Gram Staining

Bacteria are transparent organisms. To visualize them in microscope, they have to be stained. Gram staining divides bacteria into 2 types:

1. Gram Positive (Purple) bacteria
2. Gram Negative (Pink) bacteria

Principle: It is based on structure and composition of cell wall of bacteria. Gram positive bacteria have thicker peptidoglycan layer then gram negative, so crystal violet iodine dye complex cannot be washed out easily from the cell wall by decolorizer. The gram negative bacteria, on the other hand, due to thin peptidoglycan, retain the dye complex and get decolorized during decolorization, and take up the counter stain & appear pink under the microscope.

Materials / Instruments:

- Wire loop
- Clear grease free glass slide
- Bunsen burner / spirit lamp

Reagent:

- Crystal violet (Primary dye)
- Acetone alcohol (decolorizer)
- Lugol's iodine solution (Mordant)
- Safranin/ neutral red (Counter stain)

Steps of Gram Staining:

1. Make a smear dry it & then fix it by passing through a flame using burner, a couple of times.
2. Cover the slide with crystal violet solution, wait for 30 seconds.
3. Wash the slides with tap water, by lifting the slides into stream of water to wash the stain.
4. Cover the slide with lugol's iodine solution, wait for 30-60 seconds.
5. Wash iodine solution with tap water.
6. Cover the slide with acetone-alcohol. Wash immediately with clean water.
7. Cover slide with safranin, leave stain for 30 seconds.
8. Wash the slide with water.
9. Let the slide dry. Put a drop of oil over it, and see under oil immersion lens.

Result:

Gram Positive Bacteria: Purple

Yeast cells: Purple

Gram Negative Bacteria: Red

Epithelial cells: Red

Nuclei of Pus cells: Red

Reporting of Gram Staining:

Number of Bacteria: Scanty, few, moderate, many.

Gram Reaction: Positive or negative

Morphology: Cocci, diplococci, bacilli, Intracellular, extracellular.

Quality Control:

Gram positive Bacteria: *Staphylococcus aureus*

Gram Negative Bacteria: *Eschrechia Coli*

Precautions:

Wash excess dye with water. Don't leave the decolorizer for too long, it may give false results.

Ziehl-Neelson Staining

Principle: The cell surface component of some bacteria contain waxes and phospholipids (Mycolic acid) so they do not stain with ordinary stains. They can be stained by Z.N staining method.

Materials / Requirements:

Primary dye: Carbol fuschsin.

Decolorizer: HCl 3% v/v or H_2SO_4 20%.

Counter stain: Malachite green or Methylene blue.

Steps:

1. Sterilize the wire loop properly. Transfer purulent material /sputum on the slide. Spread the specimen evenly covering an area of about 15-20 mm diameter on the slide. Sterilize loop properly. Dry the smear in air and fix it by applying gentle heat or alcohol. Label the slide.
2. Cover the smear with corbol fuchsin stain.
3. Heat the stain until vapours rise. (60^0C). Do not over heat. Allow the heated stain remain on slide for 5 minutes.
4. Wash with water.
5. Cover the slide with 20% H_2SO_4 or 3% HCL, until smear is sufficiently decolorized. i.e. pale pink.
6. Wash the slide well in water.
7. Counter stain it with methylene blue or malachite green for 1-2 minutes.
8. Wash it with water.
9. Let it dry and observe under oil immersion lens for Acid Fast Bacilli.

Result:

Acid fast bacilli stain as red, straight or highly covered rods occurring singly or in small groups. While the tissue cells and other organisms are stained blue or green according to the counter stain used.

Quality Control:

At regular intervals and always, when a new batch of stain is started. Two sputum smears of known high and low AFB positively should be stained. To check that carbol fuchsin staining method and microscopical examination of smear are satisfactory.

Acid Fast Bacteria:

Mycobacterium tuberculosis
Mycobacterium ulcerans
Mycobacterium bovis

Weakly Acid Fast Organism:

1%

Certain organisms are weakly acid fast. For staining of these organisms 1HCL or 5%H_2SO_4 is used as decolorizer.

Nocardia
Mycobacterium leprae
Candida spores
Actinomycetes
Cryptosporidium

Interpretation of Results:

>10 AFB/HPF : Report as 3+++

1–10 AFB/HPF : " " 2++

10–100 AFB/100 field = +

1–9 AFB/100 field = Report the exact number.

Culture Media

CULTURE MEDIA:
Artificial food prepared in the laboratory for the growth of micro-organisms is known as culture media. Culture medium should contain substances like proteins, carbohydrates, mineral salts, water and vitamins essential for the growth of microorganisms.

PURPOSE:
The purpose of using culture techniques in microbiology is to demonstrate the presence of microorganisms which may be causing disease and, when required, to test the susceptibility of these pathogens to antimicrobial agents.

COMPOSITION OF CULTURE MEDIUM

A. **ESSENTIAL NUTRIENTS :**

- **PEPTONES**: Peptones provide amino acids and peptides and are source of nitrogen for the bacteria.
- **SUGARS:** These acts as a source of carbon and energy. They also assist in differentiation of bacteria e.g. lactose in MacConkeys Agar.
- **MINERAL SALTS**: Used for growth.
  Traces of Mg^{++}, K^{+}, Ca^{++}, iron and other elements are required for enzymatic activity of bacteria.

B. **SOLIDIFYING AGENTS :**

- **AGAR OR GELATIN**: Agar 1:2 weight/ volume % or gelatin is added to solidify the liquid medium (broth). Agar is inert and does not contribute to any nutrient.

C. **GROWTH FACTORS**

- **Haeme (X) and NAD (V)**: It is used as growth factors for *Haemophilus influenza*.
- **YEAST EXTRACTS** : It is used as bacterial growth stimulant e.g. in XLD medium (Xylose Lysine deoxycholate agar)

CLASSIFICATION OF OF CULTURE MEDIUM

A. **ON THE BASIS OF CONSISTENCY:**

1. **Liquid Media**

They are known as broth and are fluid in nature. Growth appears as turbidity.

Uses:
For enrichment, where organisms are likely to be few.
Fluid medium may also be used in biochemical testing.
Examples: BHI (Blood Heart Infusion used for blood culture).

2. Semi-solid Media:

This form is prepared by adding a small amount of agar (0.4-0.5%) to a fluid medium.

Uses:

Transport media.

Motility testing.

Biochemical tests.

3. Solid Media:

These are prepared by adding solidifying agents like 2-3% agar or gelatin to liquid medium e.g. nutrient agar. Agar is an inert polysaccharide, obtained from seaweed. It is used to solidify culture media. Its high melting temperature (98^{0}C) enables petri dishes to be kept in incubators. Bacterial growth appears as colonies.

B. ON THE BASIS OF FUNCTIONAL USE OR APPLICATION:

1. Simple Media:

Used to grow organisms which do not have special nutritional requirements .e.g. nutrient broth, nutrient agar, peptone water.

2. Enriched Media:

These are media that are enriched by some substances like blood 5-10%, serum 10%, glucose 1-2%, plasma 5-10%. These media are required for growth of fastidious micro-organisms which need extra nutrients for their growth e.g. blood agar and chocolate agar.

3. Differential Media:

Used to differentiate between two groups of bacteria.e.g. MacConkey agar to differentiate between lactose fermenters and non-lactose fermenters, Blood agar also differentiate between hemolytic and non-hemolytic bacteria

4. Selective Media:

Contain substances such as bile salts or antibiotics that inhibit growth of some organisms, but have little or no effect on the required organisms. e.g. Thayer Martin medium used for *Neisseria gonorrheae*, L J medium for *Mycobacterium tuberculosis*. *Vibrio cholerae* can be isolated on TCBS medium (Thiosulphate citrate bile salt sucrose medium).

5. Enrichment Media:

Broth or liquid media with selective properties, enriching the desired range of organisms and inhibiting others e.g. Tetrathionate broth & Selenite F broth for *Salmonella*, *Shigella*.

Types of Solid Media:

1. **Nutrient agar: (Basic/simple media, used to check sensitivity)**
   It is basic culture media, used to grow organisms that have no special nutritional requirements. It is also used for sensitivity testing of drugs.
   Composition: Beef extracts, peptone water, NaCl, agar.
   Color: Transparent, whitish pale.
   Microorganism Cultured: *Staphylococcus, pseudomonas.*

2. **Blood Agar (Enriched):**
   It is used to grow those organisms that are more difficult to be grown on ordinary media such as *Streptococcus species, Hemophillus* (fastidious bacteria). Blood agar is also used to study hemolytic property of bacteria.
   Composition: Agar, sterile defibrinated blood 5-10%, beef extract, peptone water, NaCl.
   Color: Opaque red
   Microorganisms Cultured:
   Streptocuccus pyogenes, Streptococcus viridians, *Streptococcus pneumoniae.*

3. **Chocolate Agar (Enriched):**
   When blood agar is heated, the red cells are lysed and the medium turns brown in color. It is referred to as chocolate agar. This provides extra nutrients and helps in growth of some particular organisms.
   Composition: Same as blood
   Color: Chocolate color
   Microorganisms Cultured: X and V factors are released by lysis of red blood cells. These are essential for growth of some bacteria like *H. Influenzae, Neisseria gonorrhae, Neisseria meningitides.*

4. **MacConkey's Agar (Differential/Selective):**
   It is differential as well as selective medium used to distinguish lactose and non-lactose fermenters.
   Composition: Nutrient agar, Bile salts (selective agents), lactose (differential agent), Neutral red (indicator), peptone.
   Color: Transparent pink
   Microorganisms Cultured: Organisms that withstand bile salts (enteric organisms) will grow on this agar.
   Lactose Fermenters: *Klebsiella pneumoniae, Escherichia coli.*
   Non-Lactose Fermenters: *Pseudomonas, Proteus, Salmonella.*

5. **Lowenstein Jenson (LJ) Medium (Special, Enriched & Selective)**
   LJ medium is used to isolate *Mycobacterium tuberculosis.*
   Composition: Whole egg, $MgSo_4$, Mg citrate. Penicillin (Inhibits growth of other bacteria), Malachite green (It is also anti-bacterial at low pH).
   Color: Light green always in screw capped bottles.

Microorganisms Cultured: These bacteria takes 14-21 days to produce dry, buffy, raised and waxy colonies. The incubation can be extended up to 42 days before the culture is reported as negative.

6. **Thiosulphate Citrate Bile Salt (TCBS) Agar (Selective):**
   It is a selective medium used to isolate *Vibrio cholerae* and other *Vibrio species*.
   Composition: Yeast extract powder, bacteriological peptone, Sodium thiosulphate, Sodium citrate, Ox-bile, Sucrose, NaCl, Ferric citrate, Bromothymol blue, Agar.
   Color: Light green
   Microorganisms Cultured: *Vibrio cholerae* produces yellow colonies on TCBS agar.

7. **Sabourauds Dextrose Agar (Special / Selective)**
   This medium is used to grow fungus. Its color is pale yellow. The pH is kept acidic.
   Composition: Peptone, Dextrose, Agar.
   Color: Pale yellow

8. **CLED (Differential media):**
   Cysteine lactose electrolyte deficient media. It is used to culture bacteria from urine. Some strains of *E.Coli* utilize cysteine. It differentiates between lactose fermenters (yellow) and non-lactose fermenters (blue, gray, green). It also inhibits swarming of proteus because of its electrolyte deficiency.
   Composition: Cysteine, Bromothymol blue (indicator), Lactose.
   Microorganisms Cultured: *E.coli, Staphylococcus, Candida, and Streptococcus.*

9. **SS Agar (Salmonella-Shigella agar):**
   It is a selective and differential medium used to isolate *Salmonella and Shigella* species from fecal specimens.
   Composition: Lactose, Bile salts, Sodium citrate, sodium thiosulphate, ferric citrate, brilliant green, Neutral red.
   Microorganisms Cultured: *Salmonella, Shigella.*

Examples of Liquid Media:

1. **Alkaline peptone water (Selective / Enrichment):**
   Used as selective / enrichment media for *Vibrio cholerae*. (pH 8.6-9).
   Composition: Peptone, sodium chloride, distilled water.

2. **Robertson's cooked meat medium (Enrichment media):**
   Composition: Minced meat, broth, parafine oil, NaOH (to neutralize lactic acid).
   Color: Pale yellow, with minced meat at bottom.
   Microorganisms Cultured: *Clostridium species, Bacteroides*, other Anacrobes and facultative anaerobes.

Examples of Semi-Solid Media:

Transport Media: These media contain ingredients to prevent the overgrowth of commensals and ensure survival of aerobic and anaerobic pathogens when specimens cannot be cultured immediately after collection. These include:

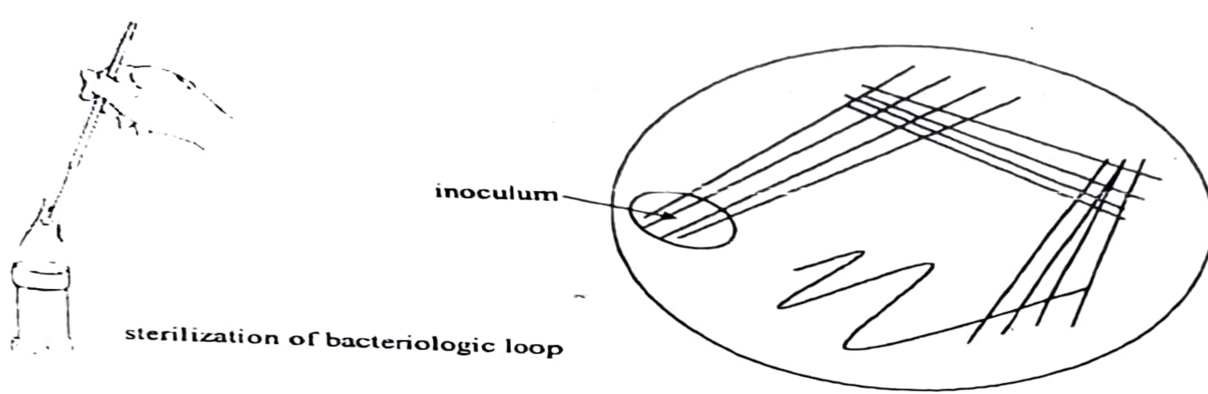
1. **Cary Blair Medium (Enteric Organisms):**
   Composition: Na thioglycolate, di-sodium hydrogen phosphate, NaCl, agar, $CaCl_2$.
   Microorganisms Cultured: *Salmonella, Shigella, Vibrio, Campylobacter jejuni.*

2. **Amies Transport Medium:** Transport of urethral swab, high vaginal swab, throat swab, tissue specimen, pus swab, including specimen meant for anaerobic culture.

INOCULATION OF VARIOUS MICOBIOLOGICAL SPECIMENS

WHAT IS INOCULATION AND WHY IS IT REQUIRED?

Inoculation is the means of growing & isolating bacteria on artificial medium. The substance used for inoculation is called inoculum.



REQUIREMENTS FOR INOCULATION

- Disposable gloves.
- Flame sterilizer.
- Wire loops.
- Pipettes or disposable syringes.
- Waste container with hypochlorite.
- Media.
- Centrifuge. → if liquid sample

DEFINATIONS:

lture: Methods of growing micro-organisms by letting them multiply in predetermined ure media under controlled laboratory conditions.

-culture: A culture made by transferring the micro-organisms to a fresh medium n a previous culture.

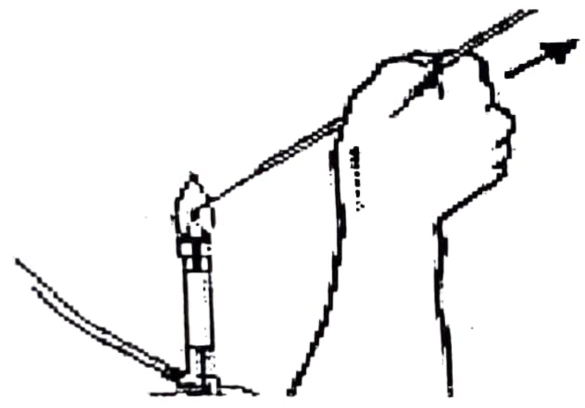
cteremia: Presence of bacteria in the blood.

epticemia: When microorganisms (mainly bacteria) circulate and multiply in the tient's blood.

SEPTIC TECHNIQUE:

- Flame sterilize the wire loops before and after use.
- Flame the mouths of specimen bottles, culture bottles before replacing the caps.

TERILIZATION OF WIRE LOOPS



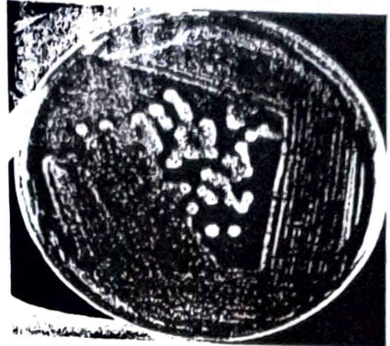
METHODS OF INOCULATION:

- **Inoculation in petri dishes.**
- **Inoculation in slopes.**
- **Inoculation in stab media.**

BEFORE INOCULATION: Bring the petri dishes at room temperature by putting in incubator. To inoculate the plate apply the inoculum or make a well with the help of wire loop. Then spread it to ensure the growth of isolated colonies.

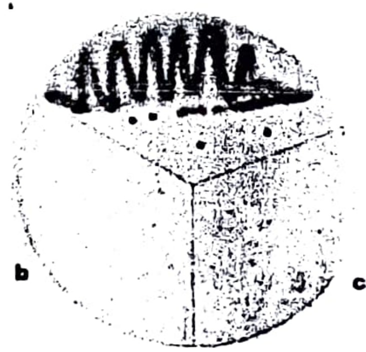
INOCULATION IN PETRI DISHES

1. Full plate inoculation.

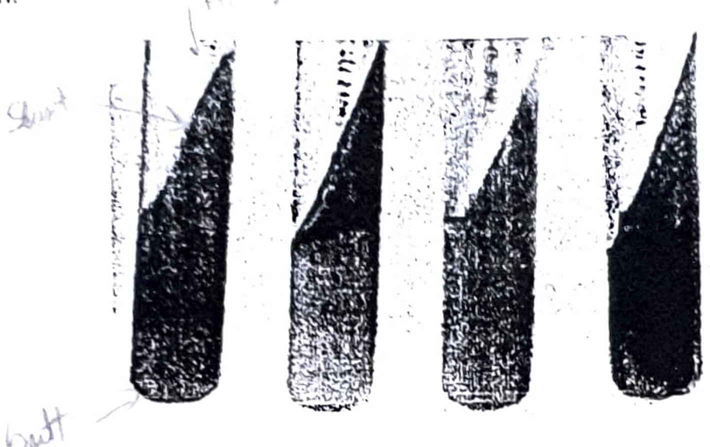


2. Half plate inoculation.

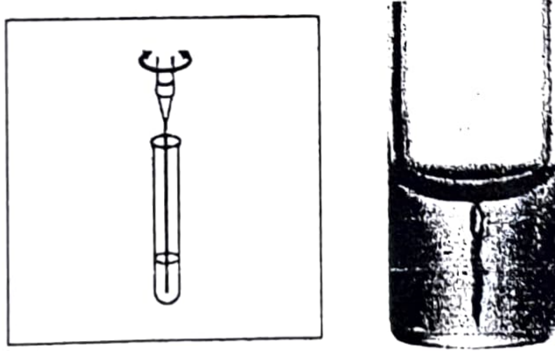
3. One third plate inoculation



INOCULATION OF SLOPES: To inoculate slope and butt e.g. TSI agar, use a sterile straight wire to stab in to butt first and then use the same wire to streak the slope in zig zag pattern.



INOCULATION OF STAB MEDIA: Use a sterile straight wire loop to stab the centre of medium, and then withdraw the wire loop along the line of inoculum.



INOCULTATION of FLUID or LIQUID MEDIUM:

Broth or other liquid media e.g. blood cultures, are inoculated using sterile wire loop or syringe.

CULTURING OR INOCULATION OF BLOOD

Blood is one of the most important specimens received by the microbiology laboratory for culture, and culture of blood is the most sensitive method for detection of bacteremia or fungemia.

INDICATIONS FOR BLOOD CULTURE:

1) Required in patients with suspicion of bacteremia or fungemia.
2) Suspected sepsis, meningitis, osteomyelitis, arthritis, endocarditis, peritonitis, pneumonia, and fever of unknown origin.
3) Pyrexia of unknown origin: Fever without any signs and symptoms.

PRE COLLECTION NOTES:

1) Specimen should be drawn, preferably PRIOR to antibiotic therapy.
2) It is generally recommended that blood cultures be drawn before and after the fever spike, when bacteria may be most likely present in the peripheral circulation.
3) Collected usually in a series, two to three blood cultures sets (aerobic & anaerobic) collected at least 1-2 hours apart.
4) Blood-to-broth ratio: Dilutions of > 1:10.
5) It is recommended that 1-2 mL of blood per culture for neonates, 2- 3 mL for infants aged 1 month to 2 years, 3-5 mL for older children and 10-20 mL for adolescents.
6) Proper site preparation and aseptic collection techniques are essential for blood culture collection.
7) If the blood culture and other laboratory blood tests need to be collected at the same time, the blood culture should always be the first bottles/tube collected to avoid contamination of the sample.

PATIENT PREPARATION:

1) Cleanse the veni-puncture site with 70% alcohol for 3 MINUTES.
2) Let dry for one minute.
3) Do not retouch the site after it has been cleaned.

7-10 day for blood culture

BLOOD CULTURING SYSTEMS:

1. **Manual blood culture systems:**

- **For Aerobes:** a. Brain heart infusion
  b. Tryptic soya broth
- **For Anaerobes:** a. Thioglycolate broth

Conventional/ manual systems are flexible and cheap. Set of Aerobic blood culture bottles and anaerobic blood culture bottles are inoculated with blood and incubated for 7 days.

Growth detection: Each bottle is examined daily for macroscopic evidence of microbial growth (e.g. hemolysis, turbidity of the media, gas production, or formation of discrete colonies). An aliquot of the contents of the aerobic bottle is gram stained and **sub-cultured** after the first overnight incubation. A **terminal subculture** is usually done at the end of the incubation period.

2. **Instrumented blood culture systems.** Commercially available intsrumented blood culture methods introduced in 1970s. BACTEC instrumented systems based on the utilization of carbohydrate substrates in the culture media and subsequent production of CO_2 by growing microorganisms. Instrument detects CO_2 in the bottle headspace, detected by infrared spectrophotometry.

Anaerobic Culture

An anaerobic atmosphere is essential for growth of strict anaerobes such as *Clostridium species, Bacteroides species and anaerobic Streptococci.* Culture of anaerobes is difficult due to their slow growth and complex growth requirements. Anaerobic conditions must be maintained during the collection, transportation, processing and identification of such specimens.
There are several techniques of obtaining anaerobic conditions. These are:

1. Conventional techniques
2. Modern techniques

1. Conventional Techniques:

A. Use of copper coated steel wool to remove oxygen:

This is a simple method of obtaining anaerobic environment, when it is not possible to obtain the commercially produced oxygen-removing sachets. It can be adapted for incubating single plates or several plates. The plates can be incubated in an airtight plastic bag. The system uses steel wool which is activated immediately before use by being dipped in acidified copper sulphate solution. The metallic copper on the surface of the iron rapidly absorbs oxygen. Anaerobic conditions are obtained more rapidly by removing some of the air from the bag before it is sealed. A source of carbon dioxide is added and also an indicator to check for anaerobic conditions.

B. Use of reducing agents in culture media:

Examples of media that contain reducing agents include:

i. **Thioglycolate broth** which is used mainly to culture anaerobes in blood. The medium contains the reducing agent sodium thioglycolate and the indicator methylene blue to show that the medium is reduced. Other reducing agents include Glucose 0.5-1%, Ascorbic acid 0.1%, Cysteine 0.1%.

ii. **Cooked meat medium** which is used to culture *Clostridium and Bacteroides* species. The anaerobes grow at the bottom of the medium among the meat particles which show effective reducing proteolytic reactions and also gas production.

iii. **Use of iron strip or iron nail:** A simple way of providing anaerobic conditions in litmus milk medium, peptone water media, and broths is by using an iron strip or an iron nail to remove the oxygen. The strip or nail is flame sterilized and while still hot it is dropped into the medium. When the medium has cooled, it can be inoculated.

2. Modern Techniques:

A. Anaerobic Cabinets:

This is a specialized cabinet which has a built in anaerobic environment. These are closed cabinets attached with vaccum, N_2 and CO_2 cylinders. These are commercially manufactured for processing of specimens & incubation of cultures in an oxygen free atmosphere enriched with 5-10%CO_2.

B. Anaerobic Jars:

In this method we have jars with reasonable lids and a disposable aluminium foil packet which is placed in jars. This foil packet contains chemicals which react to remove O_2 and replace with inert gas 5%CO_2. Specific amount of H_2O is added to the aluminium foil packet and inoculated culture plates are placed in the jar. The reaction will generate H_2 and CO_2 in the presence of palladium (catalyst). The oxygen present in the jar will combine with hydrogen to produce H_2O so that anaerobic environment is obtained for growth of anaerobic bacteria. Methylene blue is used as an indicator to check an aerobic environment.

Organisms used as control:

Aerobic Control: *Pseudomonas aeruginosa.*

Anaerobic Control: *Bactroides species.*

Possible pathogens in CSF

Gram +ve
- Strept. Pneumonia.
- Strept. Agalactiae.
- Listeria monocytogenes.

Gram -ve
- Naisseria meningitidis
- Hemophilus Influenza.

Difference b/w Viral / Bacterial / Tuberculous Meningitis

	Appearance	cells / WBCs	Protein	Glucose
Normal CSF	→ clear/colorless	Below 5x10³/L	15-40 mg/dl	45-72 mg/dl
Bacterial meningitis	Purulent/cloudy	Many pus cells (Neutrophills)	High proteins	Very low.
Tuberculous meningitis	Clear/slightly Turbid	(↑ lymphocytes)	"	Reduced
Viral meningitis	Clear/slightly Turbid	(↑ lymphocytes).	Normal proteins	Normal glucose

Biochemical Reaction

Oxidase Test: This test is used for screening of *Neisseria, Vibrio, Campylobacter* & Pseudomonas which give positive reaction. All species of *Enterobacteriaceae* give negative reaction.

Principle: A piece of filter paper is soaked with few drops of oxidase reagent. A colony of the test organism is then smeared on filter paper. If the organism is oxidase producing then phenylenediamine in the reagent will be oxidized to a deep purple color.

Material Required: Oxidase reagent (phenylenediamine) freshly prepared.

Method:

1. Place a piece of filter paper in clean petri dish.
2. Add 2-3 drops of oxidase reagent.
3. Use a sterile glass rod to remove a colony of the test organism from culture plate and smear it on filter paper. Look for development of purple blue color within ten seconds.

Results: Appearance of purple blue color = +ve test

No color or color change after 10 seconds = -ve test

Control: **+ve control:** *Pseudomonas aeruginosa*

-ve control: *E.coli*

Catalase Test: This is used to differentiate catalase producing *Staphylococci* from *Streptococci.*

Principle: Catalase is an enzyme that catalyses the breakdown of hydrogen peroxide to hydrogen and oxygen. An organism is tested for catalase production by bringing it into contact with H_2O_2. Bubbles of oxygen released demonstrate the presence of catalase if organism is catalase producing.

Care must be taken if organism is cultured in a media containing blood because RBC's contain catalase which might produce false +ve results.

Matrerial Required: 3% H_2O_2

Method:

1. Add 2ml of solution to a test tube.
2. Using a glass rod or a sterile wooden stick to remove a growth of the test organism & immerse in the H_2O_2 solution.
3. Look for immediate bubbling which indicated production of O_2.

Result: Production of active bubbling along the glass rod = +ve test.

No release of bubbles = -ve test.

Control: +ve control = *Staphylococci.*

–ve control = *Streptococci.*

Coagulase Test:

This test is used to differentiate *Staphylococcus aureus* from *Staphylococcus epidermidis* & *Staphylococcus saprophiticus.*

Principle: Coagulase causes the plasma to clot by conversion of fibrinogen to fibrin. Two types of coagulase are produced by *Staphylococcus aureus*.

1. **Free coagulase:** Coagulase converts fibrinogen into fibrin by activating coagulase reacting factors present in the plasma and is detected by the appearance of a fibrin clot in the test tube.
2. **Bound coagulase:** Converts fibrinogen directly to fibrin without requiring a coagulase reacting factor and is detected by the clumping of bacteria in the rapid test slide. Test can be performed on a glass slide or in test tube. If result is not clear on slide, always perform the test tube coagulase test.

Material Requirement: Undiluted human plasma or rabbit plasma. Plasma from EDTA or citrated blood is usually used.

1. **"SlideTest Method" (For Bound Coagulase)**

Method:

1. Place a drop of physiological saline on two slides.
2. Emulsify a colony of the test organism in both drops of saline.
3. Add a drop of plasma to one of the suspensions on the slide & mix gently.
4. Look for clumping within 10 seconds.
5. The second suspension is kept as a control to differentiate any granular appearance of the organism from the true coagulase clumping.

Result:

Positive result = clumping with loses indicates the presence of blood coagulase produced by *Staphylococcus aureus*.

Negative result = Absence of clumping means no coagulase is produced by the test organism.

2. **"Test Tube Method" (For Free Coagulase)**

Method:

1. Dilute plasma in a ratio of 1:10 in physiological saline.
2. Add 0.5ml of diluted plasma in 3 test tubes.
3. T= test organism POSITIVE=Positive control NEGATIVE= Negative control.
4. In the test tube T: add 5 drops of broth culture of test organism culture.
   In the test tube POSITIVE: add 5 drops of sterile broth of *Staphylococcus aureus*.
   In test tube NEGATIVE: add 5 drops of sterile broth.
5. After mixing gently, incubate the 3 tubes at 35-37^{0}C and examine for clotting after one hour. While looking for clotting, tilt each tube and look for gel formation.
6. If no clotting has occurred, examine at 30 minutes interval for up to 6 hrs.

Result:

+ve test: Production of fibrin clot indicates that *Staphylococcus aureus* is present.

-ve test: Absence of fibrin clot means that free coagulate is not produced.

Control: Coagulase +ve: *Staphylococcus aureus.*

Coagulase –ve: *Staphylococcus epidermidis & saphropyticus.*

"Deoxyribosenuclease Test" (DNase): This test is used to differentiate *Staphylococcus aureus* which produces the enzyme DNase from other *Staphylococci* that do not produce DNase.

Principle: DNase hydrolyses DNA present in the DNase medium. When an acid is added, it will precipitate DNA in the medium and the area will be opaque.

Material Required: DNase agar plate & HCl.

Method:

1. Divide a DNase plate into required number of strips by making the underside of the plate.
2. Using a sterile loop of swab, spot-inoculate the test and control organisms. Make sure each test area is properly labeled.
3. Inoculate the plate and incubate at 35-37^{0}C over night.
4. Cover the surface area of plate with HCl solution. Tip off excess acid.
5. Look for clearing around the colonies within 5 minutes of adding the acid. This indicates that DNase has broken the DNA in the medium, around the colonies and thus there is no opaque area. (i.e. clearing around it).

Result:

Clearing around the colonies: DNase +ve strain

No clearing around the colonies: DNase –ve strain

Control:

+ve control: *Staphylococcus aureus*

-ve control: *Staphylococcus epidermidis*

Biochemical Reaction-2

Enterobacteria are Gram negative rods belonging to the family enterobacteriaceae. All members of this family ferment glucose. A series of biochemical tests abbreviated as IMViC are helpful in indentifying different members of this family. IMViC stand for:

I = Indole test

M = Methyl red test

V = Voges proskauer's test (VP)

C = Citrate test test

1. Indole Test (Degradation of tryptophan)

Principle: The indole test screens for the ability of an organism to degrade the breakdown of amino acid tryptophan & produce indole.

Material Required: Peptone water medium and Kovac/Ehrlichs reagent. (diamino benzaldehyde)

Method: Test organism is cultured in a medium which contains tryptophan and incubated at 37^{0}C for 24 hrs & is detected by adding five drops of Kovac's reagent after 18-24 hrs.

Result: Appearance of pink colored ring near the surface within ten minutes indicates positive test. No change in color / absence of ring appearance indicates negative test.

Control: Indol +ve control: *E. Coli and Proteus*

Indol –ve control: *Enterobacter*/ KP

2. Methyl Red Test (Glucose fermentation)

Principle: Methyl red is used to identify bacteria producing stable acids by mechanisms of mixed acid fermentation of glucose. Some Enterobacteria when cultured in buffered glucose peptone water produce sufficient acidity to give red color with indicator methyl red.

Material Required: Glucose peptone broth & methyl red reagent.

Method: Inoculate a typical colony of test organism in 0.5ml of sterile glucose phosphate peptone water. After overnight incubation at 37^{0}C, put a drop of methyl red solution in it.

Result: Bright red color indicates +ve test. No change in color indicates –ve test.

Control: +ve control: *E. coli and Proteus.*

-ve control: *Klebsiella and Enterobacter.*

Voges Proskauer's Test (Glucose fermentation)

Principle: VP test is used to detect acetoin in a bacterial broth culture. Some bacteria ferment glucose with production of "Acetyl Carbinol" which can be detected by oxidation reaction.

Material required: Glucose phosphate peptone broth, sodium hydroxide & creatine powder.

Method: Test organism is cultured in glucose peptone water for 48 hrs at 37°C. Add a very small amount of creatine and Add 3ml of NaOH and shake well. Remove the bottle cap and leave at room temp for about 1 hr. Look for slow development of pink-red color.

Result: Production of pink-red color indicates +ve test.

No change in color indicates –ve test.

Control: VP +ve control: *Klebsiella pneumonia, Enterobacter & Vibrio*

VP –ve control: *E. Coli & Proteus*

Citrate Utilization Test (Citrate only carbon source)

Principle: This test is based on the ability of the microorganism to utilize citrate as the only source of carbon & ammonia as the only source of nitrogen.

Material required: Simon citrate medium (it has greenish color).

Method: Test organism is inoculted on the slant of simon citrate medium containing sodium citrate, ammonium salts & indicator bromothymol blue. It is incubated at 37°C for 24-48 hrs and checked daily for growth.

Result: Blue color of the medium & growth along the inoculums streak indicates +ve test.

Control: +ve control = *Klebsiella pneumoniae*

-ve control = *E. coli*

Urease Test (Degradation of urea)

Principle: This test is very important in differentiating members of Enterobacteriaceae. *Proteus stains, Klebsiella and Yersinia enterocolitica* show +ve urease activity.

Material required: Urease medium (Pale yellow)

Method: Test organism is cultured in urease medium containing urea and indicator phenol red. If the bacterial stain is urease producing, the enzyme will breakdown urea & release ammonia, due to which there will be a change of color in medium from yellow to reddish pink (due to alkaline change).

Result: Appearance of red pink color indicates a +ve reaction.

No appearance of red pink color indicates –ve reaction.

Control: +ve control: *Proteus vulgaris.* |KP

–ve control: *E. coli*

Motility Test

This test determines the ability of organism to move. It helps to differentiate between different organisms especially organisms with similar colony morphology e.g. *Klebsiella pneumoniae is non-motile whereas Enterobacter* is motile. Similarly *Bacillus anthracis* is non-motile member of genus bacillus while other members are motile. This test can be performed by different methods:

a. Hanging drop preparation
b. Semi solid motility medium

1. Hanging drop preparation: In this method a drop of suspension of organism is placed on a cover slip. Put wax on edges and invert a cavity or a simple glass slide over it. Now invert the slide and examine under microscope with reduced light at 10x and 40x objective lens. Motile organisms will show active movement.

2. Semi solid motility medium:

Principle: This is done in tubes containing semi solid motility medium. Thus motile bacteria can move in this semi solid medium. Motile organisms give diffused hazy growth that spread out from the line of inoculation throughout the medium rendering it slightly opaque, whereas non motile organisms have sharply defined margins with the surrounding medium clearly transparent.

Material required: Peptone water medium plus 0.2-0.4% agar.

Method: Using a sterile inoculating straight wire remove some growth from an isolated colony of 18-24 hrs culture. Inoculate motility tube by carefully stabbing the needle 3-4cm into medium & then drawing needle directly back along the same track so that a line of inoculum can be observed. Incubate the tube aerobically at 37°C for 18-24 hrs. After incubation, observe the tubes for growth in relation to the stab line.

Result: Diffused hazy growth zone that spreads out from the line of inoculation though out the medium, rendering it slightly opaque indicate +ve test.
Growth along the line of inoculation with sharply defined margins with surrounding clearly transport medium indicates –ve test.

Control: Positive control: *E. coli, Enterobacter, Vibrio, Proteus & Salmonella*
Negative control: *Klebsiella & Shigella*

Triple Sugar Iron (TSI) medium:

Tipple sugar iron medium is used to differentiate lactose fermenters from non lactose fermenters & H_2S producers from non H_2S producers.

Composition: It is a differentiated slope medium. TSI medium contains:

a. Ferric sulphate: (To detect production of H_2S)
b. Three sugars: Glucose (0.1%), lactose (1%) & sucrose (1%)
c. pH indicator (phenol red): Phenol red (yellow at acidic & pink at alkaline)

Principle: The medium in the test tube has a solid poorly oxygenated area in the bottom called Butt and a slanted well oxygenated area on the top called slant. The organism is incubated into the Butt and across the surface & across the surface of the slant & incubated at 37^0C for 24 hrs.

1. COLOUR CHANGES: Yellow acidic indicating fermentation of sugar.
   Red is alkaline.
2. CRACKS or BUBBLES: Indicating production of CO2 gas.
3. BLACKENING: Indicating production of H2S gas.

Result:

1. Reaction-1: ALKALINE SLANT/ALKALINE BUTT (red slant and red butt shown by non fermenters).

These consist of bacteria that do not ferment sugars. There is no acid production in the medium. As a result the color of the medium stays as such (pink red) in the slant and Butt along with no gas CO_2 and H_2S production.

Example: *Pseudomonas aeruginosa*

Reaction-2: ALKALINE SLANT/ACID BUTT (red slant and yellow butt shown by non-lactose fermenters)

Due to fermentation of glucose & acid is produced. The pH in the medium falls & color changes to yellow in both the slant and Butt. As the concentration of glucose is very low, the acid produced in the Butt changes the color to yellow. As the acid produced in slant is very low most of it gets evaporated as CO_2 which turns the pH back towards alkaline. Therefore pH increases & neutralizes the acid producing a pink / red color of the slant.

Example: *Shigella.*

Reaction-3: ALKALINE SLANT/ACID BUTT (black H2S gas):shown by sulphide producing non-lactose fermenters:

These organisms give similar results as above but with production of H_2S. H_2S production is seen as blackening in the medium. Blackening is due to reaction of H_2S with ferrous sulphate to produce ferrous sulphide.

Example: *Proteus* & *Salmonella* (except *Salmonella paratyphi A*)

Reaction-4: ACID SLANT/ACIDIC BUTT (shown by lactose / sucrose fermenters):

All three sugars are fermented with mixed acid production turning the entire medium yellow. Evaporating acid is insufficient to reverse the pH to alkaline in the slant. Gas is produced which causes breaks or cracks in the medium.

Example: *E. coli, Enterobacter, Klebsiella, Serratia.*

INTERPRETATION OF RESULTS:

- If lactose or sucrose is fermented; a large amount of acid is produced which turns the phenol red indicator yellow both in the Butt & the slant.
- If lactose is not fermented but small amount of glucose is fermented the oxygen deficient Butt will be yellow but on the slant the glucose will be oxidizing to CO_2 & H_2O & slant will be red (natural or red).
- If neither, glucose or lactose is fermented both slant and Butt will be red.
- Some organisms generate gases which produced bubbles in the Butt.
- If H_2S is produced the black color of ferrous sulphide is seen.

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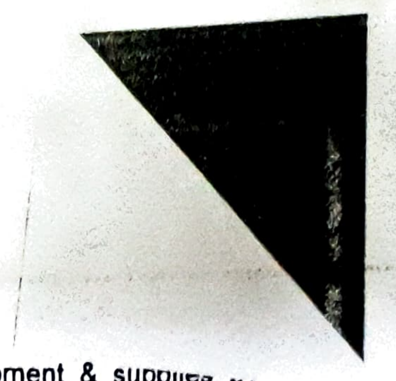


Instruments

1. Autoclave: An autoclave is a device used to sterilize equipment & supplies by subjecting them to high pressure saturated steam at 121°C for around 15-20 min. During autoclaving the contents (liquid / solid) become exposed to saturated steam at the required temp for appropriate length of time.

Principle: Pressure is used to produce high temperature steam to achieve sterilization. The temperature of saturated steam at atmospheric pressure is approximately 100°C. At a pressure of 15lb/inch^2, the temperature of saturated steam rise to 121°C. When this superheated steam falls on cooler objects, it releases thermal energy, leading to enzymatic and protein denaturation.

Use: An autoclave is used to sterilize liquid media (including agar), lancets, scalpel blades, glassware like pipette & numerous other items that can with stand high temperature & pressure.

Disadvantage of autoclave: Some plastic ware melts in high heat & sharp instruments become dull. Moreover, many chemicals breakdown during sterilization process & oiy substances cannot be treated because. They do not mix with water.

2.Hot Air Oven: Hot air ovens are electrical devices used in sterilization. The oven uses dry heat to sterilize articles. They do not require water & there is not much pressure built up within oven unlike on autoclave. Temperature 160-180 °C for 2 hours.

Principle: It uses dry heat for sterilization.

Uses: It is used for sterilizing dry powders & water free oily substances, as well as for many types of glassware, such as pipettes, flasks & syringes. Dry heat does not corrode sharp instruments as steam often does nor does it erode the ground glass surfaces of non disposable syringes.

Disadvantages: Dry heat does not penetrate materials easily & therefore long period of exposure to high temperature is necessary.

3.Incubators: (optimum temperature for growth =35-37°CMicroorganism require incubation at a particular temperature for their growth & metabolism. Incubator is fitted with an internal glass door & has hydrolytic thermostat control. It has temperature display fitted to provide continuous read out of temperature. If display is not available, insert a thermometer in vent hole through the roof of incubator.

Uses: To incubate microorganisms for cultures & tests.

4.**Centrifuge:** Centrifuge separates a heterogenous mixture of solid & liquid by spinning it. After a successful centrifugation, the solid precipitate settles to the bottom of the test tube & the solution.

Place test tube in centrifuge holder. Balance the other test tube filled to same level in the opposite holder. Close cover & turn knob. Centrifugation take a minute or more. **Note that you must turn off the centrifuge with the switch and wait for it to stop spinning to effectively separate the precipitate & solution.**

Principle: The centrifuge works using the sedimentation principle where centripetal acceleration causes denser substances to separate out along the radical direction (bottom of tube).

5.Bunsen Burner: A Bunsen burner is a common piece of laboratory equipment that produces a single open gas flame, which is used for heating, sterilization & combustion.

Use: Used to sterilize wire loop in laboratory by heating till red hot.

6.Wire Loop: An inoculation loop is a simple tool used mainly by microbiologists to retrieve an inoculum from culture of microorganisms. It is made of platinum or nichrome. The inoculation loop is sterilized in it till red hot.

Use: The loop is used in the cultivation of microbes on plates.

STERILISATION & DISINFECTION

STERILISATION

Definition:

Freeing of an article from all organisms, including viruses, bacteria, fungi etc and their spores; (both pathogenic & non-pathogenic).

USES:

a) **Clinical**
- For instruments and materials used in procedures that invade into normally sterile parts of body. i.e. Surgical operations.
- Intravenous infusions & injections.

b) **Laboratory**
- Equipments used in laboratory.
- For culture media and reagents.

STERILIZATION BY PHYSICAL METHODS:

Heat: It can be dry heat and moist heat.

1. Moist Heat

Temp above 100°C under controlled conditions kills the spores.
Coagulates and denatures enzymes and structural proteins and kills micro-organisms.

a) Autoclave:

Principle:

Pressure is used to produce high temperature steam to achieve sterilization. The temperature of saturated steam at atmospheric pressure is approximately 100°C. At a pressure of 15lb/inch^2, the temperature of saturated steam rise to 121°C. When this superheated steam falls on cooler objects, it releases thermal energy, leading to enzymatic and protein denaturation.

Uses:

Clinical
Surgical Supplies (instruments)

Laboratory
Bacteriological culture media

Controls & Indicators of autoclave:

1. **Physical Control**
   i. Automatic process control.
   ii. Recording thermometer.
   iii. Thermo-couple measurements.
2. **Chemical Control:**
   i. Browne's control tube.
   ii. Bowie Dick Tape.

3. **Biological Indicator:**
Spores of Bacillus stereothermophilus.

b) **Tyndallization:**
Intermittent exposure at 100°C at 20-30°C for three consecutive days is called as tyndallization.

c) **Pasteurization:**
Used for sterilization milk and dairy products. The recommended temperature is 63-66°C for 30 minutes or 72°C for 20 seconds.

2. Dry heat

Oxidation of essential cell components.

a) **Flaming:**
Sterilizing wire loops used for inoculation of specimen on culture media.

b) **Hot air sterilizers (Hot air Oven):**

- Used for materials that can with stand high temperature for longer time e.g. glass ware.
- Materials that are likely to be affected by steam or into which it can not penetrate. e.g. powder, oils.

Ionizing Radiation:

- X-Rays & Gamma-rays.
- Used to sterilize large amount of pre packed single-used items e.g. disposable syringes, catheters and gloves etc.

Filtration:

- Cellulose filters are used for those items which are destroyed by heating e.g. fluids & air.

STERILIZATION BY CHEMICAL METHODS:

a) **Sterilization by gases:**

- Ethylene oxide used for sterilization of heat sensitive devices e.g. prosthetic heart values.

b) **Sterilization by liquids:**
Glutaraldehyde used for fiberoptic endoscopes.

DISINFECTION:

i. **Physical**

- Ultraviolet radiation.
- Heat.

ii. **Chemical**

- Gases
- Liquids.

Disinfection by physical methods:

i. Heat:

Boiling temperature is 100°C for 20-30 minutes. It is used for disinfection of syringes.

ii. UV Radiation:

- 240-280 nm wavelength-produced by mercury lamps.
- Low energy non-ionizing radiation.
- For clothing bed sheets of patient.

Disinfection by Chemicals:

i. Gases:

- Formaldehyde
- Heat sensitive equipment like anesthetic machines, baby incubators in nurseries, etc.

ii. Liquids:

Disruption of cell membrane:

i) Alcohol (ethanol): Acts by disrupting the lipid structure in membranes. It is more effective at 70% then 100%. Alcohol used for disinfection of skin before venipuncture.

ii) Detergents: Quaternary ammonium compounds used for skin antisepsis.

Denaturation of proteins:

- Chlorine used to purify water supplies.
- Iodine skin antiseptic or tincture of iodine.
- Iodophores.
- Ethylene oxide.
- Acids & alkalis.
- Formaldehyde.
- Hydrogen peroxide.

Denaturation of Nucleic Acids:

- Crystal violet.
- Malachite green.

Urine Examination

Urine samples are excellent culture media and get contaminated easily. Their characteristics and components are unstable. Therefore all specimens should be examined within 30 minutes of collection or should be refrigerated. Random specimens are not good because constituents vary depending upon dilution. First morning specimens are more acidic and more concentrated. They are therefore best for routine urine examination.

1. **Collection of Urine Sample:**
   - Time
   - Amount
   - Container
2. **Laboratory Examination**
   - Physical
   - Chemical
   - Microscope

A. **Physical Examination of Urine:**

Normal	Abnormal	Significance
1. Color		
Pale yellow to amber. • Depends upon concentration of Urine. • In swear sweating & dehydration urine is Dark yellow. • Extensive fluid intake gives very pale color urine.	Colorless pale →	Diabetes insipidus
	Red Color →	Indicates presence of RBC e.g. glomerulonephritis, Bladder carcinoma
	Greenish →	Bile pigment (biliverdin)
	Smokey urine →	Presence of blood (Hb)
	Milky urine →	Pus cells, WBC, fat globules, bancroftian filariasis (chyluria)
	Brown Black →	Hemorrhages, mismatched blood transfusion, lead poisoning
	Yellow →	Jaundice
	Many foods, herbs, vitamins give color to urine	
2. **Specific Gravity** 1.015-1.025		
	Decreased in	Diabetes insipidus, winter, excessive fluid intake

Volume of urine	Increased in	Hypovolemic shock, conn's disease, acute viral hepatitits, hyperaldosteronism (↓ urine volume due to ↑ absorption)
pH (normal is i.e. acidic 5.0-6.5)		
Range: 4.1-8.2	**Alkaline:**	Bacterial infection, vomiting, renal failure, drugs
	Acidic:	High protein diet, diabetes mellitus, fever, starvation
Volume		
Normal adult 800-2000 ml	**Polyuria = >3000 ml in 24 hrs**	↑ Fluid intake, diuretics, chronic kidney disease, Diabetes mellitus
	Oliguria = < 500ml in 24 hrs	Dehydration, cardiac & hepatic insufficiency, shock, urinary track obstruction, chronic renal failure

B. Chemical Examination of Urine:

Principle: Tests are performed on strips nowadays. Plastic strips which bear pads having chemical/reagents. If specific analytes for these reagents are presents in urine, then these reagents undergo color change due to enzymatic reaction with metabolites. Color changes indicate presence of metabolites in urine e.g. indicator color will change in the presence of proteins, as indicator gives H^+ ions (anions) to proteins.

Method: Collect un-centrifuged urine in open mouth, clear & dry container. Remove strip from bottle & close it as soon as possible. Completely immerse the strip in urine container to avoid mixing of reagents. Compare reagents area to corresponding color blocks & match carefully. Results read at 2 min or at specified times for different reagents given by manufacturer of strips.

1.Benedict's Test: Used to detect sugar in the past. Benedict's solution ($CuSO_4$) is commonly employed for the qualitative determination of reducing sugars.

Principle: When sugars are treated with Benedict's reagent in a clean dry test tube having benedicts reagent, reducing substances, reduce $CuSO_4$ into cupric oxide & then into cuprous oxides in hot alkaline medium. Red color precipitates of cuprous oxide are formed.

2.**Enzymatic method for sugar estimation:**.This is used for glucose estimation.

Principle:

Glucose+ O_2 ⇒ Gluconic acid + H_2O_2

H_2O_2 + Orthotoluidine ⇒ Colored compound + water

C.Microscopic Examination of Urine:

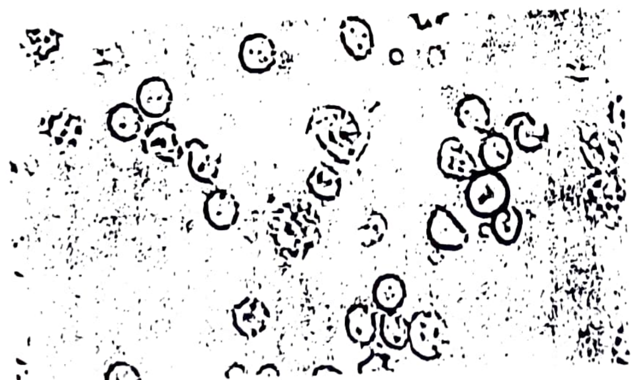
Material: Urine in wide mouth container, slides, cover slips, pipette, microscope.

Procedure:

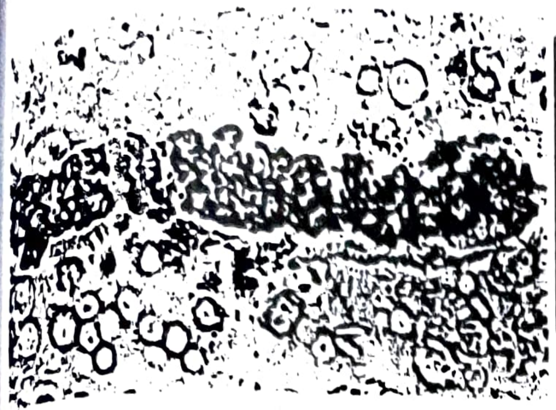
1. Take at least 5ml well mixed urine in a test tube.
2. Centrifuge the urine.
3. Discard the supernatant fluid.
4. Add 1 drop of sediment on the slide.
5. Reduce light of microscope & bring the condenser downward. First focus the slide on 4x &10x objective lens & then to comment on details of structure observes under 40x.

Normal	Abnormal	Significance
1. Cells		
WBCs		
A few WBCs can be present in normal individuals	Greater than 2 cells / high power field (HPF) in males & more than 5 cells / HPF in females	Pus cells indicate infection, trauma & stones
RBCs		
Normally absent	Any number in males & more than 2 / HPF in females	Stones, glomeruionephritis, bacterial infection or some hemolytic disease
Epithelial cells		
Absent in males Few cells be present in females		Inflammation of urinary tract or vaginal contamination of the specimens
Yeast cells		
Normally absent	Oval in shape & some yeasts structure budding	In urine of females with vaginal candidiasis & Specific of diabetes
Crystals		
Refractile appearance.	Cystine crystal →	Found in cystienuria

Normal urine containes many chemical from which crystals can form & finding of most crystals in small number has little importance	Tyrosine crystals →	Occasionally found in severe liver disease
	Cholesterol crystals →	Rarely found in severe kidney disease & when a lymphatic vessel has ruptured into renal pelvis
Calcium oxalate crystals are frequently seen in urine	Sulphonamide crystals →	Found in patients being treated with sulphonamides
	In urine (alkaline)	
	Tripple phosphate crystals	
	In urine (acidic)	
	Urate crystals	
Casts		
Casts are solidified proteins Cylindrical in shape because they are formed in kidney tubules	• Hyaline casts	Associated with damage to glomerular membrane. Few may be seen following exercise or fever
	• Granular casts	Associated with renal damage
Casts are normally not present in urine	• Cellular casts:	
	I. Red cell east	Indicates hemorrhage into renal tubules / glomerular bleeding
	II. WBC's cast	Found when there is inflammation of kidney pelvis or tubules
	• Waxy casts (Usually thicker & denser than hyaline cast)	Found in tubular damage & renal failure



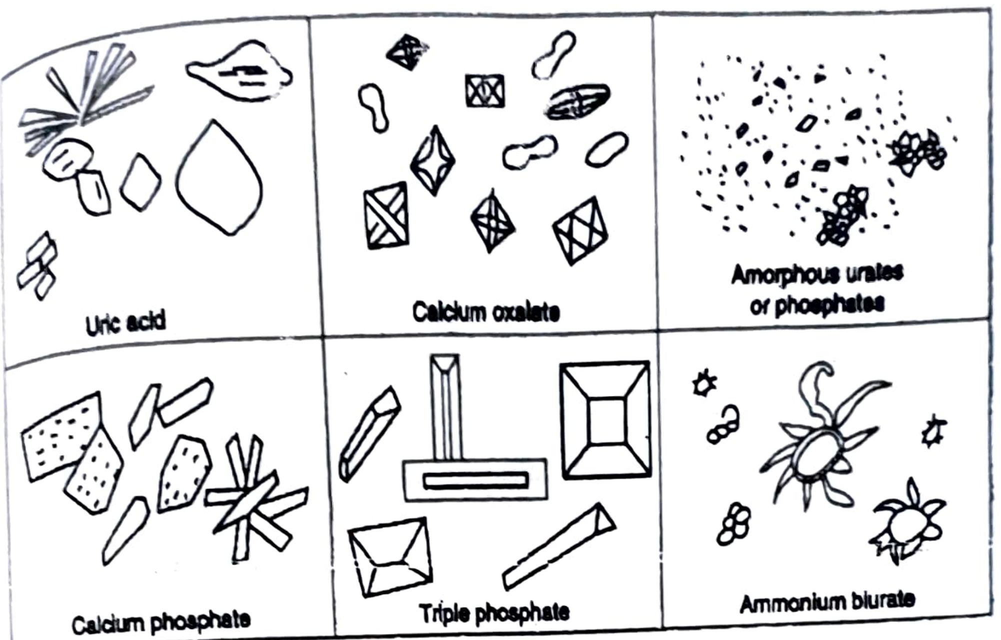
Urine sediment showing pus cells (larger granulated cells) and red cells as seen with the 40X objective



Large cellular cast, pus cells, red cells, and bacteria (bacilli in background) in urine sediment as seen with the 40X objective.

Epithelial cells, red cells and occasional pus cell in urine sediment as seen with the 10X objective

Hyaline cast in urine as seen with the 40X objective.



Appearance of urinary crystals