CHAPTER 5
Discussions
5.1 *S. aureus* AND CoNS AS NOSOCOMIAL PATHOGENS

Staphylococci are one of the most common nosocomial pathogens throughout the world. The incidence of staphylococcal infections has increased in recent years, mainly due to the spread of multidrug resistant staphylococcal strains, as well as to the growing incidence of infections in immunocompromised patients (Grosserode & Wenzel, 1991).

*Staphylococcus aureus* is responsible for diseases caused by exotoxin production (toxic shock and staphylococcal scalded-skin syndrome) and by direct invasion and systemic dissemination (bacteremia, urinary tract infections and septic shock syndrome) (Sheagren, 1984 & Brumdit & Hamilton-Miller, 1989).

Coagulase-negative staphylococci (CoNS) isolates have become a major cause of nosocomial infections (Patrick, 1990). As a group, the coagulase-negative staphylococcus species (CoNS) are among the most frequently isolated bacteria in the clinical microbiology laboratory (Peters *et al*, 1995 & Diekema *et al*, 2001). CoNS are a major component of the normal flora of the cutaneous ecosystem, including the skin and mucous
membranes (Kloos, 1986) In the cutaneous ecosystem; CoNS generally have a benign relationship with their host and function as commensal or saprophytic organisms. However, if the cutaneous organ system has been damaged by trauma, inoculation by needles, or direct implantation of foreign bodies, these organisms can gain entry to the host (Kloos, 1990). Depending upon their ability to adhere to host or foreign body surfaces, breach or avoid the host immune system, multiply, and becomes pathogen (Kloos et al, 1991).

In the present study, clinical specimens were collected (wound infections, thermal injuries, blood stream infections and urinary tract infections) from tertiary care hospitals of five different cities of Vidarbha region, Amravati, Nagpur, Akola, Wardha and Yavatmal. Out of total 3147 clinical specimens 994, (31.58%), 791 (25.14%), 637 (20.24%), 462 (14.68%) and 263 (8.36%) were collected from Amra vati, Nagpur, Akola, Wardha and Yavatmal respectively change.

From 403 clinical specimens collected from wound infections, 69.97% staphylococci were identified. The high rate of infection is due to the easy colonization of staphylococcus
species already existing in hospital environment and from visitors. The infectious process in wound begins with disruption of the host mechanical barriers to microorganisms, the availability of microorganisms, and colonization (Altemeier et al, 1984). Infections develop as organisms that previously colonized skin and gut may now invade tissues; furthermore, such an environment may allow secondary invaders (e.g., Staphylococcus aureus) to cause infection. These organisms or their liberated toxins overwhelm the local protective environment with resultant systemic sepsis (Thomson, 1984). The most common pathogens associated with nosocomial wound infections are Staphylococcus aureus, Enterococcus species, coagulase-negative Staphylococcus, Enterobacteriaceae, Pseudomonas species, and anaerobes (Horan et al, 1988).

In present study, 45.97% Staphylococci species were isolated from thermal injury specimens. Large burn size and infection are the strongest predictors of mortality among the patients suffering from thermal injuries. Infections causative agents, i.e., Staphylococcus aureus and CoNS were the major contributors in burn injuries. Kaushik et al (2001) analysed 336 samples from the patients suffering from thermal injuries. Out of
336 samples, 293 positive samples yielded 324 isolates. *Staphylococcus aureus* was the most commonly cultured organism.

Revathi *et al* (1998) collected a total of 600 pus samples from patients suffering from thermal injuries, yielding 920 isolates. Pseudomonas spp. was the most common followed by *Staphylococcus aureus*. According to Cross *et al* (1983) *Staphylococcus aureus* has become the predominant pathogen in burn centers.

Out of 414 BSI clinical specimens, staphylococci contributed 36.95% isolates. In Surveillance program conducted by SENTRY for occurrence of bacterial pathogens from bloodstream infections isolated 5,058 isolates. *S.aureus* contributed 22.8%. Weinstein *et al* (1997) reviewed 843 episodes of positive blood cultures in 707 patients with septicemia. The most common pathogens identified were *Staphylococcus aureus*, coagulase-negative staphylococci (CoNS), *Escherichia coli*, and Enterococcus species. Marshall *et al* (1998), in the SCOPE surveillance program found that Staphylococci are major causes of nosocomial blood stream
infection. Coagulase-negative staphylococci (CoNS) and *Staphylococcus aureus* were the first and second most common etiologic agents in BSI, respectively.

From 1362 clinical specimens collected from patients suffering from UTI, 28.78% staphylococci were identified. From total 3147 clinical specimens collected, maximum percentage, 40.42% of staphylococcal isolates were identified. Staphylococcal isolates were categorized as coagulase positive and coagulase negative staphylococci on the basis of tube coagulase test (TCT). On the basis of biochemical tests described in Bergey’s manual of systematic bacteriology, 17.54% of total isolates, the coagulase positive staphylococci were identified as *Staphylococcus aureus*.

The virulence of *Staphylococcus aureus* infection is remarkable, given that the organism is a commensal that colonizes the nares, axillae, vagina, and pharynx or damaged skin surfaces (Noble *et al*, 1967 & Casewell *et al*, 1986). Percentage of *Staphylococcus aureus* isolates identified from different clinical specimens was as follows; 16.30% (wound infections), 61.41% (thermal injuries), 10.51% (BSI) and
12.41% (UTI). Relatively high percentage of *Staphylococcus aureus* isolates was identified from patients suffering from thermal injuries followed by wound infections. The burn wound is particularly susceptible to bacterial colonization and infection due to the physical disruption of the normal skin barrier and the accompanying reduction in cell mediated immunity (Cetinkale et al, 1993). Asensio et al, (1996) identified six factors that were independently associated with MRSA infection, colonization, increasing age, ward type (particularly intensive care units), coma, previous hospitalization, invasive procedures and length of hospitalization. There are several ways of transfer of infection that includes bed linen, medical equipments, hands of health care workers, aerosols, hands of visitors, etc.

The overall rate of mortality from Staphylococcal bacteremia ranges from 11 to 43%. The major source of entry of Staphylococcus in blood stream is through use of catheters and broken skin or infections. The frequency of complications from Staphylococcal bacteremia is high, ranging from 11 to 53% (Mylotte et al, 1987). An increasing percentage of bacteremia infections are related to catheterization (Steinberg et al, 1996). The rate of complications is lower for catheter-related infections
than for all cases of bacteremia (24%) as is the overall mortality rate (15%) (Jernigan et al, 1993).

According to Kloos (1980) most frequently encountered CoNS species in humans includes *S.epidermidis*, *S.haemolyticus*, *S.capitis*, *S.cohnii*, *S.saprophyticus*, *S.warneri* and *S.simulans*. In the present study 720 (21.88%) isolates were identified as coagulase-negative Staphylococci. Out of 720 clinical CoNS isolates, *S.epidermidis* (33.89%), *S.capitis* (10.56%), *S.warneri* (5.14%), *S.haemolyticus* (8.47%), *S.hominis* (6.39%), *S.saprophyticus* (23.06%), *S.cohnii* (6.67%) and *S.simulans* (5.83%) were identified.

From 720 CoNS isolates, 26.67%, 14.72%, 13.47% and 45.14% were identified from wound infections, thermal injuries, BSI, and UTI respectively. Arias et al, (2003) identified a total of 177 (29.6%) isolates were identified as CoNS, of which the majority (62%) was *S.epidermidis*. Other species of CoNS identified included *S.saprophyticus*, *S.auricularis*, *S.haemolyticus*, *S.sciuri*, *S.capitis*, *S.hominis*, *S.simulans* and *S.warneri*. Most CoNS were isolated from blood, surgical wound infection and UTI.
*S. epidermidis* is widely recognized as one of the etiologic agents of bacteremia, postoperative cardiac infections and endocarditis, osteomyelitis, urinary tract infections, and peritonitis caused by ambulatory dialysis, with a frequent association with colonization of intravascular catheters and orthopedic devices (Kloos & Bannerman, 1994, Rupp & Archer, 1994).

Szewczyk & Rozalska, (2000) reported *Staphylococcus cohnii* strains dominant in the environment of investigated hospitals and isolated 420 strains of the species mainly from hospitals environments, from infants- intensive care units, patients and its medical staff. The high rate of colonization of staphylococcus in wound infections is due to the properties of the bacteria itself. Several cell wall proteins of staphylococci have been described, and specific bacterial binding mediated by these proteins to extracellular matrix molecules (e.g. fibrinogen, fibronectin, vitronectin, laminin, and collagen) have been observed (Wilkinson, 1997). Electron microscopy has revealed a fimbria-like protein structure that may play a role in attachment of coagulase-negative staphylococci to foreign materials in the host
(Veenstra et al, 1996). Hussain et al, (1997) have suggested that a 140-kD extracellular protein is important for accumulation of *S.epidermidis* on surfaces. An uncharacterized hemagglutinin also has been associated with adherence to polymer surfaces (Rupp & Archer, 1992). Various virulence factors involved in the pathogenesis of polymer-associated staphylococcal infections have been isolated and characterized during the last few years (Von Eiff et al, 1999 & Heilmann & Peter, 2000). Most important in the pathogenesis of polymer associated infection is the colonization of the foreign body surface through the formation of multilayered cell clusters embedded in an amorphous extracellular material. The colonized bacteria and the extracellular material are collectively referred to as biofilm (Peters et al, 1981 & Herrmann, 1997).

Coagulase-negative staphylococci are the most frequently reported pathogens in nosocomial bloodstream infections (Schaberg et al, 1991). During 1980's, the incidence of bloodstream infections due to coagulase-negative staphylococci increased to the point that these organisms were causing more than 25% of nosocomial bloodstream infections (Herwaldt et al, 1992). In present study 97 (13.47%) CoNS isolates out of total 720 were identified from BSI. Out of 97 CoNS isolates, majority
was *S.epidermidis* (47.42%). All other CoNS species included *S.capitis* (5.15%), *S.warneri* (4.12%), *S.haemolyticus* (7.22%), *S.hominis* (11.34%), *S.saprophyticus* (21.65%), *S.cohnii* (2.06%) and *S.simulans* (1.03%) were identified as causative agent of BSI. In case of BSI the ratio of CoNS to *Staphylococcus aureus* was 1:0.57. In BSI infections the percentage of CoNS was relatively high than that of other organisms including *Staphylococcus aureus*.

Chaudhary *et al* (1999) collected 1727 blood samples over a one-year period, in which 201(11.8%) were found positive for bacterial infection. The ratio of Gram positive to Gram-negative bacteraemia was 1:1. Coagulase negative *Staphylococcus* spp. was the most frequent organism isolated (29.8%), followed by *Pseudomonas aeruginosa* (19.9%), and *Staphylococcus aureus* (16.9%).

Among CoNS species, highest number of strains of *S.epidermidis* species were isolated. The ability of *S.epidermidis* to form biofilm on tubing etc. is major source of blood stream infections. Among CoNS, *S.epidermidis* emerged as pathogens in growing number of serious nosocomial infections in neonatal intensive care units, particularly bloodstream infections (Gaynes *et al*, 1996 & Hall *et al*, 1991). Ponce de leon *et al* (1986)
studied 50 strains of coagulase-negative staphylococci (CoNS) isolated from 50 patients with hospital-acquired bacteremia, identified by prospective surveillance at the University of Virginia Hospital between March 1981 and September 1982. Using the Staph-Ident System for speciation, identified 37 strains namely *S.epidermidis* (74%), *S.hominis* (14%), *S.haemolyticus* and *S.warneri* (6%) each.

*S.haemolyticus* and *S.hominis* are usually found as contaminants of blood cultures but could also be associated with a variety of infections (Eroeggatt et al, 1989). Monsen et al (1999) isolated 500 strains of Staphylococci from 485 blood cultures. The five most frequently isolated species were *S.epidermidis* (54.8%), *Staphylococcus aureus* (28.0%), *S.hominis* (3.4%), *S.warneri* (3.2%) and *S.haemolyticus* (2.8%).

*Staphylococcus haemolyticus* strains (n=20), responsible of blood stream infections, were consecutively isolated from patients hospitalized in two different wards at high risk of infection (Raponi et al, 2005).
Del' Alcamo et al (1999) reported species distribution in 239 coagulase-negative staphylococci (CoNS) isolates collected from blood cultures in patients admitted in a 600-bed teaching hospital as *S.epidermidis* (50.2%), *S.hominis* (12.1%), *S.haemolyticus* (10.0%), *S.cohnii* (5.9%), and isolates from other CoNS species contributed 21.8%.

In a retrospective review of 30 *S.warneri* bacteremias in children, between 1991 and 1995, at the Royal Children's Hospital, Melbourne, Australia, Buttery et al (1997) reported that organisms were viable and verified in 22 episodes, 12 representing significant bacteremias.

From total 392 Staphylococcal isolates identified from UTI patients 82.91% were identified as CoNS. Out of total 325 (45.14%) CoNS isolates identified from UTI samples, 29.54% were identified as *S.saprophyticus* and 42.46% were identified as *S.epidermidis*. *S.capitis* 11.08%, *S.warneri* 3.38%, *S.haemolyticus* 7.39%, *S.hominis* 1.54%, *S.cohnii* 3.69% and *S.simulans* 0.92%.
Coagulase-negative staphylococci are responsible for two types of urinary tract infection, infections caused by 
*S. saprophyticus*, which affect young female outpatients and nosocomial infections due to other staphylococci (mainly 
*S. epidermidis*), which occur equally in men and women (Gatterman & Crossley, 1997). Until the last decade, coagulase-
negative staphylococci occurring in urine specimens were usually considered as contaminants. In the early 1970s, i.e., more than 
ten years after the original demonstration of *Staphylococcus saprophyticus* in urine specimens, this species became 
recognized as a frequent cause of urinary tract infections (UTI). In young women, *S. saprophyticus*, after *Escherichia coli*, are the 
second-most-frequent causative agent of acute UTI (Hovelius et al, 1984). A number of proteins have been shown to be involved 
in pathogenesis of *S. saprophyticus* infections. Protein 
hemagglutinin and surface fibrillar proteins (Gatermann et al, 
1992) have been associated with attachment to urinary tract 
epithelium, and invasion of the organism has been attributed to a urease production (Gatermann et al, 1989).

Four hundred and four coagulase-negative staphylococci were isolated from 4905 urine specimens obtained from 4192
patients by Ozturkeri et al, (1994). The distribution of the strains was as follows: *Staphylococcus epidermidis* (47.8%), *Staphylococcus saprophyticus* (42.3%), *Staphylococcus haemolyticus* (7.2%), *Staphylococcus warneri* (1.2%), *Staphylococcus schleiferi* (0.7%), *Staphylococcus hominis* (0.5%) and *Staphylococcus simulans* (0.2%).

Arias et al (2003) reported major contribution of *S.epidermidis* (62%) from a total of 177 CoNS species identified in a multicentre surveillance of antimicrobial resistance in staphylococci from Colombian hospitals. *S.epidermidis* 138 (42.45%) were identified from total of 325 CoNS isolates from UTI clinical specimens.

From UTI samples the ratio of *S.aureus* to *S.epidermidis* was 1:2.06 and was the major causative organisms of UTI. Midstream specimens of urine from inpatients and out patients at King Fahd Specialist Hospital in Buraidah, Saudi Arabia, were collected over a period of 12 months. A total of 854 from 4157 specimens (20.54%) gave significant bacterial counts i.e., counts greater than $10^7$ organisms per ml. *S.aureus* and
*S. epidermidis* were found to be causative agents of UTI (Ahmad *et al*, 1995).

### 5.2 ANTIBIOGRAM

The antibiogram is the susceptibility profile of an organism to a variety of antimicrobial agents (Mayer, 1988). Antibiogram of all clinical staphylococcal isolates was determined for ten different antibiotics, penicillin, oxacillin, cephalexin, erythromycin, gentamicin, chloramphenicol, ciprofloxacin, rifampin, tetracycline and vancomycin representing β-lactam, cephalosporin, macrolide, aminoglycoside, quinolone, tetracycline, glycopeptide group of antibiotics respectively. Total 21 different antibiogram patterns were observed. Certain antibiogram patterns were found to be common in clinical isolates, identified from different cities. Isolates showing antibiogram pattern “6” were identified from all the five cities under study. Likewise, antibiogram patterns, 2, 4, 8, 9, 13, 14 were found to be common in isolates identified from more than one city. All other antibiogram patterns were found to be city specific, i.e., these antibiogram patterns (1, 3, 5, 7, 10, 11, 12, 15, 16, 17, 18, 19, 20 and 21) were restricted to specific city only.
All clinical isolates were found to be resistant to penicillin, oxacillin and cephalexin except 282 (22.16%) isolates, exhibiting antibiogram pattern 2 and 14, i.e., susceptible to β-lactam and cephalosporin. Isolates showing antibiogram pattern 20 (0.15%) showed susceptibility to all ten different antibiotics. Susceptibility to all classes of antibiotics, including β-lactams could be due to the introduction of the isolate from community to hospital environment recently. Susceptibility to vancomycin was observed in all the clinical staphylococcal isolates. Antibiograms that are identical have been used to infer relatedness between strains in epidemiological investigations (Nicolas et al, 1995 & Maki et al, 1976). The antibiograms are not entirely stable because antibiogram is influenced by genetic regulation, technical manipulation, and the gain or loss of plasmids and or transposons (Tenover et al, 1994). All clinical staphylococcal isolates, showing resistance to penicillin also showed resistance to cephalosporin antibiotic cephalexin.

*S. aureus* with confirmed resistance to oxacillin were considered to be resistant to all other β-lactam antibiotics including penicillins, cephalosporins, cephems, carbapenems, and β-lactam/β-lactamase inhibitor combinations (Simor et al, 1997).
5.3 CORRELATION BETWEEN CONVENTIONAL DETECTION OF OXACILLIN RESISTANCE AND PCR AMPLIFICATION OF \textit{mecA} GENE

Oxacillin susceptibility of clinical staphylococcal isolates was detected by conventional phenotypic methods (oxacillin disk diffusion, oxacillin agar dilution, “E” test and oxacillin agar screen) and sensitivity, specificity, positive and negative predictive values for phenotypic methods were evaluated in comparison with the results of PCR detection of \textit{mecA} gene for 552 \textit{Staphylococcus aureus} and 720 CoNS clinical isolates. PCR detection of \textit{mecA} gene in \textit{S.aureus} and CoNS isolates was considered as gold standard (Frebourg \textit{et al}, 1998 & Giusti \textit{et al}, 1999). Using PCR amplification 433 (78.44\%) \textit{S.aureus} isolates were found to harbor \textit{mecA} gene and remaining 119 (21.56\%) isolates lacked \textit{mecA} gene. For CoNS isolates, (605) 84.03\% isolates harbored \textit{mecA} gene and (115) 15.97\% isolates lacked \textit{mecA} gene.

For confirming the \textit{mecA} amplicon, southern hybridization of 25 each of \textit{mecA} positive and \textit{mecA} negative \textit{Staphylococcus aureus} and CoNS isolates was performed. Biotinylated probe internal to \textit{mecA} gene was used. All 25 \textit{mecA} positive \textit{Staphylococcus aureus} and CoNS isolates showed hybridization
signal and none of the meca negative Staphylococcus aureus and CoNS isolates showed amplification signal.

Tokue et al (1992) examined presence or absence of meca gene in 58 clinical isolates of Staphylococcus aureus by the PCR and southern blot analysis. The results were analyzed in relation to those of MIC assay of oxacillin. PCR assay results were consistent with that of Southern blot analysis of genomic DNA.

Sequencing of meca amplicon for 25 randomly selected samples each of Staphylococcus aureus and CoNS isolates was also performed in order to confirm the amplification of targeted meca gene. Sequence of amplicon was then compared with internal meca gene sequence to confirm the amplification of meca gene. In present study, all 50 samples sequenced showed perfect matching with meca gene internal sequence thus confirming the amplification of targeted meca gene with designed set of primers.

5.4 SENSITIVITY OF OXACILLIN DISK DIFFUSION

For 552 Staphylococcus aureus isolates, 76.45% isolates were found to be resistant and 23.55% found to be susceptible
using oxacillin disk diffusion method. On comparing the results of oxacillin disk diffusion with that of PCR amplification of \( \text{mecA} \) gene it was found that in the present study oxacillin disk diffusion method failed to detect 21 \( \text{mecA} \)-positive \textit{Staphylococcus aureus} strains and 10 \( \text{mecA} \) negative strains, showing least sensitivity and specificity of 95.2% and 91.6% respectively.

For 720, CoNS isolates 80.69% were found to be resistant and 19.30% found to be susceptible using oxacillin disk diffusion method. Disk diffusion for CoNS clinical isolates, showed lowest sensitivity of 95.4% and specificity of 96.5% respectively. Disk diffusion test detected 577 \( \text{mecA} \) positive strains and failed to detect 28 \( \text{mecA} \) positive strains.

\textit{Arak et al (1999)} studied methicillin resistance using disk diffusion method and correlated the result \( \text{mecA} \) PCR detection. Among 31 isolates initially characterized as MRSA by the disk diffusion test, \( \text{mecA} \) was detected in only 13 (42%) isolates. Oxacillin disk diffusion showed low sensitivity of 41.94%.
In study conducted by Prasad et al (2000), a total of 106 clinical isolates of *S. aureus* were tested for oxacillin resistance by disk diffusion and failed to detect 7 *mecA* positive strains but identified 5 *mecA* negative strains as oxacillin resistant. The sensitivity, specificity and accuracy of disc diffusion, against PCR as gold standard were as follows: 87.7, 89.9 and 88.7% respectively. The study demonstrated that disk diffusion test was least reliable when compared with PCR for detection of oxacillin resistance.

In study conducted by Atay et al (2002), oxacillin susceptibilities of 125 *S. aureus* isolates were determined by the disk diffusion as per the NCCLS guidelines, and the results were compared with those of *mecA* gene analysis. In the routine susceptibility tests, 75 isolates were found to be methicillin resistant (MRSA), whereas 50 were found to be susceptible (MSSA). Various induction tests were performed to investigate the heterogeneous resistance among methicillin-susceptible isolates. These induction tests showed that the MIC values of seven isolates reached to the resistant levels, therefore these isolates should be accepted as "borderline oxacillin resistant *S.aureus*" isolates lacking the *mecA* gene. The susceptibility
tests and meca gene analysis of the remaining isolates yielded compatible results.

York et al (1996) compared PCR detection of meca gene with oxacillin disk diffusion method for 142 CoNS strains and found low sensitivity and specificity of 84% and 89% respectively. The method for detection of resistance for oxacillin by disk diffusion test is with low accuracy and can skip various resistant cultures to be read as false negative strains. The phenotypic detection of S. aureus and CoNS clinical isolates for oxacillin resistance was comparatively low. Most of the isolates harboring meca gene were not detected by disk diffusion test and thus could escape the routine laboratory analysis. The low sensitivity of disk diffusion tests results in the treatment of patients suffering from S. aureus and CoNS carrying meca gene with β-lactam and cephalosporin antibiotics. Such infection does not respond to β-lactam and cephalosporin antibiotics as few cells in population start expressing the gene and later become a major population in infections. We recommend that the detection of meca gene by PCR amplification is more reliable compared to disk diffusion test.
5.5 SENSITIVITY OF AGAR DILUTION TEST

In present study, *Staphylococcus aureus* isolates (22.46%) had MIC ≤ 2 μg/ml and were susceptible and remaining 77.54% strains were resistant as their oxacillin MIC was ≥ 4μg/ml. Out of 428 resistant *Staphylococcus aureus* isolates, 151 had low-level MIC, 133 had moderate MIC remaining 144 had high-level MIC value. Agar dilution method correctly detected 421 *mecA* positive *S.aureus* isolates and failed to detect 21 *mecA* positive isolates, showing sensitivity of 97.2% and specificity of 94.1%.

For CoNS isolates, 120 (16.67%) isolates were found to be susceptible to oxacillin by agar dilution MIC (≤0.25 μg/ml) and 600 (83.33%) isolates were found to be resistant (≥ 0.5 μg/ml). Comparing results of agar dilution with *mecA* detection by PCR, agar dilution showed sensitivity of 98.2% and specificity of 94.8%. Out of 605 *mecA* positive CoNS isolates, agar dilution detected 594 *mecA* positive isolates and failed to detect 11 *mecA* positive isolates.

obtained by agar dilution tests showed that sensitivity of the method was 72.8%. Investigators therefore reported that the conventional susceptibility testing method, i.e., agar dilution test is not always reliable in detecting methicillin resistant staphylococci.

5.6 SENSITIVITY OF "E" TEST

_Staphylococcus aureus_ (22.46%) isolates were found to be susceptible to oxacillin with "E" test and 77.54% isolates were found to be resistant. "E" test was found to be the most sensitive method as no false negative _Staphylococcus aureus_ isolates were detected. All 433 _mecA_ positive _Staphylococcus aureus_ isolates were determined by the "E" test. Specificity with "E" test was found to 86.6% as out of total 119 _mecA_ negative _Staphylococcus aureus_ isolates, "E" test detected 16 false positive isolates.

For CoNS isolates, 16.67% were found to be susceptible and 83.33% isolates were found to be resistant. "E" test was found to be most sensitive (99%) in detecting _mecA_ positive CoNS isolates. With _mecA_ negative isolates, 114 _mecA_ negative isolates were correctly identified.
Ferreira et al, (2003) evaluated oxacillin susceptibilities of 152 coagulase-negative staphylococcal strains by “E” test and reported sensitivity and specificity of 100% and 71.4% respectively after comparing the results with PCR detection of meca gene.

Ngui-Yen et al, (1992) reported the greater sensitivity of “E” test for detecting oxacillin resistance in CoNS isolates. Ngui-Yen et al compared “E” test with standard reference methods using NCCLS recommendations for determining MICs of 96 strains of oxacillin resistant Staphylococcus aureus and 35 strains of coagulase-negative staphylococci. 100% agreement was observed with CoNS and 85% with Staphylococcus aureus. We found that E test was more reliable for detecting the MRSA and MRCoNS than disk diffusion and agar dilution test.

5.7 SENSITIVITY OF OXACILLIN AGAR SCREEN TEST

In present study, 22.28% Staphylococcus aureus clinical isolates were found to be susceptible to oxacillin using oxacillin agar screen method and 77.72% isolates were susceptible to oxacillin. Oxacillin agar screen showed sensitivity of 99%. Out of
433 meCA positive Staphylococcus aureus isolates, 4 isolates were found to be false negative. Oxacillin agar screen showed specificity of 98.3% as agar screen test detected 117 true meCA negative isolates.

For CoNS isolates, 16.39% isolates were found to be resistant and 83.61% isolates were found to be susceptible using oxacillin agar screen media, containing 4μg/ml of oxacillin after 48 hrs of incubation at 35°C. For CoNS isolates, agar screen method (agar screen media containing 4μg/ml oxacillin) showed maximum sensitivity of 98.5%. Other concentrations tested, i.e., 0.5, 1, 2 and 6μg/ml showed lower sensitivity of 90.9%, 93%, 91.9% and 89.1% respectively.

Frebourg et al, (1998) detected oxacillin resistance in 64 S.aureus isolates by oxacillin agar screen method and compared the results with PCR detection of meCA gene. All 64 S.aureus strains were found to be meCA positive. The concordance between the results of the PCR amplification of meCA gene and those of agar screening test, except for two S.aureus isolates had sensitivity of 96.9%.
Juliana et al, (2004) also reported highest sensitivity of oxacillin agar media with 4µg/ml oxacillin. The objective of study carried out by Juliana et al, was to determine the accuracy of agar screen test (0.6 and 4 µg oxacillin/ml), and to characterize methicillin resistance among CoNS isolates. One hundred and seventy five strains were analyzed, 41.1% Staphylococcus epidermidis and 59.9% other species among which 69.1% were mecA-positive. The results showed that the methods had optimal correlation with the detection of mecA gene in S.epidermidis, S.hominis and S.haemolyticus. The only 100 % accurate test was agar screening with 4µg oxacillin/ml.

Ferreira et al, (2003) evaluated sensitivity and specificity of oxacillin agar screen method for 152 coagulase-negative isolates with PCR detection of mecA gene at four (1, 2, 4 and 6 µg/ml) different oxacillin concentrations. The results revealed that the agar-screening test with 4µg oxacillin/ml and incubation for 48 hrs was superior showing sensitivity and specificity of 100%.

Kuzucu et al, (2002) determined the methicillin resistance of 112 Staphylococcus aureus and 93 coagulase negative Staphylococcus (CoNS) strains, by routine disk diffusion method,
microdilution and oxacillin agar screen test. The presence of meca gene was investigated by polymerase chain reaction in case of discordant results. All S.aureus strains (100%) and 69.9% (65/93) of CoNS strains were found resistant to methicillin by three of the methods. Of CoNS isolates, 28 strains that were found methicillin resistant by disk diffusion method were found methicillin susceptible by oxacillin agar screen method, and 27 of these were detected as meca positive. The results indicated that, the three methods tested were reliable for the detection of methicillin resistance in S.aureus strains, but oxacillin agar screen (oxacillin 6 µg/ml) revealed to be unsatisfactory for the detection of methicillin resistance in CoNS.

Out of 433 meca positive S.aureus isolates, oxacillin agar MIC correctly detected 421 isolates while 12 meca positive isolates had MIC ≤ 2 µg/ml. With 119 meca negative strains, 112 strains had MIC ≤ 2 µg/ml by agar dilution test while 7 discrepant isolates had MIC ≥ 4 µg/ml. “E” test detected all meca positive S.aureus strains. Out of 119 meca negative S.aureus clinical isolates, 103 showed MIC ≤ 2 µg/ml and 16 meca negative isolates showed discrepancy as their oxacillin MIC was ≥ 4µg/ml.
Out of total 605 meca positive CoNS isolates, agar dilution correctly detected 594 (98.2%) meca positive strains. Out of 228 meca positive strains, 4 (1.75%) meca positive *S.epidermidis* strains showed discrepancy as their oxacillin MIC according to NCCLS guidelines was ≤ 0.25 µg/ml. Likewise 2 (3.7%) meca positive strains of *S.haemolyticus*, 3 (2.19%) meca positive strains of *S.saprophyticus* and 2 (5%) meca positive strains of *S.simulans* showed discrepancy with oxacillin agar dilution MIC. *S.capitis*, *S.warneri*, *S.homlinis*, *S.cohnii* strains showed 100% correlation between oxacillin agar MIC and presence of meca gene. *S.epidermidis* (11.11%), *S.capitis* (2.94%), *S.saprophyticus* (6.67%) and *S.cohnii* (8.33%) meca negative strains showed discrepancy with oxacillin agar MIC. These discrepant isolates lacking meca gene had MIC in the range of 0.5-1 µg/ml.

Out of 605 meca positive CoNS isolates, "E" test correctly detected, 599 isolates while *S.epidermidis* (0.44%), *S.haemolyticus* (3.7%), *S.saprophyticus* (1.46%) and *S.cohnii* (2.86%) showed discrepancy with "E" test. "E" test correctly detected 114 meca negative strains, except for 1 *S.saprophyticus* strain (3.33%).
5.8 PRESENCE OF *bla-Z* GENE IN *meca* NEGATIVE DISCREPANT ISOLATES

*meca* negative strains showing discrepancy with "E" test oxacillin MIC were selected for *bla-Z* amplification. All 16 *S.aureus* and 1 *S.saprophyticus meca* negative strains amplified *bla-Z* gene by PCR. *bla-Z* amplicon was further confirmed by Southern hybridization of internal sequence of *bla-Z* amplicon with biotinylated probe. All *bla-Z* positive staphylococcal strains showed hybridization signal with the biotinylated probe and no false positive signal was observed with any *bla-Z* negative strain. Further these PCR *meca* negative and *bla-Z* positive isolates, with oxacillin MIC $\geq 4\mu g/ml$ for *S.aureus* and 0.5 $\mu g/ml$ for CoNS isolates, were selected for detection of hyperproduction of $\beta$-lactamase using amoxicillin-clavulanic acid (20 and 10 $\mu g$, respectively) disk. All 16 *S.aureus* and 1 *S.saprophyticus* strain showed zone of inhibition $\geq 20$mm and indicated hyperproduction of $\beta$-lactamase enzyme. Hyperproduction of $\beta$-lactamase explain their borderline resistance to oxacillin. The spread of *meca* among *S.aureus* and CoNS species of nosocomial origin has been attributed, but still some strains designated as $\beta$-lactamase hyperproducers have been isolated from clinical samples. These

5.9 HETEROGENEOUS EXPRESSION OF OXACILLIN RESISTANCE IN STAPHYLOCOCCUS

Phenotypic detection of oxacillin resistance among Staphylococcal isolates is difficult; mainly due to heterogeneous nature (de Lencastre et al, 1991, Hackbarth & Chambers, 1989, Ryffel et al, 1992 & Chambers, 1997). The detection of methicillin resistance in staphylococci poses a challenge for the clinical laboratory. The mecA gene is regulated by a region-designated mecR, which reduces the overall expression of resistance by negatively regulating the synthesis of PBP 2a. Very slow derepression of the mecA gene in coagulase-negative staphylococci can result in strains that appear to be susceptible by laboratory testing. Thus, special considerations must be used to detect methicillin resistance in coagulase-negative
staphylococci, including increased incubation times and lower breakpoints than those used for *S.aureus* strains.

These *mecA* positive CoNS strains are not expressing resistance phenotypically, this might be due to non induction of *mecA* gene expression or absence of *mecA* gene expression. Heteroresistance of the strains to oxacillin could be the associated reason why *mecA* positive isolates are showing susceptibility to oxacillin. The reduced β-lactam resistance relies on the down regulation of *mecA* transcription (Mempel et al, 1994) and is influenced by auxiliary genes such as *mecR, mecI* (Kuwohara-Arai et al, 1996) and the *fem* genes (de Lencastre et al, 1994a). These, cryptic oxacillin resistant strains are called preMRSA (Hiramatsu et al, 1992), and are potentially highly resistant, since they can generate highly resistant subclones *in vitro* (Tokue et al, 1992). Therefore, their detection appears to determine the choice of antibiotic therapy and relies on the detection of the *mecA* gene. Such *mecA* positive strains susceptible to oxacillin, for which MIC ranged between 0.25 to 1 μg/ml, have been reported by Huang et al, (1992) and Ramotar et al (1996). The presence of heteroresistant strains in population is not detected by phenotypic method. Since those
strains harbor meca gene, they can get activated in due course and are also capable of spread of resistance to susceptible strains.

5.10 DOT BLOT HYBRIDIZATION DETECTION OF meca AND bla-Z GENES

In present study, detection of meca and bla-Z genes was performed using dot blot hybridization. All 433 meca positive strains of S. aureus and 605 meca positive strains of CoNS showed positive hybridization signal. With 119 and 115 meca negative staphylococcal strains none of the strains showed false positive hybridization signal. Specificity and sensitivity of 100% was thus observed with dot blot hybridization. With 17 bla-Z positive strains and 20 randomly selected PCR bla-Z and meca negative strains (oxacillin susceptible) strains. All bla-Z positive strains showed hybridization signal with biotinylated probe (100% sensitivity) and none of the bla-Z negative strains showed false positive hybridization signal (100% specificity). Results of PCR detections for bla-Z and meca were in 100% correlations with that of dot blot hybridization method. However, dot blot method requires a period of 36 hrs for detection in contrast to PCR amplifications requiring time period of 12-16 hrs.
Dot blot hybridization method has advantage of detection of numerous strains simultaneously. DNA probe analysis for determining the methicillin susceptibility of staphylococci was rapid, easily interpretable, and equally accurate with nonradioactive probes, and it gave results equivalent to the most sensitive microbiologic test for all staphylococcus species studied.

Archer & Penell (1990) designed a DNA probe derived from the PBP 2a gene of the methicillin-resistant *Staphylococcus aureus* COL was compared with phenotypic microbiologic tests for its ability to identify methicillin-resistant and susceptible staphylococci. Isolates tested were both *S. aureus* and coagulase-negative staphylococci that had been recovered from a variety of geographic and clinical sources. When compared with a spread plate phenotypic test, the DNA probe gave sensitivity, specificity, and predictive values for both positive and negative tests of 100% for 204 *S. aureus* isolates (103 positive, 101 negative) and 99, 95, 99, and 95%, respectively, for 249 coagulase-negative staphylococci (210 positive, 39 negative). The probe was more sensitive than broth microdilution and more specific than agar dilution in identifying methicillin-resistant and
-susceptible coagulase-negative staphylococci; all tests were equally accurate in identifying the methicillin susceptibility of S. aureus.

Evaluating the sensitivity, specificity, positive and negative predictive values of different phenotypic methods in relation with PCR detection of mecA gene, it was found that “E” test was most sensitive method for detection of oxacillin resistance in clinical Staphylococcal isolates. According to Petersson et al, (1996) “E” test is an excellent quantitative system for detecting oxacillin resistance in staphylococcal isolates. “E” test was found to be the 100% sensitive method as no false negative Staphylococcus aureus isolates were encountered. All 433 mecA positive Staphylococcus aureus isolates were identified by the “E” test.

With 720 CoNS isolates, “E” test was again found to be most sensitive (99%) phenotypic method in detecting mecA positive CoNS isolates.

5.11 GENOTYPIC DETECTION OF RESISTANCE DETERMINANT GENES

PCR amplification and dot blot hybridization methods detected single copy of the mecA and bla-Z genes present in
genome. The molecular based methods are sensitive enough not to identify any false positive strain. Other major advantage of detecting the resistance genes by molecular based methods is that the genes, even if are not expressed are detected with same sensitivity by molecular methods. In phenotypic methods of resistance detection, expression of meca and bla-Z genes were of key importance; gene if present but not expressing itself cannot be detected by conventional methods. Genotypic methods, i.e., PCR amplification and dot blot hybridization detected the gene implicated in the resistance mechanism independent of the fact that whether gene is expressed or not. The genes involved in drug resistance mechanism were liable to dissemination between organisms via horizontal transfer; therefore resistance genes if present in the organism (expressing or not) could spread between bacterial strains and under conducive environment (in hospitals) where selective pressure of antibiotics induces the expression of genes. Therefore the major advantage of these molecular based methods is that they help in detecting those strains, which are harboring the resistance determinant genes and play a role in quarantine of these potentially dangerous clinical strains.
To increase the accuracy of resistance testing, the use of genotypic approach has been advocated (Courvalin, 1991 & Tenover, 1992). DNA based assays for detection of bacterial resistance to various other antibiotics have been developed (Ariet & Philippon, 1994, & Persing & Tenover, 1996). This approach relies on a different concept; testing for resistance instead of testing for susceptibility. Resistance of clinical staphyloccocal isolate to oxacillin has important implications necessitating patient isolation and the use of vancomycin. Despite numerous guidelines for optimization of the phenotypic detection of methicillin resistance, mecA detection became the "gold standard" for the detection of methicillin resistance (Wallet et al, 1996).

The identification of methicillin resistance in *S. aureus* represented an ideal application of nucleic acid amplification methods. Methicillin-resistant *S. aureus*, important hospital-acquired pathogens are capable of causing life-threatening infections and nosocomial outbreaks. The incidence of infections from this pathogen in hospitals has increased dramatically in the past few years. Thus, the rapid and accurate identification of the pathogen are required for patient management and for infection
control programs in hospitals (Jayaratne et al, 1999). However, the reliable detection of methicillin-resistant S. aureus using culture and susceptibility tests may be erroneous due to heterogenous expression of resistance, influenced by culture conditions, especially in strains with low-level resistance (Coudron et al, 1986) All strains of methicillin-resistant S. aureus produce a unique penicillin-binding protein (PBP2a) and encoded by a chromosomal gene, mecA.