IJBPAS, September, 2015, 4(9): 5874-5881

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

COMPARISON OF BIOFILM FORMATION BY CONVENTIONAL METHODS: MICROTITER PLATE, AIR-LIQUID INTERFACE COVER SLIP AND TUBE METHOD BY *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

S. aureus being an opportunistic microorganism has ability to form biofilm and therefore is associated with biomaterial infection. In this study, *in vitro* method, which includes air-liquid interface cover slip assay, the Tube-method and Microtiter plate assay were employed to screen 100 clinical isolates collected from *S. aureus* infected patients from different regions of Himachal Pradesh. It was observed that out of 100 samples, 60 samples showed biofilm formation by microtiter plate assay. While only few samples showed the same formation by Air-liquid interface coverslip assay method and Tube method. Therefore Microtiter plate assay can be used to assess the *S. aureus* virulence factor associated with biofilm formation and for examining the efficacy biofilm treatment modalities.

Keywords: *Staphylococcus aureus*; biofilm production; biomaterial infection; air-liquid interface cover slip assay; tube method; microtiter plate assay

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a virulent organism, which is resistant to most of the conventionally used antibiotics. Due to which it becomes difficult to treat long-term staphylococcal infections such as endocarditis, osteomyelitis and especially those infections associated with implanted medical devices. One reason of these

organisms to become capable of evading themselves from host immune systems is their capability to form biofilm [1, 2]. The bacteria get very difficult to treat clinically once biofilm has been formed. The reason behind this can be that the bacteria on the interior of the biofilm become well protected from the host immune response as well as antibiotic agents [3]. The cultural morphology of biofilm-forming bacteria is usually different from those strains which do not form biofilm. It has been observed that biofilm-forming bacteria attach themselves to solid surfaces by using their sticky appendages and by employing a rolling motion, which result in their continuous attachment and detachment to the surface and the formation of micro aggregation. First they detach from the top of micro aggregation where apparently the shearing force overcomes the attachment force; then the micro aggregations 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 adhesions 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 lack of nutrition or a heavy shearing movement, breakage and diffusion of biofilm occurs due to the formation of certain defense proteins called auto-inducing peptides (AIP) with the release and dispersal of bacteria [6].Interestingly there are evidences which suggest that increased capacity to form biofilm and adhere to epithelial cells may have been linked. Example: The spread of the so-called Brazilian MRSA clone (ST239) [17]. Although there are many techniques available for biofilm study, it is very important to standardize these techniques.

These techniques 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] and air-liquid interface cover slip assay [7,8],

Biofilm production can be considered as a marker of clinically relevant infections. Past studies have confirmed that biofilm are not only resistant to antibiotics but also contain a variety of disinfectants [15, 16]. These points emphasize that characterization of biofilm 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. 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 Tube Method, Air-liquid interface Cover slip assay method and Microtiter dish assay.

MATERIAL AND METHODS

IRB/ EC Approval

The experimental protocol was approved by institutional ethical committee of Shoolini University, Solan India under Registration Number: SUIEC/13/32.

Bacterial Isolates

Total of 100 isolates was randomly selected from different *S. aureus* infected inpatients from different region of Himachal Pradesh. The bacteria were identified on the basis of Colonial morphology, Gram-positive cluster-like appearance on staining, Positive catalase and Coagulase tests. The cultures were inoculated on trypticase soy agar (TSA) 16% (vol/vol) glycerol and kept at -20°C. Standard strains of *Staphylococcus aureus* ATCC 25923 was used as reference strains. Detection of biofilm was achieved by, Microtiter dish assay, Tube Method and Air-liquid interface cover slip assay.

Microtiter dish assay

An overnight culture of *S.aureus* was diluted 200-fold with culture medium, of which 200 µl was added to the wells of a 96-well polystyrene microtiter plate and Incubated for 24 h at 37°C. The cell density was determined at 578nm with a microtiter plate reader (Spectra Max 340; Molecular Devices). Biofilm formation in each well was analyzed by a safranin staining method. Each experiment was performed at least three times and in each experiment the samples were prepared in six wells.

Adhesion assay, an overnight culture was diluted 200-fold with 10 ml TSBg and then placed in a 9-cm petri dish. After incubation at 37°C for 24 h., the plate was tilted by placing it on the edge of a petri plate cover. It was observed that if the cells did not form a biofilm, the top portion of the plate was clear and cells presented as clumps in the medium of lower half of the plate

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 in 12 x 75 mm borosilicate test tubes 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. Afterwards, the tubes were stained with 4% solution of safranin. Each tube was then gently rotated to ensure uniform staining and 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].

Liquid-interface cover slip assay

Bacterial cultures were also analyzed for biofilm formation using the air-liquid interface cover slip assay. In this assay biofilm adhered to cover slips 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. The wells were filled to100 µl each. Sterile glass cover slips were inserted into each well to achieve a 90° angle relative to the bottom of the well, so that the meniscus of the medium was at the center of the cover slip. Plates were covered and kept in the incubator at 37°C for a period of 18 hours. Bacteria were stained by submerging cover slips in 0.1% crystal violet for 10 minutes. Excess dye was rinsed off by dipping each cover slip in two successive water baths and cover slips were allowed to air dry. Bacteria at the air-liquid interface on each cover slip were visualized under a microscope [8, 9].

RESULTS

This study is based on 100 strains of *S. aureus* which were isolated from various clinical samples. Out of 100 clinical samples of *S. aureus*:

- 60% showed biofilm positive production by Micro titer dish method.
- 20% showed biofilm positive production by cover slip assay.
- 6% showed biofilm positive production by tube method.

Biofilm formation assays

1) Tube Method

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).

2) Coverslip method

A visible line of biofilm growing more than 2 mm was seen in the cover slip at the air-liquid interface and below this line the cover slip was darkly stained showing biofilm growth in 20 out of 100 isolates (20%). Results were interpreted as no adherence, low level of adherence with only light 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 cover slip as observed under the microscope.

3) Microtiter dish assay

The qualitative microtiter dish assay adherence test depends on the visual assessment of the degree of adherence of *S. aureus* to the bottom of each well (Figure-2). *S. aureus* is non-motile and forms a biofilm on the bottom of the well.

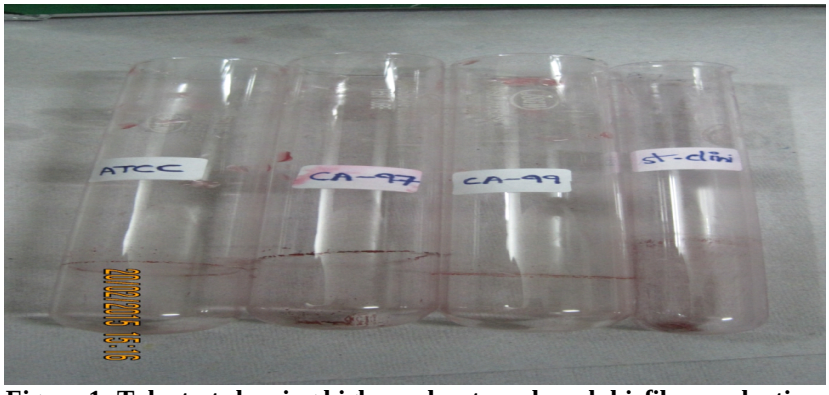

Figure-1: Tube test showing high, moderate and weak biofilm production

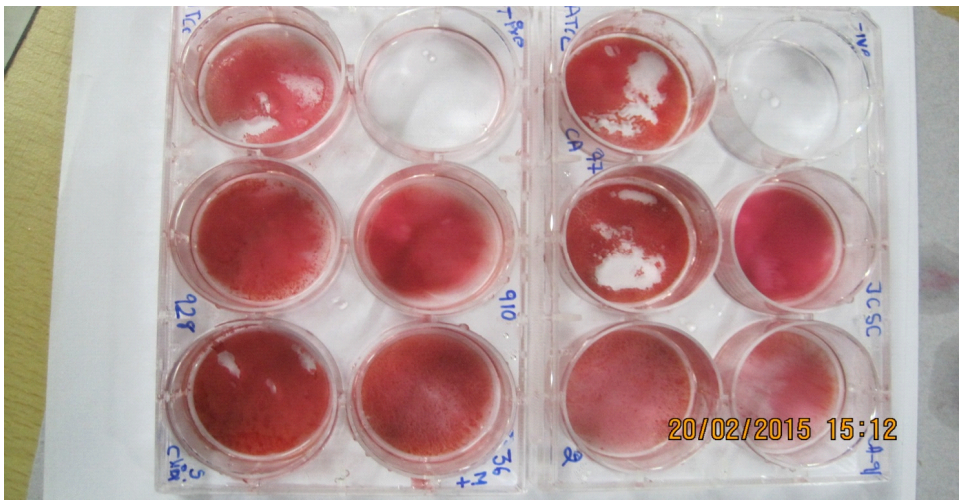

Figure-2: It indicates the presence of biofilm formation by *S.aureus* in a flat-bottom microtiter plate

DISCUSSION

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 biofilm and to understand the pathogenesis. They discovered that biofilm-producing bacteria secrete certain chemicals which 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].

The comparison of biofilm production by clinical isolates of *S. aureus* was analyzed by three conventional methods. In our study, 60 out of 100 isolates showed positive biofilm production using flat bottom microtiter plate 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 flat bottom microtiter plate are in accordance with the cover slip assay for high biofilm producing isolates; however, weak and negative isolates were difficult to differentiate on optical observations of cover slips. Using the tube method for testing biofilm production, only 25 isolates produce biofilm. Ultra-structure analysis of biofilm is therefore a practical supplement to specific *in vitro* staining procedures such as Tube method, cover slip, and the air-liquid interface cover slip assay.

CONCLUSION

In conclusion, we can say that microtiter dish assay is the most reliable method for the detection of biofilm in *S. aureus*. However the cover slip method 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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