Emergence of Antibiotic Resistance in Nosocomial Strains of Coagulase Negative Staphylococci (CNS)

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Nosocomial infections caused by Coagulase Negative Staphylococci (CNS) have reached epidemic proportions. In the present work, about 26 clinically significant CNS belonging to Staphylococcus epidermidis and S. saprophyticus, were isolated from various clinical specimens and hospital environments. A few CNS strains have shown multiple antibiotic resistance to ampicillin, cotrimoxazole, cephalexin, cefotaxime, penicillin, tetracycline, gentamicin and lincomycin. Plasmid analysis of representative clinical isolates demonstrated the presence of 2-29.9 kb sized plasmid DNA. Intra and inter-specific gene transfer was observed between different species of CNS and S. aureus which suggests the rapid emergence of multiple antibiotic resistance in CNS.

Keywords: Coagulase Negative Staphylococci (CNS), antibiotic resistance, plasmid DNA

Introduction
Coagulase Negative Staphylococci (CNS) strains have received a great deal of attention over the past century as nosocomial agents causing a wide variety of common as well as serious infections in human beings (Charles et al, 1982). They also form a relatively larger component of the cutaneous microflora. These organisms are known to colonize various indwelling medical devices such as prosthetic heart valves, orthopedic appliances and intravascular catheters. These organisms cause vascular graft infections, cerebrospinal fluid infections, post-operative infections and a variety of wound infections. S saprophyticus appears to be the predominant staphylococcal species in acute urinary tract infection (Jordan et al, 1980). The opportunistic pathogenicity is attributed to the factors like alteration of the integument of various parts of human body, which allows the bacteria to gain entry into the body. The pattern of antibiotic resistance in CNS has been reported in a few earlier studies (Wallmark et al, 1978; Aber & Mackel 1981; Marsik & Brake, 1982).

The incidence of nosocomial infections caused by CNS resistant to multiple antibiotics is very high in Bangalore hospitals. According to the survey conducted during 2001, more than 55,187 patients at 24 health centres were infected by staphylococci relating to lower respiratory tract, urinary tract, and skin infections. The current incidence of infections caused by staphylococci in some major hospitals is as high as 54% when compared to the other groups of nosocomial agents (Statistical data from-Health and Family Welfare Services, Bangalore Urban District, Bangalore). The objective of this study was to isolate the local strains of coagulase negative staphylococci and to establish their antibiotic sensitivity/ resistance patterns and to characterize antibiotic resistant plasmids in them.

Materials and Methods
Isolation and Identification
The CNS strains obtained in this study were isolated from two major hospitals in Bangalore, Victoria Hospital and Bowring & Lady Curzon Hospital.

a) Clinical relevance. Several factors were considered in determining the clinical significance of each CNS isolate. These included the source of the culture, the relative numbers of organisms isolated and clinical findings in the patients like fever, local or generalized inflammation and wounds or drained purulent materials. Associated laboratory findings included an elevated total leucocytes with a granulocyte predominance and other indicators of infection...
such as the presence of leucocytes in cerebrospinal fluid (CSF) or in the urine.

The organisms were isolated from clinical specimens of hospital patients which included mainly purulent, draining superficial wounds and burns, ear and nasal swabs, urine, blood and CSF. To study the hospital environment, samplings were made from skin and surgical ward by exposure of nutrient agar plates for 10 min.

b) Sampling and identification. The organisms isolated from hospital sources were maintained on nutrient agar slants and further identification of species of staphylococci was carried out in our laboratory. The scheme of Brun et al (1978) was followed for routine identification of CNS which included the following tests: a) Gram staining, b) catalase test, c) oxidase test, d) coagulase test, e) haemolysis, f) mannitol fermentation, g) gelatin liquefaction, h) phosphatase production and i) novobiocin sensitivity test.

c) Sensitivity test. Bacterial sensitivity test for a number of antimicrobial agents was performed by disk diffusion method (Kirby-Bauer method). Multiple antibiotic disk ring of 12 antibiotics for Gram-positive bacteria (Pasteur Bio-disc) and penicillin and methicillin individual disk (Hi-Media) were used. Sensitivity was determined after incubation for 16-18 hrs at 30°C (Methicillin) or 37°C (for all other antibiotics). The Manufacturer’s zone size criteria were used to establish sensitivity/resistance.

d) Penicillinase production. The β-lactam resistant CNS were screened for β-lactamase production by inoculating re-suspended colonies on 0.1% brain heart infusion agar (Hi-Media) followed by treatment with N-α-naphthylamino carboxy benzene (Sigma) and 20 mg of benzyl penicillin per ml as described byNovick et al (1989) with slight modification.

e) Plasmid elimination/curing. Plasmid elimination was achieved by the method devised by Anjanappa et al (1999). Ethidium bromide to a concentration of 0.1 to 0.5 mg/ml was added to sterile tryptic soy broth. The broth was inoculated with 24 hrs old slant cultures of resistant strains of staphylococci (around 12 strains exhibiting resistance to a minimum of 7 antibiotics used in the sensitivity test were chosen for plasmid curing study) and incubated at 44°C for 24-48 hrs.

f) Plasmid DNA isolation. Plasmid DNA was isolated by lysozyme/lysozyme–alkali lysis method as described by Udo and Grubb (1999) with the modification of including PEG <Sub>4000</Sub> (Sigma) precipitation at the final step of DNA extraction.

g) Restriction endonuclease digestion. Plasmid DNA samples were cleaved with restriction endonuclease EcoRI (Bangalore Genei Pvt Ltd, Bangalore) over 2-3 hrs as described by Sambrook et al (1989). HindIII-EcoRI double digest of Lambda DNA was employed as standard molecular weight marker. Plasmid DNA subjected to restriction endonuclease action was analyzed by agarose gel electrophoresis (0.8-1.0% wt/vol) and visualized with UV transilluminator and documented using Gel Doc system (Ultra-Lum make, USA).

h) Determination of plasmid phenotype. Transfer of antibiotic resistance genes between CNS strains and S. aureus (isolated in this study) was attempted by mixed culture transfer as described by Mc Donnell et al, 1983; conjugation was carried out in the presence of 40% polyethylene glycol as described by Townsend et al (1985) with modification. Plasmid specific transformation in staphylococci species was performed as specified by Lindberg et al (1972) and Harley & Prescott (1993).

Results
Antibiotic Resistance Pattern
From the biochemical test performed, 26 out of the 64 isolates were identified as CNS species, of which 20 strains (76.93%) were identified as S. epidermidis from clinical samples of superficial/purulent wounds and hospital atmosphere. S. saprophyticus strains (23.07%) were isolated from clinical samples mainly from urine. Almost all of the CNS strains were resistant to penicillin as characterized by the presence of extracellular penicillinase. Percentage of resistance to antibiotics in CNS is as shown in Fig. 1.

Plasmid Profile
In the plasmid curing experiments, 5 strains of CNS (19.23%) were found to be sensitive to Penicillin, Ampicillin, Cephallexin, Cotrimoxazole, Gentamicin and Tetracycline (Table 1) at the concentration of 0.2 mg/ml of ethidium bromide for 48-72 hrs. From the above observation it was noted that the phenotype was conferred by plasmid DNA and hence these strains were subjected to analysis of plasmid DNA.
Chromosomal encoded resistance was observed in most of the strains (80.77%) which was indicated by their ability to grow in the media containing respective antibiotics like Penicillin, Methicillin, Rifampin, Ciprofloxacin, Gentamicin, Tetracycline and Cloxacillin, even after curing of plasmids.

To examine the relatedness of phenotypically similar plasmids found in staphylococci, plasmid DNA isolated was subjected to EcoRI digestion. Restriction fragments and molecular weights of the plasmid DNA of staphylococci were documented (Fig. 2; Table 1).

**Plasmid Transfer**

The strains of CNS and *S. aureus* groups used as donor/recipient in plasmid DNA transfer were as shown in Table 2. The recipient strains were selected from plates containing selective antibiotic to which the donor strains were sensitive, or by comparison of phenotypic traits as mentioned in Table 2. As there was no plasmidless recipient strain available, the recipient strains used in the experiment may or may not originally contain plasmids. Donor and recipient used in MCT are indicated in Table 2. Results showed that most of the recipient strains acquired resistance from donor. The transfer of plasmid DNA in this method was found to be significant as there was successful transfer of resistant determinants. Resistance determinants transferred were mainly for Ampicillin, Gentamicin and Cotrimoxazole.

Conjugation was performed in the presence of 40% PEG8000, the donor and recipient strains used were as shown in Table 2. The transconjugants gained resistance to Ampicillin, Cotrimoxazole and Gentamicin as mentioned in Table 2. The frequency of transconjugants was recorded as $10^{-5}$ to $10^{-8}$. Plasmid DNA mediated transformation was carried out in the presence of 0.1 M CaCl$_2$. Under in vitro conditions, there was $10^{-14}$ transformation recorded at 500 ng of plasmid DNA concentration (Table 2), which shows

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Size(kb)</th>
<th>Restriction fragments</th>
<th>Phenotype conferred after curing</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNS-4</td>
<td>23.130</td>
<td>21.226;1.904</td>
<td>Am, Ba, Pr, Gm, Pe</td>
</tr>
<tr>
<td>CNS-7</td>
<td>29.934</td>
<td>21.226; 3.903; 2.778; 2.027</td>
<td>Am, Ba, Pr, Te, Gm,</td>
</tr>
<tr>
<td>CNS-10</td>
<td>5.434</td>
<td>3.530; 1.904</td>
<td>Te</td>
</tr>
<tr>
<td>CNS-11</td>
<td>3.183</td>
<td>2.027; 1.156</td>
<td>Te</td>
</tr>
<tr>
<td>CNS-14</td>
<td>2.027</td>
<td>2.027</td>
<td>Te</td>
</tr>
</tbody>
</table>

Am - Ampicillin; Ba - Cotrimoxazole; Pr - Cephalexin; Gm - Gentamicin; Pe - Penicillin; Te - Tetracycline
Table 2—Plasmid DNA transfer in Staphylococci

<table>
<thead>
<tr>
<th>Donor strains</th>
<th>Plasmid (kb)</th>
<th>Recipient</th>
<th>Selective agent for recipient</th>
<th>Transfer method</th>
<th>Resistance markers transferred</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNS-4</td>
<td>23.130</td>
<td>S. aureus</td>
<td>Coagulase test</td>
<td>Mixed culture</td>
<td>Am, Gm, Ba</td>
</tr>
<tr>
<td>CNS-4</td>
<td>23.130</td>
<td>CNS</td>
<td>Te</td>
<td>Conjugation</td>
<td>Am, Pr, Gm, Ba</td>
</tr>
<tr>
<td>CNS-4</td>
<td>23.130</td>
<td>CNS</td>
<td>Novobiocin</td>
<td>Transformation</td>
<td>Am, Gm, Ba</td>
</tr>
<tr>
<td>CNS-7</td>
<td>29.934</td>
<td>S. aureus</td>
<td>Coagulase test</td>
<td></td>
<td>Am, Gm</td>
</tr>
<tr>
<td>CNS-7</td>
<td>29.934</td>
<td>CNS</td>
<td>Novobiocin</td>
<td></td>
<td>Am, Pr, Gm</td>
</tr>
<tr>
<td>CNS-4</td>
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<tr>
<td>CNS-7</td>
<td>29.934</td>
<td>CNS</td>
<td>Novobiocin</td>
<td></td>
<td>Am, Gm, Te</td>
</tr>
</tbody>
</table>

Am-Ampicillin; Ba-Cotrimoxazole; Pr-Cephalexin; Gm-Gentamicin; Te-Tetracycline
** Not successful

that transformation is not the process preferred for successful transfer of plasmid DNA in a given population of staphylococci in nature.

**Discussion**

Different strains of CNS isolated from Bangalore hospitals proved to be the causative agents of serious nosocomial infections (from clinical relevance study). The most common group of individuals susceptible to infections were those belonging to pediatric age group (below 6 yrs) and adults (above 18 yrs) with wounds and burns, respiratory infections and UTI.

Plasmid analysis showed that the smallest group of plasmids were of size ranging from 2.0-3.0 kb, harbouring resistant determinants for Tetracycline resistance, which were similar to the class I family of staphylococcal plasmids reported by Novick (1989). The plasmid size 23.130 kb and 29.934 kb of CNS strains (Fig. 2) belonged to the class II α and II β family as mentioned earlier by Shalita et al (1980). These plasmid groups conferred resistance to Ampicillin, Cotrimoxazole, Cephalexin, Tetracycline, Gentamicin, Penicillin, Cefotaxime and Lincomycin as evidenced by curing studies.

Most of the strains of multiple antibiotic resistant CNS (80.77%) were endowed with the chromosomal resistance which included resistance to Penicillin, Methicillin, Rifampin, Ciprofloxacin, Ofloxacin and Pefloxacin. However, chromosomal origin of resistance to Gentamicin, Tetracycline and Lincomycin was also observed in some strains which were earlier reported to be associated with only plasmids. The possible explanation can be that the plasmid loci which can be transposable have been transposed onto the chromosome in the present strains, explaining higher level of chromosomal encoded resistance to antibiotics (Al Masaudi et al, 1997; Sullia & Geetha, 1998).

Staphylococcal R-plasmids have been transferred successfully in vitro by mixed culture transfer on solid media, thereby suggesting the importance of the normal staphylococcal habitat viz., the body surface in the emergence of antibiotic resistant organisms and that the topical use of antibiotic imposes a selective pressure on the organisms for plasmid expression. There is interspecific gene transfer reported between CNS and S. aureus.

Furthermore, plasmid mediated conjugation has been reported to be significant in transferring resistant determinants by filter mating experiments using dry absorbent surfaces simulating in vivo conditions. Therefore, transfer of resistance might also be expected to take place on surgical dressings, clothing and bedding in the hospital environment. In the transformants that have gained resistance to antibiotics (Table 2), the lower efficiency of transfer (in vitro) may be due to the production of DNases and the resultant endonuclease activity. Under in vivo conditions, there is requirement for a non-physiological CaCl₂ concentration and a short period
of competence as described by Lacey (1984), and this explains partially why transformation is not a process preferred for transfer of staphylococcal plasmids in nature.

The homogeneity of the isolates from Bangalore hospitals, with regard to their antibiograms and plasmid profiles, provides substantial evidence for the transfer of resistance genes from a common source. The current infections caused by staphylococci are likely to have arisen through a horizontal spread of genes from a single strain or its derivatives from hospital-to-hospital. Subsequent evolutionary events such as mutation and DNA insertion or deletion through transposition or recombination, might have led to the production of strain variants that are currently occurring. It is imperative that utmost care be taken to minimize the spread of staphylococci within hospital wards and between different hospitals.

Nosocomial infections with staphylococci strains resistant to multiple antibiotics have reached epidemic proportions in Bangalore, causing considerable morbidity and mortality. The emergence of this MAR (multiple antibiotic resistant) staphylococci can be due to: i) the possible role played by ubiquitous, non-pathogenic species S. epidermidis as a reservoir of plasmids carrying genes for resistance, ii) the widespread and effective inter and intra-specific genetic exchange among staphylococci and, iii) the extensive use of antibiotics. The regular use of these compounds in the hospital environment has created selective pressure leading to the de novo emergence of resistant determinants within many staphylococci as evidenced by the outbreak of resistance frequently following the introduction of an antibiotic.

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References