Relation between biofilm formation ability and antibiotics resistant in *Staphylococcus aureus* from supplicative post-operation infections

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Abstract

**Background**: Antibiotics resistance and biofilm formation among *Staphylococcus aureus* strains represents a dangerous problem of the successful treatment during infections caused by opportunists *S. aureus*. The objectives of this study were to determine the antimicrobial susceptibility patterns and biofilm production among strains of *S. aureus* isolates from hospitalized patients after surgery. **Methods**: The present study included 130 Surgical Site Infections specimens represented swabs from supplicative infected sites. Using Disc diffusion method for antimicrobial susceptibility for isolates detected and tissue culture plate method was used for detection of biofilm formation ability. **Results**: The results confirmed that MRSA isolates had high degree of biofilm formation ability and increased the role in developing antibiotics resistance in a significant manner (approximately $P<0.0001$). In addition, *Staphylococcus aureus* was found as the predominant bacterial isolate (42.30%) after culturing all swab specimens tested. **Conclusion**: Increased antimicrobial resistance and biofilm formation affected synergistically in failure of surgical wound healing and invasion of infection promoting morbidity and mortality rates of hospitalized patients.

**Keywords**: *Staphylococcus aureus*, MRSA,MSSA, Biofilm Formation, Antibiotics Resistance, Suppurative and Post-operative Infections


Introduction

Post-operative infections also named Surgical Site Infections (SSIs) as defined via the Centers of Disease Control and Prevention (CDC), these infections occur within 30 days or within one year (secondary infections) of an operation at the site of incision of the body where the surgery happened. Biofilm formation of *Staphylococcus aureus* can be due to existence in complex communities communicating among themselves, instead of growing planktonically or individually, allowing them to be more resistant to antimicrobial in the normal environment of tissue and blood[¹]. According to the WHO records [²], SSIs usually represent (20%) from total cases annually and *Staphylococcus aureus*, particularly MRSA strains, are important as the opportunistic pathogen that causes more than 50% of these infections, especially when these isolates have the ability to form biofilm. Hence, these isolates of bacteria are considered the most common causative pathogen of hospital acquired infections and a major cause of morbidity as well as mortality of complication of infections after
surgery mainly in the cases of diabetes mellitus, older age, smoke, malnutrition, immunosuppression, and obesity as higher hosts' risk factors for infections. Moreover, risk factors associated with surgical procedure and prevention, including operating room ventilation, surgical technique, sterilization techniques and antimicrobial prophylaxis, however, post-operative infections remain fundamental causes of morbidity, prolonged hospitalization and death due to contamination with the isolates under focus in the present study. The objectives of this study were to determine the antimicrobial susceptibility patterns and biofilm production among strains of *S. aureus* isolates from hospitalized patients after surgery.

Materials and Methods

- **Cases and Clinical Isolates**
  A total of one hundred thirty swabs were collected randomly from suppurative infected sites after been cleaned using sterile pads soak with alcohol 70%. Cases were adults of both genders suffering from suppurative post-operative infections post surgeries accomplished at Al-Hussein Teaching Hospital in Al-Muthanna Governorate. Swabs collection started from December 2018 to May 2019. *S. aureus* isolates were identified morphologically, microbiologically, biochemical tests and Vitek2 Compact System as in.

- **Antimicrobial Susceptibility experimentation**
  Isolated *S. aureus* was confirmed as MRSA by identification of their growth on Hi-Crome MRSA Agar Base Medium supplemented with Cefoxitin and MeReSa for more selectivity that was provided by company (Hi-Media/India) as in (Figure 1). All MRSA isolates were tested for susceptibility pattern to antimicrobial discs (11 types) provided by (Bioanalysis/Turkey). Using Disc Diffusion Method (Kirby-Bauer Method) on Mueller Hinton agar plate for screening their susceptibility; discs were incubated aerobicall y at (35-37°C) for 18-24hrs as recommended by.

- **Biofilm Formation Detection**
  Micro-Titer Plate (MTP) biofilm formation assay procedure was used according to. Isolates of *S. aureus* were inoculated in 5mL of BHI broth supplemented with 1% glucose for MRSA Isolates or with 4% NaCl for MSSA isolates according to, and then incubated overnight at 37°C. Cultures then diluted (1:100) with fresh BHI broth medium using polystyrene 96 well microtiter plates (Dragon /China), fixation step with Sodium acetate (2%) (B.D.H/ England), and then staining step was accomplished with (0.1%) crystal violet (Hi-Media/India). Biofilm formation ability was considered as positive result when a visible stained (slime film) padded the wall and the bottom of the well. Each well was examined for ability of biofilm formation was visually scored as (none, moderate or strong) depending on the intensification of resultant film. This test was repeated three times for each isolate before results recorded. Quantification of the biofilm formation was performed by using Spectrophotometer using ELISA Reader (Bio-TeK/ U.S.A.) to get Optical Density (OD) at 570nm of the stained adherent biofilm. Assay procedure was performed in triplicate and repeated three times and data were then averaged and the results were calculated according to the classification of bacterial adherence as in. Adherence recorded as (none/weak, moderate or strong) when OD values were (<0.120, 0.120-0.240, >0.240), respectively, and hence the results for biofilm formation were (Non/weak, moderately or high).
Statistical analysis

Statistical analysis of data was performed using SAS (Statistical Analysis System version 9.1). Percentages were compared by using Chi-squared test. \( P \)-value\(<0.05 \) was considered significant\(^{15} \). Moreover, Microsoft Excel (2010) was performed for demonstration of data including charts and percentage.

Ethical Consent

All authorizations were obtained before starting work in the hospital involving Ethical Consent and approval from ethical committee at Al-Hussein Teaching Hospital in Al-Muthanna Governorate.

Results

A total of 55(55\%) \textit{Staphylococcus aureus} isolates were isolated from the total collected swab samples (130) of suppurative infections. These isolates showed positive growth on Mannitol Salt Agar Base (MSAB) and appeared as golden-yellow/white colonies surrounded via yellow zone. In addition, the isolates fermented mannitol sugar (Figures 1 and 2). Further identifications by biochemical tests; Oxidase Negative, Catalase Positive and Coagulase Positive for both tube coagulase as well as slide coagulase tests(Figures 3, 4 and 5), respectively.

Figure (1): Colonies of MRSA on Hi-Crome MRSA Agar Base Medium, supplemented with Cefoxitin and MeReSa Antimicrobial Agents Vial, as shown green shiny and convex colonies after incubation at 37°C for 24-48hrs.
Figure (2): *Staphylococcus aureus* strains on MSAB medium contained (7.5%) NaCl; Golden yellow/white colonies surrounded with yellow zone as result of (Acid production due to fermentation of mannitol sugar) after incubation at 37°C for 24 hrs.

Figure (3): Oxidase test for *S. aureus* colonies. It was performed by (2-3) drops of N, N-Dimethyl p-phenylene diamine hydrochloride reagent were dropped on the test colony in filter paper No color change after 10 second indicating negative result (Oxidase-negative organisms).

Figure (4): Catalase test performed by adding 3% Hydrogen peroxide (H₂O₂) on the Colony of *S. aureus*. The appearance of bubbles indicated positive result, referred to that Catalase enzyme converts (H₂O₂) into oxygen and water. A: Control (negative control result), B: Positive result (visible bubbles).

Figure (5): (A and B) Coagulase Tube Test for *S. aureus*. A: Agglutination of the coagulase plasma Rabbit. B: Agglutination of the coagulase plasma Human; indicated for a positive result for free coagulase enzyme. C: Agglutination of the coagulase Rabbit plasma. D: Agglutination of the coagulase Human plasma; indicated for a positive result of bound coagulase enzyme.
From the total of 55 *Staphylococcus aureus* isolates, Methicillin-resistant strains (MRSA) was confirmed as 38 (69.09%) isolates. The distribution of these MRSA isolates on the clinical cases was shown in Figure (6). The highest percentage of recorded infections with MRSA was obtained from patients with amputation of diabetic foot (47.37%).

Concerning antimicrobial susceptibility tests, MRSA isolates recorded complete resistance (100%) against Oxacillin (OX), Cefamandole (MA), Carpenicillin (PY), Methicillin (ME), Erythromycin (E) and Amoxicillin (AX) (Figure 7); whereas resistance to Rifampin (RA) recorded (89%), Vancomycin (VA) (78.95%), Tetracycline (TE) (73.68%), Doxycycline (DO) (39%), and Clindamycin (DA) (79%) (Figure 8).

**Figure (6):** Methicillin-Resistant *Staphylococcus aureus* (MRSA) distribution on the clinical cases of post-operative infections.

**Figure (7):** Antibiotics Susceptibility tests for MRSA isolated from Patients with suppurative Post-operation infections. RA: Rifampin (5µg), VA: Vancomycin (30µg), TE: Tetracycline (30µg), E: Erythromycin (15µg), DO: Doxycycline (30µg), DA: Clindamycin (30µg), OX: Oxacillin (1µg), MA: Cefamandole (30µg), PY: Carpenicillin (100µg), ME: Methicillin (5µg), and AX: Amoxicillin (25µg). White arrows represent the diameters of inhibition zones measured in mm by the Caliper.
Diameter of inhibition zone for each individual antimicrobial Disc was translated in terms of sensitive, intermediate and resistant categories via comparison with the standard inhibition zone by\textsuperscript{[16]}.

Figure (8): Antibiotics Susceptibility Pattern for MRSA. S: Sensitive, I:Intermediate and R: Resistant to antibiotics.

The positive results of biofilm formation ability were confirmed in 55 isolates of both MRSA strains and MSSA strains. Biofilm forming MRSA recorded 36 isolates(94.73\%) from the total 38 (69.09) obtained MRSA isolates; whereas18(47.36\%)were strong biofilm formation producers, 18(47.36\%) were moderate producers, and (5.28\%) were non-biofilm forming isolates. On the other hand, 13(76.47\%) of MSSA isolates from the total 17 were able to produce biofilm in various levels as moderate biofilm estimated about 7(41.18\%) while 6(35.29\%) of MSSA produced strong biofilm. Non-biofilm forming of MSSA recorded 4(23.53\%) (Figures 9 and 10). The correlation between biofilm production ability of MRSA pathogens with their antimicrobial resistance revealed a significant relation (\(P<0.0001\)); whereas higher resistance to antimicrobial patternsrecorded in biofilm producers of MRSA isolates (Figure 11). Furthermore, this study confirmed that methicillin-resistant (MRSA) isolates of \textit{S. aureus} were more related to biofilm positivity than methicillin-susceptible (MSSA) isolates of \textit{S. aureus}.
Figure (9): Microtiter Plate Method (MTP) applied in screening of biofilm formation ability (blue arrow is negative control, red arrow is positive result (biofilm forming ability) and white arrow is for negative result (unable to form biofilm).

Figure (10): Biofilm formation abilities in Methicillin-Resistant *Staphylococcus aureus* (MRSA) and Methicillin-Sensitive *Staphylococcus aureus* (MSSA).

Figure (11): Biofilm forming ability and antibiotics resistance in MRSA Isolates.
Discussion

There is no doubt that *Staphylococcus aureus* represents the most dangerous bacteria that threatens patients post operation as opportunistic pathogen and, what makes the situation worse, some of these bacterial isolates are able to develop antibiotics resistance and biofilm formation which makes treatment for such infections very difficult and may lead to loss of life[17]. The present study focused on these isolates in local cases. As shown in the results, MRSA isolates represented 38(69.09%) from the total 55 *Staphylococcus aureus* isolates. Despite that, this percentage was higher than the proportion recorded by[18] but *Staphylococcus aureus* still the predominant organism of such cases. Also, [19] explained that due to the colonized health-care workers in the hospitals are considered the essential sources of MRSA strains in hospitalized patients causing higher rates of infections among them. Moreover, [20] reported that infection with MRSA isolates accounted for approximately 14(26.1) cases. Diabetic Foot Infections (DFIs) with amputations are acute health problems that negatively affect life of diabetic patients and additionally create a high economic problem for patients as well as society[21]. Similar results were obtained by [22], they reported in their study that infections of abdomen after surgery with MRSA isolates were 18% while the teamwork [23] found that osteomyelitis infections with methicillin-resistant *S. aureus* (MRSA) from the total isolates of *S. aureus*. The authors [24] during their investigation about post-operative infections also supported the present study results. The epidemiology of MRSA isolates has been continued to grow since it was first recorded more than three decades ago. Primarily, there were discontinuous reports of MRSA among nosocomial *S. aureus* isolates; whilst after that the prevalence of MRSA became a well-founded hospital-acquired pathogen as stated by [25]. The percentage of resistance to Oxacillin, Cefamandole, Methicillin, Carpenicillin, and Amoxicillin was higher than that reported by the teamwork [26]. Nevertheless, it was in partial agreement with that obtained by [27] as they found the resistance of most MRSA isolates was (100%). Moreover, [28] suggested that the dispersal of resistance can be based on the rates of gene transfer. In addition, Gram-positive bacteria have cell wall which is composed of heavily cross-linked peptidoglycan layers catalyzed by cell-wall trans-peptidases also known as penicillin-binding protein (PBP). On the other hand, β-lactam antibiotics damage peptide bonds formation by acting as competitive inhibitors to these PBPs. The latter results in formation of irreversible covalently bonded penicilloyl-enzyme complexes with weak cross-linked peptidoglycans thus facilitate bacterial lyses and dying [29]. The results of biofilm production ability that detected by phenotypic assays (Tissue Culture Plate method; TCP) were in agreement with those obtained by [30]; they observed that *in-vitro* biofilm production between MRSA strains were (43.5%) and MSSA strains were (8.7%) in phenotypic methods. The MRSA strains that were biofilm producers were observed with high resistance to most antibiotics used in the present study when compared to MRSA non-biofilm producers; this observation was consistent with the results of [31]. The latter reported a significant difference (*P*= 0.002) between biofilm producer (15 out of 50) and non-producer for isolates resistant to Erythromycin (0%) and tetracycline (10% versus 0%; *P*=0.237). Methicillin-Resistant *S. aureus* strains develop biofilm for adaptation to external factors in the environment. Biofilm limits the penetration of antibiotics and reduces treatment choices as stated by [23].

Conclusion

The present study confirmed that there was an increase in the prevalence of MRSA strains among *S. aureus* isolated from patients and these isolates were with a high degree of biofilm formation abilities in a significant manner. The latter played a role in the pathogenicity of these isolates by improving the development of
antibiotics resistant. This might lead to synergistic effect of both virulence factors on the high risk of failure in the surgical wound healing and invasion of the infection promoting morbidity and mortality of hospitalized patients.

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**Ethical Clearance**
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**Conflict of Interest**
The authors declare that they have no conflict of interest

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**References**
