**APPENDIX: I**

**Material and Equipment**

**A. List of materials**

**1. Glass wares**

Beaker Conical flask

Petri plates Measuring cylinder

Test tube Glass rod

Micropipette Micropipette tip

Glass slides Dolly rod

**2. Miscellaneous**

Bunsen burner Gloves

Hi media cotton swab Bacteriological loop

Forceps Permanent marker

Soaps Labeling tag

**3. Equipment**

Autoclave Hot air oven

Incubator Refrigerator

Compound microscope Weight balance

**4. Chemical and reagent**

Crystal violet (CV) solution Acetic acid

Methylene blue Ethanol

Lysol Microscope oil

Sodium chloride 3% H₂O₂

**5. Microbiological media**

MRS (deMan, Rogosa and Sharpe) agar

MRS (deMan, Rogosa and Sharpe) broth

MacConkey Agar

Mannitol Salt Agar (MSA)

BHI (Brain Heart Infusion) Agar

TCBS (Thiosulfate-citrate-bile salts-sucrose) Agar

SS (Salmonella Shigella) Agar

Violet Red Bile Glucose Agar

Bacillus Cereus Agar Base

Pikovskayas Agar

**6. Antibiotics Discs**

Ampicillin (AMP, 10mcg) Amoxicillin (AMX, 10mcg)

Chloramphenicol (C, 30mcg) Ciprofloxacin (CIP, 5mcg)

Ofloxacin (OF, 5mcg) Streptomycin (S, 10 mcg)

Trimethoprim (TR, 5mcg) Penicillin-G (P, 10 units)

Vancomycin (VA, 10 mcg)

**APPENDIX II**

**Bacteriological medium:**

1. **COMPOSITION AND PREPARATION OF DIFFERENT CULTURE MEDIA**:

The culture media used were from Hi-Media Laboratories Pvt. Limited, Mumbai, India. (All compositions are given in gram per litre and at 25⁰C temperature).

1. **MRS (deMan, Rogosa and Sharpe) agar:**

**Ingredients gm/litre**

 Peptone 10.0

 Beef extract 10.0

 Yeast extract 5.0

 Glucose 20.0

 Tween-80 1.0

 Na2Hpo4 2.0

 Sodium acetate 5.0

 Triammonium citrate 2.0

 MgSo4.7H20 0.2

 MnS04.4H20 0.2

 Agar 15.0

 Final pH (at 25 °C) 6.2-6.6

67.15 grams of MRS agar was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121⁰C (15 lbs pressure) for 15 minutes.

1. **MRS (deMan, Rogosa and Sharpe) broth:**

**Ingredients gm/litre**

 Peptone 10.0

 Beef extract 10.0

 Yeast extract 5.0

 Glucose 20.0

 Tween-80 1.0

 Na2Hpo4 2.0

 Sodium acetate 5.0

 Triammonium citrate 2.0

 MgSo4.7H20 0.2

 MnS04.4H20 0.2

 Final pH (at 25 °C) 6.2-6.6

55.15 grams of MRS broth was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121⁰C (15 lbs pressure) for 15 minutes.

 **3. MacConkey Agar**

**Ingredients gm/litre**

 Peptone 3.0

 Pancreatic digest of gelatin 17.0

 Lactose monohydrate 10.0

 Bile salts 1.5

 Sodium chloride 5.0

 Crystal violet 0.001

 Neutral red 0.030

 Agar 13.500

 Final pH (at 25 °C) 7.1±0.2

49.53 grams of MacConkey agar was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121⁰C (15 lbs pressure) for 15 minutes.

 **4. Mannitol Salt Agar (MSA):**

**Ingredients gm/litre**

 Peptone 5.0

 Tryptone 5.0

 HM Peptone B 1.0

 Sodium chloride 75.0

 D-mannitol 10.0

 Phenol red 0.025

 Agar 15.0

 Final pH (at 25 °C) 7.4±0.2

111.02 grams of MSA agar was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121⁰C (15 lbs pressure) for 15 minutes.

 **5. BHI (Brain Heart Infusion) Agar:**

**Ingredients gm/litre**

 HM infusion powder 12.5

 BHI powder 5.0

 Proteose peptone 10.0

 Dextrose (Glucose) 2.0

 Sodium chloride 5.0

 Disodium hydrogen phosphate 2.5

 Agar 15.0

 Final pH (at 25 °C) 7.4±0.2

52.0 grams of BHI agar was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121⁰C (15 lbs pressure) for 15 minutes.

 **6. TCBS (Thiosulfate-citrate-bile salts-sucrose) Agar:**

**Ingredients gm/litre**

 Yeast extract 5.0

 Peptic digest of animal tissue 10.0

 Sodium chloride 10.0

 Sodium thiosulphate 10.0

 Sodium cholate 3.0

 Oxgall 5.0

 Sucrose 20.0

 Sodium chloride 10.0

 Ferric citrate 1.0

 Bromo thymol blue 0.040

 Thymol blue 0.040

 Agar 15.0

 Final pH (at 25 °C) 8.6±0.2

89.08 grams of TCBS agar was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121⁰C (15 lbs pressure) for 15 minutes.

 **7. SS (Salmonella Shigella) Agar:**

**Ingredients gm/litre**

 Proteose peptone 5.0

 Lactose 10.0

 Bile salt mixture 8.5

 Sodium citrate 8.5

 Sodium thiosulphate 8.5

 Ferric citrate 1.0

 Brilliant green 0.00033

 Neutral red 0.025

 Agar 13.5

 Final pH (at 25 °C) 7.0±0.2

60 grams of SS agar was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121⁰C (15 lbs pressure) for 15 minutes.

 **8. Violet Red Bile Glucose Agar:**

**Ingredients gm/litre**

 Yeast extract 3.0

 Pancreatic digest of gelatin 7.0

 Bile salts 1.5

 Sodium chloride 5.0

 Glucose monohydrate 10.0

 Crystal violet 0.002

 Neutral red 0.030

 Agar 15.0

 Final pH (at 25 °C) 7.4±0.2

40.62 grams of Violet Red Bile Glucose agar was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121⁰C (15 lbs pressure) for 15 minutes.

 **9. Bacillus Cereus Agar Base:**

**Ingredients gm/litre**

 Peptone 1.0

 Mannitol 10.0

 Sodium chloride 2.0

 Magnesium sulphate 0.1

 Disodium hydrogen phosphate 2.5

 Potassium dihydrogen phosphate 0.25

 Sodium pyruvate 10.0

 Bromo thymol blue 0.12

 Agar 15.0

 Final pH (at 25 °C) 7.2±0.2

20.5 grams of Violet Red Bile Glucose agar was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121⁰C (15 lbs pressure) for 15 minutes.

1. **Pikovskayas Agar:**

 **Ingredients gm/litre**

 Yeast extract 0.5

 Dextrose 10.0

 Calcium phosphate 5.0

 Ammonium sulphate 0.5

 Potassium chloride 0.2

 Magnesium sulphate 0.1

 Manganese sulphate 0.0001

 Ferrous sulphate 0.0001

 Agar 15.0

31.3 grams of Pikovskayas Agar was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121⁰C (15 lbs pressure) for 15 minutes.

 **11. Muller Hilton Agar (MHA)**

 **Ingredients grams / litre**

 Beef infusion broth 30.000

 Casein acid hydrolysate 17.000

 Starch 1.000

 Agar 17.000

 pH 7.0±.0.2

38 grams of Muller Hilton Agar was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121⁰C (15 lbs pressure) for 15 minutes.

**APPENDIX: III**

**Composition and preparation of different reagent**

**1.Gram staining reagents**

**i. Crystal violet gram staining**

Crystal violet 20g

Ammonium oxalate 9g

Ethanol or methanol, absolute 95ml

Distilled water 1litre

**Preparation:**

Crystal violet is weighed and transferred to a clean bottle and absolute ethanol is added and mixed until the dye is completely dissolved.

Ammonium oxalate is weighed and dissolved in about 200ml of distilled water. Then it is added to the stain and total volume is made 1 litre by adding distilled water and mix well.

**ii. Iodine solution**

Potassium iodide 1.5g

Iodine 1.0g

Distilled water 150 ml

**Preparation:**

Potassium iodide is weighed and transferred to a clean bottle, 30-40 ml of distilled water is added to it and mixed until it is fully dissolved.

Iodine is weighed and added to the potassium iodide solution and mixed well. The final volume is adjusted to 150 ml by adding the distilled water.

**iii. Acetone- alcohol decolorizer**

Acetone 500ml

Ethanol absolute 475ml 9

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**iv. Counterstain solution**

Safranine10gm

Distilled water 1 litre

**Preparation:**

10 gm safranine is weighed and put in a clean bottle. 1 litre of distilled water is added to the it and mixed properly to dissolve safranine completely.

**v. Catalase reagent (for 100ml)**

Hydrogen peroxide solution 3ml

Distilled water 97ml

**Preparation:**

To make 100 ml solution 97ml distilled water is added to 3ml of hydrogen peroxide solution and mix well.

. **APPENDIX: IV**

**Procedure of different biochemical tests**

**1.Gram’s stain: (**Mackie and McCrtney Vol.2, 14th edition)

**Isolated colony was selected for staining:**

1. Smear was made from the pure culture by emulsifying a colony in a normal saline and heat fixed.

2. Smear flooded with crystal violet for 1 minute.

3. Wash with water.

4. Add Gram’s iodine for 1minute.

5. Wash with water.

6. Decolorize with absolute alcohol for 10-15 seconds.

7. Wash with water.

8. Flood the smear with safranine for 1 minute.

9. Wash with water, blot dry and examine under oil immersion objective of the microscope.

**2. Catalase test**

1. A small amount of isolated colony from pure culture was transferred to the surface of clean

dry glass slide.

2. A drop of 3% H2O2 was placed onto the inoculum.

3. The evolution of oxygen bubbles was recorded immediately.

4. The slide was then discarded into a disinfectant.

**APPENDIX: V**

**Antibiotics susceptibility test (Kirby- Bauer’s disc diffusion method):**

In vitro susceptibility of the pure bacterial species to fifteen different antibiotics was examined by using Kirby- Bauer’s disc diffusion technique using Muller- Hilton agar and antibiotics disc as described by the National Committee for Clinical Laboratory Standard (CLSI, 2006). One ml of each bacterial isolates prepared directly from an overnight agar plate adjusted to 0.5 McFarland standard was inoculated using sterile swab into each of the petri-dishes containing Muller- Hilton agar (MHA) and allowed to stand for 30 minutes for pre- diffusion of the inoculated organisms.

Antibiotics disc were seeded into the petri- dishes containing MHA for each bacterial isolates. The AST of the isolates towards the various antimicrobial discs was done by modified Kirby- Bauer M2-A9 disc diffusion method as recommended by Clinical Laboratory Standard Institute (CLSI) using MHA media as follows:

1.MHA was prepared and sterilized as instructed by the manufacture.

2. The pH of the medium was adjusted to the 7.2-7.4 and the depth of the medium at 4mm (about 25ml per plate) was maintained in petri dish.

3. Using a sterile loop, a single isolated colony susceptibility pattern is to be determined was touched and inoculated into MHB tube and inoculated at 370C for 2-4 hrs.

4. After incubation, the turbidity of the suspension was matched with the McFarland standard tube number 0.5 (which is equivalent to 104 organisms).

5. Using a sterile swab, MHA plate was inoculated with the matched suspension using a carpet culture technique.

6.The plate was then allowed to stand for 20-30 minutes for the pre-diffusion of the inoculated organisms.

7.Using clean and sterile forceps, the above mentioned antibiotics discs (6mm) were placed on the MHA. The discs were placed at the considerable distance apart from each other on a 90 mm petri dish. Then the plate was inoculated at 370C for 24 hrs.

8. After incubation, the plates were observed for zone of inhibition and the diameter of the inhibition zones were measured in millimeter (mm). The measurement was interpreted as a resistant and sensitive according to the manufacture standard zone size interpretative manual of CLSI (2006).

The percentage resistance was calculated using the formula PR = a/b ×100, where

PR = percentage of resistance

a = number f resistance isolates

b = number of isolates tested with the antibiotics.

The percentage of sensitivity was calculated by using formula, PS = c/d ×100, where,

PS = percentage of sensitivity

c = number of sensitive isolates

d = number of isolates tested with the antibiotics.

**Concentration of Antibiotics per discs:**

|  |  |
| --- | --- |
| **Antibiotics** | **Concᵑ /Discs** |
| 1. Chloramphenicol
 | 10µg/discs |
| 1. Ciprofloxacin
 | 5µg/discs |
| 1. Streptomycin
 | 10µg/discs |
| 1. Ampicillin
 | 10µg/discs |
| 1. Amoxycillin
 |  10µg/discs |
| 1. Ofloxacin
 | 5µg/discs |
| 1. Trimethoprim
 | 5µg/discs |
| 1. Penicillin-G
 |  10µg/discs |
| 1. Vancomycin
 |  10 µg/discs |