Compound Light Microscope

Lab1

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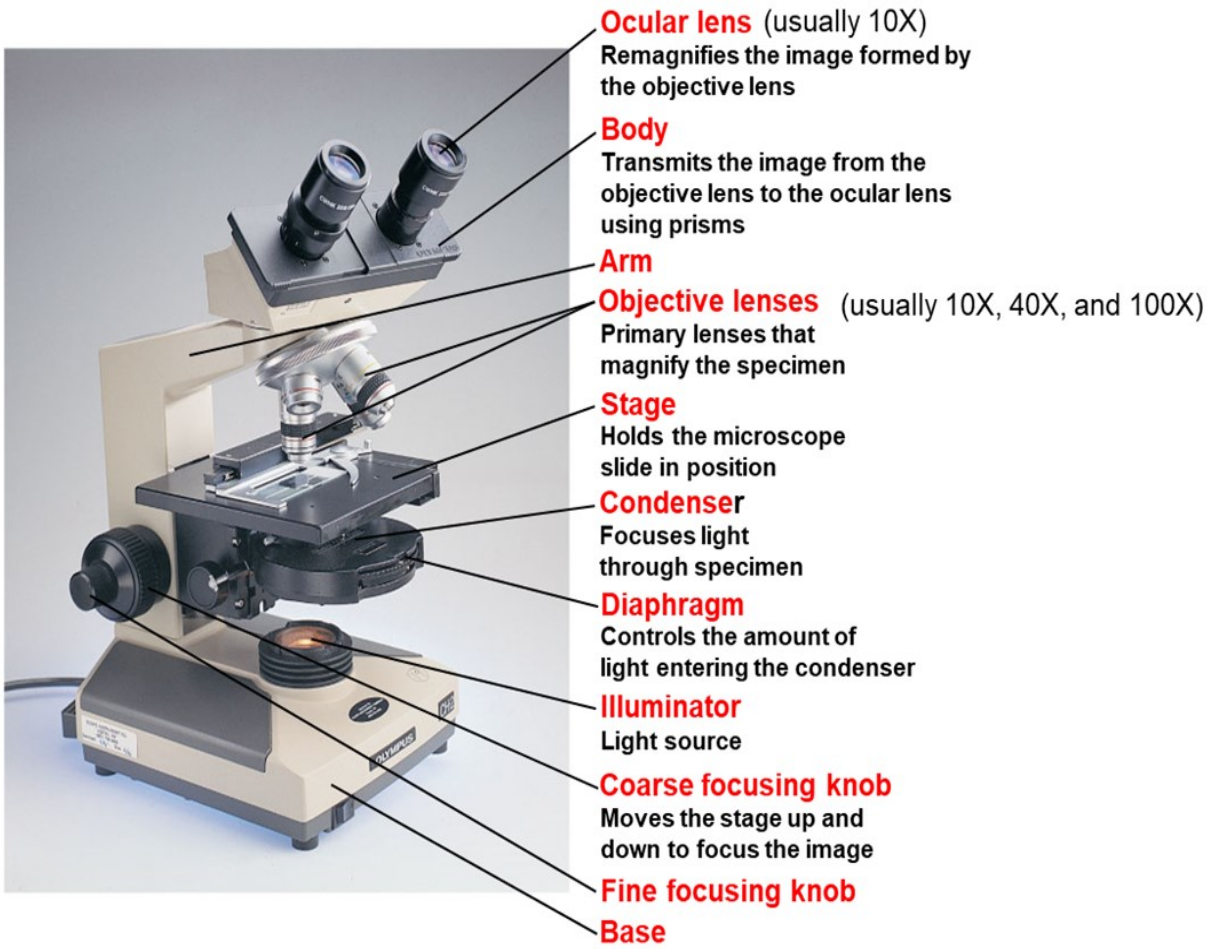
Microscopy:- is the technology of making very small things visible to the human eye.

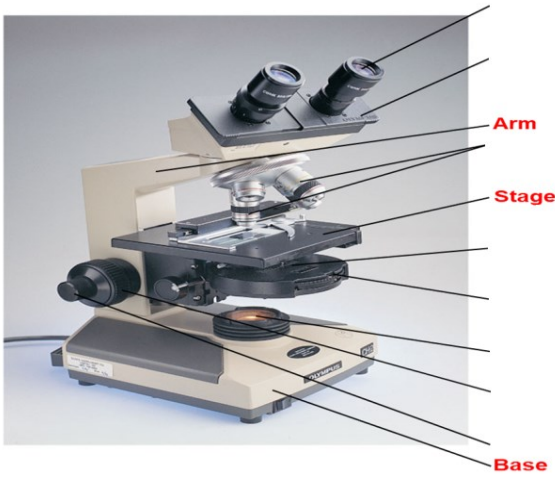
Purpose of microscope in Microbiology

- Bacteria is one of the small microorganisms that can't be seen with the naked eyes, most bacteria range in size between 0.5 - 2.0 micrometers (µm) so, there is a need to magnify the bacteria several times by using a microscope in order to see it
- There are **different types of microscopes** which are used in microbial life.
- In this lab, you will become familiar with the use of the **compound light microscope** (particularly **oil immersion microscopy**)

Basic component of compound light microscope



Parts of compound light microscope



consists of 4 main parts:-

1. **frame work.**
2. **Adjustment system.**
3. **Magnification.**
4. **Lighting System.**

1. Frame work:- Its includes three parts:

1. Base
2. Arm
3. Mechanical stage

- **Stage:-** the location of the specimen to be viewed

Stage Knobs Control

- **Top knob:-** is to move the forward and backward.
- **Bottom knob:-** is to move the stage right and left.

- **Clips:-** utilized in holding the specimen in place

2. Adjustment system:- It consists of the following parts:

1. Optical tube (body)
2. Coarse adjustment
3. Fine adjustment

Focus and Resolution Parts

- **Coarse-adjustment knob:-** is the larger of the two knobs. It is used in bringing the object into quick focus (focus of your specimen)
- **Fine-adjustment knob:-** is used for improving the clarity of the image, especially when viewing under high power (fine-tune focus)

Coarse-adjustment knob

Fine-adjustment knob

3. Magnification (Microscope lenses):-

Microscope has two sets of lenses are :

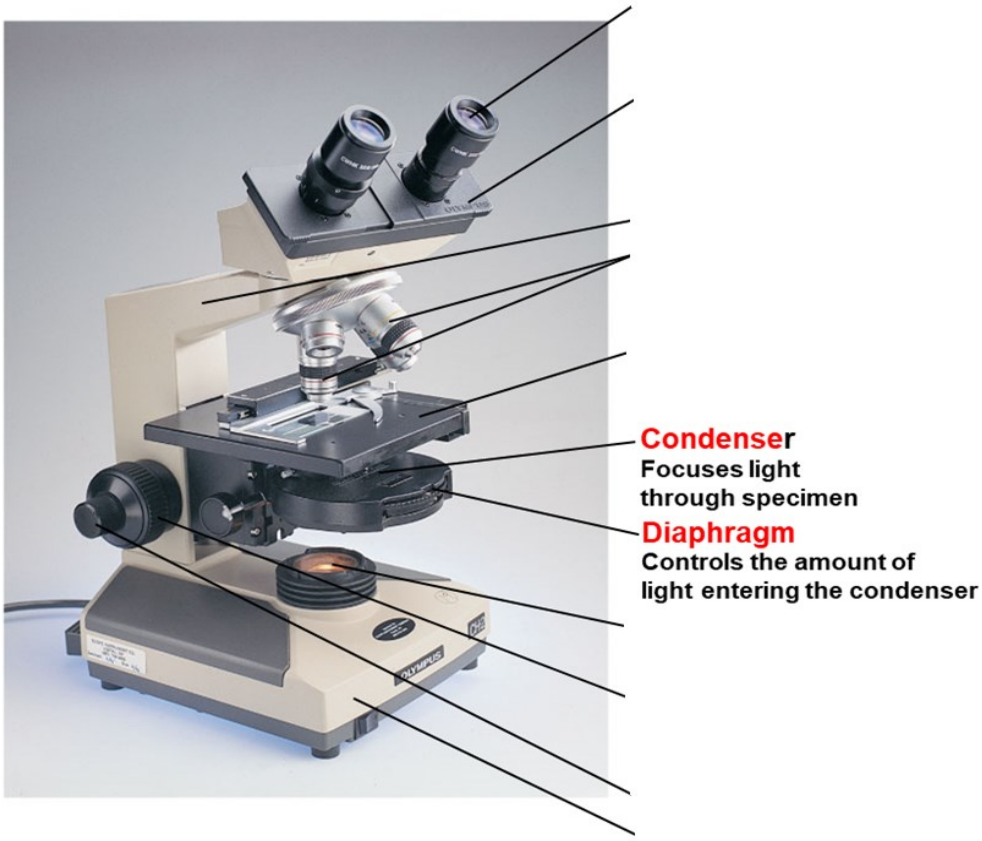
1. Ocular lens (eyepiece).
2. Objective lens.

- **Ocular lens or eyepiece**:- is used for viewing.
- **Revolving nosepiece:-** the part that holds two or more objective lenses and can be rotated to easily change power
- Usually you will find 3 or 4 objective lenses on a microscope. They almost always consist of 4X, 10X, 40X and 100X powers

- Scanning lens = 40X magnification
- Low power lens = 100X magnification
- High power lens= 400X magnification
- Oil immersion lens = 1.000X magnification

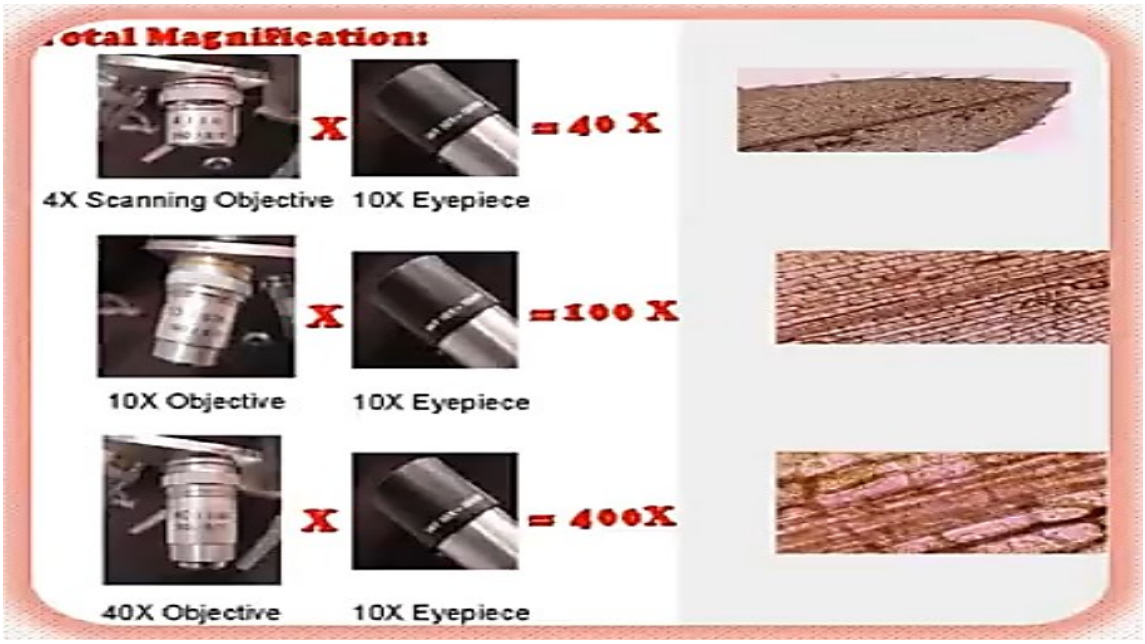
4. Lighting System.

- **Condenser**
- **diaphragm**



Total magnification:-

- **Total magnification =** powers of ocular lens(10X) × powers of objective lenses
  - if the magnification of an eyepiece is ×10 and the objective is ×4, the total magnification of the microscope is:
    Magnification of eyepiece × magnification of objective = 10 × 4 = 40x
- The image seen by the eye through a compound microscope is termed the **virtual image** and is **upside** and **reversed**.
- The surface area of view; as magnification increased , the area of view **decreased**



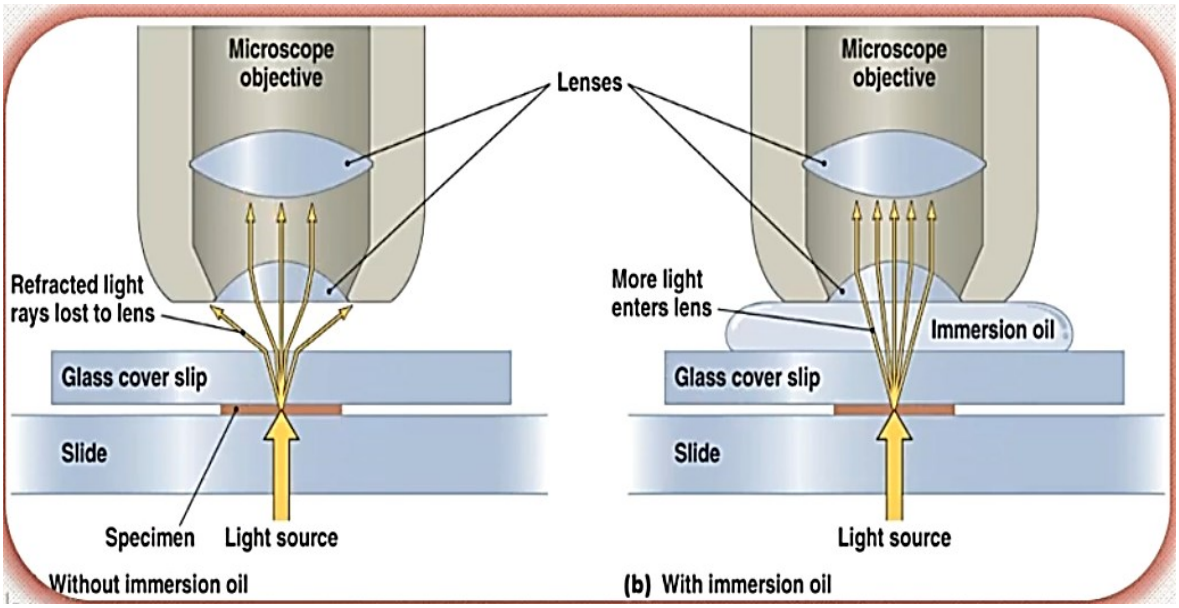
How to use Microscope (Focusing)

1. Plug in microscope and turn on illuminator. Rotate nosepiece to lock **4X objective** in place
2. Place smear on stage and center it under the 4X objective.
3. Using the **course adjustment knob**, move the objective lens to its lowest point. Look through the ocular and focus upward with the coarse adjustment **until an image comes into view.**
4. Continue with the next objective lenses and fine focus **(Fine adjustment knob)** each time
5. Look through the ocular and focus upward with the **fine adjustment** until an image comes into **view**.
6. When using oil immersion lens put a drop of oil on the slide and Rotate nosepiece and repeat step 5.

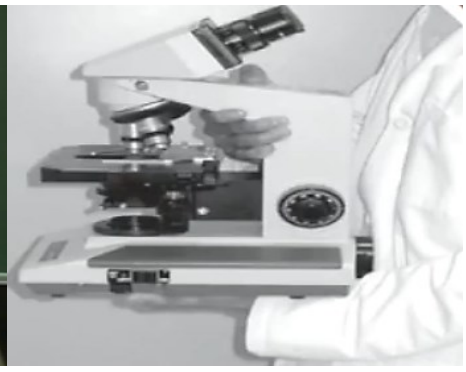
Knots

- When using **the lens (4X, 10X and 40X),** the specimen down as close to as possible **without touching it**
- When using the **oil immersion objective lens**, the specimen **touches the oil**

- Use oil **to prevent shattering of light rays**
- Oil had **refraction index equal to that of glass slide**



- Always carry a microscope **with one hand holding the arm and one hand under the base**



Harvesting question

Q1)When focusing, it is best to start with

A. lowest lens
B. scanning lens
C. high lens
D. oil immersion lens

Q2) When viewing a specimen, start focusing with the fine adjustment knob and then fine tune using the course adjustment knob

A. True
B. False

Q3) Which of the following are low power lenses?

A. 4X
B. 40X
C. 100X

Q4) Allows you to change the objective that you are viewing the specimen through.

A. Body tube
B. Stage clamp
C. Stage
D. Nose piece

Q5) This longest lens allows 1000X magnification when it is used with the ocular.

A. Scanner
B. Low Power
C. High Power
D. Oil Immersion

Q6) This part of the microscope should be turned to clarify the image seen in the microscope when it is slightly out of focus.

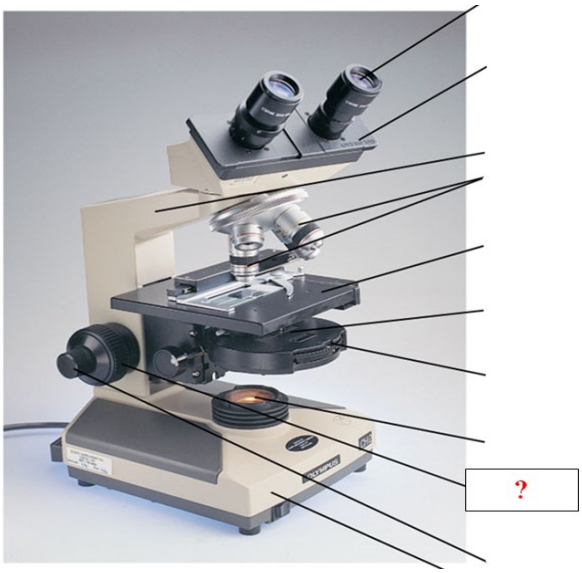
A. Coarse adjustment knob
B. Fine adjustment knob
C. Stage clamp
D. Nose piece

Q7) This part of the microscope is most commonly used with the scanner, and is sometimes needed for focusing the image when using the low power lens.

A. Coarse adjustment knob
B. Fine adjustment knob
C. Ocular lens
D. Diaphragm

Q8) This part is

A. Coarse adjustment knob
B. Fine adjustment knob
C. Ocular lens
D. Diaphragm



Dr. Sarah Abdulhussein Hasan

Glasses and Instruments in Microbiological Laboratory

Lab.2

Glasses in medical microbiology laboratory

1. Beakers: It is used for heating liquid and for preparing reagent solution

2. Conical flasks: Similar to beakers

Conical flask

Beaker

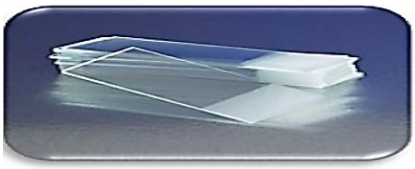
3. Petri dishes: Shallow dishes used to culture living cells and microbes

4. Graduated cylinders: Similar to beakers, they have volumetric markings to allow for monitoring of volume

Cylinder

Petri dish

5. Slides. Used to hold microorganism under a microscope for inspection and study



Slide

6. Test Tubes: They are used to heat on hold reagents for observing chemical reaction.

7. Test Tubes Racks: It is used to hold the test tube in the upright position. These are made of metal or plastics.

Test tube

Instruments in microbiological laboratory

1. **Autoclave:** is a pressurized chamber used for the process of sterilization of medical or laboratory equipment and disinfection by steam. It is commonly used for the preparation of culture media during laboratory applications.
2. **Hot Stirrer (Magnetic):** used for mixing various liquid components.

Autoclave

3. **A centrifuge:** works on the principle of sedimentation, where the high speed of the rotation causes the denser particles settle at the bottom while the lighter particles are collected at the top. It can be used for the separation of viruses, nucleic acid and blood components, etc.
4. **Oven:** can be used to sterilize materials like glassware, metal equipment, etc.

Hot stirrer

Centrifuge

Oven

5. Incubator: used for the growth and maintenance of microorganisms and cell cultures. It is providing an optimal temperature that adjusted according to the type of organism cultivated inside and maintaining a proper atmosphere for the growth of microorganisms.

Incubator

6. Sensitive balance: is a type of balance that is commonly used for the measurement of powder media, pbs (phosphate buffer saline), samples, etc.

Sensitive balance

7. Laminar Hood (biosafety cabinet): is a closed device used for processes or instruments sensitive to microbial contamination by creates a sterile environment with the flow of sterile air filter and shortwave ultraviolet germicidal lamp that sterilizes the workstation.

pH meter

8. pH meter: is used to determine the acidity or alkalinity of the solution.

9. loop: is a simple tool used to pick up and transfer a small sample (inoculum) from a culture of microorganisms, e.g. for streaking on a culture plate.

Loop

10. Bunsen burner: is connected to a gas source, it is commonly used for micro-loop sterilization

Bunsen burner

Harvesting questions

Q1) Which of the following pieces of equipment would be used to spin samples quickly to separate them based on density?

A. centrifuge
B. autoclave
C. magnetic stirrer
D. balance

Q2) A _____ is used to sterilize glassware and media in a microbiology laboratory.

A. centrifuge
B. incubator
C. autoclave
D. biosafety cabinet

Q3) Determine the name of the following piece of lab equipment.

A. Graduated cylinder
B. Beaker
C. Conical flasks
E. incubator

Q4) What piece of equipment do you need for streaking on a culture plate?

A. Graduated cylinder
B. Beaker
C. Bunsen burner
D. Loop

Q5) What lab equipment is used to accurately measure the volume of liquids?

A. Graduated cylinder
B. Beaker
C. Conical flasks
D. Sensitive balance

Q6) What lab equipment is used to measure the amount of matter in an object?

A. Graduated cylinder
B. Beaker
C. Conical flasks
D. Sensitive balance

Q7) What unit is on a graduated cylinder?

A. Kiloliter (KL)
B. Liter (L)
C. Milliliter
D. Microliter

Bacteria Morphology (staining)

Lab.3

Bacteria Morphology:- include

1. Size 2. Shape 3. Arrangement 4. Staining

1. Size (micrometer)

- 0.2 ~ 1.2 (µm) width, 0.4-14 µm length

2. Shape

- **Bacteria primarily have distinct shapes**

A. Coccus/cocci (spherical)

- Diplococci (pairs)
- Arranged in Chains (streptococcus)
- Arranged in clusters (staphylococcus)

B. Bacillus/bacilli (rod shaped)

- Cocca bacilli
- Curved (Vibrio's)

C. Spiral

- Spirila (rigid)
- Spirochetes (flexible)

3. Arrangement

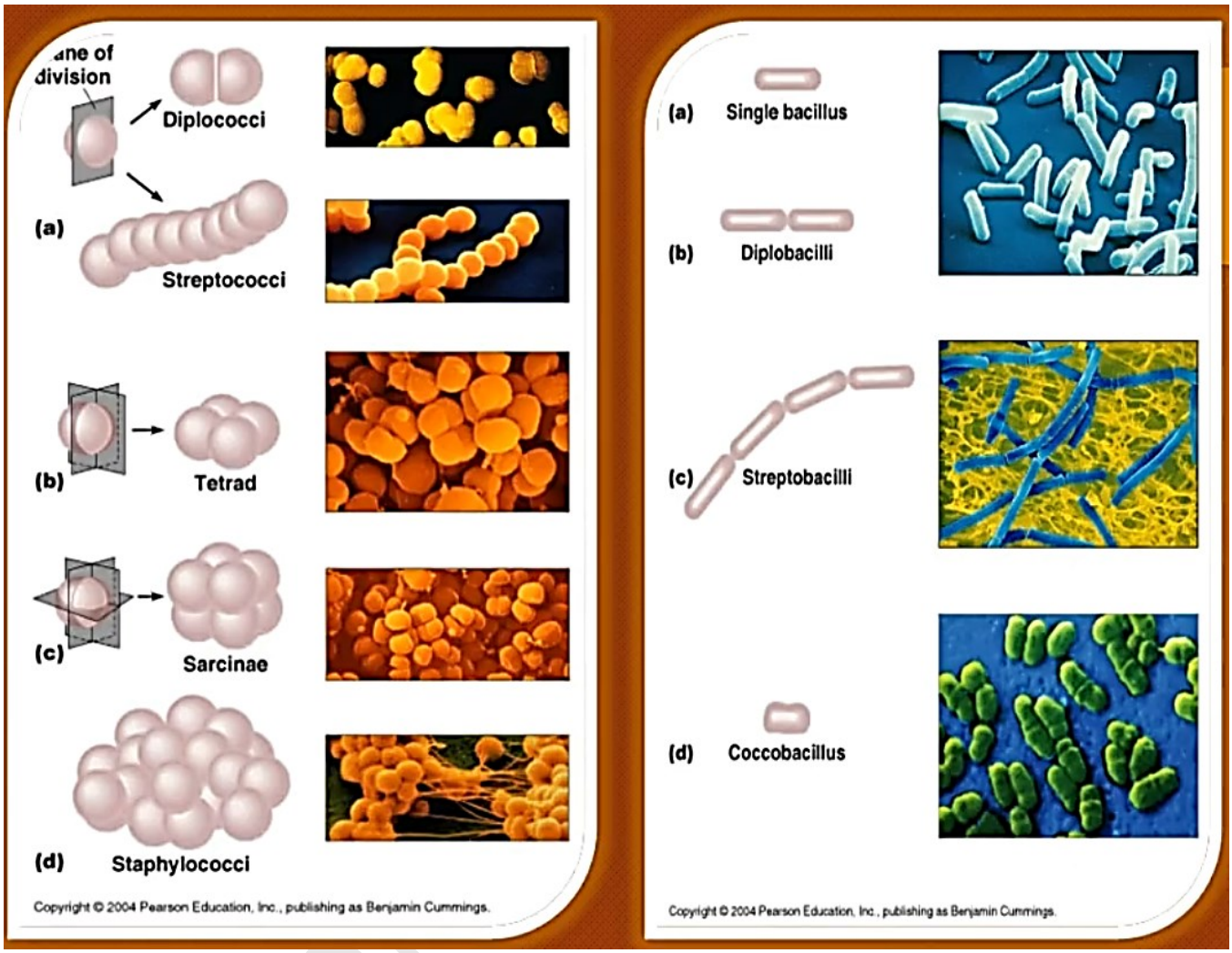
- Based on **the planes of divisions and generation time** seen in the organism bacteria may also have specific **arrangements of the cells**.

A. Plane of cell division

- Single or one plane of cell division (**single, pairs, chain**)
- More than one plane of cell division (**cluster**)

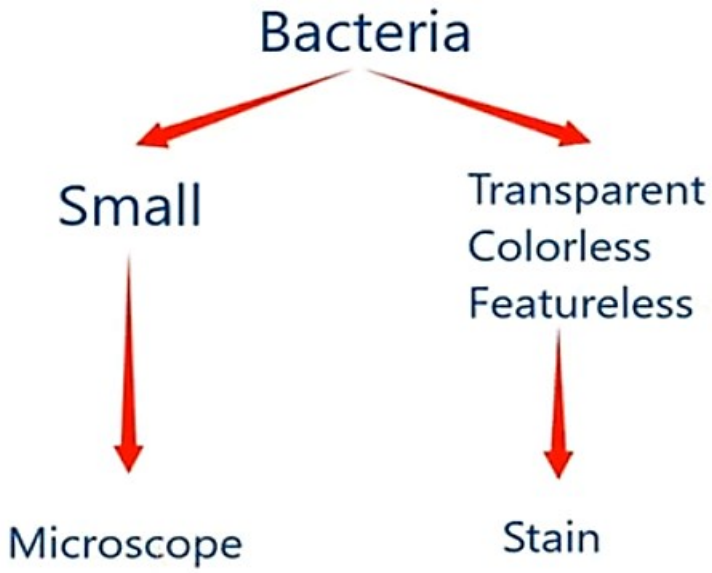
1. **Diplococci** are formed when the plane of division is vertical and the resultant two coccal cells do not completely separate from each other.
2. If the cells divide in the vertical plane continually and the cells do not separate it results in a **chain of coccal cells** called a **streptococci**/**streptobacilli**.
3. Other arrangements include: **tetrad (4), sarcina (8)**, **staphylococcus** (**irregular clusters**)

B. Generation Time (time between two successful cell division)
Separation time = generation time (pairs)



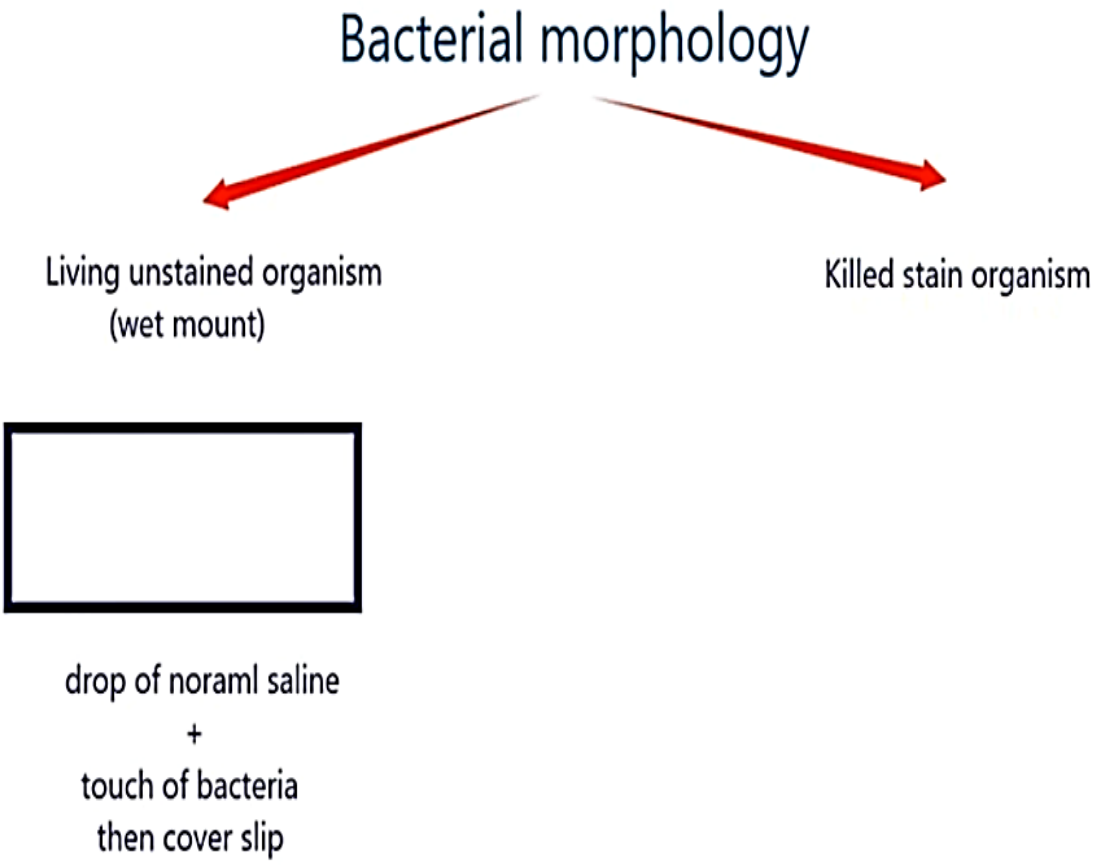
4. Staining

- The basis for staining is to study **the morphology and structure** of bacteria.
- bacteria are very small, almost **completely transparent**, **colorless** and **featureless** in their natural states. However, staining can make the structures of bacteria more pronounced.



- In our laboratory, bacterial morphology (form and structure) may be examined in two ways:

1. by observing living **un**stained organisms (**wet mount**).
2. by observing **killed** stained organisms.



A stain is a substance that adheres to a cell, giving the cell color

- **Different stains** have different affinities for different organisms, or different parts of organisms

Stains Composes From:

- Stains are a mixture of **chromogen and auxochrome**
- Chromogen = benzene derivative + chromophore (**coloring agent**)
- Auxochrome: give +ve or -ve charge to the chromogen
- The **ionized stain** is capable of binding to cell structures with **opposite charges**

A) **Basic stains** are cationic; when ionized, the chromogene exhibits a positive charge.

- Basic stains bind to **negatively charged cell structures** like nucleic acids.

Common basic stains are

1. **Methylene blue**
2. **Crystal violet**
3. **Safranin**
4. **Malachite green**
5. **Carbolfuchsin**

B) **Acidic stains** are anionic; when ionized, the chromogen exhibits a negative charge.

- Acidic stains bind to positively charged cell structures like proteins.

Common acidic stains are

1. **Picric acid**
2. **Eosin**
3. **India Ink**
4. **Nigrosin**

- Bacteria are slightly negatively charged at pH 7.0
- Basic dye stains **bacteria**

Types of staining In Microbiological Lab.

- **Different** types of staining **methods** are used to make the cells and their internal structures more visible under the light microscope.

1. **Simple stain (single stain)**
2. **Differential Stain (more than one stain)**

- Gram stain
- Acid fast Stain

3. **Special stain**

- Capsular stain
- Endospore stain
- Flagellar stain

Harvesting questions

Q1) The colorization of bacteria with more than one dye solution to distinguish between microbial cell types is called a _____.

a. wet mount
b. simple stain
c. differential stain
d. structural stain

Q2) A procedure that divides organisms into two or more groups depending on their individual reactions to the same staining procedure is referred to as _____.

a. wet mount
b. simple stain
c. differential stain
d. structural stain

Q3) The term used to describe bacteria that are oval shaped is _____.

a. coccus
b. bacillus
c. vibrio
d. coccobacillus

Q4) The term used to describe bacteria that have a round shape is _____.
a. coccus
b. bacillus
c. vibrio
d. coccobacillus

Q5) The term used to describe bacteria clump together in irregular clusters is _____.
a. streptococci
b. staphylococci
c. tetrads
d. sarcinae
e. virbriococci

Q6) Bacteria that are shaped like rigid helices are _____.
a. vibrios
b. spirilla
c. spirochetes
d. coccobacilli
e. bacilli

Q7) Cubic configurations that are formed when round bacteria remain attached to each other during reproduction are called _____.
a. streptococci
b. staphylococci
c. tetrads
d. sarcinae
e. vibrio

Q8) Bacteria that are shaped like flexible helices are _____.
a. vibrios
b. spirilla
c. spirochetes
d. coccobacilli
e. flexicetes

Q9) Curved bacteria having less than one complete turn are said to be _____.

a. spirilla
b. mycoplasma
c. vibriod
d. helical
e. sarcinae

Q10) Square planar configurations that are formed when round bacteria remain attached to each other during reproduction are called _____.

a. streptococci
b. staphylococci
c. tetrads
d. sarcinae
e. virbriococci

Q11) Numerate the common basic stains?

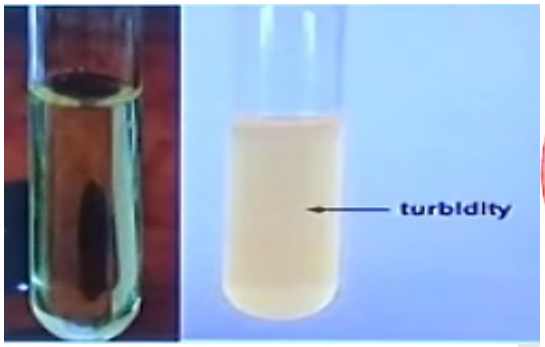
Growth Media

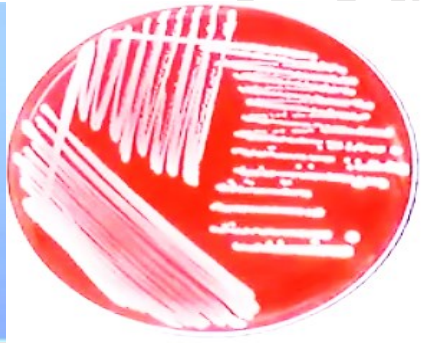
Lab.4

Bacterial Growth: refers to an increase in the numbers of individuals.

- Indicated by

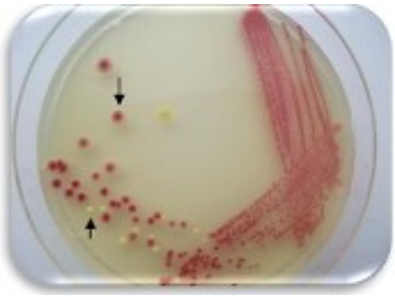
1) Turbidity of the **fluid media**
2) Colonies on **solid media** (**Macro**scopic product)





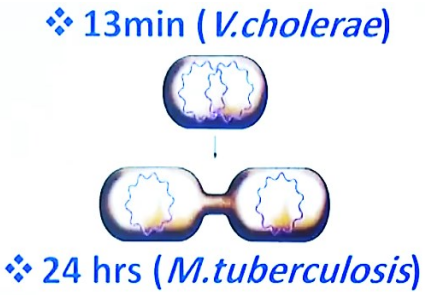
- **Colonies:** single bacterium after 20-30 division by binary fission

Single bacterium —(On solid media)→ After 20-30 division —(Binary fission)→ Colony (1 Million (2^{20}))



Generation time (doubling time)

- 13min (V.cholerae)
- 24 hrs (M.tuberculosis)



Q/ What is the time need for growth of single V. cholerae on solid media to get a visible colony?

20/4 = 5hours

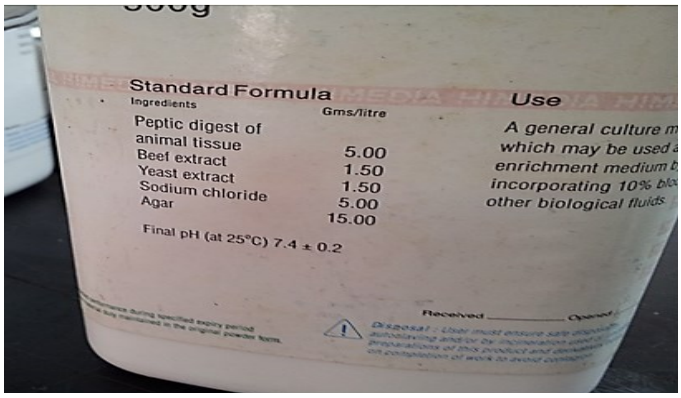
Q/What is the time need for growth of single (M.T) on solid media to get a visible Colony?

20/1 day = 20 day

30/1day = 30 day

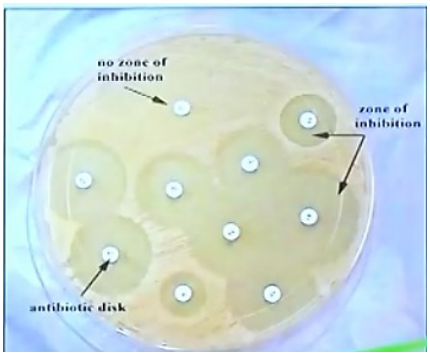
Growth Media (Culture Media): is a special medium used in microbiological laboratories to grow different kinds of microorganisms. A culture medium is composed of different nutrients (carbohydrate, lipids, amino acids, vitamins as well as inorganic compounds).

- Bacteria grow (**In vitro**)
- Need nutrients for growth (**Artificial**)



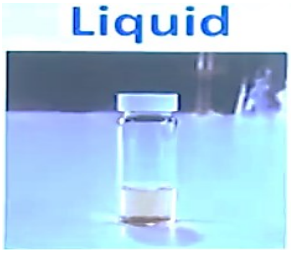
A. Purpose of culture media

1. Study Properties
2. Isolation & diagnosis (Causative agent)
3. Prepare vaccine & Other product
4. For selection proper antibiotics



B. Form or classification of culture media

1. Solid (Agar)
2. semi-solid
3. liquid media (broth media)



C. Types of media

1. Simple media
2. Enriched media
3. Selective media
4. Differential media

1) Simple media: contain basic requirement for growth of most bacteria

A) Peptone water (Peptone + 0.5% NaCl)

B) Nutrient broth (Meat extract)

C) Nutrient agar plate (Nutrient broth + 2% agar agar (Seaweed))

Nutrient agar plate

2) Enriched media: for fastidious bacteria need blood, serum for growth

a) Blood agar

(Nutrient agar heated at 45°C (semisolid) + sheep blood)

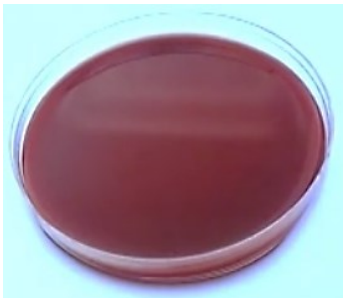
- Streptococci
- Strept. pyogenes

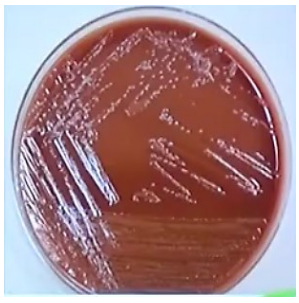
Blood agar

b) Chocolate agar

Nutrient agar heated at 100°C, add blood

- Haemophilus
- Neisseria





Chocolate agar

3) **Selective media:** which allows the growth of certain microorganisms (Selective) while inhibits the growth of the others

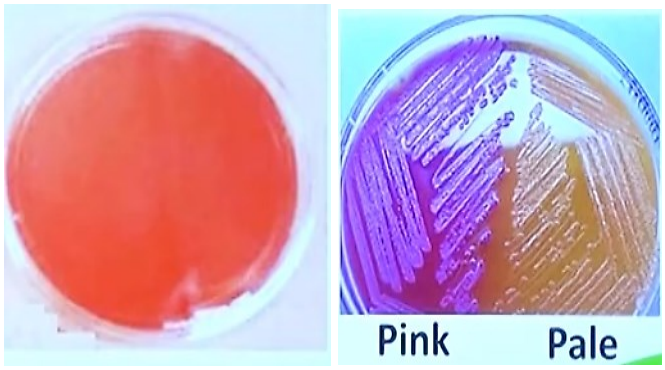
A. **Lowenstein Jensen medium**
- Malachite green
- Tubercle bacilli

B. **Blood tellurite agar**
- Potassium tellurite (C.diphtheriae)

4) **Differential media:-** Is used for differentiating between bacteria

- **Selective:** Allow a certain organism to grow
- **Indicator:** to differentiate (change in visibly)

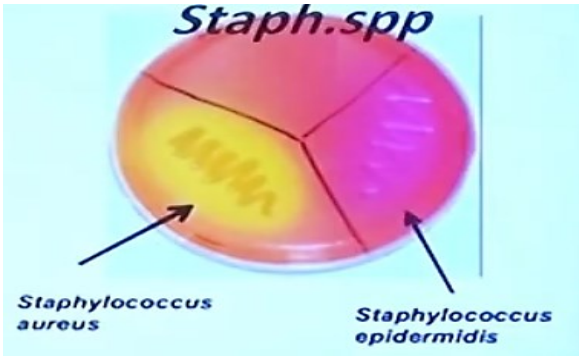
A. MacConkey's agar
- Bile (Enterobacteia)
- Lactose= test sugar
- Peptone
- Neutral red = pH indicator



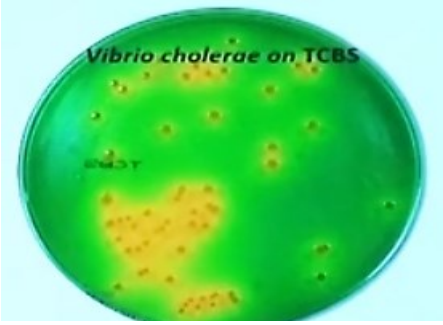
MacConkey's agar

B. Mannitol salt agar
- (high salt 7.5% NaCl)
- Phenol red =pH indicator

C. Thiosulfate-Citrate-Bile-Sucrose Agar. (TCBS)
- Thiosulphate
- Bile
- Citrate
- Sucrose as test sugar
- Bromothymol blue = indicator



Mannitol salt agar



Harvesting questions

Q1) Which one of the following is true

a) Nutrient broth is basal medium
b) Addition of selective substances in a solid medium is called enrichment media
c) Agar has nutrient properties
d) Chocolate medium is selective medium

Q2) Chococlate agar is made from blood agar

a) True
b) False

Q3) Which of the following is used as a solidifying agent for media?

a) Beef extract
b) Peptone
c) Agar
d) Yeast extract

Q4) Colony formation can be observed in liquid media broth

a)True
b) False

.

Preparation of culture media
&
Isolation Techniques
Lab.5

Preparation of culture media

1. Weightining the medium ingredients according to the direction written on its container.
2. Dissolve with little amount of D.W. then complete the volume to the volume you want and may be need using heating and stirrer for complete dissolving.
3. Check pH.
4. Dispensing the medium in to test tube by pipette.
5. Sterilization by autoclave.
6. Dispensed agar medium into petri dish when the heat reach to 45°C.

EX :prepare 500ml of N.A. medium if the direction on container wrote 8gm/liter

gm	ml
8	1000
x	500

x=8 * 500/1000 = 4 gm of media dissolve in little amount of D.W. then complete the volume to 500 ml then autoclaved and poured in plates

Method of pouring the media in plate

The sterile plates should be on the table near the burner then

- Cooling the solid medium to 45°C to avoid solidify it and to avoid forming of drop on the cover of plates
- Remove the cover (or cotton plug) and sterile the upper part by burner
- Remove the cover of plate near the burner and pouring the medium and close the cover of plate
- Moving the plate on table 5 times in two direction to distribute the media equally in plate.

Five basic techniques of culturing

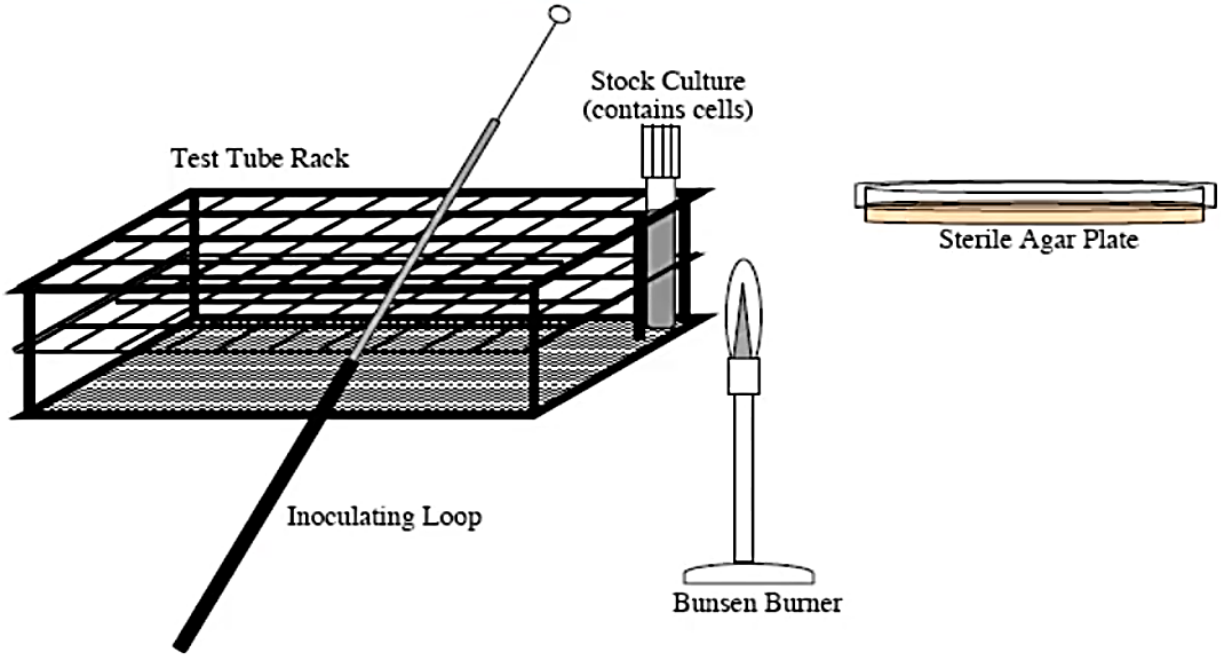
1. inoculum/ inoculation
2. Incubate
3. Isolation
4. Examination
5. Identification

- **Isolated colonies:** a population of millions of cells that are identical from a single bacteria.
- **Stock Culture:** a culture that already contains cells. It is used a source of cells from which to inoculate new cultures.

- **Aseptic technique include:**

1. Sterilization of medium
2. Sterilization of equipment
3. Proper handling

Necessary equipment

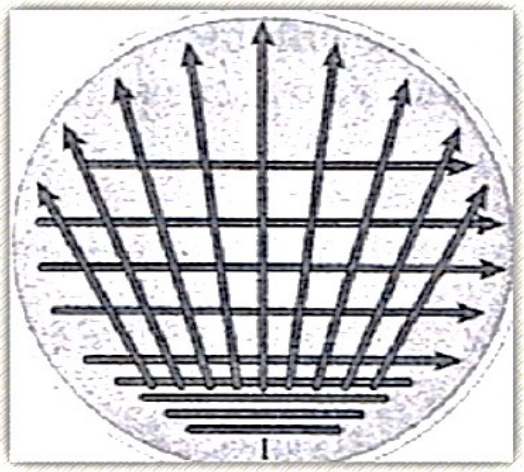


Isolation Techniques:

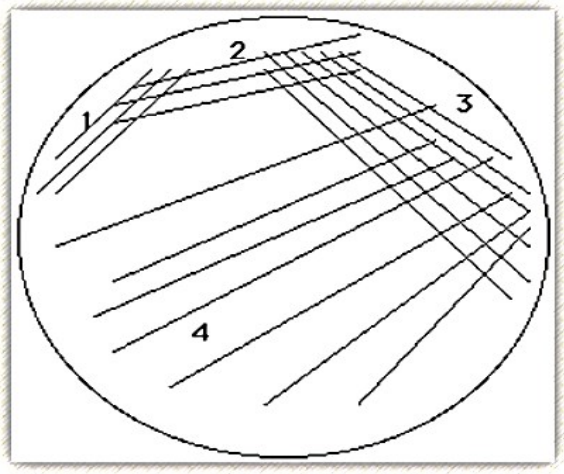
1. radiant- plate method

2. continuous - plate method (zig-zag)

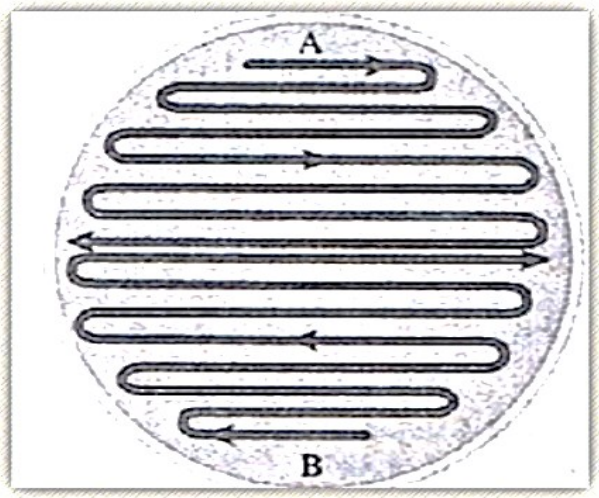
3. quadrant streak- plate method



Radiant streak



Quadrant steak

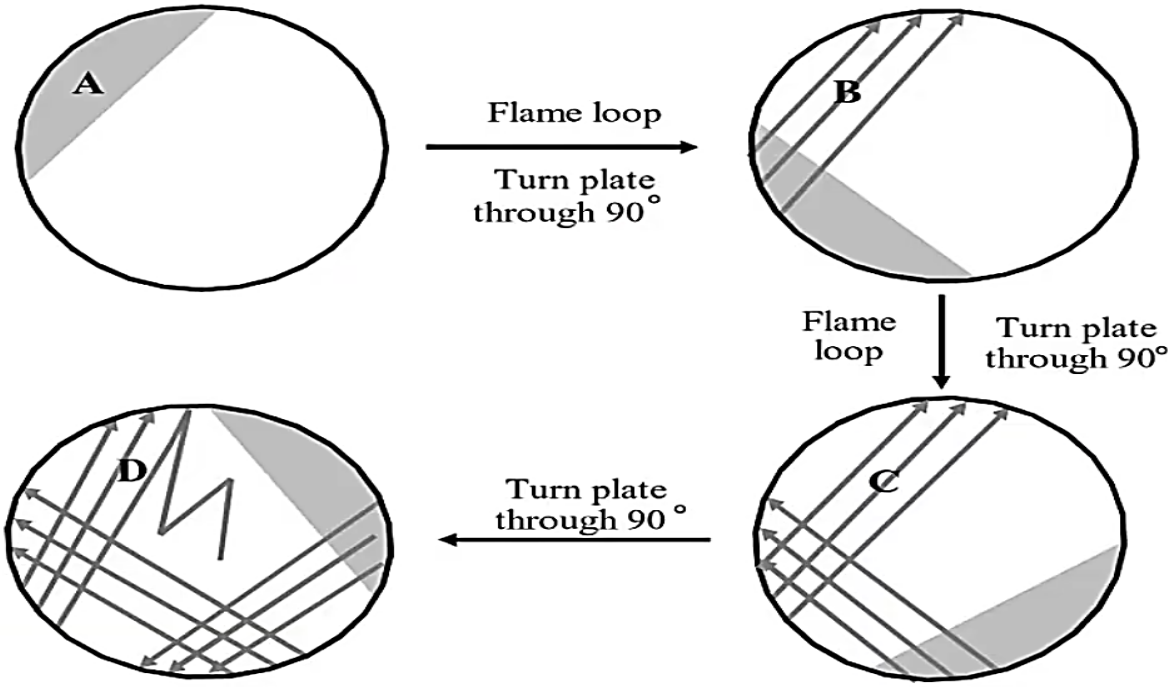


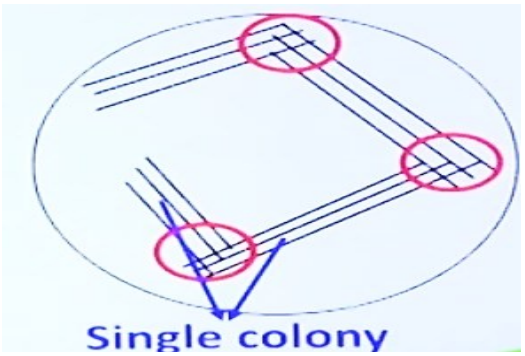
Continuous(zig-zag) streak

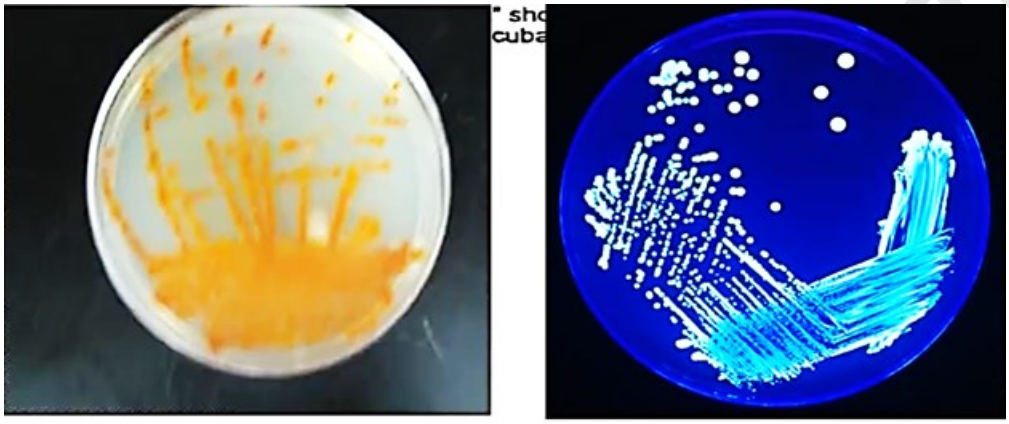
- quadrant Streak – Plate Method: To obtaining a pure culture (**Individual single colonies**) (Each colony represent one kind of bacteria)

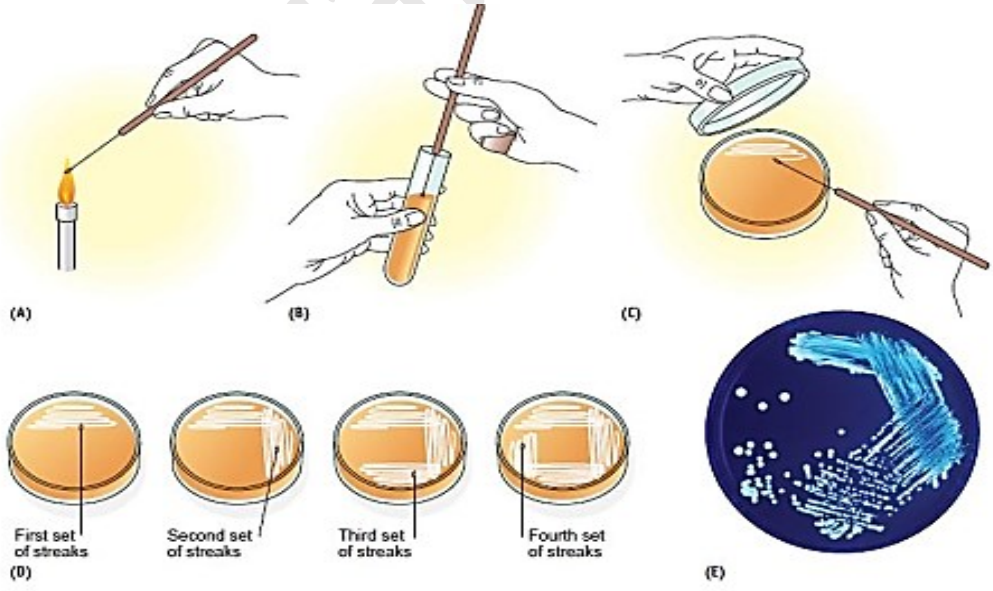
Procedure

1) A loop is sterilized, cool the loop by touching, spread the inoculum back and forth across the upper 1/4 of the plate, keeping the lines of inoculation very close together (area 1 in figure below).
2) **colonies are not expected in this area**. Do not use strong pressure, which will break the surface of the agar
3) Turn plate approximately 90°. Streak the plate as indicated in the figure (area 2) across about 1/4 of the plate. Dispose of the loop.
4) Repeat step 2 one or two times more
5) **In area 3 and/or 4 single colonies should appear**.
6) Label plates on the bottom and incubate **inverted** (upside-down to prevent condensation from splattering down onto bacteria) at 37°C.
7) Isolated and defined colonies are a successful isolation




Single colony




(A)
(B)
(C)
First set of streaks
Second set of streaks
Third set of streaks
Fourth set of streaks
(D)
(E)

Harvesting questions

Q1) Which of the following methods are the most common and routine culture methods performed in the microbiology lab for obtain Individual single colonies? Select all the correct answers

a) Streak plate culture

b) simple stain

c) direct stain

d) broth culture

Q2) Isolation of pure culture refers to separation of a single colony

A. True
B. False

Q3) A large cluster of colonies are obtained in streak plate method

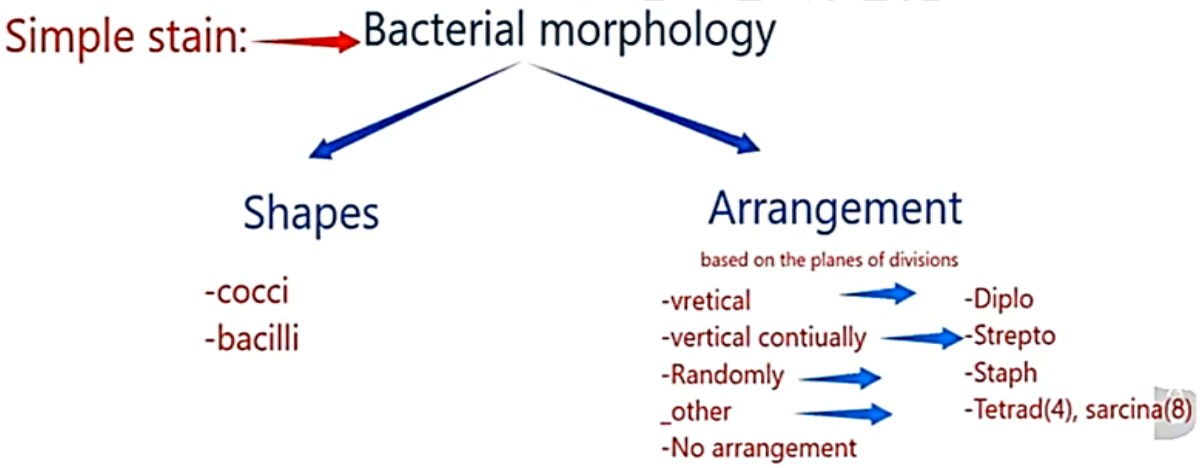
A. True
B. False

Simple Staining

Lab.6

Simple Stain (Direct stain)

- Single basic stain، is used in the procedure
- Basic stains are cationic and bind to negatively charged cell structures
- Simple stains do not usually provide any data for identification of the bacterium they simply make the bacterium easier to see
- Used to show the **general structures of some bacteria** (shape, cell arrangement)



Materials and Reagents

Reagents

Basic stain as

1. Methylene blue
2. Crystal violet
3. Carbolfuchsin
4. Saffranin
5. Malachite green

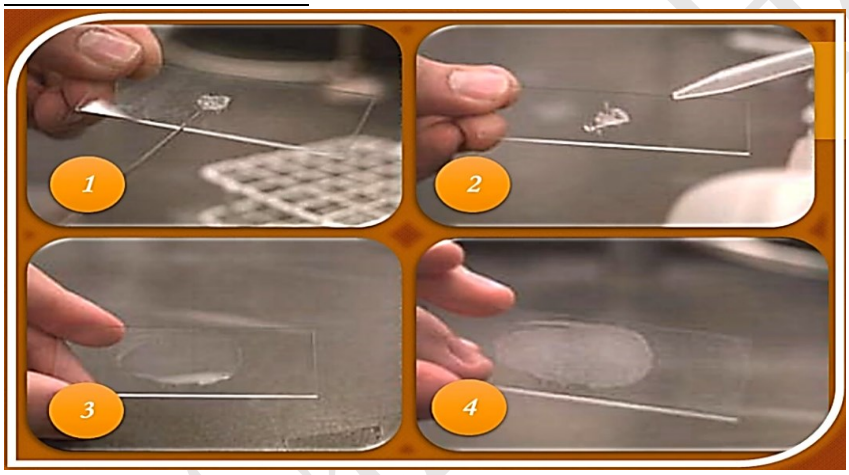
Materials

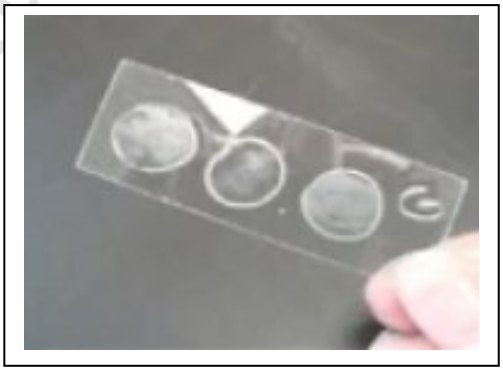
1. Inoculating loop
2. Clean glass slides
3. Sterile normal saline
4. Bunsen burner

Procedures

1. Labeling of the slides
2. Preparation of the smear

- Smear is a distribution of bacterial cells on a slide for the purpose of viewing them under the microscope.
- Method
- Place a drop of sterile normal saline solution on the slide (to maintain osmotic pressure)
- With a sterile cooled loop, pick up a very small sample of a bacterial colony (sterile technique)
- Gently stir into the drop of normal saline on the slide to create an emulsion. If the bacterial suspension is very thick, add a drop of water and mix the bacteria and the water on the slide

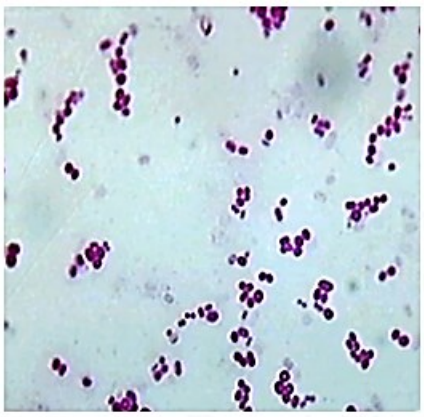




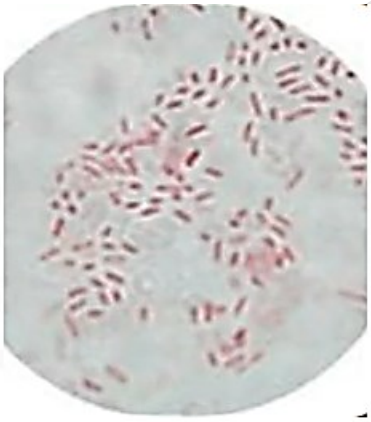
- Air drying: to prevent lysis (boiling)
- Heat fixation: Hold the slide at one end and quickly passing the slide through a Bunsen burner flame three times. This causes partial melting of the cell walls and membranes of the bacteria, and makes them stick to the slide (help the cells adhere to the slide surface)
- Do not overheat the slide as this will destroy the bacteria
- The smear is now ready for staining

3. Simple Stain Procedure

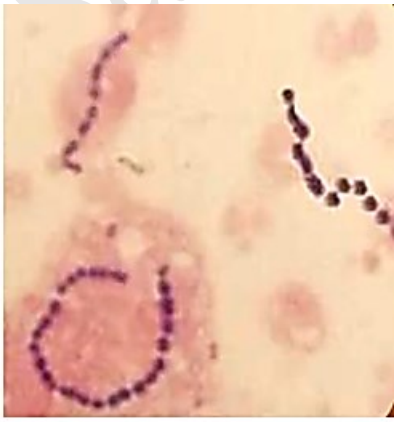
- Cover the smear with a few drops of **one of basic stain**
- Allow the stain to remain for the following periods of time:
  - Carbolfuchsin (15-30 seconds)
  - Methylene blue (1-2 minutes)
- Gently rinse the slide with tap water by holding its surface parallel to a gently flowing stream of water
- Allow the slide to air dry
- View the smear using a light-microscope under oil-immersion



diplococci



bacilli



streptococcus

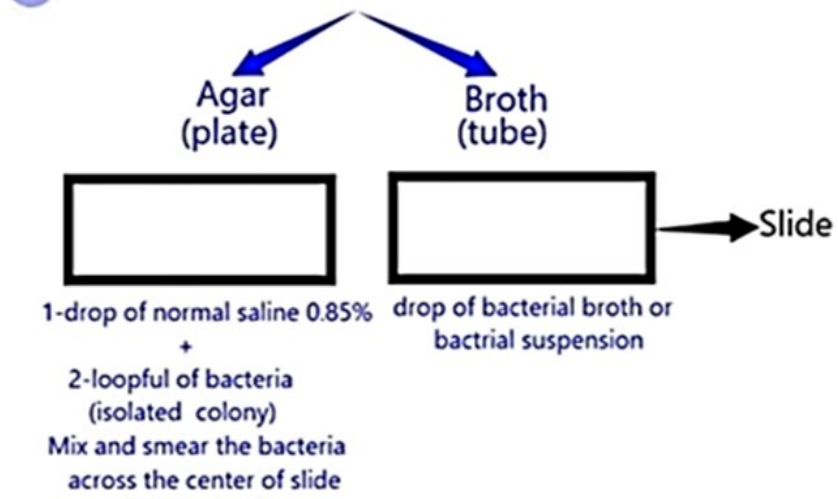
Notes

Heat Fixation:-

1. kill any bacteria that may still be a live
2. Facilitates stain penetration
3. Fixed cells to the slide so that they do not wash off when stained

Staining protocol in microbiology lab:

1. Smear preparation:



2. Air drying → To prevent lysis

Thin smear → Take another loopful of bacteria
Thick smear → Add drop of normal saline

Staining protocol in microbiology lab:

3. Heat fixation →

Why??
1. Partial melting of the cell walls and membranes of the bactrtia
2. To let bacteria stick to slide
3. To kill bacteria

X Do not overheat → This will destroy the bacteria

---

4. Stain → Simple stain
   1. Basic stain
   2. Washing
   3. Mordant (Iodine)
   4. Washing

Harvesting questions

Q1) Smear is made by spreading bacterial suspension on a clean slide and allowing it to air dry

a) True
b) false

Q2) simple staining procedures that use only one stain can be used to determine cell morphology, size and arrangements

a) True
b) false

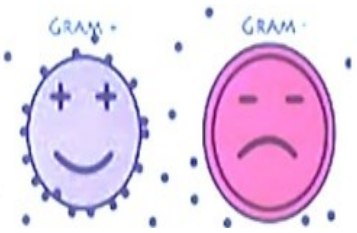
Q3) What is the correct procedure in simple-Staining?

a) heat fixation, air drying, stain, smear

b) smear, air drying, heat fixation, stain

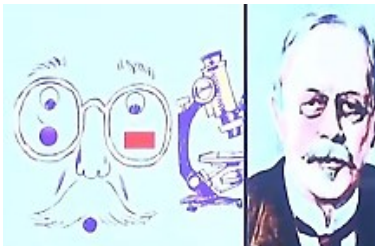
c) stain, heat fixation, smear, air drying

d) smear, heat fixation air drying, stain



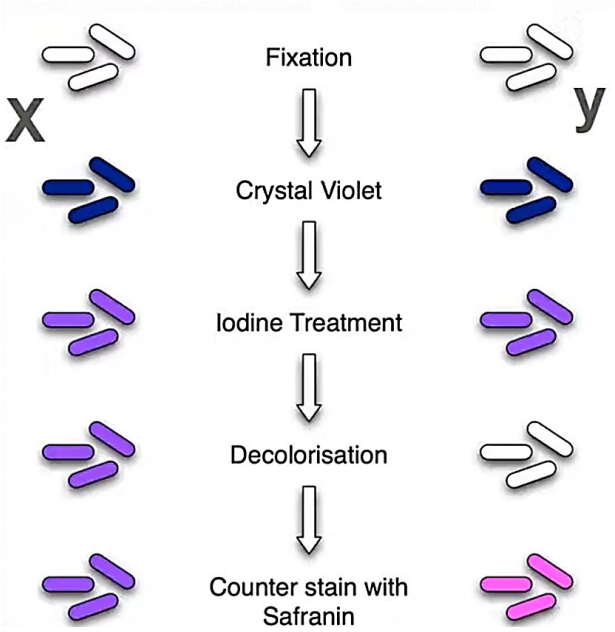
Gram stain

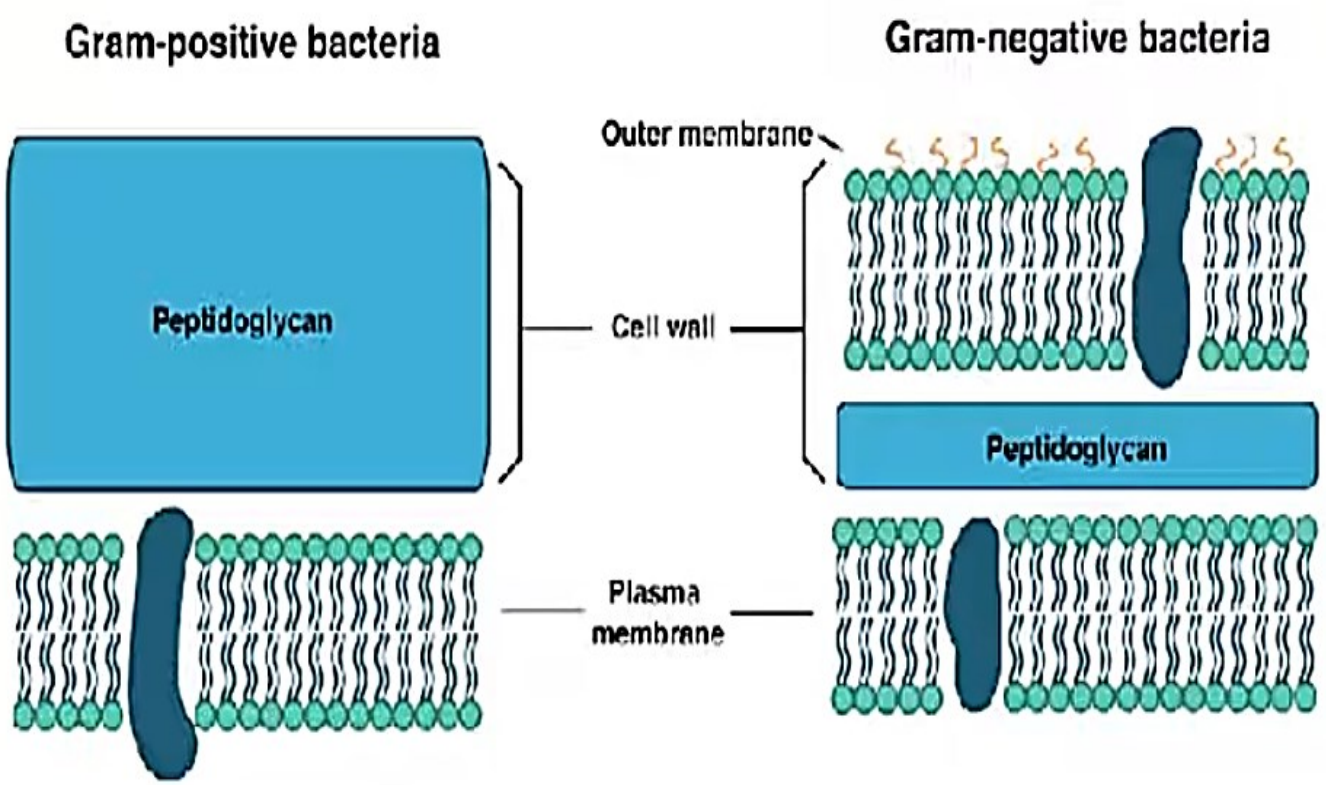
Lab.7

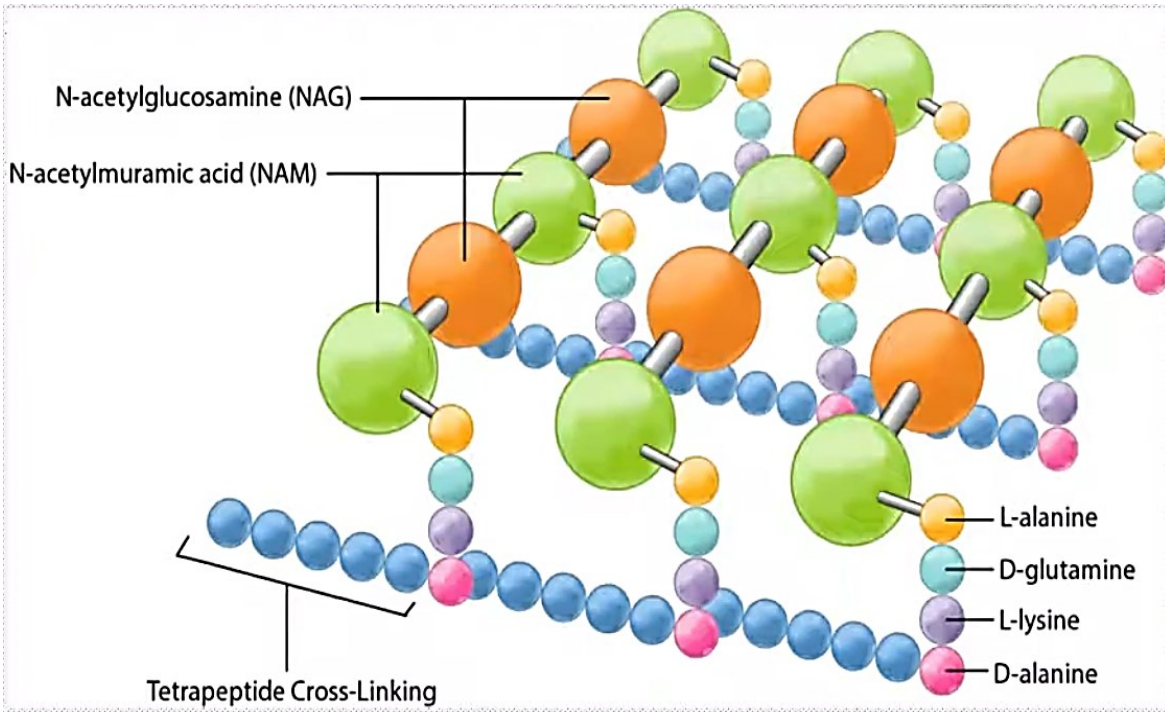


Gram Stain

- Differential stain (Hans Christian Gram, a Danish doctor). He developed a new method to stain bacteria so they can be visible in specimen samples.
- Differentiate bacteria into two large groups (the Gram Positive and the Gram negative).
- Gram status is important in medicine; the presence or absence of a cell wall will change the bacterium's susceptibility to some antibiotics.
- Almost all bacteria are described by their Gram stain characteristics.







Reagents for Gram Stain

1. Crystal Violet (purple)
   - Primary stain; positive stain
   - Stains cell wall purple
2. Iodine
   - Mordant
   - Combines with primary stain to form an insoluble complex
3. Ethanol/acetone
   - Decolorizer
   - CV-I complex washed out of Gram negative organisms because it cannot be trapped by lippopolysaccharide layer
4. Safranin (pink)
   - Counterstain
   - that provides contrasting dye
   - only the negative ones actually appear pink.

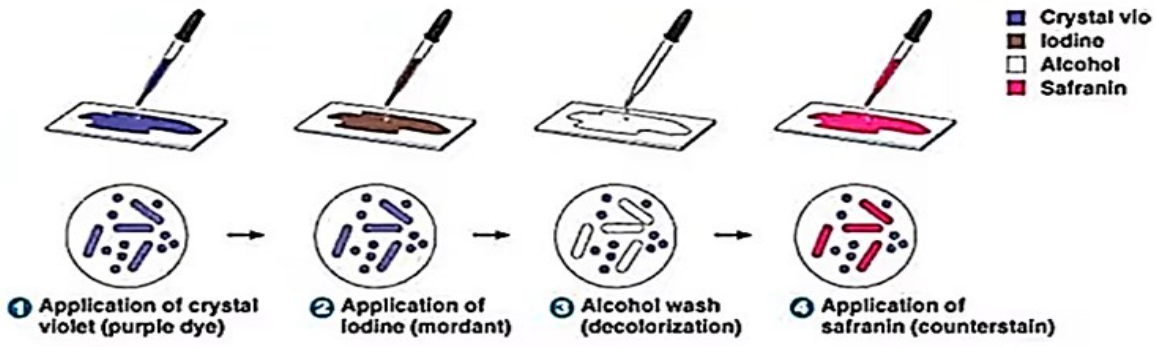
Procedures

- Prepare a smear of bacteria

Gram Stain Procedure

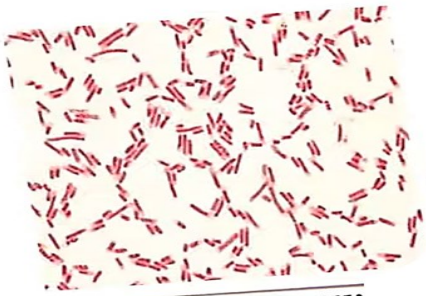
1. crystal violet (primary stain) for 1 minute
2. wash by tap water
3. Gram's iodine (Mordant) for 1minute
4. wash by tap water
5. 95% ethyl alcohol (Decolourizer) for 5 to 10 seconds
6. wash by tap water
7. safranin (Secondary Stain) for I minute
8. wash by tap water
9. air dry and examine

Gram Staining Procedure

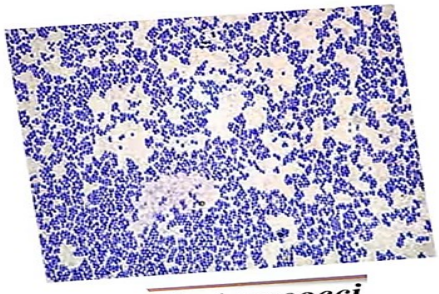


- *Time Frame*
  - *1) 1 minute 2) 1 minute*
  - *3) 15 sec 4) 1 minute*
- *Rinse with water between each step*

Results



Gm-ve bacilli



Gm+ve cocci

Errors During Staining

1. Never ever used old culture
2. Never ever used sample for patient take antibiotic
3. Time of Decolorizer:

- Over: G+ see as G –
- Low: G– see as G +

4. Time of fixation:

- Over: G + see as G –
- Low: no sample on slide

	Gram +	Gram –
Primary stain: Crystal violet	Purple	Purple
Mordant: Iodine	Purple	Purple
Decolorizing agent: Alcohol-acetone	Purple	Colorless
Counterstain: Safranin	Purple	Pink

Harvesting questions

Q1) Gram staining was developed by ----------

Q2) Gram staining is an example of

a) Acid fast stain
b) Acid stain
c) Differential stain
d) None of the above

Q3) The most common stains used in Gram staining is

a) crystal violet and methylene blue
b) crystal violet and safranin
c) crystal violet and carbol fuschin
d) safranin and methylene blue

Q4)Counter stain used in Gram staining is ----------

Q5) In Gram staining , the alcohol acts on

a) Teichoic acids

b) Periplasm

c) Membrane lipids

d) Peptidoglycan

Acid fast stain

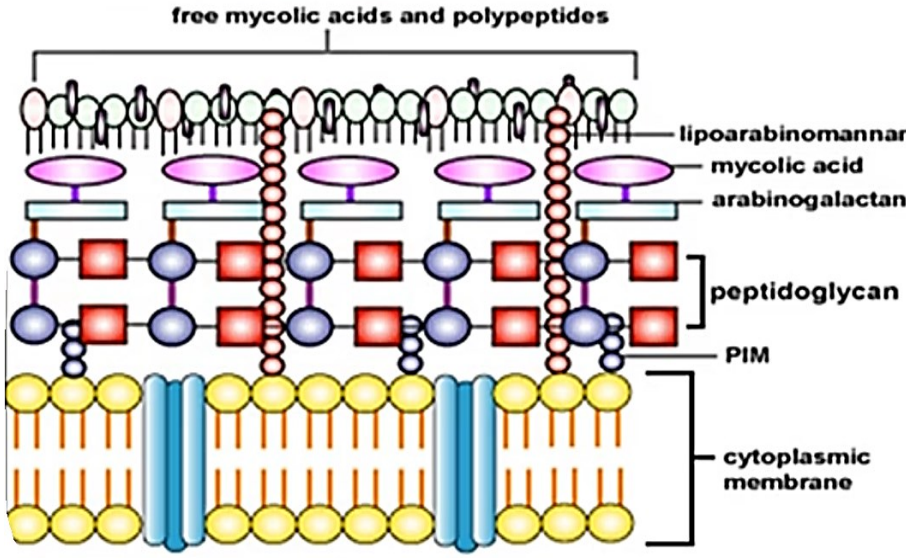
Lab.8

Acid-fast stain (Ziehl-Neelsen stain)

- The acid-fast stain is another differential staining method, that differentiated between becteria acid fast bacilli and non-acid fast bacilli
- the target cells are usually members of the genus Mycobacterium (Acid-fast bacilli) (AFB).
- The cell walls of these bacteria contain an unusually high concentration of waxy lipids, thus making conventional simple stains and Gram stains useless.

Reagents

1. A primary stain (carbol fuchsin)
2. Decolorize (3% HCL in 95% alcohol)
3. A secondary stain (methylene blue)

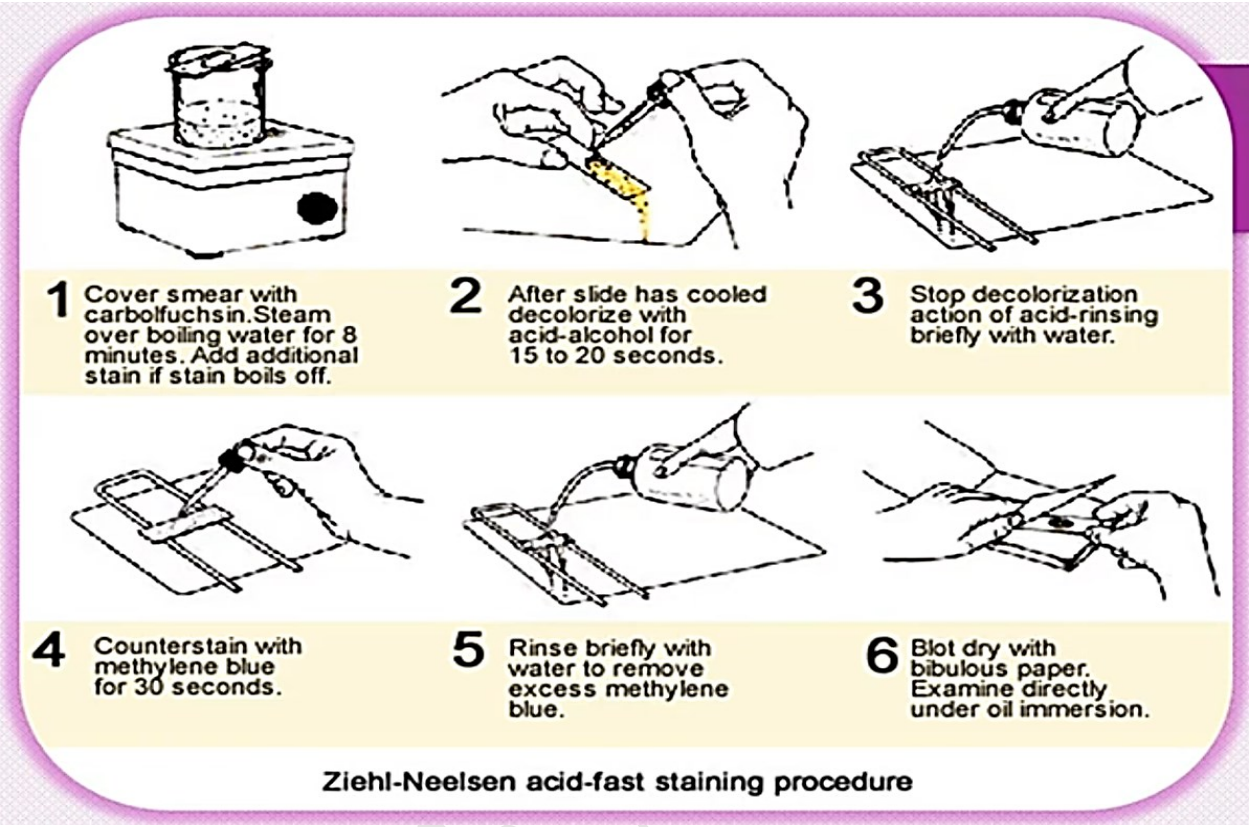


Procedures

- Prepare a smear of bacteria

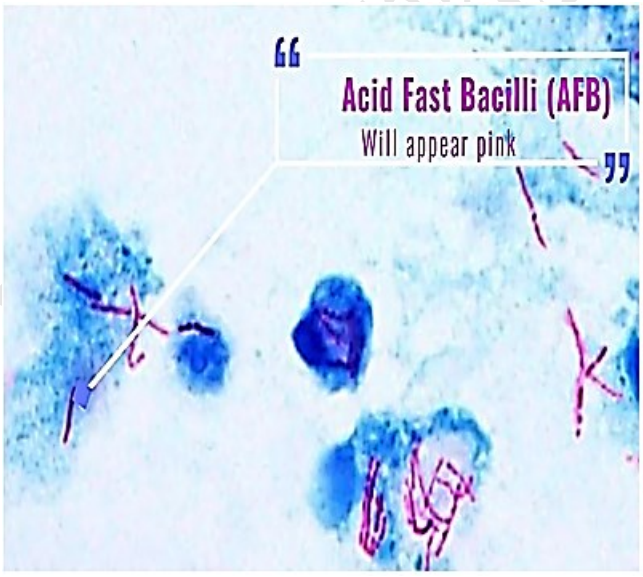
Acid Fast stain procedure

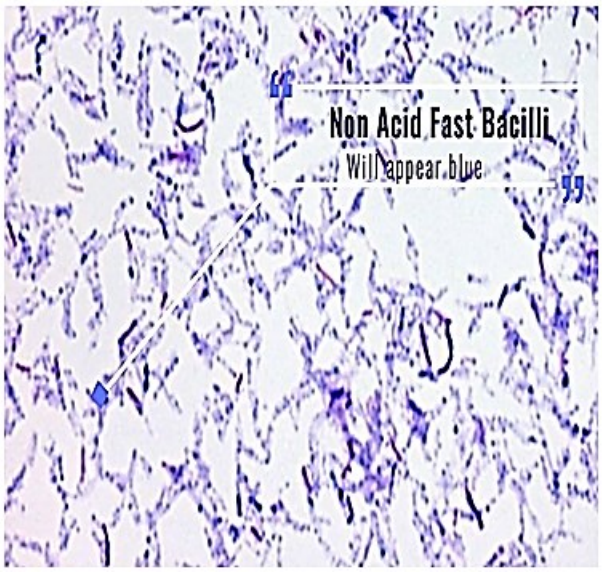
1. Carbolfuchsin (primary Stain) with heating (steaming water bath to increases the penetration of the carbolfuchsin) for 8 minutes. Apply additional stain if it begins to dry out
2. The specimen is cooled (a mordant) and decolorized with a solution of 3% hydrochloric acid and 95% ethanol (acid-alcohol) (decolorizer) for (15-20sec) drop by drop
3. Wash by water
4. Methylene blue (Secondary Stain) for 30 seconds
5. Wash by water
6. Dry and examine, Positive organisms will appear pink or red; Negative organisms will appear blue.

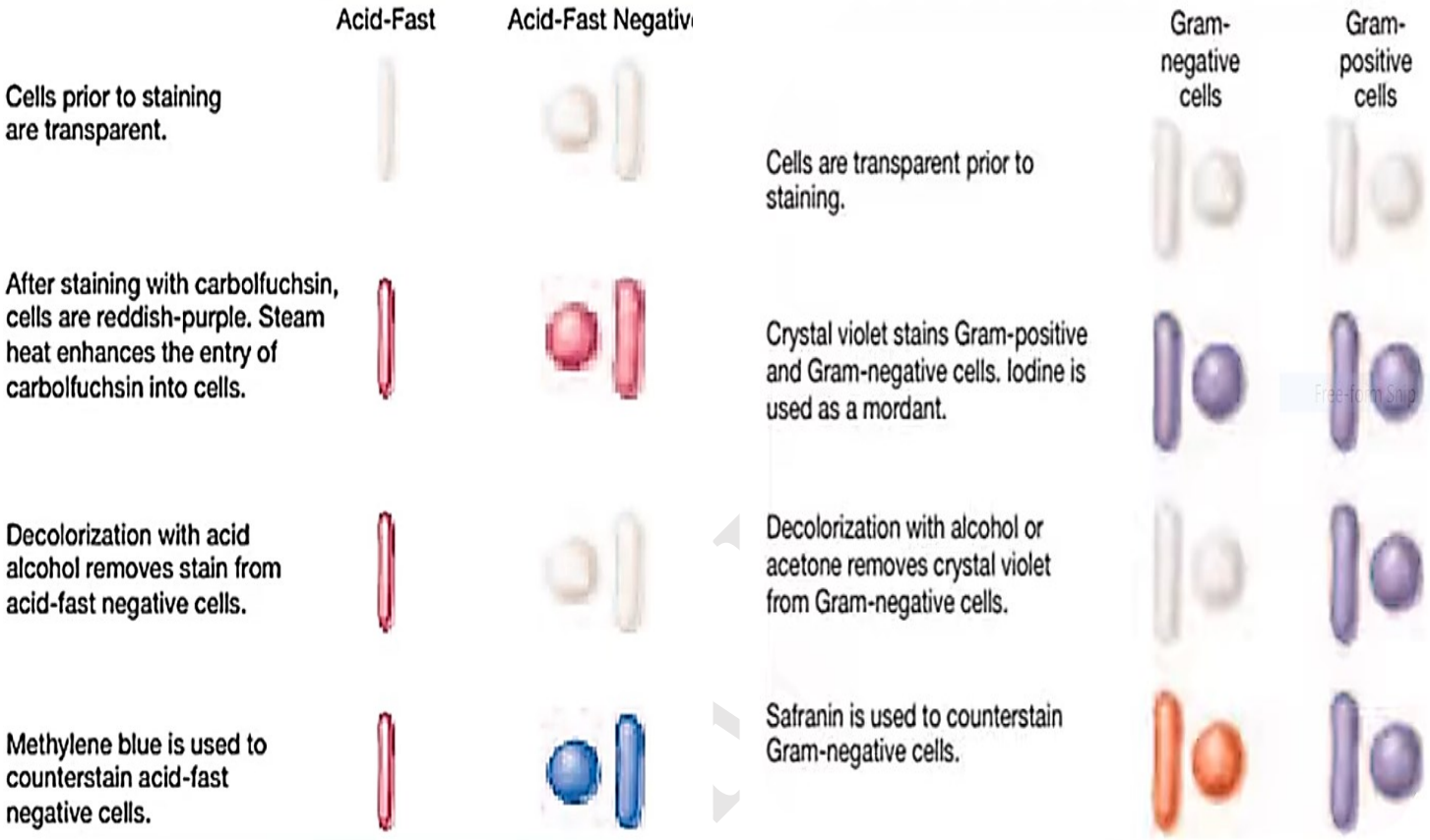


Ziehl-Neelsen acid-fast staining procedure

Results







Harvesting questions

Q1) The common stains used in Acid Fast staining is---------- and ----------

Q2) Acid Fast stain is differentiate between bacteria ---------- and --------

Q3) Acid-fast organisms such as genus of Mycobacterium resist decolorization by an acid-alcohol wash because of the high concentration of ---------- in their cell walls

Q4) In Acid Fast stain technique cooling acts as ----------

Q5) In Acid Fast stain procedure, methylene blue was exposed to steam

a) True
b) False

Q6) In Acid Fast stain technique, decolorize is ----------

Acid-fast bacilli

Mycobacterium tuberculosis (Human Tuberculosis)

Lab.9

Mycobacterium tuberculosis

Mode of transmission

- Aerosol (airborne) (droplet nuclei)
- Ingestion of infected animal (milk contaminated with M. bovis) (intestinal infection)

Laboratory diagnosis

1. **Laboratory diagnosis of open (active) pulmonary tuberculosis**

A. **Specimens** (Sputum)

- **Three early** morning sputum specimens collected on **three consecutive days**, from a deep productive cough, give the best results

B. **Direct detection**

1- **Smears** prepared directly from sputum

a. **Ziehl-Neelsen (Z-N) method** (confirmed), under the light microscope, AFB (**acid fast bacilli**) appear **pink** in a blue background.

b. Fluorochrome staining with the auramine-rhodamine stain (screening), under the microscope, AFB fluoresce **orange yellow** in a black background

- ✓ More sensitive and faster (**good negative**, bad positive)
- ✓ Less specific (positive results, **confirmed** by Z-N stain)

- **Advantages positive result**

1. early diagnosis
2. early treatment
3. protect people from infection (**noninfectious**)
4. monitoring patient

- **Negative results (PCR, cultivation)**

C. **Processing of sputum (viscous) by**

1. **liquefaction**(N-acetyl-L-cysteine)
2. **decontamination** (NaOH)
3. **concentration** (centrifuge)

D. **Cultivation: (special media)**

1. **Conventional culture media, such as Lowenstein-Jensen (L-J) medium,** colonies appear after about 2-8 weeks of incubation

- an egg-based medium
- malachite green dye (**toxic to respiratory flora**)

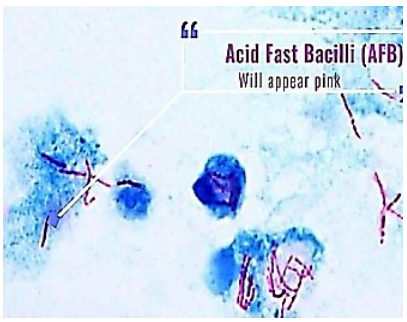
2. **Fluid medium systems** (detection of growth in 4 to 14 days)

a) **Bactec medium system: Bactec medium** (radio-labelled carbon source of palmitate acid), M. tuberculosis multiplies breaks down the palmitate and liberates CO2

b) **Mycobacteria Growth Indicator Tube (MGIT):**

- selective medium, oxygen depletion
- detected by a fluorescence sensor that fluoresces upon exposure to UV light

E. **Tuberculin and Quantiferon tests**



L-J medium

2. Laboratory diagnosis of extrapulmonary tuberculosis (CSF, urine,…)

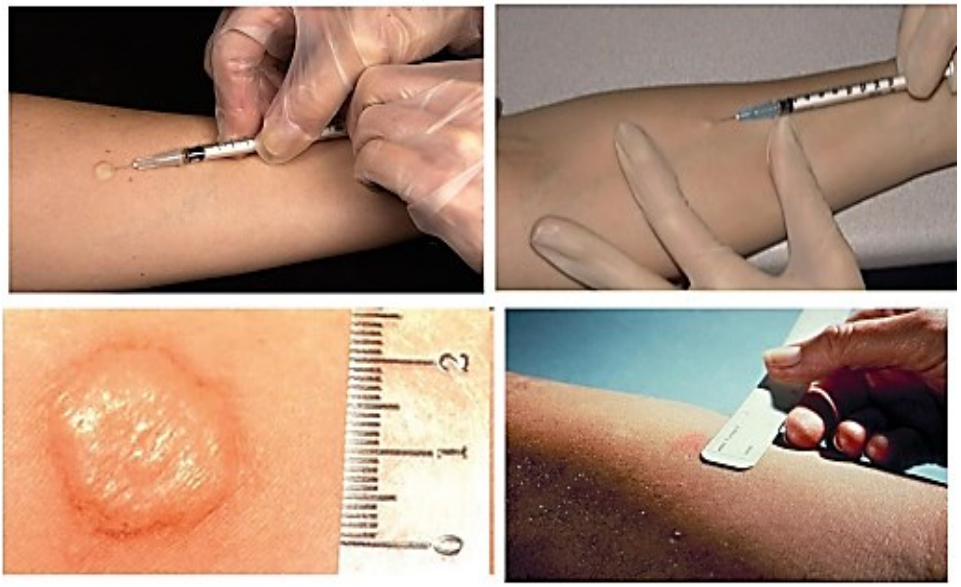
- such as tuberculous meningitis, lymphadenitie and intestinal tuberculosis, specimens collected from sterile sites, e.g. CSF, urine do not require decontamination

3. Laboratory diagnosis of latent tuberculosis

A- Tuberculin Skin Test (TST)

Technique:

- Purified protein derivative (PPD), is the antigen used in the tuberculin test
- PPD is injected **intradermally** in the skin of the anterior aspect of the forearm
- The result is read after 48-72 h by palpating for the presence of **induration**. The diameter of the induration is measured in 5-10-15 millimeter.



Interpretation of the tuberculin skin test:

An induration of 5 or more millimeters (10) 	An induration of 10 or more millimeters (15) 	An induration of 15 or more millimeters 
considered positive for: 1. People with past history of TB 2. Close contacts of infectious TB patients 3. People with HIV infection	considered positive for 1. People in endemic areas where TB is common 2. People with certain medical conditions such as diabetes 3. Unvaccinated children younger than 4 years old	considered positive even in absence of any risk factor for TB

False negative reactions:

1. Anergy:

- weakened immune system, e.g. severe TB disease, HIV infection, or cancer

2. Recent TB infection:

- After exposure, it takes 4 to 6 weeks for tuberculin test

False positive reactions:

1. Infection with non-tuberculous mycobacteria (NTM)

2. Vaccination with bacille Calmette-Guérin (BCG): for up to 5 years

B- Quantiferon TB (IFN-γ release assay)

- Principle measurement of the amount of IFN-γ released
- Compared to TST, this test is **specific for diagnosing latent tuberculosis infection** because the antigens used are not present in NTM or BCG vaccine strains

Harvesting questions

Q1) M. tuberculosis can be isolated by culture on Blood agar

a. True
b. false

Q2) M. tuberculosis can be isolated by culture on ----------

Q3) As regards the diagnosis of growth tuberculosis on Lowenstein-Jensen medium appears after 48-72 hours

a) True b) false

Q4) Tuberculin test is read after ---------- hours

Q5) ---------- test is specific for diagnosing latent tuberculosis infection, that measurement of the amount of IFN-y released