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Original Article

Assessment of Biofilm Production in Clinically Significant Isolates of *Staphylococcus epidermidis* and Comparison of Qualitative and Quantitative Methods of Biofilm Production in a Tertiary Care Hospital

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Abstract

Introduction: Coagulase-negative staphylococci (CONS) transformed from being commensals to pathogens causing a wide variety of infections. Meager nutritional requirements and ability to withstand various physical, chemical agents have made CONS a successful pathogen. Main virulence factor associated with CONS infections is biofilm formation. Biofilm helps CONS adhere to surfaces to escape the assault by immune mechanisms and antibiotics. The estimation of biofilm formation will help differentiate between commensal and pathogenic CONS.

Purpose: To determine clinically significant CONS and to ascertain their virulence using qualitative and quantitative methods of biofilm detection.

Materials and Methods: A total of 75 clinically significant isolates were taken up for the study. These isolates were segregated into two groups: Isolates with definite clinical significance (Group A - 45 isolates) and isolates with moderate significance (Group B - 30 isolates). Two qualitative methods Congo red agar method and tube method were employed. Quantitative detection of biofilm (adherence) was detected by microtiter plate (MTP) method.

Results: The more sensitive and quantitative method was MTP method. In Group A, 20 were moderate biofilm producers and 14 were strong biofilm producers. In Group B, 8 out of 30 were moderate biofilm producers and 6 were strong biofilm producers. The comparison of the three methods showed that MTP method was more sensitive in detecting of biofilm and helps in quantitative assessment on the amount of biofilm formation. Statistical significance of the difference between Group A and Group B isolates was found to be statistically significant, $P = 0.004$.

Conclusion: These methods are cost-effective and need minimal technical training. 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: Adherence, Biofilm, Congo red agar, Microtiter plate method, *Staphylococcus epidermidis*, Tube method

INTRODUCTION

Coagulase-negative staphylococci (CONS) are Gram-positive cocci living on each and every part of our body and are seen abundantly in nature. As of now, 47 species and 23 subspecies have been identified.[1] This is excluding the animal pathogens and purely environmental organisms. These organisms are sturdy; they can live on meager nutrition, do not have specific growth requirements, grow well in a wide range of temperature and pH, and can form biofilms.[2] All these characteristics have given CONS the ability to thrive in varied circumstances. The mere number present on the skin in the presence of an ever growing population with waning immunity has given this organism a formidable stature. Modern medicine has grown by leap

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and bounds and so has the use of prosthetic devices. Here, the ability of biofilm formation gives CONS the survival advantage it needs to be a successful pathogen.[3,4] CONS are no more the commensals; they were thought to be in the early 1900's. Their presence in routine cultures can no more be discarded as improper sampling or contamination. Time is ripe to regard them as part of pathogens and proceed cautiously in the light of clinical correlation. The most common species implicated in human infections are *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*. This is followed by other species such as *Staphylococcus saprophyticus, Staphylococcus lugdunensis, Staphylococcus hominis, Staphylococcus warneri, Staphylococcus cohnii, Staphylococcus simulans, Staphylococcus schleiferi, S. warneri,* and *Staphylococcus capitis*. Just like the established pathogen *Staphylococcus aureus* members of CONS are capable of causing a variety of infections. It can range from mild to moderate skin and soft tissue infections to limb and life-threatening infections such as bacteremia, native and prosthetic valve endocarditis, ophthalmic infections, prosthetic joint infections, and device-associated infection (cerebrospinal fluid [CSF] shunts, indwelling CSF catheters, intrathecal pumps, and ventriculostomy sites).[5] CONS-related urinary tract infections (UTIs) and catheter-related UTIs are on the rise. Among CONS, *S. saprophyticus* is strongly related to uncomplicated UTI in sexually active young females.

The property of adhesion by CONS was first observed by Bayston and Penny in 1972.[6] They observed mucoid colonies of CONS from CSF shunts. This was followed by many investigators using scanning electron microscopy on various prosthetic instruments and implants. These included critical devices such as peritoneal dialysis catheter,[7] intravascular catheters,[8-10,11] and pacemakers.[12,13]

Biofilms are composed of bacteria that stick to each other as well as to surfaces forming large communities. They produce an extracellular matrix comprising of polysaccharides and proteins.[14] The matrix allows the bacteria to stick to surfaces. The process of adhesion happens in a phased manner. Biofilms can be formed on biotic (like host tissue) or abiotic surfaces (like implants).[4] From here on the formation of biofilm happens in 4 steps. The initial attraction of bacteria toward a polymer surface can be due to hydrophobic interactions, van der Waal's forces, or surface charge.[1] Bacterium may also adhere via cell wall teichoic acids and proteins, such as autolysins or cell wall-associated proteins that interfere with collagen, fibronectin, or other matrix proteins. The bacteria quickly attach to biotic or abiotic surface. This is followed by rapid proliferation of bacteria and intracellular adhesion. Slowly, the biofilm matures into thick structured layer. This multilayered structure is well organized to have fluid filled cavities and channels. These channels play an important role in supply of nutrition and the much required oxygen to the proliferating bacterial cells. *S. epidermidis,* which is the most common isolate member of the CONS family, produces polysaccharide intracellular adhesion (PIA). PIA comprises 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 a hospital environment or device-associated infections differ from the commensal CONS. Nosocomial CONS form thick multilayered biofilms on polymers or metals.[3]

The amount of biofilm production in CONS can help us assess 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.[15-18] Among the slime producers, *S. epidermidis* was the most prevalent species.[16,19] Nearly, 40-50% of CONS isolates from clinical specimens can be slime producers.[19-22] Bacterial films produced by a standard slime-producing strain of CONS on plastic tissue culture plates varied with the type of fixative.[23] The incidence of biofilm production by *S. saprophyticus* is comparatively less than *S. epidermidis*.[24] The percentage of slime-producing CONS ranged from 20% in the peritoneal fluid to 66% in CSF.

A number of simple and cost-effective tests are available to detect slime production by Staphylococci. The methods include microtiter plate (MTP) method,[25] tube method (TM),[25] Congo red agar (CRA),[11,26] bioluminescent assay,[4] and light or fluorescence[27] or confocal microscopic examination.[9] Marrie and Costerton have studied the biofilm formation using transmission electron microscopy in intravenous and intra-arterial catheters.[28]

Assessment of biofilm has been tried with different methods. The CRA method and TM are qualitative methods of assessment, whereas the MTP method is a quantitative method. TM helps in detection of strong biofilm producers. It is difficult to differentiate between moderate and weak biofilm producers using TM. The technically simple CRA method has very low level of correlation when compared with other methods. The sensitivity, specificity, and accuracy using the CRA method were elucidated in previous studies done by different researchers. The sensitivity, specificity, and accuracy were 7.6%, 97.2%, and 51.3%, respectively.[29] The TM, on the other hand, showed 77.9% sensitivity, 96% specificity, and 86.8% accuracy.[25] The qualitative method of biofilm estimation done by MTP method scored much better with a sensitivity of 96.2%, specificity of 94.5%, and accuracy of 97.3%.[25] The tissue culture plate or MTP method also has the advantage of being a quantitative model to study

biofilm formation by CONS on biomedical devices.[29] This study aimed at identifying the clinically significant *S. epidermidis* isolates and compares their ability to form biofilm using qualitative and quantitative methods.

MATERIALS AND METHODS

The aim of the study was to determine the ability of CONS to form biofilms. This was done employing two different qualitative methods CRA method[11] and TM.[25] Quantitative detection of biofilm was detected using MTP method.[25]

This study was carried out in SRM Medical College Hospital and Research Centre, Kattankulathur, Kancheepuram District of Tamil Nadu. The study period was from April 2012 to March 2013. The study was carried out after obtaining the Institutional Ethics Committee approval. During the study period, 337 isolates of CONS were isolated from clinical samples. 262 samples which did not correlate with the clinical status of the patient were ruled out as contaminants/skin commensals. A total of 75 isolates were confirmed to be clinically significant isolates based on clinical and lab parameters. These isolates were segregated into two groups - isolates with definite clinical significance (Group A). The term definite significance was applied to those isolates which showed a clinical correlation in terms of signs of infection like fever and elevated white blood cell counts along with repeated isolation of the same organism. The comparison group was the isolates with moderate significance (Group B). Group B consisted of isolates which showed up on repeat cultures along with signs of infection like fever but did not show elevated white blood cell counts. Group A comprised 45 isolates of *S. epidermidis* and Group B comprised 30 isolates of *S. epidermidis* (Graph 1).

Modified CRA method - the test is based on the property of Congo red to stain polysaccharides black. If a given strain produces enough polysaccharide in the presence of Congo red in the medium, the colony formed will be black.[26] As a trial procedure to ascertain the percentage of various components that need to be added to the basal medium different concentrations of Agar (2%, 3%, 4%, and 6%) and varied concentrations of Congo red dye (0.2%, 0.4%, and 0.8%) were tried. 3% agar and 0.4% Congo red stain gave consistent results demarcating the biofilm producers and non-biofilm producers. In this study, we used trypticase soy broth as the basal media and added 5% sucrose, 3% agar, and 0.4% Congo red dye. The test samples were inoculated on the CRA plates and incubated aerobically for 24-48 h. The appearances of black-colored colonies were indicative of strong biofilm formation. Weak biofilm producers produced dark pink colonies. Non-biofilm producers were seen as red, dry colonies.

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 dried in air 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.

MTP 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 MTP with flat bottom was used. First, three wells served as media controls without addition of cultures. 2 known in house positive and 2 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 MTP, it was washed thrice 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. Optical density (OD) readings were determined using ELISA auto reader at a wavelength of 490 nm. The OD readings were considered as an index of bacteria adhering to the surface and forming biofilms.

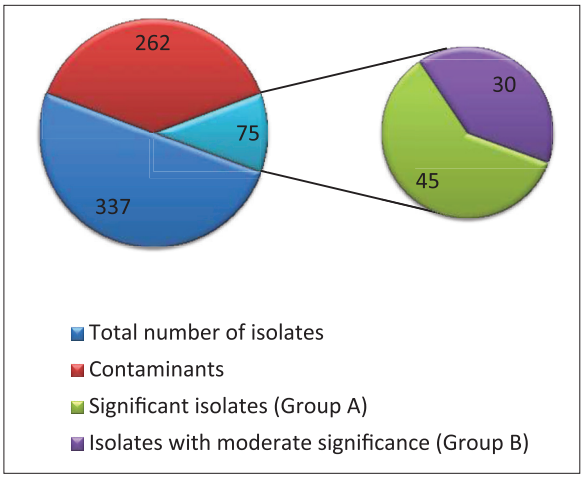
RESULTS

Biofilm production by CONS was evaluated using three different methods: Modified CRA method, TM, and MTP method. Literature suggests the use of brain heart infusion agar with addition of 5% sucrose and 0.8% of Congo red dye. In the pilot study conducted, this combination did not work well and hence a modified method comprising of 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. The final combination of trypticase soy broth with 5% sucrose, 0.4% Congo red dye, and 3% agar gave satisfactory results. Group A showed 33 of 45 isolates of *S. epidermidis* to be non-biofilm

Table 1: Comparison of biofilm detection using quantitative and qualitative methods

Groups	CRA method (%)				TM (%)				MTP method (%)			
	Non-adherent	Weak	Moderate	Strong	Non-adherent	Weak	Moderate	Strong	Non-adherent	Weak	Moderate	Strong
Group A (*n*=45)	33 (73.3)	7 (15.5)	1 (2.2)	4 (8.9)	25 (55.5)	3 (6.7)	8 (17.8)	9 (20)	0 (0)	11 (24.4)	20 (44.4)	14 (31.1)
Group B (*n*=30)	21 (70.0)	5 (16.7)	0 (0)	4 (13.3)	10 (33.3)	4 (13.3)	10 (33.3)	6 (20.0)	0 (0)	16 (53.3)	8 (26.7)	6 (20.0)

CRA: Congo red agar, TM: Tube method, MTP: Microtiter plate



Graph 1: Distribution of isolates of *Staphylococcus epidermidis* during study period

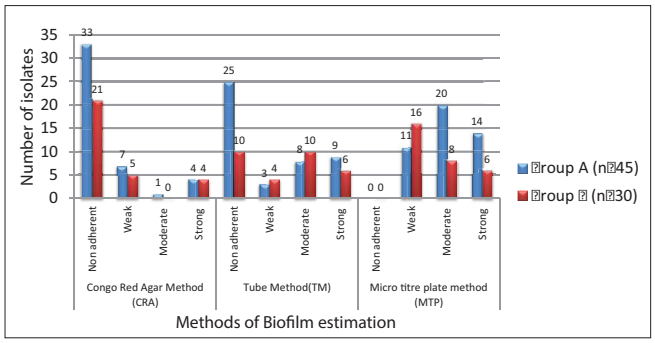
producers. 7 out of 45 isolates were weak biofilm producers, 1 isolate of *S. epidermidis* was found to be moderate biofilm producer, and 4 out of 45 isolates of *S. epidermidis* were found to be strong biofilm producers producing jet black crystalline colonies. In Group B, 21 out of 30 isolates were non-biofilm producers, 5 out of 30 isolates showed weak biofilm formation, and 4 isolates showed strong biofilm formation. 0.1% crystal violet stain was used in TM for assessment of biofilm. In Group A, 25 out of 45 isolates of *S. epidermidis* were found to be non-adherent, 3 out of 45 isolates of *S. epidermidis* were weak biofilm producers, 8 out of 45 isolates of *S. epidermidis* were moderate biofilm producers, and 9 out of 45 isolates of *S. epidermidis* were strong biofilm producers. In Group B, 10 out of 30 isolates of *S. epidermidis* were non-adherent, 4 out of 30 isolates were weak biofilm producers, 10 out of 30 isolates of *S. epidermidis* were moderate biofilm producers, and 6 out of 30 isolates were strong biofilm producers. Both modified CRA method and TM failed to provide a quantitative analysis on biofilm production. The results of both these methods were prone to observer bias (Graph 2).

The more sensitive and quantitative method of estimation of biofilm production was by MTP method. Two dyes, 0.1% crystal violet, and 0.1% safranin were used for assessment of biofilm production. 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, 20 were moderate biofilm producers, and 14 were strong biofilm producers (Figure 1). In Group B, 16 out of 30 isolates of *S. epidermidis* were weak biofilm producers, 8 out of 30 were moderate biofilm producers, and 6 were strong biofilm producers (Table 1). The comparison of the three methods showed that MTP method was more sensitive in detecting of biofilm and helps in quantitative assessment on the amount of biofilm formation.

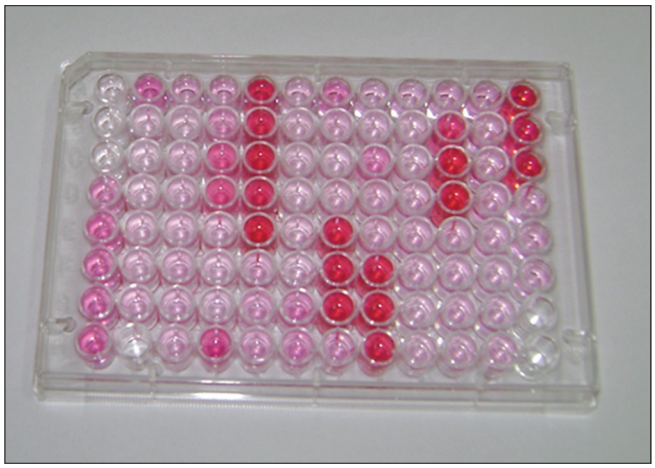
Statistical significance of the difference between Group A and Group B isolates of *S. epidermidis* with reference to the degree of biofilm production was assessed using Chi-square test and were found to be statistically significant, $P = 0.004$.

DISCUSSION

CONS are ubiquitous in nature. The mere presence in large numbers on the skin, minimal nutritional requirements coupled with very potent virulence factors such as biofilm formation provide a survival advantage to this organism. Technological innovations in the field of science have resulted in increased use of indwelling devices. This coupled with inadvertent use of antibiotics has helped this commensal become a potential pathogen. In the era of increasing immuno/immune compromised population and emerging and re-emerging infections, CONS have established itself as pathogenic bacteria. The dilemma exists in differentiating commensal CONS from the offending organism. Antibiotic resistance methicillin resistance 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 the 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 are costly, cumbersome and need technical expertise which may not be available everywhere in resource-poor countries. 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



Graph 2: Comparison of biofilm detection using quantitative and qualitative methods



Graph 3: Determination of biofilm production by *Staphylococcus epidermidis* isolates using safranin dye in microtiter plate method (quantitative method)

by the 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 via 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 the 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.

Three methods of detection of biofilm, namely, the modified CRA method, TM, and MTP 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. An alternative method using 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.

The comparison of these three methods of biofilm production leads us to conclude that the biofilm detection by MTP 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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