Detection and Inhibition of *Staphylococcus aureus* Biofilms by Chemical Agents

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**ABSTRACT**

**Introduction:** *Staphylococcus aureus*, a gram-positive pathogen, is one of the most frequent causes of biofilm-associated infections on indwelling medical devices. Biofilm is formed when bacteria live in communities and form a matrix as a survival mechanism in a generalized manner. With the emergence of *methicillin-resistant Staphylococcus aureus* (MRSA) and its biofilm forming ability, there is an urgent need to discover active agents against *Staphylococcus aureus*. One possible way to control biofilm formation on indwelling medical devices is to coat the devices with the agents that can inhibit bacterial colonization and biofilm formation. To test this concept, in this study, we used several assays to detect biofilm forming potential of a clinical isolate of *Staphylococcus aureus*, followed by testing of the anti-biofilm activity of three chemical agents.

**Materials And Methods:** *Staphylococcus aureus* clinical isolate was screened for susceptibility/resistance against eight commonly prescribed antibiotics (ciprofloxacin, chloramphenicol, ampicillin, amikacin, cephalothin, clindamycin, streptomycin, and gentamicin) using the Kirby-Bauer disk diffusion method. We applied both qualitative and quantitative assays to detect the biofilm formation by the *Staphylococcus aureus* isolate. In the next step, the antibiofilm activity of three chemical agents, namely SDS, NaOH and Tween20, was examined using qualitative and quantitative biofilm reduction assays.

**Results:** The *Staphylococcus aureus* isolate was found to be susceptible to all antibiotics except clindamycin. The individual Minimum Inhibitory Concentration (MIC) of SDS, NaOH, and Tween20 against *Staphylococcus aureus* isolate was found to be 0.05%, 1M and 5%, respectively. In biofilm reduction assay, we found a noteworthy biofilm inhibition potential of these agents, which was 89.41% for Tween20, 83.05% for SDS and 71.93% for NaOH.

**Conclusions:** These compounds are generally used in laboratories for diverse research purposes. These chemicals can be repurposed for their ability to inhibit the biofilm produced by *Staphylococcus aureus*, hence providing an effective alternative to deal with the problem of catheter-associated infections.

**Keywords:** Biofilm, *Staphylococcus aureus*, Chemical agents


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INTRODUCTION

Bacterial biofilm formation is the natural phenomenon in which bacteria colonize themselves to evade environmental stress, antibacterial drugs and disinfectants (1). Biofilm is readily formed on indwelling medical devices (IMDs) which can lead to resistant infections and ultimately treatment failure (2). Chances of device-related infections can increase when a device is left inserted into the patient for more than 48 hours (3). A survey-based study conducted from 2009-2010 concludes that 47.4% (239 of 504 infections) of all healthcare-associated infections were device-related infections (4). These device-related infections result in significant morbidity, mortality, and costs of healthcare delivery in the patients around the globe and measures are required to mitigate these infections. Biofilm formation takes place when the microbial cells get attached to the indwelling device surface and produce a matrix known as extracellular polymeric substance (EPS). Composition of EPS includes proteins, polysaccharides and extracellular DNA which performs various necessary functions like adhesion, aggregation and protection from antimicrobial agents as well as from the host immune system (5). As the biofilm ages, the organism develops a tolerance against the antimicrobial agents and the only option left, especially in the case of Staphylococcus aureus, is the removal of the device (6).

Staphylococcus aureus is one of the notorious pathogens which actively causes nosocomial infections. With the alarming increase in the prevalence of methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant Staphylococcus aureus (VRSA), the need to control the spreading of the nosocomial and device related infections are high (7). One possible way to control biofilm formation on indwelling medical devices is to coat the devices with inhibitory agents that can inhibit bacterial colonization and biofilm formation. The aim of the current study was, therefore, to use qualitative and quantitative assays to detect biofilm forming potential of the Staphylococcus aureus clinical isolate, followed by evaluation of the anti-biofilm activity of three chemical agents namely, Sodium Dodecyl Sulfate (SDS), sodium hydroxide (NaOH) and Tween20.

MATERIALS AND METHOD

Staphylococcus aureus culture

The culture of Staphylococcus aureus was obtained from our laboratories culture bank and maintained in Tryptic Soy Broth (TSB; Oxoid) at 37°C.

Determination of antibiotic susceptibility/resistance profile of Staphylococcus aureus using Kirby-Bauer disc assay

Kirby-Bauer disc assay, in accordance with Clinical and Laboratory Standards Institute guidelines 2015, was used to analyze the antibiotic susceptibility/resistance profile of Staphylococcus aureus against 8 commonly prescribed antibiotics, namely Ciprofloxacin 5μg (CIP), Gentamicin 120μg (CN), Chloramphenicol 30μg (C), Ampicillin 10μg (AMP), Clindamycin 2μg (DA), Amikacin 30μg (AK), Streptomycin 10μg (S) and Ceftalothin 30μg (KF). The lawn of Staphylococcus aureus was prepared with 0.5 McFarland (10^6 CFU/mL) on nutrient agar plates and discs of the above-mentioned antibiotics were placed over it, which were incubated at 37°C for 24 hours and zones of inhibition were measured.

Determination of Minimum Inhibitory Concentration of compounds

To determine the Minimum Inhibitory Concentration of Sodium dodecyl sulfate (SDS), Sodium Hydroxide (NaOH) and Tween20, stock solutions were prepared as 10%, 5M, and 10%, respectively. Serial dilution of 1:40 was performed in 96 well untreated sterile plates containing TSB, the culture of Staphylococcus aureus and the chemical agents. Blank (only media, no culture, no test agents) and control (Staphylococcus aureus culture but no test agent) wells were used as a reference. The plate was incubated at 37°C for 24 hours. The next day, the MIC for each agent was determined using the following criteria: the lowest concentration of the agent in each well that exhibited no visible bacterial growth (8).
Detection of Biofilm forming potential of the *Staphylococcus aureus* isolate

The biofilm forming potential of *Staphylococcus aureus* isolate was tested using the following qualitative methods: Tube Method and Air Liquid Interface Assay. Biofilm formation was quantitatively analyzed using the spectrophotometric assay.

**TUBE METHOD**

This gold standard method used for the qualitative detection of biofilm was described by Christensen et al. (9). The tube method was performed in two different ways. Firstly, 50μl of *Staphylococcus aureus* culture was inoculated in 3ml of Tryptic Soy Broth and incubated at 37°C for 48 hours. After incubation, at the air-liquid interface, a dense matt formation was observed representing the formation of biofilm. In the second method, the same procedure was performed as above but after incubation, the tube’s content was decanted in another tube for spectrophotometric analysis. Following this, the tube was washed 3 times with distilled water and allowed to air-dry. Subsequently, Crystal violet (0.1% w/v) was added by rotating the tube for 15 minutes to stain uniformly. Excess stain was removed, and tubes were washed with distilled water and kept in an inverted position to air dry. Positive biofilm formation was considered when both the wall and bottom of the tube were stained violet. Scoring was done as 1-weak/none, 2-moderate and 3-strong. The experiment was performed in triplicate and repeated three times (10).

**Air Liquid Interface Assay**

*Staphylococcus aureus* culture (20μl) of 0.5 McFarland was inoculated in 3mL of TSB in each well of 12 well plates and a coverslip was carefully placed in a vertical direction (90° angle) to the well’s surface and incubated at 37°C for 48 hours. Coverslips were washed with distilled water and placed in another well-containing 4mL of Crystal violet (0.1% w/v) for 15 minutes. Following this, coverslips were washed three times with water and placed on a glass slide and kept on a hotplate to dry. Completely dried coverslips were visualized under a high power light microscope (Olympus BX43, Japan) and pictures were taken using a digital camera. One well served as a medium control (blank) while others served as culture control (positive control) (11).

**Spectrophotometric assay for quantitative analysis of biofilm formation**

The content of the tube, from the tube method, as described above was, used to measure the O.D at 630nm for the detection of biofilm. Blank was done with TSB and cuvette was placed in a spectrophotometer (Thermo Scientific™ GENESYSTM 30 Visible Spectrophotometer) and O.D was measured. Scoring was done as: O.D<0.5 = No/weak biofilm, O.D=0.5 = moderate biofilm, O.D>0.5 = strong biofilm.

**Determination of the antibiofilm activity of the chemical agents against *Staphylococcus aureus* biofilms**

The anti-biofilm activity of the three chemical agents (SDS, NaOH, and Tween20) was measured by using tube method and air-liquid interface method as described previously, with the modification that this time chemical agents were also added according to their MIC determined in the previous assays.

Quantitative evaluation of the antibiofilm activity of the three chemicals was performed using microtiter plate spectroscopic assay (10, 11). Briefly, *Staphylococcus aureus* culture (5μl) of 0.5 McFarland was added in 200ul TSB in each of 96-well untreated flat-bottom microtitre plate (Thermo Scientific™ Nunc™ Edge 2.0) in control well while in other well agents (SDS 0.05%, NaOH 1M, and Tween20 5%) were added to have the required concentration and incubated at 37°C for 24 hours. The next day, the content of the well was discarded and gently washed with distilled water. For staining, 250μl of Crystal violet (0.1% w/v) was added for 15 minutes with lid closed and washing of plate was done by distill water and left it for air dry. Subsequently, 300μL of 95% ethanol was added in each well and the plates were incubated for 15 minutes at room temperature with the lid closed. Pipetting was...
done in each well to mix the content properly and 150μL of the Ethanol/ Crystal Violet solution was transferred into a new 96 well plate. The optical density of each well was measured at 630nm using Multiskan Sky Microplate Spectrophotometer (Cat #51119700). The experiment was performed in triplicates. One well was served as a medium control (blank) while others served as culture control (positive control). Efficacy for reduction of biofilm was calculated in percentages from blank, control and test by using the formula:

\[
\text{Percentage Reduction/Removal} = \left(\frac{(C-B) - (T-B)}{(C-B)}\right) \times 100\%
\]

Where \( B \) = absorbance of blank (only TSB), \( C \) = absorbance of the control (biofilm, no treatment) and \( T \) = absorbance of the test (biofilm and treatment) \(^{(12)}\).

RESULTS

Antibiotic resistance/susceptibility profile of *Staphylococcus aureus*

The Antibiotic resistance/susceptibility profile of *Staphylococcus aureus* revealed that the isolate was sensitive to all the antibiotics except clindamycin (Table 1).

Analysis of Biofilm forming potential of *Staphylococcus aureus* isolate:

In this study, the biofilm forming potential of Staphylococcus aureus was assessed using both qualitative methods (Tube Method, and Air Liquid Interface Assay) and quantitative method (Spectrophotometric analysis). The tube method shows a whitish matt at the liquid-air interface as well as the bottom of the tube which confirms the growth and biofilm formation of *Staphylococcus aureus* (Figure 1A). Upon staining the tube shows visible purple stain on the wall and bottom of the tube (Figure 1B). These findings were corroborated by Air Liquid Interface assay, where microbial aggregation (biofilm formation) was observed on the coverslips under an inverted microscope (Figure 1C).

Findings from the spectrophotometric analysis revealed that the *Staphylococcus aureus* isolate was a dominant biofilm former (OD=1.0).

Antimicrobial and anti-biofilm activity of the chemical agents:

The antimicrobial potential of the three chemical agents was evaluated using the MIC of each of the test agents. The minimum inhibitory concentration was found to be 0.05% (v/v) for SDS, 1M for NaOH (w/v) and 5% (v/v) for Tween20.

Qualitative analysis of the effect of three chemical agents on Biofilm forming potential of *Staphylococcus aureus*

The qualitative analysis of the biofilm formation performed using the tube method (Figure 2) and Air-liquid interface assay (Figure 3) in the presence of the three chemical agents showed significant biofilm inhibition at MIC concentration.

<table>
<thead>
<tr>
<th>ANTIBIOTICS</th>
<th>Disc Concentration (ug)</th>
<th>Zone of inhibition (mm)</th>
<th>Sensitive/Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>30</td>
<td>30</td>
<td>S</td>
</tr>
<tr>
<td>Amikacin</td>
<td>30</td>
<td>21</td>
<td>S</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>30</td>
<td>28</td>
<td>S</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>10</td>
<td>15</td>
<td>S</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>2</td>
<td>17</td>
<td>R</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>10</td>
<td>15</td>
<td>S</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>120</td>
<td>26</td>
<td>S</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>30</td>
<td>20</td>
<td>S</td>
</tr>
</tbody>
</table>

Table 1: Antibiotic resistance/susceptibility profile of *Staphylococcus aureus* isolates against eight commonly prescribed antibiotics showed that the isolate was only resistant to clindamycin.
Figure 1: Qualitative analysis of *Staphylococcus aureus* biofilm formation. 1A) A whitish matt layer at the air-liquid interface and bottom is clearly observed (highlighted with red box). 1B) Crystal violet staining of biofilm attached to the wall of the polystyrene tube. 1C) Microscopic image of Air-Liquid interface coverslip assay. Aggregation of dense matt is noticeably evident.

Figure 2. Tube method for the qualitative analysis of the anti-biofilm activity of the three chemical agents against *Staphylococcus aureus* biofilms: Tube A= Blank, containing media only shows no biofilm formation; Tube B= Positive control without any treatment shows strong biofilm attached to the surface and bottom of the tube; Tube C= 1 M NaOH, Tube D= SDS 0.05% and Tube E=Tween20 5% presenting visible inhibition when compared with control.
Figure 3. Air-Liquid Interface method for the qualitative analysis of the anti-biofilm activity of the three chemical agents against *Staphylococcus aureus* biofilms: A= Positive control without any treatment shows microbial aggregates attached to the coverslip, Coverslip B (NaOH 1 M), C (SDS 0.05%) and D (Tween20 5%) show significant inhibition.

Quantitative analysis of the effect of three chemical agents on Biofilm forming potential of *Staphylococcus aureus*

Quantitative analysis of the anti-biofilm activity also validated the qualitative results, where all three chemicals gave appreciable anti-biofilm activity: 89.41% reduction was observed with Tween20, 83.05% reduction with SDS and 71.93% reduction with NaOH (Figure 4).

Figure 4. Effect of chemical agents on inhibition of biofilm of *Staphylococcus aureus*: Biofilm reduction assay shows that these three agents exhibit strong anti-biofilm activity.
DISCUSSION

In this study, we used qualitative and quantitative assays to detect biofilm forming potential of the *Staphylococcus aureus* clinical isolate, followed by evaluation of anti-biofilm activity of three chemical agents namely, Sodium Dodecyl Sulfate (SDS), sodium hydroxide (NaOH) and Tween20.

This qualitative and quantitative analyses performed in this study showed that *Staphylococcus aureus* clinical isolate is a potent biofilm former. This study is in agreement with the previous studies (13-15) that showed that *Staphylococcus aureus* has a strong biofilm forming ability. By analyzing the antibiotic resistance/susceptibility profile of *Staphylococcus aureus* we found that this isolate was susceptible to all antibiotics except clindamycin. These results are also supported by other studies. For example, Khamash, et al., reported clindamycin resistance in *Staphylococcus aureus* that has an effect on surgical site infection, empirical management and prophylaxis (16, 17).

One possible way to control biofilm formation, especially on indwelling medical devices, is to coat the devices with inhibitory agents that can inhibit bacterial colonization and biofilm formation. In our study, we tested the anti-microbial and anti-biofilm activity of 3 chemical agents, SDS, NaOH and Tween20. Our results showed that all three chemical agents are potent anti-antimicrobial agents. Furthermore, these three chemical agents also exhibited potent anti-biofilm activity, which was 89.41% for 5% Tween20, 83.05% for 0.05% SDS and 79.93% for 1M NaOH. Our results are noteworthy in the backdrop that SDS has been used previously as an anti-biofilm agent (18) in combination with an enzyme DNase I against *Pseudomonas aeruginosa* biofilms. In another study, NaOH and Tween20 were found to exhibit antibiofilm activity (19).

We believe that these observations have important implications and utility. These can be used to coat the medical devices which could significantly reduce the incidence of device related infection and aid in the control of hospital-acquired infections caused by *Staphylococcus aureus*.

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