Biofilm production by *staphylococcus aureus* and *staphylococcus epidermidis*: an evaluation of three different screening methods

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Abstract

Purpose: The purpose of this study was to determine the rate of biofilm production by *S.aureus* and *S.epidermidis* isolated from different clinical specimens.

Methods: Quantitation of biofilm formation by *staphylococci* was carried out using three in vitro methods namely tissue culture plate (TCP), tube method (TM), and congo red agar (CRA) method.

Results: Out of 237 (94.8%) *S.aureus* isolates tested, 25 strains were found to form biofilm, out of these one (0.42%) was strong biofilm producer and 24 (10.12%) were moderate biofilm producers. In TM and CRA method 20 (8.43%) and 10 (4.2%) strains were moderate biofilm producers, rest were considered as weak or non-biofilm producers. All the 25 biofilm positive strains were *S.aureus*.

Conclusion: The TCP method is more sensitive to detect biofilm formation by *S.aureus* when compare to tube method (TM), and congo red agar (CRA) method.

Keywords: Biofilm, CRA, *S.aureus*, *S.epidermidis*, TCP, TM.

Introduction

Microbial biofilms are mechanisms used by microorganisms that cause chronic infections in humans.[1] Biofilms are associated with many diseases like osteomyelitis, cystic fibrosis, endocarditis, nosocomial diseases related to prosthetic heart valves, orthopedic devices, central venous catheters and urinary catheters.[2] Microbial biofilms develop when microorganisms adhere to surfaces and produce extracellulasr polymers that provide structural matrix which helps adhesion. The surface may be living or non-living material.

Biofilms on indwelling medical devices may be composed of bacteria or yeasts. *Staphylococcus epidermidis*, *Staphylococcus aureus*, *viridans streptococci*, *Pseudomonas aeruginosa*, *Eschrichia coli*, *Klebsiella pneumoniae* are commonly isolated from such biofilms.[2] Biofilms are characterised by the presence of extracellular polymeric substance encasing the cells. Biofilm-associated bacteria behave differently from freely suspended bacteria. With regards to growth rate and antibiotic resistance. Therefore, biofilm pose a public health problem.

Implantable medical device related infections by *S.aureus* and *S.epidermidis* are often difficult to treat.[3] virulence by *staphylococcal* is caused by a complex process that involves cell-to-cell communication through the release and response to chemical signals in a process called quorum sensing.[4] Biofilm production by staphylococci depends on the production of a polysaccharide intercellular adhesin (PIA) encoded by the *ica* operon comprising *icaA*, *icaB*, *icaC* and *icaD* genes.[5]

Biofilm associated staphylococcal infections are difficult to treat and may require antibiotic concentration 100-1000 times higher than that needed to treat freely suspended bacteria.[6]

The objectives of the study were

1. To determine the rate of biofilm production by *S.aureus* and *S.epidermidis* isolated from different clinical specimens.
2. To compare the reliability of tissue culture plate (TCP), tube method (TM) and congo red agar (CRA) method to detect the biofilm produced by *staphylococci*

Materials and Methods

A total of 250 strains of *staphylococci* were isolated from different clinical specimens like pus, exudate swab, blood, sequestrum etc. Among 250 staphylococci isolates, 237 were *S.aureus* followed by *S.epidermidis* 13. *Staphylococcus* spp. isolated from clinical specimens were identified by standard microbiological techniques. All cultures were maintained on nutrient agar slope, (Hi media Pvt. Ltd, Mumbai). The antibiotic susceptibility test was done using Kirby-Bauer disk diffusion method and interpretation was based on CLSI guidelines.[7] Antibiotics (Hi media Pvt. Ltd, Mumbai) used were - Ciprofloxcin (five µg), Co-trimaxazole (25µg) Erythromycin (15µg), Gentamicin (10µg), Netilmicin (30µg), Oxacillin (one µg), Penicillin (10u), Tetracycline (30µg), and Vancomycin (30µg). *S.aureus* ATCC 25922 was used as control.

Biofilm Detection

1. **Tissue Culture plate Method (TCP):** TCP method was done using a previously described

method.[8] Bacteria were inoculated into tryptic soy broth (Hi media, Mumbai) and incubated at 37°C for 18 h in a stationary condition and diluted 1 in 100 with fresh medium. Individual wells of tissue culture plate was filled with 0.2ml aliquots of the diluted cultures and only broth (control) to check sterility and non-specific binding of media.

The tissue culture plates were incubated for 24 at 37°C. By gently tapping the plate content of each well was removed. The wells were washed four times with 0.2ml of phosphate buffer saline (PBS pH 7.2) to remove free floating bacteria. Biofilm formed in plates was fixed with help of Sodium acetate solution (2% w/v) and stained with crystal violet (0.1 w/v). Excess stain was removed by washing with deionised water and plates were kept for drying. Optical density (OD) of stained adherent bacteria was determined with a micro ELISA autoreader at wavelength of 570nm (OD_{570}).These OD values were considered as an index of bacteria adhering to surface and forming biofilm. Classification (Table 1) of bacterial adherence by TCP method.

Table 1: Classification of bacterial adherence by TCP method

Mean OD values	Adherence	Biofilm formation
< 0.120	None	Non / Weak
0.120 – 0.240	Moderately	Moderate
> 0.240	Strong	High

2. **Tube Method:** Tube method described by Christensen et al was used.[9] Tube containing tryptic soy broth was inoculated with loopful of bacteria from overnight fresh culture and incubated for 24 h at 37°C. The tubes were decanted and washed with phosphate buffer saline (PBS pH 7.2) and dried. Dried tubes were stained with crystal violet (0.1%). Excess stain was removed and tubes were washed with deionised water. Observed for the biofilm formation after drying the tubes in inverted position.

Biofilm formation was considered positive when a visible film lined the wall and bottom of the tub. Ring formation at the liquid interface was considered negative.

Tubes were examined and biofilm formation was scored as 0- absent, 1- weak, 2 – moderate, 3 – strong.

3. **Congo red agar (CRA) Method:** This is an alternative method for screening biofilm formation by *Staphylococci*[10] The medium contains; Congo red, 0.8g; Agar, 20g and sucrose, 50g brain heart infusion broth, 1000ml. Congo red was prepared as concentrated aqueous solution and autoclaved for 15 min at 131°C Separately from other medium constituents and was added when the cooled at 55°C. After inoculation plates were incubated aerobically for 24-48 h at 37°C. The chemicals were purchased from Hi Media, Mumbai.

Positive results were indicated by black colonies with a dry crystalline consistency. Pink colonies were indicate weak slime production. A darkening of colonies with a absence of a dry crystalline colonial morphology indicates an intermediate result. The experiment was performed in triplicate and repeated three times. In all experiments, *S.epidermidis* ATCC 35984 (high slime producer) and *S.epidermidis* ATCC 12228 (non-slime producer) were used as controls.

Inoculation Procedure: Four or five colonies were picked from overnight growth on blood agar and inoculated into 5ml of nutrient broth. The tubes were incubated for 2 to 6h at 37°C and the turbidity was matched with McFarland 0.5 standard (Bacterial concentration 1.5 x 10^8 cfu/ml). The tubes were inoculated with 0.01 ml of standardized suspension, resulting in the final inoculum of approximately 10^6cfu/ml. Growth control was used with each test. And an aliquot of the inoculum was plated to check for purity and inoculum density. *S.aureus* ATCC 25923 was used as control. The tubes were incubated at 37°C for 18-24 h.

Statistics: Statistical analysis of the results was done using Wilcoxon Signed Rank, Kruskul-Wallis test, and Chi-Square test and p values <0.05 were considered significant.

Results

A total of 250 strain of staphylococci were isolated from different clinical specimens. Among 250 staphylococci isolates, 237 (94.8%) were *S.aureus* followed by *S.epidermidis* 13(5.2%). Among 237 *S.aureus* isolates majority (63.29%) were from pus, followed by exudate swab (12.65%), vaginal sawb (5.9%), blood (2.53%), nasal swab (2.53%), suction tip (2.10%), wound discharge (1.68%), tracheal aspirate (1.26%), sputum (1.26%), endotracheal tube tip (0.84%), biopsy (0.84%), ear swab (0.84%), ascitic fluid (0.42%), drain tip (0.42%), endotracheal suction (0.42%), pleural fluid (0.42%), post hernioplasty drain fluid (0.42%) and sequestrum (0.42%).

Among 13 *S.epidermidis* isolates, majority (38.46%) were from pus, followed by exudate swab (23.07%), blood (15.38%), ascitic fluid (7.69%), Foly's catheter tip (7.69%) and nasal polyp (7.69%).

The highest rate of *S.aureus* isolates (65 cases, 27.42%) was among the age group 31-40 years and the lowest (11 cases, 4.64%) among the age group 1-10years. Whereas the highest rate of *S.epidermidis* isolates (3 cases, 23.07%) among the age group 11-20 yrs and the lowest (1case, 15.38%) among the age group 31-40yrs.

The rate of *S.aureus* isolates was high in males (154; 64.97%) compared to females (83; 35.03%) and *S.epidermidis* also showed high rate in males (10; 76.92%) compared to females (3; 23.08%). The male: female was 1.8:1 and 3.3:1 respectively.

The efficiency of TCP, TM, CRA method in the detection of biofilm is shown in Table 2.

Table 2: Comparison of three methods for detection of biofilm by *staphylococci*

Species	Degree of biofilm	Method		
		TCP	TM	CRA
		Number (%)		
S.aureus (n=237)	Strong	1 (0.42%)	0 (0%)	0 (0%)
	Moderate	24 (10.12%)	20 (8.43%)	10 (4.2%)
	Weak / Non-producer	212 (89.45%)	217 (91.56%)	227 (95.78%)
S.epidermidis (n=13)	Strong	0 (0%)	0 (0%)	0 (0%)
	Moderate	0 (0%)	0 (0%)	0 (0%)
	Weak / Non-producer	13 (100%)	13 (100%)	13 (100%)

TCP: Tissue culture plate; TM: Tube method; CRA: Congo red agar method

In TCP method, out of 237strains of *S.aureus*, one (0.42%) was strong biofilm producer 24 (10.12%) were moderate and 212 (89.45%) isolates were considered as weak or non-biofilm producers. In tube method, there were no strong bioflim producer, 20(8.43%) were moderate and 217(91.56%) isolates were considered as weak or non-biofilm producers. In CRA method also there were no strong biofilm producers, 10(4.2%) were moderate and 227(95.78%) isolates were considered as non or weak biofilm producers. (Table 2) All biofilm producers were *S.aureus* and out of 13 *S.epidermidis* strains noneof the isolates were positive for biofilmformation.

The TM showed correlation with the TCP method for 5(20%) moderately biofilm forming isolates. However it was difficult to discriminate between moderate and weakly biofilm producing isolates and no correlation between colony morphology on CRA and TCP results was observed.

Among 25 biofilm producing *S.aureus* isolates, majority (56%) were from exudate swab followed by pus (20%), blood (12%), sequestrum (4%), suction catheter tip (4%) and endotracheal tube (4%) (Table 3).

Antibiotic susceptibility pattern was studied by disk diffusion method for 25 biofilm positive isolates. Among 25 isolates of *S.aureus*, maximum number (20; 80%) were resistant to pencillin and lowest to netillin (1; 4%), whereas all 25 isolates were susceptible to vancomycin, teicoplanin, rifampicin and linezolid.

Discussion

We studied 250 strains of *staphylococci* isolated from different clinical specimens for biofilm formation. Out of these 237 (94.8%) were *S.aureus* indicating prevalence of *S.aureus*. Of the 25 biofilm forming *S.aureus* strains, most of the strains were isolated from osteomyelitis, indicating the importance of biofilm in the pathogenesis of osteomyelitis. Our results with regard to isolation and role of biofilm by *S.aureus* in osteomyelitis is in correlation with previous study.[11] We tested 250 isolates of staphylococci by three different in vitro screening methods for their ability to form biofilm. In the TCP method, out of 237 strains of *S.aureus*, one (0.42%) isolate displayed a strong biofilm positive phenotype. This was in agreement with observations of other studies in which only few or no biofilm producing isolates could be detected using this medium.[12]

On the other hand TSB media supplemented with different sugars such as sucrose and glucose exhibited biofilm formation in more number of strains, by TCP method we can discriminate better between moderate and non-biofilm producing *staphylococci* which correlates well with previous studies.[12]

The tube method correlated well with the TCP method for 05 moderately biofilm producing isolates but it was difficult to discriminate between weak and biofilm negative isolates due to the variability in observing results from investigators. In the present study high variability was observed and classification in biofilm positive and negative was difficult by the tube method. These results correlates well with findings of a previous study.[13] Based on our results tube method cannot be recommended as a general screening test to detect biofilm production by bacteria.

In CRA method, out of 10 moderately biofilm positive isolates which showed no correlation with TCP and TM. Our observation is inconsistent with observations of a previous study.[14] Based on our results we do not recommend the CRA method for detection of biofilm formation by *staphylococci.*

The data of the present study indicate that the TCP method is more sensitive and superior than TM and CRA method for detection of biofilm This co-relates

with previous report.[15] It was also found to be an accurate and reproducible method for screening and this technique can serve as a reliable quantitative tool for determining biofilm formation by clinical isolates of *staphylococci*.

The biofilm formation is a complex multistep process that can be subdivided into the stages of attraction, adhesion and aggregation. These stages of adherence are mediated by a variety of nonspecific factors, such as electrostatic charge, hydrophobicity and specific factors, such as bacterial adhesins.[16]

Conclusion

The results of the present study show that biofilm can be formed by *S.aureus* and can pose problems in the treatment of biofilm associated infections. Tissue Culture plate Method (TCP) is more sensitive and reproducible method for screening and this technique can serve as a reliable quantitative tool for determining biofilm formation by clinical isolates of staphylococci.

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