APPENDIX: I

CLINICAL AND MICROBIOLOGY PROFILE

Clinical profile

Name: ______________________ **Lab No:** ____________

Age: ________________ **Date:** ______________

Sex: ________________ **Patient: OPD/Ward**

Address: _______________ **Bed no:** ___________

Brief Clinical History: __________________

Patient on antibiotics: **Yes** **No**

If Yes, Antibiotics taken: 1) ____________ **2)** ________________

Duration of treatment: ____________________

Others (if any):

Microbiological Profile

Day 1 (__ / __ / __)

Time of sample collection: ______________ **Specimen:** __________

Mode of collection: ______________________

Receiving time at the laboratory: _________________

Direct microscopic observation: 1) ___________________________

(Gram's staining) 2) ___________________________

Media inoculated: 1) _____ **2)** ______ **3)** _____

Incubated at: 1) Aerobic 2) Anaerobic 3) Microaerophilic

Day 2 (__ / __ / __)

Reading of culture plates: Colony Characters

Media	Feature	Shape	Size	Color	Texture	Opacity	Consistency
CA							
BA							
MA							

Gram staining results: ______________________

Catalase: __________ **Oxidase:** __________ **Coagulase:** __________

Provisional Identification of Organism:

Day 3 (__ / __ / __)

BIOCHEMICAL TESTS:

TSIA: ______________ **SIM:** ______________

Citrate: ______________ **Urea:** ______________

Decarboxylase tests:

Lysine: ________ **Arginine:** ________ **Ornithine:** ________

Organism identified as: ____________________

Antibiotic sensitivity test method: Kirby- Bauer Method

Antibiotics used: **1)** **2)** **3)**

4) **5)** **6)**

Antibiotics	Zone of inhibition	Interpretation

Performed by: __________ **Checked by:** __________

APPENDIX-II

2.1 MATERIALS REQUIRED

EQUIPMENTS:

1) Biological safety cabinet 2) Incubator 3) Autoclave 4) Hot air oven 5) Refrigerator 6) CO_2 Gas Jar 7) Weighing machine 8) Microscope

MEDIA USED (Hi Media):

1) Chocolate agar 2) Blood agar 3) MacConkey agar 4) Mueller Hinton agar 5) Nutrient agar 6) Biochemical media: Simmons citrate agar, Hugh and Leifson media, Triple Sugar Iron agar, Sulphur Indole Motility, Urea agar

2.2 COMPOSITION AND METHOD OF PREPARATION OF DIFFERENT CULTURE MEDIA, BIOCHEMICAL MEDIA AND REAGENT USED FOR ISOLATION AND SENSITIVITY TESTING OF BACTERIAL ISOLATED FROM SPUTUM SAMPLE

A. CULTURE MEDIA

Different types of culture media such as enrichment media, selective media and differential media were used. Composition and preparation of different types of culture media are given below. All the media used are supplied by Hi Media Company and final pH of all the Medias at 25^0C is 7.4 ± 0.2.

1. NUTRIENT AGAR

Ingredients	gm/liter
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Agar	15.0
Final pH	7.4 ± 0.2

As directed by the manufacturing company, 37 gm powder was suspended in 1000 ml distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes.

2. BLOOD AGAR

The sterilized nutrient agar medium is cooled to 50^0C and 5-10% blood was added aseptically and mixed well before pouring.

Ingredient	gm/liter
Beef heart infusion	50
Tryptose	10
Sodium chloride	5
Agar	17
Final pH	7.4 ± 0.2

Preparation: As directed by the manufacturing company, 40 grams of blood agar base was dissolved in 1000 ml distilled water and heated to dissolve the media. The media was autoclaved at 15lbs pressure at 121^0C for 15 minutes. Thus prepared medium was cooled to about 40-50^0C to which 50 ml of sterile defibrinated blood was added aseptically. The media was poured to Petri plates.

3. CHOCOLATE AGAR

The sterilized blood agar plate was heated at 75^0C in hot air oven for 30 mins until it gave chocolate color.

4. MACCONKEY AGAR

Ingredients	gm/liter
Peptic digest of animal tissue	17.0
Protease peptone	3.0
Lactose	10.0
Bile salt	1.50
Sodium chloride	5.0
Neutral red	0.075
Agar	12.0
Final pH	7.4 ± 0.2

As directed by the manufacturing company, 52 grams powder was suspended in 1000 ml distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes.

5. NUTRIENTS BROTH

Ingredients	gm/liter
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final pH	7.4 ± 0.2

As directed by the manufacturing company, 13 grams powder was dissolved in 1000 ml distilled water and sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes.

6. MANNITOL SALT AGAR

Ingredients	gm/liter
Beef extract	1.0
Protease peptone	10.0
Sodium chloride	75.0
D-mannitol	10
Phenol red	0.025
Agar	15
Final pH	7.4 ± 0.2

Preparation: As directed by the manufacturing company, 111 grams of the medium was dissolved in 1000 ml of distilled water and heated to dissolve the media. The media was autoclaved at 15lbs pressure at 121^0C for 15 minutes.

ANTIBIOTIC SENSITIVITY TESTING MEDIUM

7. MULLER HINTON AGAR

Ingredients	gm/liter
Beef infusion form	300.0
Casein acid hydrolysate	17.5
Starch	1.5
Agar	17.0
Final pH	7.4 ± 0.2

As directed by the manufacturing company, 38 grams powder was suspended in 1000 ml distilled water and then boiled to dissolve completely and the medium was sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes.

B. BIOCHEMICAL MEDIA

1. HUGH AND LEIFSON'S MEDIUM

Ingredients	gm/litre
Peptic digest of animal tissue	2.0
Sodium chloride	5.0
Dipotassium Phosphate	0.3
Bromothymol blue	0.05
Agar	2.0
Final pH	7.4 ± 0.2

As directed by the manufacturing company, 0.94 grams powder was suspended in 1000 ml distilled water. The medium was boiled to dissolve the medium completely. Dispensed in 5 ml amounts tubes and cotton plugged. Then the tubes were sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes.

2. SULFIDE INDOLE MOTILITY (SIM) MEDIA

Ingredients	gm/litre
Peptic digest of animal tissue	30.0
Beef extract	3.0
Peptonized iron	0.2
Sodium thiosulphate	0.025
Agar	3.0
Final pH	7.3 ± 0.2

As directed by the manufacturing company, 36.23 grams powder was suspended in 1000 ml distilled water. The medium was boiled to dissolve the medium completely. Dispensed in 5ml amounts tubes and cotton plugged. Then the tubes were sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes.

3. MR-VP MEDIUM

Ingredients	gm/litre
Buffered peptone	7.0
Dextrose	5.0
Dipotassium phosphate	5.0
Final pH	7.3 ± 0.2

As directed by the manufacturing company, 17.0 grams powder was suspended in 1000 ml distilled water. The medium was boiled to dissolve the medium completely. It was dispensed in 5ml tubes and cotton plugged. Then the tubes were sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes.

4. SIMMONS CITRATE AGAR

Ingredients	gm/litre
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Agar	15
Bromothymol blue	0.08
Final pH	6.8 ± 0.2

As directed by the manufacturing company, 24.2 gram was dissolved in 1000 ml distilled water and boiled to dissolve the medium completely. Medium was distributed about 3 ml in test tubes and sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes. After autoclaving tubes containing medium were titled to form slant.

5. CHRISTENSEN UREA AGAR MEDIUM

Ingredients	gm/litre
Peptic digest of animal tissue	1.0
Dextrose	1.0
Sodium chloride	5.0
Disodium phosphate	1.20
Monopotassium phosphate	0.8
Phenol red	0.012
Agar	15.0
Final pH	6.9 ± 0.2

As directed by the manufacturing company, 24 grams powder was suspended in 950 ml distilled water and sterilized at autoclaving at 115^0C (15 lbs pressure) for 20 minutes. After cooling to about 55^0C, 50 ml of 40% urea was added and mixed well. Then 5 ml was dispensed in test and set at slope position to make agar slant.

6. TRIPLE SUGAR IRON (TSI) AGAR

Ingredients	gm/litre
Peptic digest of animal tissue	10.0
Casein hydrolysate	10.0
Yeast extract	3.0
Beef extract	3.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Sodium chloride	5.0
Ferrous sulphate	0.20
Sodium thiosulphate	0.30
Phenol red	0.024
Final pH	7.4 ± 0.2

As directed by the manufacturing company, 65 grams powder was dissolved in 1000 ml distilled water and boiled to dissolve the medium completely. Medium was distributed about 5ml in test tubes and sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes. After autoclaving tubes containing medium were tilted to form slant with a butt of about 1 inch long.

7. DEOXYROBONUCLEASE (DNase) AGAR MEDIUM

Ingredients	gm/litre
Tryptose	20.0
Deoxyribonuclease	2
Sodium chloride	5.0
Agar	12.0
Final pH	7.3

As directed by the manufacturing company, 3.9 grams powder was suspended in 1000 ml distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes.

C. REAGENTS

1. GRAM'S STAINING REAGENT

a. Crystal violet stain

Composition	gm/liter
Crystal Violet	20
Ammonium oxalate	9
Ethanol (absolute)	95ml
Distilled water	to make 1000 ml

Directions: Twenty grams crystal violet was dissolved in 95 ml absolute alcohol to which 9 gm ammonium oxalate suspended in about 200 ml distilled water was added. Distilled water was added to make final volume to 1000 ml.

b. Lugol's iodine solution

Composition	gm/liter
Potassium iodide	20
Iodine	10
Distilled water	1 liter

Directions: Twenty grams of potassium iodide was dissolved in distilled water and mixed well. To it 10 grams of iodine was added and the volume was adjusted to 1000 ml by adding distilled water.

c. Ethyl alcohol decolouriser

Composition	gm/liter
Absolute alcohol	95 ml
Distilled water	5 ml

Directions: To make 100 ml of 95% ethyl alcohol decolouriser 95 ml of absolute alcohol and 5 ml distilled water was mixed well.

d. Safranine solution

Composition	gm/liter
Safranine 99% dye content	10
Distilled water	1000 ml

Directions: Ten grams of safranine was dissolved in 1000 ml of distilled water and mixed well.

2. TEST REAGENTS

i) Catalase reagent

To make 100 ml

Hydrogen peroxide	3 ml
Distilled water	97 ml

Directions: Three ml of hydrogen peroxide was added to 97 ml distilled water, mixed well and stored away from light.

ii) Oxidase reagent

To make 10 ml

Tetraethyl p-phenylene diamine dihydrochloride	0.1 gm
Distilled water	10 ml

Directions: In 10 ml distilled water, 0.1 gm tetraethyl p-phenylene diamine dihydrochloride (TPD) was dissolved and mixed well. Strips of Whatman's no. 1 filter paper was soaked in the oxidase reagent and dried before storing in a dark bottle tightly.

iii) Methyl red indicator solution

To make 50 ml

Methyl red	0.05 gm
Absolute ethanol	28 ml
Distilled water	22 ml

Directions: In 28 ml ethanol, 0.05 grams of methyl red was dissolved. To it distilled water was added and mixed well.

iv) Barritt's reagent

a. VP reagent A:

To make 100 ml

α-napthol	5 gm
Ethyl alcohol	100 ml

Directions: Five grams of alpha-Napthol was added to 100 ml of ethyl alcohol.

b. VP reagent B:

To make 100 ml

Potassium hydroxide	40 gm
Distilled water	100 ml

Directions: Forty grams of potassium hydroxide was added to 100 ml of distilled water and mixed till dissolved.

1 ml of solution B and 3 ml of solution A was added to test suspension.

v) Kovac's reagent

To make 40 ml

Isoamyl alcohol	30 ml
4- dimethylaminobenzaldehyde	2 gm
Concentrated hydrochloric acid	10 ml

Directions: Two grams of 4- dimethylaminobenzaldehyde was dissolved in 30 ml isoamyl alcohol and to it 10 ml concentrated hydrochloric acid was added and mixed well.

vi) Bile salt solution

Composition

Commercially available Sodium deoxycholate	10 gm
Distilled water	100 ml

Preparation: A 10% solution of Sodium Deoxycholate was prepared by adding 10 gm Sodium Deoxycholate powder in 100 ml distilled water and transferred into a clean bottle.

APPENDIX-III

Gram staining procedure

The test was originally developed by Christian Gram in 1884.The modification currently used for general bacteriology was developed by Hucker in 1921. Gram-stain can be used effectively to divide all bacterial species into two large groups: those that take up the basic dye, crystal violet (Gram-positive) and those that allow the crystal dye to wash out easily with the decolourizer alcohol or acetone (Gram-negative).
The following steps are involved in Gram stain:

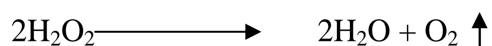
1. A thin film of the material to be examined was prepared and dried.
2. The material on the slide was heat fixed and allowed to cool before staining.
3. The slide was flooded with crystal violet stain and allowed to remain without drying for 30 to 60 seconds.
4. The slide was rinsed with tap water, shaking off excess.
5. The slide was flooded with iodine solution and allowed to remain on the surface without drying for twice as long as the crystal violet was in contact with the slide surface.
6. The slide was rinsed with tap water, shaking off excess.
7. The slide was flooded with acetone alcohol decolorizer for 10 seconds and rinsed immediately with tap water until no further colors flow from the slide with the decolorizer. Thicker smear requires more aggressive decolorizing.
8. The slide was flooded with counter stain (safranine) for 30 seconds and washed off with tap water.
9. The slide was blotted between two clean sheets of blotting paper and examined microscopically under oil immersion at 100X.

APPENDIX-IV

I) Methodology for Bio-Chemical Tests

1. Catalase Test

This test is based on the ability of bacteria to produce catalase, an enzyme that catalyses the rapid release of oxygen from hydrogen peroxide.



$$2H_2O_2 \longrightarrow 2H_2O + O_2 \uparrow$$

Method

With a sterile glass slide a small amount of pure growth from NA was transferred to a clean, grease free slide. To it a drop of 3% hydrogen peroxide solution was added and obsereved for the evolution of gas.

Interpretation

Rapid evolution of gas bubbles indicated positive test.

2. Oxidase Test

It is based on the presence of cytrochrome oxidase in bacteria that oxidizes 1% tetra-methyl-p-phenylene-diamine-dihydrochloride forming end product indophenols which is dark purple in color.

Method

A small amount of growth on agar was picked with a sterile glass rod and rubbed on a moist filter paper impregnated with oxidase reagent or oxidase papers.

Interpretation

If positive, color of filter paper changed from light to dark purple within 10 seconds.

3. Coagulase Test

S. aureus is known to produce coagulase, which can clot plasma into gel in tube or agglutinate cocci in slide. This test is useful in differentiating *S. aureus* from other coagulase-negative staphylococci. Most strains of *S. aureus* produce two types of coagulase, free coagulase and bound coagulase. While free coagulase is an enzyme that is secreted extracellularly, bound coagulase is a cell wall associated protein. Free

coagulase is detected in tube coagulase test and bound coagulase is detected in slide coagulase test. Slide coagulase test may be used to screen isolates of *S. aureus* and tube coagulase may be used for confirmation. While there are seven antigenic types of free coagulase, only one antigenic type of bound coagulase exists. Free coagulase is heat labile while bound coagulase is heat stable.

Reagents and Equipments

Test solution: Discrete bacterial colonies growing on solid medium.

Slide coagulase test: Commercially available plasma (Ethylene diamine-tetraacetic acid, EDTA added).

Tube coagulase test: Commercially available plasma (EDTA added) suitable for tube coagulase. Bacteriological loop (preferably nichrome) or disposable Pasteur pipette

Slide Coagulase Test:

Principle: The bound coagulase is also known as clumping factor. It cross-links and chain of fibrinogen in plasma to form fibrin clots that deposit on the cell wall. As a result, individual coccus sticks to each other and clumping is observed.

Procedure:

• Place a drop of distilled water on a slide

• Emulsify the test strain to obtain a homogenous thick suspension. False negative reactions will occur if the bacterial suspension is not heavy enough

• Observe for auto-agglutination

• Dip a straight wire or loop in the plasma

• Mix gently with the homogenous suspension

NB: Strains which auto-agglutinate must be tested by an alternative procedure.

Positive result: visible clumping within 10 seconds

Negative result: no visible clumping within 10 seconds

Tube Coagulase Test:

Principle: The free coagulase secreted by *S. aureus* reacts with coagulase reacting factor (CRF) in plasma to form a complex, which is thrombin. This converts fibrinogen to fibrin resulting in clotting of plasma.

Procedure: Three test tubes are taken and labeled “test”, “negative control” and “positive control”. Each tube is filled with 0.5 ml of 1 in 10 diluted rabbit plasma. To

the tube labeled test, 0.1 ml of overnight broth culture of test bacteria is added. To the tube labeled positive control, 0.1 ml of overnight broth culture of known *S. aureus* is added and to the tube labeled negative control, 0.1 ml of sterile broth is added. All the tubes are incubated at 37°C and observed up to 4 hours. Positive result is indicated by gelling of the plasma, which remains in place even after inverting the tube. If the test remains negative until 4 hours at 37°C, the tube is kept at room temperature for overnight incubation.

Positive result: formation of a clot up to 4 hours at 37°C or following overnight incubation at 22-25°C

Negative result: no clot, plasma moves freely at 4 hours and 24 hours incubation.

4) Citrate Test

This differential test determines the ability of an organism to use citrate as its sole carbon source and is used to differentiate the Enterobacteriaceae. The uninoculated medium is green. The bromothymol blue pH indicator changes to blue when an organism is able to metabolize the citrate and produce alkaline by-products. Positive: *Klebsiella pneumoniae* and *Enterobacter cloacae*. Negative: *Escherichia coli.*

Theory: The slant is inoculated and bacteria that are able to utilize citrate as a fuel will catabolize the citrate in the medium and release an end product that is basic (alkaline). The indicator Bromthymol blue is blue above pH 7.6 and green at pH values below 7.6. If citrates are utilized, the medium pH will rise and the medium will turn from green to blue. The blue color change is the result: blue is positive, green is negative.

Materials and Method

Simmons Citrate agar	Bacteria	Disinfectant
Loop , Bunsen burner	Incubator	Paper towels

Method: Citrate Agar was aseptically streaked with one of the bacteria was incubated for 48 hours.

5) Triple Sugar Iron Test

The TSI agar contains casein and meat peptones, phenol red as the pH indicator, 0.1% glucose, 1% lactose and 1% sucrose for fermentation. Ferric or ferrous ions and sodium thiosulphate are present to detect hydrogen sulfide production. Organisms that are non-lactose fermenting initially produce a yellow slant due to the production of acid from the glucose. The small amount of glucose is rapidly depleted. Oxidative metabolism continues in the slant after the low concentration of glucose has been depleted, producing an alkaline pH from the aerobic breakdown of peptone; the slant turns red. There is no oxygen penetration into the butt and no oxidative metabolism; the butt remains acid and yellow. Thus, a non-lactose fermenting organism yields an alkaline (K) slant over an acid (A) butt (K/A); red slant; yellow butt). Lactose fermenting and/ or sucrose-fermenting bacteria continue to produce a large amount of acid in the slant and butt so the reaction in both remains acid (A/A; yellow slant; yellow butt). If the slant and butt remain neutral, the organism is not capable of fermenting glucose or other sugars (K/K; red slant; red butt). Gas production from sugar fermentation is indicated by bubbles, fracturing of the medium, or displacement of the medium.

Hydrogen sulfide is produced by the action of the bacteria with sodium thiosulphate. This is detected by the reduction of ferric ions to produce a black precipitate.

Procedure: The test organism was stabbed and streaked on the surface of TSI and incubated at 37ºC for 24 hours. Acid production limited only to the butt region of the tube is indicative glucose utilization, while acid production in slant and butt indicates sucrose or lactose fermentation. The results were interpreted as follows:

a.Yellow (Acid)/ Yellow (Acid), Gas, H_2S ⟶ Lactose/Sucrose fermenter, H_2S producer.

b.Red (Alkaline)/ Yellow (Acid), No gas, No H_2S ⟶ Only Glucose, not Lactose/Sucrose fermenter, anaerogenic, no H_2S production.

c.Red (Alkaline)/ No change ⟶ Glucose, Lactose and Sucrose non fermenter.

d.Yellow (Acid)/ No change ⟶ Glucose oxidizer

6) Indole Test:

Principle: Some bacteria can produce indole from amino acid tryptophan using the enzyme, tryptophanase.

Tryptohan Indole + Pyruvic acid + NH_3

Production of indole is detected using Ehrlich's reagent or Kovac's reagent. Indole reacts with the aldehyde in the reagent to give a red color. An alcoholic layer concentrates the red color as a ring at the top.

Procedure: Bacterium to be tested was inoculated in peptone water, which contained amino acid tryptophan and incubated overnight at $37^{o}C$. Following incubation few drops of Kovac's reagent was added. Kovac's reagent consists of para-dimethyl aminobenzaldehyde, isoamyl alcohol and con. HCl. Formation of a red or pink colored ring at the top was taken as positive.

Example: *Escherichia coli*: Positive; *Klebsiella pneumoniae*: Negative

7) Methyl Red Test:

Principle: This is to detect the ability of an organism to produce and maintain stable acid end products from glucose fermentation. Some bacteria produce large amounts of acids from glucose fermentation that they overcome the buffering action of the system. Methyl Red is a pH indicator, which remains red in color at a pH of 4.4 or less.

Procedure: The bacterium to be tested was inoculated into glucose phosphate broth, which contained glucose and a phosphate buffer and incubated at $37^{o}C$ for 48 hours. Over the 48 hours the mixed-acid producing organism produced sufficient acid to overcome the phosphate buffer and remained acid. The pH of the medium was tested by the addition of 5 drops of MR reagent. Development of red color was taken as positive. MR negative organisms produced yellow color.

Example: *Escherichia coli*: Positive; *Klebsiella pneumoniae*: Negative

8) Voges Proskauer Test:

The voges-Proskauer (VP) test is used to determine if an organism produces acetyl-methyl-carbinol, a neutral end product (acetoin) or its reduction product 2, 3

butanediol during fermentation of carbohydrates. If present, acetyl-methyl-carbinol is converted to diacetyl in the presence of -naphthol, strong alkali (40% KOH), and atmospheric oxygen. The -naphthol is not the part of the original procedure but is found to act as a color intensifier by Barrit and must be added first. The diacetyl and quanidine-containing compounds found in the peptones of the broth then condense to form a pinkish red polymer. An organism of the enterobacterial group is usually either Methyl red positive, Voges-proskauer negative or Methyl red negative, Voges-proskauer positive. The Voges proskauer test for acetoin is used primarily to separate *E.coli* from *Klebsiella* and *Enterobacter* species.

Procedure: A pure colony of the test organism was inoculated into 2 ml of MR-VP medium and was incubated at 37ºC for 24 hours. After incubation, about 5 drops of Barritt's reagent was added and shaken well for maximum aeration and kept for 15 minutes, positive test is indicated by the development of pink red color.
Positive (pink-intense red): *Enterobacter, Klebsiella pneumoniae*
Negative (no color change): *Escherichia*, *Citrobacter*

9) Oxidation Fermentation Test.

This method (Hugh & Leifson 1953) depends upon the use of a semi-solid tubed medium containing the carbohydrate (usually glucose) together with a pH indicator. If acid is produced only at the surface of the medium, where conditions are aerobic, the attack on the sugar is oxidative. If acid is found throughout the tube, including the lower layers where conditions are anaerobic, the breakdown is fermentative.

Procedure: The test organism was stabbed into the bottom of two sets of tubes with Hugh and Leifson's media, bromothymol blue being the pH indicator. The inoculated medium in one of the tubes was covered with a 10 mm deep layer of sterile paraffin oil. The tubes were then incubated at 37ºC for 24 hours. After incubation the tubes were examined for carbohydrate utilization as shown by yellow color of media that denotes the acid production. Fermentative organism utilizes the carbohydrate in both the open and sealed tubes as shown by a change in color of the medium from green to yellow. Oxidative organisms, however, are able to use the carbohydrate only in the open tube.

Results:

Gram-negative rod reactions:

Oxidation…. Acid in aerobic tube only (yellow color in aerobic tube, green in anaerobic)

Fermentation….. Acid in both tubes (yellow color)

Neither fermentation nor oxidation….. No acid production (green color in aerobic tube, purple in anaerobic tube)

Gram-positive cocci reactions:

Oxidation…. Acid in aerobic tube only (yellow color in aerobic tube, purple in anaerobic)

Fermentation….. Acid in both tubes (yellow color)

Neither fermentation nor oxidation….. No acid production (purple color in both tubes)

10) Motility Test Medium

Semi-solid Motility Test medium was used to detect motility. For this SIM medium was inoculated by stabbing culture on loop straight into agar and incubated at 37ºC for 48 hours. The agar concentration (0.3%) is sufficient to form a soft gel without hindering motility. When a non-motile organism is stabbed into Motility Test medium, growth occurs only along the line of inoculation and the surrounding media remains colorless and clear. Growth along the stab line is very sharp and defined. When motile organisms are stabbed into the soft agar, they swim away from the stab line. Growth occurs throughout the tube rather than being concentrated along the line of inoculation. Growth along the stab line appears much more cloud-like fuzzy growth as it moves away from the stab.

II) Bile Solubility Test.

Introduction

The test is used specifically to differentiate between *S. pneumoniae* (bile soluble) and other -haemolytic streptococci (not bile soluble). The bile solubility test is used to determine the ability of bacterial cells to lyse in the presence of bile salts, within a specified time. *S. pneumoniae* possesses an autolytic enzyme which lyses the

cell's own wall during division. The addition of bile salts (sodium deoxycholate) activates the autolytic enzyme and the organisms rapidly autolyse. Other -haemolytic streptococci do not possess such an active system and therefore do not dissolve in bile.

Broth procedure

- A heavy suspension of a pure culture was prepared in 1.0 mL of 0.85% saline
- The suspension was divided in two tubes (one test and one control)
- 0.5 mL of 10% sodium deoxycholate was added to the test suspension and 0.5 mL of 0.85% saline to the control
- Both suspensions were gently mixed and incubated at 37°C for up to 15 minutes
- Evidence of clearing of turbidity in the tube marked test compared with the saline control was examined

Positive result: Suspension cleared in tube labelled test and remained turbid in control tube

Negative result: Suspension remained turbid in both tubes

III) Bacitracin Sensitivity

Beta-haemolytic streptococci are a diverse group of organisms. Lancefield's group A is the frequent human pathogen that causes classical acute suppurative spreading infections and sequelae (post-streptococcal diseases). Maxted introduced sensitivity to the antibiotic bacitracin as a presumptive test for the identification of Lancefield group A streptococci.

Disc containing 0.04 IU of bacitracin was placed in the Blood Agar streaked with the test organism and the plate was incubated at 37°C for overnight. After incubation, the plate was observed for the large zone of inhibition around the disk as given by group A beta haemolytic streptococci whereas most streptococci are resistant.

This test also assists in the screening of *H. influenzae* from the primary agar plate. A 10 unit Bacitracin suppresses the growth of Viridans streptococci, Niesseriae, Diptheroid bacilli and Staphylococci. But *H. influenzae* is resistant to the 10 unit Bacitracin.

A paper disk containing 10U of bacitracin was placed in the primary inoculums of a chocolate agar plate streaked with the material from the specimen and the plate was incubated at 37°C in candle jar for overnight, after incubation the plate was observed for the colonies of *H. influenzae* growing near the bacitracin disk.

IV) Optochin Sensitivity Test

This test is used to determine an organism's susceptibility to the chemical optochin (ethyl hydrocupreine hydrochloride) for the presumptive identification of *S. pneumoniae*. Optochin (ethyl hydrocupreine hydrochloride), a quinine derivative, has a detergent-like action and causes selective lysis of pneumococci.

A sterile Bacto Optochin Disc (5 µg) was placed on the first sector of an isolation plate (BA) before incubation. A zone of inhibition (area with no growth) of 14 mm. or more in diameter was seen around the disk after incubation if the organism was *Streptococcus pneumoniae*. Other alpha-hemolytic streptococci were resistant to (not killed by) optochin.

V) Mannitol Salt Agar (MSA).

MSA is both selective and differential and is used to differentiate *Staphylococcus* species from each other and from *Micrococcus* species. Because the medium contains 7.5% salt, it selects for organisms that can grow in a high salt content. Additionally, a pH indicator determines if an organism is able to ferment mannitol. Yellow indicates an acidic pH change, which is a positive indicator for mannitol fermentation. Positive growth (salt tolerance): *Staphylococcus*. Negative growth (salt intolerance): *Micrococcus.* Positive mannitol fermentation (yellow): *S. aureus*. Negative mannitol fermentation (no change in color): *S. epidermidis*, coagulase negative Staphylococci.

VI) Urease Test

The urease test is used to differentiate urease-positive *Proteus* species from other members of the Enterobacteriaceae. Urea medium, whether a broth or agar, contains urea and pH indicator phenol red. Many organisms, especially those that infect the urinary tract, have a urease enzyme, which is able to split urea in the

presence of water to release two molecules of ammonia and carbon dioxide. The ammonia combines with the carbon dioxide and water to form ammonium carbonate, which turns the medium alkaline, turning the indicator from its original orange-yellow color to bright pink.

Procedure: The test organism was inoculated in a medium containing urea and the indicator phenol red. The inoculated medium was incubated at 37ºC overnight. Positive organism showed pink red color due to the breakdown of urea to ammonia. With the release of ammonia the medium becomes alkaline as shown by a change in color of the indicator to pink.

VII) DNase Test

DNase agar is a differential medium that tests the ability of an organism to produce an exoenzyme, called deoxy ribonuclease or DNase that hydrolyzes DNA. When the DNA is broken down, it no longer binds to the methyl green, and a clear halo will appear around the areas where the DNase-producing organism has grown. This test is used to identify *S. aureus* which produce deoxyribonuclease enzymes. This test is particularly useful when plasma is not available to perform a coagulase test or when results of a coagulase test are difficult to interpret.

Deoxyribonuclease hydrolyses deoxyribonucleic acid (DNA). The test organism was cultured on a medium which contains DNA. After overnight incubation, the colonies were tested for DNAse production by flooding the plate with a weak hydrochloric acid solution. The acid precipitates unhydrolysed DNA. DNAse producing colonies are therefore seen clear areas surrounding colonies due to DNA hydrolysis.

VIII) Satellitism Test

H. influenzae is a fastidious organism requiring media containing haemin (X-factor) and Nicotinamide adenine dinucleotide (NAD, V-factor). Growth occurs on chocolate agar because NAD (V-factor is released during heating process in the preparation of chocolate agar. Haemin is available from non-haemolyzed as well as haemolyzed cells. Both X and V factor are available in chocolate agar. The amount of V factor in blood agar is usually suboptimal. If, however *S. aureus* which produces V

factor in excess of its own needs is cultured on a blood agar plate with *H. influenzae*, the factor V and the haemin released by Staphylococcal haemolysin help the growth of *H. influenzae*. This help given by *S. aureus*, forms the basis of the satellitism test which is a simple way of recognizing *H. influenzae.*

Firstly, with the help of straight wire from the pure culture of haemophilus few colonies were streaked horizontally on a blood agar plate. Then from pure culture of *S. aureus* few colonies were streaked vertically and the plate was incubated at 37°C in a CO_2 enriched atmosphere for overnight. After incubation the culture was examined for growth and satellite colonies in the neighborhoods of Staphylococcal colonies.

IX) X, V and XV factors discs test

H. influenzae was also identified by its requirements for factors X and V using factor identification discs. Saline suspension of the test organism from a primary culture was made and inoculated it on Nutrient agar plate known to lack factors X and V. Then the factors discs were placed 10-20 mm from the side of the plate, positioning each disc as follows:

Factor X……………..at 12 o'clock
Factor V……………..at 4 o'clock
Factor XV…………...at 8 o'clock

Control plate was also made using a known *H. influenzae* and both plates were incubated for overnight in a moist carbondioxide atmosphere at 37°C and examined for the growth around disc. *H. influenzae* growth was obtained around factor XV while slight growth was obtained between X and V.

APPENDIX-V

Disc Diffusion Susceptibility Methods

Introduction

When a filter paper disc impregnated with a chemical is placed on agar the chemical will diffuse from the disc into the agar. This diffusion will place the chemical in the agar only around the disc. The solubility of the chemical and its molecular size will determine the size of the area of chemical infiltration around the disc. If an organism is placed on the agar it will not grow in the area around the disc if it is susceptible to the chemical. This area of no growth around the disc is known as a "zone of inhibition".

Principle

When performing these tests certain things are held constant so only the size of the zone of inhibition is variable. Conditions that must be constant from test to test include the agar used, the amount of organism used, the concentration of chemical used, and incubation conditions (time, temperature, and atmosphere).

The amount of organism used is standardized using a turbidity standard. This may be a visual approximation using a McFarland standard 0.5 or turbidity may be determined by using a spectrophotometer (optical density of 1.0 at 600 nm).
For antibiotic susceptibility testing the antibiotic concentrations are predetermined and commercially available.

Antibiotic susceptibility testing

Kirby-Bauer method was used for antibiotic susceptibility test with following procedures:
1. Preparation of plates:

The agar plates were prepared in a way to make the thickness of medium of about 4 mm.

2. Preparation of inoculum:

For inoculum preparation, 3-4 pure culture colonies were transferred into a test-tube containing 2-3 ml of nutrient broth and were incubated at 37^0C for 2-4 hrs to obtain turbidity. The turbidity of the actively growing broth culture is adjusted with sterile saline or broth to obtain turbidity optically comparable to that of the 0.5 McFarland standards. This results in a suspension containing approximately 1 to 2 x 10^8 CFU/ml for *E.coli* ATCC 25922. To perform this step properly, either a photometric device can be used or, if done visually, adequate light is needed to visually compare the inoculums tube and the 0.5 McFarland standard against a card with a white background and contrasting black lines.

3. Inoculation:

A sterile cotton swab was dipped into the turbid solution and was streaked (by means of swabbing) on the agar surface of MHA plate. The plate was then left 3-5 mins at room temperature to dry the inoculum.

4. Application of the discs:

Using a sterile forcep, antibiotic discs were carefully placed on the agar surface of the plate with certain distance in between the discs so that the zones of inhibition do not get overlapped. Finally, the discs were pressed gently in order to make contact with media surface and the plate was left in the room temperature for 15 mins.

5. Incubation:

The plates were then incubated at 37^0C for overnight.

6. Reading of zone and its interpretation:

After incubation, the inhibition zones formed were measured and the results were interpreted on the basis of standard interpretative table given by Hi-Media Laboratories Pvt. Limited (based on NCCLS guidelines).

APPENDIX VI

ZONE SIZE INTERPRETATIVE CHART FOR ANTIBIOTIC SENSITIVITY TEST

Antibiotic	Strength	Diameter of zone of inhibition		
		Resistant	Intermediate	Sensitive
Amikacin	30mcg	14	15-16	17
Ciprofloxacin	5mcg	12	13-17	18
Cefotaxime	30mcg	14	15-22	23
Chloramphenicol	30mcg	12	13-17	18
Cortimoxazole	25mcg	10	11-15	16
Erythromycin	15mcg	13	14-22	23
Gentamicin	10mcg	12	13-14	15
Cephalexin	30mcg	13	14-18	19
Levofloxacin	5mcg	12	13-16	17
Cloxacillin	5mcg	14	15-18	19
Ampicillin	10mcg	-	-	15

Note: mcg = micro-gram

Source: Product Information Guide, Hi-Media Laboratories Pvt. Limited, Mumbai, India.