Ministry of Higher Education and Scientific Research

University of Baghdad

College of Science

Department of Biology

Practical Antibiotics

2023-2022

المرحلة الثالثة / الدراستين الصباحية والمسائية

الفصل الدراسي الأول

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LAB (1)

Introduction to antimicrobial agents

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 -Antiseptics.

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 defences will work and deal with this infection, e.g. antibiotics.

Disinfectants:

They are antimicrobial substances and mostly they are able to kill awide 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 the 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 pathway.

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 it's growth and if these cells have the suitable media and conditions available , they can resume their activity to replicate again forming new progenies.

LAB(2)

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.

Factors that control the strength of the antimicrobial agent:

1 - Characteristics of the microorganism.

2 -The environment (temperature , pH , time , substance concentration , presence of organic substance.

3 -Number of bacteria

4- Mode of action

Differences between Antibiotics and Disinfectants.

Disinfectants	Antibiotics
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 micrograms.

Note: Sometimes concentration of antimicrobial agent measured by **International Unit (IU).**

Importance of antibiotics:

1. Prevention of diseases spreading from a human being to another or from a human being to animal or vice versa.
2. Prevention of food contamination e.g. some canned food (Nicin).
3. Some antibiotics can be added to prevent lysis of skin , wood , or papers by some types of fungi.
4. In agriculture and veterinary practice.

Sterilization:

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

Disinfection : Process in which most or nearly all microorganisms (whether or not pathogenic) on clothing, hard surfaces, and/or wounds are killed through the use of chemicals, heat, or ultraviolet rays. Milk, for example, is disinfected by heating up to 100°C for at least 10 seconds to kill most microbes (but not necessarily their 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 produce an inhibitory effect. **4-**

Germicidal zone:

In this zone there are killing effects starting to appear through the end of the inhibitory zone and increased with increasing concentrations.

Major groups of antimicrobial agemts:

1-Phenol and phenolic compounds.

2-Alcohol.

3-Halogens .

4-Heavy metals.

5-Deys.

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 it's 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 it's high solubility in lipids, and it has a major killing effect on fungi, such as in case of the O-cresol.

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 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 corrosive effect on the metal surfaces.

Heavy metals:

Most of them are bacteriostatic because of their ability to combine with proteins and suppress enzymes through their effect on the (-SH) group.e.g. mercury and silver which are considered as the only inorganic ionic metals used as disinfectants.

LAB(3)

Evaluat ion of Disinfectants or comparison of antiseptics useed against microorganisms

I- Phenol coefficient:

Phenol coefficient is a method used for:

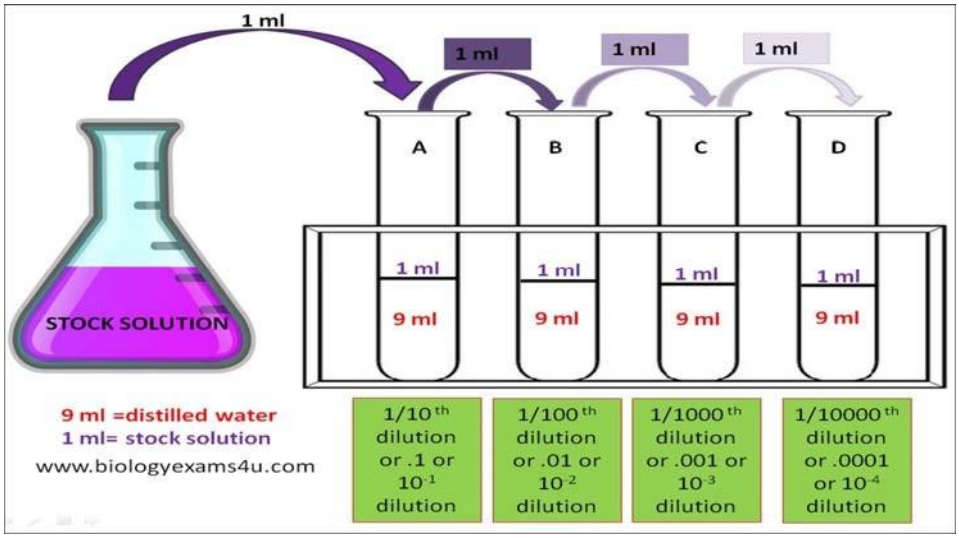
a- Evaluation of water soluble disinfectants, which is similar to phenol in chemical composition,

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

c- This method used by Food and Drug Administration (FDA).

Method :

1. Make serial dilutions from test disinfectant **(x)** in test tubes with volume of (5ml).



2. Make serial dilutions from pure phenol **(P)** .
3. Inoculate tubes with 0.5 ml from young broth culture of test organism usually ***S. aureus*** or ***P. aeruginosa*.**
4. Incubate all tubes at 20c.
5. Take loopfull (0.1) ml from both dilution (x , p) after 5 minutes , 10 minutes and 15 minutes , inoculate in sterile nutrient broth (from every tube take a loop full) to 3 new tubes after (5,10,15) minutes.
6. Incubate tubes at 37c for 24-48h. , if there is no growth (this means completely eradication) , the result is (-) , if there is a turbidity (presence of growth) the result (+).

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.

Phenol coefficient (P. C.) =

Highest diltion of (X) killing m.o during 10 min but not on 5 min.

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).**

Ex:

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

Disinfectant	Dilution	Treatment Time		
Pure phenol (P)	**Completely eradication by using highly concent**	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. 20x5 = 100 → 1 : 100 or 1/100

II: 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 over night culture , the final volume should be 10 ml.

2. after 10 minutes take a loop full from every tube to new test tubes 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 using optical density (O. D.)

3.Incubate the tubes at 37c for 18 - 24 hr.

* The result have been shown by turbidity or not.

Q:Make a comparison btween p.c. and UDT?

Q: Why do we use (D.S.N.B.) in the beginning of this test?

Q: prepare the following concentrations of bleach 12 %, 6 %?

Cone.	D.S.N.B.	Dis.	D.W.	Inoc	T. V.
6%	5ml	0.6 ml	4.3 ml	0.1 ml	10 ml
12%	5 ml	1.2 ml	3.7 ml	0.1ml	10 ml

Q prepare the following concentrations of alcohol: 10 %, 50 %, 70 %

Conc.	D.S.N.B.	Dis.	D.W.	Inoc.	T. V.
10%	5 ml	1ml	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	-	0.1ml	10 ml

LAB 4

Test of antibiotic susceptibility (sensitivity)

Microbial susceptibility to different types of antibiotics could be estimated through the ability of these antibiotics to 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 resistant 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 microbes pocess 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:

- Selective toxicity.

- inhibition of cell membrane synthesis and function
- inhibition of cell proteins synthesis.
- inhibition of cell nucleic acid synthesis.

I/ Diffusion methods of sensitivity testing

This method is done by using solid media inoculated previously with the microbe under test, the antibiotics will diffuse 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 some time considered as semi quantitative method because the inhibition zone is effected by the sensitivity of the microbe towards the antibiotic.

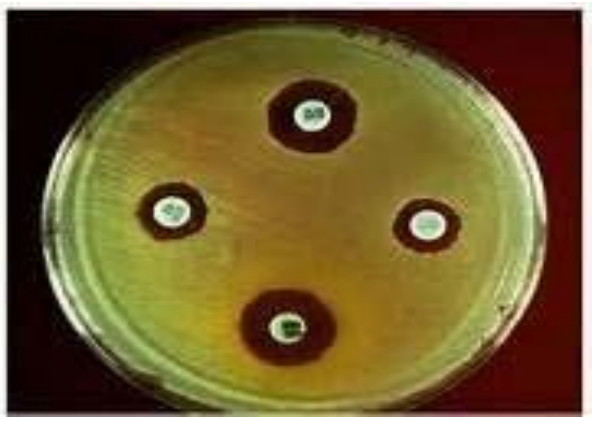
A\ Disc- diffusion method(Kirby -Bauer method)

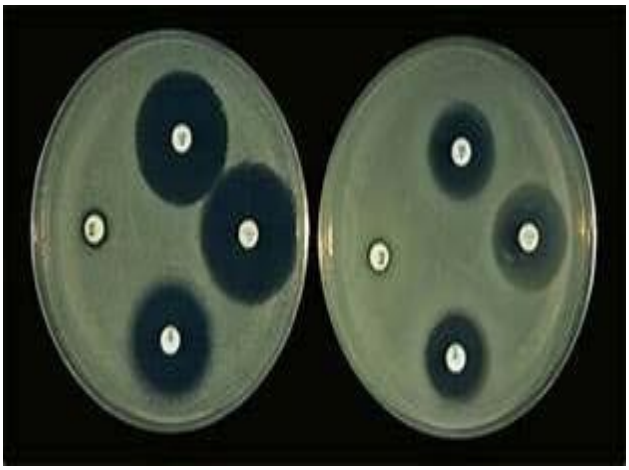
One of the most commonly methods used routinely in diagnostic laboratories. Test is done by inoculating bacteria under test on solid media in Petri dish and using blotting paper discs containing different types of antibiotics with different concentrations applied on the agar surface, during the incubation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

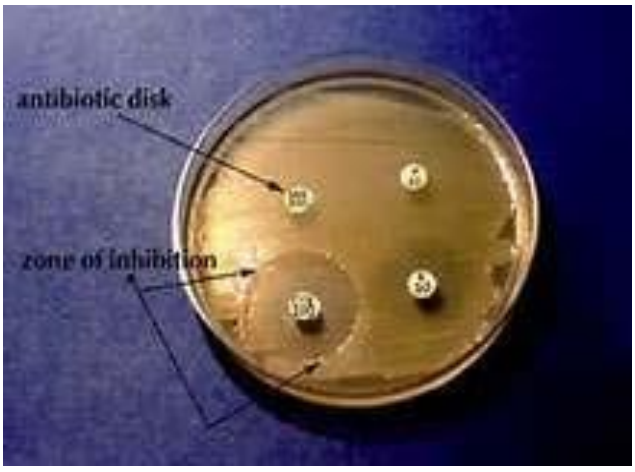
Antibiotic discs can be prepared in laboratory in any concentration by using blotting paper as following:

Filter paper is pored 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.







Methodology

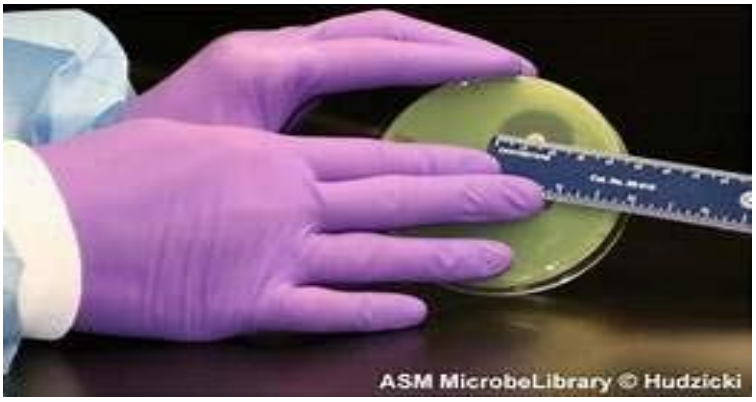
prepare Muller-Hinton agar(prepared according to FDA) as the best media for the growth of most types of pathogenic bacteria

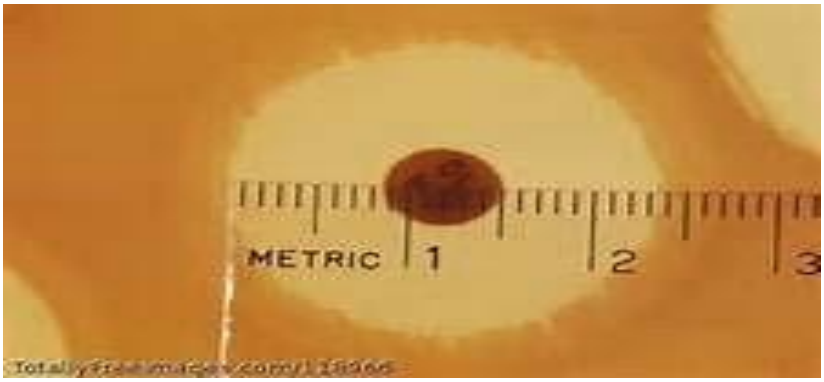
1. the media is cultured by the bacteria under test in sterile conditions (by streaking or swabbing method).

3. after drying of inoculum, 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 h.(not more than 24 hr).

5.the inhibition zone measured by mm. around each disc.





Antibiotics susceptibility testing plate . You measure the diameter of inhibition zone in millimeter, the zone seen here measures 23 mm. in diameter

LAB 5

Factors affecting the results of diffusion tests.

1.)) **Rate of drug diffusion**: depends mainly on the molecular weight of the antibiotic, for example Penicillin has low m.wt so it diffuses rapidly in the agar while Polymyxin has high m.wt so it diffuses 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 dose 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 catalize the activity of many antibiotics like Bacitracin and Novobiocin against ***Staphylococci*** and Penicillin against ***Proteus ,*** while Divalent cations decrease the inhibition zone because they interact with peptide antibiotics like Polymyxin or alkaline antibiotic like kanamycin.

D. Carbohydrates : may decrease the inhibition zone to some microbes and some antibiotics.

E. The blood: small inhibition zone are seen for some antibiotics that have peptide bonds like F'usidic 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 4mm.

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

In disc method, the perfect type for typical growth is semi-confluent growth since the inoculation method is effective in density of inoculum.

5.)) **Incubation period** : should be less than 24hr. because long incubation period less the drug activity and this allows the sensitive microorganisms thatis 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 4^0C, validity (not expired), number of discs per dish and spaces between them.

7))Type of microorganism.

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

**Ideal sensitivity testing medium:-

1. very well known ingredients
2. should not be enriched media support the rapid growth of bacteria.
3. dose not contain antagonist materials to any bacterial Spp.
4. keep it's pH stable and constant during bacterial growth.
5. should be isotonic.

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 layed 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 inoculum 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.**

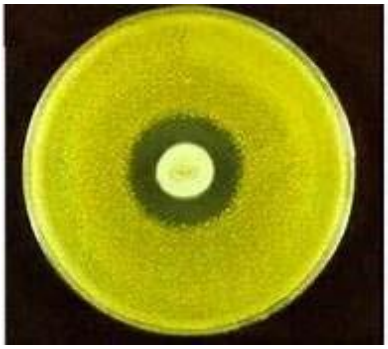
D. Spreading by spreader

E. Streaking by loop

The theory of zone formation:

The rate of drug diffusion in the media depending on it's 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. inoculum volume
2. growth rate
3. pre-incubation period
4. pre-diffusion

Critical density:The minimum density of inoculum that inhibited by the drug on zone edge and depends on both drug and organism.

If the inoculum is more than critical density , the inhibition zone will not formed , but if the inoculum 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)** .

II// the gradient plate test

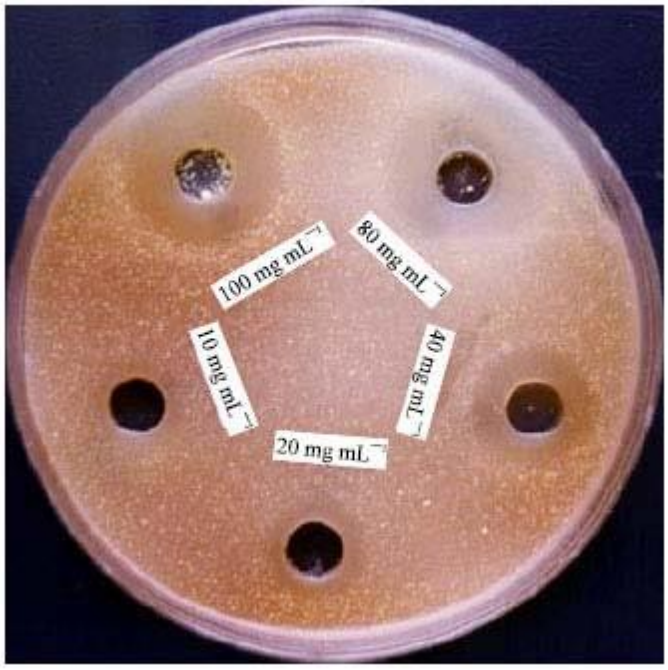
This method is used to know the sensitivity or resistance of m.o. to specific or several antibiotics, and from the gradient of concentration you can know the MIC of

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 leaving the media to solidify to get tilt surface ; then the remaining 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 layers 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 time and notice the growth of bacteria on the surface of media.

III// 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 add a suitable amount of drug (ex: 50 µl), incubate the plate at 37°C for 24 hr. then estimate the inhibition zone around the well determined by mm.

This method could be used to know the activity of certain antibiotic to several kinds of m.o and on the same Petri dish by making many streaking lines of inoculum for each m.o and using the loop from the edge of the well to the edge of the Petri-dish.



Lab 6

Dilution method

It is a quantitative method, depend on preparation of series of gradually duplicate concentration of antibiotic in a suitable medium for growth, then adding limited number of bacteria and checking the ability of antibiotic to inhibit or kill the bacteria under testing by prescence of turbidity in test tubes or not .

The diffusion test of antibiotics by using disc method is useless and do 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. generation time), using disc method give wrong results because the antibiotic diffusion in agar faster than bacterial growth, for that, the inhibition zone appears bigger than normal case (not truth).

2.In case of swarming bacteria such as ***Proteus*** , the swarming movement is considered as a hinder for antibiotic diffusion and leads 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)** which diffuses in agar slowly , for that, the inhibition will be small or will not appear because the growth of bacteria is faster than diffusion of antibiotic.

In all above cases , the dilution method is recommended and the **MIC** (minimal inhibitory concentration) and **MBC** (minimal bactericidal concentration) can be estimated.

MIC = The minimum concentration leads to inhibition of bacteria under testing ; while **MBC** = The minimum concentration that kill the bacteria under testing.

Method:- (Tube method and well method)

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

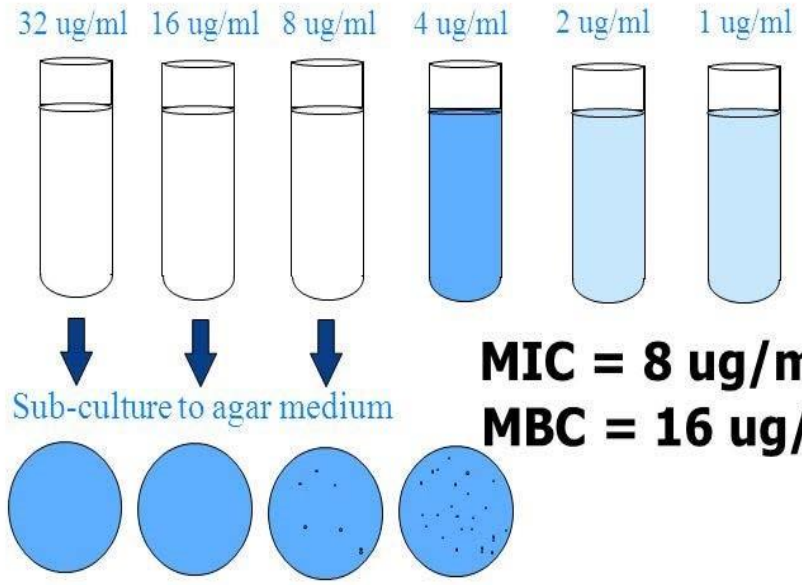
2. Add he antibiotics to tubes except tube No.1 which containing (0) concentration of antibiotic as control; tube No.2 containing lowest concentration of antibiotic; tube No,3 containing double conceatration of tube No,2 and so.

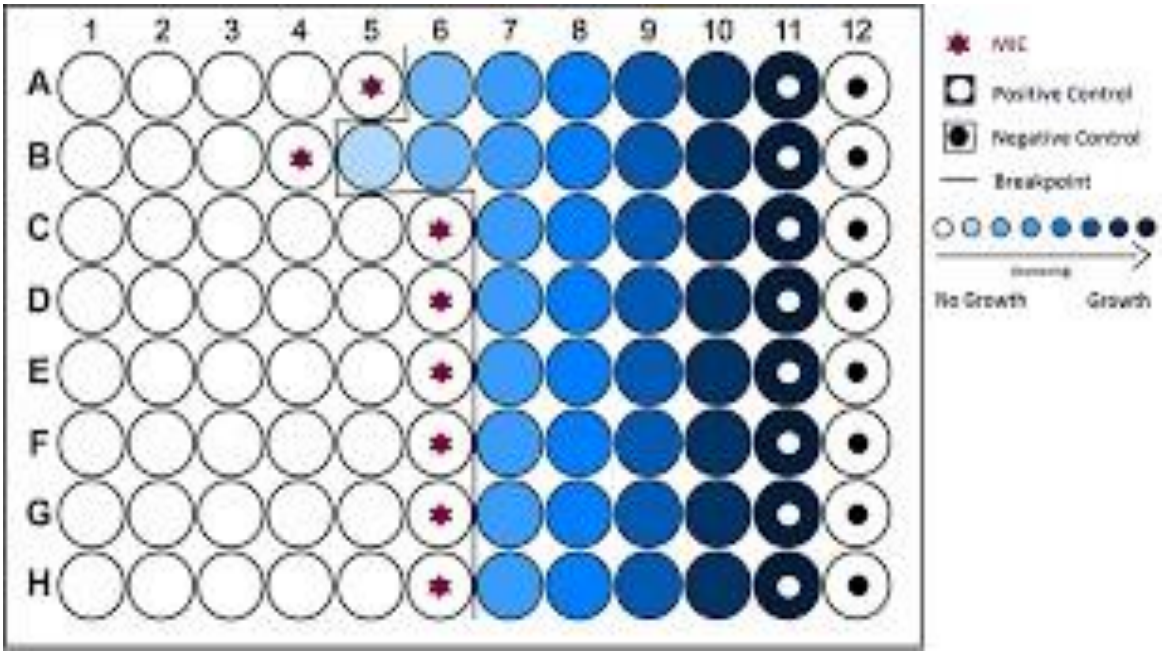
3. Incubated all tubes at optimum condition, then check ; any turbidity refer to growth of bacteria while no-turbidity (clear) refer to inhibition of bacteria by antibiotic action.

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

- **To determine MBC** , take 0.1 ml from clear tubes and transfer to pet dishes containing Mueller Hinton agar and spread on the surface of agar then incubate at 37°C for 24-48 hr and then check the growth of colonies in each plate ;the 1st plate that dose not show any colony represents the concentration of antibiotic for clear tube and is considerd MBC for example the following flow chart:

Minimal Inhibitory Concentration (MIC) vs. Minimal Bactericidal Concentration (MBC)





problem:

Prepare the following concentrations 0,4,8,16,32,64,128....µg/ml of penicillin G 250mg (capsule) , the final volume for each concentration is 10 ml. the penicillin used against *Staphylococcus aureus* .

Solution :

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

$\frac{250mg}{5ml}$ = 50mg/ml .....stock 1

The unites milligram mg convert to microgram µg

50X 1000 = 50.000 µg/ ml

Note

Preparation stock 2 should be highest and nearest than higher request concentration (higher than 128 µg/ml) let is =200 µg/ml and it's volume is 100 ml (prepared from stock 1).

C1 VI =C2 V2

50.000X V1 =200X 100

VI = $\frac{20.000}{50.000}$ = 0.4ml from stock 1 + 99.6 ml of D.W.

From stock 2 (200 µg/ml) we can prepare all above request concentrations with final volume is 10 ml in same way of 1st concentration 4 µg preparation as following .

To prepare concentration 4 µg/ml

C1 VI =C2 V2

200X V1 =4X 10

VI $= \frac{40}{200}$ = 0.2ml from stock 2 + 5 ml of D.S.M.B + 4.7 ml D.W + 0.1 ml inoculation

*(total volume should be 10 ml)

Try to prepare other concentrations and complete the following table

Final cone. of Antibiotics	Double strength Mueller Hinton Broth	Amount of antibiotic	D.W	Inoculation	Total volume
0	**5ml**	**0**	**4.9ml**	**0.1 ml**	**10 ml**
4	**5ml**	**0.2ml**	**4.7ml**	**0.1 ml**	**10 ml**
8	**5ml**			**0.1 ml**	**10 ml**
16	**5ml**			**0.1 ml**	**10 ml**
32	**5ml**			**0.1 ml**	**10 ml**
64	**5ml**			**0.1 ml**	**10 ml**
128	**5ml**			**0.1 ml**	**10 ml**
256	**5ml**			**0.1 ml**	**10 ml**

Questions: container contains 500 mg of tetracycline , prepare the following concentrations 10 mg/ml , 500 µg /ml and 50 µg/ml with final volume 10 ml to tested against *E.coli.*

Lab 7

Epsilometer test (E test) : diagnostic device used by laboratories to determine the MIC (Minimum Inhibitory Concentration) and whether or not a specific strain of bacterium or fungus is susceptible to the action of a specific antimicrobial. This type of test is most commonly used in healthcare settings to help guiding physicians in treatment of patients by indicating what concentration of antimicrobial would successfully treats an infection.

The E test consists of a thin reagent plastic strip that carries a continuous concentration gradient of stabilized and dried antibiotic with the strongest being at the top of the strip and the weakest at the bottom and providing quantitative wide-range MICs in a simple and easily reproducible manner (the E test is easy to exam) (figure1) the gradient range is equivalent to 15 dilutions by a conventional reference MIC procedure.

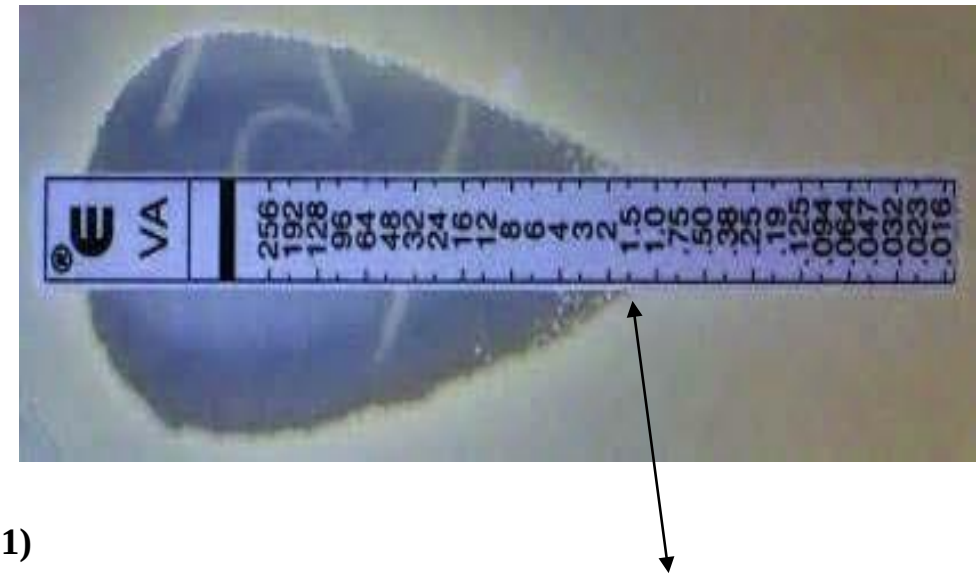


Figure (1)

MIC

E test is a quantitative technique for determining the antimicrobial susceptibility (MIC) in µg/ml of Gram-negative and Gram-positive aerobic bacteria such as **Enterobacteriaceae,** ***Pseudomonas***, ***Staphylococcus***, and ***Enterococcus*** species and fastidious bacteria, such as anaerobes, ***N. gonorrhoeae***, ***S. pneumoniae***, ***Streptococcus*** and ***Haemophilius*** species.

Method:

1.The surface of agar plate inoculated with an adjusted bacterial suspension by swab in the same manner as a disk diffusion test.

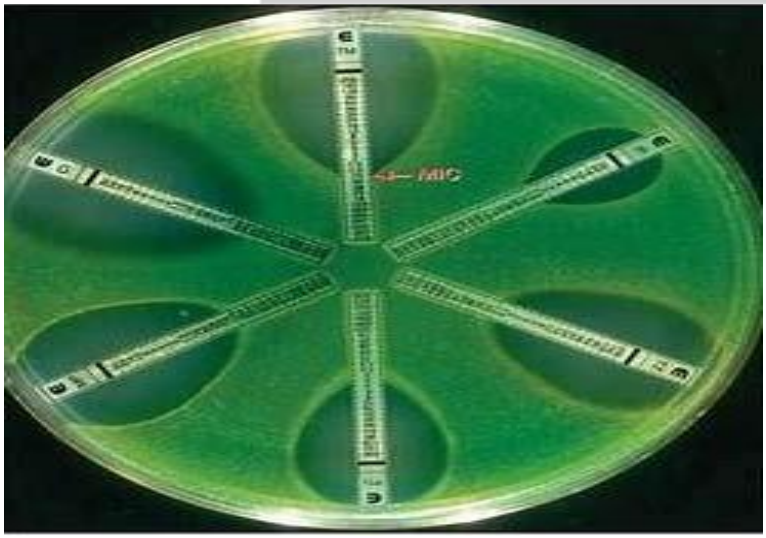
2.One or more E test strips for the antimicrobial agents to be tested are then placed on the inoculated agar surface.

3.After an overnight incubation, the interaction of the antimicrobial agent gradient and the test bacterial inoculum gives rise to elliptical inhibitory zones (figure 1)

4.The results are read in the intersection of the ellipse with a MIC scale (in µg/ml on the strip (figure 2) .

The E test inhibition ellipses were clearly demarcated, and the points of intersection of the zone edge with the strips were generally easy to interpret. Figure (3)

Figure(2)



Figure(3)

E-test can help to:-

- Determine the MIC of fastidious, slow-growing or nutritionally deficient microorganisms that have unique growth requirements and cannot be testing by automated methods, or for a specific type of patient or infection.
- Detect low levels of resistance.
- Test an antimicrobial not performed in routine use or a new recently introduced antimicrobial agent.

- Confirm an equivocal antibiotics sensitivity test results

Lab 8

Antimicrobial Drugs Used in Combination

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

1. **Undiagnosed infection :-** in patnogenic cases, the causative agent should be diagnosed firstly before treatment; but in some cases there arc unstable relationship between diseases symptoms and bacterial causative agents, ex:- Acute UTI and RTI caused by many causative agents that reveals same symptoms but there is no specific antibiotic lead lo inhibit all microbial causative agent.

2. **Mixed infections :-** there is no specific antibiotic Vs (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 antibiotic (A) in one microbe is $A=10^{-5}$and to the antibiotic(B) is $B=l0^{-7}$, the possibility resistance for both antibiotics A & 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 TB 1/2 dose of Streptomycin+ 1/2 dose Refampin

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.

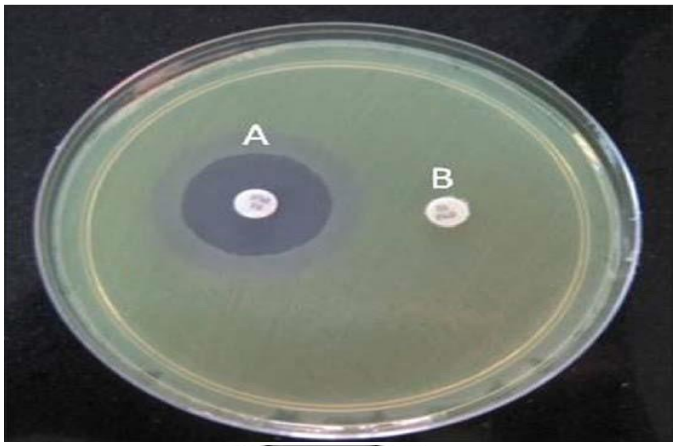
 There are many methods to observe the effects of antibiotics combination:

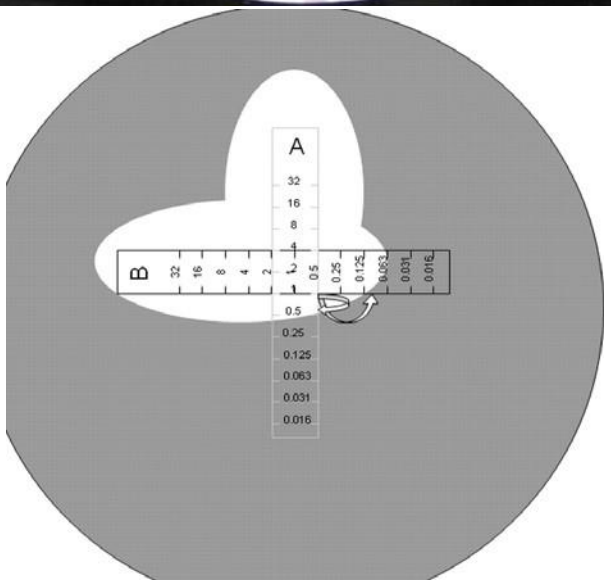
- **Diffusion method**: it is simplest method used to estimate the effect of combined antibiotics (discs or strips) on m.o; this test done in Petri dishes containing Muller Hinton agar the cultural media inoculated with bacteria , then the antibiotics applied on the agar surface in 90 degree angle (strips) and in a suitable distance (discs) by using sterilized forceps and incubated at 37°C for 18 hr. and note the results.

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.

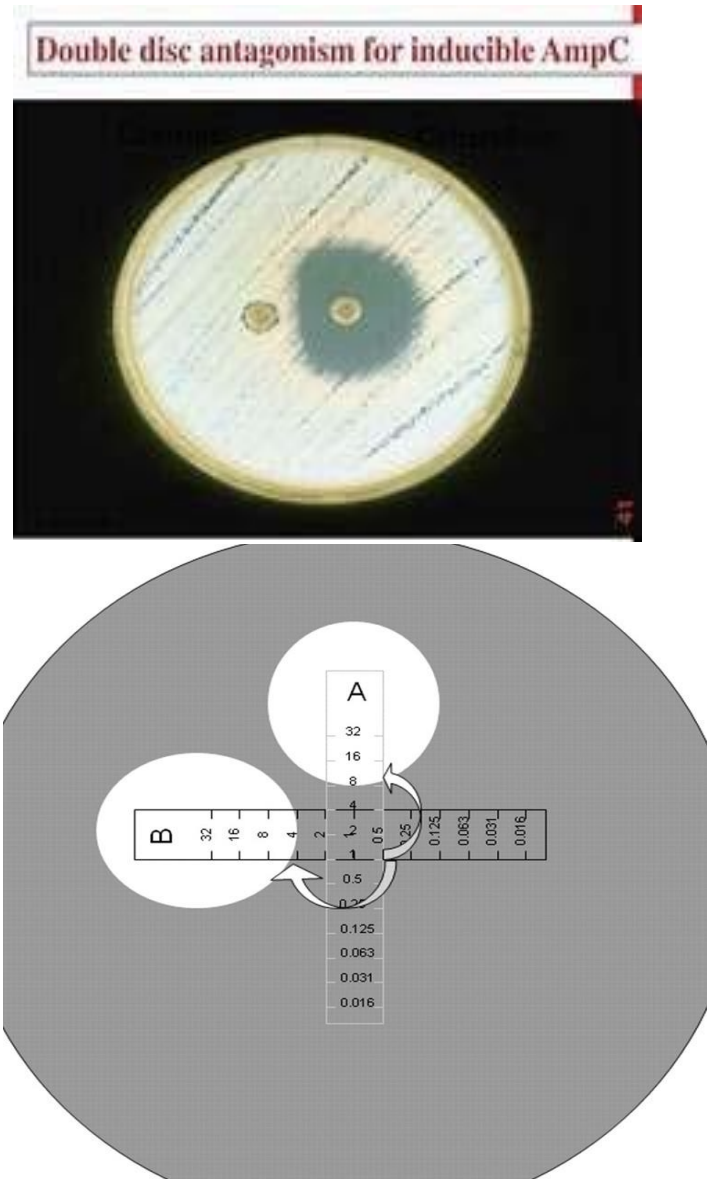




Indifference: The MIC of antimicrobial A was 1.0 mg/L when tested alone or in combination with antimicrobial B; the MIC of antimicrobial B was 0.5 mg/L when tested alone or in combination with antimicrobia

2. **Antagonistic effect** : when the activity of one drug is reduced by the preset 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

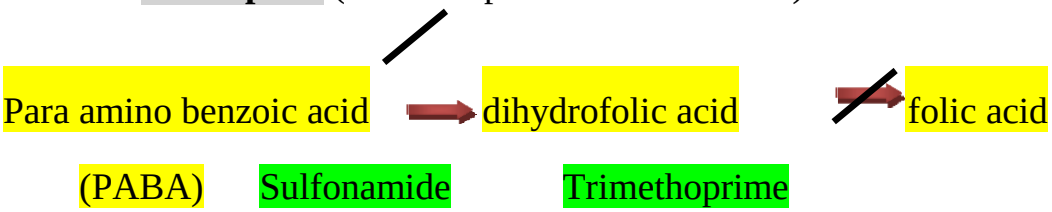
arrived to infected organ before the second one; this case shown clearly in bacteria that causes meningitis.



Antagonism: The MIC of antimicrobial A was 1.0 mg/L when tested alone, but was 8.0 mg/L when tested in combination with antimicrobial B; the MIC of antimicrobial B was 0.5 mg/L when tested alone, but was 4.0 mg/L when tested in combination with antimicrobial A.

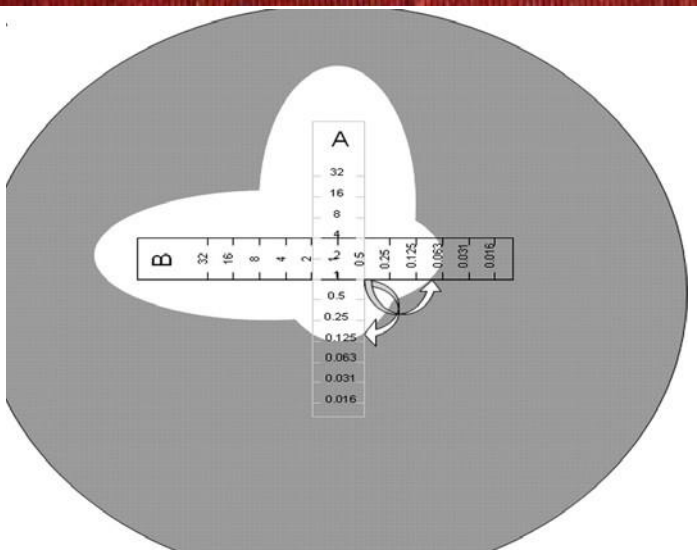
3. **Synergetic effect** :When the activity of both drugs is significantly greater than that of either acting alone in the same concentration.

EX:-**Methoprim** (Trimethoprime+ Sulfonamide)



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

the above two antibiotics have double blockage role when sulfonamid 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.



Synergy: The MIC of antimicrobial A was 1.0 mg/L when tested alone, but was 0.125 mg/L when tested in combination with antimicrobial B; the MIC of antimicrobial B was 0.5 mg/L when tested alone, but was 0.063 mg/L when tested in combination with antimicrobial A

Lab 9

Detection of B-lactamases

B- lactamsase consider the most important antimicrobial substance among drug groups against bacteria since penicillin discovering at present time. B- lactm antibiotics fall into two groups:

1) Penicillins

it was discovered by Alexndar Flaming in 1928, when he observed inhibition in growth of ***Staphylococcus aureus*** after contaminated with ***Penicillium*** ; the penicillin used clinically after purify from ***P.notatum*** to treatment world-war victims (1941).

2) Cephalosprins:

It was discovered by Bortzain 1948 from ***Cephalosporium aeromonium*** in sewage water; it's differ than Penicillins because it has Dihydrothiozin ring attached with B-lactam ring and present 2-R-side chain according to derivatives type.

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

A. Production of B-lactam degrading enzymes.

B. Changing the target side of antibiotics by Penicillin Binding Proteins (PBPs).

C. Modification of permeability barriers in outer membrane.

B-lactamase degrading enzymes:

1-B- lactamase 2-Acylase 3-Esterase

B-lactamase enzymes able to degrade amide bond of lactam ring and convert the active antibiotic to inactive, Penicillins degredation gives stable intermediate substance Penicilloic acid (dibasic acid) ; while Cephalosprins degredation gives unstable intermediate substance cephalosporic acid cleave to two fragments; One molecule of enzyme able to stop many antibiotics , because it is able to destroyed the antibiotic and return to re-binding with another antibiotic and so.

Classification of B-Iactamase according to staining of productive bacteria:

- **Gram positive B- lactamase**: it's Extracellular enzymes produced by many bacteria-such as ***Staphylococcus aureus*** and others, production of this enzymes equal 2% of weight of dried cells and fall into two types constitutive and inducible enzymes.
- **Gram negative B- lactamase** : it is present in periplasm space and fall into types , constitutive (common) and inducible increased by inducer.

There are other classification as following : -

Chromosomal B-lactamase:

It is constitutive ,controlled by chromosome and it is very low amount and not easy detected such as ***Haemophilus***; while ***Pseudomonas aeroginosa*** was heavy amount and easy detect.

Inducible enzymes:

It is secreted when inducer is found, often the inducer is B-lactam antibiotics; this enzymes coded by structural genes controlled by other genes called control genes through repressor.

Detection of B-lactamase:

The principle of B-lactamase enzymes detection is investigation of Penicilloic acid (dibasic acid compound), and the detection done by many methods such as :

1) Rapid Iodometeric 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 or MacConkey agar for 24hr.
- Transport some colonies by loop to Eppendorf tube containing 100 µl of Penicillin G suspension, and incubate for 30 minutes at 37°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 minutes refer to positive result comparatively with control tube.

2) Rapid Acidimetric method:

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

Lab 10

The Vitek System

The VITEK is an automated microbiology system utilizing growth-based technology, used for identification of m.o and antibiotics sensitivity test .The system is available in three formats (VITEK 2 compact, VITEK 2, and VITEK 2 XL) that differ in increasing levels of capacity and automation. All three systems accommodate the same colorimetric reagent cards that are incubated and interpreted automatically

There are three types:

*VITEK2
*VITEK 2 compact

*VITEK2XL

All three systems accommodate the same colorimetric reagent cards

Reagent cards

The reagent cards have 64 wells that can each contain an individual test substrate. Substrates measure various metabolic activities such as acidification, alkalinization, enzyme hydrolysis, and growth in the presence of inhibitory substances. An optically clear film present on both sides of the card allows for the appropriate level of oxygen transmission while maintaining a sealed vessel that prevents contact with the organism-substrate admixtures. Each card has a pre-inserted transfer tube used for inoculation. Cards have bar codes that contain information on product type, lot number, expiration date, and a unique identifier that can be linked to the sample either before or after loading the card onto the system.

There are four reagent cards:

1. GN - Gram-negative fermenting and non-fermenting bacilli
2. GP - Gram-positive cocci and non-spore-forming bacilli
3. YST - yeasts and yeast-like organisms
4. BCL - Gram-positive spore-forming bacilli

Culture Requirements

Include acceptable culture media, culture age, incubation conditions, and inoculum turbidity.

Suspension Preparation

A sterile swab or applicator stick is used to transfer a sufficient number of colonies of a pure culture and to suspend the microorganism in 3.0 mL of sterile saline

(aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) in a 12 x 75 mm clear plastic (polystyrene) test tube. The turbidity is adjusted accordingly (see Table 1) and measured using a turbidity meter called the DensiChekTM.

Product	McFarland Turbidity Range
GN	0.50-0.63
GP	0.50-0.63
YST	1.80-2.20
BCL	1.80-2.20

Identification cards are inoculated with microorganism suspensions using an integrated vacuum apparatus

1- A test tube containing the microorganism suspension is placed into a special rack (cassette).

2- the identification card is placed in the neighboring slot while inserting the transfer tube into the corresponding suspension tube.

3- The filled cassette is placed into a vacuum chamber station.

4- After the vacuum is applied and air is re-introduced into the station, the organism suspension is forced through the transfer tube into micro-channels that fill all the test wells.

Vitek system

Vitek card