(Lab 1) M. Ashwaq Hazem:

Preparation of culture media

Procedure :

1- weight the amount of powder using **Analytical Balances** .

2- Add the distilled water.

3- Dissolve the powder using the **magnetic stirrer**.

4- Take to boiling

5- PH as indicated using PH – meter or PH paper (If the medium is acidic ,add the base (**NaOH)** solution or the medium is basic add the acid (**HCl)** solution to get neutralization) .

6- close with cotton plugs or screw caps.

7- **Autoclave** at 121 ° C and pressure (15 psi) for 15-20 min.

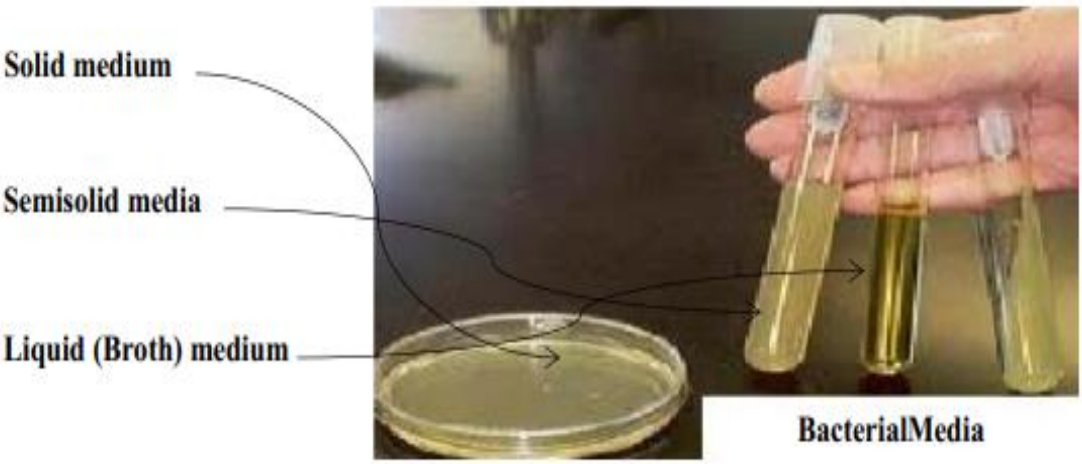
8- Cool and into petri dishes.

Classification Of Culture media on consistency:

1-Solid medium: contains agar at a concentration of (1.5-2.0%) boiled well until it becomes clear and useful for isolating colony of bacteria in petri dish or slant to save bacteria.

2. Semisolid media: They are prepared with agar at concentrations of 0.5% . **used test for motility**

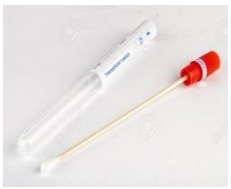
3. Liquid (Broth) medium: without solidifying agents , it is not boiled after preparation and used to grow large number of bacterial cell for use in biochemical tests.



Isolation of Microbes from the environment

"Bacteria are like living paint covering nearly all living and non-living surfaces".

Loop: is a simple tool used mostly by microbiologists to pick up and transfer a small sample from a culture of microorganisms may be made of platinum, or chrome because it heats and cools rapidly, e.g. for streaking on a culture plate .



Swab : is a piece of absorbent material on a stick, Like a mop, but it can also be small , Like a cotton swab **used for clean the ears and collect samples.**

Isolation of Microorganisms from Air: In the exposure plate technique, media plates are exposed to air for a specified duration 30 minute and the microbial flora settles down on a plate. When plates are incubated, the colonies of microorganisms develop on a plate Such as Micrococcus ,Staphylococcus and Bacillus.

Swabbing Methods:

1-Write your name and where you got your sample on the back of the plate

2-You will have one plate to work with.

3-Inoculate the plate by wetting a cotton swab in sterile water then gently wiping it across a surface where you think bacteria exists.

4-Then you will gently wipe that sample across your plate being careful, not to leave the cover off the plate to long.

(ALSO do not dig in to the agar it is really soft)

4- Streak sample on in a zigzag fashion, Incubate at 37^0 C for 24 hrs.

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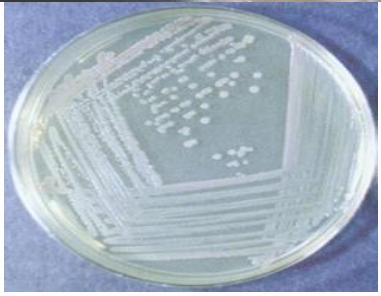
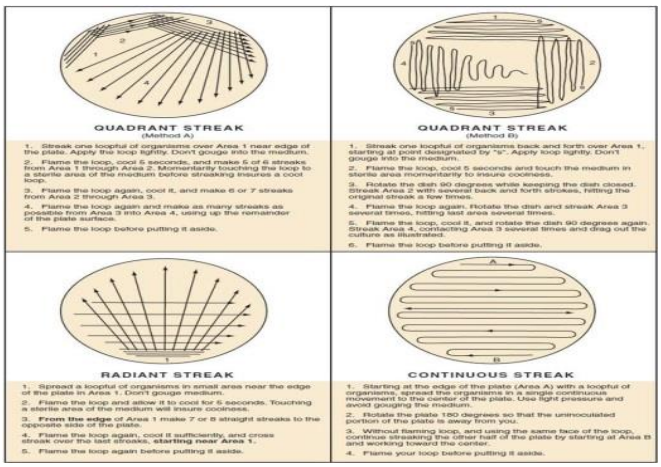
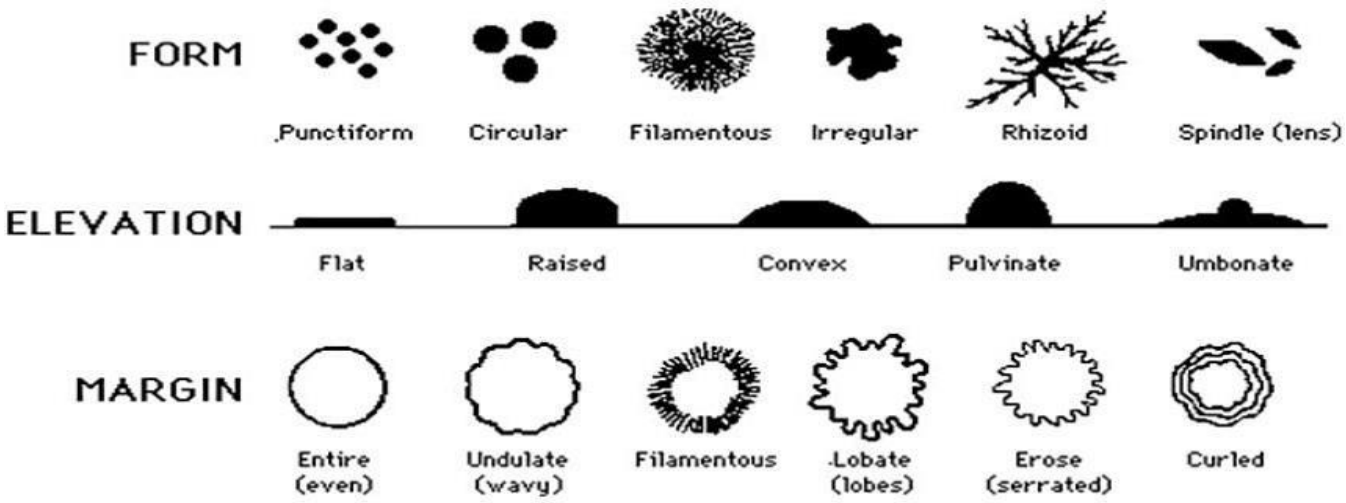




Colony: a single cell divides exponentially forming a small, visible collection of cells. Colonies are observed when bacteria are grown on a solid medium. Each colony usually contains 10^7-10^8 bacteria.

Colony morphology: Characteristics of a colony such as shape, edge, elevation, color and texture

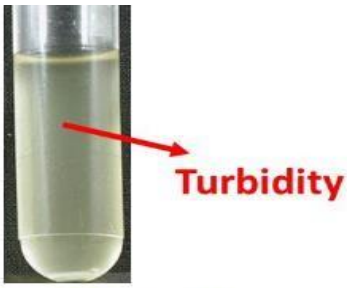


Serratia marsescence.

Pseudomonas sp.

Turbidity: cloudy appearance of a liquid medium due to the presence of bacteria.

Medium (no bacteria)



Culture (bacteria)

Differential stains

Acid-Fast -Stain(Ziehl-Neelsen)

The acidfast stain is a differential stain used to identify acidfast organisms such as members of the genus Mycobacterium . Acidfast organisms are characterized by waxlike, nearly impermeable cell walls; they contain mycolic acid and large amounts of fatty acids, waxes, and complex lipids.

A few species of bacteria in the genera **Mycobacterium and Nocardia**, acid-fast organisms require a special staining technique.

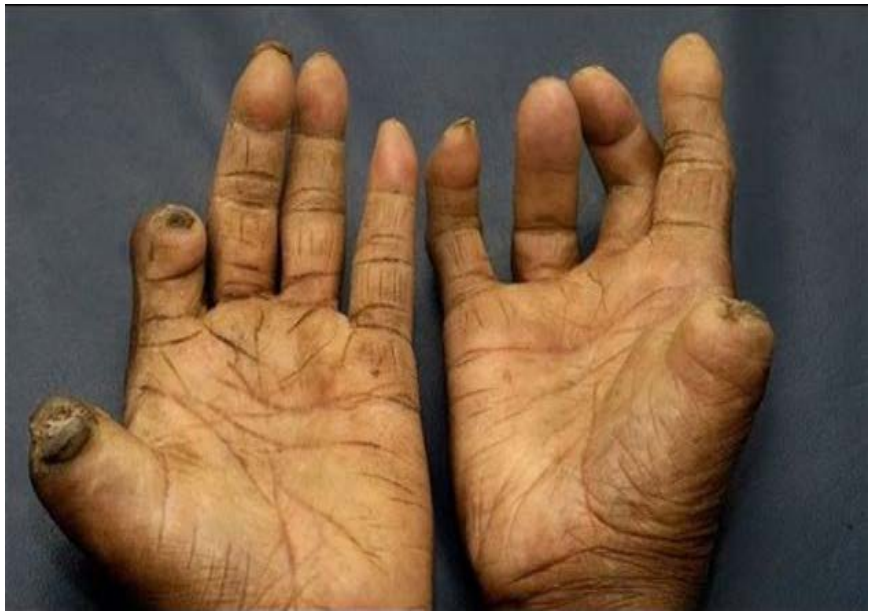
The **primary stain** used in acid fast staining, **Carbol Fuchsin**, is lipid soluble and contains phenol, which helps the stain penetrate the cell wall. This is further assisted by the addition of heat. Once the microorganisms have taken up the carbolfuchsin, they are not easily decolorized by acid alcohol, and hence are termed acid-fast.

This acid-fastness is due to the high lipid content (mycoli-acid)in the cell wall of the microorganisms. Acid-fast microorganisms will retain this dye and appear red.

In the clinical laboratory, the acid-fast stain is important in identifying bacteria in the genus Mycobacterium; specifically , *Mycobacterium leprae* (leprosy) and *Mycobacterium tuberculosis* (tuberculosis).

This differential stain is also used to identify members of the aerobic actinomycete genus *Nocardia;* specifically ,the opportune is tic pathogens ***Nocardia brasiliens*** is and ***Nocardia asteroids*** that cause the lung disease nocardiosis .

Leprosy (Hansen's disease)
Mycobacterium leprae



Primary stain :

- **0.3% Carbol Fuchsin – Dissolve 50g phenol in 100ml ethanol (90%) or methanol (95%). Dissolve 3g basic fuchsin in the mixture and add distilled water to bring the volume to 1 L.**
- **Decolorization solution: 25% Sulphuric acid**
- **Counter stain : 0.3% methylene blue .**

PROCEDURE:

1- Smear the sputum over the glass slide, and fixed the by heating .

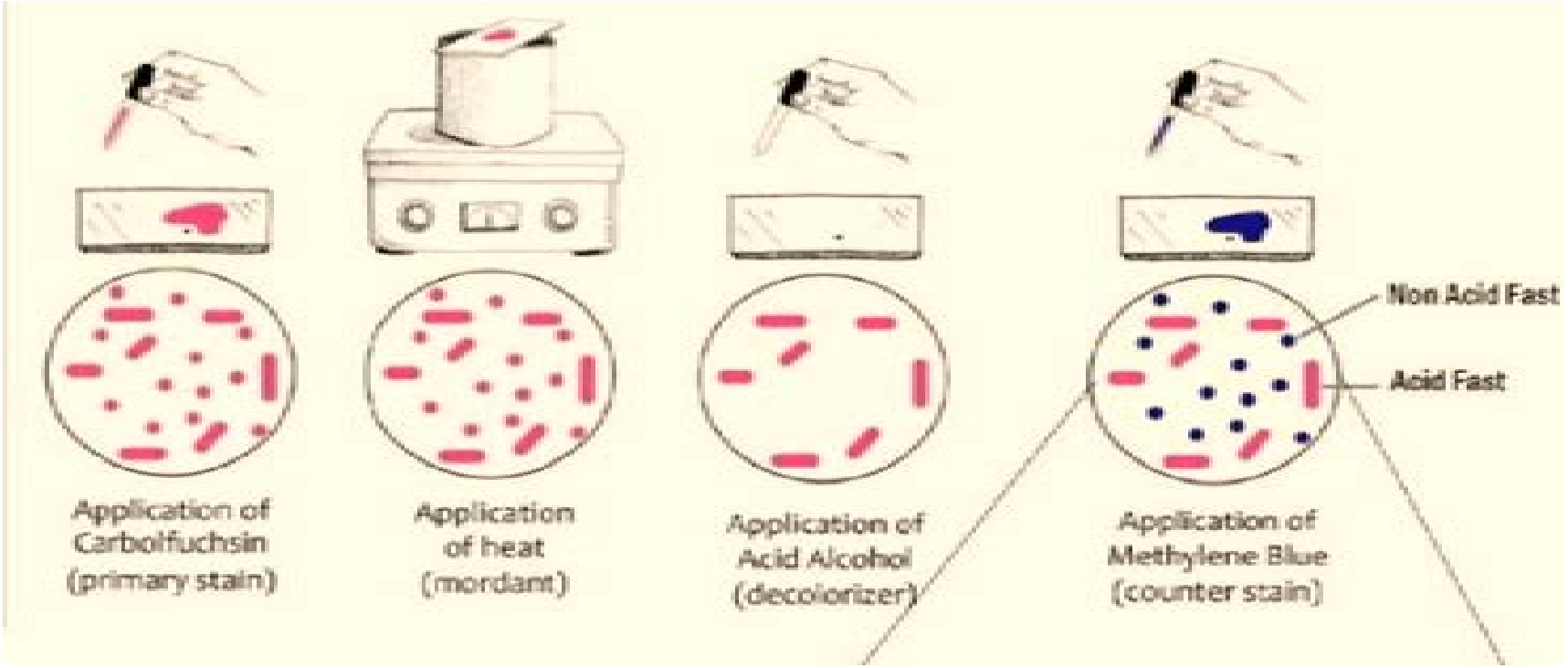
2-Pour Carbol Fuschsian (primary stain: Work in lipids + dissolved ethanol) over smear and heat gently until fumes appear. Do not over heat and allow it to stand for 5 minutes, then wash it off with water (cooling).

3-carbol Fuschsian (contains) + heat= penetrate the stain of cell (3-5min) add 25% sulphuric acid (H_2SO_4= decolorizer),wait for one minute ,and keep on repeating this step until the slide appears light pink. Wash off with water.

4. add methylene blue, wait for two minutes, again wash with water.

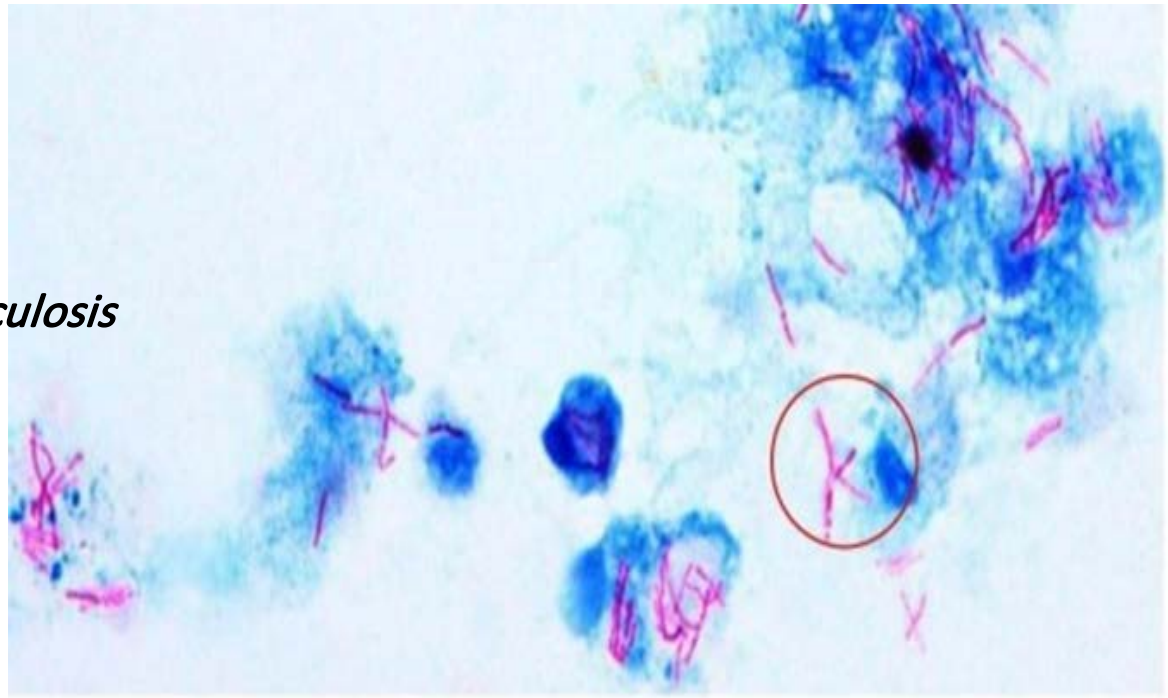
5. Allow it to air dry and examine under oil immersion lens.

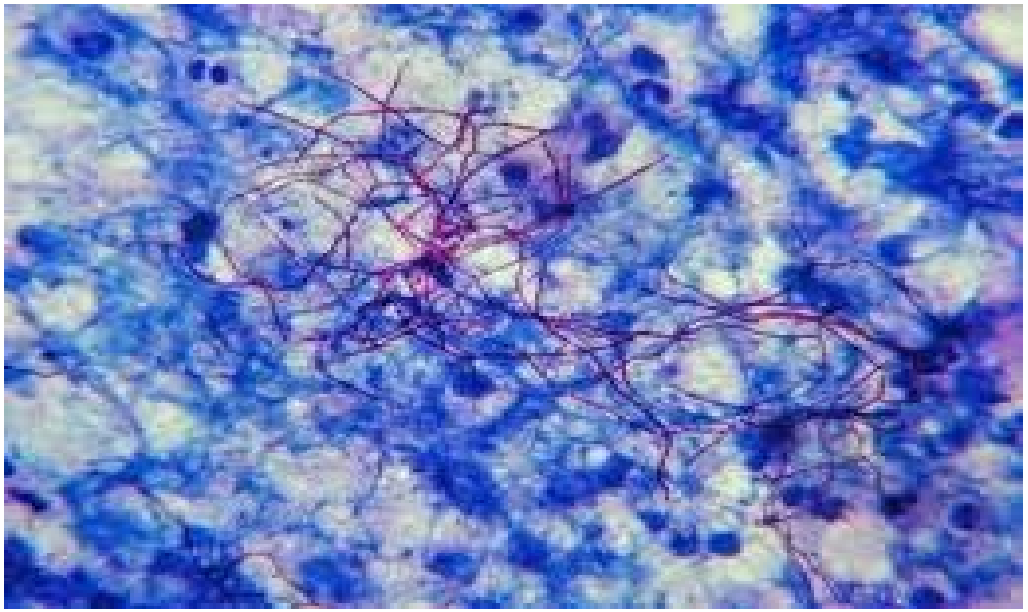
ACID FAST STAIN STEPS	Color of Acidfast + cells	Color of Acidfast – cells
1. Primary stain: Carbol fuchsin	RED	RED
2.Decolorizing agent: Acid alcohol	RED	COLORLESS
3.Counterstain: Methylene Blue	RED	BLUE



Results Acid Fast Bacilli: Red, straight or slightly curved rods, occurring singly or in small groups ,may appear beaded with Chinese letter or palisade arrangement. Mycobacterium tuberculosis: red Cells: Blue (methylene blue) Background material : Blue(methylene).

Mycobacterium tuberculosis



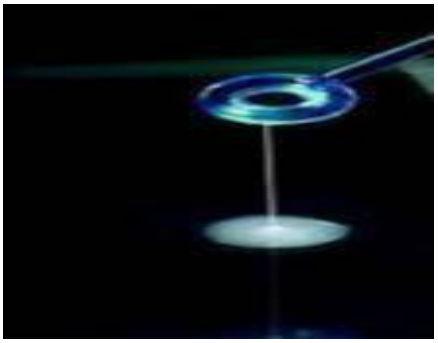


Nocardia

Differential stains : 4- Capsule stain

The capsule or glycocalyx : is a gelatinous outer layer that is secreted by the microbe and remains stuck to it. Capsules may be polysaccharides, glycoproteins, or polypeptides depending on the organism.

Capsules give colonies a mucoid glistening morphology which forms a string when pulled with a loop as shown below:



Capsulated mucoid colonies **+ve string**

Bacterial capsules are non-ionic, so neither acidic or basic stains will adhere to their surfaces. Therefore, the best way to visualize them is to stain the background using an acidic stain and to stain the cell itself using a basic stain. We use India ink and Gram crystal violet. This leaves the capsule as a clear halo surrounding a purple cell in a field of black.

The medium in which the culture is grown as well as the temperature at which it is grown and the age of the culture will affect capsule formation. Older cultures are more likely to exhibit capsule production. When performing a capsule stain on your unknown, be sure the culture you take your sample from is at least five days old.

Capsules are associated with virulence in several microorganisms ,including:

1- Klebsiella pneumoniae (Pneumonia)

2- Streptococcus mutans (tooth decay)

3- Neisseria meningitides (meningitis)

Method Procedure:

1- India Ink :-

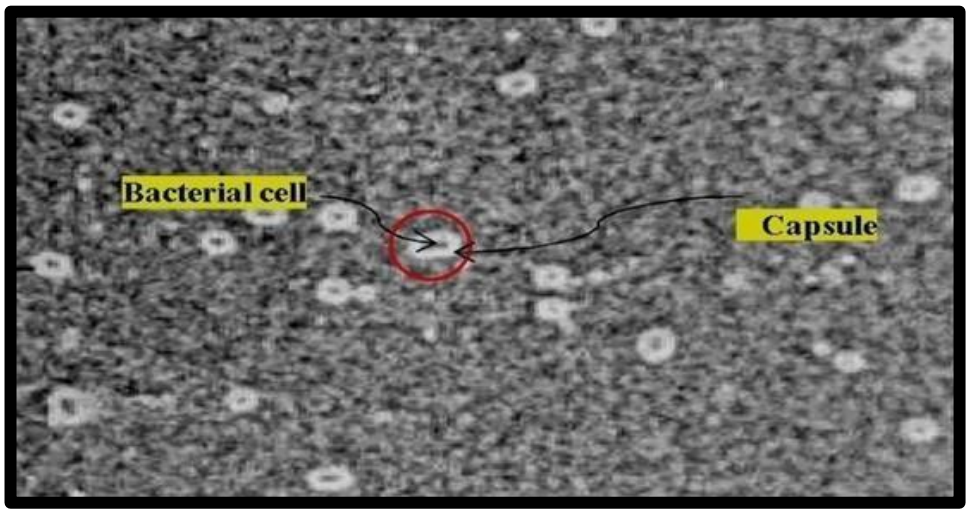
1- Place a large loopful of undiluted (India ink or Nigrosin) on slide

2- Mix into this a small portion of the bacterial colony and spread the mixture to slide.

3- Air dry DO NOT HEAT FIX because there will be capsule shrinkage

4- examine under the microscope.

Results: The capsule appears as a clear zone between the retractile cell outline and the dark background.

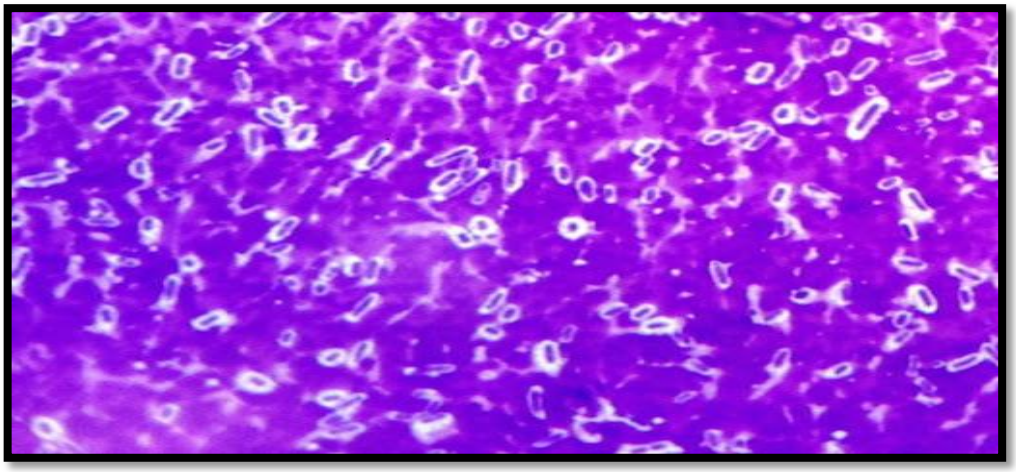


Capsule demonstrated with Indian ink method

2- Dry method:

1. Put a drop of glucose 6% solution on one end of a clean glass slide.
2. Mix into this a small portion of the bacterial colony .
3. Add a drop of Nigrosine and mix it well.
4. Spread the mixture to slide using the edge of a second microscope slide, fixing with methanol and drying with hot air.
5. Stain with crystal violet stain for one minute.
6. Wash with water ,dry, and examine under the microscope.

stained purple. The capsule (if present) will appear clear against the dark background.



Capsule demonstrated with Dry method.

C- Anthony's method : (فقط اسم الطريقة مطلوب)

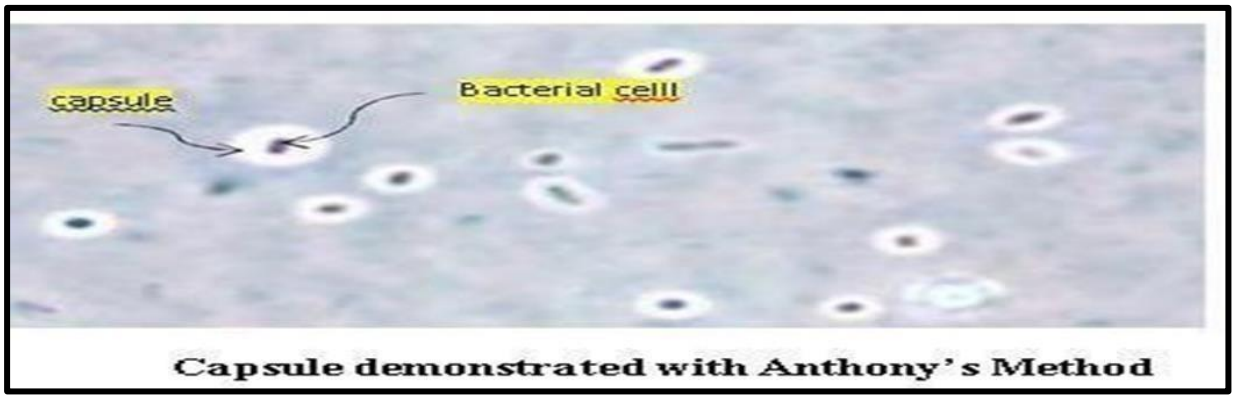
1- Add a loopful of a culture to a microscope slide

2- Spread the culture over the slide using the edge of a second microscope slide

3- Stain with 1% crystal violet for 2 minute

4- Wash with a solution of 20% copper sulfate

5- Blot dry and examine under oil immersion.



Capsule demonstrated with Anthony's method.

Result: The cells are stained a deep blue or purple. The capsules are stained a light blue. The background may appear colorless or light violet depending on the degree of destaining .

IMViC Tests

- **IMViC is a series of tests that are useful in the identification of enteric bacteria (Enterobacteriaceae.) Tests include:**

1. **I = Indole test**
2. **M = Methyl red test (MR)**
3. **V= Voges-Proskauer test (VP)**
4. **C = Citrate test**
5. **Ii = used for ease of pronunciation**

Indole test

Medium: peptone water broth is rich in the amino acid tryptophan.

Principle of Indole Test:

(Tryptophanase)

- **Tryptophan ⟶ Indole + pyruvic acid + NH_4**
  (C-source) (N-source)

- **reagent used in indole test is called :**

Kovac's reagent

Procedure of Indole Test:

1. Take a sterilized test tubes containing 4 ml of peptone water broth.

2. Inoculate the tube by taking the growth from 18 to 24 hrs. Culture.

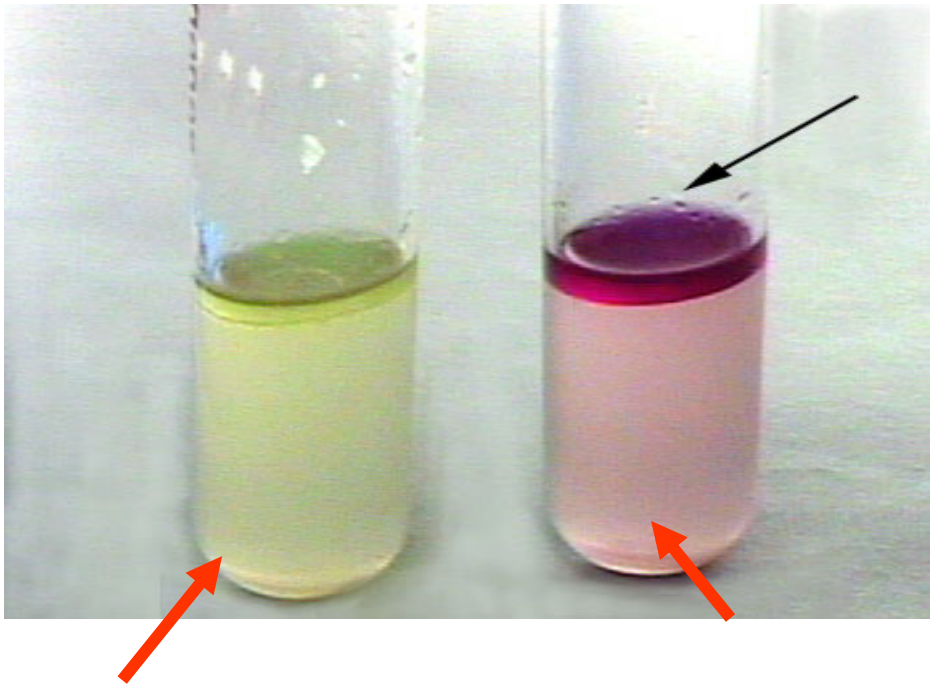
3. Incubate the tube at 37°C for 24-28 hours.

4. Add 0.5 ml of Kovac's reagent to the broth culture

Result:

A bright pink ring color in the top layer indicates the presence of indole

The absence of color means that indole was not produced indole is negative



Negative test
e.g. *Klebsiella*

Positive test e.g.
Escherichia . coli

Methyl red Test

Media:

Glucose Phosphate Peptone water (MRVP) broth (pH 6.9)

Principle:

Some bacteria have the ability to utilize glucose and convert it to a stable acid like lactic acid, acetic acid or formic acid as the end product The acid so produced decreases the pH to 4.5 or below, which is indicated by a change in the color of methyl red from yellow to red.

Voges-Proskauer (VP) test

Media :

Glucose Phosphate Peptone water (MRVP) broth (pH 6.9)

Reagents:

A: Barritt's reagent A (Alpha-Naphthol 5%)

B: Barritt's reagent B (Potassium Hydroxide (KOH) 40%)

- **Principle: is used to determine if an organism produces acetyl methyl carbinol (acetion) as a fermentation product from glucose.**

acetyl methyl carbinol If present, is converted to diacetyl in the presence of (Alpha-naphthol, strong alkali (40% KOH), and atmospheric oxygen). The Alpha - naphthol is a color intensifier and must be added first. The diacetyl compounds found in the peptones of the broth then condense to form a pinkish red.

-

Procedure MR - VP:

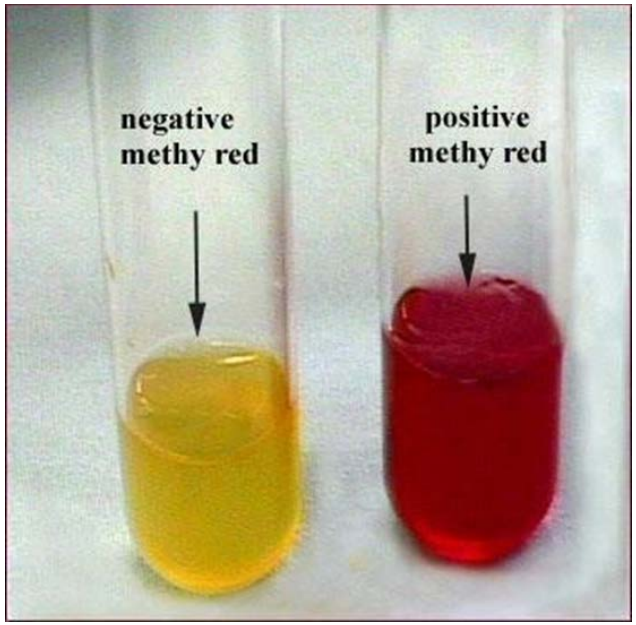
1-Inoculate the tested organism into two tube of MR-VP broth.

2-Incubate the tubes at 37°C for 24 hours

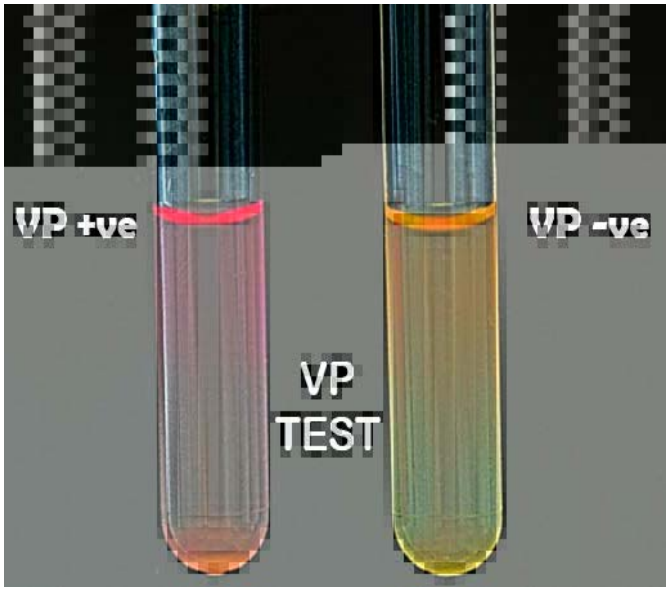
3-For methyl red: Add 2-3 drops of methyl red reagent

4-For Voges-Proskauer Following 48 hours of incubation:

Add 6 drops of Barritt's A (α-naphthol), mix,4 drops of Barritt's B (40% KOH), mix Observe for a pinkish red color at the surface within 30 min



Methyl Red test



Voges-Proskauer test

- ✓ **Red: Positive MR (*Escherichia. coli*)**
- ✓ **Yellow: Negative MR (*Klebsiella*)**
- ✓ pinkish red**: Positive VP (*Klebsiella*)**
- ✓ **No pink: Negative VP (*Escherichia. coli*)**

Citrate utilization test

The medium :Simmon's Citrate agar

Principle :

When the bacteria metabolize citrate, the ammonium salts are broken down to ammonia, which increases alkalinity. The shift in pH turns the Brom thymol blue indicator in the medium from green to blue above pH 7.6.

Method:

1-Streak at the medium slant with the organism

2-Incubate at 37°C for 24 hours

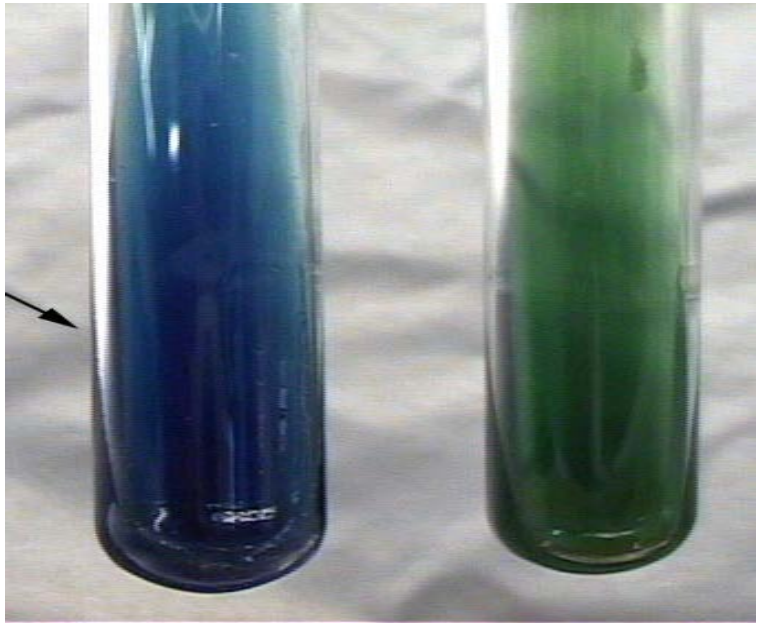
Citrate utilization test results:

1-Positive Reaction: Growth with color change from green to intense blue along the slant.

Examples: *Salmonella, Klebsiella* .

2-Negative Reaction: No growth and No color change; Slant remains green. examples: *Escherichia coli , Shigella*

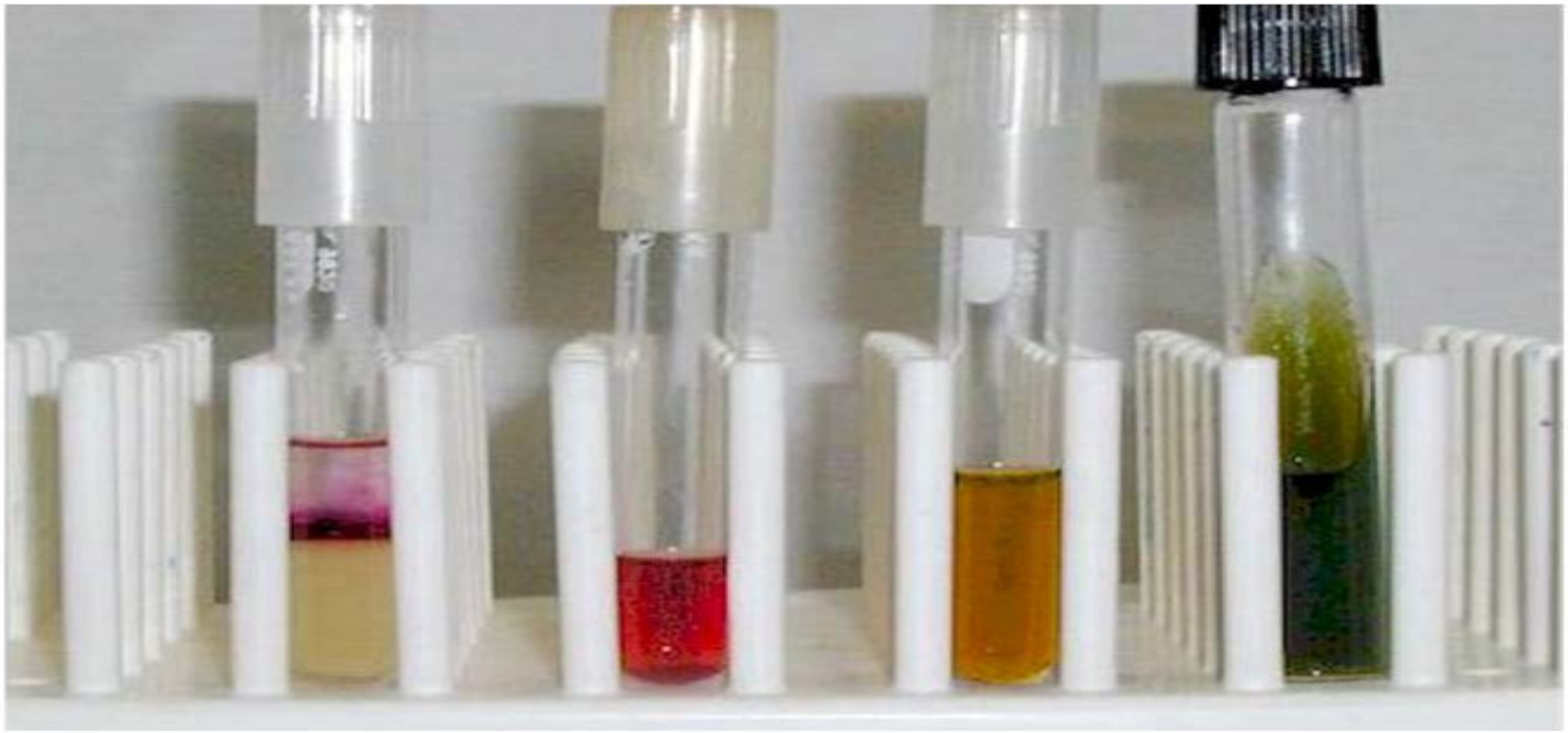
Positive
Klebsiella



Negative
Escherichia. coli

Escherichia coli

IMViC (+ + – –)



Indole Positive

MR Positive

VP Negative

Citrate Negative

Klebsiella

IMViC (- - + +)

test	media	Reagents	result
Indol	peptone water broth	**Kovac's reagent**	Positive: Formation of a pink to red color ("cherry-red ring") in the reagent layer on top of the medium within seconds of adding the reagent
Methyl red	Glucose phosphate peptone water (MRVP) broth	Methyl red	Positive Reaction: A distinct red color\ Negative Reaction: A yellow color .
Voges Proskauer	Glucose Phosphate Peptone water (MRVP) broth	Barritt's reagent A and Barritt's reagent B.	A positive test is represented by the development of a red color 15 minutes or more after the addition of the reagents.
Citrate utilization	Simmon's Citrate Agar	bromthymol blue indicator	Positive Reaction: Growth with color change from green to intense blue along the slant.\ Negative Reaction: No growth and No color change; Slant remains green.

Quantitative measurements of bacterial growth:

Measurement of Cell Numbers:

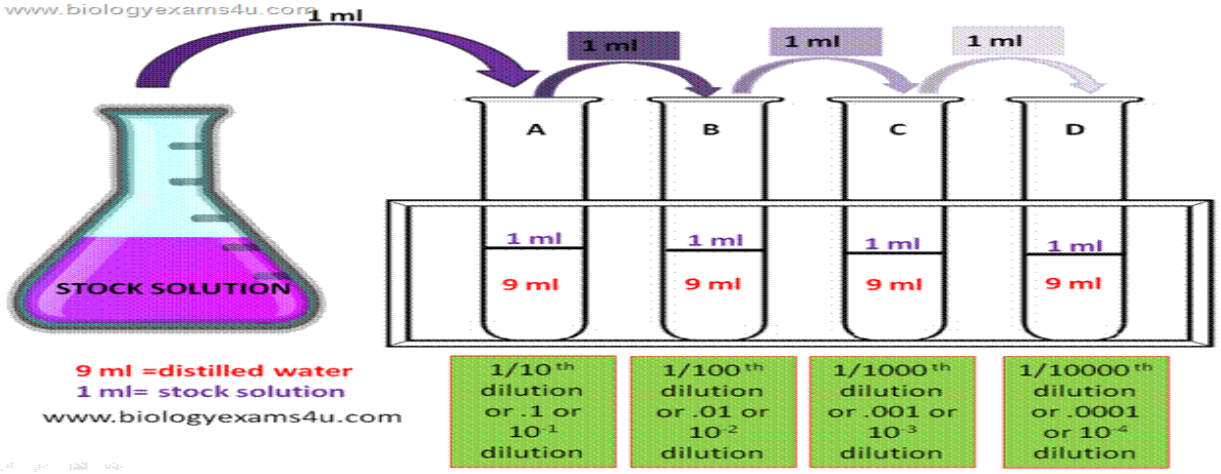
A- Direct Methods:

1-Breed Method

2-Turbidity (spectrophotometer)

3-McFarland Opacity standard tubes

4- Hemocytometer counting chamber



1-Breed Method:

Q: Calculate the number of the bacterial cells in 1ml in the second dilution when the number of the 5 filed are (2,10,4,6,3) ?

No. of cells / ml = Average no. of microbes per microscopic field x 5000 x 100 x 1/Dilution(inverted dilution)

Average= 2+10+4+6+3 =25 / 5 = 5

No. of cells / ml = 5 x 5000 x100 x 10^{2} =2,5 x 10^{8} cells / ml

2-Turbidity (spectrophotometer):

A quick and effective method of estimating the number of bacteria in a liquid medium is

to measure the turbidity or cloudiness of a culture and translate this measurement into cell

numbers.

3-McFarland Opacity standard tubes:

McFarland tubes have a mixture of specified amounts of barium chloride (BaCl2) and sulfuric acid (H2So4) together which forms a barium sulfate precipitate.

4-Hemocytometer :

Q: Calculate the number of the bacterial cells in(200ml) of sample and in the fourth dilution when the average of the six square in twelve ؟

No. cells / ml = Average no. of cells / small square x 20 x 10^6 x 1/dilution (inverted dilution)

No. cells / ml = Average no. of cells / small square x 20 x 10^6 x 10^4

= 12 x 20 x 10^6 x 10^4 x200 =4.8 x 10^{14} cells / ml

B-Indirect Methods

1- **Plate count**
2- **Dry Weight**
3-Filter membrane

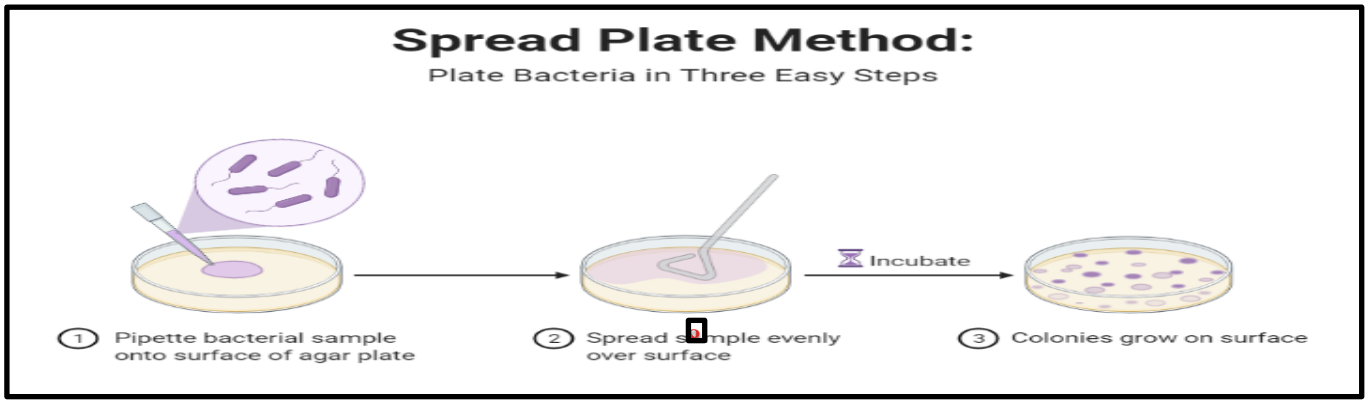
1- Total plate count:

Each colony represents a "colony forming unit" (CFU). In the methods below, ONLY viable (living cells are counted)

1-spread plate method :

1-Put 0.1 ml of bacterial suspension is placed in the center of the plate using a sterile pipet.

2- The glass rod (L ship : spreader) is sterilized by first dipping it into a 70% alcohol solution and then passing it quickly through the Bunsen burner flame.

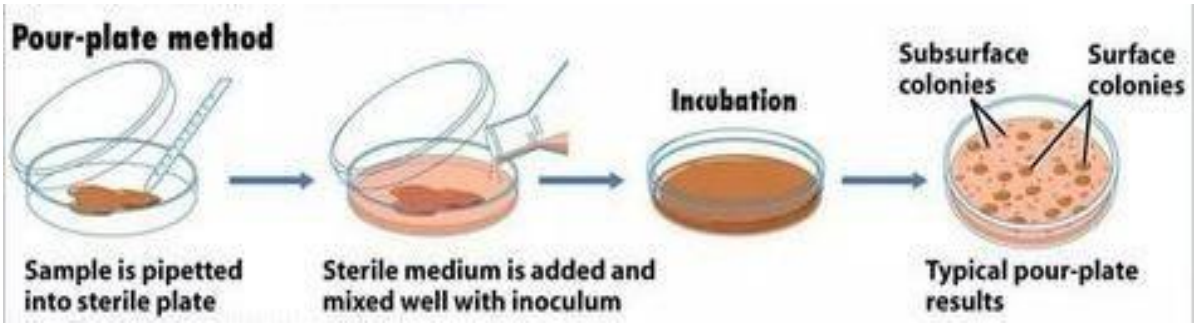


Colonies are most readily counted . Don't count plates with more than 300 or less than 30 colonies. (30-300) In the former

Bacterial Cells \ ml = No. CFU x DF x 10 (because we plated 0.1 ml)
DF= Dilution Facto

2-Pour plate method:

Using this method allows for a larger volume of the diluted sample; usually **1 ml.** This method yields colonies that form colonies throughout the agar, not just on the surface.



Bacterial Cells \ ml = No. CFU x DF

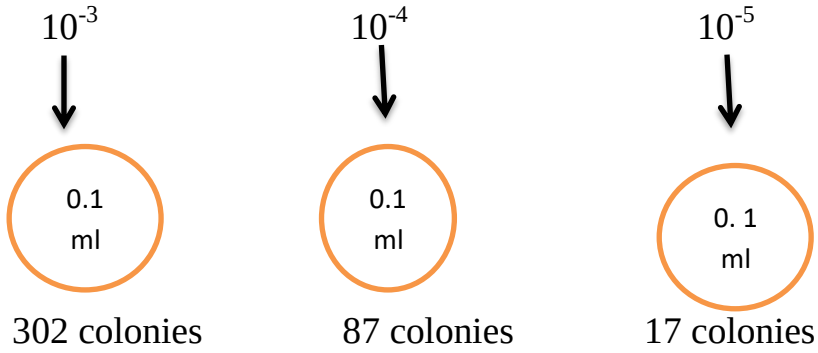
يأتي السؤال بهذا الشكل المذكور نحن نحدد الحجم (1مل او 250 مل او لتر او 10 مل و.....) ونحدد لك الطريقة اذا ذكرنا 0.1 مل نستخدم الطريقة الاولى طريقة الفرش وقانونها

Bacterial Cells \ ml = No. CFU x DF x 10

اما اذا ذكرنا 1 مل نستخدم الطريقة الثانية طريقة الصب وقانونها

Bacterial Cells \ ml = No. CFU x DF

Q: Calculate the number of bacterial cell in 1Liter of the original sample if the numbers of colonies in the last three dilutions are: 302, 87, and 17 respectively.



ذكر في السؤال 0.1 مل اذا نستخدم القانون

Bacterial Cells \ ml = No. CFU x DF x 10

اي وعدد البكتريا المسموح به ما بين (30-300) اذا كان اقل من 30 يهمل واكثر من 300 يهمل في السؤال نختار 87 ومذكور بالسؤال 1Literنضرب في 100 يكون الحل

Bacterial Cells \ ml = No. CFU x DF x 10

Bacterial Cells \ ml = $87 \times 10^4 \times 10 \times 1000$

$= 8.7 \times 10^9$ cells\ ml