

## Study on biofilm-forming properties of clinical isolates of *Staphylococcus aureus*

Yasmeen Taj<sup>1</sup>, Farhan Essa<sup>1</sup>, Faisal Aziz<sup>2</sup>, Shahana U. Kazmi<sup>2</sup>

<sup>1</sup>Dow Medical College Karachi, Karachi, Pakistan

<sup>2</sup>Immunology and Infectious Diseases Research Laboratory (IIDRL), Department of Microbiology, University of Karachi, Karachi, Pakistan

### Abstract

**Introduction:** The purpose of this study was to observe the formation of biofilm, an important virulence factor, by isolates of *Staphylococcus aureus* (*S. aureus*) in Pakistan by different conventional methods and through electron microscopy.

**Methodology:** We screened 115 strains of *S. aureus* isolated from different clinical specimens by tube method (TM), air-liquid interface coverslip assay method, Congo red agar (CRA) method, and scanning electron microscopy (SEM).

**Results:** Out of 115 *S. aureus* isolates, 63 (54.78%) showed biofilm formation by tube method. Biofilm forming bacteria were further categorized as high producers (n = 23, 20%) and moderate producers (n = 40, 34.78%). TM coordinated well with the coverslip assay for strong biofilm-producing strains in 19 (16.5%) isolates. By coverslip method, weak producers were difficult to differentiate from biofilm negative isolates. Screening on CRA showed biofilm formation only in four (3.47%) strains. Scanning electron micrographs showed the biofilm-forming strains of *S. aureus* arranged in a matrix on the propylene surface and correlated well with the TM.

**Conclusion:** Biofilm production is a marker of virulence for clinically relevant staphylococcal infections. It can be studied by various methods but screening on CRA is not recommended for investigation of biofilm formation in *Staphylococcus aureus*. Electron micrograph images correlate well with the biofilm production as observed by TM.

**Key words:** *Staphylococcus aureus*; biofilm production; electron microscopy

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### Introduction

*Staphylococcus aureus* (*S. aureus*) is a virulent organism resistant to most of the conventionally prescribed antibiotics. It is difficult to treat long-term staphylococcal infections such as endocarditis, osteomyelitis and especially those infections associated with implanted medical devices. One reason these organisms are capable of defending themselves from host immune systems is their capability to form biofilms [1,2]. The interior of the bacterial biofilms presents greater resistance to the opsonization by antibodies and to phagocytosis, which explains the chronic character of these infections [3].

The cultural morphology of biofilm-forming bacteria is usually different from those strains which do not form biofilms. It has been observed that biofilm-forming bacteria attach themselves to solid surfaces by using their sticky appendages and employing a rolling motion, which results in their continuous attachment and detachment to the surface and the formation of microaggregation. First they

detach from the top of microaggregation where apparently the shearing force overcomes the attachment force; then the microaggregations roll slightly and attach someplace downstream [4]. In case of biofilm formation with prosthetic devices such as indwelling catheters and endo-tracheal tubes, this movement helps the dissemination of bacteria inside their lumen [4]. In a biological system, biofilm formation takes place in various steps: first there is an attachment to a surface; later, microbial surface adhesins recognize adhesive matrix molecules (MSCRAMMS), start aggregating, attaching to each other and produce extra polymeric substances (EPS) that interact with host-derived components such as platelets to form a strong biofilm [5]. Under certain adverse circumstances such as deprivation of nutrition or a heavy shearing movement, breakage and dissemination of biofilm occurs due to the formation of certain defense proteins called auto-inducing peptides (AIP) with the release and dispersal of bacteria [6].

While there are many techniques available for biofilm study, it is imperative that standardized techniques should be developed. A variety of methods have been standardized in various laboratories, each having their own merits. These methods include tissue culture plate (TCP) [7], tube method (TM) [8], Congo red agar method (CRA) [9], bioluminescent assay [10], light or fluorescent microscopic examination [11,12], air-liquid interface coverslip assay [7,8], and scanning electron microscopy (SEM) [13,14]. SEM is an advanced resolution method that provides ultra-structure analysis of biofilms. The scanning electron microscope has an extensive magnification to about 210 times the limit of a light microscope. It displays whether biofilm is loosely expressed by bacteria in the surrounding medium or is definitely fixed to the bacteria allowing the bacteria to be embedded in a matrix [13,14].

Biofilm production is considered as a marker of clinically relevant infection. Previous observations have confirmed that biofilms are not only resistant to antibiotics but a variety of disinfectants [15,16] which emphasizes that their characterization is an important aspect of infection control. Furthermore, the information on the capacity of a clinical isolate to produce biofilm would help a clinician to evaluate the measure of its virulence and devise an appropriate treatment plan for the patient. The objective of this study was to observe the formation of biofilm by *S. aureus* isolated from different clinical specimens by three conventional methods, namely TM, air-liquid interface coverslip assay method, and the CRA methods. We also performed their ultra-structural analysis by SEM.

## Methodology

### *Bacterial strains*

A total of 115 *S. aureus* isolates from different clinical samples such as catheters, urine, blood, high vaginal swab, pus swab and urethral swab (Table 1) were obtained from Civil Hospital, Essa Laboratories, and Ziauddin Hospital Laboratories in Karachi, Pakistan. The organisms were identified on the basis of colonial morphology, Gram-positive cluster-like appearance on staining, positive catalase and coagulase test. The cultures were inoculated on trypticase soy agar (TSA) 16% (vol/vol) glycerol and kept at -20°C. Standard strains of *Staphylococcus epidermidis* ATCC 35984 (strong biofilm producers), *Staphylococcus epidermidis* ATCC 35983 (medium biofilm producers), and *S. epidermidis* ATCC (biofilm

nonproducers) were purchased and used as reference strains.

Detection of biofilm was achieved by CRA, TM and air-liquid interface slip assay, and the results were correlated with SEM.

### *Congo red assay method*

This method is based on the characteristic cultural morphology of biofilm-forming bacteria on Congo red medium. The medium was composed of brain heart infusion broth (BHI) (Oxoid Ltd, Hampshire, England) 37 g/l, sucrose 50 g/l, agar No 1 (Oxoid Ltd, Hampshire, England) 10 g/l and Congo red (BDH Chemical Ltd, Poole, England) 0.8g/l. Congo red stain was made ready as a strong aqueous solution and sterilized (121°C for 15 minutes) separate from the rest of the medium components and supplemented to the agar when the temperature reached 55°C. Agar plates were prepared and inoculated and kept in the incubator for 24 hours at 37°C. The production of black colonies with a dry crystalline consistency by the organisms was taken to indicate biofilm production as non-biofilm-producing strains develop red colonies [9].

### *Tube method*

*S. aureus* isolates were tested for biofilm production by a modification of the standard method of Christensen *et al.* [7] Two milliliters of trypticase-soy broth (TSB; Difco Laboratories, Detroit, MI, USA) in 12 x 75 mm borosilicate test tubes (Corning, Tewksbury, MA, USA) were inoculated with a loopful of microorganisms from overnight culture plates and incubated for 48 hours at 37°C, after which the contents were decanted and washed with PBS (pH 7.3) and left to dry at room temperature. Afterward, the tubes were stained with 4% solution of crystal violet (Merck, Darmstadt, Germany). Each tube was then gently rotated to ensure uniform staining and then the contents were gently decanted. The tubes were placed upside down to drain and then observed for biofilm formation which was considered positive when a visible film lined the wall and bottom of the tubes. Ring formation at the liquid interface was not regarded as indicative of biofilm formation. The results were scored visually as 0-absent, 1-weak, 2-moderate, 3-strong [9].

**Table 1.** Biofilm production of the 115 staphylococcal strains of human origin examined according to the source of isolation.

Source	<i>S. aureus</i> (n = 115)				Total
	Biofilm +	(%)	Biofilm -	(%)	
Catheters	11	57.89	8	42.11	19
Urine	13	65.00	7	35.00	20
Blood	8	44.44	10	55.56	18
High vaginal swab	11	61.11	7	38.89	18
Pus and wound swab	8	42.11	11	57.89	19
Urethral swab	12	57.14	9	42.86	21
<b>Total</b>	63		52		115

#### Liquid-interface coverslip assay

Bacterial cultures were also analyzed for biofilm formation using the air-liquid interface coverslip assay. In this assay biofilm adhered to coverslips were

visualized under light microscope. Cultures were inoculated into tubes containing 3-5 ml of TSB and allowed to grow to a stationary phase. The stationary phase cultures were diluted 1:100 in TSB. Diluted cultures were used to fill a well in a flat-bottom 12-well plate (Sumilon Multi-well plate, Sumitomo Bakelite Co, Ltd, Tokyo, Japan. The wells were filled to 100 µl each. Sterile glass coverslips were inserted into each well to achieve a 90° angle relative to the bottom of the well (*i.e.*, perpendicular to the bottom of the well) so that the meniscus of the medium was at the center of the coverslip. Plates were covered and kept in the incubator at 37°C for a period of 18 hours. Bacteria were stained by submerging coverslips in 0.1% crystal violet for 10 minutes. Excess dye was rinsed off by dipping each coverslip in two successive water baths and coverslips were allowed to air dry. Bacteria at the air-liquid interface on each coverslip were visualized under a microscope [8,9].

#### Scanning electron microscopy

An appropriate amount of 24-hour-old culture grown on Mueller-Hinton Agar (Oxoid Ltd, Hampshire, England) was inoculated in a sterile tube containing 3 ml TSB (Oxoid Ltd, Hampshire, England) for 24 hours at 37°C. Next 2 ml of 20% glucose was added and incubated for 24 hours further. The tube was decanted and 5 ml of 99% methanol was added as a fixative. After fixation, the specimen was rinsed gently in several changes of distilled water to remove excess fixative, followed by 0.1% crystal violet staining for 20 minutes. Specimens were air dried for 24 hours. To examine the biofilm formation on the inside surface of the tubes, longitudinal and

horizontal divides were mounted on copper stubs with the help of double-sided tape. The sample was then coated with gold film by SEM JEOL JFC-1500 Quick auto sputter coater up to 300 Armstrong. The sample was then placed in a sample chamber of SEM JEOL JSM-6380A and scanning was performed under different magnifications ranging from 6,000x to 12,500x, and voltage 5kV [13,14].

#### Results

This study is based on 115 strains of *S. aureus* which were isolated from various clinical samples including urethral swabs, urine, catheters, pus, high vaginal swabs, and blood. As shown in Table 1, 57.14% of the urethral isolates were biofilm formers, whereas 65.0% urinary, 57.89% catheter, 42.11% pus, 44.44% blood and 61.1% of the vaginal isolates were also able to form biofilms.

Comparison of biofilm production by clinical isolates of *S. aureus* by three conventional methods is given in Table 2. Out of 115 clinical samples of *S. aureus*, 63 (54.8%) showed biofilm positive production by tube method under the conditions that were optimized in the laboratory. By coverslip assay, 19 (16.52%) were positive for biofilm production, and by Congo red agar method, 4 (3.47%) were positive for biofilm production.

In the TM, strains were further grouped as strong producers (n = 23; 20%) and medium producers (n = 40; 34.78%) while in 52 (45.2%) specimens little or no biofilm was detected. The results of TM coordinated with the coverslip assay for 19 (16.5%) high biofilm-producing strains; weak producers were difficult to differentiate from biofilm negative isolates in the coverslip assay.

**Table 2.** Comparison of the production of biofilm by clinical isolates of *Staphylococcus aureus* by three conventional methods

Methods	<i>S. aureus</i> (n = 115)	
	Biofilm +	(%)
Tube method	63	54.78
Cover slip assay	19	16.52
Congo red agar method	4	3.47

#### *Study of biofilm formation by TM*

The qualitative tube adherence test depends on the visual assessment of the degree of adherence of *S. aureus* to the sides of borosilicate test tubes. Interpretation of the TM were recorded as strong adherence (+++), moderate (++), weak adherence (+) or negative (Figure 1).

#### *Study of biofilm formation by coverslip method*

A visible line of biofilm growing more than 2 mm was seen in the coverslip at the air-liquid interface and below this line the coverslip was darkly stained showing biofilm growth in 19 out of 115 isolates (16.5%) isolates. Results were interpreted as no adherence (–), low level of adherence with only sparse staining and no line of biofilm at the air liquid interface (+); intermediate level of adherence with staining below the air/liquid interface (++); and high level of adherence with a clearly defined line of staining at the air/liquid interface and staining across the lower half of coverslip (+++) as observed under the microscope.

#### *Study of biofilm formation on Congo red agar plates*

Only four (3.8%) isolates showed a black crystalline morphology indicating biofilm production was observed in plates.

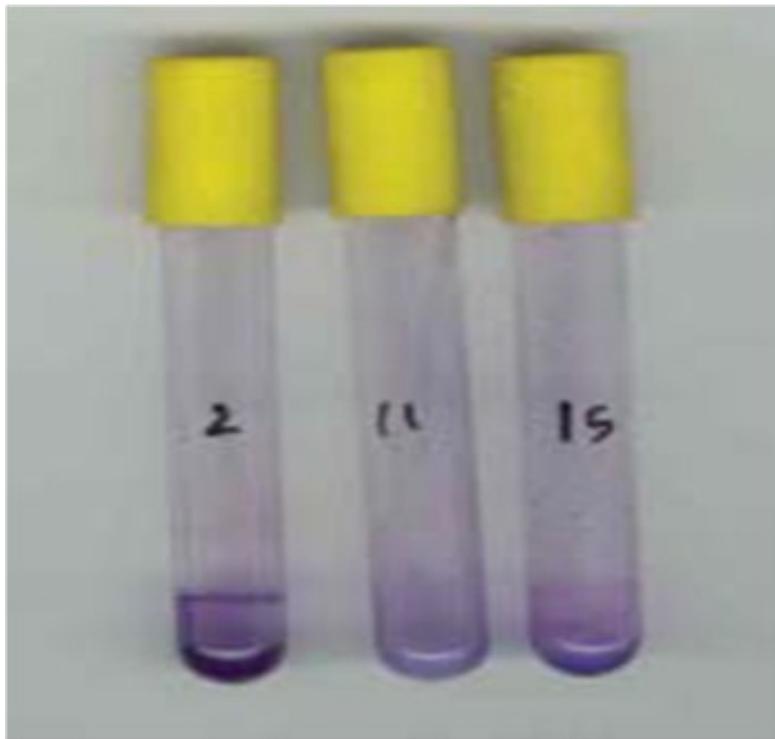
#### *Scanning electron microscopy (SEM)*

Scanning electron microscopy (SEM) showed staphylococci arranged in a matrix on propylene surfaces and it co-related with the results of the conventional methods for biofilm analysis. The electron microscopy images of negative biofilm producers showed *Staphylococcus aureus* in clusters showing no biofilm production (Figure 2). The strong producers by conventional methods were seen by electron microscopy as bacteria attached in distinct layers of biofilm (Figure 3).

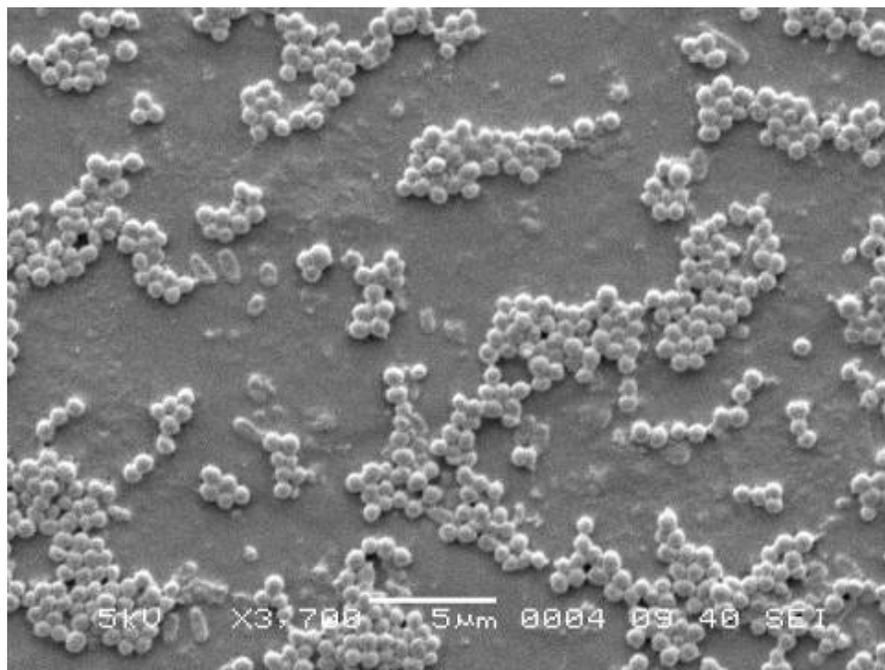
## Discussion

Biofilm and multidrug resistance have been identified as virulence factors of great magnitude in *Staphylococcus aureus* infections in clinical settings. *S. aureus* is a medically important organism associated with a vast variety of diseases; some strains can cause chronic infections and gain increased resistance to antimicrobial agents through biofilm formation [15,16,]. Researchers have investigated the strategies employed by microorganisms to produce biofilms and to understand the pathogenesis. They discovered that biofilm-producing bacteria secrete certain chemicals that protect them from disinfectants and antimicrobials, and phagocytic host immune systems [16,]. Several conventional methods of detecting biofilm production have been established, such as the standard TM [7], plate method [8,9], and coverslip assay [9]. Because these methods can give indecisive results, we attempted to detect the localization of biofilm with respect to the bacterial cell wall by SEM [13,14]. The origin of isolates obtained from different clinical samples is given in Table 1. The comparison of the production of biofilm by clinical isolates of *S. aureus* by three conventional methods is given in Table 2. In our study, 63 (54.8%) out of 115 isolates showed positive biofilm production using TM when we used TSB. Similar reports have been given by other studies when using TSB as a medium and extending the incubation time to 24 hours [17]. These results suggest that biofilm formation depends strongly on the environmental conditions [18]. The results of TM are in accordance with the coverslip assay for high bio-film producing isolates; however, weak and negative isolates were difficult to differentiate on optical observations of coverslips. Using the CRA plate method for testing biofilms production, only four isolates (3.4%) showed black crystalline morphology while 111 (96.5%) gave orange-red colonies. The CRA plate method is not recommended as a medium for biofilm production in *S. aureus* species, as researchers have only recently found that PIA/PNAG (polysaccharide intracellular adhesins/poly N-actyl glucosamine) have little input in the biofilm matrix of *Staphylococcus aureus* and cannot be detected by the CRA method. Similar results have been reported by other authors [9,19]. These reports suggest that CRA screening cannot be recommended to detect biofilm formation for *S. aureus* isolates. Scanning electron microscopy (SEM) displayed the existence of biofilm,

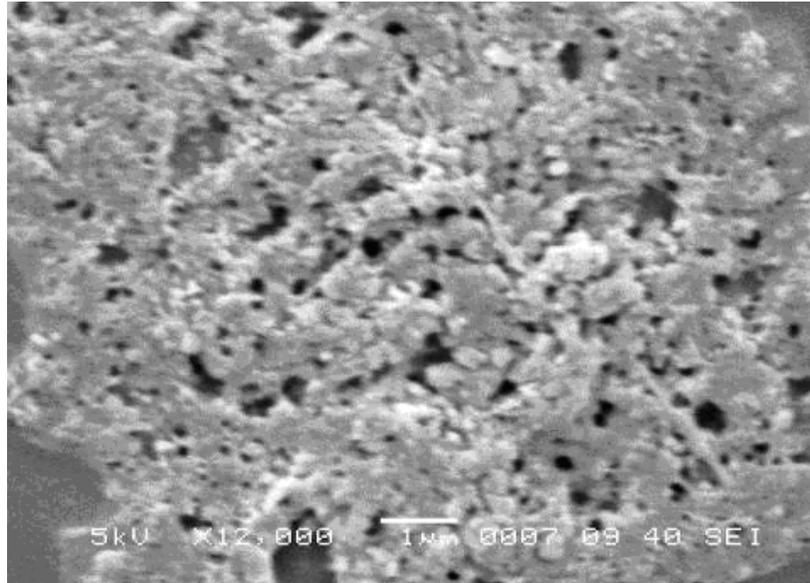
**Figure 1.** Tube test showing high, moderate and weak biofilm production



**Figure. 2** Electron scanning microscopy showed no biofilm productions by *Staphylococcus aureus* with resolution (%KUx3,700,5um,004,09,40,SEI)



**Figure 3.** Electron scanning microscopy showed high biofilm productions by *Staphylococcus aureus* with resolution (5kUx12,000,1um,0007,09,40,SEI)



directly showing whether *S. aureus* biofilm was loosely expressed in the medium or is definitely fixed to the bacteria allowing the bacteria to be embedded in a mesh network of fibrils. Ultra-structure analysis of biofilm is therefore a practical supplement to specific *in vitro* staining procedures such as TM, coverslip, and the air-liquid interface coverslip assay.

### Conclusion

In conclusion, we can say that TM is the most reliable method for the detection of biofilms in *S. aureus*. The method is also complemented by scanning electron microscopy. The Congo red method, however, is not reliable for the detection of biofilm. Biofilm formation can cause a multitude of problems in the medical field, particularly with prosthetic devices such as indwelling catheters and endo-tracheal tubes. Obtaining clinical samples from such devices for laboratory testing to identify biofilm formation can help prevent potentially fatal and persistent infections.

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**Corresponding author**

Yasmeen Taj  
Dow Medical College Karachi  
Karachi, Pakistan  
Telephone: (Cell) 0333-2203333  
Email: y.taj@hotmail.com

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