NATIONAL UNIVERSITY-SUDAN

Faculty of Medical Laboratory Sciences

Student Practical Manual- Microbiology Department

Second year, Semester (4)
Clinical Microbiology-1(MLS-CMIC-226)

Student Name: ……..

ID: …… **Batch**……...…...

Instructions

- Wear lab coat
- Wear Gloves
- Avoid swallow any chemical
- Follow the procedures provided
- Write your results in this manual

Contents

Practical No. (1):

Staphylococcus aureus

1. **Colonial Morphology:**

	Blood Agar	Nutrient Agar	CLED	MacConkey Agar
Size				
Shape				
Colour				
Fermentation				
Hemolysis				
Texture				
Elevation				

2. **Gram stain:**

Aim: To differentiate between gram positive and gram negative bacteria as an aid to their identification.

Principle: Gram-positive bacterial cell walls are rich in tightly linked peptidoglycans that enable cells to resist decolorization and keep the basic stain. Gram-negative bacterial walls have a high concentration of lipids (fats) that dissolve in the decolorizer and stain by counter stain.

Requirements: Cultured plate, Loop, Sterile saline, Crystal violet, or Methyl violet (basic stain), Gram's iodine (mordent), 70% Alcohol, Acetone alcohol, or Acetone (decolourizer), Safranin or Neutral red stain (counter stain), Microscope, Oil, Flame, and Staining rack.

Method:

1. Prepare a well, dried, fixed smear.
2. Apply crystal violet, for 1 min. Wash well with water.
3. Apply Gram's iodine, for 2 mins .Wash well with water.
4. Flood 70% alcohol and wash off within 5-10 seconds. Wash well with water.
5. Apply safranin, for 1 min .Wash well with water.
6. Blot dry the back of slide with filter paper and leave to dry.
7. Examine using 100X oil immersion lens.

Report: (Gram reaction, Shape and Arrangement)

……
……
……

3. Identification tests:

a. Rapid test:

- **Catalase test:**

Aim: This test is used to differentiate those bacteria that produce the enzyme catalase, such as staphylococci, from non-catalase producing bacteria such as streptococci.

Principle: Catalase acts as a catalyst in the breakdown of hydrogen peroxide to oxygen and water. An organism is tested for catalase production by bringing it into contact with hydrogen peroxide. Bubbles of oxygen are released if the organism is a catalase producer.

Requirements: Cultured plate, Slides, Wooden stick, Normal saline, Hydrogen peroxide (3% H_2O_2) and Flame.

Method:

1. Pour 2–3 ml of the hydrogen peroxide solution into a test tube.
2. Using a sterile wooden stick remove several colonies of the test organism and immerse in the hydrogen peroxide solution.

- ***Important:*** Care must be taken when testing an organism cultured on a medium containing blood because catalase is present in red cells. If any of the blood agar is removed with the organism, a false positive reaction may occur. Don't use wire loop for colony collection.

3. Look for immediate bubbling as shown in Plate

Results:

Active bubbling . . . . . Positive catalase test

No bubbles . . . . . Negative catalase tes

Comment:……

……

- **Coagulase test:**

Aim: This test is used to identify *S. aureu* swhich produces the enzyme coagulase.

Principle: Coagulase causes plasma to clot by converting fibrinogen to fibrin. Two types of coagulase are produced by most strains of S. aureus:

1. Free coagulase which converts fibrinogen to fibrin by activating a coagulase-reacting factor present in plasma. Free coagulase is detected by clotting in the tube test.

2. Bound coagulase (clumping factor) which converts fibrinogen directly to fibrin without requiring a coagulase reacting factor. It can be detected by the clumping of bacterial cells in the rapid slide test.

Requirements: Cultured plate, Slides, Wire loop, Normal saline, undiluted human plasma and Flame.

Procedure:

Slide method

1. Place a drop of distilled water on each end of a slide or on two separate slides.
2. Emulsify a colony of the test organism in each of the drops to make two thick homogenous suspensions.
3. Add a loopful (not more) of plasma to one of the suspensions, and mix gently. Look for clumping of the organisms within 10 seconds. No plasma is added to the second suspension. This is used to differentiate any granular appearance of the organism from true coagulase clumping.

Results:

Clumping within 10 secs . . . . . *S. aureus*

No clumping within 10 secs . . . . . No bound coagulase

Report:……

……

<u>Tube method Tube test method (detects free coagulase)</u>

1. Take three small test tubes and label:Test organism, Positive control (18–24 h S. aureus broth culture) and Negative control (sterile broth).
2. Pipette 0.2 ml of plasma into each tube.
3. Add 0.8 ml of the test broth culture to tube T.
4. Add 0.8 ml of the S. aureus culture to the tube labelled +ve.
5. Add 0.8 ml of sterile broth to the tube labeled -ve.
6. After mixing gently, incubate the three tubes at 35–37^{o}C.
7. Examine for clotting after 1 hour. If no clotting has occurred, examine after 3 hours. If the test is still negative, leave the tube at room temperature overnight and examine again.

<u>Note</u>: When looking for clotting, tilt each tube gently.

Results:

Clotting of tube contents . . . . . *S. aureus* Positive test

No Clotting or fibrin clot . . . . . Negative test

<u>Important:</u> Don't use colonies from differential media such as MacConkey, MSA or CLED as the acid produce during fermentation inactivate the coagulase Enzyme.

- **24 hour tests:**
- **Mannitol fermentation test:**

Aim: To differentiate between *Staphylococcus*s species.

Principle: Some of *Staphylococcus*s species can ferment mannitol sugar and produce acid that will change the colour of the indicator phenol Red from **<u>Pink to Yellow.</u>**

Requirements: Mannitol Salt agar, cultured plate, loop, flame.

Procedure: Inoculate the MSA plate with the tested organism and incubate at 37^{o}C for 24hr aerobically.

Results:……

……

- **DNase test:**

Aim: This test is used to help in the identification of *S. aureus* which produces deoxyribonuclease (DNAase) enzymes

Principle: Deoxyribonuclease hydrolyzes deoxyribonucleic acid (DNA). The test organism is cultured on a medium which contains DNA. After overnight incubation, the colonies are tested for DNA-ase production by flooding the plate with a weak hydrochloric acid solution. The acid precipitates unhydrolyzed DNA. DNA-ase-producing colonies are therefore surrounded by clear areas due to DNA hydrolysis.

Requirements: DNA-ase agar, Hydrochloric acid 1 mol/1 (1 N), Cultured plate, Slides, Wire loop, and Flame.

Procedure:

1. Divide a DNA-ase plate into the required number of strips by marking the underside of the plate.
2. Using a sterile loop or swab, spot-inoculate the test and control organisms. Make sure each test area is labelled clearly.
3. Incubate the plate at 35–37C overnight.
4. Cover the surface of the plate with 1 mol/l hydrochloric acid solution. Tip off the excess acid.
5. Look for clearing around the colonies within 5 minutes of adding the acid.

Results:

Clearing around the colonies . . . . . DNA-ase positive strain

Comment:……

……

- **Sensitivity to Novobiocin:**

Aim: The Novobiocin inhibition test presumptively differentiates *Staphylococcus saprophyticus* (Resistant) from other *Staphylococcus* species (sensitive)

Principle: *Staphylococcus saprophyticus* able to grow on the presence of Novobiocin while other Staphylococci didn't.

Requirements: Cultured plate, Loop, Novobiocin disc, Forceps and Flame.

Procedure: Using a pure culture of the test organism, inoculate a Blood agar plate with the bacterial suspension. Place a Novobiocin disk in the center of the inoculated Blood agar plate and incubate at 35◦C for 18–24 h.

Results: Growth of the organism around the Novobiocin disk is considered a positive test.

Comment:……

……

- **Other tests:**
- **Phosphatase test (Homework):**

Aim:……

……

……

……

Principle:**……**

……

……

……

……

Requirements:……

……

Procedure:**……**

……

……

……

……

……

Results:……

……

Practical No. (2)

Coagulase Negative Staphylococci

1. **Colonial Morphology:**

	Blood Agar	Nutrient Agar	CLED	MacConkey Agar
Size				
Shape				
Colour				
Fermentation				
Hemolysis				
Texture				
Elevation				

2. **Gram stain:**

- **Methodology:**

Preparation of the smear:

……

……

……

Gram Stain procedure:

……

……

……

……

……

Results:……

……

Comment:

……

……

3. Identification tests:

b. Rapid test:

- **Catalase test:**

Aim:……

……

……

Principle:……

……

……

……

Requirements:……

……

Procedure:……

……

……

……

……

……

……

Results:……

……

- **Coagulase test:**

Aim:……...……
……

Principle:……
……
……
……

Requirements:……
……..

Procedure:……
……
……
……
……
……

Results:……
……

- **24 hour tests:**

Mannitol fermentation test:

Aim:……...……
……

Principle:……
……
……
……

Requirements:……

……..

Procedure:**……**

……

……

……

……

……

Results:**……..…**

……

- **DNase test:**

Aim:……

……

Principle:**……..……**

……

……

……

……

……

Requirements:……..……

……

Procedure:**……**

……

……

……

……

Results:…….

……

- Sensitivity to Novobiocin

Aim:……

……

Principle:……

……

……

Requirements:……

……..……

Procedure:……

……

……

……

……

……

……..……

Results:……..…

……

……

- **Please complete this table with the right test results:**

Organism	Coagulase	DNA-ase	MSA	Novobiocin
S. aureus				
S. epidermitis				
S. saprophyticus				

Practical No. (3)

Group A streptococci

1. **Colonial Morphology:**

	Blood Agar
Size	
Shape	
Colour	
Fermentation	
Hemolysis	
Texture	
Elevation	

2. **Gram stain:**

Results:……

……

Comment:……

……

1. **Identification tests:**

- **Rapid tests:**
- **Catalase test:**

Requirements:**……**

……

Results……

……

Report:**……**

……

- **24hr tests:**
- **Sensitivity to Bacitracin:**

Aim: The bacitracin inhibition test presumptively differentiates group A streptococci (GAS) from other beta-hemolytic streptococci.

Principle: The bacitracin at concentration of 0.04 units will selectively inhibit growth of GAS.

Requirements: Cultured plate, Loop, Bacitracin disc, Forceps and Flame.

Procedure: Using a pure culture of the test organism, inoculate a Blood agar plate with the bacterial suspension. Place a bacitracin disk in the center of the inoculated Blood agar plate and incubate at 35◦C for 18–24 h.

Results: Any zone of inhibition around the bacitracin disk is considered a positive test. Uniform lawn of growth right up to the rim of the disk indicates a negative bacitracin inhibition test

Result:**……**

……

- **PYRase (PYR (pyrrolidonyl)) test:**

Aim: This detects pyrrolidonyl peptidase enzyme activity. Besides *S. pyogenes*, *Enterococcus* species and occasionally streptococci belonging to groups C and G are also PYR positive.

Principe: PYR is a chromogenic substrate (L-pyrrolidonyl-β-naphthylamide, or PYR) which when hydrolyzed by PYRase (L-pyrroglutamyl-peptide hydrolase) produces a red color upon the addition of a specific reagent. PYR is a substrate that is hydrolyzed by 100% of the enterococci and group A streptococci but not by any other streptococcal species.

Procedure:

1. Two to 4 drops of a buffer reagent is applied to the PYR test strip circle.
2. The strip is then inoculated with 3–5 colonies of the organism and incubated at room temperature for 2 min.
3. Two drops of a second reagent is applied to the test strip circle.

Results: An intense red color develops immediately around the colonies in the presence of hydrolyzed PYR.

The PYR test is negative if no color, an orange color or a weak pink-color develops.

Comment:……

……

- **Lancefield grouping**:

Aim: Lancefield grouping *S. pyogenes* belongs to Lancefield Group A. Pure growth of beta-haemolytic colonies are grouped using specific Group A antiserum to identify the A antigen extracted from the cell wall of the bacteria.

Assignment (1): ASO titration test.

Practical No. (4)

Group B streptococci

1. **Colonial Morphology:**

	Blood Agar
Size	
Shape	
Colour	
Fermentation	
Hemolysis	
Texture	
Elevation	

2. **Gram stain:**

Results:……

……

Comment:……

……

3. Identification tests:

- **Rapid tests:**
- **Catalase test:**

Requirements:**……**

……

Results……

……

Report:**……**

……

- **Sensitivity to Bacitracin:**

Requirements:……
……

Method:……
……
……
……
……

Results……
……

- **Other tests:**
- **Hippurate hydrolysis test:**

Aim: Used to differentiate *Streptococcus agalactiae* from other beta-hemolytic streptococci.

Principle: The assay is based on hydrolysis of the sodium hippurate by the enzyme hippuricase to sodium benzoate and glycine. Glycine is detected by oxidation with ninhydrin reagent that results in production of a deep-purple color.

Requirements: Sodium hippurate, Ninhydrin reagent, cultured plate, sterile tubes, and Flame.

Procedure:

1. Add 0.4ml of Sodium Hippurate in the tubes.
2. Inoculate the hippurate tubes with a heavy suspension of the organism.
3. Incubated at 35◦C for 2 h.
4. Add 0.2 mL of ninhydrin reagent and reincubated for additional 10 to 15 min.

***Note*:** A light inoculum or use of an old culture may give false-negative results

Results: The presence of a deep-purple color indicates a positive hippurate and no color change indicates a negative hippurate.

Comment:……
……..……

- **Lancefield grouping (Homework):**

Aim:……
……
……..……
……..……

Principle:……
……
……
……
……
……

Requirements:……
……

Procedure:……
……
……
……
……
……
……

Results:……..
……

- **CAMP test:**

Aim: used to differentiate between beta hemolytic Streptococci.

Principle: The CAMP test is based on the fact that group B streptococci produce a protein-like compound known as the CAMP (Christie Atkins & Munch Petersen) factor that acts synergistically with a staphylococcal beta-hemolysin (β-lysin) on sheep erythrocytes to produce an enhanced zone of hemolysis (similar to arrow head).

Requirements: Cultured organism, culture of beta-hemolytic *S. aureus*, 10% sheep Blood agar, loop, flame.

Procedure:Streak a loopful of β toxin–producing *S. aureus* in a straight line across the center of a BAP. Streak a loopful of group B streptococci perpendicular to and nearly touching the streak line of the staphylococci (positive control).Streak a loopful of group A streptococci perpendicular to and nearly touching the streak line of the staphylococci (negative control). Streak a loopful of unknown isolate perpendicular to and nearly touching the streak line of the staphylococci and incubate the plate at 35◦C for 24 h in the aerobic incubator.

Results:

An arrowhead zone of enhanced hemolysis, ……. group B streptococci.

No arrowhead of enhanced hemolysis…… not group B streptococci.

Note: Do not incubate the CAMP test plate in the presence of 5–10% CO2incubator. This may result in an incorrect interpretation.

Comment:……
……
……..

Practical No. (5)

Alpha hemolytic- Streptococci

1. **Colonial Morphology:**

	Blood Agar	Chocolate Agar Plate
Size		
Shape		
Colour		
Fermentation		
Hemolysis		
Texture		
Elevation		

2. **Gram stain:**

Results:

……

……

……

Comment:

……

……

3. Identification tests:

a. Rapid tests:

- **Catalase test:**

Requirements:……

……

Results……

……

Report:……

……

- **Quellung test: (Homework)**

Aim:……

……

……

Principle:……

……

……

……

……

Requirements:……

……

Results……

……

Report:……

……

b. 24hr tests:

- **Sensitivity to Optichin:**

Aim: The Optichin inhibition test presumptively differentiates *S. pneumoniae* (Pneumococci) from other alfa-hemolytic streptococci (Viridans Streptococci).

Principle: The Optichin at concentration of 0.04 units will selectively inhibit growth of *S. pneumoniae.*

Requirements: Cultured plate, Loop, Optichin disc, Forceps and Flame.

Procedure: Using a pure culture of the test organism, inoculate a Chocolate agar plate with the bacterial suspension. Place an Optichin disc in the center of the inoculated Chocolate agar plate and incubate at 35◦C for 18–24 h.

Results: Any zone of inhibition around the Optichin disk is considered a positive test. Uniform lawn of growth right up to the rim of the disk indicates a negative Optichin inhibition test

Comment:**……**

……..……

- **Bile Solubility test:**

Aim: Used to differentiate between *Streptococcus pneumoni*aefrom other Viridans Streptococci.

Principle: *S. pneumonia* produce autolytic enzymes which increased in their activity in the presence of bile salts (Na taucholate or Na desoxycholate) leading to lysis when treated with a 10% solution of bile salts, whereas other streptococci and Gram positive cocci are not bile soluble. Lysis occurs because bile-soluble organisms contain autolytic amidase that when activated by bile salts cleave the bond between alanine and muramic acid in the cell wall.

Requirements: Cultured plate, Loop, Normal saline, 100 g/l (10% w/v), Sodium deoxycholate, sterile small tubes and Flame.

Procedure:

Tube method:

1. Emulsify several colonies of the test organism in 2tube containing 1ml sterile Normal saline, to give a turbid suspension.
2. To one tube add 2 drops of the sodium deoxycholate reagent and mix.
3. To the other tube (negative control), add 2 drops of sterile distilled water and mix.
4. Leave both tubes for 10–15 minutes at 35–37^{o}C.
5. Look for a clearing of turbidity in the tube containing the sodium deoxycholate.

Results:

Clearing of turbidity . . . . . Probably *S. pneumoniae*

No clearing of turbidity . . . . . Organism is probably not *S. pneumoniae*

Note: There should be no clearing of turbidity in the negative control tube to which distilled water was added.

Comment:

……

……

- **Inuline fermentation test (Homework):**

Aim:……

……

Principle:……

……

……

……

Procedure:……

……

……

……

……

……

……

……

……

Result:……

……

- **Compare between *S. pneumoniae* and Viridans Streptococci in form of:**

	S. pneumoniae	**Viridans Streptococci**
Gram's stain reaction		
Quellung reaction		
Turbidity formed in broth medium		
Bile solubility		
Inuline fermentation		
Capsule formation		
Animal pathogenicity		

Practical No. (6)

Group D and Enterococci

1. **Colonial Morphology:**

	Blood Agar	MacConkey Agar	CLED
Size			
Shape			
Colour			
Fermentation			
Hemolysis			
Texture			
Elevation			

2. **Gram stain:**

Results:……

……

Comment:……

……

3. Identification tests:

- **Rapid tests:**
- **Catalase test:**

Requirements:**……**

……

Results……

……

Report:**……**

……

c. 24hr tests:

- **Litmus Milk reduction test:**

Aim: to differentiate between Enterococci and other Streptococci.

Principle: It is based on the ability of most strains of Enterococcus species to reduce litmus milk by enzyme action as shown by decolorization of the litmus. Enterococci ferment the lactose sugar in the milk and produce lactic acid will change the pH of the medium resulting in reduction of the litmus indicator. Reduction may occur within the first 4 hrs of incubation.

Requirements: Cultured plate, Loop, Litmus milk, and Flame.

Method: Suspend **2-3** colonies of Enterococci in the litmus milk and incubate at 37^{o}C for 24hr.
Results: A reduction reaction as shown by a change in colour from mauve to white or pale yellow indicating positive result.

Comment:……
……..

- **Bile aesculin hydrolysis test:**

Aim: use to differentiate between D Streptococci including *Enterococcus* spp. from other Streptococci.

Principle: Group D streptococci (including *Enterococcus* spp.) and a few other bacteria, such as *Listeria monocytogenes*, can grow in the presence of 40% bile and also hydrolyze aesculin sugar to aesculetin. Aesculetin reacts with ferric ions, supplied by ferric citrate in the agar medium, to form a diffusible black complex.

Requirements: Cultured plate, Loop, Bile aesculin azide agar slope, and Flame.

Procedure: Streak the surface of the bile aesculin agar slant with several colonies of the organism to be tested. Incubate at 35°C in for 4 hr and read for the development of black colour on the surface, re-incubate for 48 hrs.
Results: A diffuse blackening of more than half of the slant within 24 hrs is considered positive. No growth or growth without blackening of the medium after 24hrsis considered negative test**.**

Comment:……

……

- **6.5% NaCl tolerance test:**

Aim: Used to differentiate Enterococci from non-Enterococcal group D Streptococci.

Principle: *Enterococcus* spp., which are salt-tolerant (halophilic), while non-Enterococcal group D streptococci (*S. Bovis* and *S. equines*) are not salt-tolerant.

Requirements: Cultured plate, Loop, Nutrient broth with 6.5% sodium chloride and Flame.

Procedure: Inoculate the tube containing 6.5% sodium chloride with the organism and incubate at 35◦C in non-CO2for 24–48 h.

Results

Visible growth (turbidity) ……positive

No growth …… negative

Comment:……

……

- **Heat resistance test:**

Aim: used to differentiate Enterococci from non-Enterococcal group D streptococci.

Principle: *Enterococcus* spp., resist heating for 30 min at 60^{o}C, while non-Enterococcal group D Streptococci are sensitive (killed) for heating for 30 min at 60^{o}C.

Requirements: Cultured plate, Loop, 2ml Normal saline, Blood agar plate, water path and Flame.

Procedure:

1. Divide the BA plate in to two halves, label one half Non-heated and the other one heated.
2. Prepare a suspension by inoculate the tube containing Normal saline with tested organism.
3. Subculture from the suspension on the non-heated half of the BA plate (as a control).

4. Then heat the suspension for 30 min at 60^{o}C in water path.
5. Subculture from the heated suspension on the heated half of the BA plate organism and incubate at 37^{o} C for 24hr.

Results:

Non-heated	**Heated**	**Comment**
Growth	Growth	Heat resistant
Growth	No growth	Heat sensitive

Report:……

……..

Practical No. (7)

Corynebacterium diphtheriae

1. **Colonial Morphology:**

	Blood agar	**Tellurite blood agar**
Size		
Shape		

Colour		
Fermentation		
Hemolysis		
Texture		
Elevation		

1. **Gram stain:**

Results:……

……

Comment:……

……

1. Identification tests:

a. Rapid tests:

- **Catalase test:**

Requirements**……**

……

Results:**……**

……

Report:……**……**

……

- **Oxidase test:**

Aim: The oxidase test is used to assist in the identification of the organisms that produce the enzyme cytochrome oxidase.

Principle: The oxidase test is based on the production of the enzyme indophenol oxidase by organisms containing cytochrome C. Indophenol oxidase, in the presence of atmospheric oxygen, oxidizes a redox dye (N,N,N,N-tetramethyl-p-phenylene diamine dihydrochloride) to form a dark-purple indophenol compound.

Procedure: A loopful of bacteria is placed onto a disc paper impregnated with the reagent and examined for development of a violet or purple color.

Note: Wire loops containing iron may give a false-positive reaction. Colonies growing on selective media or differential media containing glucose cannot be used for oxidase determination because fermentation inhibits indophenol oxidase activity resulting in false negative results.

Result:

Development of purple colour within 5 second …….. Positive test

No color change within 5 second…….Negative test

Comment:……

……

b. Other tests:

- **Growth on Tellurite blood agar:**
- **Aim:** This medium is used as a primary medium for isolating *C. diphtheriae* from throat and nasopharyngeal swabs.
- **Principle:** *C. diphtheriae* reduces tellurite and produces grey or grey-black colonies measuring 0.5–2 mm in diameter after 48 h incubation.
- **Procedure:** Subculture the tested organism on TBA medium and incubate at 37^{o}C for 2 days.

Result:……

……

Comment:……

……

- **Volutine granules examination:**

Aim: The Albert technique is used to stain the volutine, or metachromatic granules of *C. diphtheriae.*

Principle: The granules are most numerous after the organism has been cultured on a protein-rich medium such as Dorset egg or Loeffler serum.

Requirements: Cultured plate, Dorset egg medium, loop Albert stain, and Flame.

Procedure:

1. Inoculate the Dorset egg slope with Corynebacterium
2. Incubate for 4-24hr at $37^{o}C$
3. Perform Albert stain to examine the volutine granules.

- **Albert stain method:**

Prepare of the smear a well fixed smear.

Staining procedure:

1. Apply Albert solution for 3-5 min, and then wash by Albert iodine.
2. Leave Albert Iodine for 1min, wash by tap water
3. Blot, dry, put one drop of oil and see with X100 (oil immersion lens).

Results:

Bacteria cells . . . . . Pale green

Metachromatic granules . . . . . Green-black

Report:……..……
……
……..……..

- **<u>HOMEWORK:</u> Mention other methods for staining volutine granules**
- **Urease test:**

Aim: used to detect urease producing organism.

Principle: The test organism is cultured in a medium which contains urea and the indicator phenol red. When the strain is urease producing, the enzyme will break down the urea (by

hydrolysis) to give ammonia and carbon dioxide. With the release of ammonia, the medium becomes alkaline as shown by a change in colour of the indicator to pink-red.

Requirements: cultured organism, medium contain urea, loop, flame.

Procedure: near the flame remove the cap of the tube and flame the mouth of the tube. Inoculate slope using a sterile wire loop to streak the inoculum down the centre of the slope and then spread the inoculum in a zig-zag pattern. Re-sterilize the mouth of the tube before re-capping.

Result:

Pink colour developed……..Positive test

No change (yellow)…….…….negative test

Comment:……

……

Assignment (2):

Write on the Toxigenicity testing of *C. diphtheriae.*

Practical No. (8)

Listeria monocytogenes

1. **Colonial Morphology:**

	Blood agar	**MacConkey agar**
Size		
Shape		
Colour		
Fermentation		

Hemolysis		
Texture		
Elevation		
Size		

2. **Gram stain:**

Results:……

……

Comment:……

……

3. Identification tests:

c. Rapid tests:

- **Catalase test:**

Requirements**……**

……

Results:**……**

……

Report:……**……**

……

- **Oxidase test:**

Requirements……

……

Results:**……**

……

Report:……**……**

……

- **24hr test:**

Motility test: there are two methods to test the motility of organisms:

1. **Motility test using semisolid medium.**

Aim: This test is used to detect bacterial motility.

Principle: Motility Test Agar is a semisolid medium designed to detect bacterial motility. Its agar concentration is reduced from the typical 1.5% to 0.4%—just enough to maintain its form while allowing movement of motile bacteria. It is inoculated by stabbing with a straight transfer needle. Motility is detectable as diffuse growth radiating from the central stab line. Listeria has inverted tree growth pattern at room temperature (22^oC) but not at 37^oC.

Requirements: Cultured plate, Semi-solid medium, Straight wire loop and Flame.

Procedure: inoculate 2 tubes contain semisolid medium. Use a sterile straight wire to inoculate a stab medium. Stab through the center of the medium till the 2/3 of the tube, taking care to withdraw the wire along the line of inoculum without making further stab lines. Incubate at 37^oC overnight.

Result: Diffusion of the organism around the line of the inoculation indicate positive test.

Report:……

……

2. **Motility test using wet preparation:**

Aim: This test is used to detect bacterial motility.

Principle: Listeria has a characteristic tumbling motility at room temperature but not at 37^oC.

Procedure: Inoculate two nutrient broth tubes with the tested organism. Incubate one at 37^oC and the other at 22^oC overnight.

Result:

At 22^oC	At 37^oC

Show tumbling motility	Non motile

Report:

……

……

- **Aesculine hydrolysis test:**

Requirements:……

……

Procedure:……

……

……

……

Result:……

……

- **CAMP test:**

Requirements:……

……

Procedure:……

……

……

……

……

……

Result:……

……

- **Urease test:**

Requirements:……

……

Procedure:……

……

……

……

……

……

Result:……

……

Practical No. (9)

Bacillus species

1. **Colonial Morphology:**

	Blood agar	**Nutrient agar**	**MacConkey agar**
Size			
Shape			
Colour			
Fermentation			
Hemolysis			

Texture			
Elevation			

2. **Gram stain:**

Results:……

……

Comment:……

……

3. Identification tests:

d. Rapid tests:

- **Catalase test:**

Requirements……

……

Results:**……**

……

Report:……**……**

……

- **Oxidase test:**

Requirements……

……

Results:**……**

……

Report:……**……**

……

- **Spore Stain:**

Aim: To demonstrate bacteria spore and position.

Principle: Hot Malachite green retention by thick wall spore, while bacilli stain by Safranin.

Requirements: Cultured plate, Loop, Sterile saline, malachite green, safranin or 0.05% basic fuchsin Microscope, oil, Flame, and staining rack.

Procedure:

1. Prepare a well, dried, fixed smear.
2. Cover the smear with 5% aqueous solution of malachite green.
3. Heat till vapor arise (avoid boiling of stain) let it for 5 - 10 min. Wash with clean water.
4. Apply 0.5% saffranin or 0.05% basic fuchsin for 2 min.
5. Wash, dry, put one drop of oil and see with X100 (oil immersion lens).

Result:

- Spores appear ……. green.
- Vegetative bacilli …….red.

Report:……

……

Note: Overheating of the stain leads to germination of the spores outside the bacilli.

- **24hr test:**
- **Sensitivity to penicillin:**

Aim: to differentiate between *B. anthracis* from *B. cereus*

Principle: the growth *B. anthracis* is inhibited by penicillin

Requirements: Cultured plate, Loop, penicillin disc, Mueller and Hinton agar, Forceps and Flame.

Procedure: Using a pure culture of the test organism, inoculate Mueller and Hinton agar plate with the bacterial suspension. Place a penicillin disc in the mean area of the inoculated Mueller and Hinton agar plate and incubate at 35°C for 18–24 h.

Results: Any zone of inhibition around the penicillin disk is considered a positive test. Uniform lawn of growth right up to the rim of the disk indicates a negative penicillin inhibition test

Comment:……

……..……

Tests	B. anthracis	B. cereus
Spore	+	+
Capsule (in vitro Mcfadyean test)	+	-
motility	-	+
Granules	+	+
Haemolysis on BA	-	+
Gelatin liquefaction		
Lecithinase activity	±	+
Penicillin sensitivity	+	-
McCloy's Y-phage	+	-
Pathogenicity to mouse	+	-

Practical No. (10)

Clostridium species

1. **Colonial Morphology:**

	Blood agar	**Cooked meat medium**
Size		
Shape		
Colour		
Fermentation		

Hemolysis		
Texture		
Elevation		

1. **Gram stain:**

Results:……

……

Comment:……

……

2. **Identification tests:**

- **Spore stain:**

Requirements……

……

Procedure:**……**

……

……

……

……

Report:**……**

……

- **Sormy clot (Homework):**

Aim:……

……

Principle:……

……

……

……

……

……

Requirements:……

……

Method:……

……

……

……

……

……

Results:……

……

Comment:……

……..

- **Reverse CAMP Test:**

Aim: to differentiate between *Clostridia* spp.

Principle: The reverse CAMP test is based on the fact that some organisms such as *Clostridium perfringens* completely inhibit the effect of *Staphylococcus aureus* B-hemolysin on sheep erythrocytes. The β-hemolysin inhibition zone in the form of a triangle is formed.

Procedure: A loopful of β toxin–producing *Staphylococcus aureus* is streaked in a straight line across the center of a BA plate. Tested organism is streaked perpendicular to and nearly touching the streak line of the staphylococci. The plate is incubated at 35◦C for 24 h in anaerobic jar. Following the incubation, if the test isolate demonstrates a triangular-shaped inhibition of β-hemolysis, it is a reverse CAMP test positive. If the test isolate does not demonstrate a triangle-shaped inhibition of β-hemolysis, it is a reverse CAMP test negative.

- **Nagler reaction:**

Aim:……

…….

Principle:……
……
……
……
……

Requirements:……
……

Procedure:…….
……
……
……
……
……
……
……
……
……

Result:……
……
……

Report:……
……
……

Assignment (3):

Methods of anaerobic incubation: