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COMPARISON OF QUALITATIVE AND QUANTITATIVE TECHNIQUES OF BIOFILM PRODUCTION IN *STAPHYLOCOCCUS EPIDERMIDIS*

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ABSTRACT
Staphylococcus epidermidis with its non fastidious nature has evolved as successful pathogen causing wide variety of infections. One of the main virulence determinants of *Staphylococcus epidermidis* is biofilm formation which helps escape the immune assault and antibiotics. The estimation of biofilm formation will help differentiate between commensal and pathogenic *S.epidermidis*. **Aim & Objective**: To determine clinically significant *Staphylococcus epidermidis* and to ascertain their virulence using qualitative and quantitative methods of biofilm detection. **Methodology** 76 clinically significant isolates were segregated into two groups - Isolates with definite clinical significance (Group A - 46isolates) and Isolates with doubtful significance (Group B – 30isolates).Two qualitative methods Congo red agar method, Tube method were employed. Quantitative detection of biofilm (adherence) was detected by Microtitre plate method. **Results**: The more sensitive and quantitative method was microtitre plate method. In group A 21 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 methods showed that microtitre plate method was more sensitive in detecting of biofilm and helps in quantitative assessment of biofilm formation. Difference between Group A and Group B isolates was statistically significant, p value being <0.004. **Discussion**: These methods are cost effective and need minimal technical training. Identifying Biofilm will help differentiate pathogenic and commensal CONS. The reporting of Biofilm will help the clinician plan appropriate line of therapy.

KEYWORDS: CONS, Biofilm, Congo red agar, *Staphylococcus epidermidis*.

INTRODUCTION
Staphylococcus epidermidis, once considered as mere skin commensal and innocuous has in the recent years become a pathogenic cocci. The increase in use of invasive devices, the increasing population of immunocompromised patients, community and nosocomial infections affecting extremes of age have aided the establishment of *Staphylococcus epidermidis* as a very potent pathogen. As early as 1970's the *Staphylococcus epidermidis* were noted to attach themselves to prosthetic valves, shunts, urinary catheters and intra vascular devices.[1] *Staphylococcus epidermidis* has been notorious with various infections like bacteremia, urinary tract infection, ophthalmic infections, prosthetic joint infections, native and prosthetic valve endocarditis, catheter associated urinary tract infections (CAUTI), device associated infection (CSF shunts, indwelling CSF catheters, intrathecal pumps and ventriculostomy sites). The capacity to adhere to polymer surfaces followed by biofilm production heralds the Pathogenicity of *Staphylococcus epidermidis*.[2]

Biofilms are communities of microorganisms that stick together or to the surfaces by production of extracellular matrix comprising of polysacchrides and proteins.[3] In the initial phase bacterium attach to surfaces by the use of nonspecific factors like hydrophobicity and surface charge. Bacterium may also adhere to Surfaces using 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. *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, Aap (accumulation associated proteins) and Bap/Bhp (Biofilm associated proteins). Biofilms on medical devices are difficult to eradicate as the bacteria in biofilm layers can be upto 1000 fold resistant compared to the free living forms. This can be due to restricted penetration, decreased growth rate, a distinct genetic phenotype with resistance against a particular antibiotic or resistance against host mechanism, the expression of resistance genes and the presence of biofilm persister cells.[4]

In response to infection by biofilm producers the human body mounts an immune response but due to the architecture of biofilm the immune response fails to eradicate the biofilm. More damage is imminent when the surrounding host tissue is also damaged by the immune mechanisms. The free floating or planktonic cells may be responsible in development of infection in sites far away from primary infection. This in turn leads to recurrence of infections.[5]

The differentiation of CONS based on biofilm formation will help to predict the impact of CONS in device related infections. Studies done in the past indicate that clinically significant bloodstream isolates of CONS produced slime[6,7,8,9] Among the slime producers, S. epidermidis was the most prevalent species.[7,10] Nearly 40-50% of CONS isolates from clinical specimens can be slime producers.[4,7,10,12] Bacterial films produced by a standard slime producing strain of CONS on plastic tissue culture plates varied with the type of fixative.[13]

A number of tests are available to detect slime production by *Staphylococcus epidermidis*. The methods include Micro titre plate (MTP) method[14], Tube method[14] Congo red agar[15], bioluminescent assay[16] and light or fluorescence or confocal microscopic examination. 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 micro titre plates. This acts as a quantitative model for the study of the attachment of CONS to medical devices. This will help understand the manner in which CONS can cause or enhance infection in the presence of a medical device. Previous studies indicate that the modified tissue culture plate method (TCP) was most sensitive (96.2%), specific (94.5%), and accurate (97.3%) in terms of discriminating between biofilm producers and non-producers[15]. Tube method of biofilm detection was dependable when using strong biofilm producing strains. When the same method was applied to moderate and weak biofilm producers the results were not encouraging. Tube method showed a sensitivity (77.9%), specificity (96.0%), and accuracy (86.8 %). The (CRA) Congo read agar method showed very little correlation with biofilm production. It showed a very low sensitivity (7.6 %). specificity (97.2 %), and accuracy (51.3 %) were very low.[15] 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.[17]

MATERIALS AND METHODS

This study was carried out in SRM Medical College Hospital & Research centre during April 2012- March 2013. Here two different qualitative methods, namely Congo red agar method[16] and Tube method[15] were used. Quantitative detection of biofilm production was detected by microtitre plate method[14]. A total of 337 isolates were identified during the study period. After processing the samples, clinical correlation showed 255 samples were mere contaminants and were excluded from the study. 82 clinically significant isolates based on clinical and lab parameters and were subjected to further speciation. After speciation 6 isolates belonged to *Staphylococcus hemolyticus*, *Staphylococcus saprophyticus* & *Staphylococcus capitis*. "These isolates" to be deleted. The remaining 76 isolates were segregated into two groups - Isolates with definite clinical significance (Group A) and Isolates with doubtful significance (Group B). Group A comprised of 46 isolates of *S.epidermidis* and Group B comprised of 30 isolates of *S.epidermidis*.

Modified Congo Red Agar method - The Congo red method utilizes the dye's property of staining the polysacchrides 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 colour[15]. This media comprised of Trypticase soy broth, 5% sucrose, agar 3% and Congo red dye 0.4%. The test cultures were inoculated on the Congored agar plates and incubated aerobically for 24-48 hours. Different concentration of agar (2% and 3%) were tested with varying concentration of Congo red dye (0.2%, 0.4%, 0.8%) were tried. 3% agar with 0.4% of Congored stain gave consistent results with clear demarcation between biofilm forming and negative strains. Inference of the test was based on the colour production. Strong biofilm producers developed black coloured colonies. Weak biofilm producers had dark pink colonies whereas non biofilm producers were seen as red, dry colonies.

Tube method – 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 10mins the stain was decanted and tube 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.

Microtitre 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 microtitre plate with flat bottom was used. First three wells served as media controls without addition of cultures. Two known 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 24hours of incubation the Microtitre plate was washed three times with phosphate buffer saline to remove the free floating planktonic bacteria 300µl of methanol was added to each well and allowed to stand for 15mins. The excess of methanol was discarded and the wells of tissue culture plate were stained using 0.1% safranin stain. After 20mins 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 490nm. The OD readings were considered as an index of bacteria adhering to surface and forming biofilms.

RESULTS

Modified congored agar method, tube method and microtitre plate method were evaluated. Previous studies have used the base of Brain heart infusion agar with addition of 5% sucrose and 0.8% of Congo red dye**.** In this study Trypticase soy broth was tried instead of brain heart infusion agar. Various concentrations of congored 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 46 isolates of *Staphylococcus epidermidis* were found to be non biofilm producers. 7 out of 46 isolates were weak biofilm producers, one isolate of *Staphylococcus epidermidis* was found to be moderate biofilm producer and 4 out of 46 isolates of *Staphylococcus epidermidis* were found to be strong biofilm producers producing jet black crystalline colonies. In Group B 21 out of 30 *Staphylococcus epidermidis* were found to be non biofilm producers (70%), 5 out of 30 isolates were categorized as weak bio film producers and 4 out of 30 isolates were strong biofilm producers. (Fig 1).

Biofilm production was assessed by tube method with 0.1% crystal violet stain. In group A 25 out of 46 isolates of *Staphylococcus epidermidis* were non adherent, 3 out of 46 isolates of *Staphylococcus epidermidis* were weak biofilm producers, 9 out of 46 isolates of *Staphylococcus epidermidis* were moderate biofilm producers and 9 out of 46 isolates of *Staphylococcus epidermidis* were strong biofilm producers.

In group B 10 out of 30 isolates of *Staphylococcus epidermidis* were non adherent, 4 out of 30 isolates were weak biofilm producers, 10 out of 30 isolates of *Staphylococcus epidermidis* were moderate biofilm producers and 6 out of 30 isolates were strong biofilm producers. The modified congo red agar method and tube method did not provide a quantitative analysis on biofilm production. The results of both these methods were known to have observer bias.

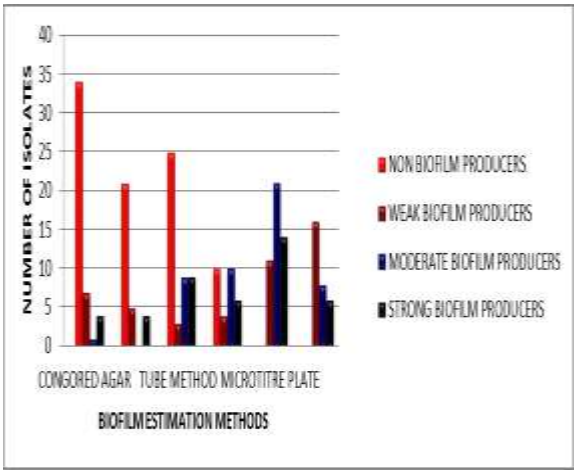

Fig: 1. Graph showing the detection of biofilm production by different methods of biofilm estimation.

Fig-2 Estimation of Biofilm production using Safranin in Microtitre plate method

The sensitive quantitative method of estimation of biofilm production was by microtitre plate method. 0.1% safranin was used to stain the biofilm formed in the Microtitre plate (Fig-2). In group A 11 out of 46 isolates of *Staphylococcus epidermidis* were found to be weak biofilm producers, 21 were moderate biofilm producers and 14 were strong biofilm producers. In group B 16 out of 30 isolates of *Staphylococcus epidermidis* were weak biofilm producers, 8 out of 30 were moderate biofilm producers and 6 were strong biofilm producers. The comparison of the three methods showed that microtitre 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 *Staphylococcus epidermidis* with reference to the degree of biofilm production was assessed using Chi square test and were found to be statistically significant, P value being <0.004.

DISCUSSION

Staphylococcus epidermidis is an excellent example for commensal to pathogen transformation. Their presence in large numbers on the skin, their minimal nutritional requirements along with potent virulence factors like Antibiotic resistance, biofilm formation provide the much needed survival advantage. The advent of newer antibiotics witnessed a change in the prescribing pattern. The evolving mindset of the patient as well as the treating physician resulted in improperly timed regimens, under dosing of antibiotics and use of irrational combinations. The net result is that we now have a hoard of drug resistant bacteria.

Improvements in patient care also meant increased use of prosthetic devices. This gave the biofilm forming strains of bacteria an immediate access into the human body. *Staphylococcus epidermidis* being abundantly present on the skin had a better opportunity to cause device associated infections. In the era of evolving drug resistance and ever increasing immunocompromised population, *Staphylococcus epidermidis* has secured its place as pathogenic bacterium. The dilemma exists in differentiating commensal from the offending organism. Antibiotic resistance (MRSE) alone cannot be taken into account for differentiating commensal from pathogens as many of the commensal CONS exhibit resistance to cefoxitin.

Molecular methods are available and can pinpoint the associated possible virulence but these methods are not cost effective. They need skilled labour and appropriate laboratory settings. The cost effective alternative 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. Among virulence factors analysis of biofilm production helps a commensal bacterium to become pathogenic under clinical settings. Biofilms are communities of microorganisms that stick to each other or to the surfaces by production of extracellular matrix comprising of polysacchrides and proteins. First the bacterium attach to surfaces by the use of nonspecific factors like hydrophobicity and surface charge. Bacterium may also adhere to surfaces using cell wall teichoic acids and proteins, such as autolysins or cell wall associated proteins that interfere with collagen, fibronectin or other matrix proteins. Once the initial phase of adherence is completed the stage of actual biofilm formation starts. Here the bacteria produce factors helping in cell to cell contact. The most commonly isolated *Staphylococcus 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, Aap (accumulation associated proteins) and Bap/Bhp (Biofilm associated proteins). Hospital associated strains or nososcomial strains form thick multilayered biofilms on polymers or metals.

Three methods of detection of biofilm namely the modified congored agar method, tube method and microtitre plate method were evaluated. An alternative method using Trypticase soy broth was tried instead of brain heart infusion agar was tried. Various concentrations of congored 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 result analysis of these three methods of Biofilm production showed that the Biofilm detection by Microtitre 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.

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 help even the grass root level microbiologist to differentiate pathogenic and commensal CONS. The reporting of Biofilm will help the clinician to decide the appropriate line of therapy. Routine reporting of Biofilm will also lessen the antibiotic associated financial burden to the patient. The net result, if properly followed will take us towards successful antibiotic stewardship.

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