

Original Article

## Biofilm formation by clinically isolated *Staphylococcus Aureus* from India

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### Abstract

**Introduction:** *Staphylococcal* biofilms are prominent cause for acute and chronic infection both in hospital and community settings across the world. Current study explores biofilm formation by *Staphylococcus aureus* isolates from clinical samples by different methods.

**Methodology:** Standard techniques used for the characterization of *S.aureus*. Qualitative and quantitative biofilm formation was assessed by Congo red Agar, Tube and Microtiter plate methods.

**Results:** A total of 188 clinical isolates of *S.aureus* were screened for biofilm formation and 72 (38.29%) of them were found to be biofilm producers, 34 (18.08%) strong, 38 (20.21%) moderate. The remaining 116 (61.7%) were weak/ non biofilm producers. Maximum biofilm formers were recorded in pus samples (39.06%), followed by isolates from blood (38.23%) and urine (34.61%). Statistical analysis for the formation of biofilm indicated that Microtiter plate method is the most sensitive and specific method for screening biofilm production.

**Conclusions:** Biofilm formation is one of the influential virulence factor in *staphylococcal* pathogenesis and persistence. Microtiter plate and Congo red agar remain as reliable methods for the qualitative and quantitative estimation of biofilm formation. Monitoring of biofilm formation in various etiological agents will help in determining the severity of infection.

**Key words:** Biofilms; *Staphylococcus aureus*; detection methods.

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### Introduction

Implantable medical devices have become indispensable in healthcare systems, however, they provide good surface for the attachment of adherent bacteria and result in device related chronic infections, which will be difficult to treat. The ability to form biofilm, an important virulence factor is expressed by many pathogenic bacteria, and the *Staphylococci* are the most common etiological agents of device related infections [1]. Biofilm associated infections are difficult to treat since the biofilm confers resistance against the host immune system as well as makes the bacterial cells impervious to the antibiotics [2]. As persists in the host, the stability to withstand stress of antimicrobial agents and host defense system has led to the emergence of multi drug resistant strains [3]. Biofilm acts as a diffusion barrier to slow down the infiltration of antimicrobial agents [4]. Alternative approach in the treatment of biofilm associated infections has become one of main concern in the current medical research. Biofilms are systematic structured sessile communities

surrounded by extracellular Polysaccharide Intercellular Adhesins composed of 1,6-linked N-acetylglucosamine residues and non-N-acetylated D-glucosaminyl residues composed of phosphate and ester-linked succinate. The *Staphylococcal* adherence on biomaterial is due to the expression of *ica ADBC* genes [5]. Morphological studies have shown the diversification of biofilm producing variants [6]. Quorum sensing concepts played an important role in understanding the diffusible signal molecules produced and released by bacteria which increases the bacterial density [7]. Various methods are available for evaluation of biofilm detection like bioluminescent assay, light or fluorescent microscopic examination, air-liquid interface cover slip assay and confocal laser scanning electron microscopy. Many reports are available for isolation of pathogens from biofilm on medical devices such as roll plate method and endoluminal brush technique [8]. Molecular analysis such as Transcriptome and proteomic studies have promising solutions in the identification and expression

of biofilm regulatory genes and progression of virulence gene expression in the biofilm formation [9]. Despite of advanced techniques, conventional methods have remained as standardized protocols because of their reliability to perform under laboratory conditions and do not require any sophisticated instruments and are cost effective. But efficient protocols are still required to overcome erroneous results. The present study evaluates the biofilm formation by clinical isolates of *S. aureus* obtained from different locations in India using three different methods namely, Congo red agar, Tube method and Microtiter plate method.

## Methodology

### *Bacterial strains*

A total of 188 *S. aureus* isolates from clinical samples such as pus, blood, and urine were obtained from various diagnostic centers in India. The isolates were enriched with Brain heart infusion broth for overnight. The isolates were confirmed as *S. aureus* by standard microbiological techniques including coagulase and Baird Parker agar tests [10]. The cultures were inoculated on tryptic soy agar containing 16% of glycerol and preserved at -20°C. Standard *S. aureus* ATCC 25923 (strong biofilm producer), *S. aureus* ATCC 20372 (moderate biofilm producer), and *S. aureus* ATCC 12228 (non-biofilm producer) were included in the study as a reference strains.

### *Detection of biofilm formation*

#### Microtiter plate method

Microtiter plate method was performed according to the method of Christensen *et al.* (1985) [11]. Biofilm production was carried out using Brain heart infusion broth with 2% of sucrose. Overnight cultures were enriched with brain heart infusion broth for 4-6 hours. Log phase cultures of 0.5 MacFarland were diluted 1:100 by in freshly prepared BHI broth. 200 µL aliquots were transferred to 96 well microtitre plates (Hi media, Mumbai, India). Sterile broth without culture served as control. The plates were incubated at 37°C for 48 hours. After incubation the contents of each well was gently decanted. The wells were washed 2-3 times with 0.2 mL of PBS (phosphate buffer saline of pH 7.2) to remove planktonic bacteria. Then air dried by inverting the plate at room temperature and stained with 0.1% of crystal violet. Further the wells were washed with distilled water for 5-6 times to remove excess stain. The stain adherent to walls was dissolved with 100 µL of 33% glacial acetic acid and absorbance (OD) was read at 570nm using a iMark™ Microplate Absorbance Reader (Bio-Rad Gurugram, India). The biofilm

formation by the *S. aureus* isolates was classified as strong, moderate and weak/none as suggested by Mathur *et al.* [12] based on OD values as follows:

#### Tube method

Tube method was performed as per the protocol suggested by Christensen *et al.* [11]. Enriched overnight cultures were inoculated aseptically in to test tubes containing 2 mL of sterile, freshly prepared Brain heart infusion (Hi media, Mumbai, India) broth with 2% sucrose and incubated at 37°C for 48 hours. Contents of the tube were decanted gently and washed with PBS (pH 7.2) 3-4 times to wash off the planktonic cells and tubes were air dried at room temperature. 0.5 mL of 1% crystal violet was used to stain the adherent bacteria. Walls of the tubes were uniformly stained by slowly tilting the test tube to ensure proper staining of the adherent bacteria. After one minute the tubes were washed with distilled water for 5-6 times to remove the excess stain. Tubes were air dried and observed for biofilm formation. Visible blue film line at the bottom of the tube indicates positive test and ring formation at the liquid interface indicates no biofilm formation. The results scored visually as 0-absent, 1-weak, 2-moderate, 3-strong.

#### Congo red agar method

Morphological detection of biofilm formation was carried out by Congo red agar as described by Freeman *et al.* [13]. The medium was prepared by using Brain heart infusion 38 g/L, 0.08% of Congo red dye and supplemented with 2% sucrose and Agar 15 g/L. Congo red dye was prepared separately and added when agar was cooled to 55°C. Inoculated plates were incubated at 37°C for 24 hours. Black colonies on media indicate positive test for strong biofilm production, grayish black to deep red indicates moderate biofilm producers and red colonies are considered as weak/non biofilm producers.

#### *Statistical analysis*

Statistical analysis was done as described by Mathur *et al.*, (2006) [12]. Data are presented as percentages and proportions. TCP was considered to be gold standard method of biofilm detection among the used methods in this study and calculated the other statistical parameters accordingly.

## Results

Biofilm formation by *S. aureus* isolates as observed by the three methods is shown in (Figure 1). A total of 72 (38.29%) *S. aureus* isolates were recorded as biofilm

**Table 1.** Number of biofilm forming *S. aureus* isolates determined using the three methods.

Biofilm formation (n = 188)	Method of biofilm determination		
	Microtiter plate (MTP)	Tube (TM)	Congo red agar (CR)
Strong	34 (18.08%)	25 (13.29%)	15 (7.97%)
Moderate	38 (20.21%)	25 (13.29%)	40 (21.27%)
Weak/none	116 (61.70%)	138 (73.40%)	133 (70.74%)

**Table 2.** Biofilm formation *S. aureus* isolates determined using the three methods.

Std isolate	Biofilm formation by various methods		
	Microtiter plate (MTP)	Tube (TM)	Congo red agar (CR)
<i>S. aureus</i> ATCC(25923)	High (OD <sub>570</sub> 2.22 ± 0.015)	High (score 3)	High (black colonies)
<i>S. aureus</i> ATCC(20372)	Moderate (OD <sub>570</sub> 0.184 ± 0.018)	Weak (score 2.1)	Weak (intermediate black + non red)
<i>S. aureus</i> ATCC(12228)	None (OD <sub>570</sub> 0.05 ± 0.016)	None (score 0)	None (red colonies)

producers by the MTP method, 18.09% strong biofilm producers and 20.21% moderate biofilm producers. Whereas with congo red agar method 55 (29.25%) isolates were found to be biofilm producers, 7.98% strong and 21.18% moderate. The same with tube method was minimum, 50 (26.6%) total, 13.30% each strong and moderate.

Biofilm formation by the *S. aureus* isolates from the current study was compared with the biofilm formation by the three standard cultures, one each for strong, moderate and non-biofilm producers (Table 1). The OD values observed in the present study are almost comparable with the OD values recorded with the standard *S. aureus* strains for strong, moderate weak/non biofilm formation. Some of the *S. aureus* isolates produced a stronger biofilm (Table 2) than the standard strain, ATCC-25923. From these results it is clear that the microtiter plate method is better than the other and can distinguish clearly between strong, weak and non-biofilm producers (Table 3).

The percentage of biofilm producing *S. aureus* isolates from different samples is shown in Table 4, which indicates higher incidence of biofilm producers (moderate to high) in pus and blood samples, while in case of urine samples though the overall biofilm producers are less, but the percentage of strong biofilm producers are comparatively higher than that recorded in pus or blood samples.

Biofilm formation by the clinical isolates of *S. aureus* obtained from different locations in India as determined by the three methods was subjected to

**Table 3.** Level of biofilm formation by *S. aureus* isolates by MTP method (OD at 570 nm).

Biofilm formation OD at 570nm	No. of isolates
Strong (> 0.251 to 2.980)	34
Moderate (0.120 to 0.199)	38
Non/ weak biofilm producers (0.000 to 0.092)	116

**Table 4.** Number of *S. aureus* isolates from different samples showing levels of Biofilm formation.

Biofilm producer	Clinical isolates from (n = 188)			
	Pus	Blood	Urine	Total
Strong	22 (17.19%)	6 (17.64%)	6 (23.07%)	34 (18.05%)
Moderate	28 (21.88%)	7 (20.59%)	3 (11.53%)	38(20.2%)
Non biofilm	78 (60.94%)	21 (61.76%)	17 (65.38%)	116 (61.7%)
<b>Total</b>	<b>128</b>	<b>34</b>	<b>26</b>	<b>188</b>

**Table 5.** Statistical evaluation of the biofilm formation by the *S. aureus* isolates.

Screening method	Test characteristics in %				
	Sensitivity	Specificity	PPV	NPV	Accuracy
MTP	93.50	98.30	97.01	95.86	96.27
TM	80.65	89.68	79.36	90.40	86.70
CRP	33.30	93.01	60.01	81.59	78.82

PPV: Positive Predictive value; NPV: Negative Predictive Value; MTP: Microtiter Plate method; TM: Tube Method; CRP: Congo red Plate method.

statistical analysis and evaluated the sensitivity and specificity of the test method [14] (Table 5). From the table it is very clear that among the three methods, microtiter plate method is the most sensitive, specific as well as accurate and can predict the positive and negative biofilm production with equal efficiency. Though, CRA method was found to be the least (33%) sensitive, but was found to have a very good (93.01%) specificity.

## Discussion

*Staphylococcal* infections are a major global burden in health care systems [3]. Antimicrobial approach in the control of staphylococcal infections has often become ineffective due to the emergence of multi drug resistance. Threats of MDR transforming to PAN drug resistance in near future requires a steady research outcome to combat these infections. Biofilm is one of the major factors in emphasizing antibiotic resistance and hence biofilm detection facilitates the investigation of severity of infection among invasive *S. aureus*. Physiological heterogeneity in biofilms involves chemical gradients, adaptation to confined environmental settings, varied genotypes and phenotypes that express distinct metabolic pathways within the population [9]. The medium used for biofilm production also significantly influences the level of biofilm formation [12].

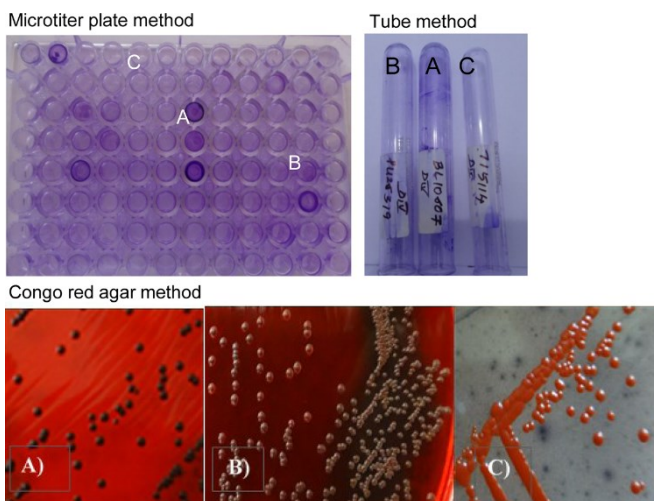
In the present study biofilm formation among the *S. aureus* isolates obtained from different parts of India is determined qualitatively and quantitatively using three conventional methods, Microtiter plate, Tube and

Congo red agar methods which showed an accuracy of 96.27%, 86.70% and 78.72% respectively. Interestingly most of the biofilm producers were from pus, followed by isolates from blood and urine (Table 1). Overall among the 188 clinical isolates 38.28% were able to produce significant amounts of biofilm (moderate to strong). A recent report from Northeast India has shown 31% *S. aureus* isolates as biofilm producers [15]. Our observation is significantly less than that reported by a recent study wherein 63% of the 92 isolates were found to be biofilm producers [16]. Mathur *et al.* (2006) [12] recorded up to 52.6% biofilm forming staphylococci from clinical isolates using BHI medium. Biofilm producing staphylococci have been found to be more frequent in medical device associated infections [9]. Higher percentage of biofilm producers among *S. aureus* isolates from urine as recorded in our study probably is indicative of the fact of their association with catheters [16].

The level of biofilm formation; strong, moderate, weak/none, is evaluated using all the three methods (Figure 1). The gradation of the biofilm formation from none to very strong was demonstrated using the variation in colours of the colonies formed on congo red agar as very red, red, bordeaux, almost black, very black, and black [6]. Maximum number of biofilm producers among the *S. aureus* isolates has been detected using the MTP method compared to the other two methods indicating that MTP method is more sensitive. Statistical analysis also has shown that the MTP method is more sensitive, specific as well as accurate with differentiation ability PPV and NPV (Table 4). Mathur *et al.* (2006) [12] have also reported in a study involving 152 clinical isolates of staphylococci that MTP (TCP) is more accurate and reproducible method for biofilm detection.

False positive and false negative results are found to be more in TM and CRA methods when compared to MTP method. However, specificity of CRA was found to be higher as 93%. Hence CRA method can be conveniently used for the qualitative estimation biofilm formation. Accurate results can be obtained by molecular analysis, a study showed that 100% specificity and sensitivity were obtained by PCR method for the detection of biofilm forming genes [17]. MTP is a very simple and cost effective method, however, takes longer time, whereas molecular methods, though require very short time, but cost intensive. Hence, the specificity, sensitivity and reproducibility of MTP is required to be evaluated along with the molecular methods on broader scale before recommending any one particular method.

**Figure 1.** Biofilm formation by *S. aureus* using the three methods.



A: strong biofilm producer; B: moderate biofilm producer and C: non-biofilm producer.

## Conclusion

Studies on *in vitro* laboratory models and similar *in vivo* methods for detection of biofilm formation enhance the understanding of adherence variation in different environments. Newer methods are required to decrease the false negative and false positive values in overcoming erroneous results. MTP has been found to be a more sensitive, specific and accurate method for the evaluation of biofilm formation among clinical isolates.

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