3.0 MATERIALS AND METHODS

This chapter deals with the materials used and research methodologies employed to achieve the specified objectives of the study. All the materials, chemicals, glassware and equipment used in the experiments are given in Annexure-I.

3.1 *Staphylococcus aureus* (isolates/strains)

3.1.1 Clinical Isolates of *Staphylococcus aureus*

A total of 135 isolates recovered from human patients at Indira Gandhi Medical College (IGMC) Shimla and confirmed as *S. aureus* isolates in our laboratory at the Department of Microbiology, Shoolini University, Solan. These isolates were recovered from human patients during the period Jan, 2011 to January, 2012 at IGMC, Shimla. The isolates originated from different clinical conditions and recovered from different samples: pus 84 (62.2%), urine 22 (16.2%), blood 19 (14.0%), and catheter associated infections 10 (7.4%). These isolates were identified on the basis of their colony characteristics, Gram’s staining, microscopic examination and biochemical characteristics (Patil *et al*., 2013) and used for further characterization according to the objectives of the study.

3.1.2 Standard/reference strains of *Staphylococcus aureus*

ATCC 29295 strain of *S. aureus* was used as a multi drug resistant (MDR) strain and ATCC 43300 strain of *S. aureus* was used as sensitive reference strain in the study.

3.2 Preservation of the isolates

The *S. aureus* isolates were preserved in 50% glycerol and stored at -20°C till their use in the study.

3.3 *In vitro* antibiotic culture sensitivity assay

3.3.1 Standard antibiotic discs used in the assay

The standard antibiotic discs (Hi-media, Mumbai-IC002) included in the assay were: cephalothin (30mcg), clindamycin (2mcg), co-trimoxazole (25mcg), erythromycin (15mcg), gentamycin (10mcg), ofloxacin (5mcg), penicillin (10units), vancomycin (30mcg), ampicillin (10mcg),...
chloramphenicol (30mcg), methicillin (5mcg), tetracycline (30mcg), oxacillin (1mcg), linezolid (30mcg), azithromycin (15mcg), amikacin (30mcg), clarithromycin (15mcg), teicoplanin (10mcg), amoxyclav (30mcg) and novobiocin (5mcg).

3.3.2 Procedure of the sensitivity assay

Isolated colonies, 3-4 in number were picked up from 24 hours blood agar culture and suspended in normal saline. The turbidity of the saline was measured by using spectrophotometer at 625nm. The optical density (OD) of the suspension was adjusted to 0.08 to 0.10 which is equivalent to 0.5 McFarland standards (Walsh et al., 2001). The suspension at this OD value contained approximately $1 \times 10^8$ cfu/ml which was further diluted ten folds in normal saline to adjust the number of bacteria at $10^7$ cfu/ml. The bacterial suspension (100µl) was inoculated on the surface of Muller Hinton agar (Hi-media, Mumbai) plates and spread uniformly under sterile conditions. S. aureus strain ATCC 29295 was included as a reference MDR strain in each batch. The inoculated agar plates were left at room temperature for 5-10 minutes. The standard antibiotic discs as mentioned in the section 3.3.1 were then aseptically placed with the help of sterile forceps over the agar surface and the plates were then incubated at $37^0$C for 24 hrs. The inhibitory activity of each antibiotic was determined by measuring the diameter of zone of inhibition and evaluated as per standard protocol of CLSI (M7-44) for all MRSA and MSSA strains.

3.4. Extraction of genomic DNA of MRSA and MSSA strains.

The genomic DNA of 40 MRSA and 20 MSSA strains originating from blood, pus, urine and catheter associated infections was extracted by the following methods and utilized for molecular studies.

3.4.1 Phenol: Chloroform method

The overnight cultures of S. aureus isolates, each in 10 ml of Luria broth were utilized for the extraction of genomic DNA. The cells were harvested by centrifugation at 12,000 rpm for 12 min. in a centrifuge (Eppendorf-5810R). The pellet was washed twice in normal saline and again centrifuged at 8000 rpm at $10^0$C for 10 min. 10 mM Tris-HCl (pH 8) in a volume of 0.5 ml and lysozyme (2.5 mg/ml) were added to the pellet followed by incubation at $37^0$C for 2 hrs. Equal
volume of lysis buffer (50 mM Tris, 100 mM EDTA, 1% SDS, pH 8) containing proteinase-K to a final concentration of 1 mg/ml was then added. The contents were mixed gently and incubated at 65°C for 1 hr in a water bath (Super Tech- Digital Water Bath). One ml of phenol: chloroform (1:1) was then added and contents mixed thoroughly for 2-3 minutes. Centrifugation was done at 12,000 rpm at 4°C for 15 min. The upper layer was transferred to a fresh sterile tube for further extraction with chloroform: isoamyl alcohol (24:1), centrifuged at 12,000 rpm at 4°C for 15 min. To the supernatant, 5 M NaCl in a volume of 50 µl and 95% ethanol twice the volume of the supernatant were then added for precipitating the DNA after overnight incubation. The pelleting of the DNA precipitates was done by centrifuging the contents at 10,000 rpm at 4°C for 15 min. The DNA pellet was dried after decanting off the supernatant and suspended in TE buffer in a volume of 50 µl to 200 µl depending upon the amount of extracted DNA. It was then incubated at 45°C in a water bath for 2-3 hours. The extracted genomic DNA was electrophoresed in 0.75% agarose gel under constant current of 90 mA and visualized using gel documentation system (Alpha InfoTech). The extracted DNA was stored at -20°C till its use in the study.

3.4.2 Extraction and purification of genomic DNA of MRSA and MSSA isolates with Axygen Kit.

The isolation bacterial genomic DNA by this kit is based upon the efficient release of genomic DNA by a special lysis buffer, buffer G-A. Following this, rapid separation of genomic DNA from the proteins, polysaccharides and lipids is achieved by a unique phase-partitioning step. Highly purified genomic DNA in the lower Phase is then selectively bound to a special miniprep column. After washing successfully with buffer W1 and buffer W2 remove residue impurities and salts, the purified bacterial genomic DNA is eluted from the miniprep column in Tris buffer or water. The bacterial genomic DNA was extracted by using DNA miniprep spin protocol which is as follows:

The overnight cultures of *S. aureus* isolates, each in 10 ml of Luria broth were utilized for the extraction of genomic DNA. The bacterial cells were harvested by centrifugation at 12,000 rpm for 30 sec. and the pallet was resuspended in 150 µl of the buffer ‘S’ containing RNase, followed by the addition of 20 µl of lysozyme. The contents were thoroughly mixed and incubated at 37°C for 30 min. 0.25 M EDTA (pH 8.0) in a volume of 30 µl was then added and the contents mixed
well and incubated on ice for 5 min. Buffer G-A in a volume of 450 µl was added to the contents followed by vortexing for 15 sec. The contents were then heated at 65°C for 10 min. in a water bath; G-B buffer in a volume of 400 µl was then added followed by 1 ml of pre-chilled buffer DV (pre-chilled to 4°C). The contents were mixed vigorously and centrifuged at 12,000g for 2 min. The upper phase was aspirated without disturbing the interphase. One ml of DV (pre-chilled to 4°C) buffer was then added to the remaining interphase and lower phase. The contents were mixed vigorously in order to achieve homogeneity followed by centrifugation at 12,000g for 2 min. The colored upper phase was discarded and lower phase was transferred to spin filter, placed into a 2 ml microfuge tube and centrifuged at 12,000g for 1 min. Spin filter was discarded and 400 µl of buffer BV was added to the filtrate and contents mixed thoroughly. A miniprep column was placed to a 2 ml microfuge tube. The binding mixture from the previous step was transferred to the miniprep column and centrifuged at 12,000g for 1 min. The filtrate was discarded from 2 ml microfuge tube and miniprep column was placed back to the 2 ml microfuge tube. W1 buffer in a vol. of 500 µl was added to the miniperp column and centrifuge at 12,000g for 1 min. The filtrate was discarded and the miniprep column was placed back to the 2ml microfuge tube. W2 buffer in a vol. of 700 µl was then added to it followed by centrifugation at 12,000g for 1 min. This washing step was repeated with a second aliquot of 700 µl of W2 buffer. The filtrate was discarded and miniprep column was placed back into 2 ml microfuge tube, centrifuged at 12,000g for 1 min. Finally, the miniprep column was transferred into a clean 1.5 ml microfuge tube. For elution of DNA, the 100-200 µl of eluent was added to the center of the membrane. This was allowed to stand for 1 min. at room temperature and centrifuged at 12,000g for 1 min. In order to improve the elution efficiency, the eluent or water was used pre warmed at 65°C. The purified DNA was collected in the micro-centrifuge tube by centrifugation at 12,000g for one min. The DNA was stored in micro-centrifuge tubes at -20°C till further use.

3.5 Amplification of virulence (coa and spa) genes and toxic shock syndrome toxin gene (tst) of MRSA and MSSA isolates

The Coagulase (coa), staphylococcal protein A (spa) and toxic shock syndrome toxin (tst) genes of 40 MRSA and 20 MSSA strains were amplified. The primer pairs used in the PCR assay along with the reaction conditions of amplification are presented in Table 3.1.
3.6 Electrophoresis of amplicons of coa, spa and tst genes
All the PCR products were electrophoresed in 1.5% agarose gel containing 0.2 μg/ml ethidium bromide (EtBr) at constant current of 90 mA. The PCR products were loaded in the wells using gel loading dye. DNA bands were visualized by gel documentation system.

3.7 Restriction enzyme analysis of amplicons of coa and spa genes

3.7.1. Coagulase (coa) gene amplicon
The PCR amplified products were digested with restriction enzyme *Hae*II. Reaction mixture (20μl) consisted of 5.0 μl PCR products, 2.0 μl react buffer, 0.5 μl *Hae*II (10 units/μl, Takara Biotech, 1052A), 12.5μl molecular grade water and incubated at 37°C for two hrs followed by snap freezing. The digests were electrophoresed in 2.0% agarose gel in order to visualize the restriction fragments.

3.7.2 Staphylococcal protein A (spa) gene amplicon
The amplicon of spa was digested with *Eco*RII. The digestion mixture (20μl) consisted of, 0.5μl PCR product, 2μl buffer, 0.25μl restriction enzyme (10 units/μl, Takara Biotech, 1052A), 12.75μl deionized water and incubated at 37°C in a water bath for 1:30 hrs. This was followed by snap freezing. The digests were analyzed by electrophoresis using 2.5% agarose gel.
Table 3.1 Amplification of coagulase (coa), staphylococcal protein A (spa) and toxic shock syndyome toxin (tst) genes by PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers used (5’-3’)</th>
<th>Reaction contents</th>
<th>Reaction conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coa</td>
<td>F: ATAGAGATGCTGGTACAGG R: GCTTCCGATTGTTCGATGC (Janwithayanuchit et al., 2006)</td>
<td>Target DNA 2 µl, 10x buffer 2.5 µl, MgCl₂ 50 mM, dNTPs 0.2 mM (each), Primers 20 pmol (each), Taq pol. 3 Unit/µl</td>
<td><strong>Step-1</strong>: Initial denaturation 94°C - 3 min (30 Cycles)</td>
</tr>
<tr>
<td>Spa</td>
<td>F: CAAGCACAAAAAGAGGAA R: CACCAGGTTTAACGACAT (Kurlenda et al., 2010)</td>
<td>Target DNA 3 µl, 10x buffer with MgCl₂ 2.5 µl, dNTPs 0.2 mM (each), Primers 20 pmol (each), Taq pol. 3 Unit/µl</td>
<td><strong>Step-1</strong>: Initial denaturation 94°C – 3 min (35 Cycles)</td>
</tr>
<tr>
<td>Tst</td>
<td>F: ATG GCA CGA TCA GCT TGA TA R: TTT CCA ATA ACC ACC CGT TT (Ghodban et al., 2006)</td>
<td>Target DNA 3 µl, 10x buffer MgCl₂ 2.5 µl, dNTPs 0.2 mM (each), Primers 50 pmol (each), Taq pol. 3 Unit/µl</td>
<td><strong>Step-1</strong>: Initial denaturation 94°C – 3 min (30 Cycles)</td>
</tr>
</tbody>
</table>
3.8 Nucleotide sequencing of the amplicons of coa and spa genes of MRSA and MSSA strains

The nucleotide sequencing of amplicons of coa and spa genes of three isolates each MRSA and MSSA originating from the blood, urine and pus was done by Xcelaris Laboratory on commercial bases. The sequences were compared with published nucleotide sequences of standard strains and their homology to them as well as amongst each other was determined. The nucleotide sequences of the amplicons of the coa and spa genes were submitted to National Centre for Biotechnology Information (NCBI) and their accession numbers were obtained.

3.9 Bacteriophage typing of MSSA isolates

A total of 42 MSSA strains were submitted to the National phage typing Centre, Dept. of Microbiology, Maulana Azad Medical College, at New Delhi. The typing was done employing international sets of phages in a routine test dilution (RTD) X 100.