Staphylococci are gram positive spherical bacteria usually arranged in grape-like clusters. Some species of *Staphylococcus* are members of the normal flora of the skin and mucous membranes of humans and animals. Others cause pyogenic infections. Staphylococci are usually divided into two groups based on the ability to produce coagulase, an enzyme that clots blood plasma. The coagulase positive group includes *Staphylococcus aureus* which is an important human pathogen. Coagulase negative group is a large and heterogeneous group with a diverse natural habitat that includes humans, birds, fishes and other animals. In recent decades coagulase negative staphylococci (CNS) have emerged as important nosocomial pathogens. They are reported as etiological agents of infections associated with catheters and other indwelling medical devices. Due to the presence of some virulence factors and multiple drug resistance, these organisms are occupying the status as emerging pathogens in clinical Microbiology. In view of the importance of these bacteria as emerging pathogens and the scarcity of reports of study from southern part of India, a study was carried out with the following objectives.

**Objectives**

- To isolate and identify coagulase negative staphylococci (CNS) from different sources.
- To study the virulence properties in the identified species of CNS
- To analyse antibiotic sensitivity pattern and plasmid profile of the isolated CNS species
- To assess the adherence of CNS to different smooth surfaces
- To test the methods to reduce or inhibit biofilm formation and surface colonization by the CNS isolates.
To explore the genetic basis of biofilm formation.

The present thesis is a detailed report on the study conducted with the above objectives. It is presented in ten chapters are described below.

Chapter 1

Introduction

Coagulase-negative Staphylococci are ubiquitous in nature. They were formerly regarded as harmless inhabitants of the skin, mucous membranes and other body sites in humans and animals; but are now recognized as major cause of significant clinical infections. The incidence of virulence properties in CNS include slime, biofilm, capsule, several toxins and enzymes (Dornbusch et al., 1976). The emergence of drug resistance among CNS is a threat to public health throughout the world. CNS produce polysaccharide slime and form biofilm, which allow them to persist on the foreign body and become less susceptible to antibiotics. In addition to the use of antibiotics, a wide variety of non steroidal anti inflammatory drugs and mucolytic agents are used to control the production of slime and thereby prevent the formation of biofilm by CNS. Antibiotic-impregnation in to medical devices significantly reduces bacterial colonization and adherence. The presence of antibiotic resistance genes along with the biofilm forming character make S. epidermidis and other CNS the potentially harmful microbes to be screened regularly.

Chapter 2

Review of literature

The relevance of study in view of the importance of CNS as emerging pathogens has been narrated. The objectives of the present work are described in detail. The taxonomical status of the organisms under study, their morphological, biochemical characteristics etc. have been detailed. The CNS species possess some virulence factors which make them potential
opportunities pathogens. A detailed review on these virulence properties has been given. Studies show that CNS species possess multiple drug resistance. The most important virulence properties include slime production and biofilm formation. Reports on studies on these virulence factors have been reviewed. In recent years efforts have been made to incorporate biocide or antibiotics in the devices such as catheters. There are a few reports on studies based on this concept. The molecular mechanism of biofilm formation has also been reviewed.

Chapter 3

Isolation and species identification of coagulase-negative staphylococci isolated from different sources

The isolates of CNS were obtained from 3 different sources, comprising clinical samples, human healthy skin and domestic animals. Out of a total 600 isolates, 200 from each of the above sources; 579 isolates were subjected to study. CNS species were tentatively identified as described by Kloose and Schleifer (1986). The following tests were used: utilization of xylose, sucrose, trehalose, maltose, fructose, lactose, mannose and mannitol, nitrate reduction, urease, ornithine decarboxylase, phosphatase and resistance to novobiocin and characterization of haemolysis.

The tentative identification of isolated CNS strains revealed the presence of 12 species of coagulase negative Staphylococci. Of the 12 CNS species isolated the predominant were S. epidermidis (27.46%), S. saprophyticus (11.9%), S. haemolyticus (11.7%) and S. hyicus (11.9%). The most predominant species was S. epidermidis in both human healthy skin and clinical samples. Two species i.e., S. lugdunensis and S. xylosus were present only in clinical samples. In domestic animals the predominant species isolated was S. hyicus (11.9%). Presence of two species i.e., S. hyicus and S. chromogen were observed only in these samples.
Comparison of species distribution in these three different sources showed statistically significant difference (p < 0.001).

**Chapter 4**

**Study of virulence properties in the identified species of coagulase-negative staphylococci**

The identified CNS species were examined for the expression of some potential virulence factors such as production of an extracellular polysaccharide known as slime, which facilitates the colonization of bacteria to host tissues, biofilm that leads to persistent infections and capsule which helps the bacteria to escape from phagocytosis. Production of siderophore, enzymes such as esterase, DNase and TNase were also studied.

The percentage incidence of various virulence properties in CNS samples showed capsule in 33.85%, slime in 41.62%, biofilm in 36.61%, siderophore in 27.46% and synthesis of enzymes including esterase in 20.38%, DNAse in 13.3% and TNase in 8.98%. Virulence properties of CNS from three different sources were compared. The statistical analysis of these results indicated that CNS isolates from different sources showed significant difference (p<0.028). Incidences of all virulence properties were higher in clinical samples than in samples from other two sources.

**Chapter 5**

**Antimicrobial susceptibility and plasmid pattern analysis of the isolated coagulase-negative staphylococci species**

The CNS species isolated were subjected to antibiotic sensitivity study. Susceptibility to 14 different antibiotics were examined by Kirby-Bauer Method. The β-lactam resistant CNS were screened for β-lactamase (Penicillinase) production by iodometric tube method. 20 strains exhibiting resistance to at least 7 antibiotics were chosen for plasmid profile analysis.
Plasmid DNA was isolated by alkali lysis method and cleaved with restriction endonuclease EcoRI as described by Sambrook et al (1989). The plasmid DNA was analyzed by agarose gel electrophoresis.

Incidence of antibiotic resistance was highest in the isolates of *S.epidermidis* compared to the isolates of other species. *S.epidermidis* showed sensitivity rate of 77% and 80% to penicillin and oxacillin respectively. Antibiotic resistances of CNS isolates were analyzed by χ² test. The result showed statistically significant difference (p < 0.001). Antibiotic susceptibility test showed that CNS isolated from human healthy skin was completely susceptible to vancomycin (100%). Among domestic animal samples 7% of *S.epidermidis*, 6% of *S.hyicus* and 5% of *S.chromogen* isolates were resistant to vancomycin.10% of isolates of *S.epidermidis*, *S. saprophyticus* and *S.haemolyticus* were resistant to vancomycin. 87% and 84% of *S.epidermidis* isolates were resistant to penicillin and oxacillin respectively. Multiple antibiotic resistance was frequent among the isolates from all the three sources. Majority of CNS species in clinical samples were resistant to most of the antibiotics except vancomycin and rifampicin. The β-lactamase test showed that of the 81 penicillin resistant strains 68(84%) were positive for penicillinase by iodometric tube method and 13(16%) were negative for this test. The incidence of antibiotic resistance was higher in clinical isolates compared to the other two sources. Different types of plasmids were obtained in the plasmid profile analysis.

**Chapter 6**

**Adherence of coagulase negative staphylococci to different smooth surfaces: - qualitative and quantitative assessment**

Twenty five strains of *S.epidermidis* isolated from clinical samples were random selected and subjected to study for the adherence of CNS to different smooth surfaces. Slime production
and adherence on to bacterial adherence to tissue culture plates, culture tubes, glass slides, and catheters were studied.

The qualitative assessment of slime production by tube method revealed that 18 (72% of the tested isolates) were slime producing, 7 strains showed negative results. The qualitative assessment of slime production on PVC catheter by SEM method showed attached bacterial cells and the cells with slime layer surrounding them. Photomicrograph of glass slide with adherent bacterial films showed deeply stained the cells with slimy extracellular material. In the spectrophotometric method 15 (60%) isolates were strongly adherent and 10 (40%) isolates of *S.epidermidis* were weakly adherent and none of them were non adherent in Trypticase soy broth (TSB) with glucose. In TSB without glucose, 13 (52%) isolates showed strong adherent bacterial films whereas all other strains were weakly adherent and none of them were non adherent.

**Chapter 7**

**Comparison of influence of N - acetyl cysteine and salicylic acid on the formation of biofilm by *Staphylococcus epidermidis***

Fifteen strains of *S.epidermidis* isolated from clinical samples were subjected to study for the influence of N-acetyl cysteine (NAC) and salicylic acid (SAL) on biofilm formation. Ten concentrations (0.003-0.2mg/ml) of NAC and SAL were evaluated. The formation of biofilm in the presence of NAC and SAL was determined spectrophotometrically by the method of Christensen *et al* (1985).

SAL showed higher influence on biofilm formation compared to NAC. At a concentration of 0.06mg/ml of SAL, 6 strains turned weakly adherent (OD ≤ 0.240) where as 0.5 mg/ml of NAC was required to get such an effect. The decrease in the OD of biofilm was directly proportional to the SAL concentrations.
Chapter 8

The *in vitro* efficacy of rifampicin – impregnation of silicon catheter to prevent surface colonization of CNS

Rifampicin-impregnation of silicon catheter was done to provide high initial and long lasting release of the drug. Two catheter pieces were removed from each test system after 30min, 3h, 12h and 24h respectively; one for culture and other for SEM study.

SEM demonstrated that the surface of the catheters removed from the bacterial suspension after 30min and 3hrs showed no sign of bacterial colonization. After 12 hrs of exposure very few bacterial cells were seen on the surface of the catheter without any sign of slime production. After 24 hrs of exposure, the surface of the catheter was covered by colonies of bacteria with slime matrices.

Chapter 9

Detection of *ica*AB and *mec*A genes in *Staphylococcus epidermidis* by combined polymerase chain reactions and sequence analysis

The results of methicillin resistance and biofilm formation of the selected *S.epidermidis* strains were confirmed by PCR based methods. Out of the 69 *S.epidermidis* samples, 20 were screened for the presence of biofilm forming (*ica*AB) and methicillin resistance (*mec*A) genes. PCR products from the selected sample were gel purified, sequenced and the sequence data were further subjected to BLAST analysis. The sequence data were also used for multiple sequence alignment and phylogenetic analysis for comparative study.

The results showed the presence of *ica*AB and *mec*A genes among most of the *S.epidermidis* strains selected for the study. This was further confirmed by sequence analysis.
One strain showed negative result in the PCR amplification. This also indicates the presence of ica independent mechanism for biofilm formation.

Chapter 10

Summary and conclusion

Studies on CNS revealed that S.epidermidis was the most predominant species isolated from human isolates. CNS isolated from humans and animals have virulence factors and might have an important role in the pathogenesis of infections. CNS isolated from clinical samples showed highest level of antimicrobial resistance. Salicylic acid (SAL) and N-acetyl cysteine (NAC) were found to be preventing the formation of biofilms and adherence of S.epidermidis to catheters and other indwelling medical devices. Studies on evaluation of catheters revealed that rifampicin impregnation is an effective procedure conferring antimicrobial protection against CNS. The PCR amplification of icaAB and mecA genes in S.epidermidis isolated from clinical samples confirmed the presence of genes for biofilm formation and methicillin resistance. The presence and expression of genes such as icaAB and mecA may help in clarifying the relevance of pathogenesis of infection caused by CNS especially the nosocomial infections. It could also be of value in the development of new preventive and therapeutic measures.

References