Assessing Antibiotic Resistance of *Staphyloccocus:*

Students Use Their Own Microbial Flora To Explore Antibiotic Resistance

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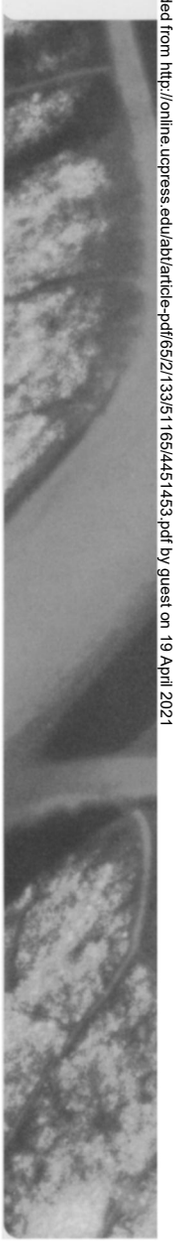
HOW-TO-DO-IT



Antibiotic resistance is a growing health problem throughout the world. It has been identified as a major public health threat and priority in the United States. Antibiotic resistance is an attractive topic to introduce students to many concepts in biology including microbial taxonomy, the biochemical basis of antibiotic action, and evolution of resistance. Laboratory exercises in antibiotic resistance for college and university majors routinely use established antibiotic resistant strains to demonstrate methods for quantifying antibiotic resistance. We were interested in broadening the appeal of a laboratory exercise by using students' own microbial flora. We found that this exercise is feasible for students with little experience in microbiological laboratory techniques. The advantage of using this approach is that students, by testing their own bacteria for antibiotic resistance, have a much greater interest in the exercise and in underlying principles of antibiotic resistance.

In this exercise, students isolate *Staphylococcus* species from their skin, nose, or ear by using a selective growth medium. Individual colonies are selected and plated for testing with commercially available antibiotic sensitivity test discs. Students measure the zones of growth inhibition and use a data table provided with the antibiotic sensitivity discs to determine the level of susceptibility of their *Staphyloccoci* to each of several different antibiotics. The data of susceptibility to each of the antibiotics for the class are tabulated (see percentages in the parentheses in Table I) and compared to the reported frequency of resistance in the population. This exercise is easily done in a standard microbiology laboratory, but we found that it is also successfully accomplished in a freshman nonmajors class.

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Procedures

First, a bacterial sample is obtained. Each student moistens a sterile swab with sterile saline and rubs it on his or her skin, in their nose, or in their ear. Streak the swab over the surface of a mannitol salt agar plate and discard the used swab in a beaker of disinfectant such as 10% bleach or 95% ethanol. Label the plate with the student's name or initials and incubate in an inverted position overnight at 35° to 37° C. Mannitol salt agar selects for halophilic organisms like *Staphylococcus* due to the high salt concentration (7.5% NaCl). *Staphylococcus aureus* isolates turn the medium yellow due to acid production from the fermentation of mannitol while most other *Staphylococcus* sp. do not ferment mannitol, leaving the medium color unchanged (Figure 1). This exercise will work with any *Staphylococcus* species.

The next day (if class meets on alternate days, the plates can be kept in the refrigerator) students pick a single or a few colonies with a sterile bacterial transfer loop and suspend the cells in 1 ml of nutrient broth. Recipes for nutrient broth and nutrient agar plates are found in the Materials section, but any general medium like nutrient broth and agar will work for this portion of the exercise. Dip a sterile swab into the bacterial suspension and thoroughly saturate the swab. Remove the excess liquid by firmly rotating the swab against the inside wall of the test tube above the bacterial suspension. Streak one side of the swab across the whole surface of a nutrient agar plate (Figure 2). Rotate the plate 60 degrees, turn the swab to an unused side, and streak again. Rotate the plate another 60 degrees and use a third side of the swab to streak again. It is important to cover the entire surface of the plate with bacteria to produce a lawn. Allow any surface moisture to be absorbed by leaving the

Table 1. Results of antibiotic resistance test.

Category of Resistance/Susceptibility

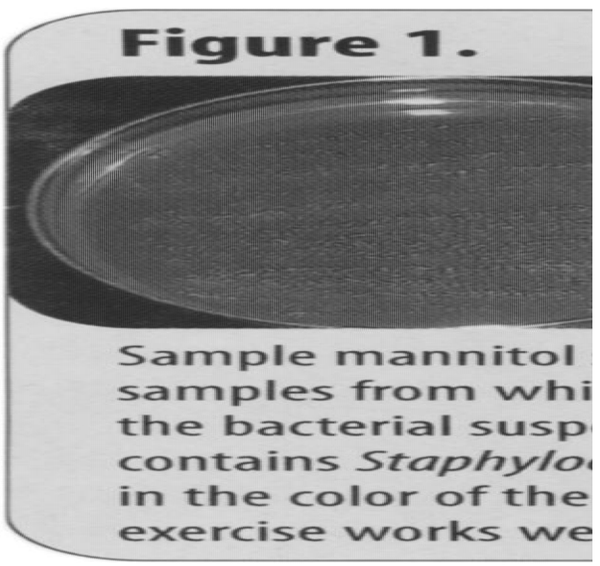
ANTIBIOTIC	RESISTANT	INTERMEDIATE SUSCEPTIBILITY	SUSCEPTIBLE
Penicillin	< 28 (100%)		> 29
Oxacillin	< 10 (50%)	11 - 12 (12.5%)	> 13 (37.5%)
Vancomycin	< 9	10 - 11 (12.5%)	> 12 (87.5%)
Cephalothin	< 14	15 - 17	> 18 (100%)
Erythromycin	< 13 (100%)	16 - 20	> 21
Amoxicillin	< 19		> 20 (100%)

First number is the zone of inhibition in mm.
% of students in class in each category in parentheses.
Source: the informational sheet from the package of antibiotic discs.

cover of the plate ajar for 3 to 5 minutes. If the plates are too wet, the antibiotic will diffuse in the liquid and give an inaccurately large zone of inhibition.

Ideally, this test should be performed with a single bacterial colony, as each colony may have variable degrees of antibiotic susceptibility. However, since the bacterial suspension should be of fairly high density, for this lab we used several colonies and obtained good results. Evidence of two bacterial strains with different antibiotic susceptibility is easily detected. For example, see the Erythromycin disc in Figure 3.

When the plates are sufficiently dry, place the antibiotic sensitivity discs on the agar surface. Using sterile forceps, place each disk at least an inch apart and no less than 1/2 inch from the edge of the agar plate. The disc must be in firm contact with the agar. To sterilize the forceps, soak them in a container of alcohol, then flame or air dry. Incubate the plates for 24 to 48 hours at 37° C. After 48 hours, the plates can be stored in the refrigerator for days before the zone of inhibition is measured (Figure 3).



We find the BBL™ self-tamping 6-disc dispenser and a standard 100 mm petri dish are easy to use. Many antibiotic sensitivity discs are available and include an interpretive standard for zones of inhibition in the packaging materials. This experiment uses six antibiotics: Penicillin, Oxacillin (a member of the methicillin family), Vancomycin, Cephalothin, Erythromycin, and Amoxicillin.

The students measure, in millimeters, the diameter of the zones of inhibition surrounding each of the six antibiotic sensitivity discs on their plates. Use the interpretive standard (see Table I) for rating the bacteria as resistant, intermediate, or susceptible to each of the antibiotics, and determine the level of resistance of the students' bacteria to each antibiotic.

All contaminated materials used in this experiment must be sterilized before disposal. The preferred method is sterilization by autoclave or, if there is no machine available, flood the tubes and plates with a disinfectant solution such as 10% bleach. After sufficient time in the autoclave (20 minutes) or exposure to the disinfectant solution for 20 to 30 minutes, the decontaminated materials are disposed of in the regular waste stream.

Materials

Sterile saline solution containing 0.9% sodium chloride is suitable and available in a grocery store or pharmacy. Look for saline wetting solution for contacts or sterile saline nasal sprays, or make a saline solution by dissolving 0.9 grams of sodium chloride in 100 milliliters of water and sterilize by boiling or autoclaving.

Mannitol salt agar plates, nutrient broth, and agar are available as dehydrated media and as commercially prepared media from many vendors. In the Carolina Science and Math Catalog, mannitol salt agar plates, item number CE-82-1702, have a list price of $14.70 for a package of 10 plates. Nutrient agar plates, CE-77-3782, have a list price of $11.85 for a package of 10 plates, and nutrient broth tubes, CE-82-6122, have a list price of $11.85 for a package of 10 tubes. This medium can also be prepared using the recipes below. Note: the key feature of mannitol salt agar is the high concentration of sodium chloride. The phenol red is an indicator dye.

Figure 2.

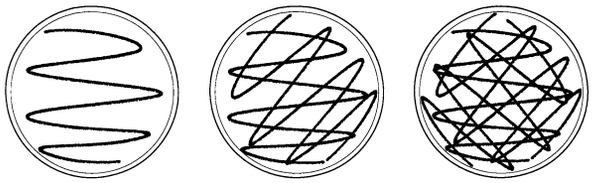
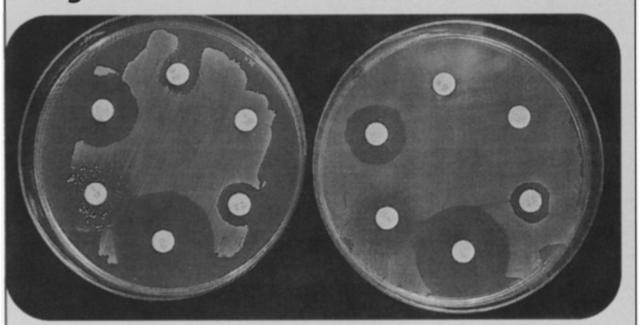


Diagram of preparation of a bacterial lawn. The circles represent agar plates. The circle on the left shows the first streak. In the center circle the plate is rotated 60° and streaked again. In the circle on the right the plate is rotated another 60° and streaked.

Figure 3.



Two sample test plates with antibiotic discs. On both plates the discs contain, starting at 12 o'clock and going clockwise, Penicillin, Oxacillin, Vancomycin, Cephalothin, Erythromycin, and Amoxicillin. The plate on the left has a poor spread of bacteria, and thus some antibiotic discs do not have a complete circle of the zone of inhibition. The plate on the right has a good spread of bacteria. The zone surrounding the Erythromycin disc demonstrates the complication of using more than a single colony of bacteria. The plate on the left shows a subset of bacterial colonies that are resistant to Erythromycin while the plate on the right shows varying degrees of Erythromycin resistance as indicated by the lack of a distinct ring.

Any standard nutrient broth and nutrient agar plate such as Luria Broth, Tryptic Soy Agar, or Mueller-Hinton Media, will work in the test plates.

To make mannitol salt agar, combine the following ingredients in 1 liter of distilled water: 10 g peptone, 1 g meat extract, 75 g sodium chloride, 10 g mannitol, 0.025 g phenol red, and 15 g agar. Heat to boiling and sterilize in an autoclave for 15 minutes at 20 psi. Cool the sterile medium to 55° C in a water bath. Pour the sterile tempered agar in 100 mm petri dishes using 15 to 20 ml per dish. To make nutrient broth, dissolve the following ingredients in 1 liter of distilled water: 3 g of meat extract, 5 g of peptone. Dispense approximately 1 ml of the broth into glass culture tubes, cap with metal caps or heat resistant plastic caps, and sterilize by autoclaving or boiling in the microwave. The exact volume of nutrient broth is not critical, so simply pour to a depth sufficient for a swab. Nutrient agar is prepared by adding 15 grams of agar to 1 liter of nutrient broth. Sterilize by boiling for 20 minutes or autoclave for 15 minutes at 20 psi. Cool the sterile medium to 55° C in a water bath and pour the tempered agar in 100 mm petri dishes using 15 to 20 ml per dish.

The BBL Sensi-disc™ dispenser is available in the Fisher Health Care catalog, product #148908, and has a list price of $316.26. The manufacturer is Becton Dickinson Microbiology and the item number is 60661. Fisher also carries Sensitivity disc cartridges. The cost is approximately $10 per 50-disc cartridge. Individual cartridge dispensers are also available. Discs can be transferred and pushed onto the plates with sterile forceps.

Extension – Class Discussion

This exercise takes 3 separate 15- to 30-minute periods. Depending upon the level of students and the class emphasis, the remainder of the class time can be used to discuss the structure of bacteria and the differences between bacteria and eukaryotic cells. The molecular mechanisms of antibiotic action and antibiotic resistance could be another discussion topic. This class time is a good point to introduce the history of antibiotic discovery and the role of antibiotics in history. In our freshman nonmajors genetics class, we discuss the evolution of bacterial resistance from a population genetics viewpoint. This leads to a discussion of practices that contribute to the rise of antibiotic resistance. As either pre-lab assignment, or post-lab research, students may explore governmental (FDA, http://www.fda.gov/ CDC, http://www.cdc.gov/) or pharmaceutical company Internet sites for reports about the frequency of antibiotic resistance.

Conclusions

This modification of a standard antibiotic resistance laboratory greatly stimulates student interest. All the students in a nonmajors class rated this exercise as Excellent to Good in the class evaluations. Students are extremely interested in how their own bacteria rate in antibiotic resistance. The most expensive parts of this exercise are the antibiotic discs and the dispenser. However, the application of this exercise to a variety of courses makes the investment well worthwhile.

Disclaimer

Although the discs used in this laboratory exercise are the same as those used for clinical semi-quantitative antibiotic susceptibility tests, we necessarily take shortcuts that make our test not accurate for diagnostic purposes. The test employs diffusion through the agar of accurately measured quantities of antibiotics on each disc. Thus, the accuracy of the test relies upon, among other factors, the moisture on the plate, and close contact of the disc with the agar. Testing labs use controls, test with a single colony of bacteria, and use cell suspensions at a specific density.