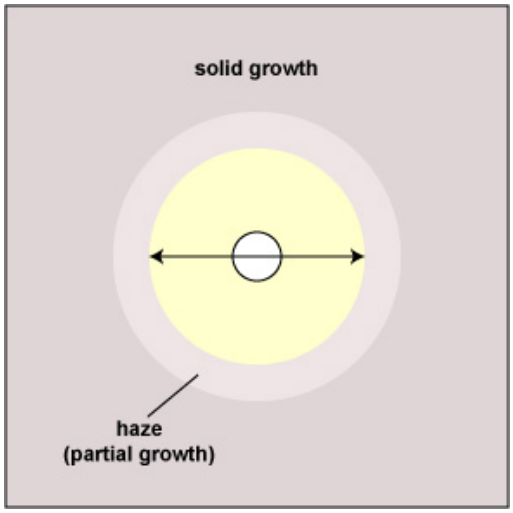
9. Determine whether each organism is **susceptible, moderately susceptible, intermediate, or resistant to each chemotherapeutic agent** using the standardized table (**Table 2**) and record your results.

Fig. 2:
Measuring the diameter of zones of inhibition in mm.



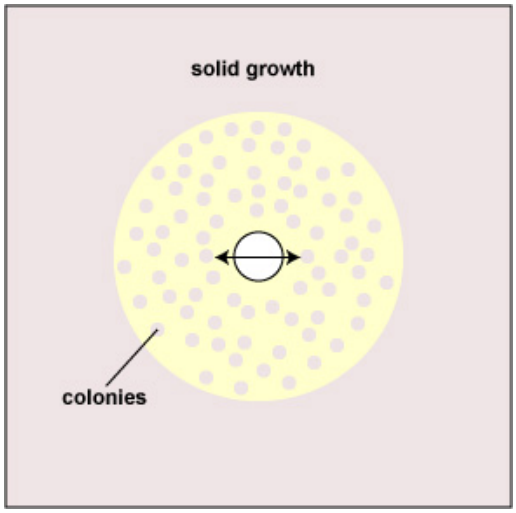
LABORATORY 18
USING CHEMICAL AGENTS TO CONTROL MICROORGANISMS

Fig. 4:
Double Zone of Inhibition



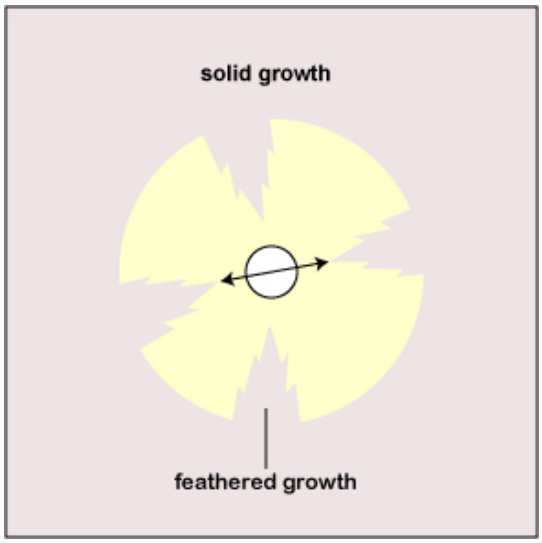
If there is a double zone of inhibition, measure the diameter of the innermost zone (arrowed line).

Fig. 5:
Zone Containing Colonies



If there is a zone containing colonies, measure the diameter of the colony free zone (arrowed line).

Fig. 6:
Feathered Zone of Inhibition



If there is a zone containing feathered growth, measure the diameter of the growth-free zone (arrowed line).

Table 2
Zone Size Interpretive Chart for Bauer-Kirby Test

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

Table 2, continued
Zone Size Interpretive Chart for Bauer-Kirby Test

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

RESULTS: MICROBIAL RESISTANCE TO ANTIMICROBIAL CHEMOTHERAPEUTIC AGENTS

Count the number of colonies on each plate. If the growth is too dense to count individual colonies, record "lawn" (bacteria cover nearly the entire agar surface).

Plate	Number of colonies	Conclusion
LB/amp "Transformed Bacteria, Experiment"		
LB "Transformed Bacteria, Positive Control"		
LB/amp "Wild-Type Bacteria, Negative Control"		
LB "Wild-Type Bacteria, Positive Control"		

RESULTS: ANTIBIOTIC SUSCEPTIBILITY TESTING: BAUER-KIRBY METHOD

Interpret the results following steps 9 and 10 of the procedure and record your results in the tables below.

Staphylococcus aureus

Disc code	Antimicrobial agent	Zone in mm	R	I	MS	S
AmC-30	Amoxicillin/ Clavulanic Acid					
CTX-30	Cefotaxime					
FOX-30	Cefoxitin					
CIP-5	Ciprofloxacin					
CC-2	Clindamycin					
E-15	Erythromycin					
K-30	Kanamycin					
OX-1	Oxacillin					
SXT	Sulfamethoxazole + Trimethoprim					
Te-30	Tetracycline					
TZP-110	Piperacillin/Tazobactum					
Va-30	Vancomycin					

R = Resistant
I = Intermediate
MS = Moderately Susceptible
S = Susceptible

Enterococcus faecalis

Disc code	Antimicrobial agent	Zone in mm	R	I	MS	S
AmC-30	Amoxicillin/ Clavulanic Acid					
CTX-30	Cefotaxime					
FOX-30	Cefoxitin					
CIP-5	Ciprofloxacin					
CC-2	Clindamycin					
E-15	Erythromycin					
K-30	Kanamycin					
OX-1	Oxacillin					
SXT	Sulfamethoxazole + Trimethoprim					
Te-30	Tetracycline					
TZP-110	Piperacillin/Tazobactum					
Va-30	Vancomycin					

R = Resistant
I = Intermediate
MS = Moderately Susceptible
S = Susceptible

Pseudomonas aeruginosa

Disc code	Antimicrobial agent	Zone in mm	R	I	MS	S
AN-30	Amikacin					
AmC-30	Amoxicillin/ Clavulanic Acid					
AM-10	Ampicillin					
CB-100	Carbenicillin					
CIP-5	Ciprofloxacin					
CTX-30	Cefotaxime					
FOX-30	Cefoxitin					
GM-10	Gentamicin					
K-30	Kanamycin					
SXT	Sulfamethoxazole + Trimethoprim					
Te-30	Tetracycline					
TZP-110	Piperacillin/Tazobactum					

R = Resistant
I = Intermediate
MS = Moderately Susceptible
S = Susceptible

PERFORMANCE OBJECTIVES
LABORATORY 18

After completing this lab, the student will be able to perform the following objectives:

A. DISINFECTANTS, ANTISEPTICS, AND SANITIZERS

1. Define the following terms: sterilization, disinfection, decontamination, disinfectant, antiseptic, and sanitizer.
2. State why chemical agents are usually unreliable for sterilization.
3. List five factors that may influence the antimicrobial action of disinfectants, antiseptics, and sanitizers.
4. Describe two modes of action of disinfectants, antiseptics, and sanitizers (i.e., how they harm the microorganisms).
5. Name two chemical agents that are reliable for sterilization.

B. EVALUATION OF DISINFECTANTS, ANTISEPTICS, AND SANITIZERS

1. State why the results of an *in vitro* test to evaluate chemical agents may not necessarily apply to *in vivo* situations.

C. EVALUATION OF HAND WASHING

1. Define transient flora and resident flora and compare the two groups in terms of ease of removal.

D. ANTIMICROBIAL CHEMOTHERAPEUTIC AGENTS

1. Define the following: antibiotic, antimicrobial chemotherapeutic chemical, narrow spectrum antibiotic, broad-spectrum antibiotic.
2. Discuss the meaning of selective toxicity in terms of antimicrobial chemotherapy.
3. List four genera of microorganisms that produce useful antibiotics.
4. Describe four different major modes of action of antimicrobial chemotherapeutic chemicals and give three examples of drugs fitting each mode of action.

E. MICROBIAL RESISTANCE TO ANTIMICROBIAL AGENTS

DISCUSSION

1. State five mechanisms by which microorganisms may resist antimicrobial chemotherapeutic agents.
2. Briefly describe R-plasmids and name four bacteria that commonly possess these plasmids.

RESULTS

1. Interpret the results of the *Escherichia coli* plasmid transformation experiment.

F. ANTIBIOTIC SUSCEPTIBILITY TESTING

DISCUSSION

1. State why antimicrobial susceptibility testing is often essential in choosing the proper chemotherapeutic agent to use in treating an infection.
2. State what is meant by MIC.

RESULTS

1. Interpret the results of a Bauer-Kirby antimicrobial susceptibility test when given a Mueller-Hinton agar plate, a metric ruler, and a standardized zone-size interpretation table.

LABORATORY 19
PARASITOLOGY

A. Parasitic Protozoans
B. Parasitic Helminths

A. PARASITIC PROTOZOANS

Protozoans are unicellular **eukaryotic** microorganisms belonging to the Kingdom Protista. They **reproduce asexually** by **fission** (one cell splits into two), **schizogony** (multiple fission; the nucleus divides many times and the nuclei are separated into daughter cells), or **budding** (pinching off of a bud from a parent cell). Some protozoans also **reproduce sexually** by fusion of haploid sex cells called gametes.

The vegetative form (motile, feeding, reproducing form) of a protozoan is called a **trophozoite**. Under certain conditions, some protozoans produce a protective form called a **cyst** that enables them to survive harsh environments. Cysts allow some pathogens to survive outside their host. Favorable conditions in a new host result in excystation that once again produces a trophozoite.

The parasitic protozoans can be divided into 4 groups based primarily on their means of locomotion.

1. **The Sarcomastigophora (Amoeboflagellates)**

a. The **amoebas** (subphylum Sarcodina) move by extending lobe-like projections of their cytoplasm called pseudopodia. Food is obtained by phagocytosis.

1. One important pathogen in this group is ***Entamoeba histolytica***, the causative agent of **amoebic dysentery**. The organism is **transmitted** by the **fecal-oral route**. Cysts are excreted in the feces of an infected individual or carrier and ingested through fecally-contaminated food, water, objects, etc. After excystation, the trophozoites penetrate the walls of the large intestines causing ulceration and frequently causing the symptoms of dysentery. Involvement of the liver and other organs may occur if the protozoan invades the blood. The disease is **diagnosed by microscopically looking for cysts of *E. histolytica* in a fecal smear** (**Fig. 1**).

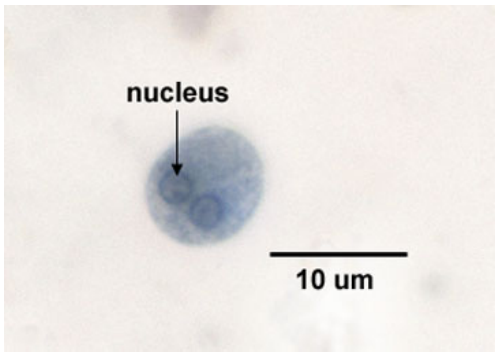
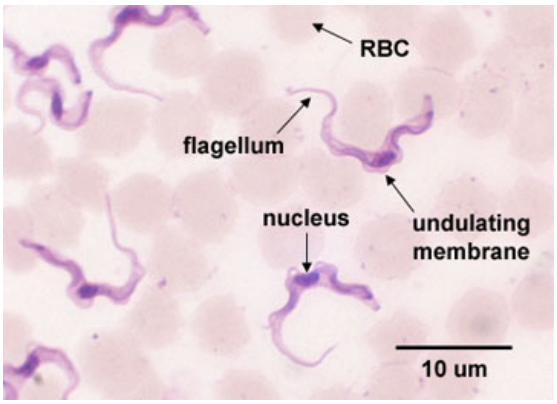
2. *Acanthamoeba*, another infectious amoeba, can infect the eye, blood, spinal cord, and brain and is transmitted by waterborne cysts picked up while swimming in contaminated water, crossing the mucous membranes.

b. The flagellates (subphylum Mastigophora) move by means of flagella. Some also have an undulating membrane. Important pathogens in this class include:

1. ***Trypanosoma gambiense, Trypanosoma rhodesiense*, and *Trypanosoma cruzi***

T. gambiense and *T. rhodesiense* cause the disease **African sleeping sickness** or **African trypanosomiasis**. They are **transmitted** to humans by the bite of an **infected tsetse fly** (a vector). The disease primarily involves the lymphatic and nervous systems of humans and is **diagnosed by microscopically looking for *Trypanosoma*** (**Fig. 2**) **in the blood, in aspirated fluid from lymph nodes, or in spinal fluid**.

T. cruzi causes **South American sleeping sickness** or Chagas' disease and is **transmitted** by infected **Triatomid bugs (kissing bugs)**.

Fig. 1: Cysts of *Entamoeba* in a fecal smear.	**Fig. 2:** Trophozoite of *Trypanosoma* in the blood.
	

2. ***Giardia lamblia***

Giardia lamblia (*G. intestinalis*) causes a gastroenteritis-type of disease called **giardiasis.** Giardiasis is the most common protozoan intestinal disease in the U.S. and is **transmitted** by the **fecal-oral route**. Cysts of the organism are ingested through fecally-contaminated food, water, etc. Giardiasis is **diagnosed by microscopically looking for cysts of *G. intestinalis* in fecal smears** (**Fig. 3**).

3. ***Trichomonas vaginalis***

This protozoan causes **genitourinary trichomoniasis**. There are an estimated 2.5 million cases per year in the U.S. In females, it usually appears as **vaginitis** with itching and a white discharge. In males it is often asymptomatic but may cause urethritis. It is **transmitted** mainly by **venereal contact** and is **diagnosed by microscopically looking for *T. vaginalis* trophozoites in vaginal discharge and urine** (**Fig. 4**).

2. **The Ciliophora**

This group of protozoans is characterized by a covering of **cilia** used for motility and direction of food particles into the mouth or cytosome. In the trophozoite, a large macronucleus, small micronucleus, cilia, and contractile vacuoles may be seen.

The only pathogen in this group is ***Balantidium coli***, which causes a diarrhea-type infection called **balantidiasis**. The protozoan is **transmitted** to humans by the **fecal-oral route** and invades the large intestines causing ulceration. It is **diagnosed by microscopically looking for *B. coli* in a fecal** smear (**Fig. 5**).

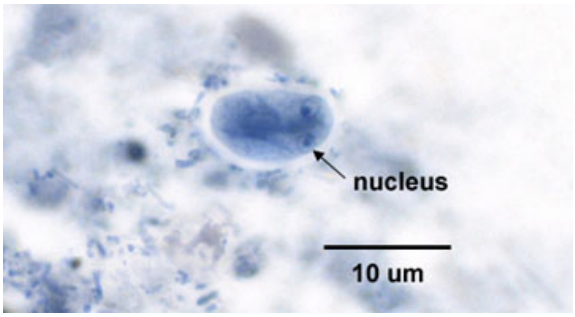
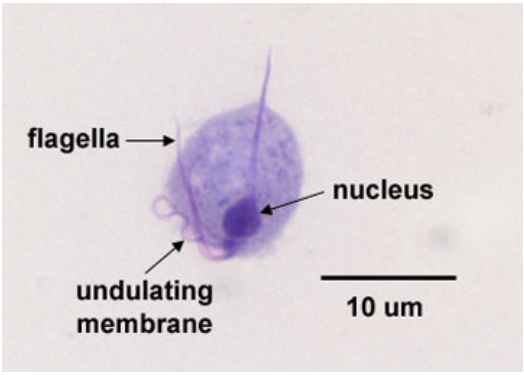
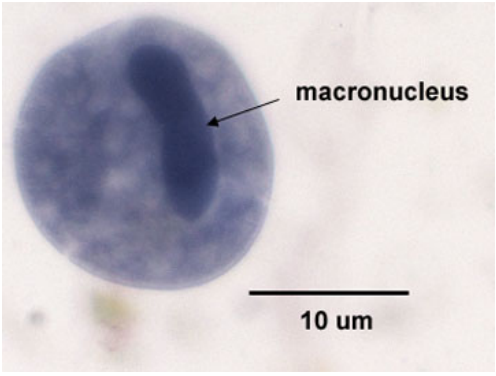
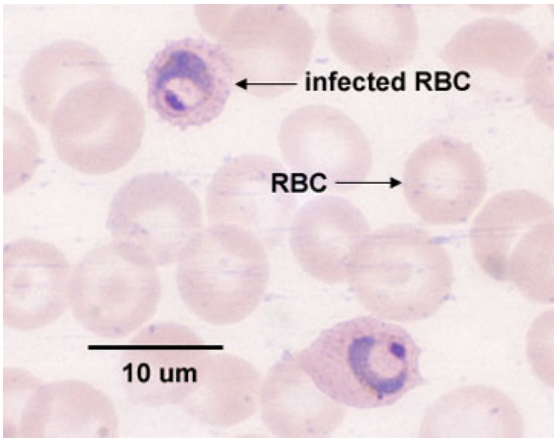
Fig. 3: Cyst of *Giardia lamblia* in a fecal smear.	**Fig. 4:** Trophozoite of *Trichomonas vaginalis.*
	

Fig. 5: *Balantidium coli* in a fecal smear.	**Fig. 7:** Blood smear showing red blood cells infected with *Plasmodium*.
	

3. **The Apicomplexans**

The **sporozoa** are **not motile** in their mature forms, reproduce both asexually and sexually, and often have complex life cycles for transmission from host to host. They possess a complex of organelles at their apex (apical complexes) that contain enzymes used in penetrating host tissues.Threecommon pathogens in this group are:

a. ***Toxoplasma gondii***

This protozoan causes the disease **toxoplasmosis**. In adults, the disease is usually mild and resembles infectious mononucleosis. However, new-born infants who contracted toxoplasmosis *in utero* commonly have severe central nervous system damage. It also causes severe disease in immunosuppressed individuals such as people with AIDS. Domestic cats, who pick up the organism from eating infected rodents, may act as carriers of *T. gondii*, and their feces may contain oocysts of the protozoan. However, the organism may be found in practically every mammal. The disease is

transmitted to humans by **ingesting raw meat of an infected mammal or by inhaling or ingesting cysts of *T. gondii* from cat feces**. Pregnant women should be especially careful to avoid raw meat and cat feces. The disease is **diagnosed** by **serologic testing and by growing the organism in cell culture**.

b. ***Plasmodium***

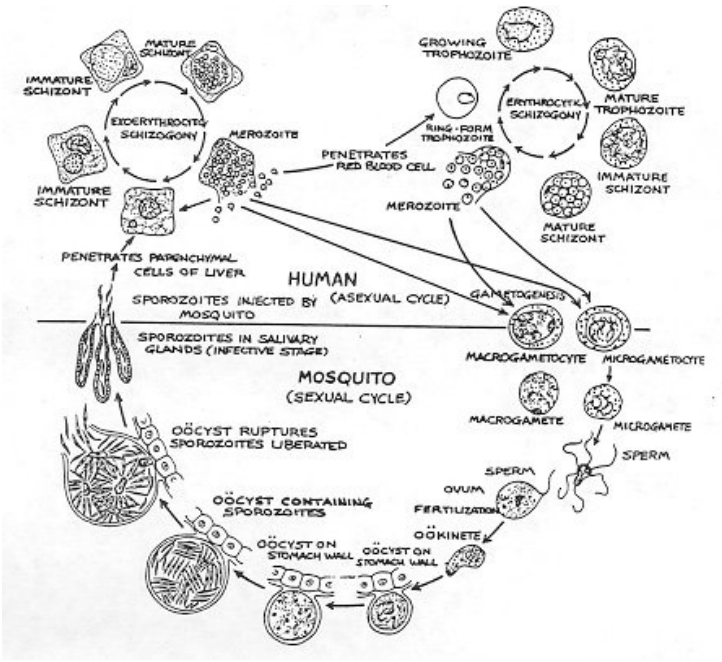
Four *Plasmodium* species, *P. falciparum, P. malariae, P. ovale,* and *P. vivax* cause **malaria**. The vector involved in the **transmission** of the disease from human to human or from animal to human is an **infected female *Anopheles* mosquito**.

Asexual reproduction (or **schizogony**) of the *Plasmodium* occurs within liver cells and red blood cells of the **infected human**. With malaria caused by *P. vivax* and *P. ovale*, a dormant form or **hypnozoite** remains in the liver and may cause later relapses. The infected cells in which the organism is reproducing by schizogony are called **schizonts** (**Fig. 7**). The **sexual cycle** (or **sporogeny**) occurs in the **mosquito**. The typical **recurring malarial fever** is a result of the lysis of the infected red blood cells, causing release of **merozoites** and their metabolic by-products. Fever cycles of 24, 48, or 72 hours usually occur depending on the infecting species. The **life cycle** is shown in **Fig. 6**. Malaria is **diagnosed** by **microscopically looking for the parasite within infected red blood cells (schizonts)**.

c. ***Cryptosporidium***

Cryptosporidium is an intracellular parasite that causes diarrhea, although in people who are immunosuppressed it can also cause respiratory and gallbladder infections. It is transmitted by the fecal-oral route.

Fig. 6:
Malaria life cycle.



B. PARASITIC HELMINTHS

Helminthology is the study of worms, or **helminths**. Helminths are multicellular, often macroscopic worms having both rudimentary organs and organ systems. We will look at three groups of pathogenic helminths: **nematodes, cestodes, and trematodes**

1. **The Nematodes (Roundworms)**

Nematodes are **elongated, unsegmented, cylindrical worms having separate sexes**. The various systems of the roundworms can be seen in **Fig. 8**, and a generalized drawing of a nematode is given in **Fig. 9**. We will look at several pathogenic nematodes.

Fig. 8
Organ systems of a typical Nematode (roundworm)

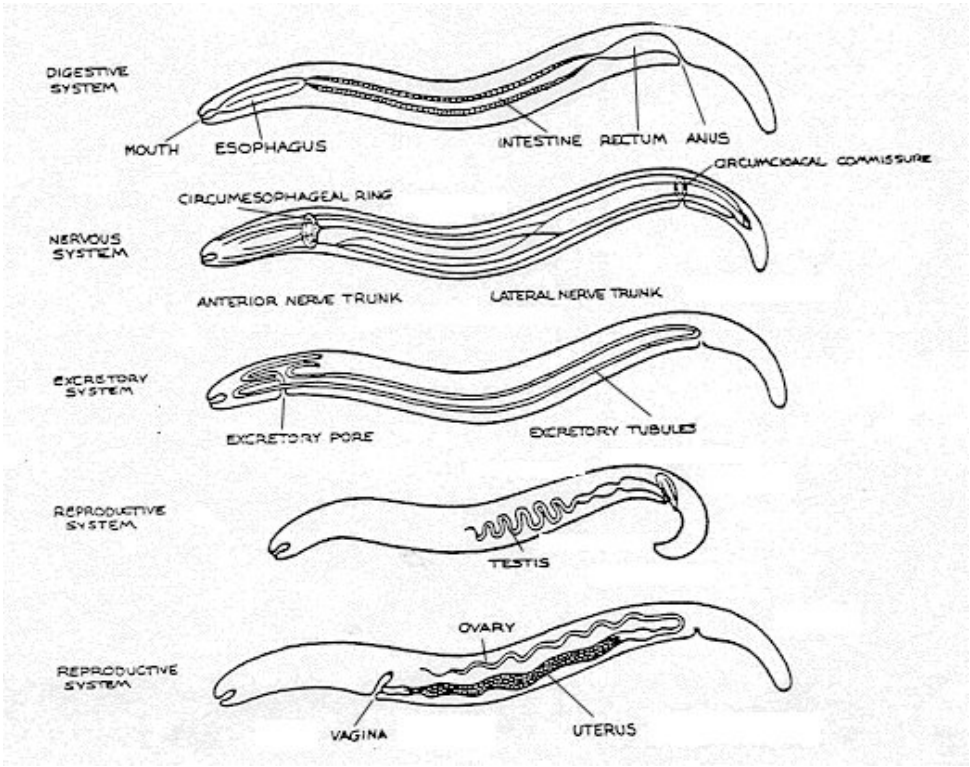
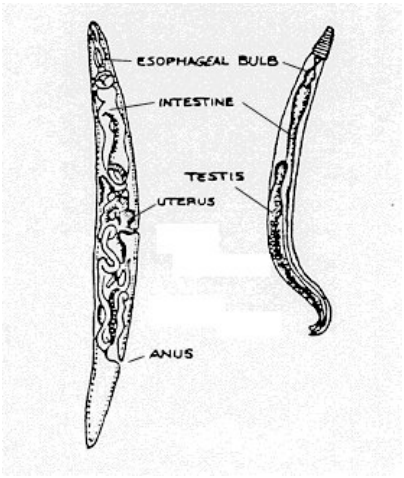


Fig. 9:
Female and male nematodes



a. ***Ascaris lumbricoides*** **(Fig. 11A)**

These worms range from 20-45 cm long and are 5 mm in diameter in the adult form, the female being larger than the male. The life cycle is seen in **Fig. 10**. The disease is called **ascariasis**. Humans become **infected** by **ingesting water or food contaminated with feces that contains *Ascaris* ova or from fingers contaminated with polluted soil**. The disease is **diagnosed by microscopically looking for *Ascaris* ova in a fecal smear**. The Ascaris ova have a bumpy or serrated edge (**Fig. 11**). A similar roundworm, ***Toxocara*****,** parasitizes dogs and cats. Visceral larva migrans is the migration of larvae of these worms in human tissues such as lung, liver, and brain, where they may cause tissue damage and allergic reactions.

b. ***Enterobius vermicularis*** **(pinworms) Fig. 13A**

E. vermicularis is a small worm, the female being 8-13 mm long and 0.3-0.5 mm wide; the male being 2-5 mm long and 0.1 mm wide. The life cycle is shown in **Fig. 12**. Humans, frequently children, become **infected** by **inhaling *E. vermicularis* ova or from transfer of ova to the mouth from fecally-contaminated fingers**. The female worm migrates to the perianal region of the infected individual, releasing masses of ova and causing an itching sensation. The disease is **diagnosed** by applying tape to the perianal region and **microscopically looking for pinworm ova that have stuck to the tape**. The pinworm ova have a smooth edge (**Fig. 13**)

c. ***Trichinella spiralis***

T. spiralis causes a disease called **trichinosis**. Humans become **infected** mainly by **eating poorly cooked infected pork** containing encysted larva 1-2 mm long (**Fig. 15**). The life cycle is shown in **Fig. 14**. The larvae excyst and develop into adult worms in the intestines. After mating, the female releases larvae which enter the blood and are distributed throughout the body where they become encysted in muscle tissue. The disease is **diagnosed by serological tests and microscopic examination of biopsy specimens**.

2. **The Cestodes (Tapeworms)**

Tapeworms are **flat, segmented worms which are hermaphroditic** (contain both male and female sexual organs). Adult tapeworms have several distinct regions. The **scolex** is a head-like structure with distinct suckers and possibly hooks used for attachment to the intestinal wall (**Fig. 16** and **Fig. 17**). Behind the scolex is a constricted neck region consisting of germinative tissue from which new segments, or **proglottids**, are formed (**Fig. 18**). Finally, there is a long **strobila** or chain of proglottids of varying stages of maturity. Proglottids containing thousands of ova are excreted in the feces. When ingested by intermediate hosts (such as cattle, pigs, and fish), the larva hatch from the ingested ova and migrate to muscle where they encyst as cysticerci. The life cycle is shown in **Fig. 19**.

Humans become **infected** with tapeworms by **eating poorly cooked infected beef, pork, or fish containing cysticerci**. *Taenia saginata*, the beef tapeworm often reaches 6 meters in length; *Taenia solium*, the pork tapeworm is normally 2-7 meters in length; and *Diphyllobothrium latum*, the fish tapeworm may reach 3-6 meters in length. These tapeworms are **diagnosed by looking for proglottids and ova in the feces**.

When humans ingest tapeworm eggs instead of cysts, embryos are released, penetrate the intestinal wall, and enter the blood. The embryos migrate to various tissues (frequently the brain) and develop into cysticerci. Humans also act as intermediate hosts for *Echinococcus granulosus* found in dogs and cats. Larva hatch from ingested ova and migrate to the liver and lungs and form hydatid cysts.

Fig. 10:
Life cycle of *Ascaris lumbricoides*

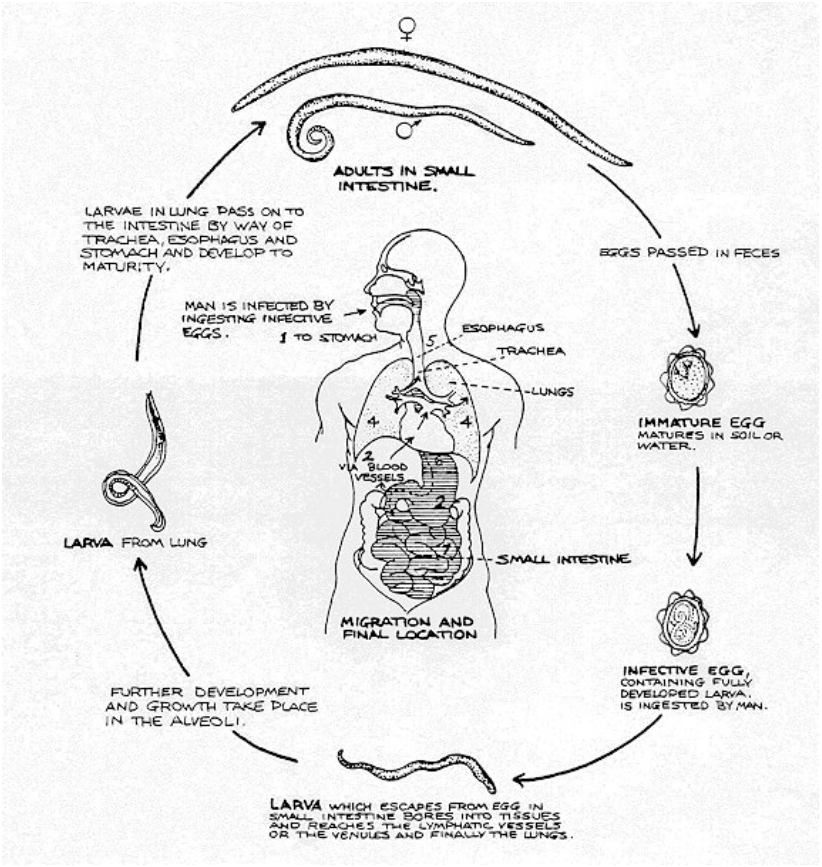


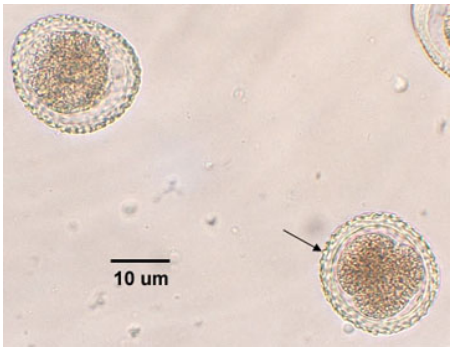
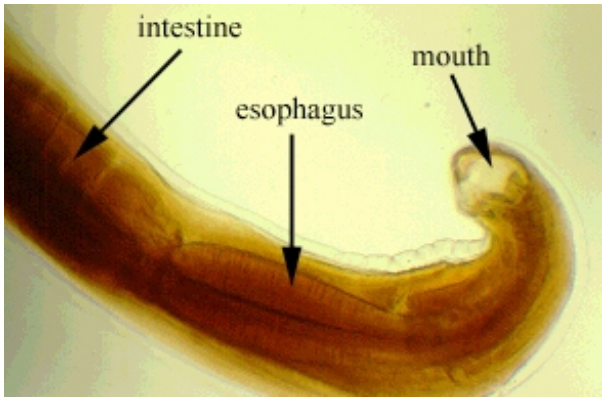
Fig. 11: *Ascaris* ova	**Fig. 11A:** *Ascaris*
	

Fig. 12:
Life cycle of *Enterobius vermicularis*

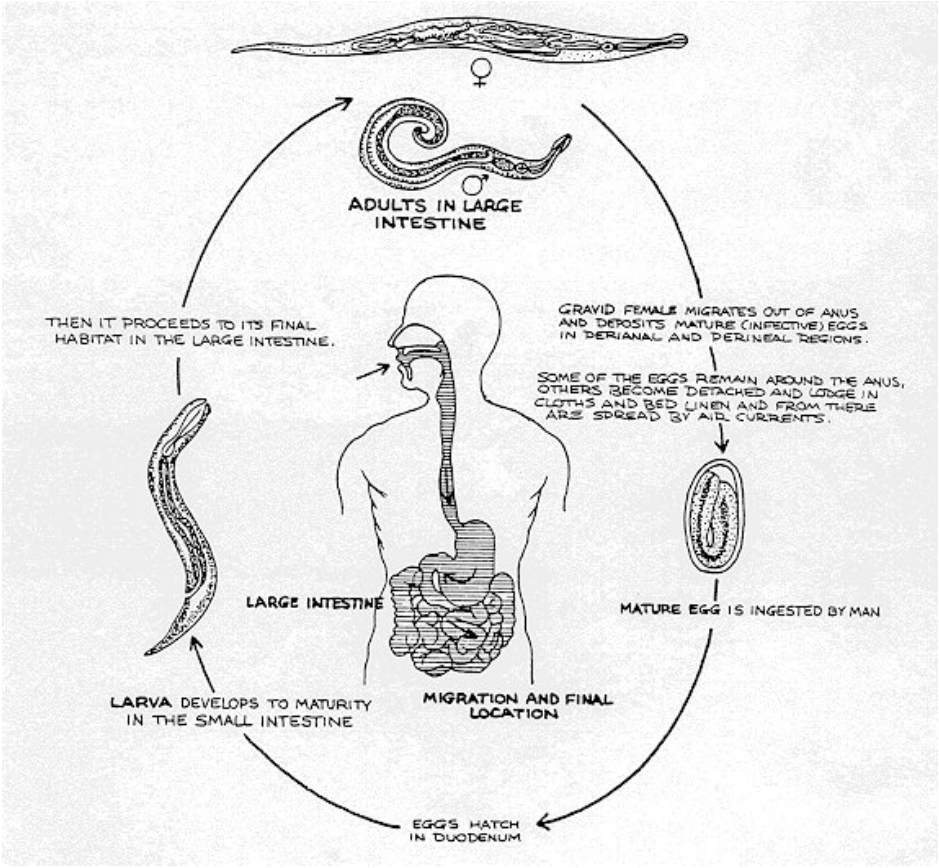


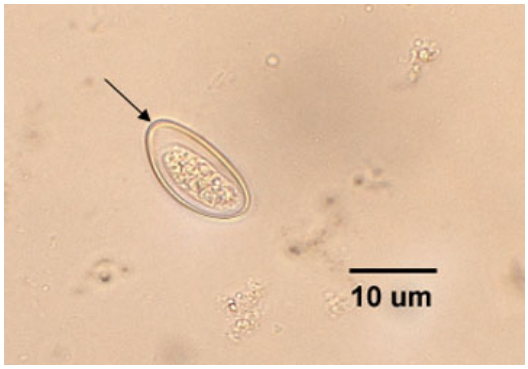
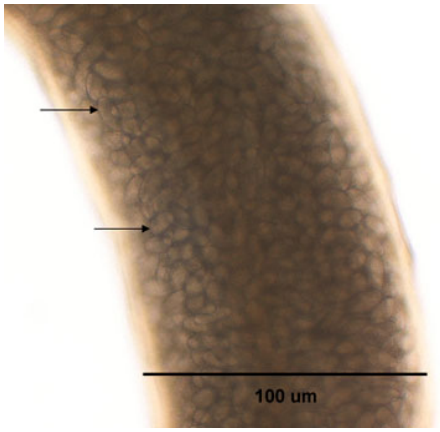
Fig. 13: *Enterobius* ovum	**Fig. 13A:** Female *Enterobius* filled with ova (arrows)
	

Fig. 14:
Life cycle of *Trichinella spiralis*

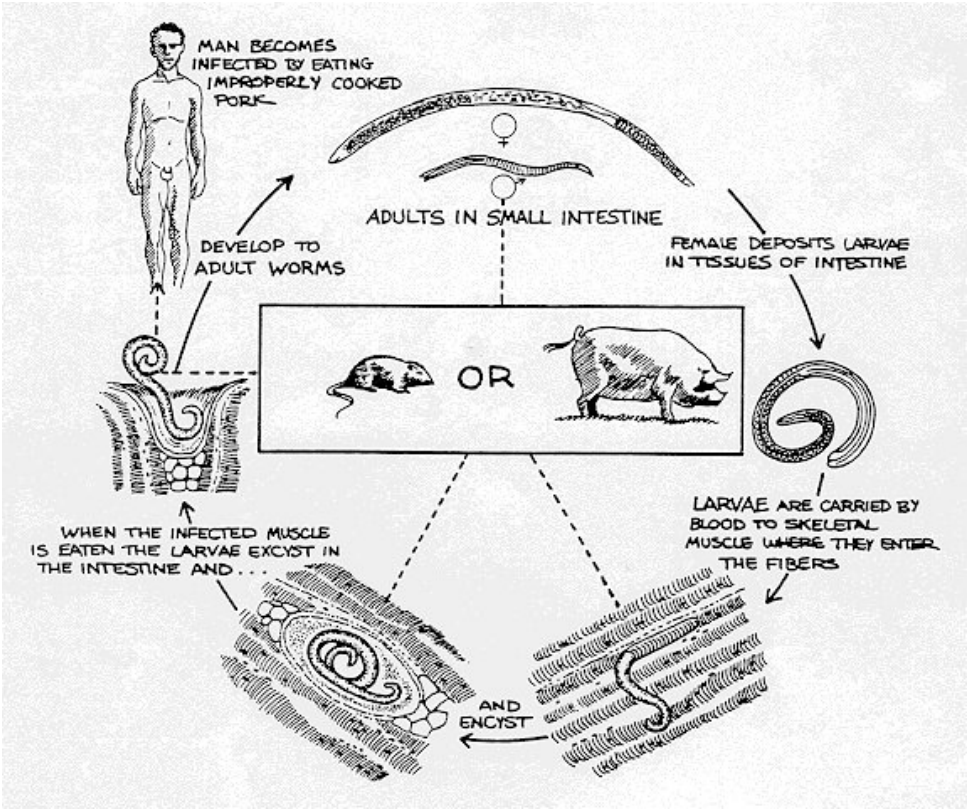


Fig. 15:
Trichinella encysted in muscle

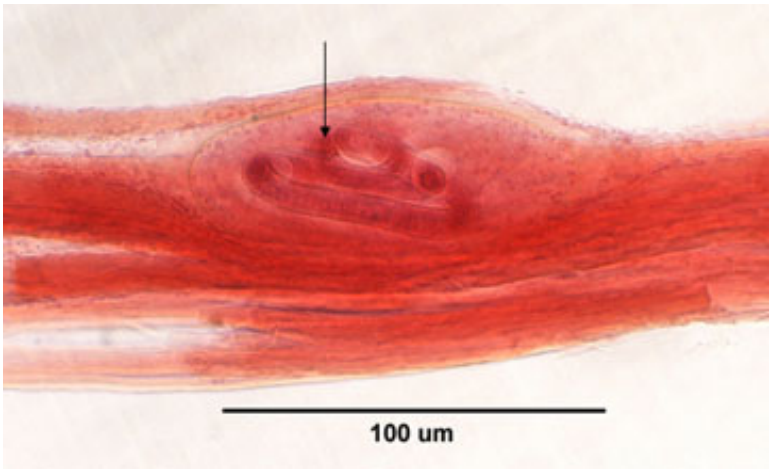


Fig. 16:
Cestode (tapeworm) morphology

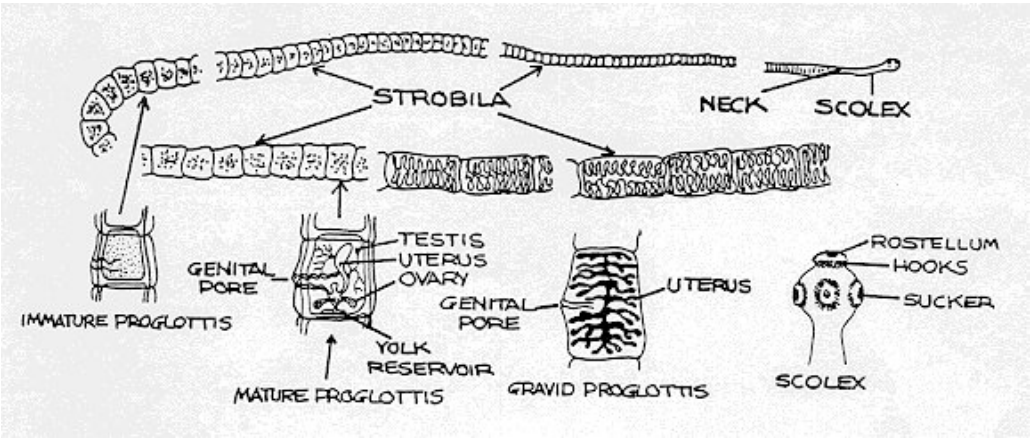


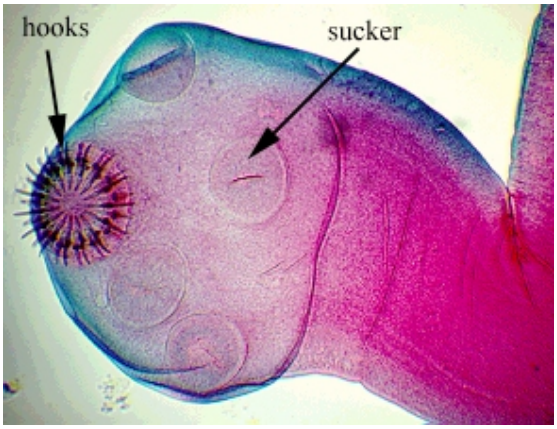
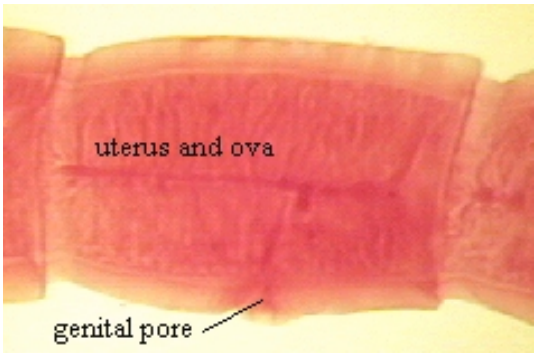
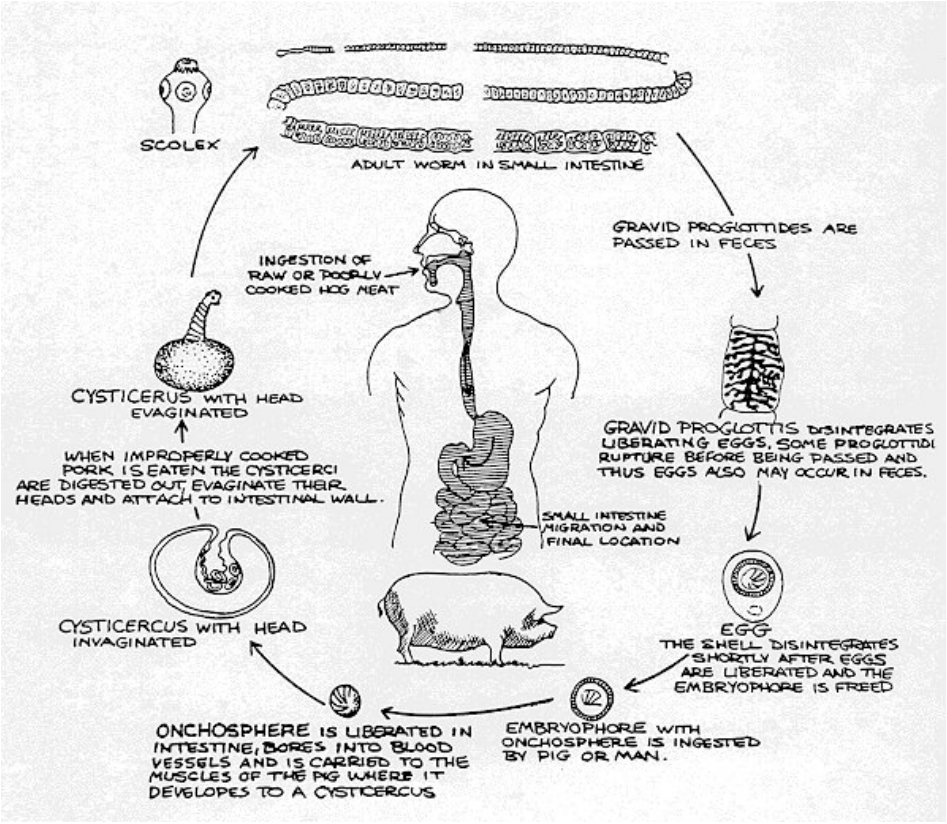
Fig. 17: Scolex of a tapeworm	**Fig. 18:** Gravid proglottid of a tapeworm filled with ova
	

Fig. 19:
Life cycle of *Taenia solium*



3. **The Trematodes (Flukes)**

Flukes are **unsegmented, flat, leaf-shaped worms** having a variety of organ systems (**Fig. 20**). Most flukes are **hermaphroditic**. They attach to the host by means of an oral sucker and a ventral sucker (**Fig. 20A**). Flukes, as adults, may infect either the portal blood vessels, intestines, liver, or lungs of humans and are named according to the tissue they infect. Humans become **infected** with liver flukes, lung flukes, and intestinal flukes by **ingesting poorly cooked fish, crayfish, crabs, snails, or water vegetables infested with flukes**. Blood flukes **directly penetrate the skin**.

In their life cycle (**Fig. 21**), fluke ova leave the body of the infected human or animal by means of feces, urine, or sputum (depending on the type of fluke). The ova enter water and infect the first intermediate host, certain species of **water snails**. A free-swimming form of the fluke called the **cercaria**, then leaves the snail and infects second intermediate hosts (fish, crayfish, water vegetables, etc.) which are ingested by humans. The cercariae of the **blood fluke** *Schistosoma* (**Fig. 22 and Fig. 22A**) can directly penetrate the skin of humans and cause **schistosomiasis**, a major problem in Africa, South America, and Asia.

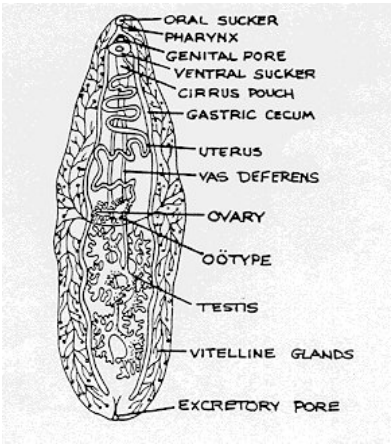
Fig. 20: Morphology of a liver fluke	**Fig. 20A:** *Fasciola hepatica* (liver fluke)
	

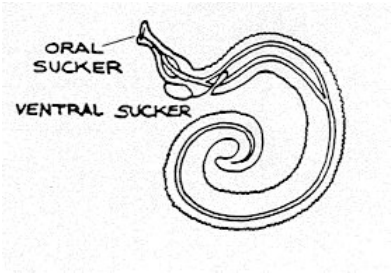
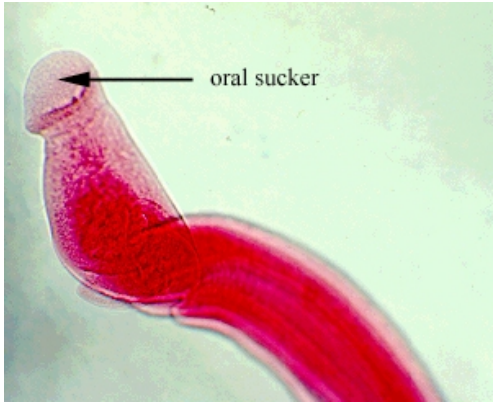
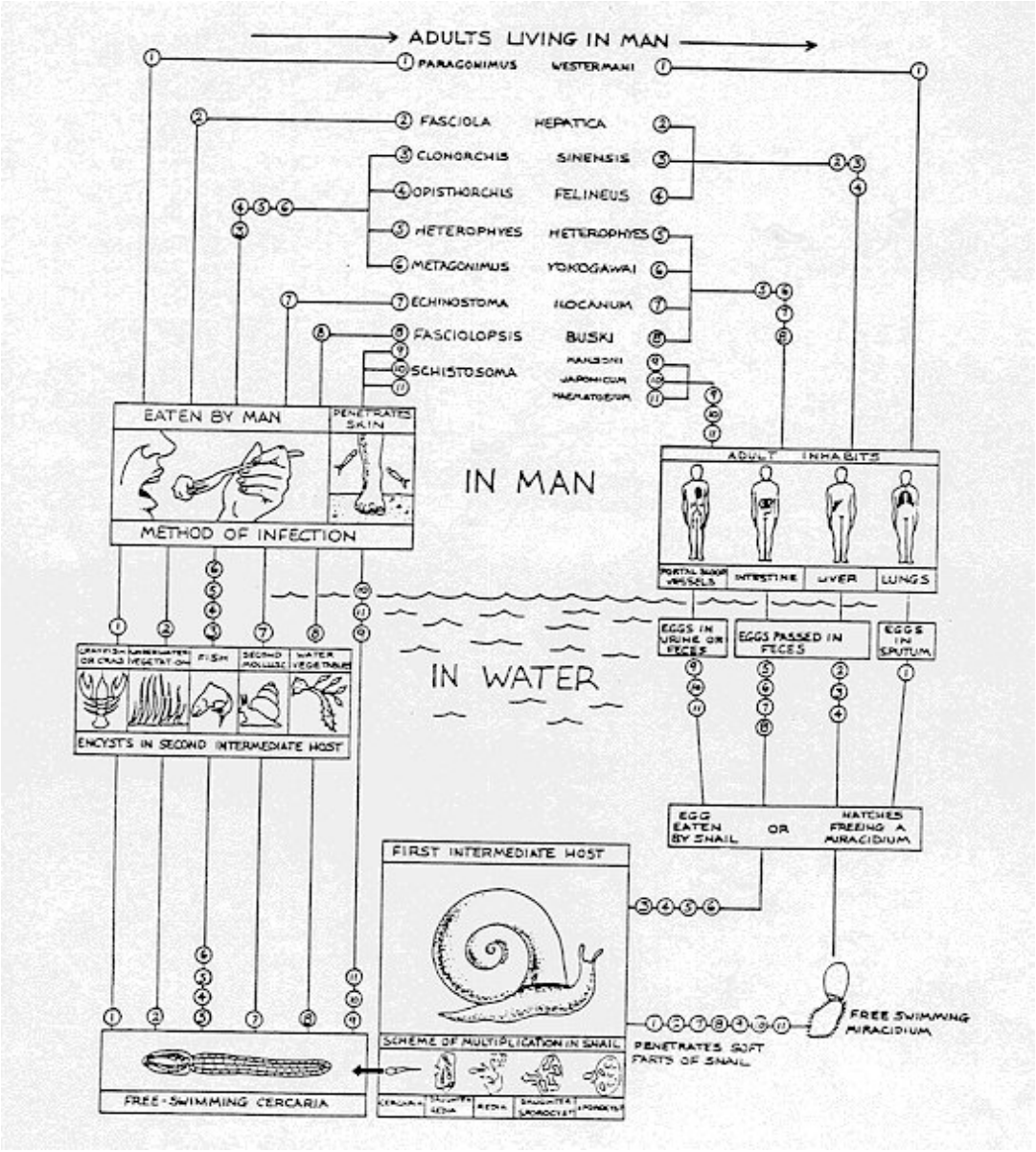
Fig. 22: Morphology of a blood fluke	**Fig. 22A:** The blood fluke *Schistosoma*
	

Fig. 21:
Life cycle of trematodes (flukes)

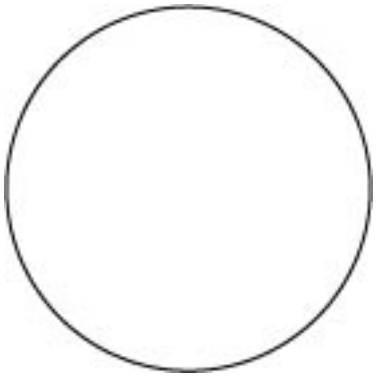


PROCEDURE AND RESULTS

1. Observe the prepared slides of the following **parasitic protozoans** and **compare them with the indicated figures in this lab exercise**.

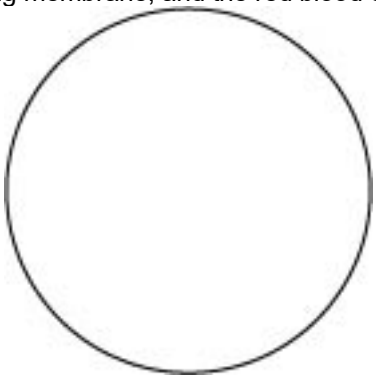
a. **The Sarcodina**

Fecal smear containing cysts of *Entamoeba histolytica* (the cause of amoebic dysentery). **Note** that it contains several nuclei. **See Fig. 1**.

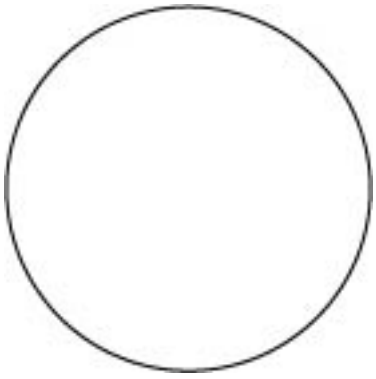


b. **The Mastigophora**

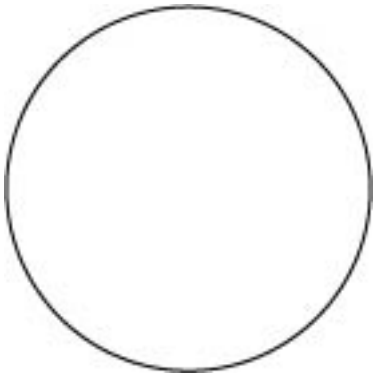
1. Blood smear containing *Trypanosoma gambiense* (the cause of African sleeping sickness). **Note** the nucleus, the undulating membrane, and the red blood cells in the background. **See Fig. 2**.



2. Fecal smear containing cysts and/or trophozoites of *Giardia lamblia* (the cause of giardiasis). **Note** the bilateral symmetry and macronuclei of the organism thay look like "eyes." **See Fig. 3.**

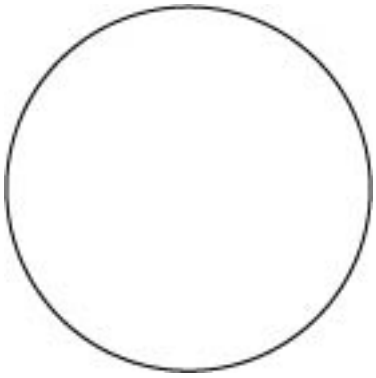


3. Vaginal discharge containing *Trichomonas vaginalis* (the cause of genitourinary trichomoniasis). **Note** the bundle of flagella, the undulating membrane, and the nucleus. **See Fig. 4**.



c. **The Ciliophora**

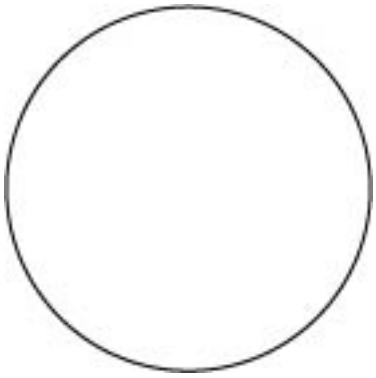
Fecal smear containing *Balantidium coli* (the cause of balantidiasis). **Note** the large dumbbell-shaped macronucleus. **See Fig. 5**.



d. **The Sporozoa**

1. Sporozoites of *Plasmodium* from the salivary glands of an infected mosquito. **See Fig. 6**.

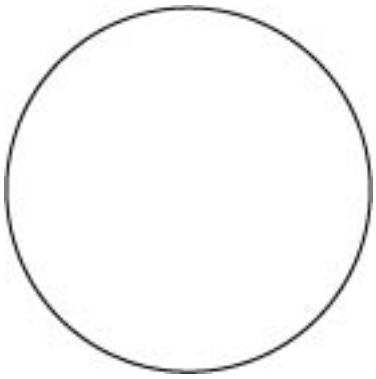
2. Blood smear containing red blood cells infected with merozoites of *Plasmodium* (the cause of malaria). **See Fig. 6 and 7.**



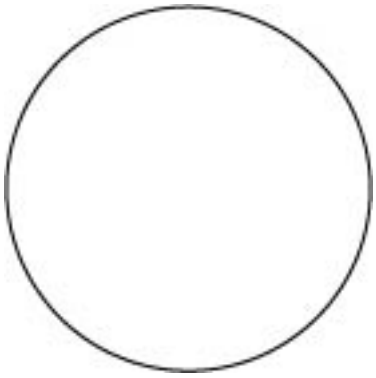
2. Observe the prepared slides of the following **parasitic helminths** and **compare them with the indicated figures in this lab exercise**.

a. **The Nematodes (roundworms)**

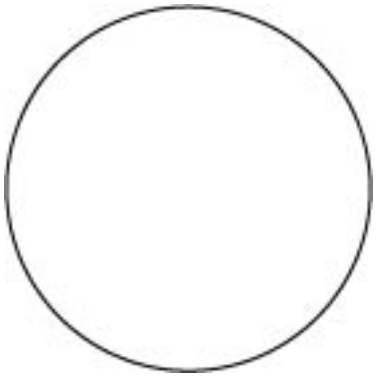
1. Fecal smear containing ova of *Ascaris lumbricoides* (the cause of ascariasis). **Note** the "bumpy" edge of the ova. See **Fig. 10 and Fig. 11**.



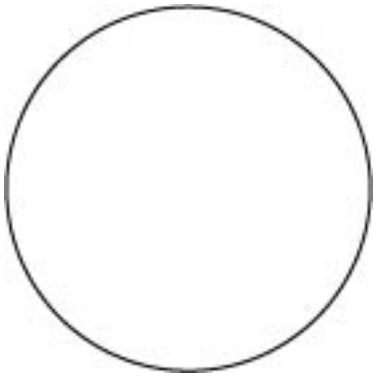
2. *Ascaris lumbricoides* larva. **Note** the organ systems. **See Figs. 8, 9, and 10**.



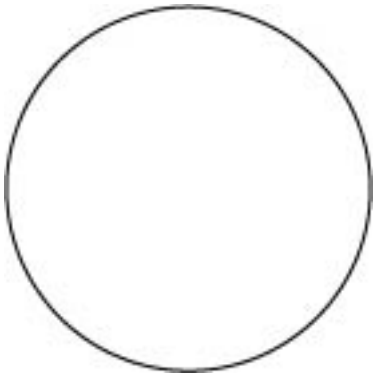
3. Fecal smear containing ova of *Enterobius vermicularis* (pinworm). **Note** the "smooth" edge of the ova. **See Fig. 13**.



4. *Enterobius vermicularis* larva. **Note** the organ systems. **See Figs. 8, 9, and 12**.

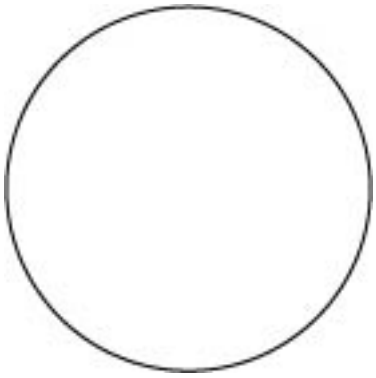


5. Muscle tissue containing encysted larvae of *Trichinella spiralis* (the cause of trichinosis). **Note** the spiral-shaped larva within the cyst. **See Fig. 14 and Fig. 15**.

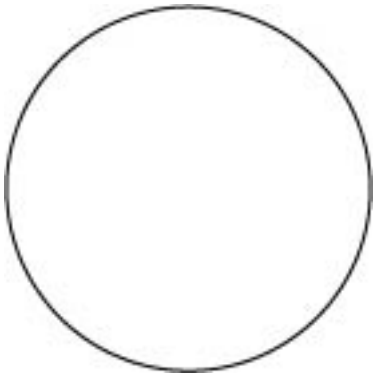


b. **The Cestodes (tapeworms)**

1. Scolex of *Taenia pisiformis* (dog tapeworm). **Note** hooks and suckers. **See Fig. 16 and Fig. 17**.

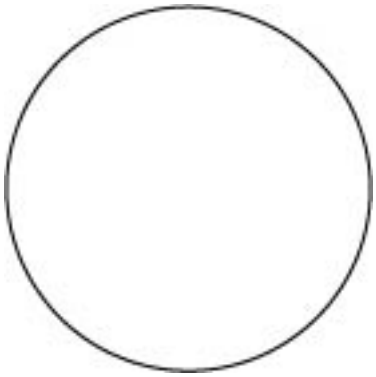


2. Gravid proglottid of *Taenia pisiformis*. **Note** the uterus and ova. **See Fig. 18**.

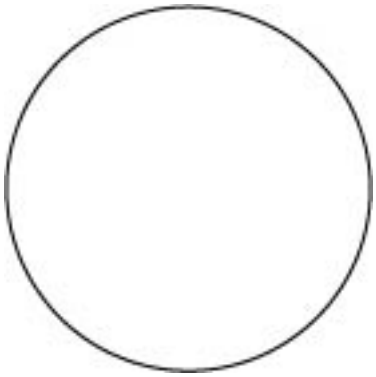


c. **The Trematodes (flukes)**

1. *Fasciola hepatica* (liver fluke). **Note** the organ systems. **See Fig. 20 and Fig. 21, and 21A**.



2. *Schistosoma mansoni* (blood fluke). **Note** the oral and ventral suckers. **See Fig. 22 and 22A**.



1. Observe the **preserved helminths**.

PERFORMANCE OBJECTIVES
LABORATORY 19

After completing this lab, the student will be able to perform the following objectives:

A. PARASITIC PROTOZOANS

DISCUSSION

1. Define the following: protozoan, trophozoite, cyst.
2. State how the following diseases may be transmitted to humans and briefly discuss how the diseases are diagnosed in the clinical laboratory:
    a. amoebic dysentery
    b. African sleeping sickness
    c. giardiasis
    d. genitourinary trichomoniasis
    e. balantidiasis
    f. malaria
    g. toxoplasmosis
3. Describe the following in terms of the malarial life cycle: sporozoite, schizont, asexual cycle, sexual cycle.
4. State what causes the recurring fever of malaria.

RESULTS

1. Recognize the following organisms when seen through a microscope and state what disease they are associated with:
    a. *Entamoeba histolytica* cysts in a fecal smear
    b. *Trypanosoma gambiense* in a blood smear
    c. *Giardia lamblia* cysts in a fecal smear
    d. *Trichomonas vaginalis* in vaginal discharge
    e. *Balantidium coli* in a fecal smear
    f. *Plasmodium* merozoites in infected red blood cells

B. PARASITIC HELMINTHS

DISCUSSION

1. Define the following: helminth, ova, hermaphroditic.
2. List the three classes of parasitic helminths and state the common name for each class.
3. State how the following diseases may be transmitted to humans and state how each disease may be diagnosed in the clinical laboratory:
    a. ascariasis
    b. pinworms
    c. trichinosis
    d. tapeworms
    e. flukes
4. Compare protozoans and helminths in terms of their size and structural complexity.

RESULTS

1. Recognize the following organisms or structures when seen through a microscope:
    a. roundworms
    b. *Ascaris* ova
    c. pinworm ova
    d. *Trichinella* in muscle tissue
    e. tapeworms
    f. scolex of a tapeworm
    g. ova and uterus in a gravid proglottid of a tapeworm
    h. flukes

FINAL LAB PROJECT

Introduction

The Final Lab Project is an active-learning, problem-based activity that enables you to work collectively in a small group to diagnose an infectious disease and apply what was learned in this course. The goal of this final project is for you and your lab partners as a group to:

1. **Come up with a valid diagnosis of the infectious disease seen in your case study and identify the bacterium causing that infection**; and

2. **Support your group's diagnosis** based on:

   a. Any relevant facts in the **patient's history**. (A reliable on-line source will be used to support this.)
   b. The patient's **signs and symptoms.** (A reliable on-line source will be used to support this.)
   c. **Each of the individual lab tests** given in your case study.
   d. All **microbiological lab tests you performed** as part of the project.

The due date for this report can be found on the class calendar. Remember, you are **working as a group to solve a problem**. Your grade for this lab is based on the **completeness of your report and written evidence of the critical thinking process that went into making and supporting your diagnosis**, therefore, it is critical that all members of the group participate, question any conclusions being made by the group, and contribute to the report. Remember, you are trying to convince your instructor that you understand how the diagnosis was made by **supporting that diagnosis with data**.

Grading:

The lab 12, lab 14, and lab 15 Lab Reports are worth 25 points each. The Final Lab Project Lab Report is worth 50 points.

These case studies are based in part on your in-class participation as part of your group. Therefore:

a. **If you were not in labs when the inoculations with your unknown were performed, 6 points from your Lab Report score for the Final Project.**

b. **If you were not in labs when the results of your lab tests were observed, 6 points from your Lab Report score for the Final Project**.

c. **For each day your Lab Report is late, 4 points from your Lab Report score for the Final Project.**

d. **Four points will be deducted if you don't include the rubric with your Final Lab Project report.**

Unlike the previous case study reports, <u>you must write your own individual lab report and submit a hard copy of that report along with a copy of the rubric</u> I will use to grade your report.

Staple the rubric to the back of your individual lab report. Do not submit the same exact lab report as other members that worked in your group. That is plagiarism! The reports must be individually written!

FINAL LAB PROJECT

You will be working in groups of 3. Your group will first choose one of the unknown bacteria provided by your instructor. Look your unknown number up in the instructor's case study key to determine which case study goes with your unknown.

For more information on writing your Lab Report, see Course evaluation for Lab (Core labs, case studies, lab quizzes) under Course Info in the menu of my BIOL 230 website or on your course Blackboard **site.**

Your unknown number: _____

Your case study letter: _____

Be sure to handle all the bacterial cultures you are using in lab today as if they are pathogens! Be sure to wash and sanitize your hands well at the completion of today's lab.

Case Studies

Case Study A

A 66-year old female with a history of recurring urinary tract infections and multiple antibiotic therapies presents with frequency and urgency of urination, dysuria, suprapubic discomfort, unilateral costovertebral angle (CVA) tenderness, fever and chills, and nausea. She currently has a temperature of 103°F. A complete blood count (CBC) shows leukocytosis with a left shift. A urine dipstick shows a positive leukocyte esterase test, a negative nitrite test. Microscopic examination of centrifuged urine shows 30 white blood cells, as well as 7 red blood cells and 18 bacteria per high-power field.

Assume that your unknown is from the urine of this patient.

Case Study B

A 61-year old male who has type 2 diabetes, a history of alcoholism, and is a heavy smoker was admitted to the hospital with a leg wound that is not healing - for which he was previously treated for two weeks ago but was subsequently released against medical advice. He was brought to the ER by a brother who noticed he was febrile and appeared confused, disoriented, and anxious. He exhibits flushing and peripheral vasodilatation. He has a temperature of 102° F, a heart rate of 130 beats per minute, a respiration rate of 42 breaths per minute, a blood pressure of 90/40 mm Hg, a urine output of only 110 cc for the last 8 hours, and a total white blood cell count of 2500/µL with a marked left shift. Lactic acid levels measure 3.5 mmol/L.

Assume your unknown is from a blood sample.

Case Study C

A 78-year old female who for several days had a severe productive cough and chest pain when breathing deeply, is admitted to the hospital by her sister with whom she lives. Symptoms include confusion and agitation, difficulty in breathing, peripheral edema, and shaking and chills. She has a temperature of 96.1° F, a heart rate of 112 beats per minute, a respiration rate of 45 breaths per minute, a blood pressure of 105/60 mm Hg, and a total white blood cell count of 13,000/µL. chest X-ray reveals a right lower lobe infiltrate.

Assume your unknown is from a blood sample.

FINAL PROJECT LAB REPORT

Your name:

Your unknown number:

Your case study letter:

Your lab section:

Others in your group:

Procedure and Results

1. Patient's history and predisposing factors

Read the case study. Explain how any relevant parts of the patient's history contributed to your diagnosis as to the type of onfectious disease seen here. You are urged to use the computers in lab to search reliable medically oriented Internet sources to support this. Reliable sources you might consider are Medscape (http://emedicine.medscape.com/infectious_diseases) and The Centers for Disease Control and Prevention (CDC) at http://www.cdc.gov/. Cite any sources you use at the end of this Patient's History section in APA style (http://www.apastyle.org/).

The **patient's history should suggest a general type of infectious disease** that is present, such as a urinary tract infection, a wound infection, gastroenteritis, pharyngitis, pneumonia, septicemia, etc. **Do not look up the bacterium you eventually identify** as the cause of this infectious disease. You do not know the causative bacterium at this point. **You need to determine the general type of infection to determine what microbiological tests to perform to identify the bacterium causing the infection**. Search at least one medically oriented reference article from a reliable site such as *Medscape* and **use this article to support your diagnosis the type of infectious disease seen here**. Don't forget to **cite any sources you used in APA style directly under this Patient's History and Patient's Symptoms sections** of this Lab Report.

2. Patient's signs and symptoms

Read the case study. Explain how the patient's signs and symptoms contributed to your diagnosis. You are urged to use the computers in lab to search reliable Internet sources to support this. Reliable sources you might consider are *Medscape* (http://emedicine.medscape.com/infectious_diseases) and the Centers for Disease Control and Prevention (CDC) at http://www.cdc.gov/. Cite any sources you use at the end of this Patient's History section in APA style (http://www.apastyle.org/). Also refer to appendix F (SIRS and Sepsis) in your lab manual for an indication as to whether or not the patient has SIRS.

The **patient's signs and symptoms should suggest a general type of infectious disease** that is present, such as a urinary tract infection, a wound infection, gastroenteritis, strep throat, pneumonia, septicemia, etc. **Do not look up the bacterium you eventually identify** as the cause of this infectious disease. You do not know the causative bacterium at this point. **You need to determine the general type of infectious disease present to determine what microbiological tests to perform to identify the bacterium causing the infection**. Search at least one medically oriented reference article from a reliable site such as *Medscape* and **use this article to support your diagnosis the type of infectious disease seen here**. Don't forget to **cite any sources you used in APA style under this Patient's History and Patient's Symptoms sections** of this Lab Report.

3. Vocabulary list for medical terms used in the case study under signs and symptoms

List and define any medical terms used in your case study that describe the patient's signs and symptoms that the average person not in the medical profession might not know.

4. Results of laboratory test given in the case study

List each lab test given and explain how the results of that test helps to contribute to your diagnosis. Also refer to **appendix C** (Complete Blood Count), **appendix D** (Urinalysis), and **appendix F** (SIRS and Sepsis) in your lab manual.

5. Gram stain

The Gram stain is discussed in **Lab 6**. Give the Gram reaction (Gram-positive or Gram-negative and how you reached this conclusion) and the shape and arrangement of the unknown you were given. **Remember that Staphylococci and Enterococci can look similar in a Gram stain when using a plate culture. If the result of the arrangement is inconclusive after seeing Gram-positive cocci, try doing the catalase tes**t described in **Lab 8** and state **specifically** how the results of the catalase test helped to confirm the arrangement. The catalase test will help you differentiate an *Enterococcus* from a *Staphylococcus*.

State how this contributed to your decision as to which microbiological tests and/or media to use next. **Make sure you check your Gram stain results with your instructor before determining which microbiological lab tests you will perform.**

.

6. Based on the results of your Gram stain, determine which of the following isolation media you will inoculate and why. Refer back to Labs 12, 14, and 15 to help determine your selection.

a. MacConkey agar (Lab 12)
b. Blood agar with NB disc (Lab 15)
c. Bile Esculin Azide agar (Lab 14)

Also **inoculate a tube of Trypticase soy broth (TSB)** with your unknown **to be used next time for antibiotic susceptibility testing**.

Inoculate each medium you chose, making sure to **streak all petri plates for isolation**. Incubate the plates upside down and stacked in the petri plate holder on the shelf of the **37°C** incubator corresponding to your lab section. Incubate the TSB and Bile esculin agar tubes in your test tube rack on the shelf of the **37°C** incubator corresponding to your lab section.

Isolation Results

Explain why you chose to **use or not use** each of the isolation media.

a. MacConkey agar (Lab 12)

Why did you choose to use or not use this medium?

If you used this medium, describe the results of the MacConkey agar plate you inoculated with the sample from the patient. **State specifically how this contributed to your decision as to what bacterium is causing the infection**.

b. Blood agar with NB disc (Lab 15)

Why did you choose to use or not use this medium and disc?

If you used this medium, describe the results of the Blood agar plate you inoculated with the sample from the patient. **State specifically how this contributed to your decision as to what bacterium is causing the infection**.

c. Bile Esculin Azide agar (Lab 14)

Why did you choose to use or not use this medium?

If you used this medium, describe the results of the Bile Esculin Azide agar you inoculated with the sample from the patient. **State specifically how this contributed to your decision as to what bacterium is causing the infection**.

7. Based on the results of your isolation media used in step 5 above, determine which of the following media you will inoculate or tests you will perform and why. Refer back to Labs 12, 14, and 15 to help determine your selection of media/tests and how to correctly perform the lab procedures.

a. Oxidase Test (Lab 12)
b. Cetrimide agar (Lab 12)
c. EnteroPluri-*Test* (Lab 12)
d. Mannitol Salt agar
e. Coagulase test (Lab 15)
f. Bauer-Kirby antibiotic susceptibility testing on Mueller-Hinton agar (Lab 18)

Incubate the plates upside down and stacked in the petri plate holder on the shelf of the **37°C** incubator corresponding to your lab section. Incubate the tubes in your test tube rack on the shelf of the **37°C** incubator corresponding to your lab section.

Results of Additional Lab Media or Lab Tests Performed

Explain why you chose to **use or not** use each of the following tests or media.

a. Oxidase test (Lab 12)

Why did you choose to use or not use this test?

If you used this test, describe the results of the oxidase test and **state specifically how this contributed to your decision of media to use**.

b. Cetrimide agar (Lab 12)

Why did you choose to use or not use this medium?

If you used this medium, describe the results of the Cetrimide agar **and state specifically how this contributed to your decision as to what bacterium is causing the infection**.

c. EnteroPluri-*Test* (Lab 12)

Why did you choose to use or not use this test?

Using your EnteroPluri-*Test*, identify the unknown you were given.

1. In the table below, **put a (+) or a (-) in the Result row for each test**.
2. **Add up the value of each positive test** in a group and put that number in the code for each group.
3. The 5-digit number is the CODICE number. Look that number up in the **Codebook** and identify your unknown.

	Group 1			Group 2			Group 3			Group 4			Group 5		
Test	**Glucose**	**Gas**	**Lysine**	**Ornithine**	**H_2S**	**Indole**	**Adonitol**	**Lactose**	**Arabinose**	**Sorbitol**	**VP**	**Dulcitol**	**PA**	**Urea**	**Citrate**
Value	**4**	**2**	**1**	**4**	**2**	**1**	**4**	**2**	**1**	**4**	**2**	**1**	**4**	**2**	**1**
Result															
Code															
CODICE NUMBER:							**Identification:**								

Genus and species from the EnteroPluri-*Test*: _____

d. Mannitol Salt agar (Lab 15)

Why did you choose to use or not use this medium?

If you used this medium, describe the results of the Mannitol Salt agar plate you inoculated with the sample from the patient**. State specifically how this contributed to your decision as to what bacterium is causing the infection**.

e. Coagulase test (Lab 15)

Why did you choose to use or not use this test?

If you used this test, describe the results of Coagulase test and **state specifically how this contributed to your decision as to what bacterium is causing the infection**.

f. Bauer-Kirby antibiotic susceptibility testing on Mueller-Hinton agar (Lab 18)

Gram-Positive Unknown

Disc code	Antimicrobial agent	Zone in mm	R	I	MS	S
AmC-30	Amoxicillin/ Clavulanic Acid					
CTX-30	Cefotaxime					
FOX-30	Cefoxitin					
CIP-5	Ciprofloxacin					
CC-2	Clindamycin					
E-15	Erythromycin					
K-30	Kanamycin					
OX-1	Oxacillin					
SXT	Sulfamethoxazole + Trimethoprim					
Te-30	Tetracycline					
TZP-110	Piperacillin/Tazobactum					
Va-30	Vancomycin					

R = Resistant
I = Intermediate
MS = Moderately Susceptible
S = Susceptible

Gram-Negative Unknown

Disc code	Antimicrobial agent	Zone in mm	R	I	MS	S
AN-30	Amikacin					
AmC-30	Amoxicillin/ Clavulanic Acid					
AM-10	Ampicillin					
CB-100	Carbenicillin					
CIP-5	Ciprofloxacin					
CTX-30	Cefotaxime					
FOX-30	Cefoxitin					
GM-10	Gentamicin					
K-30	Kanamycin					
SXT	Sulfamethoxazole + Trimethoprim					
Te-30	Tetracycline					
TZP-110	Piperacillin/Tazobactum					

R = Resistant
I = Intermediate
MS = Moderately Susceptible
S = Susceptible

Final diagnosis:

What infectious disease does the patient have?

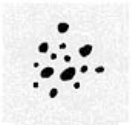
What is the genus and species of the bacterium causing this infectious disease?

Based on your Bauer-Kirby results, what antibiotics might be effective against this bacterium?

APPENDIX A
LAB 2

Colony Morphology on Agar Plate Cultures

A. Form of colony

Punctiform		Under 1 mm in diameter
Circular		
Filamentous		Long, irregular, interwoven threads
Rhizoid		Irregular, branched
Irregular		

B. Elevation of colony

Effuse		Very thin, spreading
Flat		
Raised		
Convex		
Umbonate		

APPENDIX A
LAB 2

C. Margin (or edge) of colony

Entire 

Undulate 

Erose 

Filamentous 

Curled 

D. Surface of colony

Smooth 

Contoured 

Undulating

Radiate 

Radiating ridges

Concentric 

Concentric rings

Rugose 

Wrinkled

E. Pigmentation

Specific color and solubility of pigment or lack of color

F. Optical characteristics

Opaque, translucent, dull, mucoid, etc.

APPENDIX B
LAB 4

I. Scientific Notation

When doing scientific calculations or writing, **scientific notation** is commonly used. In scientific notation, **one digit (a number between 1 and 9) only is found to the left of the decimal point.** The following examples are written in scientific notation:

3.17×10^{3}

5.2×10^{-2}

Note that **exponents** (the powers of 10) are used in these conversions.

Multiples of 10 are expressed in **positive exponents:**

$10^{0} = 1$

$10^{1} = 10$

$10^{2} = 100 = 10 \times 10$

$10^{3} = 1000 = 10 \times 10 \times 10$

$10^{4} = 10{,}000 = 10 \times 10 \times 10 \times 10$

$10^{5} = 100{,}000 = 10 \times 10 \times 10 \times 10 \times 10$

$10^{6} = 1{,}000{,}000 = 10 \times 10 \times 10 \times 10 \times 10 \times 10$

Fractions of 10 are expressed as **negative exponents:**

$10^{-1} = 0.1$

$10^{-2} = 0.01 = 0.1 \times 0.1$

$10^{-3} = 0.001 = 0.1 \times 0.1 \times 0.1$

$10^{-4} = 0.0001 = 0.1 \times 0.1 \times 0.1 \times 0.1$

$10^{-5} = 0.00001 = 0.1 \times 0.1 \times 0.1 \times 0.1 \times 0.1$

$10^{-6} = 0.000001 = 0.1 \times 0.1 \times 0.1 \times 0.1 \times 0.1 \times 0.1$

A. Procedure for converting numbers that are multiples of 10 to scientific notation

1. Convert 365 to scientific notation.

    a. Move the decimal point so that there is only one digit between 1 and 9 to the left of the point (from 365.0 to 3.65).

b. 3.65 is a smaller number than the original. To equal the original you would have to multiply 3.65 by 100. As shown above, 100 is represented by 10^2 . Therefore, the proper scientific notation of 365 would be 3.65 x 10^2 .

c. A simple way to look at these conversions is that you **add a positive power of 10** for each place the original decimal is moved to the **left.** Since the decimal was moved two places to the left to get 3.65, the exponent would be 10^2 , thus 3.65 x 10^2.

2. Convert 6,500,000 to scientific notation.

a. Move the decimal point so there is only one digit to the left of the point (6,500,000 becomes 6.5).

b. To equal the original number, you would have to multiply 6.5 by 1,000,000 or 10^6. (Since you moved the decimal point 6 places to the **left**, the exponent would be 10^6.)

c. Therefore, the proper scientific notation of 6,500,000 would be 6.5 x 10^6.

B. Procedure for converting numbers that are fractions of 10 to scientific notation.

1. Convert 0.0175 to scientific notation.

a. Move the decimal so there is one digit between 1 and 9 to the left of the decimal point (0.0175 becomes 1.75).

b. To equal the original number, you would have to multiply 1.75 by 0.01 or 10^{-2}. Therefore, the proper scientific notation for 0.0175 would be 1.75 x 10^{-2}.

c. A simpler way to look at these conversions is that you **add a negative power of 10 for each place you move the decimal to the right.** Since the decimal point was moved 2 places to the right, the exponent becomes 10^{-2}, thus 1.75 x 10^{-2}.

2. Convert 0.000345 to scientific notation.

a. Move the decimal point so only one digit (between 1 and 9) appears to the left of the decimal (0.000345 becomes 3.45).

b. To equal the original number, you would have to multiply 3.45 by 0.0001 or 10^{-4}. (Since you moved the decimal point 4 places to the **right,** the exponent becomes 10^{-4}.)

c. Therefore, the proper scientific notation of 0.000345 is 3.45 x 10^{-4}.

C. Other examples

12,420,000 = 1.242 x 10^7

21,300 = 2.13 x 10^4

0.0047 = 4.7 x 10^{-3}

0.000006 = 6.0 x 10^{-6}

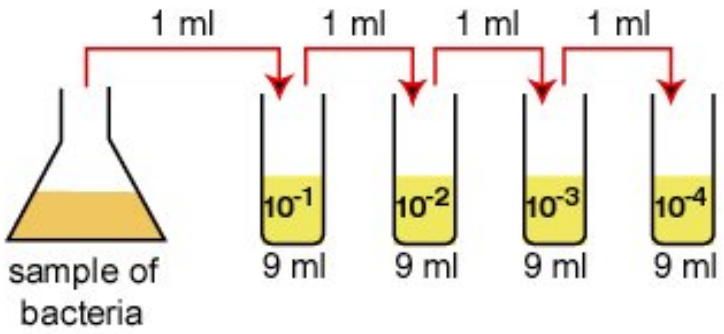
II. Dilutions: Examples

A. **1 ml of bacteria is mixed with 1 ml of sterile saline**. The total ml in the tube would be 2 ml, of which 1 ml is bacteria. This is a **1:2 dilution** (also written 1/2, meaning 1/2 as many bacteria per ml as the original ml).

B. **1 ml of bacteria is mixed with 3 ml of sterile saline**. The total ml in the tube would be 4 ml, of which 1 ml is bacteria. This is then a **1:4 dilution** (also written **1/4**, meaning 1/4 as many bacteria per ml as the original ml).

C. **1 ml of bacteria is mixed with 9 ml of sterile sali**ne. The total ml in the tube would be 10 ml, of which 1 ml is bacteria. This is then a 1:10 dilution (also written **1/10** or 10^{-1}, meaning 1/10 or 10^{-1} as many bacteria per ml as the original ml).

D. For dilutions greater than 1:10, usually serial dilutions (dilutions of dilutions) are made. The following represents a serial ten-fold dilution (a series of 1:10 dilutions):



The dilution in tube #1 would be 1/10 or 10^{-1}.
The dilution in tube #2 would be 1/100 or 10^{-2} (1/10 of 1/10).
The dilution in tube #3 would be 1/1000 or 10^{-3} (1/10 of 1/100).
The dilution in tube #4 would be 1/10,000 or 10^{-4} (1/10 of 1/1000).

The **dilution factor** is the **inverse** of the dilution. (Inverse means you flip the two numbers of the fraction; with scientific notation you use the **positive** exponent.)

For a dilution of 1/2, the dilution factor would be 2/1 or 2.
For a dilution of 1/4, the dilution factor would be 4/1 or 4.
For a dilution of 1/10 or 10^{-1}, the dilution factor would be 10/1 or 10 or 10^{1}.
For a dilution of 1/1,000,000 or 10^{-6}, the dilution factor would be 1,000,000/1 or 1,000,000 or 10^{6}.

In other words, the dilution factor tells you what whole number you have to multiply the dilution by to get back to the original 1 ml.

Appendix C: The Complete Blood Count (CBC)

The CBC test is used as a common screening test to check for a variety of disorders such as infection, anemia, and a wide variety of other diseases. It consists of a panel of tests that examines different parts of the blood and includes the following:

A. White blood cell (WBC) count: This is a count of the actual number of white blood cells per µL of blood. Both decreases in the total WBC count (**leukopenia**) and increases in the total WBC count (**leukocytosis**) can be significant. The normal range of WBCs ml of blood in both males and females is typically between 4500 and 10,000.

1. **Leukocytosis** is often seen during infections, inflammation, cancer, and leukemia.

2. **Leukopenia** is often seen during certain autoimmune conditions, bone marrow failure some severe infections, and congenital marrow aplasia where the marrow doesn't develop normally.

B. White blood cell differential count: This **determines the number of each type of leukocyte calculated as a percentage of the total number of leukocytes**. There are five different types of white blood cells: neutrophils, basophils, eosinophils, monocytes, and lymphocytes. An increase or a decrease in any of the types of WBCs can be significant.

1. **Neutrophils** are the most abundant of the leukocytes and normally accounting for between 40% and 60% of the WBCs.

a. **An increase in the percentage of neutrophils (neutrophilia**) is seen with an infection. When doing a differential WBC count, neutrophils are usually divided into segs (a mature neutrophil having a segmented nucleus) and bands (an immature neutrophil with an incompletely segmented or banded nucleus). Band forms normally account for 0% to 4% of the neutrophils, but during an active infection, people are generally producing large numbers of new neutrophils and therefore will have a higher percentage of the immature band forms. (An increase in band forms above 8% is referred to as a **"shift to the left"** because on laboratory slips used for differential WBC counts, the heading for bands is to the left of the heading for mature neutrophils or segs.)

b. A decrease in the percentage of neutrophils (neutropenia) is seen during cancer chemotherapy, aplastic anemia, radiation therapy and exposure, certain viral infections like influenza, and some widespread bacterial infections.

2. **Lymphocytes** normally make up between 20 and 40% of the WBCs circulating in the blood.

a. **An increase in the percentage of lymphocytes** is seen during viral infections (such as infectious mononucleosis, mumps, measles and infectious hepatitis), lymphocytic leukemia, chronic bacterial infections, and multiple myeloma.

b. **A decrease in the percentage of lymphocytes** is seen during chemotherapy, radiation therapy and exposure, HIV infection, leukemia, and sepsis.

3. **Monocytes** normally account for between 2% and 8% of the WBCs. **An increase in the percentage of monocytes** can be seen during parasitic infections, chronic inflammatory diseases, viral infections (such as infectious mononucleosis, measles, and mumps), and tuberculosis.

4. **Eosinophils** normally make up 1% and 4% of the WBCs. **An increase in the percentage of eosinophils** can be seen during parasitic infection, cancer, allergic reactions, and collagen vascular disease.

5. **Basophils** normally represent between 0.5% and 1% of the WBCs. **An increase in the percentage of basophils** can be seen during acute allergic reactions, chronic infections, and some forms of leukemia.

C. Red blood cell (RBC): This is a count of the actual number of red blood cells (erythrocytes) per ml of blood. Normal levels are 4.5 – 5.5 X 106 cells per ml in males and 4.0 – 4.9 X 106 per ml in females. Both decreases in the total RBC count (**erythropenia**) and increases in the total RBC count (**erythrocytosis**) can be significant.

1. **Erythropenia** is seen in the case of anemia.

2. **Erythrocytosis** is seen when too many RBCs are being made or when conditions such as diarrhea, dehydration, or burns cause excessive bodily fluid loss.

D. Hemoglobin: This test measures the amount of the oxygen-carrying protein hemoglobin in the blood. Hemoglobin levels mirror the RBC count results. Normal levels in males are between 13.5 and 16.5 Grams per deciliter (g/dL) and between 12.0 and 15.0 g/dL in females.

E. Hematocrit: This test measures the percentage of red blood cells in a given volume of whole blood. Hematocrit levels mirror the RBC count results. Normal levels in males are between 41% and 50% and between 36% and 44% in females.

F. Platelet count: This count measures the number of platelets in a given volume of blood. Normal platelet levels are between 100,000 and 450,000 per ml. Both increases and decreases can point to abnormal conditions either excess bleeding or clotting. The platelet count decreases when greater numbers are being used, such as with bleeding or when a person has certain inherited disorders, systemic lupus erythematosis, or pernicious anemia.

G. Mean platelet volume (MPV): This is a measurement of the average size of the platelets in the sample being tested; newly formed platelets are larger, so an increased MPV is seen when increased numbers of platelets are being produced.

H. Mean corpuscular volume (MCV): This is a measurement of the average size of the RBCs. Normal MCV is between 80 and 100. The MCV is elevated when RBCs are larger than normal (macrocytic), e.g., in anemia caused by a deficiency in vitamin B12; the MCV is decreased when the RBCs are smaller than normal (microcytic), e.g., in iron deficiency anemia.

I. Mean corpuscular hemoglobin (MCH): This is a calculation of the average amount of oxygen-carrying hemoglobin inside a red blood cell. Normal MCH is between 26 and 34. Because macrocytic RBCs are larger, they tend to have a higher MCH; smaller microcytic RBCs have a lower value.

J. Mean corpuscular hemoglobin concentration (MCHC): This is a calculation of the average concentration of hemoglobin inside a red blood cell. Normal MCHC is between 31% and 37%. In disorders such as such as in iron deficiency anemia where the hemoglobin is abnormally diluted inside the RBCs, decreased MCHC values (hypochromia) are seen; in conditions where the hemoglobin is abnormally concentrated inside the RBCs, such as in burn patients, increased MCHC values (hyperchromia) are seen.

K. Red cell distribution width (RDW): This is a calculation of the variation in the size of the RBCs. Normal distribution width is 11 - 15. In certain anemias, such as pernicious anemia, the amount of variation in RBC size (anisocytosis) and variation in RBC shape (poikilocytosis) causes an increase in the RDW.

Appendix D: Urinalysis

Urine Dipsticks

Because urine dipstick tests are relatively inexpensive, convenient, and simple to perform, they are often done in a physician's office. Most urine testing dipsticks have four tests in their panel that indicate the possibility of a urinary tract infection (UTI). These are:

1. Leukocyte esterase (LE)

Approximately 96% of people with UTIs have pyuria or white blood cells (WBCs) in the urine. Both living and lysed WBCs release the enzyme leukocyte esterase. (A positive LE test is equivalent to 4 or more WBCs per high power field when urine is examined microscopically.) A positive LE is a good indicator of a UTI. High concentrations of protein (500mg or more per deciliter) or glucose (1g or more per deciliter) in the urine, however, can interfere with this test.

2. Nitrite test (NIT)

The NIT test is an indirect test for bacteria in the urine (bacteriuria). Many of the Gram-negative enteric bacilli that cause UTIs will reduce dietary nitrate in the urine to nitrite. *Pseudomonas aeruginosa*, a non-fermentative Gram-negative bacillus that causes UTIs, reduces nitrate to nitrogen gas, not nitrite. *Staphylococcus saprophyticus* and *Enterococcus* species, common Gram-positive bacteria that cause UTIs do not reduce nitrate to nitrite. A positive NIT along with a positive LE is good indicator of a UTI. Low colony counts, however, can result in a false-negative NIT so a urine specimen should not be collected if the person has recently urinated. A first-morning urine sample or one with urinary retention of 4-8 hours is best.

3. Protein

A person with a UTI usually exhibits trace to 30mg of protein (mainly albumin) per deciliter indicating that protein-containing substances such as bacteria, WBCs, and mucous are in the urine.

4. Red blood cells (RBCs)

This test looks for the strong pseudoperoxidase action of RBCs and hemoglobin. Microscopic hematuria (RBCs in the urine) is common with UTIs. A trace result is equivalent to 5-10 RBCs per microliter (µl). A RBC excretion of up to 5 RBCs per µl is seen in normal urine. Levels above this warrant further diagnostic evaluation.

Microscopic analysis of urine

Microscopic analysis of urine can also be used to confirm UTIs. Along with classic symptoms of a UTI, detection of 2-5 WBCs or 15 bacteria or more per high-power microscopic field is indicative of a UTI.

APPENDIX E
LAB 18

BACTERIAL EXAMINATION OF WATER: COLIFORM COUNTS

Discussion

The purpose of the bacteriological examination of water is to determine if there is a possibility of pathogens being present. Infectious diseases such as salmonellosis, typhoid fever, shigellosis, cholera, hepatitis A, amoebic dysentery, *Campylobacter* gastroenteritis, giardiasis, and other fecal-oral route diseases may be transmitted by fecally-contaminated water. The identification of pathogens, however, is quite difficult. Pathogens may not survive long in water and are usually present only in small quantity. Therefore, one usually tests for the presence of coliforms in water.

Coliforms are Gram- negative, lactose-fermenting rods of the family Enterobacteriaceae. *Escherichia coli*, a fecal coliform, is normal flora of the intestines in humans and animals and is, therefore, a **direct indicator of fecal contamination** of the water. The presence of coliforms would then indicate the possibility of fecal pathogens being present.

Two tests are frequently performed to monitor water: the fecal coliform count and the total coliform count.

1. The **fecal coliform count** tests specifically for the fecal coliform *E. coli*. M-FC medium is used in this test and the plates are incubated at 45.5°C. This temperature is **selective for fecal coliforms** (nonfecal coliforms will not grow at this temperature) that produce blue colonies. This test, however, requires a special water bath incubator to assure a temperature of 45.5°C.

2. The **total coliform count** will detect **any coliforms (fecal and nonfecal)** present in the water. It is not as specific an indicator of fecal contamination, but is a useful **screening test**. M-coliform medium is used in this test and the plates are incubated at 37°C. **Both fecal and nonfecal coliforms will grow and produce metallic green colonies**. Coliforms would indicate the **possibility of fecal contamination of the water**.

Both of these tests use the micropore membrane filter method. Different amounts of the water sample being tested are passed through a membrane filter (Lab 19). The water passes through and the bacteria are trapped on the surface of the filter. The filter is then placed in a petri plate on pads containing either M-FC or M-coliform medium. Colonies then form on the filter. By counting the number of colonies and knowing the volume of water sample used, the number of fecal coliforms or total coliforms per ml of water can be determined.

PROCEDURE

1. Take three 50mm petri plates and aseptically place a **sterile pad** in the bottom of each. **Label** the plates 0.1ml, 1.0ml, and 10.0ml.

2. Using a **10ml pipette**, add **2.0ml of M-coliform broth** to each pad.

3. Using alcohol-flamed forceps, remove a **0.45µm pore-diameter gridded membrane filter** and place it **grid-side-up** in the filter set-up.

4. Secure the funnel to the filter set-up.

5. Pour about **one inch of sterile saline** into the filter set-up.

6. Using a **1.0ml pipette**, add **0.1ml of the water sample being tested** to the sterile water and mix.

7. **Vacuum** the water through the filter.

8. Add **another inch of sterile saline** to the funnel and swirl. This washes the bacteria off the sides of the funnel onto the filter.

9. **Vacuum**.

10. Using alcohol-flamed forceps, **remove the filter** and place it **grid-side-up** in the plate labeled 0.1ml. Make sure the entire filter **makes contact with the M-coliform-containing pad**.

11. Repeat using **1.0ml of the water sample**.

12. Repeat using **10.0ml of the water sample**.

13. Incubate the 3 plates **at 37°C** until the next lab period.

14. Observe the M-coliform plates. Both **fecal and non-fecal coliforms** will produce **metallic green colonies**. If there are a feasible number of coliform colonies for counting, determine the number of total coliforms per ml of water.

SIRS AND SEPSIS

Sepsis is an infection that leads to a systemic inflammatory response resulting in physiologic changes occurring at the capillary endothelial level. This systemic inflammatory response is referred to as **Systemic Inflammatory Response Syndrome or SIRS.**

Based on severity, there are three sepsis syndromes::

1. **Sepsis**. SIRS in the setting of an infection.

2. **Severe sepsis**. An infection with end-organ dysfunction as a result of hypoperfusion, the reduced delivery of nutrients and oxygen to tissues and organs via the blood.

3. **Septic shock**. Severe sepsis with persistent hypotension and tissue hypoperfusion despite fluid resuscitation.

A diagnosis of SIRS can generally be made when a patient **exhibits two or more** of the following:

1. A temperature above 100.4° F or below 96.8° F.

2. A heart rate above 90 beats per minute.

3. A respiration rate above 20 breaths per minute or a Pa CO_2 below 32 mm Hg.

4. A white blood cell count above 12,000/µL or below 4000/µL.

Other common symptoms include:

- hypotension
- pulmonary edema
- peripheral edema
- flushed, warm skin
- decreased urine output
- decreased bowel sounds
- altered mental status
- shaking and chills
- pain
- hyperlactemia and lactic acidosis (Patients with septic shock have lactate levels of more than 5 mmol/L, a lactate-to-pyruvate ratio greater than 10-15:1, and arterial pH of less than 7.35.)

For a tutorial on sepsis, check out the following website:
http://faculty.alverno.edu/bowneps/new%20indexes/circindex.html
and click on “Sepsis-What’s Bugging Your Patient” by Linda Bay