Lec(4) Microbiology
/10/2010

Pure Culture Techniques

Most biochemical tests made only when the culture is pure , several different methods are available to yield pure culture from mixed culture.

1- Plating out on asolid culture medium.
2- Use of selective media.
3- Special treatment of the specimen before culture.
4- Use of specific growth conditions
5- Animal inoculation.
6- Use of motility feature

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1- Plating out a solid culture medium:-

the infective material is inoculated on one of surface of a solid medium using following methods, streak plate, pour plate method, and spreading plate method after incubation period, pure separate colonies are yield.

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2- Use of selective media:-

Different types of these media are available which contain substances that inhibit the undesirable bacteria and allow the desire one to grow ,Table(1) .

NO	Medium type s	Bacteria	Inhibitor(s)	Indicator(s
1	Macconkey agar	Enterobacteriaceae	Bile salts	Neutral red
2	Mannitol salt agar	Staphylococcus	Nacl 5-7%	Phenol red
3	M Edwards Medium	Streptococcus	Crystal violate	------

4	Bismuth sulphite agar	Salmonella	Brilliant green	Bismuth sulp
5	Brilliant green agar	Salmonella	Brilliant green	Phenol
6	Eosin methylene blue	E.coil	--------	osin methylene
7	Thiogycollate medium	Clostridium	odium Thiogycollate	methylene bl
8	Tetrathionate broth	Salmonella	Brilliant green	Iodine
9	Selenite – f broth	Salmonella	Selenite	------

3- Special treatment of the specimen before culture:-

1- Heating at 65c for 30 minutes is used for isolation of clostridium.

Sputum for culture of tubercle bacilli is treated with aOH.

2-N

3- Incubating the selenite f- broth culture or Tetrathionate medium.culture at 43c for 18- 24h to isolated *Salmonella*.

4- Use of specific growth conditions:-

1- Incubation at 37c used for most medically important bacteria.

2- Uses of bacteria anaerobic condition or addition of Co2.

3- PH at 4 prevents the growth of the most bacteria and allow growing of yeast and fungi.

5- Animal inoculation:-

1-Mice are used to isolate pneumoococcus, the sputum is inoculated subcutaneously, the animal dies of pneumococcus septicemia in 12- 48 h and the organism can be obtained in pure culture from the heart blood.

2- Guinea pigs are used to isolate tubercle bacilli from contamination materials by inoculating infected specimen the tubercle bacilli can be purely isolated**.**

6- Use of motility feature:-

1- atest – tube of semi solid agar medium containing apiece of small glass tubing which projects above the surface of the agar.

2- Stabbing the inner tube by motile and non motile bacteria then incubate.

3- Motile bacteria will migrate through the semi solid medium which become turbid

preservation of bacterial cultures

Purpose of preservation bacterial cultures:-

1- to avoid sub culturing in short time.
2- keep cultures and live without any physiological or genetic changes .

Two main methods are used:-

1- Short period preservation:-

a- Aerobes are preserved for months in slant at 4c.

b- Facultative anaerobes are preserved in agar stabs and then seal the surface of the medium with sterile liqid paraffin to reduce dehydration, the cultures stored at either room temperature or at 4c in refrigerator .

c- Strict anaerobes must be cultured in reductive medium that draws o2 e.g Thioglycollatemedium . .

2- Long period preservation:-

a- Mixing with litmus milk.

b- Mixing with sterile glycerin 20% and stored at -20c or less.

c- Preservation in liquid nitrogen in caped bottle at – 100 to -200c.

e- Freeze-drying method (lyophilization).:-

Bacterial cultures are dried and kept in the dry state under negative pressure bacteria may remain viable for several years.

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Bacterial Motility

bacteria can be divided according to motility:-

1- Motile bacteria e.g Proteus
2- Non motile bacteria e.g Micrococcus

There are several types of bacteria movement, true motility(self – propulsion) .

1- Flagella :- fine hair appendage, free from one end and connected from the other end by body cell. It's length is about 5-15 µm, while it's diameter is about 10-20 µm It can not be seen by light microscope, but it can be seen by dark field microscope or by using specific dyes that precipitate (accumulate) on the surface of flagella and increase it's thickness.

2-Inflexion motility:- this type of motility is seen bacteria that have no flagella e.g a spirochaetes and the motility occurred by contraction side and reflex that produce transitional movement . It can be seen by using dark field microscope.

3- Gliding motility :- this type of motility is seen in some types of slime bacteria that doesn't have flagella hence it moves by gliding on the surface of solid media.

Brownian movement(false motility):-is a vibrational movement results from the random motion ot the water molecules bombarding

the bacteria and cause and causing them to move .

Methods of motility determination:-

1- Wet mount slide.
2- Mixing with the oil.
3- Hanging drop slides .
4 - Semisolid stabbing method.

1- Wet mount slide -:

a- it is the simplest way to determine motility when working with non pathogens.

b- Place afew loopfuls of the organism on aclean slide and cover it with a cover glass then examine under an oil immersion objective .

2- Mixing with the oil:-

a- in this procedure ,adrop of oil placed on the slide then a drop of bacteria suspension mixed with oil, the oil droplets will track the bacteria thus it can be seen clearly .

3-Hanging drop slides :-

drop of bacteria suspension is placed on cover glass, which is then placed aspecial slide that has aconcave depression in its center.the glass is held in place with Vaseline, thus forming an enclosed glass chamber that prevent drying. other method is placing Vaseline in the four corners of cover silde for fast technique.

4- Semisolid stabbing method:-

a- this mehod is widely used with pathogenic bacteria , don't use the three previous methods, to avoid infection.

b-In this procedure the organism is inoculated by stabbing the semisolid medium(Gelatin 12-15% or 0.5-1%agar) with inoculating needle.

c-Motile bacteria move away from the line of inoculation in to the un inoculated surrounding medium, non motile bacteria will be found only along the line of inoculation.

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Bacterial Morphology

1. Shape

a- Coccus

b- Short rods

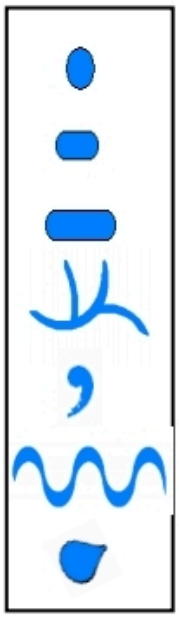
c- Long rods

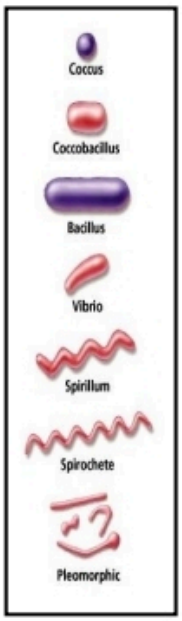
d -Filamentsd

e-Comma shape

f-Spirals

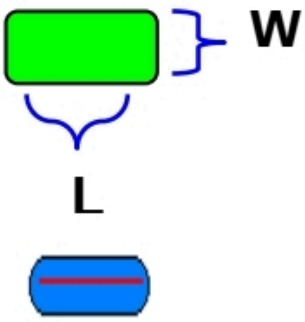
g -Club





2. Size .

Width and Length



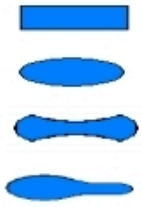
3. Axis.

a- Straight.

b-Curved.

4. Sides.

a-Parallel
b-Swollen
c-Concave
d-Irregular



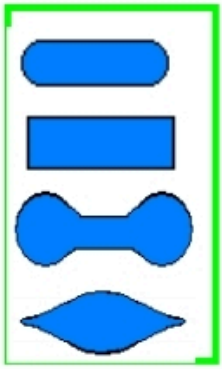
5. Ends.

a- Rounded

b-Flat ends

c-Swollen

d-Point**ed**



6- Arrangement.

a-Single cells

b-Diploids

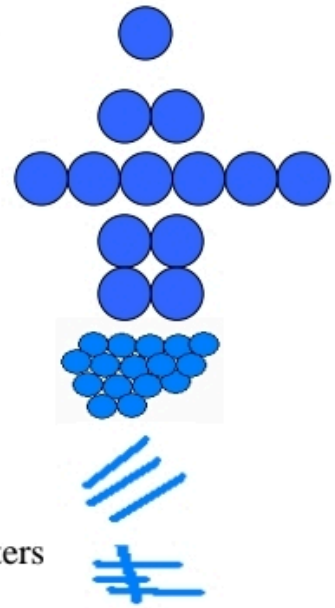
c-Chains

d-Tetrads

e-Clusters

f-Bands

g-Chinese letters



7. Motility and Flagella.

Flagella are arranged in different patterns:-

a-monotrichous 

b-Iophotrichous 

c-peritrichous 

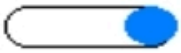
d-amphitrichous 

8. Spores.

a- Round, Central. 

b-Oval, Central. 

c-Round, sub terminal. 

d-Round, Terminal. 

e-Causes swollen. 

f-Does not causes swollen. 

9. Capsule.

a-Capsulated. 

b-Noncapsulated. 

10.Staining.

a-Even. 

b-Uneven. 

c-Bipolar. 

d-Metachromatic granules. 

11. Gram's stain.

12. Ziehel – Neelsen stain.

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Bacterial Staining

Stains are:

1-Simple stains:

a-Basic dye. **b-**Acidic dye.

2-Differential stains:

a-Gram stain . b-Acid-fast stain.

3-Structural stains:

a-Feulgen stain. b-Endospore stain. c-Cell wall stain. d-Capsule stain. e-Flagella stain

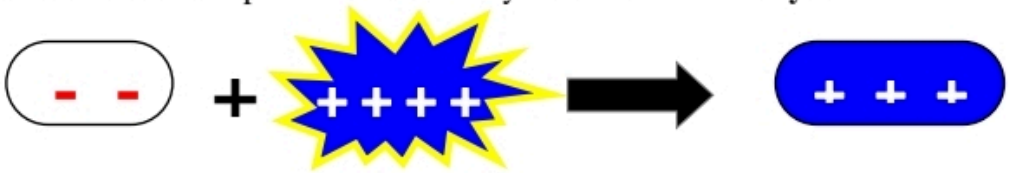
Dyes:- are generally salts in which one of the ions are colored. A salt is a compound composed of a positive and negative ions.

1-Simple stains: single stain to color the organism .

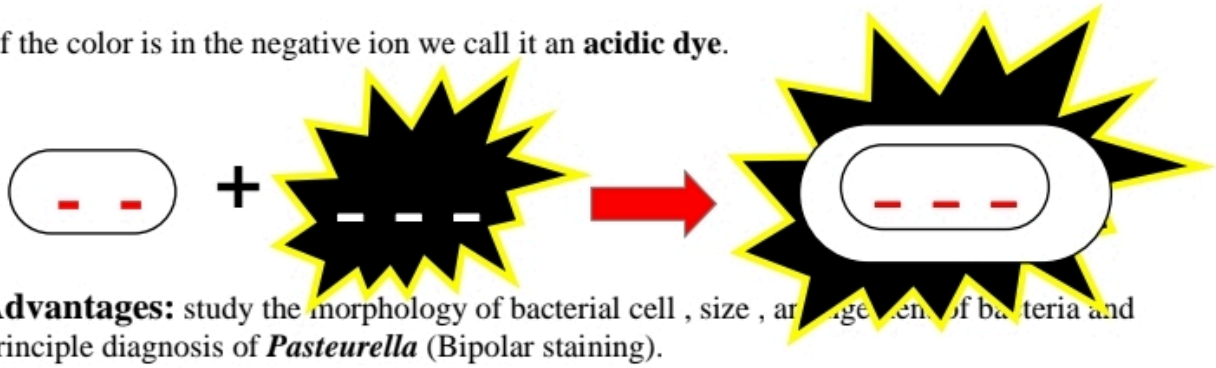
The simple dye methylene blue is the salt blue chloride:

MBCI → MB +Cl

If the color is in the positive ion of the dye we call it **a basic dye .**



If the color is in the negative ion we call it an **acidic dye**.



Advantages: study the morphology of bacterial cell , size , arrangement of bacteria and principle diagnosis of ***Pasteurella*** (Bipolar staining).

Smear Preparation:

1- Clean the slide with 50% of ethyl alcohol.

2- If the bacteria are growing in a liquid media, one starts by placing two loopful of liquid media directly on the slid. From solid media, one starts by placing one or two loopful of water on the slide and mix with one loopful of organism.

3- Spread the drop on the slide to form thin film.

4- Allow the slide to dry in the air.

5- When the film dry, pass the slide, film side up three times through the Bunsen flame.

6-Staining.

Crystal violet	Methylene blue
Safranin	Carbol fuchsin

The purpose of fixation:

is to kill the micro organisms cause it to adhere to the slide.

2-Differential stains:

a-Gram stain

It is divide bacteria in to two groups gram positive , gram negative. The difference in staining is due to the variance in surface layer of the two types of cells. Gram positive consist of thick layer of peptidoglycan which resist decolorize with alcohol so gram

positive bacteria retain a crystal violet through decolonization and appear purple, but gram negative consist of vary thin layer of peptidoglycan and lipopolysaccharide which decolorize with alcohol and take the counter stain with red dye of safranin.

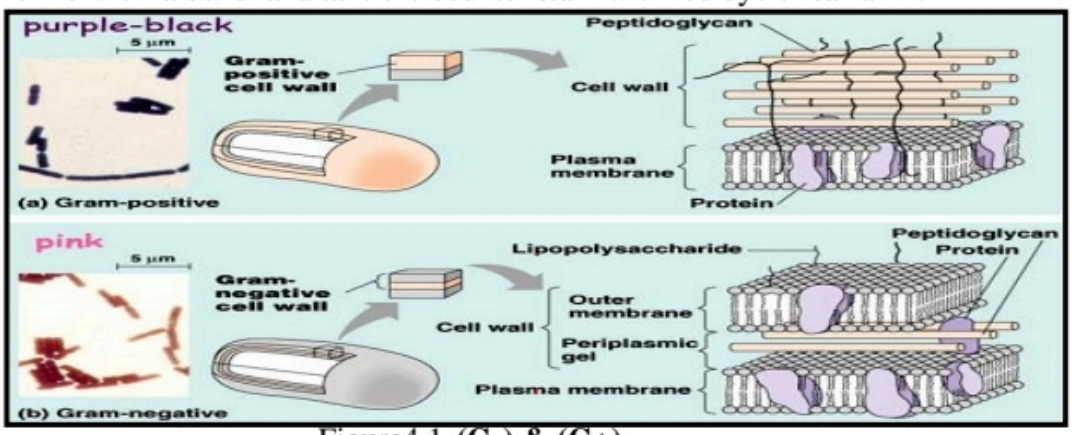


Figure4.1 **(G-) & (G+)**

Gram's stain steps

1-Prepare smear and fixed.

2-Crystal violate for 30 second and wash with water.

3-Gram s iodine for 30 second and wash with water.

4-Decolorized with alcohol 95% for 10 – 20 second and wash with water.

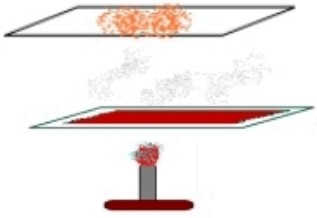
5-Counter stain (Safranin) for 30 second and wash with water and examine with oil immersion lens

b-Acid-fast stain(Ziehl-Neelsen stain).

Bacteria in the genus *Mycobacterium* and some in the genus *Nocardia* contain a waxy material in their cell wall called mycolic acid. If they are stained with carbol fuchsine and heat is applied during the staining procedure, the carbol fuchsine is able to penetrate the cell and it is not removed by subsequent washing with acid-alcohol. This method is important in diagnosis tuberculosis, and leprosy in human .

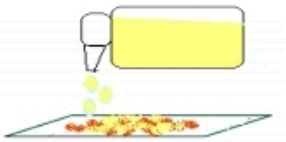
Staining Method

1-Prepare the smear of Mycobacterium sometime killed 40% formalin of sputum .

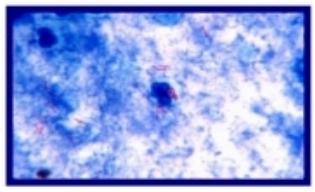


2-Carbol fuchsine and heating 5 minutes than wash with water.

3-Decolorize with acid-alcohol 95% for 15-20 second and wash with water.



4-Methylene blue or malachite green (counter stain) for 30 second and wash with water.



5-Examine with oil immersion lens.

3-Structural stains:

a- Endospore stain .

species of *Bacillus* and *Clostridium* produce a structure referred to as the endospore.

1- Schaeffer and fultons stain.

Staining Method

1- Prepare smear of Bacillus or Clostridium, and fix them with heat

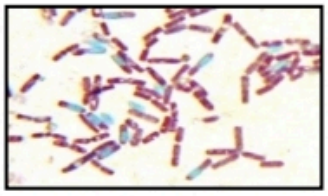
.

2-Cover the smear with 5% malachite green and continue heating for 5 minute.

3-Washing with water.

4- Safranin for 30 second , wash with water.

5- Examine under oil immersion lens.



2-Modified Ziehl-Neelsen stain.

Staining Method

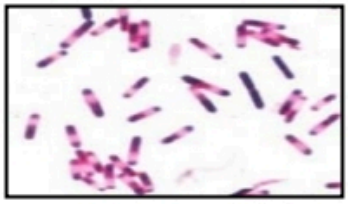
1- Prepare smear of Bacillus or Clostridium, and fix them with heat.

2-Cover the smear with carbol fuchsin and continue heating for 5 minute.

3-Washing with water.

4- Methylene blue for 30 second , wash with water.

5- Examine under oil immersion lens.



b- Capsular stain.

Some bacterial cell are surrounding by an extracellular slime layer called a capsule.

There are three main stain of capsule:-

1-Hiss stain. 2-Anthony stain. 3- Negative stain.

1-Hiss stain:-

Staining Method

1-Prepare smear and fixed.

2-Cover the smear with carbol fuchsin and continue heating for 5 minute.

3-Washing with 20% copper sulfate solution.

4-Examine under objective lens 40x.

2-Anthony stain.

Staining Method

1-Prepare smear and fixed.

2-Cover the smear with crystal violet and continue heating for 5 minute.

3-Washing with 20% copper sulfate solution.

4-Examine under objective lens 40x.

3- Negative stain.

There are two method of Negative capsule stain :-

1-Dry Negative stain.

Staining Method

1- two loopful of the organisms are mixed in a small drop of Indian ink.
2- Spread the suspension of bacteria over slide as a blood smear.
3-Dried with air and examine with oil immersion lens.

1-Wet Negative stain.

Staining Method

1- two loopful of the organisms are mixed in a small drop of Indian ink.
the suspension of bacteria with cover slide without dried --
2examine with oil immersion lens.

c- Flagellar stain:-

Solution of dye:

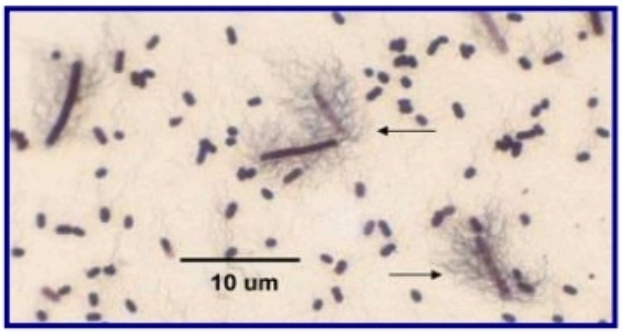
1- 1% Nacl.
2- 3% tannic acid.
3- 1.25% basic fuchsine dissolve with 95% ethanol.

Staining Method

Putting two loopful of the organisms on clean slide without mixed and dried
1- with air .

2-Cover the smear with Flagellar stain for 30 second, wash with water.

3- Methylene blue for 30 second , wash with water.



Lec(8)

Microbiology /10/2010

Bacterial count

Bacterial count are important requirement for the study and growing of bacteria in case of:

1- experimental infection.

-Food poisoning.

3- vaccine 2

preparation .

The bacterial number calculate by:

a- bacterial count: use with unicellular which easy counting like bacteria and yeast.

b- cell mass use in case accumulated bacteria and filamentous.

The bacterial count can be classified in to two parts:-

1- Viable count :calculate the live bacteria only.

2- Total count: calculate the live and dead bacteria.

1- Viable count .We can calculate the live bacteria by:-

A- Plate Method:- which have 2 ways -:

1-Spreading plate method -:

Procedure method

a- two fold or ten fold dilution to the sample in the test tubes which contain culture broth or normal saline
(sterile).

b- put 0.1ml from diluted sample (which contain bacteria) in to the nutrient agar.

c- spreaging by glass spreader,Leave nutrient agar for 5 minutes to absorbed the bacteria suspension .

e- put the plate incubator in 37c for 16-24 hrs.

bacterial count in the sample=total count of bacteria in plate x 10x reversible dilution

2-Pour plate method:-

Procedure method

a- put 0.1ml from diluted sample (which contain bacteria)in to plate.

b- add melting agar in 45c in plate then make circular movement to the plate.

c- Leave nutrient agar to rigid then put the plate in incubator in 37c for 16-24 hrs.

bacterial count in the sample=total count of bacteria in plate x 10x reversible dilution

B- Membrane filter method.

a- Counting of live bacteria by passing the sample through membrane filter which trap the bacteria cell on filter membrane.

b- Take the flter membrane and put it in suitable culture agar for growing of the bacteria. the filter membrane will absorbed thre nutrient material which lead to

growing of bacteria.

.

2- Total count: Total count less accurate method like the methods which counting the live bacteria.

Advantages: 1- easy method.
2-saving nutrient materials and time.

The important methods are:-

A-Method calculated the number of bacteria can be done.

1- Direct microscopic counting method.

2- Breed method:-

Procedure method

a-Take standard volume (usually onedrop equal to 0.025 ml which means each 1ml equal to 40 drop).

b- spreading one small spot (1cm2) of bacterial suspension on glass slide then staining by methylene blue stain after drying and fixation the smear.

c- Determine the bacterial colony by passing on ach point of square.

Total count of bacteria = Total count of bacteria in square x 40 x reversible Dilution

3-Coulter Counting method:-

This apparatus depends on measuring the bacterial numbers solution by passing the sample from small foreman which present in this counter and every unit will pass record changing in electric resistance.

Disadvantage method:-

This method is measuring all unit which passing bacteria or another unit. Big unit may be close the foreman which lead to wrong reading.

B- Method calculating the mass of bacteria.

Cell mass:- means bacterial contain of protoplasm.

1-turbid metric method:-this method depends on light scattering which direct proportion with the liquid unit .use in this method spectrophotometer and Brown tube sets .

.

2-Chemical method:-we can measuring the bacterial mass by measuring some ofbacteria contents like N, protein, P, DNA. in this method usually use spectrophotometer with standard chemical substance.

3- dry weight:- measure by the weight of the cell by washing several time with centrifugation and drying the cell.

4-Packed cell volume P.C.V.:-by using labled capillary tube and put it in the centrifuge and measure of packed cell after centrifugation.

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Figure5.1 Colony Counters.

Figure5.2 Typical Spectrophotometers.

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Antibiotic sensitivity test

Antibiotic:- substances produced from metabolic microorganism that posses the ability to inhibit the bacterial growth when used in less concentration or it is known as substances produced by rhombus organism and inhibit another live organism.

The conditions should be available in the antibiotics :-

1- it should be kill large number of organisms
2- it has no side effect on human health and animals
3- do not affect the normal flora present inside the human and animal body

Many types of bacteria ,fungi and viruses that cause diseases to human and animals show resistance to different antibiotics that used for treatment of pathological case leading to economical losses which represents:-

1- Long-Lasting treatment peroid.

2- Quantities of consumed drug.

3- Downfall of animal health.

Advantage of uses this technique:-

1- To choose more effective antibiotics which remove the causative agent.

2- To learn of non-effective antibiotics which are resistant to causative agent and changing it by more effective drug in order to decrease costs.

There are two main methods for antibiotic sensitivity test.

1- Dilution methods can done by.

A-Tube dilution test.

B- Agar dilution test.

2- Disc diffusion method.

A-Tube dilution test.

Procedure method

1- Make two fold dilution of antibiotics in broth media (Mueller Hinton broth) With standard bacterial inoculums.

2- Prepare control tube (inoculums+ broth) without antibiotics.

3- Incubate tube for 16-20h,the result was determined by turbidity in tubes, and the tube that contain upper dilution of antibiotic that inhibit bacterial growth (no turbidity) was known as (MIC) Minimum Inhibitory Concentration.

4- (MBC) Minimum Bactericidal Concentration upper dilution of antibiotic that prevent bacterial growth(even after long incubation).

B- Agar dilution test:-

Procedure method

1- Prepare antibiotic serial dilution in solid culture media ,each Petri dish contain specificdilution this serial dilution is inoculated with one drop of bacterial inoculums using standard loop without spreading some time replicating device is used.

.

2- Bacterial inoculation in this test is calculated according to McFarlands standard tube(0.5m) comparing with turbidity.

.

3- After incubation 12-16h the result is readed and MIC was determined by this way.

2- Disc diffusion method.

- The test is widely used because is vary simple and economic.
- Priciple of this test depend on use

Done by fixing the disk on the surface of agar which contain bacteria (Kirby Bauer method).

- There are two disc type

a- multi disc b- uni disc.

Procedure method

1- Satrated cotton swab with bacterial culture broth prepared by comparing with McFarland turbidity standard(0.5M).

2- The surface of agar is screening by cotton swab contain bacteria at lest 3 time this done by rotting the plate in 120 time.

3- let Petri dish for 15 min to absorbed the inoculums.

4- Sterile forceps (alcohol and flaming) was used to put uni disc or multi disc on agar surface.

5- by using forceps press on disc to be sure that it adhesive to agar surface and let Petri dish for 15 min.

6- Incubate Petri dish in 37c for 18h,in converted way ,determine zone of inhibition around the disc and according the diameter refers as resistance (R), sensitive (S), intermediate (M).

Some factor affecting on the test:-

1- Agar depth.
2- PH of media.
3-Media source.
4-Age and turbidity of bacterial media.
5-Spreading method
6- Heat.
7- Incubation time.
8-Determination.
9- Disc antibiotic concentration.

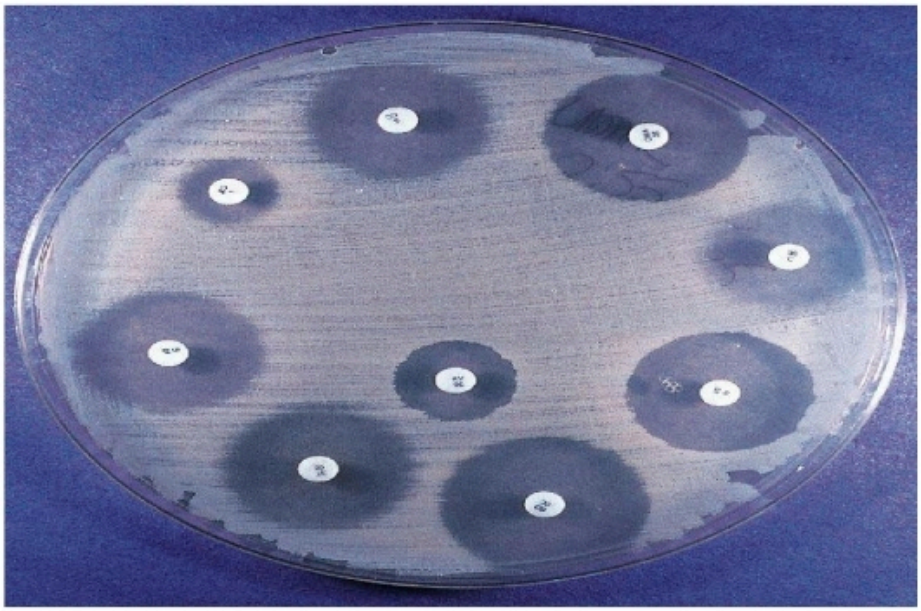

Figure5.3 Antimicrobic Sensitivity Testing: The Kirby-Bauer Method •