Principles of Microbiology Laboratory Manual

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Original Publication Year 2022

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Funding Statement

This material was funded by the Fund for the Improvement of Postsecondary Education (FIPSE) of the U.S. Department of Education for the Open Textbooks Pilot grant awarded to Middlesex College (Edison, NJ) for the Open Textbook Collaborative.

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BIO211 PRINCIPLES OF MICROBIOLOGY LABORATORY MANUAL

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BIO211 PRINCIPLES OF MICROBIOLOGY LABORATORY MANUAL

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**Module spans multiple weeks; an additional week is allocated for the practical exam*

MODULE 1: Introduction to the Microbiology Laboratory

When you hear the term *microbe*, what comes to mind? Many students think of “germs” such as bacteria and viruses. However, microbiology encompasses many other organisms, including archaea, yeasts, molds, protozoa, algae, slime molds, and even parasites and vectors. It is also a science that studies acellular entities such as viruses, viroids, and prions. What is common to these microbes is the inability of scientists to observe them without the aid of a microscope.

The *Principles of Microbiology* laboratory exercises focus primarily on the observation, cultivation, and identification of bacteria and common eukaryotic microbes such as fungi and protozoa. Over the course of the semester, you will learn various techniques for the safe handling and cultivation of microorganisms, as well as the skills and good practices necessary for working confidently in any biology laboratory.

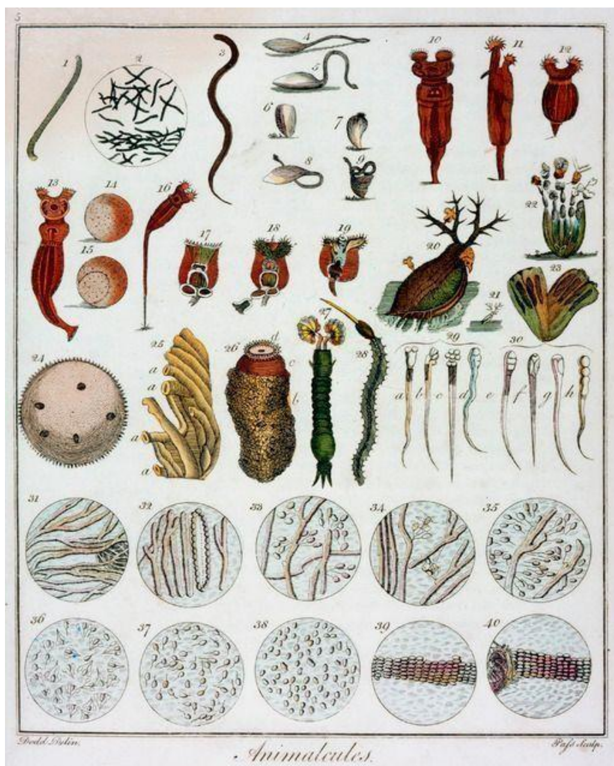


Figure 1.1: An artistic rendering from 1795 of the first observations of microorganisms or “animalcules” by Antonie van Leeuwenhoek, a self-taught scientist and pioneer in microscopy.

Middlesex College Culture Code

All stock cultures at Middlesex College are assigned a unique number. These numbers are an easy way to label tubes and plates to identify the microorganisms used in lab exercises. When completing reports, scientific names rather than culture code numbers should be used.

Just as you have two names, scientists use *binomial nomenclature* when referring to organisms. These names are based on taxonomic hierarchy where the first name is the *genus* (plural, genera) and the second is the *species*. For example, the binomial name of humans is *Homo sapiens.*

Whenever a binomial name is first used, the genus and species names should be written out in full . After this, the genus may be abbreviated by a letter, but the species is never abbreviated.

First use:	*Escherichia coli*
Subsequent use:	*E. coli*

Since multiple species may exist within a given genus, microbiologists often only use the genus, e.g., *Pseudomonas,* or the genus followed by the abbreviation "sp." or "spp." to designate any species. For example, *Pseudomonas* sp. might refer to *Pseudomonas aeruginosa, Pseudomonas fluorescens,* or another species of *Pseudomonas* entirely.

Conventional rules exist for typing or handwriting a scientific name. When typing, both genus and species are italicized but not underlined. When handwritten, both names are underlined:

When typing:	*Staphylococcus aureus*
When handwriting:	<u>Staphylococcus aureus</u>

Correctly formatting names, whether in a report or on a patient's chart, is good lab practice and avoids confusion that can lead to error. This may occur when a word that is used as a general descriptor is also a genus name (think of a person named Ms. Tall, who may or may not be tall). In microbiology, *Bacillus* is a good example. When written as a lower-case term, "bacillus" means a rod-shaped bacterial cell that is characteristic of *many* bacterial genera. Thus, while cells of the genera *Escherichia* and *Pseudomonas* are also rod-shaped bacteria, *Bacillus* refers to a <u>specific</u> genus of rod-shaped bacteria when formatted as such.

MICROBIOLOGICAL CULTURE CODE

The following is a list of the microorganisms used by the Biology Department at MCC. As a time saving device, the cultures have been assigned the following numbers:

1. Escherichia coli

2. Staphylococcus aureus

3. Staphylococcus epidermidis

4. Bacillus subtilis

5. Bacillus megaterium

6. Serratia marcescens

7. Micrococcus luteus

8. Pseudomonas aeruginosa

9. Klebsiella aerogenes

10. Streptococcus salivarius

11. Enterococcus faecalis

12. Alcaligenes faecalis

13. Proteus vulgaris

14. Saccharomyces cerevisiae

15. Streptococcus agalactiae

16. Clostridium sporogenes

19. Mycobacterium smegmatis

20. Bacillus cereus

21. Morganella morganii

22. Proteus mirabilis

23. Rhodospirillum rubrum

24. Micrococcus roseus

25. Lactococcus lactis

26. Aspergillus niger

27. Penicillium notatum

28. Agrobacterium tumefaciens

29A. Rhizopus stolonifer +

29B. Rhizopus stolonifer –

30. Chromobacterium violaceum

31. Moraxella catarrhalis

32. Escherichia coli MM294

33. Klebsiella pneumoniae

34. Enterococcus faecium

35. Geobacillus stearothermophilus

36. Salmonella typhimurium (Ames Strain)

37. Citrobacter freundii

38. Acinetobacter calcoaceticus

39. Halobacterium salinarum

40. Escherichia coli B

41. Pseudomonas fluorescens

42. Streptomyces griseus

43. Streptomyces epidermidis

44. Streptomyces venezuelae

47. Neisseria perflava

Exercise 1.1 – Safety in the Microbiology Laboratory

LEARNING OUTCOMES

1. Identify best practices and safety regulations for the microbiology laboratory.
2. List and compare the four biological safety levels.
3. Describe proper disposal of biological and non-biological laboratory waste.

You probably have taken a biology or chemistry course and are already familiar with good laboratory practices and safety protocols. The microbiology lab is a bit different because there are strict guidelines for handling and disposing of live organisms.

In the United States, multiple organizations on the local, state, and federal level work together to monitor and ensure laboratory safety. The Centers for Disease Control and Prevention (CDC) provide guidance to clinical, research, public health, and educational laboratories that handle potentially infectious agents, such as viruses and bacteria, that can spread among hosts.

Biological Safety Levels

The CDC has established four biological safety levels (BSL) to classify microorganisms based on each agent's infectivity, ease of transmission, and potential disease severity, as well as the type of work being done with the agent (Figure 1.2). Each BSL requires a different level of biocontainment to prevent contamination and spread of infectious agents to laboratory personnel and, ultimately, the community.

BSL-1 requires the fewest precautions because it applies to situations with the lowest risk for microbial infection in healthy adults. These include nonpathogenic strains of *Escherichia coli* and environmental bacteria such as *Bacillus subtilis*. Laboratory workers may work with these agents at an open laboratory bench wearing personal protective equipment (PPE) such as a laboratory coat, goggles, and gloves, as needed.

Agents classified as BSL-2 include those that include organisms that may be potentially pathogenic or infectious, and particularly those that may aerosolize or disperse through the air. Working with BSL-2 bacteria such as *Staphylococcus aureus* and *Streptococcus pyogenes*, or viruses like hepatitis, mumps, and measles, require additional precautions beyond those of BSL-1, including restricted access to the laboratory, required PPE, and the use of biological safety cabinets for certain organisms.

BSL-3 agents have the potential to cause lethal infections by inhalation. These may include pathogens such as *Mycobacterium tuberculosis*, *Bacillus anthracis*, human immunodeficiency virus (HIV), and coronavirus (SARS-CoV-2). Because of the serious nature of the infections caused by BSL-3 agents, laboratories working with them require restricted access and are equipped with directional airflow which cannot be recirculated. Laboratory personnel always wear a respirator and handle microbes in a biological safety cabinet.

BSL-4 agents are the most dangerous and often fatal. These microbes are easily transmitted by inhalation and cause infections for which there are no treatments or vaccinations. Examples include Ebola and smallpox viruses. There are only a small number of laboratories in the United States and around the world appropriately equipped to work with these agents.

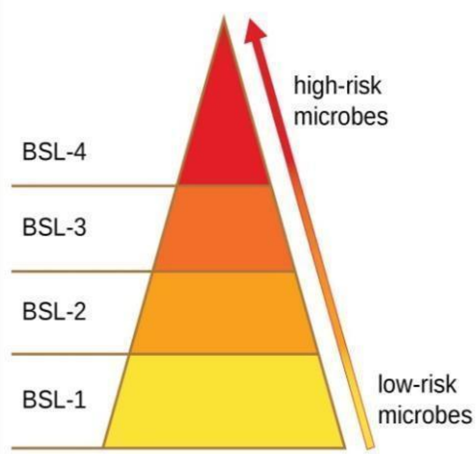
Biosafety Levels			
Biological Safety Levels	**Description**	**Examples**	**CDC Classification**
BSL-4	Microbes are dangerous and exotic, posing a high risk of aerosol-transmitted infections, which are frequently fatal without treatment or vaccines. Few labs are at this level.	Ebola and Marburg viruses	
BSL-3	Microbes are indigenous or exotic and cause serious or potentially lethal diseases through respiratory transmission.	*Mycobacterium tuberculosis*	
BSL-2	Microbes are typically indigenous and are associated with diseases of varying severity. They pose moderate risk to workers and the environment.	*Staphylococcus aureus*	
BSL-1	Microbes are not known to cause disease in healthy hosts and pose minimal risk to workers and the environment.	Nonpathogenic strains of *Escherichia coli*	

Figure 1.2: The CDC classifies infectious agents into four biosafety levels based on potential risk to laboratory personnel and the community. Each level requires a progressively greater level of precaution.

Best Practices in the Microbiology Laboratory

Departmental safety regulations will be reviewed during the first lab period. Your instructor will provide specific instructions each week for working with microorganisms, but some general points below are relevant for all microbiology sections. Please read them carefully.

1. Live bacteria, fungi, and protists in concentrated numbers are used for most exercises. Although these microorganisms are typically found in the environment or our bodies, all should be treated as potential pathogens and handled with care.

2. Wear suitable clothing and shoes for working in a laboratory and always use appropriate personal protective equipment when working with cultures.

3. Wash your hands and change gloves frequently and keep pens/pencils and fingers away from your mouth and face.

4. Disinfect the top of your work area before and after lab, wiping the back of your chair and any personal items used during lab such as your notebook, laptop, etc., as well.

5. Never pour anything in the sink or place in the regular lab trash without approval. Microbial cultures and other biological waste must be sterilized by autoclaving prior to disposal. Your instructor will provide specific instructions for clean-up each week.

6. Food containers, beverage bottles, gum, lip balm, and cell phones are all things that are used in or near your mouth. These can become contaminated with microbes and therefore are not permitted in the lab at any time.

7. For most exercises, an item that generates intense heat or an open flame is used. Never let go of a metal inoculating loop while using the incinerator and never leave an open flame unattended. Keep electrical cords from contact with hot plates and incinerators.

8. Notify your instructor immediately of any safety incident no matter how minor. Never attempt to clean up a culture spill or broken glass on your own; there are special protocols for these types of accidents that must be followed.

9. The caps of test tubes that contain bacteria are often loose, so be careful when picking up tubes and always use a test tube rack.

10. Most materials are sterile and must be kept free from contamination, so keep in mind that when an item is opened it is no longer sterile. Do not return any used or contaminated items to a rack or container with sterile materials. Likewise, do not place used pipettes or swabs back in their wrapper or in the lab trash bin.

11. Finally, always leave your lab bench clean and neat for the student who follows you.

Microbiology Laboratory Best Practices
Always wear appropriate personal protective equipment
Disinfect bench before and after lab
Have proper attire and tie hair back
Wash hands frequently
Keep cell phones away
Only necessary materials on bench top
No food, drinks, or gum in lab at any time
Follow safe disposal guidelines for all materials, cultures, and chemicals
Notify instructor of any accident immediately, no matter how minor


Exercise 1.2 – Night on the Town

LEARNING OUTCOMES

1. Describe the relationship between epidemiology and public health.
2. Identify public health agencies at the local, national, and international levels.
3. Discuss the role of contact tracing in the prevention of disease transmission.

The science of *epidemiology* is the study of disease occurrence and distribution in a defined population. Epidemiologists investigate the etiology (cause), incidence (number of new cases), prevalence (number of infected persons), and transmission of diseases with the goal of understanding and controlling transmission. The population that is at risk may be geographically defined or may be identified by other parameters such as susceptibility due to age, environment, health or nutritional status, lifestyle choices, or other related factors.

Many agencies and organizations participate in reporting and analyzing epidemiological data to keep the public safe. At the local and state level, public health officials work closely with hospitals, clinics, and medical providers when a new disease or outbreak occurs. In the United States, the Centers for Disease Control and Prevention (CDC) and the National Institutes of Health (NIH) provide information and public health guidance at the national level. Internationally, the World Health Organization (WHO) is responsible for research and oversight of health and disease on a global scale.

One essential way in which health authorities monitor disease transmission is through *contact tracing.* Contact tracing is the identification, assessment, and monitoring of infected individuals to break the chain of transmission and control the spread of disease. Contact tracing relies on information provided by infected persons regarding where they have been and with whom they had close contact. This information is kept confidential and shared only as necessary to prevent further infection.

In this exercise, the spread of disease among a population (the class) and the challenges of contact tracing are simulated. Each student will receive a test tube with water, with one tube containing the "pathogen" (sodium hydroxide solution [NaOH]; only the instructor will know who was given this tube). Students will be asked to mingle and talk for brief periods, exchanging solutions with at least two other students. At the end of the exercise, each sample will be tested for the presence of NaOH and those students who receive positive tests will be asked to recall who they exchanged solutions with and in what order. Students will then have the task of determining the original source of the outbreak from the contact tracing information.

Exercise 1.2 – Night on the Town

OBJECTIVE

Determine the source of a simulated disease outbreak.

MATERIALS

- SOLUTIONS: 0.1M NaOH (1 tube); sterile water (remaining tubes); phenolphthalein
- EQUIPMENT: Disposable plastic Pasteur pipets

PROCEDURE – STUDENTS WORK AS A CLASS

1. Each student receives a solution tube and pipet. Only the instructor will know which student receives the tube of NaOH.
2. The mingling period begins as students move about the room, socializing with others and exchanging fluids with at least two other students using the pipet. When the mingling period is over, pipets are placed in the disinfectant beaker.
3. Students bring their tubes to the “clinic” (instructor) for testing with phenolphthalein, which will react with the “pathogen” (NaOH) to turn the solution pink. Those who receive positive tests record who they exchanged solutions with, and in what order, for contact tracing data.
4. Data is analyzed to determine which student received the contaminated tube.

Exercise 1.3 – Hand Hygiene

LEARNING OUTCOMES

1. Discuss the importance of hand hygiene.
2. Describe proper handwashing technique.

Practicing good hand hygiene is important both in and outside of a health care setting. Clean hands reduce the transmission of germs to others and prevent the spread of infection. The CDC reports that each day, at least one healthcare-related infection is spread for every 31 patients.[1] Many of these infections are caused by bacteria that are resistant to treatment with multiple drugs, which has resulted in a global effort to combat antibiotic resistance.

Although microbiology teaching labs are relatively safe, they are not without potential risk of infection. Between August 2010 and June 2011, a *Salmonella* outbreak resulted in multiple hospitalizations and one death. Infected individuals were from 38 states and ranged in age from one to 91 years. The incident was eventually traced back to three students taking a community college microbiology course who had been working with the outbreak strain of bacteria.[2]

The most effective ways to ensure hand hygiene are handwashing with soap and water or using an alcohol-based hand sanitizer or similar antiseptic. When using soap and water, hands should be washed for at least 20 seconds by first wetting and then lathering with soap. Fingernails harbor greater numbers of microorganisms relative to their length and are often overlooked when scrubbing. Hands should be rinsed and dried with a clean towel or air dryer. When soap and water are not available, hand sanitizer that contains at least 60% alcohol can be used. Since most people tend to wash their hands with the same pattern, a nontoxic fluorescent product called GloGerm™ will be used in this exercise to visualize washing efficacy.

References

1. United States, Department of Health and Human Services, Centers for Disease Control and Prevention. "Hand Hygiene in Healthcare Settings." *Centers for Disease Control and Prevention*, 2019, www.cdc.gov/handhygiene/.

2. United States, Department of Health and Human Services, Centers for Disease Control and Prevention. "Salmonella Typhimurium Infections Associated with Lab Exposure." *Centers for Disease Control and Prevention*, 2017, www.cdc.gov/salmonella/typhimurium-07-17/index.html.

Exercise 1.3 – Hand Hygiene

OBJECTIVE

Determine personal handwashing pattern.

MATERIALS

- SOLUTIONS: GloGerm™ nontoxic fluorescent mineral oil; soap and water
- EQUIPMENT: Ultraviolet lamp

PROCEDURE – STUDENTS WORK INDIVIDUALLY

1. Begin with unwashed hands.
2. Add a few drops of GloGerm™ to your palm and rub in as you would lotion.
3. Wash and dry your hands at the sink NORMALLY, without removing watches or jewelry. It is tempting to over-wash, but this defeats the purpose of the exercise!
4. **Safety glasses must be worn for this step.** Shine a UV lamp over your hands to check for areas where you missed washing, which will fluoresce.

Figure 1.3. Areas of the hands where GloGerm remains will fluoresce under ultraviolet light.

BIO 211 MODULE 1 REPORT **NAME:**______________________________________
LABORATORY SAFETY **REPORT DATE:**________________________________

EXERCISE 1.1 – LABORATORY SAFETY

1. Describe appropriate attire for working in a biological laboratory:________________________

2. What two things should you always do before and after working lab class?
   - ____
   - ____

3. The Middlesex College microbiology laboratory is BSL-________. How are BSL-2 organisms different from those that are designated BSL-1?_______________________________________

4. Explain how you should properly dispose of these items at the end of lab:
   - Plastic Petri plate:____
   - Glass slide:____
   - Used gloves:____
   - Cotton swab:____
   - Paper towels:____

5. Circle the items that are permitted to be out during microbiology lab:

Lab manual	Cell phone
Laptop	Chewing gum
Closed beverage bottle	Notebook

BIO 211 MODULE 1 REPORT **NAME:**___________________________________

EXERCISE 1.2 – NIGHT ON THE TOWN

OBSERVATIONS – Pool data as a class.

STUDENT NAME	INFECTED (Y/N)	CONTACT TRACING (NAMES AND ORDER OF PARTNERS*)

**If you cannot remember a name, describe something about that person*

BIO 211 MODULE 1 REPORT **NAME:**______________________________________

QUESTIONS FOR REVIEW

1. Who carried the pathogen?____

2. What approach did you or your group use to determine the carrier?______________________

3. Name 3 factors that might influence the validity of the reported epidemiological data:

   a) ____

   b) ____

   c) ____

4. Give the full name and function of each of the following

   agencies: <u>CDC</u>

   Full name:____

   Function:____

   <u>WHO</u>

   Full name:____

   Function:____

   <u>NIH</u>

   Full name:____

   Function:____

Module 2 Introduction to Culture Techniques

LEARNING OUTCOMES

1. Identify and compare forms of solid and liquid media.
2. Define colony and colony forming unit.
3. Describe growth patterns in liquid and solid media using appropriate terminology.

Culture Media

In nature, microorganisms exist as mixed populations of distinct species of bacteria, fungi, and even viruses. To study, characterize, and identify microorganisms, it is often necessary to cultivate them at a preferred temperature using a nutrient base called a *medium* (plural, *media*). Two commonly used physical forms of growth media are in the form of liquid broth and semisolid agar.

Broth can be used to determine growth patterns and are the medium of choice for growing large quantities of organisms. Semisolid agar is essentially broth with the addition of a polysaccharide thickening agent derived from red algae called agarose. Agarose is an effective solidifying agent because it withstands the high temperature needed for sterilization of the medium and is not broken down by bacteria. Forms of semisolid media include agar plates, agar slants, and agar deeps (Figure 2.1). Agar plates are made by pouring melted media into a Petri dish and allowing it to cool. Plates can be used to separate mixtures of bacteria and to observe colony characteristics of different species of bacteria. To make agar slants or agar deeps, melted agar is poured into a test tube and then allowed to solidify on an angle (agar slant), or vertically (agar deep). Agar slants are commonly used to generate stocks of bacteria, while deeps are often used to observe bacterial motility.

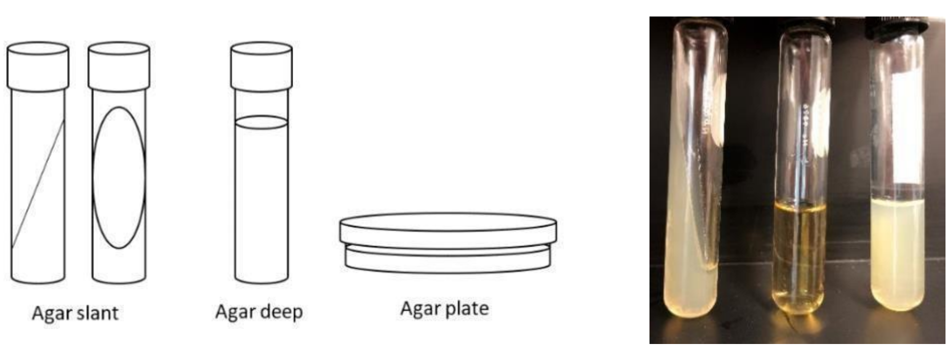


Figure 2.1: Forms of solid media (left); nutrient agar slant, broth, and deep (right).

Although individual bacteria cells are too small to be viewed with the naked eye, microorganisms form certain patterns of growth that are easily observed when they grow on or in media. These distinguishing characteristics help us to differentiate and identify organisms that are present. Figure 2.2 illustrates common growth patterns in liquid and on solid media.

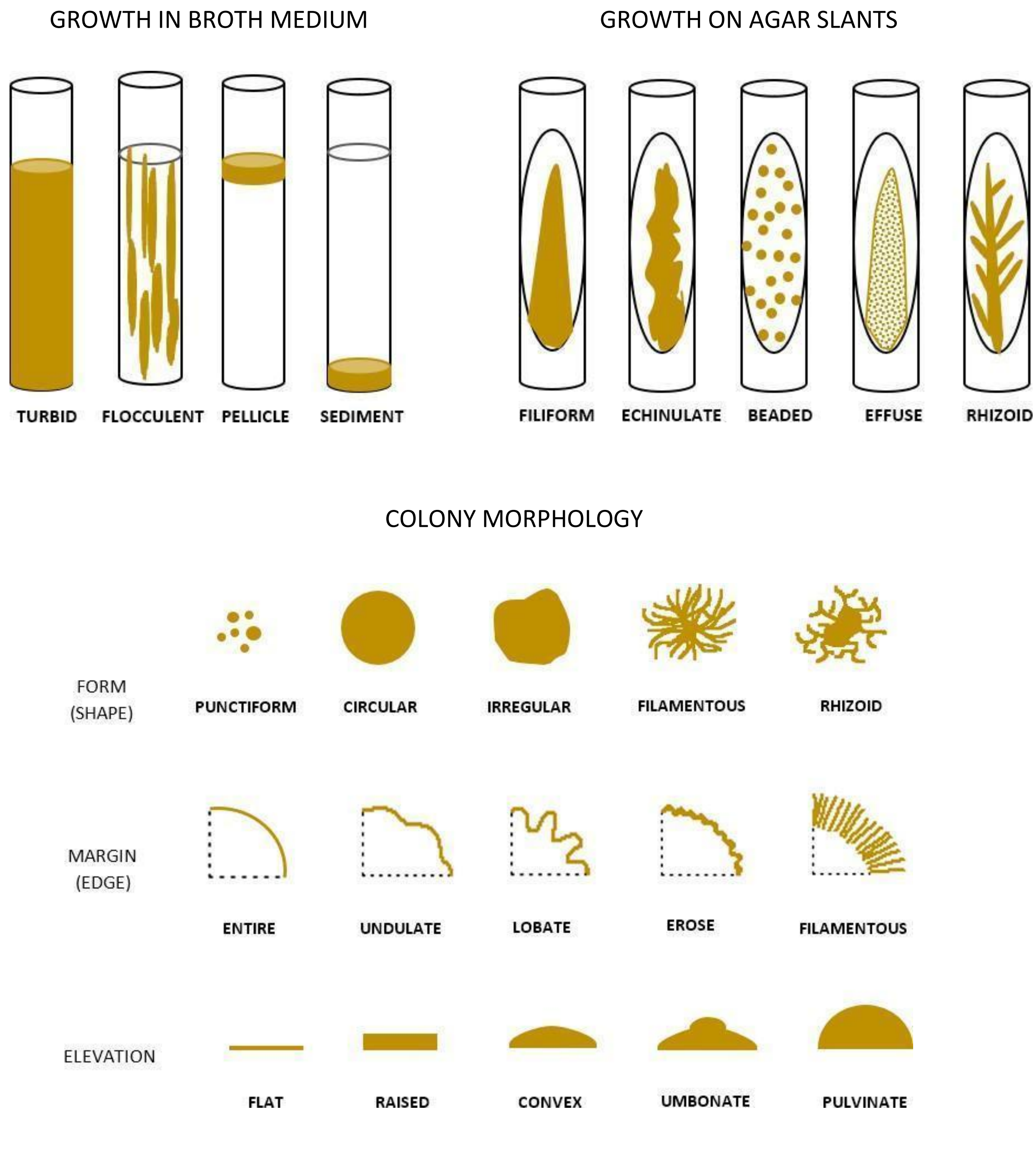


Figure 2.2: Patterns of growth in liquid and semi-solid media.

Growth Patterns in Liquid Media

Growth patterns in broth should be observed and evaluated without disrupting or shaking the tube. Many bacteria exhibit uniform *turbidity* (cloudiness) throughout the broth while others form a *sediment* at the bottom. In the latter case, the broth may be slightly turbid or clear. Some bacteria grow as a *pellicle* or film on the surface of the broth or form a ring around it. Heavy pellicles may sink a bit during incubation and appear just below the surface of the broth. Bacteria may also exhibit *flocculence,* or discrete clusters of growth, which are suspended in clear broth throughout the tube (Figure 2.3).

It is important to note that trypticase soy broth is not a reduced medium, meaning that oxygen has not been removed from the broth by chemical or other means. Since oxygen is present throughout the tube, growth patterns in non-reduced broth are not necessarily indicative of an organism's preference for oxygen. Later in the course, we will test bacteria for aerotolerance using several methods, including the use of reduced broth media.

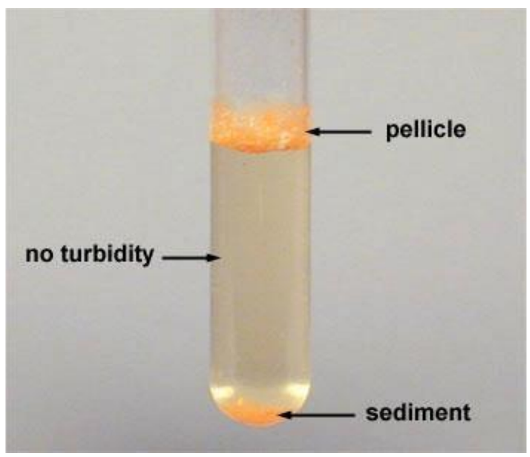


Figure 2.3. Patterns of bacterial growth in liquid media.

Growth Patterns on Semi-Solid Media

Bacteria grow on an agar surface as visible masses of cells called *colonies*. Each colony is composed of thousands to millions of cells that originated from a single bacterium or small group of bacterial cells called a *colony forming unit (CFU).* Colonies have distinguishing features including the pigment or color, the overall shape or form, the elevation of colonies when viewed from the side, the *margin* or edge, and surface texture. Evaluation of colony morphology is often subjective, which is why more than one descriptor is used (Figure 2.4).

Similar growth characteristics to those observed on plates can be seen on slants. There are additional slant characteristics that experienced microbiologists use to evaluate growth. Those observed on a slant.

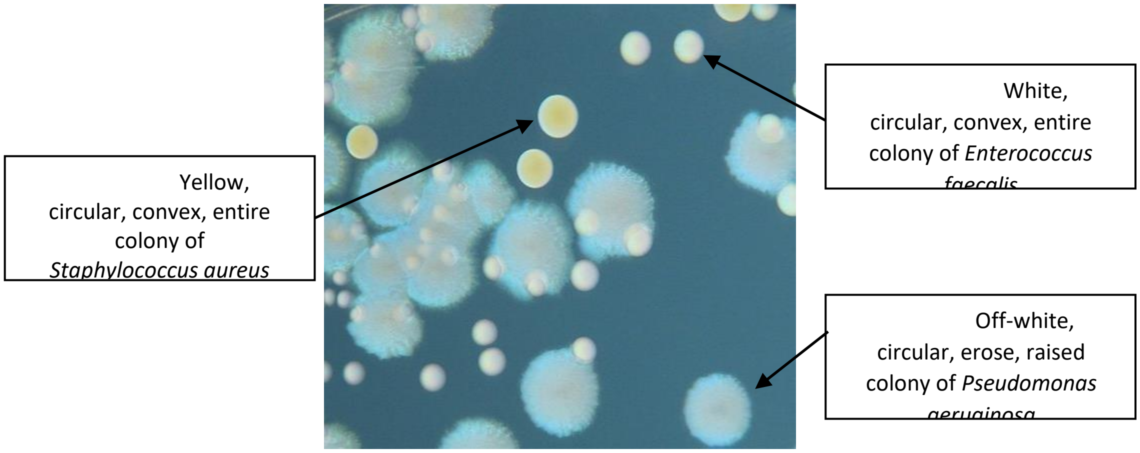


Figure 2.4: Three different colony types growing on trypticase soy agar. Colony descriptors include those for form (overall shape), elevation, and margin (edge). Colony pigment and surface texture (e.g., smooth, shiny, rough) is often evaluated as well

Exercise 2.1 – Microbial Ubiquity

LEARNING OUTCOMES

1. Discuss microbial ubiquity and factors that influence growth.
2. Name two types of all-purpose growth media.
3. State the specific temperature in Celsius for room and body incubation.
4. Properly label and prepare a Petri plate for incubation.

Microorganisms are ubiquitous, meaning that they are found almost everywhere. They are present in the air, in the environment, on and in our bodies, and may contaminate surfaces of inanimate objects or *fomites.* In fact, life on Earth would be impossible without microbes! They decompose and recycle nutrients, carry out photosynthesis and produce oxygen, and fix atmospheric nitrogen in usable form for synthesizing essential biomolecules such as proteins and nucleic acids. Microbes that exist as part of the human microbiome play a significant role in maintaining our health and protecting us from disease. Recent research has supported the hypothesis that the role of organisms in our microbiome also may have profound influence on our metabolism and behavior.

In this exercise, various surfaces are investigated for the presence of microbes. Although the procedure is simple, several important factors must be considered to ensure proper results. All materials used for the procedure must be *sterile,* or free from any microorganisms, to ensure that the only organisms recovered are those on the surface being sampled. The media used to cultivate microorganisms must also contain sufficient nutrients to support growth. Two common types of media used in the microbiology laboratory are nutrient agar (NA) and trypticase soy agar (TSA). Both provide a semi-solid surface on which many bacteria and fungi can grow.

Since the growth requirements of one organism may differ from another, the time and temperature of incubation are also factors. Organisms that are present in the environment typically grow optimally at room temperature (25^{o}C) incubation, while those associated with your microbiome prefer body temperature (37^{o}C). Most of these bacteria produce *colonies*, or visible masses of growth, on the agar surface within 18-24 hours. Other microorganisms, such as fungi, may require several days or even weeks to appear.

Exercise 2.1 – Microbial Ubiquity

OBJECTIVE

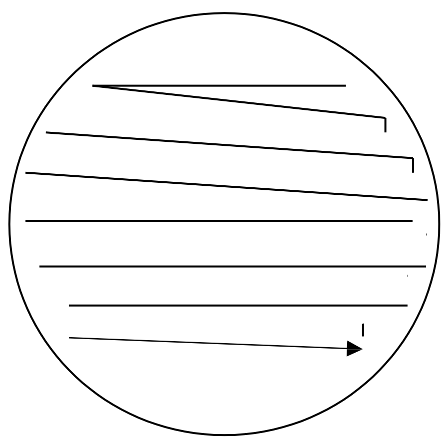
Sample a living or nonliving surface to observe different microorganisms that may be present.

MATERIALS

- MEDIA: Nutrient or trypticase soy agar plate
- SOLUTIONS: Sterile water
- EQUIPMENT: Sterile swab, marking pen, test tube rack

PROCEDURE – STUDENTS WORK INDIVIDUALLY

1. Label the bottom of a sterile agar plate with your initials, date, and what is being sampled.
2. Dip a swab in sterile water to moisten, then rub on a fomite or a specific area of your body.
3. Roll the swab over the agar surface using a tight Z pattern as shown below.
4. Dispose of the used swab in the disinfectant beaker – do not return to wrapper (why not?)
5. Invert plates and place them in a common rack for incubation.
6. Incubate for 18-24 hours at 25°C for fomite sample or 37°C for body site sample.
7. Observe results and complete the lab report.



Swab in a tight Z-pattern on the agar surface, being careful not to break the agar.

Exercise 2.2 – Aseptic Transfer

LEARNING OUTCOMES

1. Explain the importance of working aseptically when handling microorganisms.
2. Use aseptic technique to inoculate solid and liquid media from a bacterial culture.

Whenever it is necessary to transfer growing organisms to a sterile medium, microbiologists use a method called *aseptic technique*. Aseptic technique prevents the introduction of unwanted contaminants and is good lab practice for handling bacteria and other microbes in the lab.

In this exercise, you will practice using aseptic technique to transfer two microorganisms, *Escherichia coli* and *Staphylococcus aureus*, between various forms of liquid and solid media (Figure 2.5). *E. coli* is a predominant member of the enteric, or intestinal, microbiome of humans and animals. While many species of *Staphylococcus* are typically present on the skin and mucous membranes, particularly the respiratory tract, *S. aureus* is only carried by some people. It is important to note, however, that both *E. coli* and *S. aureus* are potential pathogens and frequently implicated in infections, particularly those that are healthcare associated.

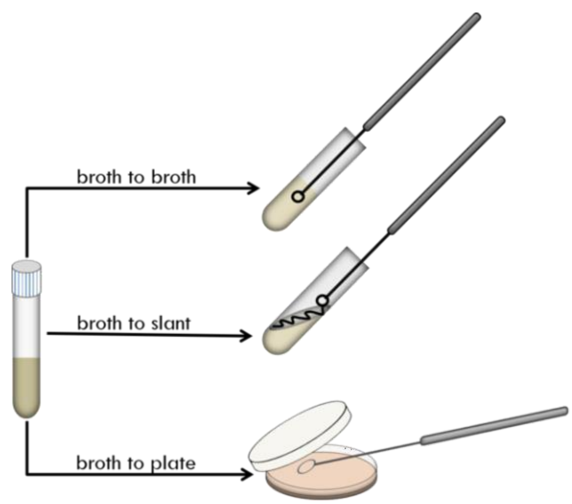


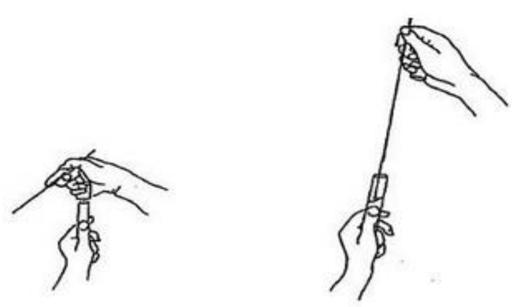
Figure 2.5: Transferring bacteria from a broth culture to liquid and semisolid media.

Exercise 2.2 – Aseptic Transfer

General Procedure for Aseptic Transfer

Note: Aseptic transfer is done without the help of a partner, so always close one tube before opening another. By doing this, you will always have a free hand with which to work.

1. Sterilize the inoculating loop (for broth cultures) or needle (for agar cultures) by inserting the wire in the incinerator for about ten seconds until it turns bright orange, then removing it. **To avoid a serious burn, never leave the loop unattended in the incinerator.**
2. Pick up the bacterial culture tube with your free hand and wrap the pinky of the hand holding the loop around the cap (Figure 2.6a). Remove the cap by turning the tube rather than the cap, always keeping the cap in your pinky (Figure 2.6b).
3. Heat the mouth of the culture tube by holding it against the incinerator opening and rotating it once, keeping the tube upright.
4. Obtain bacteria by dipping the loop just once in broth or lightly touching the needle to bacteria growing on a semisolid surface.
5. Reheat the mouth of the culture tube and recap it, turning the tube rather than the cap, then return the tube to the rack so that you have a free hand.
6. Pick up and carefully uncap the sterile media tube using the pinky of the hand holding the inoculating loop or needle with bacteria.
7. Heat the mouth of the sterile media tube by turning it against the incinerator opening.
8. Inoculate the sterile media tube by dipping the loop once into broth or by spreading the needle on the surface of an agar slant, being careful not to cut into the agar.
9. Reheat the mouth of the sterile media tube and recap it.
10. Sterilize the loop by placing it in the incinerator for 10 seconds.



(a) (b)

Figure 2.6: (a) Aseptically obtaining bacteria; (b) The cap always remains in the hand holding the loop.

Exercise 2.2 – Aseptic Transfer

OBJECTIVE

Practice aseptic transfer technique using agar slants and broths.

MATERIALS

- MEDIA: Trypticase soy agar slant and broth (2 each)
- SOLUTIONS: Sterile water
- CULTURES: *Escherichia coli* slant, *Staphylococcus aureus* broth
- EQUIPMENT: Inoculating loop and needle, incinerator, vortex, marking pen, labeling tape, test tube rack

NOTE: Loops are intended for transferring bacteria that are growing in broth while needles are used for bacteria on solid media. However, when first practicing aseptic transfer, students are often more comfortable using a loop rather than a needle for obtaining bacteria.

PROCEDURE – STUDENTS WORK INDIVIDUALLY

1. Practice with a tube of sterile water before working with bacteria.
2. When you are ready, label one sterile slant and one sterile broth tube for each organism using small pieces of labeling tape with your initials, date, and organism number and placing the tape on the glass portion of the tubes near the cap; do not write directly on tubes.
3. Tighten the cap of the *S. aureus* broth culture and vortex the tube briefly to mix.
4. Using aseptic technique, transfer a loopful of *S. aureus* to a sterile tube of broth and finger-tightening the cap to close.
5. Beginning anew, use aseptic technique to transfer a loopful of *S. aureus* to a sterile slant by dragging the loop up on the agar and finger-tightening the cap to close.
6. Repeat steps 4 through 6 to transfer *E. coli* to a sterile broth tube and agar slant.
7. Place the four inoculated tubes in a common rack for incubation at 37°C for 18-24 hours.
8. Place the two bacterial cultures and water tube in a common discard rack for autoclaving.
9. Following incubation, observe growth patterns and complete the report.

Exercise 2.3 – Isolation Streak Plate

LEARNING OUTCOMES

1. State the purpose and principle of an isolation streak plate.
2. Identify factors contributing to a poor isolation streak plate.

In microbiology, isolation streaking is a technique used to separate organisms in a mixed sample. The procedure is done by spreading the sample over several sections of an agar surface using an inoculating loop or needle. Following incubation, a colony of interest is picked to create *a subculture* by repeating the process on a sterile agar plate. Colonies on the subculture plate are identical, thus providing a pure culture for further identification and testing.

In this exercise, you will prepare a *quadrant streak plate* to separate two bacteria, *Serratia marcescens* and *Micrococcus luteus,* growing together in broth medium. Both organisms are members of the human microbiome and grow at 37^{o}C. However, while *Micrococcus* colonies appear bright yellow at all temperatures, *Serratia* colonies are red only at lower temperatures. Incubating the streak plate at 25^{o}C rather than at 37^{o}C results in colonies of two different colors, yellow and red (Figure 2.7). This contrast distinguishes or differentiates organisms in a mixed sample and demonstrates if successful isolation of each organism was achieved.

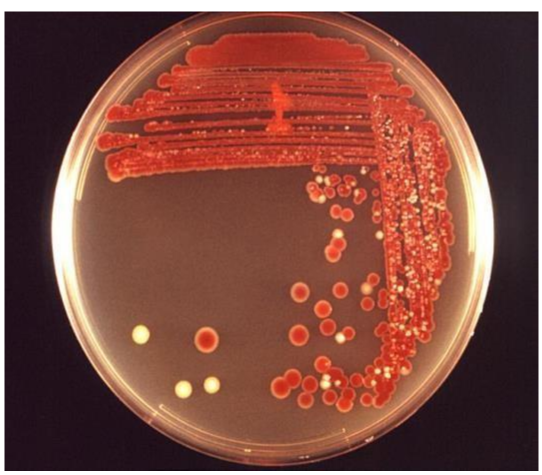


Figure 2.7: Isolation streak plate prepared from a mix of Serratia marcescens (red colonies) and Micrococcus luteus (yellow colonies) following incubation at 25^{o}C.

Exercise 2.3 – Isolation Streak Plate

OBJECTIVE

Isolate bacteria from a mixed culture using the quadrant streak method.

MATERIALS – STUDENTS WORK INDIVIDUALLY

- EQUIPMENT: Inoculating loop, incinerator, vortex, marking pen, labeling tape
- MEDIA: Trypticase soy agar plate
- CULTURES: Mix of *Escherichia coli* and *Serratia marcescens*

PROCEDURE

1. Using a marker, label the bottom of each plate with your initials, date, and culture mix.
2. Vortex the mix and aseptically obtain a loopful of broth.
3. Heat and close the culture tube before opening the plate.
4. Inoculate the first quadrant by streaking the agar as shown in Figure 2.8.
5. Close the plate and sterilize the loop, allowing it to cool for at least 5-10 seconds.
6. Rotate the plate 1/4 turn and continue the streak into the next quadrant, going back into the first quadrant with the loop several times and then continuing the streak. This separates cells to form isolated colonies.
7. Close the plate and heat the loop, allowing it to cool for at least 5-10 seconds.
8. Rotate the plate another 1/4 turn and continue the streak into the third quadrant.
9. Do not heat the loop. Rotate the plate a final 1/4 turn and streak the last quadrant, bringing the loop into the center of the plate while avoiding touching the first quadrant.
10. Invert the plate and place it in the common rack for incubation at room temperature.

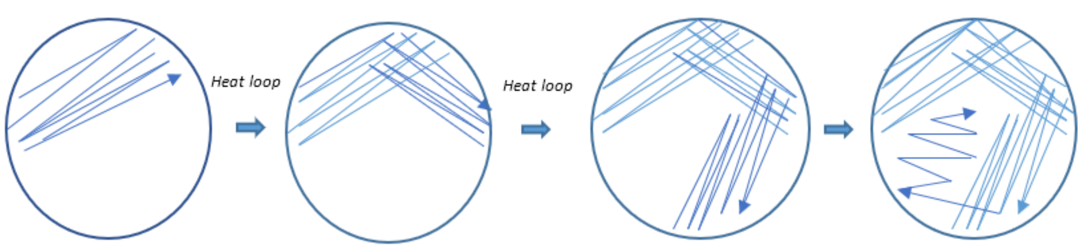


Figure 2.8: Quadrant streak plate technique.

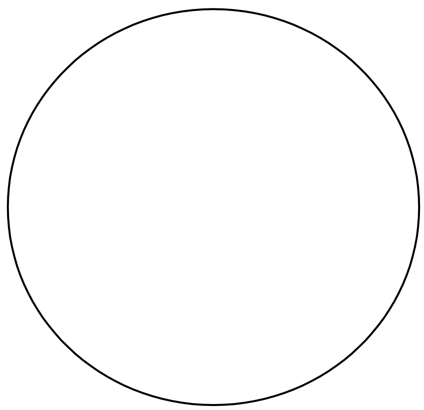
BIO 211 MODULE 2 REPORT **NAME:**____
CULTURE TECHNIQUES **REPORT DATE:**_____________**PARTNER INITIALS:**_________

EXERCISE 2.1 – MICROBIAL UBIQUITY

OBSERVATIONS

Examine your plate for growth of microbial colonies and record your observations below. If there is no growth on your plate, view another student's plate and record their initials at the top of this report. Dispose of plates in the Petri plate discard container.

SOURCE OF SAMPLE:__________________________



QUESTIONS FOR REVIEW

1. At what temperature was your plate incubated?___________For how long?________________
2. Based on appearance, how many different <u>types</u> of colonies are present in total?___________
3. If plates had accidentally been placed in the refrigerator rather than the incubator, would the number of colonies increase, decrease, or remain the same?_________________Explain.

____

____

4. Some plates may have resulted in poor to no growth even though microbes were present on the surface at the time of sampling. List three factors that could reduce the number of colonies formed. *Hint: Consider steps of the procedure and microbial growth requirements.*

   a) ____

   b) ____

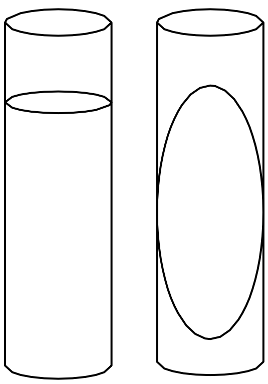
   c) ____

BIO 211 MODULE 2 REPORT **NAME:**______________________________

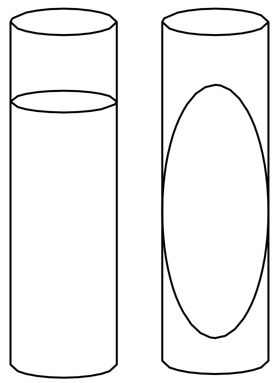
EXERCISE 2.2 – ASEPTIC TRANSFER

OBSERVATIONS

Observe growth in broth and on slants. Draw the appearance and describe using appropriate terminology. Remove tape from tubes and place them in a common rack for autoclaving when done.



BROTH SLANT



BROTH SLANT

Organism:______________________

Best descriptor for growth appearance:

In broth______________________

On slant______________________

Organism:______________________

Best descriptor for growth appearance:

In broth______________________

On slant______________________

QUESTIONS FOR REVIEW

1. A student inoculates a tube of broth and a slant with the same bacteria at the same time, but after incubation observes growth only in the broth. What is the most likely explanation?

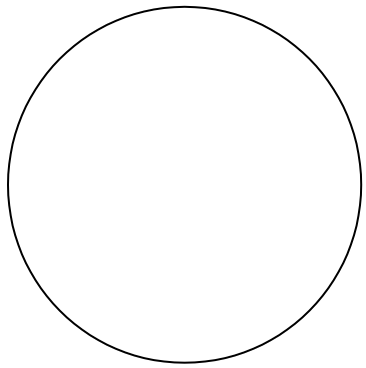
2. A large rhizoid colony is observed on slant but is not on the bacterial streak line. What type of microbe might have formed this colony, and why? ______________________________

BIO 211 MODULE 2 REPORT **NAME:**________________________________

EXERCISE 2.3 – ISOLATION STREAK PLATE

OBSERVATIONS

Use colored pencils to record the appearance of your isolation streak plate below, labeling the genus names for both organisms on the diagram and using appropriate terms to describe the growth in the table. Dispose of plates in the Petri plate discard container.



Organism	Pigment	Form	Elevation	Margin

QUESTIONS FOR REVIEW

1. Did you achieve isolated colonies of each genus on both plates?_________________________
2. Based on the results, how can your streaking technique be improved?___________________

____

3. How would the appearance of colonies change if the plate had been incubated at 37°C?_____

____

4. Explain how the appearance of a streak plate would change with the following errors:
   a) Not cooling the loop sufficiently between quadrants:_______________________________

   ____

   b) Dipping the loop into the broth before streaking each quadrant:_____________________

   ____

MODULE 3: Introduction to Staining

LEARNING OUTCOMES

1. Explain the purpose of staining in microbiology.
2. Discuss the action of basic and acidic dyes when applied to bacterial cells.
3. Name several examples of simple and differential dyes.
4. Compare and contrast simple and differential staining techniques.

Dyes Used in Staining

In their natural state, most of the cells and microorganisms that we observe under the microscope lack color and contrast. This makes it difficult, if not impossible, to detect important cellular structures and their distinguishing characteristics. Staining bacteria for viewing with a microscope provides valuable information about their size, shape, arrangement, and other cellular characteristics that assist in identification.

Dyes are selected for staining based on the chemical properties of the specimen being observed as well as the *chromophore,* which is the charged part of a dye that is responsible for color. Bacterial cells carry a net negative charge, so in most cases, it is preferable to use a basic (alkaline) dye that has a positively charged chromophore. Basic dyes are absorbed by cells to make them visible against a light background (Figure 3.1). Commonly used basic dyes include crystal violet, methylene blue, and safranin.

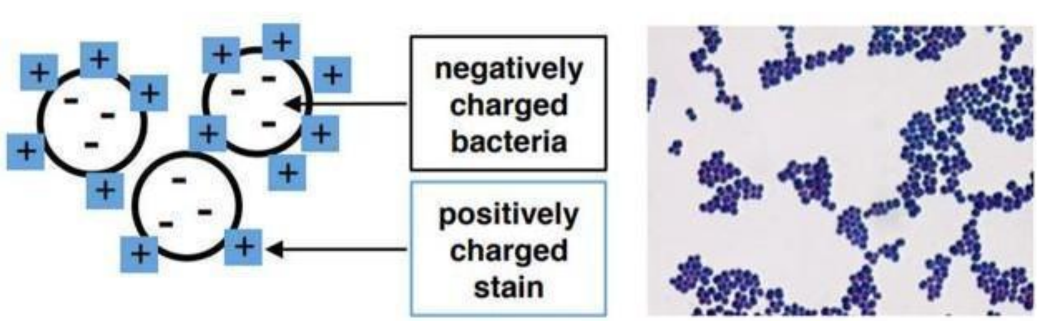


Figure 3.1: The positively charged chromophores of a basic dye such as methylene blue are absorbed by negatively charged bacterial cells.

However, there are times when it is advantageous to use an acidic dye, such as nigrosin or eosin, when staining a sample. These dyes have a negatively charged chromophore which is repelled by the cells, resulting in colorless cells against a dark background (Figure 3.2).

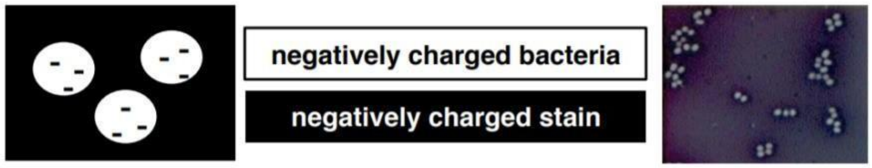
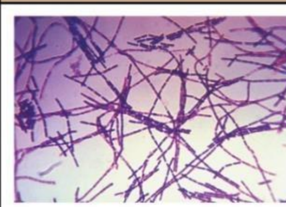


Figure 3.2: Negatively charged chromophores of an acidic dye such as nigrosin are repelled by negatively charged bacterial cells.

Simple vs. Differential Stains

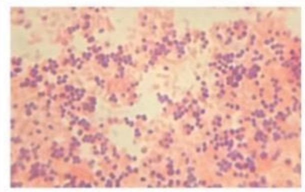
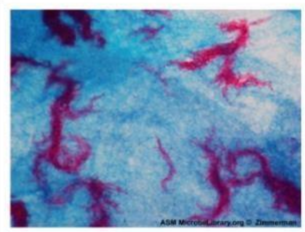
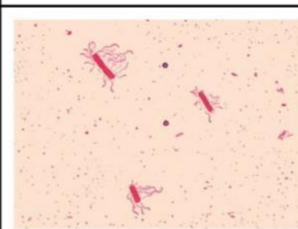
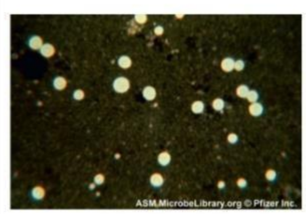
Simple staining techniques involve the application of only one dye to a sample to determine the size, shape, and arrangement of cells or to emphasize certain cellular structures. A simple stain makes all cells in a sample appear to be the same color, even if the sample contains more than one type of organism. Although it is a quick method to determine basic cellular morphology, simple staining often does not provide enough information to distinguish bacteria present in each sample. Table 3.1 shows common simple stains.

Table 3.1. Simple Stains

SIMPLE STAINS				
Stain Type	**Specific Dyes**	**Purpose**	**Outcome**	**Sample Images**
Basic stains	Methylene blue, crystal violet, malachite green, basic fuchsin, carbolfuchsin, safranin	Stain negatively charged molecules and structures, such as nucleic acids and proteins	Positive stain	
Acidic stains	Eosin, acid fuchsin, rose bengal, Congo red	Stain positively charged molecules and structures, such as proteins	Can be either a positive or negative stain, depending on the cell's chemistry.	
Negative stains	India ink, nigrosin	Stains background, not specimen	Dark background with light specimen	

In contrast to simple staining, *differential staining techniques* use multiple dyes and offer more information about cell types (Table 3.2). This may include cell wall thickness or the presence of mycolic acids, which are found in bacteria that cause tuberculosis. Since each dye of the procedure interacts with specific cellular components, this method distinguishes differences between bacteria and thus provides more details for identification. The most common differential stains performed in clinical microbiology laboratories are the Gram stain and acid-fast stains. Other differential stains include those for endospores, capsules, and flagella.

Table 3.2. Differential Stains

DIFFERENTIAL STAINS				
Stain Type	**Specific Dyes**	**Purpose**	**Outcome**	**Sample Images**
Gram stain	Uses crystal violet, Gram's iodine, ethanol (decolorizer), and safranin	Used to distinguish cells by cell-wall type (gram-positive, gram-negative)	Gram-positive cells stain purple/violet. Gram-negative cells stain pink.	
Acid-fast stain	After staining with basic fuchsin, acid-fast bacteria resist decolorization by acid-alcohol. Non acid-fast bacteria are counterstained with methylene blue.	Used to distinguish acid-fast bacteria such as *M. tuberculosis*, from non–acid-fast cells	Acid-fast bacteria are red; non–acid-fast cells are blue.	
Endospore stain	Uses heat to stain endospores with malachite green (Schaeffer-Fulton procedure), then cell is washed and counterstained with safranin.	Used to distinguish organisms with endospores from those without; used to study the endospore.	Endospores appear bluish-green; other structures appear pink to red.	
Flagella stain	Flagella are coated with a tannic acid or potassium alum mordant, then stained using either pararosaline or basic fuchsin.	Used to view and study flagella in bacteria that have them.	Flagella are visible if present.	
Capsule stain	Negative staining with India ink or nigrosin is used to stain the background, leaving a clear area of the cell and the capsule. Counterstaining can be used to stain the cell while leaving the capsule clear.	Used to distinguish cells with capsules from those without.	Capsules appear clear or as halos if present.	

Exercise 3.1 – Smear Preparation

LEARNING OUTCOMES

1. List the steps of preparing a smear for staining.
2. State the purpose of air-drying and heat-fixing a slide.

Many simple and differential staining techniques begin with a *smear.* A smear is prepared by adding bacteria from solid media to a drop of water on a glass slide or adding a loopful of bacterial broth to a slide directly (Figure 3.3). The smear is allowed to air dry completely, after which it is heat-fixed using an incinerator or Bunsen burner. Heat-fixing adheres cells to the glass, preventing them from washing away during the staining procedure. Because heat-fixing also kills bacteria, smears can be stored and stained later.

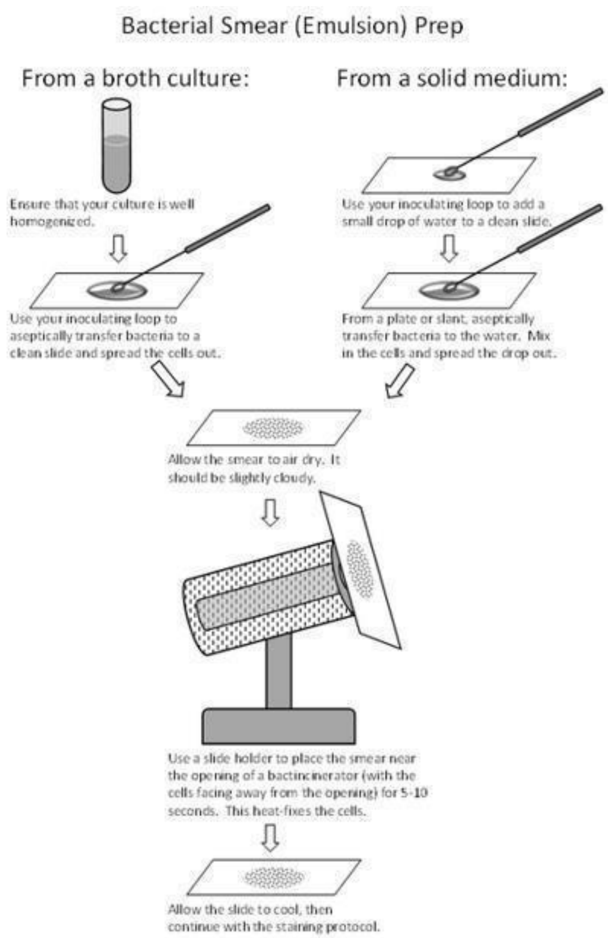


Figure 3.3. Preparation of a bacterial smear

Exercise 3.1 – Smear Preparation

NOTE: To best manage time, prepare smears first and then follow-up Module 2 exercises while smears are air-drying.

OBJECTIVE

Prepare heat-fixed smears for simple, Gram, and acid-fast staining in the next lab.

MATERIALS

- CULTURES: Slants of *Staphylococcus aureus, Escherichia coli, Bacillus cereus, Mycobacterium smegmatis,* and an unknown sample (U)
- SOLUTIONS: Deionized water in small dropper bottles
- EQUIPMENT: Inoculating loop, incinerator, glass slides, Sta-Clear paper or Kimwipes, clothespin or slide holder, slide box, pencil

PROCEDURE – STUDENTS WORK IN PAIRS

1. Obtain seven glass slides and wipe both sides with Sta-Clear paper.
2. Place slides directly on the lab bench, not on paper or a paper towel, and use a pencil to label the frosted edge of the glass with your initials, stain, and culture number:

   SS = Simple stain, two slides *B. cereus* and *S. aureus*

   GS = Gram stain, three slides *E. coli, S. aureus,* and an unknown sample (U)
   AF = Acid fast stain, two slides *M. smegmatis* and *S. aureus*

3. Add a small drop of water to the first slide.
4. Using aseptic technique, obtain bacteria on the inoculating loop and close the culture tube.
5. Mix the loop in the drop of water, spreading it out over the slide surface to facilitate drying.
6. Allow the smear to air-dry completely.
7. Repeat steps 4 through 7 for remaining slides.
8. Once slides are completely dry, use a clothespin or slide holder to hold the **back** of the slide (e.g., side of the slide without cells) against the incinerator opening for 10 seconds.

   TO AVOID AN AEROSOL, SLIDES MUST BE COMPLETELY DRY BEFORE HEAT FIXING.

9. Heat-fixed smears are ready to stain or may be stored in a slide box to stain in the future.

Exercise 3.2 – Simple Stain: Basic Dye

LEARNING OUTCOMES

1. List several basic dyes used in simple staining.
2. Describe the steps for preparing a simple stain.

One of the easiest techniques to use when visualizing cells under the microscope is to prepare a *simple stain.* Simple stains utilize one type of dye and result in cells that are all the same color, regardless of bacterial type. The most common simple stains use basic, or alkaline, dyes. These are dyes that contain a positively charged chromophore that are attracted by the negative charge of bacterial cells. When viewed microscopically, pigmented cells are visible against a white background or field. Common dyes used in simple staining are crystal violet, methylene blue, and the pink dye safranin.

Although simple staining is quick to do, the information that it provides about cells is limited. Since all bacteria are the same color upon completing the procedure, we only learn the size, shape, and arrangement of cells. Because of this, most microbiology laboratories do not perform simple staining alone and instead use a combination of dyes in multi-step differential staining procedures. These procedures tell us the same information that simple stains provide while also distinguishing between bacteria based on the type of composition of the cell wall, the presence or absence of endospores, etc. Two of the most common differential staining methods, the Gram stain and acid-fast stain, include the same dyes that we are using in this simple staining exercise.

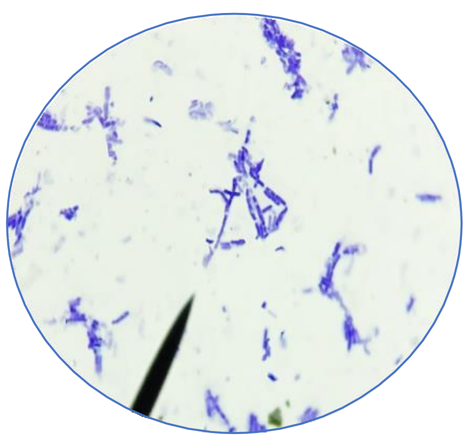


Figure 3.4: Simple stain of Bacillus megaterium using methylene blue dye (1000x)

Exercise 3.2 – Simple Stain: Basic Dye

<u>OBJECTIVE</u>

Stain cells with a basic dye to determine cell size, shape, and arrangement.

<u>MATERIALS</u>

- SLIDES: *Bacillus cereus* and *Staphylococcus aureus* heat-fixed smears
- SOLUTIONS: Methylene blue; safranin (from Gram stain kit)
- EQUIPMENT: Stain pan, rack, wash bottle, bibulous paper, clothespin/slide holder

<u>PROCEDURE – STUDENTS WORK IN PAIRS</u>

IMPORTANT: If your staining pan becomes full, empty it into the sink at the center of your bench. ***Never carry a full pan across the room****!*

1. Place the heat-fixed smear of *B. cereus* on the rack over the staining pan, smear side up.
2. Cover the smear entirely with methylene blue and let stand for five minutes.
3. Using the clothespin or slide holder, rinse both sides of the slide with water.
4. Blot the slide gently in the bibulous paper booklet and put the slide aside.
5. Dispose of the pan water in the bench sink.
6. Place the heat-fixed smear of *S. aureus* on the rack over the staining pan, smear side up.
7. Cover the smear entirely with safranin and let stand for one minute.
8. Using the clothespin or slide holder, rinse both sides of the slide with water.
9. Blot the slide gently in the bibulous paper booklet and put the slide aside.
10. Dispose of the pan water in the bench sink.
11. View the stained slides microscopically under oil immersion and complete the lab report.

Exercise 3.3 – Negative Stain: Acidic Dye

LEARNING OUTCOMES

1. List several acidic dyes used in simple staining.
2. Describe the steps for preparing a negative stain.

Some bacteria secrete a polysaccharide-rich structure external to the cell wall called a glycocalyx. If the glycocalyx is thin and loosely attached, it is called a slime layer; if it is thick and tightly bound to the cell, it is called a capsule. The glycocalyx can protect the cell from desiccation and can allow the cell to stick to surfaces like tissues in the body. They may also provide cells with protection against detection and phagocytosis by immune cells and contribute to the formation of a biofilm. In this way, the glycocalyx can serve as a virulence factor that contributes to the ability of an organism to cause disease.

Capsules can be detected using a *negative staining* procedure, in which an acidic dye stains the background rather than the encapsulated cells. Unlike simple staining with a basic dye, negative staining results in colorless cells that are easily seen against a colored background (Figure 3.5). Acidic dyes carry a negatively charged chromophore, thus they are repelled by the net negative charges on the bacterial cell. Examples of acidic dyes include the black dyes nigrosin and India ink, as well as the red dye eosin. Since capsules are destroyed by heat, this staining procedure is done without heat-fixing.

Although negative staining reveals the cellular morphology, size, and arrangement of cells, it does not differentiate between Gram-negative and Gram-positive bacteria. The term *negative* in negative staining technique refers to the negative charge of acidic dyes, while *Gram-negative* describes the type of bacterial cell wall.

Figure 3.5: Negative stain of Rhodospirillum rubrum using nigrosin dye (1000x).

Exercise 3.3 – Negative Stain: Acidic Dye

OBJECTIVE

Stain cells with an acidic dye to determine cell size, shape, and arrangement.

MATERIALS

- EQUIPMENT: Glass slides, Kimwipes or Sta-Clear paper, inoculating loop, marker
- CULTURES: Broth culture of *Rhodospirillum rubrum*
- SOLUTIONS: Nigrosin

PROCEDURE

1. Close the cap and vortex the broth culture 5-10 seconds.
2. Wipe both sides with Kimwipes or Sta-Clear paper to reduce static charge.
3. Label the frosted edge of one slide with the organism number and NS for negative stain.
4. Place a small drop of nigrosin on slide near the labeled end.
5. Aseptically obtain a loopful of bacteria and close the culture tube.
6. Emulsify the bacteria on the loop in the nigrosin drop, but do not spread it over the slide.
7. Place the second slide in the dye at a 45^{o} angle in the nigrosin.
8. Pull the spreader slide across the bottom slide to spread the nigrosin toward the other end.
9. Dispose of the second slide in the disinfectant beaker.
10. Allow the nigrosin slide to air dry.
11. Repeat steps for the second culture.
12. View the stained slides microscopically under oil immersion and complete the lab report.

Exercise 3.4 – Gram Stain

LEARNING OUTCOMES

1. List the steps for preparing a Gram stain.
2. State the purpose of the primary dye, mordant, decolorizer, and counterstain.

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

Gram stain results reflect differences in cell wall composition. These differences are reflected in the way the cells react with the stains used in the Gram stain procedure. Gram-positive bacteria have thick layers of the carbohydrate peptidoglycan in their cell walls, while the cell walls of Gram-negative bacteria are relatively thin. However, unlike Gram-positive bacteria, Gram-negative cells possess an outer membrane in addition to the plasma membrane. This outer membrane contains lipopolysaccharides (LPS) which act as endotoxins when Gram-negative cells are destroyed by the host's immune system. Endotoxins can heighten the inflammatory response in a patient and cause elevated fever. Figure 3.6 shows the major differences between the Gram positive and Gram-negative cell walls.

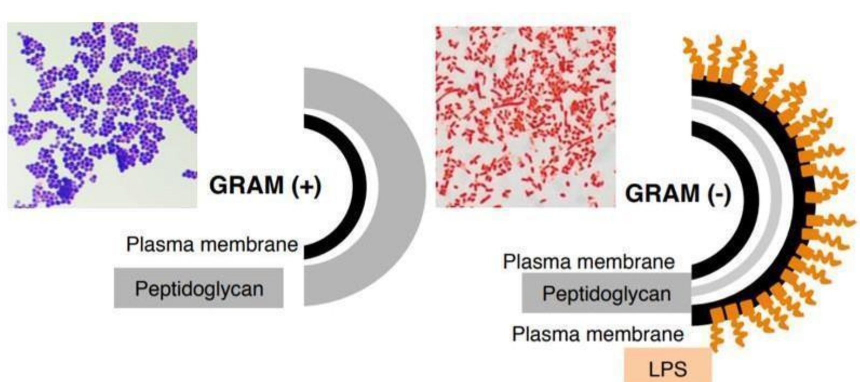


Figure 3.6. The thick peptidoglycan cell walls of Gram-positive bacteria retain crystal violet throughout the staining procedure. Gram-negative bacteria have much thinner cell walls and additional outer membranes. These cells are readily decolorized despite application of a mordant and must be counterstained with safranin to be observed microscopically.

Although most bacteria are either Gram-positive or Gram-negative, it is important to remember that not all bacteria can be stained with this procedure. For example, *Mycoplasma* bacteria, which have no cell wall, stain poorly with the Gram stain. Figure 3.7 shows a microscopic image of two cell types stained by the Gram method, where Gram-positive cells appear purple in color and Gram-negative cells appear pink (note that cell wall thickness, rather the shape of cells, determines Gram reaction).

Steps of the Gram Stain

The Gram Stain is a differential stain because it separates bacteria into two groups based on differences in their cell wall structure. The protocol involves more steps than a simple stain, but is still performed on air-dried, heat-fixed smear preps. The smear prep is critical. If the smear is too thick the bacteria will not stain evenly, nor will they decolorize evenly. This can be a major source of error in evaluating the Gram reaction of a culture.

The four steps of the Gram stain procedure are outlined in Table 3.3 and Figure 3.7. It is important to understand the purpose of every step as well as the color of cells after the application of each chemical reagent.

Table 3.3. Steps of the Gram stain.

STEP	REAGENT	PURPOSE	GRAM (+)	GRAM (-)
Primary dye	Crystal violet	Stains peptidoglycan	Purple	Purple
Mordant	Gram's iodine	Fixes crystal violet into peptidoglycan	Purple	Purple
Decolorizer	Alcohol or Acetone-Alcohol	Removes crystal violet from Gram (-) cells	Purple	Colorless
Counterstain	Safranin	Stains the colorless Gram (-) cells	Purple	Pink

STEP 1 – Primary Dye: The first, or primary, stain is crystal violet. Crystal violet is a basic dye that attaches to peptidoglycan of both Gram-positive and Gram-negative bacteria. The primary dye is applied for one minute and then rinsed off with water. Since both Gram-positive and Gram-negative cells have peptidoglycan, application of the primary dye stains all cells purple.

STEP 2 – Mordant: A mordant is a chemical that stabilizes or fixes a stain with its target. In this case, Gram's iodine is applied as a mordant, enhancing the action of the primary dye by forming strong complexes between crystal violet and peptidoglycan. Following application of the mordant and a water rinse, all cells remain purple.

STEP 3: Decolorizer – The decolorization step is the most critical step of the procedure because it differentiates Gram-positive and Gram-negative cells. If not done carefully, over-decolorization or under-decolorization can lead to incorrect or ambiguous results. An alcohol or acetone/alcohol solution is applied to the smear to dissolve the outer membrane and remove the crystal violet from Gram-negative cells. Gram-positive cells retain the crystal violet/iodine complex due to the many layers of peptidoglycan and lack of an outer membrane. The action of decolorizer is stopped by a water rinse. After decolorization, Gram-positive cells are still purple but Gram-negative cells are colorless. Stopping here would make it very difficult to observe Gram-negative cells microscopically, so a final step is needed to make these cells visible.

STEP 4: Counterstain – The second stain, or counterstain, in the Gram procedure is safranin. Safranin is a basic dye that stains cells pink and imparts color to the Gram-negative cells that lost the primary dye following decolorization. Safranin may also attach to Gram-positive cells, but since crystal violet is so strongly complexed with the cell wall it obscures any additional pink staining. Following the application of safranin and a final water rinse, Gram-positive cells are purple and Gram-negative cells are pink (Figure 3.8). Note that after all four steps of the procedure, Gram-positive cells remained purple.

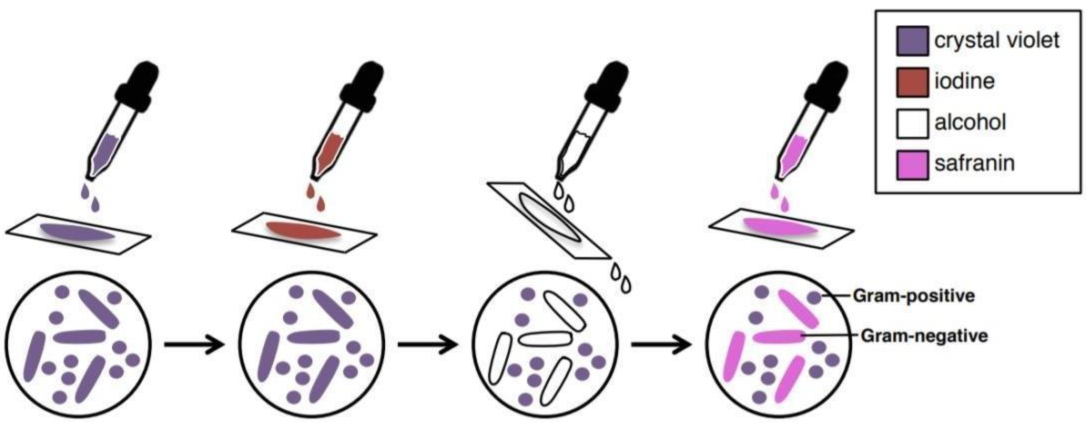


Figure 3.7: Steps of the Gram stain. Each step is followed by a water rinse.

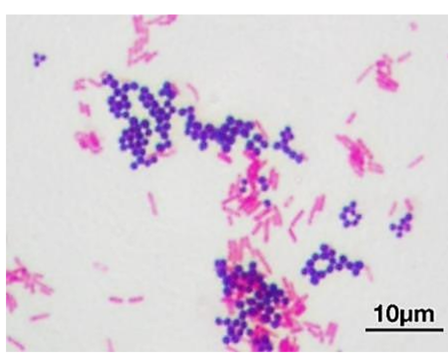


Figure 3.8: Microscopic image of a Gram stain of mixed Gram-positive cocci (Staphylococcus aureus, purple) and Gram-negative bacilli (Escherichia coli, pink). Total magnification 1000X.

Gram stains are best performed on fresh cultures—older cells may have damaged cell walls which may not produce an accurate Gram reaction. Some species of bacteria are Gram-variable, appearing as a mix of both Gram-positive and Gram-negative reactions. The decolorization step is also critical for accurate results. This step uses an alcohol/acetone mixture that disrupts the outer membrane and thin layer of peptidoglycan of Gram-negative cells. When under-decolorization occurs, the decolorizer is not left on long enough and Gram-negative bacteria retain too much of the crystal violet, causing them to appear purple instead of pink. Likewise, over-decolorization causes Gram-positive cells to lose crystal violet and appear pink after counterstaining with safranin.

Another common mistake is in the preparation of the heat-fixed bacterial smear. The main purpose of heat-fixing is to adhere the bacterial cells to the microscope slide (it also denatures the proteins, killing the cells as well). If you forget to do this step, or do it inadequately, then the cells will be washed off in all the subsequent steps of your staining process and there will be no bacteria on the slide to observe! Variable colors are also observed if bacterial cells are not spread evenly in a drop of water when preparing a smear from solid media. This could result in uneven decolorization during the Gram staining procedure and both purple and pink cells in a slide made from one type of bacteria. For example, thick areas of a smear made from Gram- negative bacteria might be under-decolorized, causing cells in those areas only to appear purple, while remaining cells are pink as expected (Figure 3.9).

Common Gram Staining Errors

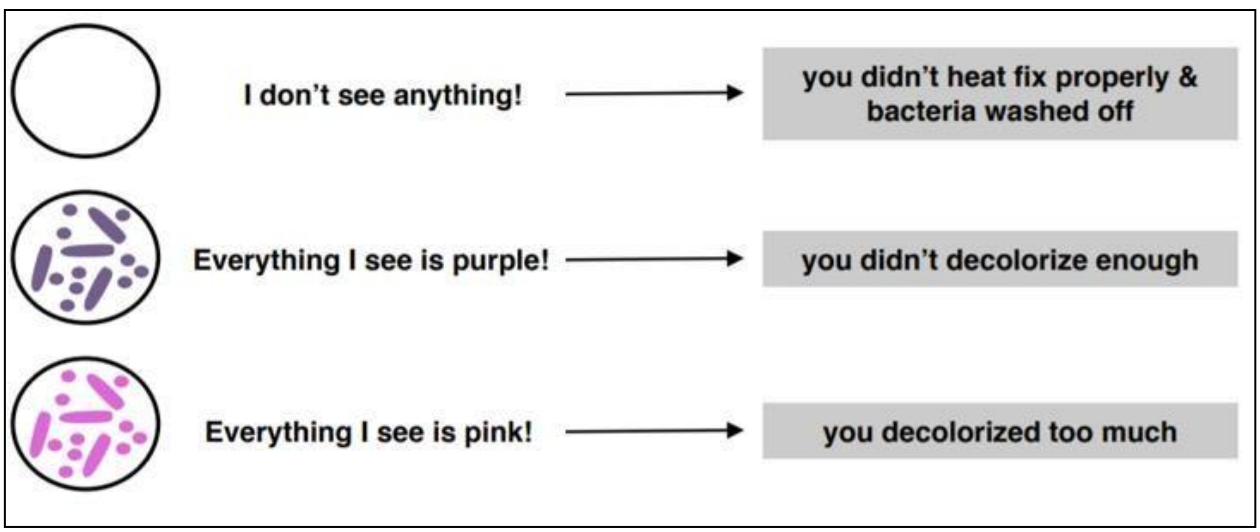


Figure 3.9: Common errors in Gram staining.

Exercise 3.4 – Gram Stain

OBJECTIVE

Stain heat-fixed smears with an acidic dye to determine cell size, shape, arrangement, and cell wall type.

MATERIALS

- SLIDES: *Staphylococcus aureus, Escherichia coli,* and *Unknown* heat-fixed smears
- SOLUTIONS: Gram stain kit (crystal violet, iodine, alcohol decolorizer, safranin)
- EQUIPMENT: Stain pan, rack, wash bottle, bibulous paper, clothespin/slide holder

PROCEDURE – STUDENTS WORK IN PAIRS

IMPORTANT: If your staining pan becomes full, empty it into the sink at the center of your bench. ***Never carry a full pan across the room****!*

1. Place the heat-fixed smears on the rack over the staining pan.
2. Cover smears entirely with **crystal violet** and let stand for one minute.
3. Using the clothespin or slide holder, rinse both sides of each slide with water; do not blot.
4. Cover smears entirely with **iodine** and let stand for one minute.
5. Using the clothespin or slide holder, rinse both sides of each slide with water; do not blot.
6. Lifting one slide at a time, apply **alcoho**l until the color just starts to run (10-20 seconds) and immediately rinse the slide with water to stop action of the decolorizer; do not blot.
7. Cover smears entirely with **safranin** and let stand for one minute.
8. Using the clothespin or slide holder, rinse both sides of each slide with water.
9. Blot slides gently in the bibulous paper booklet and put the slides aside.
10. Dispose of the pan water in the bench sink (do not carry the full pan to the room sink!).
11. View the stained slides microscopically under oil immersion and complete the lab report.

Exercise 3.5 – Acid-Fast Stain: Kinyoun Method

LEARNING OUTCOMES

1. Discuss the clinical importance of acid-fast bacteria.
2. Explain why the cell wall structure of acid-fast bacteria is unique.
3. List the steps in preparing an acid-fast smear by the cold Kinyoun method.

The acid-fast stain is a differential stain used to identify organisms that are members of the genera *Mycobacterium* and *Nocardia*. The cell walls of these bacteria contain a waxy component known as *mycolic acid* (Figure 3.10). The presence of mycolic acid makes acid-fast bacteria highly resistant to staining, antibiotics, disinfectants, and environmental factors such as desiccation or drying. Acid-fast bacteria are ubiquitous in soil and water, and they include medically important species that cause diseases such as tuberculosis and leprosy.

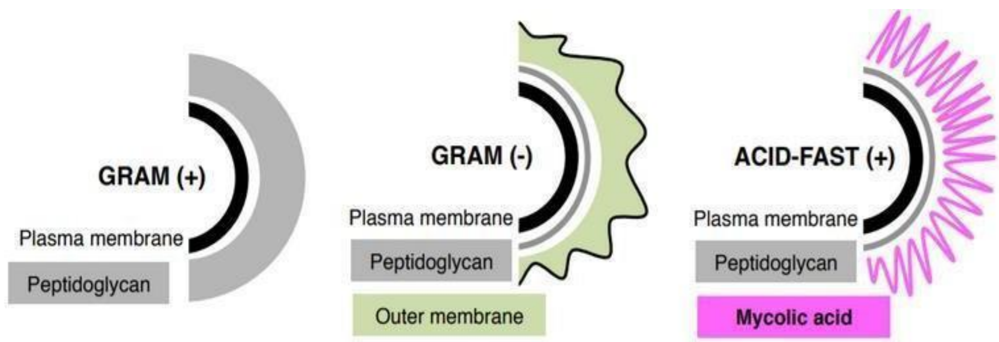


Figure 3.10: A comparison between the cell envelopes of Gram-positive, Gram-negative, and acid- fast bacteria.

Mycobacteria often takes weeks to months to cultivate on media. When a patient is suspected of having tuberculosis, an acid-fast stain prepared from sputum or other specimen confirms that mycobacteria are present. While these bacteria contain peptidoglycan, they Gram stain poorly because mycolic acid prevents dyes from entering their cell walls. The Ziehl-Neelsen and cold Kinyoun procedures are special staining techniques that penetrate this waxy component.

The Ziehl-Neelsen method uses heat to soften cell walls prior to application of the primary dye carbol fuchsin. Carbol fuchsin imparts a bright pink or fuchsia color to all cells. When slides are removed from the heat and permitted to cool, carbol fuchsin becomes trapped within the bacterial cell walls. Next, acid-alcohol is applied to decolorize non- acid-fast cells. Bacteria that retain the carbol fuchsin and do not lose their primary color are said to be acid fast (i.e., color-fast) despite decolorization. Finally, the counterstain methylene blue is applied to stain non-acid fast cells (Figure 3.11).

A cold acid-fast staining technique is the Kinyoun procedure. This method uses the same three reagents, but rather than heating cells to melt mycolic acid, additional phenol is added to the carbol fuchsin which disrupts lipids. The microscopic appearance of cells stained by the Kinyoun or Ziehl-Neelsen methods is the same (Figure 3.12).

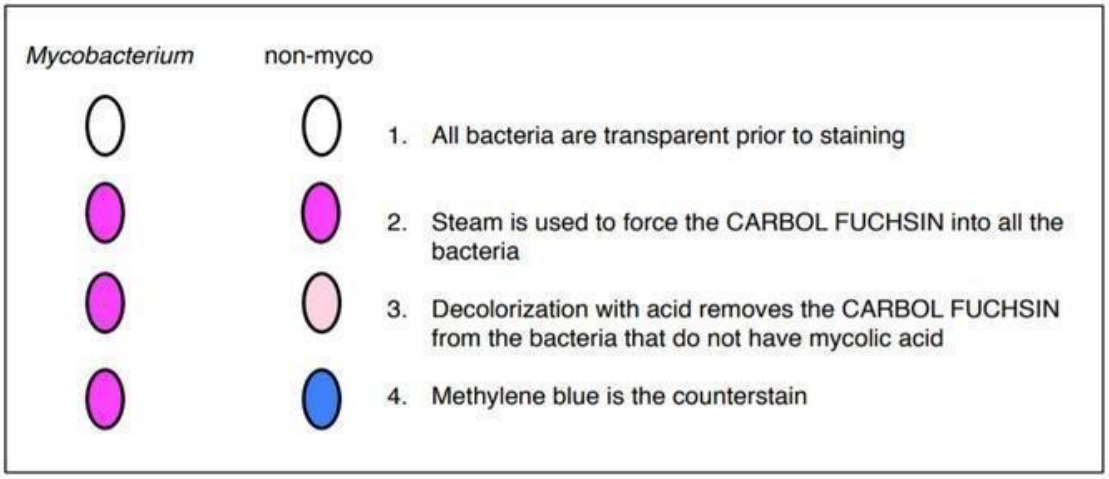


Figure 3.11. Acid-fast mycobacterial cells retain the bright pink primary dye carbol fuchsin despite decolorization with acid alcohol (hence, they are "colorfast"). Non-acid-fast bacteria are stained by the methylene blue counterstain following decolorization.

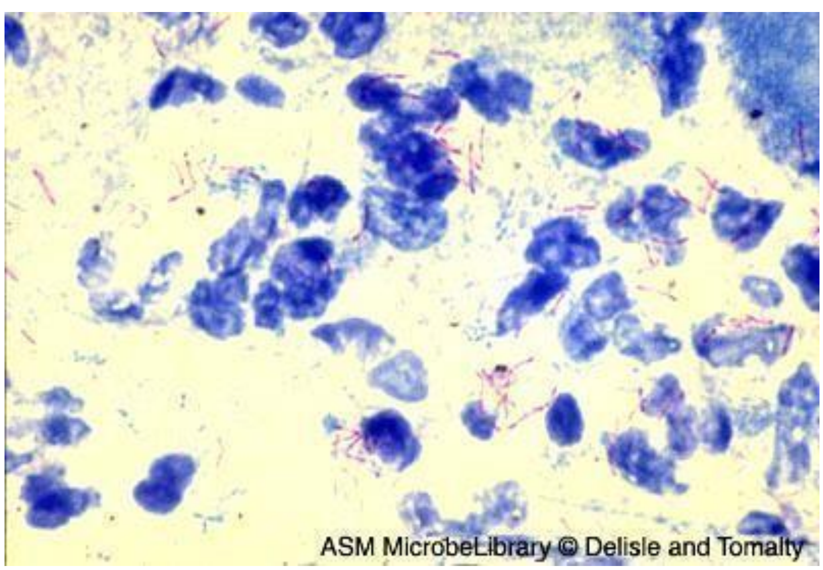


Figure 3.12: Acid-fast Stain of Mycobacterium tuberculosis in sputum. Note the reddish acid-fast bacilli among blue microbiota and white blood cells in the sputum which are not acid-fast.

Exercise 3.5 – Acid-Fast Stain: Cold Kinyoun Method

OBJECTIVE

Stain cells by the cold Kinyoun method to determine the presence of mycolic acid.

MATERIALS

- SLIDES: *Mycobacterium smegmatis* and *Staphylococcus aureus* heat-fixed smears
- SOLUTIONS: Acid-fast kit (carbol fuchsin, acid alcohol, methylene blue)
- EQUIPMENT: Stain pan, rack, wash bottle, bibulous paper, clothespin/slide holder

PROCEDURE – STUDENTS WORK IN PAIRS

IMPORTANT: If your staining pan becomes full, empty it into the sink at the center of your bench. ***Never carry a full pan across the room****!*

1. Place the heat-fixed smears of *M. smegmatis* and *S. aureus* on the rack of the staining pan.
2. Cover both smears entirely with carbol fuchsin and let stand for three minutes.
3. Using the clothespin or slide holder, rinse both sides of the slides with water. Do not blot.
4. Lifting one slide at a time, apply acid alcohol until observing the color just beginning to run (10-20 seconds)
5. Immediately rinse the slides with water to stop the action of the decolorizer. Do not blot.
6. Cover both smears entirely with methylene blue and let stand for two minutes.
7. Using the clothespin or slide holder, rinse both sides of the slides with water.
8. Blot the slides gently in the bibulous paper booklet and put the slides aside.
9. Dispose of the pan water in the bench sink.
10. View the stained slides microscopically under oil immersion and complete the lab report.

BIO 211 MODULE 3 REPORT **NAME:**____

STAINING **REPORT DATE:**_____________**PARTNER INITIALS:**_________

EXERCISE 3.1-3.5 – STAINING

Place a check for information learned about cells for each staining technique:

	Shape	Size	Arrangement	Cell Wall Type
Simple stain				
Negative stain				
Gram stain				
Acid-fast stain				

Define the use of the terms "negative" for each of the following phrases:

- Negative stain:____

  Gram-negative:____

Complete the table for each step of the following procedures:

GRAM STAIN	Which reagent is used in this step?	Color of Gram-positive cells after this step?	Color of Gram-negative cells after this step?
Primary dye			
Mordant			
Decolorizer			
Counterstain			
KINYOUN STAIN	Which reagent is used for this step?	Color of acid-fast cells after this step?	Color of non-acid-fast cells after this step?
Primary dye			
Decolorizer			
Counterstain			

Which step of the Gram and acid-fast staining procedures is most critical?___________________

Explain:____

MODULE 4: Introduction to Light Microscopy

LEARNING OUTCOMES

1. Define basic terms and principles of brightfield microscopy.
2. Describe appropriate units of measurement for microorganisms.

INTRODUCTION

The pioneers of microscopy opened a window into the invisible world of microorganisms. Early microscopes, which used visible light to illuminate cells, continued to advance in the centuries that followed. The 20th century saw the development of microscopes that leveraged nonvisible light, such as fluorescence microscopy (which uses an ultraviolet light source) and electron microscopy (which uses short-wavelength electron beams). These advances led to major improvements in magnification, resolution, and contrast.

Brightfield Microscopy

The brightfield microscope is one of the most common types of light microscopes used in microbiology laboratories. It is a compound microscope, meaning that more than one type of lens is used to magnify an image. Visible light is the source of illumination and specimens are observed against a bright field or background. Some brightfield microscopes are equipped with special attachments that change the field to appear darker than the specimens being viewed. This is known as darkfield microscopy and is often helpful when viewing live microorganisms, such as protists, that might otherwise be killed if stained (Figure 4.1).

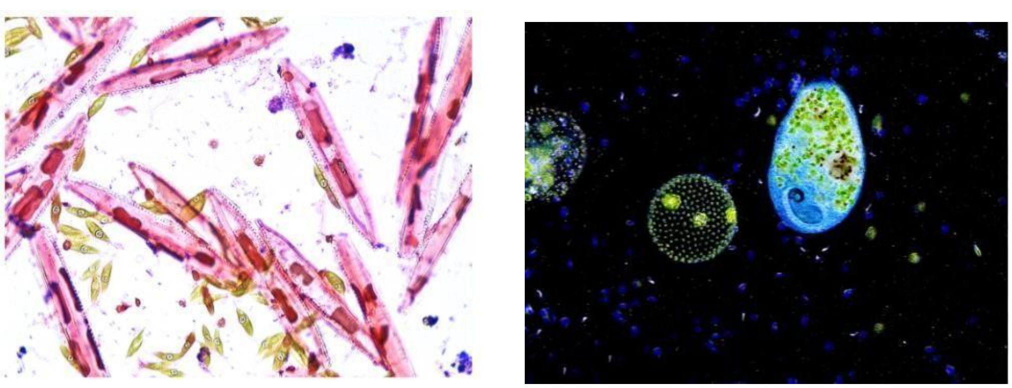


Figure 4.1: Gram stain of freshwater diatoms, euglenoids, and bacteria (left) and darkfield image of live protists (right).

The size of microbes can be hard to imagine because they are so small in comparison to what most people see day to day. Even when compared to plant or animal cells, microbes tend to be much smaller. The unit micrometer (µm), also known as a micron, is used when describing the size of bacterial cells. A micrometer is 1/1000 of a millimeter and 1/1,000,000 of a meter. To put it more tangibly, a typical cell of *Staphylococcus* bacteria measures one micrometer, or about 1/400 the size of the period at the end of this sentence.

Viruses, which are too small to be viewed with a light microscope and instead must be observed using a much more powerful electron microscope, are measured in nanometers (nm). One nanometer is 1/1000 of a micrometer. Most viruses range in size from 10 to 100 nanometers. See Figure 4.2 for a comparison of relative cell sizes.

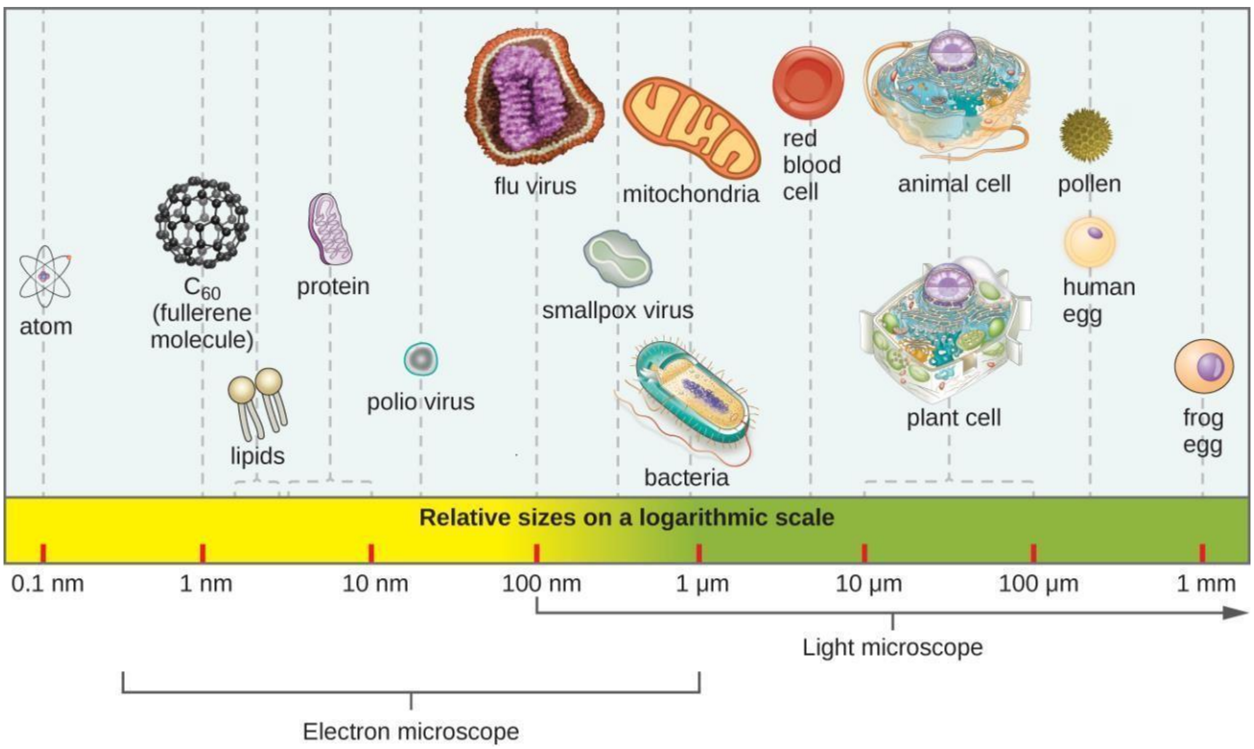


Figure 4.2: Relative sizes of various cellular and non-cellular structures. Bacteria and larger microorganisms such as protists and fungi are visible with a light microscope, while a more powerful electron microscope is required to observe most viruses.

In this module, you will use the light microscope to view bacterial smears that were previously prepared. The maximum magnification of the microscope, 1000X, will be used to observe all cells.

Exercise 4.1 – Using the Light Microscope

LEARNING OUTCOMES

1. List the ways in which a microscope is properly maintained and stored.
2. Identify and give the function of key parts of a compound light microscope.
3. Discuss the principles of magnification and resolution; define key terms.
4. Calculate total magnification.
5. Use the scanning, low power, and high-power objective lenses to focus the letter "e."

Microscope Care

Even a very powerful microscope cannot deliver high-resolution images if it is not properly cleaned and maintained. Microscopes are rather delicate instruments, and great care must be taken to avoid damaging parts and surfaces.

Each student is assigned a microscope for use during the semester. Be sure to record the number of your microscope and follow the guidelines below when obtaining and storing it.

Care and Storage of the Light Microscope
1. Carry your microscope with two hands, one on the arm and the other under the base.
2. Lift and place the microscope to reposition it on the benchtop; do not drag it.
3. Clean the stage and objective lenses before and after use with lens paper/cleaner.
4. Lower the light intensity before turning off the microscope.
5. Move the stage to its lowest position before storage.
6. Position the 4X objective to point down toward the stage before storage.
7. Return your microscope to the corresponding number compartment in the cabinet.

Basic components of the light microscope are shown in Figure 4.3.

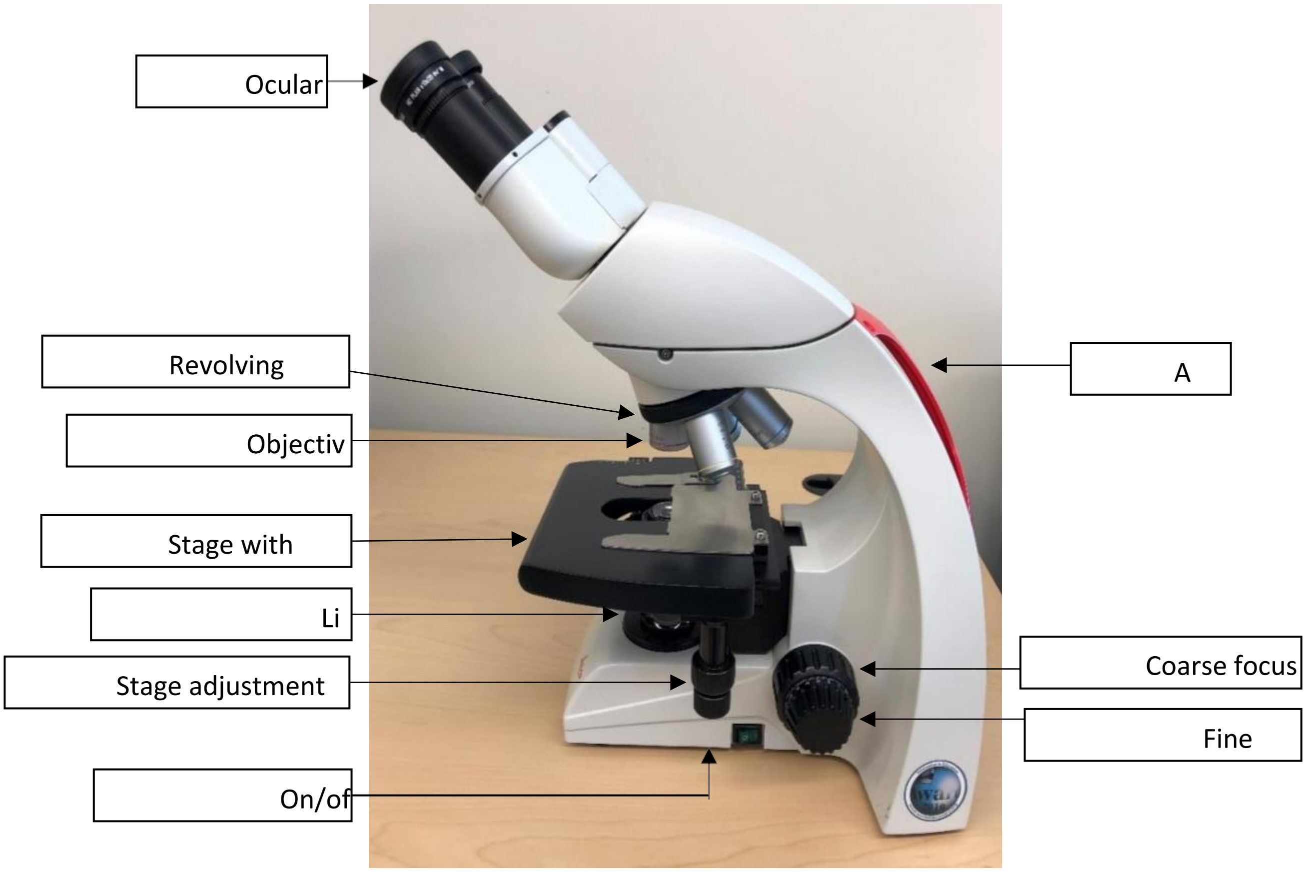


Figure 4.3a: Components of a typical brightfield microscope.

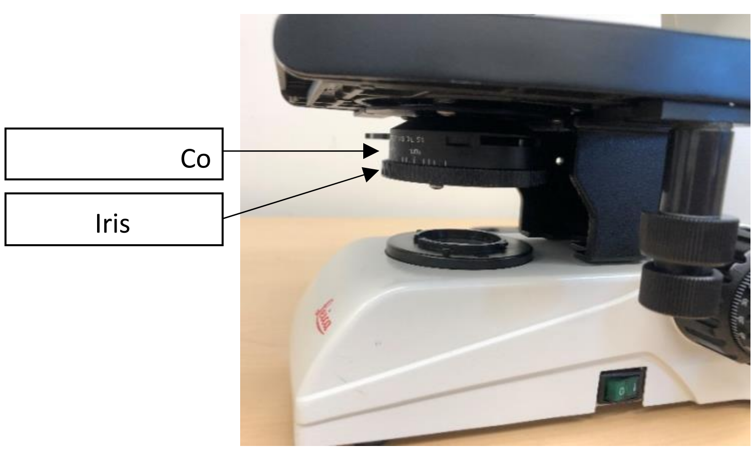


Figure 4.3b: The amount of light traveling through the condenser lens is controlled by turning the adjustment for the iris diaphragm located inside the condenser.

Summary of Microscope Components

- **Ocular lens:** Eyepiece that usually magnifies 10X; binocular microscopes have two oculars that are adjustable for interpupillary distance between the eyes. Oculars may have a pointer and/or a ruler for measuring cells called an ocular micrometer.
- **Revolving nosepiece**: Rotates to allow each objective to align in place with the ocular.
- **Objective lenses**: Seated in the nosepiece. Each objective lens has a different magnifying power: scanning (4X), low power (10X), high power (40X) and oil immersion (100X).
- **Coarse focus knob**: Outer large knob that raises and lowers the stage to bring the specimen into initial focus; used with the scanning objective lens.
- **Fine focus knob:** Smaller inner knob that raises and lowers the stage to bring the specimen into sharp focus; used with low power, high power, and oil immersion lenses.
- **Mechanical stage**: Horizontal surface on which slide is placed and held by stage clips; the stage is moved left and right by turning the x-y mechanical stage knobs.
- **Illuminator**: Light source turned on by a switch on the base and controlled by a rheostat located on the side of the base that adjusts the brightness of the light.
- **Iris diaphragm and condenser**: The iris diaphragm can be adjusted to control the amount of light passing from the illuminator through the bottom of the slide. It is located inside the condenser, which is a lens system that gathers and directs light up from the illuminator.

Magnification and Resolution

Light microscopes use visible light and a series of lenses to view microscopic specimens. The condenser lens focuses the light as it goes through the specimen and can be adjusted for optimization. The objective lenses magnify the specimen, capturing the transmitted and reflected light to create a real image of the specimen. The ocular lens further magnifies the image and creates a virtual image for viewing. This difference can be observed with a slide of the letter “e” or “p” and noting how the image changes when viewed through the ocular.

What is observed through the microscope is the *field of view*. While an entire organism might be visible in the field of view using the scanning lens, only a small portion of it may be seen under high power. Since microorganisms have a wide range of sizes, the most appropriate objective to use for each varies. For example, while a large protist such as *Amoeba* may be viewed under low power, this would not be suitable for viewing bacteria which are much smaller.

Most modern microscopes are *parfocal* in that they remain in relative focus when changing magnifications. This property eliminates the need for extensive re-focusing when switching between objective lenses.

Related to the concept of field of view are *depth of field* and *working distance*. Depth of field refers to the nearest and furthest planes of a specimen that are in focus at the same time. Depth of field depends on thickness of the specimen and decreases as magnification increases. The working distance, or space between the slide and objective lens, decreases as magnification increases. To avoid damaging the objective lenses or the slide, the coarse focus knob should only be used for initial focus when the working distance is greatest.

Calculating Total Magnification

Magnification is the process of making an object appear larger than it is. The magnification of each objective is printed on the metal portion of the lens. The *scanning* objective has a magnification of 4X and is used when first bringing an image into focus. The next objective is *low power* which magnifies 10X. The high-power objective, sometimes called the *high dry* objective because it is used without immersion oil, magnifies 40X and is used when fine focusing an image. Finally, the *oil immersion* objective has a magnification of 100X and is used when viewing bacterial cells.

The ocular and objective lenses work together to create a magnified image. Total magnification (TM) is calculated by multiplying the ocular and objective magnifications:

Total magnification = (ocular magnification) x (objective magnification)

For example, if the ocular is 10X and the 40X objective lens is selected, TM is (10X)(40X) = 400X. Total magnification using each objective lens for your microscope is given in Table 4.1.

Table 4.1. Total Magnification

Objective Lens Magnification	Ocular Lens Magnification	Total Magnification
Scanning (4X)	10X	40X
Low power (10X)	10X	100X
High power (40X)	10X	400X
Oil immersion (100X)	10X	1000X

Unlike magnification, *resolution* is the ability to distinguish two objects as separate entities. The resolving power for a light microscope is about 0.2 micrometers, meaning any that two objects that are closer than two tenths of one micrometer will be seen as a single point.

The following exercise is designed to provide practice using the light microscope to view a slide with the letter "e." Work through the steps slowly and apply the same principles when viewing stained slides in later exercises.

Exercise 4.1 – Using the Light Microscope: Viewing the Letter "e"

OBJECTIVE

Use the light microscope to practice focusing under scanning, low power, and high power.

MATERIALS

- EQUIPMENT: Light microscope, Sta-clear paper, lens paper, lens cleaner
- SLIDE: Letter "e"

PROCEDURE - *Take your time and work through steps in order.*

1. Obtain a microscope from the cabinet. Remember to carry it with two hands and reposition it on the bench by lifting rather than dragging.

2. Place the microscope directly in front of you on the bench. Sit up straight and push in your chair so that you are comfortable. Do not bend over or kneel on your chair to view slides.

   - Record the number that is found on the back of your microscope:_________________

3. Verify that the student before you stored the microscope correctly, making sure:

   ______The stage is clean, has no slides, and is free from oil.

   ______The scanning (4X) objective lens is pointing down toward the stage.

   ______The stage is lowered completely.

   ______The rheostat (light intensity dial on the base) is turned down all the way.

4. Clean the oculars and objective lenses with lens paper and lens cleaner, checking that each objective lens is securely screwed into the revolving nosepiece.

   - Record the magnification printed on the oculars:____________X

5. Plug in your microscope and turn it on using the power switch on the base.

6. Raise the light intensity by turning the rheostat to a high number on the base and adjust brightness by closing the iris diaphragm rather than lowering the rheostat.

7. Move the oculars together or apart so that you can use both eyes to view the slide. Note that one ocular will have a pointer and the other will have a micrometer for measuring cells.

   - Record the interpupillary distance between the oculars:___________________

8. Obtain a slide of the letter “e” from the slide tray and clean it using Sta-Clear paper and lens cleaner.

9. Place the slide on the stage with the label facing up and to the left, securing corners in the stage clips so that it lies flat and pushing the slide back as far as it will go.

    - Record the appearance of the letter as it appears looking at the stage:_______

10. Using the stage control knobs, position the slide so that the letter is over the light source.

11. Look through the oculars and keep turning the coarse focus knob until the image comes into focus. This may require significant rotation of the focus knob. If you go too far and miss the image, turn the knob slowly in the opposite direction.

    - Record the appearance of the letter as it appears through the oculars:_______
    - Record the total magnification using this objective:___________X
    - Circle the appearance of a “p” as it would be viewed through the oculars: p d b q

12. View the slide under low power by rotating the 10X objective in place and turning the fine focus knob until the image is clear. If necessary, adjust the iris diaphragm to lower the light.

13. If directed to do so, raise your hand for the instructor to verify your observation.

    - Record total magnification using this objective:______________X
    - Which property maintains focus while changing objectives?______________________

14. View the slide under high power by rotating the 40X objective in place and turning the fine focus knob until the image is clear. Increase light by opening the diaphragm. If the image is blurry, use lens paper to firmly clean the bottom of the objective.

    - Record total magnification using this objective:______________X
    - What happens to the field of view as magnification increases?____________________
    - Which objective lens is most appropriate for viewing the letter?__________________

15. Return the slide to the corresponding numbered slot on the tray.

16. When you are done using the microscope, prepare it for storage by ensuring:

    ______The stage is clean, has no slides, and is free from oil.

    ______The scanning (4X) objective lens is pointing down toward the stage.

    ______The stage is lowered completely.

    ______The rheostat (light intensity dial on the base) is turned down all the way.

17. Show your microscope to the instructor before returning it to the cabinet.

Exercise 4.2 – Bacterial Cellular Morphology & Arrangement

LEARNING OUTCOMES

1. Use the oil immersion objective to view stained bacterial cells.
2. Identify results from simple, Gram, acid-fast, and negative stains.
3. Name the basic shapes and arrangements of bacterial cells.
4. Measure bacterial cell size with the ocular micrometer when using oil immersion.

Using the Oil Immersion Lens

As light rays move through different media (air, glass, water, etc.), the light bends, or refracts, at a particular angle known as the *refractive index*. This explains why swim goggles are needed to see clearly underwater. As light moves from air to water it refracts, and the angle changes. Wearing goggles creates an air space in front of your eyes so that the light bends again, in essence correcting itself in terms of your ability to see clearly.

At very high magnifications, such as when viewing bacteria with the 100X objective lens, resolution may be compromised when light passes through the small amount of air between the specimen and the lens. This is due to the large difference between the refractive index of air and that of glass; the air scatters the light rays before they can be focused by the lens. To solve this problem, a drop of oil can be used to fill the space between the slide and the objective, thus forming a connection between the two through which light can travel. Since oil and glass have a similar refractive index, the light is collected rather than refracted. Thus, adding immersion oil improves the resolution or clarity of the image (Figure 4.4).

Bacterial Shapes (OpenStax)

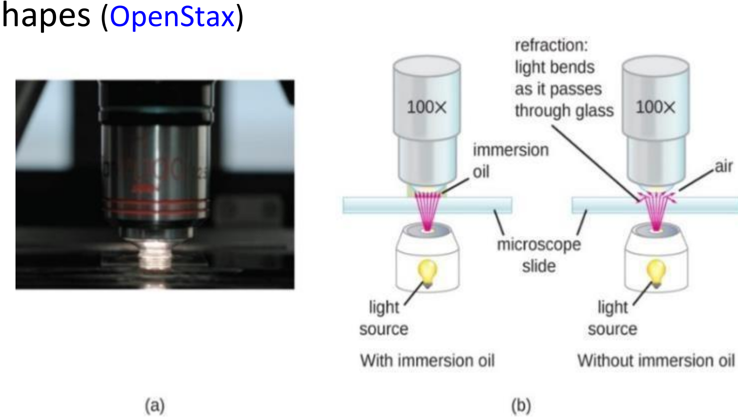


Figure 4.4: (a) Oil immersion lenses like this one are used to improve resolution. (b) Because immersion oil and glass have very similar refractive indices, there is a minimal amount of refraction before the light reaches the lens. Without immersion oil, light scatters as it passes through the air above the slide, degrading the resolution of the image.

Three basic shapes of bacterial cells are spherical *cocci* (singular, coccus), rod-shaped *bacilli* (singular, bacillus), and curved or *helical* bacilli. Cocci are generally less than one micrometer in size, but the length of bacilli can vary. Helical bacilli can be further subtyped based on the degree of cellular curvature. *Vibrio* cells are small, comma-shaped bacilli, while rigid *spirilli* have multiple curves. *Spirochetes* are highly curved flexible bacilli that have a corkscrew-like structure (Figure 4.5).

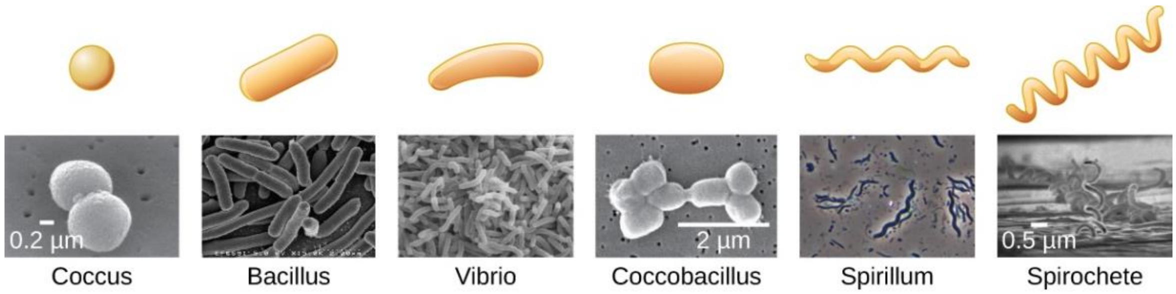


Figure 4.5: Bacterial cellular shapes.

Cellular Arrangements

Bacterial cells divide by an asexual process known as *binary fission*, where one parent cell splits to form two identical new daughter cells. Following division, daughters may separate into individual cells or remain together as a pair, chain, or clusters. The shape and arrangement of cells from stained smears helps microbiologists to preliminarily identify bacteria. In clinical settings, these results provide valuable information upon which initial treatment can be based. Common bacterial arrangements are shown in Figure 4.6.

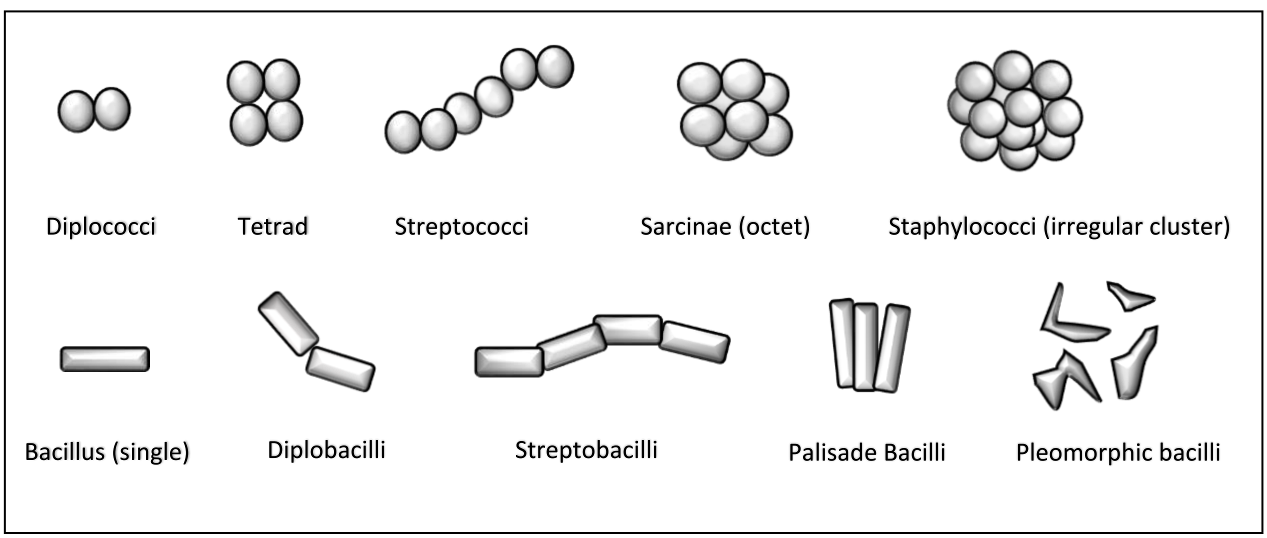


Figure 4.6: Bacterial cellular arrangements.

Exercise 4.2 – Bacterial Cellular Morphology & Arrangement

OBJECTIVE

Use the light microscope to observe and identify the shape and arrangement of bacterial cells.

MATERIALS

- EQUIPMENT: Light microscope, lens paper, lens cleaner, immersion oil, bacterial smears prepared in prior lab

PROCEDURE - *Take your time and work through steps in order.*

1. Place a stained slide of bacteria on the stage and secure it in the stage clips.
2. Follow steps from Exercise 4.1 to bring cells into focus under high power, raising the light.
3. Once the image is in focus under high power with high light, without adjusting the focus knobs, rotate the 40X objective to the side and place a large drop of immersion oil (several taps of the glass wand) directly on the slide.
4. Rotate the 100X objective lens without passing the 40X objective through the oil until it clicks into place. The oil should connect the bottom of the objective and the slide.
5. Focus the image by turning the fine focus (inner) knob **only**; using coarse focus under higher magnifications may crack the lens. Raise the rheostat light control on the base and fully open the diaphragm. You should observe pigmented cells against a white background.

   If you have trouble:

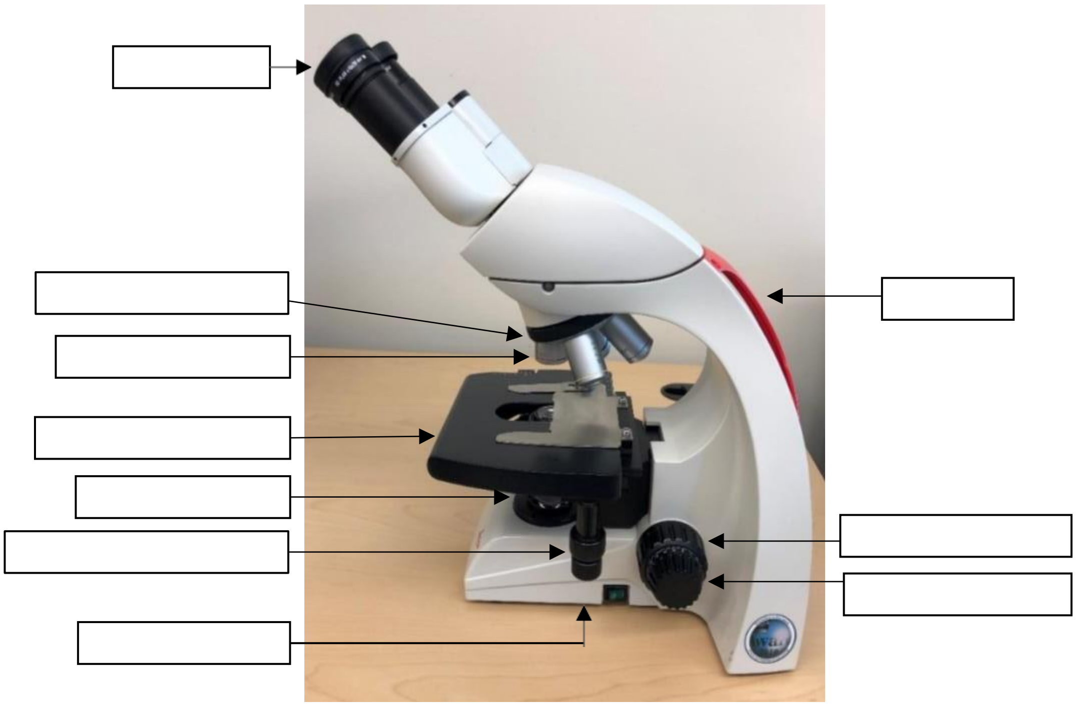
   - Make certain that the objective lens is clicked in place.
   - Check that the slide is flat on the stage and not over/under the clips.
   - Add additional oil.
   - Increase the amount of light.
   - Return to 10X and try again (no need to remove oil from the slide).

6. Once cells are in focus, use the slide adjustment knobs to observe an area near the edge of the smear that is less dense to determine the shape and arrangement of cells.
7. Cell size: Use the ocular micrometer (in one of the eyepieces) to measure cell length. Rotate the ocular to position the micrometer over a single cell. When using the oil immersion objective, each division of the micrometer is equivalent to approximately one micrometer.
8. Complete the Module 4 report.
9. When you are finished, dispose of all smears directly in the disinfectant beaker.

MICROSCOPY **DATE:_____________MICROSCOPE #:_____**

EXERCISE 4.1 – USING THE LIGHT MICROSCOPE

Label the parts of the microscope in the image below:



Complete the table:

Objective Lens	**Magnification of Objective Lens**	**Magnification of Ocular Lens**	**Total Magnification**
Scanning			
Low power			
High power			
Oil immersion			

Name five important things that you should do to properly store your microscope:

1. ______________________________
2. ______________________________
3. ______________________________
4. ______________________________
5. ______________________________

EXERCISE 4.2 – BACTERIAL CELL MORPHOLOGY

View all cells using oil and 1000X total magnification. Your instructor may ask you to raise your hand when you have an image in focus to verify that you are viewing the image accurately. Draw several large representative cells with colored pencils to depict bacterial shape and arrangement. Dispose of used slides in the disinfectant beaker.

SIMPLE STAIN 1

Organism:________________________________

Cell shape:__________________Size:_______µm

Arrangement:______________________________

Endospores (circle): Present / Absent

SIMPLE STAIN 2

Organism:________________________________

Cell shape:__________________Size:_______µm

Arrangement:______________________________

Endospores (circle): Present / Absent

GRAM STAIN 1

Organism:________________________________

Cell shape:________________Size:______µm

Arrangement:____________________________

Gram reaction (circle): Positive / Negative

GRAM STAIN 2

Organism:________________________________

Cell shape:________________Size:______µm

Arrangement:____________________________

Gram reaction (circle): Positive / Negative

UNKNOWN SAMPLE

Gram reaction (circle): Positive / Negative

Cell shape:________________Size:______µm

Arrangement:___________________________

NEGATIVE STAIN

Organism:______________________________

Cell shape:________________Size:______µm

Arrangement:___________________________

ACID-FAST STAIN 1

Organism:______________________________

Cell shape:________________Size:______µm

Arrangement:___________________________

Acid-fast result (circle): Positive / Negative

ACID-FAST STAIN 2

Organism:______________________________

Cell shape:________________Size:______µm

Arrangement:___________________________

Acid-fast result (circle): Positive / Negative

QUESTIONS FOR REVIEW

1. If a Gram-stained slide of *Staphylococcus* was over-decolorized, what color might cells appear when the slide was viewed microscopically?__________

2. Based on the Gram stain, could the unknown organism be *Escherichia coli*?_______________
   Why or why not?____
   ____

3. If safranin and crystal violet were switched for a Gram stain on a smear of Gram-negative bacteria, what color would cells appear when viewed microscopically?___________________

4. Two students used the same culture of *Mycobacterium* to prepare and stain slides by the Kinyoun method. When viewing their slides microscopically, one student observed cells, but the other student saw nothing at all on the slide. What was this student's most likely error?
   ____
   ____

5. Drainage from a post-surgical wound is sent to the micro lab for analysis. The physician suspects MRSA (methicillin-resistant *Staphylococcus aureus*), a prevalent healthcare-associated pathogen, is the cause of the infection. The initial lab report indicates that "heavy Gram-negative bacilli" are observed on the initial smear.
   Based on these results, is the physician correct?__________Explain.____________________
   ____
   ____

6. Using your knowledge from this lab, complete table regarding color of cells for each stain.

GENUS	GRAM STAIN	ACID-FAST STAIN	NEGATIVE STAIN
Staphylococcus		Blue	
Bacillus	Purple		
Escherichia			Colorless
Mycobacterium	Purple (weak)		

MODULE 5: Eukaryotic Microorganisms

LEARNING OUTCOMES

1. Summarize the major types of eukaryotic microorganisms.
2. Define the primary characteristic that distinguishes eukaryotic microbes from bacteria.

INTRODUCTION

The Domain *Eukarya* contains all eukaryotes, including unicellular or multicellular organisms such as protists, fungi, plants, and animals. The major defining characteristic of eukaryotes is that their cells contain DNA within a membrane-bound nucleus.

Eukaryotic microbes are an extraordinarily diverse group, including species with a wide range of life cycles, morphological specializations, and nutritional needs. Organisms classified in this domain include fungi (yeasts and molds), protists (protozoa and algae), helminths (flatworms and roundworms), and vectors of disease transmission such as insects and other arthropods. In this module, we will survey various eukaryotic organisms of clinical significance and review some of the major characteristics associated with each.

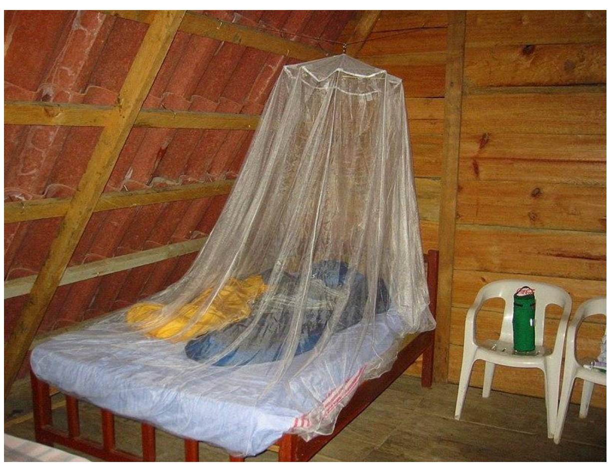


Figure 5.1: Mosquito netting is a primary defense against vector-borne illnesses like malaria, a disease caused by a eukaryotic parasite transmitted to humans by mosquitoes.

Exercise 5.1 – Fungi

LEARNING OUTCOMES

1. Discuss the beneficial role of fungi in the ecosystem.
2. Compare yeast and mold structure.
3. Identify and discuss examples of pathogenic and nonpathogenic fungi.

Fungi include unicellular yeasts and multicellular molds. The study of fungi is called *mycology*. Macroscopic fungi, such as mushrooms, may resemble plants but are quite different. Unlike plants, which are photosynthetic, fungi are heterotrophic, obtaining their nutrients from preformed organic matter. The cell walls of fungi are usually made from chitin rather than cellulose, and taxonomic classification is primarily based on reproductive strategies. Three major groups of fungi are the Ascomycota (sac fungi and yeast), Zygomycota (bread molds), and Basidiomycota (club fungi and mushrooms). The colorful but poisonous mushroom *Amanita*, known as the death cap, may appear innocuous but produces deadly toxins (Figure 5.2).

In addition to being environmental decomposers, fungi are used commercially to produce foods such as bread, cheeses, and alcoholic beverages. They are also major sources of antibiotics. While fungi exist as a normal part of the human microbiome, some cause opportunistic infections, or mycoses, particularly when a host's immune defenses are compromised.

Some fungi are dimorphic, meaning that they can exist as both yeasts and molds depending on environmental conditions. Unlike yeasts, which grow very rapidly, molds may take weeks to months to cultivate. Specialized media such as Sabouraud agar is used to cultivate molds in the clinical laboratory. Mycologists use both the microscopic appearance of sporangia, as well as the macroscopic topology (color and texture) of molds when grown on solid media, as aids in identification.

Figure 5.2: Amanita: a deadly mushroom.

Yeasts are unicellular, oval cells that reproduce asexually by budding. Cells are sometimes observed as short strands of elongated *pseudohyphae* (Figure 5.3). A common yeast that is available in dehydrated form is *Saccharomyces cerevisiae, or* brewer's yeast, which is widely used in breadmaking and in the production of alcoholic beverages.

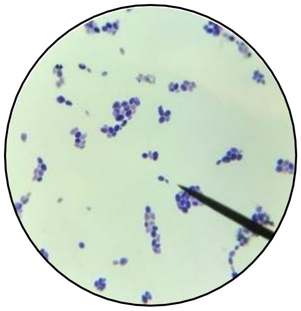


Figure 5.3: Budding Saccharomyces (left; 1000X); brewer's yeast (right).

Yeasts sometimes cause opportunistic mycoses when the immune defenses of a host are compromised, often following a primary infection. One example of an opportunistic mycosis is the overgrowth of *Candida albicans* yeast, which are present in the human microbiome. When a person is prescribed an antibiotic to treat a bacterial infection, *Candida* may overgrow on mucous membranes due to lack of competition once bacteria are killed, leading to genital yeast infection or oral thrush (Figure 5.4). Yogurt and other fermented foods that are rich in probiotic bacteria are often recommended as dietary supplements when antibiotics are prescribed.

Opportunistic yeasts such as *Pneumocystis* species can spread from person to person through the air. These yeasts are a leading cause of pneumonia among AIDS patients, as well as individuals with chronic diseases or autoimmune disorders.

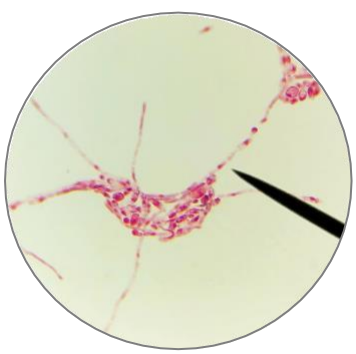


Figure 5.4: Candida albicans pseudohyphae; oral thrush (right).

Molds

Molds are multicellular fungi made of long filaments called *hyphae,* which form a visible mass called *mycelium.* Reproductive *sporangia*, which vary in color and type, grow at the end of hyphal stalks (Figure 5.5). These fungi are saprobes that feed on dead organic matter and thrive in a range of moist anaerobic environments, from soil to the dank walls of a basement. Some molds can cause allergies, while others produce disease-causing metabolites called mycotoxins. Molds are an important source of anti-microbial agents and pharmaceutical drugs. Penicillin, which is one of the most prescribed antibiotics, and cyclosporine, which is used to prevent organ rejection following a transplant, are products of molds.

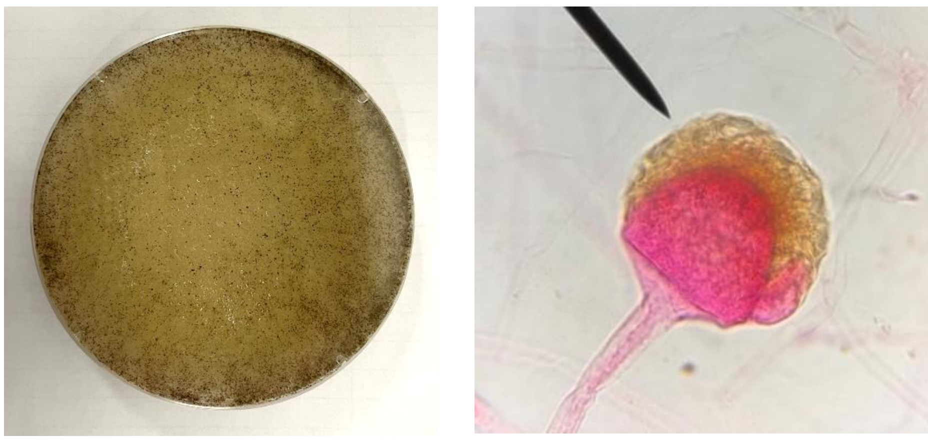


Figure 5.5: Rhizopus stolonifera mycelium (left); microscopic sporangium, 400X (right).

Molds are used in food production but can also lead to food spoilage. Species of the common bread mold *Rhizopus* are found in a wide range of foods, including jams, jellies, peanuts, and tobacco. The fermentation process by *Rhizopus* is used in the production of soy-derived foods such as tempeh. These molds are also associated with soft rot on fruits such as strawberries and tuberous vegetables like sweet potatoes.

The mold *Penicillium* includes many species of ecological, commercial, and agricultural significance. *Penicillium* species are ubiquitous in the environment and act as primary decomposers of organic matter. The first antibiotic – penicillin, a product of *Penicillium* mold – was discovered by Alexander Fleming in 1928. *Penicillium* is also a flavorful component of soft cheeses such as Roquefort, brie, and blue cheese (Figure 5.6).

Aspergillus is an opportunistic black mold that can cause lung infections and allergic reactions when spores are inhaled, and specialized remediation is necessary to remove these and other black molds from damp areas of homes (Figure 5.7). Other species of *Aspergillus* are contaminants of nuts and stored grains, producing potent toxins that can lead to cancer.

Figure 5.6: Penicillium notatum mycelium (left); microscopic sporangium, 400X (right).

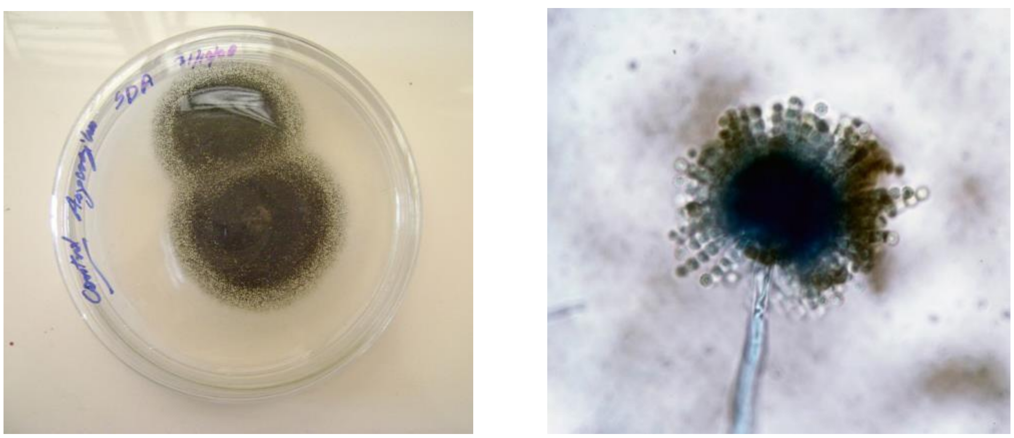


Figure 5.7: Aspergillus niger mycelium (left); microscopic conidiophore, 400X (right).

Exercise 5.1 – Fungi

OBJECTIVE

Distinguish representative organisms in the Kingdom Protista by microscopic observation.
Use proper terminology to classify and describe protozoa and algae.

MATERIALS

- EQUIPMENT: Microscope, lens paper, lens cleaner, Sta-clear paper, slides, coverslips, disposable Pasteur pipettes
- CULTURES: *Saccharomyces* broth; *Rhizopus, Penicillium, Aspergillus* on sealed Sabouraud agar plates
- SLIDES: Permanent mounts of *Candida* pseudohyphae, *Penicillium notatum, Aspergillus niger, Rhizopus* sp.

PROCEDURE – STUDENTS WORK IN SMALL GROUPS

1. Use a pipette to place a drop of *Saccharomyces* on a clean slide and add a coverslip.
2. Bring the slide in focus under high power, adjusting light as necessary to view cells.
3. Record your observations in the lab report.
4. Dispose of the slide in the disinfectant beaker without removing coverslip.
5. Obtain permanent slides of yeast and mold; clean them with lens cleaner and Sta-Clear paper.
6. Bring the organisms in focus using high power or oil immersion for yeast and low or high power for mold.
7. Record your observations in the lab report.
8. Remove oil from the slides and clean with lens cleaner and Sta-Clear paper.
9. Return the slide to the slide tray and give the plates back to the instructor.
10. Observe the topology of molds on Sabouraud agar plates (<u>do not open the plates</u>).
11. Record your observations in the lab report.

Exercise 5.2 – Protists

LEARNING OUTCOMES

1. Discuss the beneficial role of algae in the ecosystem.
2. Compare protozoa and algae regarding cell structure and nutritional mode.
3. Describe the cyst and trophozoite stages of protozoa.
4. List four groups of protozoa based on motility.
5. Identify and discuss examples of specific pathogenic and nonpathogenic protozoa.

Protists are a diverse group of unicellular eukaryotes that are not plants, animals, or fungi. Algae and protozoa are examples of protists. Algae (singular, alga) are plant-like protists that can be either unicellular or multicellular. Algae are photosynthetic organisms that extract energy from the sun and release oxygen and carbohydrates into their environment. Because other organisms can use their waste products for energy, algae are important parts of many ecosystems. Since algae are self-feeding autotrophs, they do not cause disease by breaking down tissues of other organisms for organic matter. However, some algae, such as the marine dinoflagellates, can overgrow under certain environmental conditions. When a population of dinoflagellates becomes particularly dense, an algal bloom can occur (Figure 5.8). Blooms of red algae, or "red tides," occur in coastal waterways. Neurotoxins released by algae during red tides can be harmful to humans and animals that are exposed to them.

Figure 5.8: Blue-green algae bloom on the shore of Catawaba Island, Ohio in Lake Erie.

Protozoa (singular: protozoan) are protists that make up the backbone of many food webs by providing nutrients for other organisms. Protozoa are remarkably diverse. Some protozoa are nonmotile, relying on other organisms such as insects or arthropods to carry them to their hosts. Others move with help from hair-like structures called cilia or whip-like structures called flagella. Some extend part of their cell membranes and cytoplasm into pseudopods – false feet – to propel themselves forward.

Protozoa inhabit a wide variety of aquatic and terrestrial habitats. Some protozoa are free-living photosynthetic autotrophs, while others are heterotrophic and feed on host organisms. During the growth part of their life cycle, protozoa are called *trophozoites.* While some protozoa exist exclusively in the trophozoite form, others enter an encapsulated *cyst stage* that protects the protozoa when environmental conditions become harsh (Figure 5.9).

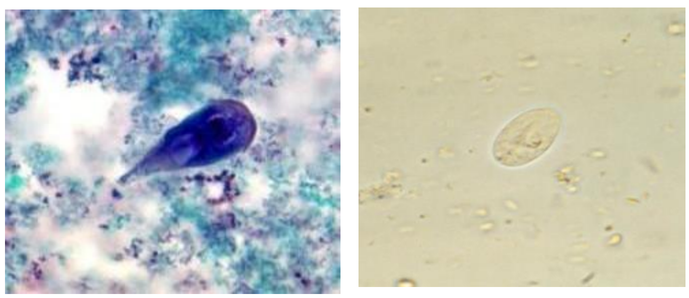


Figure 5.9: Giardia lamblia trophozoite in fecal trichrome stain (left);cyst in a fecal wet mount stained with iodine (right).

Most protozoa are harmless to humans and animals, but some are highly pathogenic. Water that is contaminated with sewage, particularly following a storm or flooding, often serves as a *vehicle* for transmission of pathogenic protozoa via the fecal-oral route to a host. Cysts may also enter the body through a cut, piercing, or surgical site. Some protozoa are carried to the host by a *vector,* such as an animal or insect, following a bite or exposure to an open wound.

Protozoans have unique organelles and sometimes lack organelles found in other cells (Figure 5.10). Some have contractile vacuoles that move water out of the cell for osmotic regulation (salt and water balance). Because they lack a cell wall, eukaryotic cells also have the unique ability to perform various types of endocytosis, the uptake of matter via enclosure by the plasma membrane to form a vacuole. When particulate matter or other cells are engulfed by endocytosis, the process is called phagocytosis, or “cell eating.” Organelles called lysosomes then fuse with the phagocytic vacuole, releasing powerful enzymes which digest the contents.

Although the classification of protists is complex and in flux, for simplicity we will divide the pathogenic protozoa by method of motility and review several representative examples.

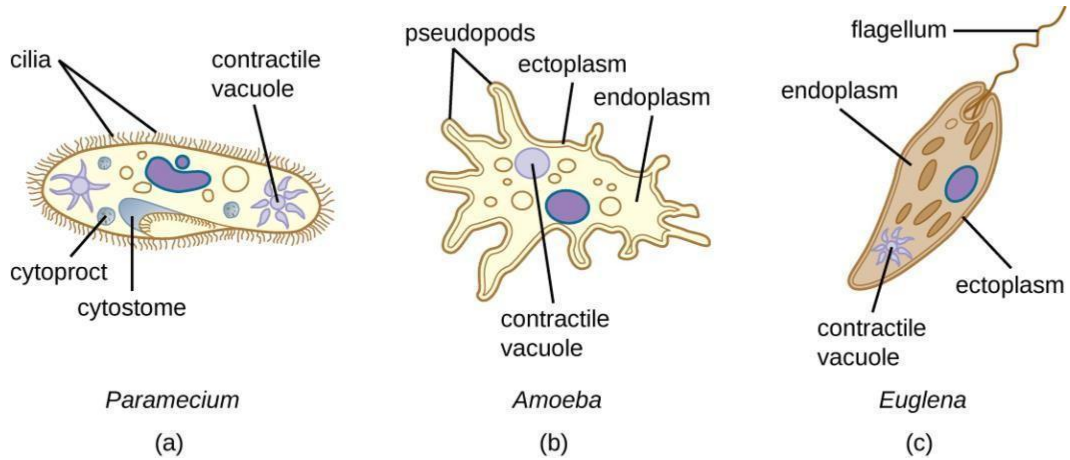


Figure 5.10: (a) A paramecium uses hair-like appendages called cilia for locomotion; (b) An amoeba uses lobe-like pseudopodia to anchor itself to a solid surface and pull itself forward; (c) A euglena uses a whip-like structure called a flagellum to propel itself.

Amoeboid Protozoa

Amoebae move via "false feet" or pseudopodia (Figure 5.11). Pseudopodia are formed by extensions of actin microfilaments into which protoplasm flows, thereby moving the organism. You may already have observed the locomotion of *Amoeba proteus*, a nonpathogenic protozoan that is often studied in high school biology labs.

Entamoeba histolytica is a pathogenic protist that causes amoebic dysentery, an intestinal disorder characterized by bloody diarrhea. It is transmitted when cysts from the feces of infected hosts are present in contaminated water. Once in the body, *E. histolytica* forms trophozoites that create flask-shaped ulcerations in the intestinal lining of the host. Scarring from dysentery often results in permanent damage to the colon and chronic colitis.

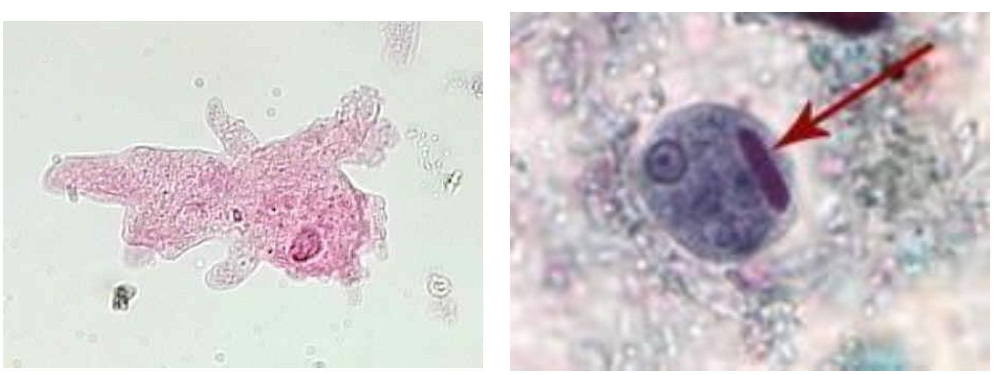


Figure 5.11: Amoeba proteus with many pseudopodia (left); Entamoeba histolytica cyst, with characteristic target-like nucleus and ribonuclear chromatoid body (right).

Ciliated Protozoa

The ciliates are a large, very diverse group characterized by the presence of short hairlike structures on their cell surfaces (Figure 5.12). Although the cilia may be used for locomotion, they are often used for feeding as well. *Paramecium caudatum* is a common nonpathogenic ciliated protozoan that lives in freshwater environments. *Balantidium coli* is the only parasitic ciliate that affects humans by causing intestinal illness, although it rarely causes serious medical issues except in immunocompromised individuals.

Figure 5.12: Paramecium caudatum with visible cilia, oral groove, and vacuoles (left); Balantidium coli isolated from the gut of a primate (right).

Flagellated Protozoa

Protozoa that move by one or more flagella include photosynthetic and non-photosynthetic species (Figure 5.13). They are widespread in the environment, and most do not cause disease. Pathogenic flagellates, such as *Giardia lamblia*, are commonly acquired via fecal-oral transmission from contaminated water. *Giardia* is a prevalent parasite in the United States and causes giardiasis, a diarrheal illness in which watery stools and vomiting lead to dehydration. Unlike *Entamoeba histolytica,* which invades the intestinal lining, *Giardia* does not cause dysentery.

Other pathogenic protozoa that move by flagella include *Trichomonas vaginalis,* a sexually transmitted parasite that infects both sexes. In females, trichomoniasis is characterized by genital odor, itching, and discharge while the disease is often asymptomatic in males. *Trichomonas* from infected individuals is occasionally observed during microscopic examination of urine. Because it is similar in size to white blood cells, it often surprises microbiologists who are viewing the specimen if it begins to swim across the field!

Flagellates of the genus *Trypanosoma* are spread by insect vectors. African sleeping sickness, caused by *T. brucei*, is transmitted through the bite of a tsetse fly, while Chagas disease (American trypanosomiasis) is associated with “kissing bugs” that carry *T. cruzi.* Both diseases lead to systemic illnesses that, if left untreated, are fatal.

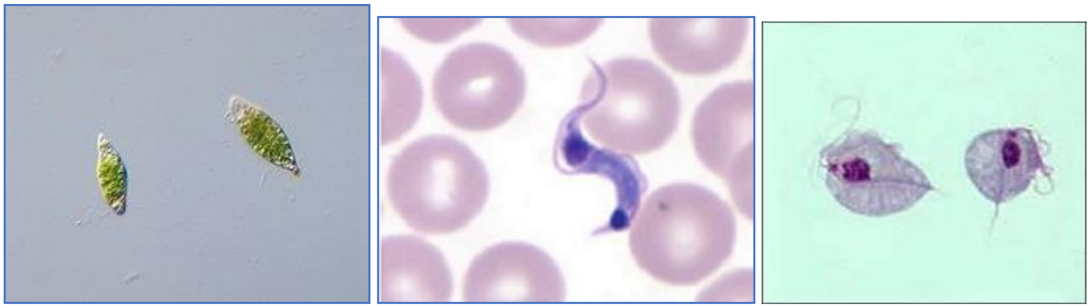


Figure 5.13: Three representative flagellates (from left): Euglena gracilis; Trypanosoma cruzi in whole blood; and *Trichomonas vaginalis.*

Apicomplexa Protozoa

The apicomplexans have complex life cycles that often depend on multiple hosts. *Toxoplasma gondii* causes toxoplasmosis, a disease transmitted from cat feces, unwashed produce, or undercooked meat. *Toxoplasma* can cross the placenta to enter the uterus and potentially cause serious fetal birth defects; therefore, handling cat litter is ill-advised during pregnancy. Recent evidence also links *Toxoplasma* with certain changes in behavior and personality traits, including suicidal ideation[1].

Plasmodium, including *P. vivax* and *P. falciparum*, is the cause of malaria. These apicomplexans undergo several stages of development in mosquitoes and humans. Following a bite, *Plasmodium* enters the host's blood and circulates to the liver where it continues to develop and is periodically released. During these episodes, the parasite is visible within infected red blood cells on smears (Figure 5.14). The illness is characterized by high fever, chills, and malaise. Despite over a century of research and clinical advancements, malaria remains one of the most challenging infectious diseases in the world to control and to treat, with most cases occurring in Africa.

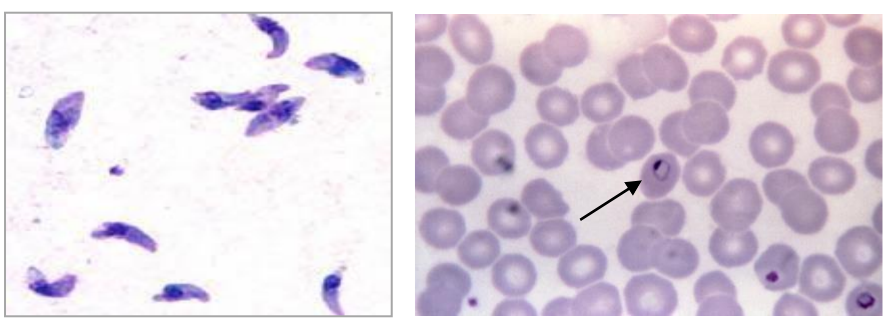


Figure 5.14: Stained whole blood smears from patients with toxoplasmosis (left) and malaria (right).

References

1. Jaroslav Flegr, Effects of *Toxoplasma* on Human Behavior, *Schizophrenia Bulletin*, Volume 33, Issue 3, May 2007, Pages 757–760, https://doi.org/10.1093/schbul/sbl074

Exercise 5.2 – Protists

OBJECTIVE

Distinguish representative organisms in the Kingdom Protista by microscopic observation.
Use proper terminology to classify and describe protozoa and algae.

MATERIALS

- EQUIPMENT: Microscope, lens paper, lens cleaner, Sta-clear paper, immersion oil, slides, coverslips, disposable Pasteur pipettes
- SOLUTIONS: Methyl cellulose
- CULTURES: Live *Amoeba, Paramecium, Euglena*
- SLIDES: Permanent mounts of various protozoa: *Entamoeba, Giardia, Trichomonas, Trypanosoma, Toxoplasma, Plasmodium*

PROCEDURE – WORK IN SMALL GROUPS

1. Use a disposable pipette to place a drop of live protozoa on a clean slide; add a coverslip (for *Paramecium,* add a drop of methyl cellulose prior to adding the coverslip to slow movement of cells).
2. **Lower the light** and scan the slide using the 4X objective. Move in a systematic direction to scan all areas of the slide as shown in Figure 5.15.

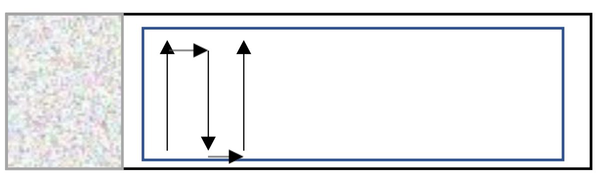


Figure 5.15. Scanning direction

3. When you find an organism, move to a higher objective where the cell is best observed; prepare additional slides if necessary.
4. Record your observations in the lab report and dispose of wet mount slides in the disinfectant beaker without removing coverslips.
5. Obtain a prepared slide of protozoa and clean it with lens cleaner and Sta-Clear paper.
6. **Raise the light** and view the slide under 1000X with immersion oil.
7. Record your observations in the lab report
8. Remove oil from the slides and clean with lens cleaner and Sta-Clear paper.
9. Return all slides to the slide tray.

Exercise 5.3 – Helminths & Arthropod Vectors

LEARNING OUTCOMES

1. Explain why helminths and vectors are included within the discipline of microbiology.
2. Identify and describe several examples of pathogenic nematodes and platyhelminthes.
3. Identify and describe several examples of insect and arachnid arthropod vectors.

Helminths and vectors are often included within the study of microbiology despite being macroscopic in appearance. Helminths are parasitic worms that are often identified by their microscopic eggs and larvae, and vectors are most commonly insects or arthropods that act as intermediate hosts for disease-causing microorganisms that can be carried to humans and animals. Both groups are multicellular eukaryotes that are classified in the kingdom *Animalia.*

Helminths

There are two major groups of parasitic helminths: the nematodes (roundworms) and the platyhelminthes (flatworms). Parasitic forms may have complex reproductive cycles with several different life stages and more than one type of host. Some are hermaphroditic, having both male and female reproductive organs.

Nematodes (Roundworms)

Nematodes are members of a diverse phylum that contains more than 15,000 species. Pinworm infection, characterized by severe anal itching, is caused by the thin, small, white roundworm *Enterobius vermicularis*. It is transmitted by the fecal-oral route and most common among children in day care and preschool settings. Diagnosis is made by observing the eggs microscopically after collection using a small paddle with cellophane tape pressed against the anus. Eggs can persist on bedding and clothing for several weeks, so good hand hygiene and meticulous laundering of potentially infected items with hot water is required during treatment.

Figure 5.16: Adult male pinworm (left) and eggs captured on cellulose tape.

Another nematode, *Ascaris lumbricoides*, is the largest nematode intestinal parasite found in humans (Figure 5.17). Females may reach lengths greater than 1 meter. It may cause symptoms ranging from relatively mild abdominal pain to severe intestinal blockage.

Figure 5.17: Ascaris lumbricoides removed from a 14-year-old patient with intestinal obstruction.

Platyhelminthes (Flatworms)

This group includes flukes, tapeworms, and planarians. Flukes and tapeworms are medically important parasites that attach to the inner walls of the intestines and other organs, causing anemia, malnutrition, abdominal pain, and sometimes death.

Tapeworms of the genus *Taenia* are segmented flatworms having a *scolex* at the head region that contains a circle of hooks and suckers which attach to intestinal wall of the host (Figure 5.18). The body of the worm is made up of segments called *proglottids* that contain reproductive structures and can detach following fertilization. The beef tapeworm *T. saginata* and the pork tapeworm *T. solium* are transmitted to humans through ingestion of contaminated undercooked meat. Some human tapeworms can grow to lengths of several meters or more.

Fig 5.18: Taenia saginata, approximately 4 meters long (left); hook-like scolex of Taenia solium (right).

Arthropods

Arthropods are biological vectors such as insects and arachnids that carry pathogenic microorganisms in or on their bodies (Figure 5.19). Organisms multiply within the vector and are introduced to the host through physical contact, usually a bite. Mechanical transmission occurs when an infectious agent is carried on outside of the body of a vector. For example, flies that land on feces can pick up bacteria on their feet and then transfer it to food.

Common insect vectors are mosquitoes, flies, and fleas. Insects have six legs and bodies that are divided into three segments: head, thorax, and abdomen. The *Anopheles* mosquito is an intermediate host to *Plasmodium* protozoa, the pathogen that causes malaria. *Glossina*, also known as the tsetse fly, carries *Trypanosoma* protozoa, which cause African sleeping sickness. Bacterial diseases are also associated with insect vectors. Bubonic plague, or Black Death, is caused by bacteria which are transmitted to humans by rat flea vectors.

In contrast to insects, arachnid vectors have eight legs and two body segments: cephalothorax and abdomen. Examples of arachnids are ticks, spiders, and mites. The bacteria that cause Lyme disease in humans are carried in the saliva of ticks. The deer tick *Ixodes* has multiple intermediate hosts, including mice and coyotes.

While treatment and/or vaccines are available for many diseases that are transmitted by vectors, controlling the vector population in the wild is an important strategy for limiting the spread of these diseases.

Figure 5.19: Insects such as the Anopheles mosquito (left) and arachnids such as the deer tick Ixodes (right) are significant disease-associated arthropod vectors.

Exercise 5.3 – Helminths & Arthropod Vectors

OBJECTIVE

Distinguish representative helminths and vectors by microscopic observation.

MATERIALS

- EQUIPMENT: Microscope, lens paper, lens cleaner, Sta-clear paper
- MORGUE: Preserved flat and roundworms
- SLIDES: *Taenia* scolex, *Enterobius* eggs, *Anopheles* mosquito, *Ixodes* tick

PROCEDURE – STUDENTS WORK IN SMALL GROUPS

1. Obtain prepared slides and clean them with lens cleaner and Sta-Clear paper.
2. Bring the organisms in focus with the scanning objective.
3. Record your observations in the lab report.
4. Return the slide to the slide tray.
5. Observe the preserved specimens of flatworms and roundworms and complete the report.

BIO 211 MODULE 5 REPORT **NAME:_______________________________**
EUKARYOTIC MICROORGANISMS **DATE:_____________MICROSCOPE #:_____**

EXERCISE 5.1 – FUNGI

YEAST – *View under high power or oil if needed. Label parent cell, bud, pseudohyphae if present.*

Genus:_____________________________ Genus:______________________________

TM:_________X /Instructor check_____ TM:__________X /Instructor check_______

MOLDS– *View under high power near edge of the mycelium. Label the hyphae with sporangium.*

Genus:_________________ Genus:_________________ Genus:__________________

TM:______X / Check_____ TM:_________X / Check_____ TM:______X / Check_______

SABOURAUD AGAR TOPOLOGY – *Describe color and texture of growth on plates.*

GENUS	SURFACE	REVERSE

BIO 211 MODULE 5 REPORT **NAME:_______________________________**

EXERCISE 5.2 – PROTISTS

LIVE – *View with scanning, then low/high power; lower the light; indicate N/A for disease.*
PERMANENT – *View with the 100X objective under oil immersion. Raise the light as needed.*

Genus:____________________
Motility:__________________
Disease:__________________
TM:________X / Check:_____

Genus:____________________
Motility:__________________
Disease:__________________
TM:________X / Check:_____

Genus:____________________
Motility:__________________
Disease:__________________
TM:________X / Check:_____

Genus:____________________
Motility:__________________
Disease:__________________
TM:________X / Check:_____

Genus:____________________
Motility:__________________
Disease:__________________
TM:________X / Check:_____

Genus:____________________
Motility:__________________
Disease:__________________
TM:________X / Check:_____

BIO 211 MODULE 5 REPORT **NAME:________________________________**

EXERCISE 5.3 – HELMINTHS & ARTHROPOD VECTORS

HELMINTHS

Genus:__________________________

Flat or roundworm:_______________

Disease:_________________________

TM:___________X / Check:_________

Genus:__________________________

Flat or roundworm:_______________

Disease:_________________________

TM:___________X / Check:_________

ARTHROPOD VECTORS

Genus:__________________________

Insect or arthropod:______________

Disease:_________________________

TM:___________X / Check:_________

Genus:__________________________

Insect or arthropod:______________

Disease:_________________________

TM:___________X / Check:_________

MODULE 6: Media and Aerotolerance

LEARNING OUTCOMES

1. Explain the importance of optimal environmental conditions for microbial growth.
2. Describe methods used to transport or cultivate microbes with specific growth requirements.

INTRODUCTION

The growth of microorganisms depends on available nutrients as well as a favorable environment. Microbes flourish where temperature, moisture, pH, and other physical conditions are optimal. These requirements significantly vary among organisms. Bacteria that grow in hot springs have different needs than those found in polar regions. Likewise, organisms such as the archaebacteria, which often require extreme environments for growth, are unlikely pathogens of humans and animals.

When cultivating organisms in a clinical setting, microbiologists must consider the type of growth medium, incubation temperature, amount of oxygen, and available water that are necessary to support growth of the suspected pathogen. For example, oxygen-free glove box chambers are designed to cultivate anaerobic organisms that might otherwise be killed if they were handled out on the bench. Commercial transport systems provide a suitable environment for most organisms that are present in a patient sample to survive between collection and delivery to the microbiology laboratory (Figure 6.1).

In this module, you will cultivate bacteria based on their oxygen requirements and nutritional need. Table 6.1 summarizes the various types of media used in these exercises.

Figure 6.1: Microbiologists use anaerobic glove boxes to work with bacteria that require oxygen-free conditions (left); a culture transport system (right).

Table 6.1: Common Microbiological Media

Medium	Purpose	Principle	Results
BAP Blood Agar Plate	Nonselective; often used to differentiate streptococci by hemolysis	TSA with 5% sheep red blood cells; bacterial hemolysins act on red blood cells in the agar	Beta (β) = complete hemolysis, clear zone Alpha (α) = partial hemolysis, brown zone Gamma (γ) = no hemolysis, no zone
MSA Mannitol Salt Agar	Selective for *Staphylococcus*; differentiates between *S. aureus* and other *Staphylococcus* sp.	Salt inhibits most non-staphylococcal; *S. aureus* ferments mannitol to turn agar yellow	*S. aureus* = growth; fermentation *Staphylococcus* sp. = growth; no fermentation Gram (-) = no growth
EMB Eosin Methylene Blue Agar	Selective for Gram-negative bacteria, particularly lactose fermenting coliforms	Eosin and methylene blue dyes inhibit Gram-positives; lactose fermentation forms dark colonies	Gram (+) = no growth Gram (-) LF = purple/green sheen Gram (-) NLF = pink
MAC MacConkey Agar	Similar to EMB	Same principle as EMB except crystal violet replaces eosin and methylene blue dyes	Gram (+) = no growth Gram (-) LF = dark pink Gram (-) NLF = colorless
CET Cetrimide Agar	Highly selective for *Pseudomonas* species	Cetrimide inhibits most bacteria; enhances growth and pigment production by *P. aeruginosa*	*Pseudomonas* = growth with pyocyanin; fluorescent under UV Non-pseudomonads = no growth
FTM Fluid Thioglycolate Medium	Enriched, reduced broth to cultivate anaerobes and determining aerotolerance	Oxic and anoxic zones; contains a resazurin indicator which turns pink where oxygen is present	Obligate anaerobes = below resazurin Obligate aerobes = growth in resazurin Facultative anaerobes = heavier in resazurin Aerotolerant = even growth throughout

Exercise 6.1 – Selective and Differential Media

LEARNING OUTCOMES

1. Name and state the purpose of all-purpose, selective, differential, and enriched media.
2. Identify bacterial growth patterns on MSA, EMB, MacConkey, and cetrimide agars.
3. Distinguish streptococcal hemolysis patterns on blood agar.

The study of microorganisms is greatly facilitated once we can culture them: that is, to keep reproducing populations alive under laboratory conditions. The number of available media with which to grow bacteria is considerable. General, or all-purpose, media support growth of many organisms. Prime examples of all-purpose media are trypticase soy broth and agar.

Specialized media are used in the identification of bacteria and are supplemented with dyes, pH indicators, or antibiotics. *Selective* media contain nutrients that support the growth of a particular organism of interest and have additional inhibitory agents, such as salts or dyes, which suppress growth of unwanted microbes. *Differential* media have an additional agent, such as sugar or blood, utilized by certain bacteria and not others. This helps microbiologists to distinguish between types of bacteria that grow on the agar.

Mannitol salt agar (MSA) is one type of medium that is both selective and differential. It contains 7.5% salt, the sugar mannitol, and a phenol red indicator. High salt inhibits Gram-negative bacteria and many Gram-positive bacteria, while selecting for salt-tolerant *Staphylococcus*. Fermentation of mannitol by *S. aureus* results in acid production, lowering the pH of the medium and turning the phenol red indicator from red to yellow. Other staphylococcal species grow on the agar but do not ferment mannitol or produce a color change (Figure 6.2).

Figure 6.2: Staphylococcus epidermidis (left) and Staphylococcus aureus (right) on mannitol salt agar. Fermentation of mannitol by S. aureus produces acid that lowers the pH, turning the phenol red indicator in the medium from red to yellow.

Two selective and differential media for Gram-negative bacteria are eosin methylene blue (EMB) and MacConkey agars. These agars contain dyes that inhibit Gram-positive bacteria, and the sugar lactose which is fermented by coliform bacteria. Coliforms are lactose-fermenting Gram-negative enteric or intestinal bacteria and grow as purple or iridescent green colonies on EMB or bright pink colonies on EMB (Figure 6.3). A high coliform count in environmental water samples after a flood or heavy storm indicates that the water is contaminated with sewage and thereby poses a public safety threat.

Figure 6.3: Escherichia coli on EMB agar (left) and MacConkey agar (center). Salmonella sp., a non- lactose fermenter, on MacConkey (right).

Enriched media contain growth factors, vitamins, and other essential nutrients to promote the growth of fastidious, or nutritionally demanding, microorganisms. Enriched agars can also be differential, as in the case of the blood agar plate (BAP). Blood agar is TSA medium with 5% sheep red blood cells. It is nonselective and is frequently used to distinguish hemolysis patterns of various streptococci (Figure 6.4).

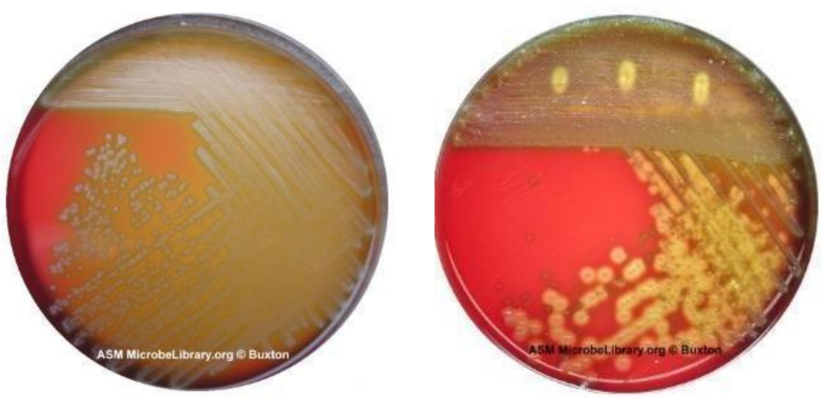


Figure 6.4: Streptococcal hemolysis patterns. Alpha hemolysis (left) is characterized by a greenish-brown zone around colonies due to methemoglobin release from partial lysis of red blood cells. Beta hemolysis (right) results in a clear zone around colonies following complete lysis.

Streptococcus pyogenes, or Group A streptococci, are associated with acute pharyngitis (commonly called "strep throat"). These bacteria produce proteins that completely hemolyze red blood cells. The breakdown of the red blood cells results in a loss of color around the colonies growing on the agar. This is called *beta* (β) hemolysis. The viridans streptococci, such as *S. mutans* and *S. salivarius,* are present on mucous membranes of the upper respiratory tract. These streptococci partially hemolyze red blood cells to release methemoglobin which changes the color of red blood cells to brownish green. We call this action *alpha* (α) hemolysis. Finally, Group D enterococci are streptococci that live in the intestinal microbiome. These bacteria, which include *Enterococcus faecalis* and *E. faecium*, do not hemolyze red blood cells. Since there is no hemolytic activity, the color of the agar around the colonies remains red. This is termed *gamma* (γ) hemolysis, which really means no hemolysis.

Some additives to media make them highly selective and differential for a particular organism of interest. Cetrimide agar inhibits most bacteria other than *Pseudomonas* species. When grown on cetrimide agar, *P. aeruginosa* produces a characteristic blue-green pigment called pyocyanin and a yellow-green pigment called pyoverdine, which exhibits fluorescence when colonies on the plate are held under ultraviolet light.

Enriched, selective, and differential media play key roles in the identification of bacteria. In this exercise, we will use mannitol salt agar and blood agars to differentiate the Gram-positive staphylococci and streptococci respectively, and eosin methylene blue (EMB), MacConkey agar, and cetrimide agars to differentiate lactose-fermenting Gram-negative bacteria and *Pseudomonas*.

Exercise 6.1 – Selective and Differential Media

OBJECTIVE

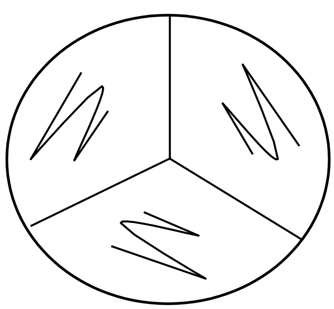
Use selective and differential agar to distinguish staphylococci and enteric bacteria.

MATERIALS

- EQUIPMENT: Inoculating loop, incinerator, marking pen
- MEDIA: Mannitol salt agar (MSA), eosin methylene blue agar (EMB), MacConkey agar (MAC), cetrimide agar (CET)
- DEMO PLATE: Blood agar plate (BAP) for follow-up – demo provided by instructor
- CULTURES: *Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis, Klebsiella aerogenes, Pseudomonas aeruginosa, Alcaligenes faecalis*

PROCEDURE – STUDENTS WORK IN PAIRS

1. Label the bottom of an MSA, an EMB, a MAC, and a CET plate with your initials and date, and divide each plate into three sections.
2. Label and inoculate each section of the plate in a "Z" pattern with the following organisms:
    - MSA plate: *S. aureus, S. epidermidis, E. coli*
    - EMB and MAC plates: *E. coli, K. aerogenes, P. aeruginosa*
    - CET plate: *E. coli, P. aeruginosa, A. faecalis*
3. Invert plates and place them in a common rack for incubation at 37ºC for 18-24 hours.



Avoid over-streaking!

Excess growth may result in a color change that spreads into the adjacent section, giving a false positive result.

FOLLOW UP

1. Observe MSA agar for growth and for mannitol fermentation, which turns the agar yellow.
2. Observe EMB and MAC agars for growth and for lactose fermentation, which darkens colonies.
3. Observe CET agar for growth and UV fluorescence.
4. BAP demo plate for alpha, beta, and gamma hemolysis (do not unwrap parafilm).
5. Record results and complete the lab report.
6. Return the BAP to the instructor and dispose of remaining plates in the discard bucket.

Exercise 6.2 – Aerotolerance

LEARNING OUTCOMES

1. Describe categories of microbes regarding oxygen and carbon dioxide requirement.
2. Identify growth patterns of bacteria in a reduced broth medium.
3. Explain principles of thioglycolate medium and the anaerobic jar for cultivating anaerobes.

Microbes have evolved different strategies for growing with or without oxygen. One oxygen-dependent pathway that many organisms use to produce energy (in the form of ATP) is aerobic respiration. During this process, unstable free radicals such as superoxide (O_2^-) and peroxide (H_2O_2) are also generated. These radicals seek electrons from DNA and other biomolecules, thus damaging cells. Organisms that grow in the presence of oxygen must produce special enzymes, such as superoxide dismutase and catalase, to neutralize any reactive species that may form.

Bacterial aerotolerance is classified into several categories based on oxygen requirements. *Obligate aerobes* require oxygen to grow, while *obligate anaerobes* are killed in its presence. *Facultative anaerobes* prefer oxygen when it is available but can grow without it. *Aerotolerant* organisms do not use oxygen, nor are they killed by it. Finally, *microaerophiles* use lower amounts of oxygen, preferring other gases such as carbon dioxide instead (Figure 6.5).

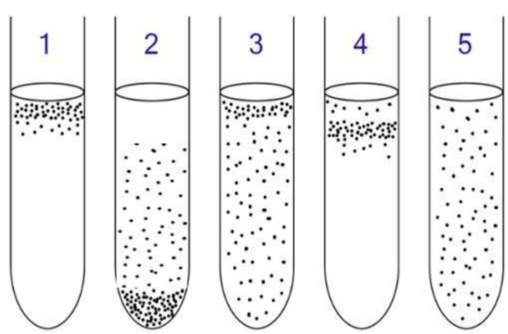


Figure 6.5: Aerotolerance patterns in reduced broth media: (1) Obligate aerobe; (2) Obligate anaerobe; (3) Facultative anaerobe; (4) Microaerophile; (5) Aerotolerant bacteria.

In this exercise, two methods for determining bacterial aerotolerance are compared. In the first procedure, bacteria are inoculated into fluid thioglycolate medium (FTM) and allowed to grow. The addition of thioglycolate to nutrient broth makes FTM a reduced medium, because thioglycolate chemically removes most of the oxygen from the broth. Following incubation, growth will be most dense where oxygen concentration is best suited for growth of each organism (Figure 6.6). Resazurin, a chemical that turns pink in the presence of oxygen, is also added to the broth as an indicator. The oxic zone appears as a pink region at the surface of the broth where oxygen has diffused from the air. The anoxic zone is the anaerobic area below the oxic zone and does not have a pink color.

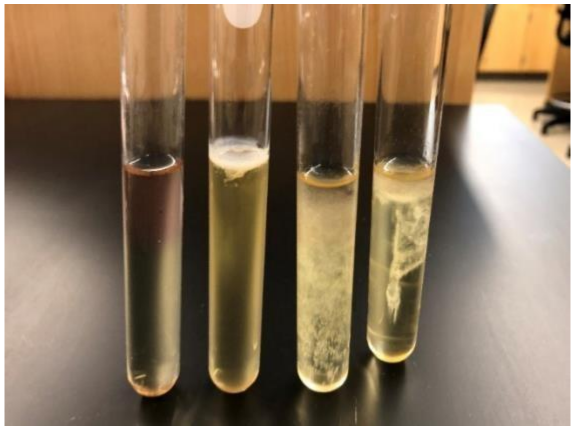


Figure 6.6: Growth patterns in fluid thioglycolate medium. From left: Uninoculated control tube, obligate aerobe, obligate anaerobe, and facultative anaerobe.

The second method uses an anaerobic jar containing a chemical pack (e.g., GasPak) that releases hydrogen and carbon dioxide gases upon activation (Figure 6.7). Hydrogen binds any oxygen inside the jar to form water, which is absorbed on moisture-wicking pellets, creating an environment in which anaerobic bacteria can grow. A methylene blue indicator strip is also added to the jar. The indicator strip is blue in the presence of oxygen but becomes colorless in its absence, thus ensuring that the conditions within the jar remain oxygen-free during incubation.

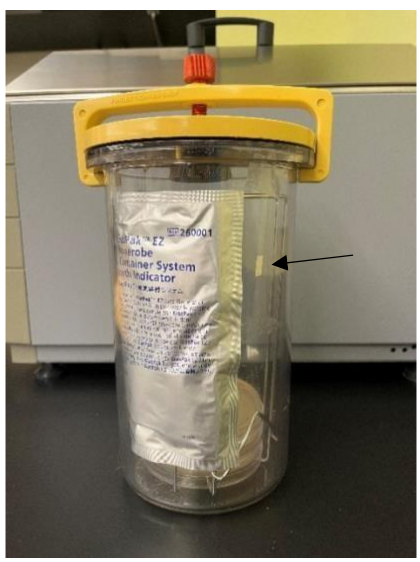


Figure 6.7: Anaerobic jar. The arrow points to the colorless methylene blue strip, indicating that conditions inside the jar are anaerobic.

Exercise 6.2 – Aerotolerance

OBJECTIVE

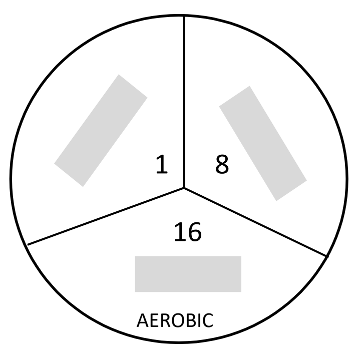
Determine bacterial aerotolerance patterns using reducing broth and anaerobic jar methods.

MATERIALS

- EQUIPMENT: Inoculating loop, incinerator, labeling tape, marking pen, anaerobic jar, GasPak catalyst with methylene blue indicator
- MEDIA: Fluid thioglycolate medium (FTM broth), TSA plates
- CULTURES: *Escherichia coli, Pseudomonas aeruginosa, Clostridium sporogenes*

PROCEDURE – STUDENTS WORK IN PAIRS

1. Using tape, label three FTM broth tubes with your initials, date, and organism number.
2. Aseptically inoculate each broth under the under the resazurin zone. Do not shake the loop!
3. Finger-tighten caps and put tubes in a common rack for incubation at 37°C for 18-24 hours.
4. Obtain a TSA plate, labeling it "aerobic" and with your initials and date, and dividing it into three sections labeled with each organism number as shown in the diagram below.
5. Aseptically spot inoculate each section of the plate by dragging the loop in a line on the agar.
6. Invert the plate and place in a common rack for incubation at 37°C for 18-24 hours.
7. The instructor will demonstrate the use of the anaerobic jar and GasPak system.



Spot inoculation of the aerobic plate

FOLLOW UP

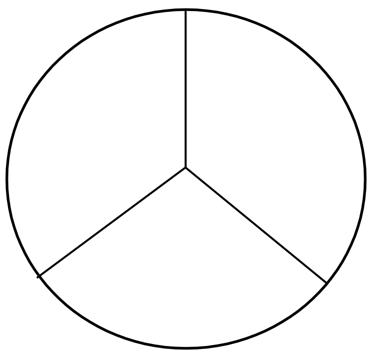
1. Evaluate growth in the FTM tubes and on the aerobic and anaerobic plates.
2. Complete the lab report.
3. When you are done, remove tape from the broth tubes and place them in a common rack for autoclaving; return the anaerobic plate to the instructor and dispose of the remaining plates in the Petri plate discard bucket.

MEDIA & AEROTOLERANCE **DATE:__________PARTNER INITIALS:_______**

EXERCISE 6.1 – SELECTIVE & DIFFERENTIAL MEDIA

OBSERVATIONS: Use colored pencils to draw the appearance of each plate, labeling all organisms.

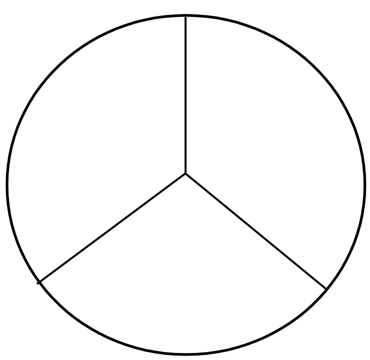
BLOOD AGAR



TYPE	ORGANISM	HEMOLYSIS APPEARANCE
Beta		
Alpha		
Gamma		

1. Which ingredient makes BAP differential?___________________Is this agar selective?________
2. Acute pharyngitis (strep throat) is associated with which streptococcal hemolysis type?_____
3. Where in the body do gamma hemolytic streptococci predominate?______________________

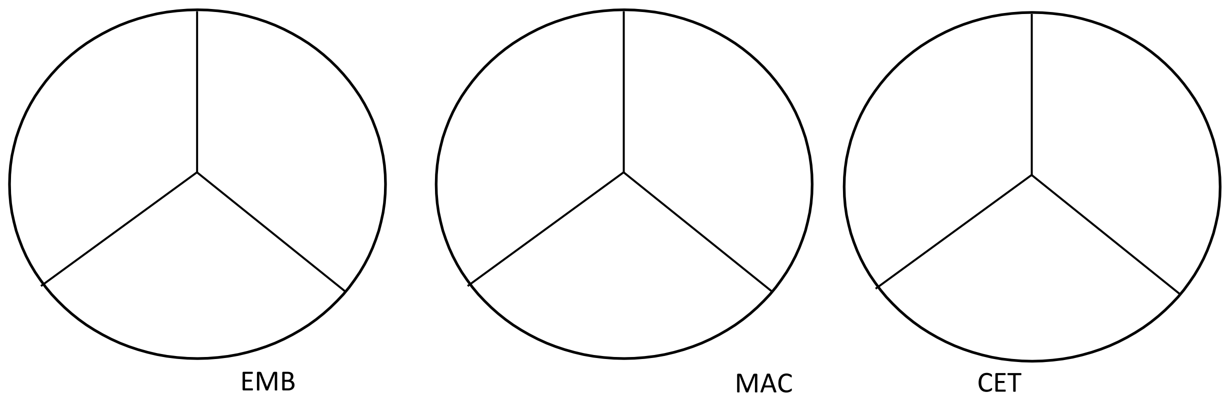
MANNITOL SALT AGAR



ORGANISM	APPEARANCE ON MSA

4. Which ingredient makes MSA selective?_________________Differential?___________________
5. What can you conclude based on the results?____

EOSIN METHYLENE BLUE, MACCONKEY & CETRIMIDE AGARS



ORGANISM	APPEARANCE ON EMB	APPEARANCE ON MAC	APPEARANCE ON CET

6. Which ingredients make EMB and MAC selective?____________________________________
7. Which ingredient makes EMB and MAC differential?__________________________________
8. Were results for growth and fermentation similar for EMB and MAC agars?______________
9. Which of the organism(s) tested are coliforms?______________________________________
10. Which ones are non-lactose fermenting, Gram-negative bacteria?______________________
11. How does the color of lactose-fermenting coliforms on EMB differ from those on MAC?_____

____

12. Would you expect a coliform to grow on blood agar?_________Why or why not?___________

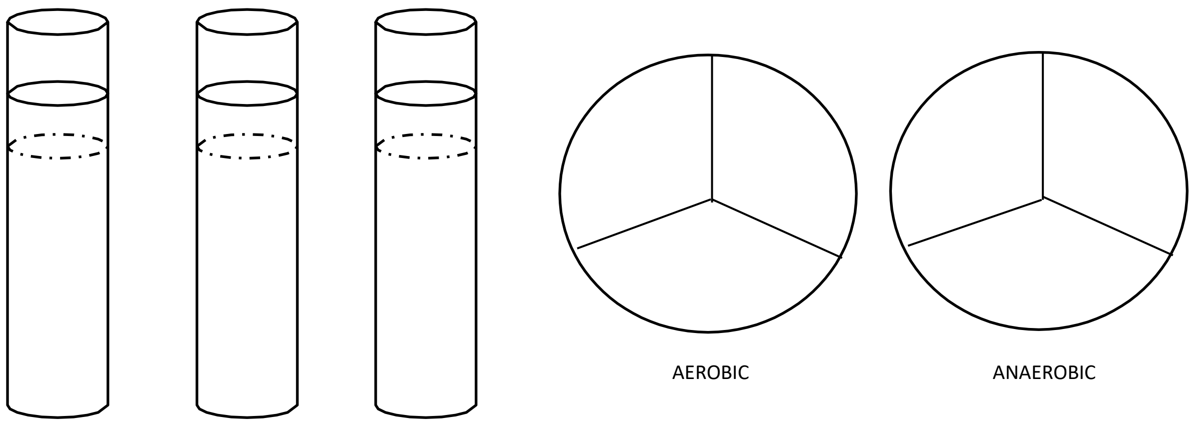
____

____

13. Is cetrimide agar selective?________________________Differential?____________________

EXERCISE 6.2 – AEROTOLERANCE

OBSERVATIONS: Draw growth patterns in FTM broth and on plates, labeling all organisms. Complete the table regarding aerotolerance type: **obligate aerobe, obligate anaerobe, facultative anaerobe**, or **aerotolerant**.



ORGANISM	AEROTOLERANCE TYPE

QUESTIONS FOR REVIEW

1. What color was the resazurin in the FTM oxic zone prior to inoculation?__________________
2. If FTM tubes were accidentally vortexed before observing, what aerotolerance type would all organisms appear to be?________________________Explain.__________________________

3. What color should the methylene blue indicator be before opening the jar?_______________ What does this indicate about conditions inside the jar?_______________________________

4. Would you expect *Pseudomonas* to cause a deep wound infection?_________Why or why not?____

MODULE 7: Control of Growth

LEARNING OUTCOMES

1. Describe various chemical and physical methods of controlling microbial growth.
2. Compare sterilization, antisepsis, disinfection, and sanitization.
3. Discuss the discovery of chemotherapeutics and the emergence of antibiotic resistance.

INTRODUCTION

To prevent the spread of human disease, it is necessary to control the growth and abundance of microbes through physical or chemical control methods. Many of these methods kill cells by dissolving membranes, disrupting osmotic balance, or denaturing proteins and/or nucleic acids. When selecting an antimicrobial method, it is necessary to consider the surface to be treated as well as the resistance level of the microorganisms targeted. For example, surgical instruments require a much higher level of cleanliness than clothes washed in a laundry machine.

The most extreme protocols for microbial control aim to achieve sterilization: the complete removal or killing of all vegetative cells, endospores, and viruses from the targeted item or environment. Sterilization can be accomplished by using very strong chemicals or gases, or by physical means, including moist or dry high heat, ionizing radiation, or ultrafiltration. Most laboratories and clinical facilities use an *autoclave* for sterilizing glassware, media, and instruments. An autoclave is a chamber that is heated to 121 °C for a minimum of 15 minutes to ensure that all microorganisms and endospores are killed. For heat-sensitive or flammable materials, ethylene oxide gas treatment is an effective means of sterilization.

Although sterilization is ideal for many medical applications, it is not always practical for other purposes. *Pasteurization* is one form of microbial control for food that kills pathogens and reduces the number of spoilage-causing microbes while maintaining food quality. This is achieved by exposing products to moderate heat treatment (typically 72°C) for short periods of time (typically 15 seconds). The process was first developed by Louis Pasteur in the 1860s as a method for preventing the spoilage of beer and wine and is still used today for many products such as milk, juices, cheese, and honey. However, because pasteurized food products are not sterile, they will eventually spoil.

To inactivate microbes on fomites or nonliving surfaces, chemicals called *disinfectants* are used. Disinfectants do not achieve sterilization because some microbes may survive treatment. Vinegar is a natural disinfectant due to its high acidity, and halogens such as chlorine bleach are routinely used to clean laboratory benches. Unlike disinfectants, *antiseptics* are antimicrobial agents that are safe for use on living tissues. Treating a cut with hydrogen peroxide is an example of antisepsis. When microbes are removed from the skin after using an alcohol swab, hand sanitizer, or betadine scrub, the process is called *degerming.*

The term *sanitization* refers to the cleansing of fomites to remove enough microbes to achieve levels deemed safe for public health. For example, commercial dishwashers used in the food service industry typically use extremely hot water and air for washing and drying; the high temperatures kill most microbes, sanitizing the dishes. Hospital rooms are commonly sanitized using a chemical disinfectant to prevent disease transmission between patients.

Various other methods are used in clinical and nonclinical settings to reduce the microbial load on items. Although the terms for these methods are often used interchangeably, there are important distinctions. Table 7.1 summarizes common protocols, definitions, applications, and agents used to control microbial growth.

Table 7.1: Common protocols for control of microbial growth

Common Protocols for Control of Microbial Growth			
Protocol	**Definition**	**Common Application**	**Common Agents**
For Use on Fomites			
Disinfection	Reduces or destroys microbial load of an inanimate item through application of heat or antimicrobial chemicals	Cleaning surfaces like laboratory benches, clinical surfaces, and bathrooms	Chlorine bleach, phenols (e.g., Lysol), glutaraldehyde
Sanitization	Reduces microbial load of an inanimate item to safe public health levels through application of heat or antimicrobial chemicals	Commercial dishwashing of eating utensils, cleaning public restrooms	Detergents containing phosphates (e.g., Finish), industrial-strength cleaners containing quaternary ammonium compounds
Sterilization	Completely eliminates all vegetative cells, endospores, and viruses from an inanimate item	Preparation of surgical equipment and of needles used for injection	Pressurized steam (autoclave), chemicals, radiation
For Use on Living Tissue			
Antisepsis	Reduces microbial load on skin or tissue through application of an antimicrobial chemical	Cleaning skin broken due to injury; cleaning skin before surgery	Boric acid, isopropyl alcohol, hydrogen peroxide, iodine (betadine)
Degerming	Reduces microbial load on skin or tissue through gentle to firm scrubbing and the use of mild chemicals	Handwashing	Soap, alcohol swab

The use of antimicrobial agents to treat infections began in the early 1900's, when Paul Ehrlich developed the chemotherapeutic drug Salvarsan to treat individuals infected with *Treponema pallidum*, the spirochete that causes syphilis. Most people associate the term "chemotherapy" with treatments for cancer. However, chemotherapy is a broad term that refers to any use of chemicals or drugs to treat disease. Chemotherapy may involve drugs that target cancerous cells or tissues, or it may involve antimicrobial drugs that target infectious microorganisms.

Antimicrobial drugs typically work by destroying or interfering with microbial structures and enzymes, either killing microbial cells or inhibiting of their growth. In 1928, Alexander Fleming observed that the mold *Penicillium* growing on agar plates could inhibit the growth of bacteria. This naturally produced antimicrobial agent was the first *antibiotic,* which was later purified into penicillin to treat disease. Penicillin is only one example of a natural antibiotic. In the 1940s, Selman Waksman, a prominent soil microbiologist at Rutgers University, led a research team that discovered several antibiotics, including actinomycin, streptomycin, and neomycin (Figure 7.1a). His work earned him the Nobel Prize in Physiology and Medicine in 1952.

Today, many organisms have evolved mechanisms to resist the action of antibiotics. The overuse and misuse of antibiotics (Figure 7.1b) are major contributing factors for the emergence of multidrug resistant "superbugs" that are a leading cause of healthcare-associated infections. Discovering novel approaches to treating infectious disease and preventing antibiotic resistance is a global health priority.

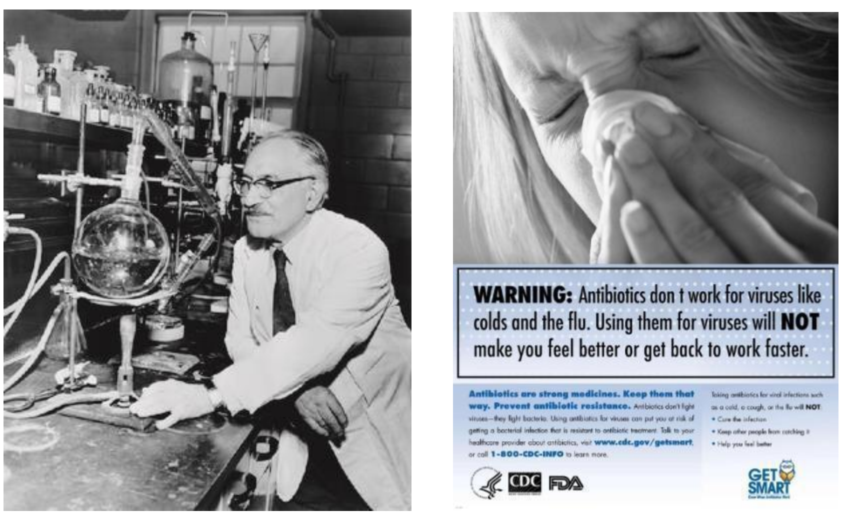


Figure 7.1: (a) Selman Waksman was the first to show the vast antimicrobial production capabilities of soil bacteria; (b) Public awareness poster for antibiotic misuse.

In this module, we will examine the action of antibiotics and the effect of ultraviolet radiation on bacteria. We will also use the scientific method to evaluate the effectiveness of various antiseptics and disinfectants and will draw conclusions from pooled data.

Exercise 7.1 – Effect of UV Radiation

LEARNING OUTCOMES

1. Define and compare ionizing and nonionizing radiation.
2. Determine the effect of ultraviolet radiation on bacterial cells.

Radiation in various forms, from high-energy radiation to sunlight, can be used to kill microbes or inhibit their growth. *Ionizing radiation* includes X-rays, gamma rays, and high-energy electron beams. This type of radiation passes into cells and alters molecular structures of the DNA and other cell components, leading to mutations and cell death.

Both X-rays and gamma rays easily penetrate paper and plastic and can therefore be used to sterilize items such as plastic Petri dishes, disposable gloves, intravenous tubing, and other latex and plastic items used for patient care. Ionizing radiation is also used for the sterilization of other types of heat-sensitive materials used clinically, including tissues for transplantation, pharmaceutical drugs, and medical equipment. Gamma irradiation of foods such as dried spices and produce eliminates microorganisms that cause spoilage and greatly extends shelf life (Figure 7.2).

(a) (b)

Figure 7.2: (a) Foods are exposed to gamma radiation by passage on a conveyor belt through a radiation chamber. (b) Gamma-irradiated foods must be clearly labeled and display the irradiation symbol, known as the "radura."

Another type of radiation used to control microbial growth is *nonionizing radiation,* which uses lower energy and longer wavelengths. Ultraviolet (UV) light is one example of this type of radiation. UV light includes three types of rays that increase in energy: UVA, UVB, and UVC. While exposure to UVA and UVB rays are associated with sunburns and skin cancer in humans, UVC is blocked by the Earth's ozone layer and is artificially produced by germicidal lamps to disinfect air, water, and nonporous surfaces (Figure 7.3b).

UVC waves range between 200-300 nm, with peak effectiveness at 260 nm. The energy from UVC excites electrons in cells and can lead to the formation of abnormal bonding between adjacent nitrogenous bases, particularly thymine, in DNA. These thymine dimers (Figure 7.3a) change the shape of the DNA molecule, causing errors in DNA replication that lead to cell death.

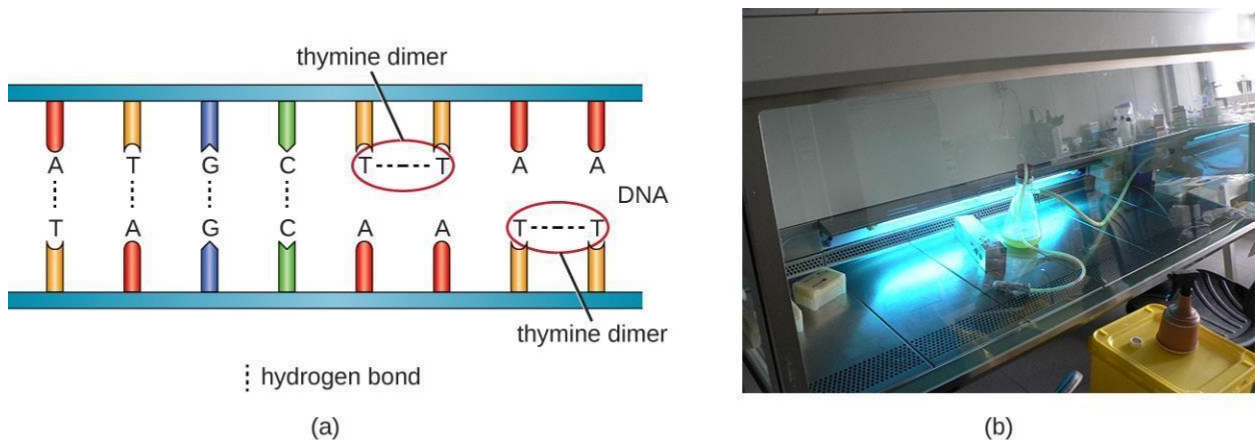


Figure 7.3: (a) UV radiation causes the formation of thymine dimers in DNA, leading to lethal mutations in the exposed microbes. (b) Germicidal lamps that emit UV light are commonly used in the laboratory to sterilize equipment.

UV lamps are now commonly incorporated into water purification systems for use in homes. In addition, small portable UV lights are commonly used by campers and hikers. These lights purify water from natural environments by killing coliforms (fecal bacteria) and protozoa such as *Giardia* that could lead to intestinal illness. Germicidal lamps are also used in surgical suites and biological safety cabinets. Because UV light does not penetrate surfaces or pass through plastics or glass, cells must be exposed directly to the light source.

In this exercise, the effect of UV radiation exposure on the growth of spore-forming and non-spore- forming bacteria is examined.

Exercise 7.1 – UV Radiation

OBJECTIVE

Determine the effect of UV light on spore-forming and non-spore-forming bacteria.

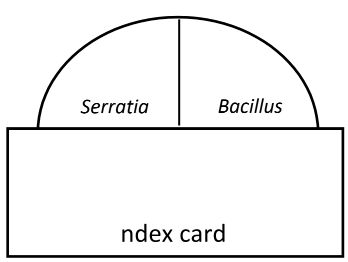
MATERIALS (WORK IN PAIRS)

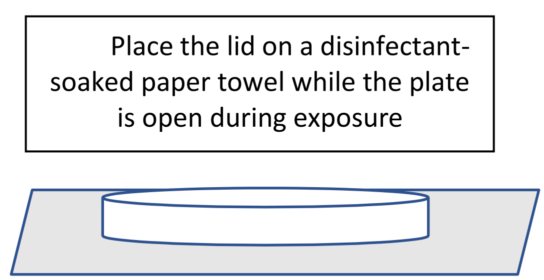
- EQUIPMENT: Sterile swabs, index card, UV lamp, timer, paper towel, disinfectant
- MEDIA: TSA plates (3)
- CULTURES: *Serratia marcescens, Bacillus cereus*

PROCEDURE – STUDENTS WORK IN PAIRS

1. Use a marker to divide the bottom of each plate in half and label with your initials, date, exposure time (1 min, 2 min, or 5 min), and organism numbers.
2. Tighten and vortex the bacterial culture tubes. Aseptically obtain bacteria by dipping a sterile swab into the broth, pressing the swab against the side of the tube to remove excess liquid, and swabbing each half of the agar confluently, leaving no gaps. Dispose of swabs in the disinfectant beaker, not in the wrapper.
3. Soak a paper towel with disinfectant and place the lid of the plate face down on the towel.
4. Cover the bottom half of the open plate with an index card as shown below and expose the plate to UV light for the designated time, then return the lid.
5. Repeat the exposure procedure for each remaining plate.
6. Invert the plates and place them in a common rack for incubation at 37^{o}C for 18-24 hours.

UV radiation can damage your eyes. Keep safety glasses on AT ALL TIMES.
To check if the lamp is on, shine it on an index card – do not look directly at the light.





FOLLOW UP

1. Count the number of colonies on each side of the plate and complete the report.
2. Dispose of plates in the Petri plate discard bucket.

Exercise 7.2 – Antibiotics: Kirby-Bauer Test

LEARNING OUTCOMES

1. Describe the various ways antibiotics kill bacterial cells.
2. Discuss the emergence of antibiotic resistant bacteria.
3. Use the Kirby-Bauer test to determine antibiotic susceptibility for select bacteria.

Since their discovery, antibiotics have saved countless lives, and they remain an essential tool for treating and controlling infectious disease. The spectrum of activity for antibacterial drugs relates to diversity of targeted bacteria. A *narrow-spectrum* antibiotic targets only specific types of bacteria, as is the case with penicillin against Gram-positive bacteria. If the pathogen is known, using a narrow spectrum drug minimizes damage to the normal microbiota. A *broad-spectrum* antibiotic, such as those that target both Gram-positive and Gram-negative bacteria, is often used to cover a wide range of potential pathogens while awaiting laboratory results. It may also be effective when a narrow-spectrum drug fails due to bacterial resistance.

Antibiotics vary in their interactions with bacteria. *Bacteriostatic* antimicrobials inhibit growth, while *bactericidal* drugs kill their targets. For the optimum treatment of some infections, two antibacterial drugs may be administered together to provide a synergistic interaction that is better than the efficacy of either drug alone. A classic example of synergistic combinations is trimethoprim and sulfamethoxazole (Bactrim). Individually, these two drugs provide only bacteriostatic inhibition of bacterial growth, but combined, the drugs are bactericidal.

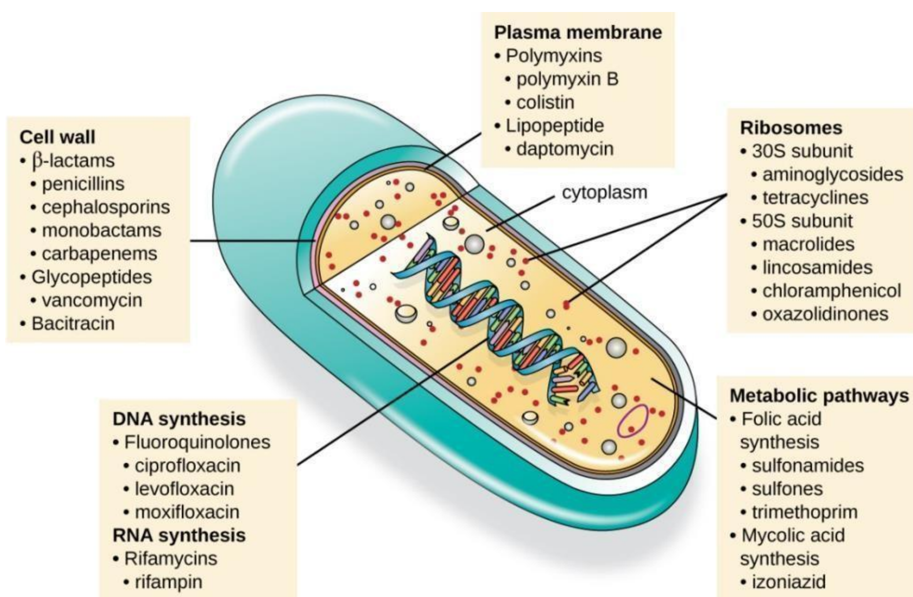


Figure 7.4: Each class of antibacterial drugs has a unique mode of action on a bacterial cell.

Each class of antibiotics has a unique mode of action (Figure 7.4). Some antibiotics inhibit peptidoglycan synthesis and compromise cell wall integrity. Others disrupt cell membranes or prevent the synthesis of nucleic acids or proteins. Antibiotics may also block essential metabolic pathways that cells use to make vitamins such as folic acid. Although life-threatening infections such as acute endocarditis require the use of a bactericidal drug, their widespread and often unnecessary use is a major contributing factor to the rise of multidrug-resistant microbial strains such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* species (VRE), and most recently, carbapenem.

The gold standard used by laboratories to determine antimicrobial susceptibility is the Kirby-Bauer disk-diffusion method**.** First developed in the 1950s, the Kirby-Bauer test uses small paper disks containing known concentrations of different antibiotics and a specialized medium called Mueller-Hinton agar. Bacteria growing in broth are confluently inoculated over the surface of the agar and the antibiotic-containing disks are placed on top. During incubation, the antibiotic diffuses away from disks into the agar. If an organism is killed or inhibited by the drug, an area of no growth called a *zone of inhibition* will form around the disk (Figure 7.5).

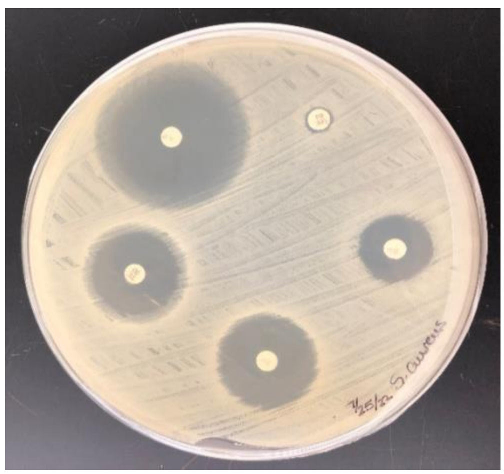


Figure 7.5: The Kirby-Bauer test. Zones of inhibition *that form around antibiotic-containing disks indicate if bacteria are susceptible, intermediate, or resistant to each drug tested.*

The diameter of each zone of inhibition is an indicator of whether bacteria are sensitive (susceptible or killed), intermediate, or resistant to an antibiotic, and a standardized table is used to interpret zone size for each drug (Table 7.2). It is important to note that since the interpretation of zone size is unique for each antibiotic, a larger zone does not always mean that an organism is effective. For example, a zone of 20 mm is interpreted as resistant to penicillin while a smaller 16 mm zone is interpreted as susceptible for gentamicin. The codes on each disk also indicate the MIC, or minimal inhibitory concentration, of the antibiotic. This corresponds with the lowest dosage of the drug that prevents bacterial growth.

Table 7.2. Standard Clinical Interpretive Values for Determining Antibiotic Susceptibility

		Zone of Inhibition (mm)		
ANTIBIOTIC	**CODE**	**RESISTANT**	**INTERMEDIATE**	**SENSITIVE**
Chloramphenicol	C-30	≤ 12	13-17	≥ 18
Ciprofloxacin	CIP-5	≤ 15	16-20	≥ 21
Erythromycin	E-15	≤ 13	14-22	≥ 23
Gentamicin	GM-10	≤ 12	13-14	≥ 15
Penicillin	P-10	≤ 28	---	≥ 29
Polymyxin B	PB-300	≤ 11	---	≥ 12
Streptomycin	S-10	≤ 11	12-14	≥ 15
Tetracycline	TE-30	≤ 14	15-18	≥ 19
Trimethoprim/Sulfa	SXT	≤ 10	11-15	≥ 16
Vancomycin	VA-30	≤ 14	---	≥ 15

Exercise 7.2 – Antibiotics: The Kirby-Bauer Method

OBJECTIVE

Evaluate the effect of selected antibiotics on bacteria using the Kirby-Bauer procedure.

MATERIALS

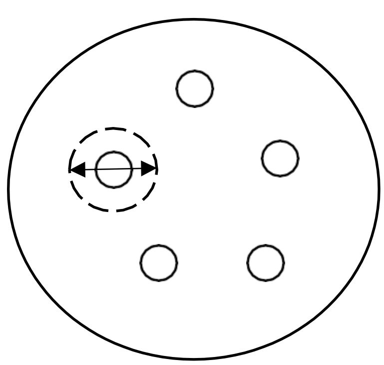
- EQUIPMENT: Sterile swabs, forceps, antibiotic disks*, 15-cm ruler, marker
- MEDIA: Large Mueller-Hinton agar plate
- SOLUTIONS: Small volume of alcohol in a beaker
- CULTURES: *Staphylococcus aureus, Pseudomonas aeruginosa*

PROCEDURE – STUDENTS WORK IN PAIRS

1. Label two large Mueller-Hinton agar plates with your initials, date, and organism numbers.
2. Tighten and vortex a bacterial culture tube.
3. Aseptically obtain bacteria by dipping a sterile swab into the broth and pressing it against the side of the tube to remove excess liquid.
4. Swab the agar surface in three confluent layers (horizontally, vertically, and diagonally) and around the rim. Dispose of the swab in the disinfectant beaker, not the wrapper.
5. Dip the tip of the forceps in alcohol and obtain an antibiotic disk from the cartridge.
6. Place the disk on the agar and gently tap with forceps, being careful not to push the disk into the agar. Follow the pattern below for all disks so that they are not too close together.
7. Repeat the procedure for the second bacterial culture.
8. Invert the plates and place them in a common rack for incubation at 37°C for 18-24 hours.

FOLLOW UP

1. Measure the diameter of the zone of inhibition in millimeters. For best results, hold the ruler against the bottom of the plate. If growth is up to or under a disk, record the zone as 0 mm. A haze of bacteria indicates developing resistance and should be considered growth.
2. Interpret susceptibility for each antibiotic using Table 7.2 to complete the report.
3. When you are done, dispose of plates in the Petri plate discard bucket.



Measure the diameter of each zone of inhibition in millimeters following growth.

Exercise 7.3 – Antiseptics & Disinfectants/Scientific Inquiry

LEARNING OUTCOMES

1. Describe the various ways chemical agents act on bacterial cells.
2. Use scientific inquiry to evaluate the efficacy of antiseptics and disinfectants on bacteria.

Antimicrobial chemicals damage cells in a variety of ways (Table 7.3). Soaps and detergents are lipids which disrupt lipids in bacterial cell membranes. Agents such as chlorine and iodine destroy cellular proteins and spores. Alcohols dehydrate cells but are unable to penetrate spore coats. Phenol compounds such as Lysol® have properties of both soaps and chemical agents. Other factors, such as temperature, type of object being disinfected, application time, and microbial target, can also influence disinfectant efficacy.

In this experiment, each group will test one assigned agent against two bacteria:

- *Staphylococcus:* Gram-positive; thick peptidoglycan; tolerates high levels of salt and sugar
- *Pseudomonas:* Gram-negative; extensive outer membrane proteins confer high resistance

Prior to testing, students will propose a hypothesis and a prediction as to the effectiveness of their assigned agent. Class data will be pooled, and the results analyzed for all agents tested.

Table 7.3. Microbial targets of common disinfectants, antiseptics, and antimicrobial chemical agents.

TYPE OF AGENT	EXAMPLES	MODE OF ACTION
Alcohol	70% alcohol, Scope® mouthwash	Dehydration; denatures proteins
Peroxide	3% hydrogen peroxide	Oxidizing agent
Halogen	10% bleach	Oxidizing agent
Phenolic	5% Lysol®, lab disinfectant	Denatures proteins
Detergent	Soap, cationic detergents	Disrupts membrane lipids
Iodophor	Betadine® scrub, tincture of iodine	Denatures proteins
Acid	Vinegar, lemon juice, hot sauce	Denatures proteins
Essential oil	Eucalyptus, Listerine® mouthwash	Denatures proteins
Solute	Karo® corn syrup, honey	Dehydration; denatures proteins

Exercise 7.3 – Antiseptics & Disinfectants/Scientific Inquiry

OBJECTIVE

Evaluate the effect of selected chemical agents on bacteria using a disk-diffusion procedure.

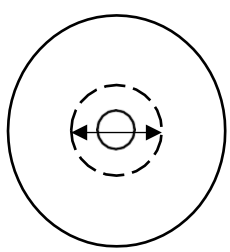
MATERIALS

- EQUIPMENT: Sterile swabs, forceps, assigned chemical agent, 15-cm ruler, marker
- MEDIA: Small Mueller-Hinton agar plates
- SOLUTIONS: Small volume of alcohol in a beaker
- CULTURES: *Staphylococcus aureus, Pseudomonas aeruginosa*

PROCEDURE – STUDENTS WORK IN PAIRS

Note: One pair of students will be assigned to set up the control disks, which contain no agent.

1. Complete the pre-lab report prior to beginning the procedure.
2. Label two small Mueller-Hinton agar plates with your initials, date, and organism numbers.
3. Tighten and vortex a bacterial culture tube.
4. Aseptically obtain bacteria by dipping a sterile swab into the broth, pressing it against the side of the tube to remove excess liquid.
5. Swab the agar surface in three confluent layers (horizontally, vertically, and diagonally) and around the rim. Dispose of the swab in the disinfectant beaker, not the wrapper.
6. Dip the tip of the forceps in alcohol and obtain a sterile paper disk.
7. Dip the disk into the chemical agent and place it in the center of the agar. Gently tap with forceps, being careful not to push the disk into the agar.
8. Repeat the procedure for the second bacterial culture.
9. Invert plates for incubation for 37^{o}C for 18-24 hours.



Measure the diameter of the zone of inhibition in millimeters following growth.

FOLLOW UP

1. Measure the diameter of the zone of inhibition in millimeters as shown above.
2. Record results on the class data table and complete the lab report.
3. When you are done, dispose of plates in the Petri plate discard bucket.

BIO 211 MODULE 7 PRE-LAB REPORT **NAME:**________________________________

EXERCISE 7.3 – ANTISEPTICS & DISINFECTANTS: SCIENTIFIC INQUIRY

After reading the procedure, consider the mode of action of the chemical agent that you were assigned and review the properties of Gram-positive and Gram-negative bacteria in your text. Use this information to propose a testable hypothesis regarding which organism will be most resistant to the agent that you are testing, and why.

Assigned chemical agent:____

How does this agent specifically destroy microbial cells?__________________________

Propose a **hypothesis** as to which organism will be most resistant to this agent, and why:_______

Predict the specific experimental results that you should observe to support your hypothesis:___

Identify the:

- **Independent variable**, manipulated by the experimenter:______________________
- **Dependent variable,** which cannot be manipulated:__________________________
- **Control treatment**, or comparative benchmark that lacks the independent variable:________
- **Controlled variables**, or factors that might affect the outcome and which must be standardized. Be specific – use “incubation time for plates” rather than simply “time”:

  1. ______________________________________
  2. ______________________________________
  3. ______________________________________
  4. ______________________________________

CONTROL OF GROWTH **DATE:__________PARTNER INITIALS:_______**

EXERCISE 7.1 – EFFECT OF UV RADIATION

OBSERVATIONS: Count and record the number of colonies on each side of each plate. Dispose of plates in the Petri plate discard container.

	NUMBER OF COLONIES FOLLOWING INCUBATION		
ORGANISM	UV Exposure Time		
	1 minute	2 minutes	5 minutes
Bacillus cereus			
Serratia marcescens			

QUESTIONS FOR REVIEW

Which genus survived the longest exposure to UV?____

What cellular feature of this genus protects DNA from UV exposure?_________________________

How does UV radiation specifically damage cells?____

Why was it necessary to cover half of the plate with a card?__________________________________

If the card were not used, would you expect more colonies or fewer?________________________

Explain:____

If the lid were not removed, would you expect more colonies or fewer?______________________

Explain:____

Which of the bacteria tested is a fecal coliform?____

Based on the data, what minimum UV exposure time is required to purify environmental water that may contain these bacteria?____

EXERCISE 7.2 – ANTIBIOTICS: THE KIRBY-BAUER METHOD

OBSERVATIONS: Record the diameter of zones in millimeters and round values to the nearest whole number. If there is a haze of growth around a disk, consider it to be growth. Refer to Table 7.2 in the exercise to interpret results as resistant (R), intermediate (I), or sensitive (S).

ANTIBIOTIC	*Staphylococcus aureus*		*Pseudomonas aeruginosa*	
	Zone Size (mm)	Interpretation (R/I/S)	Zone Size (mm)	Interpretation (R/I/S)

QUESTIONS FOR REVIEW

Which antibiotic tested has the narrowest spectrum of activity?____________________________

Based on the data, which genus is more difficult to treat?__________________________________

Which antibiotic(s), if any, could be used to treat both bacteria?___________________________

Based on the data, is the *Staphylococcus* a MRSA strain?___________How do you know?________

Examine the zone sizes for penicillin and tetracycline on the interpretive table in the procedure. Does a larger zone always mean that one antibiotic is more effective than another?__________
Explain.____

____

Circle the change to the size of the zone of inhibition if the following errors occurred:

- Under-inoculating the agar: False increase False decrease
- Delay in placing disks after inoculation: False increase False decrease
- Recording zone size in cm rather than in mm: False increase False decrease
- Using disks that are past the expiration date: False increase False decrease

EXERCISE 7.3 – ANTISEPTICS & DISINFECTANTS: SCIENTIFIC INQUIRY

OBSERVATIONS: Complete the table below by measuring the diameter of the zone of inhibition in millimeters, pooling class data. The type of agent is found in Table 7.3 of the exercise. Note that unlike antibiotics, since there is no MIC value given, zone sizes are relatively compared.

AGENT	TYPE OF AGENT	INHIBITION DIAMETER (mm)	
		Staphylococcus	*Pseudomonas*
Control disk	None		
70% isopropanol			
3% H_2O_2			
10% bleach			
5% Lysol®			
Betadine®			
Soap			
Scope®			
Listerine®			
Vinegar			
Corn syrup			
Lemon juice			

QUESTIONS FOR REVIEW

1. Which of the two bacteria was resistant to the most agents?____________________________
2. Which agent(s) are effective against both bacteria?_____________________________________
3. What type of graph best represents the data? (Circle) Bar chart Line graph Pie chart
4. Indicate the axis (y or x) used to plot each variable, and how that axis is labeled:

   Independent variable: Axis?________Label?__________________________________

   Dependent variable: Axis?________Label?__________________________________

Your instructor may ask you to prepare a graph of the data at the end of this report.

BIO211 MODULE 7 REPORT **NAME:**______________________________

5. List two limitations or weaknesses of the procedure, and how each could be corrected:
   - Limitation:____
     Correction:____
   - Limitation:____
     Correction:____

6. Which agent did you test?__________________Which organism did you hypothesize would be most resistant?________________________Was your hypothesis supported?__________
   - If your hypothesis was supported, what should be investigated next?_________________
   ____
   - If your hypothesis was not supported, what would be the new hypothesis?____________
   ____

7. Write a conclusion statement that best reflects your hypothesis:_______________________
____
____

OPTIONAL GRAPH OF DATA

MODULE 8: Identification of Gram-Positive Cocci

LEARNING OUTCOMES

1. Discuss the purpose of identifying medically important Gram-positive bacteria.
2. Name several tests used to identify staphylococci and streptococci.

INTRODUCTION

In clinical care, identification of bacterial pathogens is essential to determining appropriate treatment options for an infected patient. Gram-positive bacteria of the human microbiome are often implicated in opportunistic infections of skin, respiratory tract, and enteric regions. Some of these infections are *nosocomial,* or hospital acquired. Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE) are two predominating nosocomial pathogens in health care settings.

Staphylococci

Staphylococcus species are commonly found on the skin, with *S. epidermidis* and *S. hominis* being prevalent in the normal microbiota. *Staphylococcus aureus* is also commonly found in the nasal passages and on healthy skin in some individuals, but pathogenic strains are often the cause of a broad range of infections of the skin and other body systems.

When a staphylococcal infection is suspected, patient samples are collected, Gram stained, and cultured. Under the microscope, Gram-positive staphylococci cells are arranged as grapelike clusters; when grown on blood agar, colonies have a unique pigmentation ranging from opaque white to cream. Since the Gram reaction of staphylococci and streptococci is often similar in appearance, a catalase test is performed on colonies to initially distinguish the two types of bacteria. Catalase is an enzyme that is only produced by aerobic bacteria, including *Staphylococcus.* Streptococci are anaerobic and do not produce catalase.

The plasma-clotting protein coagulase produced by *S. aureus* is used to distinguish this species from other staphylococci. Other biochemical tests, such as growth and fermentation on mannitol salt agar, are also useful in confirming the identity of staphylococcal species.

Streptococci

Similar to staphylococci, streptococci are normally present on skin and mucous membranes. *Streptococcus pyogenes* (Group A streptococci) in the respiratory tract is a common cause of “strep throat” or acute pharyngitis, and *Streptococcus agalactiae* (Group B streptococci) in the genital region has been implicated in neonatal meningitis following vaginal delivery. Group D enterococci, particularly *Enterococcus faecalis* and *E. faecium*, reside in the large intestine.

These bacteria are often opportunistic pathogens of wounds and bedsores and can acquire resistance to vancomycin through horizontal gene transfer with other resistant bacteria.

Recall that following a Gram stain, one way to identify streptococci is by observing hemolysis patterns of colonies on blood agar. Beta hemolysis, typical of Group A and Group B streptococci, results in complete lysis of red blood cells and a clear color of the agar around colonies. Alpha hemolysis, or partial lysis, results in the release of methemoglobin and greenish brown discoloration of agar around colonies which is characteristic of viridans streptococci. Gamma hemolytic Group D enterococci do not lyse blood cells and produce no change of color in the agar around colonies.

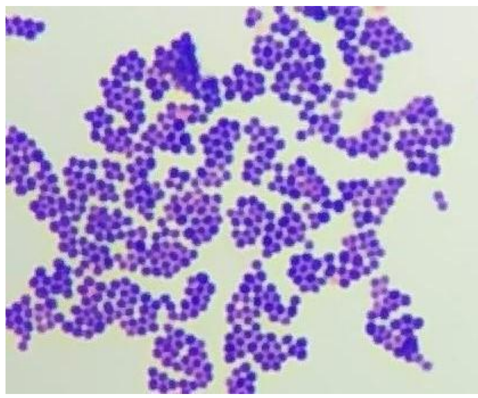


Figure 8.1: Gram staining is the initial step for the identification of Gram-positive cocci.

Two additional procedures, the CAMP and bile esculin tests, are useful for identifying Group B streptococci and Group D enterococci respectively. In the CAMP reaction, beta hemolysis of *S. agalactiae* is enhanced when grown near *Staphylococcus aureus.* The bile esculin test detects the ability of *Enterococcus* to hydrolyze esculin, a derivative of glucose, in the presence of bile.

Figure 8.2 depicts the relationship between the major tests in this module and their use in the identification of Gram-positive cocci.

DIFFERENTIAL TESTING OF GRAM-POSITIVE COCCI

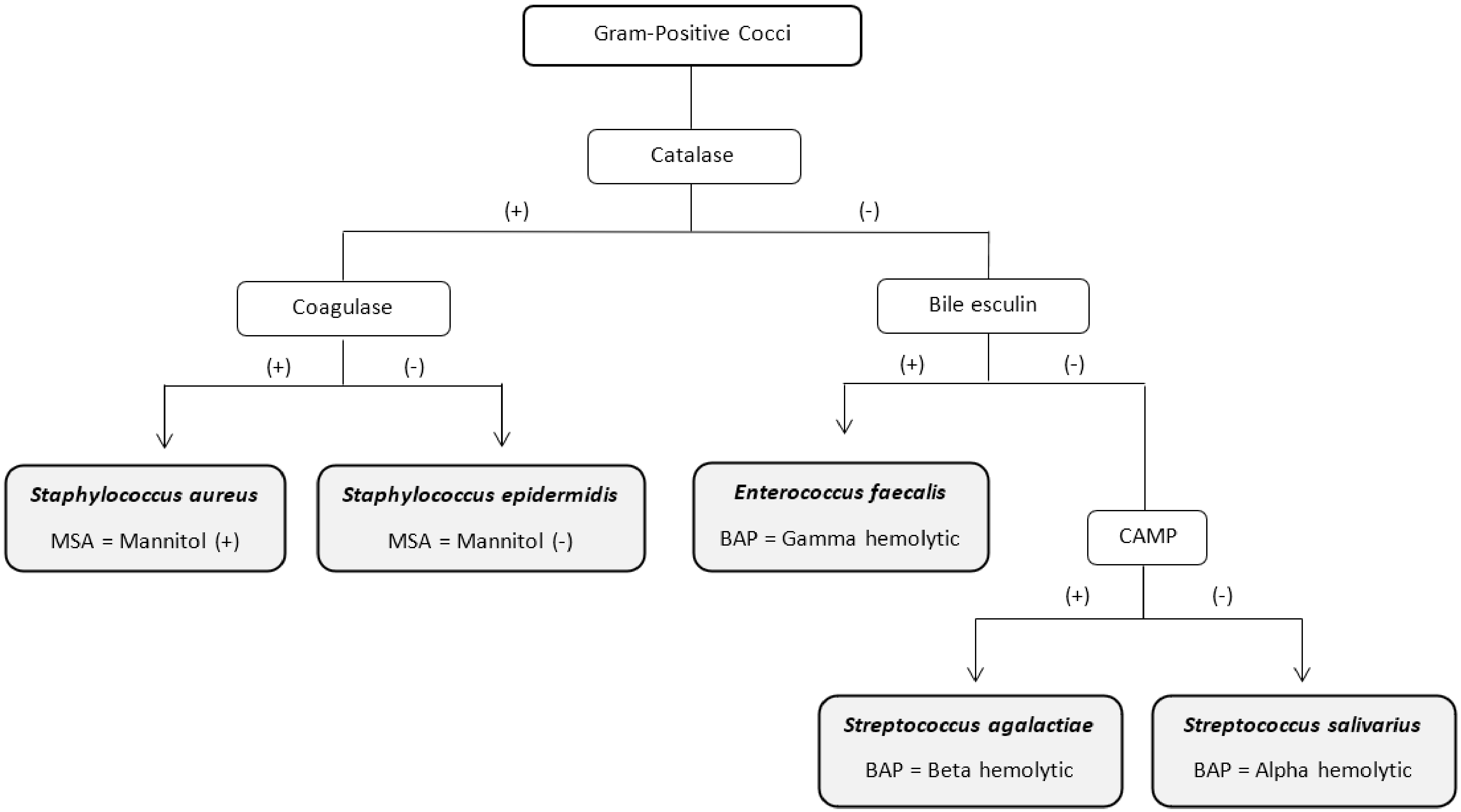


Figure 8.2. Differential testing of Gram-positive cocci.

Exercise 8.1 – Catalase Test

LEARNING OUTCOMES

1. State the principle and procedure of the catalase test.
2. Explain how the catalase test is used to distinguish staphylococci from streptococci.

Organisms that use aerobic respiration to produce energy also generate reactive oxygen species (ROS) that can cause cellular damage. One ROS produced is hydrogen peroxide, which is neutralized by the enzyme catalase to form water and oxygen:

$$\underset{\text{Hydrogen peroxide}}{H_2O_2} \xrightarrow{\text{catalase}} \underset{\text{Water}}{H_2O} + \underset{\text{Oxygen}}{O_{2\,(gas)}}$$

This reaction is the basis for the catalase test, which is useful in distinguishing aerobic *Staphylococcus* from anaerobic streptococci since both appear as Gram-positive cocci microscopically. It is important to note, however, that production of catalase is based on oxygen use and not on Gram reaction. Therefore, many Gram-negative bacteria and other Gram-positive bacteria are catalase positive as well.

The test is done on a glass slide by adding a sample from a growing bacterial culture to a drop of hydrogen peroxide. Organisms that produce catalase will produce bubbles of oxygen, indicating a positive test. Those bacteria that do not produce catalase will produce no change since the enzyme is not present to act on the peroxide (Figure 8.3).

Figure 8.3: A positive catalase test distinguishes aerobic staphylococci (left) from anaerobic streptococci (right).

Exercise 8.1 – Catalase Test

OBJECTIVE

Determine catalase production by aerobic bacteria.

MATERIALS

- EQUIPMENT: Inoculating loop, incinerator, clean microscope slides
- SOLUTIONS: 3% hydrogen peroxide in dropper bottle
- CULTURES: *Staphylococcus aureus, Enterococcus faecalis*

PROCEDURE – STUDENTS WORK IN PAIRS

1. Add a drop of hydrogen peroxide to the center of each slide.
2. Aseptically transfer bacteria to the drops and observe the reaction.
3. Dispose of slides in the disinfectant beaker.

Exercise 8.2 – Coagulase Test

LEARNING OUTCOMES

1. State the principle and procedure of the coagulase test.
2. Explain how the coagulase test is used to distinguish *Staphylococcus aureus* from other staphylococcal species.

Most strains of *Staphylococcus aureus* produce the exoenzyme coagulase, which exploits the natural mechanism of blood clotting by the host to evade the host's immune system. Normally, when blood vessels are damaged, platelets begin to plug the clot and a cascade of reactions occurs in which fibrinogen, a soluble protein made by the liver, is cleaved into fibrin. Fibrin is an insoluble, thread-like protein that binds to platelets, cross-linking them to form a clot. However, if bacteria release coagulase into the bloodstream, the fibrinogen-to-fibrin cascade is triggered in the absence of blood vessel damage. The resulting clot coats the bacteria in fibrin, protecting the *S. aureus* from exposure to phagocytic immune cells circulating in the bloodstream.

Whereas coagulase causes blood to clot, enzymes called kinases have the opposite effect by triggering the conversion of plasminogen to plasmin, which promotes digestion of fibrin clots. By digesting a clot, kinases allow pathogens trapped in the clot to escape and spread, thus worsening the infection. Examples of kinases include staphylokinases produced by *S. aureus* and streptokinases produced by *Streptococcus pyogenes*.

In this exercise, production of coagulase by *S. aureus* will be evaluated using rabbit plasma. Formation of a clot by coagulase-producing bacteria is observed after 18-24 hours of incubation at 37^{o}C (Figure 8.4).

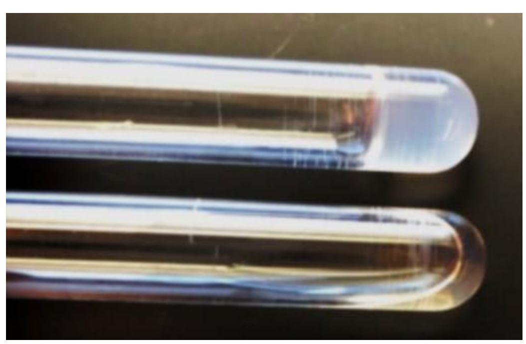


Figure 8.4: Clot formation in rabbit plasma from production of coagulase by Staphylococcus aureus (top); other species of Staphylococcus are coagulase negative (bottom).

Exercise 8.2 – Coagulase Test

OBJECTIVE

Distinguish coagulase-producing *Staphylococcus aureus* from coagulase-negative staphylococci.

MATERIALS

- EQUIPMENT: Inoculating loop, incinerator, parafilm
- SOLUTIONS: 0.5 mL tube of dilute rabbit plasma
- CULTURES: *Staphylococcus aureus, Staphylococcus epidermidis*

PROCEDURE – STUDENTS WORK IN PAIRS

1. Use tape to label each tube of plasma with your initials, date, and organism number.
2. Remove the parafilm cap and aseptically inoculate the plasma with bacteria.
3. Re-cover the tube with parafilm.
4. Place tubes in a common rack for incubation at 37^{o}C for 18-24 hours.

FOLLOW UP

1. Evaluate tubes for clotting and record results in the lab report. *Note that production of kinase may cause a clot to partially dissolve; this should be interpreted as a positive test.*
2. Discard coagulase tubes in the disinfectant beaker.

Exercise 8.3 – Bile Esculin Test

LEARNING OUTCOMES

1. State the principle and procedure of the bile esculin test.
2. Explain how the bile esculin test is used to distinguish Group D enterococci from other streptococcal species.

The bile esculin test is used in the identification of Group D streptococci, including species of *Enterococcus.* Unlike many Gram-positive bacteria, enterococci grow in 4% bile, which serves as a selective agent in bile esculin medium. This medium also contains esculin, a carbohydrate derivative. While many organisms can hydrolyze or break down esculin, few other than the enterococci are able to do so in the presence of bile. When esculin is hydrolyzed, a molecule called esculetin forms and reacts with ferric (iron) ions added to the medium. This reaction causes a black precipitate and darkening of the agar (Figure 8.5).

Recall from earlier experiments that Group D enterococci are gamma hemolytic on blood agar, meaning that they do not act on red blood cells. Therefore, a positive bile esculin reaction is useful for distinguishing these bacteria from other catalase-negative, Gram-positive cocci.

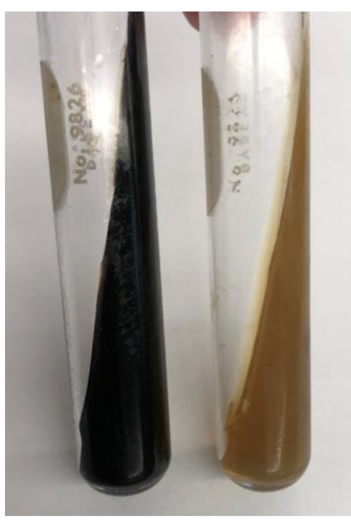


Figure 8.5: Enterococcus faecalis (left) hydrolyzes esculin in the presence of bile, forming a black complex of esculetin and ferric ions while growth of most other Gram-positive cocci is inhibited (right).

Exercise 8.3 – Bile Esculin Test

OBJECTIVE

Distinguish Group D *Enterococcus* from other streptococci based on esculin hydrolysis in the presence of bile.

MATERIALS

- EQUIPMENT: Inoculating loop, incinerator
- MEDIA: Bile esculin agar slants
- CULTURES: *Enterococcus faecalis, Staphylococcus aureus*

PROCEDURE – STUDENTS WORK IN PAIRS

1. Use tape to label each tube with your initials, date, and organism number.
2. Aseptically inoculate bacteria on the slant surface in a single line from bottom to top.
3. Place tubes in a common rack for incubation at 37^{o}C for 18-24 hours.

FOLLOW UP

1. Evaluate tubes for esculin hydrolysis by observing blackening of the agar.
2. Record results in the lab report.
3. Remove tape from tubes and place them in a common rack for autoclaving.

Exercise 8.4 – CAMP Test

LEARNING OUTCOMES

1. State the principle and procedure of the CAMP test.
2. Explain how the CAMP test is used to distinguish Group B streptococci from other streptococcal species.

The CAMP (Christie-Atkins-Munch-Peterson) reaction is used in the identification of *Streptococcus agalactiae*, which are Group B beta hemolytic streptococci. CAMP factor is a protein secreted by these bacteria that causes hemolysis to be enhanced when grown near *Staphylococcus aureus*, another beta hemolytic organism. Enhanced hemolysis is due to a synergistic effect of proteins secreted by both bacteria, producing an arrow-shaped area of hemolysis on the agar where the two meet (Figure 8.6).

It is important to note that in this test, *S. aureus* is used for the purpose of enhancing hemolysis by *S. agalactiae* only; the CAMP reaction is not used for identification of staphylococci.

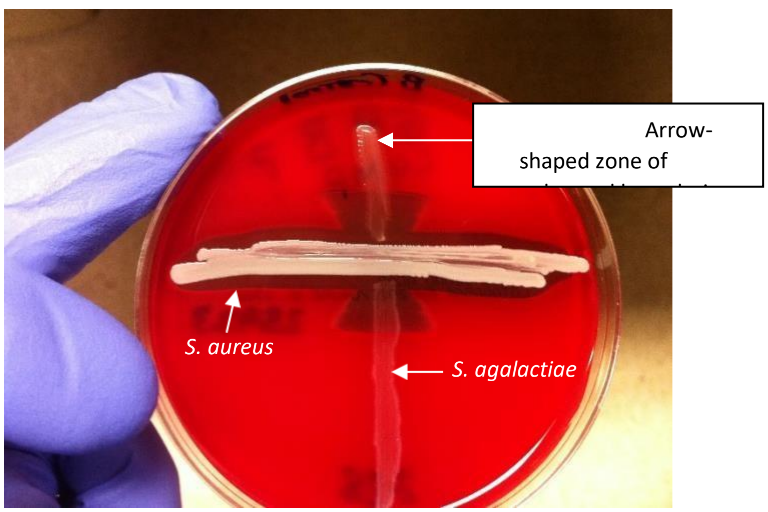


Figure 8.6: The hemolysis action of S. agalactiae is enhanced when grown near S. aureus, resulting in arrow- shaped hemolysis and positive CAMP test.

Exercise 8.4 – CAMP Test

OBJECTIVE

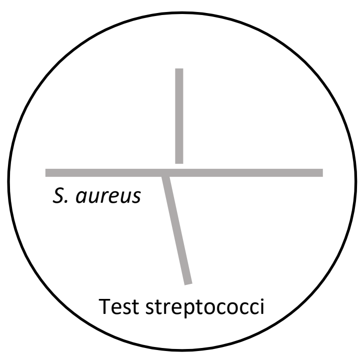
Distinguish Group B *Staphylococcus agalactiae* by enhanced beta hemolysis on blood agar.

MATERIALS

- EQUIPMENT: Inoculating loop, incinerator
- MEDIA: Blood agar plates
- CULTURES: *Staphylococcus aureus, Streptococcus agalactiae, Enterococcus faecalis*

PROCEDURE – STUDENTS WORK IN PAIRS

1. Label the bottom of two blood agar plates with your initials, data, and organism number.
2. Aseptically inoculate the agar by streaking a line of *S. aureus* across the center of the agar.
3. Rotate the plate 90^o and streak lines of *S. agalactiae* across the center of the agar just up to, but not touching, both sides of the *S. aureus* streak as in the diagram below.
4. Repeat for the procedure, using *S. aureus* and *E. faecalis.*
5. Invert the plates in a common rack for incubation at 37^{o}C with CO_2 for 18-24 hours.



Streak a single line of *S. aureus* across the agar, and then the streptococci to be tested perpendicular to each side of the *S. aureus* line

FOLLOW UP

1. Evaluate the plates for arrow-head hemolysis where the two bacteria meet.
2. Record results in the lab report.
3. Dispose of the plates in the Petri plate discard bucket.

ID OF GRAM-POSITIVE COCCI **DATE:__________PARTNER INITIALS:_______**

EXERCISE 8.1 – CATALASE TEST

OBSERVATIONS: Record the appearance of results for the catalase test below. Dispose of slides in the disinfectant beaker.

ORGANISM	APPEARANCE	INTERPRETATION (+ or -)

QUESTIONS FOR REVIEW

What is the purpose of catalase in cells?____

A student attempting to identify an unknown performs a catalase test without doing a Gram stain. The student observes bubbles and assumes that the organism must be *Staphylococcus.* Why might this be an incorrect assumption?

EXERCISE 8.2 – COAGULASE TEST

OBSERVATIONS: Record results for the coagulase tube and/or slide test in the table. Dispose of tubes and slides in the disinfectant beaker.

ORGANISM	TUBE TEST APPEARANCE	LATEX TEST APPEARANCE	INTERPRETATION (+ or -)

Which selective and differential agar confirms the results of coagulase test?___________________

What is the appearance of coagulase-positive organisms on this agar?________________________

EXERCISE 8.3 – BILE ESCULIN TEST

Record results for the bile esculin test in the table.

ORGANISM	APPEARANCE	INTERPRETATION (+ or -)

Enterococcus, or Group_________streptococci, is part of the___________________microbiome.

Which differential agar is used to confirm the results of the bile esculin test?_________________

What is the expected result for *Enterococcus* on this agar?_________________________________

Which test is used to determine whether a particular isolate is VRE?_________________________

What is the expected result? Be specific.____

EXERCISE 8.4 – CAMP TEST

Record results for the CAMP test in the table.

ORGANISM	APPEARANCE	INTERPRETATION (+ or -)

Streptococcus agalactiae, or Group______streptococci, are_____________hemolytic on blood agar, while *Enterococcus faecalis*, or Group_enterococci are_____________hemolytic.

What is the expected CAMP reaction (positive or negative) for Gram-positive bacteria that are catalase negative and bile esculin positive?______________Explain.__________________________

MODULE 9: Identification of Gram-Negative Bacilli

LEARNING OUTCOMES

1. Discuss the purpose of identifying medically important Gram-negative bacteria.
2. Name several tests used to identify enteric and non-enteric Gram-negative bacilli.

INTRODUCTION

Identification of Gram-negative bacteria is initially based on distinguishing enteric (intestinal) bacteria from those that are non-enteric. Enteric bacteria, particularly those members of the family *Enterobacteriaceae*, are important causes of urinary, wound, blood, and hospital-acquired infections. These organisms are Gram-negative bacilli and include many genera: *Escherichia, Proteus, Citrobacter, Serratia,* and *Klebsiella* to name but a few.

Following a Gram stain, biochemical identification schemes usually begin with tests to detect production of key metabolic enzymes involved in respiratory pathways. Initially, the nitrate reduction and oxidase tests separate Gram-negative bacilli into two major groups based on the fermentation or oxidation of sugar (Figure 9.3). Secondary tests to identify genus and species include the IMViC series (indole, methyl red, Voges-Proskauer, and citrate tests), production of urease, and/or growth characteristics on specialized media.

Commercial systems with multi-test capabilities, such as the Enterotube™ and API strip, provide results of many tests with one inoculation procedure (Figure 9.1). These tests have multiple wells of media and require only one inoculation of the culture. Following incubation, reactions are interpreted as positive or negative, and compared with known results of a particular organism.

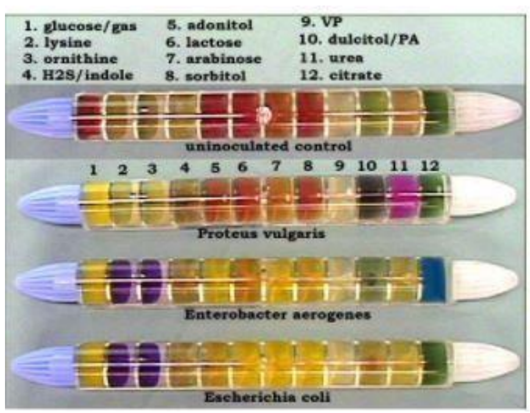
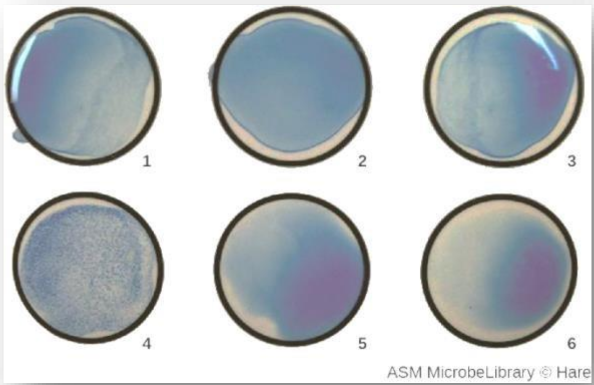


Figure 9.1: Rapid systems such as the Enterotube™ provide results of multiple biochemical reactions with a single inoculation.

Other rapid identification systems are based on principles of immunology, where antigens, or proteins found on the bacterial cell surface, are bound by specific test antibodies. Test antibodies are also proteins and usually attached to an indicator, such as a colored latex bead or fluorescent marker, in which a positive result creates a visible reaction (Figure 9.2). Direct or indirect fluorescent antibody kits utilize fluorescent microscopes to visualize bacterial antigens that are bound together by antibodies.



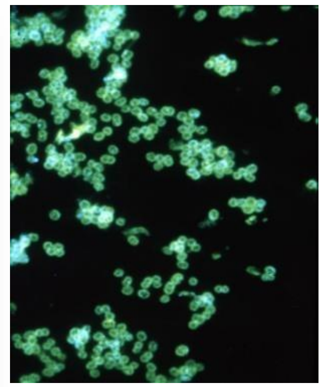


Figure 9.2: Immunological reactions include visible agglutination (left, well 4) and immunofluorescence (right).

Although identification of bacteria through biochemical testing has been traditionally used in microbiology laboratories for decades, genetic tests are now widely available for detecting many pathogens. Genetic tests are particularly useful for identifying fastidious bacteria or those that are slow growing, such as *Mycobacterium tuberculosis*, which may take weeks to months to cultivate on media.

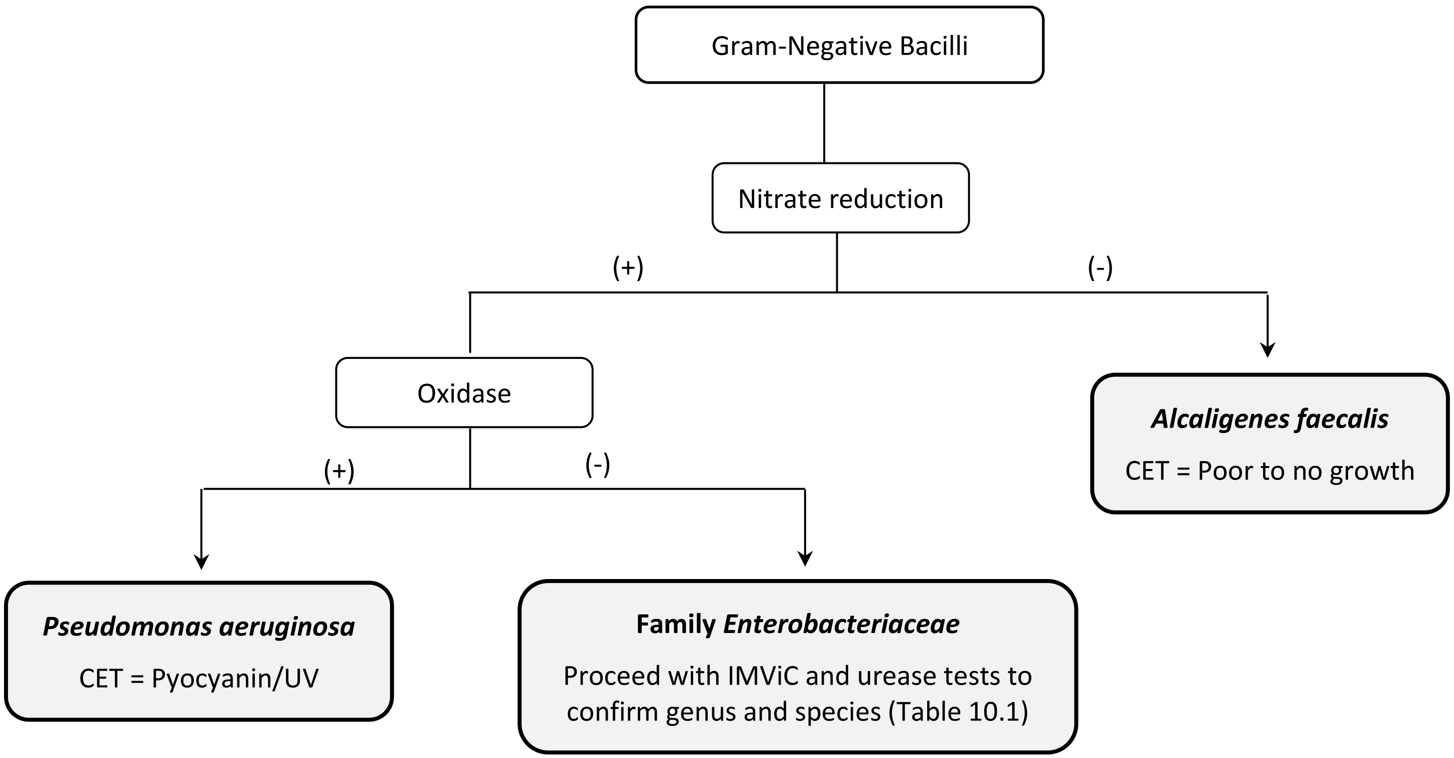


Figure 9.3. Differential testing of Gram-negative bacilli.

Exercise 9.1 Oxidase Test

LEARNING OUTCOMES

1. State the principle and procedure of the oxidase test.
2. Explain how the oxidase test distinguishes *Pseudomonadaceae* from *Enterobacteriaceae.*

Cytochrome c oxidase is an important enzyme that is found in the mitochondrial membrane of eukaryotic organisms and in the periplasmic space in aerobic bacteria. When cells undergo aerobic respiration to produce energy in the form of ATP, cytochrome oxidase is the final enzyme in the process that reduces oxygen to form water.

The oxidase test is used to distinguish *Pseudomonas, Alcaligenes,* and other oxidase-positive bacteria from oxidase-negative members of the family *Enterobacteriaceae,* including *Escherichia, Proteus, Serratia,* and related genera. It is also occasionally used to identify aerobic bacteria outside of these families, including those that are Gram-positive.

The test uses a chemical called oxidase reagent (1% tetramethyl-para-phenylenediamine dihydrochloride), which is added to bacteria on a swab or filter paper. Oxidase reagent is colorless, but it produces a deep purple color when oxidase is present (Figure 9.4).

Figure 9.4: A deep purple color forms when oxidase reagent is added to oxidase-positive bacteria on a swab (top); a negative test produces no color change (middle). The oxidase ampule is activated when the plastic sleeve is crushed, breaking the inner glass tube to release the reagent.

There are several ways to perform the oxidase test. It may be done by applying several isolated bacterial colonies to filter paper using a wooden stick (an inoculating wire should not be used as the metal can react with the test) or by collecting colonies with a sterile cotton swab. A drop of oxidase reagent is then applied directly to the bacteria on the filter paper or swab. Formation of a deep purple color within one to two minutes indicates the presence of oxidase enzyme and a positive test. A negative test produces no color change.

Oxidase reagent comes as a small plastic sleeve with a dropper cap. Inside the sleeve, the oxidase solution is contained within a glass ampule. To use the reagent, the outside of the sleeve is crushed by hand which breaks the ampule, releasing the test solution. It is important to note that the reagent reacts with oxygen, so once an ampule is activated it will eventually turn purple and should be used in a timely manner. Test results must also be read within one to two minutes; otherwise, a false positive color may form in the presence of oxygen. Depending on the age and type of bacteria being tested, varying degrees of color may be observed, and it is not unusual to have spotty or questionable results. For this reason, the nitrate reduction test is often done in tandem with the oxidase test as a confirmatory measure.

Exercise 9.1 Oxidase Test

OBJECTIVE

Determine the presence of cytochrome oxidase in Gram-negative bacteria that use oxygen as a final electron acceptor in aerobic respiration.

MATERIALS

- EQUIPMENT: Sterile cotton applicator swab
- SOLUTIONS: 1% oxidase reagent (tetramethyl-*p*-phenylenediamine dihydrochloride)
- CULTURES: *Escherichia coli, Alcaligenes faecalis, Pseudomonas aeruginosa*

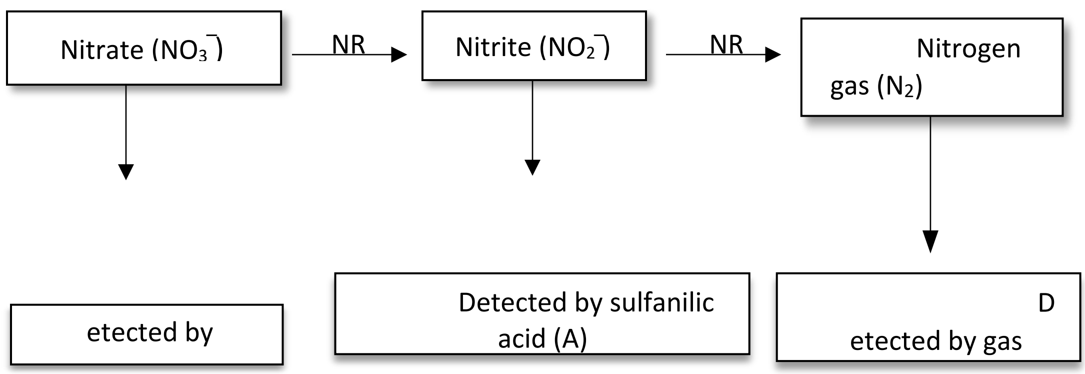
PROCEDURE – STUDENTS WORK IN PAIRS

Note: Because oxidase reagent eventually turns color in the presence of oxygen, your instructor will activate only a few common ampules for the class to share.

1. Remove a cotton applicator from the package and use the cotton end of the swab to obtain bacteria by touching several colonies that are growing on the surface of the agar or slant.
2. Gently shake the crushed ampule with the opening facing down to bring the fluid to the tip. **Without taking off the cap,** squeeze the ampule to deliver a drop of oxidase reagent to the bacteria on the swab. To avoid contamination, allow the drop to fall on the swab rather than touching the swab to the drop.
3. Read the result within one minute and record it in the lab report. A purple color indicates the presence of cytochrome oxidase, characteristic of *Pseudomonas, Alcaligenes,* and related genera. There will be no color change for aerobic organisms that do not have the enzyme, such as those in the family *Enterobacteriaceae*.
4. Dispose of the swab immediately in the disinfectant beaker; do not return to the wrapper.

Microorganisms use a variety of pathways to make energy. In aerobic respiration, oxygen serves as a final electron acceptor to ATP via an electron transport system. However, many organisms, including *Escherichia coli* and other facultative anaerobes of the family *Enterobacteriaceae,* can switch from aerobic respiration to anaerobic respiration in the absence of oxygen. In this case, oxygen is replaced by an inorganic molecule such as nitrate.

The enzyme nitrate reductase (NR) catalyzes the reduction of nitrate (NO_3^-) to nitrite (NO^-). Nitrate reductase is also used by many soil bacteria, such as *Pseudomonas aeruginosa*, to fully reduce nitrate to nitrogen gas (N_2), which then enters the atmosphere in a process called denitrification. Nitrogen-fixing bacteria that live around the roots of legume plants incorporate atmospheric nitrogen back into usable form for production of larger molecules such as proteins.



The nitrate reduction test is performed by inoculating bacteria into broth that contains nitrate as an initial substrate. A small glass tube, called a Durham tube, is added to trap any N_2 gas produced by denitrifying bacteria. Following incubation, the Durham tube is examined for presence of a bubble or broth displacement, indicating that nitrate was fully reduced. This result is typical of *Pseudomonas* species.

If no gas is observed, sulfanilic acid (reagent A) and α-naphthylamine (reagent B), are added to the broth. These reagents detect nitrite and turn the broth red if it is present, indicating that nitrate was reduced to nitrite and the test is positive. This result is typical for bacteria in the family *Enterobacteriaceae.*

Should the broth not turn red following the addition of reagents A and B, a small amount of zinc powder is added to the broth. Zinc reacts with the original substrate nitrate. If the broth turns red after adding zinc, it means that nitrate is present and was not reduced, i.e., a negative test. This result is typical for *Alcaligenes* species, which has no nitrate reductase activity. If there is no color change after zinc, nitrate was utilized by an enzyme other than nitrate reductase.

Results of the nitrate reduction test correlate with the oxidase test. *Enterobacteriaceae* are oxidase negative and reduce nitrate to nitrite; therefore, they have cytochrome oxidase c and nitrate reductase. *Pseudomonas* and *Alcaligenes* are both oxidase positive, but only *Pseudomonas* has nitrate reductase activity.

Exercise 9.2 – Nitrate Reduction Test

OBJECTIVE

Determine the denitrification of nitrate to nitrite or gaseous nitrogen by Gram-negative bacteria that possess the enzyme nitrate reductase.

MATERIALS

- EQUIPMENT: Inoculating loop, incinerator, labeling tape
- MEDIA: Nitrate broth (3)
- SOLUTIONS: Sulfanilic acid (reagent A), α-naphthylamine (reagent B), zinc powder
- CULTURES: *Escherichia coli, Pseudomonas aeruginosa, Alcaligenes faecalis*

PROCEDURE – STUDENTS WORK IN PAIRS

1. Use tape to label each tube with your initials, date, and organism number.
2. Aseptically inoculate the broth with bacteria, being careful not to introduce bubbles into the Durham tube.
3. Repeat for remaining cultures.
4. Place tubes in a common rack for incubation at 37^{o}C for 18-24 hours.

FOLLOW UP

1. Observe the Durham tube for nitrogen gas, indicating that the organism fully reduced nitrate to nitrogen gas, characteristic of *Pseudomonas.* Record results in the lab report.
2. For all tubes where gas was not present, add 5 drops each of reagent A and B to the broth. A red color that develops within a few minutes indicates that organism reduced nitrate to nitrite, characteristic of *Enterobacteriaceae.* Record results in the lab report.
3. For any remaining tubes where nitrite was not present, use a wooden applicator to add a little zinc powder directly to the broth and gently mix the tube by rolling in your hands. A red color that develops within a few minutes indicates that the organism does **not** have the enzyme nitrate reductase and that the original nitrate substrate is still present. This is characteristic of bacteria such as *Alcaligenes.* Record results in the lab report.
4. Remove tape from all tubes and place them in a common rack for autoclaving.

Exercise 9.3 IMViC Testing

LEARNING OUTCOMES

1. Name the tests that are included in the IMViC series and state the principle of each.
2. Explain how IMViC testing is useful in the identification of *Enterobacteriaceae.*

The IMViC series constitute six tests that help to differentiate bacteria within the family *Enterobacteriaceae*. Each letter of the acronym refers to a specific reaction within the series:

- I = Indole production; SIM medium also detects H_2S production and motility
- MV = Methyl red and Vogues-Proskauer tests for products of sugar fermentation
- C = Citric acid used as a sole carbon source

The IMViC reactions serve as an important aid in the identification of Gram-negative enteric bacilli, many of which are bacterial pathogens (Table 9.1). Members of this family also reduce nitrate to nitrite and are oxidase negative.

Table 9.1. IMViC reactions for select Gram-negative bacilli

TEST	*Escherichia coli*	*Serratia marcescens*	*Klebsiella aerogenes*	*Klebsiella pneumoniae*	*Proteus vulgaris*	*Proteus mirabilis*	*Citrobacter freundii*
Sulfur reduction to H_2S	-	-	-	-	+	+	+
Indole production	+	-	-	-	+	-	-
Motility	+	+	+	-	+	+	+
Methyl red test	+	-	-	+	+	+	+
Voges-Proskauer test	-	+	+	+	-	-	-
Citrate utilization	-	+	+	+	-	-	+

Sulfide-Indole-Motility (SIM) Tests

This medium is an agar deep that detects hydrogen sulfide (H_2S) production, indole production, and motility in the same tube. Bacteria are inoculated into the deep by stabbing it with a wire needle. Following incubation, the tube is examined for changes that indicate the presence of end products (Figure 9.5).

Sulfide production is determined by observing the agar for a black precipitate. Bacteria that metabolize sulfur-containing compounds in the medium (such as the amino acid cysteine) or reduce sulfate (SO_4^{2-}) during anaerobic respiration produce molecules that react with ferrous iron (Fe^{2+}) in the SIM medium. This reaction forms an insoluble black precipitate that is visible in the agar following incubation.

Indole production is indicative of an organism producing the intracellular enzyme tryptophanase. Tryptophanase catalyzes the breakdown of the amino acid tryptophan to pyruvate and indole. While pyruvate is used by the cell as a carbon and energy source, the indole is excreted as a waste product. SIM medium contains high levels of tryptophan. Following incubation, Kovacs reagent (para-dimethyl-aminobenzaldehyde) is added to the tube. If indole is present, the reagent will react with it and form rosindole, a pink compound.

Motility is assessed by growth in a semi-solid, soft agar. Motile bacteria can swim through the medium and will show diffuse growth and turbidity away from the line of inoculation.

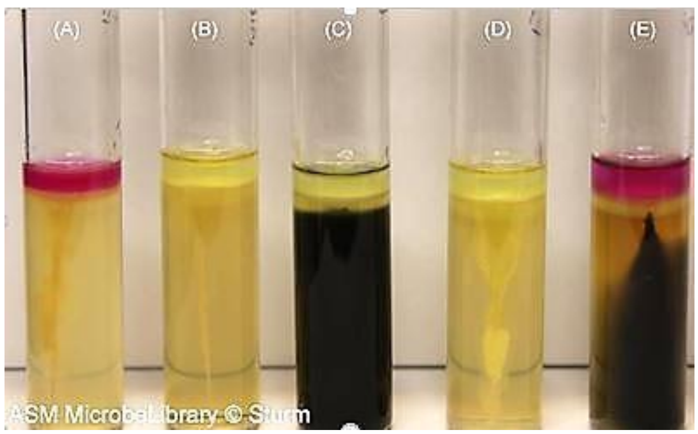


Figure 9.5: SIM test results. From left to right: (A) Escherichia coli, (B) Staphylococcus aureus, (C) Salmonella arizonae, (D) Klebsiella aerogenes, (E) Proteus vulgaris. After addition of Kovács reagent, a pink ring at the top of the tube indicates a positive indole result (A, E). Blackening of the media indicates hydrogen sulfide production (C, E). Growth feathering away from the stab line creating a cloudy appearance in the media indicates motility (A, C, D, E). Growth strictly along the stab line indicates a nonmotile organism (B).

Methyl Red & Voges-Proskauer (MRVP) Tests

MRVP testing begins by inoculating bacteria into a tube of a buffered peptone broth medium that contains glucose. Following incubation, the broth is separated into separate test tubes: one for the methyl red test and the other for the Voges-Proskauer test. Note that the two tests detect distinct products of fermentation, but most bacteria are positive for one and negative for the other (Figure 9.6).

Methyl Red Test: To detect mixed acids (lactic acid, acetic acid, etc.) from fermentation of glucose, methyl red reagent is added to the first broth tube. The reagent turns red when pH is less than 4.4, yellow when pH is above 6.2, and orange in between. The red color indicates that organic acids lowered the pH of the buffered broth, and the MR test is positive.

Voges-Proskauer Test: To detect production of less acidic products of glucose fermentation (acetoin or its precursor 2,3-butanediol), Barritt's reagents VP-A (alpha-naphthol) and VP-B (potassium hydroxide, or KOH) are added to the second tube. The formation of a red ring at the surface of the broth, which takes at least 20 minutes to form, is a positive VP reaction.

Figure 9.6: Methyl red tests (left) and Voges-Proskauer tests (right). Escherichia coli (EC) is MR+VP- while Klebsiella aerogenes (KA) is MR-VP+.

Citrate Test

Organisms that can survive using citrate (citric acid) as the sole source of carbon have a citrate permease enzyme that can transport citrate molecules into the cell. The citrate is then made into pyruvate, which can be converted into various products. Simmons citrate agar is a chemically defined medium that contains sodium citrate as the only source of carbon and the pH indicator bromothymol blue. Bromthymol blue is green at neutral pH and blue when the pH is alkaline. Bacteria that grow on this medium can survive by using citrate as the sole source of carbon and produce alkaline byproducts that will change the pH indicator in the medium from green to blue (Figure 9.7).

Figure 9.7: Citrate test. Proteus mirabilis (left) uses citric acid as a sole carbon source while Escherichia coli (right) does not utilize citrate to grow.

Exercise 9.3 IMViC Testing

OBJECTIVE

Use the IMViC series of tests (SIM, MRVP, and citrate tests) as well as the urease test to determine the genus and species of a member of the *Enterobacteriaceae.*

MATERIALS

- EQUIPMENT: Inoculating loop, inoculating needle, incinerator, labeling tape, Pasteur pipette, small serological test tube
- MEDIA: SIM deep, MRVP broth, Simmons citrate slant, urease broth
- SOLNS: Kovacs reagent, VP reagents A and B, methyl red reagent
- CULTURES: *Escherichia coli, Proteus vulgaris, Klebsiella aerogenes*

PROCEDURE – STUDENTS WORK IN PAIRS

1. Label three SIM agar deeps with your initials, date, and organism numbers (*E. coli, P. vulgaris, K. aerogenes*). Using an inoculating needle, aseptically inoculate each agar deep by stabbing the center of the agar almost to the bottom of the tube and then pulling the needle straight out.
2. Label two MRVP broths with your initials, date, and organism numbers (*E. coli, K. aerogenes*) and aseptically inoculate each using an inoculating loop.
3. Label two citrate slants with your initials, date, and organism numbers (*E. coli, P. vulgaris*) and aseptically inoculate each by streaking the loop up the surface of the slant.
4. Label two urea broths with your initials, date, and organism numbers (*E. coli, P. vulgaris*) and aseptically inoculate each with a loop.
5. Place all tubes in a common rack for incubation at $37^{o}C$ for 18-24 hours.

FOLLOW UP – Record reactions in the lab report and check results against Table 9.1.

1. SIM DEEP: Examine tubes for blackening (H_2S) and turbidity (motility). Add 5 drops of Kovacs reagent to each tube and observe for a pink color change (indole production).
2. MRVP BROTH: Use a disposable pipette to transfer half of the incubated broth into two sterile screw-top tubes. To one tube, add 5 drops of methyl red reagent and record color change. To the other tube, add 15 drops of VP-A and 15 drops of VP-B reagents. Gently mix by rolling the tubes with your hands for a few seconds. Wait 20-30 minutes to observe a positive reaction (red ring at top).
3. CITRATE SLANT: Examine the slant for a change in color from deep green to Prussian blue.
4. UREASE BROTH: Examine the broth for a change in color from golden to deep pink.
5. Remove tape from all tubes and place them in a common rack for autoclaving.

Exercise 9.4 - Urease Test

LEARNING OUTCOMES

1. State the principle and procedure of the urease test.
2. Explain how the urease test distinguishes *Proteus* spp. From non-lactose fermenting *Enterobacteriaceae.*

Urea is a product that many organisms generate from the catabolism of proteins. Bacteria that produce the enzyme urease are capable of hydrolyzing urea into ammonia and carbon dioxide. *Helicobacter pylori,* bacteria that cause gastric ulcers, use this reaction to neutralize the extremely acidic environment of the stomach. A breath test for detection of carbon dioxide produced by *H. pylori* in patients suspected of having gastric ulcers is one method of diagnosis.

In the laboratory, the urease test is used to identify certain species of *Proteus* from other non-lactose-fermenting Gram-negative enteric bacilli. Christensen's urea agar and Stuart's urea broth are two types of media used to test for the presence of urease. The medium contains urea and a phenol red indicator that changes from yellow (acid) to bright pink (alkaline). Bacteria that produce urease will break down the urea to produce ammonia, thus raising the pH of the medium and turning the indicator pink. Organisms that demonstrate a slow urease reaction, such as *Klebsiella* species, cause the medium to turn slightly orange, which is interpreted as a positive test. Urease-negative bacteria may grow in the broth and will either produce no color change or turn the medium yellow from acid production (Figure 9.8).

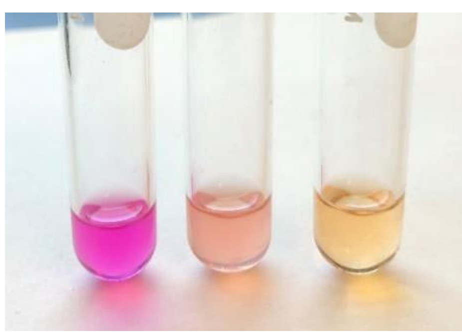


Figure 9.8: Urease test. Positive reaction (Proteus vulgaris, left); slow positive reaction (Klebsiella pneumoniae, center); negative reaction (Escherichia coli, right).

Exercise 9.4 - Urease Test

OBJECTIVE

Determine the production of urease in non-lactose-fermenting Gram-negative bacteria.

MATERIALS

- EQUIPMENT: Inoculating loop, incinerator, labeling tape
- MEDIA: Urease broth (2)
- CULTURES: *Escherichia coli, Proteus vulgaris*

PROCEDURE – STUDENTS WORK IN PAIRS

1. Use tape to label each tube with your initials, date, and organism number.
2. Aseptically inoculate each broth tube with bacteria.
3. Place tubes in a common rack for incubation at 37ºC for 18-24 hours.

FOLLOW UP

1. Examine broth for a change in color from golden to deep pink, indicating rapid hydrolysis of urea. Occasionally urease may produce a slower reaction, resulting in only a slight color change in the medium.
2. Record results in the lab report.
3. Remove tape from both tubes and place them in a common rack for autoclaving.

ID OF GRAM-NEGATIVE BACILLI **DATE:__________PARTNER INITIALS:_______**

EXERCISE 9.1 – OXIDASE TEST

OBSERVATIONS: Record the appearance of results for the oxidase test below. Dispose of swabs in the disinfectant beaker; do not return them to the wrapper.

ORGANISM	APPEARANCE	INTERPRETATION (+ or -)

QUESTIONS FOR REVIEW

Why should reactions for the oxidase test be recorded within two minutes?__________________

How is the oxidase test helpful in distinguishing Gram-negative *Enterobacteriaceae* from other Gram-negative bacilli such as *Pseudomonas* or *Alcaligenes*?________________________________

EXERCISE 9.2 – NITRATE REDUCTION TEST

OBSERVATIONS: Follow the directions in the lab exercise for follow-up testing and record results in the table.

ORGANISM	GAS IN DURHAM TUBE	APPEARANCE AFTER A&B	APPEARANCE AFTER ZINC	CONCLUSION

QUESTIONS FOR REVIEW

How do results of the oxidase test correlate with the nitrate reduction test for:

Enterobacteriaceae?____

Pseudomonas?____

Alcaligenes?____

EXERCISES 9.3 & 9.4 – IMViC & UREASE TESTING

OBSERVATIONS: Follow the directions in the exercise for follow-up tests and record results.

SIM DEEP

ORGANISM	H_2S PRODUCTION	MOTILITY	INDOLE PRODUCTION

MRVP BROTH

ORGANISM	MR COLOR	RESULT (+/-)	VP COLOR	RESULT (+/-)

CITRATE SLANT

ORGANISM	COLOR	RESULT (+/-)

UREASE BROTH

ORGANISM	COLOR	RESULT (+/-)

QUESTIONS FOR REVIEW

Which IMViC medium provides the most information to identify unknown Gram-negative bacteria?________________Explain.____

____

____

How should the result of a urease test be interpreted if the medium turns pale orange rather than bright pink?________________Explain. ____

____

MODULE 10: Identification of Bacterial Unknowns

LEARNING OUTCOMES

1. Identify two bacterial unknown cultures using a dichotomous key and standard staining and biochemical techniques.

INTRODUCTION

Identification of bacterial isolates requires careful technique, deductive reasoning, and timely decision-making on the part of the microbiologist. In this module, you will apply the skills learned in previous exercises. Your aseptic technique, time management, and ability to work independently will also be assessed in the process.

For this exercise, you will receive one Gram-positive and one Gram-negative culture listed in Table 10.1. Your task is to determine which is which, and to correctly identify the genus and species of each unknown using the techniques that you've learned in lab. A dichotomous key is provided to help you with this task. The key includes positive and negative test results and the names of the bacteria that are potentially unknowns. You may consult your notes, lab manual or other references; however, your instructor will not help you in the identification process. Working independently provides an understanding of what the microbiologist experiences in a clinical situation.

A few important tips:

- ✓ Use controls (bacteria that give known results) to compare with results for your unknowns.
- ✓ View heavier smears near the edges where cells are less crowded.
- ✓ Confirm the results of biochemical tests with selective media.
- ✓ Organisms don't always read the textbooks! Expect some atypical results.

All organisms have been examined for purity and are quality-controlled prior to distribution, but occasionally cultures become weak or nonviable. If you experience problems with the quality of your unknown, notify your instructor as soon as possible.

Practice aseptic technique! It is your responsibility to keep your bacterial cultures free from contamination. Use the same color tape for everything to be incubated, and label all tubes and plates with your initials, date, and unknown letter. When setting up biochemical tests, try to use isolated colonies for the best results. Repeated sub-culturing may lead to mutation and should be avoided.

Good luck!

Exercise 10.1 Identification of Bacterial Unknowns

LEARNING OUTCOMES

1. Apply deductive reasoning to determine appropriate tests for identification of bacteria.
2. Correctly identify two bacterial unknown isolates.

You will receive two bacterial unknown isolates from your instructor. Your task is to identify the genus and species of each unknown using the techniques and tests that you've learned throughout the semester. A dichotomous key and table of biochemical reactions is provided in this module to help. It is your responsibility to keep track of your unknown letters, reactions, and results throughout this project.

PERIOD 1

OBJECTIVE

This period is used to determine the Gram reaction and cellular morphology of each unknown, to prepare isolation streak plates for each unknown, and to perform preliminary testing.

MATERIALS

- EQUIPMENT: Inoculating loops, labeling tape, incinerator, sterile cotton applicators, glass slides, marking pen
- MEDIA: TSA plates, nitrate broth
- SOLUTIONS: Gram stain materials, 3% hydrogen peroxide, oxidase reagent
- CULTURES: Bacterial unknown and control

cultures PROCEDURE – STUDENTS WORK INDIVIDUALLY

1. Obtain two unknown cultures from the instructor and record the letters in your notes.
2. Streak each unknown for isolation onto a TSA plate and place it in a common rack for incubation at 37^{o}C for 18-24 hours.
3. Determine the Gram reaction, shape, and arrangement of cells for each unknown. When preparing slides, include additional slides with known Gram-positive and Gram-negative bacteria as controls. Examine stained slides microscopically under oil immersion and record results. You may wish to save heat-fixed or stained slides (blot excess oil) in a box.
4. Aseptically inoculate each unknown into a nitrate broth tube.
5. Time permitting, perform a catalase and oxidase test for each unknown.
6. When you are done, return unknown culture tubes to the instructor.

PERIOD 2

OBJECTIVE

This period is used to determine colony morphology and to inoculate secondary test media for each unknown.

MATERIALS

- EQUIPMENT: Inoculating loops, labeling tape, incinerator, marking pen, wooden sticks
- MEDIA: SIM deeps, MRVP broth, citrate slants, urease broth, coagulase tubes, bile esculin slants, agar plates (BAP, EMB, MAC, MSA, cetrimide)
- SOLUTIONS: Nitrate reagents (A, B, zinc powder)
- CULTURES: Bacterial unknown subculture plates from Period 1; control cultures

PROCEDURE – STUDENTS WORK INDIVIDUALLY

1. Examine unknown subculture plates for isolated colonies and record colony color, shape, and margin for each culture.
2. Examine results of the nitrate reduction test, adding reagents where appropriate. Record results for each unknown culture.
3. Follow the dichotomous key to select appropriate secondary tests required to identify each unknown based on Gram reaction, nitrate, oxidase, and catalase results for each unknown.

NOTE: Setting up all tests for both unknowns is costly and unnecessary.
Follow the dichotomous key to work deductively and inoculate only those tests which apply.

4. Use isolated colonies from each subculture plate to inoculate the appropriate secondary media. Your instructor may assign a common set of control bacteria for biochemical tests to be set up by different students rather than having each student set up controls individually.
5. Place secondary test tubes and media in a common rack for incubation at 37^{o}C for 18-24 hours. If your unknown requires incubation at 25^{o}C, let the instructor know.
6. When you are done, remove tape from the nitrate broth tubes and place them in a common rack for autoclaving; return the subculture plates for both unknowns to the instructor.

PERIOD 3

OBJECTIVE

This period is used for follow-up secondary testing on each unknown and for completing the final report.

MATERIALS

- EQUIPMENT: Disposable Pasteur pipets and small glass serological tubes, wooden stick
- SOLUTIONS: Nitrate reagents, MRVP reagents, zinc dust, UV lamp
- CULTURES: Bacterial unknown subculture plates from Period

1 PROCEDURE – STUDENTS WORK INDIVIDUALLY

1. Perform secondary tests for each unknown, adding reagents where necessary. Record results.
2. When you are done, remove tape from all tubes and place them in a common rack for autoclaving; dispose of plates in the Petri plate discard bucket. Small serological tubes for MRVP and coagulase tests should be disposed of in the disinfectant beaker.
3. Complete the final report, indicating the identity of each unknown and the test results on which the conclusion was based.

DICHOTOMOUS KEY FOR IDENTIFICATION OF UNKNOWNS

I. Gram Stain

A. Gram-positive
  1. If cocci → Go to II
  2. If bacilli → Repeat Gram stain; unknowns do not include Gram-positive bacilli

B. Gram-negative
  1. If cocci → Repeat Gram stain; unknowns do not include Gram-negative cocci
  2. If bacilli → Go to VI

II. Catalase Test

A. Catalase (+) → Go to III
B. Catalase (–) → Go to IV

III. Coagulase Test

A. Coagulase (+) → *Staphylococcus aureus*; confirm by growth and fermentation on MSA
B. Coagulase (–) → *Staphylococcus epidermidis*; confirm by growth on MSA

IV. Bile Esculin Test

A. Bile esculin (+) → *Enterococcus faecalis*; confirm by gamma hemolysis on BAP
B. Bile esculin (–) → Go to V

V. CAMP Test

A. CAMP (+) → *Streptococcus agalactiae*
B. CAMP (–) → *Streptococcus salivarius*; confirm by alpha hemolysis on BAP

VI. Nitrate Reduction

A. Nitrate (+) → Go to VII
B. Nitrate (–) → Go to VIII

VII. Oxidase Test

A. Oxidase (+) → Go to VIII
B. Oxidase (–) → Proceed with IMViC and urease testing; consult Table 10.1 to confirm

VIII. Cetrimide Agar

A. Heavy growth → *Pseudomonas aeruginosa*; confirm by UV and pyocyanin pigment
B. Poor to no growth → *Alcaligenes faecalis*

Table 10.1. Select reactions for bacterial unknowns*

TEST	*Pseudomonas aeruginosa*	*Alcaligenes faecalis*	*Escherichia coli*	*Serratia marcescens*	*Klebsiella aerogenes*	*Klebsiella pneumoniae*	*Proteus vulgaris*	*Proteus mirabilis*	*Citrobacter freundii*	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Streptococcus agalactiae*	*Streptococcus salivarius*	*Enterococcus faecalis*
Gram reaction	-	-	-	-	-	-	-	-	-	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	-	-	-
Nitrate	+	-	+	+	+	+	+	+	+					
Oxidase	+	+	-	-	-	-	-	-	-					
Lactose (EMB/MAC)	-	-	+	+	+	+	-	-	+					
MR			+	-	-	+	+	+	+					
VP			-	+	+	+	-	-	-					
H_2S			-	-	-	-	+	+	+					
Indole			+	-	-	-	+	-	-					
Motility			+	+	+	-	+	+	+					
Citrate			-	+	+	+	-	-	+					
Urease			-	-	-	+	+	+	-					
Pyocyanin / UV (CET)	+	-												
Colony pigment 25°C (TSA)				Red	Cream									
Coagulase										+	-			
7.5% NaCl (MSA)										+	+			
Mannitol (MSA)										+	-			
Hemolysis (BAP)												β	α	γ
CAMP												+	-	-
Bile esculin												-	-	+

**Shaded boxes indicate that a test is not applicable for identification. Results may vary due to strain, mutation, or other factors.*

BACTERIAL UNKNOWNS **DATE:**________________________________

	Unknown #1 Letter(s):______	**Unknown #2 Letter(s):______**
Cellular Morphology – *Circle the Gram reaction and the cell shape for each unknown*		
Gram stain (circle)	Positive Negative	Positive Negative
Cell shape (circle)	Cocci Bacilli	Cocci Bacilli
Test Results – *Indicate whether the result was (+) or (–) for **applicable** tests only*		
Catalase		
Bile esculin		
CAMP		
Coagulase		
Cetrimide – growth		
Nitrate reduction		
Oxidase		
Methyl red		
Voges-Proskauer		
Hydrogen sulfide		
Motility		
Indole		
Urease		
Confirmatory Tests		
Hemolysis (circle)	Alpha Beta Gamma	Alpha Beta Gamma
Mannitol fermentation		
Lactose fermentation		
Pyocyanin/UV		
Conclusion – *Correctly spell the genus and species names of each identified unknown*		
FINAL IDENTIFICATION		

COMMENTS

Discuss any atypical results, discrepancies, or other problems that may have affected the identification of your unknowns. Your instructor may assign a formal report for this project.

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