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INTRODUCTION

This laboratory text is designed to guide the student through basic microbiology lab techniques, procedures and experiments. Throughout this manual, the student will learn the scientific method and its application. The final portion of this manual involves the identification of an unknown microbial agent. This will allow the student to apply all knowledge gained throughout this course which will lead to the correct identification of an unknown organism.

Proper Safety Procedures

To insure safety of those working in the lab, as well as the integrity of each experiment, each of the following rules must be followed:

1. Clothing should be protected by a lab coat or apron. No shorts are allowed - you will be asked to return home and change if worn to lab class.
2. Hair that is long should always be tied back to avoid contamination as well as safety when working near the Bunsen burners.
3. Lab stations must be wiped down at the beginning of lab to lower contamination rates of cultures by organisms already on the stations as well as safety for the student. Stations must also be wiped down at the end of every lab session. Station cleaning is best accomplished with fresh 10% bleach. If there is visible contamination on the bench, wash with soap and water before the bleach.
4. Avoid direct contact with any microbes being tested by keeping all cultures well below mouth, nose, and eye regions. Microbial agents normally travel with gravity, so downward is the basic direction. Because of this movement, to insure integrity of cultures, avoid coughing, excessive talking, laughing, etc, while working with cultures while open. Keep cultures at a minimum of exposure to the air for best results.
5. Bunsen burners should be lit from the beginning of each session to the end as this decreases the risk of contamination of cultures and helps the safety of the lab worker.
6. Gloves must ALWAYS be worn when handling any microbial agent. Gloves are provided.
7. Lab stations should be kept clear of any extra materials (non-lab books, book bags, purses, keys, etc.) to avoid contamination as well as accidents.
8. All lab materials must be stored in the appropriate locations at the end of each session and gloves and other disposables placed in biohazard bags.
9. Tubes and racks should be placed in the appropriate location for autoclaving.
10. All spills should be reported IMMEDIATELY to the lab instructor for proper
cleanup. Unreported spills can result in biohazardous conditions.

Laboratory work should be fun and rewarding. To keep spills, burns, contamination and other accidents to a minimum, it is wise to stay alert and pay attention to your surroundings at all times. By following appropriate procedures, an enjoyable and safe lab can be assured.

The Scientific Method & Experimentation

Scientific experiments are designed around a question that requires an explanation. The scientific method is a guideline by which the explanation may be found and is a general procedure that is followed by all sciences. The method is modified slightly to fit each individual discipline.

General procedure for biological research:

1. Definition of the problem.
2. Review of the literature pertinent to the problem.
3. Formulation of hypothesis.
4. Statement of objectives
5. Analysis of variables
6. Design of experiment.
7. Experimental procedure.
8. Analysis of data (results).
9. Interpretation of data and conclusions from interpretations.

Some explanations and definitions of each step of the procedure are important

PROBLEM - two types:
1. Normally a fact that is assumed to be caused by some factor or factors the researcher is looking for cause and effect.
2. Involves the quantification of cause and effect. The association of the magnitude of cause related to the magnitude of effect.

HYPOTHESIS – an educated guess, a tentative theory. Anything that needs explanation is an effect. A hypothesis attempts to explain those effects by putting forth causes. A good hypothesis should:
1. Explain facts not previously explained.
2. Be consistent with all known facts.
3. Account only for phenomena in question.
4. Aid in producing new facts and relations.
5. Be susceptible to verification or refutation.

OBJECTIVE – This should be a carefully thought out and worded statement that covers the cause and effect variables whose relationship is being determined. The objective should be highly specific. The attainment of the objective will support or refute the hypothesis and therefore should solve the problem under study.
VARIABLES – A thing that may vary or is able to vary. Refers to both cause and effect.

1. Primary – variables on which the experiment will focus. (cause and effect)
2. Secondary – variables which may influence or be influenced by the primary.
   These are not of primary interest. (side effects)
3. Independent – variables that are not dependent upon other variables.

4. Dependent – variables that depend upon other variables for magnitude.
   These may be grouped together as (1) primary independent variable (cause);
   (2) primary dependent variable (effect); (3) secondary independent variable (extraneous variable of influence); and (4) secondary dependent variable (side effect).

The research should identify ALL variables that may concern or influence the experiment.

The general procedure for handling variables is:
1. Control the secondary independent variables.
2. Manipulate and evaluate or select and evaluate the primary independent variable(s).
3. Evaluate the resulting variations in the primary dependent variable(s).
4. Ignore the secondary dependent variable(s).

EXPERIMENT – A trial or test. The process of learning through observation (Webster’s, 181)

Most scientific experiments are controlled or “pure” experiments which are defined by (1) a predetermined plan; (2) objective of confirmation or refutation of a cause and effect relationship or degree of effect between two variables; (3) elimination, equalization, or evaluation of influencing, extraneous variables; and (4) assumption that the independent variable (cause) will be actively manipulated.

ANALYSIS – May or may not be statistical in nature. Often involves averages of observations.

INTERPRETATION/CONCLUSIONS – Meaning of facts, underlying causes, their affects and any other implications should be discussed. Researchers should avoid the most common errors in logic (illusions, generalizations, prejudices, biases, etc.)
Microbiology Laboratory Skills

A student successfully completing basic microbiology will demonstrate the ability to:

1. **Use a bright field light microscope** to view and interpret slides, including
   a. correctly setting up and focusing the microscope
   b. proper handling, cleaning, and storage of the microscope
   c. correct use of all lenses
   d. recording microscopic observations

2. **Properly prepare slides** for microbiological examination, including
   a. cleaning and disposing of slides
   b. preparing smears from solid and liquid cultures
   c. performing wet mount and/or hanging drop preparations
   d. performing Gram stains

3. **Properly use aseptic techniques** for the transfer and handling of microorganisms and instruments, including
   a. sterilizing and maintaining sterility of transfer instruments
   b. performing aseptic transfer
   c. obtaining microbial samples

4. **Use appropriate microbiological media and test systems**, including
   a. isolating colonies and/or plaques
   b. maintaining pure cultures
   c. using biochemical test media
   d. accurately recording macroscopic observations

5. **Estimate the number of microbes** in a sample using serial dilution techniques, including
   a. correctly choosing and using pipettes and pipetting devices
   b. correctly spreading diluted samples for counting
   c. estimating appropriate dilutions
   d. extrapolating plate counts to obtain the correct CFU or PFU in the starting sample

6. **Use standard microbiology laboratory equipment correctly**, including
   a. using the standard metric system for weights, lengths, diameters, and volumes
   b. lighting and adjusting a laboratory burner
   c. using an incubator
Microbiology Laboratory Thinking Skills

A student successfully completing basic microbiology will demonstrate an increased skill level in

1. **Cognitive processes**, including
   a. formulating a clear, answerable question
   b. developing a testable hypothesis
   c. predicting expected results
   d. following an experimental protocol

2. **Analysis skills**, including
   a. collecting and organizing data in a systematic fashion
   b. presenting data in an appropriate form (graphs, tables, figures, or descriptive paragraphs)
   c. assessing the validity of the data (including integrity and significance)
   d. drawing appropriate conclusions based on the results

3. **Communication skills**, including
   a. discussing and presenting lab results or findings in the laboratory

4. **Interpersonal and citizenry skills**, including
   a. working effectively in teams or groups so that the task, results, and analysis are shared
   b. effectively managing time and tasks allowing concurrent and/or overlapping tasks to be done simultaneously, by individuals and within a group
   c. integrating knowledge and making informed judgments about microbiology in everyday life
Microbiology Laboratory Safety

A student successfully completing basic microbiology will demonstrate ability to explain and practice safe

1. **Microbiological procedures**, including
   a. reporting all spills and broken glassware to the instructor and receiving instructions for clean up
   b. methods for aseptic transfer
   c. minimizing or containing the production of aerosols and describing the hazards associated with aerosols
   d. washing hands prior to and following laboratories and at any time contamination is suspected
   e. using universal precautions with blood and other body fluids and following the requirements of the OSHA Bloodborne Pathogen Standard
   f. disinfecting lab benches and equipment prior to and at the conclusion of each lab session, using an appropriate disinfectant and allowing a suitable contact time
   g. identification and proper disposal of different types of waste
   h. reading and signing a laboratory safety agreement indicating that the student has read and understands the safety rules of the laboratory
   i. good lab practice, including returning materials to proper locations, proper care and handling of equipment, and keeping the bench top clear of extraneous materials

2. **Protective procedures**, including
   a. wearing long pants or dresses (NO shorts); no sandals
   b. tying long hair back, wearing personal protective equipment (eye protection, coats, gloves; glasses may be preferred to contact lenses), and using such equipment in appropriate situations
   c. always using appropriate pipetting devices and understanding that mouth pipetting is forbidden
   d. never eating or drinking in the laboratory
   e. never applying cosmetics, handling contact lenses, or placing objects (fingers, pencils, etc.) in the mouth or touching the face

3. **Emergency procedures**, including
   a. locating and properly using emergency equipment (eye wash stations, first aid kits, fire extinguishers, chemical safety showers, telephones, and emergency numbers)
   b. reporting all injuries immediately to the instructor
   c. following proper steps in the event of an emergency
Microbiology Laboratory Skills Checklist

A student successfully completing basic microbiology will demonstrate ability to:

**Use a bright field light microscope** to view and interpret slides, including
  _ a. correctly setting up and focusing the microscope;
  _ b. proper handling, cleaning, and storage of the microscope;
  _ c. correct use of all lenses;
  _ d. recording microscopic observations.

**Properly prepare slides** for microbiological examination, including
  _ a. cleaning and disposing of slides;
  _ b. preparing smears from solid and liquid cultures;
  _ c. performing wet mount and/or hanging drop preparations;
  _ d. performing Gram stains.

**Properly use aseptic techniques** for the transfer and handling of microorganisms and instruments, including
  _ a. sterilizing and maintaining sterility of transfer instruments;
  _ b. performing aseptic transfer;
  _ c. obtaining microbial samples.

**Use appropriate microbiological media and test systems**, including
  _ a. isolating colonies;
  _ b. maintaining pure cultures;
  _ c. using biochemical test media;
  _ d. accurately recording macroscopic observations.

**Estimate the number of microbes** in a sample using serial dilution techniques, including
  _ a. correctly choosing and using pipettes and pipetting devices;
  _ b. correctly spreading diluted samples for counting;
  _ c. estimating appropriate dilutions;
  _ d. extrapolating plate counts to obtain the correct number of CFU (Colony Forming Units) in the starting sample.

**Use standard microbiology laboratory equipment correctly**, including
  _ a. using the standard metric system for weights, lengths, diameters, and volumes;
  _ b. lighting and adjusting a laboratory burner;
  _ c. using an incubator.
LAB: 1 THE COMPOUND MICROSCOPE

Materials
Compound light microscope
Prepared slides of 3 types of bacteria
Prepared freshly stained bacteria slides
Immersion oil
Lens paper

Primary Objective
Utilize all powers of magnification on the compound microscope.

Other Objectives
Be able to identify all the parts of a compound microscope.
Know the rules for proper microscope care.
Accurately measure appropriate specimens using micrometer (μm = 0.001 mm) scale in the scope lens.

Introduction
The most fundamental skill of microbiology is the use of the microscope. The definition of a microscope is a device for magnifying objects that are too small to be seen with the naked eye.

The development, evolution, and the inventors involved in the microscope is quite interesting. Prior to 1500, the magnifying glass was the best form of magnification and was not necessarily that advanced. In 1500, Zacharias Janssen and Hans, his son, invented a microscope that used two sets of lenses, a forerunner to the compound microscope. They used sunlight to illuminate the objects being studied. This earliest microscope magnified objects up to 100 times the objects actual size.

Today’s microscopes often employ more than two sets. The largest and most accurate microscope is the electron microscope that uses electrons in a focused beam to illuminate objects rather than light. The electrons pass through the specimen being viewed and are electrically focused. Normally the image is then ‘broadcast’ to a television monitor or photographed. A scanning electron microscope gives a three-dimensional image of the specimen. An electron microscope is capable of specimen magnification millions of times the actual size. This type of microscopy has allowed the viewing of virus particles that are so small a few million could fit on the head of a pin.
Calculation of Magnification

Total magnification requires multiplication of the magnification of the objective lens times that of the ocular lens (which is typically 10x).

\[
\text{(Objective magnification)} \times \text{(Ocular magnification)} = \text{Total magnification}
\]

i.e. \((4x \text{ objective}) \times (10x \text{ ocular}) = 40x \text{ total magnification}\)

The problem is, the greater the magnification of an object the more blurred it is. This is known as empty magnification. Biconcave lens split and bend light causing a blurred image characteristic known as chromatic aberration. To solve these problems, the focus of light must occur at the microscopist’s eye.

Ernst Abbe, a German mathematician and physicist determined that resolving fine detail depends upon the amount of light that is gathered by a lens. More light equals better resolution. Abbe found that immersion oil, which has the ability to bend light equivalent to that of glass, allows more light to be gathered and allows a greater amount of resolution.

Numerical Aperture

Abbe is also responsible for the development of the concept of numerical aperture. This is a mathematical description of a lens system’s light gathering power. The NA of an objective is dependent upon two factors.

1. The medium between the specimen and the lens.
2. The size of the light cone a lens can receive.

(See http://micro.magnet.fsu.edu/primer/anatomy/anatomy.html for more info.)

The numerical aperture is normally stamped on the objective.

What are the numerical apertures of the four lenses on the scopes provided for this lab? ___  ____  ____  __

Resolving Power

The resolving power (RP) is, “the ability of a lens to show two adjacent objects as discrete entities, when the two objects appear as one, it has lost resolution” (Microbiology: A Laboratory Manual, Cappuccino & Sherman, Benjamin Cummings, 2002, pg 30). RP is dependent upon the wavelength of light (\(\lambda\), or nm) that is employed and the numerical aperture (NA). 550 nm is an average wavelength of visible light. Using the normal numerical aperture for a low-power (4x) lens (0.10) the smallest object than can be seen clearly through a low-power objective, theoretically is 0.00275 mm.

Some Other Features

Working Distance - this is the distance separating a specimen and the objective lens. Each time the magnification is increased, the working distance is DECREASED. This is
an important relationship to remember when working with the microscope. If the stage is a great distance away from the objective when the higher powers are used, the microscope has been adjusted incorrectly.

Microscopic Field - this is the area one can observe while looking through the oculars. As the magnification increases this will also decrease.

Parfocality - this refers to the ability of a microscope to need only minor focusing adjustments after the specimen is found and focused using the lowest power. A microscopist should use the coarse tuning knob only when originally finding and focusing the specimen. Once the original focus is made, the only adjustment should be with the fine tuning knob as the magnification is increased. Most microscopes are considered parfocal.

Parts, Care and Procedures
The parts of the microscope are labeled on the diagram on the following page. Knowing the names of the parts and their functions is an important part of working with microscopes. Many microscopes have only one ocular, these are termed monocular. Those with two oculars are termed binocular.

Microscopes should always be carried with one hand under the base and the other hand holding the arm of the scope securely, keeping the cord out of the way of feet. The lenses should always be wiped down with lens paper and the cord should be kept out of the way of the working area.

When beginning an examination of a specimen the scope should be on the lowest power with the stage raised as high as it will go. The slide should be placed between the stage clips and all placement of slide and stage objectives should be done BEFORE looking into the oculars. Once all placement is ready, adjustment should be done while looking through the ocular. Adjustment should begin with the coarse adjustment, once the specimen is spotted then the fine tuning adjustment can be used. Because of parfocality, once the specimen is in focus on the lowest power there should be no need to adjust the coarse adjustment even with increased magnification.
Light Microscope

Parts

- Oculars
- Body
- Nosepiece
- Objective Lenses
- Stage
- Condensor
- Iris Diaphragm
- Fine focus
- Coarse Focus
- Light source
- Base
Procedure 1 - Using the Microscope
1. Obtain a slide from the instructor.
2. Place the slide on the stage of the microscope.
3. Turn the revolving nosepiece so that the scope is on the 4x objective (low).
4. Roll the stage up to its highest point.
5. Move the stage until the specimen is in the middle of the stage.
6. Focus on the specimen by rolling the stage down slowly using the coarse adjustment.
7. Ask the instructor to verify that the sample is in focus and sign the report sheet.

Procedure 2 - Examining Stained Bacteria
1. Obtain one slide of stained bacteria.
2. Place the slide on the stage and follow proper procedure for focus, etc.
3. Once the high dry objective (40x) has been reached and focus is maintained, apply one drop of immersion oil to the slide and move to the oil-immersion objective (100x). Refocus if necessary and draw what is in the two fields on the report sheet.

Lab 1 Report Form

Procedure 1: □ Low (4x) □ Medium (10x) □ High dry (40x)

Procedure 2: (Oil immersion)
LAB: HAND WASHING

Materials
Wax pencil or Sharpie (permanent marker)
One Tryptic Soy Agar (TSA) plate
(1) Scrub Brush
Anti-microbial soap
Hand sanitizer (62% Ethyl alcohol)

Primary Objective
Analyze the validity of the statement ‘wash your hands’.

Other Objectives
Evaluate normal skin flora.
Identify sources of contamination of hands beyond normal flora.

Introduction
Humans are taught from a young age that hand washing is a must for cleanliness and to avoid disease. With the advent of the ‘antibacterial’ craze, it has been found that most antibacterials do no more than normal soap or in some cases a thorough rinsing with water. This lab examines the validity of hand washing as a limit to disease causing agents.

Procedure - Hand Washing Analysis
1. Obtain a TSA plate.
2. Turn the plate upside down and use a wax pencil or Sharpie to make a graphic that divides the plate into 5 sections. Label the sections – 1, 2, 3, 4, 5. The numbers will correlate with the following actions: 1. Unwashed; 2. Water rinse; 3. Soap and water; 4. Brush, soap and water; 5. Sanitizer.
3. On #1 lightly stroke a finger across (inoculate) the media. Rinse your hands briefly with water, flick off excess water, and inoculate #2. Wash your hands with soap and water, flick off excess water – inoculate #3. Use the brushes provided and scrub your hands well, pat dry, and inoculate #4. Last of all, rub your hands thoroughly with the alcohol-based hand sanitizer, allow to air dry, then inoculate #5.
4. Incubate the plates for 48 hours at 35 - 37_C.
5. Hypothesize which section of the plate will have the most growth, which will have the least.
6. Apply Glo Germ® lotion to hands – work around fingernails, between fingers etc.
7. Wait 5 minutes and examine hands under black light.
8. Wash hands thoroughly using scrub brush, dry, and reexamine under the black light.
Second session
1. Retrieve the plates with finger imprints.
2. Evaluate the growth on each plate and check the hypotheses made.
Record all findings and answer the questions on the report sheet.

Report Form

Session 1

Hypothesis: I expect the section to have the most growth to be # _____.
I expect the section to have the least growth to be # _____.

Session 2

Please sketch the growth results below:

![Diagram of a circle divided into five sections: 1, 2, 3, 4, 5.]

Were your hypotheses correct? If not then write what the correct information below.

Report on hand washing after using Glo Germ®.
LAB: ASEPTIC TRANSFER TECHNIQUES
(Visit Transfer Techniques Movie on my Web Page)

Materials (per group)
- Tryptic soy broth (TSB) culture Escherichia coli
- Tryptic soy broth (TSB) culture Staphylococcus epidermidis
- Tryptic soy agar slant (TSA) culture E. coli
- Tryptic soy agar slant (TSA) culture S. epidermidis
- (1) sterile 1 mL pipet
- (2) sterile Tryptic Soy Agar (TSA) slants
- (3) sterile Tryptic Soy Broth (TSB) tubes
- Forceps
- (1) capped test tube with 5 mL of sterile water
- Pipettors (blue - use with 1 mL pipets, green - use with 10 mL pipets)
- Small beaker with alcohol for flaming
- Vial with sterile paper disks

Primary Objective
Learn, understand, and practice aseptic transfer techniques.

Other Objectives
Identify the aseptic procedures used in lab, both before and after work with microbes.
Demonstrate these techniques successfully by transferring and maintaining pure cultures.
Define pure culture, sterile, and contamination.

Introduction
This exercise is designed to teach the proper techniques in aseptic transfer.
These techniques are very important when working with ALL microbes. The integrity of the culture is essential because it insures that outcomes of tests are valid and that findings, therefore, are accurate and are repeatable.

Aseptic transfer is the transference of bacteria or other microbial cultures from one container to another while maintaining purity of the culture. Pure cultures consist of only one type of bacteria ideally the descendants from a single bacterial cell. Because microbes are present everywhere - in the air, the work area, clothes, bodies, etc., - it is important to follow the rules for aseptic transfer at all times. This is the only way of controlling contamination.
Rules for Aseptic Transfer Techniques

Before Work
1. Remove all unnecessary items from the work area.
2. Keep hair under control.
3. Put on lab coat.
4. Wash hands.
5. Glove up.
6. Disinfect work area.

During Work
Sterilize instruments before touching culture and after.
Do not lay culture tubes on work area.
Work near flame to allow rising air currents to keep microbes & dust away.
Keep Petri dish lids as barrier between mouth and nose or hold entire dish minus lid upside down for inoculation.
Work quickly and avoid excess talking while cultures are open.

After work
Sterilize all instruments again.
Put gloves and petri dishes in biohazard bags when finished.
Wash hands thoroughly.
Disinfect work area.
Put used tubes in racks to be autoclaved.

In general, keep the work area as clean as possible. Avoid producing excess aerosols by talking, laughing, etc. while cultures are open. Keep cultures closed as much as possible, working quickly and accurately is essential. Keep all items away from mouth, avoid food or drink in laboratory. Always wash hands and work area before leaving. Leave any clothing cover used in the lab to avoid taking any extra microbes out or bringing any in.

Aseptic Transfer
Aseptic techniques are practiced because they are procedures that:
  - Protect the culture.
  - Protect the worker
  - Protect the environment

Maintaining purity of culture is essential in microbiology if the biologist is to be able to identify bacteria, test for antibiotic sensitivity, or maintain stock cultures. Often in nature a pure culture is impossible to come by because species live together. The scientist is left working with mixed cultures. Pure cultures can be derived from mixed cultures through isolation of cultures and this also requires that sterile (aseptic) techniques to be used.
Normally transference is done from colonies. A colony consists of usually several million cells that are assumed to be the descendants from one cell. Inoculations from one media to another, therefore, is usually done by removal of a few million cells from one colony into a new environment. This must be done with the integrity of all colonies remaining intact. Through the use of sterile techniques, this can be accomplished successfully.

There are a number of tools that are used for inoculation procedures. **Inoculating loops** are used when transferring members of a broth culture to another broth, plated media or an agar slant. **Inoculating needles** are used when inoculating a broth culture from a colony on plated media or when making a stab in an agar deep or agar slant from broth or solid media. **Forceps** are used to place sterile disks containing some testing agent in a broth culture or on a solid media culture. **Pipets** are used when transferring liquids into other liquids or onto solid media.

**Flaming** is used to incinerate any microbes left on loops and needles. **Alcohol flaming** is used to sterilize forceps. When flaming inoculating loops and needles, care should be taken avoid burning the plastic handle at the end of each. The metal of the loop or needle should glow red hot and then be allowed to cool before dipping it into any cultures - if the metal is too hot it will kill the organisms that are to be used for inoculation. Alcohol flaming for the forceps is done by dipping the forceps into a small amount of alcohol and then burning the alcohol off. The forceps should be dipped and burned three times. Care should be taken to avoid alcohol running up toward the hand. The flame will follow the alcohol and burns will result.

Pipets normally used in lab are prepackaged, sterile, disposable pipets. Sometimes glass pipets are used and these are stored in cans. The glass pipets are discarded into a pipet jar filled with disinfectant. Disposable pipets are deposited in biohazard bags. It is important that pipettors are always used and pipetting by mouth is prohibited.

**Procedure 1 - Sterile Pipetting with Pipettors**

1. Obtain one tube of sterile TSB and one tube of sterile water. Place in a tube rack.
2. Label the TSB tube ‘0.5 mL H₂O’ then write your initials & the date.
3. Loosen the lids on the 2 tubes.
4. Open a prepackaged, sterilized 1 mL pipet and attach it to the blue pipettor.
5. Remove the lid from the water tube, flame the lip of the tube, insert the 1 mL pipet and withdraw 0.5 mL of water. Re-flame the lip and recap.
6. Remove the lid from the TSB tube, flame the lip of the tube, deposit 0.5 mL of water into the TSB, re-flame the lip and recap.
7. Return the pipet to the package and discard in the biohazard bag. Place the tube with the water in the rack designated for autoclaving.
8. Incubate the TSB tube at 35°C for 48 hours until next lab session
9. Return the stock culture to the front desk.
**Procedure 2 - Sterile Transfer of Bacteria from Broth to TSA Slant**

1. Obtain a tube of TSB containing *E. coli* or *S. epidermidis* and 1 TSA slant. Place all tubes in tube rack, keeping the rack near a lit Bunsen burner.
2. Label the sterile TSA slant with the appropriate organism name, procedure #, your initials & the date.
3. Flame the mouth of the TSB tube and the sterile slant. Use the inoculating loop to remove 1 loop full of bacteria from the stock TSB tube and carefully streak the sterile slant from bottom to top. ONE STREAK ONLY!!!! Be careful to not gouge the agar. Re-flame the mouths of both tubes.
4. Flame the inoculating loop and place tube in incubator at 35°C for 48 hours.
5. Return the stock culture to the front desk.

**Procedure 3 - Sterile Transfer of Bacteria from TSA Slants to Broth with a Stab/Needle**

1. Obtain a slant inoculated with *S. epidermidis* or *E. coli*
2. Obtain a sterile tube of TSB.
3. Label the sterile TSB tube with the appropriate organism name, procedure #, your initials & the date.
4. Flame the inoculating needle and use the needle to remove a small amount of growth from the stock slant. A light touch is suggested, it does not take a large amount of inoculum to obtain copious amounts of organisms. Deposit the inoculum in the TSB tube. BE SURE to flame the lip of the TSB tube and the TSA slant before and after inoculation.
5. Roll the TSB tube between the hands to thoroughly mix the organisms. (This is called ‘subbing’ out the organism. It is a procedure to keep the original culture fresh.)
6. Incubate at 35°C for 48 hours.
7. Return the stock culture to the front desk.

**Procedure 4 - Sterile Transfer Using Alcohol-Flamed Forceps**

1. Obtain 1 tube TSB, forceps, small beaker of alcohol, and vial of sterile disks.
2. Label the TSB tube ‘Sterile Disk’, initial and date. Loosen the lid of the tube and place in the tube rack.
3. Dip the tips of the forceps into the alcohol and, holding the forceps HORIZONTALLY, place them in the flame. Be careful not to let the alcohol run towards the hand.
4. Open the sterile disk tube and flame the lip. Remove 1 sterile disk with the sterilized forceps.
5. Open TSB tube, flame the lip, insert the sterile disk into the tube.
6. Re-flame and re-cap both the sterile tube and the TSB tube.
7. Incubate the tube at 35°C for 48 hours.
Second Session

1. Inspect each of the broths and slants.
2. Check for growth, may be seen as turbidity, or cloudiness in the broths.
3. Record your results in the chart below and answer the questions for each procedure.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Growth</th>
<th>No growth</th>
<th>Repeat?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sterile water to broth</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Organism broth to slant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Organism slant to broth</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Sterile disk to broth</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Procedure 1

Should there be growth in this broth? _____ Yes _____ No

If there is no growth, yell ‘Whoopee!!’ and dispose of the tubes in the rack designated for autoclaving.

If there is growth, record below the possible problems that could have caused the growth:

Perform the procedure again if there is growth.

Procedure 2

If there is growth, yell ‘Whoopee!!’ and dispose of the tubes in the rack designated for autoclaving. If there is no growth, tell what went wrong; perform the procedure again.

Procedure 3

If there is growth, yell ‘Whoopee!!’ and dispose of the tubes in the rack designated for autoclaving. If there is no growth tell why: perform the procedure again.
**Procedure 4**

Look for any growth that might be occurring around the disk in the TSB. Describe any growth below.

What are some of the possible areas of contamination if growth is present? (Answer this question even if growth is not present.)

Put tube in autoclaving rack.

Perform the procedure again if there is growth.
LAB: PREPARING A SMEAR AND SIMPLE STAIN

Materials:
(1) TSB of \textit{Escherichia coli}
(1) TSA slant of \textit{Staphylococcus epidermidis}
Stain rack
Bibulous paper
Reverse osmosis water (lab quality) wash bottle [marked H$_2$O]
Lens paper
(2) Microscope slides
Inoculating loop
Inoculating needle
Bunsen burner
Striker (to light Bunsen burner)
Grease pencil
Methylene blue stain
Clothes pin
Immersion oil

Primary Objective
Prepare and stain bacterial smears made from broth and solid media. Evaluate cell morphology.

Other Objectives
Define aniline dyes.
Be able to heat fix organisms without overheating and shrinking of organisms.
List the general characteristics of bacterial morphology.

Introduction
Bacterial smears are stained to allow easier viewing of cells. Bacteria cells are virtually transparent when smeared on a slide and placed under the bright-field microscope. Little to no detail is distinguishable. Stains enhance the contrast and allow the microscopist to view the cell more distinctly.

Normally, in Microbiology, \textit{aniline dyes} are employed to stain the cells. These dyes are a coal tar derivative, derived from benzene. The dye adheres to the bacteria and \textit{morphology} (shape, arrangement, structure, size) can be more readily seen.

Morphology of Bacteria
Stained bacterial shapes are usually identified as \textit{rods} (bacilli), \textit{cocc}i (sphere), or \textit{spirals} (spirillum & spirochetes). Normally short rods are termed \textit{coccobacilli} and bacteria that are shape-changers are termed \textit{pleiomorphic}. Individual cell shape is a good beginning determiner for bacterial identification.

Another determiner is the \textit{arrangement} of bacteria. The arrangement is the physical placement of one cell to another within the same species. Some of the more common arrangements are \textit{singles}, \textit{pairs} (diplococci or diplobacilli), \textit{chains}, \textit{tetrads}, or \textit{clusters}.

Pleiomorphic rods are often found in \textit{palisades} (picket fence) formation. Some
rods also form ‘X’ and ‘Y’ formations.

Morphologically, cell size is important. **Micrometers** on the ocular should be used to measure cells. Different species have different cell sizes. Cell structures are also viewed using stains. Different staining techniques that are employed to highlight various structures are discussed and used in the following chapters.

Some bacteria do not stain evenly and this may result in a **beaded** look which can be mistaken for a short chain of cocci. **Bipolar staining** is also a phenomenon. The stain darkly spots the ends of each cell leaving the middle much lighter. This is a common occurrence in some strains of *E. coli*.

### The Chemistry of Staining

Staining is based on the principle that opposite charges attract and that like charges repel. Most bacteria, when placed in an aqueous environment with the pH at about 7, have a net electrical charge that is **negative**. These negatively charged cells will attract positively charged molecules and repel those molecules that are negative.

Each dye that is used in microbiology is a salt that contains two **ions** (charged particle). One ion, the **cation**, has a positive charge and the other ion, the **anion**, has a negative. Either one of these ions can be the **chromophore** (portion of the molecule that is brightly colored). In most commonly used dyes (**basic** dyes), the cation is the chromophore. Basic dyes include **methylene blue**, **crystal violet**, and **safranin**. These are used to prepare a **simple** stain. If a dye is repelled by the bacteria, they are normally **acid** dyes. These dyes color the background and leave the cells colorless. An example of an acid dye is **nigrosine**.

### Preparing Stains

When preparing a stain, a perfectly clean microscope slide must be used. New slides are usually the best, however if used slides are used, great care should be taken to clean all greasy film from the slide. Cleanliness can be tested by dropping a drop of water on the slide. If it spreads over the entire slide, the slide is clean. Any beading of the water indicates the presence of a greasy film.

**A thin film of bacteria should be spread upon the slide.** If the smear is too thick, it is difficult to see anything because there will be little light passing through. The smear should be thin and allowed to dry. Once the smear has dried, the slide should be passed over a lit Bunsen burner several times to affix the organisms. This procedure is known as **heat fixing**. There is a slight shrinkage of cells during this process which is normal, but it helps the bacterial cells to adhere to the slide through several rinses.

**If the slide is overheated, the cells will warp and structure will be indistinguishable.** If heat is applied to the cell before the smear is dry, there will be **distortion**.

A properly stained bacterial smear should be slightly difficult to see to the naked eye. If there are dark splotches of color, the bacteria are piled on top of each other. Preparing stains and smears takes practice. Often it takes several tries when first learning to prepare a smear.

**Finished stained smears will last for months stored in a cool dark place provided no oil is present on the stain.** There are solvents, such as **xylol**, that can be used to remove excess oil from slides that are to be saved. Solvents, however, strip any
markings made by wax pencils, so re-labeling is important.

**Prep 1 - Preparing Smears from Broth Cultures**

1. Prepare the slide. A circle made with a grease pencil will provide an area in which to apply the smear. The slide may be turned over so that the markings of the pencil are on the bottom of the slide. This keeps any wax from getting into the smear and causing a viewing problem.

2. Obtain a tube of TSB containing *E. coli*.

3. Resuspend the bacteria in the broth by rolling the tube between the hands.

   **Bacteria must always be resuspended before removing any inoculum.**

4. Using aseptic techniques, transfer a loop full of bacteria from the tube to the labeled circle on the slide. Keep the slide and the tube near the flame. Avoid inhaling any aerosols. Flame the loop after transfer. If the tube culture is not excessively turbid (a pencil can be seen when held behind the tube), use up to six loops full to make the smear. Flame the loop after each transfer.

5. While the smear is drying, **Prep 2** may be started.

6. When the smear is completely dry pass the slide through the top of the Bunsen burner flame several times to heat fix the organisms. **Use the clothes pin to hold the slide while heat fixing to save finger burns.** Then proceed to Procedure 1.

**Prep 2 - Preparing Smears from Solid Media**

1. Obtain and prepare a slide by drawing 3 dime-sized circles on the slide. Once again, turn the slide upside-down to keep the wax out of the smear.

2. Obtain a TSA slant inoculated with *S. epidermidis*.

3. Place a drop of water in each of the three circles.

4. WITHOUT GOUGING THE AGAR, obtain a small amount of bacteria and mix with the water on the first circle. Flame the needle.

5. Flame the loop and dilute the bacteria in the first circle by mixing it into the water. Transfer a loop of suspended organisms from the first circle into the second circle, mix and flame off loop. Transfer a loop from the second circle into the third circle, mix and flame off loop.

6. Place the slide off to the side to dry. Retrieve the slide from Prep 1 and begin the staining process, Procedure 1.

7. After the slide from this prep has dried, heat fix the organisms as was done in Prep 1. Then repeat the staining process, Procedure 1.
**Procedure 1 - Simple Staining**

Be sure of the following before staining begins: the work area is cleared of anything that is not needed; the stain rack is in place on the sink; stain bottle and clothes pin are readily accessible.

1. Place the slides on the stain rack over the sink.
2. Cover the slides with methylene blue. Allow the stain to stay on the slide for one full minute.
3. Once the one minute is up, pick the slide up with the clothes pin and tilt the slide to the side to drain off excess stain into the sink (other lab facilities often use stain pans). While holding the slide still tilted to the side, begin to rinse with deionized water from the supplied water bottles. Aim around the smear and remove all excess stain. Do not aim right at the smear as it may result in the removal of the smear.
4. Shake all excess water from the slide. Water is itself a decolorizer and should not be allowed to sit on the slide for very long.
5. Slides can be air dried, but to avoid any chance of decolorization by water, you may blot the slides dry in the book of bibulous (absorbent) paper. **DO NOT RUB!!!!!!!!!**
6. Examine the stained smears on the microscope. The smears should be examined on every power including the oil immersion lens.
7. Draw what is seen in the field of view on the oil immersion lens below. Once done, clean up work area and dispose of gloves and slides in a biohazard bag.

\[ E. \text{coli} \quad S. \text{aureus} \]

What is the morphology (shape) of each type of bacteria?
LAB: GRAM STAINING

Materials
TSB tube of organism #1 (E. coli or Klebsiella)
TSB tube of organism #2 (Staphylococcus epidermidis or Streptococcus bovis)
2 clean microscope slides

Gram’s staining set including:
- Gram’s crystal violet [primary stain]
- Gram’s iodine [mordant - makes 1_ stain fix to cell wall]
- Decolorizer (95% ethyl alcohol) [washes stain out of cell walls with high lipid content]
- Gram’s safranin [counterstain]

Clothes pin
Inoculating loop and needle
Bunsen burner
Striker
Immersion oil
Wash bottle
Bibulous paper, Lens paper
Methanol (CMS)

Primary Objective
Prepare and interpret a Gram stain.

Other Objectives
Identify primary stain, mordant, decolorizer, and counterstain.
Identify organisms as gram-positive or gram-negative.
Define gram-variable and gram-non-reactive and identify genera that display each.
Identify eight items, including cell wall structure, that can alter Gram stain results.

Introduction
Hans Christian Gram, circa 1884, was studying the etiology of respiratory disease. Working with Streptococcus pneumoniae from human lung tissue, he discovered a staining procedure that differentiated this organism at autopsy. This revolutionized the microbiology world and Gram’s staining procedures are done millions of times daily worldwide.

Gram’s procedure divides the vast array of bacterial organisms including cocci, bacilli, and spirilli into two broad groups. Gram-positive bacteria appear purple after a gram staining procedure, while gram-negative organisms appear pink. Most organisms are gram-negative except for gram-positive bacteria, a few molds, and yeasts.

Gram staining is normally the first step towards identifying an unknown pathogenic agent.
Common diseases caused by gram-positive bacteria are:
- wound infections (positive = purPle)
- boils
- diphtheria
- septic sore throat
- gas gangrene
- scarlet fever
- some pneumonias

Common diseases caused by gram-negative bacteria are:
- Typhoid (negative = piNk)
- bubonic plague
- dysentery

There are many other diseases associated with both gram-negative and gram-positive that exist. These do not include the many others that microbiologists are aware of or the many that are emerging almost daily.

In the past, slides with samples of organisms or body fluids/secretions were heated to fix the material onto the slide so it wouldn’t wash off during staining (used for our simple staining procedure). However, in recent years, an organism fixation technique using methanol has been found to be highly effective during Gram and other staining techniques. This fixation technique will be used along with heat fixation in this laboratory.

**Gram Staining Technique**

Gram staining is not a simple stain, but rather is known as a differential technique. A differential technique is a process that distinguishes between a variety of microbial organisms based on the ability of their cell wall to hold certain dyes. The Gram staining technique depends upon the ability of a microbial cell wall to resist decolorization.

The Gram stain consists first of a primary stain, normally crystal violet, applied to a heat-fixed or alcohol-fixed smear. A substance that increases the reaction between the stain and the cells (mordant) is then applied, normally Gram’s iodine. This is then followed with a quick water rinse and the application of a decolorizer (usually 95% ethyl alcohol). This will remove color from the cells. Gram-negative cells should decolorize whereas gram-positive organisms will not. The application of decolorization is then followed by another quick water rinse. A counterstain, usually safranin, is then applied. Excess safranin is often rinsed off, but it should be noted that water is itself a mild decolorizer and long, excessive rinsing can alter the results of the staining procedure.

**Cell Wall Structure and Other Factors Affecting Gram Stain Results**

Gram-positive bacteria’s cell walls have a distinctly different structure than that of the gram-negative bacteria cell. The gram-positive cell wall has a multitude of layers of peptidoglycan (up to 40) which resists decolorization better than the thin (often only 2 layers) gram-negative cell wall. The gram-negative cell wall also contains lipoprotein and lipopolysaccharide that can be verified through chemical analysis. The two other groupings that should be noted at this time are gram-variable and gram-non-reactive.
Genera like *Neisseria* and *Moraxella* are gram-negative chemically but resist decolorization, therefore they are considered gram-variable. The genus *Mycobacterium* is often gram-non-reactive due to a waxy cell wall. Some members of this genus do accept gram stain and are normally gram-positive. Other genera, such as *Mycoplasma*, lack cell walls and gram staining is considered inappropriate for those (an acid-fast stain is used for these organisms, such as looking for *M. tuberculosis* in a sputum sample).

Other factors that can affect the gram staining procedure include the following:

- Using cells (from an old culture) that cannot resist decolorization.
- Intrusion of stain crystals into smear or clumping of stain and bacteria.
- Not allowing enough time for each stain to sit or allowing too much time for decolorizer and/or water to sit on slide.
- Using old stain reagents.
- Using thick vs. thin smears. Thin is normally much better.
- Overheating the cells during fixation.

To avoid problems from these factors, be sure not to shake reagent bottles, keep reagents fresh, do not over rinse or overdo the decolorization in the step, keep smears thin, do not overheat when heat-fixing organisms, allow plenty of time for each stain to sit on the slide (about 1 minute per each is best).

**Procedure - Preparing and Gram Staining**

(Gram positive cocci & Gram negative rods)

1. Prepare 2 slides using the TSB stock culture tubes of organism #1 (E. coli or *Klebsiella*) and organism #2 (*Staphylococcus aureus* or *Streptococcus bovis*). Each slide should have a sample of organism #1 and organism #2 on it.
2. Allow the slides to air dry. Procedure 2 can be prepared during this time as well.
3. After the slides have air dried, heat-fix one slide.
4. Alcohol fix the other slide by flooding the slide with methanol for 30 seconds. DO NOT ALLOW THE METHANOL TO DRY. Rinse with water.
5. Place the slides on the stain rack:
   a. apply Gram’s crystal violet, let set 1 minute;
   b. dump any excess crystal violet off and rinse briefly with water;
   c. apply Gram’s iodine, let set 1 minute;
   d. rinse briefly with water;
   e. apply decolorizer and rock slide for 1-2 seconds.
   f. rinse briefly with water
   g. apply Gram’s safranin; let set 1 minute.
6. Rinse excess safranin away with water and blot slides dry. Place under the microscope for observation, adjust the scope accordingly and, using the oil immersion lens for viewing, draw/describe what is observed on the report. Describe any difference you observe between the slide that was heat fixed and the one that was fixed with methanol.
Before leaving lab for the day, get two tryptic soy agar plates or nutrient agar plates and sub-culture both organisms, #1 and #2, to a plate. The instructor will demonstrate how to streak the plates.
LAB: ACID-FAST STAINING

Materials
(2) Acid-fast Bacteria QA slides
(1) Acid-fast stain reagent ‘set’
   - Carbolfuchsin
   - Acid-alcohol (3% HCl in 95% ethanol)
   - Methylene blue
Paper towel
Stain Rack
Clothes pin
Bunsen burner
Inoculating needle and loop
Water bottle
Striker
Bibulous paper, Lens Paper

Primary Objective
Learn to prepare and interpret Ziehl/Neelsen acid-fast stains with efficiency and reliability.

Other Objectives
Know the names of the genera that include acid-fast species.
Know the differences between acid-fast staining techniques.
Name three diseases caused by acid-fast species and in what clinical specimens they can be found.
Know the theoretic role that mycolic acids play in acid-fast staining.

Introduction
The acid-fast stain was developed in 1882 by Paul Ehrlich. Ehrlich was working with Mycobacterium tuberculosis, the bacilli responsible for tuberculosis, and found a technique that renders M. tuberculosis distinguishable from nearly all other bacteria. Acid-fast staining is, therefore, known as a differential stain.

Mycobacterium and some Nocardia species are considered acid-fast because, during the acid-fast procedure, they are able to retain the primary dye even when decolorized by a powerful solvent known as acid-alcohol. Most other bacterial genera are easily decolorized by acid-alcohol.

Ziehl/Neelsen Acid-Fast Staining Procedure
In later years, Ehrlich’s technique was improved upon by two microbiologists, Ziehl and Neelsen. Like Ehrlich, Ziehl and Neelsen’s procedure requires heat to force the primary dye through the cell wall. Another microbiologist, Kinyoun, developed a procedure in which a detergent is used as a wetting agent and heating becomes unnecessary. Other modifications have been added to accommodate the use of
fluorescence microscopy. For general laboratory use, the Ziehl/Neelsen technique is still widely taught and used today.

The Ziehl/Neelsen technique uses a red primary dye called carbol fuchsin. This dye is made of basic fuchsin, ethanol, phenol, and water. This solution is steamed for about 5 minutes allowing the dye to penetrate the cell walls. The smear is then cooled, rinsed with deionized water, and decolorized using acid-alcohol which is a 95% ethanol base with 3% hydrochloric acid. After decolorizing, the only cells that should be red are the acid-fast cells. A counterstain of methylene blue is applied to give greater contrast between those cells that are acid-fast and those that are not. We will use cold Kinyoun method.

**Acid-Fast Bacteria Cell Wall Structure and Significance**

The cell walls of genera that are considered acid-fast contain lipid-based acids called mycolic acids. Usually these lipids act as a barrier to most other dye chromophores and prevent the cell from being colored. *Mycobacterium tuberculosis* cells contain an enormous amount of mycolic acid and are virtually impossible to stain with the normal gram staining. Some other members of this genus (*M. smegmatis* and *M. phlei*) have less mycolic acid and are normally found to be gram-positive. Except for *Nocardia* and *Mycobacterium*, mycolic acids are not normally found in the cells of other bacterial genera. It is theorized that heat enables the dye to break through the mycolic acid barrier, but upon cooling, the mycolic acids coalesce forming a fairly impenetrable barrier against decolorizer. Other bacteria do not have this barrier forming ability.

Acid-fast bacteria hold great significance medically speaking. The member species of *Mycobacterium* and *Nocardia* are responsible for leprosy (Hansen’s disease), tuberculosis, and nocardiosis. Most of these species are resistant to disinfectants and should always be handled with care. These bacteria can be contained in every organ in an infected human body and contact with any infected area, particularly sputum and nasal mucous, is considered a strong contagion. Because of these health risks, we will be using prepared slides of bacteria genetically altered to be harmless. These are normally used in labs for quality assurance.
**Kinyoun Cold Acid-Fast Stain**

**PRINCIPLE**
Certain organisms, most notably the mycobacteria, retain stain even after attempts at decolorizing with acids, acid-alcohol, or acid-acetone solutions. This property, called acid-fastness, is attributed to a cell wall containing mycolic acid (a lipid) in mycobacteria and close related organisms and to undefined impermeability factors for endospores and Cryptosporidium. Since these factors make staining the organism more difficult, heat, organic solvents or detergents are needed to facilitate stain penetration.

**II. REAGENTS**
1. Carbolfuchsin stain
   - basic fuchsin 4 gm
   - ethyl alcohol (95%) 20 ml
   - Dissolve and add slowly while shaking.
   - distilled water 100 ml
   - liquefied phenol (melted crystals) 8 gm

2. Decolorizer
   - ethanol (95%) 97 ml
   - concentrated hydrochloric acid 3 ml

3. Counterstain
   - methylene blue chloride 0.3 ml
   - distilled water 100 ml

**III. PROCEDURE** (When a package insert is available, the procedure outlined in the flyer should be used instead of the procedure below.)

1. Prepare smear; fix with gentle heat.
2. Stain with Kinyoun carbolfuchsin for 3 minutes (do not heat).
3. Rinse gently with running water.
4. Decolorize with acid-alcohol until no more color appears in the washing (about 2 minutes).
5. Rinse gently with running water.
6. Counterstain with methylene blue for 30 seconds.
7. Rinse gently with running water. Air dry.

Acid-fast organisms appear **red**, and background material appears **blue**.

See the following web sites for good pictures:
http://www.nottingham.ac.uk/pathology/protocols/afb.html
http://images.google.com/images?q=acid fast+bacteria+picture
LAB: ENDOPORE STAINING

Materials
(1) 3 day culture of Clostridium or Bacillus
(2) Microscope slides
Schaeffer-Fulton endospore staining reagents:
   Malachite green, 5% aqueous (aq.)
   Safranin, 5% aq.
Paper towel
Acid-alcohol
Bunsen burner, Striker
Inoculating needle
Clothes pin
Water bottle
Bibulous paper, Lens paper

Primary Objective
Learn to stain endospore-forming rods using the Schaeffer-Fulton endospore staining technique and be able to stain non-endospore-forming cocci using the same technique and learn to interpret the procedure accurately.

Other Objectives
Identify the bacterial genera that commonly form endospores.
Define refractile, sporangium, saprophytes, cryptobiotic state.
Identify the various characteristics of cells while they are contained within an endospore.
Identify 4 endospore-formers that cause disease and 4 antibiotics that are effective against them.

Introduction
This lab investigates the varying properties of bacterial endospores. Endospores are hard dry structure which form inside certain cocci and rod genera. When the vegetative (dividing) cell dies, the endospore remains and preserves life.

What Is An Endospore?
Endospores contain may hydrophobic (water fearing) amino acids. Endospores exist in what is known as a cryptobiotic state, this is a state with no measurable metabolic activity. These tiny ‘containers’ have large amounts of dipiolinic acid and calcium that are not normally present in vegetative cells. Most endospores have an outer spore coast known as exosporia.
Possibly because of their status as a metabolically inert life-form, endospores are resistant to drying, ultraviolet radiation, and can survive for a time in strong bases, acids, and disinfectants. They tend to resist staining with the normal aniline dyes used and when unstained are highly refractile (shiny, light reflective).
Endospores can be located terminally, subterminally, or centrally. If the endospore diameter is larger than the vegetative cell that holds (sporangium), the vegetative cell is swollen. The endospore containment characteristics are stable for
each species and therefore are identification characteristics.

Endospore-Forming Genera and Their Medical Importance

There are several genera that are capable of forming endospores in the bacterial kingdom. There are four common rod genera that form endospores - *Bacillus*, *Sporolactobacillus*, *Clostridium*, and *Desulfotomaculum*. There are two common cocci genera that form endospores - *Sporosarcina* and *Oscillospira*.

Of these genera, many are harmless soil saprophytes (decomposers), but there are a few that are extremely pathogenic and are considered very important. *Clostridium botulinum* is the producer of what is considered the most deadly biological nerve toxin. *Clostridium tetani* produces a toxin that causes tetanus (lockjaw) that kills through paralysis. *Clostridium perfringens* causes gas gangrene as can other clostridia. Gas gangrene can also be deadly. *Bacillus anthracis* causes anthrax which is deadly to livestock and humans. Other bacilli and clostridia members cause gastroenteritis and other forms of food poisoning. Normal cooking does not destroy endospores and can leave them ready to germinate. Refrigeration does slow germination, but does not fully stop it.

*Clostridium* and *Bacillus* are also important to the pharmaceutical industry. These genera supply many of the antibiotics used to kill or inhibit other microbial growth. These antibiotics include gramicidin, tyrocidin-type antibiotics, polymyxin, and the bacitracins. These antibiotics can either alter cell membranes, as do the first three, or can inhibit synthesis of the cell wall, the bacitracins are responsible for this.

Schaeffer-Fulton Endospore Stain

This technique, like the Ziehl-Neelsen technique, requires that heat be applied to drive the primary dye (malachite green) into the endospore. Once an endospore is dyed it is difficult to decolorize it. Vegetative cells that are present are stripped of the primary dye by a water rinse. The counterstain of safranin then dyes all non-endospore-containing cells pink.

Work in pairs!!! This procedure takes two sets of hands!

**Procedure - Schaefer/Fulton Staining of Clostridium or Bacillus**

1. Make a smear from a TSA slant or TSB of *Clostridium* or *Bacillus*.
2. Allow to air dry.
3. Heat-fix the organisms.
4. Place a piece of paper towel on the slide, securing with a clothes pin.
5. Apply malachite green to the slide and begin heating. Heat 5 minutes. **DO NOT ALLOW STAIN TO BOIL**. Add extra stain if it begins to dry out!
6. Cool briefly, remove the paper towel and rinse with deionized water for about 15-30 seconds or until the water runs clear with no green present.
7. Apply safranin and allow to set for 1 minute. Rinse briefly with water.
8. Blot dry with bibulous paper. Remove any burned on dye with acid-alcohol.

**Observe each slide through the microscope on oil immersion (100x).**
Lab: MOTILITY TEST

Purpose and Procedure Summary

The motility test is often used to distinguish certain bacteria. The motility determines the presence of flagella, external appendages used by bacteria for movement. Bacteria with flagella, such as *Proteus mirabilis*, are called **motile**, while bacteria without flagella, such as *Staphylococcus epidermidis*, are called **nonmotile**.

**Motility test medium**, used to detect bacteria with flagella, contains beef extract and peptone to support growth, and 0.5% agar. The medium is semisolid because of the low concentration of agar, allowing movement of motile organisms through the medium. **SIM medium** is another semisolid medium used to determine bacterial motility.

An isolate is inoculated into a tube with a sterile transfer needle. The needle is inserted and withdrawn in a straight line in the center of the medium. The tube is incubated at 35°C for 24-48 hours before examining the growth along the line.

Tips for Success

*Hold the tubes up to the light* for better contrast when examining growth.

*Compare results to an uninoculated tube* held up to the light.

**Procedure -**

1. Get 2 tubes of SIM or motility media (work in pairs).
2. Label one *E. coli* and one *Staphylococcus epidermidis*.
3. Sterilize your needle, obtain a small bit of growth from the stock culture and stick it straight down into the appropriate media, going straight to the bottom and back out.
4. Flame the needle and repeat step 3 for the Staph.
5. Incubate at 35°C for 48 hours.

**Expected Results**

Bacteria with flagella spread away from the line of inoculation. When the tube is held up to the light, growth is seen macroscopically as turbidity extending through the semisolid medium. Growth away from the line of inoculation indicates that the organism is motile. Bacteria without flagella do not spread away from the line of inoculation, so their growth does not extend into the medium. Growth along the line of inoculation only indicates that the organism is non-motile.

**Positive test:** motility test medium...growth spreads away from the line of inoculation (motile)

Example: *E. coli*, *Proteus mirabilis*

**Negative test:** motility test medium...growth occurs only along line of inoculation (nonmotile)

Example: *Staphylococcus epidermidis*, *S. bovis*
LAB: CULTURE TECHNIQUES/CHARACTERISTICS

Materials
(1) TSB culture of *Bacillus subtilis*
(1) TSB culture of *Streptococcus bovis* or *Streptococcus equi*
(1) TSB culture of *Staphylococcus aureus*
(1) TSB culture of *Proteus mirabilis*
(1) TSB tube
(2) TSA plates
(1) microscope slide
Inoculating loop
Bunsen burner
Striker
Gram staining reagents
Water bottle
Clothes pin

Primary Objective
Study the growth patterns of specified organisms in broth and on varying forms of solid media so that rough identification of each organism can be made using colony growth patterns.

Other Objectives
Demonstrate proper inoculating procedures for broths and plates.
Understand and use the descriptive vocabulary used in microbiology.
Reinforce Gram staining procedures.
Describe accurately growth patterns in broth, on slants, and on plates.
Learn the proper procedure for spreading plates and demonstrate this technique by obtaining even growth covering the medium completely.

Introduction
Microorganisms are everywhere (air, food, beverages, etc.) therefore they are termed ubiquitous. The microbiologist must be able to identify organisms that appear quickly and easily. If molds or other microbes are present other than bacteria, specific characteristics are observed that will be covered in later lab sessions. This lab deals specifically with the growth characteristics of bacterial organisms.

Microbial Growth Terminology
Microbes (not just bacteria) require appropriate conditions of incubation. These may include the correct nutrients, chemicals, temperatures, etc. When the conditions are right, microbes are able to multiply and will form colonies. A colony is the only way to see microorganisms with the unaided eye and it should be remembered that colonies are large groups of microbes, not individuals. Using nutrients, chemical, pH, temperature, salinity, and other conditions of incubation, a microbiologist is provided with the first clue to what a particular organism might be.
For accurate reporting, the microbiology student must learn the descriptive vocabulary of the microbiologist. Descriptions of colonies must be concise and be phrased in such a manner that other scientists will know what is being described. The terms used in this lab are employed in *Bergey’s Manual of Systematic Bacteriology* which consists of four volumes and is the most used source book for bacterial taxonomy.

Descriptions of growth patterns for varying media are given on the following pages along with diagrams. There are also other characteristics that can be used from colony growth on media, however, most other diagnostic characters are physiochemical in origin and will be explained in the following labs.

**Broth**

Bacteria, when suspended in broth, may cause cloudiness or **turbidity**. If there are floating clumps it is known as **flocculent**. Often bacteria will form a **ring** at the top or will float like a heavy island known as a **pellicle**. Cells that sink to the bottom of the tube become **sediment** and those that float in wisps down from the surface are termed **streamers**.

**Plates**

On plates, an isolated colony is best used for descriptive purposes. The following are commonly used terms to describe colonies as viewed on an agar plate:

- **Size** - diameter of colony measured in mm or cm
- **Whole colony shape** - circular, rhizoid, punctiform, irregular, lenticulate
- **Edge or margin** - lobate, entire, undulate, filamentous, erose
- **Elevation** - raised, flat, umbonate, convex, pulvinate, crateriform
- **Surface** - wrinkled, rough, concentric rings, dull, waxy, glistening, etc.
- **Pigmentation**:
  - **Color** - red, cream, white, yellow, none, etc.
  - **Water-solubility** - water soluble tints the colony and the agar OR non-water-soluble only the colony is colored.
- **Opacity** - transparent, translucent, opaque.

**Odor** can also be used as a diagnostic tool, however, this characteristic should only be used sparingly and if the odor is distinctive - sweet, putrefying, fruity, etc.

**Preparing a Spread (Lawn) Plate**

The exact process requires an inoculation from the original culture to be spread over the primary area. The loop is flamed and cooled and, after turning the plate 90 degrees, a few bacteria are spread over in the secondary area from the primary area. The loop is flamed, cooled, and the plate again turned and a few bacteria are spread from the secondary area into the tertiary area. If the inoculum is heavy, the primary area should be made smaller so fewer organisms are distributed. If the inoculum is lighter, the primary area should be made larger so that more organisms might be introduced. Spread plates are often used when test reactions are needed. Testing antiseptics,
antibiotics, and infecting the bacteria with a **bacteriophage** (virus that infects bacteria) are just some of the examples in which spread plates are employed.

The procedure is fairly simple in that a sterile cotton-tipped swab is placed in the culture and then is applied to a plate with media. The spreading technique requires that the entire surface of the media be covered with inoculum. A smooth spread is what is considered best. The illustration below shows the proper manner in which to swab. 90 degree turns, as used in the streak-plate technique, are implemented, but NO thinning of organisms should result.

BE SURE TO go all the way to the edges of the plate. No Agar should be seen! (after growth occurs.)

**Procedure 1 - Gram Staining Organisms**

1. Obtain 1 TSB culture of *S. aureus*, *P. mirabilis*, *B. subtilis*, or *S. bovis*,
2. Obtain a slide and proceed to Gram stain two of the four bacterial samples according to Lab #4 Gram staining procedure. (You may put 2 samples per slide- make sure that you stain all four organisms between you and your lab partner.)
3. Record the findings below.

<table>
<thead>
<tr>
<th><em>Staphylococcus aureus</em></th>
<th><em>Bacillus subtilis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptococcus bovis</em> (or <em>equi</em>)</td>
<td><em>Proteus mirabilis</em></td>
</tr>
</tbody>
</table>

**Procedure 2 - Describing Growth Patterns in Broth, & on Agar Plates**

1. Obtain 1 sterile TSB tube and 1 sterile TSA plate.
2. Flame the inoculating loop.
3. Open 1 uninoculated TSB tube and inoculate with 3 loops full of one of the organisms that you Gram stained in Procedure 1. Flame the stock tube lip and new TSB tube lip before and after inoculation as well as the inoculating loop.
4. Open 1 uninoculated TSA plate, hold it so that air cannot get to the agar and, using 1 loop full of the organism you used in #3, streak for isolation using the quadrant method. Flame the lip of the tube before and after inoculation as well as the inoculation loop. Each person will do one organism and make sure that all four organisms are used on your bench.
5. Incubate at 35°C for 24-48 hours. Be sure plates are turned so that agar is on top. This is the appropriate way for agar plates to incubate.
Second Session
Retrieve all slants, broths, and plates.
Observe growth patterns and record patterns your organism, then borrow plates/tubes from your peers so that you see all four organisms. Use appropriate microbiological descriptions (see spiral bound lab book for more information).

**Staphylococcus aureus:**
- Broth
- Plate

**Bacillus subtilis**
- Broth
- Plate

**Proteus mirabilis:**
- Broth
- Plate

**Streptococcus bovis (or equi):**
- Broth
- Plate

**Procedure 3 - Preparing a Spread Plate (work in groups of 2)**
1. Obtain a TSA plate and a TSB tube of organism from Procedure #2.
2. Prepare a spread plate of each as per the techniques described.
3. Incubate for 48 hours at 35_C.

Second session:
Observe for even growth on the plate, extending to the very edges.
LAB: POUR-PLATE TECHNIQUES

Groups of Four

Materials
(1) TSB tube of *Staphylococcus aureus* or
(1) TSB tube of *Escherichia coli*
Water bath
(2-3) TSA deeps
(3) Sterile petri plates
(2) 1 mL pipettes
(1) Sterile test tube with lid
Bunsen burner
Striker
Inoculating loop
Blue pipettor
Test tube rack

Primary Objective
Properly prepare and pour loop-dilution plates.

Other Objectives
Identify the use of loop-dilution with pour plates and the significance of pure vs. mixed cultures.
List and apply the significance of Koch’s postulates.
Identify what deems a plate countable vs. uncountable.

Introduction
Agar is poured into Petri plates to either prepare sterile media for inoculation or to suspend diluted bacteria. The techniques of pouring plates with diluted bacteria is one of the common ways to isolate individual colonies of bacteria. The other way is through streak plate techniques.

Loop-Dilution Series
A *loop-dilution series* is a technique used to isolate specific bacterial colonies. This lab uses a *serial dilution* which is performed using an inoculating loop. To prepare the bacteria for dilution a mixed culture must be made from the two pure cultures. This is done by placing equal numbers of different bacteria in a sterile empty test tube. The equal numbers are approximate and turbidity is used to judge the numbers present in the pure culture. The mixture must be of equal proportions otherwise a species whose numbers are disproportionately low will, through dilution, reach the *extinction point* before a countable plate is reached. Provided aseptic techniques have been used, only two colony types should appear in the countable plates.
Colony Growth

Three plates are used to dilute the colonies down to achieve a countable plate. A plate that is considered countable has between 30 and 300 colonies. Less than 30 colonies can be accounted for through chance events and greater than 300 is impossible to accurately count.

Because each colony grows from a single bacterial cell, counting colonies after a controlled loop-dilution can aid the researcher in determining the bacterial load in a given fluid. Exact amounts of broth are used to make the loop dilutions and the exact numbers of organisms can be obtained. This lab will concentrate on learning how to do a loop-dilution properly, and how to cultivate pure cultures from mixed.

When a plate is counted, if there is less than 30 colonies the plate is labeled NG for no growth or TFTC can be used (too few to count). If there are over 300, the plate is labeled TNTC (too numerous to count).

Because the plates are poured, the bacteria are distributed evenly throughout the media. Lenticulate colonies may be observed trapped in the agar, larger colonies are usually on the surface, and any region of crowding will result in smaller colonies as organisms compete for food.

Koch’s Postulates (Why pure cultures are important!)

Bacteria is normally found existing with other bacteria and other organisms that live in the community the bacteria inhabit. The diverseness of bacteria will also be great, not just species, but many different genera as well. To identify a particular species a pure culture of that species must be used. It is particularly important in identification of disease causing bacteria.

To begin identification, the microbiologist must culture a pure culture (one that comes from a single bacterial cell) from the variety obtained from whatever media is under scrutiny. Robert Koch, one of the fathers of medical microbiology, was the first to put great emphasis on the pure culture. The procedure defined below that enables a specific pathogenic organism to be identified is known as Koch’s Postulates.

Koch’s Postulates:

1. The researcher must isolate a pure culture.
2. The isolated organisms must show up in every case of the disease.
3. The same organisms must be recovered in pure culture from a previously healthy, test animal that was exposed to said organisms and then contracted the disease.

Other reasons for needing pure cultures include physiological characteristics. These characteristics include the ability to use specific substrates and produce specific substance. These can only be determined from a pure culture. Any contamination of another organism can alter the results of physiological tests.
Procedure - Loop-Dilution Pour Plates (Please work in pairs)

1. Obtain a broth culture of either *S. aureus* or *E. coli*.
2. Obtain 3 sterile Petri plates and label them with the organism name and #1, #2 and #3. Date and initials should be written on the plates as well.
3. Remove 1 liquefied, tempered agar deep from the water bath and transfer 1 loop full of the selected culture to the tube and mix well.
4. Remove another agar deep from the water bath and transfer 1 loop full from the *first agar deep tube* to the second and mix. Pour the first tube into Plate #1, lightly swirl to cover bottom of plate, then add one to two more tubes of liquified agar to fill the plate and allow the agar to cool (takes @ 20 minutes for agar to solidify).
5. Remove another agar deep from the water bath, add one loopful of sample from tube #2 into the new tube and mix. Pour the second tube into Plate #2 and swirl to cover bottom, add one to two more tubes of liquified agar to fill the plate and allow the agar to cool.
6. Pour the last tube into Plate #3 and swirl to cover bottom, add one to two more tubes of liquified agar to fill the plate and allow the agar to cool.

Second session

1. Observe plates 1, 2 & 3.
2. Record the approximate # of colonies in each plate.
LAB: ANTISEPTICS & DISINFECTANTS

Groups of Three

Materials
(1) TSB culture of *Staphylococcus aureus*  
(1) TSB culture of *Escherichia coli*  
(1) TSB culture of *Bacillus subtilis*  
(3) TSA plates  
(3) sterile cotton-tipped applicators  
Sterile disks  
Bunsen burner

Betadine  
Anti-microbial soap  
95% Ethyl Alcohol  
1 M Hydrochloric Acid  
3% Hydrochloric Acid  
Disinfectant (from home)  
Alcohol for flaming

Primary Objective
Observe and evaluate the effects of disinfectants and antiseptics on vegetative bacterial cells, both endospore and non-endospore forming.

Other Objectives
Identify the factors that can influence disinfectant and antiseptic efficiency.  
Identify the difference between antiseptics and disinfectants.  
Define phenol coefficient, bacteriostasis, and bacteriocidal.  
Identify the factors involved in selecting the appropriate antiseptic and disinfectant.

Introduction
The emergence of anti-microbial soaps, lotions and other products has seen a huge increase over the last few years. The number of choices is excessive and the consumer is often unaware that many of the anti-microbial agents are no more effective than basic soap and water. The effectiveness of an anti-microbial is dependent upon many factors such as the concentration of the antimicrobial agent, the amount of contamination, the sensitivity of the contaminating organisms, temperature, and length of exposure. This exercise evaluates the influence that specific antimicrobial agents may or may not have on bacterial growth.

Application, Selection, and the Phenol Coefficient of Antimicrobial Agents
To understand antimicrobial agents one must first understand the difference between antiseptics and disinfectants. Antiseptics are normally safe for application to living tissue such as the human skin and throat. Antiseptics normally are more bacteriostatic in that they prevent bacterial multiplication, but do not kill the organism. Disinfectants, however, are considered germicidal or bacteriocidal. Germicides are chemicals that are usually lethal to bacteria and are meant to be used on non-living areas such as floors, work benches, etc.

There are specific characteristics that identify an ideal antimicrobial. A food antiseptic often is chosen for its ability to work on specific organisms effectively. Several antiseptics as well as disinfectants are listed on the next page. An ideal disinfectant should be highly effective even when diluted, nontoxic, colorless, odorless, stable in any concentration, harmless to all surfaces, biodegradable, inexpensive and, if
it is a phenolic, a good **phenol coefficient**.

### Antimicrobial Solutions

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Examples</th>
<th>Action</th>
<th>Effectiveness</th>
<th>Selected Apps</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohols</td>
<td>Ethanol</td>
<td>Denature proteins, dissolves lipids,</td>
<td>Vegetative cells killed, not endospores</td>
<td>Disinfect instruments clean skin</td>
</tr>
<tr>
<td></td>
<td>Isopropanol</td>
<td>dehydrates molecules</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkylating agents</td>
<td>Formaldehyde</td>
<td>Inactivates proteins, nucleic acids</td>
<td>Vegetative cells and endospores killed</td>
<td>Embalming and vaccinations Antiseptic (Cidex)</td>
</tr>
<tr>
<td></td>
<td>Glutaraldehyde</td>
<td>See above</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Halogens</td>
<td>Iodine</td>
<td>Inactivates proteins</td>
<td>Vegetative cells and some endospores killed</td>
<td>Antiseptic; surgical preparation (Betadine)</td>
</tr>
<tr>
<td></td>
<td>Chlorine</td>
<td>Oxidizing agent</td>
<td>Vegetative cells and endospores killed</td>
<td>Disinfectant for water, dairies, restaurants</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenolics</td>
<td>Cresols</td>
<td>Denature proteins, alters membranes</td>
<td>Vegetative cells killed, not endospores</td>
<td>Preservatives</td>
</tr>
<tr>
<td></td>
<td>Hexachlorophene</td>
<td>See above</td>
<td></td>
<td></td>
</tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quaternary ammonium compounds</td>
<td>Zephran, Roccal</td>
<td>Denature proteins, alters membranes</td>
<td>Most vegetative cells killed, does not kill</td>
<td>Sanitization of labs, industrial sites, restaurants</td>
</tr>
<tr>
<td></td>
<td>cationic detergents</td>
<td></td>
<td><em>M. tuberculosis</em>, <em>Pseudomonas aeruginosa</em>,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>or endospores or unenveloped viruses</td>
<td></td>
</tr>
</tbody>
</table>

#### Procedure (work in groups of 3)

1. Obtain 3 plates of TSA and 3 sterile cotton applicators.
2. Label each plate - one for each species (one per person).
3. Using a sterile cotton-tipped applicator, spread each plate with the labeled species.  
   **BE SURE AND USE A FRESH APPLICATOR FOR EACH PLATE!!!**
4. Obtain each of the antiseptics/disinfectants and a vial of sterile disks.
5. Soak one disk per solution per plate in each of the antiseptics/disinfectants. Use sterile forceps for the transfer of disks to solutions.
6. Using sterile forceps place the disks on the plates and label each disk.  
   **Be sure all disks are evenly spaced.**
7. Incubate at 35°C for 24-48 hours.
Second session
1. Obtain the 3 plates made in the first lab session.
2. Using a metric ruler measure the zone of inhibition of bacterial growth
   IN MILLIMETERS!!
3. Measure the diameter unless unable to, then measure the radius and multiply by two.
4. Record all findings below. Try to determine which are bacteriocidal and which are
   bacteriostatic.

<table>
<thead>
<tr>
<th>Antiseptic</th>
<th>Active ingredients</th>
<th>Diameter of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S. aureus E. coli B. subtilis</td>
</tr>
<tr>
<td>Betadine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Anti-microbial soap</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>95% Ethanol</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1M HCl</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>3% Hydrogen peroxide (H₂O₂)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Lab bleach</td>
<td></td>
</tr>
</tbody>
</table>
LAB 12: SELECTIVE VS. DIFFERENTIAL MEDIA

Materials (Groups of Two)
Stock culture of *Escherichia coli*
Stock culture of *Proteus mirabilis*
Stock culture of *Staphylococcus aureus*
Stock culture of *Salmonella typhimurium*
(2) TSA plates
(1) Hektoen Enteric (HE) plate
(1) Simmons Citrate tube
(1) Mac agar plate
(1) EMB agar plate
Inoculating loop
(1) Sterile cotton-tipped applicator
Bunsen burner
Striker

Primary Objective
Utilize both selective and differential media in growth and identification of bacteria.

Other Objectives
Define all-purpose (complex), selective, and differential media.
List six compounds that can be used to inhibit growth of unwanted organisms in selective media.
List five media that are considered all-purpose.
Identify which media is best used for growth of specific organisms in this lab.

Introduction
There are many different media that can be used in the microbiology lab setting. Some, such as Tryptic Soy Agar (TSA), are considered all-purpose (complex) in that a very broad range of bacterial genera can be grown on it. Other media are selective and inhibit some genera and not others. Still other media are considered differential. These differential media enable the researcher to easily distinguish between different genera using growth characteristics (especially color differences). This lab investigates all three types of media.

All-Purpose (Complex) Media
There is no media that is considered universal in that it can be used for all microbial growth. Conditions of incubation come into play as well as the nutrients needed. There are, however, all-purpose media. Tryptic Soy Agar and Broth, Nutrient Agar and Broth, Blood Agar, and several others are classified as such and can be used to grow a wide variety of soil microbes, pathogens, and human body flora.

Selective Media
Media that contains dyes and other chemicals that can inhibit the growth of certain microbes is termed selective. There are several media that are considered selective and this lab employs Levine EMB Agar. Levine EMB Agar inhibits the growth of gram-positive
bacteria and selects for the growth of gram-negative. Levine EMB can also be considered differential.

**Differential Media**

Media that simplifies the task of distinguishing between various types of bacteria is considered **differential**. This can be done by changing colonies various colors or by the action of the colonies on the media. Levine **EMB Agar** is different in that it distinguishes between *Enterobacter aerogenes* (colonies pink to buff with black centers), *Escherichia coli* (colonies have a green metallic sheen), and those that remain colorless (*Pseudomonas, Salmonella, Shigella*). **MacConkey Agar** is employed in this lab and distinguishes between pathogenic coliform and enteric bacteria versus non-pathogenic coliform and enteric bacteria. Those that are pathogenic can generally ferment lactose and will have pink to red colonies while those that cannot ferment lactose will be transparent. MacConkey Agar is often also considered a selective media. **Hektoen Enteric (HE) agar** is also used to isolate & distinguish between pathogenic coliform and enteric bacteria versus non-pathogenic coliform and enteric bacteria. It is particularly helpful in isolating *Salmonella* and *Shigella* in stool samples. HE agar is both selective and differential. Lactose-fermenting bacteria will have an orange pigment and organisms that produce hydrogen sulfide (H\(_2\)S) will turn the media black. **Citrate agar** is used to detect if bacteria utilize citrate as their sole source of carbon. The agar starts out green but will turn blue if citrate is utilized. This biochemical reaction helps to identify the organism – along with other biochemical reactions such as indole, urease, glucose, etc.

Blood Agar is an all-purpose media that can also be used for differentiating between groups and species of *Streptococcus*. *Streptococcus* is well known for its hemolytic properties. Checking inoculated blood agar for alpha, beta, or gamma hemolysis helps discern what Strep species might be involved.

**Procedure 1 - All-Purpose (Complex) Media**

Obtain (2) TSA plates.

Split each TSA plate into (2) sections. Use each section to streak for isolation with *E. coli* & *S. aureus*; and *P. mirabilis* & *S. typhimurium*.

Incubate the plates at 35_C for 48 hours.

**Procedure 2 - Selective and Differential Media**

Obtain (1) MacConkey and (1) EMB plate.

The **east side** of the class divide the MacConkey and EMB plates into two sections.

Innoculate one side with *E. coli* and the other side with *P. mirabilis*.

The **west side** of the class divide the MacConkey and EMB plates into two sections.

Innoculate one side with *S. typhimurium* and the other side with *S. aureus*.

Streak for isolation in each section using the appropriate organisms.

Incubate plates at 35_C for 24-48 hours.
Procedure 3 - Application of Selective Media

Obtain (1) HE Agar plate.

Divide the HE plate into two sections.

The **west side** of the class (nearest the door) label one side with *E. coli* and the other with *S. typhimurium*. Inoculate each for isolation.

The **east side** of the class (nearest the refrigerator) label one side with *P. mirabilis* and the other with *S. aureus*. Inoculate each for isolation.

Procedure 4 – Biochemical test

Obtain (1) Simmons Citrate tube.

The **first two benches on the east side of the class** will label their tube *E. coli*,

the **last two benches on the east side** will label their tube *P. mirabilis*,

the **first two benches on the west side** will label their tube *S. typhimurium*

and the **last two benches on the west side** will label their tube *S. aureus*

Each pair will use a stab/needle to inoculate their tube with the appropriate organism. Just obtain some organism on the stab and push it straight down into the media.
<table>
<thead>
<tr>
<th></th>
<th>E. coli</th>
<th>P. mirabilis</th>
<th>S. typhimurium</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSA plate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MacConkey plate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EMB plate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HE plate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate agar</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
LAB: QUANTIFYING BACTERIA

Materials (groups of four)
(1) TSB culture of E. coli
(2) TSA plates
(4x3) TSA deeps (liquid) [don’t remove all at the same time]
(4) Sterile Petri plates
(4) Sterile 1.0 mL pipettes
(4) Sterile tubes with 9 mL of water
Pipettor
Bunsen burner
Striker
Tube rack
Quebec colony counter
(8) Sterile 1 mL pipettes

Primary Objective
Be able to quantify bacteria.

Other Objectives
Be able to calculate and prepare bacterial dilutions.
Identify when and why a bacterial count might be needed.
Be able to correctly operate a Quebec Colony Counter and a spectrophotometer.
Construct and evaluate a standard curve using optical density vs. number of cells in suspension.

Introduction
This exercise allows the researcher to determine the number of bacteria/mL in a specific solution. Any work done in the areas of public health, medicine, or industry will require the microbiologist to be able to conduct such analyses. This procedure is normally used when the concentration of LIVING cells in a liquid is needed.

Counting Viable Microbes
It is almost impossible to distinguish a living cell from a dead cell when viewing through a microscope. Viable cells can reproduce and dead ones cannot. When employing dilution plate counts, a diluted amount of microbes can be incubated and, using mathematical manipulation, the number of microbes present per milliliter can be figured. There are two techniques for preparing dilution plates that are the most common. There is the pour-plate technique and the spread-plate technique. In the pour-plate technique, a sample from a dilution of bacteria that has been accurately done is pipetted into a Petri dish. An agar medium is then poured over the liquid and mixed. When the spread-plate technique is employed, usually a 0.1 mL sample of the dilution is pipetted over the surface of already poured plates. After incubation, colonies are counted, and results are then averaged. Often times the bacteria will clump and a colony will come from more than one cell therefore researchers normally say a colony has come from a colony forming unit rather than a single cell.
Dilutions
Dilutions are normally prepared in 9 mL of water (if larger amounts are needed 99 mL are used). When 1 mL of bacterial suspension is pipetted into 9 mL of water a 1:10 dilution is made. This may be written as $10^{-1}$. If further dilution is needed, 1 mL from the 1:10 dilution is used in another 9 mL of water. This makes a $10^{-2}$ dilution or a 1:100. Further dilutions can be done. What dilution is produced if 1 mL of 1:1000 is added to 9mL of water?

Calculations
The following formula is used to find the number of bacteria in the original solution. One should remember that a countable plate is a plate that contains between 30 and 300 colonies.

\[ \text{# bacteria/mL of original solution} = \text{# of colonies on plate} \times \text{dilution factor} \]

(\text{the dilution factor is the reciprocal of the dilution})

Example:

- 80 colonies on a dilution plate of 1:1,000,000

\[ 80 \times 1,000,000 = 80,000,000 \text{ bacteria/mL} \]

Quantifying Living & Dead Bacteria
When a total concentration of dead and living microbes in a solution is needed, measurement of numbers, constituents, or mass is needed. Numbers can be measured with a direct microscopic count using a counting chamber. Constituents (protein, peptidoglycan, etc.) can be measured and the microbial concentration can be determined. Cell mass can also be estimated by dry weight (works well with fungi).

Normally, however, cell mass of bacteria is indicated by the ability of a suspension containing bacteria to scatter light. Scattering light is a characteristic of turbidity. Turbidity actually reflects mass of cells, not numbers. Microbes that are larger, even when in smaller numbers, will reflect as much light as microbes that are smaller existent in higher quantities. To determine the mass in a given volume, turbidometric assay is used. This procedure measures the density of a suspension and then mass can be figured by \( d = m/v \).

To perform the assay, controls and a serial dilution is prepared. Then the spectrophotometer is used to measure the optical density (the amount of light absorbed and scattered by cells in solution) of suspension in each tube. Turbidity is then graphed by placing the optical density on the vertical \( Y \text{ axis} \) and the dilution of the microbes on the \( X \text{ axis} \). The plate count analysis will have given the number of bacteria per mL of each dilution and this is plotted on the X axis. The readings from the spectrophotometer are then plotted on the Y axis. The graph can be used to estimate cell concentration when under the same conditions. The same wavelength of light must be used and the same species must also be used.

When these conditions are met the numbers in suspension of a given organism can be estimated and plate counts can be avoided.
**Procedure 1 – Pour Plate Count**

1. Obtain (4) TSA deeps, liquefied and tempered.
2. Obtain *E. coli* in broth and (4) sterile water blanks, each containing 9 mL of water.
3. Transfer 1 mL using a sterile pipette from original culture into the first tube of 9 mL of water to make a 1:10 dilution (tube 1).
4. Transfer 1 mL from tube 1 to the second tube containing 9 mL of water to make a 1:100 (tube 2).
5. Transfer 1 mL from tube 2 to the third tube containing 9 mL of water to make a 1:1,000 (tube 3).
6. Transfer 0.1 mL from tube 3 to the fourth tube containing 9.9 mL of water to make a 1:100,000 (tube 4).
7. Obtain (4) sterile Petri plates and label them P1, P2, P3 and P4 (with initials and date also).
8. Transfer 1 mL from each tube into the corresponding plate. Pour 2-3 agar deeps over each plate, swirl to mix, and incubate at 35°C for 24-48 hours.

**Procedure 2 – Spread Plate Count**

1. Obtain (4) TSA plates. Label them S1, S2, S3 and S4 (with initials & date also).
2. Transfer 0.1 mL from each tube (prepared in Procedure 1) onto the appropriate plate. Rotate the plate to coat the surface of the agar with the sample. Incubate at 35°C for 48 hours.

**Second session**

1. Inspect all pour and spread-plates.
2. Count all countable plates and calculate the number of bacteria in the original solution.
3. Record and compare results from Procedure 1 to those in Procedure 2.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Pour plates</th>
<th>Spread plates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:1,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:100,000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
LAB: OXYGEN

*Perform in designated groups

Materials
(1) Culture *Campylobacter jejuni*
(1) Culture *Pseudomonas aeruginosa*
(1) Culture *Bacteroides fragilis*
(1) Culture *Neisseria gonorrhoea*
(4) CVA plates
(4) TSA plates
(4) Chocolate Agar plates
(4) Fluid Thioglycollate medium tubes without bicarbonate
(4) TSA deeps
(4) TSA slants
Candle jar
Anaerobic gas-pak
Campy gas pak
10 mL pipette
Pipetter
Bunsen burner
Inoculating loop
Inoculating needle
Striker

Primary Objective
Utilize 3 methods for culturing of organisms, analyze and compare the results.

Other Objectives
List 5 microbial groupings according to oxygen tolerance.
Explain how organisms use catalase and superoxide dismutase to survive in oxygenated environs.
Compare the microbes of each group according to their use for respiration and/or fermentation.
Identify $O_2$ and $H_2O$.
Define reduced environment and oxygen reduction potential.
**Introduction**
Louis Pasteur was the first to note bacteria that could not live in the presence of oxygen. He noticed clostridia dying when they came in contact with oxygen on the edges of wet mounts. Pasteur used the term **anaerobic** when describing organisms poisoned by oxygen. This exercise is designed to look at how oxygen affects bacterial growth.

**Oxygen Requirements**
Most humans assume that since they and other animals require oxygen to survive, members of the microbial world would too. Since the earth is covered in vast quantities of liquid environments, however, the first statement is not true. In fact, oxygen is not highly soluble in water and therefore microbes needing oxygen are at a disadvantage in nature. Most require only water and do not need oxygen. There is such a diversity of the amount of oxygen required and/or tolerated by organisms that 5 groupings have been named to attempt to recognize all requirement levels.

**Aerobes** - Must have oxygen to live. Oxygen is the last acceptor of electrons released when foods are burned for energy. Use aerobic respiration.

**Microaerophiles** - Organisms respire and require oxygen, but these organisms require less oxygen and more carbon dioxide than is found in air.

**Facultative Anaerobes** - These have the enzymes needed to use oxygen, it is not a requirement. Facultative anaerobes can substitute nitrate (NO$_3^-$) and sulfate (SO$_4^{2-}$) or other molecules when the environment is oxygen poor. Most of this group can also perform fermentation in which organic compounds are broken down and some energy is released.

**Aerotolerants & Anaerobes** - Neither of these utilize oxygen and **obligative anaerobes** (true or strict) are killed by oxygen. Anaerobes lack both catalase and superoxide dismutase can help break down H$_2$O$_2$ into harmless H$_2$O and O$_2$. Without these enzymes, anaerobes cannot process oxygen toxins and will die in the presence of oxygen. Aerotolerants will not die, but neither will they utilize oxygen.

**Reactions using superoxide dismutase and catalase**
Superoxide dismutase takes unstable oxygen, binds it to hydrogen and makes hydrogen peroxide and stable oxygen. Catalase then will break down hydrogen peroxide into water and oxygen. Aerobes, microaerophiles, facultative anaerobes, and aerotolerants all have superoxide dismutase. Aerobes, microaerophiles, and facultative anaerobes also can have catalase. Anaerobes have neither enzyme and aerotolerants do not have catalase.

\[
2 \text{O}_2^{2-} + 2 \text{H}^+ \xrightarrow{\text{Superoxide Dismutase}} \text{H}_2\text{O}_2 + \text{O}_2
\]

\[
2 \text{H}_2\text{O}_2 \xrightarrow{\text{Catalase}} 2 \text{H}_2\text{O} + \text{O}_2
\]
Oxygen Reduction Potential

Oxygen reduction potential is a means of expressing an affinity by a compound for electrons. This affinity is then compared with the attraction of H₂ for electrons. If an environment is oxidized, it has a high affinity for electrons therefore it has a high oxygen reduction potential and thus there are fewer electrons. If an environment is reduced there is a higher proportion of H₂ than is in H₂O which makes a negative oxidation reduction potential. An environment that is rich in electrons is therefore a reduced environment. Usually oxidation reduction potential is simply termed redox potential.

Cultivating Anaerobes

There are several techniques that can be used to make an oxygen negative environment. This exercise will look at a few of the more common techniques including boiling oxygen out of media and making an agar shake culture and an agar stab culture. This lab also employs the use of Fluid Thioglycollate Medium which is made specifically to cultivate anaerobes, an anaerobic environmental chamber, and a candle jar. The environmental chamber uses chemical packages make specifically to bind to free oxygen and make the environment reduced. Fluid Thioglycollate Medium relies upon sodium thioglycollate to bind to free oxygen and rid the environment of oxygen. Both the chamber and the thioglycollate medium rely on dyes that turn from white to pink to indicate the loss of oxygen. The candle jar uses a candle to burn up oxygen and make a CO₂ rich environment. The campy pak produces a microaerophilic environment with 5% to 10% CO₂ and a residual atmosphere of 5% to 15% O₂.

Bacterioides organisms are anaerobic, Gram negative rods. They will not grow in regular TSA but will thrive in the Thio broth as long as it is in a reduced state (anaerobic environment, little or no pink on top).

Campylobacter organisms are curved, oxidase positive, non-spore forming microaerophilic gram-negative rods*; they are motile by means of a single, polar flagellum. They are non-fermentative. Recognized as one of the leading causes of bacterial diarrhea worldwide.

Campylobacter grows best in an atmosphere containing 5%-10% O₂, & 10% CO₂. Optimum growth temperature is 42_C.

*When gram-staining, use carbolfushin as a counter stain instead of safranin. Campylobacter doesn’t hold safranin well.

P. aeruginosa is a highly aerobic, gram-negative rod. It is a non-fermenter. It produces pyocyanin, a water soluble, chloroform soluble green (blue-green) phenazine pigment. It is the only organism in the clinical laboratory to produce this pigment. It also produces fluorescein, a feature it shares with P. fluorescin.

Neisseria are aerobic, gram-negative cocci that inhabit the mucous membranes of humans and other animals. While some species are considered normal flora, N. gonorrhea and N. meningitidis are pathogenic and infect only humans, causing
gonorrhea and meningitis, respectively. Although the species is aerobic, \textit{N. gonorrhea} and \textit{N. meningitidis} grow best at 3% to 7% CO$_2$.

**Procedure 1 - Agar Shake and Stab cultures**

Obtain TSA deeps from the water bath as needed.

The \textbf{shake cultures} will be made using the liquefied TSA tubes. Transfer 1 loop of \textit{P. aeruginosa} to one of the liquefied tubes and disperse the bacteria by rolling the tube between the hands. Leave lids loose on the tubes. Repeat for each of the other 3 species.

The \textbf{stab cultures} will be made using the TSA slants. Transfer one needle of \textit{P. aeruginosa} to the solidified agar by stabbing the needle down into the middle of the agar. Repeat for each of the other 3 species.

Incubate at 35°C for 48 hours.

**Procedure 2 - Fluid Thioglycolate Medium Cultures**

1. Obtain (4) tubes of fluid thioglycollate. \textbf{Reazurin} dye turns pink in the presence of oxygen. Tubes should be less than 30% pink.
2. Transfer (2) loops of \textit{P. aeruginosa} to one cooled tube of medium and repeat with the other 3 species.
3. Incubate at 35°C for 48 hours.

**Procedure 3 - Aerobic, Anaerobic, Microaerophilic Environment and Candle Jar**

1. Obtain (4) TSA plates, (4) Chocolate plates, (4) CVA plates
2. Split each of the four plates into 4 quadrants. Label with the names of the four organisms (\textit{C. jejuni, P. aeruginosa, B. fragilis & N. gonorrhea})
   Inoculate the plates with the appropriate organism.
3. Label one plate AER and incubate aerobically at 35°C.
4. Label one plate CAMPY and incubate in a jar with a campy pak at 42°C.
5. Label one plate ANA and incubate in the anaerobe jar with a gas pack at 35°C.
6. Label one plate CAN and incubate in a candle jar at 35°C.
**Second Session**
Observe tubes and plates from each procedure and describe growth below. Pay particular attention to ‘where’ the organism grows in the broths (top, middle, bottom). If there is no growth, just put the letters NG in the box.

<table>
<thead>
<tr>
<th></th>
<th>Deep</th>
<th>Stab/Slant</th>
<th>Thio</th>
<th>Aerobic</th>
<th>Campy</th>
<th>Anaerobic</th>
<th>CandleJar</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. fragilis</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>C. jejuni</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>N. gonorrhea</strong></td>
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<td></td>
</tr>
<tr>
<td><strong>P. aeruginosa</strong></td>
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</tr>
</tbody>
</table>
LAB: TEMPERATURE

Materials
(1) Culture of B. subtilis
(1) Culture of S. epidermidis
(1) Culture of E. coli
(4) TSA plates
Refrigerator (6-10 C)
35 C incubator
55 C incubator
Endospore staining reagents
   • Malachite green
   • Safranin
Other staining supplies (stain rack, clothespin, wash bottle, etc.)
Inoculating loop
Bunsen burner, Striker
Hot plate

Primary Objective
Research and analyze the affects of temperature on various species of bacteria

Other Objectives
Identify 5 physical factors that can alter microbial growth patterns.
Identify the natural environments and temperature requirements of psychrophiles, thermophiles, and mesophiles.

Introduction
There are five physical factors that can alter microbial growth patterns. These five are pH, temperature, osmotic pressure, radiation, and oxygen tension. Microbes do not have hemostatic (maintenance of equilibrium) mechanisms and are therefore directly affected by any changes in the environment. This exercise allows the researcher to examine the influence of temperature on some representatives of the microbial groups.

Temperature Range
Every species of microbe has its favorite temperature that is known as the optimum temperature and thus an optimum temperature range. The range of species can be either narrow or broad and this is normally dependent upon genetic potential and physical/chemical stress factors. The five physical factors can interact with each other and narrow or broaden the temperature range.
**Groupings by Temperature**

There are 3 temperature groupings observed by microbiologists.

- **Psychrophiles** - This group multiplies best at a range of 0°C to around 20°C. Members of this group are often found causing spoilage of food in the refrigerator.

- **Mesophiles** - Members of this group live in a range of temperatures from 20°C to about 40°C. Most human pathogens are members of this temperature group as well as normal animal flora. They are also found in abundance in topsoil.

- **Thermophiles** - These microbes thrive at high temperatures. Normally found in hot springs, spas, desert soils, and other areas that are hot environments, they multiply well in temperatures in excess of 40°C.

*If a microbe can survive at these excessive heats but can not reproduce, they are termed **thermodurics**. Normally endospore formers are thermoduric since any genus that can form endospores can usually endure boiling for 10 minutes.*

**Effects of Temperature on Bacterial Growth**

The following procedures will allow the researcher to look at how temperature effects bacterial growth. The procedure will examine the ability of three different species of bacteria to multiply in varying temperatures.

**Procedure 1 - Growth at Varying Temperatures**

Obtain (4) TSA plates.

Divide the plates in thirds and label with each species name.

Using a sterile inoculating loop, transfer one streak of *B. subtilis, S. epi, E. coli* to each of the four plates.

Place one plate in a refrigerator (approximately 10°C), place another in an incubator set at 35°C, place another in an incubator set at 55°C, and leave the fourth plate out at room temperature (approximately 25°C) as a control.

5. Sub the *B. subtilis* to a TSB and also incubate at 55°C.

6. Let the plates and tube incubate for **48 hours**.

**Second session**

Examine the four plates.

Draw the growth patterns for each species on each plate.

Pick a species from each plate that grew poorly or not at all and simple stain each sample. Describe/diagram the findings on the report sheet.

Endospore stain the *Bacillus subtilis* that was incubated at 55°C.
## Report - Growth characteristics

<table>
<thead>
<tr>
<th></th>
<th>10°C</th>
<th>25°C</th>
<th>35°C</th>
<th>55°C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td><strong>S. epidermidis</strong></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>B. subtilis</strong></td>
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</table>

**Endospore Stains of B. subtilis**

55°C plate  
Tube growth
LAB: STAPHYLOCOCCI

**Materials**
(Please work in pairs)
1. TSB/TSA culture of *Staphylococcus epidermis*
2. TSB/TSA culture of *Staphylococcus aureus*
3. TSB/TSA culture of *Streptococcus pyogenes*
4. Coagulase plasma
5. Mannitol Salt agar plate
6. Microscope slide
7. Hydrogen peroxide (H$_2$O$_2$)
8. Inoculating loop
9. Bunsen burner
10. Striker
11. 1 mL pipettes

**Primary Objective**
Perform and evaluate tests on two species of *Staphylococcus*.

**Other Objectives**
Identify sites on the human body where staphylococci is normally located, areas where clinical specimens can be collected and some pathological conditions. List the shared characteristics of all the members of the Family Micrococcaceae. Compare and contrast *Staphylococcus aureus* and *Staphylococcus epidermis* for their pathogenic potentials and their microscopic and macroscopic characters.

*Staphylococcus* is one of four genera in the Family Micrococcaceae. The other three genera are *Micrococcus*, *Stomatococcus*, and *Planococcus*. These are all gram-positive cocci, but only *Micrococcus* and *Staphylococcus* are medically significant. The commonly shared characteristics of these four genera include non-endospore forming, facultatively anaerobic or aerobic, catalase-positive, nonmotile, and chemoorganotrophic (carbon and energy from organic compounds). The DNA and RNA sequences of these four genera are very different as well as their cell wall composition and thus should probably separate them from the same family. This lab looks specifically at two members of the genus *Staphylococcus* and their importance in the clinical field.

*Staphylococcus*

Nineteen species comprise the genus *Staphylococcus*. Among these members that are common flora on the human skin like *Staphylococcus epidermis*. This species is normally considered a typical resident, but is also an opportunistic pathogen and can infect when tissue is compromised. This is one of the more
common causes of post-operative infection as well as infection among immunosuppressed patients.

Staphylococcus aureus is a very formidable pathogen. This species contains enzymes that allow it to invade healthy tissue and cause disease. It is one of the most common causes of wound infection, both non-surgical and surgical. S. aureus is responsible for impetigo, enterocolitis, pneumonia, urogenital tract infections, endocarditis and food poisoning causing gastroenteritis.

Staphylococcus aureus is characterized by four key points:
- Coagulase production.
- Mannitol fermentation that yields acid. (Turns Mannitol Salt yellow.)
- Heat-stable DNA is produced.
- Beta hemolysis of blood is produced by most strains.
Some other characteristics shared by most all members of this genus include cell arrangement in clusters, catalase-positive, oxidase-negative, and positive for lysis by lysostaphin.

Catalase

Catalase is responsible for the breakdown of hydrogen peroxide into oxygen and water. Usually those organisms lacking catalase are unable to live in the presence of oxygen because of the formation of hydrogen peroxide that results from cellular respiration. Two of the more common aerobic bacteria that do not contain catalase and can withstand oxygen are Streptococcus and Lactobacillus.

Procedure 1 - Catalase Testing

1. Obtain one clean microscope slide.
2. Mark two circles on a slide and place a sample of Staphylococcus epidermidis (from broth) in one circle and Streptococcus pyogenes (from broth) in the other.
3. Add a drop of 3% hydrogen peroxide to each organism.
4. Watch for a bubbling reaction and record findings on report sheet.

Procedure 2 - Coagulase Testing

1. Make up a bottle of coagulase plasma (number of bottles of plasma needed depends on size of class).
2. Obtain (2) tubes and pipet 0.5 mL of Coagulase plasma into each.
3. Label one tube of coagulase S. epi and inoculate with 0.1 mL of S. epidermis. Label the other tube of coagulase S. aure and inoculate with 0.1 mL of S. aureus.
4. Incubate at 35°C for 24 hours.
Second session
Retrieve the two tubes containing the Coagulase plasma.

Check for coagulation of plasma in the tube. If the mixture is still liquefied, the species is coagulase negative. If the mixture is coagulated (solid or very lumpy), then the species is coagulase positive.

Record all information on the report sheet.

Procedure 3 - Mannitol Salt Agar Testing
Obtain one Mannitol Salt agar plate. Divide the plate into two halves, inoculate one streak down the middle of each respective half using
S. epidermidis and S. aureus.
Incubate at 35°C for 48 hours.

Second session
Examine the Mannitol Salt agar plates. Growth indicates the species has a tolerance for 7.5% NaCl (salt). This is a shared characteristic by staphylococci that is not shared by most gram-positive cocci. If there is a color change to yellow, then the species was able to ferment mannitol and therefore release acid that alters the phenol red to yellow. Record all results on the report sheet.

REPORT SHEET - STAPHYLOCOCCI

Procedure 1 - Catalase Testing
Staphylococcus epidermidis

Streptococcus pyogenes

Procedure 2 - Coagulase Testing
Staphylococcus epidermidis

Staphylococcus aureus

Procedure 3 - Mannitol Salt Agar Testing
Staphylococcus epidermidis

Staphylococcus aureus
LAB: ALPHA-, BETA-, AND GAMMA-HEMOLYTIC STREPTOCOCCI

Materials
(1) culture of *Streptococcus agalactiae* (Group B)
(1) culture of *Streptococcus pyogenes* (Group A)
(1) culture of *Streptococcus pneumoniae*
(1) culture of *Enterococcus faecalis* (Group D, enterococcal)
(1) culture of *Staphylococcus aureus*
(1) culture of *Streptococcus bovis* (Group D, non-enterococcal)
Blood agar plates (BAP)
Bacitracin disks
Optochin disks
Bile esculin stab
Inoculating loop
Forceps
Alcohol for flaming
Bunsen burner & Striker

Primary Objective
Identify and differentiate between alpha-, beta-, and gamma-hemolytic streptococci.

Other Objectives
Compare and contrast the major ways to divide streptococci into groups.
Identify five GABHS infections.
Define hemolysis, CAMP test, GABHS, bacitracin, sequelae.
Properly perform a bacitracin sensitivity test.
Properly perform an optochin sensitivity test.

Introduction
The genus *Streptococcus* is a member of the Family Deinococcaceae. There are several distinguishing characteristics of the genus. *Streptococcus* is catalase-negative, oxidase negative, homofermentative (yield only one end product) resulting in lactic acid, cytochrome system negative which make them strict fermenters, and have single or chain cell arrangement. The genus normally exhibits some type of hemolysis (breakdown of red blood cells), either alpha (partial hemolysis – green zone), beta (complete hemolysis – clear zone), or gamma (no hemolysis). The beta-hemolytic streptococci contain several pathogenic members. In the 1930’s Rebecca Lancefield identified five distinct antigenic groups and named them A, B, C, D, and E. There have since been 13 more groups identified, but the Lancefield groupings are the most common. The clinical significance of species varies from group to group as does the resistant to certain antibiotics. This lab will look at all three hemolytic types and the tests that help identify them.
**Streptococcus**

Streptococci are found in a variety of areas from the nasopharyngeal tract to the intestinal tract and wound infections. There are several medically important alpha-hemolytic species. *Streptococcus pneumoniae* is the common cause of pneumonia. It is listed among the top 10 causes of death in the U.S. It is spread by carriers through the nasopharynx. It can also be one of the causes of bacterial meningitis and otitis media. *Streptococcus mitis* is another alpha-hemolytic streptococci, but it is normally considered harmless in the upper respiratory tract. It can cause subacute bacterial endocarditis.

One of the most recognized beta-hemolytic members is *Streptococcus pyogenes*. It is the species responsible for **pharyngitis** (septic sore throat, strep throat). Before rapid diagnosis was available, *S. pyogenes* caused many **nonspurative sequelae** (non-pus-forming, post-infection conditions) such as rheumatic fever, scarlet fever, and glomerulonephritis. *S. pyogenes* is the only group A, beta-hemolytic streptococci and is termed **GABHS** (**group A, beta-hemolytic Streptococcus**). In more recent years, the bacteria known as the “flesh-eating bacteria” has also been found to be a possible mutant member of the GABHS. Several other beta-hemolytic species are members of other groups. *S. agalactiae* is a member of the group B streps. *S. zooepidemicus* is a member of group C and is the cause of wound infections and endocarditis.

There are several distinguishing factors between the species of streptococci and the four species dealt with in this lab exhibit most of the factors:

<table>
<thead>
<tr>
<th>Group Species</th>
<th>Hemolysis</th>
<th>Sensitivity</th>
<th>Pathogenic Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. pyogenes</em></td>
<td>Beta</td>
<td>Bacitracin (PYR positive)</td>
<td>Pharyngitis, septicemia, etc.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(CAMP reaction negative)</td>
<td></td>
</tr>
<tr>
<td>Group B</td>
<td></td>
<td>None</td>
<td></td>
</tr>
<tr>
<td><em>S. agalactiae</em></td>
<td>Gamma or Beta</td>
<td>None (PYR negative)</td>
<td>Pneumonia, endocarditis etc.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(CAMP reaction positive)</td>
<td></td>
</tr>
<tr>
<td>Group D</td>
<td></td>
<td>None</td>
<td></td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>Alpha</td>
<td>None (PYR positive)</td>
<td>Peritonitis, urinary tract infections, endocarditis, etc.</td>
</tr>
<tr>
<td><em>S. bovis</em></td>
<td>Alpha</td>
<td>None (PYR negative)</td>
<td></td>
</tr>
<tr>
<td>No Group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. pneumo</em></td>
<td>Alpha</td>
<td>Optochin</td>
<td>Pneumonia, meningitis, etc.</td>
</tr>
</tbody>
</table>
The sensitivity testing that is done centers around several different antimicrobics and bile salts. **Optochin** is an antimicrobial that most alpha-hemolytic streptococci are resistant to. **Bacitracin** is another antimicrobial that works well on GABHS and SXT works on beta-hemolytic members of group C (S. equis, S. equisimilis). Another species indicator is known as the **CAMP reaction**. This is not an antimicrobial, but a reaction in which group B streptococci enhances the beta-hemolytic property of *Staphylococcus aureus*. The antibiotic disks can be used across from each other and determine the identity of unknowns. Group A will avoid Bacitracin, but grow over SXT. Group B is resistant to both Bacitracin and SXT. Group C will grow over Bacitracin, but avoid SXT. Group A will also be PYR positive whereas Group B will be negative. Optochin is normally more useful on alpha-hemolytic and not beta-hemolytic. There are also rare strains of groups B and C that can be sensitive to Bacitracin as well. The researcher must be aware that these possibilities do exist and not use one test only to conclude the species that is being dealt with.

There are two basic types of Group D strep: Enterococcus and non-enterococcus. At one time, *Enterococcus faecalis* was listed as *Streptococcus faecalis*. *Streptococcus bovis* is a non-enterococcus strep. All Group D streps will be bile esculin positive but only the enterococcus group will by PYR positive.

**Procedure 1 - Alpha-, Beta-, and Gamma-Hemolytic Streptococci Testing**

Obtain (2) Blood Agar plates.
Split plates in threes.
Label one plate *S. pyogenes*, *S. agalactiae* and *S. aureus*.
Label the second plate with *S. pneumoniae*, *E. faecalis*, and *S. bovis*.
Inoculate each plate with the appropriate bacteria streaking for isolation.
Incubate the plates at 35°C for 48 hours.

**Procedure 2 - Bacitracin Sensitivity**

Obtain one BAP
Label one half *S. pyogenes* and one half *S. agalactiae*.
Streak for isolation using the appropriate organism.
Drop a Bacitracin disk on each half, pressing lightly to make contact with the agar.
Incubate at 35°C for 48 hours

**Procedure 3 - Optochin Sensitivity**

Obtain one BAP
Label one half *S. pneumoniae* and one half *E. faecalis*.
Streak for isolation using the appropriate organism.
Drop an Optochin disk on each half, pressing lightly to make contact with the agar.
Incubate at 35°C for 48 hours
Procedure 4 - The CAMP Reaction

Obtain one BAP.
Make one line of *S. aureus*.
Make one line perpendicular to the line (made in step 2) of *S. pyogenes*, and one line of *S. agalactiae*.
Incubate for 48 hours at 35°C.

Procedure 5 – Bile Esculin Hydrolysis

Obtain 1 Bile esculin stab.
After labeling, Inoculate one with either *S. pyogenes*, *E. faecalis*, or *S. bovis*.
Incubate for 48 hours at 35°C.

Second Session

Read Procedures 1 – 5 and record below.

Procedure 6 – The PYR test

Using growth from Procedure 1 plates, perform the following steps on *S. pyogenes*, *S. agalactiae*, *S. bovis*, and *E. faecalis*:

1. Place three PYR disks on a paper towel and label the appropriate species below each disk.
2. Moisten the PYR disk slightly with distilled or deionized water.
   Do not saturate.
3. Using a sterile loop, pick 2-3 well isolated, 18-24 hour colonies and rub into a small area of the PYR disk so that here is a visible paste. After the test organism has been inoculated onto the disk, allow it to react for two (2) minutes.
4. After the incubation period, add one drop of PYR reagent. A pink or cherry red color will appear within one minute if the test is positive. A negative test is indicated by no color change.
5. Record your results below. (See lab book page 78 for flow chart.)
<table>
<thead>
<tr>
<th>Group</th>
<th>Hemolysis</th>
<th>Bacitracin</th>
<th>Optochin</th>
<th>CAMP</th>
<th>Bile esculin</th>
<th>PYR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. pyogenes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. agalactiae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. pnemoniae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>E. faecalis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. bovis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. aureus</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
LAB: IMViC TESTS

Materials
(1) Culture of Escherichia coli
(1) Culture of Klebsiella pneumoniae
(2) SIM tubes
(2) MR-VP broth tubes
(2) Citrate agar slants
(2) EMB Plates
(2) Capped test tubes, nonsterile
(2) 1 mL pipets
Pipettor
Inoculating loop
Bunsen burner
Striker
Kovac's reagent
Methyl red pH indicator
Barritt’s reagents A and B

Primary Objective
Properly perform and interpret the IMViC tests.

Other Objectives
Define IMViC and name what reagents are used with each portion of the tests.
Compare and contrast the positive and negative test results between E. coli and K. pneumoniae
Define mixed-acid fermentation, butanediol fermentation, coliform, enteric.

Introduction
The term ‘IMViC’ is an acronym for four tests and the order in which they are normally run. These tests are normally run in order to distinguish between varying coliform bacteria. The definition of coliform bacteria is gram-negative, facultatively anaerobic or aerobic, non-endospore forming, bacilli (rod shaped). these usually produce gas and acid from lactose fermentation. Most of the coliforms are placed with their relatives in the family Enterobacteriaceae. This group is more commonly known as the enterics. Most of these bacteria exist in the intestines of humans and other animals. The IMViC tests are good for differentiating between the fecal bacteria Escherichia coli and Enterobacter cloacae which can be found in feces, but is most commonly found in soil and decaying vegetation that is free of fecal material. IMViC stands for I - Indole, M -Methyl Red, Vi - Voges-Proskauer, and C - Simmons Citrate.

Indole
**Indole** is one of the products produced when the amino acid **tryptophan** is hydrolyzed. The other two products are ammonia and pyruvic acid. Some coliforms are able to break tryptophan down because they contain **tryptophanase** (the enzyme responsible for hydrolyzing tryptophan). 1% tryptone in water is used to determine
whether a coliform has tryptophanase or not. Indole can often be degraded by certain bacteria and therefore this test should never sit longer than four to five days. Following incubation, Kovac’s reagent is added to the tryptone broth. If the reagent forms a red ring, indole has been produced and thus the organism contains tryptophan. If the ring is green and stays green even after sitting 15 minutes, it can be assumed that there was no indole produced, therefore the organism does not contain tryptophanase. SIM media may also be used to detect indole production. SIM stands for sulfur, indole and motility. Kovac’s reagent is added after the SIM is incubated for 24-48 hours.

**Methyl Red and Voges-Proskauer**

To properly perform these tests, the organisms must be grown in **MR-VP medium**. MR-VP has the key ingredient of dextrose. Most of the coliform bacteria convert some dextrose to acids when initially placed in the medium. After the initial conversion, however, some genera are able to go on and produce more acid in what is known as **mixed acid fermentation** while still others yield much more neutral end products in what is known as **butanediol fermentation**. These are good reactions for determining the identity of an enteric genus. Both the methyl red and the Voges-Proskauer must have incubation times of a two-day minimum. Two to five days is normally recommended for incubation. **Methyl red** is a pH indicator that turns red if the pH is 4.4 or below, indicating acid formation. **Voges-Proskauer** used two reagents (**Barritt’s Reagents A and B**) which react with acetyl methyl carbinol which is a precursor to 2,3-butanediol. The VP test will also turn red if the precursor is present. It sometimes takes up to 30 minutes before the reagents will react.

**Citrate**

**Citrate** is a source of carbon and is the only source in the synthetic medium known as **Simmons Citrate agar**. Because this is a synthetic, the source of nitrogen is also controlled and consequently the nitrogen is supplied only by **ammonium salts**. The medium also contains the pH indicator **bromthymol blue** which indicates alkaline conditions of pH above 7.6 by turning a royal blue. A characteristic of all coliforms is the ability to metabolize citrate when it is inside the bacterial cell, however, they must produce transport enzymes that can bring the citrate into the cell. The only coliform bacteria that grows well in Simmons Citrate medium are those that can transport the citrate and use the ammonium ions as their source of nitrogen. If these two things are accomplished, alkaline conditions are produced and the Simmons Citrate will become bright, royal blue.
Procedure 1 - Indole Testing

Obtain two tubes of SIM media.
Obtain one culture of *E. coli*, and one culture of *K. pneumoniae*. Label one SIM tube for one species and the other tube for the other.
Inoculate the SIM tubes with one loop full of each species. Placing the appropriate species in the appropriate tube.
Incubate the tubes for 48 hours at 35°C.

Second session

Retrieve the SIM tubes from the incubator.
Observe tubes for motility and H₂S production.
Add 2-3 drops of Kovac’s reagent to each tube. Roll each tube between the palms to mix.
Place the tubes back in the tube rack and observe the reaction after 15 minutes.
Record results on report sheet.

Procedure 2 - Methyl Red, Voges-Proskauer Testing

1. Obtain two MR-VP broths and label as was done with the SIM tubes.
2. Using the same two cultures of *E. coli* and *E. cloacae*, inoculate the MR-VP tubes with three loops of the appropriate species for each tube.
3. Incubate the tubes at 35°C for a minimum of 48 hours.

Second session

1. Retrieve the inoculated MRVP tubes from the incubator and obtain four empty test tubes.
2. Label each of the tubes, two for each species (MR & VP) and obtain two 1 mL pipettes.
3. Pipette 1 mL from each culture into two appropriately labeled empty tubes. One tube will be used for the Voges-Proskauer test and the other for the Methyl Red test.
4. To each MR tube, add 8-10 drops of Methyl red reagent. If the medium turns red indicating acid production, then the test is positive. If the medium turns yellow or orange, there has been no acid produced and the test is negative.
5. To the VP tubes containing 1 mL of each species add 18 drops of VP reagent A (Bacti-drops) and then 6 drops of VP (Bacti-drops) reagent B.
6. Mix the organism periodically to add oxygen and record findings after 30 minutes. A red ring is considered positive, green or yellow or no ring is considered negative.
7. Record all findings on report sheet.
Procedure 3 - Simmons Citrate Testing

1. Obtain two Simmons Citrate agar slants.
2. Again using the same cultures used in procedures 1 and 2, inoculate the slants with one loop full of each organism to its appropriate tube. Be sure the tubes are labeled.
3. Incubate at 35°C for 48 hours.

Second session

1. Retrieve the Simmons Citrate slants and observe any color change that may have occurred.
2. If the medium is a bright royal blue, the test is positive for the use of citrate as the sole carbon source and the use of ammonium salts as the sole nitrogen source.
3. If there is growth, but no color change the test is negative.
4. Record all findings on the report sheet.
<table>
<thead>
<tr>
<th></th>
<th>Indole (SIM)</th>
<th>Methyl Red</th>
<th>V-P</th>
<th>Citrate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>K. pneumoniae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
LAB: ENTEROTUBE

Materials
(1) Culture of *Escherichia coli*
(1) Culture of *Klebsiella pneumoniae*
(1) Culture of *Enterobacter cloacae*
(1) Culture of *Pseudomonas aeruginosa*
BBL Enterotube II
Inoculating loop
Bunsen burner
Striker

Primary Objective
Innoculate, observe and record reactions of organisms using an Enterotube.

Other Objectives
Explain how the Enterotube can be used to identify an unknown organism.

Compare the manual testing done to date with the multi-test approach used by the Enterotube system.

Introduction
The Enterotube system is a tube which contains several compartments with various biochemical media and tests. The system is particularly useful in identifying Gram negative, enteric organisms.

(See product insert pages for a more thorough description of the Enterotube II.)

Procedure
See product insert page 4.

1. Did the test results match the organism you inoculated?
2. How did this procedure compare to the manual methods we’ve used in class?
LAB: ANTIBIOTIC SENSITIVITY TEST

Materials
(1) TSB culture of *Staphylococcus aureus*  
or  
(1) TSB culture of *Escherichia coli*  
(2) Mueller - Hinton agar plates  
(2) Sterile cotton-tipped applicators  
Vials containing antibiotic disks of the following:  
  - Ampicillin  
  - Bacitracin  
  - Chloramphenicol  
  - Erythromycin  
  - Kanamycin  
  - Levafloxacin  
  - Neomycin  
  - Penicillin G  
  - Streptomycin  
  - Tetracycline  
  - Triple Sulf

Primary Objective  
Observe and evaluate the effects of antibiotics on gram-negative and gram-positive vegetative bacterial cells, both endospore and non-endospore forming.

Other Objectives  
Define antibiotics (narrow-spectrum and broad-spectrum) and chemotherapeutic agents.  
Identify the 3 genera that produce the majority of antibiotics and at least one antibiotic produced by each.  
Explain how the majority of antibiotics accomplish selective toxicity.

Introduction
Chemicals that are used to treat disease are termed **chemotherapeutics**.  
**Antibiotics** are members of this group of chemicals. Antibiotics were originally produced through bacterial and fungal metabolic reactions and were named as such because they were found to stop or inhibit the growth and reproduction of microorganisms. There are two bacterial genera that produce most antibiotics - *Streptomyces* and *Bacillus*. The fungal genus *Penicillium* is responsible for many other antibiotics. Those chemotherapeutics that are produced by a chemical lab, like
sulfa drugs, are not considered antibiotics. However, with the advent of synthetic drug production and the manipulations that have occurred to those agents produced by living organisms, the line between antibiotics and those that are synthesized has blurred and the term antibiotic is used when dealing with a chemical that counteracts a biotic (life form).

**Chemotherapeutics and Sensitivity Testing**

Ehrlich, in the 1800's, discovered that chemotherapeutics were the only drugs that displayed selective toxicity and were therefore useable when treating disease in humans. In other words, the drug harmed the germs more than it harmed the patient. Normally this harm to the microbe comes through the chemotherapeutic blocking some biochemical pathway (i.e. Penicillin=s action on cell walls). Antibiotics can be categorized as broad-spectrum which denotes effectiveness against a wide range of bacteria or narrow-spectrum which indicates effectiveness against very few species.

The **Kirby-Bauer Antibiotic Disk Diffusion Test** is the most widely used test to observe the sensitivity of a pathogen to various chemotherapeutic agents. This is a standardized test that involves comparing the turbidity of a bacterial saline suspension with that of the McFarland No. 5 turbidity standard and then seeding Mueller-Hinton agar plates with the bacterial solution. After drying, disks containing a specific amount of antibiotic is placed on the agar and the plates are then incubated at 35°C for 16-18 hours. After incubation, zones of inhibition are measured and the diameter is compared to a standardized table and bacteria are grouped into three categories – ‘R’ Resistant, ‘S’ Sensitive (susceptible), ‘I’ Inconclusive. The table below gives the measurements in millimeters that should be used when checking for antibiotic sensitivity.

**Evaluation Table**

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Content</th>
<th>Zones of Inhibition Distances</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Resistant mm or less</td>
<td>Inconclusive mm</td>
</tr>
<tr>
<td>Ampicillin (gram-negative)</td>
<td>10g</td>
<td>14</td>
<td>15-16</td>
</tr>
<tr>
<td>Ampicillin (gram-positive)</td>
<td>10g</td>
<td>11</td>
<td>12-13</td>
</tr>
<tr>
<td>Bacitracin</td>
<td>10 units</td>
<td>20</td>
<td>21-28</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>30g</td>
<td>12</td>
<td>13-17</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>15g</td>
<td>13</td>
<td>14-17</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>30g</td>
<td>13</td>
<td>14-17</td>
</tr>
<tr>
<td>Neomycin</td>
<td>30g</td>
<td>12</td>
<td>13-16</td>
</tr>
<tr>
<td>Penicillin G (other microbes)</td>
<td>10 units</td>
<td>11</td>
<td>12-21</td>
</tr>
<tr>
<td>Penicillin G (staphylococci)</td>
<td>10 units</td>
<td>20</td>
<td>21-28</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>10g</td>
<td>11</td>
<td>12-14</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>30g</td>
<td>14</td>
<td>15-18</td>
</tr>
<tr>
<td>Triple Sulfa</td>
<td>300g</td>
<td>12</td>
<td>13-16</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>30g</td>
<td>9</td>
<td>10-11</td>
</tr>
</tbody>
</table>

This lab is a variation of the Kirby-Bauer method as there is no standard used aside from the comparisons of broth culture turbidity between species used.
Procedure
1. Obtain 2 Mueller-Hinton agar plates and label appropriately.
2. Spread one organism on each of the two plates specified for that species using the cotton-tipped applicators.
3. Place 5 antibiotic disks on one plate and 5 on the other making certain they are well spaced and that every two plates contains all 10 antibiotics (chemotherapeutics).
4. Place the 4 plates in the incubator at 35°C for 24 hours.

Second session
1. Measure the zones of inhibition on each plate and record findings.
2. Using the evaluation table, determine resistant, susceptible, or inconclusive for each species and each antibiotic.
3. Record the results on the report sheet.

Antibiotic Sensitivity Report

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Inhibition Zone</th>
<th>‘R’, ‘I’, or ‘S’</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. coli</td>
<td>S. aureus</td>
</tr>
<tr>
<td>Bacitracin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythromycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kanamycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Levafloxacin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neomycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptomycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triple Sulfa</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
LAB: ENZYMES WITH AMINO ACID SUBSTRATES

Materials
Stock culture of *Proteus vulgaris*
Stock culture of *Escherichia coli*
Stock culture of *Pseudomonas aeruginosa*
Stock culture of *Shigella boydii*
Triple Sugar Iron (TSI) agar slants
Lysine Decarboxylase broth tubes
Phenylalanine deaminase (PAD) agar slants
Inoculating loop
Bunsen burner, Striker
Ferric Chloride (FeCl) reagents

Primary Objective
Utilize, observe, and evaluate tests used for some enzymes that alter amino acids.

Other Objectives
Identify the functional groups that can be altered by carboxylases, desulfhydrases and deaminases.
Explain why ferrous sulfide is used to indicate production of hydrogen sulfide.
Explain why ferric chloride is used to indicate the production of phenylalanine deaminase.

Introduction
When identifying varying bacteria, knowing the specific enzymes that a species contains can be helpful. Facultatively anaerobic, gram-negative rods can often be identified by their ability to degrade amino acids. If amino acids are altered, then the exact groups of amino acids altered can be a good identification character. This lab examines three enzyme groups - desulfhydrases, carboxylases, and deaminases.

Amino Acid Degradation - Desulfhydrases, Carboxylases, and Deaminases
When studying the structure of an amino acid and the ability of an organism to break the structure apart, the researcher must look at the radical (R) group that is part of the amino acid. The R group is different for each of the 20 essential amino acids. **Desulfhydrases** deal with the removal of the sulfhydryl R groups (—SH), **decarboxylases** remove the carboxyl group (COOH), and **deaminases** remove the amine group (NH₂).
Cysteine desulphydrase removes both the sulphydryl group and the amine group from the amino acid cysteine. This removal results in the formation of pyruvic acid, hydrogen sulfide \((H_2S)\), and ammonia \((NH_2)\). While hydrogen sulfide will have the distinctive odor of rotten eggs, it also reacts with heavy metals. In the presence of a heavy metal, hydrogen sulfide will leave a black precipitate. Triple Sugar Iron agar is used when testing for cysteine desulphydrase. Once inoculated, if the triple sugar iron agar develops a black precipitate, \(H_2S\) is definitely present.

Triple Sugar Iron (TSI) also tests for the presence of sucrose &/or lactose in the slant and glucose in the butt. The default color is pink but the test is positive for sugar utilization if the pink changes to yellow due to a change in the pH.

Lysine decarboxylase is able to remove the carboxyl group from lysine. When the carboxyl group is removed, carbon dioxide \((CO_2)\) gas and an amine known as cadaverine is produced. The durham tube in the broth will indicate the presence of gas and the pH indicator dye present in the broth will indicate the formation of cadaverine. The response when cadaverine is formed is acidic.

If bacteria are able to remove an amine group, they contain a deaminase enzyme. Those that can remove the amine group from phenylalanine contain phenylalanine deaminase. The removal results in ammonia and phenylpyruvic acid. Phenylpyruvic acid turns green in the presence of ferric chloride. Phenylalanine agar contains phenylalanine and the addition of 12% ferric chloride and 0.1N HC1 will result in a green color change.

Procedure 1 - Triple Sugar Iron (TSI)

a. Obtain two Triple Sugar Iron agar slants.
b. Obtain one culture of *Proteus vulgaris* and one culture of *Pseudomonas aeruginosa*.
c. Stab and streak each slant and incubate at 35°C for 48 hours.

Second session

a. Examine the Triple Sugar Iron agar slants looking for acid production due to sugar usage and black precipitate indicating \(H_2S\) production.
b. Record findings for the slant/butt and \(H_2S\) on report sheet.

Procedure 2 - Lysine Decarboxylase Testing

a. Obtain two tubes of Lysine Decarboxylase broth.
b. Inoculate one tube with *S. boydii* and inoculate the other tube with *E. coli*
c. Overlay each tube with @ 2 mL of mineral oil.
d. Incubate at 35°C for 48 hours.

Second session

a. Check tubes* for gas formation and acid formation (pretty purple color).
b. Record findings on report sheet.
*This can be observed for four days every day and each finding recorded. Often the amount of time required for lysine decarboxylase to break lysine apart is an indicative character as well.
Procedure 3 - Phenylalanine Deaminase Testing (PAD)
   a. Obtain two Phenylalanine agar slants.
   b. Inoculate the two slants, one with *P. vulgaris* and the other with *E. coli*.
   c. Incubate tubes for 48 hours at 35°C.

   Second session
   a. Observe growth in each slant.
   b. Apply ferric chloride reagent
   c. After one to five minutes record any color changes (green color formation) and record on the report sheet.

Procedure 4 - Urease Testing
   a. Obtain two tubes of urease broth.
   b. Inoculate one tube with *K. oxytoca* and inoculate the other tube with *E. coli*.
   c. Incubate at 35°C for 48 hours.

   Second session
   a. Check tubes for color change. (If +, color should change from a slight peach to bright pink!)
   b. Record findings on report sheet.

Amino Acid Degradation Report Sheet

<table>
<thead>
<tr>
<th></th>
<th>Cysteine Desulphhydrase (TSI)</th>
<th>Lysine decarboxylase</th>
<th>Phenlyalanine deaminase (PAD)</th>
<th>Urease</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. vulgaris</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. boydii</em></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>P. vulgaris</em></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>
The following guidelines are for the final report summarizing the identification of your unknown. The report should be a minimum of one page in length. It should be typed, double spaced, and professional in appearance. Correct spelling and proper grammar are essential (spell checked)

The report is actually a step by step account of your identification process. It should include the following:

1) Genus & species of your unknown (10 points);
2) Gram reaction and morphology (5 points);
3) Description, step by step, of:
   a. The media you set up and the tests that you performed (15 points)
   b. Why you performed each step (15 points)
   c. What the results were (15 points) and
   d. How the results led to the identification of your unknown (20 points)
4) Medical and/or historical importance of your unknown organism (15 points) and
5) At least one reference besides the book or lab manual (5 points).

The primary objective of this unknown investigation is not the correct identification of the bacterium, but the process itself, however, the correct id is important as well (10% of your grade). You are providing evidence that you can successfully apply the lecture and laboratory experiences in a new situation. An interpretation of the previous statements might be, if you don’t know what the procedure determines or why you might want to use the procedure, DON’T DO IT!! Take the time to gather information and make a logical evidence-based decision.

Tabulate results similar to the Proteus mirabilis report on my web page. (There is an error in the explanation part in this report)