Komar University of Science and Technology (KUST)

Introduction to Microbiology Lab Manual (MLS2405C)

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Preface

This “in-house” laboratory manual has been prepared mainly in light of two excellent manuals namely **Harley–Prescott: Laboratory Exercises in Microbiology, Fifth Edition, 2002** and **Morello–Mizer–Granato: Laboratory Manual and Workbook in Microbiology, seven edition, 2003.** Some modifications has been applied based on the direction and requirements of the Medical Laboratory Science (MLS) department curriculum and study program.

This manual composed of 13 exercises in total focused on the basic concepts of microbiology and assists students to get necessary knowledge, skills, and practice in this field. In addition, students will learn how to work safely in microbiology labs. Each exercise composed of a short background about a specific topic followed by the principle, purpose, required material, and procedures needed to perform that exercise. The results of each exercise will be recorded by the students and together with the answers of some review questions will be submitted to the lab instructor in the form of lab reports.

Exercises are written in a way to be easy to follow yet informative to the students. The overall laboratory experience reinforces the concepts of the theoretical lectures and together provide a comprehensive knowledge to the students in the field of microbiology.

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Orientation to the Microbiology Laboratory

Introduction

In the laboratory individuals are exposed to hazards not found in a regular classroom. It is essential that students follow all rules established by the lab instructor, lab manager, or lab assistant to ensure the safety of all individuals in the class. Failure to follow established rules may result in dismissal of the individual from the class.

Laboratories have certain standard safety equipment. These typically include a general-purpose fire extinguisher, eyewash, safety shower and cut-off switches for electrical and gas outlets. It is the responsibility of the student to locate and know how to use the general safety equipment in the laboratory. Additionally, students should be aware of exits from the room in case of emergency, the location of the nearest fire call box, how to summon Campus Security, and how to obtain emergency medical assistance.

The microbiology lab has some additional safety considerations. Since individuals work with potentially pathogenic organisms care must be taken to prevent possible infection or transmission of the organisms from the laboratory. Students must wear protective clothing (lab coats) while working the laboratory. Lab coats may not be worn outside the laboratory. Intact skin is an adequate barrier against microorganisms so gloves are not necessary in lab. Gloves will be provided and students may wear gloves when handling cultures if they so desire. Table tops must be disinfected before and after lab using the disinfectant provided. Instruction in aseptic technique will be provided. Aseptic technique must be followed while working with microorganisms.

Handwashing is a simple and effective way to prevent the transmission of disease. While antibacterial soap may provide some additional protection the major effect of handwashing is the mechanical removal of microbes from the skin. Friction when washing hands is important to mechanically remove organisms from the surface of the skin. Using a paper towel to turn off the water prevents recontamination of the hands with microorganisms. Hands must be washed whenever the student leaves the lab.

Microbiology Laboratory Safety Rules

1. All materials and clothes other than those needed for the laboratory are to be kept away from the work area.
2. A lab coat or other protective clothing must be worn during lab. The lab clothing is not to be worn outside of the laboratory.
3. Clean the lab table before and after lab with the disinfectant solution provided.
4. Wash hands before leaving lab.
5. Any item contaminated with bacteria or body fluids must be disposed of properly. Disposable items are to be placed in the BIOHAZARD container. Reusable items are to be placed in the designated area for autoclaving prior to cleaning. Sharps are to be disposed of in the appropriate container.
6. Reusable items should have all tape and marks removed by the student before being autoclaved.
7. Because organisms used in this class are potentially pathogenic, aseptic technique must be observed at all times. NO eating, drinking, application of cosmetics or smoking is allowed. Mouth pipetting is not allowed.
8. Cuts and scratches must be covered with Band-Aids. Disposable gloves will be provided on request.
9. Long hair should be tied back while in lab.
10. All accidents, cuts, and any damaged glassware or equipment should be reported to the lab instructor immediately.
11. Sterilization techniques will involve the use of Bacticinerators that are fire and burn hazards. Bacticinerators reach an internal temperature of 850^{o}C or 1500^{o}F. Keep all combustibles (capable of catching fire) away from the Bacticinerators. Do not leave inoculating loops or needles propped in the Bacticinerator.
12. Microscopes and other instruments are to be cared for as directed by the instructor.
13. It is the responsibility of the student to know the location and use of all safety equipment in the lab (eyewash, fire extinguisher, etc).
14. Cultures may not be removed from the lab. Visitors are not allowed in the lab.
15. Doors and windows are to be kept closed at all times.

16. For the best lab experience, read labs before coming to class. Make notes as necessary. Wait for a laboratory introduction by the instructor before starting work.

Every student must singe and return a copy of these Laboratory Safety Rules to the laboratory instructor at the end of class.

I have read and understand the above rules and agree to follow them.

Signed …….. Date……..

Name ……

Laboratory reports preparation and submission

For each exercise or two (in some cases) in this lab manual a laboratory report is required. The report needs to be prepared according to the template provided by the instructor (see appendix 1). The report is designed to make sure that students understood the logic and principles of each exercise through recording their results in the form of tables, drawings, illustrations etc. In addition students are also requested to answer some review questions. The required result format and review questions are accompanied at the end of each exercise.

The lab report of a previous week must be submitted to the lab instructor at the beginning of the next lab work. Late submission leads to deduction of 1 grade of your total report grade for each hour lateness. No report sheets will be collected after 10 hours from the submission deadline. Finally, each student should submit a report sheet for his/her work individually. Group submission is not accepted unless there is a strong justification.

Exercise 1: Sterilization, Disinfection, and Antisepsis

Techniques needed to control the growth of microorganisms has been shaped by both cultural and scientific advances. From the days of early food preservation using fermentation of milk products and smoking of meats to extend the shelf life of foods, practical needs have contributed to the development of these techniques.

Formal development of clinical medical settings led to an awareness of the cause and effects of disease. Society continues to shape our needs today, with the widespread evolution of drug-resistant microbes, a high nosocomial-infection rate in patient-care facilities, and the development of exploratory and permanently inserted medical devices.

Our requirements for sterilization, antisepsis, and sanitizing thus go beyond the historical needs of the research and clinical laboratories and commercial production requirements. This lesson not only reviews commonly used laboratory techniques, but also specifically addresses their limitation when used to reduce or eliminate microorganisms.

Sterilization:

Sterilization is the removal of all microbes, including endospores, and can be achieved by mechanical means, heat, chemicals, or radiation (Table 1-1). When using heat, it may be either dry heat or moist heat. Traditionally, moist heat under pressure is provided by autoclaving and dry heat by ovens (see Table 1-1). Steam sterilization or dry heat can be monitored by the use of biological indicators or by chemical test strips that turn color upon having met satisfactory conditions. These indicators are widely available. Usually the spores of species of *Geobacillus* or *Bacillus* spp. are used in either a test strip or suspension, as these organisms are more difficult to kill than most organisms of clinical interest. Growth of the spores in liquid media after the cycle of sterilization is complete indicates the load was not successfully sterilized. Standard laboratory methods based on different and lower autoclave packing levels and lower density materials may not be effective.

Table 1-1: Routine methods of sterilization.

Method [Ref.]	Temperature	Pressure	Time	Radiation (Mrad)
Dry heat [24]	150–160°C 302–320°F		>3 h	
Dry heat [24]	160–170°C 320–338°F		2–3 h	
Dry heat [24]	170–180°C 338–356°F		1–2 h	
Moist heat	135°C 275°F	31.5 psig	40 min	
Boiling—indirect [24]			1 hr	
Boiling—direct[2]			2 min	
Radiation—cobalt 60[3] [24]	Ambient		hours	2–3
Radiation—cesium 137[3] [24]	Ambient		hours	2–3
Electronic accelerators[4] [24, 25]			<1 sec	2.5
Ozone [26, 27]		See cited references	See cited references	

[1] Steam under pressure (i.e., autoclave).

[2] Boiling point of liquids- e.g., cumene (isopropylbenzene) 152°C (306°F).

[3] Dependent on curies in source.

[4] Electrostatic (Van de Graaff), electromagnetic (Linac), direct current, pulsed transformer.

Other common methods of sterilization include **gases** such as ozone, radiation, or less commonly electronic accelerators (see Table 1-1). Solutions containing heat-labile components require a different approach. **Filtration** is generally the most accepted and easiest method. The FDA and industry consider 0.22 µ filters sterilization grade based on logarithmic reductions of one of the smaller bacteria *Brevundimonas diminuta*.

The removal of contaminants from air may be necessary in the case of fermentation or drug manufacturing in chemical reactions requiring some form of gas. **Various types of filters** are available for the removal of organisms from air. One such filter, as an example, the Aerex 2 (Millipore), can withstand 200 steam-in-place cycles at 293°F (145°C), resists hydraulic pressure at 4.1 bard (60 psid), and has the ability to retain all **phages**. It is important to note that all filter sterilization is relative. While a 0.22 µ filter will remove most bacteria, it will not remove viruses, mycoplasma, prions, and other small contaminants (see Table 1-2).

Table 1-2: Removal of particulates, some bacteria, yeast, and filamentous fungi using filters.

Size (µ)	Purpose
0.1	Mycoplasmal removal
0.22	Routine bacterial removal
0.45	Plate counts water samples
>0.45	Removal of particulates, some bacteria, yeast, and filamentous fungi

Disinfection and Antisepsis:

Disinfection is the process that eliminates most or all microorganisms, with the exception of endospores. Disinfectants can be further subcategorized as **high-level disinfectants**, which kill all microorganisms with the exception of large numbers of endospores with an exposure time of less than 45 min; **intermediate-level disinfectants**, which kill most microorganisms and viruses but not endospores; and **low-level disinfectants**, which kill most vegetative bacteria, some fungi, and some viruses with exposure times of less than 10 min. **Antiseptics** destroy or inhibit the growth of microorganisms in or on **living tissues** and can also be referred to as biocides. **Disinfectants are used on inanimate objects** and can be **sporostatic** but are not usually sporocidal.

Many factors influence the effectiveness of chemical disinfectants and antiseptics. The **microbicidal** (to kill) or **microbiostatic** (to inhibit) efficiency of a chemical is often determined with respect to its ability to deter (discourage) microbial growth. Disinfectants are often tested against cultures of the following bacteria: *Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella typhuimuium, Mycobacterium smegmatis, Pevotella intermeida, Streptococcus mutans, Actinobacillus actinomycetemcomiticans, Bacteriodies fragilis, and Escherichia coli*. One common approach is to use the broth dilution method, wherein a standard concentration of the organism is tested against increasing dilutions of the disinfectant. The **minimum inhibitory concentration (MIC)** of the organism is then determined. While there are many reports of liquid disinfectant activity against liquid cultures, **biofilms** of the aforementioned organisms have survived when the liquid culture of the same organism has been killed. Therefore, biofilm disinfection must be evaluated separately. Better efficacy in biofilm

prevention and removal has been demonstrated by the use of anti-biofilm products compared to detergent disinfectants containing quaternary ammonium compounds. One **problem** with most disinfectants and antiseptics is their **short effective life span**. Hospitals and laboratories today are challenged with **multiple drug-resistant** organisms that may be transmitted from surfaces. These surfaces may be routinely recontaminated by either new sample processing or patient/visitor/staff activity. Therefore, continuous development of new agents/strategies of disinfection is required.

1.1. Aseptic Techniques

1.1.1. Background:

The proper handling of laboratory materials requires special skills that you must master. It is the purpose of this exercise to provide you with procedures that will become routine as you progress through this course. It's all about aseptic technique. When working with microorganisms it is desirable to work with a **pure culture**. Apure culture is composed of only one kind of microorganism. Occasionally a mixed culture is used. In a mixed culture there are two or more organisms that have distinct characteristics and can be separated easily. In either situation the organisms can be identified. When unwanted organisms are introduced into the culture they are known as **contaminants**.

Aseptic technique is a method that prevents the introduction of unwanted organisms into an environment. When changing wound dressings aseptic technique is used to prevent possible infection. When working with microbial cultures aseptic technique is used to prevent introducing additional organisms into the culture.

Microorganisms are everywhere in the environment. When dealing with microbial cultures it is necessary to handle them in such a way that environmental organisms do not get introduced into the culture. Microorganisms may be found on surfaces and floating in air currents. They may fall from objects suspended over a culture or swim in fluids. Aseptic technique prevents environmental organisms from entering a culture.

Doors and windows are kept closed in the laboratory to prevent air currents which may cause microorganisms from surfaces to become **airborne.** Once these microbes are airborne they are more likely to get into cultures. Transfer loops and needles are sterilized before and after

use in the Bacticinerator to prevent introduction of unwanted organisms. Agar plates are held in a manner that minimizes the exposure of the surface to the environment. When removing lids from tubes, lids are held in the hand and not placed on the countertop during the transfer of materials from one tube to another. These techniques are the basis of laboratory aseptic technique. In this laboratory exercise the location of environmental organisms will be explored and how microorganisms can be transmitted through contact with contaminated surfaces.

1.1.2. Principles:

As mentioned above, aseptic transfer of a culture from one culture vessel to another is successful only if no contaminating microorganisms are introduced in the process. A transfer may involve the transport of organisms from an isolated colony on a plate of solid medium to a broth tube, or inoculating various media (solid or liquid) from a broth culture for various types of tests. **The general aseptic procedure can be summarized as follows:**

Work Area Disinfection: The work area is first treated with a disinfectant to kill any microorganisms that may be present. This step destroys vegetative cells and viruses; **endospores**, however, are not destroyed in this brief application of disinfectant.

Loops and Needles: The transport of organisms will be performed with an inoculating loop or needle. To sterilize the loop or needle prior to picking up the organisms, heat must be applied with a Bunsen burner flame, rendering them glowing red-hot.

Culture Tube Flaming: Before inserting the cooled loop or needle into a tube of culture, the tube cap is removed and the mouth of the culture tube flamed. Once the organisms have been removed from the tube, the tube mouth must be flamed again before returning the cap to the tube.

Liquid Medium Inoculation: If a tube of liquid medium is to be inoculated, the tube mouth must be flamed before inserting the loop into the tube. To disperse the organisms on the loop, the loop should be twisted back and forth in the medium. If an inoculating needle is used for stabbing a solid medium, the needle is inserted deep into the medium.

Final Flaming: Once the inoculation is completed, the loop or needle is removed from the tube, flamed as before, and returned to a receptacle. These tools should never be placed on the table top. The inoculated tube is also flamed before placing the cap on the tube.

Petri Plate Inoculation: To inoculate a Petri plate, no heat is applied to the plate and a loop is used for the transfer. When streaking the surface of the medium, the cover should be held diagonally over the plate bottom to prevent air contamination of the medium.

Final Disinfection: When all work is finished, the work area is treated with disinfectant to ensure that any microorganisms deposited during any of the procedures are eliminated. To gain some practice in aseptic transfer of bacterial cultures, three simple transfers will be performed here in this exercise:

1. Broth culture to broth.
2. Agar slant culture to agar slant.
3. Agar plate to agar slant.

1.1.3. Purpose:

To appreciate the importance of aseptic techniques in microbiology laboratories and learn how to apply it.

1.1.4. Transfer from broth culture to another broth:

Do a broth tube to broth tube inoculation, using the following technique. Figure 1-1 illustrates the procedure for removing organisms from a culture, and Figure 1-2 shows how to inoculate a tube of sterile broth.

1.1.4.1. Materials:

- Broth culture of *Escherichia coli*
- Tubes of sterile nutrient broth
- Inoculating loop
- Bunsen burner
- Disinfectant for desktop and sponge
- China marking pencil

1.1.4.2. Procedures:

1. Prepare your desktop by swabbing down its surface with a disinfectant. Use a sponge.
2. With a marking pencil, label a tube of sterile nutrient broth with your initials and *E. coli.*
3. Sterilize your inoculating loop by holding it over the flame of a Bunsen burner until it becomes bright red. The entire wire must be heated. See illustration 1, Figure 1-1**)**
4. Using your free hand, gently shake the tube to disperse the culture (illustration 2, Figure 1-1).
5. Grasp the tube cap with the little finger of your hand holding the inoculating loop and remove it from the tube. Flame the mouth of the tube as shown in illustration 3, Figure 1-1.
6. Insert the inoculating loop into the culture (illustration 4, Figure 1-1).
7. Remove the loop containing the culture, flame the mouth of the tube again (illustration 5, Figure 1-1), and recap the tube (illustration 6). Place the culture tube back on the test-tube rack.

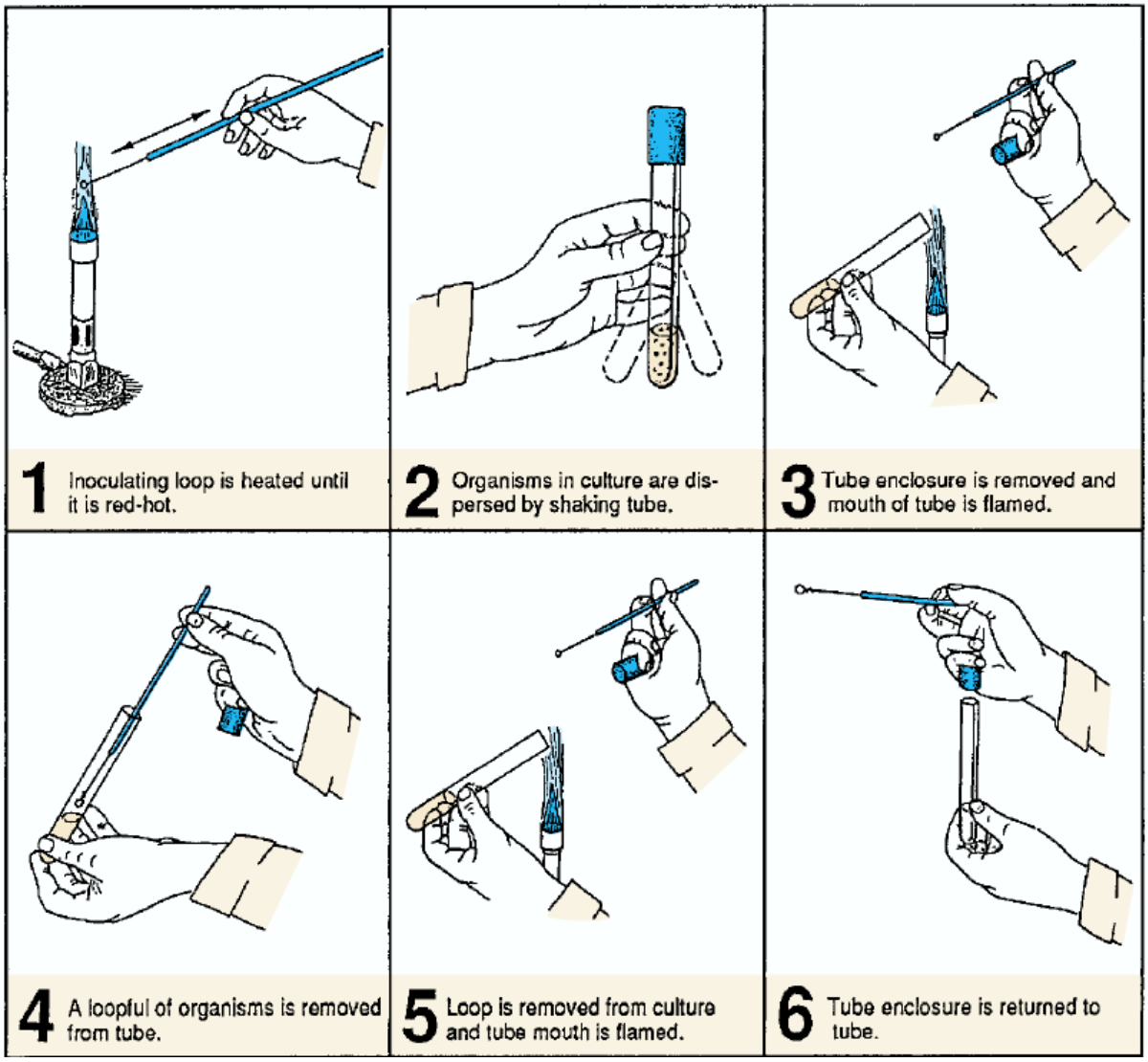


Figure 1-1: Procedure for removing organisms from a broth culture with inoculating loop.

8. Grasp a tube of sterile nutrient broth with your free hand, carefully remove the cap with your little finger, and flame the mouth of this tube (illustration 1, Figure 1-2).
9. Without flaming the loop, insert it into the sterile broth, inoculating it (illustration 2, Figure 1-2). To disperse the organisms into the medium, move the loop back and forth in the tube.
10. Remove the loop from the tube and flame the mouth (illustration 3, Figure 1-2). Replace the cap on the tube (illustration 4, Figure 1-2).
11. Sterilize the loop by flaming it (illustration 5, Figure 1-2). Return the loop to its container.
12. Incubate the culture you just inoculated at 37° C for 24–48 hours.

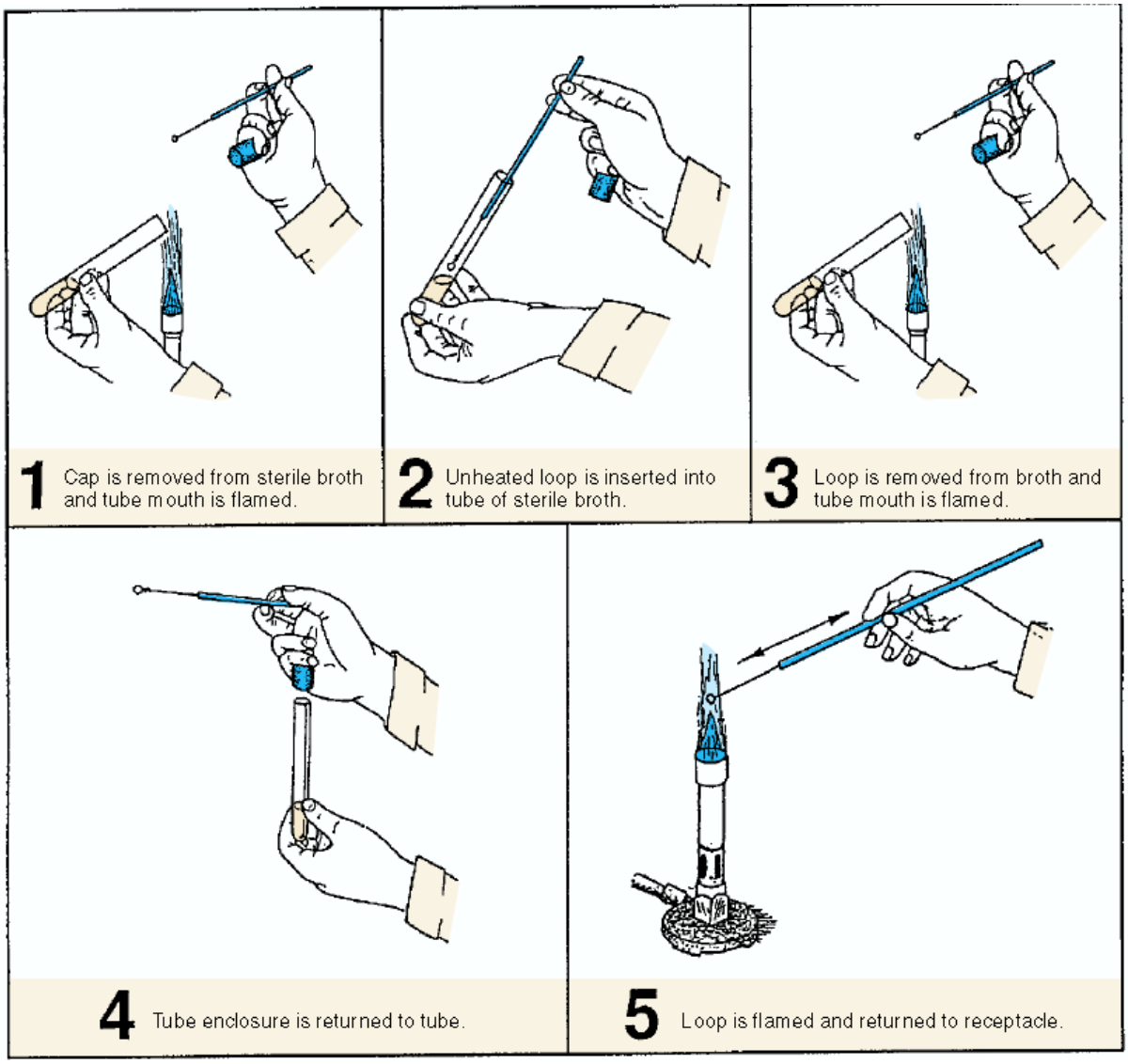


Figure 1-2: Procedure for inoculating a nutrient broth.

1.1.5. Transfer of bacteria from slant to slant:

To inoculate a sterile nutrient agar slant from an agar slant culture, use the following procedure. Figure 1-3 illustrates the entire process.

1.1.5.1. Materials:

- Agar slant culture of *E. coli*, sterile nutrient agar slant
- Inoculating loop, Bunsen burner, China marking pencil

1.1.5.2. Procedures:

1. Prepare your desktop by swabbing down its surface with a disinfectant.
2. With a marking pencil label a tube of nutrient agar slant with your initials and *E. coli*.

3. Sterilize your inoculating loop by holding it over the flame of a Bunsen burner until it becomes bright red (illustration 1, Figure 1-3). The entire wire must be heated. Allow the loop to cool completely.
4. Using your free hand, pick up the slant culture of *E. coli* and remove the cap using the little finger of the hand that is holding the loop (illustration 2, Figure 1-3).
5. Flame the mouth of the tube and insert the cooled loop into it. Pick up some of the culture on the loop (illustration 3, Figure 1-3) and remove the loop from the tube.
6. Flame the mouth of the tube (illustrations 4 and 5, Figure 1-3) and replace the cap, being careful not to burn your hand. Return tube to rack.
7. Pick up a sterile nutrient agar slant with your free hand, remove the cap with your little finger as before, and flame the mouth of the tube (illustration 6, Figure 1-3).
8. Without flaming the loop containing the culture, insert the loop into the tube and gently inoculate the surface of the slant by moving the loop back and forth over the agar surface, while moving up the surface of the slant (illustration 7, Figure 1-3). This should involve a type of serpentine motion.
9. Remove the loop, flame the mouth of the tube, and recap the tube (illustration 8, Figure 1-3). Replace the tube in the rack.
10. Flame the loop, heating the entire wire to red-hot (illustration 9, Figure 1-3), allow to cool, and place the loop in its container.
11. Incubate the inoculated agar slant at 30° C for 24–48 hours.

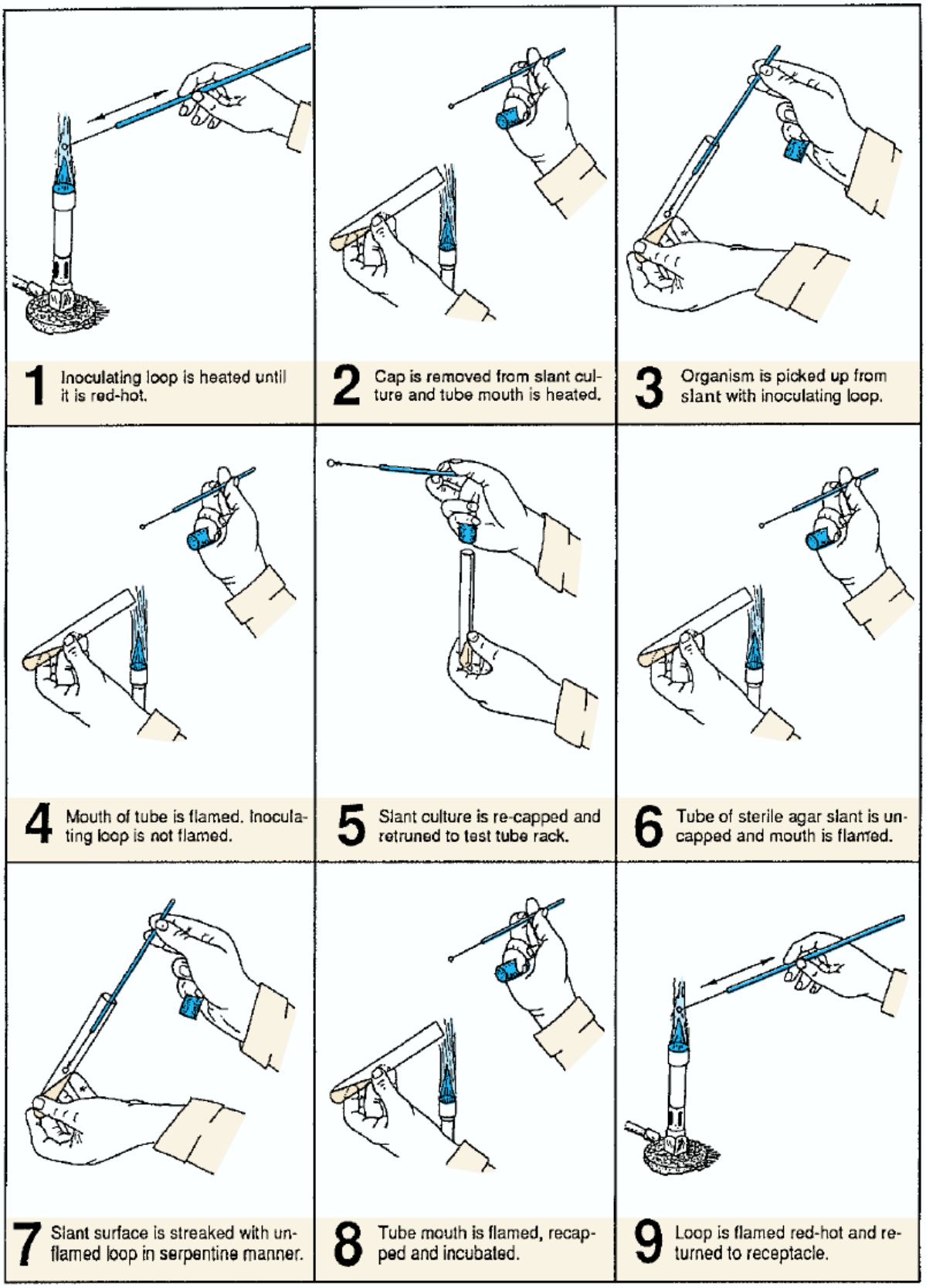


Figure 1-3: Procedure for inoculating a nutrient agar slant from a slant culture.

1.1.6. Working with agar plates (Inoculating a slant from a Petri plate):

1.1.6.1. Materials:

- Nutrient agar plate with bacterial colonies
- Sterile nutrient agar slant
- Inoculating loop
- China marking pencil

1.1.6.2. Procedures:

1. If you have not done so, swab your work area with disinfectant. Allow area to dry.
2. Label a sterile nutrient agar slant with your name and organism to be transferred.
3. Flame an inoculating loop until it is red-hot (illustration 1, Figure 1-4). Allow the loop to cool.
4. As shown in illustration 2, Figure 1-4, raise the lid of a Petri plate sufficiently to access a colony with your sterile loop.

Do not gouge (scrape) into the agar with your loop as you pick up organisms, and do not completely remove the lid, exposing the surface to the air. Close the lid once you have picked up the organisms.

5. With your free hand, pick up the sterile nutrient agar slant tube. Remove the cap by grasping the cap with the little finger of the hand that is holding the loop.
6. Flame the mouth of the tube and insert the loop into the tube to inoculate the surface of the slant, using a serpentine motion (illustration 3, Figure 1-4). Avoid disrupting the agar surface with the loop.
7. Remove the loop from the tube and flame the mouth of the tube. Replace the cap on the tube (illustration 4, Figure 1-4).
8. Flame the loop (illustration 5, Figure 1-4) and place it in its container.
9. Incubate the nutrient agar slant at 37° C for 24–48 hours.

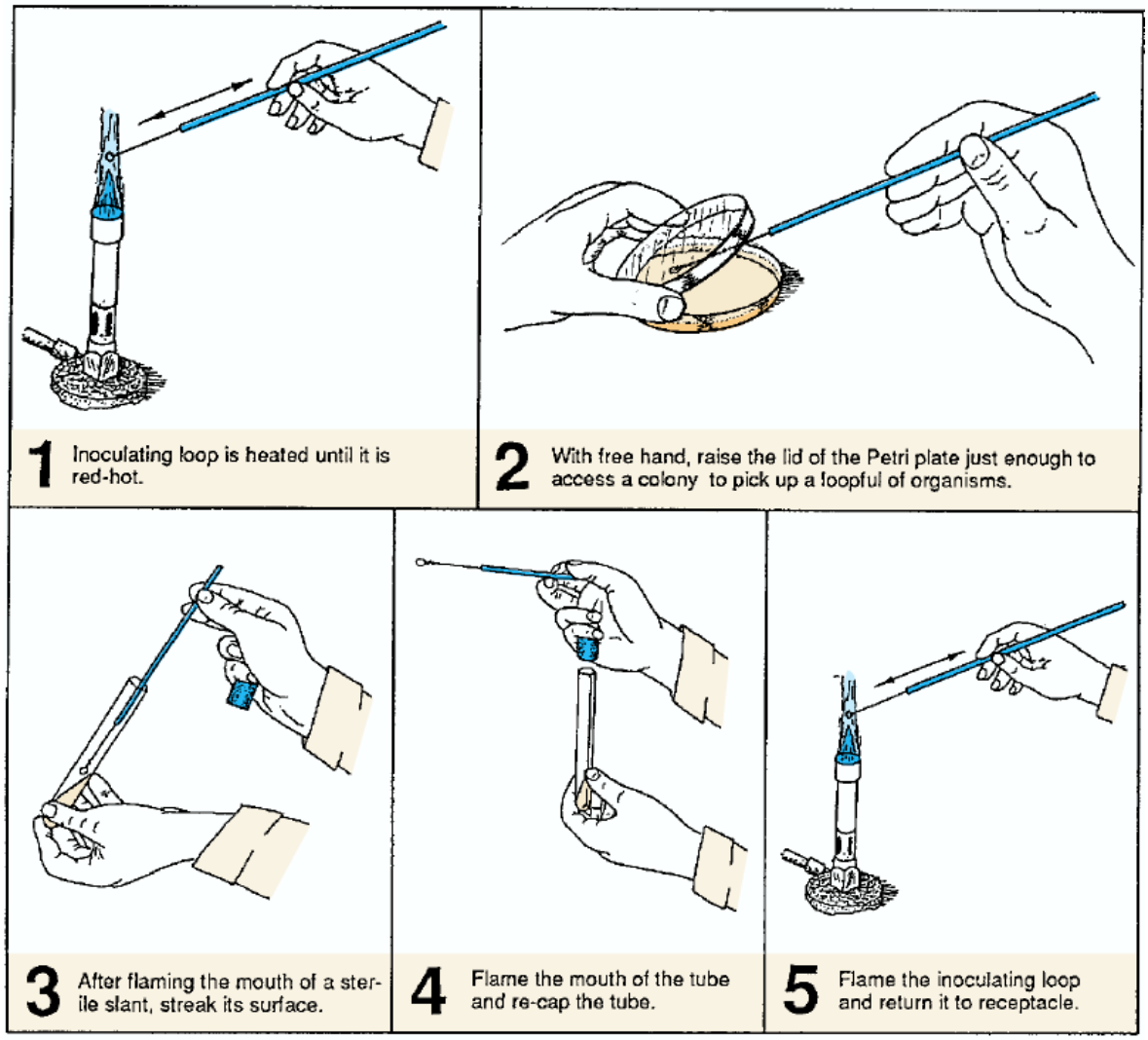


Figure 1-4: Procedure for inoculating a nutrient agar slant from an agar plate.

1.1.7. Results:

1. Were all your transfers successful?
2. What evidence do you have that they were successful?
3. What evidence do you have that a transfer is unsuccessful?

1.1.8. Review Questions:

1. What kinds of organisms are destroyed when your desktop is scrubbed down with a disinfectant?
2. Are bacterial endospores destroyed?
3. How hot should inoculating loops and needles be heated?
4. Why is it necessary to flame the mouth of the tube before and after performing an inoculation?

Exercise 2: Microscopic Techniques I

2.1. Wet-Mount and hanging drop Preparations

2.1.1. Background:

Microbiologists employ a variety of light microscopes in their work: bright-field, dark-field, phase-contrast, and fluorescence are most commonly used. In fact, the same microscope may be a combination of types: bright-field and phase-contrast, or phase-contrast and fluorescence. You will use these microscopes and the principles of microscopy extensively in this course as you study the form, structure, staining characteristics, and motility of different microorganisms. Therefore, proficiency in using the different microscopes is essential to all aspects of microbiology and must be mastered at the very beginning of a microbiology course. **(for more details about the different types of microscopes see Lecture 2 of the theory part).**

2.1.2. Principles:

The simplest method for examining living microorganisms is to suspend them in a fluid (water, saline, or broth) and prepare either a “hanging drop” or a simple “wet mount”. The slide for a hanging drop is ground with a concave well in the centre; the cover glass holds a drop of the suspension. When the cover glass is inverted over the well of the slide, the drop hangs from the glass in the hollow concavity of the slide (**Error! Reference source not found.**, step 4). M icroscopic study of such a wet preparation can provide useful information. Primarily, the method is used to determine whether or not an organism is motile, but it also permits an undistorted view of natural patterns of cell groupings and of individual cell shape. Hanging-drop preparations can be observed for a fairly long time, because the drop does not dry up quickly. Wet-mounted preparations are used primarily to detect microbial motility rapidly. The fluid film is thinner than that of hanging-drop preparations and therefore the preparation tends to dry up more quickly, even when sealed. Although the hanging drop is the classical method for viewing unstained microorganisms, the wet mount is easier to perform and usually provides sufficient information.

2.1.3. *Purpose:*

To observe bacteria in a simple wet mount and determine their motility.

2.1.4. Materials:

- 24-hour broth culture of *Proteus vulgaris* mixed with a light suspension of yeast cells.
- 24-hour broth culture of *Staph. epidermidis* mixed with a light suspension of yeast cells.
- Microscope slides.
- Cover glasses.
- Capillary pipettes and pipette bulbs.
- Permanent marking pen.
- Clear nail polish (optional).

2.1.5. Safety precautions:

Be careful with the Bunsen burner flame. Slides and coverslips are glass. Do not cut yourself when using them. Dispose of any broken glass in the appropriately labelled container. Discard contaminated depression slides in a container with disinfectant.

2.1.6. Procedure for wet-mount preparation:

1. Using a pipette bulb, aspirate a small amount of the *Proteus* culture with a capillary pipette and place a small drop on a clean microscope slide (Figure 2-1, step 1).
2. Carefully place a clean cover glass over the drop, trying to avoid bubble formation (Figure 2-1, step 2). The fluid should not leak out from under the edges of the cover glass. If it does, wait until it dries before sealing.
3. If you examine the slide immediately, you don't need to seal the coverslip. Otherwise, seal around the edges of the coverslip with a thin film of clear nail polish (Figure 2-1, step 3). Be certain the nail polish is completely dry before examining the slide under the microscope.
4. Place the slide on the microscope stage, cover glass up. Start your examination with the low-power objective to find the focus. You may find it helpful to focus first on the left-hand edge of the coverslip. **The light should be reduced with the iris diaphragm and, if necessary, by lowering the condenser for increased contrast**. You should be

able to focus easily on the yeast cells in the suspension. If you have trouble with the focus, ask the instructor.

5. Continue your examination with the high-dry and oil-immersion objectives (be very careful not to break the cover glass with the latter). Although the yeast cells will be obvious because of their larger size, look around them to observe the bacterial cells.
6. Make a wet-mount preparation of the *Staphylococcus* culture, following the same procedures just described.
7. Record your observations of the size, shape, cell groupings, and motility of the two bacterial organisms in comparison to the yeast cells.
8. Discard your slides in a container with disinfectant solution.

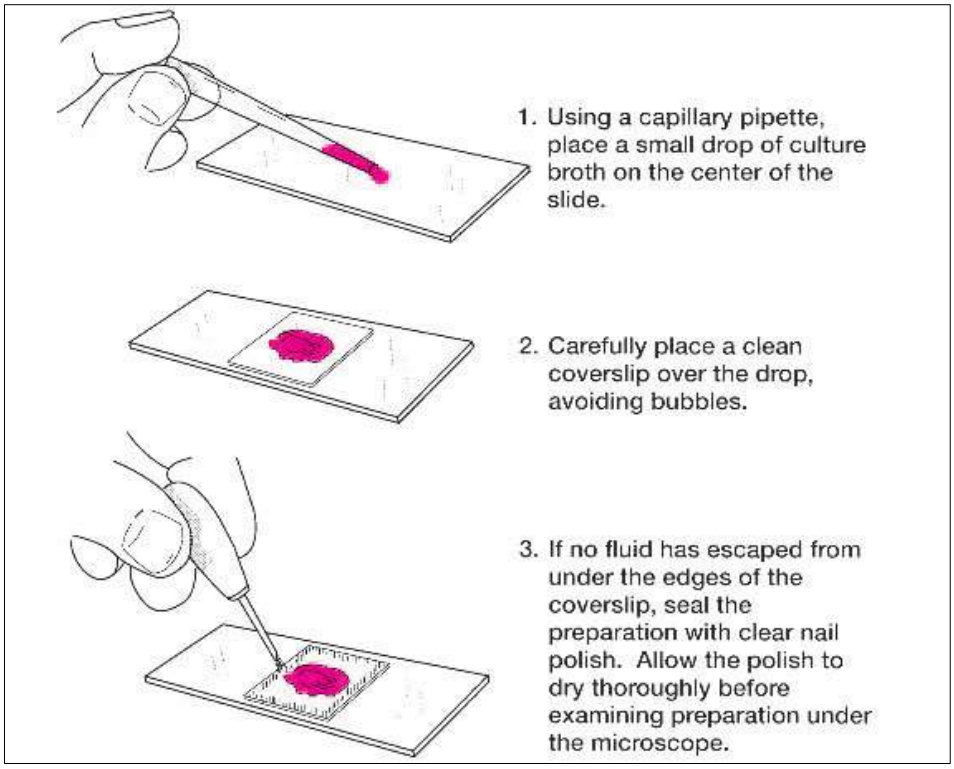


Figure 2-1: Wet-mount preparation.

Procedures

1. Take a cover glass and clean it thoroughly, making certain it is free of grease (the drop to be placed on it will not hang from a greasy surface). It may be dipped in alcohol and polished dry with tissue, or washed in soap and water, rinsed completely, and wiped dry.

2. Take one hollow-ground slide and clean the well with a piece of dry tissue. Place a thin film of petroleum jelly around (not in) the concave well on the slide (Figure 2-2, step 1).
3. Gently shake the broth culture of *Proteus* until it is evenly suspended. Using good aseptic technique, sterilize the wire loop, remove the cap of the tube, and take up a loopful of culture. Be certain the loop has cooled to room temperature before inserting it into the broth or it may cause the broth to "sputter" and create a dangerous aerosol. Close and return the tube to the rack.
4. Place the loopful of culture in the center of the cover glass as in Figure 2-2, step 2 (do not spread it around). Sterilize the loop and put it down.
5. Hold the hollow-ground slide inverted with the well down over the cover glass (Figure 2-2, step 3), then press it down *gently* so that the petroleum jelly adheres to the cover glass. Now turn the slide over. You should have a sealed wet mount, with the drop of culture hanging in the well (Figure 2-2, step 4).
6. Place the slide on the microscope stage, cover glass up. Start your examination with the low-power objective to find the focus. It is helpful to focus first on one edge of the drop, which will appear as a dark line. The light should be reduced with the iris diaphragm and, if necessary, by lowering the condenser. You should be able to focus easily on the yeast cells in the suspension. If you have trouble with the focus, ask the instructor for help.

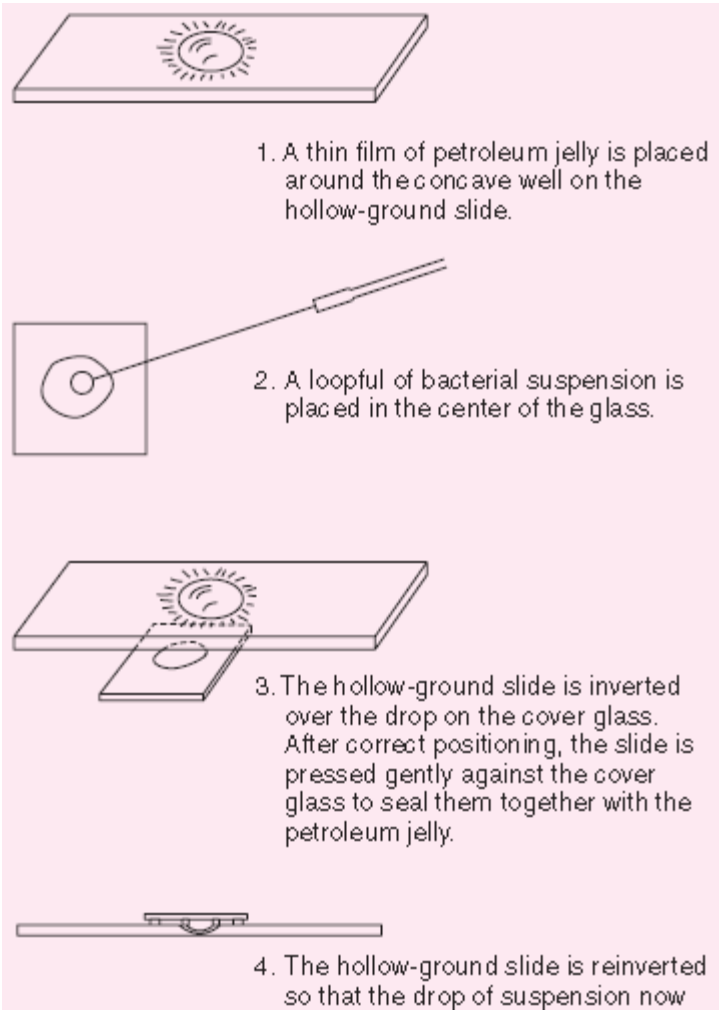
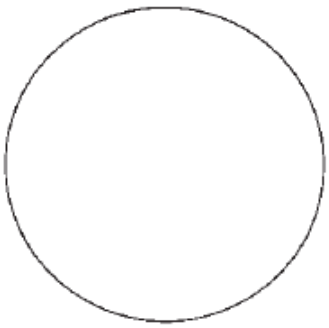


Figure 2-2: Hanging-drop preparation using petroleum jelly to seal the cover glass to the slide.

7. Continue your examination with the high-dry and oil-immersion objectives (be very careful not to break the cover glass with the latter). Although the yeast cells will be obvious because of their larger size, look around them to observe the bacterial cells.
8. Make a hanging-drop preparation of the *Staphylococcus* culture, following the same procedures just described.
9. Record your observations of the size, shape, cell groupings, and motility of the two bacterial organisms in comparison to the yeast cells.
10. *Discard your slides in a container with disinfectant solution.*

2.1.7. Results:

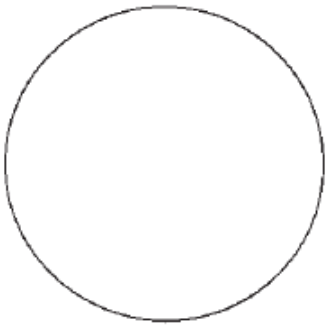
1. Make drawings in the following circles to show the shape and grouping of each organism. Indicate below the circle whether it is motile or nonmotile. How does their size compare with that of the yeast cells in the preparation?



Proteus vulgaris

Motile _____

Nonmotile _____



Staphylococcus epidermidis

Motile _____

Nonmotile _____

2.1.8. Review Questions:

1. How does true motility differ from Brownian movement?

2. What morphological structure is responsible for bacterial motility?

3. Why is a wet preparation discarded in disinfectant solution?

4. What is the value of a hanging-drop preparation?

5. What is the value of a wet-mount preparation?

Exercise 3: Microscopic Techniques II

3.1. Simple Staining and Smear Preparation

3.1.1. Background:

As we have seen in the previous exercise, wet mounts of bacterial cultures can be very informative, but they have limitations. Bacteria bounce about in fluid suspensions with Brownian movement or true motility, and are difficult to visualize sharply. We can see their shapes and observe their activity under a cover glass, but it is difficult to form a complete idea of their morphology. An important part of the problem is the minute size of bacteria. Because they are so small and have so little substance, they tend to be transparent, even when magnified in subdued light. The trick, then, is to find ways to stop their motion and tag their structures with something that will make them more visible to the human eye. Many sophisticated ways of doing this are known, but the simplest is to smear out a bacterial suspension on a glass slide, “fix” the organisms to the slide, then stain them with a visible dye.

3.1.2. Principles:

A bacterial smear is a dried preparation of bacterial cells on a glass slide. In a bacterial smear that has been properly processed, (1) the bacteria are evenly spread out on the slide in such a concentration that they are adequately separated from one another, (2) the bacteria are not washed off the slide during staining, and (3) bacterial form is not distorted. In making a smear, bacteria from either a broth culture or an agar slant or plate may be used. If a slant or plate is used, a small amount of bacterial growth is transferred to a drop of water on a glass slide (Figure 3-2a) and mixed. The mixture is then spread out evenly over a large area on the slide (Figure 3-2b).

One of the most common errors in smear preparation from agar cultures is the use of too large an inoculum. This invariably results in the occurrence of large aggregates of bacteria piled on top of one another. If the medium is liquid, place one or two loops of the medium directly on the slide (Figure 3-2c) and spread the bacteria over a large area (Figure 3-2d). Allow the slide to air dry at room temperature (Figure 3-2e). After the smear is dry, the next step is to attach the bacteria to the slide by heat-fixing. This is accomplished by gentle heating (Figure 3-2f), passing the slide several times through the hot portion of the flame of a Bunsen burner. Most bacteria can be fixed to the slide and killed in this way without serious distortion of cell structure. The use of a single stain or dye to create contrast between the bacteria and the background is referred to as simple staining. Its chief value lies in its simplicity and ease of use. Simple staining is often employed when information about cell shape, size, and arrangement is desired. In this procedure, one places the heat fixed slide on a staining rack, covers the smear with a small amount of the desired stain for the proper amount of time, washes the stain off with water for a few seconds, and, finally, blots it dry. Basic dyes such as crystal violet (20 to 30 seconds staining time), carbolfuchsin (5 to 10 seconds staining time), or methylene blue (1 minute staining time) are often used. Once bacteria have been properly stained, it is usually an easy matter to distinguish their overall shape. Bacterial morphology is usually uncomplicated and limited to one of a few variations. For future reference, the most common shapes are presented in Figure 3-1.

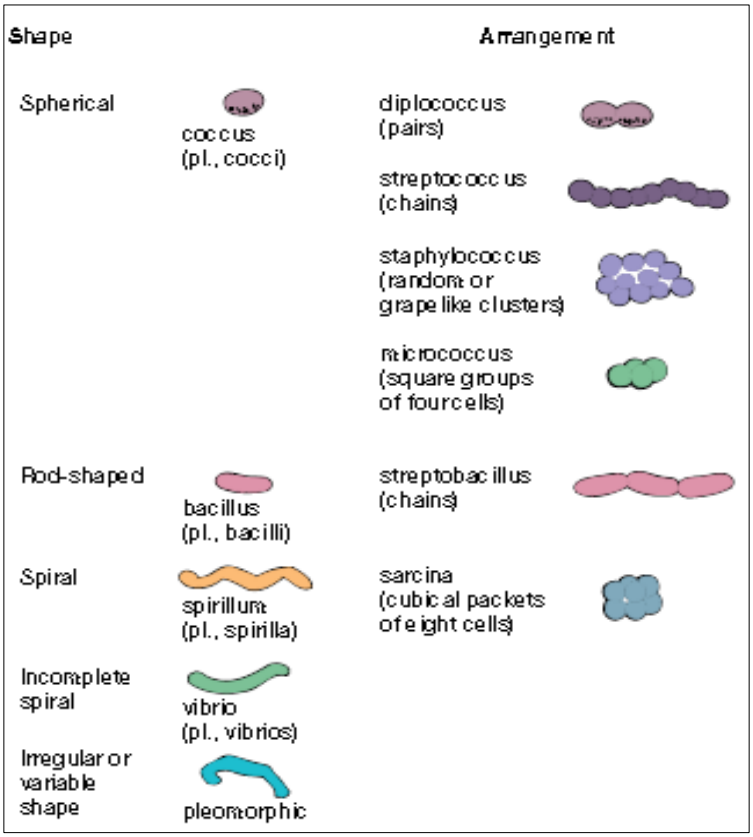


Figure 3-1: Common Bacterial Shapes.

3.1.3. Purpose:

To learn the value of simple stains in studying basic microbial morphology.

3.1.4. Materials:

- 24-hour agar culture of *Staphylococcus epidermidis*.
- 24-hour agar culture of *Bacillus subtilis*.
- 24-hour agar culture of *Escherichia coli*.
- Prepared stained smear of a spiraled organism.
- Methylene blue.
- Absolute methanol (if bacterial incinerator used).
- Safranin.
- Toothpicks.
- Slides.
- Permanent marking pen.

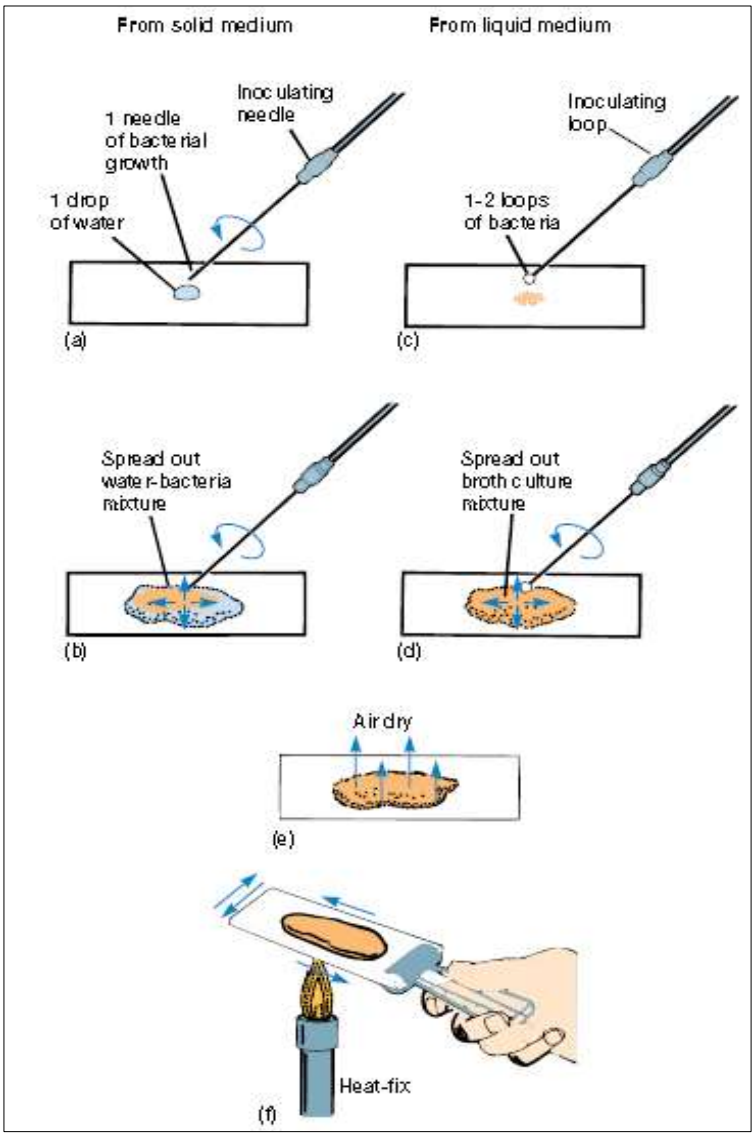


Figure 3-2: Bacterial Smear Preparation.

3.1.5. Safety precautions:

Always use a slide holder or clothespin to hold glass slides when heat-fixing them. Never touch a hot slide until it cools. If a glass slide is held in the flame too long, it can shatter. Be careful with the Bunsen burner flame. If the stains used in this experiment get on your clothing, they will not wash out. Always discard slides in a container with disinfectant.

3.1.6. Procedures:

Smear Preparation

1. With the wax pencil, mark the name of the bacterial culture in the far left corner on each of three slides.

2. For the broth culture, shake the culture tube and, with an inoculating loop, aseptically transfer 1 to 2 loopfuls of bacteria to the centre of the slide. Spread this out to about ½ inch area. When preparing a smear from a slant or plate, place a loopful of water in the centre of the slide (Figure 3-2). With the inoculating needle, aseptically pick up a very small amount of culture and mix into the drop of water. Spread this out as above. (Three slides should be prepared; one each of *B. subtilis* or *Staphylococcus epidermidis*, *Escherichia coli*).
3. Allow the slide to air dry, or place it on a slide warmer or carefully move it over a gas burner 2-3 times.
4. Pass the slide through a Bunsen burner flame three times to heat-fix and kill the bacteria.

Simple Staining

1. Place the three fixed smears on a staining loop or rack over a sink or other suitable receptacle (Figure 3-3a).
2. Stain one slide with alkaline methylene blue for 1 to 1.5 minutes; one slide with carbolfuchsin for 5 to 10 seconds; and one slide with crystal violet for 20 to 30 seconds.
3. Wash stain off slide with water for a few seconds (Figure 3-3b).
4. Blot slide dry with bibulous paper (Figure 3-3c). Be careful not to rub the smear when drying the slide because this will remove the stained bacteria.
5. Examine under the oil immersion lens and complete the report for this exercise.
6. You may want to treat smears of the same bacterium with all three stains in order to compare them more directly. It is also instructive to cover bacterial smears for varying lengths of time with a given stain in order to get a feel for how reactive they are and the results of overstaining or understaining a slide preparation.

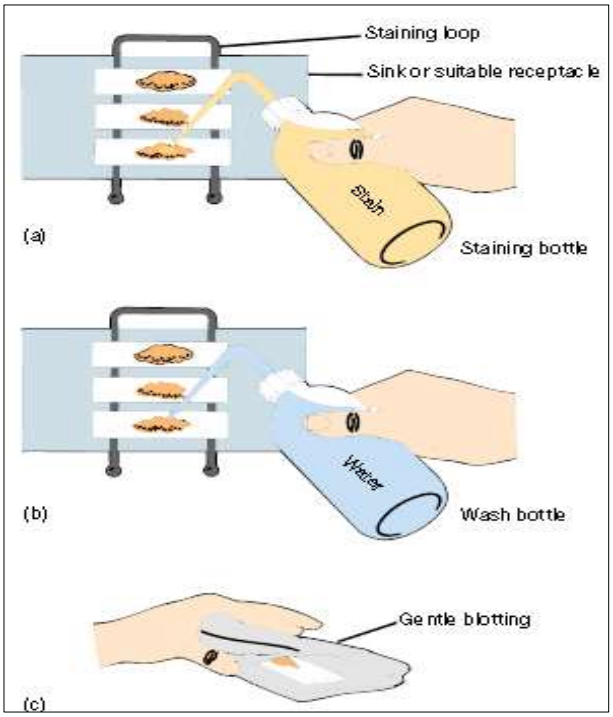


Figure 3-3: Simple Staining Procedure.

3.1.7. Results:

Organism in Broth Culture	*Stain*	*Color*	*Coccus, Rod, or Spiral*	*Cell Grouping*	*Diagram*
S. epidermidis					
B. subtilis					
E. coli					

3.1.8. Review Questions:

1. What are the two purposes of heat fixation?
2. What is the purpose of simple staining?
3. Why are basic dyes more successful in staining bacteria than acidic dyes?
4. Name three basic stains.
5. Why is time an important factor in simple staining?
6. How would you define a properly prepared bacterial smear?
7. Why should you use an inoculating needle when making smears from solid media? An inoculating loop from liquid media?

Exercise 4: Differential staining I

4.1. Gram stain:

4.1.1. Background:

The simple staining procedure performed in the previous exercise makes it possible to see bacteria clearly, but it does not distinguish between organisms of similar morphology. In 1884, a Danish pathologist, **Christian Gram**, discovered an important staining procedure (Gram stain) which is used till now. The Gram stain is one of the most useful tools in the microbiology laboratory and is used universally. In the diagnostic laboratory, it is used not only to study microorganisms in cultures, but it is also applied to smears made directly from clinical specimens. Direct, Gram-stained smears are read promptly to determine the relative numbers and morphology of bacteria in the specimen. **This information is valuable to the physician in planning the patient's treatment before culture results are available**. It is also valuable to microbiologists, who can plan their culture procedures based on their knowledge of the bacterial forms they have seen in the specimen.

4.1.2. Principles:

Simple staining depends on the fact that bacteria differ chemically from their surroundings and thus can be stained to contrast with their environment. **Bacteria also differ from one another chemically and physically and may react differently to a given staining procedure.** This is the principle of differential staining. Therefore, differential staining can **distinguish** between **types of bacteria**.

The Gram stain is the most useful and widely employed differential stain in bacteriology. It divides bacteria into two groups; gram negative and gram positive. The first step in the procedure involves staining with the basic dye crystal violet. This is the primary stain. It is followed by treatment with an iodine solution, which functions as a mordant; that is, it increases the interaction between the bacterial cell and the dye so that the dye is more tightly bound or the cell is more strongly stained. The smear is then decolorized by washing with an agent such as 95% ethanol or isopropanol-acetone. Gram-positive bacteria retain the crystal violet-iodine complex when washed with the decolorizer, whereas gram-negative bacteria lose their crystal

violet-iodine complex and become colorless. Finally, the smear is counterstained with a basic dye, different in color than crystal violet. This counterstain is usually safranin. The safranin will stain the colorless, gram-negative bacteria pink but does not alter the dark purple color of the gram-positive bacteria. The end result is that gram-positive bacteria are deep purple in color and gram-negative bacteria are pinkish to red in color.

The Gram stain does not always yield clear results. Species will differ from one another in regard to the ease with which the crystal violet-iodine complex is removed by ethanol. Gram-positive cultures **may often turn gram negative if they get too old**. Thus, it is always best to Gram stain young, vigorous cultures rather than older ones. Furthermore, some bacterial species are gram variable. That is, some cells in the same culture will be gram positive and some, gram negative. Therefore, one should always be certain to run Gram stains on several cultures under carefully controlled conditions in order to make certain that a given bacterial "strain" is truly gram positive or gram negative. **Indistinct Gram-stain results can be confirmed by a simple test using KOH**. Place a drop of 10% KOH on a clean glass slide and mix with a loopful of bacterial paste. Wait 30 seconds, then pull the loop slowly through the suspension and up and away from the slide. A gram-negative organism will produce a **mucoid string**; a gram-positive organism **remains fluid**.

4.1.3. Purpose:

To learn the Gram-stain technique and to understand its value in the study of bacterial morphology.

4.1.4. Materials:

- 24-hour agar culture of: *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Neisseria sicca*, *Saccharomyces cerevisiae* (yeast), *Bacillus subtilis*, *Escherichia coli*, *Proteus vulgaris*.
- Specimen of simulated pus from a postoperative wound infection.
- Hucker's crystal violet.
- Gram's iodine.
- Ethyl alcohol, 95%.
- Safranin.
- Slides.
- Marking pen and slide labels.

4.1.5. Safety precautions:

Be careful with the Bunsen burner flame. Volatile and flammable liquids (ethanol, isopropanol-acetone) are used in this experiment. Do not use them near an open flame. If the stains used in this experiment get on your clothing, they will not wash out. Discard slides in a container with disinfectant. Hold all slides with forceps or a clothespin when heat-fixing. Gram crystal violet, safranin, and iodine can cause irritation to the eyes, respiratory system and skin. Avoid contact with skin and eyes. Do not breathe spray. Wear suitable protective gloves. Always keep the containers tightly closed.

4.1.6. Procedures:

1. Prepare heat-fixed smears of *E. coli*, *S. aureus*, and the mixture of *E. coli* and *S. aureus* (see Figure 3-2).

2. Place the slides on the staining rack.
3. Flood the smears with crystal violet and let stand for 30 seconds (Figure 4-1a).
4. Rinse with water for 5 seconds (Figure 4-1b).
5. Cover with Gram's iodine mordant and let stand for 1 minute (Figure 4-1c).
6. Rinse with water for 5 seconds (Figure 4-1d).
7. Decolorize with 95% ethanol for 15 to 30 seconds. Do not decolorize too long. Add the decolorizer drop by drop until the crystal violet fails to wash from the slide (Figure 4-1e).
8. Rinse with water for 5 seconds (Figure 4-1f).
9. Counterstain with safranin for about 60 to 80 seconds (Figure 4-1g). Safranin preparations vary considerably in strength, and different staining times may be required for each batch of stain.
10. Rinse with water for 5 seconds (Figure 4-1h).
11. Blot dry with bibulous paper (Figure 4-1i) and examine under oil immersion. Gram-positive organisms stain blue to purple; gram-negative organisms stain pink to red. There is no need to place a coverslip on the stained smear.

Control Procedure:

1. Prepare two heat-fixed slides of the mixed culture of *E. coli* and *S. aureus.*
2. Stain one with crystal violet only (steps 3 to 6).
3. Carry the second slide through the decolorizing process (steps 3 to 8).
4. Examine these two slides and compare with the mixed culture slide that was carried all the way through the staining procedure (steps 1 to 10). Your observations should help you understand how the Gram stain works.

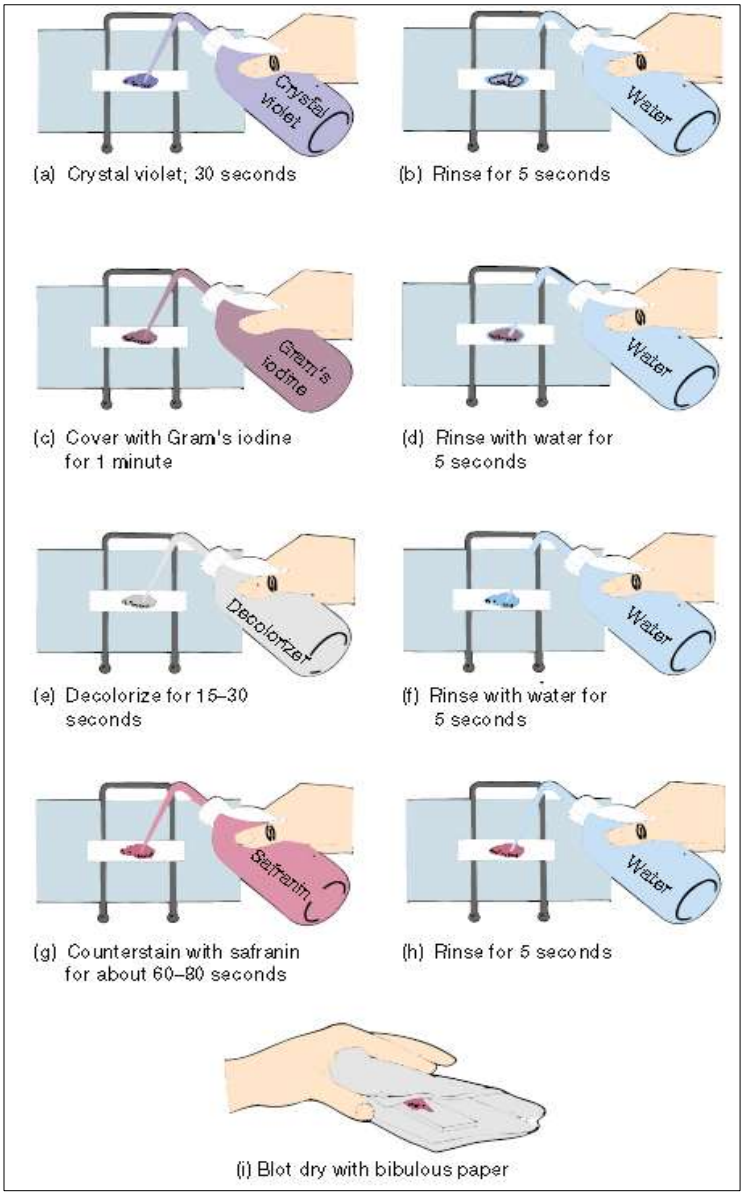
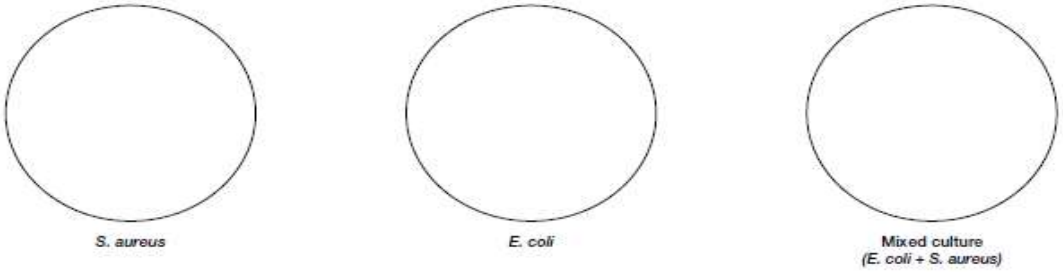


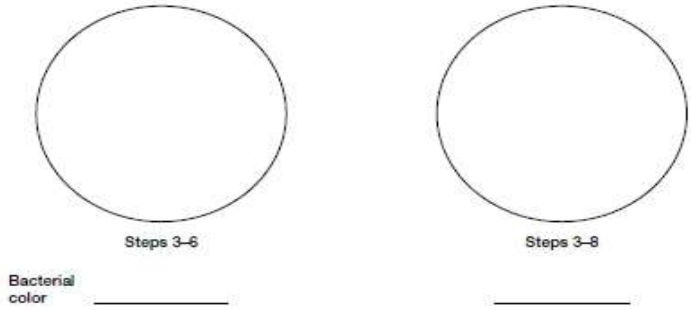
Figure 4-1: Gram-stain Procedure.

4.1.7. Results:

1. Draw the Gram-stained bacteria in the following circles.



2. Control Gram-stain results.



4.1.8. Review Questions:

1. What is the function of the iodine solution in the Gram stain? If it were omitted, how would staining results be affected?
2. What is the purpose of the alcohol solution in the Gram stain?
3. What counterstain is used? Why is it necessary? Could colors other than red be used?
4. On the basis of Gram reaction, can you distinguish species of:
   - *Staphylococcus* and *Streptococcus*?
   - S*taphylococcus* and *Neisseria*?
   - *Escherichia* and *Proteus*?
   - *Escherichia* and *Bacillus*?
5. What is the size of staphylococci in micrometers? In centimeters?
6. What is the advantage of the Gram stain over the simple stain?
7. In what kind of clinical situation would a direct smear report from the laboratory be of urgent importance?
8. What is the current theory about the mechanism of the Gram-stain reaction?
9. Describe at least two conditions in which an organism might stain gram variable.

Exercise 5: Differential staining II

5.1. Acid-Fast Stain

5.1.1. Background:

Members of the bacterial genus *Mycobacterium* contain large amounts of lipid (fatty) substances within their cell walls. These fatty waxes resist staining by ordinary methods. Because this genus contains species that cause important human diseases (the agent of tuberculosis is a *Mycobacterium*), the diagnostic laboratory must use special stains to reveal them in clinical specimens or cultures. The original technique for applying carbolfuchsin with heat is called the Ziehl-Neelsen stain, named after the two bacteriologists who developed it in the late 1800s. The later modification of the technique employs more concentrated carbolfuchsin reagent rather than heat to ensure stain penetration and is known as the Kinyoun stain. A more modern fluorescence technique is used in many clinical laboratories today. In this method, the patient specimen is stained with the dye auramine, which fluoresces when it is exposed to an ultraviolet light source. Because any acid-fast bacilli take up this dye and fluoresce brightly against a dark background when viewed with a fluorescence microscope, the smear can be examined under 400 X (high-dry) magnification rather than 1,000 X (oil-immersion) magnification. As a result, the slide can be screened more quickly for the presence of acid-fast bacilli.

5.1.2. Principles:

A few species of bacteria in the genera Mycobacterium and Nocardia, and the parasite Cryptosporidium do not readily stain with simple stains. However, these microorganisms can be stained by heating them with carbolfuchsin. The heat drives the stain into the cells. Once the microorganisms have taken up the carbolfuchsin, they are not easily decolorized by acid-alcohol, and hence are termed acid-fast. This acid-fastness is due to the high lipid content (mycolic acid) in the cell wall of these microorganisms. The Ziehl-Neelsen acid-fast staining procedure (developed by Franz Ziehl, a German bacteriologist, and Friedrich Neelsen, a German pathologist, in the late 1800s) is a very useful differential staining technique that makes use of this difference in retention of carbolfuchsin. Acid-fast microorganisms will retain this dye and appear red. Microorganisms that are not acid-fast, termed non-acid-fast, will appear blue or brown due to

the counterstaining with methylene blue after they have been decolorized by the acid-alcohol. A modification of this procedure that employs a wetting agent (Tergitol No. 7) rather than heat to ensure stain penetration is known as the Kinyoun staining procedure (developed by Joseph Kinyoun, German bacteriologist, in the early 1900s).

5.1.3. Purpose:

To learn the acid-fast technique and to understand its value when used to stain a clinical specimen.

5.1.4. Materials:

- A young slant culture of *Mycobacterium phlei* (a saprophyte), 24-hour broth culture of *Bacillus subtilis*
- A sputum specimen simulating that of a 70-year-old man from a nursing home, admitted to the hospital with chest pain and bloody sputum
- Gram-stain reagents
- Kinyoun's carbolfuchsin and Acid-alcohol solution
- Methylene blue
- Slides and Slide rack
- Diamond glass-marking pencil
- Marking pencil or pen
- 2 x 3-cm filter paper strips
- Forceps

5.1.5. Safety precautions:

A volatile and flammable liquid (acid-alcohol) is used in this experiment. Do not use near an open flame. If the carbolfuchsin or methylene blue get on your clothing, they will not wash out. Note: when carbolfuchsin is heated, phenol is driven off. Phenol is poisonous and caustic. Thus, always use a chemical hood with the exhaust fan on for the hot plate or boiling water bath setup.

Discard slides in a container with disinfectant. No mouth pipetting. Mycobacteria should be handled in a safety cabinet to prevent dissemination in case the human pathogen *Mycobacterium*

tuberculosis should occur among the cultures. Infected material should be disinfected by heat because mycobacteria are relatively resistant to chemical disinfectants.

5.1.6. Procedures:

Ziehl-Neelsen (Hot Stain) Procedure:

1. Prepare a smear consisting of a mixture of *E. coli* and *M. phlei*.
2. Allow the smear to air dry and then heat-fix (**Error! Reference source not found.**).
3. Place the slide on a hot plate that is within a chemical hood (with the exhaust fan on), and cover the smear with a piece of paper towelling that has been cut to the same size as the microscope slide. Saturate the paper with Ziehl's carbolfuchsin (Figure 5-1a). Heat for 3 to 5 minutes. Do not allow the slide to dry out, and avoid excess flooding! Also, prevent boiling by adjusting the hot plate to a proper temperature. A boiling water bath with a staining rack or loop held 1 to 2 inches above the water surface also works well. (Instead of using a hot plate to heatdrive the carbolfuchsin into the bacteria, an alternate procedure is to cover the heat-fixed slide with a piece of paper towel. Soak the towel with the carbolfuchsin and heat, well above a Bunsen burner flame).
4. Remove the slide, let it cool, and rinse with water for 30 seconds (Figure 5-1b).
5. Decolorize by adding acid-alcohol drop by drop until the slide remains only slightly pink. This requires 10 to 30 seconds and must be done carefully (Figure 5-1c).

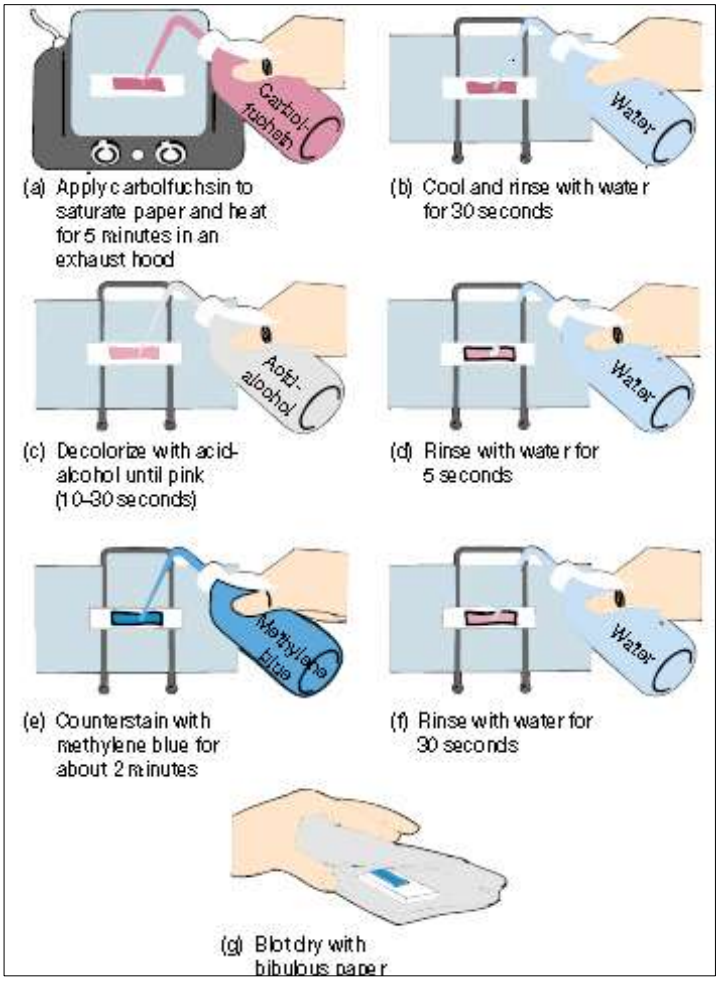


Figure 5-1: Acid-fast Staining Procedure.

6. Rinse with water for 5 seconds (Figure 5-1d).
7. Counterstain with alkaline methylene blue for about 2 minutes (Figure 5-1e).
8. Rinse with water for 30 seconds (Figure 5-1f).
9. Blot dry with bibulous paper (Figure 5-1g).
10. There is no need to place a coverslip on the stained smear. Examine the slide under oil immersion and record your results in the report for this exercise. Acid-fast organisms stain red; the background and other organisms stain blue or brown.
11. Examine the prepared slide of *Mycobacterium phlei*.

Kinyoun (Cold Stain) Procedure:

(This may be used instead of or in addition to the Ziehl-Neelsen procedure).

1. Heat-fix the slide as previously directed.
2. Flood the slide for 5 minutes with carbolfuchsin prepared with Tergitol No. 7 (heat is not necessary).
3. Decolorize with acid-alcohol and wash with tap water. Repeat this step until no more color runs off the slide.
4. Counterstain with alkaline methylene blue for 2 minutes. Wash and blot dry.
5. Examine under oil. Acid-fast organisms stain red; the background and other organisms stain blue.

5.1.7. Results:

Name of Organism	*Visible in Gram Stain* (Yes, No)*	*Gram-Stain Reaction (If Visible)*	*Visible in Acid-Fast Stain*	*Color in Acid-Fast Stain*	*Acid-Fast Reaction (If Visible)*
Cultures					
Sputum specimen					

*Note: Some saprophytic mycobacteria may stain weakly gram positive or appear beaded in Gram-stained smears.

5.1.8. Review Questions:

1. What is a differential stain? Name two examples of such stains.

2. Is a Gram stain an adequate substitute for an acid-fast stain? Why?

3. When is it appropriate to ask the laboratory to perform an acid-fast stain?

4. In light of the clinical history and your observations of the Gram and acid-fast smears, what is your tentative diagnosis of the patient's illness? How should this preliminary laboratory diagnosis be confirmed?

5. Are saprophytic mycobacteria acid fast?

6. Does the presence of acid-fast organisms in a clinical specimen always suggest serious clinical disease?

7. How should the acid-fast stain of a sputum specimen from a patient with suspected pulmonary Nocardia infection be performed?

Exercise 6: Special Stains

Some bacteria have characteristic surface structures (such as capsules or flagella) and internal components (e.g., endospores) that may have taxonomic value for their identification. When it is necessary to demonstrate whether or not a particular organism possesses a capsule, is flagellated, or forms endospores, special staining techniques must be used.

6.1. Endospore Staining:

6.1.1. Background:

Among bacteria, endospore formation is most characteristic of two genera, Bacillus and Clostridium. The process of sporulation involves the condensation of vital cellular components within a thick, double-layered wall enclosing a round or ovoid inner body. The activities of the vegetative (actively growing) cell slow down, and it loses moisture as the endospore is formed. Gradually, the empty bacterial shell falls away. The remaining endospore is highly resistant to environmental influences, representing a resting, protective stage. Most disinfectants cannot permeate it, and it resists the lethal effects of drying, sunlight, ultraviolet radiation, and boiling. It can be killed when dry heat is applied at high temperatures or for long periods, by steam heat under pressure (in the autoclave), or by special sporicidal (endospore-killing) disinfectants. Because bacterial endospore walls are not readily permeated by materials in solution, the inner contents of the endospores are not easily stained by ordinary bacterial dyes. When sporulating bacteria are Gram stained, the endospores forming within the vegetative cells appear as empty holes in the bacterial bodies. Depending on their location within the cell, the endospores are referred to as terminal (at the very end of the vegetative cell), subterminal (near, but not at, the end of the cell), or central. Free endospores are invisible when stained with the Gram stain or appear as faint pink rings. To demonstrate the inner contents of bacterial endospores, you must use a special staining technique that can drive a dye through the endospore coat.

6.1.2. Principles:

Endospores do not stain easily, but, once stained, they strongly resist decolorization. This property is the basis of the Schaeffer-Fulton (Alice B. Schaeffer and MacDonald Fulton were

microbiologists at Middlebury College, Vermont, in the 1930s) or Wirtz-Conklin method (Robert Wirtz and Marie E. Conklin were bacteriologists in the early 1900s) of staining endospores. The endospores are stained with malachite green. Heat is used to provide stain penetration. The rest of the cell is then decolorized and counterstained a light red with safranin.

6.1.3. Purpose:

To learn a technique for staining bacterial endospores.

6.1.4. Materials:

- 3- to 5-day-old agar slant culture of *Bacillus subtilis*
- 24-hour-old slant culture of *Staphylococcus epidermidis*
- Malachite green solution
- Safranin solution
- Slides
- Diamond glass-marking pencil
- Slide rack
- 500-ml beaker
- Tripod with asbestos mat
- Forceps

6.1.5. Safety precautions:

Be careful with the Bunsen burner flame and boiling water bath. If either malachite green or safranin get on your clothes, they will not wash out. Discard slides in a container with disinfectant.

6.1.6. Procedures:

1. With a wax pencil, place the names of the respective bacteria on the edge of four clean glass slides.
2. Aseptically transfer one species of bacterium with an inoculating loop to each of the respective slides, air dry (or use a slide warmer), and heat-fix.

3. Place the slide to be stained on a hot plate or boiling water bath equipped with a staining loop or rack. Cover the smear with paper toweling that has been cut the same size as the microscope slide.
4. Soak the paper with the malachite green staining solution. Gently heat on the hot plate (just until the stain steams) for 5 to 6 minutes after the malachite green solution begins to steam. Replace the malachite green solution as it evaporates so that the paper remains saturated during heating (Figure 6-1a). Do not allow the slide to become dry.
5. Remove the paper using forceps, allow the slide to cool, and rinse the slide with water for 30 seconds (Figure 6-1b).
6. Counterstain with safranin for 60 to 90 seconds (Figure 6-1c).
7. Rinse the slide with water for 30 seconds (Figure 6-1d).
8. Blot dry with bibulous paper (Figure 6-1e) and examine under oil immersion. A coverslip is not necessary. The spores, both endospores and free spores, stain green; vegetative cells stain red.

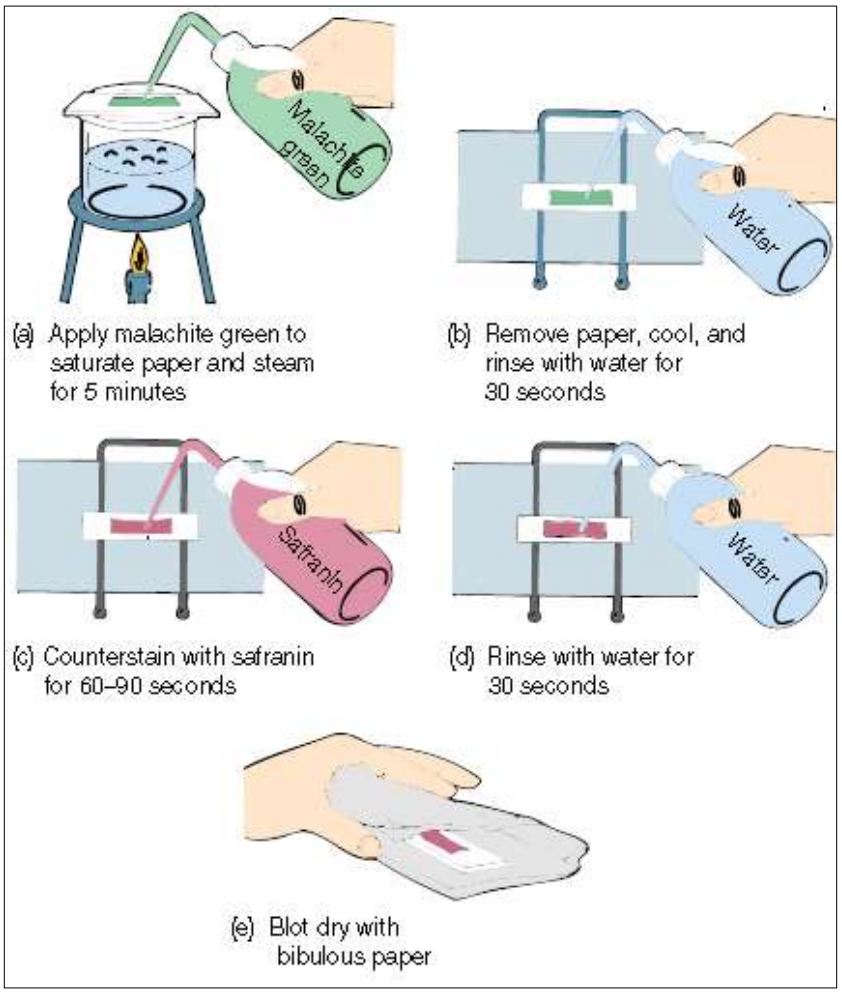
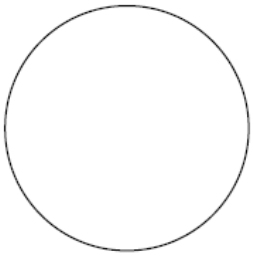


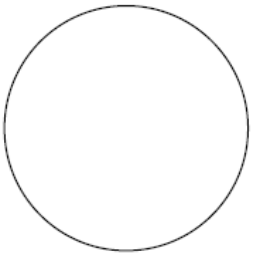
Figure 6-1: Endospore Staining Procedure.

6.1.7. Results:

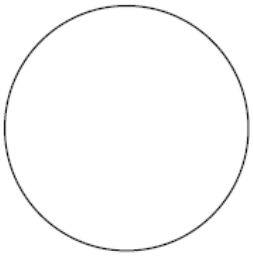
1. Make drawings and answer the questions for each of the bacterial endospore slides.



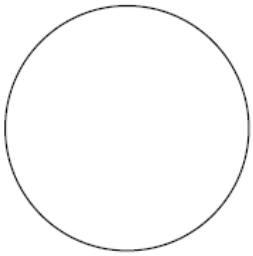
B. megaterium



B. macerans



B. circulans



C. butyricum

Bacterium	_____	_____	_____	_____
Magnification	× _____	× _____	× _____	× _____
Bacterium other than above	_____	_____	_____	_____
Spore color	_____	_____	_____	_____
Color of vegetative cell	_____	_____	_____	_____
Location of endospore (central, terminal, subterminal)	_____	_____	_____	_____

2. Are you satisfied with the results of your endospore stain? _____ If not, how can you improve your results the next time you prepare an endospore stain?

6.1.8. Review Questions:

1. Why must special stains be used to visualize bacterial capsules, flagella, and endospores?

2. Why is it important to know whether or not bacterial cells possess capsules, flagella, or endospores?

3. What do endospore stains have in common with the Ziehl-Neelsen acid-fast stain?

4. Is bacterial sporulation a reproductive process? Explain.

5. Why is it important to determine the location of the endospore within the bacterial cell?

6. Can you relate endospore staining to endospore survival in hospital or other environments?

Exercise 7: Microbiological Culture Media Preparation and Sterilization

7.1. Culture Media:

7.1.1. Background:

Once the microscopic morphology and staining characteristics of a microorganism present in a clinical specimen are known, the microbiologist can make appropriate decisions as to how it should be cultivated and what biological properties must be demonstrated to identify it fully. First, a suitable culture medium must be provided, and it must contain the nutrients essential for the growth of the microorganism to be studied. Most media designed for the initial growth and isolation of microorganisms are rich in protein components derived from animal meats. Many bacteria are unable to break down proteins to usable forms and must be provided with extracted or partially degraded protein materials (peptides, proteoses, peptones, amino acids). Meat extracts, or partially cooked meats, are the basic nutrients of many culture media. Some carbohydrate and mineral salts are usually added as well. Such basal media may then be supplemented, or enriched, with blood, serum, vitamins, other carbohydrates and mineral salts, or particular amino acids as needed or indicated.

7.1.2. Principles:

In the laboratory, the nutrient preparations that are used for culturing microorganisms are called media (singular, medium). Three physical forms are used: liquid, or broth, media; semisolid media; and solid media. The major difference among these media is that solid and semisolid media contain a solidifying agent (usually agar), whereas a liquid medium does not. Liquid media, such as nutrient broth, or brain-heart infusion broth (Figure 7-1a), can be used to propagate large numbers of microorganisms in fermentation studies and for various biochemical tests. Semisolid media can also be used in fermentation studies, in determining bacterial motility, and in promoting anaerobic growth. Solid media, such as nutrient agar or blood agar, are used (1) for the surface growth of microorganisms in order to observe colony appearance, (2) for pure culture isolations, (3) for storage of cultures, and (4) to observe specific biochemical reactions.

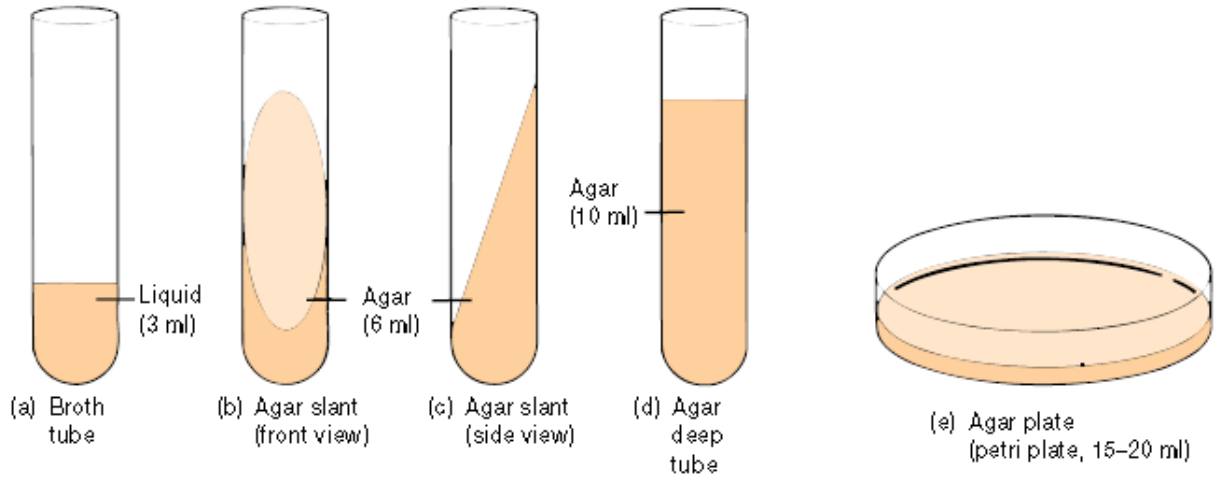


Figure 7-1: Culture Media. Different forms of culture media with the proper volume in each.

While in the liquefied state, solid media can be poured into either a test tube or petri plate (dish). If the medium in the test tube is allowed to harden in a slanted position, the tube is designated an agar slant (Figure 7-1b, c); if the tube is allowed to harden in an upright position, the tube is designated an agar deep tube (Figure 7-1d); and if the agar is poured into a petri plate, the plate is designated an agar plate (Figure 7-1e). Agar pours (the same as Agar deeps) containing about 15 to 16 ml of media are often used to prepare agar plates.

Microorganisms may be cultured using two different types of media. Chemically defined, or synthetic, media are composed of known amounts of pure chemicals (Table 7-1). Such media are often used in culturing autotrophic microorganisms such as algae or nonfastidious heterotrophs. In routine bacteriology laboratory exercises, complex, or nonsynthetic, media are employed (Table 7-2). These are composed of complex materials that are rich in vitamins and nutrients. Three of the most commonly used components are beef extract, yeast extract, and peptones. The preparation of media from commercial dehydrated products is simple and straightforward. Each bottle of dehydrated medium has instructions for preparation on its label. For example, to prepare a liter of nutrient broth, suspend 30 g of the dehydrated medium in 1,000 ml of distilled water. Mix thoroughly in a 2-liter Erlenmeyer flask (always use a flask that holds twice the volume of media you are preparing). Dispense and sterilize for 15 to 20 minutes at 121°C (15 lbs pressure). As noted, the amount of powder for 1,000 ml of water will be indicated. If the medium lacks agar, the powder will usually dissolve without heating. If it contains agar, you must heat the medium until it starts to simmer or boil in order to completely dissolve the agar. Specific heating

instructions are given for each type of medium. For example, to prepare a liter of Vogel-Johnson agar, suspend 61 g of the dehydrated medium in a liter of distilled water. Mix until a uniform suspension is obtained. Heat with constant agitation and simmer for 1 minute. Dispense in 100-ml amounts into 250-ml flasks and sterilize by autoclaving at 121°C for 20 minutes.

Table 7-1: A Chemically Defined Medium.

Ingredient	Quantity
Dipotassium phosphate, K_2HPO_4	7 g
Potassium phosphate, monobasic, KH_2PO_4	2 g
Hydrated magnesium sulfate, $MgSO_4 \cdot 7H_2O$	0.2 g
Ammonium sulfate, $(NH_4)_2SO_4$	1 g
Glucose	5 g
Distilled water	1 liter

Table 7-2: A Complex (Undefined) Medium—Nutrient Broth.

Ingredient	Quantity
Casein peptone	17 g
Soybean peptone	3 g
NaCl	5 g
Dipotassium phosphate, K_2HPO_4	2.5 g
Glucose	2.5 g
Distilled water	1 liter

7.1.3. Purpose:

To learn how culture media are prepared for use in the microbiology laboratory.

7.1.4. Materials:

- Dehydrated nutrient agar
- Dehydrated nutrient broth
- A balance, and weighing papers
- A 1-liter Erlenmeyer flask, cotton plugged or screw capped
- A 1-liter glass beaker

- A 1-liter graduated cylinder
- Glass stirring rods (at least 10 cm long)
- 10-ml pipettes (cotton plugged)
- Test tubes (screw capped or cotton plugged)
- Petri dishes
- Aspiration device for pipetting

7.1.5. Safety precautions:

Several types of heat that can cause severe burns are used in this experiment. Do not operate the autoclave without approval from your instructor. Always wear heat-proof (Zetex) gloves when unloading the autoclave. Zetex fabric gives all of the protection of asbestos with none of the health hazards. In like manner, boiling agar can cause severe burns if spilled on your hands. Be especially careful with Bunsen burners, hot plates, and boiling water baths. All are potentially hazardous and can cause burns. If you are burned, seek immediate treatment. Do not pipette by mouth.

7.1.6. Procedures:

1. Read the label on a bottle of dehydrated nutrient agar. It specifies the amount of dehydrated powder required to make 1 liter (1,000 ml) of medium. Calculate the amount needed for 1/2 liter and weigh out this quantity.
2. Place 500 ml of distilled water in an Erlenmeyer flask. Add the weighed, dehydrated agar while stirring with a glass rod to prevent lumping.
3. Set the flask on a tripod over an asbestos mat. Using a Bunsen flame, slowly bring the rehydrated agar to a boil. Stir often. An electric hot plate may be used instead of a Bunsen burner.
4. When the agar mixture is completely dissolved, remove the flask from the flame or hot plate, close it with the cotton plug or cap, and give it to the instructor to be sterilized in the autoclave.
5. While the flask of agar is being sterilized, prepare 500 ml of nutrient broth, adding the weighed dehydrated powder to the water in a beaker for reconstitution and dissolution.

6. Bring the reconstituted broth to a boil, slowly. When fully dissolved, remove from flame or electric burner and allow to cool a bit.
7. The instructor will demonstrate the use of the pipetting device. Do not pipette by mouth. Using a pipette, dispense 5-ml aliquots of the broth into test tubes (plugged or capped). The instructor will collect the tubes and sterilize them.
8. When the flask of sterilized agar is returned to you, allow it to cool to about 50°C (the agar should be warm and melted, but not too hot to handle in its flask). Remove the plug or cap with the little finger of your right hand and continue to hold it until you are sure it won't have to be returned to the flask. Quickly pour the melted, sterile agar into a series of petri dishes. The petri dish tops are lifted with the left hand, and the bottoms are filled to about one-third capacity with melted agar (Figure 7-2). Replace each petri dish top as the plate is poured. When the plates are cool (agar solidified), invert them to prevent condensing moisture from accumulating on the agar surfaces.
9. Place inverted agar plates and tubes of sterilized nutrient broth (cooled after their return to you) in the 35°C incubator. They should be incubated for at least 24 hours to ensure they are sterile (free of contaminating bacteria) before you use them in the next exercise.

Figure 7-2: Preparing a plate of agar medium by pouring melted sterile agar into it.

7.1.7. Results:

After at least 24 hours of incubation at 35°C, do your prepared plates and broths appear to be sterile?

Record your observation of their physical appearance:

Plates:

Broths:

7.1.8. Review Questions:

1. Define a culture medium.

2. Discuss some of the physical and chemical factors involved in the composition, and in the preparation, of a culture medium.

Nutrient ingredients:

pH and buffering:

Heat (to reconstitute):

Heat (to sterilize):

Other:

3. At what temperature does agar solidify? At what temperature does agar melt?

4. What would happen to plates poured with agar that is too hot? Could they be used?

5. What would happen to plates poured with agar that is too cool? Could they be used?

6. Why are culture media sterilized before use?

7. Discuss the relative value of broth and agar media in isolating bacteria from mixed cultures.

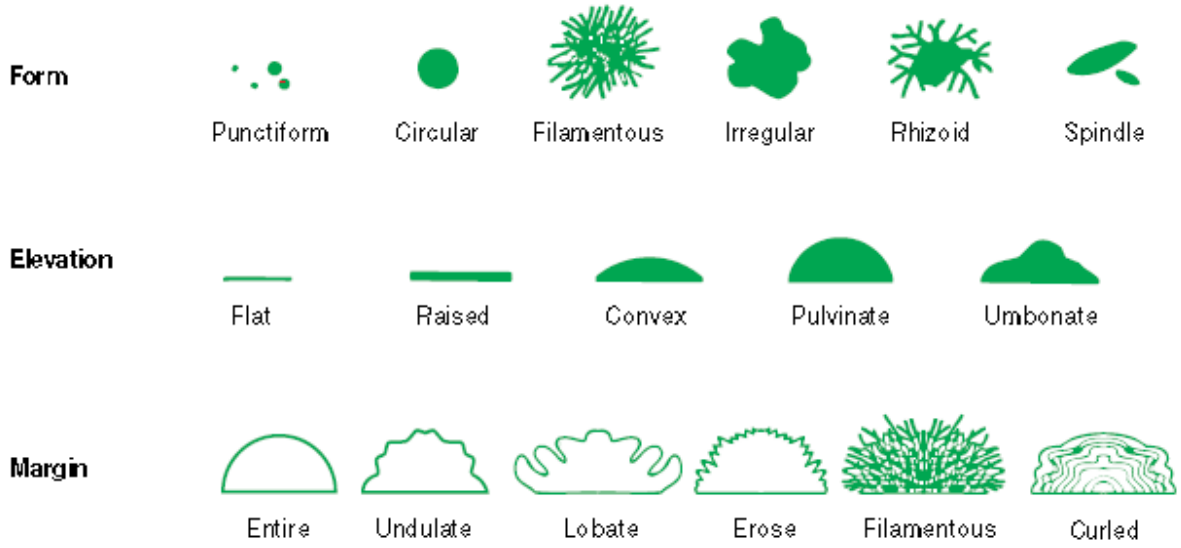
8. Are nutrient broths and agars, as you have prepared them, suitable for supporting growth of all microorganisms pathogenic for humans? Explain your answer.

Exercise 8: Culturing and Isolation Techniques I

Microorganisms must have a constant nutrient supply if they are to survive. Free-living organisms acquire nutrients from the environment and parasitic organisms acquire nutrients from their host. When trying to grow microbes in the lab adequate nutrition must be provided using artificial media. Media may be liquid (broth) or solid (agar). Any desired nutrients may be incorporated into the broth or agar to grow bacteria. Agar is the solidifying material used in solid media. It is an extract of seaweed that melts at 100 ^{o}C and solidifies at about 42 ^{o}C. Most pathogenic bacteria prefer to grow at 37^{o}C so agar allows for a solid medium at incubator temperatures. Since agar remains solid until reaching 100 ^{o}C, thermophiles (heat-lovers) that prefer temperatures above 50 ^{o}C for growth can still be grown on solid media. Organisms grown in broth cultures cause turbidity, or cloudiness, in the broth. On agar, masses of cells, known as colonies, appear after a period of incubation. Certain techniques will allow bacterial cells to be widely separated on agar so that as the cell divides and produces a visible mass (colony), the colony will be isolated from other colonies. Since the colony came from a single bacterial cell, all cells in the colony should be the same species. Isolated colonies are assumed to be pure cultures. Among the techniques used to obtain pure culture are spreading and streaking techniques.

Spread-Plate Technique:

The spread plate technique is an easy, direct way of achieving this result. In this technique, a small volume of dilute bacterial mixture containing 100 to 200 cells or less is transferred to the centre of an agar plate and is spread evenly over the surface with a sterile, L-shaped glass rod. The glass rod is normally sterilized by dipping in alcohol and flamed to burn off the alcohol. After incubation, some of the dispersed cells develop into isolated colonies. A colony is a large number of bacterial cells on solid medium, which is visible to the naked eye as a discrete entity. In this procedure, one assumes that a colony is derived from one cell and therefore represents a clone of a pure culture. After incubation, the general form of the colony and the shape of the edge or margin can be determined by looking down at the top of the colony (Figure 8-1). After a well-isolated colony has been identified, it can then be picked up and streaked onto a fresh medium to obtain a pure culture. We are not practicing this technique in the lab.



- Appearance: Shiny or dull
- Optical property: Opaque, translucent, transparent
- Pigmentation: Pigmented (purple, red, yellow)
- Nonpigmented (cream, tan, white)
- Texture: Rough or smooth

Figure 8-1: Bacterial Colony Characteristics on Agar Media as Seen with the Naked Eye. The characteristics of bacterial colonies are described using the following terms.

8.1. Streaking Technique to Obtain Pure Cultures

8.1.1. Background:

The skin and many mucosal surfaces of the human body support large numbers of microorganisms that comprise the normal, or indigenous, flora. When clinical specimens are collected from these surfaces and cultured, any pathogenic microorganisms being sought must be recognized among, and isolated from, other harmless organisms. Colonies of the pathogenic species must be picked out of the mixed culture and grown in isolated pure culture. The microbiologist can then proceed to identify the isolated organism by examining its biochemical and immunological properties. Streaking technique is one of the common methods to obtain pure culture which is critical to successful and accurate identification of microorganisms.

8.1.2. Principles:

A mixed culture contains two or more bacterial species that are known and can be easily separated based on cultural or biochemical characteristics. Culturing techniques provide a means for maintaining adequate nutrition for the organisms so they can continue to survive. As

organisms grow in a culture they consume the available nutrients and periodically need to be transferred to fresh media to continue to grow. Certain culturing techniques not only provide the organisms with a fresh supply of nutrients but also allow for the separation of bacterial cells to obtain isolated colonies. These culturing procedures are known as isolation techniques. Streak plates allow for the growth of isolated colonies on the surface of the agar. An isolated colony is a colony that is not touching any other colonies and is assumed to be a pure culture. These colonies are easily accessible for performing staining and identification procedures. They also show colonial morphology that may be useful in identifying the organism. Part of the identification of any organism includes a description of colonial morphology. Since organisms may grow differently on different media, the type of media used must be included as a part of any colonial morphology. Other elements of a colonial description include colony color, hemolysis (if grown on blood agar), form, elevation and margin.

8.1.3. Purpose:

To understand and practice the purpose of the streaking technique though isolating pure cultures from a specimen containing mixed flora.

8.1.4. Materials:

- Nutrient agar plates
- Blood agar plates
- A mixed broth culture containing *Serratia marcescens* (pigmented), *Escherichia coli*, and *Staphylococcus epidermidis*
- A demonstration plate culture made from this broth, showing colonies isolated by good streaking technique
- Glass slides
- Gram-stain reagents

8.1.5. Safety precautions:

Be careful with the Bunsen burner flame and the hot water baths.

8.1.6. Procedures:

1. Make certain the contents of the broth culture tube are evenly mixed.

2. Place a loopful of broth culture on the surface of a nutrient agar plate, near but not touching the edge. With the loop flat against the agar surface, lightly streak the inoculum back and forth over approximately one-eighth the area of the plate; do not dig up the agar (Figure 8-2, area A).
3. Sterilize the loop and let it cool in air.
4. Rotate the open plate in your left hand so that you can streak a series of four lines back and forth, each passing through the inoculum and extending across one side of the plate (Figure 8-2, area B).
5. Sterilize the loop again and let it cool in air.
6. Rotate the plate and streak another series of four lines, each crossing the end of the last four streaks and extending across the adjacent side of the plate (Figure 8-2, area C).
7. Rotate the plate and repeat this parallel streaking once more (Figure 8-2, area D).
8. Finally, make a few streaks in the untouched center of the plate (Figure 8-2, area E). Do not touch the original inoculum.
9. Incubate the plate (inverted) at 35°C.

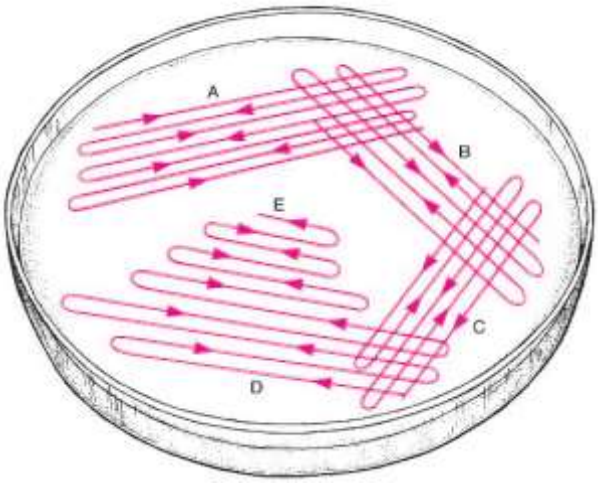
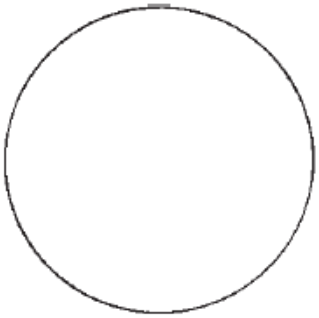


Figure 8-2: Diagram of plate streaking technique. The goal is to thin the numbers of bacteria growing in each successive area of the plate as it is rotated and streaked so that isolated colonies will appear in sections D and E.

8.1.7. Results:

1. Examine the incubated nutrient agar plate carefully. Make a drawing showing the intensity of growth in each streaked area.
2. Describe each different type of colony you can distinguish.
3. Select one discrete colony, describe it, and identify the bacterium it contains.



Bacterium	
Colony form	
Colony elevation	
Colony margin	
Colony size	
Colony color	

4. Draw your streaking patterns. Did you obtain isolated colonies? If not, what went wrong?

8.1.8. Review Questions:

1. When an agar plate is inoculated, why is the loop sterilized after the initial inoculum is put on?
2. Define a pure culture, a mixed culture.
3. Define a bacterial colony. List four characteristics by which bacterial colonies may be distinguished.
4. In the streak-plate technique, how are microorganisms diluted and spread out to form individual colonies?
5. Why should a petri dish not be left open for any extended period?
6. Why does the streaking method you used to inoculate your plates result in isolated colonies?
7. Why is it necessary to isolate individual colonies from a mixed growth?
8. Which area of a streak plate will contain the greatest amount of growth? The least amount of growth? Explain your answers.

Exercise 9: Culturing and Isolation Techniques II

9.1. Pour-Plate Techniques

9.1.1. Background:

An alternative method for using agar plates to obtain isolated colonies, other than streaking or spreading their surfaces, is to prepare a "pour plate." In this case, an aliquot of the specimen to be cultured is placed in the bottom of an empty, sterile petri dish, then melted, cooled agar is poured over it. Quickly, before the agar cools, the plate is gently rocked to disperse the inoculum. When the agar has solidified and the plate is incubated, any bacteria present in the specimen will grow wherever they have been embedded within the agar layer or localized on its surface. Their colonies will be isolated and can be removed from subsurface positions by inserting the inoculating loop or a straight wire into the agar. To prepare pour plates, the inoculum must be a liquid specimen or culture. If it is not, it must be suspended in sterile fluid before being placed in the petri dish. Another method for preparing a pour plate is to inoculate the specimen or culture directly into the tube of melted, cooled, but not yet solidified agar. Mix it by rolling it back and forth between the outstretched fingers of both hands, and pour the inoculated agar into a sterile petri dish. These steps must be performed quickly before the agar cools enough to harden.

9.1.2. Principles:

The original sample is diluted several times to reduce the microbial population sufficiently to obtain separate colonies upon plating (Figure 9-1). The small volumes of several diluted samples are added to sterile petri plates and mixed with liquid nutrient agar that has been cooled to about 48° to 50°C. Most bacteria and fungi will not be killed by the brief exposure to the warm agar. After the agar has hardened, each cell is fixed in place and will form an individual colony if the sample is dilute enough. Assuming no chaining or cell clusters, the total number of colonies are equivalent to the number of viable microorganisms in the diluted sample. To prepare pure cultures, colonies growing on the surface or subsurface can be inoculated into fresh medium.

9.1.3. Purpose:

To learn the pour-plate technique for obtaining isolated colonies.

9.1.4. Materials:

- 24- to 48-hour mixed nutrient broth culture of *Escherichia coli*, *Serratia marcescens*; *Micrococcus roseus* also can be used), and *Bacillus subtilis*
- 3 nutrient agar pour tubes
- Three 9-ml sterile 0.9% NaCl (saline) blanks
- 48° to 50°C water bath
- Boiling water bath
- Wax pencil
- 3 petri plates
- Inoculating loop
- Bunsen burner
- 3 sterile 1-ml pipettes with pipettor

9.1.1. Safety precautions:

Be careful with the Bunsen burner flame and the hot water baths. Do not use your mouth to pipette.

9.1.2. Procedures:

1. With a wax pencil, label three sterile saline blanks 1 to 3.
2. Melt the nutrient agar deeps in a boiling water bath and cool in a 48° to 50°C bath for at least 10 to 15 minutes.
3. With a wax pencil, label the bottoms of three petri plates 1 to 3, and add your name and date.
4. Inoculate saline tube 1 with 1 ml of the MIXED bacterial culture using aseptic technique and MIX thoroughly. This represents a 10^{-1} dilution (Figure 9-1).
5. Using aseptic technique, immediately inoculate tube 2 with 1 ml from tube 1; a 10^{-2} dilution.
6. Using aseptic technique, mix the contents of tube 2 and use it to inoculate tube 3 with 1 ml; a 10^{-3} dilution.

7. After tube 3 has been inoculated, mix its contents, remove the cap, flame the top, and aseptically transfer 1 ml into petri plate 3. Then inoculate plates 1 and 2 in the same way, using 1 ml from tubes 1 and 2, respectively (Figure 9-1).
8. Add the contents of the melted nutrient agar pours to the petri plates. Gently mix each agar plate with a circular motion while keeping the plate flat on the bench top. Do not allow any agar to splash over the side of the plate! Set the plate aside to cool and harden.
9. Incubate the plates at 30°C to 37°C for 24 to 48 hours in an inverted position.
10. Examine the pour plates and record your results in the report for this exercise.

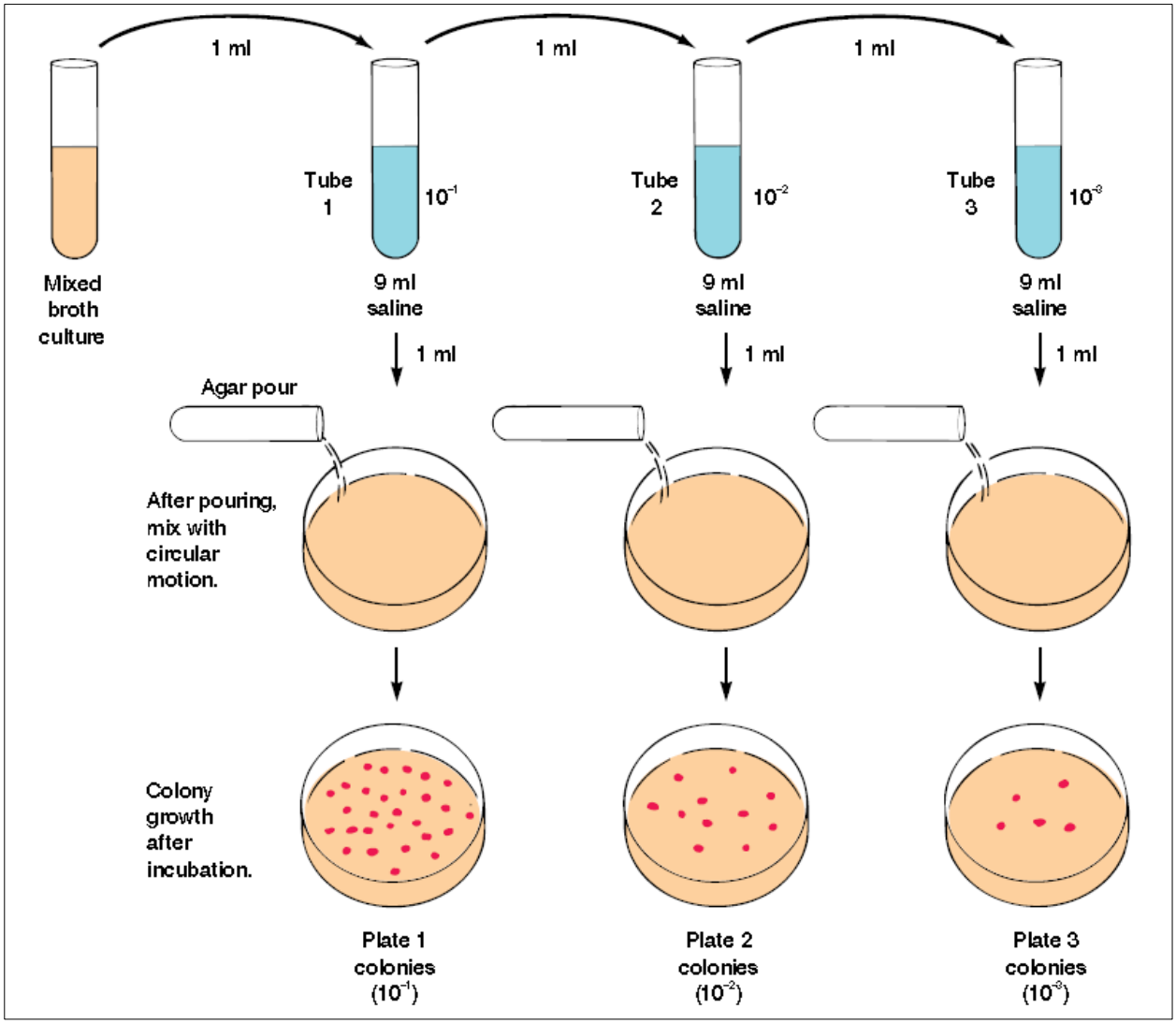


Figure 9-1: The Pour-Plate Technique. The original sample is diluted several times to decrease or dilute the population sufficiently. 1 ml of each dilution is then dispensed into the bottom of a petri plate. Agar pours are then added to each plate. Isolated cells grow into colonies and can be used to establish pure cultures. The surface colonies are circular and large, subsurface colonies are lenticular or lens-shaped and much smaller.

9.1.3. Results:

1. Examine each of the agar plates for colony distribution and amount of growth. Look for discrete surface colonies and record your results. Do the same for the subsurface colonies. Color each species of bacterium a different color or label each. Fill in the table.

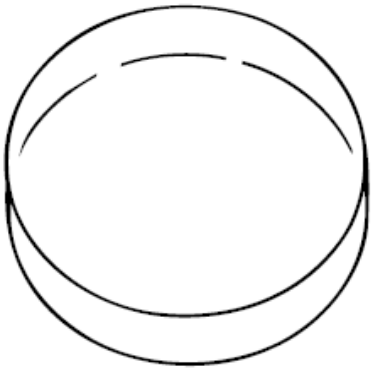


Plate 1

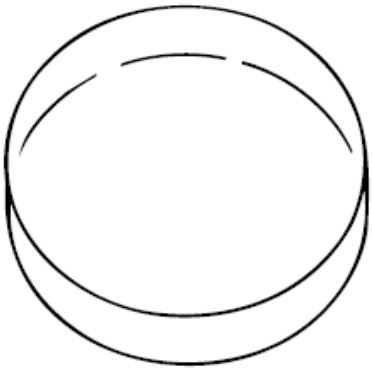


Plate 2

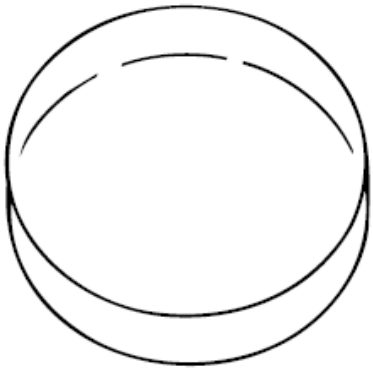


Plate 3

Surface Colonies

Form

Elevation

Margin

Number

Subsurface Colonies

Form

Elevation

Margin

Number

9.1.4. Review Questions:

1. Discuss the relative convenience of pour- and streak-plate techniques in culturing clinical specimens.
2. Why are plate cultures incubated in the inverted position?
3. How do you decide which colonies should be picked from a plate culture of a mixed flora?

4. When more than one colony type appears in a pure culture, what are the most likely sources of the extraneous organisms?
5. What is the main advantage of the pour-plate method over other methods of bacterial colony isolation? What are some problems?
6. Why are the surface colonies on a pour plate larger than those within the medium?
7. Why doesn't the 48° to 50°C temperature of the melted agar kill most of the bacteria?
8. Explain how the pour-plate method can be used to isolate fungi.

Exercise 10: **Determination of Bacterial Numbers**

10.1.1. Background:

Many studies require the quantitative determination of bacterial populations. The two most widely used methods for determining bacterial numbers are the standard, or viable, plate count method and spectrophotometric (turbidimetric) analysis. Both of the methods are discussed in this manual and the standard method is set to be practiced.

10.1.2. Principles:

Although the two methods mentioned above are somewhat similar in the results they yield, there are distinct differences. For example, the standard plate count method is an indirect measurement of cell density and reveals information related only to live bacteria. The spectrophotometric analysis is based on turbidity and indirectly measures all bacteria (cell biomass), dead or alive. The standard plate count method consists of diluting a sample with sterile saline or phosphate buffer diluent until the bacteria are dilute enough to count accurately. That is, the final plates in the series should have between 25 and 250 colonies. Fewer than 25 colonies are not acceptable for statistical reasons, and more than 250 colonies on a plate are likely to produce colonies too close to each other to be distinguished as distinct colony-forming units (CFUs). The assumption is that each viable bacterial cell is separate from all others and will develop into a single discrete colony (CFU). Thus, the number of colonies should give the number of live bacteria that can grow under the incubation conditions employed. A wide series of dilutions (e.g., 10^{-4} to 10^{-10}) is normally plated because the exact number of live bacteria in the sample is usually unknown. Greater precision is achieved by plating duplicates or triplicates of each dilution. Increased turbidity in a culture is another index of bacterial growth and cell numbers (biomass). By using a spectrophotometer, the amount of transmitted light decreases as the cell population increases. The transmitted light is converted to electrical energy, and this is indicated on a galvanometer. The reading indirectly reflects the number of bacteria. This method is faster than the standard plate count but is limited because sensitivity is restricted to bacterial suspensions of 10^7 cells or greater.

10.1.3. Purpose:

To learn different ways to quantify the number of bacteria in a given sample such as the standard plate count technique and spectrophotometric analysis.

10.1.4. Materials:

- 24-hour nutrient broth culture of *Escherichia coli*
- 4 sterile 99-ml saline or phosphate buffer blanks
- 1-ml or 1.1-ml pipettes with pipettor
- 6 petri plates
- 6 agar pour tubes of nutrient agar (plate count agar)
- 48° to 50°C water bath
- Boiling water bath
- Bunsen burner
- Cuvettes
- Spectrophotometer
- 4 tubes of nutrient broth

10.1.5. Safety precautions:

No mouth pipetting. Be careful with the Bunsen burner flame and water baths.

10.1.6. Procedures:

Standard Plate Count

1. With a wax pencil, label the bottom of six petri plates with the following dilutions: 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9}. Label four bottles of saline or phosphate buffer 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8}.
2. Using aseptic technique, the initial dilution is made by transferring 1.0 ml of liquid sample or 1 g of solid material to a 99-ml sterile saline blank (Figure 10-1). This is a 1/100 or 10^{-2} dilution, cap the bottle.

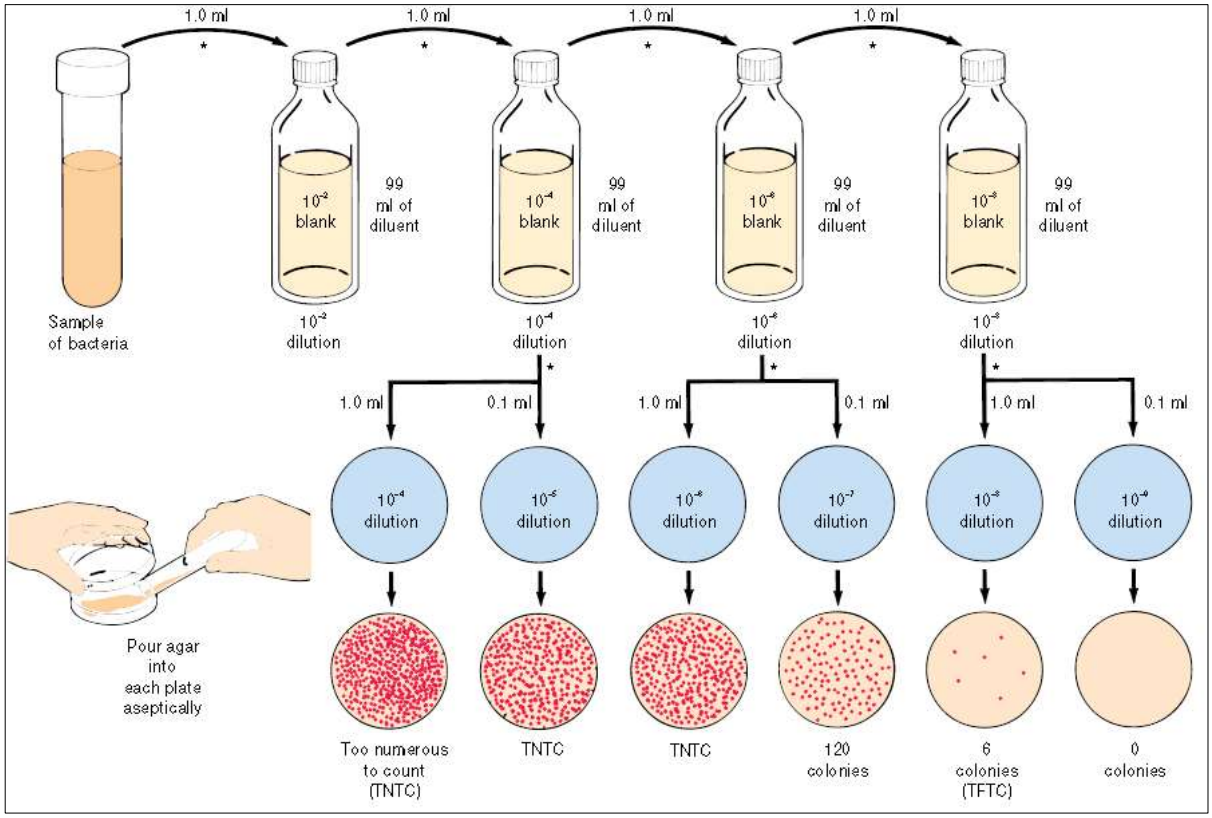


Figure 10-1: Quantitative Plating Procedure.

3. The 10^{-2} blank is then shaken vigorously 25 times by placing one's elbow on the bench and moving the forearm rapidly in an arc from the bench surface and back. This serves to distribute the bacteria and break up any clumps of bacteria that may be present.
4. Immediately after the 10^{-2} blank has been shaken, uncap it and aseptically transfer 1.0 ml to a second 99-ml saline blank. Since this is a 10^{-2} dilution, this second blank represents a 10^{-4} dilution of the original sample. Cap the bottle.
5. Shake the 10^{-4} blank vigorously 25 times and transfer 1.0 ml to the third 99-ml blank. This third blank represents a 10^{-6} dilution of the original sample. Cap the bottle. Repeat the process once more to produce a 10^{-8} dilution.
6. Shake the 10^{-4} blank again and aseptically transfer 1.0 ml to one petri plate and 0.1 ml to another petri plate. Do the same for the 10^{-6} and the 10^{-8} blanks (Figure 10-1).
7. Remove one agar pour tube from the 48° to 50°C water bath. Carefully remove the cover from the 10^{-4} petri plate and aseptically pour the agar into it. The agar and sample are immediately mixed by gently moving the plate in a figure-eight motion while it rests on the table top. Repeat this process for the remaining five plates.

8. After the pour plates have cooled and the agar has hardened, they are inverted and incubated at 35°C for 24 hours or 20°C for 48 hours.
9. At the end of the incubation period, select all of the petri plates containing between 25 and 250 colonies. Plates with more than 250 colonies cannot be counted and are designated too numerous to count (TNTC). Plates with fewer than 25 colonies are designated too few to count (TFTC). Count the colonies on each plate. If at all possible, a special counter such as a Quebec colony counter should be used. Your instructor will demonstrate how to use this counter or a handheld counter.
10. Calculate the number of bacteria (CFU) per milliliter or gram of sample by dividing the number of colonies by the dilution factor. For example, suppose the plate of the 10^{-6} dilution yielded a count of 130 colonies. Then, the number of bacteria in 1 ml of the original sample can be calculated as follows:

Bacteria/ml = (130) ÷ (10^{-6}) = 1.3×10^{8} or 130,000,000.

11. Record your results in the report.

10.1.7. Results:

Record your observations and calculated bacterial counts per milliliter in the following table.

Petri Plate	Dilution	ml of Dilution Plated	Number of Colonies	Bacterial Count per ml of Sample[a]
1				
2				
3				
4				

[a] This value is also expressed as colony-forming units per milliliter (CFU/ml).

10.1.8. Review Questions:

1. Why is the viable plate count technique considered to be an indirect measurement of cell density, whereas the turbidimetry method is not a “count” at all?
2. Why is it necessary to perform a plate count in conjunction with the turbidimetry procedure?
3. What is a CFU?
4. How would you prepare a series of dilutions to get a final dilution of 10^{-10}? Outline each step.

Exercise 11: Environmental Factors Affecting Growth of Microorganisms

11.1. Temperature, pH, Osmotic Pressure

11.1.1. Background:

The growth of microorganisms is greatly affected by the chemical and physical nature of their environment. An understanding of the environmental factors that promote microbial growth aids in understanding the ecological distribution of microorganisms. Therefore, the nature of some of these influences will be surveyed in this part of the manual. Some of these factors include temperature, pH, oxygen, and osmotic pressure. These same environmental factors that maximize microbial growth can also be manipulated to inhibit or retard the growth of unwanted microorganisms.

Temperature

Temperature is one of the most important factors influencing the activity of bacterial enzymes. Unlike warm blooded animals, the bacteria lack mechanisms that conserve or dissipate heat generated by metabolism, and consequently their enzyme systems are directly affected by ambient temperatures.

Hydrogen ion concentration (pH)

Aside from temperature, the hydrogen ion concentration of an organism's environment exerts the greatest influence on its growth. The concentration of hydrogen ions, which is customarily designated by the term pH ($-\log 1/H^+$), limits the activity of enzymes with which an organism is able to synthesize new protoplasm. As in the case of temperature, there exists for each organism an optimum concentration of hydrogen ions in which it grows best. The pH values above and below which an organism fails to grow are, respectively, referred to as the minimum and maximum hydrogen ion concentrations. These values hold only when other environmental factors remain constant. If the composition of the medium, incubation temperature, or osmotic pressure is varied, the hydrogen ion requirements become different.

Oxygen (Cultivation of Anaerobic Bacteria)

One of the environmental factors to which bacteria and other microorganisms are quite sensitive is the presence of O_2. For example, some microorganisms will grow only in the presence of O_2 and are called obligate aerobes. Facultative anaerobes will grow either aerobically or in the absence of O_2, but better in its presence. Strict obligate anaerobes will grow only in the absence of O_2 and are actually harmed by its presence. Aerotolerant anaerobes are microorganisms that cannot use O_2 but are not harmed by it either. Finally, microorganisms that require a small amount of O_2 for normal growth but are inhibited by O_2 at normal atmospheric tension are called microaerophiles. These variations in O_2 requirements can be easily seen by inoculating a tube of molten agar with the bacterium in question, mixing the agar thoroughly without aerating it, and allowing it to solidify. The bacteria will grow in the part of the agar deep culture that contains the proper O_2 concentration. The damaging effects of O_2 on anaerobic bacteria create difficult culturing problems. Ideally, one should not only provide an O_2-free environment, but one that has an adequate amount of moisture for bacterial growth. It is also necessary to have CO_2 present for the growth of many anaerobic bacteria. There are a number of ways in which anaerobic bacteria may be cultured. Four of the most useful will be described. One of the most convenient approaches is to employ a specially designed commercial anaerobic broth. Two of the most useful are cooked meat medium and thioglycollate broth. Thioglycollate medium can be purchased with methylene blue or resazurin as an oxidation-reduction indicator. When this medium begins to turn bluish or reddish, it is becoming too aerobic for the culture of anaerobic bacteria.

Another way of culturing bacteria anaerobically on plates is the GasPak Anaerobic System. In the GasPak System, hydrogen and CO_2 are generated by a GasPak envelope after the addition of water. A palladium catalyst (pellets) in the chamber lid catalyzes the formation of water from hydrogen and O_2, thereby removing O_2 from the sealed chamber. For greater convenience and visibility, GasPak pouches can be used instead of the regular GasPak incubation chamber. In this procedure, a special activating reagent is dispensed into the reagent channel. Inoculated plates are then put into the pouch. The anaerobic environment is locked in with a sealing bar and the pouch incubated. Growth can be observed at one's convenience.

Osmotic Pressure

Growth of bacteria can be profoundly affected by the amount of water entering or leaving the cell. When the medium surrounding an organism is hypotonic (low solute content), a resultant higher osmotic pressure occurs in the cell. Except for some marine forms, this situation is not harmful to most bacteria. The cell wall structure of most bacteria is so strong and rigid that even slight cellular swelling is generally inapparent. In the reverse situation, however, when bacteria are placed in a hypertonic solution (high solute content), their growth may be considerably inhibited. The degree of inhibition will depend on the type of solute and the nature of the organism. In media of growth-inhibiting osmotic pressure, the cytoplasm becomes dehydrated and shrinks away from the cell wall. Such plasmolyzed cells are often simply inhibited in the absence of sufficient cellular water and return to normal when placed in an isotonic solution. In other instances, the organisms are irreversibly affected due to permanent inactivation of enzyme systems.

Organisms that thrive in hypertonic solutions are designated as halophiles or osmophiles. If they require minimum concentrations of salt (NaCl and other cations and anions) they are called halophiles. Obligate halophiles require a minimum of 13% sodium chloride. Osmophiles, on the other hand, require high concentrations of an organic solute, such as sugar.

In this exercise we test the effect of different temperature on growth of bacteria.

11.1.2. Principles:

Each microbial species requires a temperature growth range that is determined by the heat sensitivity of its particular enzymes, membranes, ribosomes, and other components. As a consequence, microbial growth has a fairly characteristic temperature dependence with distinct cardinal temperatures—minimum, maximum, and optimum. Minimum growth temperature is the lowest temperature at which growth will occur; maxi-mum growth temperature is the highest temperature at which growth will occur; and optimum growth temperature is the temperature at which the rate of cellular reproduction is most rapid. The optimum temperature for the growth of a given microorganism is correlated with the temperature of the normal habitat of the microorganism. For example, the optimum temperature for the growth of bacteria pathogenic to humans is near that of the temperature of human blood (35° to 37°C).

Most bacteria can be classified into one of three major groups based on their temperature requirements. Psychrophiles can grow at 0°C and have an optimum growth temperature of 15°C or lower; the maximum is around 20°C. Mesophiles have growth optima between 20° and 45°C. The majority of bacteria fall into this category. Thermophiles can grow at temperatures of 55°C or higher.

Boiling is probably one of the easiest methods of ridding materials of harmful bacteria. However, not all bacteria are equally sensitive to this high temperature. Some bacteria may be able to survive boiling even though they are unable to grow. These bacteria are termed thermoduric. Many of the spore formers (such as *B. subtilis*) can withstand boiling for 15 minutes because of their resistant endospores. Thus, both temperature and the species of bacteria will affect the disinfection of certain specimens. This is important to know when trying to kill pathogenic bacteria with heat.

11.1.3. Purpose:

1. Understand how microorganisms are affected by the temperature of their environment.
2. Carry out an experiment that differentiates between several bacteria based on temperature sensitivity.
3. Classify these same bacteria based on their temperature preference for growth.

Determine the effects of heat on bacteria.

11.1.4. Materials:

- 24- to 48-hour nutrient broth cultures of *Escherichia coli*, *Bacillus stearothermophilus*, *Bacillus globisporus*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and spore suspension of *Bacillus subtilis*. (To produce endospores, grow *B. subtilis* for 48 hours at 35°C on endospore agar, nutrient agar plus 0.002% $MnCl_2 . 4H_2O$. Resuspend the paste in at least 7 ml of sterile diluent.)
- 12 nutrient agar slants
- Bunsen burner and Inoculating loop
- 15 nutrient broth tubes (9.9 ml per tube) and Test-tube rack
- 18 sterile 1-ml pipettes with pipettor and 3 sterile test tubes
- Refrigerator set at 4°C

- Incubators or water baths set at 4°, 23° to 25° (Room temperature), 60°, 85°, and 100°C (The instructor or students are not limited to these temperatures. Modifications can be instituted based on incubators or water baths available)
- Wax pencil and sterile water

11.1.5. Safety precautions:

Be careful with the Bunsen burner flame. No mouth pipetting. Use caution with the hot water baths. Keep all culture tubes upright in a test-tube rack or in a can.

11.1.6. Procedures:

First Period

1. Work in groups of three to four students. Each group of students will be assigned one temperature to study: 4°, 23° to 25°, 60°, 85°, or 100°C.
2. Label each of the nutrient agar slants with the name of the test bacterium to be inoculated (*E. coli*, *B. stearothermophilus*, and *B. globisporus*), your name, and date.
3. Using aseptic technique, streak the surface of each slant with the appropriate bacterium. Incubate the slants for 24 to 48 hours at the temperature assigned to your group.
4. Take three sterile test tubes and label one *S. aureus*, the second *B. subtilis* spores, and the third *P. aeruginosa*. Add your name and date.
5. With a sterile pipette, aseptically add 1 ml of bacterial culture or spore suspension to the respective tubes.
6. Subject your tubes to the temperature you are studying for 15 minutes (i.e., either place them in the refrigerator, let them stand at room temperature, or put them in one of the water baths).
7. After 15 minutes, let the samples cool or warm up to room temperature. For each bacterial sample, make a dilution series as follows:
   a. Pipette 0.1 ml of the incubated sample into the 9.9 ml nutrient broth (10^{-2} dilution). Mix the tube thoroughly. With a fresh pipette, transfer 0.1 ml of this 10^{-2} dilution into 9.9 ml of broth (10^{-4} dilution) and mix. In the same way, prepare a 10^{-6}, 10^{-8}, and 10^{-10} dilution.
8. Incubate all dilutions at 35°C for 24 to 48 hours.

Second Period

1. At the end of incubation, observe the slants for the presence of growth. Record your observations and those of your classmates; use a + for the presence of growth and a – for the absence of growth in Part 1 of the report for this exercise.
2. Observe your dilution series to see which tubes have bacterial growth as indicated by turbidity. The logic of this procedure is that reproduction will have occurred in each tube that received at least one living bacterium. The greater the number of bacteria present in the particular sample, the more such a sample can be diluted and still contain bacteria in the aliquot transferred. Thus, if bacteria A are less susceptible to heat than bacteria B, bacteria A will require more dilutions in order to obtain a sterile sample as indicated by no growth.

From your results and those of your classmates, complete Part 2 of the report for this exercise by indicating the last dilution in which growth occurred.

11.1.7. Results:

1. Based on your observations and those of your classmate, complete the following table.

Bacterium	Growth (+) or (–)					Temperature Classification
	4°C	23° to 25°C	60°C	85°C	100°C	
E. coli						
B. globisporus						
B. stearothermophilus						

2. Based on your observations and those of your classmate, complete the following table, showing range of surviving bacteria.

Temperature for 15 Minutes	Last Dilution in Which Growth Occurred		
	S. aureus	*P. aeruginosa*	*B. subtilis*
4°C	_____	_____	_____
Room temperature (23° to 25°C)	_____	_____	_____
60°C	_____	_____	_____
85°C	_____	_____	_____
100°C	_____	_____	_____

11.1.8. Review Questions:

1. How can you be sure that the turbidity produced in the broth tubes was caused by the bacteria used for the inoculation?
2. How can you determine experimentally whether a bacterium is a psychrophile or a mesophile?
3. What limitations are there for using boiling water as a means of sterilizing materials?
4. Is *S. aureus* a mesophile? Explain your answer.
5. Describe the three cardinal temperatures.
6. Which bacterium had the widest range of temperature tolerance? The narrowest range of temperature tolerance?
7. What are thermoduric bacteria?

Exercise 12: Determination of a Bacterial Growth Curve

12.1. Classical and Two-Hour Methods

12.1.1. Background:

Bacterial population growth studies require inoculation of viable cells into a sterile broth medium and incubation of the culture under optimum temperature, pH, and gaseous conditions. Under these conditions, the cells will reproduce rapidly and the dynamics of the microbial growth can be charted by means of a population growth curve, which is constructed by plotting the increase in cell numbers versus time of incubation and can be used to delineate stages of the growth cycle. It also facilitates measurement of cell numbers and the rate of growth of a particular organism under standardized conditions as expressed by its generation time, the time required for a microbial population to double. The stages of a typical growth curve are:

1. **Lag phase:** When the cells are adjusting to their new environment.
2. **Exponential phase:** Under optimum nutritional and physical conditions, the physiologically robust cells reproduce at a uniform and rapid rate by binary fission.
3. **Stationary phase:** During this stage, the number of cells undergoing division is equal to the number of cells that are dying.
4. **Death phase:** Because of the continuing depletion of nutrients and build-up of metabolic wastes, the microorganisms die at a rapid and uniform rate.

12.1.2. Principles:

Classical Growth Curve

The four phases (lag, logarithmic, stationary, and death or decline) of growth of a bacterial population can be determined by measuring the turbidity of the population in a broth culture. Turbidity is not a direct measure of bacterial numbers but an indirect measure of biomass, which **can be correlated with cell density during the log growth phase**. Since about **10^7 bacterial cells**

per milliliter must be present to detect turbidity with the **unaided eye**, a spectrophotometer can be used to achieve increased sensitivity and obtain quantitative data.

The construction of a complete bacterial growth curve (increase and decrease in cell numbers versus time) requires that aliquots of a shake-flask culture be measured for population size at intervals over an extended period. Because this may take many hours, such a procedure does not lend itself to a regular laboratory session. Therefore, the first part of this exercise has been designed to demonstrate only the lag and log phases of a bacterial growth curve. The bacterial population will be plotted on graph paper by using both an indirect and direct method for the measurement of growth. The resulting growth curve can be used to delineate stages of the growth cycle.

The indirect method uses spectrophotometric measurements of the developing turbidity in a bacterial culture taken at regular intervals. These samples serve as an index of increasing cellular mass. The graphical determination of generation time is made by extrapolation from the log phase, as illustrated in Figure 12-1. For example, select two points (0.2 and 0.4) on the absorbance (A) scale that represent a doubling of turbidity. Using a ruler, extrapolate by drawing a line between each absorbance on the ordinate, and the plotted log or exponential phase of the growth curve. From these two points, draw perpendicular lines to the time intervals on the abscissa. From these data, the generation time can be calculated as follows:

Generation time = t (A of 0.4) – t (A of 0.2)

Generation time = 90 minutes – 60 minutes = 30 minutes

The same graphical generation time determination can be done with a plot of population counts. The growth rate constant can also be determined from the data. When the $\log_{10}$ of the cell numbers or absorbance is plotted versus time, a straight line is obtained, the slope of which can be used to determine the value of g and k. The dimensions of k are reciprocal hours or per hour. The growth rate constant will be the same during exponential growth regardless of the component measured (e.g., cell biomass, numbers). The growth rate constant provides the microbiologist with a valuable tool for comparison between different microbial species when standard growth and environmental conditions are maintained.

Once the growth rate constant is known, the mean generation time (doubling time) can be calculated from the following equation:

$$g = 1/k$$

This equation also allows one to calculate the growth rate constant from the generation time. As mentioned previously, the generation time can be read directly from the bacterial growth curve plot, and the growth rate constant then determined. To calculate the generation time (g) from these data with an equation, use the following formula:

Generation time = $0.301t/\log_{10}Nt - \log_{10}N0$

Where $N0$ = bacterial population at point B or any other point at the beginning of the log phase

Nt = bacterial population at point b or any other point at or near the end of the log phase

t = time in minutes between b and B (Figure 12-1).

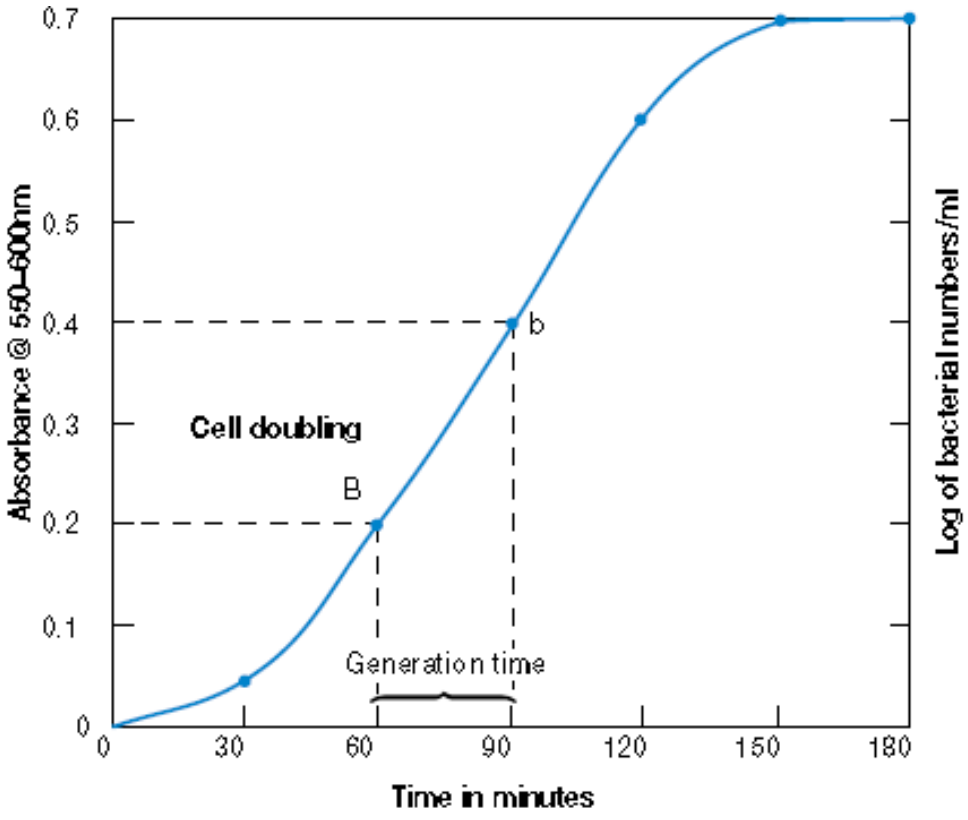


Figure 12-1: Indirect Method of Determining Generation.

From the previous equation, one can also determine the specific mean growth rate constant (k) for any culture during unrestricted growth. During this time, the rate of increase of cells is

proportional to the number of cells present at any particular time. In mathematical terms, the growth rate is expressed as

$$k = n/t$$

Where n is the number of generations per unit time. The symbol k represents the mean growth rate constant. Converting the equation to logarithms:

$$k = \log Nt - \log N0/0.301t$$

Two-Hour Method

Vibrio natriegens is a facultative anaerobic rod with a single polar flagellum. It was first isolated in 1958 from a salt marsh on Sapelo Island, Georgia. The rapid growth of *V. natriegens* (a generation time of less than 10 minutes) makes this bacterium useful for determining a complete bacterial growth curve. The growth cycle, from lag through log and stationary phases, can be measured in approximately 2 hours.

12.1.3. Purpose:

1. Understand the growth dynamics of a bacterial culture
2. Identify the typical phases of a bacterial growth curve
3. Use a spectrophotometer
4. Measure bacterial growth and turbidity
5. Plot a growth curve and determine the generation time of a culture of *E. coli* and/or *Vibrio natriegens*

12.1.4. Materials:

Classical method:

- 10- to 12-hour (log phase) nutrient broth
- Cultures of *Escherichia coli*
- Cultures can be maintained in log phase by immersion in an ice-water bath
- 100 ml of brain-heart infusion in a 250-ml
- Erlenmeyer flask
- 21 99-ml saline saline blanks

- 3 100-ml bottles of nutrient agar
- 37°C water bath with shaker or
- temperature Controlled shaker incubator
- Spectrophotometer
- 13 × 100 mm cuvettes
- Nephelo culture flasks (Bellco Glass Co., 340 Edrudo Rd., Vineland, NJ 08360; 800–257–7043; www.bellcoglass.com)
- Colony counter
- 28 petri plates
- 1-ml and 10-ml sterile pipettes with pipettor
- Bunsen burner, Wax pencil, 1,000-ml beaker, Ruler

Two-Hour Method:

- 6-hour starter culture of *Vibrio natriegens*
- 500-ml flask containing approximately 300 ml of *V. natriegens* medium (3.7% [37 g/liter] brain-heart infusion + 2% [20 g/liter] NaCl, final pH 7.4)
- 1- and 10-ml pipettes with pipettor
- Water bath or incubator at 37°C

12.1.5. Safety precautions:

Be careful with the Bunsen burner flame. No mouth pipetting.

12.1.6. Procedures:

Procedure (Classical Method):

1. Separate the 21 sterile saline blanks (99 ml each) into seven sets of three each. Using the wax pencil, label each set as to the time of inoculation (t = 0, t = 30, t = 60, t = 90, t = 120, t = 150, and t = 180) and the dilution in each blank (10^{-2}, 10^{-4}, and 10^{-6}) (Figure 12-2).
2. Using the wax pencil, label seven petri plates with your name, the time of inoculation (use the same times as in step 1), and the dilution (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}) to be plated.
3. Melt three tubes of nutrient agar in a water bath and cool to 45°C.

4. Using a sterile pipette, transfer 5 ml of the log phase E. coli culture to the flask containing 100 ml of brain-heart infusion broth. Label with your name, time, and date. The approximate absorbance (A) of this broth should be about 0.1 at 550 to 600 nm.
5. After the initial A has been determined, shake the culture and aseptically transfer 1 ml to the 99-ml water blank labelled 10^{-2} and continue to serially dilute to 10^{-6} (Figure 12-2).
6. Place the culture flask in the shaker water bath or incubator, set at 37°C and 120 rpm. **If a shaker bath is not available, the flask should be shaken periodically**.
7. Plate the 0 time dilutions into the appropriately labelled petri plates, using the amounts indicated in Figure 12-2. Pour 15 ml of the melted agar into each plate and mix by gentle rotation on a flat surface.
8. Thereafter, at 30-minute intervals, transfer 5 ml of the broth culture to a cuvette and determine the A of the culture at 550 to 600 nm. Be sure to suspend the bacteria thoroughly each time before taking a sample.
9. At the same time interval, transfer 1 ml of the culture into the 10^{-2} water blank of the set labelled with the appropriate time (see step 1). Complete the serial dilution once again as indicated in Figure 12-2 and plate into the labelled (see step 2) petri plates. Add melted agar as per step 7.
10. When the media in the petri plates hardens, incubate them in an inverted position for 24 hours at 35°C.

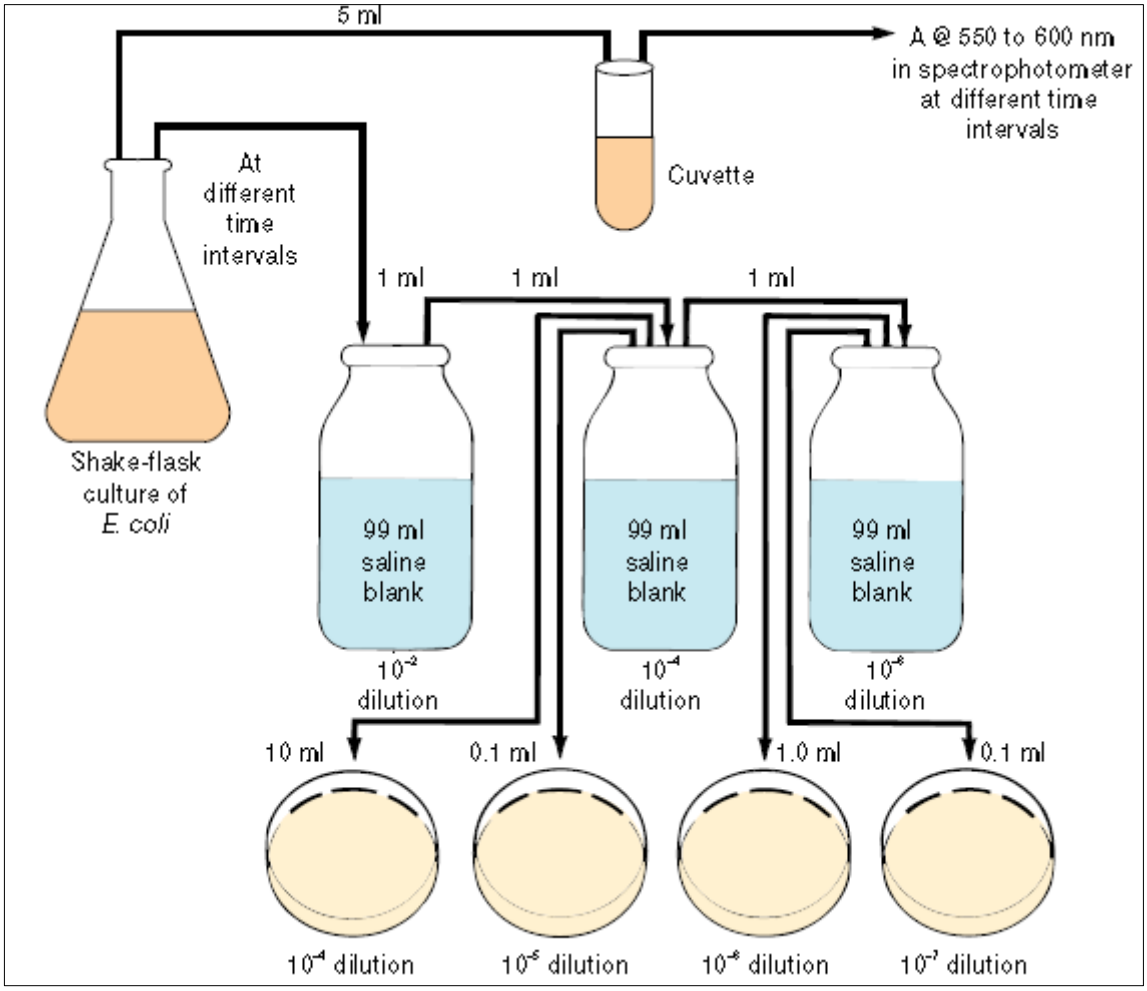


Figure 12-2: Dilution Plating and Spectrophotometric Procedure for Constructing Bacterial Growth Curves.

Second Period:

1. Perform colony counts on all plates and record all measurements and corresponding bacterial counts in the table in the report for this exercise.
2. On the paper provided, plot the following:
    a. Log absorbances on the ordinate, and incubation times on the abscissa. Use Figure 12-1 as an example.
    b. Log_{10} values of the bacterial counts on the ordinate, and incubation times on the abscissa. Connect the points with a ruler.
    c. Also construct a graph of the data using semilog graph paper. Calculate generation time and mean growth rate constant. Employ both the graphical method and growth equations.

Procedure (Two-Hour Method):

1. Zero the spectrophotometer at 550 to 600 nm with the *V. natriegens* medium from the flask.
2. Place the flask containing the brain-heart infusion medium in the 37°C water bath or incubator for 15 minutes.
3. While slowly agitating the flask in the water bath, inoculate it with 10 ml of 6-hour *V. natriegens* culture.
4. Read and record the %T of this initial culture (0 time) and every 10 minutes thereafter for about 2 hours. Be sure to suspend the bacteria thoroughly each time before taking a sample. %T (transmittance) must be accurately convert to A (absorbancy). If your spectrophotometer has a digital readout, measure the absorbance directly rather than calculating it from the %T.
5. Construct a growth curve by plotting A against time on semilog graph paper. Calculate the mean generation time and growth rate constant using both equations and the graphical method.

12.1.7. Results:

Classical Method

1. Based on your data on absorbance and plate counts (bacterial cells per milliliter), complete the following table.

Incubation Time in Minutes	Absorbance @ 550 to 600nm	Plate Counts, Bacteria/ml	Dilution Factor	Log of Bacteria/ml
0				
30				
60				
90				
120				
150				
180				

2. Calculate the generation time for this *E. coli* culture by the indirect method, using the formula given in the Principles section and by the indirect method using your growth curve and extrapolations from the absorbances for doubling. Show all calculations in the space provided.
    a. From formula
    b. From growth curve
3. What is the *k* value for your *E. coli* culture?

Two-Hour Method

1. Based on your data on absorbance, complete the following table.

Incubation Time in Minutes	*%T*	*A*
0	_____	_____
20	_____	_____
40	_____	_____
60	_____	_____
80	_____	_____
100	_____	_____
120	_____	_____

12.1.8. Review Questions:

1. Define generation time.

2. When following bacterial growth, why is absorbance plotted instead of percent transmission?

3. Can generation time be calculated from any phase of the growth curve? Explain your answer.

4. What is occurring in a bacterial culture during the lag phase? During the growth phase?

5. What is the significance of a k value?

6. What is meant by the turbidity of a culture?

7. How can the mean generation time be determined for a bacterial culture?

Exercise 13: **Antimicrobial susceptibility test**

13.1.1. Background:

An important function of the diagnostic microbiology laboratory is to help the physician select effective antimicrobial agents for specific therapy of infectious diseases. When a clinically significant microorganism is isolated from the patient, it is usually necessary to determine how it responds in vitro to medically useful antimicrobial agents, so that the appropriate drug can be given to the patient. Antimicrobial susceptibility testing of the isolated pathogen indicates which drugs are most likely to inhibit or destroy it in vivo. Susceptibility testing has shown that bacteria are becoming increasingly resistant to a wide variety of antimicrobial agents. Although new antibiotics continue to be developed by pharmaceutical manufacturers, the microbes seem to quickly find ways to avoid their effects. Two important bacteria that have developed resistance to multiple antimicrobial agents are *Staphylococcus aureus* strains, especially those resistant to the drug methicillin and its relatives, and Enterococcus spp. resistant to vancomycin. These organisms are referred to as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE), respectively. Antibiotic-resistant strains of both organisms play important roles in infections acquired by hospitalized patients. The laboratory must use methods to detect this resistance so that special precautions are quickly instituted to prevent transfer of the resistant bacteria among patients.

13.1.2. Principles:

The testing method most frequently used is the standardized filter paper disk agar diffusion method, also known as the NCCLS (National Committee for Clinical Laboratory Standards) or Kirby-Bauer method. In this test, a number of small, sterile filter paper disks of uniform size (6 mm) that have each been impregnated with a defined concentration of an antimicrobial agent are placed on the surface of an agar plate previously inoculated with a standard amount of the organism to be tested. The plate is inoculated with uniform, close streaks to assure that the microbial growth will be confluent and evenly distributed across the entire plate surface. The agar medium must be appropriately enriched to support growth of the organism tested. Using a disk dispenser or sterile forceps, the disks are placed in even array on the plate, at well-spaced

intervals from each other. When the disks are in firm contact with the agar, the antimicrobial agents diffuse into the surrounding medium and come in contact with the multiplying organisms. The plates are incubated at 35°C for 18 to 24 hours.

After incubation, the plates are examined for the presence of zones of inhibition of bacterial growth (clear rings) around the antimicrobial disks. If there is no inhibition, growth extends up to the rim of the disks on all sides and the organism is reported as resistant (R) to the antimicrobial agent in that disk. If a zone of inhibition surrounds the disk, the organism is not automatically considered susceptible (S) to the drug being tested. The diameter of the zone must first be measured (in millimetres) and compared for size with values listed in a standard chart (Table 13-1). The size of the zone of inhibition depends on a number of factors, including the rate of diffusion of a given drug in the medium, the degree of susceptibility of the organism to the drug, the number of organisms inoculated on the plate, and their rate of growth. It is essential, therefore, that the test be performed in a fully standardized manner so that the values read from the chart provide an accurate interpretation of susceptibility or resistance. In some instances, the organism cannot be classified as either susceptible or resistant, but is interpreted as being of “intermediate” or “indeterminate” (I) susceptibility to a given drug. The clinical interpretation of this category is that the organisms tested may be inhibited by the antimicrobial agent provided that either (1) higher doses of drug are given to the patient, or (2) the infection is at a body site where the drug is concentrated; for example, the penicillins are excreted from the body by the kidneys and reach higher concentrations in the urinary tract than in the bloodstream or tissues. When an interpretation of I is obtained, the physician may wish to select an alternative antimicrobial agent to which the infecting microorganism is fully susceptible or additional tests may be necessary to assess the susceptibility of the organism more precisely.

Table 13-1: Interpretation of Inhibition Zones of Test Cultures.

Disk Symbol	Antibiotic	Disk Content	Diameter of Zones of Inhibition (mm)		
			Resistant	Intermediate	Susceptible
AM	Ampicillin[a] when testing gram-negative microorganisms and enterococci	10 µg	16 or less		17 or more
AM	Ampicillin[a] when testing staphylococci and penicillin G-susceptible microorganisms	10 µg	28 or less		29 or more
B	Bacitracin	10 units	8 or less	9–12	13 or more
CB	Carbenicillin when testing *Proteus* species and *E. coli*	50 µg	19 or less	18–22	23 or more
CB	Carbenicillin when testing *P. aeruginosa*	50 µg	13 or less	14–16	17 or more
C	Chloramphenicol (Chloromycetic®)	30 µg	12 or less	13–17	18 or more
CC	Clindamycin[c] when reporting susceptibility to clindamycin	2 µg	14 or less	15–20	21 or more
CC	Clindamycin[c] when reporting susceptibility to lincomycin	2 µg	16 or less	17–20	21 or more
CL	Colistin[d] (Coly-mycin®)	10 µg	8 or less	9–10	11 or more
E	Erythromycin	15 µg	13 or less	14–22	23 or more
GM	Gentamicin	10 µg	12 or less	13–14	15 or more
K	Kanamycin	30 µg	13 or less	14–17	18 or more
ME	Methicillin[e]	5 µg	9 or less	10–13	14 or more
N	Neomycin	30 µg	12 or less	13–16	17 or more
NB	Novobiocin[f]	30 µg	17 or less	18–21	22 or more
OL	Oleandomycin[g]	15 µg	11 or less	12–16	17 or more
P	Penicillin G. when testing staphylococci[h]	10 units	28 or less		29 or more
P	Penicillin G. when testing other microorganisms[h,i]	10 units	14 or less		22 or more
PB	Polymyxin B[d]	300 units	8 or less	9–11	15 or more
R	Rifampin when testing *N. meningitidis* susceptibility only	5 µg	16 or less	17–19	20 or more
S	Streptomycin	10 µg	6 or less	7–9	10 or more
S	Sulfonamides	300 µg	12 or less	13–16	17 or more
T (TE)	Tetracycline[j]	30 µg	14 or less	15–18	19 or more
VA	Vancomycin	30 µg	14 or less	15–16	17 or more

13.1.3. Purpose:

To learn the agar disk diffusion technique for antimicrobial susceptibility testing

13.1.4. Materials:

- Nutrient agar plates (Mueller-Hinton if available)
- Tubes of sterile nutrient broth or saline (5 ml each)
- Antimicrobial disks (various drugs in standard concentrations)
- Antimicrobial disk dispenser (optional)
- McFarland No. 0.5 turbidity standard
- Sterile swabs
- Forceps
- 24-hour plate cultures of *Staphylococcus epidermidis* and *Escherichia coli*

13.1.5. Safety precautions:

Be careful with the Bunsen burner flame. Always handle cultures with care since they may be potential pathogens. The ethyl alcohol that is used to sterilize the forceps is flammable.

13.1.6. Procedures:

1. Touch 4 to 5 colonies of *S. epidermidis* with your sterilized and cooled inoculating loop. Emulsify the colonies in 5 ml of sterile broth or saline until the turbidity is approximately equivalent to that of the McFarland No. 0.5 turbidity standard. This can also be determined by using a spectrophotometer with Optical density of 1 at 600 nm wavelength. http://vlab.amrita.edu/?sub=3&brch=73&sim=1628&cnt=1
2. Dip a swab into the bacterial suspension, express any excess fluid against the side of the tube, and inoculate the surface of an agar plate as follows: first streak the whole surface of the plate closely with the swab; then rotate the plate through a 45°C angle and streak the whole surface again, rotate the plate another 90°C and streak once more. Finally, run the swab around the edge of the agar. This procedure ensures that the whole surface has been seeded. Allow the culture to dry on the plate for 5 to 10 minutes at room temperature with the top in place (Figure 13-1). Discard the swab in disinfectant.
3. Repeat steps 1 and 2 with the *E. coli* broth culture on a second nutrient agar plate.
4. Heat the forceps in the Bunsen burner flame or bacterial incinerator, and allow to cool.
5. Pick up an antimicrobial disk with the forceps and place it on the agar surface of one of the inoculated plates. Press the disk gently into full contact with the agar, using the tips of the forceps.
6. Heat the forceps again and cool.
7. Repeat steps 5 and 6 until about eight different disks are in place on one plate, spaced evenly away from each other. (If an antimicrobial disk dispenser is available, all disks may be dispensed on the agar surface simultaneously. Be certain to press them into contact with the agar using the forceps tips).
8. Place a duplicate of each disk on the other inoculated plate, using the same procedures.
9. Invert the plates and incubate them at 35°C for 18 to 24 hours.

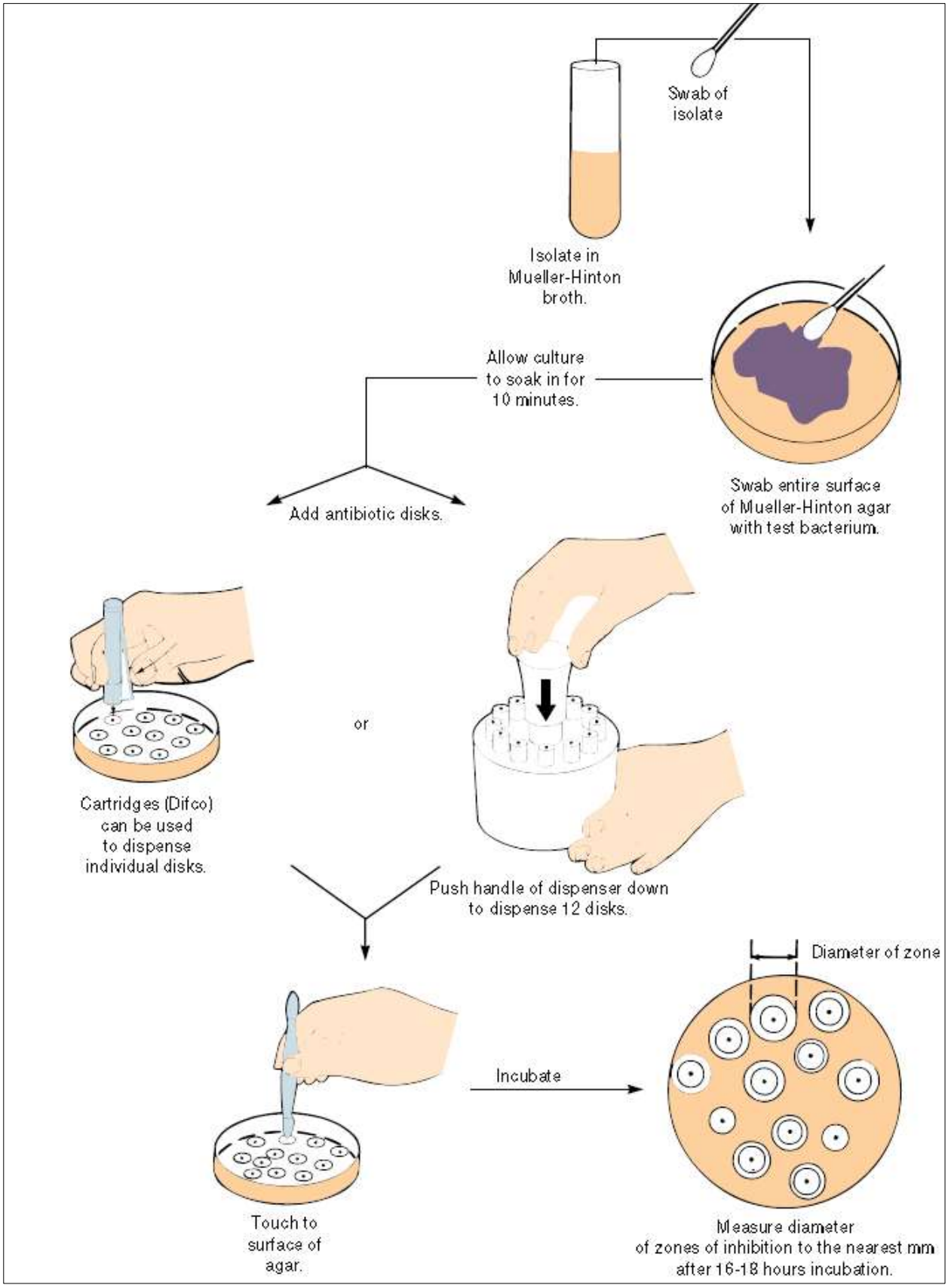


Figure 13-1: Antimicrobic Sensitivity Testing.

13.1.7. Results:

1. Observe for the presence or absence of growth around each antimicrobial disk on each plate culture. Using a ruler with millimetre markings, measure the diameters of any zones of inhibition and record them in the chart. If the organism grows right up to the edge of a disk, record a zone diameter of 6 mm (the diameter of the disk).

Antimicrobial Agent	*Concentration*	*Zone Diameter*		*E. coli (S, I, or R)*	*S. epidermidis (S, I, or R)*
		E. coli	*S. epidermidis*		

2. From the above table, which antibiotic (antimicrobic) would you use against each of the following?

S. epidermidis _____

E. coli _____

13.1.8. Review Questions:

1. Define an antimicrobial agent.

2. What is meant by antimicrobial resistance? Susceptibility?

3. Why are pure cultures used for antimicrobial susceptibility testing?

4. Would it be acceptable to use a mixed culture for this test? Why?

5. List three factors that can influence the accuracy of the test.

6. Could an organism that is susceptible to an antimicrobial agent in laboratory testing fail to respond to it when that drug is used to treat the patient? Explain.

7. Are antibacterial agents useful in viral infections? Explain.

8. Describe a mechanism of bacterial resistance to antimicrobial agents.

9. If the laboratory isolates *S. aureus* from five patients on the same day, is it necessary to test the antimicrobial susceptibility of each isolate? Why?

10. In which growth phase is a bacterium most sensitive to an antibiotic?

Appendix I: Laboratory report template

Exercise (no. & name): ……

Student name: ……

Submission Date

Grade

Results:

Review Questions:

References:

1. **Harley–Prescott**, *Laboratory Exercises in Microbiology*. 5th ed. 2002, The McGraw–Hill Companies. 449 p.
2. **Morello–Mizer–Granato**, *Laboratory Manual and Workbook in Microbiology*, 7th ed. 2003. The McGraw–Hill Companies. 285 p.
3. **Benson**, *Microbiological Applications Lab Manual*, 8th ed. 2001, The McGraw–Hill Companies. 455 p.
4. **Emanuel G., and Lorrence H. G.**, *Practical Handbook of Microbiology*, 2nd ed. 2009, CRC Press, Taylor & Francis Group. 876 p.