CHAPTER 3

3.0 MATERIALS AND METHODS

In the present study, used various chemicals, fine chemicals, enzymes, and quality glasswares manufactured from standard companies. (ABgene, Bio-Tech, Bio-Rad, Borosil, eppendorf, Hi media, Labomed, Medox, Olimpus, Ranbaxy, Sigma, SRL).

Reference cultures were obtained from MTCC, India and Curtain University, Western Australia for the comparison work of the MRSA isolates and characterization.

3.1 MICROBIOLOGICAL STUDIES

3.1.1 Epidemiology

Various clinical laboratories selected for the collection of methicillin resistant S.aureus prevalence of Tamilnadu, India.

3.1.2 Sample collection: (Mackie and Mc Cartney, 1989)

Various clinical laboratories at various places in northern and southern districts of Tamilnadu, India were chosen for sample collection (Clinical samples includes blood, pus, urine, sputum, throat swab, ear swab, nasal swab, ET, catheter tube, pleural fluid, peritoneal fluid, IND, umbilical swab, drain tube), patients belonging to various age groups 8 categories (viz, below 1 year, 1-10 year, 11-20 year, 21-30 year, 31-40 year, 41-50 year, 51-60 year, and above 60) were chosen for sample collection.
3.1.3 Identification

The clinical samples collected from patients belonging to various age groups were thoroughly analyzed for the presence of gram positive cocci using grams staining technique.

Clinical samples showing positive with gram staining technique were separated and cultured on nutrient agar plates. The plates were incubated at 37°C for 24 hours.

a) Nutrient agar media composition

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Grams/ litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptic digest of animal-</td>
<td>5.0</td>
</tr>
<tr>
<td>Tissue</td>
<td></td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>Beef extract</td>
<td>1.5</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1.5</td>
</tr>
<tr>
<td>Agar agar</td>
<td>15.0</td>
</tr>
<tr>
<td>Final pH (at 25°C)</td>
<td>7.4 ± 0.2</td>
</tr>
</tbody>
</table>

b) Hemolytic activity

The colonies formed on the nutrient agar were transferred into blood agar medium containing 7% of defibrinated sheep blood for the identification of haemolytic activity.
Media composition

Blood agar media

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Grams/ litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptic digest of animal-</td>
<td>5.0</td>
</tr>
<tr>
<td>Tissue</td>
<td></td>
</tr>
<tr>
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<td>5.0</td>
</tr>
<tr>
<td>Beef extract</td>
<td>1.5</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1.5</td>
</tr>
<tr>
<td>Agar agar</td>
<td>15.0</td>
</tr>
<tr>
<td>Defibrinated blood</td>
<td>70 ml</td>
</tr>
</tbody>
</table>

Final pH (at 25°C) 7.4 ± 0.2.

c) Coagulase test

Colonies exhibiting a hemolytic activity in the blood agar medium were subjected to a coagulase test for identification of *Staphylococcus aureus*, which produces coagulase enzyme that forms colloidal nature of plasma. The isolates were inoculated into plasma containing tube.

Tube test is the more reliable of the two tests and should be done in all doubtful cases. It requires readings at intervals up to 6 hours and again after standing overnight, so that its use may delay the reporting of results or a decision as to whether the culture should be tested for antibiotic sensitivities. (Mackie and McCartney, 1989).
d) Gelatinase test

Principle

Gelatin will not by itself support the growth of many pathogens and it is added to a liquid nutrient medium to produce a firm gel called nutrient gelatin.

The organisms are inoculated into the nutrient gelatin medium. Incubation is carried out at 37°C for 24 hours. After the incubation, kept it in refrigerator (4°C) for 30 minutes.

Media composition

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Grams/lit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5.0</td>
</tr>
<tr>
<td>Beef extract</td>
<td>3.0</td>
</tr>
<tr>
<td>Gelatin</td>
<td>120.0</td>
</tr>
<tr>
<td>Final pH</td>
<td>6.8</td>
</tr>
</tbody>
</table>

e) Enzyme Linked Immunosorbent Assay (ELISA)

This test especially used for the identification of penicillinase enzyme producing microbes.

Principle

Penicillinase enzyme binds to blue colored starch iodine complex, finally it decolorizes the blue color.
Procedure: (Indigenous)

Nutrient broth containing 7% NaCl prepared and 1 gram of starch and 1 ml of iodine were added and sterilized at 121°C and 15 bounds pressure for 20 minutes.

- MRSA isolates and MTCC 87 cultures are inoculated, one tube considered as a control.
- Incubated at 37°C for overnight.
- Results were noted.

f) Mannitol Salt Agar

1. The MRSA isolates and MTCC 87 were streaked on mannitol salt agar media.
2. The results were noted.

Media Composition

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Grams/lit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient broth</td>
<td>1000ml</td>
</tr>
<tr>
<td>Mannitol</td>
<td>50</td>
</tr>
<tr>
<td>Neutral red</td>
<td>2.5</td>
</tr>
<tr>
<td>pH</td>
<td>11.0</td>
</tr>
</tbody>
</table>
g) **Antibiotic assay (Sundararaj 1997)**

**Media composition**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Grams/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef infusion</td>
<td>300ml</td>
</tr>
<tr>
<td>Casein hydrolysate</td>
<td>17.5</td>
</tr>
<tr>
<td>Starch</td>
<td>1.5</td>
</tr>
<tr>
<td>Agar</td>
<td>10.0</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000ml</td>
</tr>
<tr>
<td>Final pH</td>
<td>7.4</td>
</tr>
</tbody>
</table>

**Procedure**

The Methicillin resistant *S. aureus* cultured in the nutrient broth were transferred into Muller-Hinton agar containing 7% NaCl. Various antibiotic discs were used for antibiotic assay as methicillin (5µg), penicillin G (10U), tetracycline (30µg), gentamycin (10µg), bacitracin (10µg), ampicillin (10µg), novobiocin (30µg), doxycycline (30µg), amikacin (30µg), (Varaldo *et al.*, 1984), were placed on the Muller-Hinton agar with 7% NaCl, and incubated for 12 hours at 35º C for the screening of Methicillin resistant *S. aureus* (MRSA).

3.1.4 **Pathology (Paulhebeisen *et al.*, 2001)**

3.1.4.1 **Test animal**

Albino mice obtained from KSR College of Arts & Science, Thiruchengode, Tamilnadu. Weighing 30 to 36 g. 3 weeks old were taken for this study.
3.1.4.2 Test sample

Methicillin resistant *Staphylococcus aureus* (MRSA) isolates.

3.1.4.3 Experimental septicemia in mice

Septicemia was induced in outbred Swiss albino mice (weight, 30 to 36 g). Mice were infected by intravenous (i.v.) injection of diluted overnight cultures of the test organisms (Methicillin resistant *Staphylococcus aureus* (MRSA) isolates.

3.1.4.4 Experimental Rheumatoid arthritis in mice

Rheumatoid arthritis was induced in outbred Swiss albino mice (weight, 30 to 36 g). Mice were subjected to intra cutaneous (i.c) injection of diluted overnight cultures of the test organisms (Methicillin resistant *Staphylococcus aureus* (MRSA) isolates.

3.2 BIOTECHNOLOGICAL STUDIES

3.2.1 SDS-Page

3.2.2 Preparation of cell surface antigenic protein (Hussain et al., 2000)

1. To prepare cell surface proteins, MRSA isolates and MTCC87 were grown in 5 ml Brain Heart Infusion broth).

2. Incubated at 37° C for 18 hour.

3. Then centrifuged at 6000 rpm for 5 minutes.

4. The pellet was resuspended in extraction buffer at 10 μl/mg (wet weight) of pellet to adjust for differences in cell number.

5. Then heated at 95° C for 3 minutes.

6. Then centrifuged at 10,000 rpm for 3 minutes. Supernatant was collected, and remove the SDS using dialyzed against distilled water.
Purification

1. Antigenic cell surface protein suspended with 20 µl of Tris-Glycine buffer (pH 8.0).
2. Then added equal volume of 10% TCA (TriCholoro Acetic acid). Precipitated proteins are collected.
3. Then added 20µl of Petroleum Ether were added and removed.

Composition of Brain Heart Infusion broth:

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Grams/lit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf brain, infusion form</td>
<td>200.00</td>
</tr>
<tr>
<td>Beef heart, infusion form</td>
<td>250.00</td>
</tr>
<tr>
<td>Protease peptone</td>
<td>10.00</td>
</tr>
<tr>
<td>Dextrose</td>
<td>2.00</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.00</td>
</tr>
<tr>
<td>Disodium phosphate</td>
<td>2.50</td>
</tr>
<tr>
<td>Final pH (at 25°C)</td>
<td>7.4 ± 0.2</td>
</tr>
</tbody>
</table>

3.2.3 Estimation of protein (Lowry et al., 1951)

To estimate the Antigenic protein content to evaluate the purification and for quantification. Therefore, determination of total antigenic protein concentration was done by the procedure of Lowry et al., 1951. This method is sensitive enough to give constant volume and hence routinely followed. Antigenic protein content is determined by this method.
Materials required

Solution A (100ml)

- 0.5 gm CuSO₄·5H₂O
- 1gm Na₃C₆H₅O₇(2H₂O)
- Distilled Water 100ml
  (Stored at room temperature)

Solution B (1000ml)

- 20 gm Na₂CO₃
- 4 gm NaOH
- Distilled Water 1000 ml
  (Stored at room temperature)

Solution C (51ml)

- 1ml Solution A
- 50 ml Solution B
  (Stored at room temperature)

Solution D (20ml):

- 10 ml Folin’s reagent
- 10 ml Distilled Water
  (Stored at 7°C)
Principle

Protein reacts with Folin’s reagent to give blue colored complex. The color so formed is due to the reaction of alkaline copper with protein at the reduction of phosphomolybdate by tyrosine and tryptophan present in protein. The intensity of the color depends on the amount of aromatic amino acid present.

3.2.4 Estimation of antigenic protein

Procedure

1. 0.2, 0.4, 0.6, 0.8 and 1mg/ml of working standards were pipetted out into a series of test tubes.

2. 20μl of MRSA isolates and MTCC 87 proteins were transferred to fresh sterile test tubes.

3. Volume was made up to 1ml in all the test tubes with the use of sterile distilled water. Another tube with 1ml sterile distilled water serves as the blank.

4. 5ml of reagent C was added to each tube including the blank. Mixed well and allowed to stand for 10 minutes.

5. 5ml of reagent D was added, mixed well and allowed incubate at room temperature in the dark for 30 minutes. Blue color was developed.

6. Readings were taken after 30 minutes at 660 nm.

7. Standard graph was drawn and the amount of protein in the sample was calculated.
3.2.5 Sodium-Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis. (SDS-PAGE) (Laemmli, 1970)

A) Materials

Pasteur pipettes and bulbs
Micropipettes, tips Glass pipettes,
10µl of molecular weight protein marker (indigenous)
Gel apparatus with Power pack, gel plates, comp, spacers
Gloves & syringes.

B) Buffers and reagents used

a) Stock solution 1.5M Tris Hcl pH (8.8) 100 ml

Tris buffer 9.06 gm was added to 50 ml of distilled water. 1 N Hcl was added
to equilibriate pH 8.8. Distilled water was added to a total volume of 100ml.

b) 0.5M Tris Hcl pH (6.8) 100 ml

Tris buffer 30.28 gm was added to 50 ml of Distilled water. 1N Hcl was
added to equilibriate pH 6.8. Distilled water was added to a total volume of 100ml.

c) 10% SDS W/V 100ml:

SDS - 10 gm
Distilled Water - 100ml

Stored at room temperature.
d) **Monomer 100ml 30% w/v**

- Acrylamide: 29.2gm
- Bis acrylamide: 0.8gm

Stored at 7 °C

Warmed distilled water was added to make 100ml and stirred until completely dissolved. Worked under Hood and kept covered with Para film until it was completely dissolved.

e) **4X Separating or Resolving gel buffer 100 ml (pH 8.8)**

- 1.5 M Tris Hcl: 75ml
- 10% SDS: 4ml
- Distilled Water: 21ml

It is stable for months in refrigerator.

f) **4% of stacking gel buffer 100ml (pH 6.8)**

- 0.5M Tris Hcl: 50ml
- 10% SDS: 4ml
- Distilled Water: 46ml

It is stable for months in refrigerator

h) **Sample buffer 100ml (pH 8.8)**

- Tris chloride: 0.25 M
- Glycerol: 20 ml
SDS   -  4 gm
Bromophenol blue -  2 mg
β- Mercaptoethanol -  10 ml
Distilled Water -  100 ml

i) **Tank Buffer**  
1Litre (pH 8.3)

Tris chloride -  3 gm
Glycine -  14.4 gm
SDS -  1 gm
Distilled Water -  1 Litre

It is stable indefinitely at room temperature.

j) **Staining solution**  
1Litre

Coomassie Blue R- 250 - 1gm
Methanol - 100 ml
Glacial Acetic acid - 100ml
Distilled Water - 800 ml

k) **Destaining Solution**  
1Litre

Glacial Acetic acid - 70 ml
Distilled Water - 930 ml
Procedure

There are two components to the gel we used, a separating gel on bottom and a stacking gel on top.

**Separating gel (12%)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dis. water</td>
<td>3.3 ml</td>
</tr>
<tr>
<td>1.5M Tris Hcl</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Monomer</td>
<td>4.0 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.004 ml</td>
</tr>
</tbody>
</table>

Pour or pipette between the glass plates. Layer enough water over the surface of the acrylamide to form about a mm layer.

When the separating gel has fully polymerized (20-40 minutes), carefully remove the water and unpolymerized gel.

**Stacking gel solution 5% (pH 6.8)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dis. Water</td>
<td>1.4 ml</td>
</tr>
<tr>
<td>0.5M Tris Hcl</td>
<td>0.25 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.33 ml</td>
</tr>
<tr>
<td>Monomer</td>
<td>0.02 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.02 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.002 ml</td>
</tr>
</tbody>
</table>
Poured down the side of the gel apparatus near one of the spacers. Poured slowly and carefully to avoid the formation of bubbles. Placed the comb at a slant between the two glass plates and lower gradually.

Allowed the gel to polymerize for 20-40 minutes.

While the polymerizing, prepared samples for loading.

Heated the samples and molecular weight markers at 100°C in the water bath for 3 minutes just before loading. Stand to the sink and rinsed with buffer to remove any non-polymerized acrylamide. Then removed the comb by pulling it straight up, slowly and gently. Rinsed the wells with distilled water at the sink using a syringe.

Removed the gel assembly from the casting stand and snap it into the cooling core assembled of the gel in the tank, so that the two gels are in one unit. The tank is filled with running buffer.

Samples are loaded. Placed the lid on top of the lower buffer chamber. Attached the electrical leads to the gel apparatus. Attached the leads to the power supply and turned on power. Set the power at 50 Volts.

The gel was run for approximately 45 minutes or until the dye just begins to run out the bottom. Turned the voltage to zero and then turned off the power supply. After removed the gel lid and pull inner cooling core out of the lower chamber. Pour off upper buffer into the sink and flush with running water. Disassembled the units to obtain the glass sandwich out. Carefully rip off the gel by grappling two corners and lifting off. These gels are very thin and map rip easily. This is easier to do with wet fingers. Nicked one edge of the separating gel for orientation. Place the in Coomassie blue stain. Stained the gel for 30 minutes. Transferred the gel from the
stain to the de stain solution. Cover the container. Poured Coomassie blue back into its original bottle using the funnel. Leave the gel in the destaining solution on the shaker for 30 minutes – 3 hrs. After destaining transfer the gel. Poured the destain back into the “used destain bottle” for recycling or disposal.

Observed the bands using with transluminator and measured.

**SDS-PAGE of proteins**

**Separation of proteins under denaturing conditions**

Sodium dodecyl sulphate (SDS) is anionic which denatures proteins by “wrapping around” the polypeptide backbone and SDS binds to proteins fairly specifically in a mass ratio of 1:4:1. In doing, so SDS confers a negative charge to the polypeptide in proportion to its length. The denatured polypeptide becomes “rods” of negative charge cloud with equal charge densities per unit length. It is usually necessary for separation by size. This is done with β mercaptoethanol or dithiothreitol. In denaturing SDS-PAGE separations therefore migration is determined not by intrinsic electrical charge of the polypeptide, but by molecular weight.

**Continuous and discontinuous buffer systems**

There are two types of buffer systems in electrophoresis, continuous and discontinuous. A continuous system has a only single separating gel uses the same buffer in the tanks and the gel. In a discontinuous system, a non restrictive large pore gel called a stacking gel is layered on top of a separating gel called resolving gel. Each gel is made with different buffers, and the tank buffers are different from the gel buffers. The resolution obtained in a discontinuous system is much greater than that obtained with a continuous system.
Hazards

Acryl amide has been shown to be a NEUROTOXIN. TEMED is a flammable substance and vapors are irritating to mucous membranes and eyes. Acetic acid is an irritant. Ammonium per sulphate is harmful if swallowed or inhaled.

Flow- chart -1
SDS - PAGE (DISCONTINUOUS)
(LAEMMLLI 1970)

Ag Preparation (Hussain et al 2000.)

Ag purification

Discontinuous SDS - PAGE

50 Volts (3hrs)

Staining (CBB)

Destaining
3.2.5 Western/ Immunoblotting (Wolf 1954)

Primary antibodies were raised in healthy rabbits, following the procedure of Wolf (1954).

When the gel was approaching the end of its run, rinsing of the graphite plates with distilled water and wiping of any beads of liquid that had adhered to them with Kim wipe or other non absorbent tissues were done.

1. Wearing gloves, six pieces of Whatman No 3 mm paper and one piece of nitrocellulose filter paper (Millipore HAWP or equivalent) to the exact size of SDS-PAGel were cut; one corner of the filter was marked with a soft lead pencil. The gloves are worn to prevent the transfer of oil and secretions from the skin to the filter paper.

2. The nitrocellulose filter was floated on the surface of a tray of deionized water, and allowed to wet from beneath by capillary action. Then, submerged the filter in the water for at least 5 minutes to displace trapped air bubbles.

3. The six pieces of 3 mm paper were soaked in a shallow tray containing a small amount of transfer buffer.

Transfer buffer

39 mM Glycine

48 mM Tris base

0.037% SDS (electrophoresis grade)

20% methanol
4. To prepare one litre of transfer buffer (pH 8.3), mix 2.9 g of glycine, 5.8g of Tris base, 0.37 gm of SDS and 200ml of methanol.

5. Wearing gloves, the transfer buffer apparatus was set as follows:

   a) Laying the bottom electrode (which will become the anode) was laid float on the bench graphite side up.

   b) On the electrode three sheets of 3mm paper that has been soaked in transfer buffer was placed. The sheets were stacked one on top of the other so that they were exactly aligned using a glass pipette as a roller, air buffles were squeezed out.

   c) Placing the nitrocellulose filter on a stack of 3 mm paper. The filter was exactly aligned and verified that no air bubbles were trapped between it and the 3mm paper.

   d) The glass plates holding the gel was removed from the electrophoresis tank, the gel was transformed briefly to a tray of deionized water, and placed exactly on top of the nitrocellulose filter. The gel was adjusted. So that the mark on the filter corresponded to the bottom left hand corner of the gel. Any trapped air bubble if present was squeezed out with a glove hand. To avoid the possibility of short circuit the bottom left hand corner of the gel was not cut off.

   e) The final three sheets of 3 mm paper on the gel were placed, again making sure that they were exactly aligned and that no air bubble was trapped.

6. The upper electrode (which would become the cathode) was placed on top pf the stack, graphite side down. The electrical leads (positive or red lead to the
bottom graphite electrode) were connected. Apply a current of 0.65 mA/sq.cm of gel for a period of 1.5 – 2 hour.

7. The electric current was turned off and the leads were disconnected. The transfer apparatus was disassembled from the top down, peeling off each layer in turn. The gel was transferred, to a tray containing coomassie brilliant blue, and stains it. This would allow checking whether electrophoretic transfer is complete.

8. The bottom left-hand corner of the filter was cut. This served as insurance against obliteration of the pencil mark (step 2). The filter was stained with ponceau S.

3.2.6 Staining proteins immobilized on the NCP

Staining with ponceau S does not interfere with detection of antigens by chromogenic reactions catalyzed by antibody-linked enzymes such as alkaline phosphatase or horseradish peroxides.

3.2.6.1 Ponceau S stain

a. Stock solution of ponceau S: ponceau S 2g, trichloro acetic acid 30g water100 ml.

3.2.6.2 Staining procedure

1. After the nitrocellulose filter paper had been dried, it was floated on the surface of a tray of deionized water and allowed to wet from beneath by capillary action. Then it was submerged in the deionised water for at least 5 minutes to displace trapped air bubbles.

2. The filter was transferred to a tray containing working solution of ponceau S stain and incubated the filter for 5 – 10 minutes with gentle agitation.

3. When the bands of protein were visible, the nitrocellulose filter was in several changes of deionised water of room temperature.

4. The positions of proteins were marked using the molecular weight marker as standards with waterproof black ink.

5. Immunological probing was then proceeded blocking binding sites were immunoglobulins on the NCP.

6. The sensitivity of western blotting depends on reducing back round of nonspecific binding by blocking potential binding sites with irrelevant proteins. The best and least expensive blocking solution is non fat dried milk. It is easy to use and is compatible with all of the immunological detection systems in common.

3.2.6.3 Blocking solution

5 % wt/v non fat dried milk

0.01% antifoam A

0.02 % sodium azide in PPS
3.2.6.4 Blocking procedure

1. The nitrocellulose filter was transferred to a plastic bag (sears seal- A-meal), and added with 0.01 ml of blocking solution per square cm of NCP. The bag was sealed, leaving as few air bubbles as possible, and incubated for 1 – 2 hour at room temperature with gentle agitation on a platform shaker.

2. The plastic bag was cut and open. The blocking solution was discarded. Immediately the filter was incubated with an antibody directed against the target protein.

3.2.7 Binding of the primary antibody to the target protein

Virtually all western blots are probed in two stages: An unlabelled antibody specific to the target protein is first incubated with the nitrocellulose filter paper in the presence of blocking solution. The filter is then washed and incubated with a secondary reagent – anti – immunoglobulin coupled to an enzyme such as horse radish peroxidase or alkaline phosphatase. After further washing, the antigen – antibody – anti-antibody complexes on the nitrocellulose filters are located by in situ enzymatic reaction.

3.2.8 Incubating the NCP with the primary antibody

1. The nitrocellulose filter paper was placed in a heat – sealable plastic bag (sears seal- A-meal), and added with 0.1 ml blocking solution per square cm of NCP and appropriate quantity of primary antibody raised in rabbits. The test dilutions of the polyclonal antibodies were 1:500.

2. The bag was sealed, leaving as few air bubbles as possible, and incubated for 1 – 2 hour at room temperature with gentle agitation on a platform shaker.
Longer incubation time (up to 18 hour room temperature) increases the sensitivity of detection of target antigens. However the background of nonspecific binding also increases with the time and temperature of incubation the NCP with secondary immunological reagent.

1. The NCP was transferred from the final wash in PBS to a tray containing 200 ml of 150 mM NaCl, 50 mM Tris Cl (pH 7.5) the filter was incubator for 10 minutes at room temperature with gentle agitation.

2. The filter was transferred to a heat sealable plastic bag or shallow tray, containing 0.1 ml of phosphate free, azide free blocking solution 5 % wt/v not fat dried milk, 150 mM NaCl, 50 mM Cl (pH 7.5)

3. The enzyme – coupled secondary reagent as horseradish peroxidase was added before sealing the bag. The secondary reagent was diluted to 1:1000.

4. The filter with the enzyme coupled secondary reagent was incubated for one hour at room temperature with gentle agitation (This step was repeated 3 more times using NaCl / Tris Cl solution each time).

5. The filter was transferred to a tray containing 200 ml of 150 mM NaCl and 50 mM Tris Cl (pH 7.5) the filter was incubated for 10 minutes at room temperature with gentle agitation.

6. Chromogenic substrate was added with enzyme-coupled antibodies.

**Chromogenic substrate solution**

6 mg of Di amino Benzidine Tetra hydrochloride was dissolved in 9 ml of 0.01 M Tris Cl (pH 7.6) 1ml of 0.3 % of (w/v ) NiCl₂ or CoCl₂ was added. The solution of Di amino benzidine was freshly prepared and filtered through whatman
No 1 to remove precipitate. 10 µl of 30 % H₂O₂ was added and mixed well and the mixture was used immediately.

This most sensitive substrate immuno coupled horseradish peroxidase was used (3, 3¹-Diamino benzidine) which converted in situ in to a brown precipitate. The reaction was stopped as soon as the specifically bands clearly visible.

7. Transferring the washed nitrocellulose filter to shallow tray was done. 0.1 ml of substrate solution was added per square cm of filter. The NCP was incubated at room temperature with gentle agitation.

8. Monitoring the progress of reaction carefully, when the bands were desired intensity, the NCP was washed briefly in water then transferred to a tray containing 250 ml of PBS.

3.3 FIELD INVERSION GEL ELECTROPHORESIS - RESTRICTION ENDONUCLEASE ANALYSIS (FIGE-REA)

All strains of methicillin resistant *S. aureus* isolates and MTCC 87, which could not able to be differentiated by physical and biochemical parameters, were collected from Tamilnadu and they were selected as the subject for conducting the RFLP study.

3.3.1 Maintenance of the Culture

All strains of methicillin resistant *S. aureus* and MTCC 87 were maintained in nutrient agar slant with 7% NaCl and 5µg/ml Oxacillin and it was stored at 4°C.
3.3.2 Preparation of the Nutrient Agar Medium

Ingredients | Grams/litre
---|---
Peptone | 5g
Beef Extract | 3g
Yeast Extract | 2g
Sodium Chloride | 5g
Agar | 15g
Distilled water | 1000ml
pH | 7.2 to 7.4

3.3.4 Propagation of the Culture

The organisms were propagated in Luria Bertani (LB) Broth and were incubated at 37°C for 24-48hrs.

3.3.5 Preparation of Luria Bertani Broth

Ingredients | Grams/litre
---|---
Tryptone | 10g
Yeast Extract | 5g
NaCl | 10g
Distilled water | 1000ml
pH | 7.2
The growth of the culture could be monitored by reading the OD value at 660nm. Harvesting the culture by centrifuge at 5000rpm for 10mins and the pellet was taken for a genomic DNA isolation.

3.3.6 Chromosomal DNA isolation (Lysozyme extraction) (Murray and Thompson, 1980)

Materials Required

- Isolates and MTCC 87
- Centrifuge tubes
- Eppendorff tubes
- Micropipette
- Phenol-chloroform
- 70% ethanol

a. **Solution A**

   25mM Tris Hcl – 30.3mg
   50mM glucose- 900mg
   Lysozyme – 100mg
   Distilled water- 100ml
   pH 12.5

b. **Solution B**

   0.2M NaOH-0.8g
   SDS-1g
Distilled water- 100ml
pH – 7.8

c. Solution C

3M Sodium acetate – 24.6g

Distilled water – 100ml, pH ± 4.8

Procedure

1) 1.5ml of overnight broth culture was transferred and it was subjected to
centrifugation at 5000 rpm for 10mins.

2) The supernatant was discarded and the pellets were collected and
suspended in 100µl of solution A and it was kept in ice for 30mins.

3) To this, 200µl of fresh Solution B was added and it was vortexed and
kept gently in ice for 5mins.

4) 150µl of solution C was added and vortexed and again kept in ice for one
hour.

5) This mixture was centrifuged at 10000 rpm for 10mins.

6) The pellet was removed and 100µl phenol-chloroform (1:1) was added.
The mixture was mixed well and phenol-chloroform was removed after
centrifugation at 10000 rpm for 10mins.

7) Then added 50µl of 70% ethanol. The mixture was centrifuged at 10000
rpm for 10mins.

8) The supernatant was discarded and the pellet was suspended in TE buffer
and it was run through AGE at 50V for 30mins.
FLOW CHART – 2

CHROMOSOMAL DNA ISOLATION  (Murray and Thompson, 1980)

Transferred 1.5 ml of overnight culture

Centrifuged at 5000 rpm for 10 minutes

Suspended the pellet in 100 l of solution A

Kept on ice condition for 30 minutes

Added 200µl of fresh solution B

Vortexed gently and kept it in ice for 5 minutes

Added 150 l of solution C, vortex gently and kept on ice for 1 hour

Centrifuged at 10,000 rpm for 10 minutes.

Removed the pellet and phenol: chloroform (1:1) and add 70% ethanol

Centrifuged at 10,000 rpm for 10 minutes at 4°C

Discarded the supernatant and suspended the pellet with TE buffer

Ran on AGE at 50 V for 30 – 40 minutes
3.3.7 Setting Up Digestions With Restriction Enzymes

Requirements

Genomic DNA from MRSA Strains, Agarose, BSA 10mg/ml, 20x Dithiothrietol, EtBr, Restriction enzyme as Sma I, Erlenmayer flask, gloves. Ice cubes, eppendorf tubes, and micropipette. Micro tips.

Restriction Enzyme Buffer (10X)

2X KGB (Hanish and Mc Clelland et al., 1988)

200mM Potassium glutamate

50 mM Tris acetate (pH 7.5)

20 mM Magnesium acetate

100 mg/ml BSA

1mM β- mercaptoethanol

Stop Buffer

0.5 M EDTA (pH 8.0)

6X gel loading buffer

40 g of sucrose in 60 ml distilled water and add 250 mg of bromophenol blue mixed by stirring for 20 minutes and stored at 4°C.

Procedure

Genomic DNA was isolated from MRSA isolates and MTCC 87 was placed in a sterile microfuge tube, and it was mixed with sufficient deionized water to give
a volume of 18µl. Then 2 µl of 10% restriction digestion enzyme buffer was prepared with 1-2 Units of restriction enzyme was added.

The mixture was mixed well by tapping the tube and the mixture was incubated at 37°C for one hour. After incubation the reaction was stopped by adding 0.5 M EDTA to a final concentration of 10mM. Then 6µl gel loading buffer was added and mixed by gentle vortexing and the digest was loaded into the gel slot.

**FLOW CHART - 3**

**DNA DIGESTION WITH RESTRICTION ENZYME**  
*(Maniatis * et al., 1989)*

Placed the DNA solution in a sterile microfuge tube  
↓

Mixed with sufficient deionized water to give volume 18µl  
↓

Added 1 to 2 Units of Restriction Enzyme  
↓

Mixed by tapping the tube  
↓

Incubated the mixture at 37°C for an hour  
↓

Stop the reaction by adding 0.5 M EDTA to a final Concentration of 10 mM  
↓

Added 6µl of gel loading buffer  
↓

Mixed by brief vortexing  
↓

Loaded the digest into the gel slot
Deprotenisation of digested DNA (Maniatis et al., 1989)

Concentrated DNA was taken after the reaction has been stopped by the addition of EDTA. 6 volumes of 5mM ammonium acetate and 2 volumes of ethanol were added. The tubes were stored on ice for 5 minutes. The tubes were at centrifuged 10,000 rpm for 5 minutes at 4° C. Supernatant which contained most of the proteins was discharged. The pellet was allowed to dry at room temperature. DNA pellet was then dissolved in 10µl of TE buffer.

If the digested DNA has to be purified further more. It should be extracted once with phenol: chloroform (1:1) and once with chloroform and the DNA was precipitated with ethanol. Before loading buffer was added to the sample 10µl of 6% gel loading buffer was added to the sample, further it was heated at 65° C for 2 minutes to dissociate the DNA aggregates.

3.3.8 Agarose Gel Electrophoresis (Maniatis et al., 1989)

Procedure

The mini gel electrophoretic apparatus at the experiment table was assembled. 240 mg of agarose was melted in 30 ml of 1XTAE buffer at 55° C in a water bath. The through was filled with the molten agarose and the comb was inserted immediately. Then the 7.5mm thickness gel was allowed to solidify at room temperature for 30 minutes.

After the solidification of the agarose the comb removed gently and also the tape from the trough. The buffer reservoir was filled with 5X TAE buffer until it just covered the gel.

Then DNA sample mixture heated at 90° C for one minutes, and
Poured to wells of the agarose gel. Ran at using PFGE-FIGE (BMERF, INDIA). The banding patterns of DNA fragments were analyzed.

**FLOW CHART - 4**

**AGARASOE GEL ELECTROPHORESIS**

*(Maniatis et al., 1989)*

Assembled the minigel electrophoretic apparatus

Melted 240 g of agarose in 30 ml 1X TAE buffer at 55 C in a water bath

Filled the trough with agarose and immediately insert the comb

Thickness of gel should be 7.5 mm

Gently removed the comb

Removed the tape from the trough

Filled the buffer reservoir with 5X TAE buffer until it just covered the gel

Samples mixture poured into appropriate well

Gel apparatus connected to PFGE power pack
3.4 PCR AMPLIFICATION OF meca GENE (Olivera and Ramos 2002):

Bacterial strains (MRSA isolates)

Prepared chromosomal DNA

Oligonucleotides

5' - GTT GTA GTT GTC GGA TTT GG - 3' (upstream)

5' - CTT CCA CAT ACC ATC TTC TTT AAC - 3' (down stream)

Finzyme PCR kit (Finland)

Name of the Instrument: eppendorf master cycler

PCR conditions

The DNA extract (600ng) was amplified by PCR in a final volume of 50µl, containing 0.25mM of each dNTP, 40pmol of each primer, 0.5 IU Taq DNA polymerase and buffer provided by the manufacturer. The denaturation was performed for 1 min at 95°C; the annealing was for 1 min at 58°C, and the primer extension for 2 min at 72°C with total of 30 cycles. A sample of 10µl from each reaction was analysed by gel electrophoresis in a 0.8% agarose gel with ethidium bromide, and results were observed.

3.4.1 PCR – Amplification of 16S rDNA Sequence

To isolate and sequence the 16S rDNA of MRSA isolates named as SMKV-1 and SMKV-2, a single colony was transferred into nutrient agar broth (Oxoid CM3 agar) supplemented with 0.04% (wt/vol) KH₂PO₄ and 0.24% (wt/vol) Na₂HPO₄.H₂O (pH 6.8) and incubated at 28°C for 48h. The cell pellets from 1.5 ml cultures were obtained by centrifugation at 13000 rpm for five minutes and washed with TE
buffer (10 mM Tris-HCL; 1 mM EDTA, pH 8.0). The cell pellets were then dissolved in 200 µl of TE. Cell lysis was obtained at 37°C after treated with lysozyme (2mg/ml; final concentration) for 30 min and by using SDS (1%). The lysate was extracted twice with phenol/chloroform followed by two extractions with chloroform/isoamyl alcohol (24:1). After adding 1/10 volume of sodium acetate (3 M, pH 5.2) and 0.5 volume of isopropanol, the supernatant was incubated at -20°C for 30 min. The precipitated nucleic acids were then sedimented by centrifugation at 13000 rpm for 20 min and the resulting pellet was washed with 70% ethanol before drying under vacuum. The nucleic acid pellets were then dissolved in 100 µl TE and used as template for PCR amplification of 16S rDNA gene.

The Primers 27f (5’-GAGTTTGATCCTGGCTCAG-3’) and 1525r (5’-AGAAAGGAGGTGATCCAGCC-3’) were used (Rainey et al., 1996). The 100 µl reaction mixture containing 200 µM deoxynucleoside triphosphates (Perkin-Elmer, France), 0.1 µM concentrations of primers (Sigma), 20 mM Tris-Hcl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 2.5 U of Taq polymerase (Qiagen), and 10 µl of template DNA or water for the negative control. An initial denaturing step of 95°C for 10 min was followed by 30 cycles of amplification (1 min at 94°C, 1 min at 55°C, and 2 min at 72°C) and a final extension step at 72°C for 10 min. DNA amplification was checked by electrophoresis of 5 µl of PCR product in a 1% agarose gel in Tris-borate-EDTA (TBE) buffer (pH 8.3) and by staining with ethidium bromide.

The PCR product was purified by using the Nucleotrap PCR extraction kit (Macherey Nagel) and sequenced (ABI Prism 377 DNA sequencer, Perkin-Elmer, Biosystems) using the following primers 27 f (5’-GAGTTTGATCCTGGCTCAG-3’), 343r (5’-CTGCTGCCTCCCGTA-3’), 357f (5’-TACGGGAGGCAGCAG-3’), 519r [5’-G(T/A) ATTACCGCGGC (T/G) GCTG-3’], 536f [5’-AGC(C/A)
GCCGCGGTAA (T/A) C-3’], 803f (5’-ATTAGATACCCCTG GTA G-3’), 907r (5’-CCGTCATTCATTTGAGTTT-3’), 1114f (5’-GCAACGAGCGCAA CCC-3’), 1385r[5’-CGGTGTGT(A/G) CAAGGCC-3’) and 1525r (5’-GAAAGGAGG TGATCCAGCC-3’).

For the phylogenetic analyses, related 16S rRNA gene sequences within the genus *Staphylococcus* were included. 16S rDNA sequences were aligned by using the MEGALIGN program of DNASTAR. An evolutionary distance matrix was generated as described by Jukes & Cantor (1969). The evolutionary tree for the datasets was inferred from the neighbor-joining method of Saitou & Nei (1987) by using the neighbor-joining program of MEGA version 2.1 (Kumar *et al*., 2001). The stability of relationships was assessed by performing bootstrap analyses of the neighbor-joining data based on 1000 resamplings.

### 3.5 WHOLE GENOME SEQUENCE STUDIES

Complete Genome Sequence of two highly pathogenic related methicillin resistant *S. aureus* strains isolates such as MRSA1, MRSA2 (SMKV1 and SMKV2) were done with the help of shot gun random sequencing. The open reading frames were identified by use of GAMBLER and GLIMMER programs, and annotation of each was done with a BLAST homology search, motif analysis and protein localization prediction.
3.6 PHYTOCHEMICAL STUDIES

3.6.1 Extraction Procedure

Materials required

1. Ethanol
2. Soxlet apparatus
3. Thimbles
4. Heating mantle
5. Whatman No 1 filter paper
6. Muller – Hinton agar medium
7. Muller – Hinton broth

Procedure

1. The medicinal plants of *Azadirachta indica* juss, *Duranta plumeneri jacq* and *Punica granatum* Linn Leaves, bark and seeds, pulp and epicarp were collected and dried in room temperature of 10 days.(Plate 17, Plate 18, Plate 19)

2. After 10 days the dried plant materials were converted in to powder form with the help of mixer grinder.

3. The medicinal plant materials were filled in the thimbles separately (per thimble containing 8gm of dried powder). Then the powder filled thimbles were kept in the Distillation unit is functioned with the help of heating mantle (temperature about 80 °C). The extraction was carried out with the help of ethanol.
3.6.2 Antibiotic disc preparation (Sundararajan 1997)

Procedure

1. The sterile whatman No1 filter paper was taken and disc was prepared.

2. The disc was impregnated with the medicinal plant extractions (5µg, 10µg, 15µg, 20µg, 25µg, 30µg). And discs were dried in sterile condition. And stored in sterile vials at 4º C

3.6.3 Analysis of the antibacterial activity using medicinal plants extractions

Procedure

Prepare the Muller-Hinton agar plate to perform the disc diffusion method.

1. MRSA isolates swabbing is done on MHA Plate and plant extractions discs were placed on a plate.

2. Incubated the plate for 12 hrs at 35 C. And the zone growth inhibition measured and the antibacterial activity was identified.

3.6.4 Tube-dilution technique (Annear. 1968)

Procedure

1. Sterile Muller-Hinton broths were prepared and 5ml transferred to sterile test tubes.

2. A loopful organism was transferred to MH broth tube.

3. One tube acts as a control.

4. Then our extracts are added to MH broth containing organism respectively.

5. Tubes are incubated at 37ºC for overnight.
6. After incubation organisms were serially diluted and spreaded on MHA plate with the help of “L” rod.

7. MIC values of medicinal plants extract were calculated.

3.6.5 Thin Layer Chromatography: (VWR International limited 2004)

Materials required:

1. TLC plates
2. TLC spreader
3. cellulose powder(TLC grade)
4. Ethanol
5. Acetic acid
6. Hot air oven
7. Tannic acid
8. Samples
9. Vortex mixer

Procedure

1. Good antibacterial activities of plant extract were only selected for TLC analysis.

2. *Punica granatum* Linn epicarp have good antibacterial activity against MRSA.

3. 25 gram of cellulose powder (TLC grade), weighed and thoroughly mixed with 50 ml of sterile distilled water.(60-90 S electric mixer at slow stirring)
4. Then poured to TLC plate and spreaded with the help of spreader. The thickness of the layer was 250mm and allowed air dry for 60 minutes. After drying layer thickness was 100mm (Stationary phase)

5. Then plate activated at 120° C for 30 minutes.

6. Activated plate used for further purpose.

7. 0.1 µl of samples and standard markers (Tannic Acid) were properly loaded on TLC plate.

8. The loaded plates were transfer to chromatographic jar containing acetic acid and distilled water [1:9 Ratio] (Mobile phase)

9. After running of the sample NaN02 were sprayed.

10. Finally the spots were identified. And the Rf values were calculated.

3.6.6 UV spectrophotometric analysis (Hitachi UV 100)

Materials required:

1. Fractionated samples
2. Methanol
3. Sterile distilled water
4. Sterile cuvettes
5. Tannic acid

Procedure

1. Standard marker, fraction A, B, C was dissolved in methanol (1 mg/ml).
2. The mixed marker and samples were transferred to cuvette.
3. And it was placed in to the UV spectrophotometer (210nm- 550nm).
4. Maximum OD values were observed.
3.6.7 High Performance Liquid Chromatography

(HPLC Shimadzu (SPD- 6AV))

Materials required

1. Fractionated samples
2. Methanol
3. Sterile distilled water
4. Sterile 1ml Glass syringe
5. Tannic acid

Procedure

1. Standard marker (Tannic Acid) and fractions A, B, C were dissolved in methanol (1 mg/ ml)
2. 20 µl of marker fraction A, B, C injected in to RP 18 column of HPLC Shimadzu (SPD- 6AV) instrument.
3. Methanol: Water (2:1 ratio) were act as a mobile phase
4. The flow rate was 1min/ml
5. Results were observed.

3.6.8 Hydrogen Nuclear Magnetic Resonance : (Bruker VC 200 U.S.A, 200 MHz)

Materials required

1. Fractionated samples
2. Methyl - d₃ - alcohol- d
3. Sterile distilled water
4. Sterile 1ml Glass syringe
5. Tannic acid
Procedure

1. Standard marker (Tannic Acid) and fractions A, B, C were dissolved in methyl d₃ alcohol-d.

2. Then samples are transferred to cuvette and put in inside of NMR apparatus. (Bruker VC 200 U.S.A 200 MHz).

3. Results were observed.