Detection of Biofilm formation among *Staphylococcus aureus* isolated from hospital-admitted patients' wounds and their relationship with antibiotic resistance

Abu baker H. Abduelrhman

Department of Medical Laboratory Technology, High Institute of Science and Medical technology, EL-Garahbolii, Libya

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ABSTRACT

**Background:** Biofilm is known to be formed by microorganisms. The biofilms are commonly found in chronic wound infections, surgical site infections, implants, and other places. They can cause recalcitrant infections and are known to have high antibiotic resistance. The aim of this research was to prevalence and compare the *in vitro* biofilm-forming ability of *Staphylococcus aureus* isolated from wounds of hospitalized patients', as well as their association with antibiotic resistance.

**Materials and methods:** Using standard microbiological techniques, 57 clinical isolates of *S. aureus* were obtained from 128 wounds samples. *S. aureus* was identified by VITEK2 system. Tissue culture plate (TCP) analysis was used to detect biofilm formation in these isolates. The modified Kirby–Bauer disk diffusion method was used to test antibiotic sensitivity in accordance with Clinical and Laboratory Standards Institute guidelines.

**Results:** *S. aureus* was identified by VITEK2 system. The Biochemical characteristics and antibiotics resistance by VITEK2 system with excellent probability 99%. Biofilm formation occurs in 26 (31, 32%) of *S. aureus* isolates using the TCP method. When compared to biofilm nonproducers, biofilm-producing *S. aureus* had a higher incidence of antimicrobial resistance (*P*<0.05). **Conclusion:** An in vitro method revealed that *S. aureus* isolated from hospitalized patients' wound infections has a high degree of biofilm-forming ability. Antibiotic resistance is highly prevalent in biofilm-producing strains.

**Keywords:** *Staphylococcus aureus*, biofilm, Antibiotic resistance, *in vitro*

1. Introduction

The aggregation of bacteria in a self-produced extracellular matrix of exopolysaccharides (EPSs), proteins, and some micromolecules such as DNA is known as a biofilm. They can grow on biotic as well as abiotic surface (Gowrishankar *et al*., 2012).

Biofilm is an important virulence factor that provides a safe environment for organisms to survive (Shashikala *et al*., 2001). Biofilm-associated infections are difficult to treat because the biofilm confers resistance against the host immune system and renders the bacterial cells antibiotic-resistant (Høiby *et al*., 2010).

*S. aureus* is an opportunistic pathogen that has been linked to the majority of skin and soft tissue infections. It can be found in the nasopharynx, skin, eyes, and intestine (Ziebuhr *et al*., 2001). In *S. aureus* isolates, biofilm formation occurs via polysaccharide intercellular adhesion (PIA) as well as microbial surface components that recognize adhesive matrix molecules (MSCRAMMs) (Andhale *et al*., 2016). These structures mediate *S. aureus* initial attachment to both host tissues and biomaterials (Hassan *et al*., 2005). Biofilm formation disrupts the innate immune system's bacterial recognition and killing mechanisms (Andhale *et al*., 2016).
Antibiotic resistance mechanisms also include delayed antimicrobial agent penetration through the biofilm matrix, altered growth rates of biofilm forming organisms, and other physiological changes that occur during biofilm growth. Many, and chronic recurrent infections (Hassan et al., 2005).

As a result, our objective is to investigate the ability of \textit{S. aureus} isolated from hospitalized patients’ wounds to form biofilms \textit{in vitro} and their relationship to antibiotic resistance.

2. Materials and Methods

I. Preparation of clinical pus.

The cross-sectional observational research was performed at the Department of medical technology at higher institute of science and technology. A total of 128 routine pus samples were collected from wounds at various body sites patients of Msallata hospitals from March 2019 to July 2019. Approximately, 0.5 g of wound samples were resuspended in phosphate-buffered saline (PBS) with 5 ml of 10\% (w/v) in PBS (0.01M Tris solution (pH 7.5), 14.5mM, NaCl and 10mM, CaCl2). Wound solutions were vortexed and clarified by centrifugation at 3000 rpm for 10 min. The clarified supernatant (1.5-2.0 ml) was collected, and stored at 4-8\(^\circ\)C for short term storage until use.

II. Detection of \textit{S. aureus} by free coagulase:

Coagulase causes plasma to clot by converting fibrinogen to fibrin. Tested by: Slide coagulase test (to detect bound coagulase). One hundred microliters of brain heart infusion broth culture of each tested isolate was added to 0.5 ml of 5-fold dilution fresh citrated rabbit plasma. The tubes were incubated at 35-37\(^\circ\)C and examined for clotting after 1, 2, 3, and 24h. The results were interpreted as follows: Score of 2+ (small organized clot), 3 + (large organized clot), 4+ (entire content of the tube coagulates and is not displaced when the tube is inverted). The results were considered positive evidence of coagulase production.

III. Isolation and enumeration of \textit{S. aureus} (FDA, 2002).

Isolation and enumeration of \textit{S. aureus} in was carried out by spreading 0.1(ml) of each sufficient dilution onto the surface agar. Baird parker media (Oxoid; CM0275) supplemented with egg yolk and potassium tellurite solution. Plates were incubated at 37\(^\circ\)C for 48h, then the presence of typical colonies, which appears gray-black, shiny and convex with a narrow white margin surrounded by a clearing zone. The numbers of typical colonies were counted. Suspected \textit{S. aureus} were isolated by culturing on brain heart infusion agar (Oxoid;CM225) slants medium for further identification.

IV. Purification and identification of \textit{S. aureus}.

\textit{S. aureus} colonies obtained from all previously mentioned media were chosen and picked up according to variation in culture characteristics and colony formation then purified by streak-plate method on Nutrient agar medium. Pure isolates were maintained on slants of the same medium at 4\(^\circ\)C for subsequent identification. \textit{S. aureus} was identified by VITEK2 system (Funke et al., 1998).

The reagent cards have 64 wells that can each contain an individual test substrate. Substrates measure various metabolic activities such as acidification, alkalinization, enzyme hydrolysis, and growth in the presence of inhibitory substance. There are currently four reagent cards available for the identification of different organism classes as follows:

V. Suspension preparation:

A disposable bacterial needle used to transfer a single colony of a pure culture and suspended in 3.0 ml of sterile saline (aqueous 0.45\% to 0.50\%NaCl, pH4.5 to 7.0) in a 12x75 mm clear plastic (polystyrene) test tube. The turbidity was adjusted according Table (1) and measured using a turbidity meter called the DensiChek.

Identification cards were inoculated with \textit{S.aureus} suspensions using an integrated vacuum apparatus. A test tube containing \textit{S. aureus} suspension was placed into special rack (cassette) and the identification card was placed in the neighbor in gloat while inserting the transfer tube into the corresponding suspension tube.
Table 1: Suspension turbidities used for card inoculation

<table>
<thead>
<tr>
<th>Product</th>
<th>McFarland Turbidity Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>GN</td>
<td>0.50-0.63</td>
</tr>
<tr>
<td>GP</td>
<td>0.50-0.63</td>
</tr>
<tr>
<td>YST</td>
<td>1.80-2.20</td>
</tr>
<tr>
<td>BCL</td>
<td>1.80-2.20</td>
</tr>
</tbody>
</table>

VI. Tissue Culture Plate method

Christensen et al. (1985) described this quantitative gold standard method for biofilm detection. In a nutshell, a *S. aureus* colony was isolated from a fresh agar plate and inoculated in 2 mL of tryptase soy broth. Overnight at 37°C, the broth was incubated. With fresh medium, the culture was diluted to 1:100. 200 L of the diluted culture was poured into a sterile individual plate with 96 flat-bottom polystyrene wells. In the same way, the control organisms were processed.

The plate was incubated for 24 hours at 37°C. The contents of each well were gently tapped out after incubation. To remove free-floating bacteria, the wells were washed with 200 L of phosphate buffer saline (pH 7.3). Biofilms formed by bacteria attached to the wells were fixed with 99 percent methanol and stained with 0.1 percent crystal violet (CV). The excess stain was gently washed away, and the plate was set aside to dry. A micro ELISA auto-reader (HUMAN) was used to measure the optical density of the stained adherent biofilm at a wavelength of 570 nm.

The experiment was carried out three times. Biofilm production was interpreted using the criteria described by Stepanovic et al. (2007), and bacteria were classified as biofilm nonproducers, weak, moderate, or strong biofilm producers.

VII. Antimicrobial Sensitivity

The clinical isolates were tested for antibiotic sensitivity using the standard disk diffusion technique (modified Kirby–Bauer method) in Müller–Hinton agar (MHA), and the results were interpreted according to Clinical and Laboratory Standards Institute guidelines (CLSI-G 2002). Antibiotics that were tested included: gentamicin (10 µg), ciprofloxacin (5 µg), rifampicin (5 µg), vancomycin (30 µg), erythromycin (15 µg) and cotrimoxazole (25 µg) (HiMedia Laboratories, Mumbai, Maharashtra, India). *S. aureus* ATCC 25923 was used as the control organism.

2.1. Statistical analysis

The Chi-square analysis was used to measure categorical data. A *P*-value of 0.05 was considered statistically significant.

3. Results

3.1. *S. aureus* identification:

*S. aureus* was incidence in clinical pus and isolated and enumerated onto plates of blood media. Shaped of colony were Raised, circular, entire. Biochemical Identification of isolates which belonging to *S. aureus* showed Smooth Texture, Golden yellow Pigmentation, Shape Cocci in clusters & pairs, no motility, Beta Hemolysis on blood agar, facilitative anaerobic, positive Gram, positive Catalase, Coagulase and Urease negative Oxidase. On blood agar plates, colonies of *S. aureus* often cause β-hemolysis. *S. aureus* formed large yellow colonies surrounded by wide yellow zones and turned the color of the medium from pink to yellow. *S. aureus* was a Gram-positive spherical bacterium approximately 1 µm in diameter and formed grape-like clusters.

The Biochemical characteristics of *S. aureus* was confirmed with excellent probability 99% after full Biochemical Identification by VITIK2 system as well as the susceptibility information was provided as shown in table (2).

3.2. Biofilm formation

Among some of the 57 clinical isolates of *S. aureus* obtained from the wounds, biofilm formation was observed in 26 (45.61%) isolates tested. Only five isolates (19.23%) were classified as strong biofilm producer, while eight (30.76%) of the clones were classified as moderate producers. More than
half of the producers (50%) were found weak biofilm producers. A total of 31 (54.38%) of the samples showed no evidence of biofilm producers (table 3).

Table 2: Biochemical characteristics of *S. aureus* by VITEK 2

<table>
<thead>
<tr>
<th>Selected Organism</th>
<th>AMY</th>
<th>APPA</th>
<th>LeuA</th>
<th>AlaA</th>
<th>dRIB</th>
<th>NOVO</th>
<th>dRAF</th>
<th>OPTO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>PIPLC</td>
<td>CDEX</td>
<td>ProA</td>
<td>TyrA</td>
<td>ILATk</td>
<td>NC6.5</td>
<td>O129R</td>
<td>Installed VITEK 2® Systems Version: 08.01</td>
</tr>
<tr>
<td></td>
<td>dXYL</td>
<td>AspA</td>
<td>BGUR</td>
<td>dSOR</td>
<td>LAC</td>
<td>+</td>
<td>+</td>
<td>010402062663231</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>BGAR</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>99% Probability:</td>
</tr>
<tr>
<td>Bionumber</td>
<td>ADH1</td>
<td>BGAL</td>
<td>AMAN</td>
<td>URE</td>
<td>NAG</td>
<td>dMAL</td>
<td>PUL</td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>POLYB</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>009</td>
</tr>
<tr>
<td></td>
<td>AGLU</td>
<td>PHOS</td>
<td>BGUR</td>
<td>dGAL</td>
<td>BACI</td>
<td>-</td>
<td>ADH2s</td>
<td>008</td>
</tr>
</tbody>
</table>

Table 3: The MTP method was used to analyze biofilm production.

<table>
<thead>
<tr>
<th>Degree of biofilm formation</th>
<th>No. of <em>S. aureus</em> (N=57 )</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strong</td>
<td>5</td>
<td>19.23</td>
</tr>
<tr>
<td>Moderate</td>
<td>8</td>
<td>30.76</td>
</tr>
<tr>
<td>Weak</td>
<td>13</td>
<td>50</td>
</tr>
<tr>
<td>None</td>
<td>31</td>
<td>54.38</td>
</tr>
</tbody>
</table>

3.3. Antibiotic sensitivity of *S. aureus* isolate

As a result, the aim of the research at the pattern of antibiotic resistance, when compared to biofilm producing to non-producers was associated with a higher incidence of antimicrobial resistance, as shown in table 3, gentamicin ( 53.84 % vs 6.45 % P=0.0007 ), ciprofloxacin ( 80.76 % vs 9.67 % P=0.0001 ), ampicillin ( 92.30 % vs 35.48 % P= ), erythromycin ( % vs % P=0.00011 ) and cotrimoxazole (7.69 % vs 3.10 % P= 0.049 ) (Table 4).

Table 4: Comparison of antibiotics resistance pattern in biofilm-positive and biofilm-negative *S. aureus*

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>BP (N=26)</th>
<th>BN (N=31)</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentamicin 10 µg</td>
<td>14(53.84)</td>
<td>2(6.45)</td>
<td>*0.0007</td>
</tr>
<tr>
<td>Ciprofloxacin 5µg</td>
<td>21(80.76)</td>
<td>3(9.67)</td>
<td>0.0001*</td>
</tr>
<tr>
<td>Rifampicin 5 µg</td>
<td>0(0)</td>
<td>0(0)</td>
<td>NA</td>
</tr>
<tr>
<td>Vancomycin 30 µg</td>
<td>0(0)</td>
<td>0(0)</td>
<td>NA</td>
</tr>
<tr>
<td>Ampicillin 10 g</td>
<td>24(92.30)</td>
<td>11(35.48)</td>
<td>0.00011*</td>
</tr>
<tr>
<td>Erythromycin 15 µg</td>
<td>11(42.30)</td>
<td>1(3.10)</td>
<td>0.0003*</td>
</tr>
<tr>
<td>Cotrimoxazole 25µg</td>
<td>2(7.69)</td>
<td>1(3.10)</td>
<td>0.049*</td>
</tr>
</tbody>
</table>

Abbreviation: BP. Biofilm positive, BN. biofilm negative
Note: Statistically significant (P< 0.05)
NA, no applicable.

4. Discussion

*S. aureus* is a zoonotic pathogen that can cause a wide range of symptoms in both animals and humans. *S. aureus* that produces biofilms is a major public and animal health concern. The identification of *S. aureus* adherence property has revealed associations between biofilm formation and degree of pathogenicity, with the organism's virulence property found to vary with its ability to adhere to the surface (Baddour et al., 1984).

In this study, we discovered that *S. aureus* isolated from hospitalized patients' wounds can form biofilms in vitro and that this ability is associated with drug resistance. TCP method in vitro screening procedures were used to assess the ability of 57 *S.aureus* isolates to form biofilm.
In the present research, the prevalence of biofilm production was higher (45.61%) percent detected by TCP. Our findings, which were based on wound samples, are similar to those of a previous study, which found that 50% of blood samples formed biofilms (El-Nagdy et al., 2020). Biofilm formation is influenced by a variety of factors, including the environment, nutrient availability, geographical origin,
specimen types, surface adhesion characteristics, and the organism's genetic makeup (Poudel et al., 2015).

As a result, the adherence property of biofilm producers was classified as strong, moderate, or weak. In the current study, (19.23%) of the S. aureus were highly virulent and exhibited high adherence. Our findings were consistent with those of another Algerian study (Lotfi et al., 2014). showed five (19.23%) strongly adherent, eight (30.76%) moderately adherent, half (50%) weakly adherent, and 31 (68.84%) nonadherent isolates (Lotfi et al., 2014).

Because bacteria in biofilms are resistant to antibiotic compounds, biofilm infections are clinically significant (Doebbeling, 1995). S. aureus that produces biofilms was more prevalent. Biofilm producers are more resistant to antimicrobials than nonproducers of biofilms (Mathur et al., 2006). Biofilm producers were resistant to erythromycin, gentamicin, tetracycline, chloramphenicol, and teicoplanin in our research, whereas none of the biofilm nonproducers were ($P<0.05$). Biofilm producers had significantly higher rates of resistance to cotrimoxazole and ciprofloxacin than nonproducers ($P<0.05$). It has previously been reported that Gram-positive bacteria that produce biofilms are more resistant to erythromycin, cotrimoxazole, and ciprofloxacin (Hassan et al., 2011) and (Mishra et al., 2014). Our findings corroborate those of a previous study conducted (Ansari et al., 2014; Neopane et al., 2018).

As a result, antibiotic resistance was found to be higher among biofilm-producing S. aureus than among nonproducers in the current study. These findings suggest that biofilm formation could be a key factor in increasing resistance to commonly used antibiotics. Our results are consistent with those of a prior study (Neopane, et al., 2018).

Vancomycin is reported to be the most effective antibiotic for Gram-positive bacteria accordingly, our data also showed that all the isolates including biofilm producers were sensitive to vancomycin which is similar to others studies (Harika et al., 2020 and Neopane et al., 2018).

5. Conclusion

One of the most important virulence factors of S. aureus is biofilm production, which allows them to resist or inhibit the effects of various antibiotics. vancomycin is the best antibiotics to use against S. aureus in wound infections. As a result, we recommend that biofilm formation and antibiotic resistance profiles in S. aureus wound isolates be monitored on a regular basis. This may aid in the formulation of an effective antimicrobial policy for the treatment of wound infection in the early stages.

References


