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Research Article

ANTIMICROBIAL SUSCEPTIBILITY AND PLASMID PATTERN ANALYSIS OF COAGULASE NEGATIVE STAPHYLOCOCCI ISOLATED FROM DIFFERENT SOURCES

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ABSTRACT

Coagulase negative staphylococci (CNS) from different sources i.e. human healthy skin, clinical samples and domestic animals were identified and the antimicrobial susceptibility and plasmid pattern were analysed. Incidence of antibiotic resistance was highest in the isolates of *S. epidermidis* compared to other species. Antibiotic susceptibility test showed that CNS isolated from human healthy skin was completely susceptible to vancomycin (100%). 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. 10% clinical isolates of *S. epidermidis, S. saprophyticus and S. haemolyticus* were resistant to vancomycin. 7% *S. epidermidis*, 6% *S. hyicus* and 5% *S. chromogenes* from domestic animal samples showed resistance to vancomycin. 84% of the penicillin resistant CNS strains were positive for β-lactamase test. In plasmid pattern analysis 8 out of 20 strains of CNS showed plasmids by agarose gel electrophoresis. Phenotypically similar plasmids were found in two strains of *S. epidermidis*. Chromosomal encoded resistance towards penicillin, methicillin, rifampicin and ciprofloxacin was also observed. Comparison of results of the present study with the earlier reports shows an increase in multiresistance among the CNS isolates.

Keywords: Coagulase negative staphylococci, antimicrobial susceptibility, plasmid pattern analysis.

INTRODUCTION

Coagulase negative staphylococci formerly regarded as harmless commensals are now recognized as a major cause of significant clinical infections. CNS are also involved in animal diseases. They are more often associated with bovine subclinical mastitis. CNS are a group of microorganisms that are increasingly implicated as pathogens in both health care settings and communities around the world. They are associated with bacteremia, wound related infections, intravascular catheter-related infections and a variety of post-operative infections. They have the ability to develop resistance to all hitherto described antibiotics. They have become a serious problem due to associated antibiotic resistance, leading to significant limitations in therapeutic options. Multiple antibiotic resistance and the presence of plasmid which contain one or more resistance genes are common among CNS[1]. The spread of antibiotic resistance and increased use of medical devices are the two main reasons for the alarming rate of CNS infections in recent years. The emergence of drug resistance in CNS is an important cause of morbidity and mortality in developing countries. Over the past several years, resistance of CNS to many classes of antimicrobial agents has emerged. The glycopeptide vancomycin has been regarded as one of the last resorts for treatment of infections due to methicillin resistant *Staphylococcus aureus* and methicillin resistant coagulase negative staphylococci. Increased use of vancomycin in developing countries has led to the emergence of CNS with decreased susceptibility to this antibiotic. Unrestricted use of antibiotics is leading to the emergence of resistant determinants within many species of CNS in countries like India also. Hence a study was carried out to analyse the antibiotic sensitivity pattern and the plasmid pattern of CNS isolated from Kerala, a southern state of India.

MATERIALS AND METHODS

The CNS isolates from 3 different sources comprising clinical samples, human healthy skin and domestic animals were subjected to antibiotic sensitivity tests. A total of 579 CNS strains isolated and identified were used in this study. The clinical samples were collected from a tertiary care hospital in Kerala, over a period of 4 years. Out of a total 600 isolates, 200 from each of the above sources, 579 isolates were subjected to study. Isolates from clinical samples included 74 from blood cultures, 51 from urine cultures and 68 from other samples including cunjunctival swabs, drain tips, catheter tips and I.V.cannulas. 196 strains of CNS were isolated from normal human healthy skin both from male (99) and female (97).190 strains of CNS isolated from domestic animal samples were also studied. They comprised 72 isolates from cow, 58 isolates from cat and 60 isolates from dog. Samples from animals were obtained by swabbing the lateral udder surface and upper flank of the cows and skin of cats and dogs. The CNS species were tentatively identified as described by Kloos and Schleifer (1995)[2].

Antimicrobial susceptibility

Susceptibility to antimicrobial agents were determined by the disc diffusion method (Kirby-Bauer Method) on Mueller-Hinton Agar. For this an inoculum of 0.5 McFarland standards was inoculated in to a tube containing 5ml sterile nutrient broth and incubated for 18-24hrs at 37°C. Commercially available antibiotic discs (Himedia) (penicillin,10µg; oxacillin,1µg; gentamycin,10µg; streptomycin,10µg; tetracycline,30µg; clindamicin,2µg; chloramphenicol,30µg; ciprofloxacin,5µg; norfloxacin,10µg; nalidixic acid,30µg; ampicillin,10µg; tobramycin,10µg rifampicin5µg and vancomycin30µg) were used. The plates were incubated over night at 37°C.

Penicillinase production

The penicillin resistant CNS were screened for β-lactamase (Penicillinase) production by iodometric tube method[3]. A heavy inoculum from culture plates were added to 0.5 ml of penicillin solution, mixed well and left at room temperature for an hour. Then two drops of 1% soluble starch solution was added followed by the addition of one drop of iodine solution. This was then mixed well, incubated at room temperature and observed for discoloration at different time intervals.

Plasmid isolation

Plasmid isolation was carried out by the method described by Birnboim and Dolly (1979)[4] with slight modifications. For the plasmid isolation, twenty strains which were found to have resistance to minimum of 7 antibiotics were selected. This include 10 strains of *S. epidermidis,* 5 strains of *S. saprophyticus and* 5 strains of *S. haemolyticus* isolated from human clinical samples for plasmid isolation study.

Agarose gel electrophoresis

A 0.8% agarose gel was prepared in 0.5X TBE electrophoresis buffer and mixed with ethidium bromide at a concentration of 50 µg/100 mL. The plasmid DNA samples were mixed with 6X sample loading buffer to a final concentration of 1X and loaded into the wells and resolved at 8 V/cm. The electrophoresis was stopped and the gel removed when the bromophenol blue in the tracking dye reached 2/3rd of the gel. The plasmid DNA in the gel was visualized with UV transilluminator and photographed.

Restriction endonuclease digestion

For plasmids of identical size, restriction endonuclease fragment analysis will provide additional information about their identity. Plasmid DNA samples were cleaved with restriction endonuclease *Eco*RI (Bangalore Genei Pvt Ltd, Bangalore) over 2-3 hrs as described by Sambrook *et al.,* (1989)[5]. The restriction enzyme digestion was carried out in a final volume of 20µL containing 5 units of *Eco*RI, 1µg of plasmid DNA and 1X *Eco*RI buffer. The reaction was incubated for 2 h at 37°C. The digested DNA was resolved on a 1.2% agarose gel along with molecular size markers. HindIII-EcoRI double digest of Lambda DNA was employed as standard molecular weight marker.

Plasmid curing

Plasmid elimination was achieved by the method described by Anjanappa *et al*., (1999)[6]. Using the isolates which were found to have the presence of plasmids were subjected to study. Ethidium bromide at 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 selected isolates and incubated at 44°C for 24-48 hrs. Cell broth was then suitably diluted and spread on TSA plates at 30°C for 24 hrs. The plasmid cured isolates were further subjected to plasmid isolation to confirm the absence of plasmid. These isolates were further subjected to antibiotic sensitivity test by disc diffusion method.

RESULTS

Susceptibility of 579 CNS isolates to 14 antibiotics were studied. Antibiotic resistance of CNS isolated from three sources are shown in Table1. Incidence of antibiotic resistance was highest among the isolates of *S. epidermidis* compared to the isolates of other species. *S. epidermidis* isolates showed resistance rate of 55% to penicillin and 50% to oxacillin. At the same time 83% of *S. xylosus* isolates and 67% of *S. lugdunensis* isolates were resistant to penicillin. Antibiotic resistances of CNS isolates were analyzed by χ^2 test. The result showed statistically significant difference ($p < 0.001$).

CNS Species	Total No. of isolates	P	OX	G	S	T	CD	C	CF	NX	NA	A	TB	R	VA
S. epidermidis	159	88(55%)	80(50%)	56(35%)	46(29%)	39(24%)	58(36%)	48(30%)	59(37%)	54(34%)	65(41%)	52(33%)	47(29%)	34(22%)	9(6%)
S. saprophyticus	69	34(49%)	26(38%)	21(30%)	20(29%)	16(23%)	24(35%)	18(26%)	24(35%)	28(40%)	26(38%)	20(29%)	22(32%)	15(22%)	3(4%)
S. haemolyticus	68	31(45%)	26(38%)	19(28%)	17(25%)	18(26%)	22(32%)	21(31%)	20(29%)	18(26%)	28(41%)	23(34%)	17(25%)	17(25%)	3(4%)
S. hominis	44	21(48%)	17(39%)	11(25%)	12(27%)	5(11%)	10(22%)	10(23%)	12(27%)	9(20%)	15(34%)	11(25%)	11(25%)	7(15%)	0
S. lugdunensis	12	8(67%)	6(50%)	6(50%)	6(50%)	5(42%)	3(25%)	6(12%)	5(42%)	5(42%)	7(58%)	6(12%)	5(42%)	7(58%)	0
S. warneri	30	10(33%)	8(26%)	8(27%)	7(23%)	2(7%)	5(17%)	7(23%)	5(16%)	6(20%)	7(23%)	8(27%)	8(27%)	6(20%)	0
S. capitis	24	10(42%)	7(29%)	5(21%)	7(29%)	5(21%)	6(25%)	4(17%)	6(25%)	8(33%)	8(33%)	5(21%)	4(17%)	6(25%)	0
S. xylosus	12	10(83%)	9(75%)	6(50%)	7(58%)	5(42%)	4(33%)	4(33%)	5(42%)	4(33%)	5(42%)	5(42%)	5(42%)	4(33%)	0
S. simulans	22	11(50%)	6(27%)	7(32%)	5(23%)	4(18%)	4(18%)	4(18%)	4(18%)	4(18%)	5(23%)	6(27%)	6(27%)	3(14%)	0
S. cohini	31	15(48%)	11(35%)	8(26%)	8(25%)	5(16%)	7(22%)	7(22%)	8(26%)	7(22%)	11(35%)	9(29%)	8(26%)	4(13%)	0
S. hyicus	69	27(39%)	20(29%)	17(25%)	15(21%)	12(17%)	8(11%)	7(10%)	6(9%)	8(11%)	12(17%)	7(10%)	6(9%)	5(7%)	4(6%)
S. chromogenes	39	13(33%)	11(28%)	8(20%)	8(20%)	6(15%)	5(13%)	5(12%)	4(10%)	6(15%)	9(23%)	4(10%)	4(10%)	3(8%)	2(5%)

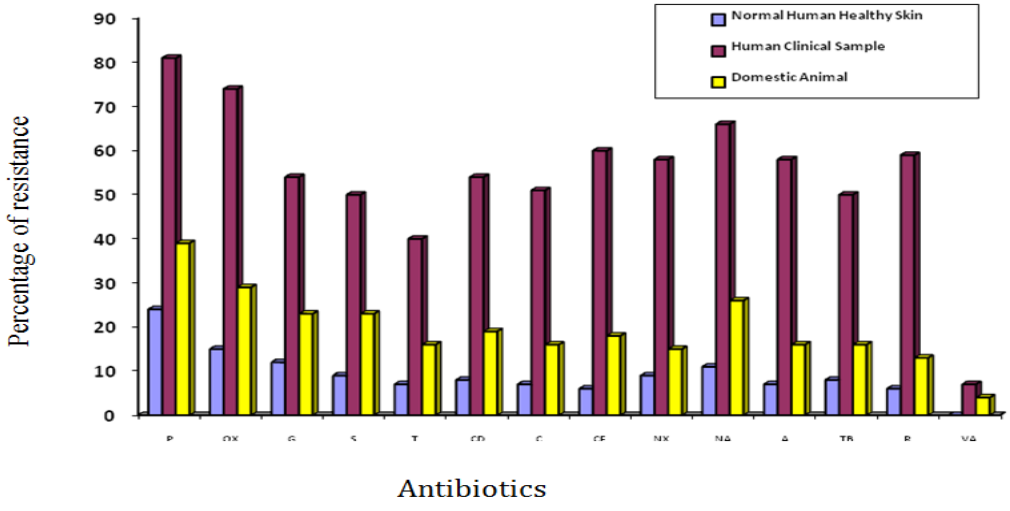

Figure 1: Antibiotic resistance pattern of CNS isolated from 3 different sources

P- Penicillin, OX-Oxacillin, G-Gentamycin, S-Streptomycin, T-Tetracycline, CD- Clindamicin, C-Chloramphenicol, CF- Ciprofloxacin, NX-Norfloxacin, NA-Nalidixic acid, A- Ampicillin,TB- Tobramycin, R- Rifampicin, VA- Vancomycin

Of the 196 numbers of CNS isolates from human healthy skin sample *S. epidermidis* showed sensitivity rate of 77% and 80% to penicillin and oxacillin respectively. These isolates were completely susceptible to vancomycin (100%). A high sensitivity rate of 80-97% was shown towards rifampicin by the CNS isolates. At the same time, of 193 CNS isolates from human clinical sources, 10% of the isolates of *S. epidermidis, S. saprophyticus* and *S. haemolyticus* were resistant to vancomycin. *S. epidermidis* showed resistance rate of 87% and 84% to penicillin and oxacillin respectively. Sensitivity towards rifampicin varied from 33-52% among the different species of CNS tested. When considering the 190 numbers of CNS isolates from domestic animal samples 7% of *S. epidermidis*,

6% of *S. hyicus* and 5% of *S. chromogenes* were resistant to vancomycin. *S. epidermidis*, *S. hyicus* and *S. chromogenes* were resistant to penicillin at a rate of 47%, 39% and 33% 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 penicillin resistant strains from the three sources were subjected to extracellular penicillinase test. The result showed that among the penicillin resistant strains 84% were positive for penicillinase test.

Percentages of resistance to antibiotics in CNS from three different sources are compared in Figure 1. Clinical samples showed highest antibiotic resistance compared to the other two sources. Of the other two sources the domestic animal samples showed higher resistance compared to the human healthy samples.

In order to study the presence of plasmids among CNS isolates resistant to multiple antibiotics, plasmid isolation was conducted. Out of the 20 selected isolates used for the study, 8 were found to have the presence of plasmid. In that 6 strains were *S. epidermidis*, one *S. saprophyticus* and the other was *S. haemolyticus,* as confirmed by agarose gel electrophoresis. Phenotypically similar plasmids present in CNS E-13 and CNS E-20 strains was confirmed by the *Eco*RI digestion (Figure 2). These strains were isolated from the neonates. Plasmid curing and further analysis proved the possible plasmid mediated tetracycline resistance in those isolates with a plasmid size ranging from 2-3kb were present initially. Also in the plasmid curing experiments, CNS were found to be sensitive to ampicillin, gentamicin, cephalosporin and tetracycline. Chromosomal encoded resistance was also observed among the strains, which was indicated by their ability to grow in the media containing respective antibiotics like penicillin, methicillin, rifampicin and ciprofloxacin even after curing of plasmids.

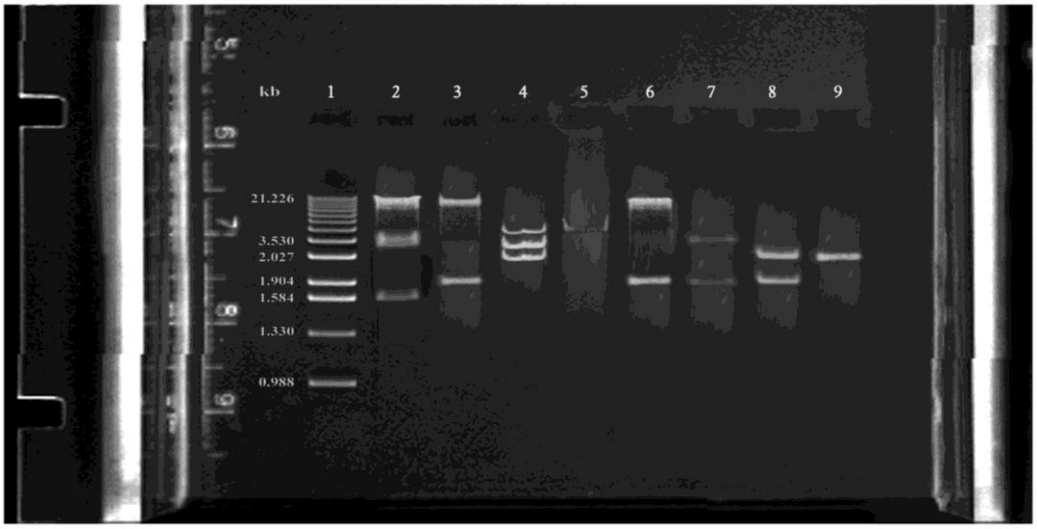

Figure 2: Agarose gel electrophoresis of plasmid DNA of CNS

Agarose gel electrophoresis analysis of plasmid DNA digested with *Eco*R1; Lane 1 is the molecular size standard (in kilobase), Lane 2-7 *S. epidermidis* strains. Lane (2) CNS E-6, Lane (3) CNS E-13, Lane (4) CNS E-17, Lane (5) CNS E-19, Lane (6) CNS E-20, Lane (7) CNS E-23 Lane 3 and 6 showed similar plsmid pattern, Lane 8 is *S. saprophyticus*, CNS S-22, Lane 9 is *S. haemolyticus.*CNS H-31.

DISCUSSION

In the present study antimicrobial susceptibility pattern of each species of CNS from 3 different sources were evaluated. Incidence of antibiotic resistance was highest among the isolates of *S. epidermidis* and it showed a resistance rate of 55% to penicillin and 50% to oxacillin. Isolates from clinical sources showed highest antibiotic resistance. A high frequency in penicillin and methicillin resistance of 77% and 80% in human healthy skin and 87% and 84% in clinical samples were observed in the present study. Some studies reveal rates of oxacillin resistance in CNS approaching 90%[8]. In reports from different parts of Europe, the oxacillin resistance in CNS varies between 70 and 80% [9] and similar high rates of resistance are also reported from the United States, Canada and Latin America [10, 11.] Studies of CNS from a variety of sources showed that 26-74 % were resistant to penicillin[12]. In certain population methicillin resistant was found in 67% of the isolates[13]. The frequency of methicillin resistance in various species of CNS in the present study has in agreement with the finding of Thore *et al,* (1990)[14]. In the present study 84% of penicillin resistant CNS strains were positive for β-lactamase test and 16% showed negative result. In India, different strains of CNS 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 these nosocomial infections were those belonging to pediatric age group and adults with wounds and burns, respiratory and urinary tract infection[15.]

It is important to note that vancomycin resistance was also found in CNS used for the present study. 10% of *S. epidermidis, S. saprophyticus and S. haemolyticus* isolated from clinical specimens were resistant to vancomycin. The emergence of vancomycin resistance in clinical strains can be accounted for indiscriminate use of the drugs in clinical practice. Vancomycin treatment of clinically defined coagulase negative staphylococcal bacteremia may frequently be unnecessary. There is a tendency for clinicians and some clinical microbiologist to overuse antibiotics in patients with CNS from blood cultures. One third of the patients with contaminating CNS were treated with specific anti - staphylococcal antibiotics. A study by Souvenir *et al* (1998)[16] found that one - half of the patients with contaminating coagulase negative staphylococci were treated with antibiotics and 34 % with vancomycin.

Penicillin- resistance found in isolates from domestic animal study was higher (39%) than that in the earlier reports[17]. In domestic animal samples 7% of *S. epidermidis*, 6% of *S. hyicus* and 5% of *S. chromogenes* were resistant to vancomycin. Increasing antimicrobial resistance has become a serious concern worldwide and antimicrobial use in animals is under scrutiny[18]. The effectiveness of therapeutic methods depends on the organism, their antibiotic susceptibility, dosage and duration of treatment and how early the treatment is initiated[19]. The CNS have adapted to survive in the udder, they shed in the milk and establish mastitis infections, which serves as a source of infection for the healthy bovine[20]. The CNS bacteria lives on teat skin can colonize into the teat canal. Anything that decreases the potency of the teat sphincter, facilities the occurrence of infections and may lead to both sub-clinical and clinical mastitis forms. CNS infections are associated with damage to milk secretory tissue stroma and moderate decreases in milk production. The high resistance to penicillin and other antibiotics found in this study emphasize the importance of presence of CNS when a bovine clinical mastitis is present. Antimicrobial

susceptibility patterns should be identified for CNS, and a susceptibility data is necessary to select appropriate antibiotics for a successful treatment. CNS are emerging as important minor mastitis pathogens and can be the cause of substantial economic losses.

Plasmid pattern analysis is a valuable tool for characterization and epidemiologic studies of CNS[21]. This is also suitable for screening of relatively large numbers of isolates. The two *S. epidermidis* strains CNS E-13 and CNS E-20 isolated from the neonates showed similar plasmid pattern as confirmed by *Eco*R1 digestion. The similarity with regard to their antibiograms and plasmid profiles provides possible evidence for the transfer of resistance genes from a common source. In this study plasmid analysis suggest that a coagulase negative staphylococcal clone was carried by two babies and most of the isolates from clinical source were multiple antibiotic resistant, implying that CNS can be endemic to the unit. Understanding the spread of CNS between babies and from site to site on the same baby may facilitate the design of effective interventions for reducing the incidence of systematic coagulase negative staphylococcal infections in these patients. However, such measures are clearly inadequate in preventing colonization of infants by antibiotic resistant CNS endemic to a unit. Several authors have observed unpredictable day to day variation in the numbers and antibiotic resistance profiles of CNS isolated from neonatal skin[22].

The infections caused by CNS are likely to have arisen through a horizontal spread of genes from a single strain or its derivatives from hospital-to-hospital. It is imperative that utmost care be taken to minimize the spread of CNS within hospital wards and between different hospitals. Nosocomial infections with CNS strains resistant to multiple antibiotics have reached epidemic proportions. The emergence of this MAR (multiple antibiotics resistant) CNS can be due to the possible role played by ubiquitous, non-pathogenic species *S. epidermidis,* as a reservoir of plasmid carrying genes for resistance. The antibiotic profile and plasmid pattern analysis adds important information in specific clinical situations.

The regular use of antibiotics in the hospital environment has created selective pressure leading to the *de novo* emergence of resistant determinants within many CNS. The present study reveals that there is an increase in the prevalence of drug resistance among CNS isolates, due to this reason it may be difficult to treat infections by CNS in the near future. Hence effective measures to postpone resistance development or to resolve resistance problem are to be developed. It is high time to implement effective surveillance and control strategies to solve this problem.

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