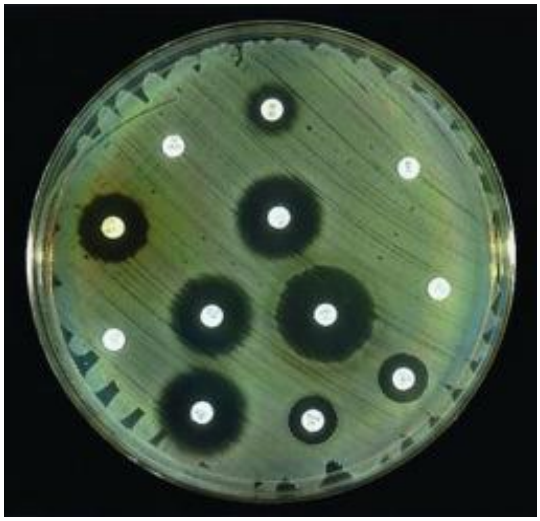
Practical Antibiotics

3rd Level



TOPICS	
Lab 1	Antimicrobials
Lab 2	Evaluation of Disinfectants or comparison of Antiseptics used against M.O.
Lab 3	Test of antibiotic susceptibility (sensitivity)
Lab 4	Dilution method
Lab 5	Antimicrobial drugs used in combination
Lab 6	Methods of antimicrobial drugs combination
Lab 7	Detection of β-lactamase

First Exam	
Second Exam	

By: Assis. Lec. Ammar B. Al-Asadi

Antimicrobials

Antimicrobial agents: they are antimicrobial substances used to kill or suppress growth of microorganisms, and they are classified into:

1. **Chemotherapeutic agents.**
2. **Disinfectants.**
3. **Antiseptic.**

Chemotherapeutic agents:

They are antimicrobial substances given systematically to treat infection and they are usually either kill bacteria (Bactericidal) or suppress bacterial growth (Bacteriostatic).

In both conditions the purpose from these substances is to prevent replication of the infective microorganism, so that the body defenses will work and deal with this infection, e.g. antibiotics.

Disinfectants:

They are antimicrobial substances and mostly they are able to kill a wide range of microorganisms but not necessarily kill spores , they are usually used with non-living substances , such as alcohols and detols (high concentration).

Antiseptics:

They are antimicrobial substances used to control and get rid of bacterial infection, these substances have antimicrobial characteristics similar to that of disinfectants except they are used with skin and other living tissues, and they don't have the spectrum of activity that disinfectants have , but is specific for microorganisms that cause skin infection e.g. detol (low concentration) because these substances don't cause injury to the tissues.

Note: most substances or chemotherapeutic agents have both killing and suppressing characteristics of bacterial growth but their action depend largely on the concentration of substance which is used.

Bactericidal (the term mean: killing bacteria)

By another words if the bacterial cells put in a suitable nutritional media will be unable to replicate and produce new cells; which mean the bacteria is dead.

Death could be caused by one of the followings:

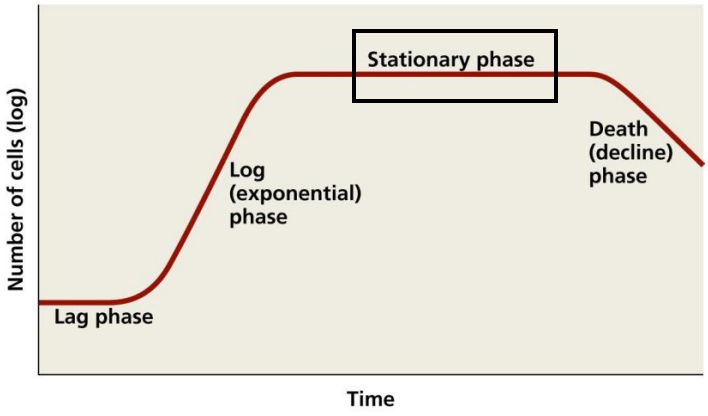
1. Protein denaturation.
2. Enzyme inactivation.
3. Damage of membrane.
4. Blocking of an essential metabolic path.

Bacteriostatic (the term mean: Suppressing bacterial growth)

By another word; each cell in the bacterial colonies is prevented from growth by the action of the substance used to suppress its growth and if these cells have the suitable media and conditions available, they can resume their activity to replicate again forming new progenies.

Antibiotics

They are chemical substances, considered as secondary metabolites produced by microorganisms during the stationary phase, they have a low molecular weight and are able to kill and suppress growth of other microorganisms.



Importance of antibiotics:

1- Prevention of spreading of diseases from a human being to another or from a human being to animal or vice versa.
2- Prevention of contamination of food e.g. some canned food.
3- Some antibiotics can be added to prevent lyses of skin, wood , or papers by some types of fungi.

Sterilization: Substances will be free from all microorganisms and eradication of M.O. whether they are bacteria, fungi or viruses or even spores.

Disinfectant spectrum:

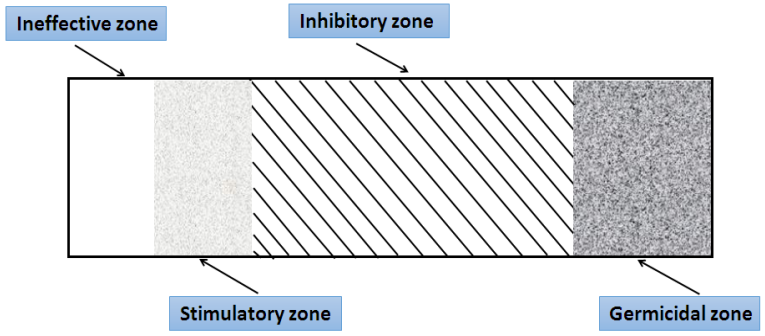
When a microorganism is subjected to an increasing high concentration of antibacterial agents , many effects are produced with different degrees ranging from stimulation to lethal degree , these different degrees of antimicrobial agent activity are known as disinfectant spectrum or zonal effects and these degrees are divided into:

1- Ineffective zone: Started from zero to the highest level when no antimicrobial effect appears on the microorganism.

2- Stimulatory zone: There is a slight stimulation zone, during adding concentrations to a limited degree it might produce slight stimulation in growth.

3- Inhibitory zone: Adding more antimicrobial concentration which produce inhibitory effect.

4- Germicidal zone: In this zone there are killing effects starting to appear through the end of the inhibitor zone and increased with increasing concentrations.



Factors that control the **strength** of the antimicrobial agent:

1- Characteristics of the microorganisms.
2- The environment (temperature, pH , time , substance concentration, presence of organic substances).
3- Number of bacteria.
4- Mode of action.

Disinfectant	Antibiotic
1 - Synthetic chemical compounds	1- Biological compounds (Secondary metabolites of microorganism)
2- Non selective Might have an effect on wide spectrum of microorganisms.	2- Selective Act on one type or limited groups of Microorganisms
3- Used outside the human body or on external surfaces (in vitro).	3- Used inside the human body (in vivo).
4- Used in high concentration such as detol (5 % for e.g.)	4- Concentration that used are small measured in microgram.

Major group of antimicrobial agents:-

1. Phenols and phenols compounds.
2. Alcohol
3. Halogens.
4. Heavy metals.
5. Dyes
6. Detergents
7. Quaternary ammonium compounds.
8. Acids and Alkaloid.
9. Gluteraldehyde.

10. Gases.

Phenol and it's compounds

Phenol (Carboxylic acid) it's effect is being through its active part which is the free carboxylic group, and it acts on:

1- Destruction of cell wall.
2- Precipitation and coagulation of proteins.
3- Suppression of a number of enzymes.

Phenol is considered highly effective against Mycobacteria because of its high solubility in lipids , and it has a major killing effect on fungi.

Alcohols:

Active against vegetative cells of bacteria and fungi with no effect on spores , their effect is through melting of lipids in the cell wall, and protein denaturation, in addition to their effect of a drying substance (dehydration).

Ethyl alcohol is used in sterilization in a concentration of 50 - 90 % but the best killing concentration to bacteria is 70 %.

Halogens:

Include chloride , iodine and fluoride , and their effects is by being a very strong oxidizing substances act to suppress proteins through oxidation of sulfhydryl group (-SH) and forming (S-S) bands and thus changing the shape of the protein and suppressing it's activity.

Chloride is used in sterilization of drinking water , from their side effects is their harmful effect on the metal surfaces.

Evaluation of Disinfectants or comparison of Antiseptics used against M.O.

A- Phenol coefficient: is method used for:

a- Evaluation of water soluble disinfectants which is similar to phenol in chemical composition.

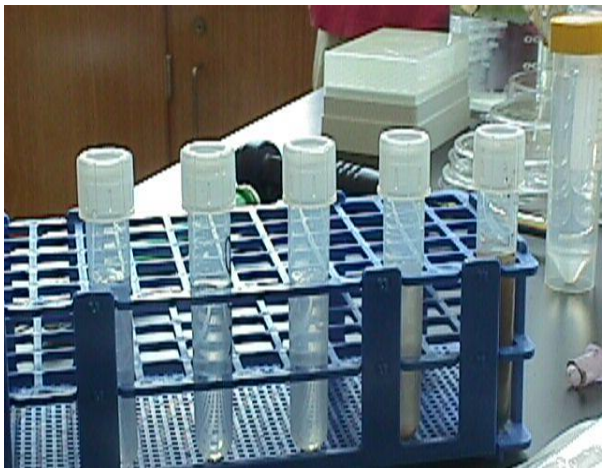
b- The principle of evaluation is a comparison between test disinfectants and pure phenol under standard practical condition.

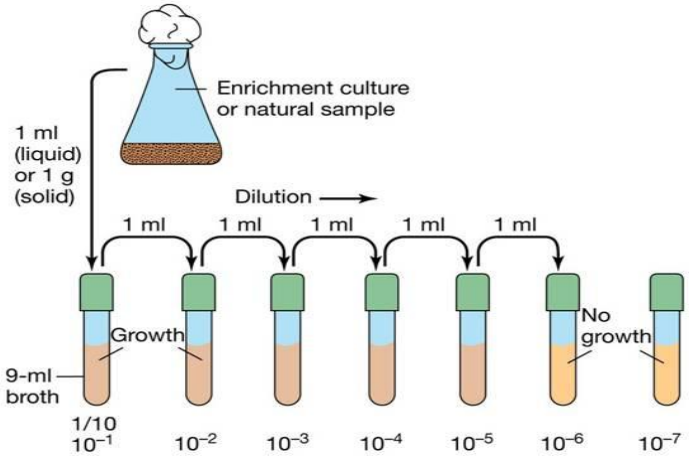
c- This method used by food and drug administration (FDA)

Method

1- Make serial dilution from test disinfectant (X) in test tube with volume of (5 ml)

2- Make serial dilution from pure phenol (P)





3- Inoculate tube with 0.5 ml from young broth culture of test organism usually *S. aureus* or *P. aeruginosa*

4- Incubate all tube at 20 ^{o}C

5- Take loop full (0.5) ml from both dilution (X , P) after 5 minutes, 10 minutes and 15 minutes, incubate in serial nutrient broth (from every tube take a loop full to 3 new tubes after (5,10,15) minutes

6- Incubate tubes at 37 ^{o}C for 48 hr, if there is no growth (this mean completely eradication), the result mean us (-), if there is turbidity, the result considered as (+).

Phenol coefficient calculated by:

Make ratio between highest dilution of (x) which kill the microbe in 10 minutes but not kill them at 5 minutes to corresponding dilution of phenol .

$$\text{Phenol coefficient (P.C.)} = \frac{\text{Highest dilution of (x) killing M.O. during 10 min. but not in 5 min}}{\text{corresponding dilution of phenol}}$$

To find the dilution that used from (x) disinfectant , you should know that it must be 20 times higher than of P.C. (that mean P.C. X 20 = dilution of (x).

Disinfectant	Dilution	Treatment time		
Test Disinfectant (X)	Completely eradication by using highly conc.	5 min	10 min	15 min
	1:350	-	-	-
	1:400	+	-	-
	1:450	+	-	-
	1:500	+	+	-
	1:550	+	+	-

Disinfectant	Dilution	Treatment time		
Phenol (P)	Completely eradication by using highly conc.	5 min	10 min	15 min
	1:70	-	-	-
	1:80	-	-	-
	1:90	+	-	-
	1:100	+	+	-

P.C. = 1:450 / 1:90 = 5 X dilution is higher than P.C. in 20 time

20 X 5 = 100 1:100 or 1/100

B- Use Dilution Test (UDT):

1- this method based on the dilution of the disinfectant in a test tubes contain double strength nutrient broth (D.S.N.B) and then inoculated by 0.1 ml from overnight culture, the final volume should be 10 ml.

2- after 10 min take a loop full from every tube to new test tube contain single strength nutrient broth (S.S.N.B)

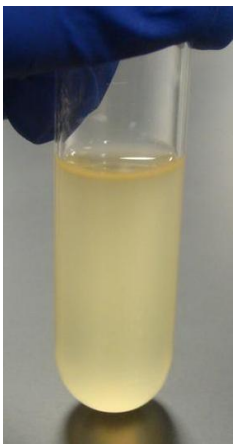
This step called **Neutralization** the purpose of neutralization is to:

a- test the ability of bacteria to grow or not after transfer to a suitable growth media

b- neutralize the disinfectant

c- remove the action of disinfectant on bacteria

d- enumerate bacteria by use optical density (O.D.)



3- incubate the tubes at 37 C for 18 -24 hr.

4- the results have been shown by turbidity or not.

Q) Prepare the following concentration of bleach **6%** and **12%**

Con.	D.S.N.B	Disinfectant	D.W.	Inoculums	Total Volume
6%	5 ml	0.6 ml	4.3 ml	0.1 ml	10 ml
12%	5 ml	1.2 ml	3.7 ml	0.1 ml	10 ml

Q) Prepare the following concentration of alcohol: **10%** , **50%** and **70%**

Con.	D.S.N.B	Dis.	D.W.	Inoculums	T.V.
10%	5 ml	1 ml	3.9 ml	0.1 ml	10 ml
50%	4.9 ml	5 ml		0.1 ml	10 ml
70%	2.9 ml	7 ml		0.1 ml	10 ml

Q) Prepare the following concentration of bleach **13%** and **39%** (homework)

Con.	D.S.N.B	Disinfectant	D.W.	Inoculums	Total Volume
13%					
39%					

Q) Complete the following table (homework)

Con.	D.S.N.B	Disinfectant	D.W.	Inoculums	Total Volume
		2.2		0.1 ml	
		1.7			

Toxicity of disinfectant:

You can estimate the toxicity of disinfectant by many ways, but the best one is by mixing the disinfectant with living cells like (WBC) or living tissues and observed the smallest quantity of disinfectant that stop the viability of this cells in test tubes in special time .

Test of antibiotic susceptibility (sensitivity)

Microbial susceptibility to different types of antibiotics could be estimated through the ability of these antibiotics inhibit the microbial growth, sensitivity could be measured by many methods qualitative and quantitative.

The purpose of this test is to know if the organism either sensitive or resistance to some antibiotics that used to treat the patients, before applying a sensitivity test we should notice the following:

1- The genetic background for microbe sensitivity in vitro since some microbe's posses mutations.
2- The sensitivity range of strains under test in comparison with the same species.
3- Information about the antibiotic under test: toxicity, chemical composition, absorption by the body and mode of action.
4- Patients' immune state.

Antimicrobial drugs act in one of several ways:

1- Sensitive toxicity
2- Inhibition of cell membrane synthesis and function
3- Inhibition of cell proteins synthesis.
4- Inhibition of cell nucleic acid synthesis.

A- Diffusion methods of sensitivity testing

This method is done by using solid media inoculated previously with the microbe under test, the antibiotics will diffused to the agar during incubation period, if the microbe was sensitive to that antibiotic, an inhibition zone of growth will be formed; this method is qualitative and sometime considered as semi quantitative method because the inhibition zone is effected by the sensitivity of the microbe towards the antibiotic.

Disc-Diffusion method (Kirby-Bauer method)

One of the most commonly methods used routinely in diagnostic laboratories. Test is done by inoculating bacteria under test on solid in Petri dish and using blotting paper discs containing different types of antibiotics with different concentrations applied on the agar surface, during the incubation period the antibiotic will diffuse from the disc to the media and Inhibition zone will form according to the microbe sensitivity.

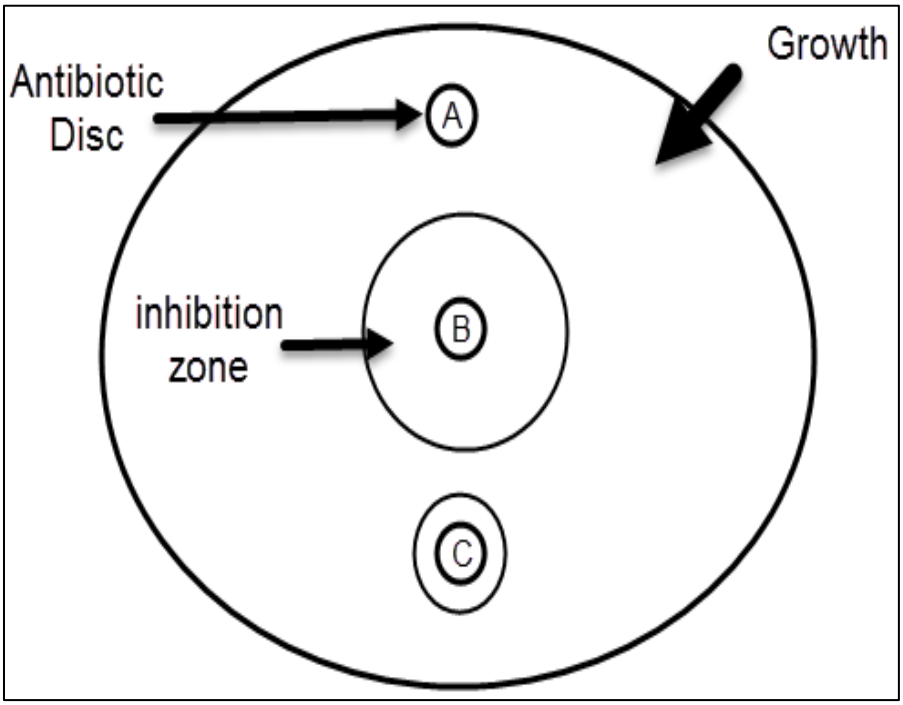


Figure (1): In Disc (**A**) Bacteria are susceptible to antibiotics, Disc (**B**) are resistant to antibiotics, Disc (**C**) intermediate resistance to antibiotics

Antibiotic discs can be prepared in laboratory in any concentration by using blotting paper as following:

Filter paper is poured in a 5 mm in diameter (filter paper No.3). Spread in a sterile Petri dish then immersing in a specific concentration of previously prepared antibiotic dried in sterile conditions then the discs used for sensitivity testing by putting them on a surface of media inoculating with bacteria previously.

Method

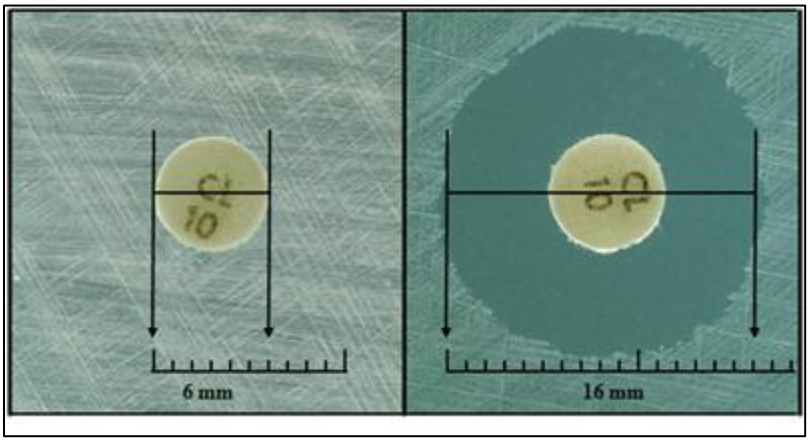
1- Prepare Muller-Hinton agar (prepared according to FDA) as the best media for the growth of most types of pathogenic bacteria

2- The media is cultured by the bacteria under test in sterile conditions (by streaking or swabbing method).

3- After drying of inoculums, and by using sterile forceps (sterilized by alcohol), the discs are putted on the medium surface but not more than 6 discs/dish.

4- Incubated at 37°C for 18 hr. (not more than 24 hr.).

5- The inhibition zone measured by mm around each disc.



Antibiotic susceptibility testing plate. You measure the diameter of zones of inhibition in millimeters. The zone seen here measures 16 mm in diameter.

Methods for inoculation:

The way of inoculation is affecting on the balance and spreading of growth on the media and the best results are obtained by flooding or agar overlay methods of culturing.

a- flooding: in this method the bacterial suspension is over laved on the agar surface in a proper volume by dropping and distributed by Pasteur pipette until the inoculums cover all the surface " the media is turning from side to side to cover the surface and then turning the media to dispose the excess inoculums and this done by Pasteur pipette. This

method is dangerous because it may produce aerosols during the pipetting and this should be avoided when using highly virulence organisms

b- Agar over lay: Bacterial suspension is diluted and mixed with agar at 45°C then poured on previously agar layer in Petri dish. This method give good results when compare with other methods

c- Swabbing by swab

d- Spreading by spreader

e- Streaking by loop

The theory of zone formation:

The rate of drug diffusion in the media depending on its m.wt and chemical composition for both drug and medium.

The drug concentration decrease from the disc to the edge of inhibition zone gradually, this disc contents is known as absolute amount.

Critical concentration: the less concentration of the drug that can inhibit the microorganism under testing at the zone edge and it depend on microbe sensitivity to the drug and Minimal inhibitory Concentration (MIC).

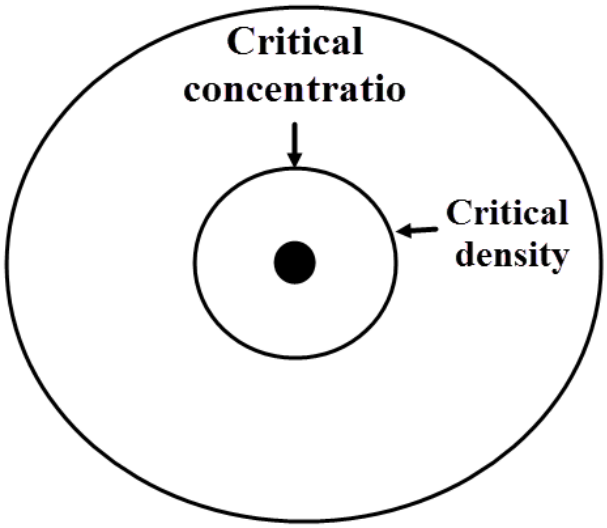
The distance between disc to zone edge depend on

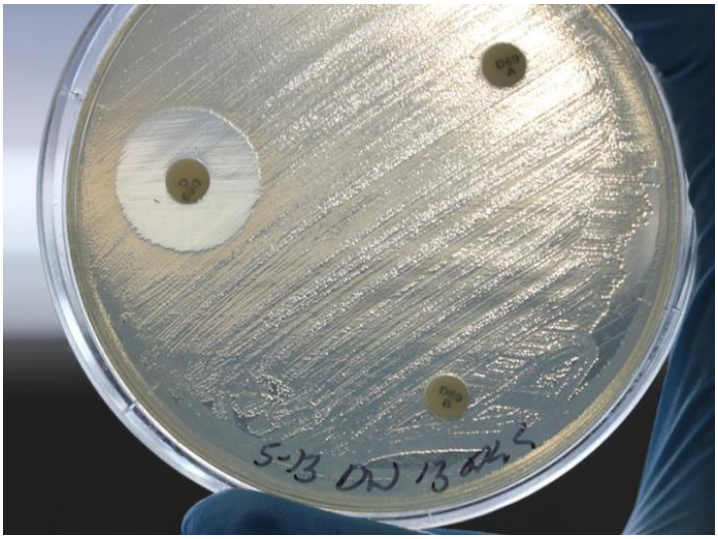
1. inoculums volume
2. growth rate
3. pre-incubation period
4. pre-diffusion

Critical density: density of inoculums that inhibited by the drug on zone edge and depend on both drug and organism.

If the inoculums is more than critical density, the inhibition zone will not formed, but if the inoculums is less than critical density so the inhibition zone will be very wide.

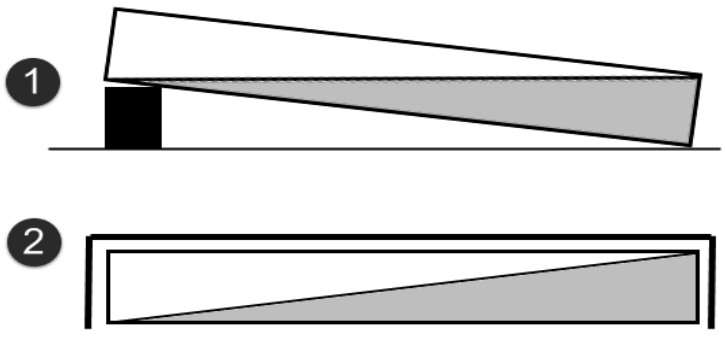
In case of slow growing organism, the drug diffusion will be faster and inhibition zone will be bigger (not truth)





B- The gradient plate test

This method is used to know the sensitivity or resistance of M.O. to specific or several antibiotics, and from the gradient of concentration you can know the MIC of antibiotic to microorganisms.; this method is done by preparing and pouring half amount of nutrient agar (at 45-50°C) in a Petri dish, then turning it and leaved the media to solidify to get tilt surface ; then they remained amount of nutrient agar wormed at 45°C (still liquid) and mixing with the antibiotic under test that prepared in certain concentration and poured over the first layer, then leave to solidify to get plane surface . So two layer will be formed in the Petri dish and get clear gradient of antibiotic concentration then the media will streaked by bacteria and incubated in suitable and notice the growth of bacteria on the surface of media.



C- Well method or the agar cup method

This method is used for evaluation of liquid and semi-solid sample of antibiotics. Media is poured in 4 mm thickness then inoculated with bacteria by streaking or swabbing, then make a well in the center of the dish (agar) by sterile cork porer (more than one well could be done by this method in order to use more than one concentration or more than one kind of antibiotic). In proper diameter 5-10 mm under a sterile conditions and the well depth should not reach the bottom of plate, then added a suitable amount of drug (ex: 50 µl). The plate incubated at 37°C for 24hr then estimate the inhibition zone around the well and determined by mm.

Factors affecting the results of diffusion tests

1- **Rate of drug diffusion:** depend mainly on the molecular weight of the antibiotic, for example Penicillin has low m.wt so it diffuse rapidly in the agar while Polymyxin has high m.wt so it diffuse slowly. Sometime the diffusion rate may be affected by the chemical interaction between media and drug.

2- **The culture media**

   a- **Media constituents:** the perfect medium should not contain any antagonist materials that interfere with antibiotic activity. Some media does not contain the proper nutrients for the microbial growth in order to perform the sensitivity test.

   b- **pH of the medium:** affecting on activity of many antibiotics (many test preferred to be done on pH=7.3 (the pH of human body).

   c- **Minerals and salts:** monovalent cataions. increase canalize the activity of many antibiotics like Bacitracin and Novobiocin against *Staphylococci* and Penicillin

against *Proteus* , while Divalent cations decrease the inhibition zone because it interact with peptide antibiotics like Polymyxin or alkaline antibiotic like kanamycin.

d- Carbohydrates: may be decrease the inhibition zone to 50 ml/microbes and some antibiotics.

e- The blood: small inhibition zone are seen for some antibiotics that have peptide bonds like Fusidic acid and Novobiocin:

3- Depth of medium: inhibition zone increase in size when agar depth is thin, very thin layer media must be avoided and the optimum depth is 4 mm.

4- Density of Inoculum: the density of inoculum affecting on the inhibition zone for example a very light inoculum may result in a difficulties in evolution for zone edge and a large inhibition zone may formed if the inoculum was very light.

5- Incubation period: should be less than 24hr because long incubation period less the drug activity and this allowing the sensitive microorganisms that inhibited by drug to grow again so, this method is used for rapidly growth microorganisms (have short generation time).

6- The discs: must be stored under proper conditions at 4°C validity (not be expired). No, of discs per dish and spaces between them.

7- type of microorganism

Control cultures: types of bacteria that well known about their response to antibiotics and they used compare with other results such as *P. aeruginosa, E.coli, S. aureus*.

Ideal sensitivity testing medium:

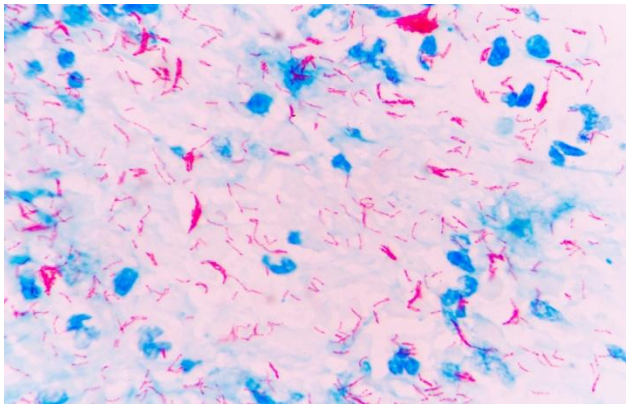
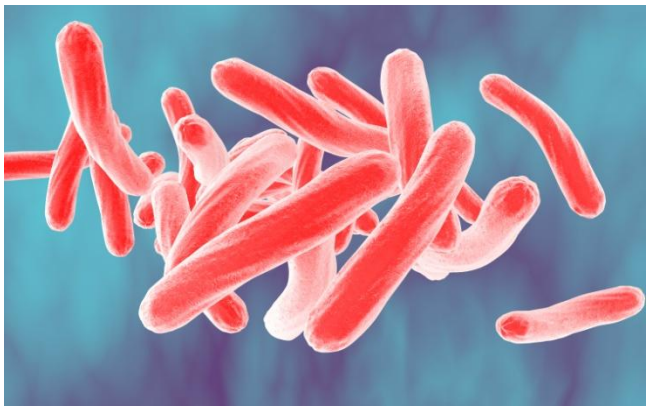
1- very well-known ingredients
2- Should not be enriched media support the rapid growth of bacteria.
3- Does not contain antagonist materials to any bacterial factor.
4- Keep its pH stable and constant during bacterial growth.
5- Should be isotonic

Dilution method

Its quantitative method; depend on preparation of series of gradually duplicate concentration of antibiotic in suitable medium for growth, than added limited number of bacteria and check the ability of antibiotic to inhibit or kill the bacteria under testing by formed or unformed turbidity in test tube.

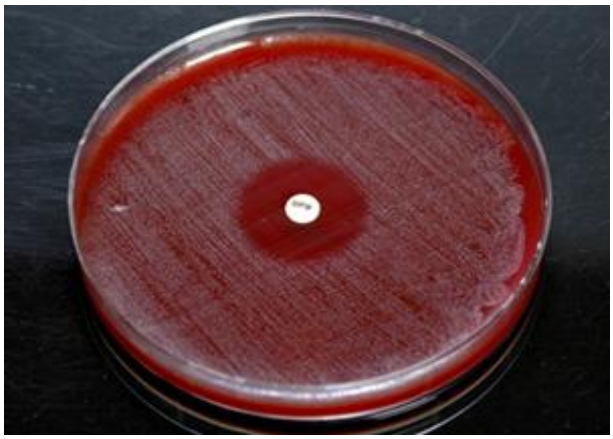
The diffusion test of antibiotics by using disc method is useless and did not give clear information about the bacterial sensitivity, as following:

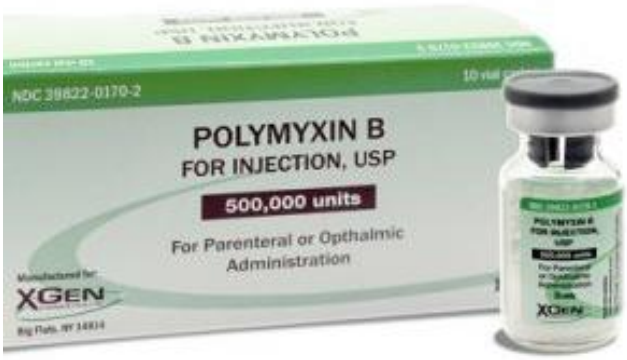
1- In case of using slow growth rate microorganism such as *Mycobacterium tuberculosis* (has 48 hr. as generation time), using disc method give wrong results because the antibiotic diffusion in agar faster than bacterial growth, for that, the inhibition zone appeared biggest than normal case (not truth).

2- In case of swarming bacteria such as *Proteus*, the swarming movement considers hinder for antibiotic diffusion and lead to make inhibition zone smaller than the normal case.


3- In case of using antibiotics with high molecular weight such as Bacitracin and Polymyxin B it is diffused in agar slowly, for that, the inhibition zone may small or not appeared because the growth of bacteria is faster than diffusion of antibiotic.





In all above case, the dilution method is recommended and the **MIC** (Minimal Inhibitory Concentration) and **MBC** (Minimal Bactericidal Concentration) can be estimated.

MIC: it is mean the minimum concentration lead to inhibition of bacteria under testing.

MBC: it is mean the minimum concentration that can kill the bacteria under testing.

Method

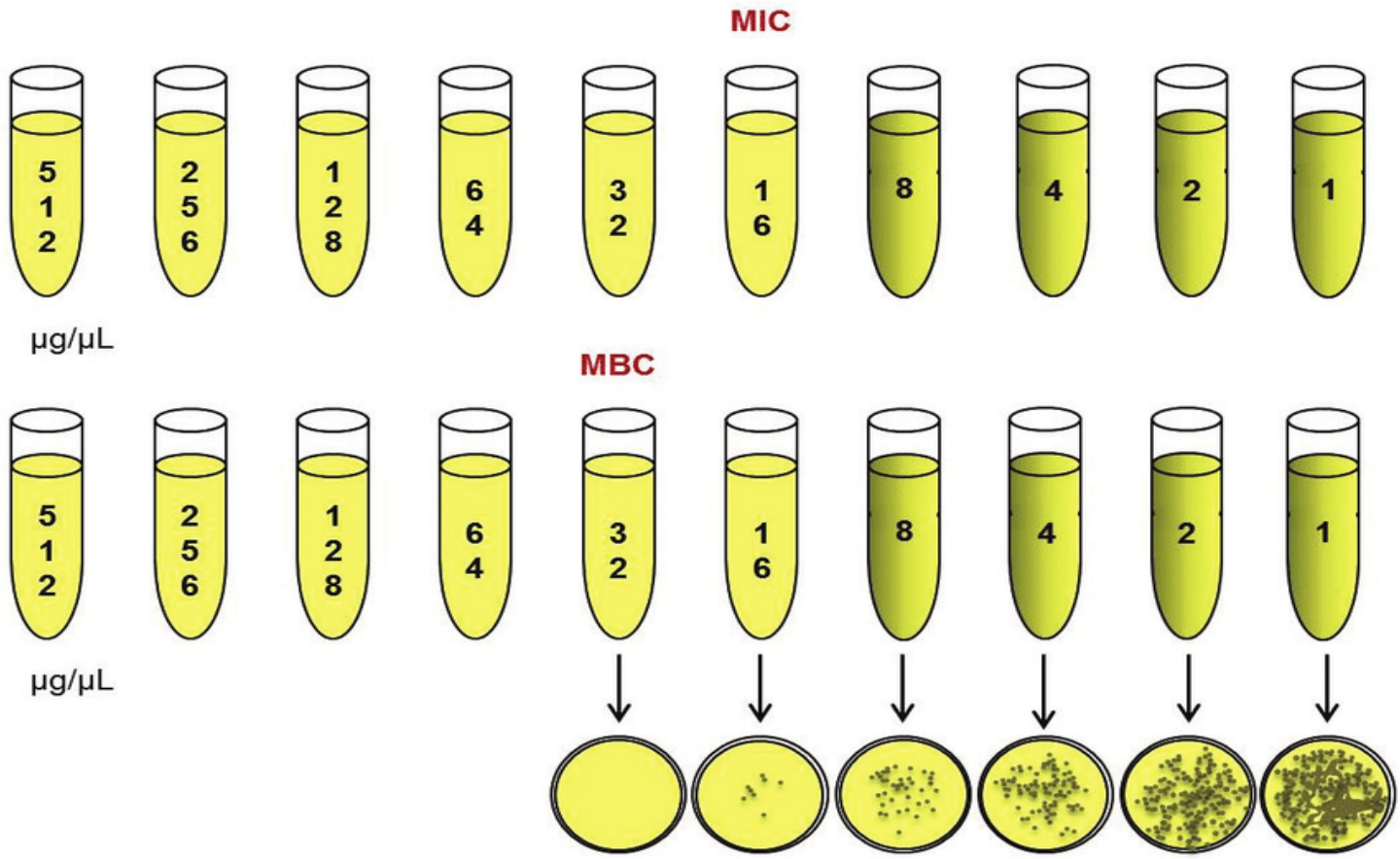
1- Cultured 10^5 - 10^6 cell/ml of bacteria under testing in sterile test tubes containing Muller-Hinton agar or other suitable medium with different requested concentration of antibiotics.

2- The antibiotics adding to the tubes, tube No. 1 containing (0) concentration of antibiotic as control; tube No. 2 containing lowest concentration of antibiotic; tube No. 3 containing double concentration of tube No. 2 and so, and so.

3- All tubes incubated at optimum condition, then check; any turbidity refers to growing of bacteria while no turbidity (clear) refers to inhibition of bacteria by antibiotic action.

To determine MIC, select the 1st clear tube (no bacterial growth) ranked at serial turbidity tubes, this tube are containing MIC of antibiotic.

To determine MBC, taking 0.1 ml from clear tubes and transfer to petri dishes containing Muller-Hinton agar and spreading on surface of agar then, incubated at 37 ^{o}C for 24 – 48 hr and then check the growth of colonies in each plate the 1st plate that does not shown any colony it represent the concentration of antibiotic for clear tube and consider MBC. For example:



Q) Container contain 250 mg of tetracycline; prepare the following concentration 10 mg/ml and 25 µg/ml with final volume 10 ml to tested against *E. coli*

The content capsule dissolved in 5 ml of D.W.

250/5 = 50 mg/ml ---------- stock 1

C1 V1 = C2 V2

50 x V1 = 10 x 10

V1 = 10 x 10 / 50

V1 = 2 ml from stock 1 + 0.1 ml (inoculation) + 5 ml (D.S.N.B.) + 2.9 ml (D.W.) = 10 ml

To prepare 100 µg/ml, the units "mg" should be converted to µg

50 x 1000 = 50000 µg/ml

C1 V1 = C2 V2

50000 x V1 = 80 x 10

V1 = 800 / 50000 = 0.016 ml from stock 1 (too small to be pipetting)

Note: Prepare stock 2 from stock 1, stock 2 should be higher and nearest than higher requested concentration (higher than 80 µg/ml) suppose it is 100 µg/ml and its volume 100 ml.

C1 V1 = C2 V2

50000 x V1 = 100 x 100

V1 = 0.2 ml from stock 1 + 99.8 ml (D.W.) = 100 ml (final volume of stock 2)

To prepare 80 µg/ml

C1 V1 = C2 V2

100 x V1 = 80 x 10

V1 = 8 ml from stock 2 + 0.1 ml (inoculation) + 1.9 ml (D.S.N.B.) = 10 ml

To prepare 25 µg/ml

C1 V1 = C2 V2

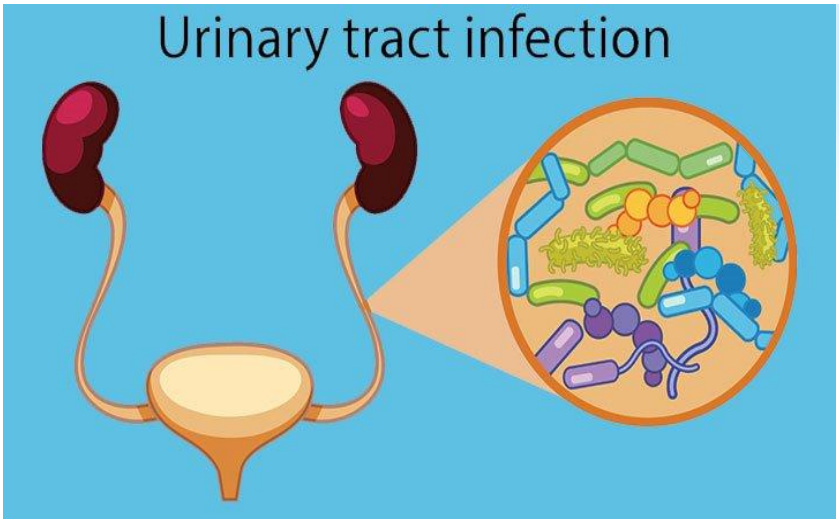
100 x V1 = 25 x 10

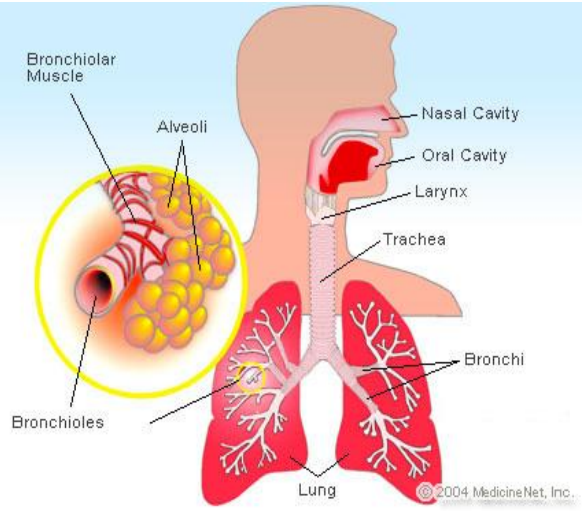
V1 = 2.5 ml from stock 2 + 0.1 ml (inoculation) + 5 ml (D.S.N.B.) + 2.4 D.W. = 10 ml

Antimicrobial drugs used in combination

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 firstly before treatment; but in some cases there are unstable relationship between diseases symptoms and bacterial causative agents, Ex: Acute UTI and RT caused by many causative agent that reveals same symptoms but there is no specific antibiotic lead to inhibit all microbial causative agent.





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

3- Preventing or delaying development of antibiotics resistance: in some pathogenic cases may recommended two antibiotics to reduce the possibility developed microbial strain resistant to antibiotics, because the frequency of the resistant codes for more antibiotics has different mechanism for single antibiotic resistance Ex: if the possibility of the resistance for the resistance for the antibiotic (A) in one microbe is $A=10^{-5}$ and to the antibiotic (B) is $B=10^{-7}$ the possibility resistance for both antibiotics A and B is $10^{-5} + 10^{-7} = 10^{-12}$ (it is rare frequency).

4- Synergetic effect: in some sever infections such as (Septicemia), it is may used two or more antibiotics together in order to eliminate the disease.

5- To reduce the toxicity of some highly toxic antibiotics: the effect of low dosage of two combined antibiotics (Synergetic), it is same effect for any dosage of antibiotics (non-Synergetic).

In the treatment of T.B. 1/2 dose of Streptomycin + 1/2 dose Rifampin

↓ ↓

Cause renal damage Cause liver damage

Using full dose of Streptomycin causing damage in kidney, while Rifampin causing damage in liver, for that, we recommended to use half dose for each above of antibiotics in order to appear same effect and reduced side effects.

Mechanisms of combination

When 2 drugs act together their combined effect may be:

1- Indifferent effect: when the activity of both combined drugs is unaffected by the presence of the other

2- Antagonistic effect: when the activity of one drug is reduced by the presence the other, when the activity of one antibiotic affected by the second antibiotic the antagonist effect will appeared Ex: mixing bacteriostatic drug such as (Chloramphenicol and Tetracycline) with bactericidal drugs such as (Aminoglycoside), the antagonistic will occurred when the first antibiotic arrived to infected organ before the second one: this case shown clearly in bacteria that causes meningitis.

3- Synergetic effect: when the activity of both drugs is significantly greater than that of either acting alone in the same concentration.

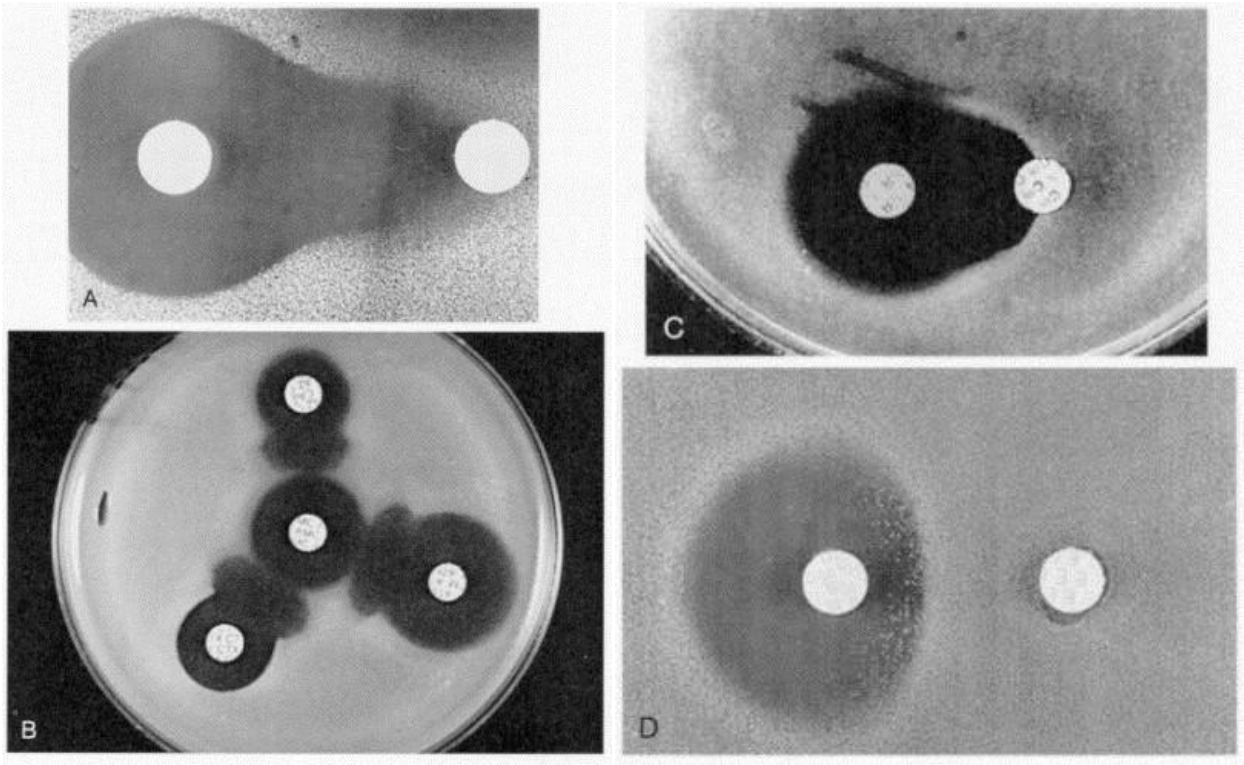
Ex: Methoprim (Trimethoprime + Sulfonamide)

Para amino benzoic acid ⟶ **dihydrofolic acid** ⟶ **folic acid**

PABA **Sulfonamide** **Trimetheoprime**

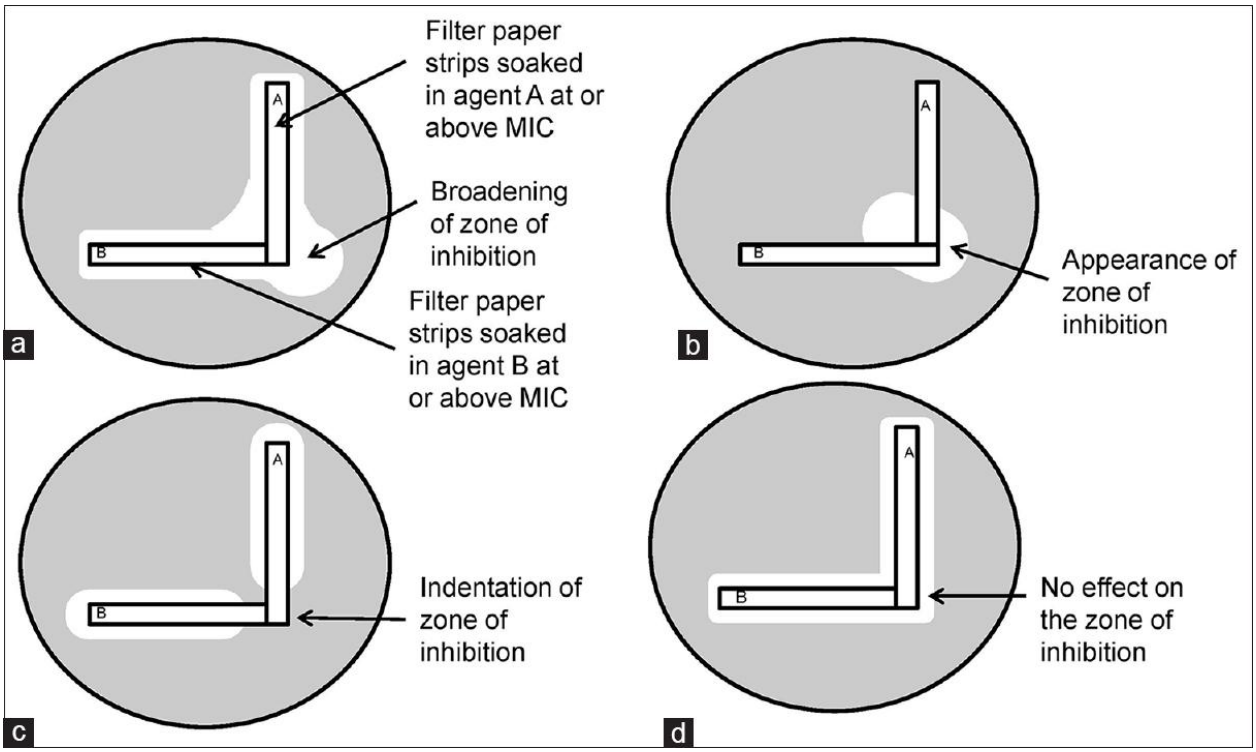
The effect and activity of synergetic antibiotics it is greater than activity of antibiotic alone, also the synergetic antibiotics may causing blockage in microbial metabolism pathway.

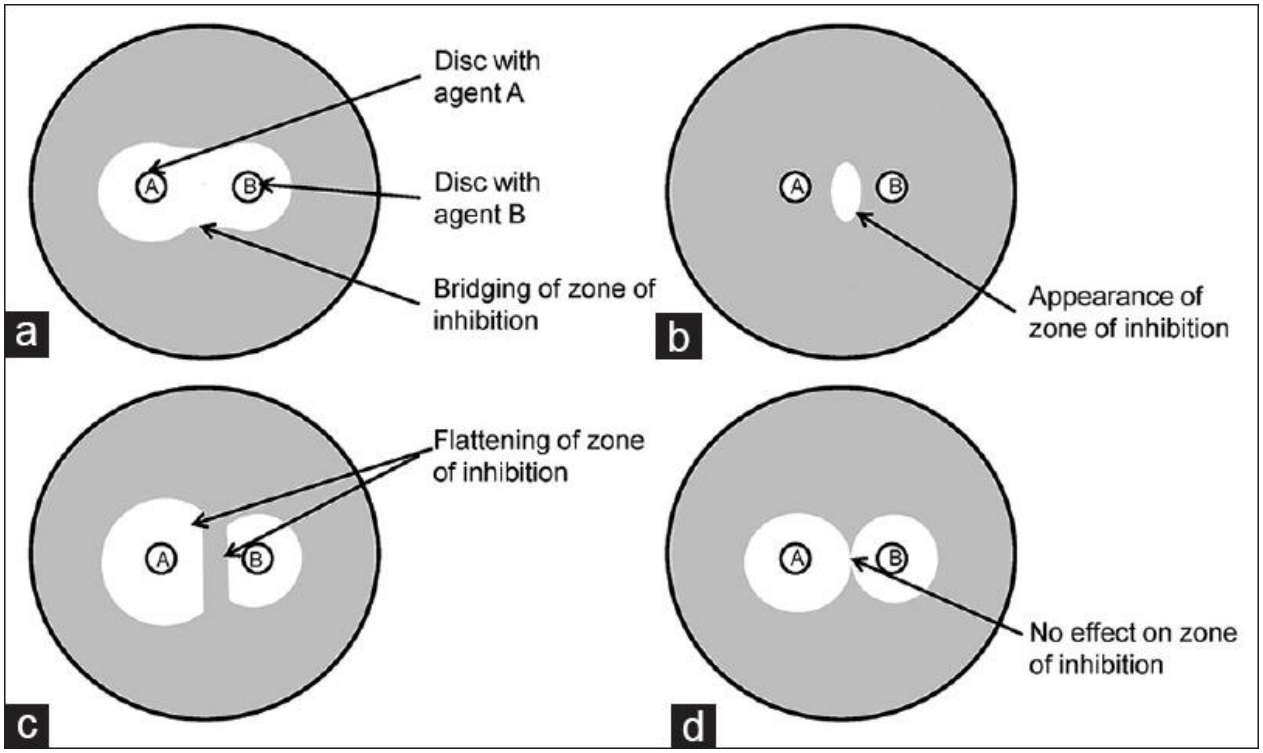
The above two antibiotics have double blockage role when sulfonamide interfere with converting PABA to dihydrofolic acid (intermediate metabolite); while Trimethoprime prevent converting the dihydrofolic acid to folic acid, for that, inhibition action occurred in two sites of the biological or metabolism pathways.



There are many methods to observe the effects of antibiotics combination:

Diffusion method: it is simplest method used to estimate the effect of combined antibiotics on M.O. by blotting paper strip; this test done in petri dish containing Muller-Hinton agar where the sterilized strip soaked in MBC concentration of antibiotics, the cultural media inoculated with bacteria, then the strips applied on the agar surface in 90 degree angle by sterilized forceps and incubated at 37 ^{o}C for 18 hr and note the results. The effect of each antibiotic alone noted at end of strip, while combined action noted at meeting point of strips by fixed on the surface of inoculated agar previously, the disc attached on the agar at suitable distances.


Filter paper strips soaked in agent A at or above MIC
Broadening of zone of inhibition
Filter paper strips soaked in agent B at or above MIC
a
A
B
Appearance of zone of inhibition
b
Indentation of zone of inhibition
c
No effect on the zone of inhibition
d


Disc with agent A
Disc with agent B
Bridging of zone of inhibition
a
A
B
Appearance of zone of inhibition
b
Flattening of zone of inhibition
c
No effect on zone of inhibition
d

Methods of antimicrobial drugs combination

Dilution method: there are **two** methods

1- Checker board titration

2- The half chess board titration

Checker board titration

It is a quantitative method where the overlap of two antibiotics action together, MIC is determined accurate by checker board titration method. The two antibiotics diluted in broth medium and their concentration together of each antibiotics. The result were measured according to horse walking on the chessboard as inverted L-shape.

Experience:

Prepare series of double concentrations for two antibiotics A and B starting from zero (control tube) at requested concentrations (µg/ml); for example, the concentrations of two antibiotic A and B are 2-128 µg/ml. the concentration of drug B arranged in horizontally while drug A arrange in vertically as fallowing table:

	2B	4B	8B	16B	32B	64B	128B	Nil
2A	2A 2B	2A 4B	2A 8B	2A 16B	2A 32B	2A 64B	2A 128B	2A
4A	4A 2B							4A
8A	8A 2B							8A
16A	16A 2B							16A
32A	32A 2B							32A
64A	64A 2B							64A
128A	128A 2B							128A
Nil	2B	4B	8B	16B	32B	64B	128B	Nil

The last horizontally row of the table is to estimate the value of MIC of the drug B while the last column row of the table is to estimate the value of MIC of drug A; the tube in the lower right corner in this experiment not contain A or B drug represents the control tube (drug free

tube). Inoculate these above tubes with 0.1 ml of bacterial suspension overnight culture with bacterial density about 10^6 cell/ml and then incubated at 37˚C degree for period (does not exceed 48 hours) the incubation period based on the appearance of turbidity in the drug free tube the vertical tubes (row) are used to estimate the value of MIC for drug A while the horizontal tubes (row) are used to estimate the value of MIC for drug B; the estimate of MIC value for synergic action should be less than four steps of the value of MIC for both A & B antibiotics. EX: if the MIC value for drug A is (128 µg/ml) and drug B is (46 µg/ml), the MIC value of the two drugs A+B = 8A 4B that means (8 µg/ml) of drug A and (4 µg/ml) of drug B.

The half checkerboard titration

This method is necessary when to be tested more than two antibiotics, especially against *Staphylococcus* infection (inflammation of cardiac valve).

The experiment:

Use only one concentration for each antibiotics and tested alone then tested all concentrations for each antibiotics under testing we can used several antibiotics used. Although the result are complex but the rearrangement all tubes in half chessboard is facilitate the process of the test and read the result. The test of a individual concentration of each antibiotic is randomly and may differ from antibiotic to another of the same M.O. under testing, should be notice the used concentrations should be within the limits that do not causing side effects when used inside the human body.

The test (use 8 drugs) as follows:

1- Rank 36 tubes in the tubes holder on a semi chessboard the horizontal top row contains 8 tubes also vertical row of the right hand side contains 8 tubes.
2- Use Muller Hinton broth (ex: volume 10 ml) and inoculated with 0.1 ml of bacterial suspension (overnight culture) (each tube contains 10^6 cells/ml).
3- Put 1 ml of the first antibiotics (Cloxacillin) to each tube of the upper horizontal row.

4- Put 1 ml of the second antibiotic (Gentamycin) to the second tube the upper horizontal row. That containing 1 ml of Cloxacillin; and1 ml to lower tube that represent 1st tube of the second horizontal row and keep doing to all tubes in both rows.

5- Start with the third drug and put 1 ml of Erthromycin in the 3ed tube of 1st row and in second tube also 1st tube of the 3ed row, the antibiotics add for each tubes among these rows, and then keep doing to all tubes in both rows.

6- Mix well and incubate at 37 ˚C for overnight, the control tube use to comparison

7- Take 0.1 ml of each tube to the center solid media in petri dish (culture media) to growing, the results are recorded after incubation as follows.

Control	Cloxacilin	Gentamycin	Erythromycin	Clindamycin	Refampicin	Fusidic acid	Streptomycin	Novobiocin
Cloxacilin								
Gentamycin								
Erythromycin								
Clindamycin								
Refampicin								
Fusidic acid								
Streptomycin								
Novobiocin								

(-) = no growth (cidal)

(+) = Very few colonies …….. Static action

+ = Few colonies ……. Static action

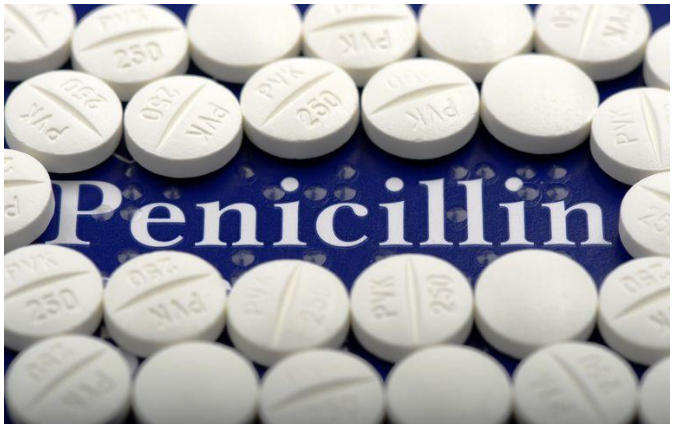
++ = as control or antagonistic effect

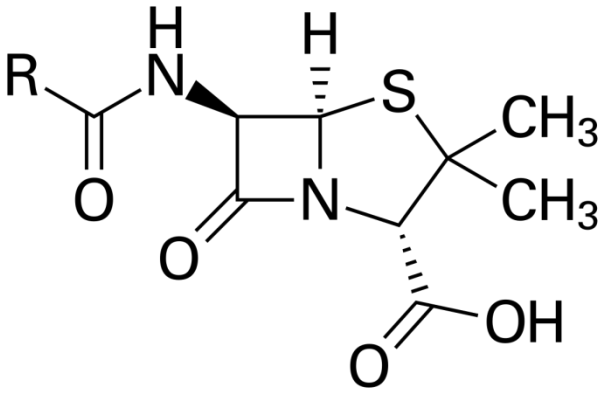
Detection of β-lactamase

β-lactamase consider most important antimicrobial substance among drug groups against bacteria since penicillin discovering at present time. β-lactam antibiotics fall into two groups:

1- Penicillin

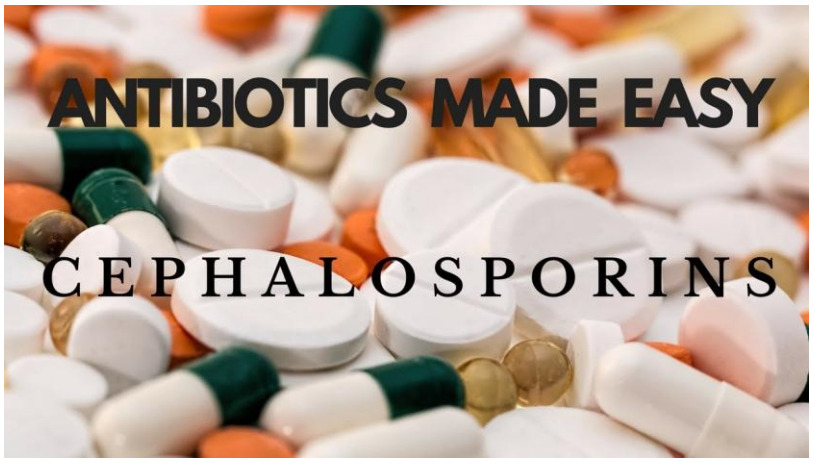
It was discovered by Alexnder Flaming in 1928, when he observed inhibition growth of *Streptococcus aureus* after contaminated with penicillin, the penicillin used clinically after purify from *P. notatum*.

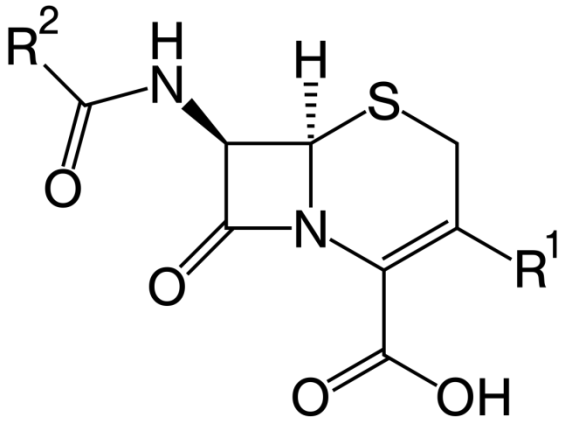




2- Cephalosporin

It was discovered by Bortza in 1948 from *Cephalosporium aeromonium* in sewage water, its differ from penicillin because it has Dihydrothiozin ring attached with β-lactam ring.





Bacterial resistance to β-lactam antibiotics by the following mechanisms:

1- Production of β-lactam degrading enzymes.
2- Changing the target side of antibiotics by penicillin binding proteins (PBPs).
3- Modification of permeability in outer membrane.

β-lactam degrading enzyme:

1- β-lactamase 2- Acylase 3- Esterase

β-lactamase enzymes able to degrade amide bond of lactam ring and convert the active antibiotic to inactive, Penicillin degradation gives stable intermediate substance penicilloic acid; while Cephalosporin degradation gives unstable intermediate substance cephalosporic acid cleave to two fragments; one molecule of enzyme able to stopping many antibiotics, because it is able to destroyed the antibiotic and return to re-binding with another antibiotic and so.

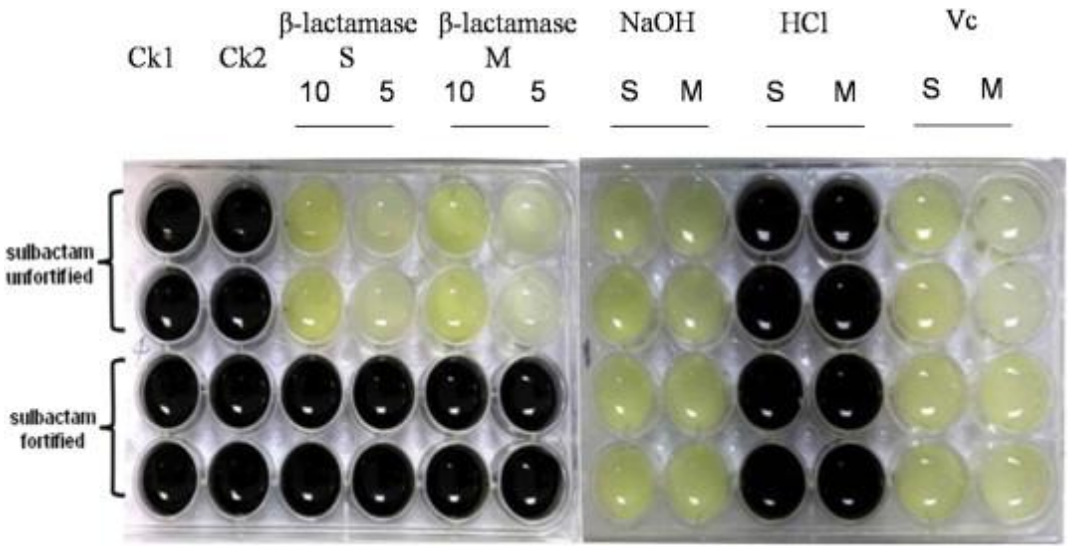
Detection of β-lactamase:

The principle of β-lactamase enzyme detection is investigation of penicilloic acid, and the detection done by many methods such as:

1- Rapid iodometric method:

Iodine able to make color complex (blue) with starch, penicilloic acid able to iodine reduction and loss their ability to form this complex as following:

- Prepare bacterial suspension of *S. aureus* growth on M.H. agar for 24 hr.
- Transports some colonies by loop to Eppendrof tube containing 0.1 ml of Penicillin G suspension, and incubate for 30 minutes at 37 ^{o}C.
- Add 50 µl of (1%) starch solution and mix well.
- Add 20 µl of iodine solution until blue color will form, the tubes shaking well, disappear blue color less than 1 min. refer to positive result comparatively with control tube.



2- Rapid acidometric method:

Alkaline red phenol solution converted to yellow color when found penicilloic acid that formed from Penicillin degradation; Penicillin detection by this method done with several mechanisms, but capillary tube method is easily.