APPENDIX I

LIST OF EQUIPMENTS AND MATERIALS USED DURING THE STUDY

A. EQUIPMENTS

Hot air oven (Ambassador)
Incubator (Ambassador)
Autoclave
Microscope (Olympus CX21)
Centrifuge (Remi)
Water bath (Ocean medical technologies)
Micropipettes
Refrigerator (Meiling, & Sanyo)
Semi automated biochemical analyzer (Italy, Model-Clin check plus, Code: RM2020 serial n.7877)
Weighing Machine

B. MICROBIOLOGICAL MEDIA

Blood agar base
MacConkey agar
MR-VP medium
Mueller Hinton agar
Nutrient agar
Mueller Hinton broth
Simmons Citrate agar
Sulphur Indole Motility agar
Triple Sugar Iron agar
Urea broth

C. CHEMICALS AND REAGENTS

3%Hydrogen peroxide
Barritt's reagent
Crystal violet
Kovac's reagent
Gram's Iodine
Barium chloride
Absolute alcohol (95%)
Normal saline
GOD-POD reagent
Safranine
Sulphuric acid
Normal saline
Pyragallol reagent
Turk's reagent

D. ANTIBIOTIC DISCS

All the antibiotics discs used for the susceptibility tests were from Hi-Media Laboratories Pvt. Limited, Bombay, India. The antibiotics used were as follows

Cloxacillin (1mcg)
Amikacin (30mcg)
Cotrimoxazole (1.25/23.75mcg)
Cefotaxime (30mcg)
Erythromycin (15mcg)
Ceftriazone (30mcg)
Chloramphenicol (30mcg)
Ofloxacin (5mcg)
Ciprofloxacin (5mcg)
Penicillin (10 mcg)
Ceftazidime (30mcg)

E. IDENTIFICATION DISCS/OTHER DISCS

Bacitracin, Optochin

F. MISCELLANEOUS

Conical flasks, Cotton, Distilled water, Droppers, Forceps, Glass slides and cover slips, Immersion oil, Inoculating loop, Inoculating wire, Lysol, Measuring cylinder, Petri dishes, Pipettes, Plastic containers, Spatula, Test tubes, Wooden applicator sticks, Test Tube Rack, Micropipette tip, Forceps, Tissue paper.

APPENDIX II

A. COMPOSITION AND PREPARATION OF DIFFERENT CULTURE MEDIA

The culture media used were from:

Hi-Media Laboratories Pvt. Limited, Bombay, India.

(All compositions are given in grams per liter and at 25^0C temperature)

1. Blood agar base (Hi Media Laboratories)

Ingredients	**gm/L**
Protease peptone	15.0
Liver extract	2.5
Yeast extract	15.0
Sodium Chloride	5.0
Agar	15.0
Final pH (at 25^0C) 7.4±0.2	

Blood agar base (infusion agar) + 5-10% sheep blood

Direction: 42.50 grams of the blood agar base medium was suspended in 1000 ml distilled water, dissolved by boiling and sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes. After cooling to 45-50^0C, 7% sterile defibrinated sheep blood was added aseptically, then mixed with gentle rotation and immediately poured in sterile petriplates.

2. MacConkey Agar (Hi Media Laboratories)

Ingredients	**gm/L**
Peptic digest of animal tissue	1.5
Casein enzymic hydrolysate	1.5
Pancreatic digest of gelatin	17.0
Lactose	10.0
Bile salts	1.50
Sodium chloride	5.0
Crystal violet	0.001
Neutral Red	0.03
Agar	15.0
Final pH (at 25^0C) 7.1±0.2	

Direction: 51.5 grams of the medium was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes. The medium was poured into sterile petriplates.

3. Mueller Hinton Agar (Hi Media Laboratories)

Ingredients	**gm/L**
Beef, Infusion form	300.0
Casein Hydrolysate	17.5
Starch	1.5
Agar	17.0
Final pH (at 25^0C) 7.4±0.2	

Direction: 38 grams of the medium was suspended in 1000 ml distilled water and the medium was warmed to dissolve completely. It was sterilized by autoclaving at 121ºC (15 lbs pressure) for 15 minutes and poured into sterile petriplates.

4. Nutrient Agar (Hi Media Laboratories)

Ingredients	**gm/L**
Peptic digest of animal tissue	5.0
Sodium Chloride	5.0
Beef Extract	1.5
Yeast Extract	1.5
Agar	15.0
Final pH (at 25^0C) 7.4±0.2	

Direction: 28 grams of the medium was suspended in 1000 ml of 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. Mueller Hinton Broth (Hi Media Laboratories)

Ingredients	**gm/L**
Beef, Infusion form	300.0
Casein Hydrolysate	17.5
Starch	1.5
Final pH (at 25^0C) 7.4±0.2	

Direction: 21 grams of the medium was dissolved in 1000 ml of distilled water, boiled and dispensed into small containers. It was then sterilized by autoclaving at 121˚C for 15 minutes.

6. Nutrient Broth (Hi Media Laboratories)

Ingredients	**gm/L**
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.50
Yeast extract	1.50
Final pH (at 25°C) 7.4±0.2	

Direction: 13 grams of the medium was dissolved in 1000 ml of distilled water, boiled and dispensed into small containers. It was then sterilized by autoclaving at 121˚C for 15 minutes.

B. COMPOSITION AND PREPARATION OF DIFFERENT BIOCHEMICAL TESTS MEDIA

1. MR-VP Medium (Hi-Media laboratories)

Ingredients	**gm/L**
Peptone	5.0
Dextrose	5.0
Dipotassium Phosphate	5.0
Final pH (at 25^0C) 6.9±0.2	

Direction: 15 gm powder was dissolved in 1000 ml of distilled water & mixed well. 3 ml of medium was distributed in each test tube and autoclaved at 121^0C for 15 minutes

2. Sulphide Indole Motility (SIM) medium (Hi Media Laboratories)

Ingredients	**gm/L**
Beef exract	3.0
Peptone	30.0
Peptonized iron	0.2
Sodium Thiosulphate	0.25
Agar	3.0
Final pH (at 25°C) 7.3±0.2	

Direction: 36 grams of the medium was suspended in 1000 ml of distilled water and dissolved completely. Then it was distributed in tubes to a depth of about 3 inches and sterilized by autoclaving at 121ºC for 15 minutes.

3. Simmon's Citrate Agar (Hi Media Laboratories)

Ingredients	**gm/L**
Magnesium Sulphate	0.2
Mono-ammonium phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Agar	15.0
Bromothymol Blue	0.08
Final pH (at 25^0C) 6.8±0.2	

Direction: 24.2 grams of the medium was dissolved in 1000ml of distilled water. 3ml medium was distributed in test tubes and sterilized by autoclaving at 121^0C for 15 minutes. After autoclaving, tubes containing medium were tilted to form slant.

4. Triple Sugar Iron Agar (TSI) (Hi Media Laboratories)

Direction: 65 grams of the medium was dissolved in 1000ml of distilled water and sterilized by autoclaving at 15 lbs (121^0C) pressure for 15 minutes. The medium was allowed to set in sloped form with a butt about 1 inch of length.

Ingredients	**gm/L**
Peptone	10.0
Tryptone	10.0
Yeast extract	3.0
Beef extract	3.0
Dextrose	1.0
Lactose	10.0
Ferrous sulphate	0.2
Sodium chloride	5.0
Sodium thiosulphate	0.3
Phenol red	0.024
Agar	12.0
Final pH (at 25^0C) 7.4±0.2	

Direction: 65 grams of the medium was dissolved in 1000ml of distilled water and sterilized by autoclaving at 15 lbs (121^0C) pressure for 15 minutes. The medium was allowed to set in sloped form with a butt about 1 inch of length.

5. Urea Base Agar

Ingredients	**gm/L**
Peptone	1.0
Dextrose	1.0
Sodium chloride	5.0
Dipotassium phosphate	1.2
Monosodium phosphate	0.8
Phenol red	0.012
Agar	15.0
Final pH (at 25^0C) 7.4±0.2	

Direction: 24 grams of the medium was suspended in 950 ml of distilled water and sterilized by autoclaving at 121^0C for 121 minutes. After cooling to about 45^0C, 50 ml of 40% urea solution was added aseptically, mixed well and distributed 5 ml amount in sterile test tubes.

C. COMPOSITIN AND PREPARATION OF DIFFERENT STAINING AND TESTS REAGENTS

1. For Gram's Stain

(a) Crystal Violet solution

Crystal Violet	20.0g
Ammonium Oxalate	9.0g
Ethanol or Methanol	95ml
Distilled Water (D/W) to make 1 Liter	

Direction: In a clean piece of paper, 20 gm of crystal violet was weighed and transferred to a clean brown bottle. Then, 95 ml of ethanol was added and mixed until the dye was completely dissolved. To the mixture, 9 gm of ammonium oxalate dissolved in 200 ml of D/W was added. Final volume was made 1 liter by adding D/W.

(b) Lugol's iodine

Potassium Iodide	20.0g
Iodine	10.0g
Distilled water	1000ml

Direction: To 250 ml of D/W, 20 gm of potassium iodide was dissolved. Then 10 gm of iodine was mixed to it until it was dissolved completely. Final volume was made 1 litre by adding D/W.

(c) Acetone-Alcohol Decoloriser

Acetone	500 ml
Ethanol (Absolute)	475 ml
Distilled Water	25 ml

Direction: To 25 ml D/W, 475 ml of absolute alcohol was added, mixed and transferred into a clean bottle. Then immediately, 500 ml acetone was added to the bottle and mixed well.

(d) Safranin (Counter Stain)

Safranin	10.0 g
Distilled Water	1000 ml

Direction: In a clean piece of paper, 10 gm of safranin weighed and transferred to a clean bottle. Then 1 liter D/W was added to the bottle and mixed well until safranin dissolved completely.

2. Normal saline

Sodium Chloride	0.85 g
Distilled Water	100 ml

Direction: The sodium chloride was weighed and transferred to a leak-proof bottle premarked to hold 100 ml. Distilled water was added to the 100 ml mark, and mixed until the salt was fully dissolved. The bottle was labeled and stored at room temperature.

3. Biochemical Test Reagents

(a) Catalase Reagent (For Catalase test)

Hydrogen peroxide	3 ml
Distilled Water	97 ml

Direction: To 97 ml of D/W, 3 ml of hydrogen peroxide was added and mixed well.

(b) Oxidase Reagent (impregnated in a Whatman's No. 1 filter paper) (For Oxidase Test)

Tetramethyl *p*-phenylene diamine dihydrochloride (TPD)	1 gm
Distilled Water	100 ml

Direction: This reagent solution was made by dissolving 1 gm of TPD in 100 ml D/W. To that solution strips of Whatman's No. 1 filter paper were soaked and drained for about 30 seconds. Then these strips were freeze dried and stored in a dark bottle tightly sealed with a screw cap.

(c) Kovac's Indole Reagent (For Indole Test)

Isoamyl alcohol	30 ml
p-dimethyl aminobenzaldehyde	2.0 g
Conc. Hydrochloric acid	10 ml

Direction: In 30 ml of isoamylalcohol, 2 g of *p*-dimethyl aminobenzaldehyde was dissolved and transferred to a clean brown bottle. Then to that, 10 ml of conc. HCl was added and mixed well.

(d) Methyl Red Solution (For Methyl Red Test)

Methyl red	0.05 g
Ethyl alcohol (absolute)	28 ml
Distilled Water	22 ml

Direction: To 28 ml ethanol, 0.05 gm of methyl red was dissolved and transferred to a clean brown bottle. Then 22 ml D/W was added to that bottle and mixed well.

(e) Barritt's Reagent (For Voges-Proskauer Test)

Solution A	
α-naphthol	5.0 g
Ethyl alcohol (absolute)	100 ml

Direction: To 25 ml ethanol, 5 g of α-naphthol was dissolved and transferred into a clean brown bottle. Then the final volume was made 100 ml by adding D/W.

Solution B	
Potassium hydroxide	40.0 g
Distilled Water	1000 ml

Direction: To 25 ml D/W, 40 gm of KOH was dissolved and transferred into a clean brown bottle. Then the final volume was made 100 ml by adding D/W.

(f) Turk's Reagent

Glacial acetic acid	1.5ml
Distilled water	98.5ml

Direction: To 98.5 ml of distilled water, 1.5ml of Glacial acetic acid was added and mixed well. Then 2-3 drops of Crystal violet was added.

APPENDIX III

GRAM-STAINING PROCEDURE

First devised by Hans Christian Gram during the late 19th century, the 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 was out easily with the decolorizer alcohol or acetone (Gram-negative). The following steps are involved in Gram-stain:

A thin film of the material to be examined was prepared and dried.

The material on the slide was heat fixed and allowed to cool before staining.

The slide was flooded with crystal violet stain and allowed to remain without drying for 1 minute.

The slide was rinsed with tap water, shaking off excess.

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.

The slide was rinsed with tap water, shaking off excess.

The slide was flooded with alcohol acetone decolorizer for 10 seconds and rinsed immediately with tap water until no further color flows from the slide with the decolorizer. Thicker smear requires more aggressive decolorizing.

The slide was flooded with counter-stain (safranine) for 1 minute and washed off with tap water.

The slide was blotted between two clean sheets of bibulous paper and examined microscopically under oil immersion at 100X.

APPENDIX IV

A. MEASUREMENT OF CSF PROTEIN

The patients CSF were received in the laboratory. Two tubes were taken one for Blank and labelled as “B”, and other for test labelled as “T”. 2ml of Pyragallol solution was pipette in both tubes. 40µL of CSF was put into tube labelled “T”. Both tubes were incubated for 5 minutes at 37°C Waterbath. The Blank was set in a Semi-Automatic Analyzer. Then the reading was taken at 600nm.

B. MEASUREMENT OF CSF GLUCOSE

The CSF sample from patient was received in laboratory. Two tubes were taken and levelled as “B” for Blank and “T” for Test. 2ml of GOD-POD reagent was pipette into both tubes. 20µL of CSF was put into Test Tube “T”. Both tubes were incubated for 10min at 37°C Waterbath. Then the reading was taken at 520nm, first inserting Blank and then Test as directed by analyzer.

C. MEASUREMENT OF BLOOD GLUCOSE

Blood sample was collected in a tube in the laboratory. The tube was allowed to incubate at 37°C in waterbath for 5 minutes. The tube was centrifuged at 3000 rpm for 5min. Supernatant was collected. Two tubes labelled “T” for Test and “B” for Blank was taken. 2ml of GOD-POD reagent was pipette into both tubes. 20µL of serum supernatant so collected was put into Test Tube “T”. Both tubes were incubated for 10min at 37°C Waterbath. Then the reading was taken at 520nm, first inserting Blank and then Test as directed by analyzer.

APPENDIX V

METHODOLOGY OF BIOCHEMICAL TESTS USED FOR IDENTIFICATION OF BACTERIA

A. Catalase test

This test is performed to demonstrate the presence of catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide.During aerobic respiration, in the presence of oxygen, microorganisms produces hydrogen peroxide, which is lethal to the cell itself. The enzyme catalase is present in most cytochrome containing aerobic and facultative anaerobic bacteria, the main exception being *Streptococcus* spp.

Procedure: A small amount of a culture from Nutrient Agar plate was taken in a clean glass slide and about 2-3 drops of 3% H_2O_2 was put on the surface of the slide. The positive test is indicated by the formation of active bubbling of the oxygen gas. A false positive reaction may be obtained if the culture medium contains catalase (e.g. Blood Agar) or if an iron wire loop is used.

B. Oxidase test

This test is performed for the detection of cytochrome oxidase in bacteria which catalyzes the transport of electrons between electron donors. In the presence of redox dye Tetramethyl-*p*-phenylene diamine dihydrochloride, the cytochrome oxidase oxidizes it into a deep purple colored end product Indophenol which is detected in the test. The test is used for screening species of *Neisseria, Alcaligenes, Aeromonas, Vibrio, Campylobacter* and *Pseudomonas* which give positive reactions and for excluding the Enterobacteriaceae, all species of which give negative reactions.

Procedure: A piece of filter paper was soaked with few drops of oxidase reagent (Whatman's No. 1 filter paper impregnated with 1% tetramethyl-p-phenylene diamine dihydrochloride). Then the colony of the test organism was smeared on the filter paper. The positive test is indicated by the appearance of blue-purple color within 10 seconds.

C. Indole Production test

This test detects the ability of the organism to produce an enzyme: 'tryptophanase' which oxidizes tryptophan to form indolic metabolites: indole, skatole (methyl indole) and indole acetic acid. The enzyme tryptophanase catalyses the deamination reaction attacking the tryptophan molecule in its side chain and leaving the aromatic ring intact in the form of indole.

Procedure: A smooth bacterial colony was stabbed on SIM (Sulphide Indole Motility) medium by a sterile stab wire and the inoculated media was incubated at 37°C for 24 hours. After 24 hours incubation, 2-3 drops of Kovac's reagent was added. Appearance of red color on the top of media indicates indole positive. Indole if present combines with the aldehyde present in the reagent to give a red color in the alcohol layer. The color reaction is based on the presence of the pyrrole structure present in indole.

D. Methyl Red test

This test is performed to test the ability of an organism to produce and maintain stable acid end product from the fermentation of glucose to give a red color with the indicator methyl red and to overcome the buffering capacity of the system. Medium used in the study was Clark and Lubs medium (MR/VP broth, pH 6.9). Methyl red is an indicator which is already acid and will denote changes in degree of acidity by color reactions over a pH range of 4.4- 6.0.

Procedure: A pure colony of the test organism was inoculated into 2 ml of MRVP medium and was incubated at 37°C for 24 hours. After incubation, about 5 drops of methyl red reagent was added and mixed well. The positive test was indicated by the development of bright red color, indicating acidity and negative with yellow color.

E. Voges-Proskauer (VP) test

The principle of this test is to determine the ability of some organisms to produce a acetyl methyl carbinol, a neutral end product (acetoin) or its reduction product 2, 3-butanidiol during fermentation of carbohydrates. An organism of the Enterobacteriaceae group is usually either methyl red positive and Voges- proskauer- negative or methyl red negative and 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 MRVP medium and was incubated at 37°C 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.

F. Citrate Utilization test

This test is performed to detect whether an organism utilizes citrate as a sole source of carbon for metabolism with resulting alkalinity. The medium used for citrate fermentation (Simmon’s Citrate medium) also contains inorganic ammonium salts. Organisms capable of utilizing citrate as its sole carbon source also utilizes the

ammonium salts present in the medium as its sole nitrogen source, the ammonium salts are broken down to ammonia with resulting alkalinity.

Procedure: A loopful of test organism was streaked on the slant area of Simmon's Citrate Agar medium and incubated at 37°C for 24 hours. A positive test was indicated by the growth of organism and change of media by green to blue, due to alkaline reaction. The pH indicator bromothymol blue has a pH range of 6.0-7.6, i.e. above pH 7.6; a blue color develops due to alkalinity of the medium.

G. Motility test

This test is done to determine if an organism was motile or non-motile. Bacteria are motile by means of flagella. Flagella occur primarily among the bacilli; however a few cocci forms are motile .Motile bacteria may contain a single flagella. The motility media used for motility test are semisolid, making motility interpretations macroscopic.

Procedure: Motility of organism was tested by hanging drop and cultural method. In cultural method, the test organism was stabbed in the SIM medium and incubated at 37ºC for 48 hours. Motile organisms migrate from the stabline and diffuse into the medium causing turbidity. Whereas non-motile bacteria show the growth along the stabline, and the surrounding media remains colorless and clear.

H. Triple Sugar Iron (TSI) Agar Test.

The TSI agar is used to determine the ability of an organism to utilize specific carbohydrate incorporated in the medium (glucose, sucrose and lactose in concentrations of 0.1%, 1.0% and 1.0% respectively), with or without the production of gas (indicated by cracks in the media as well as an air gap at the bottom of the tube) along with determination of possible hydrogen sulfide production (detected by production of black color in the medium). A pH indicator (phenol red) included in the medium can detect acid production from fermentation of these carbohydrates and it gives yellow reaction at acidic pH, and red reaction to indicate an alkaline surrounding.

Procedure: The test organism was streaked and stabbed on the surface of TSI and incubated at 37°C for 24 hours .Acid production limited only to the butt region of the tube is indicative of glucose utilization, while acid production in slant and butt indicates sucrose or lactose fermentation. The results are 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, not aerogenic, No H_2S production.

c. Red (Alkaline) / No Change → Glucose, Lactose and Sucrose non-fermenter.

d. Yellow (Acid)/ No Change → Glucose- Oxidiser.

e. No Change / No Change →Non-fermenter.

I. Urea Hydrolysis test:

This test demonstrates the urease activity present in certain bacteria which decomposes urea, releasing ammonia and carbon dioxide. Ammonia thus produced changes the color of indicator (phenol red) incorporated in the medium.

Procedure: The test organism was inoculated in a medium containing urea and the indicator phenol red. The inoculated medium was incubated at 37°C overnight. Positive organism shows 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.

J. Coagulase test

This test is used specifically to differentiate species within the genus *Staphylococcus*: *S. aureus* (usually positive) from *S. saprophyticus, S epidermidis* (negative). A positive coagulase test is usually the final diagnostic criterion for the identification of *S. aureus.* Free coagulase and bound coagulase are the two types of coagulase possessed by this organism; most strains possess both free and bound coagulase.

Slide Coagulase Test

Bound coagulase (Clumping Factor) is detected by slide test. The bound coagulase is bound to the bacterial cell wall and reacts directly with fibrinogen. This results in alteration of fibrinogen so that it precipitates on the staphylococcal cell, causing the cells to clump when a bacterial suspension is mixed with plasma.

Procedure: For slide coagulase test, a drop of physiological saline was placed on three places of a slide, and then a colony of the test organism was emulsified in two of the drops to make thick suspensions. Later a drop of plasma was added to one of the

suspensions and mixed gently. Then a clumping was observed within 10 seconds for the positive coagulase test. No plasma was added in second suspension. This was used for the differentiation of any granular appearance of the organism from true coagulase clumping. The third drop of saline was used for a known strain of coagulase positive staphylococci.

Tube Coagulase Test

This test is carried out to detect production of free coagulase. Plasma contains coagulase reacting factor (CRF) which activates free coagulase. The activated coagulase acts upon prothrombin thus converting it to thrombin. Thrombin converts fibrinogen into fibrin which is detected as a firm gel (clot) in the tube test. Tube test is performed when negative or doubtful results are obtained in slide coagulase test.

Procedure: In the tube coagulase test, plasma was diluted 1:10 in physiological saline. Four small tubes were taken, one for test organism, one for positive control, one for negative control, and one to observe self clotting of plasma. Then 0.5 ml of the diluted plasma was pipetted into each tube and 0.5 ml of test organism, 0.5 ml of positive control (*S. aureus* culture), and 0.5 ml negative control (*S. epidermidis* culture) was added to three tubes, to the fourth tube, 0.5 ml sterile broth was added. After mixing gently, all tubes were incubated at 37^0C on a water bath for 6 hours and observed for gel formation in every 30 minutes. The clotting is observed by gently tilting the tube for positive coagulase test.

APPENDIX VI

A. NORMAL VALUES OF CSF GLUCOSE

Age group	mmol/L
Newborn	1.8 - 6.6
45 days-14 years	3.3 - 4.4
Adults (15 years-50 years)	2.2 - 4.4

B. NORMAL VALUES OF CSF PROTEIN

Age group	mg/dL
≤ 1 week	15 - 170 mg/dL
≤ 45 days	15 - 150 mg/dL
46 days-14 years	15 - 40 mg/dL
14 years-60 years	15 - 60 mg /dL
>60 years	15 – 65 mg/dL

C. NORMAL CSF LABORATORY VALUES OF LEUKOCYTES

Age group	Newborn	Adults
Leukocytes (per µL)	0-30	0-10
Predominant cell type (%):	Lymphocytes (63-99%)	
	Monocytes (3-37%)	
	PMN (0-15%)	

(Marshall & Bangert, 1995; & Burtis *et al*., 2006).

APPENDIX VII

CHARACTERISTICS OF BACTERIA ISOLATED FROM CSF SAMPLE

Bacteria	Morphological Characteristics	Cultural Characteristics
Streptococcus pneumoniae	Gram positive ovoid or lanceolate cocci arranged in pairs 1-3µm×0.4-0.7µm size, aerobic and anaerobic, nonsporing, motile, capsulated.	On BA: Raised, circular about 1mm in diameter, grow well when supplemented with CO_2. The colonies are alpha (α) haemolytic. On MA: No growth
Neisseria meningitidis	Gram negative oval or spherical cocci 0.6-0.8µm in size, typically arranged in pairs with adjacent sides flttened, capsulated, nonsporing and non motile rods.	On BA: Smooth, small (about 1mm in diameter) translucent, round, convex, typically lenticular colonies are obtained. On MA: No growth
Escherichia coli	Gram negative rod of 1-3µm×0.4-0.7µm size, aerobic and anaerobic, nonsporing, motile, noncapsulated	On BA: Large 1-4 mm in diameter, grayish white, moist, smooth, convex and opaque. The colonies may appear mucoid and some strains are haemolytic. On MA: Bright pink colonies due to lactose fermentation, smooth, glossy and translucent.
Staphylococcus aureus	Gram positive, spherical cocci, 0.8-1 µm in diameter, non sporing, facultative anaerobe, non-motile, except for rare strains, non capsulated. They are arranged in characteristics grape	On BA: Large, 2-4 mm diameter. Circular, smooth with glistening surface, entire edge, soft butyrous consistency and opaque. The pigmentation is golden yellow to cream coloured.Some strains are beta-haemolytic when grown aerobically.

	like clusters or in small groups, pairs, singles and short chain(less than five cocci in line).	On MA: Small (pin head size), 0.1-0.5mm, pink or pink orange due to lactose fermentation. Some strains are non-lactose fermenting.
Klebsiella spp.	Gram negative, short and thick rods of 1-2µm × 0.8µm size, nonsporing, nonmotile and capsulated.	Large dome shaped moist and usually viscid or mucoid colonies when cultured on BA and MA. Most Klebsiella species are lactose fermenting.
Pseudomonas aeruginosa	Gram negative slender rods with 1.5-3µm x 0.5µm size, actively motile by polar flagellum, non sporing, most of the strains produce slime.	On NA: Large, opaque, irregular colonies with distinctive, musty odor and metallic sheen. On BA: Large flat colonies showing haemodigestion. On MA: Pale, nonlactose fermenting, colorless translucent colonies.

APPENDIX VIII

DISTINGUISHING CHARACTERSTICS OF ENTEROBACTERIACEAE

Species	Test/ substrate											
	lac	mot	gas	ind	VP	cit	PDA	ure	lys	H_2S	inos	ONPG
E. coli	+	+	+	+	-	-	-	-	+	-	-	+
Shigella groups A, B, C	-	-	-	±	-	-	-	-	-	-	-	-
Sh. sonnei	-	-	-	-	-	-	-	-	-	-	-	+
Salmonella (most serotypes)	-	+	+	-	-	+	-	-	+	+	±	-
S. typhi	-	+	-	-	-	-	-	-	+	+	-	-
S. paratyphi A	-	+	+	-	-	-	-	-	-	-	-	-
C. freundii	±	+	+	-	-	+	-	±	-	±	-	+
C. koseri	±	+	+	+	-	+	-	±	-	-	-	+
K. pneumoniae	+	-	++	-	+	+	-	+	+	-	+	+
K. oxytoca	+	-	++	+	+	+	-	+	+	-	+	+
E. aerogenes	+	+	++	-	+	+	-	-	+	-	+	+
E. cloacae	+	+	+	-	+	+	-	±	-	-	-	+
Hafnia alvei	-	+	+	-	+	-	-	-	+	-	-	+
Serratia marcescens[b]	-	+	±	-	+	+	-	-	+	-	±	+
P.mirabilis	-	+	+	-	±	±	+	++	-	+	-	-
P. vulgaris	-	+	+	+	-	-	+	++	-	+	-	-
M. morganii	-	+	+	+	-	-	+	++	-	±	-	-
Providencia rettgeri	-	+	-	+	-	+	+	++	-	-	+	-
P. stuartii	-	+	-	+	-	+	+	±	-	-	+	-
P. alcalifaciens	-	+	+	+	-	+	+	-	-	-	-	-
Yersinia enterocolitica[c]	-	-	-	±	-	-	-	±	-	-	±	+
Y. pestis	-	-	-	-	-	-	-	-	-	-	-	±
Y. pseudotuberculosis	-	-	-	-	-	-	-	+	-	-	-	±

[a] lac, inos, fermentation of lactose, inositol; mot, motility; gas, gas from glucose; ind, indole production; VP, Voges-Proskauer; cit, Citrate utilization (Simmons'); PDA, phenylalanine deaminase; ure, urease; lys, lysine decarboxylase; H_2S, H_2S produced in TSI agar; ONPG, metabolism of *o*-nitrophenyl-β-D-galactopyranoside. [b] Some strains of Serratia marcescens may produce a red pigment [c] *Yersinia* are motile at 22°C.{Key: +, ≥85% of strains positive; -, ≥ 85% of strains negative; 16-84% of strains are positive after 24-48 hour at 36°C}
(Source: Collee *et al.,* 1996)

APPENDIX IX

TABLE: GUIDELINES FOR THE INTERPRETATION OF RESULTS FOLLOWING HEMATOLOGICAL AND CHEMICAL ANALYSIS OF CSF FROM CHILDREN AND ADULTS

Clinical Setting	Appearance	Leukocytes per mm^3	Predominant cell type	Protein (mg/dL)	Sugar (mg/dL)
Normal	Clear and colorless	0-5	-	15-45	45-80
Purulent (pyogenic)meningitis	Cloudy or purulent, may contain clots	5-20000 (mean of 800)	Mostly Neutrophils	Elevated (>100)	Reduced
Viral (aseptic) meningitis	Clear or slightly cloudy	2-2000 (mean of 80)	Mostly lymphocytes	Normal or slightly elevated	Normal or slightly reduced
Tuberculous meningitis	Clear or slightly cloudy, fine clot may form	5-2000 (mean of 100)	Mostly lymphocytes	Elevated (>50)	Reduced
Cryptococcal meningitis	Clear or slightly cloudy	5-2000 (mean of 100)	Mostly lymphocytes	Elevated (>50)	Reduced
Leptospiral meningitis	Clear or cloudy	> 25	Mostly lymphocytes	Elevated (>50)	Normal
L. monocytogenes meningitis	Slightly cloudy or purulent	>200	Mostly lymphocytes	Elevated (>50)	Reduced
Neurosyphilis	Usually clear, may contain fine clots	>20	Mostly lymphocytes	Normal or slightly elevated	Normal or slightly reduced

(Source:- Cheesbrough, 1984; Baron *et al.*, 1994).

APPENDIX X

CALCULATION OF SPECIFICITY AND POSITIVE PREDICTIVE VALUE

A. Value of sugar related with suspected cases of bacterial meningitis.

CSF glucose	Bacterial meningitis		Total
	present	absent	
low	11 (a)	18 (b)	29 (a+b)
average	0 (c)	133 (d)	133 (c+d)
Total	11 (a+c)	151 (b+d)	162

Specificity = d/b+d × 100% = 88.07%
PPV = a/a+b × 100% = 37.93%

B. Value of protein related with suspected cases of bacterial meningitis.

CSF protein	Bacterial meningitis		Total
	present	absent	
High	11 (a)	24 (b)	35 (a+b)
average	0 (c)	127 (d)	127 (c+d)
Total	11 (a+c)	151 (b+d)	162

Specificity = d/b+d × 100% = 84.10%
PPV = a/a+b × 100% = 31.42%

C. Value of CSF / serum glucose ratio in cases suspected of bacterial meningitis.

CSF/serum glucose ratio	Bacterial meningitis		Total
	present	absent	
Low	11 (a)	5 (b)	16 (a+b)
Average	0 (c)	146 (d)	146 (c+d)
Total	11 (a+c)	151 (b+d)	162

Specificity = d/b+d × 100% = 96.68%
PPV = a/a+b × 100% = 69.43%

APPENDIX XI

STATISTICAL ANALYSIS

A. χ^2 Test to determine the association of Meningitis with Gender

Cases	**Male**	**Female**	**Row Total**
Patient with meningitis	7	4	11
Patients without meningitis	82	69	151
Total	89	73	162

Solution, since a cell frequency is less than 5, we should apply Yates correction for calculating χ^2 , for this, add 0.5 to cell frequency which is less than 5 and adjust the remaining frequencies by fixing row total and column total. Thus, adjusted 2×2 contigency table is presented in following table
The value kept after Yates correction:

Cases	**Male**	**Female**	**Row Total**
Patients with meningits	6.5	4.5	11
Patients without meningitis	82.5	68.5	151
Column Total	89	73	162

Null hypothesis H_O: Patient with BM is not associated with gender of patient.
Alternative hypothesis H_1: Patient with BM is associated with gender of patient.

Thus,
$\chi^2 = N (ad-bc)^2 / (a+b) (c+d) (a+c) (b+d)$

Calculated value of $\chi^2 = 0.0822$

Degree of freedom = (r-1) (c-1) = 1

Tabulated value of χ^2 at 5% level of significance for 1 d.f is 3.84.

Decision: Since the calculated value of χ^2 is less than tabulated value of χ^2 , the null hypothesis H_0 is accepted.

Thus there is no association of the cases of meningitis and gender of the patients.

B. Table showing association in between higher count of Leukocyte and its association with meningitis.

Leukocyte range	Patient with Meningitis	Patients without Meningitis	Total
0 - 30	0	123	123
30 - >11,000	11	28	39
Total	11	151	162

Solution,
Since a cell frequency is less than 5, we should apply Yates correction for calculating χ^2 For this 0.5 is added to cell frequency which is less than 5 and the remaining frequencies are adujusted by fixing row total and column toal. Thus, adjusted 2×2 contigency table is presented in the following table

Leukocyte range	Patient with Meningitis	Patient without Meningitis	Total
0 - 30	0.5	122.5	123
30 - >11,000	10.5	28.5	39
Total	11	151	162

Null hypothesis H_o: Higher count of Leukocyte is not associated with cases of meningitis.
Alternate hypothesis H_1: Higher count of Leukocyte is associated with cases of meningitis

Calculation of χ^2

O	E=RT×CT/N	O-E	$(O-E)^2$	$(O-E)^2/E$
0.5	8.33	-7.85	61.6	7.38
122.5	114.65	7.85	61.6	0.53
10.5	2.65	7.85	61.6	23.24
28.5	36.35	-7.85	61.6	1.69
total				32.84

Thus calculated $\chi^2 = 32.84$

Degree of freedom = (r-1) (c-1) = 1

Tabulated value of χ^2 at 5% level of significance for 1 degree of freedom (d.f.) is 3.84.

Decision: Since the calculated value of χ^2 is greater than tabulated value of χ^2 , the null hypothesis H_0 is rejected.

Thus there is association of the higher Leukocyte count in CSF and cases of meningitis.

C. Table showing Correlation in between isolation of organism in Gram stain of fresh CSF sample and growth of an organism in the culture medium.

S.N	Gram stain X_1	Culture X_2	$x_{1=} X_1$-a	$x_2= X_2$-b	x_1^2	x_2^2	$x_1.x_2$
1	3	3	1.33	1.33	1.77	1.77	1.77
2	2	2	0.33	0.33	0.11	0.11	0.11
3	1	0	-0.67	-1.67	0.44	2.8	1.12
4	1	2	-0.67	0.33	0.44	0.11	-0.22
5	1	1	-0.67	-0.67	0.44	0.44	0.44
6	2	2	0.33	0.33	0.11	0.11	0.11
Total	10	10			3.31	5.34	3.33

We have,

$a= \sum x_1 / n = 10/6 = 1.67$

$b= \sum x_2 / n = 10/6 = 1.67$

Karl Pearson correlation is given by;

$r_{12} = \sum x_1 x_2 \ / \ \sqrt{\sum x_1^2} \ \sqrt{\sum x_2^2}$

Thus, $r_{12}=3.33 / \sqrt{3.31} \ \sqrt{5.34} = 0.79$

Corr Coeff (r_{12}) = 0.79

Hence there is high degree of positive correlation in between the organism isolated in Gram stain of fresh CSF sample and growth in culture media.

APPENDIX XII

A. ZONE SIZE INTERPRETATIVE CHART

Product code	Antimicrobial agent	Symbol	Disc content	Resistant ≤ mm	Intermediate mm	Sensitive ≥mm
SD063	Amikacin	AK	30mcg	14	15-16	17
SD040	Cefotaxime	CE	30mcg	14	15-22	23
	For *S. pneumoniae*			14	15-22	26
SD062	Ceftazidime	CA	30mcg	14	15-17	18
SD065	Ceftriaxone	CI	30mcg	13	14-20	21
SD006	Chloramphenicol	C	30mcg	12	13-17	18
	For *S. pneumoniae*			17	18-20	21
SD060	Ciprofloxacin	CF	5mcg	15	16-20	21
SD143	Cloxacillin	CX	10mcg	12	12-13	14
SD010	Co-Trimxazole	CO	1.25/23.75mcg	10	11-15	16
	For *S. pneumoniae*			15	16-18	19
SD013	Erythromycin	E	15mcg	15	16-20	21
SD087	Ofloxacin	OF	5mcg	12	13-15	16
	For *S. aureus*			14	15-17	18
SD028	Penicillin G	P	10 units	19	20-27	28

B. ANTIMICROBIALS TO TREAT BACTERIAL MENINGITIS

Agent (generic name)	Route	Dose (adults)	Dose (Children)	Duration (days)
Penicillin G	IV	3-4 MU q. 4-6h	400,000 U/kg	≥4
Ampicillin or Amoxicillin	IV	2-3 g q. 6h	250mg/kg	≥4
Amoxicillin	oral	2-3 g q. 6h	250mg/kg	≥4
Chloramphenicol	IV	1 g q. 8-12	100mg/kg	≥4
Chloramphenicol (oily)	IM	3 g single dose	100mg/kg	1-2
Cefotaxime	IV	2 g q. 6h	250mg/kg	≥4
Ceftriaxone	IV	1-2 g q. 12-24 h	50-80mg/kg	≥4
Ceftriaxone	IM	1-2 g single dose	50-80mg/kg	1-2

Duration of treatment of meningococcal disease: conventional - at least 4 days; short-single dose 1 or 2 days. (IV = Intravenous, IM = Intramuscular).

APPENDIX XIII

A. Bile solubility test for identification of *Streptococcus pneumoniae* (tube method)

A loop of suspect strain from fresh growth on blood agar plate was taken and bacterial suspension was prepared in 0.5 ml of sterile saline. The suspension of bacterial cell was made cloudy similar to that of 0.5 or 1.0 Mc Farland standard. The suspension was divided into equal amount in two sterile tubes, 0.25mL of saline was added onto one tube and 0.25mL of 2% Sodium Deoxycholate to another tube (2% concentration of bile salt was made by adding 0.2gm of Sodium Deoxycholate to 10ml of saline). Tubes were shaken gently and incubated at 37°C for 2 hrs. The clearing of tube or loss in turbidity is positive result; reported as bile soluble and when turbidity remains same as that in saline control tube; reported as negative for bile solubility (bile resistant).

B. Optochin Susceptibility Test

The Optochin Susceptibility Test is performed with 6mm, 5µg Optochin disk and is used to differenciate between *S. pneumoniae* and viridians Streptococci; Optochin Susceptibe strains can be identified as *S. pneumoniae.* The suspect α-hemolytic colony was touched with sterile bacteriological loop and streaked on blood agar plate in straight line. Optochin disk 6mm diameter (containing 5µg ethylhydrocupriene) was placed aseptically on the streak of inoculums. Plates incubated in candle jar at 37°C for 18-24 hrs. The results were interpretated: A hemolytic strains with zone if inhibition of growth greater than 14mm in diameter are pneumococci, the α hemolytic strains with zones of an inhibition ranging between 9mm and 13mm should be tested for bile solubility for further characterization and identification.