CHAPTER III
MATERIALS AND METHODS
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3.1 Literature Search

The following resources were searched for the writing of introduction, review of
literature, material & methods and for the data analysis and discussion.

3.1.1 Online resources

- Google search engine: http://www.google.com
- World Health Organization (WHO): http://www.who.int/en/
- Centres for Disease Control and Prevention (CDC): http://www.cdc.gov/
- Various Journals websites.
- Various national and international newspaper websites.
- India Meteorological Department, Ministry of Earth Sciences, Govt. of India:
  http://www.imd.gov.in/
- http://www.weather.com/

3.1.2 Offline resources

- MATS University Library, Raipur.
- National Medical Library, New Delhi.
- Books and articles
3.2 Sample Collection and Processing

The study included the samples of patients of Bastar tribal region attending Maharani Hospital, a tertiary care hospital in Jagdalpur, Bastar district of Chattisgarh state. The patients were mostly the tribals and belonging to the in and around area of Jagdalpur under Bastar district only. The study found the subjects of wound and soft tissue infection, bacteraemia, sepsis, urinary tract infection, and throat infection. Permission for sample collection was taken from the Superintendent of the hospital. Informed consent was taken from the patients or their relatives to take samples for microbiological investigations. Those samples which were drawn for the microbiological investigations under hospital were also included. The following samples were collected and processed accordingly.

3.2.1 Pus and wound swab

Sterile screw capped polypropylene tube containing sterile swab stick were purchased from HiMedia Lab. The pus and wound samples were taken by swabbing. The swab stick was replaced in the tube and screwed with the cap. The swabbed samples were inoculated on nutrient agar, blood agar and MacConkey agar media in Petri dishes. The plates were placed inverted in incubator at 37°C for 24 hours.

3.2.2 Urine

Freshly voided midstream urine samples were obtained in sterile wide-mouth screw capped universal containers. The urine specimens were well mixed and inoculated onto blood agar, MacConkey agar and cysteine lactose electrolyte deficient (CLED) agar media in Petri dishes. The culture plates were incubated at 37°C for 24 hours and observed for growth.
3.2.3 Blood

Blood-culture bottles (125 ml) with a pre-perforated screw-cap and a rubber diaphragm were used. The bottles were filled with 50 ml of brain-heart infusion broth (BHI) medium (HiMedia) and then the screw-cap was left loosen half a turn. Covering the cap with a square piece of aluminium foil, the bottles were autoclaved for 20 minutes at 120ºC. Immediately after autoclaving, while the bottle and the medium were still hot, the cap was securely tighten without removing the aluminium foil.

After disinfecting the top of the cap, the bottles were inoculated with 3-5 ml of freshly drawn blood from patients. Covering the bottle-caps with aluminium foil, the bottles were incubated at 37 ºC. The bottles were routinely inspected twice a day (at least for the first 3 days) for signs of microbial growth for the maximum of 7 days. A sterile culture usually shows a layer of sedimented red blood cells with clear transparent broth. Growth is evidenced by:

- A floccular deposit on top of the blood layer
- Uniform or subsurface turbidity
- Haemolysis
- Coagulation of the broth
- A surface pellicle
- Production of gas
- White grains on the surface or deep in the blood layer.

Whenever visible growth appeared, a small amount of broth was aseptically removed by a sterile syringe piercing the rubber diaphragm through the hole in cap. The aspirated broth was examined microscopically by Gram-staining, and sub-cultured onto blood agar, MacConkey agar, and mannitol salt agar media in Petri dishes. The blood cultures were considered negative, where there were no growths occurred after 7 days.
3.2.4 Throat swab

This was done by the physician. The patient was told to sit facing a light source. While the tongue was kept down with a tongue depressor, a sterile cotton-wool swab was rubbed vigorously over each tonsil, over the back wall of the pharynx, and over any other inflamed area. Care was taken not to touch the tongue or buccal surface. Two swabs were taken from the same areas; one to prepare a smear for Gram staining, while the other to inoculate onto blood agar, chocolate agar and MacConkey agar plates.

3.3 Culture Media and Methods

3.3.1 Media preparation

All the culture media were purchased as ready-made dehydrated powder by HiMedia Laboratory. The media used for the research are described in Table. The media were prepared according to the manufacturer’s instructions. As per the written instructed quantities, each medium was weighed, added to the volume of distilled water in a conical flask, mixed to dissolve, and maintained for the pH. The mouth of the flask was closed with a cotton plug and then wrapped with a piece of aluminium foil. The media were then sterilized by autoclaving at 120°C for 20 minutes. The agar media were maintained molten by keeping the media flasks at 45°C in water-bath. For the blood agar preparation, 5% defibrinated sheep blood was aseptically added to and mixed with nutrient agar medium maintained at 45°C in water-bath, whereas for chocolate agar, the blood agar media was gradually heated to 70°C. All the media were dispensed aseptically in sterile test tubes and Petri dishes. For agar slant, the tubes containing molten agar media (about 1/3rd filled) were properly closed by a cotton plug or screw-cap and then left laid side vise at an angle on a flat platform; once cooled the agar formed a slant surface in the tubes. For agar-deep, the tubes having molten agar (about 1/3rd filled) were
closed by a cotton plug or screw-cap and left to cool in the test tube rack vertically. For the plates, the molten agar media were poured in the sterile Petri plates to about 4 mm height and left ajar to cool in a safety cabinet.

**Figure 3.1.** Media plates and tubes: (a) agar medium plate, (b) agar-deep tube, (c) agar slant, and (d) broth medium tube.
**Table 3.1.** Composition of dehydrated culture media (HiMedia) used in the present research.

<table>
<thead>
<tr>
<th>Media</th>
<th>Ingredients</th>
<th>Quantity in distilled water (gram/Litre)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nutrient agar:</strong></td>
<td>Peptic digest of animal tissue</td>
<td>5.00</td>
</tr>
<tr>
<td>(pH 7.4±0.2 at 25°C)</td>
<td>Sodium chloride</td>
<td>5.00</td>
</tr>
<tr>
<td></td>
<td>Beef extract</td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td>Yeast extract</td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td>Agar</td>
<td>15.00</td>
</tr>
<tr>
<td><strong>Blood agar:</strong></td>
<td>Add 5% defibrinated sheep blood</td>
<td></td>
</tr>
<tr>
<td>(pH 7.4±0.2 at 25°C)</td>
<td>to nutrient agar.</td>
<td></td>
</tr>
<tr>
<td><strong>Chocolate agar:</strong></td>
<td>Heat blood agar gradually to 70 ºC.</td>
<td></td>
</tr>
<tr>
<td>(pH 7.3±0.2 at 25°C)</td>
<td>Peptones (meat and casein)</td>
<td>3.00</td>
</tr>
<tr>
<td><strong>MacConkey agar:</strong></td>
<td>Pancreatic digest of gelatin</td>
<td>17.00</td>
</tr>
<tr>
<td>(pH 7.1±0.2 at 25°C)</td>
<td>Lactose monohydrate</td>
<td>10.00</td>
</tr>
<tr>
<td></td>
<td>Bile salts</td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td>Sodium chloride</td>
<td>5.00</td>
</tr>
<tr>
<td></td>
<td>Crystal violet</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Neutral red</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>Agar</td>
<td>13.500</td>
</tr>
<tr>
<td><strong>Mannitol salt agar:</strong></td>
<td>Proteose peptone</td>
<td>10.00</td>
</tr>
<tr>
<td>(pH 7.4±0.2 at 25°C)</td>
<td>Beef extract</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>Sodium chloride</td>
<td>75.00</td>
</tr>
<tr>
<td></td>
<td>D-Mannitol</td>
<td>10.00</td>
</tr>
<tr>
<td></td>
<td>Phenol red</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td>Agar</td>
<td>15.00</td>
</tr>
</tbody>
</table>
Table 3.1. Cont’d

<table>
<thead>
<tr>
<th>Media</th>
<th>Ingredients</th>
<th>Quantity in distilled water (gram/Litre)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cystine-Lactose-Electrolyte-Deficient</strong> (C.L.E.D.) agar: (pH 7.10- 7.50 at 25°C)</td>
<td>Peptic digest of animal tissue</td>
<td>4.000</td>
</tr>
<tr>
<td></td>
<td>Casein enzymic hydrolysate</td>
<td>4.000</td>
</tr>
<tr>
<td></td>
<td>Beef extract</td>
<td>3.000</td>
</tr>
<tr>
<td></td>
<td>Lactose</td>
<td>10.000</td>
</tr>
<tr>
<td></td>
<td>L-Cystine</td>
<td>0.128</td>
</tr>
<tr>
<td></td>
<td>Bromothymol blue</td>
<td>0.020</td>
</tr>
<tr>
<td></td>
<td>Agar</td>
<td>15.000</td>
</tr>
<tr>
<td><strong>Brain-heart infusion broth:</strong> (pH 7.4±0.2 at 25°C)</td>
<td>Calf brain infusion</td>
<td>200.000</td>
</tr>
<tr>
<td></td>
<td>Beef heart infusion</td>
<td>250.000</td>
</tr>
<tr>
<td></td>
<td>Proteose peptone</td>
<td>10.000</td>
</tr>
<tr>
<td></td>
<td>Dextrose</td>
<td>2.000</td>
</tr>
<tr>
<td></td>
<td>Sodium chloride</td>
<td>5.000</td>
</tr>
<tr>
<td></td>
<td>Disodium phosphate</td>
<td>2.500</td>
</tr>
<tr>
<td></td>
<td>Agar</td>
<td>15.000</td>
</tr>
<tr>
<td><strong>Müller Hinton Agar:</strong> (pH 7.3±0.1 at 25°C)</td>
<td>Beef, infusion from</td>
<td>300.000</td>
</tr>
<tr>
<td></td>
<td>Casein acid hydrolysate</td>
<td>17.500</td>
</tr>
<tr>
<td></td>
<td>Starch</td>
<td>1.500</td>
</tr>
<tr>
<td></td>
<td>Agar</td>
<td>17.000</td>
</tr>
</tbody>
</table>

**Note:** To prepare broth from any of the above mentioned agar media, agar was not added during the preparation.
Figure 3.2. Various agar media plates used in research: (a) nutrient agar plate, (b) blood agar plate, (c) chocolate agar plate, (d) MacConkey agar plate, (e) mannitol salt agar plate, (f) C.L.E.D. agar plate, and (g) Müller Hinton agar plate.

3.3.2 Subculturing and maintenance of the isolates

The growth obtained from the sample culture was subcultured on appropriate agar media. The subculture was done in two different patterns; one as quadrant streaking (or four-way streaking) and the other as a single zig-zag in a triangular area on agar. The quadrant streaking was done on nutrient agar, blood agar, mannitol salt agar, and MacConkey agar plates. One or two separate colonies were picked by the sterile inoculation loop and touched on the nutrient agar medium for spot-inoculation in plate near to periphery. The loop was sterilized by red-hot method in Bunsen flame, and cooled. The loop was touched to a peripheral sterile area on agar surface to ensure if it was cool, and then the spot inoculated point was touched by the loop and streaked heavily near periphery in right direction to inoculate area-1 (Figure 3). The loop was sterilized under flame and cooled. The plate was turned anticlockwise to 90° angle, the loop was touched to the distal end of area-1, and streaked in area-2. The plate was again turned anticlockwise to 90° angle, and the unsterilized loop was touched on the distal end of area-2 and area-3.
was inoculated by streaking. Again the plate was turned at right angle, the unsterilized loop was touched on distal area-3, and a single zig-zag streak was made in area-4. The plates were kept inverted in incubator at 37°C for duration between 12 to 24 hours. The identified pure colonies of \textit{S. aureus} were used to inoculate the triangular area in a fresh agar plates. One 90 mm diameter brain-heart infusion agar plate was used for maximum of four \textit{S. aureus} isolates from different culture positive samples. The plate was divided in four areas by etching lines at the back of plate by permanent marker pen; each area was labeled with the respective isolate numbers. The isolate single colony was picked by sterilized loop and inoculated carefully on the concerned area by single zig-zag streak. The plates were incubated overnight at 37°C.

For the storage and transportation of the isolates, a single pure colony of \textit{S. aureus} isolate was picked by sterile inoculating needle and inoculated in brain heart infusion (BHI) agar deep vials by stab-inoculation. The vials were closed by their caps, and incubated overnight at 37°C for growth. The vials were then kept at room temperature.

\textbf{Figure 3.3.} Streaking patterns of inoculation used in the research.
3.4 Identification of S. aureus Isolates

The isolates of S. aureus were identified by Gram staining, catalase test, and coagulase test. The identification was further confirmed by a latex agglutination test; Dry Spot Staphytec Plus (Oxoid), and by mannitol fermentation on mannitol salt agar. The identification scheme used is illustrated in Figure-2.

3.4.1 Gram staining

The Gram staining was first devised in 1884 by a Danish physician Hans Christian Gram. It is a differential staining procedure that divides bacteria into two classes: gram positive and gram negative. The bacteria that resist decolorization of primary stain; crystal violet, appear violet, and hence are gram positive, whereas those in which the primary stain is decolorized from cell wall and take the color of counterstain (pink), are gram negative.

3.4.1.1 Procedure

For direct microscopy, the swab was rolled on the centre of clean grease-free glass slide, dried in air and then by passing 3 to 4 times across the flame. From the subculture, a small portion of pure growth was picked by touching a separate bacterial colony with sterile inoculation needle. The growth was mixed and emulsified properly in a drop of sterile normal saline onto the centre of a clean, grease-free glass slide. The drop was spread concentrically by the inoculation needle to make a thin uniform film. The smear was first air-dried and then heat-fixed by passing the slide, smear side up, 3 to 4 times on Bunsen flame. The slide was left to cool.

The slides bearing smears were arranged in staining rack. The smear was treated with crystal violet for 1 minute. The stain was then decanted and rinsed with distill water. The water traces were rinsed by pouring and decanting the Gram’s iodine solution. The
iodine solution was flooded on slide and left for 1 minute. The iodine was decanted and
the slides rinsed with distill water. To decolorize the primary stain, absolute ethanol was
added on slide and left for 30 seconds. The alcohol was decanted and slide was rinsed
with distilled water. The smear was counterstained by flooding the slide with safranin for
1 minute. The counterstain was decanted and the slide was rinsed with distilled water.
The water traces from slides were removed by blot-drying using tissue papers. The smear
was observed microscopically under oil-immersion objective. Purple or violet color cells
were classified as gram positive, whereas pink or red as gram negative. The morphology
revealed whether the bacterial cells were cocci or bacilli. The cellular arrangements
traced the gram positive cells as staphylococci, streptococci and diplococci. The species
of genus *Staphylococci* are gram positive spherical cells arranged in irregular cluster
(i.e.staphylococci).

**Figure 3.4.** Microscopic view of Gram stained smears under oil-immersion
objective (magnification = 1000X): (a) Gram positive staphylococci, and (b) Gram negative bacilli.
Figure 3.5. Microbiological strategy used in research to identify *S. aureus* isolates.
3.4.2 Catalase test

The hydrogen peroxide, produced by microorganisms during aerobic respiration, is extremely toxic to them and its accumulation will result in the death of the organism. Certain bacteria, for their survival, produce catalase enzyme to degrade hydrogen peroxide into water and oxygen. *Staphylococcus spp* is catalase positive that differentiate them from streptococci.

\[
\text{Catalase: } 2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2\uparrow
\]

3.4.2.1 Procedure

Using sterile inoculating needle, growth was picked from the center of a separate bacterial colony on nutrient agar and transferred to the surface of a clean grease-free glass slide. To the growth on glass slide was added a drop of 3% H$_2$O$_2$. The drop was observed for bubble formation. The growth was deemed catalase positive if bubble formation occurred.

![Figure 3.6. Slide catalase test: (a) catalase positive reaction (bubble formation), and (b) catalase negative reaction.](image)
3.4.3 Coagulase test

*Staphylococcus aureus* produces coagulase protein that has a prothrombin-like activity, and can convert fibrinogen into fibrin as a distinct clot in citrated human or rabbit plasma. The plasma used in the coagulase test may be fresh human or rabbit plasma obtained with ethylenediamine tetraacetic acid (EDTA). It should be stored in the refrigerator in small amounts (1 ml), and its performance checked with control cultures of *S. aureus* and *S. epidermidis*, run in parallel.

3.4.3.1 Procedure

The blood was obtained from healthy volunteers with an informed consent. Plasma was obtained by centrifuging the EDTA blood at 1100 RCF for 10 minutes at room temperature. The plasma was aseptically transferred to sterile Bijou containers. The test was performed on clean grease-free glass slide, however in doubtful results tube coagulase test was done.

**Slide test**

The test is to detect bound coagulase or clumping factor attached to the bacterial cell wall. Fibrin strands are formed between the bacterial cells when suspended in plasma (fibrinogen), causing them to clump into visible aggregates. A drop of sterile normal saline was placed on a clean glass slide. With a wooden applicator stick single separate colony from nutrient agar plate was picked and emulsified in the drop to make a fairly thick and uniform suspension. Sterile inoculating needle was dipped into plasma and the same was used to stir the drop of bacterial suspension on slide. The slide was observed for clumping in the suspension within 15 seconds. Where there was no clumps observed even after 2 minutes, the test was considered negative. If the slide test was negative for
an isolate that seemed to be pathogenic on other grounds (pigment, clinical source), the coagulase reaction was re-examined in the tube test.

**Tube test**

The test detects free coagulase, a thrombin-like substance present in culture filtrate. The test is considered positive if any degree of clotting is noted, as fibrinolysins produced by the organism may also dissolve the clot soon after formation. In a sterile 12 × 75 mm tube, 0.5 ml of plasma was added. A small amount of the colony growth was picked with inoculation needle and emulsified in the plasma in tube. The tube was incubated at 35°C for 4 hours and observed for clot formation by gently tilting the tube. If no clot was observed at that time, the tube was reincubated at room temperature and examined after 18 hours.

![Image of coagulase test](image)

**Figure 3.7.** Coagulase test: (a) slide test (left negative and right positive), and (b) tube test (top positive and bottom negative).
3.4.4 Mannitol salt agar (MSA) culture

Mannitol salt agar is a selective and differential medium for the detection and enumeration of staphylococci from samples and cultures. It is a nutritive medium due to its content of peptones and beef extract, which supply essential growth factors, such as nitrogen, carbon, sulfur and trace nutrients. The 7.5% concentration of sodium chloride makes the media selective, and results in the partial or complete inhibition of bacterial organisms other than staphylococci. Mannitol fermentation, as indicated by a change in the phenol red indicator, aids in the differentiation of staphylococcal species.

One separate colony was picked from pure culture in brain-heart infusion agar using sterile inoculating loop and aseptically inoculated onto mannitol salt agar in plate. The plate was placed inverted in incubator at 37°C for 24 hours. Growth or colonies of Staphylococcus aureus were yellow or white surrounded by yellow zone in medium.

3.4.5 Latex agglutination test

A latex agglutination test kit, Dry Spot Staphytect Plus®, for S. aureus was purchased from Oxoid. It is a latex slide agglutination test for the differentiation of S. aureus (222) by detection of clumping factor, Protein A and certain polysaccharides found in methicillin resistant S. aureus (MRSA) from those Staphylococci that do not possess these properties. The test uses blue latex particles coated with both porcine fibrinogen and rabbit IgG including specific polyclonal antibodies raised against capsular polysaccharides of S. aureus. The reagent is dried onto the reaction card. When the reagent is mixed on the card with colonies of S. aureus emulsified in saline, rapid agglutination occurs through the reaction between (i) fibrinogen and clumping factor, (ii) Fc portion of IgG and Protein A (iii) specific IgG and capsular polysaccharide. On the test card, one drop (50 µl) of sterile normal saline was added to the small rings at the bottom of each oval in both the test and control reaction areas ensuring that the liquid did
not mix with the dried latex reagent spots. Using sterile inoculating loop, 5 average-sized (2-3 mm diameter) colonies of *S. aureus* ATCC®25923 (positive control) from nutrient agar plate were picked and carefully emulsified in the saline drop in control oval to make a smooth suspension. The loop was sterilized by red-hot in flame. Once the loop was cooled, 5 average-sized (2-3 mm diameter) suspected colonies of staphylococci (test organism) from nutrient agar plate were picked and carefully emulsified in the saline drop in test oval to make a smooth suspension. In the control oval, using a sterile inoculating loop, the suspension was mixed into the dry Control Latex spots until completely suspended and spread to cover the reaction area. Using a fresh sterile loop, the same was done in test oval. The card was rocked for up to 20 seconds and observed for agglutination under normal light. Agglutination in the reaction was deemed positive for *S. aureus*. For negative control *Staphylococcus epidermidis* ATCC®12228 strain was used. The cards used were properly discarded in disinfectant solution of sodium hypochlorite.

![Figure 3.8. Latex agglutination test, Dry Spot Staphytect Plus (Oxoid).](image)
Figure 3.9. Culture on different media used in the research: (a) β-haemolytic growth of *S. aureus* on blood agar plate, (b) non-lactose fermenting (NLF) growth of *S. aureus* (right side), and lactose-fermenting (LF) growth (left side) on MacConkey agar plate, (c) growth of *S. aureus* on C.L.E.D. agar plate, and (d) growth of *S. aureus* on mannitol salt agar (MSA) plate.
3.5 Detection of Antibiotic Resistance

3.5.1 MRSA screening

3.5.1.1 Oxacillin screen agar

Mueller-Hinton agar (MHA) plates containing 4% NaCl and 6 μg/ml of oxacillin were prepared. Plates were inoculated with 10 μL of 0.5 Mc Farland suspension of the isolate by streaking in one quadrant and incubated at 35°C for 24 h. Plates were observed carefully in transmitted light for any growth. Any growth after 24 h was considered oxacillin resistant. (67, 223)

3.5.1.2 Cefoxitin disk diffusion test

All the isolates were subjected to cefoxitin disk diffusion test using a 30 μg disk. A 0.5 Mc Farland standard suspension of the isolate was made and lawn culture was done on MHA plate. Plates were incubated at 35°C for 18 h and zone diameters were measured. An inhibition zone diameter of ≤ 21 mm was reported as oxacillin resistant and ≥ 22 mm was considered as oxacillin sensitive. (67, 224-226)

3.5.2 Antibiotic susceptibility testing

3.5.2.1 Disk diffusion test by Kirby-Bauer method

The antibiotic susceptibility pattern of all isolates was determined by Kirby Bauer disk diffusion method against the following antibiotics (HiMedia Laboratories Pvt. Ltd, India): penicillin-G (10 U), ampicillin (10 μg), ampicillin/sulbactam (10/10 μg), erythromycin (15 μg), azithromycin (15 μg), clindamycin (2 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), linezolid (30 μg), vancomycin (30 μg), tetracycline (30 μg), gentamycin (10 μg), ciprofloxacin (5 μg), gatifloxacin (5 μg), and chloramphenicol (30 μg). For the isolates cultured from urine samples, norfloxacin (10
μg), nitrofurantoin (300 μg), sulfisoxazole (300 μg) and trimethoprim (5 μg) were tested in addition to the above mentioned antibiotics except erythromycin, azithromycin, clindamycin and chloramphenicol. All the tests were performed on Müeller Hinton agar, and were interpreted after incubation for 24 h at 37°C. Following CLSI criteria, the susceptibility was noted as per the zone diameter measured around each disk.\(^{(67)}\)

### 3.5.2.2 Etest method

Minimum inhibitory concentration (MIC) of vancomycin and linezolid was determined using Etest (AB Biodisk, Solna, Sweden) as per manufacturer’s instructions\(^{(227, 228)}\). Following CLSI, 2010 breakpoints,\(^{(67)}\) the susceptibility was noted.

![Antibiotic susceptibility testing](image)

**Figure 3.10.** Antibiotic susceptibility testing: Kirby-Bauer disk diffusion method (left side), and Etest Method (right side).