CLINICAL DISTRIBUTION OF BIOFILM FORMING STAPHYLOCOCCUS AUREUS AND ITS SENSITIVITY AGAINST SOME ANTIBIOTICS

Poonam Verma* and Sunita Singh

1School of Biotechnology, IFTM University, Moradabad, Uttar Pradesh, India
2Department of Microbiology, King George Medical University, Lucknow, India

ABSTRACT

Staphylococcus aureus is one of the most versatile nosocomial (i.e. acquired in hospital) and dangerous human pathogen. In spite of the introduction of antimicrobial agents and improvements in the frequency and morbidity of staphylococcal diseases in the twentieth century, staphylococci have persisted as an important hospital and community pathogen. Thereafter, methicillin-resistant S. aureus emerged as a major pathogen worldwide. A total of 38 positive clinical isolates from various clinical samples received from different hospitals of Dehradun included from March 2014 to August 2014. 38 samples had bacterial growth, among these isolates 17(44.7%) were Staphylococcus aureus. The present study was designed to investigate antibiotic susceptibility pattern and the role of biofilm in isolates of various clinical samples (Urine, Blood, Semen and Pus), by examining the ability of isolates to form biofilm and produce signaling molecules and by developing a wound model, to relate laboratory findings with in vivo activity by exploring the possibility of detecting biofilm markers in dressings removed from chronic infections. The presence of biofilm was confirmed by specialized microscopy techniques or by detecting biofilm markers. Various antibiotics had a greater effect on viability when used at higher antibiotic concentrations (≥100 mg/L) and on younger (6h) biofilms. The antibiotics used for antibiotic susceptibility testing were Ofloxacin, Erythromycin, Amoxicillin, and Ciprofloxacin.

KEY WORDS: Biofilm, Nosocomial infection, Antibiotics, Signaling molecules.
INTRODUCTION

Biofilm has three-dimensional (3D) structured, heterogeneous community of microbial cells enclosed in an exopolysaccharide matrix (also called glycocalyx) that are irreversibly attached to an inert or living surface. In the case of human health, a number of microbial infections are associated with surface colonization not only on live surfaces (Sinusitis, pulmonary infection in cystic fibrosis patients, periodontitis, etc.) but also on medical implants (contact lenses, dental implants, intravascular catheters, urinary stents) etc. Biofilm formation represents a protected mode of growth that allows bacterial cells to stay alive in both hostile natural environments as in the human host, and enables them to disperse and colonize new niches whenever needed. Embedded micro-organisms on any surface constitute profound implications for the host, because the sessile micro-organisms that are surviving in these matrix-enclosed aggregates are recalcitrant to antibiotic treatment and demonstrate persistence in spite of sustained host defenses. Biofilm formation has not only been demonstrated for *S. aureus*, but also for numerous other pathogens. Bacteria in biofilm also coordinate behavior by cell-to-cell communication using secreted chemical signals, often referred as quorum. *Staphylococcus aureus* typically appears as Gram stain-positive cocci in grape-like clusters. It consists of an environment-resistant cell wall (peptidoglycan) surrounded by a microcapsule that determines the serotype. *S. aureus* consists of 13 capsular types, among which type 5 and 8 accounts for 85% of clinical isolates. The bacterium produces a battery of surface proteins involved in host colonization, several enzymes engaged in tissue invasion, and a multitude of toxins. Taken together, these factors make *S. aureus* one of the most pathogenic germs in humans. The second most important characteristic of *S. aureus* is its ability to colonize a broad range of host tissues and to persist intracellular or in biofilms formed on prosthetic materials or on human tissues. Such a biofilm is a structural community of staphylococcal cells enclosed in a self-produced matrix, with or without host constituents, that is adherent to a surface.

*S. aureus* is an adaptable, pathogenic organism. In the presence of environmental challenges, *S. aureus* can alter its genotype and/or phenotype to adapt to its surroundings. An example of genotypic change is the acquisition of the β-lactamase gene conferring penicillin resistance. The formation of biofilm is an example of phenotypic change. Formation of a biofilm is the hallmark characteristic of *S. aureus* infection which consists of multiple layers of bacteria encased within an exopolysaccharide glycocalyx. Presence of glycocalyx protects the
enclosed bacteria from host defenses and impedes delivery of antibiotics.\[15\] In fact Biofilms can resist antibiotic concentration 10-10,000 folds higher than those required to inhibit the growth of free floating bacteria.\[16\]

*S. aureus* and several other staphylococcal species colonize the human skin, nails and nares and disseminate among recipient host populations via physical contact and\[6\] the massive consumption of antibiotics over the past 50 years has led to the selection of drug-resistant strains, designated MRSA for methicillin-resistant *S. aureus*.\[7\]

The development of biofilm is thought to consist of four consecutive stages: (i) *Initial attachment* to a foreign body or tissue; (ii) *Accumulation* of extracellular polymeric substances and aggregation; (iii) biofilm *Maturation* including expansion and channel formation; and (iv) *Dispersal*. Dispersal involves the release of microbes from the biofilm, which might start all over again the vicious cycle.\[18\]

Biofilm formation in *S. aureus* is regulated by expression of Polysaccharide Intracellular Adhesion (PIA) which mediates cell to cell adhesion and is the gene product of *icaADBC*.\[19\] According to a publication by the National Institutes of Health, more than 80% of all infections involve biofilms.\[20\] Within a biofilm, bacteria communicate with each other by production of chemotactic particles or pheromones, a phenomenon called quorum.\[21\]

Biofilms are associated with many medical conditions including indwelling medical devices, dental plaque, upper respiratory tract infections, peritonitis, and urogenital infections.\[22\] Both Gram-positive and Gram-negative bacteria have the capability to form biofilms. The ability of biofilm-embedded *S. aureus* to resist clearance by antimicrobial agents points to the importance of a continuous search for novel agents that are effective against bacteria in this mode of growth or work in synergy with the currently available myriad of antimicrobial agents.\[23\]

**MATERIALS AND METHODS**

**Collection of samples**

A total of 38 positive clinical isolates used in this study were collected from Urine, Blood, Semen (in sterile sample collection bottles) and Pus (using sterile swabs) from different hospitals of Dehradun (UK) from March 2014 - August 2014.
**Transport of samples:** Urine, blood and semen samples were transported in sterile sample bottles as early as possible but Pus samples were transported in Stuart transport medium.

**Processing Of Samples:** Samples were processed for the isolation and identification of the organism preferably within 2 hrs after the collection from the hospitals. Samples were stored at 4°C till further processing.

**Isolation and Identification of Staphylococcus aureus:** All the collected samples were inoculated on to Nutrient Agar, MacConkey Agar, Blood Agar and, Mannitol salt agar (MSA) and incubated at 37°C for 24-48 hours. Isolates obtained from plates were identified on the basis of cultural, morphological, and biochemical characteristics as per Bergey’s Manual of Systemic Bacteriology.[24]

**Phenotype analysis of biofilm formation of Staphylococcus aureus:** Ability of the *S. aureus* isolates to form biofilm was determined according to the protocol described previously with modification.

**Tube method:** A loopful of the isolate from overnight culture plate was inoculated in 12 x 75 mm borosilicate test tubes containing 2ml of trypticase-soy broth (TSB) and incubated for 48 hours at 37°C. The contents of each tube were decanted and washed with phosphate buffer saline (PBS) pH 7.3 and left to dry at room temperature. Afterward, the tubes were stained with 4% solution of crystal violet. Each tube was 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.[25]

**Tissue culture plate method:** The TCP assay described by[25] used for detection of biofilm formation. We screened all isolates for their ability to form biofilm by TCP method with a modification in duration of incubation which was extended to 24 hours.

**Antibiotics susceptibility test**

**Disc diffusion test:** Antibiotic susceptibility test was performed on all Biofilm forming *S. aureus* isolates using Disc Diffusion method described by.[26] The antimicrobial agents tested were Amoxicillin 30 µg, Ofloxacin 30 µg, Erythromycin 30 µg, and Ciprofloxacin 30 µg.
**Agar well diffusion test:** Petri plates containing 20 ml Nutrient medium were seeded with 24 hr culture of Biofilm forming *S. aureus* strains. Wells were cut and 20 μl of the antibiotics (namely aqueous Amoxicillin, Ofloxacin, Erythromycin, and Ciprofloxacin) were added. The plates were then incubated at 37°C for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well.[27]

**RESULTS AND DISCUSSION**

![Fig 1: Colonies of S. aureus on Mannitol salt agar plate](image1.png)

![Fig 2: Colonies of S. aureus on Nutrient agar plate](image2.png)

![Fig 3: Tube method by detection of Biofilm formation by S. aureus](image3.png)
Fig 4: Tissue Culture method for detection of Biofilm formation by *S. aureus*

Fig 5: Antibiotics susceptibility (Disc diffusion) test of Biofilm forming *S. aureus*

Fig 6: Antibiotics susceptibility (Well diffusion) test of Biofilm formation by *S. aureus*

This study is based on 38 strains of *S. aureus* which were isolated from various clinical samples including urine, blood, semen, and pus. In Table 1, showed distribution of various *S. aureus* isolates in different samples. Most of isolates were isolated from pus samples. In this study 44.7% of the test isolates were biofilm formers Table 2.
Table 1: Distribution of \textit{S. aureus} isolates in different sample sites

<table>
<thead>
<tr>
<th>Total no. of isolates</th>
<th>Samples sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urine (%)</td>
</tr>
<tr>
<td>38</td>
<td>2 (5.26%)</td>
</tr>
</tbody>
</table>

Table 2: Incidence of Biofilm-forming \textit{S. aureus} in clinical samples

<table>
<thead>
<tr>
<th>Total Positive Samples</th>
<th>Biofilm forming \textit{S. aureus} (%)</th>
<th>Non-Biofilm forming \textit{S. aureus} (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>38</td>
<td>17 (44.7%)</td>
<td>21 (55.3%)</td>
</tr>
</tbody>
</table>

In the Tube method, 5 (13.89%) strains were grouped as strong biofilm producers and 13 (34.21%) medium biofilm producers while little or no biofilm was detected in 20 (55.56%) (Table 3, Fig 3). With respect to the observations made in the present study most literature report a similar result of Tube method of strong biofilm formation in \textit{S. aureus} ranging from 12.30% - 19%\textsuperscript{[25,28,29,30]}, whereas non biofilm formation in \textit{S. aureus} ranging from 51% - 58.55%\textsuperscript{[25,28,29]} reported similarity with result.

In present study results of Tissue Culture plate method showed 13 (34.21%) were high biofilm-producer, 9 (23.68%) were weak biofilm producers while 16 (44.44%) were non biofilm producer (Table 4, Fig 4). Other studies reported strong biofilm formation in \textit{S. aureus} ranging from 14.40% - 20.10%\textsuperscript{[25,28,30,31]}, whereas non biofilm formation in \textit{S. aureus} ranging from 35.11% - 46%\textsuperscript{[25,28,29,30]}

Table 3: Biofilm ability of different \textit{S. aureus} strains in Tube method (TM)

<table>
<thead>
<tr>
<th>S. No</th>
<th>Total no. of isolates</th>
<th>Biofilm production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Weak (%)</td>
</tr>
<tr>
<td>1.</td>
<td>38</td>
<td>20 (55.56%)</td>
</tr>
</tbody>
</table>

Table 4: Biofilm ability of different \textit{S. aureus} strains in Tissue culture method (TCM)

<table>
<thead>
<tr>
<th>S. No</th>
<th>Total no. of isolates</th>
<th>Biofilm production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Weak (%)</td>
</tr>
<tr>
<td>1.</td>
<td>38</td>
<td>16 (44.44%)</td>
</tr>
</tbody>
</table>

The present study was designed to identify the susceptibility and the resistance profile of the Biofilm forming \textit{S. aureus} isolates from various clinical samples (Urine, Blood, Semen, and Pus). Results revealed an increasing trend towards development of antibiotic resistance. The resistance corresponded to more for Erythromycin and Amoxicillin, whereas Ofloxacin and Ciprofloxacin were more sensitive against Biofilm forming \textit{S. aureus} (Table 5, Fig 5). In this
study, the most effective antibiotic for *Staphylococcus aureus* was Ofloxacin showing 71.05% efficacy followed by Ciprofloxacin with 68.4% efficacy, erythromycin with 31.5% efficacy, and Amoxicillin with 28.9% efficacy by disc diffusion method.\(^{[32]}\)

Efficacy (%) = \(\frac{\text{Sensitive isolates no}}{\text{Total isolates no}}\) x 100

**Table 5: Antibiotic susceptibility pattern of Biofilm forming S. aureus (Disc diffusion test)**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Antibiotic Disc</th>
<th>Conc. (µg)</th>
<th>Biofilm forming S. aureus</th>
<th>Efficacy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sensitive</td>
<td>Resistance</td>
</tr>
<tr>
<td>1</td>
<td>Ofloxacin (Of)</td>
<td>30</td>
<td>27</td>
<td>11</td>
</tr>
<tr>
<td>2</td>
<td>Erythromycin (Er)</td>
<td>30</td>
<td>12</td>
<td>26</td>
</tr>
<tr>
<td>3</td>
<td>Amoxicillin (Am)</td>
<td>30</td>
<td>11</td>
<td>27</td>
</tr>
<tr>
<td>4</td>
<td>Ciprofloxacin (Cf)</td>
<td>30</td>
<td>26</td>
<td>12</td>
</tr>
</tbody>
</table>

In Agar well diffusion test, the resistance corresponded to more for Erythromycin and Amoxicillin, whereas Ofloxacin and Ciprofloxacin were more sensitive against Biofilm forming *S. aureus* (Table 6, Fig 6). Several studies have documented a similar pattern of resistance among *S. aureus* isolates.\(^{[32]}\) In our study, the most effective antibiotic for *Staphylococcus aureus* with well agar diffusion was Ciprofloxacin showing 84.2% efficacy followed by Ofloxacin with 81.5% efficacy, erythromycin with 55.2% efficacy, where as Amoxicillin with 26.3% efficacy.

**Table 6: Antibiotic susceptibility pattern of Biofilm forming S. aureus (Agar well diffusion test)**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Antibiotic Disc</th>
<th>Conc. (µg)</th>
<th>Biofilm forming S. aureus</th>
<th>Efficacy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sensitive</td>
<td>Resistance</td>
</tr>
<tr>
<td>1</td>
<td>Ofloxacin (Of)</td>
<td>30</td>
<td>31</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>Erythromycin (Er)</td>
<td>30</td>
<td>21</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>Amoxicillin (Am)</td>
<td>30</td>
<td>10</td>
<td>28</td>
</tr>
<tr>
<td>4</td>
<td>Ciprofloxacin (Cf)</td>
<td>30</td>
<td>32</td>
<td>6</td>
</tr>
</tbody>
</table>

**CONCLUSION**

Our results indicate that the TCP method is an accurate and reproducible method for detection of biofilm producing Staphylococci. In the study most of the biofilm producing *Staphylococcus aureus* were collected from the pus samples had greater ability to produce biofilms than isolates taken from the other sites. The differentiation with respect to its biofilm
phenotype might help to modify the antibiotic therapy and to prevent infection related to biomedical devices. In the study Ofloxacin was more sensitive to most of the antibiotics used for biofilm forming *S. aureus* by Disc diffusion test, Whereas Ciprofloxacin was more sensitive to most of the antibiotics used for biofilm forming *S. aureus* by Agar well diffusion test.

**ACKNOWLEDGEMENT**

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**REFERENCES**


