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Original Articles

Species Distribution, Antimicrobial Susceptibility and Virulence Factors of Coagulase-negative Staphylococci Isolated from Different Clinical Specimens

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Abstract

Background: Eighty coagulase-negative Staphylococci (CoNS) isolates from surgical wound, pus from infected skin lesions, burn exudates and diabetic ulcer exudates of patients in Mymensingh Medical College Hospital, Bangladesh, was evaluated in order to see their species distribution, pattern of antimicrobial resistance and virulence factors.

Methods: The study was carried out in the department of Microbiology, Mymensingh Medical College during the period from July 2010 to May 2011 and it was a cross sectional descriptive type study. The eighty CoNS isolates were subjected to identification up to species level, antimicrobial resistance pattern and detection of virulence factors.

Results: Out of 80 CoNS isolates, 16 species were identified and *S. epidermidis* were the highest in number among them. Most of the isolates were resistant to oxacillin 36 (45%) and all were sensitive to imipenem and vancomycin. Virulence factor *mecA* gene was detected in 45 (56%) strains of CoNS by PCR. No PVL gene was found.

Conclusion: Considering various findings of this study, it can be concluded that CoNS are the emerging nosocomial pathogens in MMC. As MRCoNS are multidrug resistant, therefore antibiotic sensitivity must be done prior to treatment in infections caused by them.

Key words: Coagulase-negative Staphylococci, HiStaph™ Identification Kit, Antimicrobial susceptibility test

Introduction

Staphylococci are gram positive, non motile bacteria that characteristically divide in more than one plane to form irregular cluster. Nearly all are facultative anaerobes that grow better under aerobic than anaerobic condition[1]. Staphylococci are widely spread in various niches such as clinical environments and food plants. Thirty-six validated described species, including 21 subspecies, belong to the *Staphylococcus* genus according to the List of Bacterial Names with Standing in Nomenclature, updated 3 December 2004[2]. Species are classified as coagulase-positive *Staphylococcus aureus* and coagulase-negative staphylococci eg, *Staphylococcus epidermidis, Staphylococcus saprophyticus*.

Coagulase negative Staphylococci (CoNS), previously dismissed as contaminants are now emerging as important potential pathogens with the increase in number of severely debilitated patients and increased use of implants in hospitals. More than 30 species of CoNS are recognized but only a few are commonly incriminated in human infections. Multidrug resistant strains are common[3]. Infections due to CoNS can be caused by either community or hospital acquired isolates [4]. Skin of patients and health care workers, medical equipment, clothing of personnel and environment surfaces can be sources of antibiotic resistant *S. epidermidis* strains[5]. Skin contact affords easier

transmission between hosts[6]. The colonization pressure has an impact on both the risk for cross-transmission between patients and the risk for patients to acquire a nosocomial infection[7]. CoNS are a group of micro-organisms that are increasingly implicated as a cause of significant infection. They are one of the main causal agents of bacteraemia in patients with indwelling medical devices such as central and peripheral venous catheters, valvular prostheses, artificial heart valves, pace-makers and orthopedic prostheses and other infections involving biofilm formation on implanted biomaterials. The species most frequently associated with bacteraemia is *Staphylococcus epidermidis*[8].

In last two decades, CoNS have also emerged as significant pathogens, especially in immunocompromised patients, premature newborns, and patients with implanted biomaterials. The most frequently encountered CoNS species associated with human infections is*Staphylococcus epidermidis*, in particular in association with intravascular catheters. In addition, *S. epidermidis* is the predominant agent of nosocomial bacteremia, prosthetic-valve endocarditis, surgical wounds, central nervous system shunt infections, intravascular catheter-related infections, peritoneal dialysis-related infections, and infections of prosthetic joints. The second most frequently encountered CoNS species is*S.haemolyticus.* Other CoNS species are involved in a variety of infections. For example, *S. saprophyticus* is an important pathogen in human urinary tract infections, especially in young, sexually active females, and *S.lugdunensis* has been implicated in arthritis, catheter infections, and prosthetic joint infections. Because of the increasing clinical significance of CoNS, accurate species identification of CoNS is highly desirable to permit a more precise determination of host-pathogen relationship of CoNS[9]. Now a days they become important prevalent pathogens involved in hospital-acquiredinfections. The costs related to infections caused by themin the hospital setting are enormous and represent a major healthcare burden[10]. CoNS are increasing in importance as causes of hospital-acquired infections, particularly nosocomial bacteremias. The National Nosocomial Infection Survey (NNIS) of USA found that from 1980 to 1989, the incidence of CoNS as a cause of nosocomial bacteremias increased from 9 to 27% to become the single most common cause of these infections. Furthermore, NNIS data revealed that during this same period the proportion of nosocomial CoNS resistant to methicillin, oxacillin, or nafcillin increased from 20 to 60%. Most of these methicillin-resistant CoNS were also resistant to multiple additional antimicrobial agents. Thus, there is an association between the dramatic increase in CoNS as a cause of nosocomial bacteremias and the resistance of these pathogens to antimicrobial agents[11].

Considering the importance of CoNS as a causal agent of nosocomial infections, in the present work we tried to study on species distribution, antimicrobial susceptibility and some virulence factors of CoNS isolated from different clinical specimens.

Materials And Methods

Place and period of study: Department of Microbiology, Mymensingh Medical College from July'10 to May'11. Cases were randomly selected from in-patient departments of Mymensingh Medical College Hospital (MMCH).

Study design: Cross sectional descriptive study.

Study population: A total of 300 clinically diagnosed patients irrespective of age and sex were selected from in patient departments of MMCH.

Specimens: Specimens comprising of wound swab, pus, exudates from diabetic ulcer, exudates from burn ulcer and swab from stitch infection were collected with proper aseptic precaution.

Microscopy: All the specimens were subjected to gram staining and examined microscopically for initial identification of organism according to their

gram reaction and morphology.

Isolation of organism (Culture)

All the specimens were inoculated into Nutrient agar, Blood agar and Mannitol salt agar media and then incubated at 37°C for 18-24 hours aerobically. After incubation, colony morphology was noted in different culture media.

Identification of organisms

The isolates were screened by tube coagulase test and then CoNS were identified upto species level by colony morphology, Grams staining and by commercial biochemical test kit (HiStaph™ Kit).

HiStaph Identification Kit

HiStaph™ Identification Kit of HiMedia Laboratories was used for identification and differentiation of CoNS upto species level. The tests were done according to manufacturer's instructions.

Interpretation of results

The results were interpretated as per the standards given in the identification index (Table-1).

Table-1: HiStaph kit is a standardized, colorimetric identification system utilizing 12 conventional biochemical tests

No.	Test	Reagents to be added after incubation	Principle	Original color of medium	Positive reaction	Negative reaction
1.	Voges Proskauer's	2 drops of Baritt Reagent	Detects acetoin Production	Colorless	Pinkish red	Colorless
2.	Alkaline Phosphatase	2 drops of 40% NaOH	Detects ability of organism to produce phosphatase enzyme	Cream	Pink	Cream
3.	ONPG		Detects ² -galactosidase activity	Colorless	Yellow	Colorless
4.	Urease		Detects Urease activity	Orangish Yellow	Pink	Orangish yellow
5.	Arginine Utilization		Detects Arginine decarboxylation	Olive green	Purple	Yellow
6.	Mannitol		Carbohydrate utilization	Pinkish red	Yellow	Red / Pink
7.	Sucrose		Carbohydrate utilization	Pinkish red	Yellow	Red / Pink
8.	Lactose		Carbohydrate Utilization	Pinkish red	Yellow	Red / Pink
9.	Arabinose		Carbohydrate utilization	Pinkish red	Yellow	Red / Pink
10.	Raffinose		Carbohydrate utilization	Pinkish red	Yellow	Red / Pink
11.	Trehalose		Carbohydrate utilization	Pinkish red	Yellow	Red / Pink
12.	Maltose		Carbohydrate utilization	Pinkish red	Yellow	Red / Pink

Table-2: Showing differentiation of coagulase-negative Staphylococci (CoNS) species (provided byHiMedia Laboratories LTD)

Tests	VP	Alk. Phos.	ONPG	Ure Ase	Argi Nine	Man nitol	Sucr -ose	Lact -ose	Arabi -nose	Rafi nose	Treh alose	Malt -ose
S .epidermidis	+	+	-	+	+w	-	+	v	-	-	-	+
S.saprophyticus	+	-	V	+	-w	V	+	v	-	-	+	+
S. haemolyticus	V	-	-	-	+	v	+	v	-	-	+	+
S. caprae	+	+	-	+	+	V	-	+	-	-	+	+
S. cohnii	V	-	-	-	-	v	-	-	-	-	+	v
S. hominis	V	-	-	+	v	-	+	v	-	-	v	+
S. simulans	W	+	+	+	+	+	+	-	-	-	-	-w
S. capitis	+	-	-	V	-	-	+	+	-	-	+	+
S .lugdunensis	V	-	-	-	+	+	+	-	-	-	-	-
S. carnosus	V	-	-	+	V	v	+	v	-	-	+	-
S. chromogens	+	+	+	-	+	+	-	-	-	-	-	-
S. hyicus	-	+	-	V	+	+	+	+	-	-	+	v
S. warneri	+	-	-	+	V	V	+	+	-	-	+	+

Antimicrobial susceptibility test

Antimicrobial susceptibility test of all the isolated CoNS were done by disk diffusion method using the Kirby-Bauer technique (Bauer et al, 1966) and as per recommendation of CLSI, 2007.

Detection of 16s rRNA gene, mecA gene and PVL gene of CoNS by PCR

Detection of 16s rRNA gene and *mecA* gene corresponds well with the evidence of Staphylococcus and methicillin resistance in coagulase-negative Staphylococci (Graham et al. 2000, Snell 1994, Wallet et al. 1996).

Detection of 16s rRNA gene, *mecA* gene and PVL gene

Following methods were applied for the detection of 16s rRNA, *mecA* and PVL gene.

Extraction of DNA

Each strain was subcultured overnight at 37^0C in Nutrient agar. Few colonies from the plate was suspended in 100 µl TNE buffer (a buffer solution containing a mixture of Tris-Hcl, Nacl and EDTA in eppendorf tube and centrifuged at 10,000 rpm for one minute. After centrifugation, supernatant was discarded and 10µl acromopeptidase was added to it and mixed well by pipetting. Then the mixture was incubated at 40-42^0C in waterbath for 10 minutes. The suspensions were cooled at room temperature, 50 µl of 0.5M KOH was added in the tube and mixed well by vortexing. The sample was kept at room temperature for five minutes; 50 µl of 1M-Tris-HCl (pH 6.76) was added and mixed by vortexing. Finally centrifuged at 10,000 rpm for one minute. The supernatant contained the DNA, which was used as template for PCR.

Preparation of PCR Mixture

To prepare a total of 50 µl reaction mixture, 5 µl 10X PCR buffer (Takara, Japan), 4 µl dNTP (dATP, dTTP, dGTP, dCTP), 1 µl Primer mixture, 1 µl DNA Template, 0.5 µl Taq Polymerase (Takara, Japan) and 39.5 µl nuclease free water was taken in a 0.2 ml PCR tube and mixed well by gentle pipetting and take care not to be produced any bubble.

Thermal cycle

A thermal cycler (Eppendorf, Germany) was used to amplify DNA.

Primers

Three sets of primers MecA 1, MecA 2, Luk-PV-1, Luk-PV-2, 16S-1 and 16S-2 were used for the detection of mecA gene, PV-L gene and Staph gene in our study .

Preparation of 1% Agarose gel for electrophoresis

Two grams agarose powder (Tanaka, Japan) was dissolved in 200 ml TAE buffer in a flask and was heated by microwave oven for 1-2 minutes until the mixture became transparent. Then 20 µl of 10 mg/ml ethidium bromide was added and was poured on the gel-casting tray and the comb was placed and allowed to solidify (one hour at room temperature).

Amplified DNA sample preparation and electrophoresis

The comb was gently removed. The gel was transferred to the submarine gel electrophoresis chamber containing sufficient amount of TBE buffer (a buffer solution containing a mixture of Tris base, Boric acid and EDTA) with 30µl ethidium bromide. Loading buffer (as required) placed on a piece of parafilm using adjustable micropipette (0.5 to 20 µl).Then 1µl of 100 bp DNA ladder marker was mixed with loading buffer and was loaded to the first hole of the gel. After that 10 µl of amplified DNA product mixed with loading buffer was loaded in the next hole and so on. The electrophoresis chamber was covered and the electrophoresis apparatus was connected to the power supply. Electrophoresis was carried out at 110v for 35 minutes and transferred the gel to the transilluminator.

Documentation of the DNA amplified product

The gel was taken out carefully from gel chamber and gently placed on the UV transilluminator. The image was viewed on the monitor.

Results

Among the 300 clinical specimens, 240 (80%) showed growth of different isolates in Blood agar, Nutrient agar and Mannitol salt agar. A total of 240 bacterial isolates were isolated from theclinical specimens. Among them 80 (34%) were coagulase-negative Staphylococci (CoNS), 110 (45%) were *Staphylococcus aureus,* 35 (14%) were *Pseudomonas spp* and 15 (7%) were *E. coli* and they were found as single isolate.

Table 1. Distribution of bacterial isolates from different specimens (n=240)

Type of specimens	CoNS (%)	*S. aureus* (%)	*Pseudomonas spp*(%)	*E.coli* (%)
Surgical wound (n=110)	31 (28)	49 (45)	20 (18)	10 (9)
Pus (n=80)	30 (37)	35 (44)	10 (12)	5 (6)
Exudates from burn ulcer (n=30)	9 (30)	16 (53)	5 (17)	0 (0)
Exudates from diabetic ulcer (n=20)	10 (50)	10 (50)	0 (0)	0 (0)
Total (n=240)	80 (34)	110 (45)	35 (14)	15 (7)

A total of 16 species of CoNS were identified by HiStaphTM Kit consisting of 12 conventional biochemical tests. These were S. epidermidis,S. saprophyticus, S. caprae,S. haemolyticus, S. simulans,S. xylosus, S. hyicus, S. hominis, S. warneri,S. auricularis, S. lugdunensis, S. felis, S. capitis, S. chromogenes, S. carnosusand S. gallinarum.

S. epidermidis was found highest in number among the species identified. Of which 6 isolates were from surgical wound, 5 isolates from pus and 2 from burn exudates.

Antimicrobial susceptibility pattern of CoNS species by Disk diffusion method were shown in Figure 1. Predominant resistance were shown to oxacillin 36 (45%), followed by gentamicin 32 (40%), cefuroxime 25 (31%), ceftriaxone 24 (30%) and ciprofloxacin 18 (22%). All isolates of CoNS were sensitive to imipenem and vancomycin.

Table 2. Comparison of detection rate of MRCoNS among disk diffusion method and PCR

Strains	Disk diffusion method (%)	PCR (%)	Total
MRCoNS	36 (45)	45 (56)	81
MSCoNS	44 (55)	35 (44)	79
Total	80	80	160

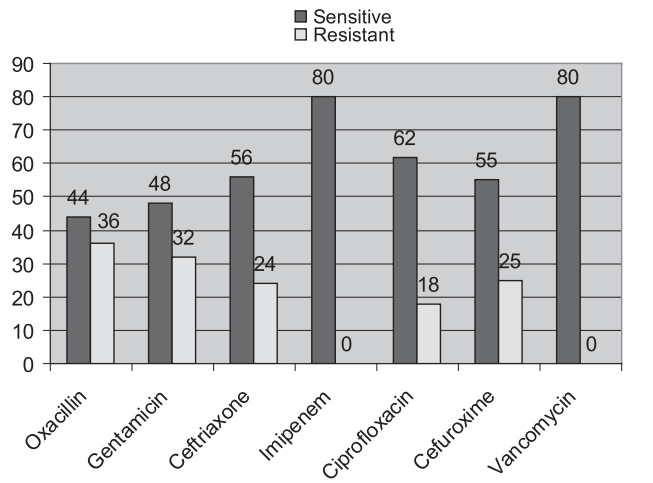

Figure 1. Antimicrobial susceptibility pattern of CoNS isolated from different clinical specimens

There was no significant difference in detection of methicillin resistance by PCR from that of Disk Diffusion Method statistically.

Chi - square value = 2.02, p value = >0.05, degree of freedom (df) = 1

Discussion

Coagulase negative Staphylococci have emerged in recent years as pathogens in a growing number of serious nosocomial infections. Hospitals and communities have been struggling with increasing number of multi drug resistant MRCoNS. Treatment is especially difficult due to biofilm formation and frequent antibiotic resistance. However, virulence mechanisms of these important opportunistic pathogens have remained poorly characterized[12]. Distinguishing clinically significant pathogenic strains from contaminant strains is one of the major challenges facing clinical microbiologists. Because many isolates are multiple antibiotic resistant, their infections are difficult to treat and can even be fatal. A detailed characterization of isolates of coagulase-negative Staphylococci through speciation, genetics and antibiotic susceptibility may be necessary to distinguish infecting from contaminating isolates and to plan suitable therapy[13].

In the present study, 300 samples were collected from different clinical specimens. Out of these, 240 (80%) were culture positive for any bacterial isolates. This result was almost similar to another study done in the same institute, where they found 140 (70%) as culture positive out of 200 samples[14]. Another study from Turkey by Boynukara[15] revealed 52% (120/280), which was much lower than this study. These sorts of variation are not unexpected, because it depends upon some external factors like socioeconomic conditions, hygienic status, environmental factors, level of education and genetic factors.

Among 240 different bacterial isolates, 34% were

CoNS, 45% S. aureus, 14% Pseudomonas spp. and 7% Escherichia coli. This finding was almost similar with Haque[14], where they isolated 23% S. epidermidis, 49% S. aureus, 19% Pseudomonas spp. and 9% Escherichia coli from different clinical specimens.

In this study, we isolated 34% of CoNS (table-2). The result was almost similar to other studies done in Turkey[16] and in Nigeria[17], where they found 54.2%, 31% and 31.4% CoNS from different clinical specimens respectively.

Out of 240 culture positive bacterial isolates, wound swabs (n=110) (Table-1) were predominant in our study that was in agreement with the study from the University of West Indies, Trinidad & Tobago[18]. Higher number of wound specimens found in this study might be due to higher number of wound infection in hospitalized patients. Due to prolonged postoperative hospital staying with exposure to substandard hygienic environment, overcrowding and human microbial flora had been evident in patients developing surgical site infections[19,20].

In the present study, majority (37%) of CoNS were isolated from pus (table-1) that raise an impression that CoNS ranked first among the aetiological agent of infected wound. Similar type of results were found in other studies done in Latvia[21] and in Italy[22], where they denoted isolation rate of CoNS from pus were 30% and 35.5% respectively.

A total of 80 CoNS strains belonging to 16 species were identified in this study. S. epidermidis, S. haemolyticus, S. hominis, S. saprophyticus, S. lugdunensis, S. warneri, S. capitis, S. caprae, and S. simulans were among them. This finding was in agreement with the study from Rio de Janeiro, Brazil[23]. They found 12 species in total of 152 CoNS strains and the species were also similar.

The prevalence of Methicillin Resistant Coagulase Negative Staphylococci (MRCoNS) varies among different countries of the world. In this study we found detection rate of MRCoNS by disk diffusion method among hospitalized patient was 36 (45%) (table-2). Detection of MRCoNS in our study was in accordance with other studies done in Tunisia[24], in Mexico[25] and in India[26,27] with 41.6%, 53.4%, 61% and 62.7% respectively. The detection rate of MRCoNS in our study was almost similar to an international survey performed in Europe, Asia, and Latin America revealed that approximately 60% of the S. epidermidis strains circulating in the hospital environment are resistant to methicillin[28].

In the present study the detection of mecA gene by PCR among CoNS was 56% (table-2). This finding was very much similar to other studies done in Canada[29], in Brazil[30] and in Turkey[31] where they found 54%, 71% (169/238) and 59% (19/32) of total CoNS strains as mecA positive by PCR. No PVL gene was documented in this study by PCR. PVL gene usually found in methicillin sensitive Staphylococcus aureus (MSSA). We have no report yet about the findings of PVL gene in CoNS. The relatively increased rate of MRCoNS isolates in our study might be due to the fact that our specimens were taken from hospitalized patients where antibiotic policy to treat patients is lacking. Inadequate practice of antibiotic policy, indiscriminate use of antibiotics becomes common. In addition, environmental factors and hygienic conditions of the hospital were not adequate. Overcrowding of patients and attendants favour the spread of infectious agents. In such hospitals acquired infection in different wards should be quite high. As a result patients become infected during hospital staying were under antibiotic therapy for prolonged period.

In this study, 45 strains were detected as MRCoNS by PCR and 36 strains were detected by disk diffusion method (table-2). The explanation behind this dissimilar result might be due to some factors such as proper incubation time and temperature, oxygen and carbondioxide concentration, osmolarity, protein concentration and P^H of the

environment in case of disk diffusion method. Moreover, the phenotypic expression of gene responsible for methicillin resistance may not always present.

Antimicrobial resistance pattern of CoNS was done by Kirby Bauer disk diffusion method in this study. Antibiotic resistance pattern of 80 CoNS isolates were as follows: oxacillin (45%), gentamicin (40%), ceftriaxone (30%), ciprofloxacin (22%), cefuroxime (31%), imipenem (0%) and vancomycin (0%) (Figure-1). Similar pattern of antibiotic resistance was reported by a study done in India in which they found 52% of the isolates as methicillin resistant followed by ciprofloxacin (36%) and gentamicin (34%). All the isolates were susceptible to vancomycin and imipenem[32].

Phenotypic and genotypic methods were used to determine the relatedness of these methods for the strains of CoNS recovered from different clinical specimens. S. epidermidis was the most frequently isolated strains as they are to be of epidemiological value[33]. The transfer of CoNS strains and the significant number of isolates expressing phenotypic and genotypic characteristics shown to promote prolonged hospital staying or life threatening infections suggests the development of endemic virulent strains[34].

Conclusion

This study suggests that finding of CoNS as a single isolate is important and not to be neglected as non pathogenic as these may threaten life. So if we keep all these things in mind, it will help us for better control of infection. If we could take more clinical specimens in this study it could have been better reflection of the scenerio.

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