



Student Activities

Measuring Antibiotic Resistance (Kirby-Bauer)

Introduction:

You might be aware that antibiotics were once thought of as a “magic bullet;” a nearly perfect drug for combating bacteria. We should have known that things are never that simple. If we use the three domains model for classification (bacteria, archaea, and eucarya), then there are more than a dozen different kingdoms of Bacteria. Thinking this way, we readily see that bacteria are too diverse for any one drug to kill them all. Some antibiotics come close, though. These are called “wide-spectrum” antibiotics. Other kinds of antibiotics are fairly specific in the type of bacteria against which they are lethal. (Refer to **Beyond Confronting the Microbe Menace DVD: Chart: Antibiotics (T5C26)**).

One of the easiest ways to tell a fundamental difference between bacteria is with the Gram stain. Bacteria will stain either Gram-positive or Gram-negative based on structural differences between the two classes of cell walls. The Gram-positive cell wall contains a thick layer of peptidoglycan while the Gram-negative cell wall contains a thinner layer of peptidoglycan surrounded by an outer membrane. (Refer to **Beyond Confronting the Microbe Menace DVD: Slide: Normal Flora of the Mouth Bacteria Gram Stained (T5C9)**). In addition to causing differences in staining, the different cell wall structures lead to differences in susceptibility to antibiotics. Some antibiotics penetrate the Gram-positive cell wall better, while others penetrate the Gram-negative cell wall better.

Some antibiotics, such as those in the penicillin group, work by inhibiting cell wall synthesis. Penicillins work better on Gram-positive bacteria due to the thicker cell wall in Gram-positive bacteria as well as the reduced ability of penicillin to cross the Gram-negative cell wall. Penicillin derivatives such as ampicillin can work on either Gram-positive or Gram-negative bacteria.

Some broad-spectrum antibiotics work by interfering with protein synthesis within the bacterial cell. This is not harmful to us because the bacterial ribosome (the site of protein synthesis) is different from our ribosome. Some antibiotics that inhibit protein synthesis, such as erythromycin, are better able to cross the Gram-positive cell wall and therefore, will work better on Gram positive bacteria. (Refer to **Beyond Confronting the Microbe Menace DVD: 1. Chart: Antibiotics (T5C26); 2. Chart: How to Combat infectious Diseases (T5C20); 3. Chart: antibiotics (T5C21); and, 4. Chart: Antibiotic Mechanisms (T5C23)**). Other antibiotics that inhibit protein synthesis, such as streptomycin, cross the Gram-negative cell wall better and thus work better on Gram-negative bacteria.

To compare how effective one antibiotic is to another, or to measure the degree of antibiotic resistance in a bacterium, a procedure called the Kirby-Bauer test can be done. To do this, a pure strain of bacteria is isolated from an infected person. This pure strain is then spread over the surface of a special medium, called Mueller-Hinton agar, to create a lawn, or carpet, of bacteria. Small filter paper discs, impregnated with standardized amounts of antibiotic, are gently pressed on to the surface of the

agar. While the plates are incubating overnight, the antibiotic diffuses from the disc and into the agar. This antibiotic diffusing into the agar will inhibit the growth of susceptible bacteria. (Refer to **Beyond Confronting the Microbe Menace DVD**: 1. Picture *E. coli* on a plate Disk Diffusion Kirby-Bauer (T5C24)); 2. Picture: *E. coli* on plate that is antibiotic resistant (T5c27); 3. Chart: Antibiotic Resistance (T5C38); 4. Demonstration of Super Bug (antibiotic resistance) (T5C29); and, 5. Chart: Antibiotic Resistance (Super Bug) (T5C29)).

Problem: The purpose of this lab is to investigate the effectiveness of several antibiotics to one another or the degree of antibiotic resistance in a bacterial species using a Kirby-Bauer test.

Predictions: If a bacterial colony is susceptible to an antibiotic, then a zone of inhibition will form around an antibiotic disk placed on an agar plate. If a bacterial colony is resistant to a particular antibiotic, then no zone of inhibition will form around the antibiotic disk. If a bacterial colony is somewhat susceptible (“intermediate susceptibility”) to an antibiotic, then the zone of inhibition will measure in-between that of a susceptible and resistant colony.

Use the letters S, for sensitive, I, for intermediate, and R, for resistant, to predict the relative sensitivities of each of the bacteria to each of the antibiotics in the data tables found in the Results section.

Materials:

broth cultures of *S. epidermidis* and *E. coli*

4 plates of TSA

sterile cotton swab

forceps

fine tipped marking pen

soap

Parafilm

Bunsen burner

matches

70% isopropanol

bent glass rod

antibiotic impregnated discs (see the chart in the Results section for details)

Safety:

1. A microbiology lab is potentially a very dangerous place. For this reason it is extremely important that you follow all safety guidelines and always practice sterile technique when handling microbes, unless instructed otherwise.
2. There should be no books or papers at your workstation except this lab packet.
3. Never have any food or drink at your workstation.
4. Always thoroughly wash your hands with disinfectant soap or alcohol before leaving your workstation.
5. Never open a Petri dish after you have inoculated it and have allowed it to incubate overnight.
6. Always dispose of used material in the biohazard bag, unless instructed otherwise.

Procedure:

1. Clean your work area and sterilize it with 70% isopropanol.
2. Swirl the contents of the broth culture of *S. epidermidis* until it is equally murky throughout. Use the sterile cotton swab and glass rod to create a lawn, or carpet, of *S. epidermidis* on 2 of the plates of TSA. (Don't forget to hold the cap in your pinky and to flame the mouth of the test tube before and after dipping the sterile swab into it).
3. Label the bottom of the Petri dish with your name, the date, *S. epi* and Gram +. Be sure to write small and only around the very edges of the bottom petri dish.
4. Use the second sterile swab to repeat step two, except substitute *E. coli* for *S. epidermidis*. Also, label this *E. coli* dish as Gram -.
5. Select two different antibiotic discs. Place them on opposite sides of a petri dish containing *S. epidermidis*, with the code side facing up. Tap them gently with sterile forceps to stick them to the agar.
6. Using the second plate, repeat step 5, again using two different antibiotic discs. You should now have four different antibiotic discs on two plates of *S. epidermidis*.
7. Repeat steps 5 and 6, using the plates of *E. coli*. Be sure to use the same types of antibiotic discs as were used in steps 5 and 6.
8. Wrap Parafilm around all four plates and place the plates on their lids and in the incubator at 37°C for 24 hours.
9. After 24 hours in the incubator, check for the presence of antibiotic activity. This is done by looking for a clear area, called a zone of inhibition, surrounding a paper disc. Remember to

never open the dish for a better view. To determine the affectivity of an antibiotic, measure the diameter, in millimeters, of the zone of inhibition. Gather class data and then find the average zones of inhibition for each of the different antibiotics before recording your data.

- After recording the average class data for the diameters of the zones of inhibition, use the SIR table to determine whether each bacteria is susceptible (sensitive), unaffected (resistant) or somewhere in between (intermediate) for each of the antibiotics.

Results:

Use class data to complete the following table.

Average Zones of Inhibition for <i>S. epidermidis</i> (a Gram positive bacterium)			
Disc Code (Antibiotic Name)	Prediction Sensitive (S) Intermediate (I) Resistant (R)	Ave. Diameter of Zone of Inhibition (mm)	Sensitive (S) Intermediate (I) Resistant (R)
AM (ampicillin)			
E (erythromycin)			
P (penicillin)			
S (streptomycin)			
SXT (sulfamethoxazole plus trimethoprim)			
Te (tetracycline)			

Average Zones of Inhibition for <i>E. coli</i> (a Gram negative bacterium)			
Disc Code (Antibiotic Name)	Prediction Sensitive (S) Intermediate (I) Resistant (R)	Ave. Diameter of Zone of Inhibition (mm)	Sensitive (S) Intermediate (I) Resistant (R)
AM (ampicillin)			
E (erythromycin)			
P (penicillin)			
S (streptomycin)			
SXT (sulfamethoxazole plus trimethoprim)			
Te (tetracycline)			

Analysis/Conclusion:

Use class data to answer the following questions.

1. *S. epidermidis* was sensitive to which antibiotics?
2. *S. epidermidis* was resistant to which antibiotics?
3. *E. coli* was sensitive to which antibiotics?
4. *E. coli* was resistant to which antibiotics?
5. Based on the average class data you have gathered, which type of bacterium is resistant to a larger number of antibiotics? Use data to support your answer.
6. Which antibiotic would you consider the “best”, given your current amount of information? Explain.

7. Use the class data to evaluate your predictions.

8. Streptomycin can damage the kidneys and the inner ear. Under what circumstances would you, as a doctor, consider prescribing streptomycin? Explain.

9. Suggest a reason why the different antibiotics were allowed different diameters of their zones of inhibition on the table of antibiotic sensitivity.

10. Describe how you could design an experiment to test your answer to question #9. Be sure to include the independent and dependent variables and a control in your description.

Diameter of Zones of Inhibition (SIR Table)				
Antibiotic	Potency of Disk (mg)	Diameter of Zone of Inhibition (mm)		
		Susceptible	Intermediate	Resistant
Ampicillin (<i>E. coli</i>)	10	14 or more	12-13	11 or less
Ampicillin (<i>S. epi</i>)	10	29 or more	21-28	20 or less
Erythromycin	15	18 or more	14-17	13 or less
Penicillin G (<i>E. coli</i>)	10	29 or more	21-28	20 or less
Penicillin G (<i>S. epi</i>)	10	22 or more	12-21	11 or less
Streptomycin	10	15 or more	12-14	11 or less
Tetracycline	30	19 or more	15-18	14 or less
SXT	1.25/23.75	16 or more	11-15	10 or less

- **Note: Results based on Mueller-Hinton agar. Class results may vary with use of different agars.**

Answers to analysis/conclusion questions:

1. *S. epidermidis* was sensitive to which antibiotics?

Answers will vary depending on results

2. *S. epidermidis* was resistant to which antibiotics?

Answers will vary depending on results.

3. *E. coli* was sensitive to which antibiotics?

Answers will vary depending on results.

4. *E. coli* was resistant to which antibiotics?

Answers will vary depending on results.

5. Based on the average class data you have gathered, which type of bacterium is resistant to a larger number of antibiotics? Use data to support your answer.

Answers will vary depending on results.

6. Which antibiotic would you consider the “best”, given your current amount of information? Explain.

Answers will vary depending on results.

7. Use the class data to evaluate your predictions.

Answers will vary depending on results.

8. Streptomycin can damage the kidneys and the inner ear. Under what circumstances would you, as a doctor, consider prescribing streptomycin? Explain.

Answers may vary; however, look for answers that talk about different strains of streptococcal bacteria and the related body damage/diseases caused by an uncontrolled outbreak.

9. Suggest a reason why the different antibiotics were allowed different diameters of their zones of inhibition on the SIR table.

Answers may vary depending on results. Concentration of the antibiotic on the disks is an important factor as well as the size of the affected bacterial colonies. Specific factors that may affect your laboratory results are: the temperature of incubation, the length of incubation, the thickness of the agar in the plates, the age of the culture used to create the lawn, the density of the culture used to create the lawn, the age of the antibiotic disks, and the laboratory technique of the student groups.

10. Describe how you could design an experiment to test your answer to question #9. Be sure to include the independent and dependent variables and a control in your description.

Answers will vary. Look for use of control v. dependent/independent variables.