CHAPTER 4

RESULTS

4.1 EPIDEMIOLOGY

Epidemiological results are tabulated and showed in figures. (Table 1, to 6.2, Text figures 1 to 6).

The clinical samples collected from southern region laboratories of Tamilnadu, India were divided into 8 age groups and assayed for pus, urine, blood, sputum, EnteroTracheal tube, throat swab, CerebralSpinalFluid, peritoneal fluid, skin swab, ear swab for methicillin resistant \textit{S.aureus} strains.(Tables 1-3).

The clinical samples collected from northern region laboratories of Tamilnadu, India were divided into 8 age groups the samples were assayed from pus, urine, blood, sputum, EnteroTracheal tube, throat swab, Cerebral Spinal Fluid, peritoneal fluid, skin swab, ear swab for methicillin resistant \textit{S.aureus} strains. (Tables 4-6)

Samples of the \textit{S.aureus} overall results showed more in the age group below 1. Infection is more in male infants compare to females. In table 1.1 showed \textit{S.aureus} was more in age group below 1. Males were affected than females. Table 1.2 showed only MRSA prevalence noticed age group below 1 again in male child.

In table 2.1 \textit{S.aureus} was more in age group below 1-blood samples. Males were affected more than females. In table 2.2 only MRSA prevalence noticed in age group 1 again in male child. Table 3.1 showed showed \textit{S.aureus} were more in
age group below 1 males were affected than females. The **table 3.2** only MRSA prevalence noticed age group below 1 again in below male child.

In **table 4.1**, *S.aureus* was more in age group below 1. Male infant were affected than female. **Table 4.2** only MRSA prevalence noticed in the age group of below 1 year olds again in male childrens. **Table 5.1 and 5.2** also showed *S.aureus* and MRSA prevalence noticed in the age group below 1, again in male children. The year 2003 also showed similar prevalence of MRSA in the age group of below 1 year olds as evidenced in tables **6, 6.1, 6.2**.

Table 7-12 and text figure 1-6 were showed the percentage of MRSA prevalence in Tamilnadu, India from 2001-2003.

### 4.2 CULTURE ISOLATION AND IDENTIFICATION

All MRSA cultures are Gram-Positive and are grown at 35 to 37°C on nutrient agar media. Most of the colony appeared as yellow in color. All colonies were smooth, circular and convex. Some of the cultures are appeared as white in color. Most of the isolates well grown on 7-15% of NaCl containing nutrient agar media. All isolates of MRSA were highly resistant to methicillin (5µg), penicillin G (10U), tetracycline (30µg), gentamycin(10µg),bacitracin(10µg),ampicillin(10µg), novobiocin(30µg),doxycycline(30µg), amikacin(30µg), *(Plate 1-3)*

All isolated colonies produced β-hemolysis *(Plate 4)*, where as some colonies produced δ-hemolysin. All isolates of MRSA were coagulase positive; it forms the colloidal nature of plasma. When all colonies were transferred to gelatin medium, it become liquid in nature at 4°C. Isolates produce golden yellow color colonies on this selective media (Mannitol-Salt Agar) media *(Plate 5)* and they are non motile.
All MRSA cultures are pencillinase enzyme producers. Pencillinase enzyme productions where identified by indigenous ELISA, all isolates decolorize the starch iodine blue color complex. The degraded starch powder settled on the bottom of the tube, (Plate 6).

4.3 PATHOLOGY and CLINICAL SYMPTOMS

The mice inoculated with 48 hours of MRSA cultures were affected by rheumatoid arthritis. The back legs of the experimental animal became bent (Plate 7). The legs had lesions-containing pus (Plate 8). The MRSA cultures were isolated from the pus of infected mice leg as per Koch’s postulate procedure.

The nasal region of experimental animals released sputum like liquid after 48 hours of inoculation. The MRSA cultures isolated from nasopharyngeal region, loss of weight, low food intake, the deserted experimental animal liver has more number of white colored lesions like patches of liver. Liver cells and kidney cells which were highly damaged and showed the formation of necrosis. (Plate 9-14)

All isolates of MRSA can be easily grown on tributyrine agar medium. That produces yellow colored colonies on TBA medium and produced a lipase enzyme finally that shows clear opaque zones of TBA medium.

4.4 PROTEIN ESTIMATIONS

110 µgm/ml of cell wall proteins were taken for SDS-PAGE study, all proteins samples are purified with 10% of TCA.
4.4.1 SDS – PAGE

All MRSA isolates bands were found in between 5.8kDa and 79.5kDa regions, compared with indigenous standard marker having the bands at 5.8kDa, 22.5kDa, 24.2kDa, 46.3kDa and 79.5kDa regions.

There is no distinct band observed for the isolates of all MRSA, banding pattern of 25 kDa 24.5 kDa, 20.8 kDa, 23 kDa and 5.8 kDa, regions belongs to all isolates of MRSA. (Plate15)

4.4.2 Immuno Blot

Polyclonal antibody was highly sensitive and specific to cell wall antigenic protein. The polyclonal antibody expressed remarkable precipitin arc in both rocket and counter immunoelectrophoresis (Plate16 and 17).

Nitrocellulose paper showed bands in the regions of 5.8 kDa (Plate 18 and 19).

4.5 FIGE - REA of MRSA

FIGE-REA on AGE had showed FOUR types of banding pattern. Totally all isolates divided into FOUR categories. One organism from each group was selected for further analysis named as MRSA1, MRSA2, MRSA3 and MRSA4.

MTCC 87 (Standard) showed 8 bands from 5148 bp to 1492 bp. MRSA 1 showed 7 bands from 5148 bp to 983bp and MRSA 2 showed 6 bands ranging from 5148 to 983 bp, MRSA 3 strains showed 7 bands from 21226 bp to 983 bp, and MRSA 4 showed 9 bands ranging from 21226 bp to 983 bp.
All strains showed some common bands in the bp range of 5148bp, 4277bp, 3530bp and 1904bp regions. MTCC 87 had showed some different bands as 4973bp, 3827bp, 2027bp and 1494bp regions. MRSA1 have three different bands in the region of 4277bp, 1480bp, and 983 bp. MRSA 2 showed no different bands and appears as a strand. MRSA 3 showed three different bands in the region of 21226bp, 4973bp, 2027bp. MRSA 4 has showed four different bands on 21226bp, 3827bp, 831bp and 564bp. MRSA 1 and MRSA 2 showed almost same banding patterns. MRSA 3 and MRSA 4 had almost similar banding pattern. (Plate 20) (Text fig 7).

4.5.1 mec A gene amplification

The present PCR results showed the banding pattern in the region of 310bp in all the strains amplified. (Plate 21)

4.5.2 PCR – Amplification and 16S rDNA Sequence

In the phylogenetic analysis of the 16S rRNA gene sequences, all known species within the genus Staphylococcus were included. Phylogenetic analysis was carried out using 16S rRNA gene sequences of gene bank strain and 1,390 nucleotides from two strains were used in this study, corresponding to the nucleotides 60 - 1,466 of Escherichia coli 16S rDNA sequence (Gene Bank No. J01695) (Brosius et al., 1978). The strains SMKV-1 and SMKV-2 showed 100% 16S rDNA sequence similarities to each other. The present study also showed 100% sequence similarity to Staphylococcus aureus strain MSSA476 (BX571857) and Staphylococcus aureus subsp. aureus MRSA252 (BX571857) (Holden et al., 2004) (100%), followed by 99.86% similarity with Staphylococcus aureus (L36472) (Green, C.J. and Vold, B.S.), followed by 99.79% similarity with Staphylococcus aureus (X68417) (Ludwig et al., 1992), followed by 99.65% with Staphylococcus
*aureus* (X70648) (Bentley *et al.*, 1993), followed by 98.59 with *Staphylococcus* sp. H780 (AB 177644).

### 4.5.3 Genome Sequence

Nucleotides of the first DNA strain namely **SMKV 1** as the length 3511507bp, and **SMKV 2** as the length 3511505bp respectively.

### 4.6 ANTIBACTERIAL ACTIVITY

*Azardicta indica juss* and *Duranta pulmeneri jasq* and *Punica granatum linn* extracts produce various levels of growth inhibition zones were shown in [table14].

*Punica granatum* epicarp’s ethanolic extract had good antibacterial activity, 24µg containing disc had produced 13mm zone against all MRSA isolates (Plate 22-26).

#### 4.6.1 Thin Layer Chromatography

Thin layer chromatography plates showed three fractions named as fraction A, B, C. These fractions were produced three different colors as pink, brown and brownish yellow. The fractions had shows different Rf value (Plate 27)

*P.granatum* epicarp extract, fraction B & C were produced growth inhibition zone (13mm) (Plate 28).

**TLC plate’s fractions were analyzed in HPLC**

Optical Density read value was 7.000 at 310 nm using UV Spectrophotometer.
4.6.2 High Performance Liquid Chromatography

HPLC exhibited significant peak. All fractions were 100% similar to that of standard marker peak value (Plate 29 A, B, C, D). Standard marker peak value was 4.056 Fraction A 4.065, B 4.217, and C 4.424.

4.6.3 H$^1$ Nuclear Magnetic Resonance

All fractions of the extract were confirmed that contains tannic acid compounds.

H$^1$ NMR results gets, standard fractions A, B and C were found in aromatic compound region. (Plate 30 A, B, C, D)