Antimicrobial Disc Diffusion Testing - A Visual Guide

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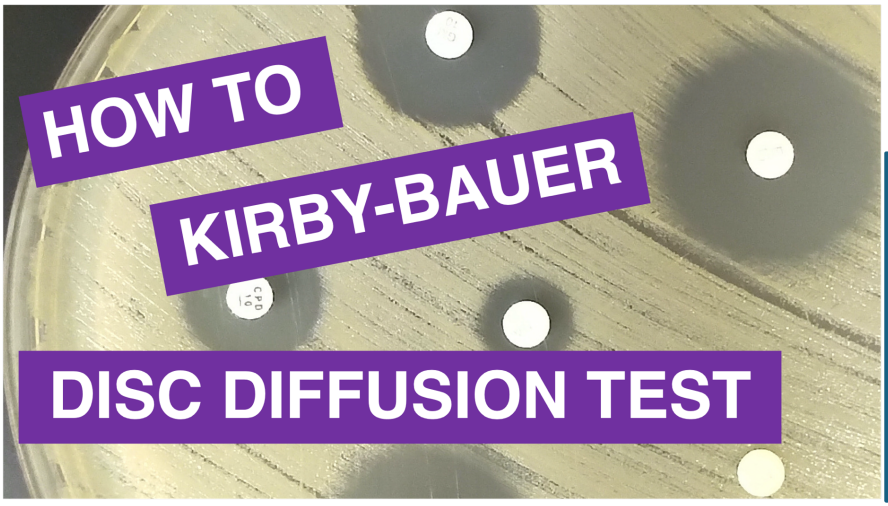


For a video demonstration of the test, see the following link/QR code:
https://youtu.be/M-szotkpT00

Antimicrobial susceptibility testing is highly standardized. Our ability to interpret the test requires that the assay is conducted according to the highly prescriptive guidelines from the Clinical and Laboratory Standards Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST). A video demonstration of this method is available by accessing the link or QR code above. Procedural and reagent factors which are standardized include:

- Test media (including pH, cation concentration)
- Size of bacterial inoculum tested
- Antimicrobial disc content
- Incubation temperature
- Incubation time

Because clinical breakpoints are designed to predict therapeutic outcomes (ex. susceptible indicates a high likelihood of treatment success and resistant predicts treatment failure), test results are specific to the scenario for which interpretive criteria were developed. Breakpoints are specific to:

- Animal species
- Each organism-drug combination (the zone diameter indicating resistance may be different for each drug)
- Indication/type of infection
- Dosing regimen

1 Preparing Test Media

1. For most non-fastidious bacteria, disc diffusion testing must be done using Mueller-Hinton agar
2. Prepare agar plates with a depth of 4mm
    a. Plates with incorrect thickness will **NOT** yield interpretable results
        i. Isolates will appear to be more susceptible on thin agar
        ii. Isolates will appear to be more resistant on thick agar
    b. The volume of agar required to have a 4mm plate depth can be calculated using the following formula:

Example: a 100mm (10cm) diameter standard plate.

Volume = ml (cm^3)
0.4 = final agar thickness in centimeters (4mm)
π = 3.1415
diameter = internal diameter of the plate in cm

$$Volume\ ml = (0.4cm)(\pi)\left(\frac{diameter}{2}\right)^2$$

$$Volume\ ml = (0.4cm)(3.1415)\left(\frac{10cm}{2}\right)^2$$

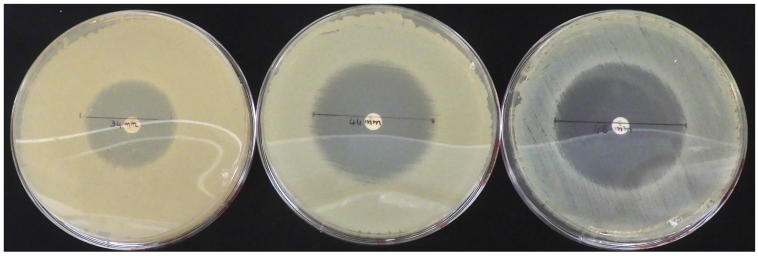
$$Volume\ ml = (0.4cm)(3.1415)(5cm)^2$$

$$Volume\ ml = (31.4cm)^3$$

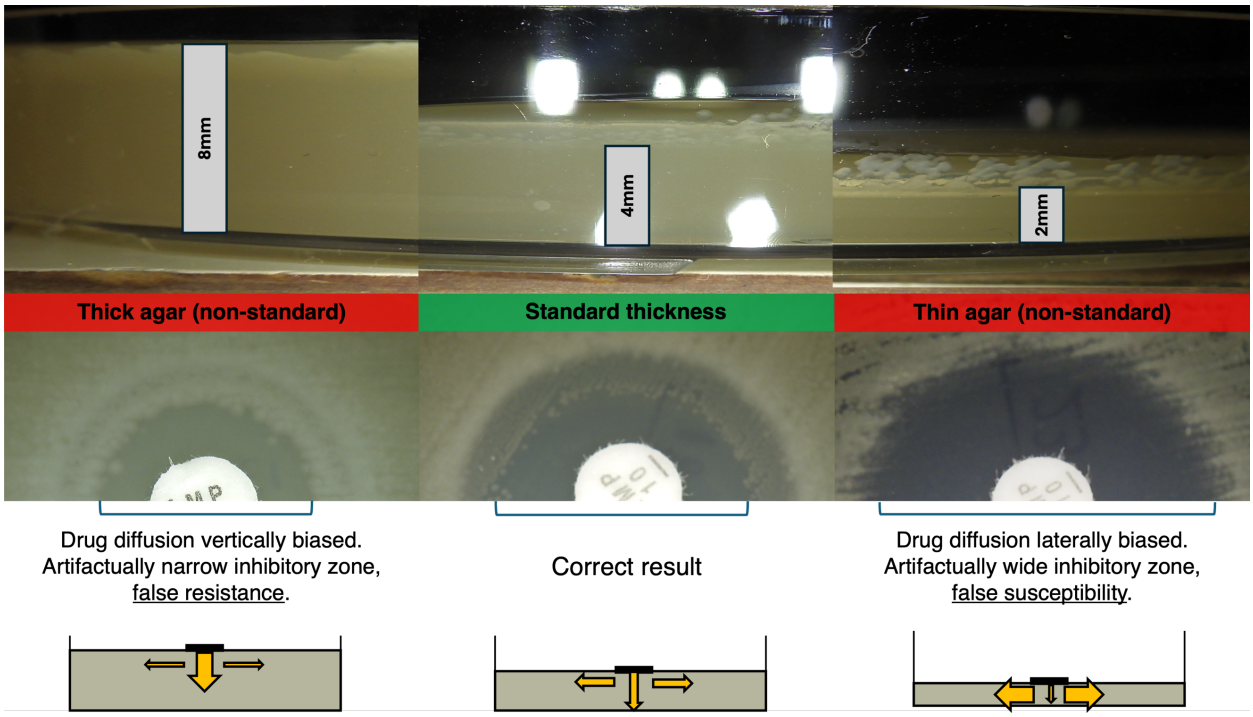
$$= 31.4ml$$

Agar volume required for plates of common diameters with 4mm thickness

Plate Diameter	Volume
75mm	17.7ml
100mm	31.4ml
150mm	70.7ml



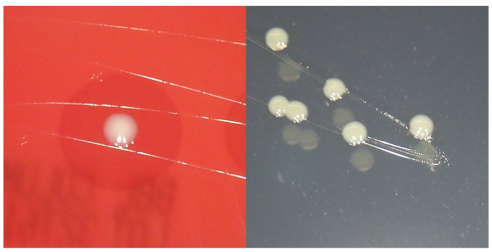
Ciprofloxacin disc-diffusion test done on one strain of *E. coli*
8mm (left), 4mm-standard (centre) and 2mm (right) thick Mueller-Hinton agar.



2 Preparing Your Isolate

3. Susceptibility testing must **ALWAYS** be done on pure cultures
    a. At least one sub-culture from the primary plate is required.
    b. The results of testing mixed cultures cannot be interpreted. If a sample contains more than one bacteria of interest (including multiple strains of the same species), each must be individually tested.
    c. When sub-culturing a bacteria for purity, it is recommended to use blood agar. Organisms of different species are more likely to exhibit distinct morphologies on blood agar compared to other media (ex. nutrient agar).
        i. See images below of *S. aureus* and *E. coli* colonies on blood and nutrient agar. While these species are readily differentiated on blood agar (colony colour, size, hemolysis, texture etc.) they are very similar on nutrient agar, and may not be recognized as distinct in a diagnostic setting.

4. Susceptibility testing must be done on <u>fresh, overnight cultures</u> of your organism on blood agar.
    a. Susceptibility testing is calibrated for rapidly growing, metabolically active bacteria. The use of older cultures does not give accurate results.
    b. When testing an old culture (a plate up to 1-week old) which has been stored in the fridge, freshly subculture an individual colony to blood agar, incubate overnight and perform disc diffusion testing on the following day.

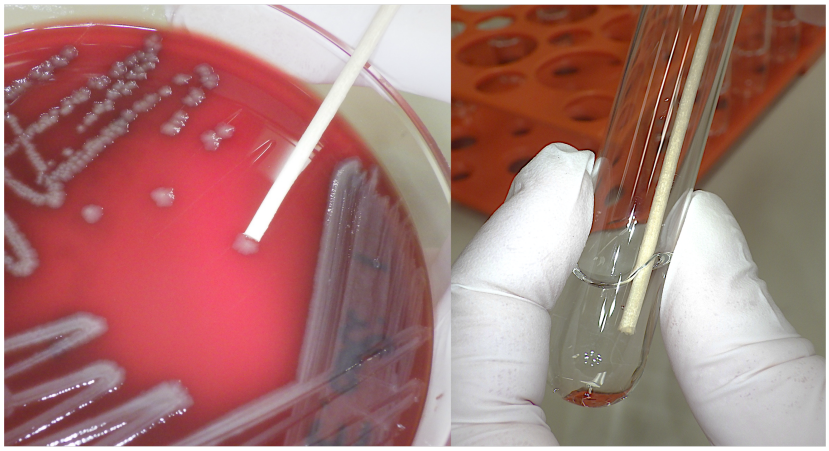


S. aureus on blood agar (left) and nutrient agar (right)

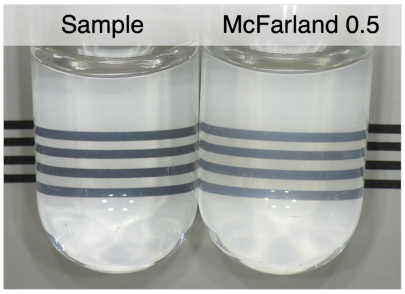
E. coli on blood agar (left) and nutrient agar (right)

3 Making Suspension

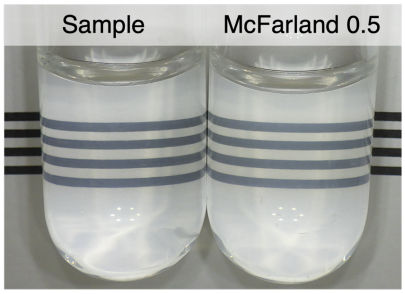
5. Prepare a McFarland 0.5 suspension of the bacteria by picking isolated colonies and suspending them in sterile water.



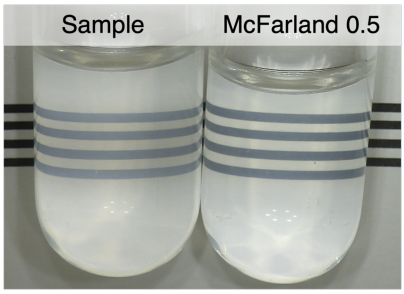
6. Depending on the organism tested, and the volume of suspension prepared, a different number of colonies will be required. Start with a single colony, add additional bacteria as required to reach the McFarland 0.5 density. This corresponds to 1-2 X 10^8 colony forming units/ml. Vortex mix the bacterial suspension before measuring its density.



Sample not dense enough (more bacteria required)



Sample approximately the correct density (test this suspension)

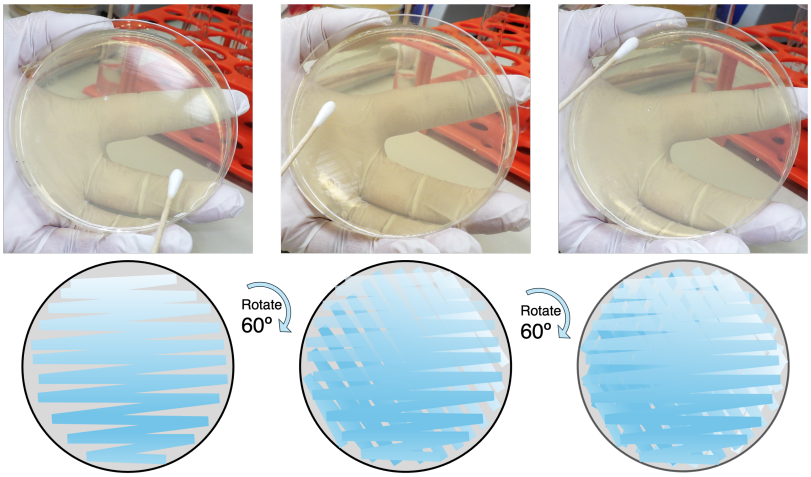


Sample too dense (remake suspension with fewer colonies)

7. The density of the suspension can be assessed using a McFarland 0.5 standard.
    a. Manual assessment of culture density is possible by visually comparing the distortion of black lines drawn on card placed behind the tubes.
    b. An equivalent level of fuzziness/distortion indicates that the bacterial suspension is the same density as the McFarland 0.5 standard.
    c. McFarland 0.5 standards can be purchased from commercial sources, prepared in-house or by a reference institution.
8. Alternatively, an instrument which measures optical density (such as a nephelometer or densitometer) can be used.

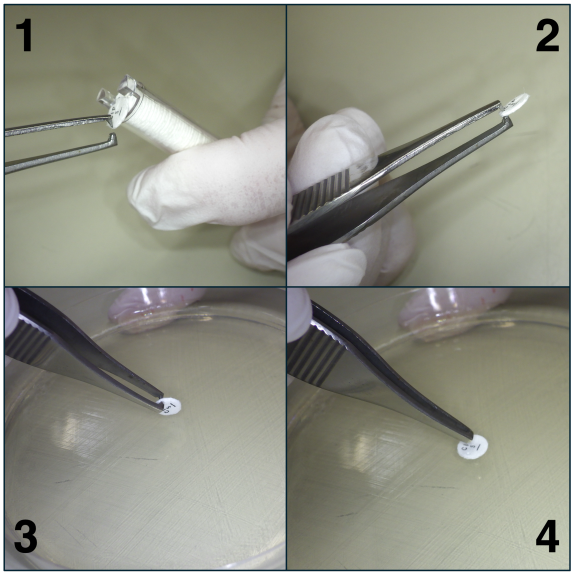
4 Inoculating Plates

9. Fully immerse a sterile swab into the bacterial suspension and saturate it.
10. Press the tip of the swab against the inside of the tube to squeeze out excess liquid. The swab should **not** be dripping wet when used to inoculate the plate.
11. Use the swab to inoculate the surface of the plate. Gently run the swab over the agar back and forth covering the entire surface.



While swabbing the plate, it is also important to rotate the swab to evenly distribute bacteria.

12. After the entire surface has been inoculated once, rotate the plate 60° and repeat the swabbing.
    a. Do **NOT** put the swab back into the bacterial suspension. The objective is to evenly distribute the organisms which have already been deposited on the surface of the plate.
13. Rotate the plate 60° and repeat the swabbing a 3rd time.
    a. The goal is to have a uniform lawn of bacteria over the entire surface of the plate.
14. Allow the plates to sit for up to 10 minutes prior to placing discs. This will allow any liquid droplets to be absorbed into the media.
15. Using **sterile** forceps, remove an antimicrobial disc from the cartridge
16. Place the disc on the surface of the agar and gently press on the centre of the disc
17. Once the discs are placed on the plate, they should not be moved
18. If the forceps become contaminated (touch the plate), re-sterilize them prior to placing the next disc.



5 Incubating Plates

19. Incubate plates inverted (agar side up) at 35°C for 16-20 hours.
20. Following incubation, measure the diameter of the inhibitory zone using a ruler.
21. Compare the measured diameter to the appropriate guideline for interpretation.
    a. Interpretive criteria are specific to each drug-organism-animal species combination.
    b. Interpretive criteria have **NOT** been developed for every drug-organism combination, in these cases it is not possible to classify an isolate as susceptible or resistant.

6 Important Resources

Freely Available CLSI AST Documents

https://clsi.org/all-free-resources/

EUCAST Open-source AST Methods

https://www.eucast.org/ast_of_bacteria

About this Visual Guide

This resource was developed by Drs. Rubin, Weerasooriya and Madalagama. The content of this manual was specifically curated to assist the regional labs within the Department of Animal Production and Health of Sri Lanka in improving their susceptibility testing procedures.

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