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DETECTION OF BIOFILM IN COAGULASE NEGATIVE STAPHYLOCOCCUS: COMPARISION OF PHENOTYPIC METHODS

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ABSTRACT

Coagulase negative Staphylococci (CoNS) are a major component of the normal flora of the cutaneous ecosystem and act as commensal or saprophytic organisms. The ability to form biofilm plays an important role in contributing virulence of Coagulase Negative Staphylococcus (CoNS). It is considered as normal commensal and shown to cause infections in neonates and immunocompromised patients, especially with indwelling medical devices. The ability to form biofilm plays an important role in contributing virulence of CoNS. Also CoNS is found to be associated with nosocomial infections. The present study has been planned to detect biofilm production and also to evaluate three phenotypic methods. **Aim:** To detect biofilm production in CoNS from the clinical samples phenotypically by using: Tissue culture plate method (TCP), Tube adherence method, Congo red agar method (CRA). **Materials and Methods:** 100 CoNS isolates were subjected to species identification by standard biochemical tests. Biofilm detection of all isolates was done by TCP, CRA and tube method. **Results:** Of the total 1058 culture positives isolates 100(9.45%) were identified as CoNS**.** Of the total 100 isolates 54% were identified as S. epidermidis, 14 (14%) were identified as S. haemolyticus, 10 (10%) were identified as S. capitis, 8 (8%) as S. cohinii, and 14 (14%) as S. Saprophyticus. Of the total isolates biofilm production was seen in 62(62%) isolates by Tissue culture plate (TCP). By Congo red agar method biofilm production was 63 (63%). In tube method biofilm production was in 66 (66%) isolates **Conclusions:** Amongst phenotypic methods CRA was more sensitive and can be easily performed in the routine laboratories laboratory and can be used as a screening test for detection of biofilm.

KEY WORDS: Coagulase negative Staphylococci (CoNS) detection of biofilm.

INTRODUCTION

Coagulase negative Staphylococci (CoNS) are a major component of the normal flora of the cutaneous ecosystem and act as commensal or saprophytic organisms.[1] However, they may develop into a pathogen if they invade the host tissue through trauma of the cutaneous barrier, inoculation by needles, or implantation of medical devices. In the past few decades, these organisms have gained importance as causative agents of human disease.[2] CoNS acts as a significant pathogen in immunocompromised patients, premature newborns, and patients with implanted biomaterials[3] and its pathogenicity and bacteremia is mainly due to the ability to form biofilms.[4] Once formed, these biofilms render the cells less accessible to the defense system of the organism, thus impairing the action of antibiotics and, in turn, represents basic survival strategies of these microorganisms.[5]

Various methods are currently in use for the detection of the biofilm production. Qualitative methods such as tube adherence test described by Christensen *et al.*[6] and the Congo red agar method described by Freeman *et al.*[7] and the quantitative method such as tissue culture plate assay described by Christensen *et al.*[8] can be used in the routine laboratories.

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], also biofilm producing strains are more resistant to the antibiotics hence present study has been carried out to identify the CoNS at species level and to compare the phenotypic methods of biofilm formation.

MATERIAL AND METHODS

This study was carried out in the Department of Microbiology JLN Hospital and Research Centre, Bhilai in Collaboration with CCM Medical College from Jan 2015 to Dec 2017. In this prospective laboratory based study Coagulase negative Staphylococci isolated from the clinical samples of patients attending and admitted in Tertiary care hospital were collected and compared by various phenotypic and genotypic methods to detect

biofilm formation in Coagulase negative Staphylococci (CoNS).

Study duration: 2015 to 2017.

Isolation: All isolates of CoNS from sterile clinical samples during study period were included in the study. A total of 100 clinical isolates from Jan 2015 to Dec 2017 were recovered from clinical samples of patients who attended the tertiary care hospital. All samples after culturing on the agar plates were further incubated for 24 hours at 37°C. All growths on the next day were processed as per standard guidelines.

Identification of Coagulase negative Staphylococci
CoNS were identified as per standard guidelines by putting various biochemical tests Antibiotics susceptibility testing was done by Kirby-Bauer disc diffusion method. Discs potency used were as per CLSI recommendation.[10]

Table 1: Antibiotic discs and potencies used.

Sr.	Antibiotics	Potency
1	Penicillin	10U
2	Erythromycin	15µg
3	Ciprofloxacin	5µg
4	Trimethoprim Sulphamethoxazole	1.25 µg/23.75 µg
5	Oxacillin	1µg
6	Linezolid	30µg
7	Gentamicin	10µg
8	Novobiocin	30µg

Biofilm detection: Using the growth obtained from the cultures, the organism's ability to form biofilm was detected by.

1. Tube method: This is a qualitative method for biofilm detection. A loopful of test organisms was inoculated in 10 ml of trypticase soy broth with 1% glucose in test tubes. The tubes were incubated at 37ºC for 24 h. After incubation, tubes were decanted and washed with phosphate buffer saline (pH 7.3) and dried. Tubes were then stained with crystal violet (0.1%). Excess stain was washed with deionized water. Tubes were dried in inverted position. The scoring for tube method was done according to the results of the control strains. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. The amount of biofilm formed was scored as 1-weak/none, 2-moderate and 3-high/strong.

2. Congo red agar method
The test was done on Congo red agar medium.

Composition

Brain heart infusion broth	-37 g
Sucrose	-50 g
Agar	-10 g
Congo red	-0.8 g

Congo red stain was prepared as a concentrated aqueous solution and autoclaved (121°C for 15 minutes) separately from the other medium constituents, and was then added when the agar had cooled to 55°C. Plates of the medium were inoculated and incubated aerobically for 24 hours at 37°C.

A positive result was indicated by black colonies with a dry crystalline consistency. Non-slime producers usually remained pink.

3. Tissue culture plate method
This quantitative test described by Christensen *et al.* is considered the gold-standard method for biofilm detection. This test was done to compare the two phenotypic methods.

Organisms isolated from fresh agar plates were inoculated in 10 ml of trypticase soy broth with 1% glucose. Broths were incubated at 37ºC for 24 h. The cultures were then diluted 1:100 with fresh medium. Individual wells of sterile 96 well-flat bottom polystyrene tissue culture treated plates were filled with 200 µL of the diluted cultures. The control organisms were also incubated, diluted and added to tissue culture plate. Negative control wells contained inoculated sterile broth. The plates were incubated at 37ºC 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 and stained by crystal violet (0.1%). Excess stain was removed by using deionized water and plates were kept for drying. Optical density (OD) of stained adherent biofilm was obtained by using micro ELISA auto reader at wavelength 570 nm.

Table 2: Interpretation of biofilm production.

Average OD value	Biofilm production
≤ODc/ ODc < ˜ ≤ 2x ODc	Non/ weak
2x ODc <˜ ≤ 4x ODc	Moderate
>4x ODc	Strong

ODc – Optical density cut-off value = average OD of negative control + 3 x standard deviation of negative control.

Statistical Analysis
Statistical analysis was done by using chisquare value, Yate's Continuity corrections, Predictive values and Kappa Statistics. The software used in the analysis were SPSS 17.0 and Graphpad Prism 5.0 version and $p<0.05$ is considered as level of significance ($p<0.05$).

RESULTS
Of the total 1058 culture positives isolates 100(9.45%) were identified as CoNS.

Table 3: CoNS isolation according to age group.

Age group	Isolates (n=100)
Neonates	51
Children	30
Adults	19

Table. 4: Species distibution of CoNS isolates.

Species	Total	%
S. Epidermidis	54	54.00%
S. Haemolyticus	14	14.00%
S. Capitis	10	10.00%
S. Cohinii	8	8.00%
S. Saprophyticus	14	14.00%
Total	100	100.00%

Of the total 100 isolates 54% were identified as S. epidermidis, 14 (14%) were identified as S. haemolyticus, 10 (10%) were identified as S. capitis, 8 (8%) as S. cohinii, and 14 (14%) as S. Saprophyticus.

BIOFILM PRODUCTION

Table 5: Table showing all phenotypic methods included in the study.

Sr.	Species	Total	Phenotypic methods		
			TUBE	CRA	TCP
	Total	100	62	63	66

Of the total isolates biofilm production was seen in 62(62%) isolates by Tissue culture plate (TCP). By Congo red agar method biofilm production was 63 (63%). In tube method biofilm production was in 66 (66%) isolates.

Table 6: Comparison of biofilm detection methods.

Method		TCP		Sensitivity	Specificity	PPV	NPV
		Producer	Non producer				
Tube	Producer	60	2	90.91%	94.12	96.77%	84.21 %
	Non producer	6	32				
CRA	Producer	62	1	93.94%	97.06 %	98.41%	89.19 %
	Non producer	4	33				

TCP-Tissue culture plate method,
CRA- Congo red agar method
PPV- Positive predictive value
NPV- Negative predictive value

Of the 100 CoNS isolated 84 (84%) were multidrug resistant, of which 56 (66.66%) were Biofilm producers and 28 (33.33%) were non biofilm producers

DISCUSSION

Coagulase-negative Staphylococci (CoNS) has been identified as the etiological agent in various infections and most frequently isolated organism in nosocomial infections and very little is known about the virulence factors produced by CoNS that contribute to the pathogenesis of infections caused by these microorganisms.[12,13] A detailed characterization of isolates of coagulase-negative *Staphylococci* through speciation, genetics and antibiotic susceptibility may be necessary to distinguish between infecting from contaminating isolates and to plan suitable therapy.[14] The most important virulence factor is the ability to adhere on the surface of biomaterials and form biofilms.[15]

In routine laboratories for detection of biofilm in clinical samples qualitative methods, such as the tube adherence test described by Christensen *et al.*[6], Congo red agar (CRA) method described by Freeman *et al.*[7] and quantitative methods such as the tissue culture plate (TCP) assay described by Christensen *et al.*[8] are used in routine laboratories. In our study out of 1058 culture positive samples isolation rate of CoNS was 9.45% which was near about similar with the isolation rate of study by Silvia Natoli *et al.*[16] which was 9.24%.

In our study isolation of CoNS from neonates was 51 (51%), from paediatric age group it was 30 (30%) while in adults it was less 19(19%). These figures were different from the study by Rajeevan *et al.*[17] in which isolation of CoNS from adults was 94.12% and in pediatrics age group it was 5.88%. This high incidence of CoNS isolation from newborn was maximum may be due to maximum no of samples were received from the neonatal age group.

Many clinical microbiology laboratories do not identify CoNS to the species level even though they are detected in sterile fluids like blood or cerebrospinal fluid. However, due to increasing significance of CoNS, and for true correlation it is important to identify them to the species level to learn more about the epidemiology and pathogenic potential of individual species. This may be particularly important in case of blood culture isolates, since it is often difficult to determine the clinical significance of an individual isolate.[18]

In various studies percentage of biofilm production varies from 14% to 88%.[19,20,21,22,23,24] In our study Biofilm production was seen in 62(62%) isolates by Tissue culture plate (TCP). By Congo red agar method

biofilm production was 63 (63%). In tube method biofilm production was in 66 (66%) isolates.

Statistically significant association was found between Tube method and TCP The sensitivity of Tube method with TCP was 90.91%, specificity was 94.12%, positive predictive value was 96.77%, negative predictive value was 84.21%. Kappa statistics shows very good agreement between Tube method and TCP Statistically significant association was found between CTA and TCP The sensitivity of CRA with TCP was 93.94%, specificity was 97.06%, positive predictive value was 98.41%, negative predictive value was 89.19%. Kappa statistics shows very good agreement between CRA and TCP Comparing CRA and Tube method, CRA method was more sensitive and more specificity was same. Accuracy of the CRA test was more as compared to TCP. In a study by Knobloch *et al*.[25] Screening on Congo red agar displayed a strong correlation with the TCP. Aricola C.R. *et al*.[26] recommend CRA method to confirm the phenotypic expression.

CONCLUSION

Considering TCP as a gold standard method Tube and CRA shows good statistical correlation in biofilm formation. CRA method is easy to perform in the routine laboratory and can be used as a screenings test for detection of biofilm. To confirm these findings extensive studies are required in the hospitals to look for the biofilm formation in CoNS.

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