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 nasopharingeal 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 tributyrne 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. (Plate21)

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 A4.065, B4.217, and C4.424.

4.6.3 $^1$H Nuclear Magnetic Resonance

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

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

5.1 MICROBIOLOGICAL STUDIES

Use of antibiotics to control the infectious diseases was originated with the discovery of penicillin in the first and later many more antibiotics. One among than *Staphylococcus* infections, which were resistant to common antibiotics. Now this species *Staphylococcus aureus* which was found frequently on the skin or nose of healthy people, occasionally cause infection on human beings. Earlier these species can be controlled with methicillin has now became resistant. Methicillin Resistant *Staphylococcus aureus* (MRSA) is methicillin and other member of the β-lactam class of antibiotics such as penicillin, oxacillin and amoxicillin. These infections are commonly break out among patients in hospitals and the carriers for the spread of the disease and with hospital contaminations. Increasingly *Staph* infections and resistant *Staph* infections are occurring now in communities too. Now, MRSA has emerged as a community infection in the present study.

The present study most of the MRSA strains were screened from Infants. In the year 2001, 57.69% and 51.72% of MRSA were isolated from the age group below 1 of southern and northern regions of Tamilnadu respectively (Text Fig. 1 and 4). In the year 2002, MRSA were isolated from 67.53% below the age group 1, and 57.70% from southern and northern region of Tamilnadu respectively. (Text Fig. 2 and 5). In the year 2003, below age group 1 childrens were affected by MRSA infection in southern regions was 84.61% and in northern regions of Tamilnadu has 79.74% respectively in blood samples (Text Fig. 3 and 6).

The present study, exactly showed an increase in the percentage of MRSA infection in male children than female from 2001 to 2003. The percentage of MRSA
has increased from 51.72% to 84.61% in Tamilnadu, India. This is a fast growth rate recorded in three years. Which is an alarming one. One should be cautious about this organism and it should be monitored for future trends.

MRSA is more prevalent and increasing both in southern and northern region below one age group childrens. Similarly above age 61 patients were highly affected in northern region. Because northern region of Tamilnadu had more percentage of educated peoples. They were used different types antibiotics, which induced the formation of multidrug resistant \textit{S.aureus}. They need a lot of powerful antibiotics. Age groups 21 to 30 consider as teenage group. They were affected in more percentage in both southern and northern region. Because those peoples were physically contact to community. (For example sports peoples, young males and females).

\textit{S.aureus} is a common species that is found in food poisoning. Because it produces enterotoxin against human and animals. It is also involved in community-acquired infections and surgical wound infections (Javetz \textit{et al.}, 1989).

In 1970, strains of Methicillin resistant \textit{S. aureus} (MRSA) had spread epidemically throughout the Australian metropolitan hospitals and have since persisted in most of these centers. They are clinically significant group because apart from their generalized resistance to the β-lactam antibiotics, they are also resistant to wide range of other antibiotics, 78 strains of MRSA obtained from Australian hospitals in 1981 showed that in general they were similar to the strains that were obtained in other countries (Wilkinson \textit{et al.}, 1987).

In India, Salaria and Singh (2001) reported that, 1600 employees in various places with direct exposure to infected or colonized inpatients who were surveyed for nasal carriage and 2% of them were found to be positive for MRSA. They
observed that 76% of MRSA isolates were not susceptible to clindamycin and it caused an epidemic in the intensive care unit. Vancomycin has been considered as the antibiotic of choice for MRSA. There is paucity of literature on use of vancomycin for MRSA.

In India, so far no cases of vancomycin resistant have been reported (Pulmood et al., 1996). India has reported susceptible to vancomycin drugs as high as 84.2%. However, Pal and Ayyagari (1991) studies have shown susceptible to vancomycin drug as high as 97%.

235(32%) MRSA out of 739 cultures isolated by Mehta et al.,(1996) in India, 27% from Bombay, 42.5% from Delhi and 47% from Bangalore. Their report strongly supports the present epidemiological study.

Pavillard et al., (1982) reported an epidemic spread of MRSA in Australian hospitals, leading to the suggestion that those strains were different from the other strains of MRSA. Gedney and Laccy (1982) analyzed the isolates of MRSA from Australia and concluded that they were closely related to the MRSA strains from the other countries and decided that they come from the original clone. However, genetic analysis has shown that the Australian strains differ markedly in many aspects (Townsend et al., 1985).

A Staphylococcal borderline susceptibility to oxacillin were found to be widely disseminated among U.S. hospitals and disproportionately isolated from wound infection of the patients who had been given Cefazolin prophylaxis. This data suggested that in the preoperative setting, in-vivo, degradation of Cefazolin may enable BSSA – 5 strains to survive beyond the time of initial lodgment in the wound tissues and ultimately to cause infection (Kerndole et al., 1998).
Oxacillin resistant *Staphylococci* are major nosocomial pathogens with frequent multiple resistance leading to the over use of glycopeptides in therapy (Frebourg *et al.*, 1998). Towards the end of 1982 it became apparent that a Methicillin – resistant strain of *S. aureus* had become epidemic in London, subsequently this strain had spread throughout the Thames region (National Health Service Administrative Divisions) and occasional isolates usually associated with patient movement were received from other regions (Richardson *et al.*, 1998).

Since 1981, strains of MRSA causing severe infection have been isolated from hospitalized patients in many countries including Australia, Ireland, England and U.S.A. In Royal Prince Altered Hospital (RPAH), five prevalent strains of MRSA had been identified by phage typing. (Peglar *et al.*, 1988).

Since 1970, Methicillin resistant *S. aureus* (MRSA) had become the main cause of nosocomial infection worldwide. As on date, Vancomycin was the only antibiotic effective against it. But in 1997 vancomycin resistant *S. aureus* was isolated. Now it is made clear that there is a threat of MRSA without having developed any antibiotics with greater activity than vancomycin (Hiramatsu *et al.*, 2001).

Community acquired MRSA typically affects children and young adults and it causes a range of infections similar to those caused by community acquired methicillin susceptible *S. aureus* (MSSA). MRSA strains circulate beyond nosocomial settings relocated community acquired MSSA as the flora of healthy human beings in some communities (Hiramatsu *et al.*, 2002).

In the present study, MRSA infection usually acquired in hospitalized patients who are already very sick or who have an open wound or a tube going into their body such as urinary catheter or intravenous catheter, and health care setting
can be severe. In addition, some factors can put some patients at higher risk for MRSA including prolonged hospitalization, receiving broad spectrum antibiotics, in an intensive care for burns, moving very closely with the other patients with MRSA or those who have undergone recent surgery or carrying MRSA in the nose.

In the present study, cases of MRSA in the community have been associated with those who recently took antibiotics, shared contaminated items, having active skin diseases and living in crowded settings. Community associated MRSA infections are typically skin infections, but also cause severe illness as in the cases of four children who died of community associated MRSA. Similar report was submitted by Sabat et al., (2000).

In the present study 96 to 99% of S. aureus isolates were resistant to penicillin, methicillin and other antibiotics such as oxacillin. Semi synthetic penicillin was successful in treating penicillin – resistant S. aureus infection. In 1992, the emergence of methicillin resistant S. aureus became epidemic in many hospitals in southern and northern regions of Tamil Nadu, India.

Flowers, Watters and Levy independently isolated 1,249 pure cultures of Staphylococci from various hospitals in Germany. Similar study was done in Japan by Mitsuhasi. In comparison with our studies the percentage (84%) of resistance pattern were found to be gradually increasing in the studies of MRSA isolates obtained during the years 2001-2003.

Barber and Waterworth in 1963 were unable to confirm the difference in the destruction of the isoxazolylpencillins by the Stewart’s strain, compared with methicillin sensitive Staphylococci. Most workers have found that the cultures of the resistant Staphylococci are composed of a mixture of a cells, the majority showing sensitivity to methicillin and much smaller percentage representing highly resistant
variants. But the present study clearly differentiates between MRSA and MSSA culture isolates. Most of the workers introduced 5% NaCl in growth medium. The osmotic pressure was used for the identification of MRSA and MSSA. All isolates showed methicillin sensitive in the absence of 5% NaCl. But when 5%NaCl was added to the medium, MRSA showed resistance to methicillin (Plate 1 and 2).

Strains of Staphylococci with multiple drug resistance are widespread. They appear and spread rapidly after the introduction of new antimicrobial agents. Dornbusch et al., (1969) reported, S. aureus is the most frequent infectious agent in chronic mastitis of cattle. In Switzerland, S. aureus were recovered from 61.5% of mastitic milk samples derived from 104 cows in 22 different farms. Further more, the spread of S. aureus suggested an epidemic infection on cattle (Baumgartner et al., 1984). The present results also reveal their studies.

The prevalence of community acquired methicillin resistant S. aureus infections showed an increase at the University of Chicago Childrens Hospital (UCCH) from 10 per 1, 00,000 admissions from 1988 to 1990 to 259 per 1, 00,000 admissions from 1993 to 1995. Because this increase might have represented a one-time occurrence or a limited disease outbreak. Previous observations at UCCH were updated in the year 1998 and 1999 to find whether this trend had continued. In the present epidemiological studies, it showed that 84% of children were infected by methicillin resistant S. aureus. These results are in accordance with other earlier studies.

Hussain et al., (2000) reported that twenty-three hospitalized children had MRSA, isolated during the one-year study period, ten were community acquired, equally distributed between children with predisposing risk factors and those without the overall prevalence of community acquired MRSA was 208 per 1,00,000
admissions. Present results showed most of the isolates of MRSA were from newborn babies and children below one year. The result of Hussain et al., (2000) also supports the results of this study.

Recent case reports of vancomycin treatment failures in the United States, Japan and France have prompted a retrospective analysis as 42 cases of septicemia caused by epidemic methicillin resistant, *S. aureus strain* 15. The most prevalent epidemic strain of Methicillin resistant *S. aureus* in the United Kingdom, between 1994 and 1998 mortality was low (4%) in patients with rifampicin treatment (Burnie et al., 2000). This epidemiological study also showed the same low (2%) percentage of mortality rate.

Khan et al., (2000) reported, the presence of *mec A* gene in 65 strains of MRSA isolates. They were resistant to penicillin, tetracycline, erythromycin, amoxicillin and clavulanic acid and variably resistant to gentamicin, ofloxain, trimethoprim, chloromphenicol and all isolates were susceptible to vancomycin. The findings demonstrated the existence of a common epidemic MRSA clone in Thailand. The present isolated strains also resistant to penicillin, tetracycline, erythromycin, amoxicillin, clavulanic acid and variably resistant to gentamicin, ofloxacin and trimethoprin and all MRSA isolates were susceptible to vancomycin. The present result findings are 100% similar to their study.

A five-fold increase from 11 to 58% in the prevalence of methicillin-resistance was observed in 1994-95 among clinical isolates of *S. aureus* in the State clinical hospital No. 2 in SZCZECIN, one of the largest hospitals in Pomeranian region of Poland. (Bilska et al., 2000). Their reports also support the current study.

The epidemiological data showed three fold increases in the prevalence of MRSA isolates from 53 to 83% during the year 2001-2003. In their study, 20% of
nasal swab isolates are from 12 MRSA patients. The MRSA isolates were susceptible to vancomycin, showed upward shifts indicating decreased vancomycin susceptibility. But the present isolates are completely different; they will not decrease their susceptibility to vancomycin.

The principal mode of transmissions of MRSA is by the transfer of the organism from a carrier or an infected patient to individuals. In the year 2001 two patients had undergone surgery, they had surgical wound infections caused by MRSA, (Wang et al., 2001).

The current studies shows the oral carriage of MRSA may act as a reservoir for re-colonization of other body sites or for cross infection to other patients or health care workers. At least two cases have been reported of cross infection from a general dental practitioner to patients. Nursing homes are another important source of colonization and infection and two cases of MRSA acute patients have been reported.

Javetz et al., (1989) described the cultural characters of S. aureus. They are spherical in nature about 1µm in diameter, arranged in irregular clusters and strongly Gram-Positive. It is non-motile, white, and yellow to golden yellow colored colonies. Cruickshank (1972) described Staphylococcus will grow in the presence of 10-15 % of Sodium chloride. It is highly selective for S. aureus. Cruickshank 1972 reported that S. aureus produced hemolytic on sheep blood or rabbit blood agar. The present isolated strains also exhibited similar characters such as β-hemolytic on the blood agar plates (Plate 4).
Nutrient Agar containing one percentage of glycerol monoacetate has been used to enhance pigmentation and allow differentiation of *S. aureus* into groups showing different kinds of pigmentation (Williams and Turner 1963).

More (1960) reported that one indicator medium that containing low concentration of mercuric chloride, strains are resistant to this medium it would be resistant to antibiotics. Present strains fermented number of sugars like glucose, mannitol, maltose, and lactose also. All strains are MRVP positive. They also produced β-hemolysis on 7% human blood agar. The present results were relevant to Ajuwape *et al.*, (1977). The strains of MRSA isolates were collected from a brain tumor patient, considered to be sensitive to methicillin. Those results were similar to Ahlm *et al.*, (2000). But that patient later developed a brain abscess that was used for the isolation of MRSA.

The rabbit plasma tube coagulase test has been recommended as the reference method for the identification of *S. aureus*. This test has been the method of choice in bacteriology for many years. DNase test is recommended for the identification of MRSA (Eriksen *et al.*, 1964). All isolated strains have shown similar results.

The isolated strains resembled with that of other isolates methicillin resistant *Staphylococci* in many properties and these findings are certainly consistent with the hypothesis of a single clone for those strains, produced β hemolysins (Lacey 1974).

The MRSA isolates were treated with acriflavin that convert methicillin susceptibility occurred in 4.2 to 12.3%, similar to Dornbusch *et al.*, (1969). MRSA strains were also resistant to mupirocin (Pawa *et al.*, 2000). Immunodominant structures, which were expressed in-*vivo* during sepsis caused by MRSA (Lorenz *et
The present MRSA isolates were susceptible to methicillin after added 4.2 to 12.3% of acriflavin and all isolates were resistant to mupirocin.

Rybak and Akins (2001) reported, an MRSA can produce β-hemolysins on 7% human blood agar and it produces Golden yellow color pigment on Mannitol Salt agar with 6µg/ml of oxacillin. The MRSA are easily grown on 5%sodium chloride containing medium. The present results were similar to their work.

Methicillin resistant *S.aureus* is frequently isolated from burns, wound, surgical wound, urine, blood and tracheal samples. The Kirby-Bauer disk diffusion agar tests, the most widely used antimicrobial susceptibility testing systems in American hospitals. The present study of MRSA isolates also shows similar result, because it cannot produce a zone of growth inhibition on Muller Hinton agar medium.

Annear (1968), Hewitt et al., (1969), Turner et al., (1967) used MHA containing 5% of sodium chloride and incubated at 30°C for 18 hours or 37°C for 48 hours. In this study MRSA isolates also produced a zone of growth inhibition on MHA containing 5% Sodium Chloride only if incubated at 35°C for 18 hours or 37°C for 48 hours. The current study exactly parallel with their work. These protocols were highly used for the isolation and identification of MRSA in various clinical samples.

Thornsbery et al., (1973) and Drew et al., (1972) determined that the incubation of plates at 35°C for 18 hours or overnight resulted in an adequate detection of MRSA strains. Varaldo et al., (1984) decided to test the effect on the *in-vitro* susceptibility to β-lactam antibiotics, mostly used in diagnostic laboratories to enhance the expression of methicillin resistance in the presence of 5% Sodium
chloride in the test medium. The National Committee for Clinical Laboratory Standards (NCCLS) recommended three manual methods for MRSA detection. They included screening on an agar plate containing oxacillin (6µg/ml) disk-disk diffusion and tube macro and micro dilution. In the year 2000 Rohani et al., tested 13 different antibiotics by disk diffusion method as recommended by the NCCLS. The MRSA isolates in this present study, antibiotic assay results completely (100%) resembled to NCCLS recommended procedure.

*Staphylococcus aureus* produces a wide variety of pathogenic factors that contribute its ability to colonize and cause infection. Hiramatsu et al., (2001), classified the factors into four categories namely adhesins, exo enzymes, exo toxins and others. They identified almost all known *S. aureus* pathogenic factors with in the strains N315 and Mu 50. In the present study results were closely resembled to their work. Because MRSA isolates expressed good clinical symptoms. All isolates of MRSA can easily grow on Tributyrne agar medium. They produce yellow colored colonies on TBA medium and produce a lipase enzyme finally that shows clear opaque zones of TBA medium

After 48 hours of MRSA cultures inoculated into the mice, they were affected by Rheumatoid Arthritis. The back legs of the experimental animal became bent. The legs had lesions-containing pus. The MRSA cultures were isolated from the pus of infected mice leg as per Koch’s postulate procedure.

After 48 hours the nasal region of experimental animals released sputum like liquids. The MRSA cultures isolated from nasopharingeal region. Weight loss, low food intake, the dessected experimental animal liver has more number of white colored lesions like patches.(personal contact, Dr.P.M.Subramanian M.B.B.S.,MD., Pathologist, Salem,Tamilnadu).
*S. aureus* expresses various surface proteins, some of which contain adhesins that act as specific receptors for extra cellular matrix proteins of the host tissue. Production of collagen binding protein is strongly associated with pathogenesis of osteomyelitis and septic arthritis and of *Staphylococcus* in patients with Rheumatoid Arthritis and showed that significantly light proportion of these patients carried oral *Staphylococcus* than control. Present pathological study results coincide to Levy *et al.*, (2001) report because most of the MRSA cultures isolated from Rheumatoid Arthritis patients were from the oral cavity region.

Sarafian, (1987), demonstrated the production of TSST-1 by batch and continuous culture of *S. aureus* strain 1169 in a carbohydrate free chemically defined medium. In continuous culture, oxygen and arginine limitation were required for steady state TSST-1 synthesis. Aeration suppressed toxin synthesis. The Mg$^{++}$ ion concentration had no effects on the specific-toxin in anaerobic conditions. In aerobic conditions, specific toxin increased C-23 fold and the Mg$^{++}$ ion concentration increased to 0.4mM. Further increased in the Mg$^{++}$ ion concentration resulted in reduction in the specified toxin.

In recent reviews, techoic acid and protein A are described as the most important surface structures which interfere either the defense mechanism of the host. It is stated that a few strains are encapsulated but encapsulation may be more common among freshly isolated clinical *S. aureus* (Sompolinsky *et al.*, 1985). Most of the MRSA isolated strains were positive of capsule staining.. Because the fresh cultures of MRSA has capsule. That capsule proteins is also associated with initiation of bone infection. The present pathological study also resulted in the formation of septic arthritis and osteomyelitis. The present results were similar to Hiramatsu *et al.*, (2001) study.
Extra cellular matrix binding protein Emb initiates sub acute bacterial endocarditis by mediating bacterial attachment to the extra cellular matrix of the damaged heart valve. The results didn’t resemble to their study. Levy et al., (2001) reported that, *Staphylococci* are the most important cause of prosthetic joint infections. *Staphylococcus* is a significant pathogen in acute arthritis, affecting both native and prosthetic joints. Recent study investigated oral carriage is used for escaping from the host animal phagocytic system. So the encapsulated MRSA can cause various infections and produce toxins.

In the present study, 85% of the hospitalized inpatients were carriers of methicillin resistant *S. aureus*. Death of five patients was due to the cause of MRSA infection, two in Salem, one in Chennai and two in Trichy. Patel et al., (2000) reported that ninety patients with significant underlying medical illness were treated at 63 centers in five countries. The most common indication was bone and joint infection (44%) and skin structure infection (16.4%). The most common nonvenous adverse events related to study medication were anthralgias (10.8%) myalgias (8.6%) and nausea (8.6%).

Santosh et al., (2000) reported that MRSA in children can be community acquired and can cause otitis externa, otitis media with otorrhea or acute mastitis. Intravenous therapy that includes vancomycin is necessary for resolution. In the study, it was found that children aged below one year were highly affected with MRSA infection. Particularly newborn babies carry a methicillin resistant *S. aureus*.

Thankluong et al., (2002) reported in their study, the gene expression of cap gene is very essential for the production of polysaccharide capsules. Because most of the young cultures of MRSA had polysaccharide capsules. Those capsules are used for escaping from the hosts immune system. The present study also matches with their study.
5.2 BIOTECHNOLOGICAL STUDIES

The present SDS-PAGE study showed cell wall Ag protein banding pattern was between 5.8 kDa region and 79.5 kDa region (Plate 15). The protein molecular weighing 5.8 kDa had good immunogenic activity on immunoblot study (Plate 19).

Clink and Pennington, (1987) studied that Staphylococcus whole cell polypeptide evaluation could be as a taxonomic and typing tool analysis. WCP obtained by SDS-PAGE complete concordance of results from both techniques was achieved. Visual analysis of the polypeptide patterns and comparison by the use of co-efficient of Dice showed minor differences in band patterns among the same species strains. Their results recommended that the same procedure for the identification of S.aureus by SDS-PAGE could be beneficent for the present study.

Castas and Owen, (1987) characterized 22 different strains of S.moniliformis by SDS-PAGE of cell proteins. Analysis showed that Haver hill fever strains can be clearly distinguished from rat-bite fever strains. Proteins band differences amongst the latter strains corresponded with different geographical locations. They concluded that high resolution PAGE combined with computerized analysis of protein profiles provides the basis for clinical isolates of S.moniliformis.

Antigenic composition of an endocarditic associated isolate of S.faecalis and identification of its glycoprotein antigens by ligand blotting with selections. A major envelope protein antigen of 53 kDa detected with patient’s serum was also present in three urinary strains of S.faecalis and a laboratory strain of S.faecalis, but not in S.aureus. Only 2 major antigens of 56 and 53 kDa reacted with sera from endocarditic patients. These antigens may therefore, be of diagnostic or protective
potential. (Elieen et al., 1986). Their study was similar to the present study, since *S.aureus* protein has exhibited high antigenic properties.

Nitrocellulose paper showed bands in the regions of 5.8 kDa. The banding pattern observed in this present study was similar to that of the work carried out by Julie et al., (2002). They identified 2 conserved immunogenic *S.aureus* cell wall proteins, of 40 and 87KDa, expressed under iron-restricted growth conditions.

Krikler et al., (1986) in their study, examined the extra cellular proteins produced by *S.aureus* strains by western blot analysis with blood donor plasma and some of the antibodies comparison of epidemiological related strains showed strong concordance between blot pattern and some of phage type.

A series of 133 isolates of MRSA was finger printed by immunoblot technique by Woellee and J.P Burnie, (1988). By their typing method confirmed the existence of an epidemic strain that accounted for 102 isolates. Remaining 31 isolates were grouped in to a further seven types which correlated with results of phage typing and antiograms.

Gaston et al., (1988) during evaluation of electrophoretic methods for typing methicillin-resistant *S.aureus* strains plasmid profiles, whole-cell protein profiles and immunoblotting profiles were compared with phage typing and they concluded that both WCPP and IP provide valuable epidemiological data on MRSA and IP and it is the easiest of three methods to interpret. These results support the current study.

Karakatoa et al., (1985) described a method for typing *S.aureus* capsular polysaccharide that is based on direct bacterial cell agglutination and immunoprecipitation of cell extracts with mono specific antisera encapsulated
strains were identified by their in agglutinability with teichoic acid antisera the typing sera reacted specifically with extract of eight prototype stains.

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. (Plate 16&17).

Monoclonal antibodies of *S.aureus* capsular polysaccharide types 5 and 8 were used in an ELISA to serotype 74 and 42 coagulase-negative isolated from cow and goat milk, respectively by Pourtel *et al.*, (1990) 18 isolates were typable 13 *S.haemolyticus*, 1*S.hyticus*, 1*S.similan*s and 1*S. warneri* from bovine origin. Type 5 was predominant, accounting for about 87% of typable isolates reactivity with monoclonal Ab’s varied considerably according to isolates.

Catherinebranger *et al.*, (1987) during their study in esterase electrophoretic polymorphism of methicillin sensitive and methicillin resistant strains of *S.aureus*, three kinds of esterase bands designated A, B and C was were defined by their ranges of activity toward five synthetic substrate and their resistance to di-isopropyl fluorophosphates. Five allozymes of esterase A, 4 of esterase B and form of esterase. 35 and 19 represented two major lymotypes respectively; where as other lymotypes were represented by one or at most seven strains. Most of the methicillin resistant strains are represented by the 2 major lymotypes, which differed from each other by the electrophoretic behavior of the three esterases their results indicated that, on the basis of esterase electrophoretic polymorphism, methicillin resistance is expressed in genetically different strains.

A series of 133 isolates of MRSA was finger printed by immunoblot technique by Woellee and J.P Burnie, (1988). Their typing method confirmed the existence of an epidemic strain that accounted for 102 of the isolates. Remaining 31
isolates were grouped into a further seven types which correlated with results of phage typing and antibiograms.

FIGE-REA on AGE had showed FOUR types of banding pattern. Totally all isolates were divided into FOUR categories. One organism from each group was selected for further analysis named as MRSA1, MRSA2, MRSA3 and MRSA4. (Plate 20). The following reports support the above study.

Blanc et al., (2001) analyzed the PFGE patterns of MRSA isolates from nosocomial infections. The number of fragments showed 0 to 6 fragments differences between the first isolates and subsequent isolates in long-term carriers and the other group had 14 to 24 fragment differences. This study also showed similarity to their work. The isolate has showed 6 to 11 fragments.

Olivera et al., (2001) stated that, the MRSA strains were typed using antibiograms, bacteriophage typing and pulsed field gel electrophoresis. The analysis of genomic DNA by PFGE showed that 65 isolates indicated the presence of an epidemic MRSA clone widely disseminated throughout Brazilian hospitals.

The present results were similar to Murono et al., (2002) work. They reported that the PFGE patterns of 56 isolates were classified into nine types including Type A, its sub type A1 and A2 and Type B through to the G. Seventeen isolates belonged to type A or its Subtype. The predominant strain of MRSA isolates in the pediatric ward was a certain strain that have been originated from the same clone cross infection control.

MRSA isolated from 41 patients undergoing periodical dialysis and genetic analysis was performed by pulsed field gel electrophoresis. When *S. aureus* was identified at the exit site the clonal identity of nasal and exit site isolates was
demonstrated in 50% of the *S. aureus* carriers, nasal isolates were genetically constant over time, in the 50%, a change of *S. aureus* strains was observed (Kreft *et al.*, 2001). Lange *et al.*, (1999) studied sixty six isolates of *S. aureus* obtained from milk of dairy cows differing from sub clinical mastitis with different molecular typing methods and the comparison of pulsed field gel electrophoretically separated genomic small fragment patterns. The present isolates fragment patterns was relevant to their earlier study.

Tenover *et al.*, (1995) interpreted the DNA fragment patterns generated by Pulsed Field Gel Electrophoresis (PFGE) and transformed them into random genetic events that can alter the patterns. Most commonly random genetic elements including point mutations, insertions and deletions of DNA altered PFGE patterns when fewer bands are detected. Their reports were strongly relevant to our isolates fragments patterns.

Mazurek *et al.*, (1986) studied the RFLP of MRSA. RFLP results had been showed the number of fragments produced by typical restriction enzymes used. An alternative approach, which limited the number of restriction fragments used infrequently. It reported that a total of 128 MRSA isolated from a burns unit in the year between 1992 and 1997 were studied by plasmid analysis and PFGE. All MRSA isolates produced β-Lactamase and high MICs to methicillin. All MRSA gene isolates from both years carried *mec A* gene in Smα I fragment. A banding pattern consisting of readily discernible number of discrete bands usually about 2000 less in number was preferable fragments in the 1-10 Kb sized range enabled short separation times. (Jordan 1991).

The present study, PCR results showed the banding pattern in the region of 310 bp. The banding pattern was in accordance with that of the following
experimental studies. The genome types represented stable differences in the investigated *S. aureus* strains. Since DNA fragment patterns remain unchanged even after numerous DNA preparations. Similar findings had been reported by Provost *et al.*, in the year of 2000. Grithuyen *et al.* (1999) amplified 298 – bp fragment of the mec A gene with the primers $5^1$ – GTT GTA GTT GTC GGA TTT GG – $3^1$) and $5^1$ – CTT CCA CAT ACC ATC TTC TTT AAC – $3^1$ specific for the mec A gene.

A second set of primers was included in each reaction mixture to amplify a polymorphic region of the coagulase gene that varied between approximately 350 to 600 bp. The coagulase primers specific for the coagulase gene were $5^1$ – CTG GTA TCC GTG AAT A – $3^1$ (upstream) and $5^1$ – TTG TAT TGA CTG TAT GTC TTT GGA – $3^1$(downstream). The later primers provided an internal control to check the presence of *S. aureus* DNA and for the absence of PCR inhibitors. MRSA isolates yielded two PCR products, the CoA amplicon and the 298 – bp mec A amplicon. Their results differ from the present study and got 310 bp band. (Plate 21).

Xiao xue Ma (2002) and his coworkers amplified DNAs encompassing the entire SCC mec sequence by long- range PCR with several sets of primers. The region from the left extremity to the CCR genes. (L-C region) was covered by primer sets $\alpha$ 5 and cLs1 (CA05) or CL 26(8/6-3p). Primers $\alpha$ 6 and mc R8 were used to 1-R region. (Ito et al., 1999, Ito *et al.*, 2001 and katayamma., 2000) Ito *et al.*, (2001) spanned a region from Tn 554 to the right extremity of SSC mec, which was amplified by long range PCR with two sets of primers, TnpA 1016 and mN13 and cR1.

Baba and his coworkers (2002) established the whole genome sequence specific of two MRSA strains, N315 and Mu50, both of which are strains associated
with health care. Two further hospital acquired MRSA strains have been sequenced by others (COL, E-MRSA-16 [strain252]).

Ito and his co-workers (2001) designed the primer mN12 and mN13 on the basis of the nucleotide sequence of the right extremely of SCC mec of 85/3907 during their research work in structural comparison of three types of Staphylococcal cassette chromosome mec integrated in the chromosome in methicillin resistant S. aureus.

In the phylogenetic analysis of the 16S rRNA gene sequences, all of the 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 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 present strains isolated in this study named as SMKV-1 and SMKV-2 showed 100% 16S rDNA sequence similarities to each other. Our strains 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) (Fig:7).

The genome sequencing for isolated strains namely SMKV1 and SMKV2 (MRSA 1 & 2) was performed. First strain has 3511507bp, second strain has 3511505bp respectively. The present genomic sequence results were similar to Hiramatsu et al., (2001) study. They reported that the S.aureus genomes were
composed of a complex mixture of genes, many of which seem to have been acquired by lateral gene transfer. Most of the antibiotic resistance genes were carried either by plasmids or by mobile genetic elements including a unique resistance island. Three classes of new pathogenicity islands were identified in the genome, 1) Toxic Shock Syndrome Toxins island family, 2) Exotoxin islands 3) Enterotoxin islands. In the later two-pathogenicity islands, clusters of exotoxin and enterotoxin genes were found closely linked with other gene clusters encoding putative pathogenic factors.

5.3 PHYTOCHEMICAL STUDIES

In this study *Duranta pluemeneri jacq, Azardicta indica juss* plant extract and *Punica granatum epicarp* extract were used against methicillin resistant *S.aureus*. *D. pluemeneri jacq, A. indica juss* could not produce effective clearing zones as that of *P.granatum*. The *P.granatum* was effective at 24 µg/ml concentrations against MRSA.

Pharmacological investigation of medicinal plant has provided important advances for the therapeutic approach to several pathologies as well as extremely useful tools for the theoretical study of physiology and pharmacology. Essavi and Srour (2000) submitted antibacterial activity of fifteen plants against both gram positive and gram negative bacteria. But the present study was focused on communicable dangerous organism such as methicillin resistant *S.aureus*. Samy and Ignasimuthu (2000) reported that twenty plants showed antibacterial activities against more species of bacteria used for their antibiotic assay. Most of the organisms as *B.subtilis* and *S. aureus* growth were inhibited by *Cassia occidentalis* and *Cassia auriculata*. This study was similar to that of the present study. Indian tribal healers used many Indian folklore medicinal plants. Thirty medicinal plant
species were used for treatment against Bacillus subtilis, E. coli, K. aerogens and S. aureus. Leaves of Duranta plumneri jacq originated from tropical regions of America. The plant leaves are used as cure for snakebite. The family is of most economic value. It is used as an ornamental plant. In the present study the plant extract of Duranta plumneri jacq, Azardicta indica juss leaves were produced only 2mm to 3mm at the concentration of 30µg/ml. These results were not sufficient for the complete eradication of MRSA. More quantity of leaves is needed compared with the P. granatum epicarp extract.

In earlier studies 10mg containing plant extract discs were used to produce antibacterial activity in the current study plant extracts of P. granatum epicarp(rind) had 24µg / ml enough for the growth inhibition of MRSA. So P.granatum epicarp extract is found to be very powerful drug to kill the MRSA. The whole fruit juice is good for heart ailments.

Calli et al., (2000) reported that one nasal ointment contains the 4% tea tree oil and 5% tea tree oil body wash and 2% mupirocin nasal ointment for the eradication of methicillin resistant S.aureus carriage. P.granatum extract containing tannin and also can be used as a nasal ointment and body wash for the eradication of MRSA from carriers.

Machado et al., (2003) reported that Punica granatum leaf extract was used for the eradication of MRSA and MSSA. But the extract needs additional antibiotic chemicals as glycerol and Vaseline. In the present study, P.granatum epicarp can eradicate the methicillin resistant and sensitive S. aureus. So this extract was highly efficient to kill the MRSA than the other.
Most of the *P. granatum* extracts were tested by Fadula *et al.*, (1975) against intestinal pathogens. They reported that the fruit rind of *P. granatum* was non-toxic to human beings. The extract collected from *P. granatum* epicarp was being already used by the ancestors for body wash. Das *et al.*, (1999) used the *P. granatum* for the cure of astringent, haemostatic, as a remedy for diabetes and antihelminthic. Gunther *et al.*, (1996), reported that several commercial preparations could be made from pomegranates. But it needs certain chemical substances to increase their quality. Astringents are prepared from highly economically important plants such as Oak, Catechu, Nut mug, Areca nut (betal nut). But *P. granatum* epicarp is considered as a waste material. So it is highly useful for the preparation of Astringents. They are the substances that precipitate proteins, but do not penetrate cells, thus affecting a superficial layer only. They toughen the surface making it mechanically stronger and decreases exudation. It is also used for curing burns. Because it forms a crust under which bacteria cannot grow and will not affect the skin. So this preparation was very good when compared with other preparations. TLC fractions, A, B & C has showed the presence of Tannic acid. But fraction A cannot produce antibacterial activity against MRSA (Plate 28). This study had acetic acid and water as a mobile phase, and Cellulose TLC grade as a stationary phase. Preparative HPLC was very useful for the isolation, identification and purification of separated compounds. (Plate 29 A, B, C and D). The findings furnished all the needed supportive points.

Fernandez *et al.*, (2005) reported that the common bean contains phytochemicals, including phenolic compounds, which can provide health benefits to the consumer. They used 100% methanol extract from seed coats that were subjected to different chromatographic fractionation methods. But HPLC-MS gave a better separation of phytochemicals. Ding *et al.*, 2004 reported that the identification of tannin component in rhubarb was carried out by High Performance Liquid
Chromatography (HPLC) and Mass Spectrometry (MS). The structure of the main tannin components were gallic acid, catechin, the dimmer, trimer, tetramer and pentamer of catechin. This study also support to the present tannic acid isolation study.

Ellagic acid hydrolysable ellagitannins were present in *P.granatum* fruit. These components were separated by the use of HPLC-UV analyzer. (Seeram *et al.*, 2004).

NMR study gave different spectra depending on their location and adjacent molecules are surrounded by electron clouds which changes the encompassing magnetic field and thereby alter the absorption frequency and finally confirming the presence of Tannin.

In this study, the presence of tannic acid chemical component in *P. granatum* epicarp (TLC region B & C) was thoroughly analyzed and detected using with NMR. The present results showed tannic acid presence in *P.granatum* epicarp extract, which is showing the peak at aromatic region (6ppm) (Plate 25 A, B, C and D).

But the current study found that the tannic acid is responsible for the activity against a medically important pathogenic strain as methicillin resistant *S.aureus* in both hospital and the community.

Moore *et al.*, (2005) reported that, the purified polyphenol compound assigned to be 3,4,5 tri-o-galloylquinic acid from the $^1$H and $^{13}$C one-and two-dimensional NMR spectra. The $^1$H-NMR spectrum displayed three singlets in the aromatic region, characteristic of the ortho protons of galloyl groups; the remaining proton signals were attributed to the methane and methylene protons of the quinic
acid spin system, H-2-H-6 was followed from the connectivities revealed by the COSY spectrum. Although the upfield methylene proton signals at 2.48 and 2.26 ppm may be due to the H-2’s or H-6’s, these protons could be assigned unambiguously to H-2ax and H-2eq respectively from chemical shifts and coupling data. Analysis of the coupling patterns of the protons and the determination of coupling constants for the well-resolved H-4 resonance established the relative stereochemistry of the methane protons in the quinic acid ring.

Kraus et al.,(2003) reported that, their study was collection of purified tannins from the foliage of nine species growing in the pygmy forest of the northern California coast were examined with $^{13}$C NMR. Their studies are also similar to the present $^1$HNMR study.

A new ellagitannin, methyl (S)-flavogallonate along with fourteen known compounds, gallic acid,methyl gallate, ethyl gallate, 2,3-di-O-[(S)-4,5,6,4’,5’,6’-hexahydroxybiphenyl-2,2’-diyl]dicarbonyl]-(alpha/beta)-D-glucopuranose, vitexin, isovitexin, orientin, kaempferol 3-O-beta-D-rutinoside, rutin, neosaponarin, ellagic acid, flavogallonic acid and (alpha/beta)-punicalgin have been isolated from the leaves of Terminalia myriocarpa Heurck (Marzouk et al., 2002). But in the present study, tannin isolated from P.granatum epicarp and the compound is yet to be identified.

The native Indian pomegranate fruit epicarp contains large quantities of tannic acid, when compared to the Brazilian study MIC value of 250µg/ml was reported in Brazilian study, where as in the present study MIC value is only 24µg/ml, which is found to be approximately ten times more concentrated and effective against MRSA.
SUMMARY AND CONCLUSION

*Staphylococci* are a group of Gram-positive bacteria known to cause infection in humans and animals. One of the most important species is *Staphylococcus aureus*.

*S. aureus* forms colonization in the body and major regions including nasal membranes skin of the warm-blooded animals. The prevalence of *S. aureus* is widespread throughout the world and also a major causative factor of nosocomial infections in human.

Due to the use of different antibiotics against this bacterium, it has developed a multidrug resistance. Methicillin resistant *S. aureus* (MRSA) strains have been shown to be responsible for large number of infections in almost all the countries throughout the world, including India. In this study an epidemiological survey was conducted in Tamilnadu, India to identify the regions that are more prone to MRSA infections, including various hospitals sources. The MRSA resistant nature was identified and confirmed by the NCCLS methodology using Kirby-Bauer assay and tube dilution techniques. Further the organism was confirmed by the coagulase test and mannitol utilization test.

In this study mice was used as an animal model to test the nature of pathogenicity caused by the MRSA isolates. After 48 hours on inoculation the mice were found to be affected with lesion in the leg with pus accumulation and animals were found to exhibit rheumatoid arthritis conditions.
Further to study the antigenic properties, the cell wall proteins were isolated from the MRSA strains. The proteins were estimated and concentrated 110µg/ml of protein were taken for the SDS-PAGE analysis. The banding patterns were found in between 5.8kDa and 79.5kDa regions when compared with indigenous standard marker.

Immunoblot analysis were conducted using the isolated proteins from SDS-PAGE were determined for antigenic properties. Polyclonal antibody expressed remarkable precipitin arc in both rocket and counter current immunoelectrophoresis. The banding pattern was observed in the regions of 5.8 kDa.

There is no major banding pattern of MRSA cell wall protein in SDS-PAGE analysis. So all MRSA strains were subjected to PFGE-FIGE-REA on AGE showed four types of banding pattern named as MRSA1, MRSA2, MRSA3 and MRSA4. The MRSA1 showed 8 bands, MRSA2 showed 6 bands, MRSA3 showed 9 bands and MRSA4 showed 11 banding patterns confirming different strains of MRSA.

The study on mec A gene amplification was performed using multiplex PCR. The PCR product was run on AGE and a characteristic banding pattern of 310 bp was obtained.

The phylogenetic analysis was carried out by using RT-PCR with “16s r RNA” gene sequence. The isolates designated as SMKV1 and SMKV 2 (MRSA1 and MRSA2) strains showed 100% sequence similarity with Gene bank S.aureus strains MSSA 476(BX 571857), S.aureus MRSA 252(RX 571857) and 99% sequence similarity with other S.aureus species.

The antibacterial activity of Azardicta indica juss, Duranta pulmeneri jasq and Punica granatum Linn were analyzed against the MRSA isolates. Antibacterial
assay was performed by Kirby-Bauer disc diffusion method and tube dilution technique. Of these three plants, *Punica granatum* Linn extract showed high antibacterial activity. To determine the exact chemical compound present in the crude extract of *P.garanatum* L, TLC, and the eluted compound was further analyzed by HPLC. The HPLC fraction was found to be Tannic acid. The TLC fractions were further analyzed by H1 NMR to confirm the presence of standard molecular weight and structure of Tannic acid present in our *P.garanatum* L extract. The MIC value of *P.granatum* L was found to be 24 µg/ml, which exhibited high antibacterial activity against the MRSA isolate.

Staph infections, resistant to common antibiotics are appearing in communities. Epidemiological studies reveal that children below age group of one year are most affected in Tamilnadu. 16s r DNA sequencing reveal that the MRSA strain 1and 2 similar to gene bank strains MSSA 476(BX 571857), *S.aureus* MRSA 252 (RX 571857). Histopathological studies on mice confirms that the strain are pathogenic. Phytochemical studies of *P.granatum* rind reveals the presence of tannic acid whose MIC values was found to be 24 µg/ml to be able to control the disease effectively.
Phylogenetic position of strains SMKV-1 and SMKV-2 within the genus *Staphylococcus* on the basis of 16S rRNA gene sequences. The phylogenetic tree was constructed by the neighbor-joining method (Saito & Nei, 1987), and the 16S rRNA gene sequence of *Macrococcus caseolyticus* ATCC13548\(^T\) (D83359) was used as the out group. The numbers at the nodes indicate the levels of the bootstrap support based on a neighbor-joining analysis of 1,000 resampled data sets. The bootstrap values below 60% were not indicated.