Incidence of Coagulase Negative Staphylococci in Various Clinical Samples in Tertiary Care Hospital, Nepal

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ABSTRACT

Objectives: To determine incidence of coagulase negative *Staphylococci* (CoNS) in various clinical samples along with the study of their multi drug resistance, methicillin resistance and biofilm formation.

Methods: A hospital based cross-sectional study was conducted which included 1875 clinical samples. The isolation and identification of isolates was done following standard microbiological protocol. The CoNS were identified phenotypically on the basis of gram staining, slide and tube coagulase test and through various carbohydrates fermentation tests. Antibiotic susceptibility test was done by Kirby Bauer disk diffusion method (Clinical and Laboratory Standards Institute 2020) whereas biofilm production was determined by Tissue Culture Plate (TCP) technique.

Results: A total of 32 CoNS, comprising of 6 species were identified. *S. epidermidis* (43.8%) was the most common species isolated followed by *S. saprophyticus* (28.1%), *S. haemolyticus* (15.6%), *S. hominis* (6.3%), *S. lugdunensis* (3.1%) and *S. cohini* (3.1%). Further, 27 (84.4%) of CoNS were found to be multidrug resistant, 22 (68.8%) methicillin resistant and 8 (25%) showed positive D- test. Strong biofilm production was detected in 9 (28.1%) isolates of CoNS, 10 (31.3%) were moderate biofilm producers and 13 (40.6%) non/weak biofilm producers. The equal distribution, 9 (33.3%) each of strong, moderate and non/weak biofilm producers were found among 27 isolates of MDR. Among 22 methicillin resistant isolates, 9 (40.9%), 7 (31.8%) and 6 (27.3%) were strong, moderate and non/weak biofilm producers respectively. All isolates were sensitive against Linezolid followed by Cotrimoxazole.

Conclusion: The increasing multi drug resistance among CoNS should be rationally approached with the use of proper antibiotics while treating the patients.

Key Words: CoNS, antibiotic susceptibility, multidrug resistance, induced clindamycin resistance, biofilm

INTRODUCTION

Coagulase Negative *Staphylococci* (CoNS) are ubiquitous colonizer which were previously dismissed as contaminants of clinical samples (Malik and Ravishekhar 2012), however, with time CoNS have emerged as crucial potential pathogens associated in number of severely debilitated patients and increased use of implants in hospitals (Javadpour and Karmostaji 2010) most notably prosthetic valve endocarditis and prosthetic joint infections, because of their propensity to form a protective biofilm (Ziebuhr et al. 2006).

S. aureus is typically known to be more virulent than CoNS and are present as indolent form rather than acute forms. However, CoNS are reported both in community and hospital acquired infections. CoNS have been addressed as the causative agents in urinary tract disease, catheter related infections, shunt infections, pneumonia, endophthalmitis (Wu

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et al. 2006), surgical wound infections, osteomyelitis, and native valve endocarditis (Chu et al. 2008). The frequently isolated CoNS include *S. epidermidis, S. haemolyticus, S. hominis*, and *S. saprophyticus* from human samples resulting in diseased condition. Other species such *as S. warneri, S. lugdunensis, S. capitis, S. simulans, S. cohnii, S. saccharolyticus*, and *S. xylosus* are relatively associated with opportunistic infections (Bouchami and Ben Hassen 2011).

It has been reported that *S. aureus* is more susceptible to antimicrobial agents such as lactam antibiotics, whereas CoNS is less susceptible (Becker et al. 2020). Though the specific virulence factors have not been clearly defined in CoNS, factors such as bacterial polysaccharide, and their ability to form biofilm for attachment and persistence on foreign materials seems to be the essential reasons for virulence (Oliveira and Cerca 2013, Singh 2015). Other factors include adhesion molecules, exoenzymes, antibiotics, modulins, delta toxins (Rupp and Fey 2014, Michal et al 2020). Formation of biofilms result in persistent infections which cannot be cured easily with standard antibiotic treatments (Hasanvand et al. 2019), due to slow diffusion of conventional antibiotics through the extracellular polymeric substance (Sheikh 2019) and often leads to removal of the foreign body for cure (Hasanvand et al. 2019). Studies have supported that CoNS have precise features and an antimicrobial susceptibility pattern (Kürekci 2016). Thus, the biofilm formation ability and the resistance to antimicrobial therapy can be intimately related (Oliveira and Cerca 2013).

Therapeutic options for the treatment of CoNS are limited because the vast majority of clinically recovered isolates are methicillin resistant (Becker et al. 2014). Even the macrolide antibiotic resistance in CoNS has been studied which may be due to an active efflux mechanism or may be due to ribosomal target modification, affecting macrolides, lincosamides, and type B streptogramins (MLSB resistance) (Becker et al. 2020). Therefore, treatment of CoNS comes with major challenges to physicians as multi drug resistance and methicillin resistant can result in untreatable conditions as well as take longer time to recover. Even economic burden can't be ignored (Soumya 2017).

Hence, the present study was done to demonstrate the ability of CoNS to produce biofilm, along with their antimicrobial susceptibility pattern from various clinical samples and identification of MDR, methicillin resistant CoNS to help in preventive and therapeutic management of staphylococci infection and developing new strategies in their treatment.

MATERIALS AND METHODS

A cross-sectional study was carried out in Microbiology laboratory of Nepal Armed Police Force Hospital, Balambu, Kathmandu, Nepal from February 2022 to October 2022. A total of 1875 clinical samples (pus/ wound swab, blood, urine, semen and body fluids/ tips) were included, collected in sterile container and having proper requisition form filled for routine culture. The samples received were subjected to gram staining and culture.

Isolation and identification:

Urine samplewas inoculated on cysteine lactose and electrolyte deficient (CLED) agar media and incubated at 37°C for 24 hours of aerobic incubation. Whereas, pus/wound swab, semen and body fluids were inoculated on MacConkey agar (MA) and Blood agar (BA) media and incubated at 37°C for 48 hours aerobically. Blood sample was poured in brain heart infusion (BHI) broth in 1:10 ratio and sub cultured after 24 hours of enrichment at 37°C aerobically on MA and BA media for consecutive 7 days.

For central venous catheter and catheter tips, the tips were collected in sterile container and then mixed with 2 ml of nutrient broth (NB). After mixing by vertexing, loop-full of the suspension was streaked on MA and BA media and incubated at 37°C for 48 hours of aerobic incubation. Isolates which grew white opaque colonies, Gram-positive cocci in clusters on Gram staining, produced catalase, were slide and tube coagulase negative, and did not ferment mannitol were identified as CoNS. The various species of CoNS were identified phenotypically through various biochemical tests and various carbohydrate fermentation tests (Cunha et al. 2004, Kloos and Schleifer 1975).

Antibiotic susceptibility test: The antibiotic susceptibility testing was performed by Kirby Bauer disc diffusion method following the Clinical and Laboratory Standards Institute (CLSI) 2020 guidelines against Gentamicin (10µg), Azithromycin (30 µg), Ciprofloxacin (5 µg), Levofloxacin (5 µg), Norfloxacin (10 µg), Clindamycin (2 µg), Cotrimoxazole (25 µg), Chloramphenicol (30 µg), Ampicillin (10 µg), Linezolid (30 µg) and Ceftriaxone (30 µg).

MDR analysis: Resistance to at least one antimicrobial agent in three or more classes of antibiotics was considered as multi drug resistant isolates (Magiorakos et al. 2012).

Detection of methicillin resistant CoNS: Methicillin resistant CoNS were detected on the basis of an inhibition zone diameter shown by cefoxitin disc (30 µg) on MHA plate. An inhibition zone diameter of ≤ 24 mm for CoNS was considered as cefoxitin resistant and reported as MRCoNS whereas ≥ 25 mm were methicillin sensitive coagulase negative *Staphylococci* (MSCoNS).

Induced clindamycin resistance: Screening of inducible clindamycin resistance was made by double disc diffusion test or D zone test outlined in CLSI guideline 2020. Erythromycin (15 µg) disc was placed at a distance of 15 mm to 22 mm (edge to edge) from clindamycin (2 µg) on MHA plates previously inoculated with 0.5 McFarland bacterial suspension. Plates were analyzed after 24 hours of incubation at 37 °C. Interpretation of the inhibition zone diameters was made as: If an isolate was erythromycin resistant and clindamycin susceptible, with a D-shaped inhibition zone around the clindamycin disc, it was considered positive for inducible resistance (D-test positive, iMLSB phenotype). If the isolate was erythromycin resistant and clindamycin susceptible, with both zones of inhibition showing a circular shape, the isolate was considered to be negative for inducible resistance (D test negative, MS phenotype). If the isolate was resistant to both drugs, it was considered to have the macrolide–lincosamide– Streptogramin B constitutive (cMLSB phenotype) (Steward et al. 2005).

Screening of biofilm production: Tissue Culture Plate (TCP) method was used to screen biofilm producers. At first, isolates were inoculated in 10 ml of trypticase soy broth with 1% glucose and incubated at 37°C for 24 hours.The cultures were then diluted 1:100 with fresh medium and individual wells of sterile TCPs were filled with 200 µl of the diluted culture including negative controls (sterile media) and positive control. Plates were incubated at 37°C for 24 hours, after which contents of each well were removed by gentle tapping. The wells were washed with 0.2 ml of phosphate buffered saline (pH 7.2) for four times followed by fixing of wells by 200 µl of 2% sodium acetate for 10 minutes and discarded. 200 µl of 0.1% crystal violet was filled in each well to stain the biofilm formed for 30 minutes. Excess stain was removed by using deionized water and plates were dried. Optical density of stained adherent biofilms was read by micro-ELISA auto reader (model 680, Biorad, UK) at a wavelength of 570 nm (Christensen et al. 1985). The interpretation of biofilm production was done according to the criteria of Stepanovic et al. The test was performed in triplicate for each test organism in a microtitre plate and tests were repeated for 3 times.

Average OD value	Biofilm Production
OD ≤ ODc / ODc< OD ≤ 2*ODc	Weak/ non- biofilm production
2*ODc< OD ≤ 4*ODc	Moderate biofilm production
4*ODc< OD	Strong biofilm production

Optical density cut-off value (ODc) = Average OD of negative control + 3* standard deviation (SD) of negative control.

Data analysis: The data gathered from the log entry and laboratory analysis were managed in Microsoft Excel for Windows 10.

Ethical approval: Ethical approval was received from Nepal Health Research Council (Ref. No. 727) and consent was obtained from patients.

RESULTS

Out of 1875 clinical samples, 415 (22.1%) showed bacterial growth among which 166 (40%) were *Staphylococci* spp while remaining 249 (60%) were gram negative rods.

Among 166 *Staphylococci* spp, 134 (80.7%) isolates were found to be *S. aureus* and remaining 32 (19.3%) were CoNS. Among CoNS, the highest isolation was from urine sample 12 (37.5%).

Identification of CoNS: The identification of species of CoNS was made phenotypically based on biochemical and various carbohydrate fermentation tests. Six species of CoNS, *S. epidermidis* (14, 43.8%), *S. saprophyticus* (9, 28.1%), S. *haemolyticus* (5, 15.6%), *S. hominis* (2, 6.3%), *S. cohini* and *S. lugdunensis* (1, 3.1%) each were identified.

S. epidermidis was identified mostly from Pus/Wound swab 5 (35.7%) whereas *S. saprophyticus* from urine sample 8 (88.9%). (Table 1)

Table 1: Distribution of CoNS species among clinical samples

Species	Pus/Wound Swab	Urine	Blood	Body fluids/tips	Semen	Total
S. *epidermidis*	5(35.7)	4(28.6)	3(21.4)	1(7.1)	1(7.1)	14(43.8)
S. *saprophyticus*	0	8(88.9)	0	0	1(11.1)	9(28.1)
S. *haemolyticus*	3(60)	0	2(40)	0	0	5(15.6)
S. *hominis*	2(100)	0	0	0	0	2(6.3)
S. *lugdunensis*	0	0	0	1(100)	0	1(3.1)
S. *cohini*	0	0	1(100)	0	0	1(3.1)
Total	**10(31.3)**	**12(37.5)**	**6(18.8)**	**2(6.3)**	**2(6.3)**	**32(100)**

Antibiotic susceptibility test of species of CoNS

Among 32 isolates of CoNS, 32 (100%) were sensitive to Linezolid whereas 32 (100%) showed resistance against Ampicillin. (Table 2)

Table 2: Antibiotic resistance pattern of individual species of CoNS [N(%)]

Antibiotics	*S. epidermidis* N = 14	*S. saprophyticus* N = 9	*S. haemolyticus* N = 5	*S. hominis* N = 2	*S. lugdunensis* N = 1	*S. cohini* N = 1
Gentamicin	2(14.3)	2(22.2)	1(20)	2(100)	1(100)	1(100)
Azithromycin	11(78.6)	8(88.9)	5(100)	2(100)	1(100)	1(100)
Ciprofloxacin	4(28.6)	1(11.1)	1(20)	2(100)	1(100)	1(100)
Levofloxacin	4(28.6)	1(11.1)	4(80)	1(50)	1(100)	0
Norfloxacin	8(57.1)	3(33.3)	0	2(100)	1(100)	1(100)
Clindamycin	4(28.6)	5(55.6)	4(80)	1(50)	1(100)	0
Cotrimoxazole	7(50)	2(22.2)	0	0	0	1(100)
Chloramphenicol	4(28.6)	5(55.6)	3(60)	0	1(100)	1(100)
Ampicillin	14(100)	9(100)	5(100)	2(100)	1(100)	1(100)
Linezolid	0	0	0	0	0	0
Ceftriaxone	4(28.6)	2(22.2)	1(20)	1(50)	1(100)	0

Prevalence of multidrug resistant and methicillin resistant CoNS

Out of 32 species of CoNS, 27 (84.4%) were identified as multidrug resistant (MDR), and 22 (68.8%) were methicillin resistant CoNS (MRCoNS). (Table 3)

Table 3: Identification of MDR and MRCoNS among species of CoNS

Isolates	MDR (%)	Non-MDR (%)	Methicillin Resistant (%)	Methicillin Sensitive (%)
S. *epidermidis*	11(40.7)	3 (60)	9(40.9)	5(50)
S. *saprophyticus*	7(25.9)	2 (40)	6(27.3)	3(30)
S. *haemolyticus*	5(18.5)	0	3(13.6)	2(20)
S. *hominis*	2(7.4)	0	2(9.1)	0
S. *lugdunensis*	1(3.7)	0	1(4.5)	0
S. *cohnii*	1(3.7)	0	1(4.5)	0
Total	27 (84.4)	5 (15.6)	22 (68.8)	10 (31.2)

Induced clindamycin resistance

Among 32 species of CoNS, 8 (25%) were positive D-test (inducible MLSB), 15 (46.9%) constitutive MLSB, 5 (15.6%) MSB and 4 (12.5%) were susceptible. (Table 4)

Table 4: Inducible clindamycin resistance among species of CoNS

Phenotypes	D-Test	*S.epidermidis* (N= 14)	*S.saprophyticus* (N= 9)	*S.haemolyticus* (N= 5)	*S.hominis* (N= 2)	*S.lugdunensis* (N= 1)	*S.cohnii* (N= 1)	Total
Inducible MLSB	+	3(37.5)	2(25)	1(12.5)	1(12.5)	0	1(12.5)	**8 (25)**
Constitutive MLSB	_	4(26.7)	5(33.3)	4(26.7)	1(6.7)	1(6.7)	0	**15(46.9)**
MSB	_	4(80)	1(20)	0	0	0	0	**5(15.6)**
Susceptible	_	3(75)	1(25)	0	0	0	0	**4(12.5)**

Biofilm production among species of CoNS

Altogether 9 (28.1%) species of CoNS were strong biofilm producers, 10 (31.3%) moderate biofilm producers and 13 (40.6%) were non/weak biofilm producers.

The maximum number of strong biofilm producers were *S. saprophyticus* 4 (44.4%), whereas maximum moderate and non/weak biofilm producers were *S. epidermidis* 4 (40%) and 8 (61.5%) respectively. (Table 5)

Table 5: Biofilm production by various species of CoNS

Biofilm Formation	*S. epidermidis*	*S. saprophyticus*	*S. haemolyticus*	*S. hominis*	*S. lugdunensis*	*S. cohnii*	Total
Strong	2 (22.2)	4 (44.4)	2 (22.2)	0	1 (11.1)	0	**9(28.1)**
Moderate	4 (40)	1 (10)	2 (20)	2 (20)	0	1 (10)	**10(31.3)**
Non/Weak	8 (61.5)	4 (30.8)	1 (7.7)	0	0	0	**13(40.6)**

Biofilm production among multidrug and methicillin resistant isolates of CoNS

Out of 27 MDR isolates of CoNS, 9 (33.3%) each were strong, moderate and non/weak biofilm producers. (Table 6)

Table 6: Biofilm production among MDR isolates of species of CoNS

Biofilm Formation	*S. epidermidis* N= 11	*S. saprophyticus* N= 7	*S. haemolyticus* N= 5	*S. hominis* N= 2	*S. lugdunensis* N= 1	*S. cohnii* N= 1	Total N= 27
Strong	2(22.2)	4(44.4)	2(22.2)	0	1(11.1)	0	**9(33.3)**
Moderate	3(33.3)	1(11.1)	2(22.2)	2(22.2)	0	1(11.1)	**9(33.3)**
Non/Weak	6(33.3)	2(22.2)	1(11.1)	0	0	0	**9(33.3)**

Likewise, among 22 methicillin resistant isolates of CoNS 9 (40.9%) were strong biofilm producers, 7 (31.8%) moderate and 6 (27.3%) were non/weak biofilm producers. (Table 7)

Table 7: Biofilm production among methicillin resistant isolates of species of CoNS

Biofilm Formation	*S. epidermidis* N= 9	*S. saprophyticus* N= 6	*S. haemolyticus* N= 3	*S. hominis* N= 2	*S. lugdunensis* N= 1	*S. cohnii* N= 1	Total N= 22
Strong	2(22.2)	4(44.4)	2(22.2)	0	1(11.1)	0	**9(40.9)**
Moderate	3(42.9)	0	1(14.3)	2(28.6)	0	1(14.3)	**7(31.8)**
Non/Weak	4(66.7)	2(33.3)	0	0	0	0	**6(27.3)**

DISCUSSION

This study was carried out to study the incidence pattern of commonly isolated CoNS in clinical samples along with their antimicrobial susceptibility testing and analysis of biofilm formation. Since, CoNS are the constituents of normal flora of skin and mucous membrane, it becomes important to identify between clinically significant and contaminant CoNS in etiology of suspected infections. (Asante et al. 2020)

In this study, 1875 clinical samples were included, among which 415 (22.1%) were culture positive. Out of total culture positive, 166 (40%) *Staphylococci* spps were identified among which 134 (80.7%) were *S. aureus* and remaining 32 (19.3%) were CoNS isolates. The higher prevalence of *S. aureus* has been reported previously in Nepal in the studies conducted by Kumari et al. 2008, Upreti et al. 2018 and Pandey et al. 2020. In this study, the CoNS were isolated from various clinical samples such as pus/wound swab, urine, blood, body fluids/tips and semen. This suggests the ability of CoNS to cause nosocomial and community-acquired infections, which includes skin and tissue infections, pneumonia, endocarditis, and septicemia (Becker et al. 2014).

Here, 6 species of CoNS were identified consisting *S. epidermidis* (14, 43.8%), *S. saprophyticus* (9, 28.1%), *S. haemolyticus* (5, 15.6%), *S. hominis* (2, 6.3%), *S. cohnii* and *S. lugdunensis* (1, 3.1%) each. *S. epidermidis* was the common highest isolates even in the studies of Shrestha et al. 2017 and Manandhar et al. 2018. The *S. epidermidis* in our study was isolated most from pus/wound swab (35.7%), suggesting its higher dominance in skin and mucosa in human body, as well as its capacity to cause

maximum human diseases among CoNS (Becker 2014 and Otto 2009). Shrestha et al. 2017 reported higher isolation and identification of *S. saprophyticus* whereas the percentage of *S. haemolyticus* was almost similar. *S. saphrophyticus* was dominantly isolated from urine relating to its ability to adhere to the urinary tract and its higher incidence among UTI patients (Rupp and Fey 2010).

In the clinical setting, the effectiveness of conventional antibiotics is decreasing due to global emergence of MDR bacterial pathogens (Mandal et al. 2014). MDR infections are related to the frequent exacerbations of the disease and worse treatment outcomes (Michalik et al. 2020). In our study 32 (100%) CoNS isolates were sensitive against Linezolid, whereas all isolates were resistant towards Ampicillin. Similar findings were concluded in the studies of Tayyar et al. 2015, Shrestha et al. 2017 and Bathala et al. 2021. CoNS are noted for their ability to develop antibiotic resistance against commonly used antibiotic classes such as β-lactams, aminoglycosides, and macrolides, with exceptionally high reported methicillin resistance rates (Asante et al. 2020) as well as resistance to antibiotics of last resort such as the glycopeptides (May et al. 2013) which could be due to the extensive exploitation of therapeutic agents (Deurenberg and Stobberingh 2008).

MDR in CoNS is problematic in countries with low or medium income due to the high cost of alternative treatment and the limited access to effective antibiotics (Asante et al. 2020).The prevalence of MDR, MRCoNS and induced clindamycin resistance in this study were 84.4 %, 68.8% and 25% respectively. In a study conducted by Singh et al. 2016, they reported 49.2% of CoNS isolates were MDR, which is around a half percentage lesser than ours and the prevalence of MRCoNS ranges from 48.2% to 60% in India which is in consistent to our findings. Likewise, as per Sader et al. 2007 and Koksal et al. 2009, the prevalence of MRCoNS in different countries were UK (53.3%), Switzerland (65.6%), Turkey (74.4%), France (71%), Greece (83.3%), Ireland (66.7%), Israel (80%) and Germany (67.4%). Manandhar et al. 2021 and Suneel et al. 2022 demonstrated inducible clindamycin resistance in 11.7% and 10.1% of CoNS isolates which is lesser than our finding whereas, the report of Schreckenberger et al. 2004 showed double our study (50%).

Clindamycin can be considered as one of the drugs of choice for treatment due to its excellent tissue penetration, good oral absorption and is an alternative to penicillin allergic patients. However, the increasing rates of inducible clindamycin resistance among strains of *Staphylococci* increases the chances of treatment failure if clindamycin is used for strains showing inducible clindamycin resistance. Therefore, the D test is helpful to determine inducible clindamycin resistance to guide in the treatment of the infections caused by *Staphylococci* (Belbase et al. 2017).

The tissue culture plate method remains among the widely used assays for investigation of biofilm with a number of modifications for the in vitro cultivation and study of bacterial biofilms (Stepanovic et al. 2007). With regards to the biofilm formation, our study demonstrates 28.1 % isolates were strong biofilm producers, 31.3% moderate and 40.6% were non/ weak biofilm producers. The findings of strong biofilm producers were higher than Tuladar 2018, Pandey et al. 2020 and Manandhar et al. 2021. Among MRCoNS and MDR isolates, 40.9% and 33.3% respectively were strong biofilm producers. Ando et al. 2004 and Melake et al. 2016 also showed methicillin resistance was higher in biofilm producers than non-slime producers. The study from Northern Thailand has demonstrated an association between biofilm-associated genes and the biofilm phenotype of MRCoNS isolates (Kitti et al. 2019) indicating the ability of MRCoNS to form biofilms. The proximity of cells within a biofilm helps in exchange of plasmid that helps to develop multi microbial resistance. Further, large polysaccharides formed on the surface of biofilm prevents antimicrobial agents to penetrate inside it. Also the rate of cell multiplication of organisms in the biofilm slows down. All these contribute to the development of chronic and recurrent infections with increase in resistance to commonly used antibiotics (Sudheendra and Basavaraj 2018, Pramodhini et al. 2012).

CONCLUSIONS

The isolation and identification of *S. epidermidis*, *S. saprophyticus* and *S. haemolyticus* as common CoNS isolates should not be neglected while considering the treatment of immune compromised and device implant patients. The incidence and association of CoNS with multidrug resistance, methicillin resistance and biofilm production certainly brings a number of challenges to the treatment of patients in health care setting. Therefore, the appropriate use of antibiotics against

CoNS is important.

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CONFLICT OF INTEREST

Authors declared no conflict of interest.

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