جامعة بغداد

كلية العلوم

قسم التقنيات الاحيائية

المضادات الحياتية / الجزء العملي

المرحلة الثالثة

الفصل الثاني

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2022-2021

Antibiotics

Lab#1 (Introduction)

2021

learning objectives :

- A review about classification of bacteria
- What is an antibiotic?
- Differences between antibiotics and other antimicrobials (Antiseptics and Disinfectants)
- Sources of antibiotics
- Antibiotic classification
- Roles of Antibiotics: Bacteriostatic vs. Bactericidal
- Resistance to antibiotics

Bacterial classification

When bacteria are classified, there are many different considerations such as:

1. Morphology: for example, are the bacteria cocci or bacilli (rod-shaped) or coccobacilli.

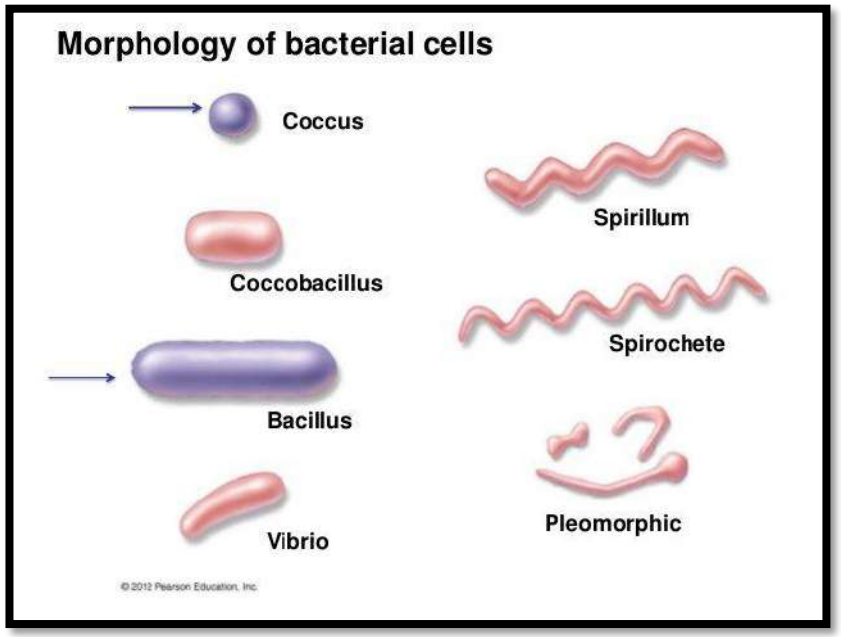
2. Staining:(ex: Gram stain) gram positive, gram negative, or not staining.
   - Gram - positive cocci: *Staphylococcus aureus*
   - Gram - negative rods : *E. coli*
   - Non- staining*: Mycoplasma*

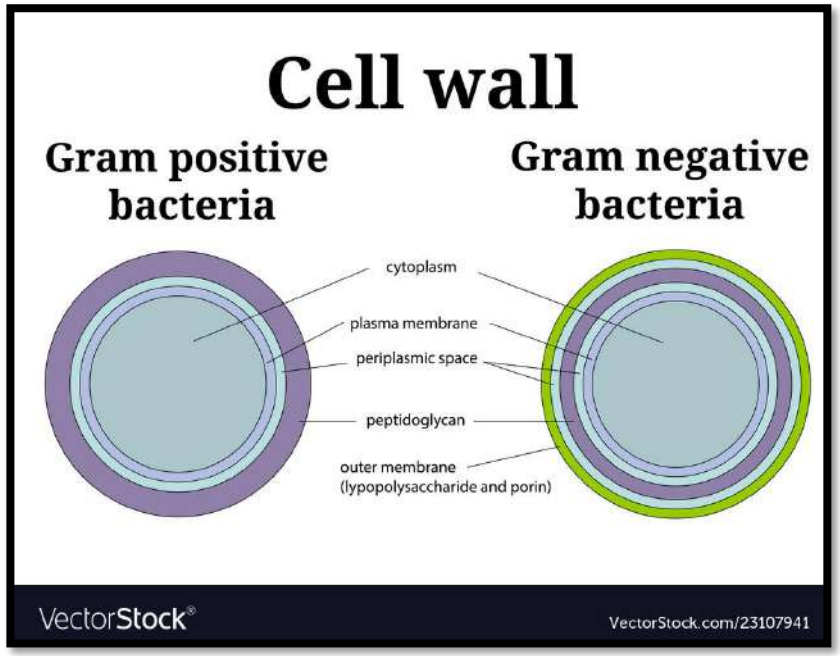
3. Growth requirements: for example, aerobic bacteria, anaerobic bacteria, or facultative anaerobic .

4. Biochemical reactions : for example, lactose fermenting or non-fermenting.

5. **Antibiotic resistance patterns** : for example, methicillin sensitive vs. methicillin resistant strains.

6. Others.





Antimicrobials

Antimicrobial: Any substance that inhibits the growth and replication of a bacterium or kills it.

Antibiotics: Any type of antimicrobial designed to target bacterial infections within (or on) the body.

❖**Antibiotics differ from other kinds of antimicrobials, such as below antimicrobials:**

- **Antiseptics:** are antimicrobials used to sterilize surfaces of living tissue when the risk of infection is high, such as during surgery.

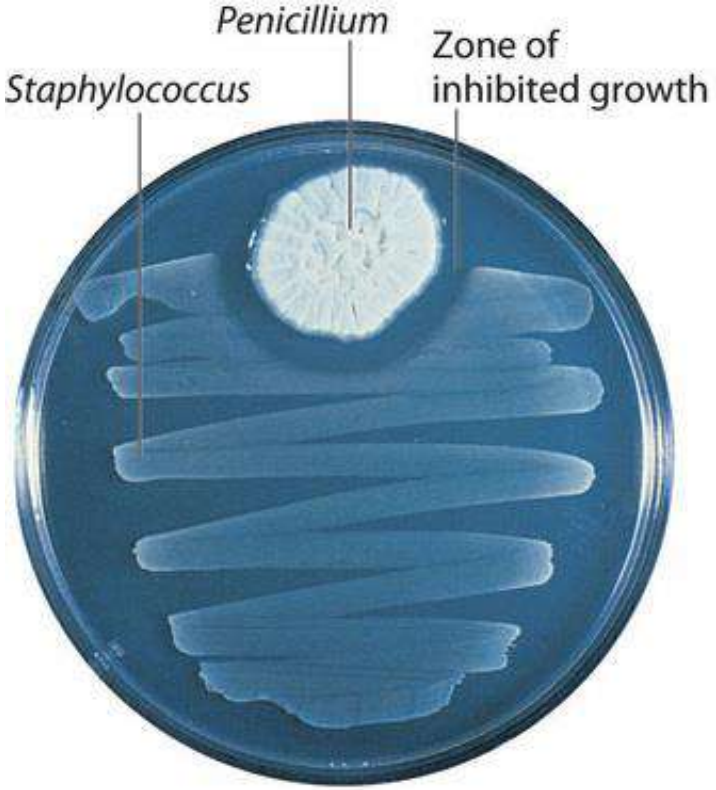
- **Disinfectants:** are non-selective antimicrobials, killing a wide range of micro-organisms including bacteria. They are used on non-living surfaces, for example in hospitals and laboratories.
- An **antiseptic** is applied to the body, while **disinfectants** are applied to nonliving surfaces, such as **countertops** and **doorknobs**. In a surgical setting, for example, a doctor will apply an **antiseptic** to the surgical site on a **person's body** and use a **disinfectant** to sterilize the **operating table**.

Antimicrobials

- Only substances that target bacteria are called **antibiotics.**
- Antibiotics **don't** affect **viruses**, **fungi**, or **parasites** - they only bind to bacterial cell targets, so they only affect bacterial cells.
- Fungi are targeted by **antifungals**.
- Viruses are targeted by **antivirals.**
- The name **antimicrobial** is a term for anything that inhibits or kills microbial cells including antibiotics, antifungals, antivirals and chemicals such as antiseptics.

First discovered antibiotic

- In 1928, Alexander Fleming discovered the first antibiotic "**Penicillin"**
- He noticed that colonies of the bacterium *staphylococcus aureus* wouldn't grow near some fungus (*penicillium notatum).*
- The fungus was making small molecules which leaked into the petri gel around it. He realized that the mold juice was killing the bacteria in the area!



Sources of antibiotics

- **Natural**:
  - Fungal sources (Penicillin)
  - Bacterial source (polymyxin isolated from some Bacillus sp.)

- **Semi synthetic**: chemically altered natural compound (ex: Ampicillin)

- **Synthetic**: chemically designed in the lab (ex: Moxifloxacin)

Antibiotic classification

Antibiotics are classified based on:

1. **Structure** (ex: B-lactams antibiotics contain Beta-lactam ring)
2. **Mode of action**
    - Disrupt bacterial cell envelope
    - Inhibit bacterial proteins synthesis
    - Inhibit bacterial DNA replication
3. **Spectrum of activity**:
    - Narrow spectrum
    - Broad spectrum

Antibiotics nomenclature

An antibiotic can have many different trade names depending on the manufactures. Therefore, antibiotics can be identified by at least three names:

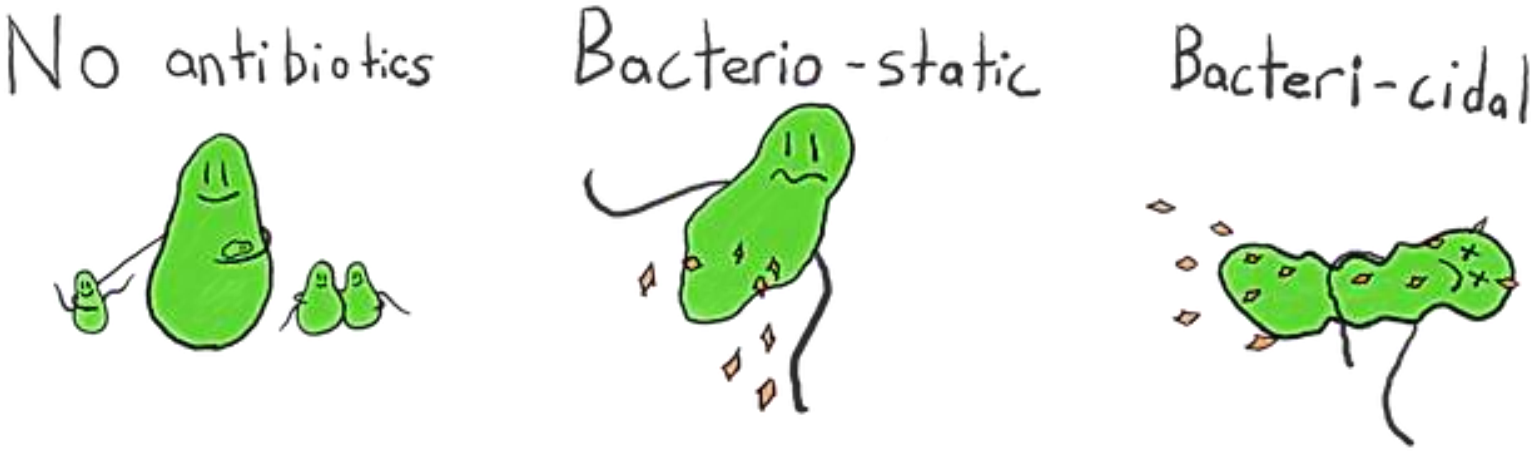
1. Chemical name: used in scientific and medical literature (usually long form name)

2. Common name: used commonly (usually shorter than chemical name).

3. Brand name: the name given by the manufacture to distinguish it from other companies' product.

Roles of Antibiotics

- **Bacteriostatic** effects: to prevent growth or reproduction of bacteria. Examples:
    - Tetracyclines
    - Trimethoprim
- **Bactericidal** effects: to kill (destroy) the bacteria. Examples:
    - Cephalosporins
    - Penicillin



Resistance to antibiotics

When bacteria start to adapt (developed resistance mechanisms) to the antibiotics and become harder to kill. That's called **antibiotic resistance**

- Some bacteria can naturally resist certain kinds of antibiotics.
- Some bacteria can become resistant if their genes change or they get drug-resistant genes from other bacteria.

The main resistance mechanisms are:

1. Modify the antibiotic
2. Modify the target of the antibiotic
3. Destroy the antibiotic
4. Prevent the antibiotic to get into the cell
5. Actively remove the antibiotic from the cells

Antibiotic Sensitivity Testing

Lab-2-

Antibiotic Sensitivity Testing

Bacteria demonstrate two kinds of resistance to antibiotics

- **Intrinsic resistance:** bacteria naturally resist certain kinds of antibiotics.
- **Acquired resistance:** bacteria was originally susceptible to an antibiotic, but later became resistant either by mutation or through getting drug-resistant genes from other bacteria.

- Because of the acquired resistance, bacterial isolates must be subjected to antibiotic susceptibility/sensitivity testing.
- Bacteria showing reduced susceptibility or resistance to an antibiotic implies that it should not be used on the patient.

Antibiotic Sensitivity Testing

- Sensitivity testing, also called susceptibility analysis:

  A test determines the “sensitivity” of bacteria to an antibiotic. It also determines the ability of the drug to kill the bacteria.

The purposes of antibiotic sensitivity test:

- To determine bacterial susceptibility to the antimicrobial agents.
- Help doctor to determine which drugs are most effective in treating infection.

Antibiotic sensitivity test types

1- **Phenotypic methods**

Testing based on exposing bacteria to antibiotics uses agar plates or dilution in agar or broth, such as:

- Disc diffusion (The Kirby-Bauer Disc Method)
- Broth dilution (The Minimum Inhibitory Concentration (MIC) Method)

2- **Genetic methods**

Genetic testing used to detect whether bacteria possess genes which confer antibiotic resistance, such as:

- Polymerase chain reaction (PCR)
- DNA microarray

Disc diffusion (The Kirby-Bauer Disc Method)

Agar disc diffusion method, is one of the oldest methods of antimicrobial susceptibility testing and remains one of the most popular manual techniques in clinical microbiology laboratories.

The main advantages are **simplicity**, **reproducibility**, the possibility for **use as a screening test against numerous isolates**, and **low cost**.

In this method the bacterial isolate is spread on an agar (frequently Mueller Hinton agar) plate and then paper disc containing specific concentration of antibiotics are placed and incubated at 37°C overnight.

Mueller Hinton Agar

It is more commonly used for the routine susceptibility testing. Mueller Hinton Agar has become the standard medium for the Bauer Kirby method.

Principle of Mueller Hinton Agar

Mueller Hinton Media contains **Beef Extract**, **Acid Hydrolysate of Casein**, **Starch** and **Agar**. Beef Extract and Acid Hydrolysate of Casein provide nitrogen, vitamins, carbon, amino acids, sulphur and other essential nutrients. Starch is added to absorb any toxic metabolites produced. Starch hydrolysis yields dextrose, which serves as a source of energy. Agar is the solidifying agent.

Disc diffusion (The Kirby-Bauer Disc Method)

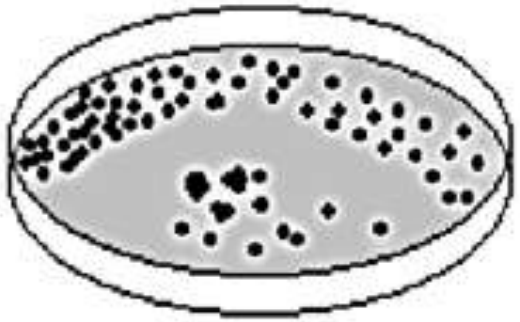
- If the bacterial isolate is susceptible to the antibiotic, it does not grow around the disk thus forming a **zone of inhibition**.
- If the bacterial isolate is resistant to the antibiotic, it grows up to the margin of disk.
- The diameter of zone of inhibition must be measured and result read from the Kirby Bauer chart as **sensitive**, **intermediate**, or **resistant.**

Laboratory supplies

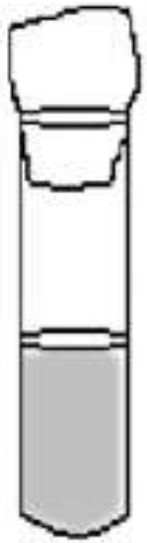
Kirby-Bauer:

- Petri dishes with Mueller-Hinton agar
- Bacterial cultures
- Test tube
- 95% Ethanol
- forceps
- Antibiotic discs 1 of each kind
- Sterile Swabs

Disk diffusion method

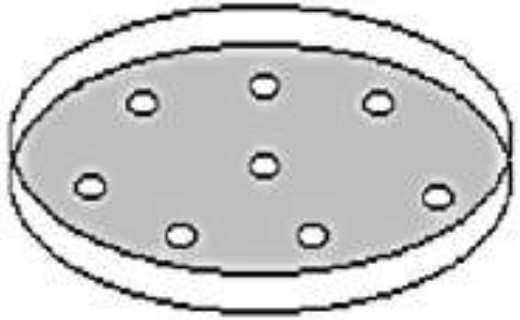


(1) 2-3 identical colonies are picked from the plate and transferred to the broth



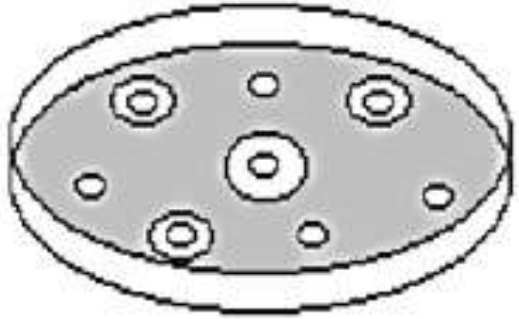
(2)
The tube is incubated for the bacteria to grow.
The inoculum density is standardized using McFarland standard

(3)
A cotton swab dipped in the inoculum suspension is swabbed over the entire surface of agar to give a lawn culture



(4)
Filter paper disks containing known antibiotic in known concentration is placed on the surface of inoculated agar

Plate incubated at 37°C overnight



(5)
Zone diameter around the disk are measured and result read from Kirby Bauer chart

www.microrao.com

Disc diffusion (The Kirby-Bauer Disc Method)

1. Obtain 2 plates contain the cultures of E. coli and Staph. aureus.

2. Obtain a swab and dip it into the E. coli broth culture. Roll the swab against the inside of the tube to remove excess liquid.

3. Streak one of the plates with the swab in even strokes to obtain a uniform growth pattern across the entire surface of the plate. Rotate the plate 90 degrees and using the same swab, streak the plate again. Rotate the plate 45 degrees and reswab. Replace the lid. Discard the swab. Label the plate.

4. Repeat the above procedure for Staph. aureus with a new plate.

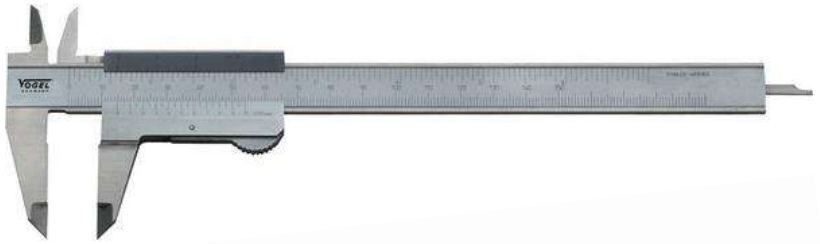
5. Allow the plates to dry for 2-5 minutes.

6. Remove the forceps from the alcohol beaker and pass through the flame of a Bunsen burner. When all the alcohol has burned off, use the sterile forceps to aseptically remove one of each antibiotic disc from the dispenser and place it on each plate. You can draw pie lines on the back to divide each plate into 6 sections. The antibiotic discs used are: gentamicin, tetracycline, penicillin G, chloramphenicol, ampicillin and erythromycin.

7. Repeat the alcohol-flame sterilization of the forceps and tap each disc gently onto the plate.

8. Replace the lid and invert the plate. Complete the label at the bottom of plates and incubate at 37°C for 2 days.
9. Record the results by measuring the diameters of the zone of inhibition (ZOI). The data is recorded and interpreted using tables supplied at the introduction section of this lab exercise.

Disc diffusion (The Kirby-Bauer Disc Method)



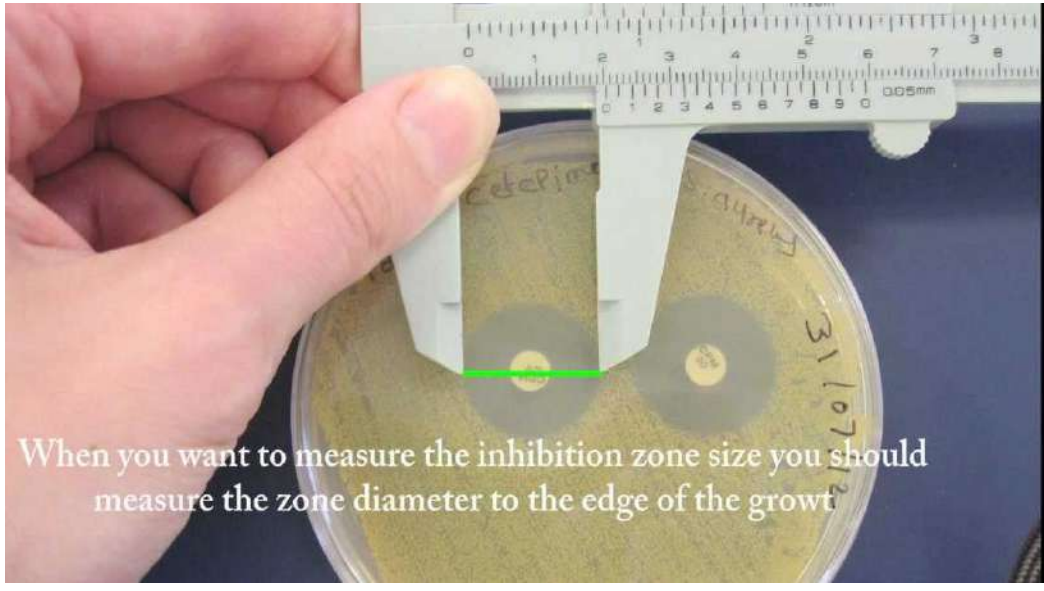
Sliding calipers

Reading and Interpretation

After incubation, the plates are examined and the diameter of the zones of inhibition is measured to the nearest whole millimeter by use of **sliding calipers**, a ruler, or a template prepared for this purpose.

When supplemented medium is used, the measuring device is held on the back of the petri plate, which is illuminated with reflected light. The end point by all reading systems is complete inhibition of growth as determined visually.

The zone diameters for individual antibiotics are translated into prefixed **susceptible**, **intermediate,** or **resistant** categories by referring to an interpretative table.



Interpretation of zones of inhibition (in mm) for Kirby-Bauer antibiotic susceptibility test.

Antibiotic	Disk Conc.	Diameter of zone of inhibition (ZOI)		
		Resistant	Intermediate	Susceptible
Amikacin	10 μg	≤11	12-13	≥14
Ampicillin	10 μg	≤11	12-13	≥14
Bacitracin	10 units	≤8	9-11	≥13
Cephalothin	30 μg	≤14	15-17	≥18
Chloramphenicol	30 μg	≤12	13-17	≥18
Clindamycin	2 μg	≤14	15-16	≥17
Erythromycin	15 μg	≤13	14-17	≥18
Gentamicin	10 μg	≤12	13-14	≥15
Kanamycin	30 μg	≤13	14-17	≥18
Lincomycin	2 μg	≤9	10-14	≥15
Methicillin	5 μg	≤9	10-13	≥14
Nalidixic acid	30 μg	≤13	14-18	≥19
Neomycin	30 μg	≤12	13-16	≥17
Nitrofurantoin	0.3 mg	≤14	15-16	≥17
Penicillin				
vs. staphylococci	10 units	≤20	21-28	≥29
vs. other organisms	10 units	≤11	12-21	≥22
Polymyxin	300 units	≤8	9-11	≥12
Streptomycin	10 μg	≤11	12-14	≥15
Sulfonamides	0.3 mg	≤12	13-16	≥17
Tetracycline	30 μg	≤14	15-18	≥19
Vancomycin	30 μg	≤9	10-11	≥12

- https://www.youtube.com/watch?v=BXr_kcki4Ag

Antibiotic Sensitivity Testing

The Minimum Inhibitory Concentration (MIC) Method

Lab-3-

2021

Minimum **I**nhibitory **C**oncentration (**MIC**): The lowest concentration of antimicrobial agent that still inhibits the growth of a particular organism

- MIC can be determined using **serial dilution methods**

❑Importance of MIC:

✓To establish the concentration of an antibiotic that is effective in preventing the growth of the pathogen.

✓To give an indication of the dosage of that antibiotic that should be effective in controlling the infection in the patient.

✓It is important to use the lowest effective concentration of the antibiotic to avoid toxicity in patient.

Methods for determination of MIC

1- Serial dilution method:

a. Broth dilution method

b. Agar dilution method

2- Agar diffusion method

a. Cup plate technique

b. Gradient plate technique

c. Ditch plate technique

a. Broth dilution method

Dilution & Dilution Factor (DF)

- **Dilution:** Refers to the process of adding additional solvent to a solution to decrease its concentration.

- Dilution process keeps the amount of solute constant, but increases the total amount of solution, thereby decreasing its final concentration.

- **Dilution factor:**

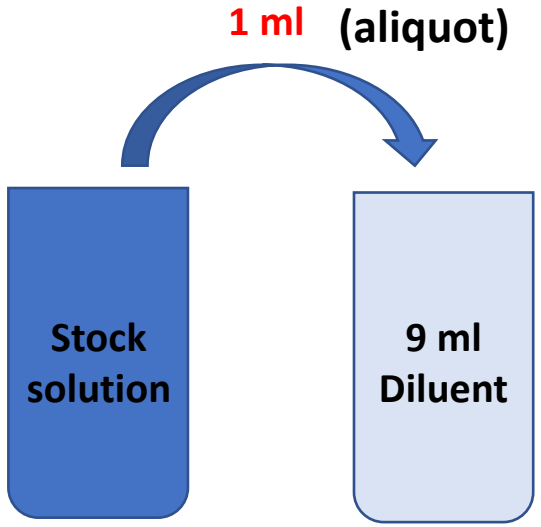
Refers to the ratio of the volume of the initial (concentrated) solution (V1) **(called an aliquot)** to the volume of the final (diluted) solution (V2). The ratio of V1 to V2, or, V1 : V2.

- A dilution factor (DF) can be calculated: **DF = V2 ÷ V1**

Note:

Stock solution = Concentrated solution = solute
Working solution = diluted solution
Solvent = diluent (such as: water, diluent broth, etc.)

Dilution examples



Volume of Stock Solution (aliquot) = 1 ml

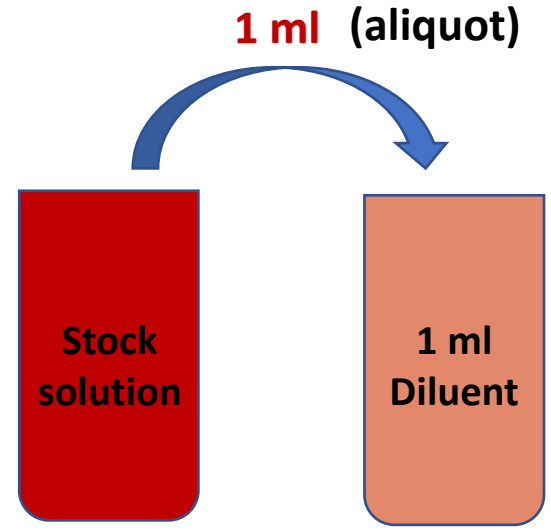
Final vol. (stock + diluent) = 1+ 9 ml = 10 ml

DF = V2 ÷ V1, DF= 10 ÷ 1 = 10

You have diluted the sample by a factor of 10.

DF of 10 means a 1:10 dilution, **(Ten-fold dilution)**

A **ten-fold dilution** reduces the concentration of a solution by a factor of ten that is to one-tenth the original concentration. A series of ten-fold dilutions is described as ten-fold serial dilutions.



Volume of Stock Solution (aliquot) = 1 ml

Final vol. (stock + diluent) = 1+ 1 ml = 2 ml

DF= 2÷ 1 = 2

You have diluted the sample by a factor of 2.

DF of 2 means a 1:2 dilution, **(Two-fold dilution)**

A **two-fold dilution** reduces the concentration of a solution by a factor of two that is reduces the original concentration by **one half (½).** A series of two-fold dilutions is described as two-fold serial dilutions.

a. Broth dilution method

MIC Principle: A standardized microbial inoculum is added to the tubes containing serial dilutions of an antibiotic, and the growth of the microorganism is monitored as a change in turbidity.

Antibiotic

(Dilution series)

+

Bacterial suspension

⬇

Growth???

a. Broth dilution method

Serial dilution: Basics

MIC Laboratory supplies

- Sterile Mueller Hinton Broth (why Mueller Hinton?)
- Micropipette
- Empty sterile test tubes
- Tube of 4ml antibiotic (at **1000 µg/ml**)
- Tube of E.coli culture
- Make a basic serial dilution across 5 test tubes

❑ Prepare antimicrobial agent stock solutions at concentrations of at least 1000 µg/ml.

❑ Preparation of inoculum: Prepare the inoculum by making a direct broth suspension of isolated colonies selected from an 18-24 hour agar plate.

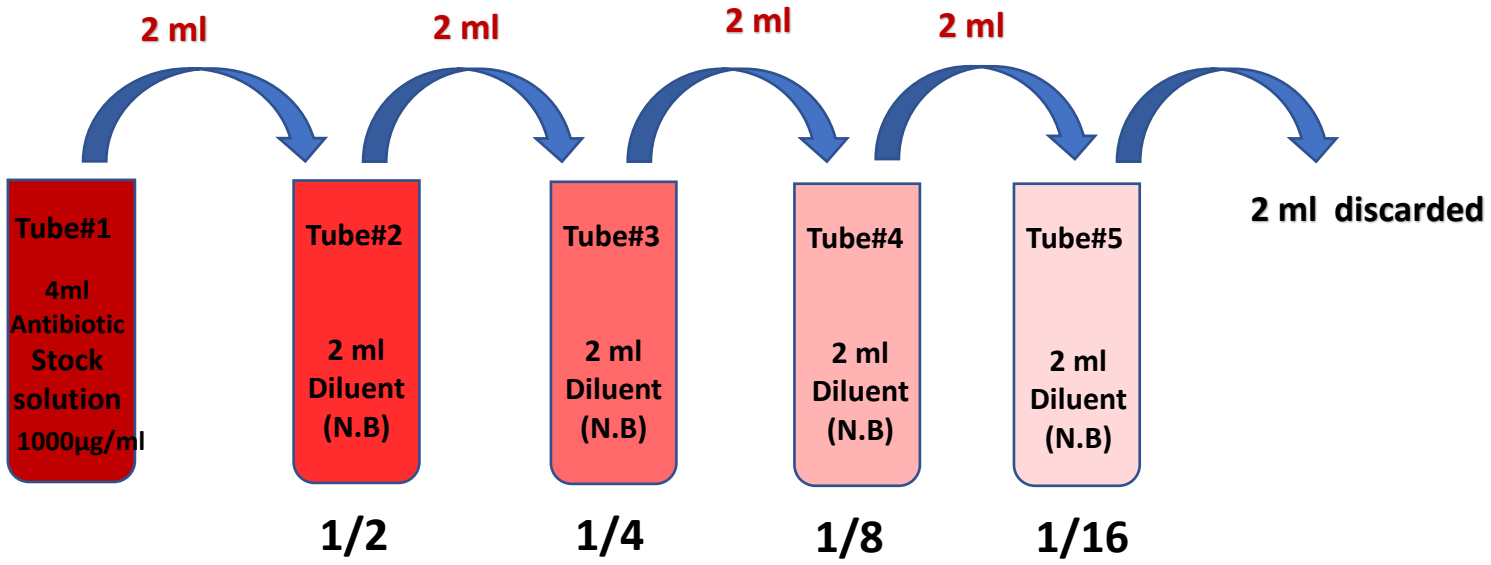
Protocol:

1- Label the antibiotic test tube as tube # 1 (which is the solute or the concentrated)

2- Label the other empty test tubes as 2, 3, 4, 5, +Ve control, -Ve control.

3- Prepare **two-fold serial dilution f**or the antibiotic as bellow:

- Transfer 2ml of nutrient broth (N.B) into each empty tube that labeled as 2, 3, 4, 5, +Ve control, and – Ve control.
- Transfer 2 ml from tube 1 to tube 2, mix well and from 2 to 3 , mix well and from 3 to 4, mix well and from 4 to 5 , mix well then discard 2 ml from test tube 5.



DF= 4 ÷ 2 = 2

DF of 2 means a 1:2 dilution, **(Two-fold dilution)**

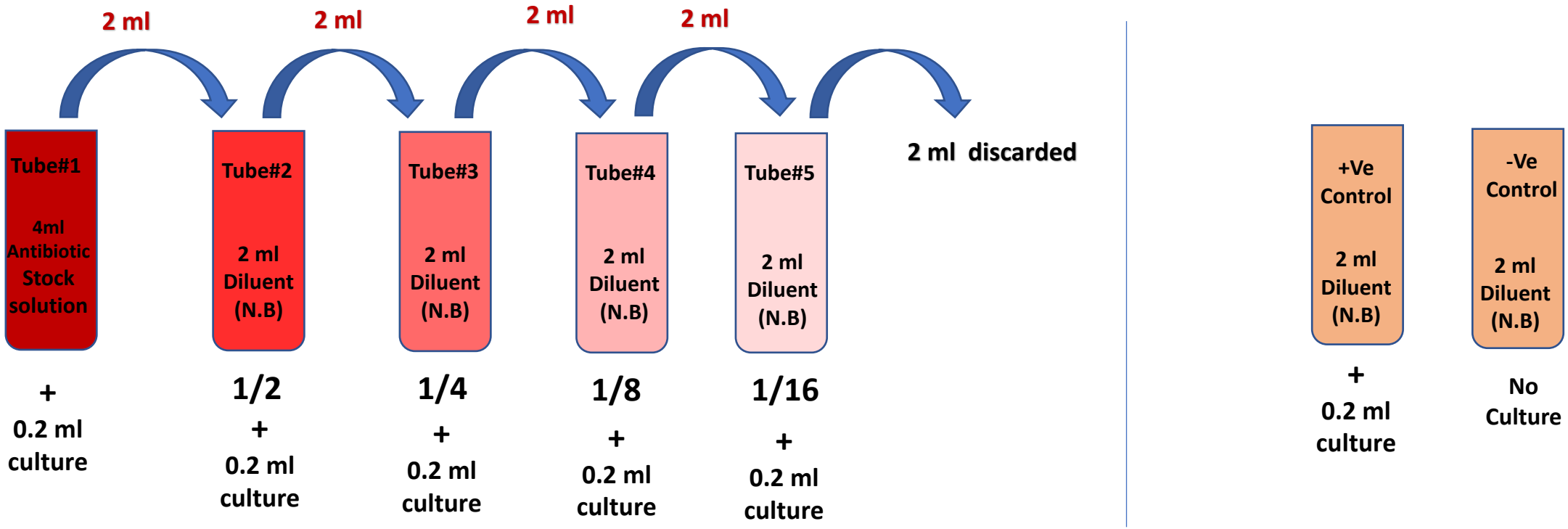
A **two-fold dilution** reduces the concentration of a solution by a factor of two that is reduces the original concentration by **one half (½).** A series of two-fold dilutions is described as two-fold serial dilutions.

Protocol:

4- Add 0.2 ml culture to all tubes (tubes 1, 2, 3, 4, 5, & +Ve control) except –Ve control.

5- Incubate the tubes at 37°C for 24 hours and determine the bacterial growth.

6- Calculate the MIC.



Controls

❑Positive control

- Broth + culture
- Test the growing ability of the microorganism and the medium
- Result: turbid

+Ve Control

2 ml Diluent (N.B)

+

0.2 ml culture

❑Negative control

- Broth only
- Test the sterility of the medium and used equipment
- Result: clear

-Ve Control

2 ml Diluent (N.B)

No Culture

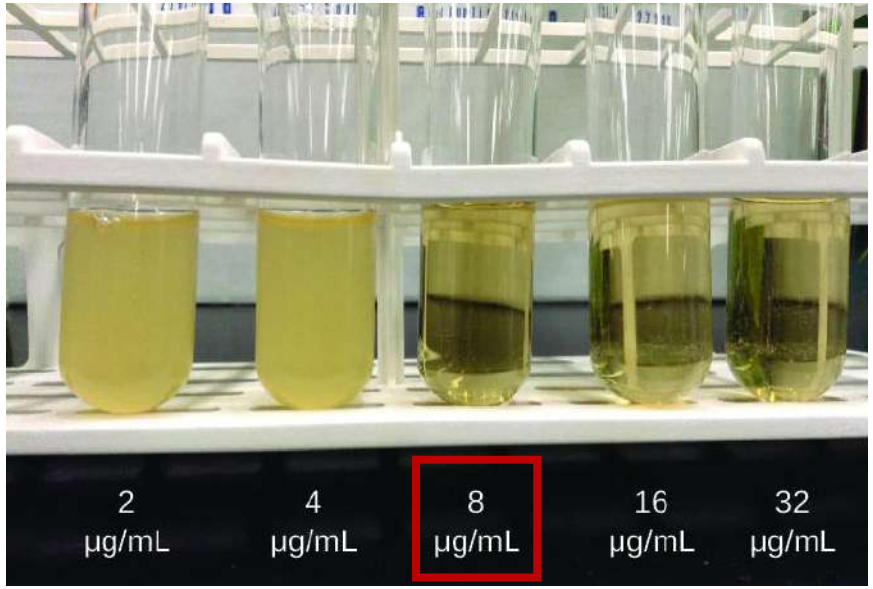
Reading results and interpretation

- After incubation, broth tubes that appear turbid are indicative of bacterial growth while tubes that remain clear indicate no growth.

- The amount of growth in each tube is compared with that in the positive control and the MIC recorded as the lowest concentration of the antibiotic that completely inhibits growth.

- A lower MIC value indicates that less drug is required for inhibiting growth of the organism; therefore, drugs with lower MIC scores are more effective antimicrobial agents.

Example: In a dilution test, the lowest dilution that inhibits turbidity (cloudiness) is the MIC. In this example, the MIC is 8 µg/mL.
Broth from samples without turbidity can be inoculated onto plates lacking the antimicrobial drug.



https://chromoscience.com/2020/04/26/the-dilution-tests-of-antimicrobials/

Trouble shooting

- If all tubes **turbid**:
  - MIC is higher than the highest concentration of the antibiotic in tube #1?
  - Resistant organism?
  - Antibiotic not working?

- If all tubes **clear** except +Ve control tube
  - MIC is less than the lowest concentration of the antibiotic in tube #5?

Control of Microorganisms growth by Chemical Agents

Disinfectants and Antiseptics

Lab # 4

2021

Antimicrobial agents: disinfectants and antiseptics

From Lab #1

❖**Antibiotics differ from other kinds of antimicrobials, such as below antimicrobials:**

- **Antiseptics:** are antimicrobials used to sterilize surfaces of living tissue when the risk of infection is high, such as during surgery.

- **Disinfectants:** are non-selective antimicrobials, killing a wide range of micro-organisms including bacteria. They are used on non-living surfaces, for example in hospitals and laboratories.
- An **antiseptic** is applied to the body, while **disinfectants** are applied to nonliving surfaces, such as **countertops** and **doorknobs**. In a surgical setting, for example, a doctor will apply an **antiseptic** to the surgical site on a **person's body** and use a **disinfectant** to sterilize the **operating table**.

Factors that affect the antimicrobial action of disinfectants & antiseptics

1. Concentration and potency of disinfectants or antiseptics.

2. Physical and chemical factors can affect the decontaminate procedures, such as:

- **Temperature:** The temperature at which the agent is being used. Generally, the lower the temperature, the longer it takes to disinfect or decontaminate.
- **pH:** The pH influences the antimicrobial activity by altering the disinfectant molecule or the cell surface. An increase in pH improves the antimicrobial activity of some disinfectants (ex. glutaraldehyde) but decreases the antimicrobial activity of others (ex. iodine).
- **Relative humidity:** is the only important factor affecting the activity of gaseous disinfectants/sterilant, such as chlorine dioxide, and formaldehyde.

3. The kinds of microorganisms: Endospore producers' microorganisms are harder to eliminate. **An endospore** is a dormant, tough, and non-reproductive structure produced by some bacteria such as Bacillus species and Clostridium species. It is a dormant form to which the bacterium can reduce itself, that is usually triggered by a lack of nutrients.

4. Microorganisms number: The more microorganisms present, the harder it is to decontaminate.

5. Nature of the material that bearing the microorganisms. For example, organic matter in the form of blood, pus, or fecal can interfere with the activity of an antimicrobial agent. Interference may occur by a chemical reaction between the antimicrobial agent and the organic matter resulting in a complex that is less effective. Chlorine and iodine disinfectants are examples to such interaction.

6. Duration of Exposure: Items must be exposed to the antimicrobial agent for the proper time period.

Antimicrobial modes of action (disinfectants and antiseptics)

1. The active ingredients in antiseptics and disinfectants interact with the microorganism cell surface followed by penetration into the cytoplasm and action on cellular targets.

2. They may damage the lipids and/or proteins of the semipermeable cytoplasmic membrane of microorganisms resulting in leakage of cellular materials which are important for microorganism life.

3. They may denature enzymes and other proteins of the microorganisms, usually by disrupting the hydrogen and disulfide bonds that give the protein its three-dimensional functional shape. This blocks metabolism of the microorganism.

Chemical agents used as disinfectant or antiseptics

Some groups are listed below:

1. **Phenol and phenol derivatives:** Phenol and phenolics (phenol derivatives) alter membrane permeability and denature proteins. Such as Bisphenols and biguanides.

2. **Soaps and detergents**

Soaps aid in the mechanical removal of microorganisms by breaking up the oily film on the skin and reducing the surface tension of water so it spreads and penetrates more readily.

Detergents, such as laundry powders, mechanically remove microorganisms but are not very microbicidal. Some detergents alter membrane permeability and denature proteins.

3**. Alcohols:** 70% solutions of ethyl or isopropyl alcohol are effective in killing vegetative bacteria, enveloped viruses, and fungi. Alcohols denature membranes and proteins.

4. **Acids and alkalies:** alter membrane permeability and denature proteins and other molecules.

5. **Heavy metals:** such as mercury, silver, and copper, can denature proteins of microorganisms.

6 . **Chlorine:** Denature microbial enzymes. Chlorine is used in the chlorination of drinking water, swimming pools, & others.

7. **Iodine:** Iodine denatures microbial proteins.

8**. Aldehydes:** denature microbial proteins, such as formaldehyde and glutaraldehyde.

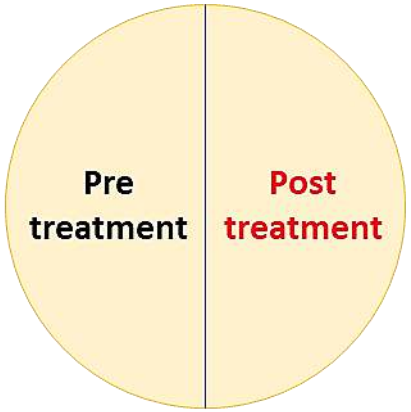
9. **Peroxygens:** such as hydrogen peroxide and peracetic acid.

- **Hydrogen peroxide** is broken down into water and oxygen by the enzyme catalase in human cells and is not that good of an antiseptic for open wounds but is useful for disinfecting inanimate objects. The high concentrations of hydrogen peroxide overwhelm the catalase found in microbes.
- **Peracetic acid** is a disinfectant that kills microorganisms by oxidation and subsequent disruption of their cytoplasmic membrane. It is widely used in healthcare, food processing, and water treatment.

Experiment (Testing the Effectiveness of some antimicrobial agents)

Supplies per group: 3 agar plates, 2 sterile cotton swabs, 1 test tube of broth

Protocol: (Day 1)



1. Prepare lab bench and clean surface with table disinfectant.
2. Divide your plate in half, labeled one half as pre-treatment, the other half as post treatment.
3. Dip one cotton swab into the broth and press the swab against the inside of the tube to remove excess broth.
1. Rub sterile cotton swab over your palm and between your fingers of one of your hands.
2. Roll the cotton swab over the half of plate labeled pre-treatment.
3. Select an antiseptic treatment (each group will have one washing with soap treatment and/or one hand sanitizer treatment). Treat your hand and let dry for few minutes.
4. Use the second cotton swab, as above, dip, squeeze and then swab the second hand (the one that you treated) following similar steps you did with the first hand.

5. Roll the swab over the other half of the plate labeled post-treatment.

6. Incubate all plates at 37℃ for 2 days or 25℃ for 5 days.

After 2 days:

1. Obtain your plate from the incubator.
2. Count all colonies on the half-labeled pre-treatment. Record your result.
3. Count all colonies on the half-labeled post-treatment. Record your result.
4. Calculate the **percent reduction** from pre-treatment to post-treatment.

You can determine this by taking the difference in colony numbers and dividing by the number in the pre-treatment sample. Then multiply by 100 to get the percent reduction.

Example: If you had 10 colonies from the pre-treatment, and 6 colonies from the post treatment, then you would have the difference of 4 colonies ➡ 10 – 6 = 4 ➡ Take 4 divide by 10 ➡ 4/10=0.4 , multiply by 100 0.4*100= 40 this would be **40% reduction**.

5. Compare your result with the other treatments in your group.

Beta Lactam Antibiotics

Lab #5

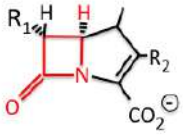
2021

1

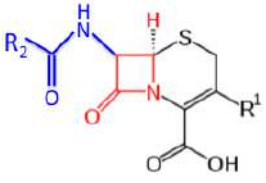
β-lactam antibiotics (beta-lactam antibiotics)

A class of antibiotic consisting of all antibiotic agents that contain a beta-lactam ring in their molecular structures. Beta-lactam antibiotics include:

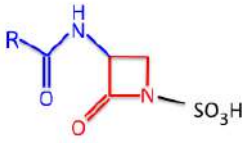
penicillins



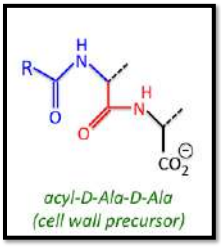
carbapenems



cephalosporins



monobactams



acyl-D-Ala-D-Ala
(cell wall precursor)

2

β-lactam antibiotics mode of action

- Act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls.
- The peptidoglycan layer is important for cell wall structural integrity.
- The final step in the synthesis of the peptidoglycan is facilitated by penicillin binding protein (PBP, an enzyme), removes the terminal alanine (D-Ala-D-Ala) in the process of forming a cross-link with a nearby peptide.
- The structural similarity between β-lactam antibiotics and d-alanyl-d-alanine facilitates their binding to the active site of PBPs.
- This irreversible inhibition of the PBPs prevents the final crosslinking (transpeptidation) of the nascent peptidoglycan layer, disrupting cell wall synthesis.

3

Enzymatic hydrolysis of the β-lactam ring

- ß-Lactamases are the main cause of bacterial resistance to penicillins and cephalosporins
- If the bacteria produces the enzyme β-lactamase, the enzyme will hydrolyse the β-lactam ring of the antibiotic, making the antibiotic ineffective.
- Bacteria often develop resistance to β-lactam antibiotics by synthesizing a β-lactamase.
- To overcome this resistance, β-lactam antibiotics are often given with β-lactamase inhibitors such as clavulanic acid.

4

Beta-lactamase inhibitors

- Resemble B-lactam antibiotic structure
- Bind to B-lactamase and protect the antibiotic from destruction
- Three important in medicine:
  - Clavulanic Acid
  - Sulbactam
  - Tazobactam

5

Detection of Beta-lactamase

Biochemical tests such as:

- Acidometric method
- Iodometric method
- Nitrocefin method

6

Iodometric tests: Hydrolysis of penicillin yields penicilloic acid, which reduces iodine, decolorizing starch-iodine complex. This reaction can be used to detect β-lactamase activity in tubes, microtiter plates, or on paper strips. These tests are particularly sensitive for staphylococcal penicillinase but are less sensitive than nitrocefin for most of the β-lactamases from Gram-negative bacteria.

Tube or microtiter method:

- 6 mg/ml of Benzylpenicillin in 0.1 M phosphate buffer (pH 6.0), is distributed in 0.1 mL quantities in tubes or a microtiter plate.
- Bacterial growth from agar (not broth) is suspended in these solutions until they are heavily turbid (10 cfu/ml).
- The suspensions are held at room temperature for 30-60 min, then 20 µL volumes of 1% (w/v) soluble starch in distilled water are added, followed by 20 µL of 2% (w/v) iodine in 53% (w/v) aqueous potassium iodide.
- β-lactamase activity is indicated by decolorization of the iodine within 5 min.
- Positive and negative controls are vital, as extraneous protein reduces iodine, and over heavily inoculated tests may give false-positive results.

*** **cfu:** A colony forming unit, or cfu, is a unit commonly used to estimate the concentration of microorganisms in a test sample. The number of visible colonies (CFU) present on an agar plate can be multiplied by the dilution factor to provide a CFU/ml result.

7

Iodometric Method

Heavy suspension of test organism from overnight culture is made in phosphate buffer with 6 mg/ml of penicillin

↓

0.1 ml into tube or microtiter plate at room temperature for 30-60 min.

↓

2 drops of starch solution

↓

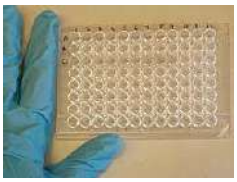
One drop of iodine

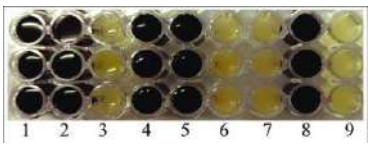
↓

Loss of blue color

↓

Positive test





(1 and 2) Negative control-containing penicillin, (3) positive, (5-9) samples

8

Result Interpretation

A positive beta lactamase production means that the test organism is resistant to following antibiotics:

- Penicillin
- Amoxycillin
- Ampicillin
- Piperacillin
- Mezlocillin
- Carbenicillin

9

Alternatives to antibiotics

Antibiotics lab (7)

Antibiotics alternatives

Antibiotics are one of the greatest advances in medicine. However; alternatives to antibiotics ought to be considered in some cases for several reasons:

- Antibiotics are over-used and abused that lead to increase drug-resistant bacteria.
- Antibiotics do not discriminate between pathogenic bacteria and bacteria of the normal flora, so could kill bad and good bacteria and change the intestinal microbiome of individuals.
- Antibiotics side effects, like diarrhea, are therefore common since a disrupted normal flora provides opportunistic bacteria with a chance to colonize.
- Some people are allergic to some types of antibiotics.

The alternatives to antibiotics

- **Probiotics:**

  Products containing live microorganisms that can help to establish or maintain the n ormal flora and thus prevent or treat mild infections.

- **Bacteriophage therapy:**

  Bacteriophages are viruses that infect bacteria, which potentially could be utilized fo r therapeutic purposes.

- **Naturally-occurring antimicrobial agents:**

  Plants and natural products

Probiotics:

- Preventive medicine more than therapeutic. Useful for preventing: urinary tract infections & diarrheal diseases.

- Bacteria & yeasts have been used. Species of Lactobacillus & Bifidobacterium are the most utilized probiotics.

- They are generally regarded as safe.

The main aim of probiotic is to :

1. To eliminate the harmful microbial population in the Gastro-intestinal tract (GIT).

2. To stabilize and enhance the beneficial microbial populations.

Mode of Action of Probiotics

Aims of using probiotics are achieved by any of the following mechanisms:

- Competitions for sites of attachment on the intestinal. Due attachment of beneficial bacteria there is a decrease in the binding sites for pathogenic organisms leading to their peristaltic removal. Mostly Lactobacillus spp act through this mechanism.

- Competition for nutrients (available carbohydrate) between favourable & unfavourable microorganisms.

- Production of antimicrobial compounds like bacteriocins.

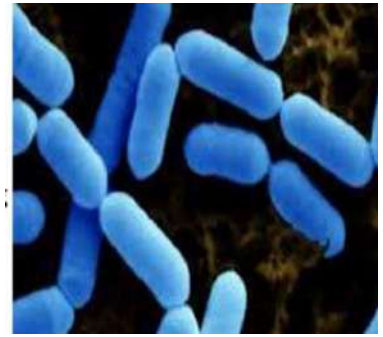
- Production of antibacterial metabolites like hydroxyperoxides & organic acids which leads to reduction of pH in intestine thus inhibit the growth and multiplication of pathogenic microorganisms.

Characteristic of Good Probiotics

- It should be a strain, which is capable of exerting a beneficial effect on the host.
- It should be non-pathogenic and non toxic.
- It should be present as viable cells preferably in large numbers.
- It should be a capable of surviving and metabolizing in gut environment eg. resistant to low pH and organic acids.
- It should be stable and capable of remaining viable for periods under storage and field condition.

Example of probiotic microorganisms

Lactobacillus

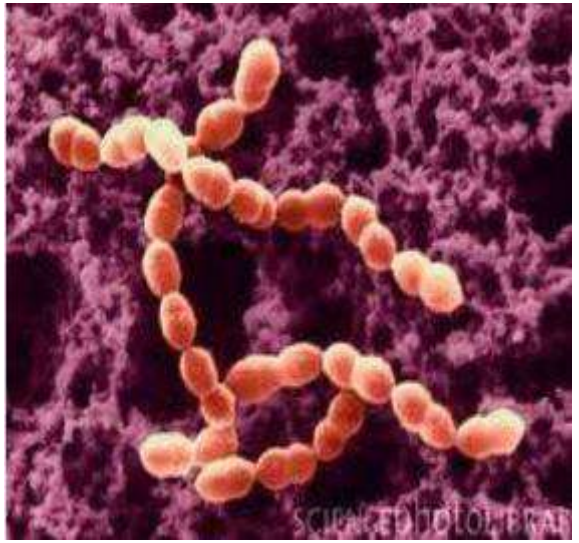


- More than 50 species of lactobacilli
- Contained in fermented food, like yogurt
- Helps in preventing yeast infection, traveler diarrhea, UTI….etc

Bifidobacteria

- More than 25 species of bifidobacterial
- Found in the intestinal tract within days of birth, especially in breast feed i nfants
- Helps in the improvement of abdominal pain, bloating, straining, …etc.

Streptococcus



- Produces large quantities of the enzyme lactase.
- Helps in the prevention of lactose intolerance.

Saccharomyces

- The only yeast probiotic.
- Effective in treating diarrhea associated with the use of antibiotics and t ravelers diarrihoea

Alternatives to antibiotics

Antibiotics lab (8)

The alternatives to antibiotics

- Probiotics
- Bacteriophage therapy
- Naturally-occurring antimicrobial agents

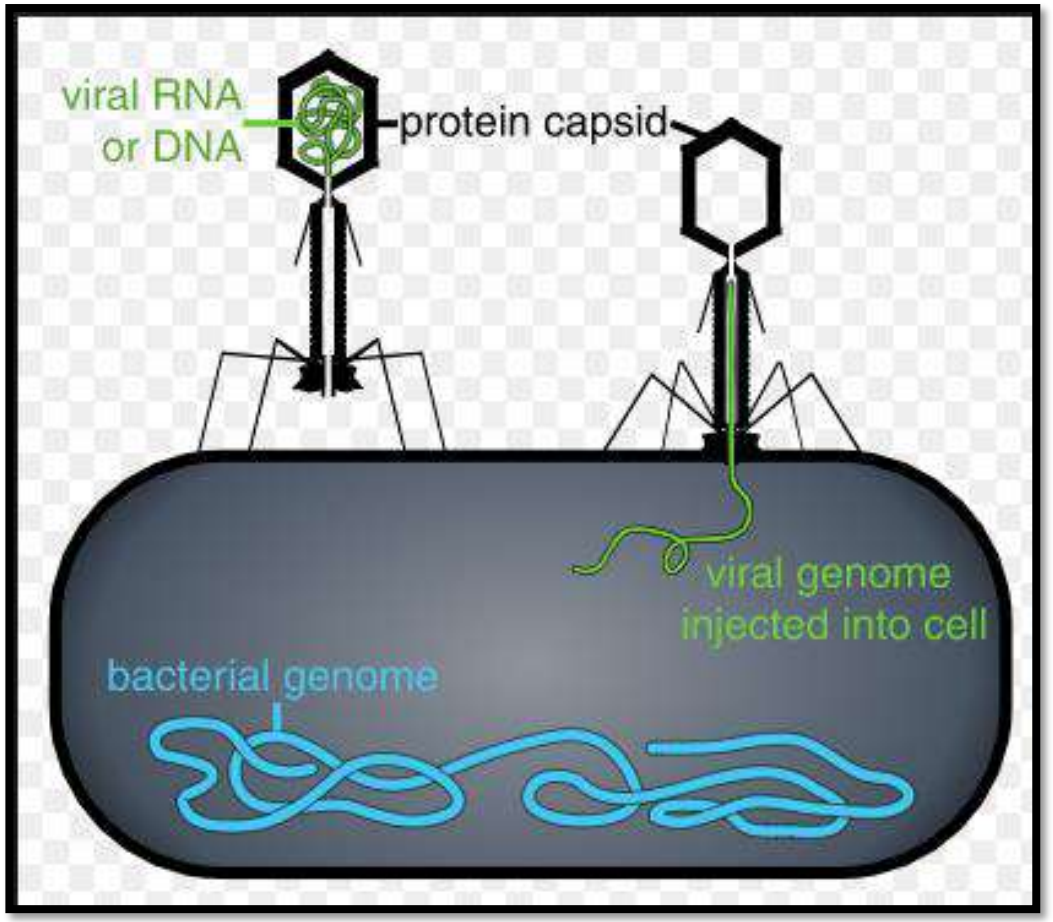
Bacteriophage therapy: Bacteriophages are viruses of bacteria that can kill and lyse the bacteria they infect, which potentially could be utilized for therapeutic purposes.

Bacteriophage therapy advantages:

- Highly specific
- Effective against multidrug resistant bacteria
- Cost of development relatively cheap
- Deleterious effects on eukaryotic cells

Bacteriophage therapy issues:

- Bioavailability
- Safety concerns, such as: Toxins in preparation
- Development of resistance



Naturally-occurring antimicrobial agents
(Plants and natural products)

Plants/Herbs

- There is a number of natural plant/herbal antibiotics that can be used as a treatment.

- Such as: Garlic, ginger, cranberry, ….etc.

- Medicines prepared from the natural herbs can be used, which are:
  - Relatively inexpensive
  - Can be stored for a year or more at room temperature

Garlic (*Allium sativum*)

- ❑Used by French and English in WWI to treat infected wounds
- ❑Antimicrobial properties attributed to allicin which is produced from alliin (alliinase)
- ❑Active against fungi including **dermatophytes**.
- ❑Active against many forms of bacteria, including **Salmonella** and ***E. coli.***
- ❑Has anti-MRSA activity. Ex. use against **multi-drug resistant tuberculosis**.

Garlic issues

Concentrated garlic may:

- Increase the risk of bleeding. This can be dangerous for people facing surgery or taking blood thinners.
- Reduce the usefulness of HIV medications.

(Natural products)

There is a number of natural products that can be used as a treatment, such as:
Honey, tea tree oil ….etc.

Honey

- Has been used as an ointment that helps wounds to heal or prevents infection.
- Has antibacterial activity against range of microorganisms such as: Staphylococcus, E. coli, Pseudomonas….. etc
- In addition to its antibacterial properties, honey may help wounds to heal by providing a protective coating.

- **Activity attributed to:**
  - High osmolarity
  - Low pH
  - Hydrogen peroxide content & others

Tea tree oil (*melaleuca alternifolia*)

- Pale yellow, viscous fluid, derived from the leaves of the Australian tea tree.
- Has antimicrobial properties.
- Incorporated as the active ingredient in many topical formulations used to treat cutaneous infections, such as: acne, fungal infections of the nail, and others.
- Only suitable for topical use.

Why haven’t these treatment options been widely explored further?

- Often no protection for a pharmaceutical company (no patent)
- Poor quality products
- High cost of clinical trials
- Lack of good data
- Safety issues

When to use prescribed antibiotics

Due to the current increase in drug-resistant diseases, most doctors do not prescribe antibiotics unless they are effective and necessary. Therefore **antibiotics are most often prescribed to:**

- Prevent the spread of infectious diseases
- Prevent a condition from becoming more serious or fatal
- Speed recovery from illness or injury
- Prevent development of complications

Entire dosage of a prescribed antibiotic should be taken as directed. Especially in people with a higher risk of bacterial infection, or people who at greater risks if they become ill, such as people who are:

- Scheduled for surgery
- Receiving chemotherapy
- Living with heart failure

Antibiotic Combination

Lab # 9

A **combination antibiotic** is one in which two ingredients are added together for additional therapeutic effect.

There are five cases that used two antibiotics or more in treatment such as:

1- Undiagnosed infection: in pathogenic cases the causative agent should be diagnosed first before treatment; but sometime there is unstable relationship between disease symptoms and bacterial causative agent ex: Acute UTI caused by many causative agents that reveals same symptoms but there is no specific antibiotic lead to inhibit all microbial causative agents.

2- Mixed infection: there is no specific antibiotic against all microbial causative agents.

3- Preventing or delaying antibiotic resistance: Two antibiotics may administrate to reduce the development of microbial resistant to antibiotics.

4- Synergetic effects: when two or more antibiotics are used simultaneously to treat an infection. In the synergistic response, the applied antibiotics work together to produce an effect more potent than if each antibiotic were applied singly.

5- To reduce toxicity of some highly toxic antibiotics: the effect of using any antibiotic dosage (not synergetic) is the same as the effect of using two combined antibiotics (synergetic) with low dosage. Ex using full dose of Streptomycin causes kidney damage,while using Rifaampin causing liver damage .so the best way for treatment is reducing the dosage for each antibiotic to half to deduce side effects .

Mechanisim of combination

The combined effect of two or more drug ma appeare as:

1-Indifferent effect: the activity of both combined drug is not affected by the presence of each other.

2- Antagonistic effect: **when a drug hinders the effect of another drug**,when the activity of one antibiotic affected on the other **.ex**: mixing drug such as (Chloramphenicol and Tetracycline)(bacteriostatic) with (Aminoglcoside)0(bactericidal).

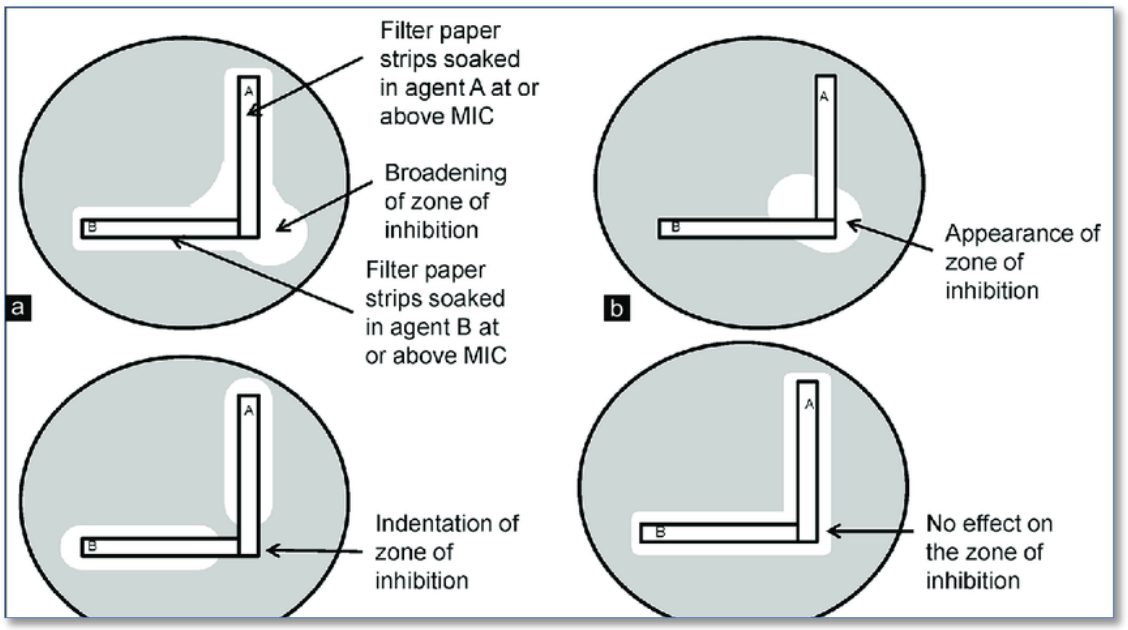
3-Synergetic effect: when the combine effect of two drugs is greater than the sum of their effects when given separately.**ex:** Methoprime (Trimethoprim-Sulfonamides)

Trimethoprim-sulfonamides act synergistically to inhibit the synthesis of folic acid, a compound that is required for microbial DNA production.

Method to observe the antibiotic combination effects:

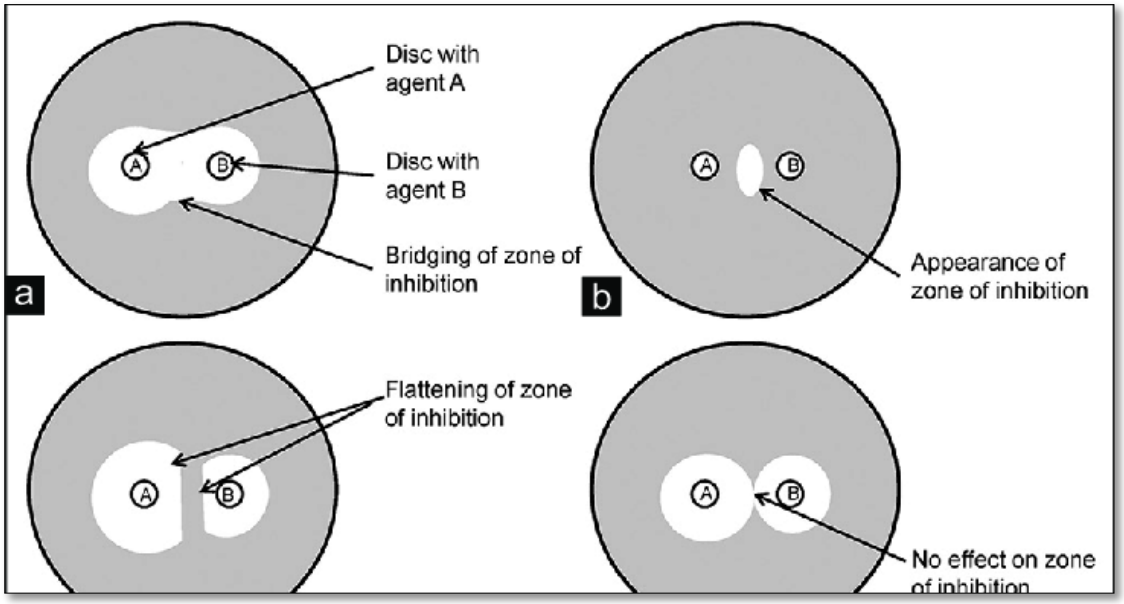
Diffusion methods: it's the simplest method to estimate the effect of combined antibiotics on microorganism by paper stripes; this test is performed on petridishes containing Muller Hinton agar where sterilized stripes soaked in the concentration of antibiotics. The culture media is inoculated with bacteria, then the soaked stripes is applies on the agar surface in90 degree angle by sterilize forceps and incubated for 3 C for 18 hrs .

the effect of each antibiotic alone noted at strip end, while combined effect



noted at strip meeting point .the test can done also by using disk instead of strip .

Paper strip diffusion test, (a) synergy (broadening of zone of inhibition at the angle); (b) synergy (appearance of zone of inhibition at the angle); (c) antagonism (indentation and narrowing of zone of inhibition at the angle); (d) indifference/additive (no effect in the zone of inhibition)



Double disk synergy test. (a) Synergy (bridging of zone of inhibition); (b) synergy (appearance of zone of inhibition in between agent A and B); (c) antagonism (flattening of zone of inhibition); (d) indifference/additive (no effect on zone of inhibition)

Etest (The Epsilometer test)

Lab #10

Etest (previously known as the **Epsilometer test**) is a way of determining antimicrobial sensitivity by placing a strip impregnated with antimicrobials onto an agar plate. A strain of bacterium or fungus will not grow near a concentration of antibiotic or antifungal if it is sensitive. For some microbial and antimicrobial combinations, the results can be used to determine a minimum inhibitory concentration (MIC).

Etest is a quantitative technique for determining the antibiotic sensitivity and minimum inhibitory concentration (in µg/ml) of some bacteria including Gram-negative and Gram-positive aerobic bacteria and fastidious bacteria, such as anaerobes. It can also be used to determine MICs against certain fungi.

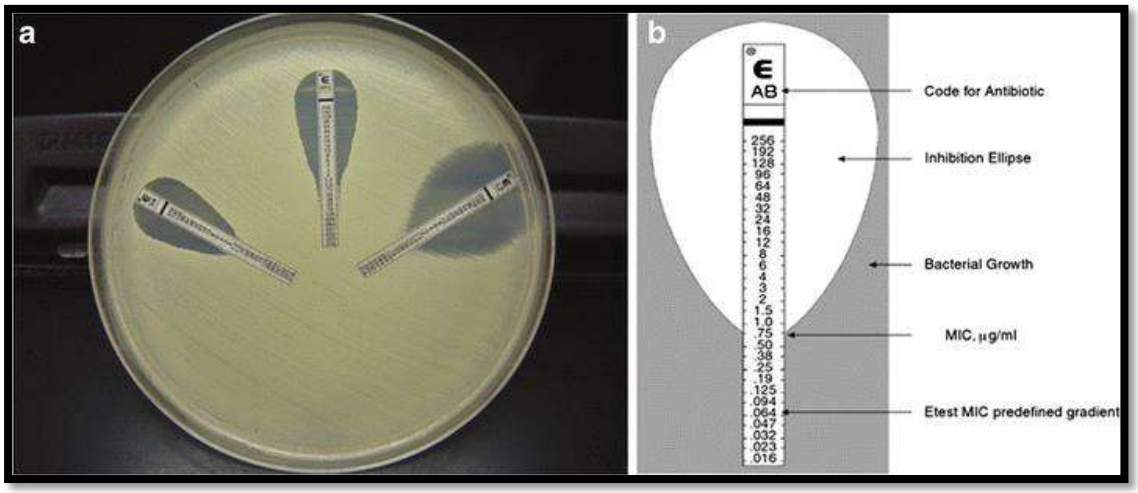
Procedure

Etest is a pre-prepared non-porous plastic reagent strip with a pre-defined gradient of antibiotic, covering a continuous concentration range. It is applied to the surface of an agar plate inoculated with the test strain, where there is release of the antimicrobial gradient from the plastic carrier to the agar to form a stable and continuous gradient beneath and in nearby to the strip.

1) Remove the E-test package from the freezer (-20°C) at least 30 minutes before required.
2) Suspend well-isolated colonies from an agar plate to obtain a turbidity of 0.5 McFarland (confluent growth should be obtained after incubation).
3) Dip a sterile swab into the inoculum suspension and squeeze it against the wall of the test tube to remove excess fluid.
4) Allow excess moisture to be absorbed so that the surface is completely dry (15 - 20 minutes) before applying the strips.
5) Apply the strip to the agar surface with the scale facing up and the strip code facing the outside of the plate, pressing it with sterile forceps onto the agar surface and ensuring that all of the length of

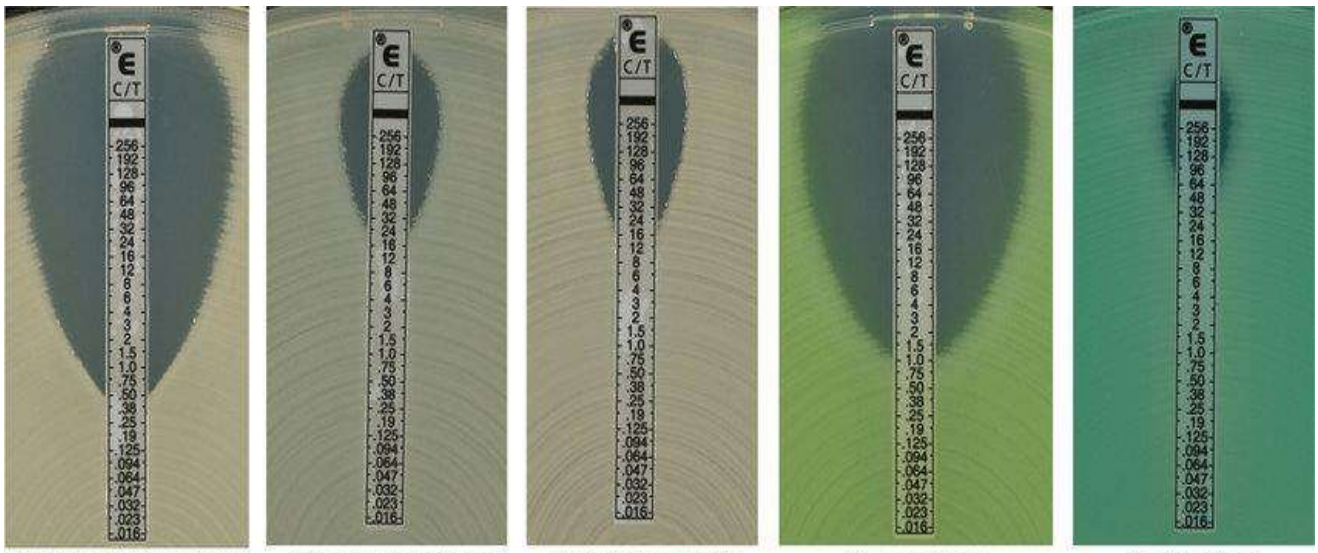
the antibiotic gradient is in full contact with the agar surface. Once applied, do not move the tape.

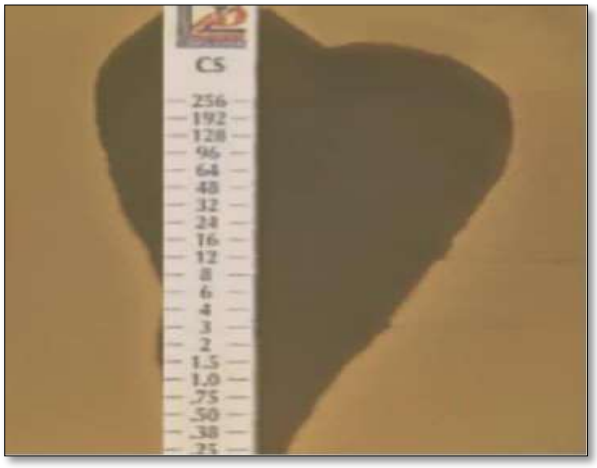
6) If air bubbles are trapped under the strip, gently move them to the edge using sterile forceps, taking care not to move the strip over the agar. When removing air bubbles, start at the lowest concentration and work your way up.
7) Incubate the agar plates in the inverted position at 35 ± 2 ° C.
8) After 18 hours of incubation or more, a symmetrical inhibition ellipse centered along the strip is formed. The MIC is read directly from the g/ml scale at the point where the edge of the inhibition ellipse intersects the MIC test strip.



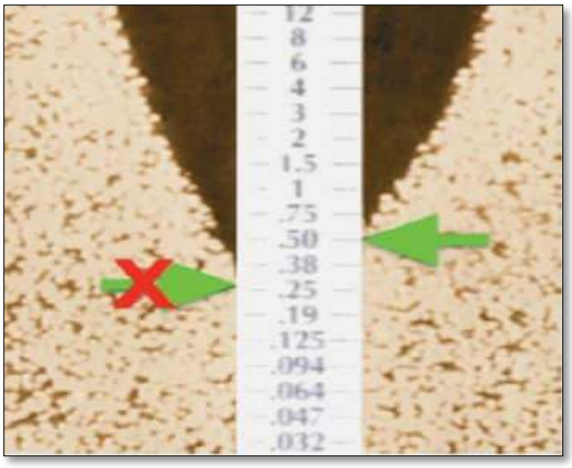
Reading strips

After 18 hours of incubation or more, a symmetrical inhibition ellipse centered along the strip is formed. The MIC is read directly from the g / ml scale at the point where the edge of the inhibition ellipse intersects the MIC test strip.

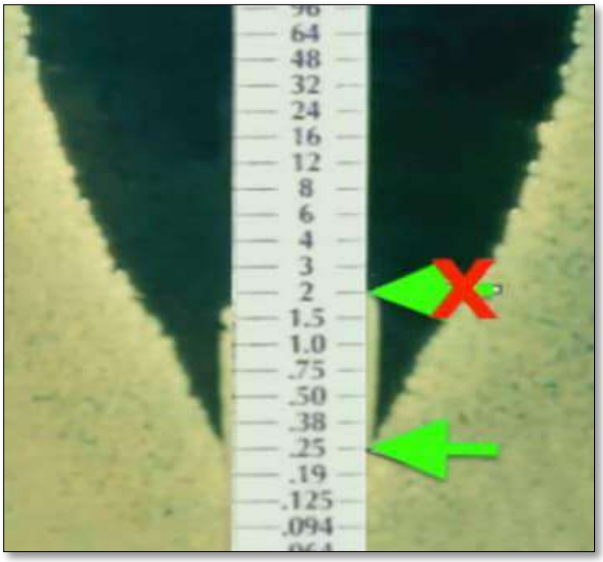




Wet surface, deformed ellipse, repeat the test

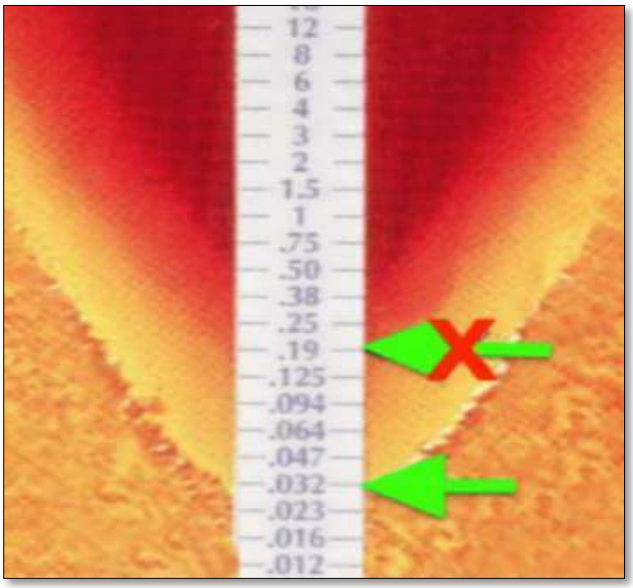


In case of uneven results. Read the upper value.



Ignore the growth line along the strip

Partial regrowth at edge Read MIC with complete inhibition



***Streptococcus* spp : ignore hemolysis**

Strip placed upside down: Invalid (repeat the test)

Advantages of the E test

1. It is very easy to perform, requires minimal training for test performance, and easy to execute.
2. Contamination can be easily recognized.
3. It is a less time-consuming and very convenient method of the determination of the Minimum Inhibitory Concentration (MIC) and applicable to a wide array of drugs and microorganisms.
4. It is useful to detect some phenotypes resistance.
5. It is an adequate method to detect potentially resistant strains to Amphotericin B.
6. It helps to confirm or detect low-level or new resistance mechanisms.
7. It can be used to investigate any synergistic potential of combination therapies.