# Purpose:

There are a number of basic techniques you must master in order to work successfully with microorganisms. These are aseptic technique, preparing a bacterial smear, staining bacteria, inoculation of bacterial growth media and the enumeration of bacteria. This lab will give the student an opportunity to learn and practice several of these techniques.

***Background***

In the early days of surgery the most important consideration when choosing a surgeon was how fast he could perform the surgery. There was no anesthesia or sterile operating rooms. Surgery was messy and often patients died of shock or more commonly post-­‐operative infections. Where did the infections come from you might ask? Our current understanding of the ubiquitous nature of microorganisms and the role that some play in disease was unknown at the time, so surgeons had no concept that by not properly sterilizing their instruments or preparing the patient they were contributing to the spread of disease and death. It would not be until the mid to late 1800’s before our modern germ theory came to be widely accepted and the consequences of not using aseptic technique were fully understood.

The word aseptic literally means without sepsis. Sepsis is a word that means infection. Therefore if a surgeon were practicing aseptic technique they would be operating in such a manner as not to transmit an infectious agent or cause an infection to occur. In today’s operating rooms, aseptic techniques are strictly adhered so as to prevent infection. The techniques include the use of antiseptics, which are substances when applied to the tissues of the body prevent infections, proper sterilization of surgical instruments, proper hand washing, disinfection of the operating room and the use of barriers such as gloves, masks and gowns. These measures have no doubt reduced suffering and saved many lives.

In the laboratory the aseptic technique is less concerned with the transmission of infectious agents as it is with the contamination of laboratory cultures and personnel working in the lab.

Contamination is the introduction of an unwanted organism or organisms into a culture, environmental space, other organism (such as your lab partner) or yourself. You will be working with a variety of bacterial cultures during the course of the semester. It is important that you work with the organisms safely and not contaminate them or yourself. To accomplish this you will need to keep a few things in mind.

You must be aware of the sources of contamination. The first is the environment. We are literally living in a sea of microorganisms. There are billions of bacteria on most environmental surfaces as well as fungal spores, viruses and perhaps a few protozoan parasites. If these organisms were to enter your culture they may begin to grow and your pure culture will no longer be pure. In the laboratory exercises we will be conducting it is important that we have pure cultures to achieve proper results.

A second source of contamination is what we refer to as cross contamination. This occurs when organisms from one culture are introduced into another. This can be deliberate or unintended, but the result is the same, a mixed culture. You will need to see that this does not happen.

The last thing to remember is that if you spill any of your organisms onto your lab bench or other surface you must properly clean up the spill immediately. This is contamination of the environment. While the bacteria we will be working with are not likely to cause problems if properly handled, they have the potential to cause infections if you contaminate yourself with them. **Remember safety first. You must properly contain and clean up any spilled cultures. Please seek assistance from the instructor should you spill a culture.**

# Basic Principles of Aseptic Technique:

There are a few basic considerations you need to keep in mind when working with microorganisms. If you learn these

good results in the lab. It should be noted that these principles might be extended to the medical and health care arenas, as well as the kitchen, bathroom, and to some extent the bedroom.

The first principle is that you should consider **EVERYTHING** to be contaminated with microorganisms. This is not meant to frighten you, as most bacteria and fungi you will encounter in your day-­‐to-­‐day life are harmless to you, however if these critters are not accounted for, they could contaminate a laboratory culture. In the medical world, some of these organisms if given the opportunity could cause an infection.

The second principle is to never work with microorganism unless you know that the tools you are working with and the medium you are using are sterile. In this lab you can assume that the media I will supply is sterile, but there are times it may become contaminated, so look it over before you use it. Sterile un- inoculated culture medium should not be fuzzy or come with bacterial colonies on the surface and broths should be clear, not cloudy.

The third principle is that cultures should be axenic, which means they contain only one species of bacteria. On occasion mixed cultures may be used. Since most of the cultures are axenic when given to you, they should remain that way.

# Aseptic Technique

Now that you have a background concerning the basic principles of aseptic technique, it is time to outline how you are to apply these principles in the laboratory.

1. the first thing you do upon entering the laboratory is to wipe down your work area with disinfectant. This will not eliminate all the microorganisms on the lab bench, but remove most, particularly any potential infectious agents and contaminants that can get into your cultures.
2. the second thing you do is to pay careful attention to all instructions given concerning the laboratory procedures you will be asked to perform. Most problems arise when a student does not follow the directions.
3. when you begin to work with the microorganisms, you need to following the following steps and protocols:
4. light your Bunsen burner. Be sure it is in a safe place and will not cause a fire. You will be instructed on the proper use of the Bunsen burner.
5. obtain your inoculating loops and flame them. The entire wire portion of the loop must be allowed to remain in the flame until it glows red. The loop should be held on a slight angle; see Figure 1



Figure 1 Flaming the loop

1. once the loop is flamed, it is sterile. **DO NOT** touch the loop, set it down on the lab bench, or allow it to come into contact with anything. It is now ready to be placed into the culture to remove a small amount of the organisms.
2. carefully remove the cap on the culture. **DO NOT** set the cap onto the lab bench; hold onto it in such a manner as it is held right side up.
3. pass the mouth of the culture tube through the flame several times. The idea here is to remove any microorganisms on the lip of the tube and to warm the tube. **DO NOT** leave the tube in the flame until it glows red. If you do, you will not like the results. Also, **DO NOT** hold the tube vertical. Always keep the tube on a slight slant.
4. once the tube has been passed through the flame, insert the sterile loop into the culture tube and down into the culture. Gently remove a loopful of the culture. The loop is now referred to as being charged and contains thousands of microorganisms**. DO NOT** touch anything with the loop or shake it around.
5. before putting the cap back onto the culture tube, pass the mouth of the tube through the flame several times. Replace the cap.
6. at this point, you are ready to transfer the bacteria on the loop to a microscope slide or to culture medium.
7. after the transfer, you must flame the loop again to remove any remaining microorganisms. Once flamed the loop can be set aside. Remember it is hot. **DO NOT** touch the loop or touch anything with it that could be burned, i.e. your lab partner or lab manual, etc...
8. each time you are going into or out of a culture to either remove organisms or to inoculate the medium, you must flame the loop and the mouth of the tube.
9. after you have finished all the transfers and are ready to leave the laboratory, wipe down your work area with disinfectant and wash your hands. It is good practice to wash your hands every time you leave the laboratory. You really do not want to take any of the microorganisms with you. They do not make good pets.

# Exercise in Aseptic Technique

This exercise is designed to help you practice your aseptic technique.

1. obtain two tubes of medium from the instructor.
2. label the tubes #1 and #2 with your wax pencil or marking pen.
3. open tube #1 and insert your loop into the tube without flaming it or the tube mouth.

There is no need to be careful here. You may even allow the tube to remain uncapped for several minutes if you like. The idea is to allow contaminants to enter the tube.

1. using the aseptic technique described above repeat the process in step 3. This time be careful to flame the loop, and tube. Do not allow the tube to remain uncapped for any longer than necessary to place the loop into the medium and remove it.
2. place both tubes into a rack as instructed. The tubes will be incubated overnight and examined for growth.

# Day Two

1. examine the tubes for growth. Growth will be evidenced by cloudiness in the medium.
2. record your results in Table 1.

# Table 1 – Results

|  |  |
| --- | --- |
| **Tube #1** | **Tube #2** |
|  |  |

Was there a difference between the two tubes?

If there was a difference, how can this be explained?

# Notes:

**Exercise in making a bacterial smear**

This exercise is designed to help you practice making a smear of bacteria on a microscope slide. It is very important you learn to make proper smears.

1. obtain a culture of bacteria from the instructor.
2. prepare the slide as instructed. Be very careful as you chip off the upper right hand corner of the slide using a forceps. The instructor will demonstrate this.
3. using aseptic technique and a culture of bacteria apply a loopful of bacteria to the slide. Be sure to not add too much.
4. add a loopful of water to the slide and mix the bacteria into a thin emulsion evenly over an area approximately the size of a dime (*note we will be working a culture grown on an agar-­‐based medium. A similar process is followed when using bacteria grown in broth with the exception that no water is added to slide as the cells are already suspended in the culture medium*) The slide should look cloudy where you inoculated but you should be able to see through the smear.
5. allow the slide to air dry. It is important to not over heat the slide during the drying process as this can damage the bacterial cells.
6. heat fix the slide as instructed. This step insures the bacteria will remain on the slide during the staining process
7. time permitting a simple stain such as methylene blue can be applied to the smear and the slide observed using the light microscope. The instructor will explain the staining procedure.

# Exercise in inoculating microbial medium

This exercise is designed to help you practice inoculating a plate of agar-­‐based medium.

There are many types of agar-­‐based media used in the microbiology laboratory and it is essential that you learn how to properly inoculate bacteria onto agar plates.

Depending on your desired outcome there are several simple and useful techniques you should know.

1. First, there is the simple inoculation. This is a technique where you are trying to simply get bacteria onto the surface of the plate and do not care about individual colonies or isolation of different species of bacteria. This technique can be done using a loop and aseptic technique or a sterile swab. The sterile loop or swab is dipped into a culture of bacteria and then used to inoculate the sterile plate of medium. In a simple inoculation the idea is to introduce bacteria to the surface of the medium. No attempt needs to be made in trying to isolate individual colonies of bacteria. In fact, you can create pretty much any design on the surface of the medium you would like. You might write your name, draw a smiley face or some other design. The key is to not push too hard and rip up the surface of the medium. Label this plate

#1.

1. Second, there are techniques for the isolation of specific type or species of bacteria from a mixed culture. These techniques rely on spreading out the bacteria in the culture over the surface of the medium thereby diluting the amount of bacteria over a large portion of the medium surface. The fastest, simplest and most used technique to accomplish isolation is the four-­‐phase streaking technique. The first step is aseptically inoculating a loop and using this loop spread the bacterial over about one quarter of the plate. The next several steps are critical and if not followed properly the desired isolation will not be accomplished.
2. After completing the first inoculation of the plate, flame the loop. Cool the loop by stabbing it into the medium in an area near the side of the plate away from where you just inoculated. **WITHOUT** obtaining more bacteria from the original culture, take the cooled loop and gently glide it over a small portion of the agar you just inoculated several times. The idea is to pick up some of the bacteria you

put onto the plate during the first inoculation. Carefully with out going back into the area you first inoculated, inoculate at a 90-­‐degree angle to the first area inoculated approximately another quarter of the plate. At this point you will flame the loop and repeat the process describes above two more times. The instructor will demonstrate this technique. Please pay careful attention to the demonstration. Label this plate #2.

1. Third, there is a technique to create a lawn of bacteria on the surface of an agar plate. There are occasions when an even growth is required. There are several ways to accomplish this; the easiest is to use a sterile swab. Simply dip a sterile swab into a culture of bacteria and gently rub it over the entire surface of the agar plate being careful to not rip or tear the medium. Label this plate #3.
2. Once you have inoculated your three plates, place them into the incubator as instructed, upside down. The reason for inoculating the plates upside down is to prevent the accumulation of moisture on the lid of the plate. Also, be sure to label the plates on the bottom as instructed.

# Day Two

1. examine the plates for growth.
2. record your results in Table 2.

# Table 2 – Results

|  |  |
| --- | --- |
| **Plate #1** |  |
| **Plate #2** |  |
| **Plate #3** |  |

Was there a difference between the three plates?