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RESEARCH ARTICLE

Characterization of biofilm producing methicillin resistant coagulase negative Staphylococci from India

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ABSTRACT

Methicillin-resistant coagulase-negative staphylococci (MR-CoNS) cause infectious diseases due to their potential to form biofilm and further colonization in hospital materials. This study evaluated the antibiotic susceptible phenotypes, biofilm-producing ability, and biofilm-associated genes (*mecA, ica*AD, *bap*, *cna*, and *fnb*A). Biofilm formation was detected through Congo red agar (CRA) method and MTP method. The presence of biofilm and associated genes in MR-*CoNS* were detected by PCR. A total of 310 (55.95%) isolates produced the biofilm. Among these isolates, *Staphylococcus haemolyticus* (34.83%), *Staphylococcus epidermis* (31.93%), *Staphylococcus capitis* (16.77%), *Staphylococcus cohnii* (10.96%), and *Staphylococcus hominis* (5.48%) were identified. The antimicrobial susceptibility pattern of CoNS isolates indicated resistance to cefoxitin (100%), erythromycin (94.8%), ciprofloxacin (66.7%), sulfamethoxazole/trimethoprim (66.7%), gentamicin (66.12%), and clindamycin (62.9%). Resistance rate to mupirocin was 48.5% in *S. epidermidis* and 38.9% in *S. haemolyticus* isolates. All isolates were sensitive to vancomycin and linezolid.

The prevalence rates of *icaAD, bap, fnbA*, and *cna* were 18.06%, 12.5%, 47.4%, and 27.4%, respectively. *ica*AD and *bap* genes were detected in 18.06% and 12.5% of MR-CoNS isolates. *fnb*A and *cna* genes were detected in 47.41% and 27.41% of MRCoNS isolates. icaAD positive strains exhibited a significant increase in the biofilm formation compared with those that lacked *ica*AD (0.86 (0.42, 1.39) versus 0.36 (0.14, 0.75), respectively; $P < 0.001$).

In conclusion, the majority of MR-CoNS isolates were biofilm producers, and *S. capitis*, which possessed *icaAD* genes, ranked as the great biofilm producer than other *Staphylococcus*. The study's findings are important to form a strategy to control biofilm formation as an alternative strategy to counter the spread of MR-CoNS in healthcare settings.

KEYWORDS

MR-CoNs, CoNs, biofilm, virulence

INTRODUCTION

Coagulase Negative *Staphylococci* are commensal bacteria of skin, anterior nares, ear canals, respiratory and gastrointestinal mucous membranes of humans [1, 2]. CoNS develop resistance to various antimicrobial agents posing challenges in treatment strategies [3]. Biofilm production by CoNS is identified as an important factor of pathogenesis and resistance against antibiotics and evasin from the immune system [4]. Among MR-CoNS, *Staphylococcus haemolyticus* and *Staphylococcus epidermidis* cause nosocomial infections, challenging to treat as they form biofilms on medical devices [5]. Methicillin resistance in coagulase negative satphylococci (MR-CoNS) is an increasing problem worldwide [6, 7].

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Three major proteins involved in biofilm development are polysaccharide intercellular adhesin (PIA), biofilm-associated protein (*Bap*), and fibronectin-binding protein (*FnbA*). PIA is encoded by *ica*ABCD genes located within the intracellular adhesion (*ica*) operon, and the *Bap* is encoded by the *bap* gene involved in the initial attachment and ica [8–11]. *fnb*A plays an important role in the accumulation phase of biofilm development through homophilic interactions or binding of proteins to the surface receptors of the adjacent cells [11]. Therefore, the process of biofilm formation of MR-CoNS isolated from different sources of clinical specimens should be studied. This will be important to develop newer therapeutic strategies for the effective treatment of CoNS infections. The current study aimed to identify the association between antibiotic resistance and biofilm formation in MR-CoNS isolated from the clinical samples and the distribution of biofilm-associated genes.

MATERIALS AND METHODS

The current study was carried out at the Department of Microbiology, Rajiv Gandhi Institute of Medical Sciences (RIMS), Government Medical College & Hospital, Kadapa, Andhra Pradesh, India, from July 2018 to February 2020. Institutional Ethical Committee approved the study procedure. Institutional ethical committee [Approval number: 3087/2017–18] approval taken before initiation of the study.

Identification and characterization of CoNS

Coagulase-negative staphylococci (CoNS) bacterial isolates identified by Gram's staining and biochemical tests (Catalase test, Tube coagulase, and Mannitol test). All tests were performed according to standard microbiological protocols using specific tests [12, 13].

Species identification of methicillin-resistant staphylococci

All isolates were confirmed as *Staphylococci* by PCR using 16S RNA primer[14]. Methicillin resistance was confirmed by Cefoxitin disk (30 µg) on Mueller-Hinton agar and *mec*A gene detected by PCR. Colonies were harvested from the Mueller-Hinton agar and suspended in 100 µl of water. One µl of suspension deposited on the plate (Bruker Daltonics, Germany) in 2 replicates, and 1 µl of ethanol (Merck) was added to dry at room temperature. 1 µl of matrix, α-cyano-4-hydroxycinnamic acid (Bruker Daltonics) dissolved in solution [50% acetonitrile, 2.5% trifluoroacetic acid, and 47.5% water] (Sigma-Aldrich, USA). MALDI-TOF-MS was analyzed to distinguish the species within MR-CoNS. MALDI-TOF-MS Spectrometer and FlexControl software (Bruker Daltonics, Germany) were analyzed to detect the species' protein. A score of 2.000–3.000 indicates the species-level identification. The score from 1.700 to 1.999 indicates genus-level identification, and a score of <1.700 was unreliable [15, 16].

Determination of antimicrobial susceptibility pattern

Disc diffusion method analyzed with 12 antibiotics:, chloramphenicol (C; 30 µg), clindamycin (DA; 2 µg), erythromycin (E; 15 µg), gentamicin (CN; 10 µg), sulfamethoxazole/trimethoprim (SXT; 1.25/23.75 µg), 30 µg), rifampicin (RD; 5 µg), cefoxitin (FOX; 30 µg), ciprofloxacin (CIP; 5 µg) (Oxoid, Basingstoke, UK), vancomycin (VA:30 µg), linezolid (Lz:30 µg) and fusidic acid (10 µg). Results were interpreted according to the Clinical and Laboratory Standard guidelines [CLSI, (2018)].

Study of biofilm formation

Tube adherence method [18]: A loopful of bacterial suspension inoculated into Trypticase Soy Broth of 1% glucose and incubated for 24 h at room temperature. Tubes were poured down and washed with PBS(pH 7.3), after drying, tubes stained with 0.1% crystal violet observed for biofilm formation.

Congo red agar method [18]: Congo red agar [37 gm of Brain heart infusion broth (HiMedia)+ 50 gm sucrose (HiMedia)+ 0.8 gm congo red dye +10 gm agar +1 liter water]. CoNS isolates were plated and incubated for 24 h at room temperature. The biofilm developing strains given the black colonies, and non-forming strains given the red colonies.

Microtiter plate (MTP) method: The biofilm formation was also detected by Microtiter plate (MTP) method and as described earlier [19]. MR-CoNS isolates were cultured overnight in 96-well polystyrene tissue culture microtiter plates at 37°C, with trypticase soy broth supplemented with 0.25% glucose medium. The culture medium was removed, and the adherent cells were fixed with 95% ethanol and then stained with 1% crystal violet. Absorbance reading was done at 570_{nm}. Isolates are considered biofilm-positive if they have $OD_{570\ nm} > 0.1$. Biofilm formation interpreted as: highly positive ($OD_{570\ nm} > 1$), low grade positive ($0.1 < OD_{570nm} < 1$), or negative ($OD_{570\ nm} < 0.1$).

Molecular detection of biofilm-associated genes

Genomic DNA isolation from the strong (S) and moderate biofilm-forming MR-CoNS isolates was carried out using bacterial DNA kit (QIAamp DNA Micro Kit). The presence of *ica*AD, *fnb*A, and *bap* genes identified by PCR using primers [20]. The primer sets were designed from the GenBank Sequence Database NCBI (http://www.ncbi.nlm.nih.gov).

Primers to the conserved region of each gene were manually designed by Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) PCR assay was performed in a thermal cycler (BioRad, USA). For PCR, total reaction volume consists of 1 µl of forward primer (10 pmol), 1 µl of reverse primer (10 pmol), 4 µl of DNA extract, 12.5 µl master mix, and final volume of 25 µl with molecular grade water. The PCR conditions as per their Tm annealing (°C) of gene (Table 1). The PCR products were stained with DNA safe and electrophoresed in 1% agarose gel at 80 V for 30 min. The amplified PCR products were finally analyzed on 1.5% agarose gel. The PCR products were sequenced and were confirmed by the amplicon size.

Table 1. List of primers used in PCR to analyze biofilm-associated genes in clinical isolates

Target gene	Primer	Size (bp)	Tm (°C)
16S rRNA	F: CGAAAGCCTGACGGAGCAAC R: AACCTTGCGGTCGTACTCCC	528	52
mecA	F: GTG GAA GTT AGA TTG GGA TCA TAG R: GTC AAC GAT TGT GAC ACG ATA GC	544	65
icaAD	F: GACAGTCGCTACGAAAAG R: AATAAGCTCTCCCTAACTA	211	55
fnbA	F: CCCTCTTCGTTATTCAGCC R: CAGGAGGCAAGTCACCTTG	422	58
bap	F: GGCGCAAGCAGCAGAATTA R: CATAGTTCTTTGTGGTGTTGC	901	63
cna	F: AAAGCGTTGCCTAGTGGAGA R: AGTGCCTTCCCAAACCTTTT	192	55

Biochemical characterization of biofilm

CoNS isolates inoculated into 5 mL TSB medium (HiMedia, Mumbai, India) and incubated at room temperature for 24 h, and transferred to 96-well tissue culture plates and incubated at 37°C for 12 h. Matured biofilms washed 3x times with PBS and treated with 200 µl of 40 mM NaIO4 (HiMedia, Mumbai, India), 0.1 mg mL-1 proteinase K (Genei, Bangalore, India) in 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, or 0.5 mg mL^{-1} deoxyribonuclease I (Sigma-Aldrich Chemical Pvt Limited, Bangalore, India) in 5 mM MgCl2 for 24 h at 37°C. Negative control without treatments contains only bacterial cells washed with PBS included in this experiment. The wells were washed 3x times with PBS, stained with 0.4% (w/v) crystal violet solution, and measured OD at 490_{nm} using a Multimode Microplate Reader Varioskan (Thermo Fisher Scientific) [21]. Assays were repeated 4x times, and the mean biofilm absorbance was analyzed.

Statistical analysis

Analysis was performed through SPSS version 22 software (SPSS version 22, SPSS Inc, Chicago, IL). A one-way ANOVA test analyzed the comparison of biofilm biomass production between the groups. The Student's *t*-test was performed to identify the significance between two different species groups. Categorical values subjected to chi-square test. *P*-value set significance at <0.05.

RESULTS

Determination of biofilm formation

Isolates show biofilm production detected through Congo red agar-based screening (CRA) and MTP methods, as shown in Table 2. Biofilm production was detected in 310 (55.95%) isolates by CRA, and 299 (53.97%) isolates were detected by MTP assay. Congo red agar-based method identified 310 (55.95%) isolates out of 554 MR-CoNS to have biofilm production. Using CRA method, it was observed that 37 isolates out of 310 (11.93%) of MR-CoNS isolates formed red colonies, 156 isolates (47.74%) formed black colonies, and 117 isolates (37.7%) formed intensely black colonies. Slime-positive isolates produced the reddish-black colonies with rough, dry, and crystalline consistency on CRA, and the negative slime strains produced pinkish-red, smooth colonies. MTP assay demonstrated that 299 (53.97%) out of the 554 MR-CoNS isolates were biofilm producers, out of which 106 (34.19%) isolates showed low-grade positivity, 193 (62.27%) isolates showed high-grade positivity.

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 and PCR. 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%), and *S. hominis* ($n = 17$; 5.48%) (Fig. 1).

Antimicrobial susceptibility testing

All MR-CoNS biofilm producing isolates ($n = 310$) were tested for the susceptibility against 12 antibiotics. The isolates were resistant to cefoxitin ($n = 310$; 100%), erythromycin ($n = 294$; 94.83%), ciprofloxacin ($n = 207$; 66.77%), sulfamethoxazole/trimethoprim ($n = 207$; 66.77%), chloramphenicol ($n = 40$; 12.9%), rifampicin ($n = 62$; 20.0%), gentamicin ($n = 205$; 66.12%), fusidic acid ($n = 65$; 20.96%), clindamycin ($n = 195$; 62.9%), and mupirocin ($n = 117$; 37.7%), but, all isolates were sensitive to linezolid and vancomycin (Table 3).

Analysis of biofilm-associated genes

The icaAD gene was detected in 18.06% ($n = 56$) of MR-CoNS isolates belonging to *S. epidermidis* 13 (23.21%) and *S. capitis* 43 (76.79%) species. Clinical isolates of *S. haemolyticus*, *S. cohnii*, and *S. hominis* did not possess this gene. The presence of the bap gene was found in 12.5% ($n = 39$) of MR-CoNS isolates that belonged to *S. capitis* ($n = 20$; 51.28%), *S. cohnii* ($n = 15$; 38.4%) and *S. epidermidis* ($n = 2$;

Table 2. Prevalence of biofilm produceres and detection of biofilm adhesion genes in methicillin-resistant coagulase-negative Staphylococci

	Clinical samples					
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%)
Adhesion genes						
icaAD (%)	0	13 (23.21%)	43 (76.79%)	0	0	56 (18.06%)
bap (%)	0	2 (5.12%)	20 (51.28%)	15 (38.4%)	0	39 (12.5%)
fnbA (%)	62 (42.17%)	66 (44.9%)	0	19 (12.9%)	0	147 (47.47%)
cna (%)	18 (21.17%)	47 (55.29%)	0	20 (23.5%)	0	85 (27.41%)
mecA	108 (34.83%)	99 (31.93%)	52 (16.77%)	34 (10.96%)	17 (5.48%)	310 (100%)

CRA: Congo red agar, MTP: Microtiter plate.

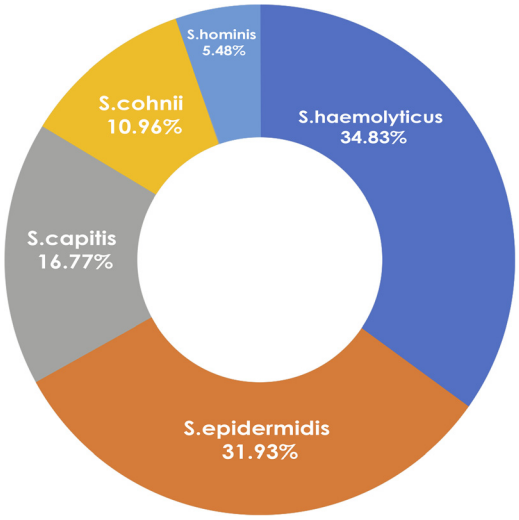

Fig. 1. Distribution of biofilm producing species in MR-CoNS obtained from the hospital samples. The pie chart shows the comparison of *Staphylococcus haemolyticus*, *Staphylococcus epidermidis*, *Staphylococcus capitis*, *Staphylococcus cohnii* and *Staphylococcus hominis* among MR-ConNs measured at OD_{570nm}

5.12%). However, the bap gene was not identified in *S. haemolyticus* and *S. hominis*.

The *fnb*A gene was present in 47.41% (n = 147) of MR-CoNS isolates and belonged to *S. epidermidis* (n = 66; 44.9%), *S. cohnii* (n = 19; 12.9%), and *S. haemolyticus* (n = 62; 42.17%), respectively. The *cna* gene was present in 85 (27.41%) of the MR-CoNS isolates, harbored by *S. haemolyticus* (n = 18; 21.17%), *S. epidermidis* (n = 47; 55.29%), and S.*s cohnii* (n = 20; 23.5%) respectively (Table 2). In the present study, the icaAD and bap genes were detected in 18.06% and 12.5% of MR-CoNS isolates. *fnb*A and *cna* genes were detected in 47.41% and 27.41% of MRCoNS isolates. *S. haemolyticus* and *S. hominis* does not possess *ica*AD and *bap* genes.

Association between biofilm genotypes and biofilm biomass in MR-CoNS isolates

The biofilm biomass of *S. epidermidis* and *S. cohnii*, *S. capitis*, and *S. hominis* significantly higher than *S. haemolyticus* (P < 0.05). The presence of biofilm-associated genes in MR-CoNS were significantly produced more biofilm biomass than the strains without biofilm-associated genes. *ica*AD exhibited a significant increase in the biofilm formation compared with those that lacked *ica*AD (0.86 (0.42, 1.39) versus 0.36 (0.14, 0.75), respectively; P < 0.001). 37 isolates out of 310 (11.93%) of MR-CoNS isolates formed red colonies, 156 isolates (47.74%) formed black colored colonies, and 117 (37.7%) isolates formed intensely black colored colonies. MTP assay demonstrated that 299 (53.97%) in 554 MR-CoNS isolates produced biofilm, out of which 106 (34.19%) isolates showed low-grade positivity, 193 (62.27%) isolates showed high-grade positivity. Biofilm production detected in 310 (55.95%) isolates by CRA method, and 299 (53.97%) isolates were detected by MTP assay.

Table 3. Species wise resistance pattern of Methicillin Resistant Coagulase negative Staphylococci (MR-CoNS)

Antibiotic	*S. haemolyticus* (n = 108)	*S. epidermidis* (n = 99)	*S. capitis* (n = 52)	*S.cohnii* (n = 34)	*S. hominis* (n = 17)
Vancomycin	108 (0%)	99 (0%)	52 (0%)	34 (0%)	17 (0%)
Linezolid	108 (0%)	99 (0%)	52 (0%)	34 (0%)	17 (0%)
Cefoxitin	0 (100%)	0 (100%)	0 (100%)	0 (100%)	0 (100%)
Erythromycin	9 (8.33%)	5 (5.05%)	0 (100%)	1 (2.94%)	1 (5.88%)
Clindamycin	2 (1.85%)	1 (1.01%)	7 (13.46%)	3 (8.82%)	2 (11.76%)
Ciprofloxacin	5 (4.62%)	1 (1.01%)	51 (98.07%)	29 (85.29%)	17 (0%)
Co-trimoxazole	3 (2.77%)	0 (100%)	49 (94.23%)	34 (0%)	17 (0%)
Chloramphenicol	17 (15.74%)	1 (1.01%)	10 (19.23%)	8 (23.52%)	4 (23.52%)
Rifampicin	77 (71.29%)	68 (68.68%)	52 (0%)	34 (0%)	17 (0%)
Gentamicin	15 (13.88%)	5 (5.05%)	47 (90.38%)	27 (79.41%)	11 (64.40%)
Mupirocin	42 (38.88%)	48 (48.48%)	52 (0%)	34 (0%)	17 (0%)
Fusidic acid	63 (58.33%)	79 (79.79%)	52 (0%)	34 (0%)	17 (0%)

DISCUSSION

In our current study, among the 310 clinical isolates of MR-CoNS belong to 5 species, in which *S. haemolyticus* was the predominant, followed by *S. epidermidis*, *S. cohnii*, S.s *capitis, S. hominis*, isolates respectively. These findings agreed with Teeraputon et al., reported the prevalence of MR-CoNS distribution, *S.s haemolyticus* recorded as the most predominant species (37.5%), followed by *S. epidermidis* (21.8%), *Staphylococcus saprophyticus* (11.7%), and *S. hominis* (11.3%), respectively [22, 23]. Our study shows the higher antibiotic resistance, especially to cefoxitin (100%), followed by penicillin (96.77%), and erythromycin (94.83%), similar to another study [23].

All 310 MR-CoNS isolates were susceptible to vancomycin and linezolid; This result is consistent with Shrestha et al.[24]. Biofilm production is an important mechanism of pathogenicity in MR-CoNS [17]. Our study found 55.95% of MR-CoNS isolates were biofilm producers. These results were comparable to other study findings by Oliveira A and Cunha Mde L, which revealed 75% of MR-CoNS clinical isolates were biofilm producers [25]. MTP method determined the biofilm production by highly positive in 53.97% of the isolates and low-grade positive in 34.19% isolates in MR-CoNS. Seng R et al. reported the prevalence of 26.3% isolates were highly positive, and 66.1% were low-grade positive [20].

This study observed that the biofilm-producing ability of MR-CoNS obtained from various species was different. The biofilm-producing ability of *S. epidermidis* was higher than *S. haemolyticus* ($P < 0.05$). This result correlated with the study by Thilakavathy et al., which reported that the biofilm production was the highest in *S. epidermidis* (38.54%) followed by *S. saprophyticus* (1.04%) [26]. Our study result observed that CRA method is better for biofilm detection than the MTP method. Several other studies demonstrated that the nosocomial infections caused by staphylococci were associated with the presence of biofilm-associated genes [9]. In our present study, the icaAD and bap genes were identified in 18.2% and 12.7% of MR-CoNS isolates. *S. haemolyticus* and *S. hominis* did not retain the icaAD and bap genes. The prevalence of *S. haemolyticus* was high, with the tendency to develop the resistance to multiple antibiotics; *S. epidermidis* is the predominant bacteria among the CoNS because of its ability to forms the biofilm on different surfaces [27].

All *S. capitis* isolates which possessed *ica*AD genes to produce the biofilm. At the same time, *S. haemolyticus*, *S. hominis*, and *S. cohnii* have the ability to produce the biofilm assessed by the CRA method lacks the *ica*AD gene. The icaAD gene was found to be involved in biofilm production. The *bap, fnbA*, and *cna* genes play the main role in attachment to biotic or abiotic surfaces, representing the first step of biofilm formation [28]. The biofilm-forming ability of some isolates in absence of *ica*AD gene, as detected by CRA method, indicates they produce biofilm through icaAD-independent mechanisms [28].

In conclusion, the present study identified the high prevalence of MR-CoNS producing biofilm from the hospital samples. Biofilm production was the highest in *S. epidermidis*. The presence of biofilm associated-genes were detected in the MR-CoNS isolates, and thus the association between biofilm-forming genes and the biofilm phenotype of MR-CoNS isolates were observed. The study also suggests that periodical monitoring of the association and co-occurrence of biofilm forming genes in inducing virulence among clinical isolates in order to curb the menace of the complications caused by MR-CoNs.

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