Modifying the Kirby-Bauer Antibiotic Susceptibility Exercise to Promote Active Learning

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Abstract

The Kirby-Bauer antimicrobial susceptibility test can be modified to provide students the opportunity for enhanced learning by comparing their data and discussing the ramifications of their results. The active learning helps students to identify and correct several common misconceptions. The misconceptions addressed include: that the particular organism they tested will give invariable results, that any culture of a species is identical to other cultures of that species, that differences in technique will not be reflected in the experimental results, and that sensitivity testing is not a necessary adjunct to appropriate treatment.

Activity

Invitation for User Feedback. If you have used the activity and would like to provide feedback, please send an e-mail to MicrobeLibrary@asmusa.org. Feedback can include ideas which complement the activity and new approaches for implementing the activity. Your comments will be added to the activity under a separate section labeled "Feedback." Comments may be edited.

Introduction.

Learning Objectives.

At the completion of this activity, students will be able to:

a) perform a Kirby-Bauer antimicrobial susceptibility test including application of antibiotic discs, measurement of zones of inhibition, and interpretation of the results.

b) discuss the competing processes of antibiotic diffusion and microbial growth that lead to the formation of a zone of inhibition for a susceptible organism.

c) describe the function of the Kirby-Bauer antibiotic susceptibility test.

d) analyze and interpret the results for the class as compared to individual data.

e) recognize that there is a wide range of variability within a bacterial species.

Background.

Students must have mastered the basics of aseptic technique before performing the Kirby-Bauer exercise. Students also must be able to measure using the metric system and interpret data tables.

Procedure.

A. Bacterial cultures
Most lab courses utilizing the Kirby-Bauer test have students perform it using the same strain of bacteria. The modification in this exercise is that students use a variety of strains of the same bacterial species. This results in students obtaining data that vary from student to student. For a class of fewer than 10 students, consider using several strains of only one species of bacteria. For a larger class, two or three strains of several different species would be appropriate.

Preparation before lab starts
Bacterial cultures must be grown, then cell density must be adjusted to an appropriate concentration. Each student will receive one tube of bacteria to perform the Kirby-Bauer antimicrobial sensitivity test. Students may be required to prepare this themselves, or the lab instructor (or technician) may prepare it. As presented here, it is expected that the lab instructor will prepare the cultures so they are ready to inoculate onto the petri plate. The culture tubes should be labeled to identify the species, with a code to indicate which strain it is. Each strain can be used by multiple students simply by being split into multiple tubes. The volume of culture required in the test tubes is minimal, a single milliliter is sufficient. Sufficient sterile-capped test tubes should be provided for the cultures.

Sources for bacterial strains
Although in practice the Kirby-Bauer test is used on strains of clinical origin, the strains used in class do not have to be of clinical origin. Many organisms are available from biological supply companies and molecular biology companies.

The strains utilized in field testing at Rogers State University included *Escherichia coli*, *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Of the six *E. coli* strains utilized, one is a *lux* HSL reporter strain, four are normally utilized in demonstrating *lac* operon action, and one is our common lab strain. Three of the five *S. marcescens* strains (KWN, Nima, and db11) would be available from biological supply companies or from other various sources indicated in the literature. Two of the *S. marcescens* strains were clinical in origin. Three of the *P. aeruginosa* strains were clinical isolates, while four were isolates from various hot tubs, and the last was the commonly utilized strain PAO-1. The three *Staphylococcus* strains actually included our lab strains of *Staphylococcus aureus* and *S. epidermidis* and one clinical strain of *S. aureus* that was antibiotic susceptible.

A variety of *Escherichia coli* strains may be purchased from the American Type Culture Collection. Preceptrol strains are the most reasonably priced strains from the ATCC ($33 in 2007). A current list of the preceptrol strains is available at: www.atcc.org/common/documents/pdf/preceptrol.pdf. A number of the preceptrol strains are used in quality control for antimicrobial sensitivity testing. To obtain more information about these strains, search the ATCC site (by ATCC number) for the individual strains; references are cited when known. For example, the page for *Escherichia coli* ATCC 25922 gave a number of references, one of which was a full length *Antimicrobial Agents and Chemotherapy* paper (1) which utilized several of the preceptrol strains and presented zone of inhibition data.

New England Biolabs will also provide cultures of various *E. coli* strains for the cost of shipping. Many of these *E. coli* strains have inherent or plasmid-acquired antibiotic resistances. Genotypes of these bacteria are supplied at the New England Biolabs website. Different species and strains of bacteria can be purchased from other biological supply companies, including Wards and Carolina. Selective media could also be used to isolate strains from various environments. Additionally, many microbiologists may be able to provide strains from their strain collections.

B. Growing the bacterial cultures
Prepare overnight (12 to 20 hour) broth cultures of the bacteria to be used in the exercise. Cultures for most species can be grown up during the week before the experiment and refrigerated until needed; few of the bacteria will die in that short period of time. Inoculate broth tubes with bacteria from three to five well-isolated colonies of the same morphological type. The tubes should contain 4 to 5 ml of sterile broth. Several different broth media could be used to grow the bacteria for the exercise. These include trypticase soy broth, nutrient broth, or Mueller-Hinton broth. Several companies manufacture these media. Powdered media or prepared media made by BD Diagnostic Systems can be purchased from a variety of scientific supply sources. Cultures are grown without shaking at the appropriate temperature. For example, *S. aureus*, *E. coli*, *P. aeruginosa*, and *S. marcescens* are all grown at 35 to 37°C.

C. Adjust concentration of bacteria to provide standardized inocula
If the culture densities are not adjusted to the correct concentration, the exercise will still yield results, although the results will not be optimal. If eyeballing concentrations is attempted, err on the side of lighter concentrations rather than higher concentrations.

Materials

- Visible light spectrophotometer and sterile cuvettes to measure A_{625} or a McFarland standard of 0.5
- Sterile pipettes, 5-ml pipettes are adequate, approximately three per culture
- Sterile cuvettes, one per culture
- Pipette bulb
- Sterile 0.9% saline, approximately 10 ml per culture
- Discard container—a plastic bag, covered autoclaveable container, or similar container

Utilizing a visible light spectrophotometer to adjust bacterial concentration
After warming up a sufficient length of time and setting the wavelength for 625 nm, the spectrophotometer should be zeroed by setting the absorbance to "infinite" when nothing is present in the wavepath. A blank should then be set up, consisting of a cuvette holding uninoculated bacterial broth, and the machine should be set to read an absorbance of zero. Instructions for a commonly used spectrophotometer, the Spec 20, are available online at http://biology.clc.uc.edu/fankhauser /Labs/Microbiology/Growth_Curve/Spectrophotometer.htm. Once the machine is set, the absorbance of a cuvette filled with bacteria can be read and adjusted utilizing the sterile saline. A final absorbance between 0.08 and 0.10 is recommended.

Utilizing a McFarland standard to adjust bacterial concentration
The turbidity of the McFarland standard should be compared to the inoculum tube by holding them against a white card with contrasting black lines. More details on procedures for using McFarland standards can be found in a variety of textbooks, e.g., *Microbiology Laboratory Theory and Application* (2), and web sources, such as the Centers for Disease Control and Prevention webpage www.cdc.gov/ncidod/dbmd/diseaseinfo/cholera/ch9.pdf. McFarland standards can be obtained from VWR; item 29447-318 costs approximately $8 each.

The following recipe for preparing your own McFarland standard is as described in the package insert for the BBL antimicrobial susceptibility test discs.

To prepare, add 0.5 ml of 0.048 M $BaCl_2$ (1.175% (wt/vol) $BaCl_2 \cdot 2H_2O$) to 99.5 ml of 0.18 M [0.36 N] H_2SO_4 [1% (vol/vol)]. The turbidity should be verified by using a spectrophotometer with a 1 cm light path and matched cuvettes. The absorbance at 625 nm should be 0.08 to 0.10. This is roughly equivalent to 1 x 10^8 to 2 x 10^8 CFU/ml (CFU, colony forming units).

D. First lab period
During the first lab period, students will select a culture to test, inoculate their plates, add antibiotic discs, and return the plates for incubation. The plates are incubated bottom side up. Incubation for 24 hours is optimal, followed by refrigeration of the plates until the next lab period.

Materials

- Bacterial cultures
- Petri plates with Mueller-Hinton agar, one per student
- Antimicrobial sensitivity test discs
- Forceps, one per pair of students
- 70% alcohol in a beaker
- Bunsen burner
- Striker or lighter
- Container to discard contaminated materials, e.g., a small plastic kitchen waste bag; one per student group
- Marking pen; one per student group
- Masking or other tape; one roll per class. Used to tape plates together, especially useful with large student groups.
- Bacterial incubator set between 35 and 37°C, one per class

Preparation of materials
1. Petri plates filled with Mueller-Hinton agar
Mueller-Hinton is the standard agar for this test; if this media is not available nutrient agar or trypticase soy agar may be substituted, although the results will not be clinically relevant. Mueller-Hinton agar, nutrient agar, and trypticase soy agar are manufactured by several companies, including BD Diagnostic Systems. Powdered Mueller-Hinton agar or prepared plates may be purchased from many scientific suppliers.

Petri plates may be either 100-mm or 150-mm in diameter; 100-mm petri plates require 25 ml of agar, while 150-mm plates need 75 ml of agar to fill them.

2. Antimicrobial susceptibility test discs
Antimicrobial susceptibility test discs may be utilized either in cartridge, disc dispenser, or prealiquoted into a sterile petri plate. The number of discs required depends on the size of petri plate used for the exercise. Five different antibiotic discs are used on 100-mm petri plates and up to 12 discs are used on 150-mm petri plates.

A wide variety of discs are available from many sources, including Carolina, Wards, VWR, and Fisher. Discs should be stored in the refrigerator and kept with their desiccator packets.

E. Second lab period
During the second lab period, students will observe their plates, measure the zones of inhibition, and use the interpretation tables to interpret their results.

Materials

- Lab report form, including data sheet for students to compile class data, and thought questions. Students will discuss results with classmates at their lab station and respond to them in writing as their lab report.
- Ruler marked in mm, one per student or pair of students
- Interpretation table (available in lab manuals, package insert for antibiotic discs, and for selected antibiotics, as an appendix for this exercise); one per student or pair of students
- Blackboard and chalk to allow students to compile class data
- Appropriate discard location for contaminated materials

After lab is completed, a method is required for the appropriate disposal of contaminated materials.

Student Version.

Available as a pdf

Instructor Version.

Available as a pdf.

References.

1. **Boyle, V. J., M. E. Fancher, and R. W. Ross, Jr.** 1973. Rapid modified Kirby-Bauer susceptibility test with single, high-concentration antimicrobial discs. Antimicrob. Agents Chemother. **3:**418–424. http://aac.asm.org/cgi/reprint/3/3/418?view=long&pmid=4790600.

2. **Leboffe, M. J., and B. E. Pierce.** 2006. Microbiology laboratory theory and application, 2nd ed. Morton Publishing Co., Englewood, CO.

Safety Issues.

Students should have access to the appropriate personal protective equipment for the biosafety level of bacteria being utilized in the exercise. If biosafety level 2 organisms such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* are utilized, appropriate precautions should be in place. Information on biosafety levels and appropriate precautions are available at http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm.

Antibiotic selection
Some students may express concern because they will be placing a filter paper disc containing an antibiotic to which they are allergic. This is not necessarily a problem. Students will not be touching the antibiotic directly, either while placing it on the plate or when analyzing the results. The amount of antibiotic in the disc is very small. There is not aerosolization of antibiotic powder or liquid. Students may also wish to wear disposable gloves to lessen their exposure even more.

Bacteria selection
The choice of bacteria for this experiment is dictated by several things: the student and instructor expertise, the condition of the laboratory, and the ability to contain the organisms. Instructors should take care to use bacterial species and strains which fit their situation. Information is provided above regarding organism sources.

Use of alcohol to flame forceps
Students should be instructed and monitored to ensure that they understand that the alcohol, not the flame, is the disinfectant. Safety issues such as how to extinguish a flame and where protective gear is located should be reviewed before the students proceed.

Suggestions for Determining Student Learning.

Student learning was assessed both formally and informally by examining student work.

Informal assessment methods

1. Observe student inoculation techniques, results, and analysis.

2. Listen to the students as they discuss the results.

3. Evaluate student participation.

Formal assessment methods
1. Data and analysis in the lab report were evaluated to determine if the student used appropriate methods to measure and determine susceptibility or resistance. The lab report contained the student's own data and analysis, as well as a compilation of the class data and analysis for susceptibility or resistance.
2. Student responses to thought questions from the student handout were evaluated to determine if students understood the objective of the Kirby-Bauer assay and were able to understand the clinical implications of the test.
3. Student responses to questions on the lecture exams regarding the set up, interpretation, and utility of the Kirby-Bauer assay were evaluated.

Field Testing.

The genesis of this project was a discussion with Dr. Kathryn Leyva, when we both taught at Midwestern University in Glendale, Arizona. We did a trial run with undergraduate students in the degree completion program and presented this concept as a poster at the ASM Conference for Undergraduate Educators in 2001.

The exercise, as presented here, has been used with students twice at Rogers State University. Both times it was used in Microbiology (BIOL 2124). The only prerequisite for this course is the introductory biology course. Microbiology is a prerequirement for students entering the A.S. nursing program at Rogers State University. Other students may also take the course, especially those students desiring to obtain an A.S. in biology, as well as those students who will be transferring to other degree programs, such as optometry and pharmacy. In the 2004 fall semester, it was used with two sections of Microbiology. Eighteen of 40 students enrolled in the class agreed to participate in the field testing. In the 2007 spring semester, 35 of 44 students agreed to participate in the field testing.

In fall 2005, we used several strains of *Staphylococcus* (two of these strains were really *Staphylococcus epidermidis*), *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Escherichia coli*. In 2007, only *P. aeruginosa*, *S. marcescens*, and *E. coli* strains were used.

Both times, slightly more than 50% of the students presented conclusions which indicated that they realized that bacterial species could have variability and that testing of individual bacterial isolates would be essential to selecting the appropriate antibiotic for treatment. Students received no information during the lab, in the handout, or during the lectures to indicate that different strains of bacteria might have different antimicrobial susceptibilities. Those students who realized this was the case made the mental leap by examining their data and discussing what might cause these results. Those students who did not reach these conclusions examined their lab procedures and results and reached conclusions that the different results were spurious. No guiding questions were asked.

Feedback from other faculty at the ASM Conference for Undergraduate Educators indicated several reservations about using this approach in their classrooms. Some indicated that they did not have a sufficient culture collection to support this exercise. In the materials section, I have indicated several ways of resolving this issue. These include obtaining strains of *E. coli* which have antibiotic resistance plasmids, isolating strains from the environment using standard approaches, and requesting attenuated or known strains of bacteria from culture collections, researchers, or other universities. Other faculty liked this approach, but felt that they did not have the time to do it as a wet exercise. In order to facilitate this, photographs of Kirby-Bauer plates from the 2007 exercise are being submitted. This includes photographs of replicate plates for students to analyze how variable the results might be, as well as photographs of each of the strains used by the students. Data from these exercises is also being submitted as appendices to this exercise.

Student Data.

Measurements of zones of inhibition are included in the list of appendices.
The table below contains examples of student responses on their lab reports.

TABLE 1. Examples of student responses on their lab reports

Did the student perform the measurements and analyze the results	Student comments regarding analysis of data and hypothesis	Did the student describe the concept of different strains having different antibiotic sensitivities	Did the student relate results to clinical use	Student lab report comments
yes	pretty much on track	yes	yes	no comments
yes	differences due to errors	no	no	no comments
yes	differences due to errors	no	no	administer a drug that was resistant to the organisms
yes	differences due to contamination	no	no	could cause different reactions in people
yes	differences due to contamination	no	no	could cause different reactions in people
yes	diluted or control broths free of bacteria?	yes	yes	different susceptibilities of bacteria would need to be addressed
yes	majority were consistent	yes	yes	varied results on different organisms; results specific to the organism causing the infection need to be used
yes	significant changes in resistance or susceptibility between the different strains of the bacteria	yes	yes	misworded, but concept is there; one needs to be particular in choosing the appropriate strains of susceptibility
yes	variance in microbe susceptibility within cultures	yes	yes	test microbe susceptibility each time to understand which antibiotic will work best
yes	cultures are important in patient care because the bacteria typing is important to determine successful treatment of infections	yes	yes	student error could've happened during inoculation but there could be differences within each culture

Suggestions to Expand the Exercise.

This exercise could be expanded in several directions.

1. Statistical analysis could be applied to the student data.

2. Information regarding mechanisms of antibiotic resistance and transfer could be introduced.

3. The Kirby-Bauer assay depends on standardization of several variables, including agar type, depth of agar in a plate, incubation temperature, and media pH. Students could examine these variables, either during a discussion or in actual lab practice.

4. A pre- and post-measure of student conceptions would be useful.

Appendices and Answer Keys.

Answer Key to Questions on Student Handout

Consolidated Student Data

Representative Kirby-Bauer Plates

Zone Diameter Interpretive Chart for Selective Antibiotics

Review of Antibiotics and Their Range of Effectiveness

Illustrative Figures and Troubleshooting

Media Recipes

The Kirby-Bauer Antimicrobial Susceptibility Test

Introduction

Today you will perform a frequently used procedure called the Kirby-Bauer antimicrobial susceptibility test. Be sure to keep a record of which culture you have selected. The instructor will demonstrate how to inoculate an agar plate with a cotton swab in order to evenly distribute the culture over the surface of the agar. You will inoculate your own plate of Mueller-Hinton agar. To the inoculated plate, you will then aseptically add sterile filter paper discs which contain known concentrations of antibiotics. As soon as the antibiotic discs touch the agar, the antibiotics will begin to diffuse into the surrounding agar. Meanwhile, the bacteria you inoculated onto the agar will begin to grow. As the antibiotics diffuse and the bacteria grow, organisms which are inhibited by the antibiotic will fail to grow where the antibiotic is highest in concentration, leaving a clear area called the zone of inhibition. When performed in a clinical microbiology laboratory, the Kirby-Bauer test is a very standardized procedure. For instance, the petri plates are filled with a standard volume of Mueller-Hinton agar. The bacterial inoculum is also standardized by adjusting it to the optimal concentration of 1×10^8 to 2×10^8 colony forming units per ml. Several methods could be used to produce an inoculum of the proper concentration. One method uses a spectrophotometer. The inoculum will be at the right concentration at a final absorbance between 0.08 and 0.10 measured at 625 nm. A second commonly used method compares the inoculum density to a turbidity standard known as a 0.5 McFarland standard. Either method will result in a bacterial concentration between 1×10^8 and 2×10^8 colony forming units per ml.

During the next lab period, you will observe your plates, measure the diameter of each zone of inhibition, and use a standard interpretation table to interpret your results. Organisms may be susceptible, intermediate in resistance, or resistant, to the antibiotic. After you obtain results from your own plate, you will join the rest of your class to pool data and discuss the results.

First Lab Period - Materials and Procedure

1. Select a culture to test. This broth culture has been diluted to the appropriate concentration, equivalent to the 0.5 McFarland standard. Record the number of the culture you have selected here:

   _____

2. You will need the following materials at your workbench:
   - Container of sterile cotton swabs
   - Discard container for contaminated swabs
   - Petri plate containing Mueller-Hinton agar (one per student)

- Sterile filter paper discs impregnated with antibiotics
- Forceps
- Beaker containing 70% alcohol (The alcohol will be used to disinfect the forceps. **Your instructor will discuss how to flame your forceps safely.**)
- Marking pen
- Masking tape (optional)

3. Before inoculating your petri plate, examine the surface of the agar and the sides of the plate carefully. Be sure there are no visible colonies or drops of water on the agar. If there are colonies, the plate is contaminated and should be discarded. If there is water on the plate, use some sterile cotton swabs to gently remove the water.

4. Resuspend the bacteria in your culture tube as you gently dip a sterile dry cotton swab into the culture. Remove the excess liquid from the swab by rolling the swab tip around the test tube above the level of the broth as you take the swab out of the tube.

5. Inoculate your Mueller-Hinton agar plate in three directions to ensure even spreading of bacteria over the surface. The pictures used to illustrate this process only show you the direction of the swab's zig zag. The zig zags you will make on your plate should be much closer than illustrated and should cover the plate completely to create an even lawn over the plate.

   a) Pick up your Mueller-Hinton agar plate and zig zag your bacteria-damp cotton swab over the surface of the agar, covering it thoroughly from one end to the other.

   b) Without dipping the swab into your culture again, rotate the plate approximately 30 degrees (1/3 around) and repeat the zig-zag inoculation in the new direction.

   c) Finally, rotate the plate 30 degrees once again, and inoculate it for a third time. This three-way inoculation results in an even confluent growth of bacteria called a lawn.

d) Discard your contaminated cotton swab into the proper container at your workbench.

6. Allow the plate to absorb the liquid for 2 to 3 minutes, then begin placing the filter paper antibiotic discs onto the surface of the agar. Five different antibiotic discs will fit in a 100-mm petri plate. Place one in the middle and space the other four discs evenly midway between the edge of the plate and the center disc. Use the illustration on the final page of this handout as a guide for disc placement.

7. Regardless of the method you use to place the discs onto the agar surface (a disc dispenser, a cartridge, or forceps), you will tap the discs onto the agar surface using the tip of a disinfected forceps.
   Disinfecting and flaming forceps is different than flaming a transfer loop and needle. When preparing the forceps, alcohol is the disinfectant, and while you do pass the forceps through the flame, the purpose is to merely burn the alcohol off, rather than to sterilize through heat. Place the forceps into the alcohol for several minutes, then pass them quickly through the flame only long enough to ignite the alcohol. **Be careful to keep the forceps away from the alcohol in the beaker and also be careful not to tip the forceps up so that a drop of flaming alcohol runs onto your fingers.**

8. Label the agar side of the plate as instructed. Generally, your name or initials and the organism are required. If there are multiple lab sections, include your section identification. The plate will be incubated at 37^{o}C for 24 hours and then refrigerated until your next lab period, when you will observe the results.

Second Lab Period - Materials and Procedure

1. Obtain your petri plate and observe the bacterial growth on the plate.

2. Clear areas where bacteria did not grow around the antibiotic discs are called zones of inhibition. Turn the plate so that you can place a ruler against the back of it and measure the diameter of each zone of inhibition in millimeters. Record your data in Table 1 below.

3. Using the zone diameter interpretative chart, determine how susceptible your organism is to each antibiotic.

TABLE 1. Individual culture antibiotic susceptibility

Antibiotic	Disc code	Diameter of zone of inhibition (mm)	Interpretation: susceptible, intermediate, or resistant

4. Bring your data to the blackboard and enter them into the tables drawn there. Record the class data in the tables on the following pages. Discuss these results with your classmates.

Analysis

1. For each species, identify resistance or susceptibility to the antibiotics used in the exercise.

2. Analyze the data you have for each species.
   Are the data consistent for each species?
   If the data are not consistent, what hypothesis can you derive?
   If the data are not consistent, what conclusions can you make?

3. Describe how patient treatment would be affected by results such as these.

TABLE 2. Kirby-Bauer antimicrobial susceptibility test student data and observations

Organism:

Code	Zone of inhibition diameter (mm)				
	Antibiotic:	Antibiotic:	Antibiotic:	Antibiotic:	Antibiotic:

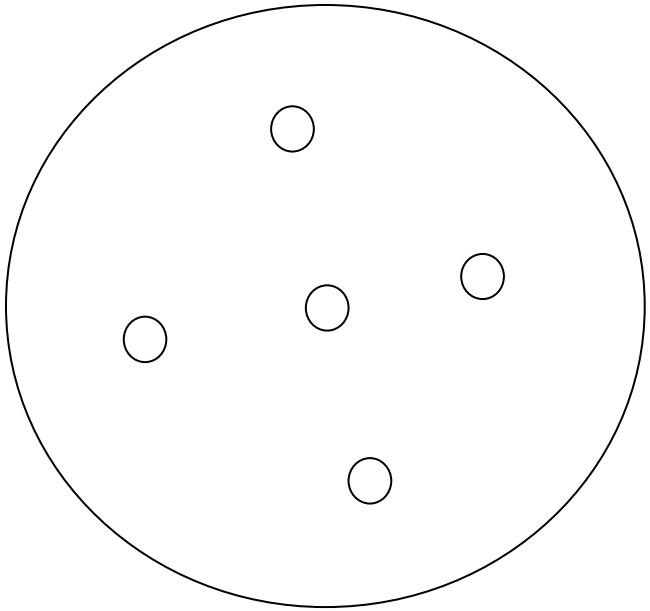

FIG 1. Guide to antibiotic disc placement in the petri plate.

The Kirby-Bauer Antimicrobial Susceptibility Test
Instructor Version

The Kirby-Bauer antimicrobial susceptibility test is a standard exercise which can be performed at several levels, depending on the student population and the time available for the lab. Virtually all microbiology laboratory manuals contain some version of this protocol. The protocol continues to be incorporated into lab manuals and utilized in the teaching laboratory because it is such a bedrock of clinical microbiology. But because it is so frequently used, instructors often do not realize that students have not made the connection between interpreting their data and and understanding real-life situations. The innovation in this exercise is the use of multiple strains of bacteria, so students find that the results they achieve on their plate are different than the results other students have for the same bacteria.

Bacterial cultures

For a class of fewer than 10 students, consider using one species of bacteria. For a larger class, two to three different species would be more appropriate. These strains do not have to be of clinical origin. For example, a variety of *Escherichia coli* strains are available from biological supply companies or from the American Type Culture Collection (www.ATCC.org). Many of these strains have inherent or plasmid-acquired antibiotic resistances. Selective media could also be used to isolate strains from various environments. Additionally, many microbiologists may be able to provide strains from their strain collections. Details are presented in the "Materials" section of this exercise.

Many of these strains can be stored in a -20°C freezer or in silica gel stocks. Details on strain storage are presented as an Appendix to this exercise. Begin growing the organisms for this lab exercise the week before they are needed to ensure that the cells are ready.

Those organisms used regularly in laboratory are subcultured several times a semester and can bestored in small culture flasks in the refrigerator. The evening before the lab, add between one and three drops of bacteria from the refrigerator stocks to sterile culture tubes which have approximately 3 ml of culture media in them. Incubate the tubes overnight at 37^{o}C, without shaking, and check the culture density in the morning before lab. Using a spectrophotometer or McFarland standard is helpful to ensure that student cultures are not too dense. Details are provided in the "Materials" section of this exercise. A final absorbance at 625 nm should be 0.08 to 0.10, the same as a McFarland standard of 0.5. This is roughly equivalent to 1×10^8 to 2×10^8 CFU/ml (CFU, colony forming units).

Before class, prepare the tubes for the students, coding them to identify the species and numbering each (e.g., the tube labeled "*Escherichia coli* 3" contains *E. coli* strain number 3). Use the coding so that results for each strain can easily

be tracked. (Author note: Interestingly, although the students knew that I was up to something unusual with this lab, both times that I have used this procedure, they did not realize that the number meant anything specific and assumed that each tube of *E. coli* was identical to the others.)

Antibiotics

There are many different antibiotics available to use in this exercise. We used penicillin which was ineffective, as well as kanamycin, which was effective. The diameters of the zones of inhibition varied widely for kanamycin and the other three antibiotics (novobiocin, streptomycin, and tetracycline). While in one respect, picking penicillin as one of the five antibiotics was a waste, the students felt it was a good demonstration that penicillin was not effective against gram-negative bacteria. Students figured out quickly that it did not matter if there was no listing for gram-negative bacteria in the interpretation table for penicillin, since there was no zone of inhibition to measure. Interpretation tables for antibiotics are widely available and an Appendix presents the interpretations for the antibiotics which were used for this exercise.

Introducing new techniques and lab safety

Before students begin the lab, there are several techniques which they may not be familiar with which should be described and demonstrated. These may include:

a) Inoculating the Mueller-Hinton agar plate. The student handout describes this procedure, but students understand it better when it is demonstrated.

b) Disposing of contaminated cotton swabs. There are several ways to handle this. A beaker of diluted bleach or a biohazard bag are each acceptable, as are small plastic kitchen waste bags.

c) Disinfecting forceps with alcohol and removing the alcohol by flaming. Students can confuse the technique for flaming loops with the flaming technique used for forceps. In flaming forceps, it is the alcohol which disinfects, not the flame. The volatile alcohol is removed by ignition, but it is important to emphasize to the students that the alcohol should be kept apart from the flame, that the alcohol flame is hard to see, and that forceps should be held so that drops of flaming alcohol do not fall onto their skin.

d) Placing antibiotic discs onto the agar. It has been my choice to not use the disc dispensers. We have six lab tables with at least four students per table, the disc dispensers are expensive and the disc cartridges have been remodeled so that they do not fit older dispensers. You can obtain a single cartridge dispenser,

if desired. However, it is also easy to use sterile forceps to remove the discs from the cartridge and distribute them into sterile petri plates so that each table gets one plate with all the discs needed for that table.

Handling the inoculated petri plates

After the petri plates are inoculated and the antibiotic discs are placed on the agar surface and gently tamped down, the plates can be inverted and incubated between 35 and 37^{o}C. Zones of inhibition are visible before 12 hours for most organisms, and plates should be refrigerated after 24 hours.

Possible problems and misconceptions

During the second lab period, students observe their plates, measure the zones of inhibition, and use the interpretation tables to interpret their results.

Common problems during this lab period include:

- measuring in inches or centimeters instead of millimeters.
- measuring the radius instead of the diameter of the zone of inhibition.
- having difficulty in distinguishing between regions of colored bacterial growth and regions with no growth.
- having difficulty in determining where to measure when zones of inhibition overlap.
- measuring through the lid instead of from the back, leading to inaccurate measurements.
- forgetting to identify their plates or not writing down their code.
- not understanding how to interpret their results (i.e., not knowing what susceptible, resistant, and intermediate mean).

During the discussion, which might done as a class or in small groups, students may voice a number of misconceptions. Some of these misconceptions arise from misuse of terminology, while others arise from student misunderstanding of the process. The questions in the handout, while simple, are designed to stimulate students to compare, analyze, and interpret their results.

Answer Key to Questions on Student Handout

1. For each species, identify resistance or susceptibility to the antibiotics used in the exercise.

Resistance or susceptibility to some of the antibiotics will be consistent for all strains of each species used. There may be some strains which vary. Students may respond without taking the varying strains into account, simply looking at the response of the majority of strains. Other students may respond in detail, listing the conclusions for each tube of each species.

2. Analyze the data you have for each species.
   Are the data consistent for each species?
   If the data are not consistent, what hypothesis can you derive?
   If the data are not consistent, what conclusions can you make?

Students should recognize that, while many strains give similar responses, not all strains are identical. This should be the point at which they begin to realize that one *Escherichia coll* is not identical to another *E. coll*. It is at this point that the students may also recognize that replicates of plates may not give identical results and that some of the differences might be due to student error.

Students should propose that the organisms that the class used were not identical, although their vocabulary might not be adequate to state their hypothesis precisely. Their conclusions should indicate recognition that species can have variable characteristics.

3. Describe how patient treatment would be affected by results such as these?

Antibiotic selection for many infections is not dictated solely by the identity of the infecting bacterial species. Appropriate treatment involves both identification of the species and testing to ensure that the proper antibiotic is selected for treatment.

		Staphylococcus aureus s trains			
		Diameter of zone of inhibition (mm)			
	1	1	2	3	3
Antibiotic					
Tetracycline	43	37	0	41	32
Kanamycin	25	30	21	27	24
Penicillin	32	24	35	45	35
Neomycin	21	24	20	25	21
Streptomycin	15	18	12	16	11
	Zone diameter interpretation (mm)				
Antibiotic	Resistant	Intermediate	Susceptible		
Kanamycin	≤ 13	14-17	≥ 18		
Neomycin	≤12	13-16	≥17		
Tetracycline	≤ 14	15-18	≥ 19		
Penicillin	≤ 28		≥ 29		
Streptomycin	≤11	12 - 14	≥15		

		Serratia marcescens strains											
				Diameter of zone of inhibition (mm)									
	1	1	2	2	2	3	4	5	5	5	5	5	
Antibiotic													
Tetracycline	10	13	8	5	15	9	10	14	8	7	6	9	
Kanamycin	25	25	21	18	35	17	30	27	25	23	24	24	
Penicillin	0	0	0	0	0	0	0	0	0	0	0	0	
Neomycin	20	19	18	11	20	19	18	18	15	15	13	16	
Streptomycin	21	7	12	8	22	22	21	0	0	0	0	0	
	6	6	7	7	8	9	10	10	10				
Antibiotic													
Tetracycline	18	18	16	18	11	11	0	20	0				
Kanamycin	25	25	23	22	25	27	21	30	30				
Penicillin	0	0	0	0	0	0	ND	0	0				
Neomycin	20	20	19	ND	14	18	20	17	17				
Streptomycin	23	ND	23	23	7	16	24	20	15				
		ND, no data. This indicates either that the disc was not used or that it fell off the plate during incubation.											
		The table includes student data from both years when this exercise was used. Different strains were used for the 2 years.											
		The same strain number indicates duplicate plates were made and analyzed.											
			Zone of inhibition diameter interpretation										
				(mm)									
		Antibiotic	Resistant	Intermediate	Susceptible								
		Kanamycin	≤13	14–17	≥18								
		Neomycin	≤12	13–16	≥17								
		Tetracycline	≤14	15–18	≥19								
		Penicillin	≤28		≥29								
		Streptomycin	≤11	12–14	≥15								

	Escherichia coli strains										
			Diameter of the zone of inhibition (mm)								
		1	1	1	2	3	4	5	5	6	7
Antibiotic											
Tetracycline		15	15	15	23	28	25	31	30	20	21
Kanamycin		30	30	30	15	34	33	25	25	30	28
Penicillin		0	0	0	0	0	9	10	0	0	0
Neomycin		20	23	16	19	17	10	17	18	15	20
Streptomycin		30	17	17	0	18	18	20	18	10	15
		8	8	9	10	11	11				
Antibiotic											
Tetracycline		25	34	25	6	17	17				
Kanamycin		21	22	23	30	30	30				
Penicillin		0	11	0	0	0	0				
Neomycin		18	18	20	15	17	17				
Streptomycin		19	15	10	15	18	18				
		Note: the same strains were not used for both years when this exercise was done.									
		Zone of inhibition diameter interpretation									
			(mm)								
	Antibiotic	Resistant	Intermediate	Susceptible							
	Kanamycin	≤13	14–17	≥18							
	Neomycin	≤12	13–16	≥17							
	Tetracycline	≤14	15–18	≥19							
	Penicillin	≤28		≥29							
	Streptomycin	≤11	12–14	≥15							

Pseudomonas aeruginosa strains										
	Diameter of the zone of inhibition (mm)									
	1	1	1	2	3	3	4	4	5	5
Antibiotic										
Tetracycline	7	11	11	18	8	13	21	15	16	15
Kanamycin	22	22	24	10	30	32	0	0	36	35
Penicillin	0	0	0	0	0	0	0	0	0	0
Neomycin	15	14	14	15	18	18	12	9	20	20
Streptomycin	11	12	12	17	21	21	10	0	0	15
	5	6	6	7	8	9				
Antibiotic										
Tetracycline	15	8	10	0	10	15				
Kanamycin	35	21	23	27	22	45				
Penicillin	0	0	0	0	0	0				
Neomycin	20	13	13	15	14	25				
Streptomycin	20	10	11	15	13	25				
		Note: different strains were used in the different years this exercise was done.								
		Zone of inhibition diameter interpretation								
			(mm)							
	Antibiotic	Resistant	Intermediate	Susceptible						
	Kanamycin	≤13	14–17	≥18						
	Neomycin	≤12	13–16	≥17						
	Tetracycline	≤14	15–18	≥19						
	Penicillin	≤28		≥29						
	Streptomycin	≤11	12–14	≥15						

Representative Kirby Bauer Plates


P 10
S 10
Carolina Biological Supply Company
Burlington, North Carolina 27215
Gladstone, O
Serratia marcescens # 1

FIG. 1.

FIG. 1. Representative plate for *Serratia marcescens* strain 1.
Antibiotics on this plate are: (center) kanamycin (K), (clockwise from upper right) penicillin (P 10), streptomycin (S 10), tetracycline (TE 30), and neomycin (N 30).

Note the resistant colonies around the edge of the zone of inhibition and in the center of the zone around the kanamycin disc. The zone around the tetracycline disc is small, with a diffuse area of growth that students might mismeasure. This photograph is distinct enough for students to measure when printed in black and white.

When students measured this plate, they measured a very small (7 mm) zone of inhibition for the streptomycin. On another replicate of this strain, the students measured a larger zone of inhibition for streptomycin.

TABLE 1. Zone of inhibition measurements for *S. marcescens* strain 1

Antibiotic	Zone of inhibition diameter (mm)	Interpretation
Kanamycin	25	Susceptible
Penicillin	0	Resistant
Streptomycin	0	Resistant
Tetracycline	9	Resistant
Neomycin	18	Susceptible

FIG. 2.

FIG. 2. Representative plate for *Serratia marcescens* strain 2, a pigmented strain. Note that this strain has a distinct white fringe around the zones of inhibition. Students should not include the fringe in their measurements. This figure will print in black and white clearly enough to be measured.

Antibiotics on this plate are: (center) neomycin (N 30), (clockwise from the top) tetracycline (TE 30), kanamycin (K), streptomycin (S 10), and penicillin (P 10).

TABLE 2. Zone of inhibition measurements for *S. marcescens* strain 2

Antibiotic	Zone of inhibition diameter (mm)	Interpretation
Neomycin	18	Susceptible
Tetracycline	14	Resistant
Kanamycin	33	Susceptible
Streptomycin	13	Intermediate
Penicillin	0	Resistant

Note that the student data indicates that the streptomycin zone was 22 mm, an incorrect measurement for this plate.

FIG. 3.

FIG. 3. Representative plate for *Serratia marcescens* strain 3. Antibiotics tested on this plate include: (center) streptomycin (S 10), (clockwise from upper right) penicillin (P 10), tetracycline (TE 30), kanamycin (K), and neomycin (N 30).

This photograph is suitable for students to measure when printed in color, but in black and white the edge of the tetracycline zone of inhibition is indistinct.

TABLE 3. Zone of inhibition measurements for *S. marcescens* strain 3

Antibiotic	Zone of inhibition measurement (mm)	Interpretation
Tetracycline	9	Resistant
Kanamycin	33	Susceptible
Penicillin	0	Resistant
Neomycin	23	Susceptible
Streptomycin	24	Susceptible

FIG. 4.

FIG. 4. Representative plate for *Serratia marcescens* strain 4. Antibiotics tested on this plate include: (clockwise from top) kanamycin (K), neomycin (N 30), tetracycline (TE 30), streptomycin (S 10), and penicillin (P 10).

Due to the ragged edges of the zones of inhibition around all but the penicillin disc, this photograph is not suitable for students to measure when printed in black and white.

TABLE 4. Zone of inhibition measurements for *S.* marcescens strain 4

Antibiotic	Zone of inhibition measurement (mm)	Interpretation
Kanamycin	29	Susceptible
Neomycin	17	Susceptible
Tetracycline	9	Resistant
Streptomycin	17	Susceptible
Penicillin	0	Resistant

FIG. 5.

FIG. 5. Representative plate for *Serratia marcescens* strain 5. This highly pigmented strain of *S. marcescens* produces nonpigmented white edges around most of the zones of inhibition. Because of the white zone edges, this plate is difficult to measure when printed in black and white. Antibiotics tested on this plate include: (center) neomycin (N 30), (clockwise from top right) tetracycline (TE 30), kanamycin (K), penicillin (P 10), and streptomycin (S 10).

TABLE 5. Zone of inhibition measurements for *S.* marcescens strain 5

Antibiotic	Zone of inhibition measurement (mm)	Interpretation
Neomycin	19	Susceptible
Tetracycline	11	Resistant
Kanamycin	30	Susceptible
Penicillin	0	Resistant
Streptomycin	9	Resistant

FIG. 6.

FIG. 6. Representative plate for *Pseudomonas aeruginosa* strain 1. Antibiotics on this plate are: (center) penicillin (P 10), (clockwise from top) streptomycin (S 10), kanamycin (K), tetracycline (TE 30), and neomycin (N 30). This figure will print in black and white clearly enough to be measured.

TABLE 6. Zone of inhibition measurements for *P.* aeruginosa strain 1

Antibiotic	Zone of inhibition measurement (mm)	Interpretation
Penicillin	0	Resistant
Streptomycin	11	Resistant
Kanamycin	25	Susceptible
Tetracycline	10	Resistant
Neomycin	16	Intermediate

FIG. 7.

FIG. 7. Representative plate for *Pseudomonas aeruginosa* strain 2. Antibiotics tested on this strain include: (center) kanamycin (K), (clockwise from top) penicillin (P 10), streptomycin (S 10), tetracycline (TE 30), and neomycin (N 30). This photograph is distinct enough to measure when printed in black and white.

TABLE 7. Zone of inhibition measurements for *P. aeruginosa* strain 2

Antibiotic	Zone of inhibition measurement (mm)	Interpretation
Kanamycin	30	Susceptible
Penicillin	0	Resistant
Streptomycin	21	Susceptible
Tetracycline	12	Resistant
Neomycin	18	Susceptible

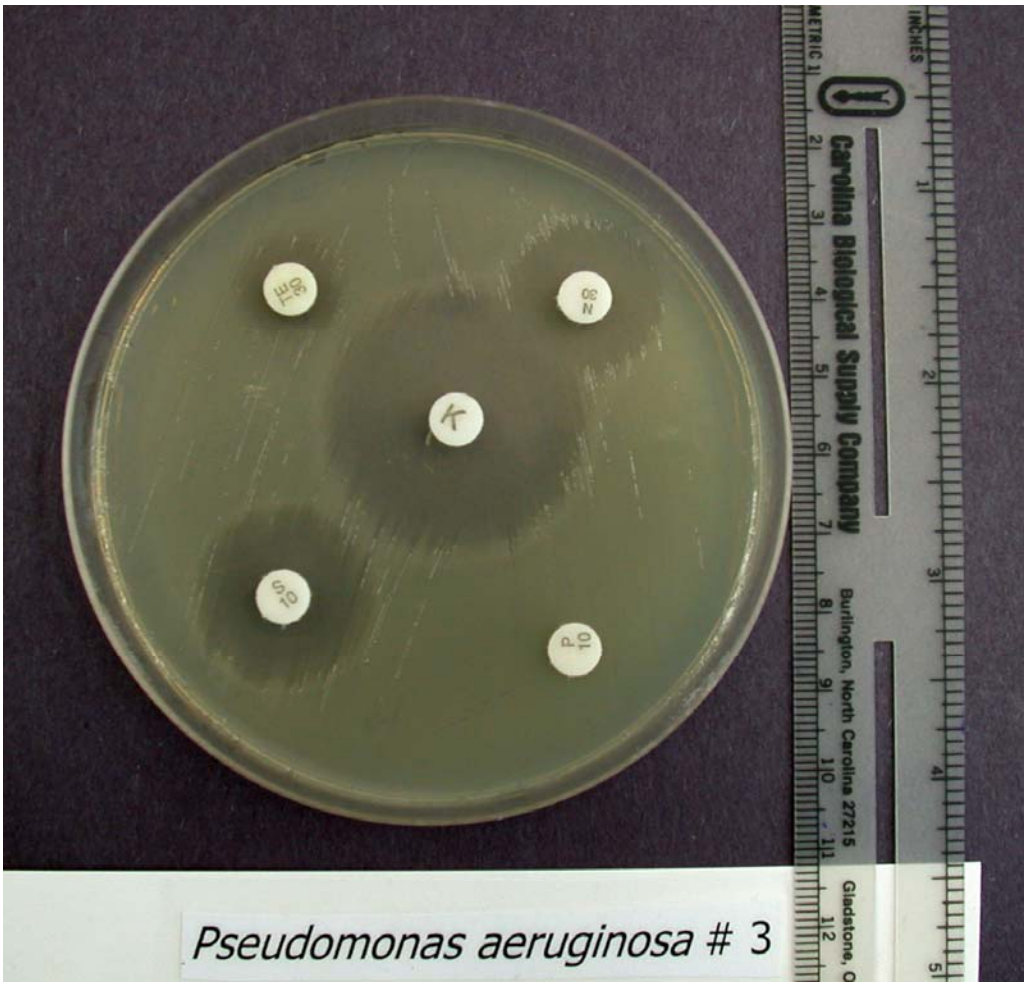
TE 30
N 30
K
CIP 5
P 10
Carolina Biological Supply Company
Burlington, North Carolina 27215
INCHES
METRIC
Pseudomonas aeruginosa # 3

FIG. 8.

FIG. 8. Representative plate for *Pseudomonas aeruginosa* strain 3. Antibiotics on the plate include: (center) kanamycin (K), (clockwise from the right top) neomycin (N 30), penicillin (P 10), streptomycin (S 10), and tetracycline (TE 30). This figure will print in black and white clearly enough to be measured by students.

TABLE 8. Zone of inhibition measurements for *P. aeruginosa* strain 3

Antibiotic	Zone of inhibition measurement (mm)	Interpretation
Kanamycin	32	Susceptible
Neomycin	19	Susceptible
Penicillin	0	Resistant
Streptomycin	21	Susceptible
Tetracycline	9	Resistant

FIG. 9.

FIG. 9. Representative plate for *Escherichia coli* strain 1.
Antibiotics tested on this strain include: (clockwise from top of figure) penicillin (P 10), streptomycin (S 10), neomycin (N 30), tetracycline (TE 30), and kanamycin (K). Students had difficulty in correctly measuring the diameter of the zone of inhibition around the streptomycin disc. The edges of the zones around the neomycin and kanamycin discs are also rough. This photograph is distinct enough to allow students to measure the zones of inhibition when printed in black and white.

TABLE 9. Zone of inhibition measurements for *E. coli* strain 1

Antibiotic	Zone of inhibition measurement (mm)	Interpretation
Penicillin	0	Resistant
Streptomycin	0	Resistant
Neomycin	21	Susceptible
Tetracycline	14	Resistant
Kanamycin	30	Susceptible

FIG. 10.

FIG. 10. Representative plate for *Escherichia coli* strain 2. Antibiotics tested on this plate include: (center) streptomycin (S 10), (clockwise from upper right) tetracycline (TE 30), kanamycin (K), neomycin (N 30), and penicillin (P 10). The kanamycin disc was probably moved after it touched the agar, leaving the asymmetric zone of inhibition. This photograph is distinct enough to allow students to measure the zones of inhibition when printed in black and white.

TABLE 10. Zone of inhibition measurements for *E.* coli strain 2

Antibiotic	Zone of inhibition measurement (mm)	Interpretation
Streptomycin	0	Resistant
Tetracycline	26	Susceptible
Kanamycin	30	Susceptible
Neomycin	19	Susceptible
Penicillin	0	Resistant

FIG. 11.

FIG. 11. Representative plate for *Escherichia coli* strain 3.
Antibiotics tested on this strain include: (center) kanamycin (K), (clockwise from upper right) penicillin (P 10), streptomycin (S 10), neomycin (N 30), and tetracycline (TE 30). This photograph will not print clear enough in black and white to be measured.

Note the indistinct margins and lighter growth at the edges of the kanamycin, streptomycin, and neomycin zones.

TABLE 11. Zone of inhibition measurements for *E. coli* strain 3

Antibiotic	Zone of inhibition measurement (mm)	Interpretation
Tetracycline	30	Susceptible
Kanamycin	40	Susceptible
Penicillin	0	Resistant
Neomycin	21	Susceptible
Streptomycin	20	Susceptible

FIG. 12.

FIG. 12. Representative plate for *Escherichia coli* strain 4.
Antibiotics tested on this plate include: (clockwise from upper right disc) neomycin (N 30), tetracycline (TE 30), streptomycin (S 10), penicillin (P 10), and kanamycin (K). This photograph is not distinct enough for all the details to be evident when printed in black and white.

Note in the student data that the students measured a small zone of inhibition around the penicillin disc.

TABLE 12. Zone of inhibition measurements for *E.* coli strain 4

Antibiotic	Zone of inhibition measurement (mm)	Interpretation
Kanamycin	36	Susceptible
Neomycin	16	Intermediate
Tetracycline	22	Susceptible
Streptomycin	22	Susceptible
Penicillin	0	Resistant

Zone diameter interpretive chart for selected antibiotics[a]

Antimicrobial agent and organism(s)	Code	Disc potency	Zone diameter interpretive standards (mm)		
			Resistant	Intermediate	Susceptible
Ampicillin *Enterobacteriaceae*	AM-10	10 µg	≤13	14–16	≥17
Ampicillin *Staphylococcus*	AM-10	10 µg	≤28		≥29
Bacitracin	B-10	10 units	≤8	9–12	≥13
Cephalothin	CF-30	30 µg	≤14	15–17	≥18
Chloramphenicol *Enterobacteriaceae*, *Pseudomonas aeruginosa*, *Acinetobacter*, staphylococci, enterococci	C-30	30 µg	≤12	13–17	≥18
Ciprofloxacin *Enterobacteriaceae*, staphylococci	CIP-5	5 µg	≤15	16–20	≥21
Gentamicin *Enterobacteriaceae*, *P. aeruginosa*, *Acinetobacter*, staphylococci	GM-120	120 µg	≤12	13–14	≥15
Kanamycin	K-30	30 µg	≤13	14–17	≥18
Neomycin	N-30	30 µg	≤12	13–16	≥17
Penicillin staphylococci	P-10	10 units	≤28		≥29
Streptomycin *Enterobacteriaceae*	S-10	10 µg	≤10	11–14	≥15
Tetracycline *Enterobacteriaceae, P. aeruginosa, Acinetobacter,* staphylococci	Te-30	30 µg	≤14	15–18	≥19

[a] For complete information on zone diameter interpretation, Becton Dickinson has the complete package insert available at: http://www.bd.com/ds/technicalCenter/inserts/8840621(0904).pdf.

Review of Antibiotics and Their Range of Effectiveness

When the Kirby-Bauer Antibiotic Susceptibility exercise was used at Rogers State University, students worked with strains of the following organisms: *Staphylococcus* using both *S. aureus* and *S. epidermidis*, *Pseudomonas aeruginosa*, *Serratia marcescens,* and *Escherichia coli*. Clinical strains can be expected to have different antibiotic sensitivities or resistances than environmental isolates.

TABLE 1. Survey of antibiotics used in antibiotic sensitivity testing in teaching laboratories

Antimicrobial agent	Antibiotic category	Response of organisms tested		
		Staphylococci (*S. aureus and S. epidermidis*)	Pseudomonas (*P. aeruginosa*)	Enterobacteriaceae (*Serratia* and *Escherichia*)
Ampicillin	Penicillin	S[a]	R[b]	S
Cephalothin	Cephem	S	R	S
Chloramphenicol	Phenicol	S	S	S
Ciprofloxacin	Fluoroquinolone	S	S	S
Erythromycin	Macrolide	S	R	R
Gentamicin	Aminoglycoside	S	S	S
Kanamycin	Aminoglycoside	S	R	S
Neomycin	Aminoglycoside	ND[c]	ND	ND
Penicillin	Penicillin	S	R	R
Streptomycin	Aminoglycoside	R	R	S
Tetracycline	Tetracycline	S	S	S

[a] S, susceptible. This antibiotic is used by the Clinical and Laboratory Standards Institute, and infections with this organism are treated with this antibiotic. The strain may be susceptible.

[b] R, resistant. This antibiotic is not used in treatment of infections caused by the organism. Resistance is assumed.

[c] ND, no data. Data for this antibiotic were not included in the Clinical and Laboratory Standards Institute's information.

Troubleshooting Figures

These figures illustrate some of the details, errors, or problems seen on Kirby-Bauer plates. Other figures in this group illustrate some of the different zones of inhibition that students may observe.

FIG. 1. The center filter paper disc (S10) containing streptomycin has been pushed into the agar, breaking the surface. Since a zone of inhibition was visible, the students were still able to use data from this plate.

FIG. 2. Penicillin (P10) and neomycin (N30) antibiotic discs were placed too close to each other on the plate. Data was obtained only because penicillin was ineffective against this organism.

FIG. 3. Penicillin (P10) is ineffective against this gram-negative bacteria, *Serratia marcescens*. The red color on the disc comes from the bacterial pigment, prodigiosin.

FIG. 4. The kanamycin (K) antibiotic disc was placed too close to the edge of the petri plate. However, students could still acquire useable data from this plate by measuring the radius of the zone of inhibition and doubling the radius measurement to obtain the diameter.

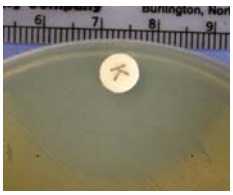

FIG. 5. These kanamycin (K) and neomycin (N30) antibiotic discs were placed too close to each other. Their zones of inhibition have merged. However, the diameter of each zone of inhibition can still be measured.

FIG. 6. This figure illustrates that some organisms have altered appearance when exposed to antibiotics. Students must make sure to measure the region of no growth, rather than from red edge to red edge. The antibiotic disc here is streptomycin (S10).

FIG. 7. There is a clear demarcation between the area of growth and the inhibited region around this tetracycline (TE 30) disc.

FIG. 8. The bacterial growth is less dense close to the tetracycline (TE 30) disc. Students may not realize that there is no measurable zone of inhibition, since there is slight bacterial growth up to the disc itself.

FIG. 9. This neomycin (N30) antibiotic disc is marked with an X, indicating it is the last disc in the antibiotic cartridge.

FIG. 10. A high proportion of cells of this bacterial strain are resistant to the streptomycin (S10) disc on the right. This results in a clear zone of inhibition with many resistant bacteria growing inside the rim. The edge of the zone of inhibition around the neomycin (N30) disc (on the left) is also ragged, again indicating that the population of bacteria contains some cells which are less resistant than others.

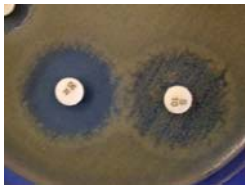

FIG. 11– 13. Each of these figures shows an antibiotic disc that was moved after it touched the agar. Enough antibiotic transferred to the agar even in the brief moment before the disc was moved to create (11) the clear area next to this neomycin (N30) disc and (12, 13) the asymmetric zones of inhibition.

11

12

13

Media Recipes

1. Mueller-Hinton agar

Commonly referred to as Mueller-Hinton agar, the current version of the medium is Mueller-Hinton II agar made by Becton, Dickinson and Company, as are all of the other bacterial media mentioned below.

The Clinical and Laboratory Standards Institute (3) specifies using Mueller-Hinton agar for the following reasons: acceptable batch-to-batch reproducibility; low in sulfonamide, trimethoprim, and tetracycline inhibitors; gives satisfactory growth for most organisms; and has been used successfully by many people.

Ingredients per liter

Beef extract	2.0 g
Acid hydrolysate of casein	17.5 g
Starch	1.5 g
Agar	17.0 g

Final pH 7.3 +/- 0.1

Preparation

Dissolve 38 g of agar in 1 liter of boiling purified water (purified water is obtained through reverse osmosis, distilled, or deionized) and mix thoroughly.

Stir the mixture and heat to boiling; allow it to boil for 1 minute to fully dissolve the powder.

Sterilize by autoclaving at 121°C for 15 minutes, do not overheat.

Pour into plates to a depth of 4 mm per plate, allow agar to cool and solidify.

Refrigerate plates if they will not be used that day. Wrap plates in plastic to delay drying. Plates can be kept for longer than 7 days.

2. Mueller-Hinton broth

Mueller-Hinton broth is used as a general medium to cultivate bacteria.

Ingredients per liter

Beef extract	3.0 g
Acid hydrolysate of casein	17.5 g
Starch	1.5 g

Preparation
Suspend 22 g of the powder in 1 liter of purified water (as above). Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Sterilize by autoclaving at 121°C for 10 minutes. Do not overheat.

3. Nutrient broth

Ingredients per liter

Pancreatic digest of gelatin	5.0 g
Beef extract	3.0 g

Preparation
Suspend 8 g of the powder in 1 liter of purified water (as above). Mix thoroughly. Warm slightly if necessary to dissolve. Sterilize by autoclaving at 121°C for 15 minutes.

4. Trypticase soy agar

Ingredients per liter

Pancreatic digest of casein	15.0 g
Papaic digest of soybean meal	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g

Preparation
Suspend 40 g of powder in 1 liter of purified water (as above). Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Sterilize by autoclaving at 121°C for 15 minutes. Do not overheat. Cool until agar can be handled (at approximately 55°C) and pour into petri plates. Let solidify.

Storing bacterial strains

There are several methods for storing bacteria. Much information is available in *Methods for General and Molecular Bacteriology* (1). Using two or more methods to preserve cultures enhances the chance that strains can be retained for a long period of time.

1. Long term storage on agar slants covered with sterile mineral oil and stored in the refrigerator

Mineral oil is available from the local pharmacy, and it can be sterilized by placing it in a heat resistant container with a heat resistant lid and baking in an oven at 180°C for 2 hours. Recover bacteria by removing a loopful of broth through the oil.

2. Anhydrous silica gel

The anhydrous silica gel method was published by Dr. John Lennox (2) and a quick description is presented here. It is a modification of the procedure presented in *Methods for General and Molecular Bacteriology* (1). In our hands, most cultures prepared according to his instructions and stored in the -20°C freezer survive for 5 years or even much longer. Materials required include glass vials and lids which can be sterilized in a dry oven (as described above), anhydrous silica gel (available from Aldrich, 21,442-6), instant nonfat dry milk (made up as a 7.5% solution and autoclaved to sterilize, make twice as much as desired due to coagulation of the milk proteins), slant cultures in stationary phase, freezer, pipettes (either regular or Pasteur pipettes) and a pipette bulb, dust mask and gloves (to protect from silica gel dust), and labeling materials. Prepare the silica gel by placing it into the small vials (borosilicate tubes with autoclavable screw caps, e.g., Kimble 60915-D, 2 dram vials). Many vials can be prepared, sterilized, and kept tightly closed in the freezer to be used when cultures need to be preserved. Vials must be cold when the bacteria are added since absorption of the liquid generates heat. Add 2 to 3 ml of sterile milk to each slant of bacteria, rinse the bacteria from the slant and then add them dropwise to the cold sterile silica gel. Add until the liquid is no longer absorbed. Allow to sit with lids slightly loose for 4 to 6 days, then tightly cap, label well, parafilm the lids shut, and place in the -20°C freezer. Do not use a frost-free freezer due to the freeze-thaw cycles.

3. Cooked meat medium

Cooked meat medium contains broth and small meat pellets. It works well as a medium to store anaerobes and facultative anaerobes, but has limited usefulness for strict aerobes. To prepare the medium, pour 1.25 grams of the medium into a screw-capped test tube and add 10 ml of purified water (see below). Allow the tube to stand for 10 minutes before autoclaving. Sterilize by autoclaving for 15 minutes at 121°C. Inoculate the tube allow bacteria to grow for 24 to 48 hours, then add 600 µl of sterile 80% glycerol (see below), mix well, cap loosely, place in the freezer until frozen, then cap tightly. Maintain in a non–frost-free freezer at

-20°C or -80°C. To revive the culture, scrape with a sterile wooden stick or loop while still frozen and touch to broth or solid media.

4. Freezing media

A web search, literature search, or molecular biology book review will provide a large number of similar recipes for freezing bacteria. Some procedures utilize dimethylsulfoxide (DMSO) to create a flexible membrane, while others utilize glycerol.

One hundred percent glycerol is very anhydrous and does not autoclave adequately, nor does it mix well with water. If using glycerol for freezing, it is best to prepare a less anhydrous solution. To make eighty percent glycerol, add 20 ml of water to 80 ml of glycerol, mix, and autoclave at 121°C for 15 minutes in an autoclavable screw-capped bottle. The final concentration of glycerol in the frozen bacteria is usually between 5% and 15%.
Bacteria to be frozen should be fresh broth cultures, just at stationary phase. Cells are not usually frozen in media that contains antibiotics. Add the glycerol to the culture, mix, and aliquot into sterile microcentrifuge tubes. Seal and label the tubes. Freeze at -20°C in a non–frost-free freezer. After freezing at -20°C, cells can be moved to -80°C. To start a new culture, remove the tube from the freezer, keep it from thawing, open the tube, scrape with a sterile wooden stick or sterile toothpick, and inoculate a broth or agar culture. Consider making a backup set of the freezer stocks and storing them in another location.

DMSO is usually used at 7.5% concentration. If DMSO is used, it should be from a freshly opened bottle, a bottle resealed as it was flushed with gaseous argon or nitrogen (DMSO is relatively unstable), or aliquots from a freshly opened bottle that were frozen at -20°C or -70°C until use. As with glycerol, DMSO stabilizes the membrane of a cell. DMSO is added to stationary-phase cultures of bacteria to a final concentration of 7.5%, and the bacteria are sealed into microcentrifuge tubes and frozen. New stocks are started by scraping with a sterile wooden stick and inoculating into broth or agar media. DMSO is extremely volatile and even when used in a biological safety cabinet, the user will taste garlic, an indicator that DMSO is being absorbed through the gloves and skin.

References.

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