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Original Article

Comparison of Three Methods of Biofilm Detection by Clinically Significant Coagulase Negative Staphylococci

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Abstract

Introduction: Coagulase negative staphylococci (CoNS) have risen from commensal or laboratory contaminants to pathogens causing variety of infections. Their meager nutritional requirements and ability to withstand various physical and chemical agents have made CoNS a successful pathogen. One of the main virulence factor associated with CoNS infections is biofilm formation. Biofilm helps CoNS adhere to surfaces and escapes the assault by immune mechanisms and antibiotics. The estimation of biofilm will help identify pathogenic CoNS.

Aims: The aim of the study was to determine clinically significant CoNS and to ascertain their virulence using qualitative and quantitative methods of biofilm detection.

Material and Methods: A total of 82 clinically significant isolates were identified for the study. These isolates were segregated into two groups – Isolates with definite clinical significance (Group A – 45 isolates), and isolates with doubtful significance (Group B – 30 isolates). Qualitative methods Congo red agar method and Tube method were employed. Quantitative detection of biofilm was detected by microtiter plate method.

Results: More sensitive and quantitative were microtiter plate method. In Group A, 21 were moderate biofilm producers and 14 were strong biofilm producers. In Group B, eight out of 30 were moderate biofilm producers and six were strong biofilm producers. The comparison of the three methods showed that microtiter plate method was more sensitive in detection and quantitative assessment of biofilm. Statistical significance of difference between Group A and Group B isolates was found to be statistically significant, *P* value being 0.004.

Conclusions: Methods employed are cost-effective and need minimal training of laboratory staff. The detection of biofilm production will help differentiate pathogenic and commensal CoNS. The reporting of biofilm will help the clinician to plan the appropriate line of therapy.

Key words: Biofilm, Coagulase negative staphylococci, CONS, Congo red agar, Microtitre plate method, *Staphylococcus epidermidis*

INTRODUCTION

Coagulase negative staphylococci (CoNS) were considered as commensals and until recently their isolation in clinical samples would merely mean contamination. However, over a period of time, CoNS have established themselves as pathogens. The role of CoNS as nosocomial pathogens has been documented over the past five decades. This increase is mainly due to the increase in use of devices in patient care and increasing population of immunocompromised people. CoNS are successful in establishing themselves as pathogens mainly due to their ability to adhere to surface of medical devices, form biofilms, and their non-fastidious nature. The most common species implicated in human infections are *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Staphylococcus hemolyticus*, *Staphylococcus lugdunensis*, *Staphylococcus hominis*, *Staphylococcus warneri*, *Staphylococcus cohnii*, *Staphylococcus simulans*, *Staphylococcus schleiferi*, *Staphylococcus warneri,* and *Staphylococcus capitis*. CONS are implicated in

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variety of conditions which include Bacteremia, native and prosthetic valve endocarditis, urinary tract infection, ophthalmic infections, prosthetic joint infections, catheter-related urinary tract infections, and device associated infection (CSF shunts, indwelling CSF catheters, intrathecal pumps, and ventriculostomy sites). The abundance of CoNS in the skin and mucous membrane of the patient and the deadly combination of multidrug resistant CoNS strain and its transmission by hands of health-care workers make CoNS a successful nosocomial pathogen. The capacity to adhere to polymer surfaces and consequent biofilm production adds to the pathogenicity of CoNS.[1,2,3,4]

Biofilm and CoNS

Biofilms are communities of microorganisms that stick together or to the surfaces by production of extracellular matrix comprising of polysaccharides and proteins.[5] In the initial phase, bacterium attaches to surfaces by the use of non-specific factors such as hydrophobicity and surface charge. Bacterium may also adhere to surfaces through cell wall teichoic acids and proteins, such as autolysins or cell wall associated proteins that interfere with collagen, fibronectin, or other matrix proteins.[6] After this initial phase of adherence comes the stage of actual biofilm formation where the bacteria produce factors helping in cell to cell contact. The most commonly isolated CONS; *S. epidermidis* produces polysacchride intracellular adhesion (PIA). PIA comprises of ß-1, 6-linked glucose aminoglycan substituted with different side groups. Other factors that mediate biofilm are surface associated proteins, accumulation associated proteins (Aap), and biofilm associated proteins (Bap/Bhp). CoNS in hospital environment or in device associated infections differ from the commensal CoNS. Nosocomial CoNS form thick multilayered biofilms on polymers or metals.

The differentiation of CoNS with respect to its biofilm may help in assessing the impact of CoNS in relation to device associated infections. Studies done in the past indicate that clinically significant bloodstream isolates of CoNS produced slime.[7,8,9,10] Among the slime producers, *S. epidermidis* was the most prevalent species.[8,11] Nearly, 40–50% of CoNS isolates from clinical specimens can be slime producers.[11,12,13,14] Bacterial films produced by a standard slime producing strain of CoNS on plastic tissue culture plates varied with the type of fixative.[15] The incidence of biofilm production by *S. saprophyticus* is comparatively less than *S. epidermidis*.[16] The percentage of slime-producing CoNS ranged from 20% in peritoneal fluid to 66% in CSF.

A number of tests are available to detect slime production by staphylococci. The methods include microtiter plate (MTP) method,[17] Tube method (TM),[18] Congo red agar,[2,19] bioluminescent assay, and light or fluorescence or confocal microscopic examination.

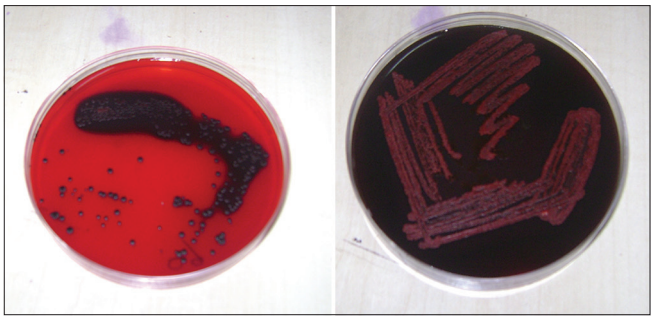

Figure 1: *Staphylococcus epidermidis* biofilm by congo red agar method

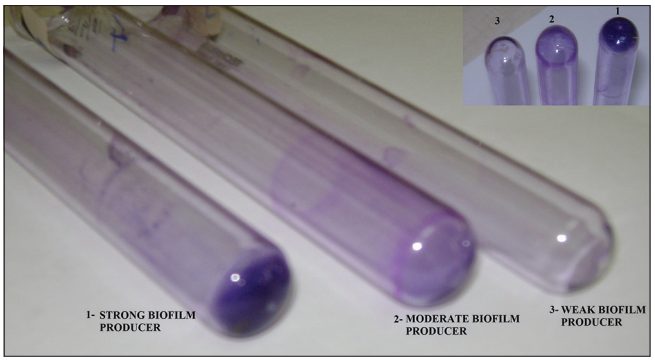

Figure 2: Biofilm procdution by *Staphylococcus epidermidis* using tube method

Figure 3: Biofilm procdution by *Staphylococcus epidermidis* using microtitre plate method

The adherence of CoNS to smooth surfaces has been estimated by measuring the optical densities of stained bacterial films adherent to the floors of plastic MTPs. The optical densities of bacterial films adherent to plastic MTPs act as a quantitative model for the study of the adherence of CoNS to medical devices. This may correlate with pathogenesis of infections in the presence of medical devices. The modified tissue culture plate method was most sensitive (96.2%), specific (94.5%), and accurate (97.3%) in terms of discriminating between biofilm producers and non-producers. In the case of TM, though the strong

biofilm producers could be easily detected, the difficulty in differentiating between moderate and weak biofilm forming isolates affected its performance in terms of sensitivity (77.9%), specificity (96.0%), and accuracy (86.8%). The Congo read agar method showed very little correlation with corresponding methods and the parameters of sensitivity (7.6%), specificity (97.2%), and accuracy (51.3%) were very low.[18] The tissue culture plate or MTP method also has the advantage of being a quantitative model to study the adherence of staphylococci on biomedical devices.[19]

SUBJECTS AND METHODS

The study was conducted to determine the ability of CoNS to form biofilms. This was studied employing two different qualitative methods, namely, Congo red agar method[7] and TM.[18] Quantitative detection of biofilm (adherence) was detected by microtiter plate method.[17]

In this study, 337 isolates of CoNS were isolated from clinical samples.[18] Two hundred and fifty-five samples were ruled out of the study as contaminants. A total of 75 isolates were considered as clinically significant isolates based on clinical and laboratory parameters and were taken up for the study. These isolates were segregated into two groups – Isolates with definite clinical significance (Group A) and isolates with doubtful significance (Group B). Group A comprised of 45 isolates of *S. epidermidis* and Group B comprised of 30 isolates of *S. epidermidis*.

Modified Congo red agar method – The Congo red test is based on the ability of this dye to stain polysaccharides black. Hence, if a strain is able to synthesize capsular polysaccharide and if the Congo red is incorporated in the culture medium, the colony will be black in color (Freeman *et al.* 1989). The media comprised of Trypticase soy broth, 5% sucrose, agar 3%, and Congo red dye 0.4%. The test cultures were inoculated on the Congo red agar plates and incubated aerobically for 24–48 h. Different concentrations of agar (2% and 3%) were tested with varying concentration of Congo red dye (0.2%, 0.4%, and 0.8%) and were tried. About 3% agar with 0.4% of Congo red stain gave consistent results with clear demarcation between biofilm forming and negative strains.[18]

The appearance of black colored colonies was indicative of biofilm formation and isolates producing black colonies were considered as strong biofilm producers. Weak biofilm producers produced dark pink colonies. Non-biofilm producers were seen as red and dry colonies (Figure 1).

TM – Test isolates were inoculated in Trypticase soy broth and incubated overnight at 37°C. After incubation, the tubes were decanted and washed thrice with phosphate buffer saline (pH 7.3). The tubes were air dried and stained with 0.1% crystal violet. After incubation for 10 min, the stain was decanted and washed with phosphate buffer saline. The tubes were dried in inverted position and observed for biofilm formation. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Tubes were examined and the amount of biofilm formation was scored as absent, weak, moderate, or strong. Ring formation at the liquid interface was not indicative of biofilm formation (Figure 2).

Microtiter plate method – Test isolates were inoculated in Trypticase soy broth. The tubes were incubated overnight aerobically at 37°c. The broth culture was diluted 1:10 with freshly prepared Trypticase soy broth. A 96 well microtiter plate with flat bottom was used. First three wells served as media controls without addition of cultures. Two known in house positive and two negative controls were inoculated in each plate. The test organism diluted in Trypticase soy broth was inoculated in triplicate and incubated overnight at 37°C aerobically. After 24 h of incubation on microtiter plate was washed 3 times with phosphate buffer saline to remove the free floating planktonic bacteria, 300 μl of methanol was added to each well and allowed to stand for 15 min. The excess of methanol was discarded and the wells of tissue culture plate were stained using 0.1% safranin stain. After 20 min of staining, the excess stain was discarded and washed with phosphate buffer saline. Finally, 33% glacial acetic acid was added to fix the stain. OD readings were determined using ELISA autoreader at a wavelength of 490 nm. The OD readings were considered as an index of bacteria adhering to surface and forming biofilms (Figure 3).

RESULTS

Three methods of detection of biofilm, namely, the modified Congo red agar method, TM, and microtiter plate method were evaluated. Many authors have suggested brain heart infusion agar with addition of 5% sucrose and 0.8% of Congo red dye. However, this combination of brain heart infusion agar, 5% sucrose, and 0.8% Congo red dye did not work well in our hands. Hence, Trypticase soy broth was tried instead of brain heart infusion agar. Various concentrations of Congo red dye (0.2%, 0.4%, and 0.8%), sucrose (2%, 4%, and 6%) and agar (2%, 3%, and 4%) were tried. A combination of Trypticase soy broth with 5% sucrose, 0.4% Congo red dye, and 3% agar gave satisfactory results. Using this method among Group A isolates, 34 of 45 isolates of *S. epidermidis* were found to be non-biofilm producers. Seven out of 45 isolates were weak biofilm producers, one isolate of *S. epidermidis* was

found to be moderate biofilm producer and four out of 45 isolates of *S. epidermidis* were found to be strong biofilm producers producing jet black crystalline colonies. Among Group B isolates, 22 out of 30 *S. epidermidis* were found to be non-biofilm producers (70%), five out of 30 isolates were categorized as weak biofilm producers, and three out of 30 isolates were strong biofilm producers. TM of assessment of biofilm was evaluated using 0.1% crystal violet stain. In Group A, 25 out of 45 isolates of *S. epidermidis* were found to be non-adherent, three out of 45 isolates of *S. epidermidis* were weak biofilm producers, nine out of 45 isolates of *S. epidermidis* were moderate biofilm producers, and nine out of 45 isolates of *S. epidermidis* were strong biofilm producers. In Group B, 11 out of 30 isolates of *S. epidermidis* were non-adherent, four out of 30 isolates were weak biofilm producers, ten out of 30 isolates of *S. epidermidis* were moderate biofilm producers, and six out of 30 isolates were strong biofilm producers. Both modified Congo red agar method and TM did not provide a quantitative analysis on biofilm production. The results of both these methods were prone to observer bias.

The more sensitive and quantitative method of estimation of biofilm production was by microtiter plate method. Two dyes, namely, 0.1% crystal violet and 0.1% safranin were tried. In our study, 0.1% safranin yielded better results. In Group A, 11 out of 45 isolates of *S. epidermidis* were found to be weak biofilm producers, 21 were moderate biofilm producers, and 14 were strong biofilm producers. In Group B, 17 out of 30 isolates of *S. epidermidis* were weak biofilm producers, eight out of 30 were moderate biofilm producers, and six were strong biofilm producers. The comparison of the three methods showed that microtiter plate method was more sensitive in detecting of biofilm and helps in quantitative assessment on the amount of biofilm formation.

Statistical significance of difference between Group A and Group B isolates of *S. epidermidis* with reference to the degree of biofilm production was assessed using Chi-square test and was found to be statistically significant, *P* value being 0.004.

DISCUSSION

CONS are ubiquitous in nature. Their presence in large numbers on the skin and their minimal nutritional requirements coupled with very potent virulence factors such as biofilm formation provides a survival advantage to this Gram-positive cocci. The increased use of indwelling devices and inadvertent use of antibiotics have helped this commensal become a potential pathogen. In the era of increasing immunocompromised population and emerging and re-emerging infections, CoNS has established itself as a pathogenic bacteria. The dilemma exists in differentiating commensal CoNS from the offending organism. Antibiotic resistance (MR-CoNS) alone cannot be taken into account for differentiating commensal from pathogenic CoNS as many of the commensal CoNS exhibit resistance to cefoxitin. The cost-effective alternative available is assessment of biofilm formation. Biofilm if present would mean that the antibiotics may not be fully effective as bacteria are not exposed to the action of antibiotic. The use of nucleic acid amplification techniques for detection of biofilm associated genes is costly, cumbersome, and need technical expertise which may not be available everywhere. Biofilm production is one of the major characters which help a commensal bacterium to become pathogenic under appropriate situations. Biofilms are communities of microorganisms that stick to each other or to the surfaces by production of extracellular matrix comprising of polysaccharides and proteins. First, the bacterium attaches to surfaces by the use of non-specific factors such as hydrophobicity and surface charge. Bacterium may also adhere to surfaces through cell wall teichoic acids and proteins, such as autolysins or cell wall associated proteins that interfere with collagen, fibronectin, or other matrix proteins. After this initial phase of adherence comes the stage of actual biofilm formation where the bacteria produce factors helping in cell to cell contact. The most commonly isolated CoNS, *S. epidermidis* produces PIA. PIA comprises of ß-1,6-linked glucose aminoglycan substituted with different side groups. Other factors that mediate biofilm are surface associated proteins, Aap,and Bap/Bhp. CoNS in hospital environment or in device associated infections differ from the commensal CoNS. Nosocomial CoNS form thick multilayered biofilms on polymers or metals.

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The comparison of these three methods of biofilm production leads us to conclude that the biofilm detection by Microtiter plate method is more sensitive and also helps in qualitative assessment of biofilm formation. In our

study, 30.4% of isolates causing infections were strong biofilm producers.

CONCLUSION

The above mentioned methods are cost-effective and need minimal training of laboratory staff and do not require any special instruments. The procedure can be carried out along with the routine bacteriological workup of a laboratory. The detection of biofilm production will be an added tool in the hands of a microbiologist to differentiate pathogenic and commensal CoNS. The reporting of biofilm will help the clinician to plan the appropriate line of therapy. Routine reporting of biofilm will create an atmosphere where the microbiologist and clinician can join hands toward successful antibiotic stewardship.

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