EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 5
Issue: 3
609-615
Year: 2018

DETECTION OF BIOFILM PRODUCING POTENTIALS OF VARIOUS SPECIES OF COAGULASE NEGATIVE *STAPHYLOCOCCI* UNDER DIFFERENT ENVIRONMENTAL CONDITIONS AND ITS CLINICAL RELEVANCE

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Article Received on 10/01/2018 **Article Revised on 30/01/2018** **Article Accepted on 20/02/2018**

ABSTRACT
Background: In recent years Coagulase Negative *Staphylococci* (CoNS) has been recognised as potential pathogen. CoNS are known to produce various virulence factors; slime (biofilm) production is one of such virulence factor associated with several nosocomial infections related to prosthetic devices and indwelling catheter. Slime producing CoNS are known to cause chronic infections, which require a prolonged period of therapy to eliminate the infection. **Materials and methods:** Total of one hundred clinically significant CoNS were further subjected to speciation by standard phenotypic methods. Antibiotic susceptibility testing was performed on all isolates by Kirby Bauer disc diffusion method. Slime production was determined by two different methods namely tissue culture plate method (TCP) and tube method. Effect of different physiochemical conditions on biofilm production was determined by both methods. **Results:** CoNS were identified as S.schleiferi, S.heamolyticus, S.lugdunensis, S.epidermidis and S.saprophyticus. Of these, 44 were female and 56 were male cases. A total of 15 and 20 isolates were detected as biofilm positive phenotypes by tube and TCP assays respectively. Biofilm positivity varied greatly from 60% by S.epidermidis to nil produced by S.saprophyticus.

KEYWORDS: Coagulase Negative *Staphylococci*, speciation, biofilm formation, antibiotic susceptibility.

1. BACKGROUND
Coagulase negative *Staphylococci* (CoNS) were long considered innocuous commensal microorganism, has recently been recognised as potential pathogen associated with neonatal sepsis, infective complications following surgical, vascular grafts, catheter associated infections and the implantation of prosthetic devices.[1,7] Though they are collectively called as CoNS different species have their own spectrum of disease; S.epidermidis and S. haemolyticus being the most significant species associated with infections in preterm new-borns and foreign body-related infections. While S. saprophyticus being isolated frequently from symptomatic acute urinary tract infections (UTIs). Whereas, S. lugdunensis has preponderance over other CoNS to cause native valve endocarditis like S. aureus. Though CoNS possess fewer virulence properties than S. aureus, owing to methicillin-resistant strains and increasing numbers of isolates with less susceptibility to glycopeptides they are considered important.[1]

Coagulase negative *Staphylococci* produce various virulence factors; biofilm formation is one of the major virulence factor associated with prosthetic devices and indwelling catheter infections.[8]

Many strains of CoNS form an adherent bacterial film after initial contact and attachment to a polymer surface, which is composed of water, exopolysaccharide, extracellular DNA, proteins, and lipids.[9,10] It is estimated that biofilms are associated with about 65% of nosocomial infections.[11,12] CoNS also form biofilms on tissue surfaces which contribute to a variety of infections such as endocarditis of native valve and otitis media.[8,13,15]

Biofilm producing bacteria resists the conditions of the surrounding environment and generally produce chronic infections, which are difficult to treat, as a result, higher doses of antibiotics are needed or sometimes a prolonged period of administration to eliminate the planktonic

bacteria of exactly the same strain.[16,2] Antibiotic susceptibility and pathogenic potentials vary among different species of CoNS, therefore, it is important to know the epidemiology, virulence factors and antibiotic resistance of CoNS to institute an appropriate therapy at an early stage.[17,18] The present study was aimed to identify CoNS to species level, to determine their antimicrobial susceptibility and their potential to form a biofilm.

2. MATERIALS AND METHODS

2.1. Sample collection

This is a cross-sectional study which was done for a period of one year. All *Staphylococci sp.* isolated from the clinical material received for routine bacteriological investigation were tested for the presence of free coagulase (tube coagulase test) and bound coagulase (slide agglutination test) by standard methods. A total of one hundred clinically significant (yields pure growth on primary plating media, $>1x10^5$ colonies/ml and isolated same type of bacteria from the repeat specimen) coagulase (tube coagulase) negative *Staphylococci sp.* were considered for further processing.

2.2. Speciation and Antibiotic susceptibility testing

These isolates were characterized and speciated according to Murray's Manual of clinical microbiology.[6] The following tests were performed to characterize the isolates: macroscopic colony appearance, Gram-staining, production of acid from mannitol salt agar (HiMedia, Mumbi, India), catalase activity, novobiocin resistance, polymyxin B resistance, sucrose and mannose fermentation, and tube and slide coagulase tests. Antibiotic susceptibility testing was performed on all isolates by Kirby Bauer disc diffusion method as per CLSI standards [CLSI 2011].

2.3. Detection of biofilm formation

After preliminary identification and speciation, all isolates were tested for biofilm formation by two different methods namely tissue culture plate method and tube method, under different environmental conditions.

2.3.1. Tissue culture plate method

All isolates were screened for their ability to form biofilm under different physiochemical conditions by TCP method as described by Rachid et al.[19] with a modification in the duration of incubation which was extended to 24 hrs at 37°C. BHI (Hi-Media, Mumbi), BHI with 1% glucose and BHI with 4% NaCl were used to detect the ability of CoNS to form biofilm under different environmental conditions. Briefly Individual wells of tissue culture plate (polystyrene 96 well flat bottom, Tarsons, Kolkata, India) were filled with 0.2 ml aliquots of the medium containing test organism and un-inoculated broth without organisms served as a control to check sterility and nonspecific binding of media. Contents of each well were removed after 24 hours of incubation at 37^0C and washed three times with 0.2 ml of distilled water and fixed by drying the plate for one hour at 60 °C in an inverted position, as recommended by Gelosia et al.[20], and stained with 0.4% crystal violet solution. The optical density of stained adherent bacterial biofilm was determined with a micro ELISA plate reader (Model 780, BioRad) at a wavelength of 490 nm. These O.D values were considered as an index of bacteria adhering to surface and forming a biofilm. The experiment was performed in duplicate and the data was then averaged. To compensate for background absorbance OD readings, O.D obtained from media control well was subtracted from all the test O.D values. Based on O.D values obtained, biofilms were classified into one of the following categories: non/weak (O.D <0.120), moderate (O.D=0.120-0.240) and high (O.D>0.240).[21]

2.3.2. Tube method

A qualitative assessment of biofilm formation was determined as previously described by Christensen et al.[22] Briefly, three sets of tubes each containing 1 ml of BHI, BHI with 1% glucose and BHI with 4% NaCl were inoculated with a 1 to 2 colonies from overnight culture plates and incubated for 24 hours at 37°C. The tubes were decanted and washed with distilled water and dried at 60^0C for one hour in an inverted position and then stained with 0.4% crystal violet. Tubes were washed with distilled water, then dried in an inverted position and examined for biofilm formation. Based on the intensity of biofilm observed, the isolates were categorised as Negative (score'0', no biofilm formation or ring formation), weak positive (score'1', Very thin biofilm formation at bottom), Moderate positive (score'2', thin biofilm formation at bottom and on side walls) and strong positive (score'3', Thick biofilm formation on both bottom and on side walls.[21]

2.4. Statistical analysis

Data was prepared and compiled in Microsoft Office Professional Plus 2013, spreadsheet (Excel) for windows. Shapiro-Wilk test was used to check the distribution of data. All continuous variables that are normally distributed were expressed as mean±SD. All categorical variables were analysed using Chi-square test. All the statistical tests were performed using IBM SPSS Statistical software for Windows, Version 21.0. Armonk, NY: IBM Corp.

3. RESULTS

A total of one hundred clinically significant coagulase negative *Staphylococci* were selected for further characterization. Of these 44 were female and 56 were male cases. The age of the selected cases ranges from 12 to 81 years with mean age of 44±16 years (mean±SD). The isolates obtained from these specimens were characterized and identified to species level (table-1) and tested for biofilm production (table-2), of which 15 and 20 isolates were detected as biofilm positive phenotypes by tube and TCP assays respectively. All isolates positive by tube method were also positive by TCP assay, in addition, 5 more isolates were also detected as

biofilm positive phenotypes by TCP assay [fig. 1 and 2]. Among these 20 positive phenotypes, 11 (n=53), 4 (n=17), 2 (n=12), 2 (n=9) and 1 (n=9) were isolated from urine, pus, blood, sputum and catheter tip specimens respectively.

In the standard TCP assay, only 5 of 100 tested isolates were displayed biofilm positive phenotype in BHI medium, with the addition of 4% NaCl and 1% glucose in BHI medium, the number of biofilm producing isolates were increased to 7% and 18% respectively (table 2).

Antibiotic susceptibility testing was performed on all isolates (table 3). Biofilm positive isolates showed increased resistance to penicillin and fluoroquinolone group of antibiotics, whereas biofilm negative phenotypes showed decreased susceptibility to macrolides, aminoglycosides and trimethoprim/Sulfamethoxazole antibiotics. However, the difference was not statistically significant (p=0.40). Interestingly few (22%) isolates of coagulase negative *Staphylococci* were resistant to linezolid (in this study all intermediately susceptible isolates were also considered as resistant after repeating the test with a new batch of antibiotic discs). Though this study showed the observable difference between these two groups, it is not statistically significant (p=0.40) and this might be due to less number of isolated tested in each group.

Table 1: Distribution of various Coagulase Negative *Staphylococci sp.* with respect to specimens.

S.No.	Species	Nature of specimen					Total
		Urine	Pus	Blood	Catheter tip	Sputum	
1.	*S. schleferi*	15	04	03	03	03	28
2.	*S. haemolyticus*	14	05	04	03	01	27
3.	*S. lugdunensis*	15	03	02	02	03	25
4.	*S. epidermidis*	07	03	02	01	02	15
5.	*S. saprophyticus*	02	02	01	Nil	Nil	05
Total		53	17	12	09	09	100

Table 2: Effect of different physicochemical conditions on biofilm formation by Coagulase Negative *Staphylococci* sp.

S. No.	Type of Species (n)	TCP Method			Tube Method			Total (%)	'p' value
		BHIB	BHIB/G	BHIB/N	BHIB	BHIB/G	BHIB/N		
1	*S.epidermidis* (15)	3	8	4	4	8	7	9 (60)	
2	*S.lugdunensis* (25)	2	6	2	Nil	5	2	7 (28)	
3	*S.haemolyticus* (27)	Nil	3	1	Nil	1	2	3 (11)	
4	*S.schleferi* (28)	Nil	1	Nil	Nil	1	Nil	1 (3.5)	
5	*S.saprophyticus* (5)	Nil	Nil	Nil	Nil	Nil	Nil	Nil	
Total (100)		5	18	7	4	15	11	20	

BHIB-brain heart infusion broth; BHIB/G- brain heart infusion broth with 1% glucose; BHIB/N- brain heart infusion broth with 4% NaCl.

Table 3: Antibiotic susceptibility pattern among biofilm positive and negative phenotypes of Coagulase Negative *Staphylococci* sp.

Type of isolate	Percentage of antibiotic resistance									
	PEN	AMP	OXA	CIP	TMP/ SXT	GEN	DOX	ERY	LZD	VAN
S. epidermidis (15)	100	73	53	86	53	53	46	60	13	0
S. haemolyticus (27)	89	85	59	85	59	70	19	81	15	0
S. lugdunensis (25)	84	72	64	88	60	56	40	84	8	0
S. schleferi (28)	100	67	61	82	54	71	25	67	11	0
S. saprophyticus (05)	80	60	60	60	60	20	20	100	20	0
Biofilm positive isolates	100	90	70	95	40	50	30	55	10	0
Biofilm negative isolates	92	70	58	81	62	62	30	80	12	0

PEN; Penicillin, AMP; Ampicillin, OXA; Oxacillin, CIP; Ciprofloxacin, TMP/ SXT; Trimethoprim/ Sulfamethoxazole, GEN; Gentamicin, DOX; Doxycycline, ERY; Erythromycin LZD; Linezolid, VAN;Vancomycin.

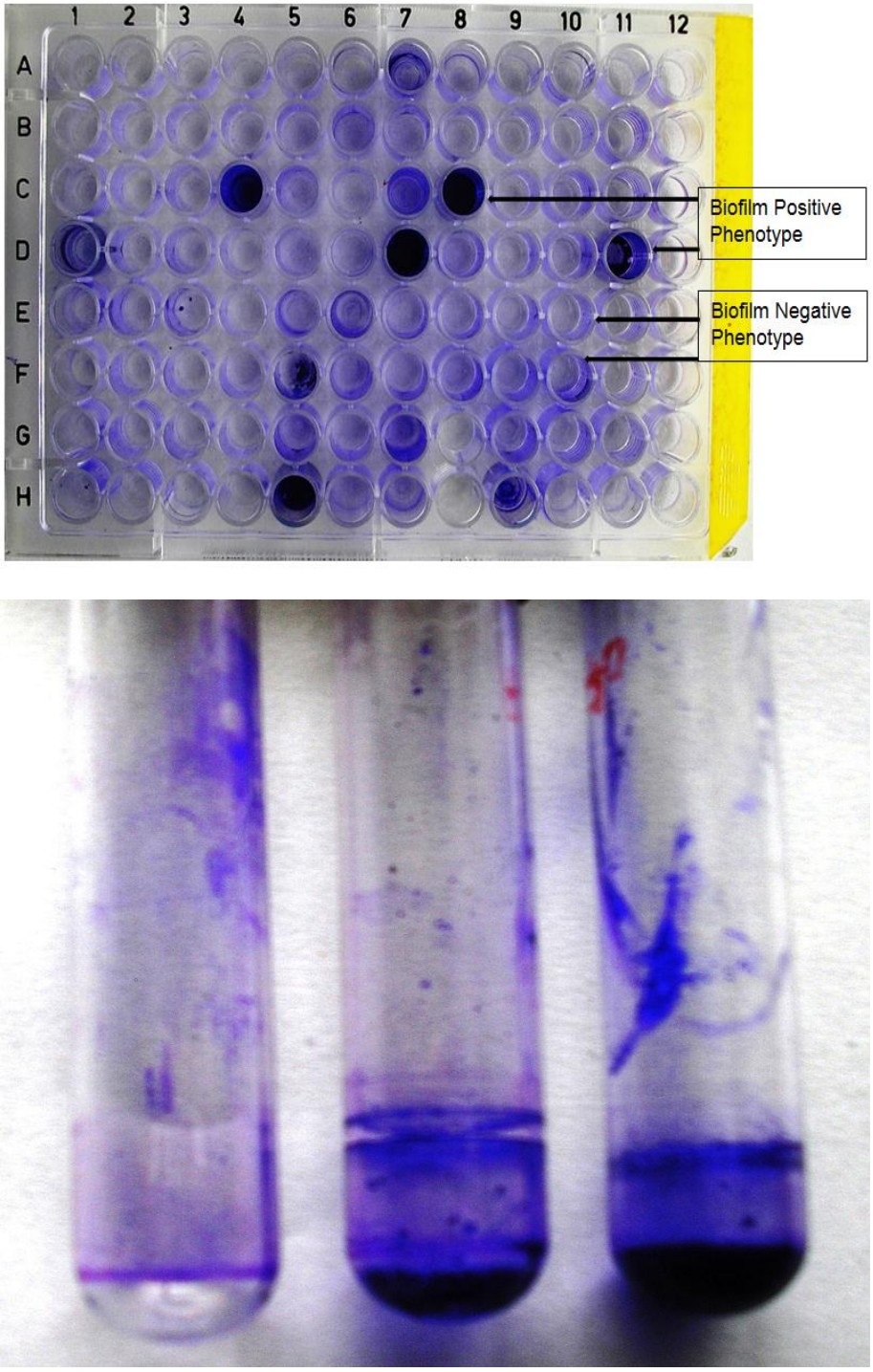

Figure 1: Tube method for biofilm detection.

4. DISCUSSION

The study was conducted on one hundred clinical isolates of CoNS and the species identified were S.schleiferi (28%) and S.heamolyticus (27%) followed by S.lugdunensis (25%), S.epidermidis (15%) and S.saprophyticus (5%) which are well-known species of CoNS causing hospital acquired infections. In contrast to the present findings, several studies reported that S. *epidermidis, S.saprophyticus, S.haemolyticus, S.hominis and S.cohnii* were predominant sp. Isolated.[3,4] Another study by Worthington *et al.*[23] and Eliezer M[24] showed that *S .epidermidis*, and *S. haemolyticus* were the major species CoNS isolated. In one study conducted on evaluation of neonatal sepsis in low birth weight preterm neonates and reported CONS as the most common organism responsible for neonatal sepsis (50%, n=151), followed by Candida spp. (15.8%, n=151).[25] Other hand authors have shown that implementation of several infection-prevention initiatives surprisingly brought down the number of CoNS associated neonatal sepsis cases from 31% during 2004 to 2009 to no cases in 2011. This findings once again emphasizes the importance of

implementation of appropriate preventive strategies especially among risk population.[26] However in our study we did not tested any neonatal samples.

In this study we observed slight male predominance for S.lugdunensis (male=17, female=08) and S.saprophyticus (male=04, female=01); whereas for other species, there is no difference observed between male and female gender. However, these difference is not statistically significant (p=0.37). This could be due to that the colonizing species slightly varies with gender and geographical region as well. *Staphylococcus heamolyticus* and *S.schleiferi* were predominantly isolated from blood (33%, 25%), central-venous catheters (33%, 33%) and pus samples (29%, 23%); whereas, *S.lugdunensis* was outnumbered from sputum (33%) and urine samples (28%). For other sp., there was no sample predominance observed. Overall there was no statistically significant difference observed for sp. distribution among various clinical material (p=0.97). In concordance with these findings Sabe et al., *reported S. lugdunensis* as the principal pathogen associated with infective endocarditis (both native and prosthetic valve infections).[27]

Staphyloccus epidermidis was more prevalent species producing detectable biofilm (60%), followed by *S.lugdunensis* (28%), *S.haemolyticus* (11%) and *S.schleiferi* (4%), and are well-known species of CoNS causing hospital acquired infections. None of the *S. saprophytic* exhibited biofilm phenotypes; this might be due to a lower number of isolates tested. Out of 293 CoNS reported by Allori *et al.*, *S.epidermidis* (84, 37%, 32%, 15%), *S.haemolyticus* (73, 13%, 25%, 35*%)*, *S.saprophyticus* (27, 0%, 7%, 20%), *S.hominis* (15%, 5%, 15%) and *S.cohnii* (65%, 0%, 28%) from blood, catheters and urine samples respectively showed biofilm production.[19]

A study by Allori *et al.*[28] and Zilevica A *et al.*[29] reported that 30% of their CONS showed biofilm formation *In Vitro*. Similar findings were obtained from our study where *S. epidermidis* was found to be the most prolific producer of biofilm (60%), followed by *S. lugdunensis* (28%).

It was observed that biofilm development was significantly induced by glucose as well as NaCl in all the isolates. The effect of glucose on the formation of biofilm was higher compared to NaCl. The addition of NaCl enhances the biofilm production, which may be due to biofilm production induced by osmotic stress. NaCl is a known activator of the "ica" operon transcription[30] and consistent with this biofilm formation under the influence of NaCl is apparently ica ADBC dependent and involves the production of PIA / PNAG.[19,31]

Mathur *et al.*[21] investigated the different isolates of *Staphylococci* from different tertiary care centres in India. They showed that 4.6% and 46% of isolates were producing biofilm in BHI and BHI (Glu) medium respectively after incubation for 24 hrs at 37^0 C. In their study, the tube method showing good correlation with the TCP assay for strong biofilm forming isolates and total 12% of the isolates were strong biofilm positive and 29% of isolates were moderate biofilm producers.

In the standard TCP assay, only 5% of 100 isolates tested displayed biofilm positive phenotype in BHI medium in our study. Mathur *et al.*[21] also detected 4.6% of positive phenotypes in their standard TCP assay in BHI medium. In our study, with the addition of 4% NaCl, the number of positive phenotypes increased to 7%, which may due to biofilm production induced by osmotic stress. With the addition of 1% glucose in BHI medium, the number of biofilm producing isolates increased to 18%.

It has been suggested that in many isolates of *Staphylococci*, the "ica" expression may be significantly suppressed and an optimal concentration of NaCl (1-5% in various studies) causes derepression of these genes. These observations suggested that a strong dependence between growth condition and biofilm in CoNS and various supplements are essential for biofilm formation.

In the light of the present study, it is clear that CoNS that readily produce biofilm are associated with all acute and chronic infections. It is clear that *S. epidermidis* has the highest potential to produce biofilm (60%), followed by *S. lugdunensis* (28%) and *S. haemolyticus* (11%), all of which are associated with device related chronic infections as well as nosocomial infections. These species are also more resistant than other CoNS. This feature, which has been well documented in various reports, has also been elaborated in the present study. In this study, all biofilm positive phenotypes showed decreased susceptibility to Penicillin, Ampicillin, Oxacillin and Ciprofloxacin as compare to their rivals. This could be explained by well-established factors such as, failure of antibiotics to reach the extracellular polymer substance embedded biofilm cells, a microenvironment unfavourable to antimicrobial activity, slow bacterial growth, activation of stress responses within biofilms, phenotypically resistant persister cells and genotypically resistant cells selected by antibiotic exposure in biofilms.[32,33]

These biofilm associated infections require prolonged treatment and are further complicated by the emergence of antibiotic-resistant pathogens, which contributes significantly to the morbidity and mortality of hospitalized patients.[2,28] Thus, it is important that CoNS should be identified to species level and detect their association with biofilm to give a better idea about their prevalence and to institute more appropriate therapy for the patients as well as to control the spread of resistant strains among the different species as different species of bacteria in biofilm matrix can exchange antibiotic resistance and virulence factors encoding genes.

5. CONCLUSION

Various sp. of CoNS are frequently isolated from clinical samples from local and systemic infections. A significant number of isolates are capable of producing biofilm on plastic surfaces in-vitro. Several physicochemical factors influence the production of biofilm; the addition of NaCl and Glucose increased the production of slime. Biofilm positive phenotypes were more resistant to most commonly used β-lactam antibiotics.

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