AST Antibiotic Susceptibility Testing

Learning Objectives

The student will

- Use aseptic techniques in the safe inoculation of various forms of media.
- Follow oral and written instructions and manage time in the lab efficiently.
- Apply correct terminology regarding microbiological techniques, instruments, microbial growth, biochemical testing, media types and forms and epidemiology when making observations.
- Correctly perform various inoculation techniques including the quadrant streak and the T streak techniques and describe each technique's purpose.
- Use antibiotic resistance testing to identify pathogens and determine an appropriate antibiotic treatment.

Background/Theory

The **Kirby-Bauer disc diffusion test** has long been used as a starting point for determining the susceptibility of specific microbes to various antimicrobial drugs. It can also be used to determine an antibiotic's range of effectiveness.

The Kirby-Bauer assay starts with a Mueller-Hinton agar plate on which a **confluent lawn** is inoculated with a patient's isolated bacterial pathogen. Filter paper discs impregnated with known amounts of antibacterial drugs are placed on the agar surface. The antibiotic diffuses from the disc into the agar creating a concentration gradient. The drug's concentration is highest near the disc and gets more dilute the greater the distance from the disc. The microbial cells interact with the drug at these varying concentrations. The minimum concentration that will keep the bacterial cells from growing is called the **minimum inhibitory concentration, MIC**. At concentrations above the MIC, the organism will not grow. After incubation, this antibacterial activity is observed as a clear circular **zone of inhibition** around the drug-impregnated disc. The diameter of the zone of inhibition, measured in millimeters and compared to a standardized chart, determines the susceptibility or resistance of the bacterial pathogen to the drug.

There are multiple factors that determine the size of a zone of inhibition in this assay, including drug solubility, rate of drug diffusion through agar, the thickness of the agar medium, and the drug concentration impregnated into the disc and the MIC. To ensure reliable, repeatable results, one must follow specific protocol which standardizes these variables.

- Mueller-Hinton agar plates are used.
- Agar is poured to a depth of 4 mm.
- The plates are incubated 18-24 hours at 37C.
- The pure broth cultures being tested must have the same turbidity (estimate of the cell concentration), specifically, a McFarland Standard of 5 or an OD at 600 nm of approximately 0.1.

Mechanisms of action of various antibiotics can be found at this link or QRS code.

(OpenStax CNX, 2018)

Experiment/Exercise

Materials per student pair

4 sterile cotton swabs
4 Mueller-Hinton Agar plates
Antibiotic Discs chloramphenicol, ciprofloxacin, penicillin, trimethoprim
Forceps
Jar of EtOH 95% with lid

Cultures

Fresh overnight cultures diluted to an OD at 600 nm of 0.1 (McFarland Standard 0.5)
E. coli
Staphylococcus epidermidis
Staphylococcus aureus BSL-2
Pseudomonas aeruginosa BSL-2

Procedure lab 1

1. Assemble all materials. Label each plate with all 5 components, one organism per plate.
2. On the bottom of each plate, place four small dots equidistant from each other. This will guide the placement of the antibiotic discs.
3. It is not necessary to add the name of each antibiotic to the label. Each disc has a code that denotes the antibiotic and its concentration.
4. Inoculate each plate with a different culture using the cotton swab to obtain a uniform **bacterial lawn** as follows.
    a. Determine which end of the swab package contains the cotton bulb. Open the package at opposite end exposing only a short length of the stick. Remove one of the swabs.
    b. Aseptically insert the swab into the diluted culture.
    c. As the swab is removed, press the bulb against the side of the culture tube to press out excess liquid. Aseptically replace the test tube cap and set the tube in the rack.
    d. With the plate right side up, hold the lid above the plate as a shield. Starting at the point farthest away from your dominant hand (figure 1A), swab the agar surface streaking back and forth from one side of the plate to the other. Attempt to cover the entire surface of the agar without space between the streaks (figure 1B).

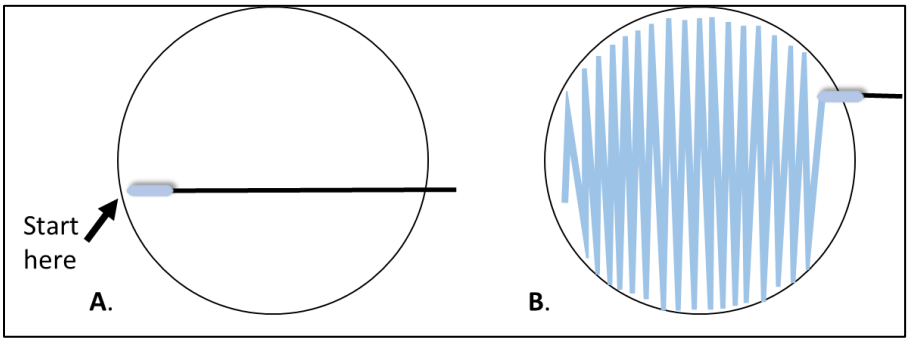


Figure 1. Swabbing to produce a bacterial lawn, for the right handed

e. Rotate the plate approximately 60°, and repeat with the same swab (figure 2).

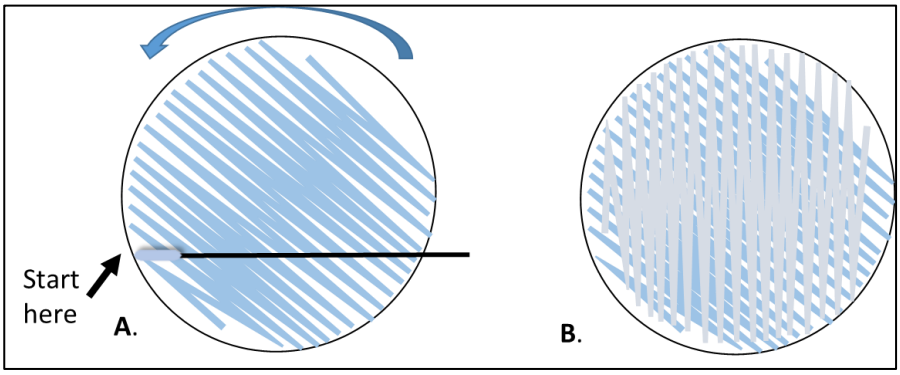


Figure 2 Second pass of the swab fills in some gaps.

f. Rotate 60° again, swab one more time (figure 3).

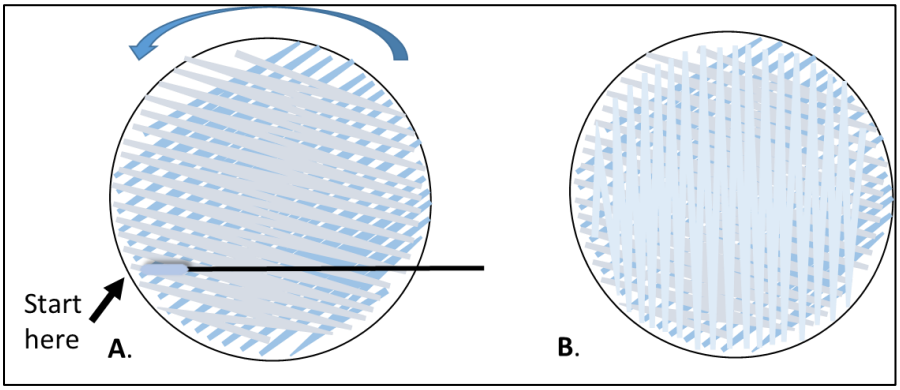


Figure 3 Third pass of the swab

g. Dispose of the swab in the disposal container on the bench top.

5. After you have inoculated each plate with the respective organism, place one disc of each antibiotic on each plate as follows.
   a. Place one plate right side up on a white piece of scratch paper so that you can see the placement dots.
   b. The forceps will be sterilized using alcohol as you did with the spreading rod in the VPC exercise. Remember that the flame simple ignites the alcohol. Keep all paper away from the area. Replace the lid on the alcohol jar immediately after taking the forceps out and before passing them through the flame. Do not shake the forceps.
   c. Using alcohol dipped and flamed forceps, remove a disc from one of the antibiotic magazines and carefully place it on the agar surface over a dot. Make sure that the paper disc makes good contact with the surface by gently tapping the disc with the forceps. Do not break the surface of the agar.
   d. Resterilize the forceps in the alcohol, and place the second antibiotic on the plate in the same way.
   e. Place the other discs on the plate in the same way. Be sure to sterilize the forceps <u>for each disc</u>.
6. Place each of the 4 antibiotic discs on the other plates using the same procedure.
7. Place the plates up-side-down in the location designated for cultures to be incubated.
8. They will be incubated for 24 hours at 37°C.

Procedure lab 2

1. Observe your plates by measuring and recording the diameters of each zone of inhibition in millimeters.
2. Compare each diameter to the interpretive chart and determine each organism's susceptibility to each antibiotic.

Zone Diameter Interpretive Chart
Adapted from (BD BBL Sensi-Disc Antimicrobial Susceptibility Test Discs package insert)

			Zone Diameter Interpretive Standards (mm)		
Antimicrobial agent	code	Disc Potency	Resistant	Intermediate	Susceptible
Chloramphenicol Enterobacteriaceae *P. aeruginosa, Staphylococci, Enterococci*	C-30	30 µg	≤12	13-17	≥18
Ciprofloxacin Enterobacteriaceae *P. aeruginosa, Staphylococci, Enterococci*	CIP-5	5 µg	≤15	16-20	≥21
Penicillin *Staphylococci*	P-10	10 µg	≤28		≥29
Penicillin *Enterococci*	P-10	10 µg	≤14		≥15
Streptomycin Enterobacteriaceae	S-10	10 µg	≤11	12-14	≥15
Tetracycline Enterobacteriaceae *P. aeruginosa, Staphylococci, Enterococci*	Te-30	30 µg	≤14	15-18	≥19
Trimethoprim Enterobacteriaceae *Staphylococci*	TMP-5	5 µg	≤12	13-17	≥18

Lab Report: Antibiotic Susceptibility Testing

Name _____
Lab Section _____

Data and Observations

Antibiotic →								
Organism ↓	Zone of inhibition (mm)	S/R/I	Zone of inhibition (mm)	S/R/I	Zone of inhibition (mm)	S/R/I	Zone of inhibition (mm)	S/R/I

S = susceptible; R = resistant; I = intermediate

Post Lab Questions

1. What is the difference between a confluent **lawn** and confluent growth as seen in the first or second area of a t-streak? Why is a lawn important for this particular test?

2. As you will see in your results, most organisms are inhibited to some degree by the antibiotics. The question is: "Does this agent inhibit the organism to the extent that the antibiotic would be useful clinically?" To determine if the organism is clinically susceptible or resistant to each antibiotic you must do what two things?

3. Which antibiotic was most effective for each organism?

4. Classify each antibiotic used in this exercise by its general mechanism of action listed below.
   Interferes with bacterial cell wall formation

   Targets the bacterial ribosome

   Interferes with bacterial DNA synthesis

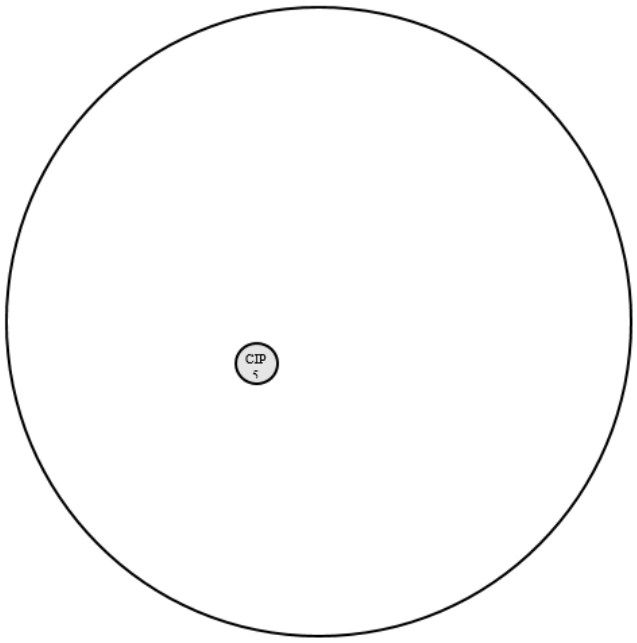
   Interferes with folic acid synthesis

   Targets the bacterial plasma membrane

5. Is there a correlation between the mechanism of action of each antibiotic and its effect on Gram positive versus Gram negative organisms? Explain.

6. Explain why an antibiotic with a greater zone of inhibition, may not be the best choice to treat an infection.

7. The large circle to the right represents a Mueller-Hinton Agar plate and the small circle represents a disc infused with ciprofloxacin 5 µg.
   a. Draw the result after incubation if the organism is <u>susceptible</u> to the drug. Shade the area of the plate that would show growth.
   b. Then label the location where the MIC is reached.
   c. Which area(s) have a drug concentration lower than MIC?
   d. Which area(s) have a drug concentration higher than MIC?



References

BD BBL Sensi-Disc Antimicrobial Susceptibility Test Discs package insert. (n.d.). *Zone Diameter Interpretive Chart*.

OpenStax Microbiology, Microbiology . OpenStax CNX. Mar 19, 2018 http://cnx.org/contents/e42bd376-624b-4c0f-972f-e0c57998e765@4.24. (n.d.).