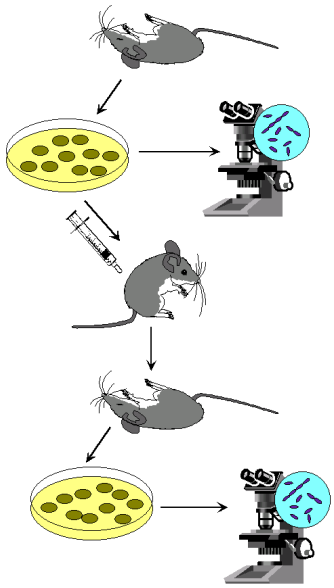
Microbiology Laboratory 4

Koch's Postulates

While it seems obvious to us today, the concept that microorganisms cause disease was hotly debated some time ago. Dr. Robert Koch was the first to conclusively link certain diseases with specific microorganisms. He developed a four step process necessary to link a disease with a specific microorganism. These became known as **Koch's Postulates**.

- **Associate -** The suspected causal organism must be constantly associated with the disease.
- **Isolate** (identify) - The suspected causal organism must be isolated from an infected individual (host) and grown in pure culture then identified.
- **Inoculate -** When a healthy susceptible host is inoculated with the pathogen from pure culture, symptoms of the original disease must develop.
- **Re-isolate -** The same pathogen must be re-isolated from the newly infected host and cultured.



Lab Assignment

Over the next few labs we will reproduce Koch's postulates with an infected "patient". You must:

- Note the disease symptoms of the infected "patient"
- Culture the suspected causative organism from an infected host.
  - Using alcohol sterilized forceps remove a but of infected tissue and spread on a PDA (Potato Dextrose Agar) plate.
  - Incubate your plates inverted on top of the incubator.
- After your culture has grown, inoculate a healthy test subject with your grown culture.
  - Inoculate a healthy hose
    - Mentally divide your host in half for a control and experimental.
    - Using alcohol sterilized forceps transfer some sterile water to the control half.
    - Using alcohol sterilized forceps transfer a small bit of your test culture to the experimental half.
    - Incubate on top of the incubator.
  - Make a wet mount of your cultured microorganism and examine it under the microscope.
- When you experimental host has developed the same symptoms as the original "patient" culture some of the suspected organism onto a new PDA plate and incubate on top of the incubator.
- Finally examine your isolated culture to ensure it is the same organism that was cultured from the original "patient". You may also microscopically examine your newly grown culture to further ensure it is the same microorganism.

Aseptic Technique: Transfer of microorganism

In order to study what microorganisms need to survive and what metabolic activities they can do we must work with individual species of organisms. In the culture medium, there must be only the organism under study with no contaminating organisms. To accomplish the different experiments we will need to transfer the organism from one environment (medium) to another. You must accomplish this task without contaminating the original culture, the new medium, and without contaminating yourself or your workspace. The techniques required are collectively referred to as **aseptic techniques**. Remember bacteria catch rides around on dust particles. Constantly be thinking how can I prevent dust particles from entering my culture medium. Most of the time you want to:

- keep media covered when not in use
- only open a little as possible (petri dish lid)
- keep open containers at an angle
- open for as short as time as possible

There are three common forms used to grow bacteria in the laboratory:

- Agar petri dish (plate)
- Agar slant in a test tube
- Liquid broth culture - this can be in a test tube, a flask, or a bottle

Transfer Techniques

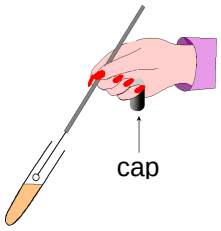
- Obtain the necessary components: culture (to transfer from), petri dish, or test tube (what transferring to), loop, and lit Bunsen burner.
- Loosen but do not open all caps..
- Use your dominate to hold and use the loop.
- Use your nondominate hand to manipulate the culture and then the transfer target.

Using a petri dish:

- The goal is to keep the dish open for as little time as possible.
- Either crack the lid and stick your loop into the dish or
- Keep the dish upside down and pick up the bottom, invert it only as long as it takes to pick up your culture or streak your culture and then immediately replace it in the lid.
- Finally flame your loop

Using a test tube:

- If you are working with a liquid culture it is often suggested that before opening the tube that you shake it to suspend the bacteria from the bottom of the tube where they may have settled.
- Always hold the test tube at an angle.
- By wrapping the little finger of your dominant hand (while still holding onto the loop) around the cap of the test tube, remove the cap.
- Pass the top of the test tube through the flame to kill any contaminating organisms that may have fallen in the opening of the test tube.
- Insert your loop into the test tube to pick up or transfer culture.
  Note: If picking up culture in a liquid there is no need to swirl your loop around.
- After you are finished withdraw your loop and again flame the top of the test tube.
- Replace the lid of the test tube and set the test tube in your test tube rack.



Lab Assignment 1

Following the procedure above transfer bacteria from a petri dish to a slant.
The difference is that the slant surface is streaked with the loop in a serpentine manner starting at the far end of the slant and pulling to the top of the slant.
Notes: Petri dishes (plates) are never flamed only glass test tubes, bottles, and flasks.
Be sure to label everything before placing it in the incubator.

Lab Assignment 2

Following the procedure above transfer bacteria from a petri dish to a liquid culture.
In all cases don't forget to label your new cultures.

Spread Plate Technique

The spread plate technique is one method of distributing bacteria evenly over the surface of an agar plate. Basically a small volume of a bacterial suspension (100 µl) is spread evenly over the agar surface using a sterile bent glass rod (hockey stick) or in some cases sterile glass beads are used. In the spread plate usually only a single species of organism is used.

Spread plates are typically used for two purposes:

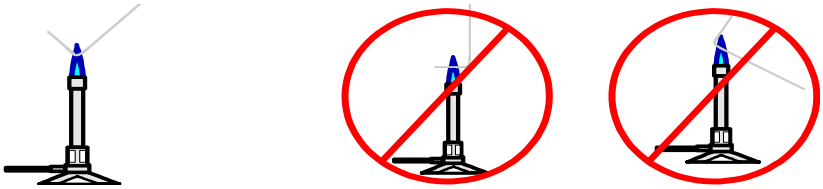
- To separate all the bacteria that will form colonies for counting
- To evenly place so many bacteria on a plate that the colonies overlap each other. In this way, a lawn of bacteria is produced that can be used to test antimicrobial substances.

Preparation

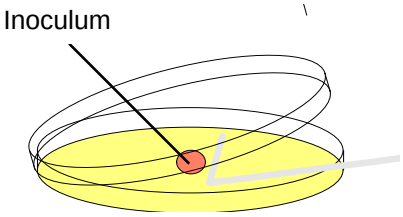
- Obtain a beaker with 70% alcohol and place it on one side of your work station.
- Obtain a bent glass rod (hockey stick) and place it in the alcohol.
- On the other side of your work station light a Bunsen burner.
- Obtain the grown culture you will transfer and a pipet that you will use to transfer the liquid.
- Finally, get a petri dish(s) you will grow your culture on.

Procedure

- Loosen the cap of the bottle/ test tube containing the broth culture.
- Place a sterile tip on the end of your micropipetter.
- Hold the micropipetter in your dominant hand and the bottle/ test tube containing the broth culture in your non-dominant hand.
- Remove the cap/ plug of the bottle/ test tube with the little finger of your dominant hand and pass the neck through the flame.
- Draw up 100 µl of broth culture into the tip of the micropipetter.
- Flame the neck of the bottle/ test tube and replace the cap/ plug.
- With your non-dominant hand, partially lift the lid of your petri dish and dispense the liquid to the center of the petri dish.
- Replace the lid of the Petri dish and discard the micropipetter tip into the appropriate container to be sterilized.
- Take the hockey stick from the alcohol and remove the alcohol by passing the hockey stick through the Bunsen burner at a 45° angle. Be careful how you hold the hockey stick.
  Allow the alcohol to fully burn away and allow time for the hockey stick to cool.
  Be careful the alcohol flame is hard to see.



- Crack open the petri dish lid and place the hockey stick on agar that does not have the liquid culture on it in case the hockey stick is not quite cool yet.
- Spread around the liquid culture till it covers all the agar in the plate
- Place the hockey stick back in the alcohol
- Allow the plate to dry before inverting it for incubation.



<u>Lab Assignment 3</u> - Spread saliva on a Snyder Test agar plate

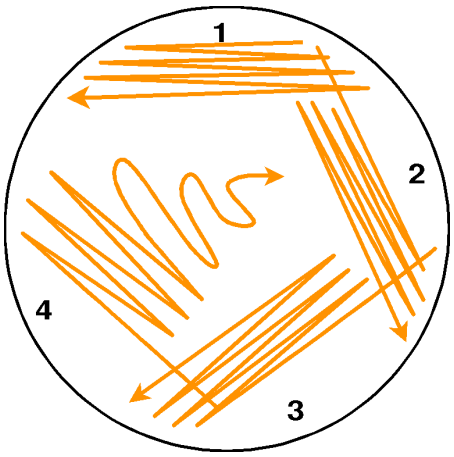
- Obtain a Snyder Test agar plate and label the underside.
- Spit into the plate, yes actually spit into the plate.
- Spread your saliva around the plate using a hickey stick as described above.
- Allow the plate to dry somewhat with the lid on before inverting it.
- Incubate your plate, inverted, in the 37°C incubator.

The Snyder Test agar contains 2% glucose and the pH indicator bromocresol green. The pH of the agar is ~ 4.8, which inhibits the growth of most organisms, but it is ideal for acidophiles such as *Lactobacillus species*. The *Lactobacillus* will ferment the glucose to lactic acid which causes the pH to fall below 4.8. In the presence of the low pH, the bromocresol green turns yellow. After incubation look for colonies with a yellow ring around them in the agar.

Streak Plate Technique

It is rare to find a single species of microorganism by itself. Most of the time microorganisms are found mixed with other microorganisms. As a result, it is important for the microbiology student to learn how to separate microorganisms so a colony of a single species will grow. The most used method in the laboratory is the streak plate method. While there are several methods to producing a streak plate we will use the quadrant method as described below.

Process



- With a flamed loop remove some bacteria from the mixed culture test tube.
- Streak one loopfull of organisms back and forth over one quadrant of the petri dish (area 1). Be careful not to gouge into the agar.
- Flame your loop till it is glowing red then let it cool for a few seconds.
- Rotate your petri dish ~70° while your loop if cooling.
- Touch your loop to the agar in the new quadrant (area 2). Without lifting your loop off the agar drag it into area 1 which contains organisms. Then, again without lifting you loop, drag it back and fourth in area 2 crossing the original streak a few times at first.
- Flame your loop again. While your loop cools, rotate the petri dish another ~70° and streak area 3 several times, crossing into the last area (2) a few times at first.
- Flame your loop again. Rotate your petri dish a final ~70° again. Streak the last quadrant several times while crossing into area 3 a few times. (Be careful not to cross into area 1.) Finally without lifting your loop drag out the culture as illustrated into the center of the dish.
- Flame your loop before putting it down.

Lab Assignment 4

- Obtain an incubated mixed liquid culture of bacteria and a NA (nutrient agar) petri dish.
- Label the under side of your petri dish.
- Transfer bacteria from a mixed liquid culture to a petri dish, then streak for isolation.
- Incubate inverted in the 37°C incubator till next lab.

Smear Technique

Before staining and observing a microbe under a microscope, a smear must be prepared. The goal of smear preparation is to place an appropriate amount of cells on a slide and then cement them there so they are not washed off during the staining procedure. Most of the time when students are having trouble observing stained bacteria it is the result of a poorly prepared smear. Caution: your goal is to produce a thin smear. Smears that are too thick obscure details about individual cells and can even trap stain which would not be removed during the wash steps of staining.

Procedure

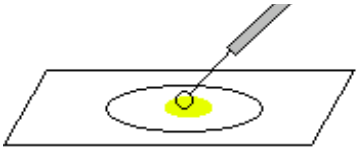
Obtain a clean slide. The smear will not spread out properly if the slide is dirty or even slightly oily. If in doubt, wash slide well with soap and water, polish with clean paper towel or a Kimwipe.

To provide a target on the slide for your smear, make a ½" circle (about the size of a dime) or oval on the *bottom* side of your slide. Later when you are more skilled you may wish to omit this step.

<u>From liquid culture</u>

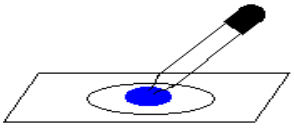
Shake the culture vigorously.

With a flamed loop, transfer one or two loopfuls of liquid culture to the center of the microscope slide.

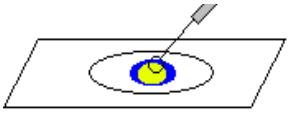


<u>From solid culture</u>

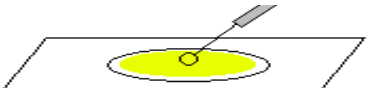
Place a small drop of water on the center of your slide.



With your flamed loop aseptically pick up a very small amount of culture and mix into the drop of water.

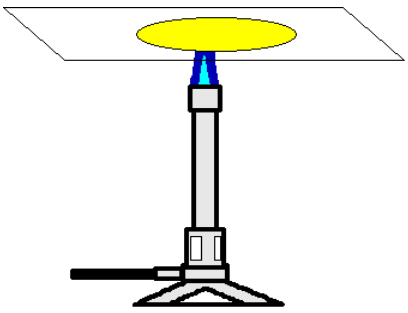


Spread the organisms over the slide to fill your target or to about the size of a dime.



Allow the smear to completely dry at room temperature.

Finally Fix your smear by passing it through your Bunsen burner flame several times to heat-kill and fix the organisms to the slide. Use of a clothespin is suggested.



Simple Stain Technique

The use of a single stain to color bacteria is called a simple stain. Some of the most common dyes used are methylene blue, basic fuchsin, and crystal violet. These dyes are composed of positively charged ions which bind the negatively charged bacteria. Positively charged dyes are called **basic dyes**. Methylene blue contains the positively charged methylene dye and a negatively charged chloride ion. Dyes that are negatively charged are called **acidic dyes**. Eosin contains a positively charged sodium ion and a negatively charged eosinate dye. Eosin will not normally stain bacteria because of the electrostatic repulsion.

Staining is done on a smear that has fully air dried and then been fixed.

Staining trays are located in the second drawer down at your lab bench. Be sure you dispose of any liquid down the sink before returning the staining trays to the cabinet.

Procedure

- Flood the fixed smear with methylene blue for 1 minute.
- Holding the slide at an angle rinse the smear briefly with water. Squirt the water above the smear and allow it to run over the stain and wash away excess stain.
- Blot off the water drops with bibulous paper. Be sure to only blot and not rub.
- Proceed to observe the bacteria under the microscope starting with low power and working your way up to the oil immersion lens.

Lab Assignment 5

- Prepare a smear and fix it of the mixed liquid culture.
- Simple stain the smear with methylene blue.
- Observe your stain under the microscope.
- How many cell morphologies do you observe?