

## ANTIBIOTIC SUSCEPTIBILITY TEST

### Principle and Purpose

Antibiotic susceptibility, sometimes termed antibiotic sensitivity, is the responsiveness of a microbe to an antibiotic. Depending upon the antibiotic employed, susceptibility can vary between species as well as among strains of the same species. Susceptibility of a microbe to a particular drug may have profound implications on treatment of an infection. Hence, antibiotic susceptibility testing (AST) is conducted to determine which antibiotic will be most successful in treating an infection. There are several types of AST methods that make this assessment. In the present exercise, students will perform a classic AST, the Kirby-Bauer method.

The Kirby-Bauer method is a semi-quantitative procedure in which small paper disks containing different antibiotics are placed on the surface of an agar medium that has been previously seeded with the microbe of interest. The standard medium, Mueller-Hinton Agar (MHA), is usually employed in this assay. The antibiotic in the disk will diffuse in the surrounding area. If the microbe is sensitive to the antibiotic, a clear ring, or zone of inhibition, forms around the disk indicating the absence or inhibition of growth (Fig. 1). Over the years, studies have determined that the diameter of this zone is suggestive of a microbe's susceptibility or resistance to the particular antibiotic.

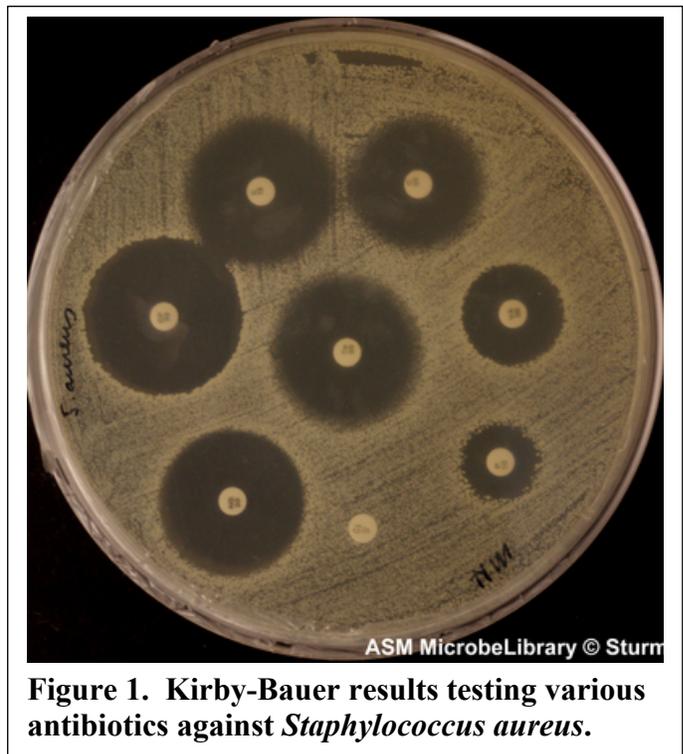
As described below, students will assess the susceptibility of a number of bacteria to a selected group of antibiotics. From the results obtained, students will determine the resistance pattern of these bacteria based upon a standard AST chart

(<http://crcooper01.people.yasu.edu/microlab/ast-chart>) listing the known resistance and susceptibility of different microbes to these antibiotics.

### Learning Objectives

Upon completion of this exercise, a student will be able to demonstrate the ability to:

- Understand the principle of the antibiotic susceptibility test;
- Properly conduct the antibiotic susceptibility test;
- Determine the validity of the test using known standards; and
- Accurately interpret the results of this test to assess the susceptibility of bacterial isolates to selected antibiotics.



**Figure 1. Kirby-Bauer results testing various antibiotics against *Staphylococcus aureus*.**

## Materials Required

The following materials are necessary to successfully conduct this exercise:

Organisms - The following organisms should be provided as 24-hour TSB cultures:

- *Enterococcus durans* (ATCC 7080) [abbreviated as 7080]
- *Enterococcus faecalis* (ATCC 19433) [abbreviated as *E. faecalis*]
- *Escherichia coli* (ATCC 25922) [abbreviated as *E. coli*]
- *Escherichia coli* (Mill Creek isolate) [abbreviated as Mill Creek]
- *Staphylococcus aureus* (ATCC 25923) [abbreviated as *S. aureus*]
- *Staphylococcus aureus* (ATCC 43300) [abbreviated as 43300]
- *Streptococcus pneumoniae* (ATCC 49619) [abbreviated as *Strept. pneumoniae*]
- *Streptococcus pyogenes* (ATCC 19615) [abbreviated as *Strept. pyogenes*]

### Media and Reagents

- Mueller-Hinton Agar (MHA) plates (15 x 150 mm; Cat. No. G45; Hardy Diagnostics; [https://catalog.hardydiagnostics.com/cp\\_prod/Content/hugo/MuellerHintonMed.htm](https://catalog.hardydiagnostics.com/cp_prod/Content/hugo/MuellerHintonMed.htm))

### Materials

- Sterile cotton swabs
- Alcohol wipes
- Forceps
- Antibiotic disk cartridges, various types (Hardy Diagnostics or other suppliers that provide cartridges that fit a multi-disk dispenser; for suggestions, see [https://catalog.hardydiagnostics.com/cp\\_prod/Content/hugo/HardyDiskASTProceduresandChart.pdf](https://catalog.hardydiagnostics.com/cp_prod/Content/hugo/HardyDiskASTProceduresandChart.pdf))
- Antibiotic Susceptibility Chart (<http://crcooper01.people.yzu.edu/microlab/ast-chart.pdf>)

### Equipment

- Multi-disk Antibiotic Dispenser (BBL™ Sensi-Disc™ 12-place dispenser [or like instrument])
- Vortex

## Procedures

### Laboratory Safety Considerations

It is important to wholly recognize that this particular exercise shall include the handling of potential pathogens. While chances of injury are very low, it is nonetheless essential that appropriate precautions be taken when warranted (e.g., wearing gloves, proper disposal of materials, caution with open flames, disinfection of work areas, etc.). Students are urged to ask questions should any portion of the following procedure not be clear, especially with regard to the handling and disposal of materials.

The following instructions describe the procedure for assessing the susceptibility of a single bacterium against a variety of antibiotics. A single Mueller-Hinton Agar plate shall be used for each organism to be tested. Your laboratory instructor will assign a certain number of bacteria

per working group of students. Hence, the procedure outlined below should be performed for each strain assigned to a working group.

It is important to note *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 must be employed in this exercise. These bacteria are the standards used to validate the results.

### Day 1 Activities

- 1) Obtain a Mueller-Hinton Agar (MHA) plate (15 mm x 150 mm in size). If not already at room temperature, set it on the bench to warm.
- 2) Label the bottom (agar side) of the plate with the organism to be tested and other appropriate information. Place the labels near the edge of the plate so that the markings will not interfere with subsequent observations.
- 3) Thoroughly mix an overnight TSB culture of the assigned bacterium using a vortex or by rolling the culture tube between both palms ten times or more to suspend any sediment of cells that may have formed. Roll the tube quickly, but not so harshly that the broth splashes onto the tube cap or such that it rolls out of the hands causing leakage or breakage.
- 4) Immerse a sterile cotton swab into the culture tube. Remove excess fluid from the swab by briefly and gently pressing it against the inside of the tube. The swab should be saturated with fluid, but not dripping wet. Do not “squeeze” the swab to dryness!
- 5) Using this swab, thoroughly streak the entire surface of the appropriately labeled MHA plate three times turning the plate about 60° each time. Also, rub the swab along the inner edge of the agar plate. Allow the MHA plate to dry with the lid slightly ajar for 3-5 minutes, then close the lid.

Immediately discard the swab into the appropriate waste bin.

**Note:** The size of the MHA plate makes it difficult for most persons to perform the streak while holding the plate. It is fine to place the MHA plate on the bench surface and completely remove the lid to perform the streaking process. Be sure, however, to place the lid on an area that has been disinfected and to replace the lid on the plate as appropriate to prevent any outside contaminants falling onto the agar.

- 6) Antibiotic-impregnated disks shall be placed on the surface of each MHA agar plate using a multi-disk dispenser as described below.
  - a) Remove the disk dispenser from its container located in the refrigerator (or -20°C freezer). Allow it to warm to room temperature.

**Note:** Antibiotic disks should be stored at -20°C unless otherwise indicated by their vendor. If the multi-disk dispenser holds antibiotic disk cartridges, it should be stored long term at -20°C in its container. For short-term storage, the dispenser holding cartridges can be placed at 4°C (refrigerator). If dispenser does not hold antibiotic disk cartridges, it can be stored at room temperature in its container.

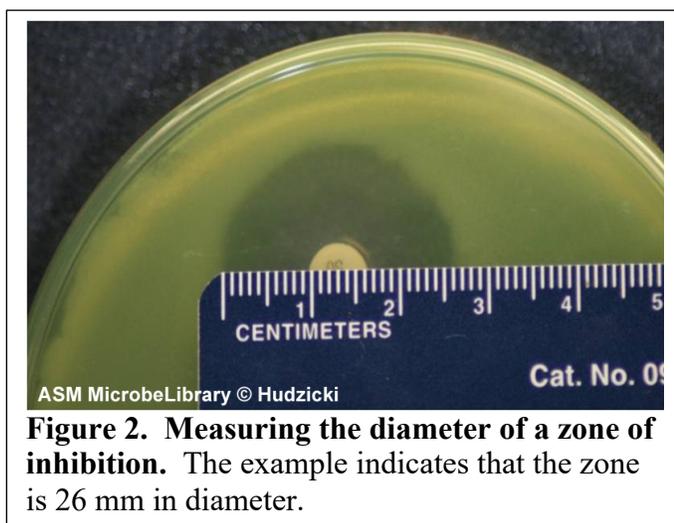
- b) Place the inoculated MHA plate on the top of the lab bench and remove the lid.
- c) Place the dispenser directly over the MHA plate. Firmly, *but not forcefully*, press the plunger once (and only once!) to dispense the disks onto the surface of the plate. Move the dispenser away from its location directly over the MHA plate. If not needed for distributing disks on other plates, place the dispenser in its storage container and return the instrument to the refrigerator (or freezer).

**Note:** The disk dispenser contains twelve disk cartridges of selected antibiotics. The laboratory instructor will provide the identities of the antibiotics used in this exercise.

**CAUTION:** Follow the instructions for use the dispenser. Improper use of the dispenser can jam the mechanical workings, causing further difficulties in completing this exercise as well as wasting materials. Please be sure to use the dispenser appropriately.

- d) Before replacing the lid of the MHA agar plate, use forceps sterilized by cleaning them with an alcohol pad to *gently* touch each disk on the plate to ensure that it is in complete contact with the agar surface. *Do not* press the disk into the agar and do not move the disk once it has been placed on the agar surface.

**Note:** Do not physically move a disk once it has contacted the agar surface except to make sure it lays flat. The drug in the disk will begin to diffuse immediately upon contact with the agar.



**Figure 2. Measuring the diameter of a zone of inhibition.** The example indicates that the zone is 26 mm in diameter.

- 7) As soon as practical, incubate the plates at 37°C with the agar side down – *not* inverted as per standard procedure.

#### Day 2 Activities

- 8) Remove and examine the plates after 16-18 hours of incubation.
- 9) Using a ruler, measure to the nearest millimeter (mm) the diameter of each zone of inhibition surrounding each antibiotic disk, i.e., across the entire zone including the disk (Fig. 2). Perform this measurement from the underside of the plate.

*Record any observations on the data report sheet attached to this document.*

**Note:** MHA plates testing *Staphylococcus* species against oxacillin or vancomycin require 24 hours of incubation at 37°C. Therefore, if the surface of the MHA plate has disks of the above antibiotics on it, measure the zones of inhibition of all the other antibiotics, then return the agar plates to the 37°C incubator. After an additional 6-8 hours of incubation, measure the zones of inhibition surrounding the oxacillin and/or vancomycin disks.

- 10) Use the antibiotic susceptibility chart provided available through the following URL (<http://crcooper01.people.ysu.edu/microlab/ast-chart>) to determine if the isolate being tested is resistant to a particular antibiotic, is susceptible to this antibiotic, or exhibits intermediate susceptibility. This chart also contains the expected measurements for the control strains, *E. coli* and *S. aureus*. If the control strains do not fall within the expected range for a given antibiotic, then the results for that particular drug is considered invalid.

**Note** The chart provided for this exercise is based upon the Hardy Diagnostics “Disk Diffusion Zone Diameter Chart” that is available via the following URL: [https://catalog.hardydiagnostics.com/cp\\_prod/Content/hugo/HardyDiskASTProceduresandChart.pdf](https://catalog.hardydiagnostics.com/cp_prod/Content/hugo/HardyDiskASTProceduresandChart.pdf).



