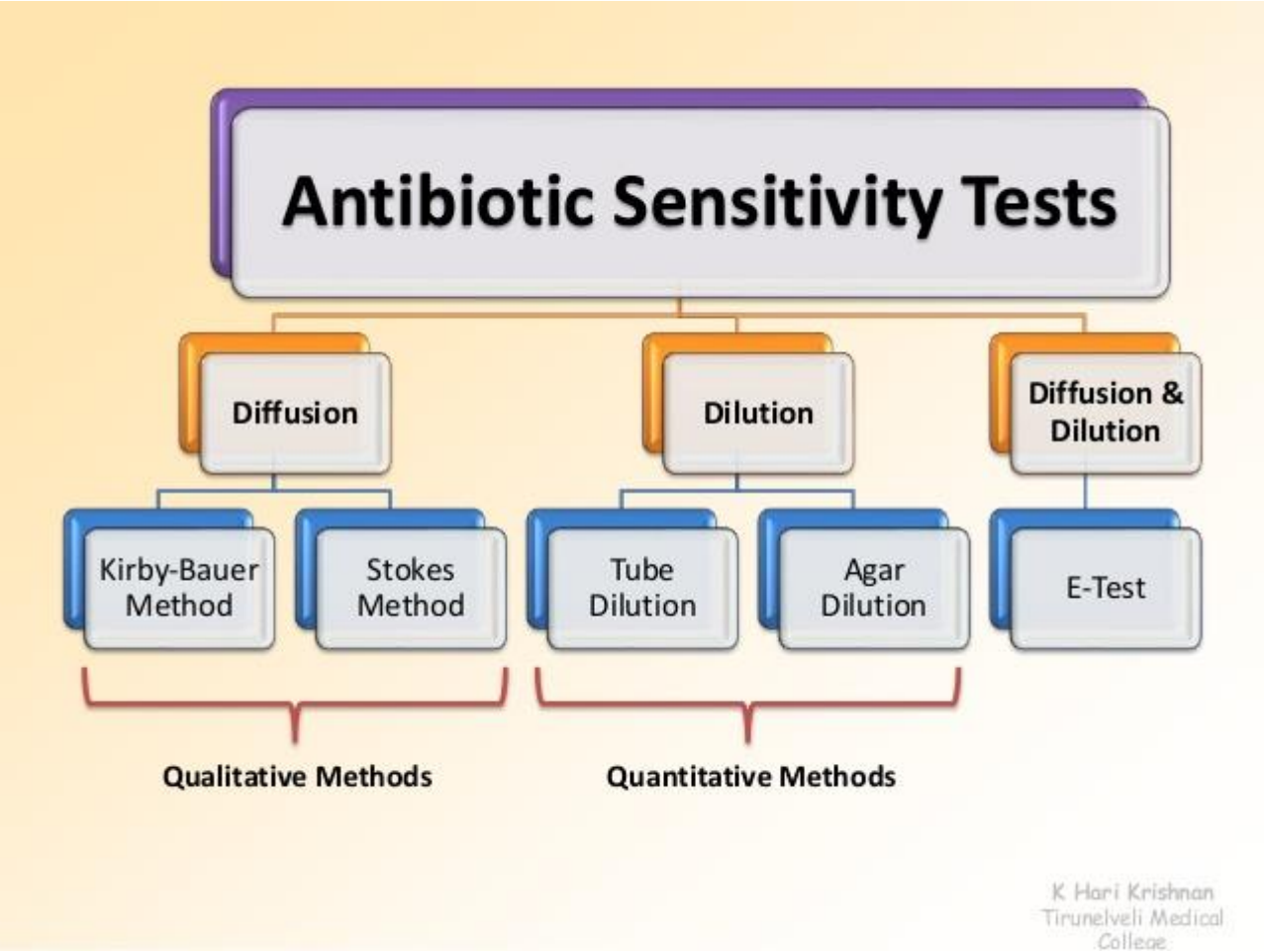
Lab 11+12 – Technique of antibiotic sensitivity



Methods of Antimicrobial Susceptibility Testing

1. Disk Diffusion method

2. Dilution that include (tube dilution method and agar dilution method)

3. Dilution and Diffusion method that include E- Test

4.1 Disk Diffusion Method

2. Reagents for the Disk Diffusion Test

1. Mueller-Hinton Agar Medium

2. Preparation of antibiotic stock solutions

Antibiotics may be received as powders or tablets. It is recommended to obtain pure antibiotics from commercial sources, and not use injectable solutions. Powders must be accurately weighed and dissolved in the appropriate diluents (Annexure III) to yield the required concentration, using sterile glassware. Standard strains of stock cultures should be used to evaluate the antibiotic stock solution. If satisfactory, the stock can be aliquot in 5 ml volumes and frozen at -20ºC or -60ºC.

Stock solutions are prepared using the formula:

$$W = \frac{1000}{P} X\ V\ x\ C$$

Where P = potency of the antibiotic base, **V**=volume in ml required, **C**=final concentration of solution and **W**=weight of the antimicrobial to be dissolved in V.

Preparation of dried filter paper discs

Whattman filter paper no. 1 is used to prepare discs approximately 6 mm in diameter, which are placed in a Petri dish and sterilized in a hot air oven. The loop used for delivering the antibiotics is made of 20 gauge wire and has a diameter of 2 mm. This delivers 0.005 ml of antibiotics to each disc.

Storage of commercial antimicrobial discs

Cartridges containing commercially prepared paper disks specifically for susceptibility testing are generally packaged to ensure appropriate anhydrous conditions. Discs should be stored as follows:

* Refrigerate the containers at 8°C or below, or freeze at -14°C or below, in a no frost-free freezer until needed. Sealed packages of disks that contain drugs from the ß-lactam class should be stored frozen, except for a small working supply, which may be refrigerated for at most one week. Some labile agents (e.g., imipenem, cefaclor, and clavulanic acid combinations) may retain greater stability if stored frozen until the day of use.
* The unopened disc containers should be removed from the refrigerator or freezer one to two hours before use, so they may equilibrate to room temperature before opening. This procedure minimizes the amount of condensation that occurs when warm air contacts cold disks.
* Once a cartridge of discs has been removed from its sealed package, it should be placed in a tightly sealed, desiccated container. When using a disc-dispensing apparatus, it should be fitted with a tight cover and supplied with an adequate desiccant. The dispenser should be allowed to warm to room temperature before opening. Excessive moisture should be avoided by replacing the desiccant when the indicator changes color.
* When not in use, the dispensing apparatus containing the discs should always be refrigerated.
* Only those discs that have not reached the manufacturer's expiration date stated on the label may be used. Discs should be discarded on the expiration date.

The test antibiotic immediately begins to diffuse outward from the disks, creating a gradient of antibiotic concentration in the agar such that the highest concentration is found close to the disk with decreasing concentrations further away from the disk. After an overnight incubation, the bacterial growth around each disc is observed. If the test isolate is susceptible to a particular antibiotic, a clear area of "no growth" will be observed around that particular disk.

The zone around an antibiotic disk that has no growth is referred to as the zone of inhibition since this approximates the minimum antibiotic concentration sufficient to prevent growth of the test isolate. This zone is then measured in mm and compared to a standard interpretation chart used to categorize the isolate as susceptible, intermediately susceptible or resistant. MIC measurement cannot be determined from this qualitative test, which simply classifies the isolate as susceptible, intermediate or resistant.

The agar diffusion method is widely used in industry for testing the sensitivity of micro-organisms to antibiotics, antiseptics, toothpaste, mouthwashes, disinfectants, etc. The method involves preparing a pour or spread plate of a test micro-organism, adding small amount of test substance to either a well cut in the agar medium or (preferably) a paper disc which is then placed on the agar surface. After incubation, an inhibitory effect on the test organism is indicated by a clear zone (no growth) around the test substance; microbial growth is visible to the naked eye in areas of the plate that are unaffected.

Materials

- Take a nutrient agar pour or spread/lawn plate of e.g. *Bacillus subtilis*, *Micrococcus luteus*, *Escherichia coli* on malt agar.
- Sterile filter paper discs
- Sterile distilled/demineralised water (control)
- Samples to be tested, 3 (e.g. mouthwashes, selected for a range of active ingredients)
- Bunsen burner
- Forceps
- Alcohol (70 % IDA) in a small beaker covered in foil (**Caution**: flammable, should be kept covered away from flames)
- Incubator at 25–30 °C (if available)

Procedure

Aseptic technique should be used throughout.

1. Mark and label four sections on the base of the Petri dish, for the three different samples and control (sterile water).

2. Using sterile forceps (flamed with alcohol and cooled) remove one filter paper disc. Dip into the first test sample, drain on the side of the container and place firmly onto the appropriate section of the seeded agar plate.

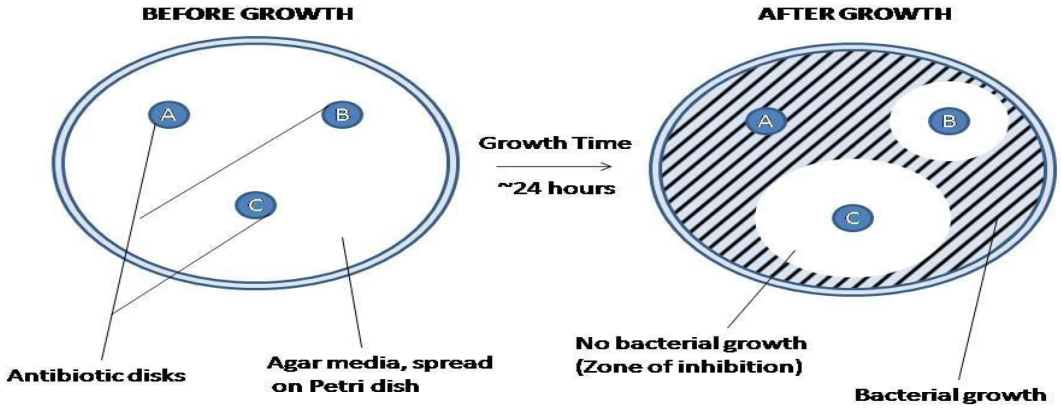
3. Wash the forceps free of the sample.

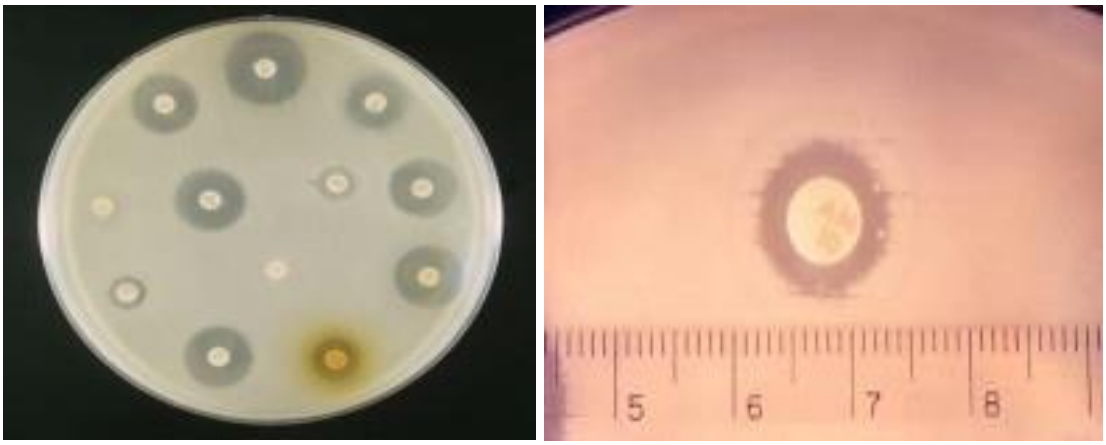
4. Repeat for the remaining samples and the control (sterile water). Remember to rinse and sterilise the forceps between each sample and to open the plate for the minimum possible time.

5. Seal the lid to the base with tape. *Incubation of the plate.*

6. Invert the plate and incubate at 25–30 °C or at room temperature for 48 hours.

7. Examine the plate (without opening). Measure and record the size of any zones of inhibition around the filter paper discs. Consider what factors might be affecting the size of the zones of inhibition.





On this agar plate, a bacterial isolate is tested for resistance to each of twelve different antibiotics. The clear zones around each disc are the zones of inhibition that indicate the extent of the test organism's inability to survive in the presence of the test antibiotic. (A)The disk shows a large zone of inhibition; whereas (B) shows no zone of inhibition, indicating resistance of the isolate to the test antibiotic.

Presence of zone of inhibition is not automatically interpreted as susceptibility to the antibiotic; the zone width has to be measured and compared against a reference standard which contains measurement ranges and their equivalent qualitative categories of

susceptible, intermediately susceptible or resistant. For example, this *E.coli* isolate on the right has a zone of inhibition of 10.1mm around ampicillin (AM); since the zone diameter interpretation chart is as follows:

Resistant: 13mm or less
Intermediate: 14-16 mm
Susceptible: 17 mm or more (this particular E.coli isolate is read as resistant to ampicillin).

2. Dilution method of antibiotic sensitivity test (Quantitative methods)

4.2 Dilution Methods

Dilution susceptibility testing methods are used to determine the minimal concentration of antimicrobial to inhibit or kill the microorganism. This can be achieved by dilution of antimicrobial in either agar or broth media. Antimicrobials are tested in $\log_2$ serial dilutions (two fold).

- **Minimum Inhibitory Concentration (MIC)**

Diffusion tests widely used to determine the susceptibility of organisms isolated from clinical specimens have their limitations; when equivocal results are obtained or in prolonged serious infection e.g. bacterial endocarditis, the quantitation of antibiotic action vis-a-vis the pathogen needs to be more precise. Also the terms 'Susceptible' and 'Resistant' can have a realistic interpretation. Thus when in doubt, the way to a precise assessment is to determine the MIC of the antibiotic to the organisms concerned. There are two methods of testing for **MIC**:

(a) Broth dilution method
(b) Agar dilution method.

1. Broth dilution method

The Broth dilution method involves subjecting the isolate to a series of concentrations of antimicrobial agents in a broth environment. Micro dilution testing uses about 0.05 to 0.1 ml total broth volume and can be conveniently performed in a microtiter format. Macro dilution testing uses broth volumes at about 1.0 ml in standard test tubes. For both of these broth dilution methods, the lowest concentration at which the isolate is completely inhibited (as evidenced by the absence of visible bacterial growth) is recorded as the **M**inimal **I**nhibitory **C**oncentration or (**MIC)**. The MIC is thus the minimum concentration of the antibiotic that will inhibit this particular isolate. The test is only valid if the positive control shows growth and the negative control shows no growth. The Broth Dilution method is a simple procedure for testing a small number of isolates, even single isolate. It has the added advantage that the same

tubes can be taken for MBC tests also.

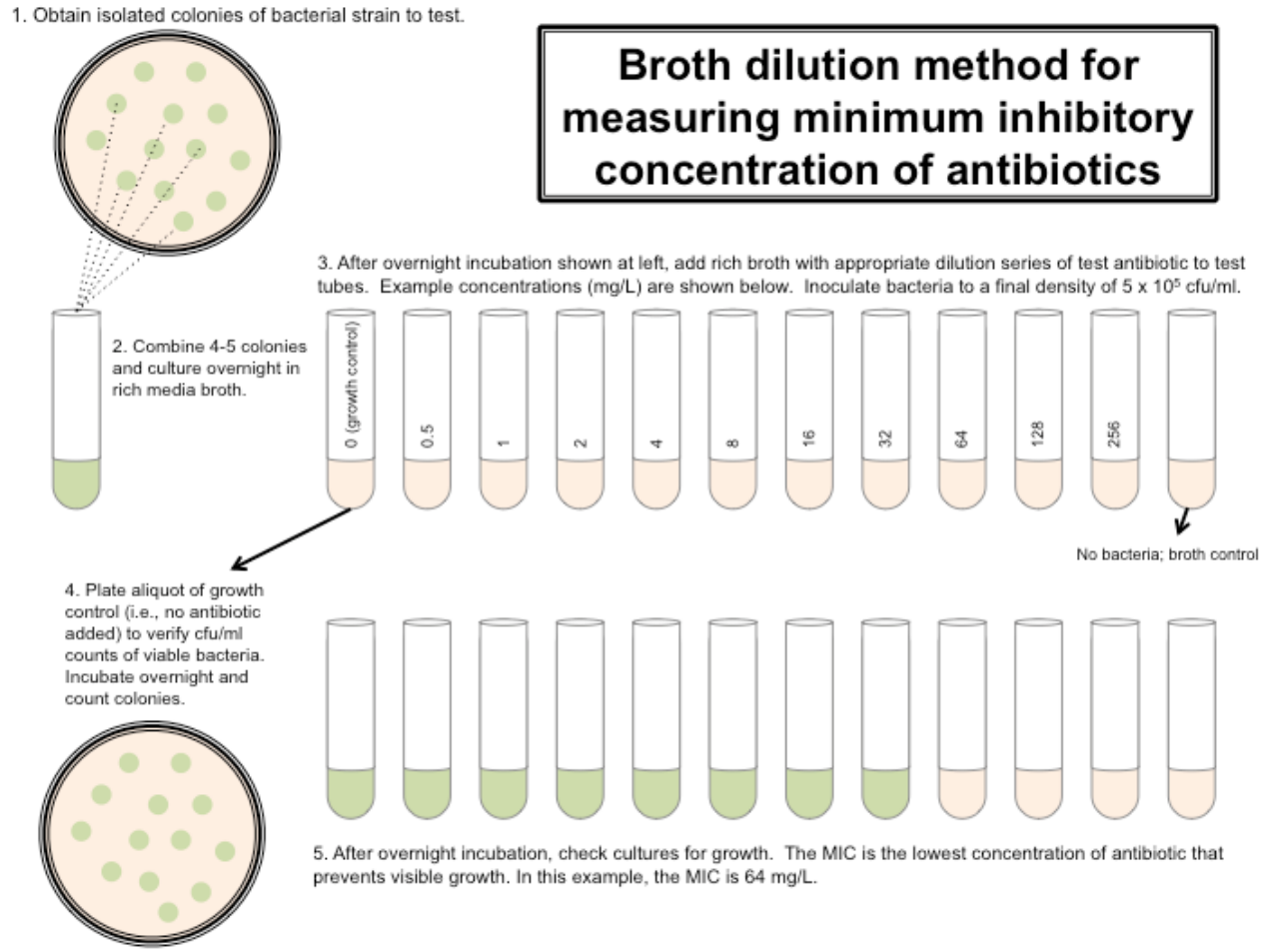


Figure 2: Tube dilution method

Minimum Bactericidal Concentrations (MBC)

The main advantage of the 'Broth dilution' method for the MIC determination lies in the fact that it can readily be converted to determine the MBC as well.

❖ Micro-broth dilution test

This test uses double-strength Mueller-Hinton broth, 4X strength antibiotic solutions prepared as serial two-fold dilutions and the test organism at a concentration of $2x10^6$/ml. In a 96 well plate, 100 µl of double-strength MHB, 50 µl each of the antibiotic dilutions and the organism suspension are mixed and incubated at 35°C for 18-24 hours. The lowest concentration showing inhibition of growth will be considered the MIC of the organism.

2. Agar dilution method

A procedure similar to broth dilution is agar dilution. Agar dilution method follows the principle of establishing the lowest concentration of the serially diluted antibiotic concentration at which bacterial growth is still inhibited.

3. E-test

E-test also known as the epsilometer test is an 'exponential gradient' testing methodology where 'E' in E test refers to the Greek symbol epsilon. The E test (AB Bio disk) which is a quantitative method for antimicrobial susceptibility testing applies both the dilution of antibiotic and diffusion of antibiotic into the medium.

This method provides for a convenient quantitative test of antibiotic resistance of a clinical isolate. However, a separate strip is needed for each antibiotic, and therefore the cost of this method can be high. E test can be used to determine MIC for fastidious organisms like S. pneumoniae, ß-haemolytic *streptococci*, N.*gonorrhoeae*, *Haemophilus* sp. and anaerobes. It can also be used for **n**one **f**ermenting **G**ram **N**egative **b**acilli (NFGNB) for e.g. *Pseudomonas* sp. and *Burkholderia Pseudomallei.*