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Biofilm Production and Antibiotic Sensitivity Pattern of Methicillin Resistant Coagulase Negative Staphylococcus (MR- CoNS) Isolates from Various Clinical Samples from a Tertiary Care Centre

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ABSTRACT
CoNS are typically considered as contaminants in clinical samples but have also been linked to clinically significant illnesses such as urinary tract infections, endocarditis, bloodstream infections (including neonatal sepsis) infections due to foreign bodies. This hospital study evaluated the prevalence of various species, capacity of CoNS to form biofilm their pattern of antibiotic sensitivity. Biofilm production was detected using the Congo red agar (CRA) method the Microtiter plate (MTP) method. 56% of the tested isolates developed biofilm. The isolates discovered are Staphylococcus haemolyticus (34.83%), Staphylococcus epidermis (31.93%), Staphylococcus capitis (16.77%), Staphylococcus cohnii (10.96%), Staphylococcus hominis (5.48%). The CoNS isolates exhibited resistance to cefoxitin (100%), erythromycin (94.8%), ciprofloxacin (66.7%), sulfamethoxazole/trimethoprim (66.7%), gentamicin (66.12%), clindamycin (62.9%) according to their antimicrobial susceptibility profile. The resistance rate to mupirocin was 48.5% in S. epidermidis 38.9% in S. haemolyticus isolates. All isolates showed susceptibility to vancomycin linezolid. The study's results are essential for creating a plan to control biofilm development as an alternative method to address the spread of multidrug-resistant CoNS in healthcare settings.

INTRODUCTION

Coagulase-negative staphylococci (CoNS) are the primary skin mucous membrane colonizers, they are often isolated in microbiology laboratories. While CoNS are commonly seen as contaminants in clinical samples, they have been connected to serious disorders such as urinary tract infections, endocarditis, bloodstream infections (including infant sepsis), infections caused by foreign bodies[1].

Staphylococcus epidermidis is the most common source of infection in CoNS species, followed by Staphylococcus haemolyticus, the second most frequently found strain in clinical samples[2].Additional species, including Staphylococcus saprophyticus, Staphylococcus hominis, Staphylococcus lugdunensis, Staphylococcus warneri, Staphylococcus capitis, have been associated to several systemic diseases[3]. Diekema *et al*. found that global methicillin resistance rates for CoNS were between 75% 90%[4]. Pal Ayyagiri found a 15% frequency of MR-CoNS in hospital settings in India. Sharma *et al*[5]. Vysakh *et al* [6]. thereafter recorded several outbreaks of MR-CoNS in hospitals across India. Methicillin-resistant coagulase-negative staphylococci (MR-CoNS) are resistant to a variety of antibiotic classes, including macrolides, lincosamides, tetracyclines, aminoglycosides, chloramphenicol, fluoroquinolones, sulphonamides, mupirocin, fusidic acid. CoNS resistance to recently developed antimicrobial medicines such as streptogramins, tigecycline, linezolid has been described[7,8,9]. In India, there have been few cases of linezolid resistance in CoNS species, as recorded by Gupta *et al*[10]. Rajan et al[11].

S.epidermidis biofilm production begins with bacterial cell attachment to surfaces via autolysin (atlE gene), followed by accumulation via polysaccharide intercellular adhesin (PIA) from the icaADBC locus proteinaceous components such as accumulation-associated protein (aap gene)[12]. The current study aims to determine the species distribution, antimicrobial susceptibility profile, biofilm generation of isolated CoNS from clinical samples of patients at a tertiary care center.

MATERIALS AND METHODS

The present study is performed at Department of Microbiology, Index Medical College Hospital and Research Centre Indore. A total of 554 MR CoNS bacterial isolates from different types of clinical samples, including pus (n = 148), wound swab (n = 111), blood (n = 87), urine (n = 47), catheters (n = 91), sputum (n=70), were preliminarily characterized as CoNS by colony morphology, Gram's staining, tube coagulase test other stard biochemical reactions. Antibiotic sensitivity test is performed on the Muller Hinton agar plate (Hi Media Mumbai) by disc diffusion method. Various antibiotics used are given in (Table 1). Cefoxitin disc is used to differentiate the methicillin resistant (MR) strains among the isolates of CoNS. Biofilm detection among the isolates were done by Congo red tissue culture plate method were analysed.

Microtiter Plate(MTP): This quantitative test described by Christensen *et al*[13]. is considered as the gold-stard method for biofilm detection[14]. Organisms isolated from fresh agar plates were inoculated in10 mL of trypticase soy broth with 1% glucose. Broths were incubated at 37 for 24 h. The cultures were then diluted in 1:100 with fresh medium. Individual wells of sterile 96 well flat bottom polystyrene tissue culture treated plates (Sigma-Aldrich, Costar, USA) were filled with 200 µL of the diluted cultures. The control organisms were also incubated, diluted added to tissue culture plate. Negative control wells contained inoculated sterile broth. The plates were incubated at 37 for 24 h. After incubation, contents of each well were removed by gentle tapping. The wells were washed with 0.2 mL of phosphate buffer saline (pH 7.2) four times. This removed free floating bacteria. Biofilm formed by bacteria adherent to the wells were fixed by 2% sodium acetate stained by crystal violet (0.1%). Excess stain was removed by using deionized water plates were kept for drying. Optical density (OD) of stained adherent biofilm was obtained by using micro-ELISA auto reader (model 680, Bio-Rad, UK) at wavelength 570 nm. The experiment was performed in triplicate repeated three times. The interpretation of biofilm production was done according to the criteria of Stepanovic *et al*[15].

Congo Red Agar (CRA): This is a simple qualitative method to detect biofilm production by using Congo Red Agar (CRA) medium[16]. CRA medium was prepared with brain heart infusion broth (Oxoid, UK) 37 g/L, sucrose 50 g/L, agar No. 1 (Oxoid, UK) 10 g/L Congo Red indicator (Oxoid, UK) 8 g/L. First Congo red stain was prepared as a concentrated aqueous solution autoclaved (121 C for 15 mins)separately from the other medium constituents. Then it was added to the autoclaved brain heart infusion agar with sucrose at 55 C. CRA plates were inoculated with test organisms incubated at 37 for 24

Table 1: List of antibiotics and the disc potency used for the antibiogram profiling of the CoNS.

Antibiotic	Disc potency
Penicillin (P)	10 units
Chloramphenicol (C)	30 µg
Clindamycin (DA)	2 µg
Erythromycin (E)	15 µg
Gentamicin (CN)	10 µg
Sulfamethoxazole/trimethoprim (SXT)	1.25/23.75 µg
Rifampicin (RD)	5 µg
Cefoxitin (FOX)	30 µg
Mupirocin	5 µg
Ciprofloxacin (CIP)	5 µg
Vancomycin (VA)	30 µg
Linezolid (LZ)	30 µg

Table 2: Frequency in the distribution of bacterial pathogens characterized from the clinical samples under study

Organism	Pus and body fluid	Swab	Blood	Urine	Catheter tip	Sputum	Total
CoNS	148 (26.71%)	111 (20.03%)	87 (15.70%)	47 (8.48%)	91 (16.42%)	70 (12.63%)	554

Table 3: Susceptibility pattern of Methicillin Resistant Coagulase Negative Staphylococci (MR- CoNS) by species.

Antibiotic	S. haemolyticus (n = 108)	S. epidermidis (n = 99)	S. capitis (n = 52)	S. cohnii (n = 34)	S. hominis (n = 17)
Vancomycin	108	99	52	34	17
Linezolide	108	99	52	34	17
Erythromycin	69	54	43	21	9
Clindamycin	2	1	7	3	2
Ciprofloxacin	5	1	51	29	17
Co- trimaxazole	3	0	49	34	17
Chloramphenicol	17	1	10	8	4
Rifampicin	77	68	52	34	17
Gentamicin	15	5	47	27	11
Mupirocin	42	48	52	34	17
Fusidic acid	63	79	52	34	17

Table 4: Incidence of biofilm producing strains among various clinical isolates of CoNS

Specimen	Pus and BF	Swab	Blood	Urine	Catheter	Sputum	Total
No of isolates producing biofilms	103	71	18	24	68	26	310
No of isolates not producing biofilms	45	40	69	23	23	44	244
Total no of isolates	148	111	87	47	91	70	554

Table 5: Different species of methicillin-resistant coagulase-negative Staphylococci producing biofilm by CRA and MTP methods

	Clinical isolates					
Biofilm formation	S. haemolyticus (n=108) (34.83%)	S. epidermidis (n=99) (31.93%)	S. capitis (n=52) (16.77%)	S. cohnii (n=34) (10.96%)	S. hominis (n=17) (5.48%)	Total Biofilm formers (n=310) (57.09%)
CRA						
Red (%)	0	11 (11.1%)	11 (21.15%)	20 (58.82%)	0	37 (11.93%)
Black (%)	68 (62.96%)	8 (43.56%)	20 (38.46%)	7 (20.58%)	11 (64.7%)	156 (47.74%)
Very black (%)	40(37.1%)	80 (80.80%)	21 (40.38%)	7 (20.58%)	6 (35.29%)	117 (37.7%)
MTP						
Highly positive (%)	101 (93.51%)	49 (49.49%)	25 (48.07%)	27 (79.41%)	11 (64.7%)	193 (62.27%)
Low-grade positive (%)	7 (6.48%)	36 (36.36%)	27 (51.92%)	7 (20.58%)	6 (35.29%)	106 (34.19%)
negative (%)	0	11 (11.11%)	0	0	0	11 (3.54%)

hours aerobically. Black colonies with a dry crystalline consistency indicated biofilm production[17]. The experiment was performed in triplicate the mean value was recorded.

RESULTS AND DISCUSSIONS

The isolation rate of CoNS from various clinical samples were described in the (Table 2).

Antibiotic susceptibility pattern of CoNs Isolates: All clinical isolates (n = 554) were tested for the susceptibility against 12 antibiotics. The isolates were

resistant to cefoxitin (n=310; 55.95%), oxacillin (n=303, 54.69%), penicillin (n = 300, 54.15%), erythromycin (n = 114, 36.77%), ciprofloxacin (n = 207, 37.36%), sulfamethoxazole/trimethoprim (n=207; 37.36%), chloramphenicol (n = 40, 7.22%), rifampicin (n=62, 11.19%), gentamicin (n=205; 36.46%), fusidic acid (n = 65, 11.73%), clindamycin (n=152; 49.03%), mupirocin (n=117; 21.11%), but, all isolates were sensitive to linezolid vancomycin (Table 3).

Detection of methicillin susceptibility: 554 isolated clinical isolates were tested for methicillin susceptibility with cefoxitin disc (30 µg) by Kirby Bauer's disc diffusion method. Among 554 isolates 224 (40.43%) isolates were susceptible to cefoxitin 310 (55.95%) isolates were resistant to cefoxitin. (MR-CoNS).

Species distribution in biofilm-producing MR-CoNS: Among 554 MR- CoNS clinical samples processed; 310 isolates were identified as biofilm producing bacteria (n = 310) (55.95%). Species level characterization was performed by biochemical tests. The 5 various species identified were S.haemolyticus (n = 108, 34.83%), S.epidermidis (n = 99, 31.93%), S.capitis (n = 52, 16.77%), S.cohnii (n = 34, 10.96%) S. hominis (n = 17, 5.48%).

Detection of biofilm production: Isolates resistant to cefoxitin (n = 310) were tested for biofilm production, detected through Congo red agar-based screening (CRA) MTP methods, as shown in (Table 4 and 5.). Biofilm production was detected in 310 (55.95%) isolates by CRA 299 (53.97%) isolates were detected by MTP assay. Using CRA method, it was observed that 37 isolates out of 310 (11.93%) of MR- CoNS isolates formed red colonies, 156 isolates (50.32%) formed black colonies, 117 isolates (37.74%) formed intensely black colonies. Slime-positive isolates produced the reddish-black colonies with rough, dry, crystalline consistency on CRA, the negative slime strains produced pinkish-red, smooth colonies. MTP assay demonstrated that 299 (53.97%) out of the 554 CoNS isolates were biofilm producers, out of which 106 (34.19%) isolates showed low-grade positivity, 193 (62.27%) isolates showed high-grade positivity. In our current study, 310 (55.95%) clinical isolates of CoNS

were MR-CoNS belonging to 5 species, with Staphylococcus haemolyticus (n = 108, 34.83%) being the predominant, followed by Staphylococcus epidermidis (n = 99, 31.93%), Staphylococcus capitis (n = 52, 16.77%), Staphylococcus cohnii (n = 34, 10.96%), Staphylococcus hominis (n = 17, 5.48%) isolates. Previous studies have documented the distribution of MR-CoNS, with Staphylococcus haemolyticus being the most prevalent species (34.5%), followed by S. epidermidis (21.8%), S. saprophyticus (11.7%), S. capitis (11.7%), S.cohnii (n = 34, 10.96%), S. hominis (11.3%), respectively. Furthermore, antibiotic resistance is a major human health issue, our study found the highest antibiotic resistance, particularly to cefoxitin (100%), followed by penicillin (96.77%) erythromycin (63.22%), which is consistent with many studies of Becker Shresta *et al*[18,19].All 310 MR-CoNS isolates were sensitive to vancomycin linezolid, the findings were consistent with Shrestha *et al*.[20]

This examination included a methicillin resistance analysis utilizing Kirby Bauer's disc diffusion method, with the results indicating that 55.95 percent of the isolates were methicillin resistant. This finding is consistent with previous studies by Ferreira *et al*. Hussain *et al*. Secchi *et al*.[21,22,23] Hira *et al*. reported a higher rate of MR-CoNS (87%)[24]. Less research, however, has confirmed a modest frequency of methicillin resistant positives as well[25-27]. The fact that the majority of the samples used in our study came from intensive care units that nearly all of the patients admitted to our hospitals were treated in primary care hospitals after being referred to our centre may explain the high prevalence of methicillin-resistant positive isolates in our study.

Biofilm formation is a major pathogenic mechanism in MR-CoNS. Our investigation indicated that 55.95% (n = 310) of MR-CoNS isolates produced biofilm. These findings were consistent with other studies, which found that 75% of MR-CoNS clinical isolates produced biofilm[28]. The MTP approach assessed biofilm generation as very positive in 53.97% of the isolates low-grade positive in 34.19% of the isolates in MR-CoNS. Another investigation found that 26.3% of isolates were extremely positive, whereas 66.1% were low-grade positive strains. This study also documented variations in the biofilm-producing ability of MR-CoNS found across different species. S.epidermidis has a stronger biofilm-producing ability than S.haemolyticus. Our findings indicate that the CRA method outperforms the MTP method for biofilm detection. The emergence of drug resistance against CONS strains is a source of severe worry, necessitating continuous monitoring of antimicrobial susceptibility against CoNS in health care settings.

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