Molecular Investigation of Fibronectin-Binding Protein Genes and Capacity of Biofilm Production in *Staphylococcus Aureus* Isolated From Clinical Samples

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Research Article

Keywords: Staphylococcus aureus, Biofilm, MDR, fnbA, fnbB

Posted Date: June 24th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-640880/v1

Abstract

Biofilm production increases *Staphylococcus aureus* resistance to antibiotics and also host defense mechanisms. The current study aims to evaluate the biofilm formation by *S. aureus* and to determine the prevalence of fibronectin-binding protein genes, also its correlation with drug resistance. In this study, 100 clinical isolates of *S. aureus* were collected. The antibiotic susceptibility pattern of the isolates was evaluated by the disk agar diffusion method. The ability of biofilm formation in the studied isolates was also determined by microplate colorimetric assay. Then, all isolates were screened by polymerase chain reaction for the *fnbA* and *fnbB* genes. Out of 100 clinical isolates of *S. aureus*, the highest and lowest antibiotic resistance rates were against penicillin (94%) and vancomycin (6%). Thirty-two cases were found to be multi-drug resistant (MDR) among the all strains. The ability of biofilm production was observed in 89% of the isolates. The PCR results showed that the prevalence of *fnbA* and *fnbB* genes were 91% and 17%, respectively. Moreover, 100% and 21.8% of the MDR strains harbored the *fnbA* and *fnbB* genes respectively. The ability to form biofilm in MDR isolates of *S. aureus* is more than non-MDR isolates, especially *fnbA* positive ones. As the bacteria in the biofilm are difficult to kill by antibiotics, attention to the removal or control of the biofilm production seems to be necessary.

Introduction

Staphylococcus aureus is one of the most important bacterial infectious agents in hospitals and also the most common cause of food poisoning(1). It causes a wide range of infections ranging from simple skin infections such as boils, furuncle, carbuncle, sty, and abscesses to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, and septicemia(2).Moreover, this organism is capable to produce many toxins including enterotoxins, Panton-valentine toxin, and exfoliative toxin(3). Also, this Gram-positive coccuscan produce polysaccharides and adherent protein factors, which are involved in biofilm production and adherence of *S. aureus* to the surfaces(4). Therefore, the presence of biofilm genes in these bacteria is considered as one of their important pathogenic factors and their investigation is very important(5). Biofilm is a structure composed of a bacterial population that is enclosed by an exopolymeric matrix produced by the bacterium(6). This property gives the bacterium the ability to bind to different levels as well as increase the intrinsic resistance to different antibiotics(6). Biofilms comprise a group of microorganisms that interact with a network of internal channels in the extracellular glycoprotein and polysaccharide matrix called extracellular polymeric material(2). The extracellular polymeric material is composed of polysaccharides, proteins, phospholipids, teichoic acid and other hydrated polymeric substances with 85 to 95% of water and thus can cause binding of various pathogens (especially bacteria) to live tissues and surfaces of medical equipment(7). This property enhances the bacterial resistance to a variety of antibiotics and host defense mechanisms, as well as facilitates metabolism gene transfer and resistance to antibiotics and disinfectants(7). Promoting the bacterial survival in harsh environmental conditions, playing a role in pathogenesis and causing chronic diseases, and influencing the development and enhancement of drug resistance through impermeability to antibiotics in the polymer matrix, are the most important features of

biofilm(8). *S. aureus* adhesion genes involved in the cellular accumulation of bacteria in biofilms include fib, *fnbA*, *fnbB*, eno, *icaADBC*, *sasG*,*sasC* and *pls*(9). However, fibronectin-binding proteins A and B (FnBPA and FnBPB) are encoded by the *fnbA* and *fnbB* genes, respectively(10). These proteins can be covalently attached to the bacterial cell and play an important role in initiating the biofilm production process by binding to fibronectin receptors(9). On the other hand, the production of fibronectin-binding proteins (FnBP) is essential for the invasion of this organism to eukaryotic cells(11). The ability to form biofilms is mediated by intercellular adhesin polysaccharides (PIAs) encoded by *IcaA*, *IcaB*, *IcaC*, and *IcaD* genes, which thicken the biofilm layers(2, 9).In contrast, fibronectin-binding proteins (FnBPA and FnBPB) play important roles in the accumulation, binding and invasion of *S. aureus* to surfaces(12).So, the current study aims to evaluate the biofilm formation by *S. aureus* and to determine the prevalence of fibronectin-binding protein genes, also its correlation with drug resistance.

Materials And Methods

Sample collection

In this study, 100 non-duplicate *S. aureus* isolates were collected during 10 months (March to December 2019) from different clinical specimens (blood, urine, ulcer, pus, body fluids, trachea, sputum, etc.) of patients admitted to Zare hospital of Sari city (a burn center) and Imam Khomeini hospital of Behshahr city (a general center).The isolates were transferred to the Microbiology Laboratory and cultured in Blood Agar (Merck, Germany) and incubated at 37°C for 24 hours. Then, all isolates were identified by standard microscopic and biochemical methods such as gram staining, catalase and coagulase assays, mannitol fermentation and DNase assay(13), and were confirmed by PCR using *nuc* gene-specific primers. *S. aureus* ATCC 25923 was used as a control strain for diagnostic tests.

Antimicrobial susceptibility testing (AST)

The antibiotic susceptibility pattern of the isolates against 6 antibiotics including penicillin (10 µg), vancomycin (30 µg), erythromycin (15 µg), tetracycline (30 µg), ciprofloxacin (5 µg), and clindamycin (2 µg) (Roscoe, Denmark) was determined by Kirby-Bauer method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI)(15). We chose the *S. aureus* ATCC 25923 as a control strain in AST. Also, micro broth dilution method based on the CLSI guidelines was used to evaluate the minimum inhibitory concentration (MIC) of vancomycin, and *S. aureus* ATCC 29213 was used as a standard strain in this study(15).

Phenotypic assessment of biofilm production

The capability of biofilm formation in the isolates was investigated by microtiter plate method(16). Briefly, 180 µl of TSB (trypticase soy broth) containing 1% glucose was poured into 96 well microplate wells.Then, 20 µl of 0. 5 McFarland's equivalent bacterial suspension was added to TSB medium in each well. Next, the microplates were incubated at 37°C for 20 h. After the contents were thoroughly discharged from the wells and washed three times with 0.15M PBS (phosphate-buffered saline), the microplates were

completely air-dried. Then, the wells were stained with 0.1% crystal violet.After dye evacuation, we washed the wells three times with distilled water and added 200 µl alcohol-acetone (1:4 ethanol to acetone) to the wells in order to release the dye on the wall of bacteria producing biofilm and attached to the well. The amount of dye released at each well was evaluated using the ELISA reader (Biotech, USA) at 590 nm. The OD of the samples were then compared with the OD of the control (ODC) and the results were analyzed using the cut-off method. The isolates which showed OD ≤ ODC, were considered as no biofilm producers, while the results as ODC < OD ≤ 2×ODC, 2×ODC < OD ≤ 4×ODC, and 4×ODC < OD were reflected as weak, moderate, and strong biofilm producer isolates, respectively. The TSB medium containing 1% glucose was used as the negative control, while *S. aureus* ATCC 35556 was used as a positive control (biofilm-producing strain) in this test(16).

Molecular analysis of *nuc*, *fnbA* and *fnbB* genes

Genomic DNAs were extracted from clinical isolates of *S. aureus* using a DNA extraction kit (SinaClon, Iran) according to manufacturer's instructions. To confirm the purity of the extracted DNAs, a Nanodrop machine (Thermo Scientific, USA) at 260 nm was used, and the DNAs were electrophoresed on 1.5% agarose gel (Wizbiosolutions, South Korea). PCR was used to identify the *nuc* gene (for the final confirmation of *S. aureus* isolates) and to detect the presence of *fnbA* and *fnbB*genes in clinical isolates. Primers sequences for the identification of target genes have been described previously (8, 14). The PCR reaction was performed in a final volume of 25 µl, consisting of 12.5 µl of premix (Denmark, Ampliqon), 10 picomoles of each primer, 1 µl of Taq DNA polymerase, 5 µl of distilled water, and 300 ng of template DNA. A thermal cycler (SensoQuest GmbH, Germany) was used to amplify the mentioned genes. The PCR reaction consisted of an initial denaturation step at 95°C for 2 minutes and 30 cycles of denaturation at 95°C for 25 seconds, followed by 30 s of the annealing stage at 53°C for the *nuc* gene, 52°C for the *fnbA* gene, and 55°C for the *fnbB* gene, and extension at 72°C for 30 s, along with a final amplification step at 72°C for 5 min. PCR products were electrophoresed on a 1.5% agarose gel (Wizbio, Korea) along with a DNA fragment length marker (GeneDireX, Taiwan) to investigate the presence of the target genes.

Statistical Analysis

Data were analyzed by SPSS software (version 22) and mean of quantitative data was analyzed using Descriptive software and were presented as Mean ± SD. Also, the significance level was evaluated by two-tailed and chi-square tests and P-*value*< 0.05 was considered statistically significant.

Results

Out of 100 clinical isolates of *S. aureus* in this study according to the presence of *nuc* gene, 50 isolates were obtained from Zare Hospital and 50 others were collected from Imam Khomeini Hospital. The mean age of the patients was 42.59 ± 24.94 years. The mean age for men and women was 47.04 ± 24.05 and 38.15 ± 25.25, respectively. There was no significant difference between the two groups in terms of mean age (P = 0.07).The distribution of the isolates, in terms of hospital wards, was as follows: ICU (29%), burn

(23%), reconstructive surgery (13%), pediatric (12%), internal (11%), gynecological surgery (5%), male surgery (3%), emergency (2%) and CCU (2%).

Also, there was a significant difference (P = 0.000) in the distribution of clinical specimens from different wards between two hospitals, however, the highest frequency of clinical specimens in Imam Hospital included ICU (16 samples, 32%), and internal and Pediatrics (11 samples, 22%), while the most frequent isolates in the Zare Hospital belonged to burn ward (23 samples, 46%) and ICU (13 samples, 26%).In general, the frequency of clinical specimens in the present study was as follows: wounds (36%), urine (29%), blood (21%), trachea (9%), surgical samples (2%), ascites, pulmonary secretions and sputum each (1%).According to the evaluation of antibiotic resistance pattern of the isolates in this study, the highest antibiotic resistance rate was observed against penicillin (94%), tetracycline (50%) and erythromycin (42%), while vancomycin, with 6% resistance rate, was the most effective antibiotic in this research (Table 1). The MIC range of vancomycin against the isolates was 0.25-32 µg/ml, which was consistent with the results of the disk agar diffusion method. In this study, 4%, 9%, 42%, 32%, 7%, and 6% of the isolates showed a MIC range of 0.25, 0.5, 1, 2, 4, and 32 µg/ml, while any of the isolates exhibited a MIC range of 8-16 and ≥ 64 µg/ml. Statistical analysis of antibiotic resistance results by the chi-square test showed no significant difference between antibiotic resistance pattern of *S. aureus* isolates in two sex groups, different parts of hospitals and different clinical samples (P> 0.05).Moreover, 32% of our clinical isolates showed multidrug resistance (MDR) phenotype. The antibiotic resistance pattern of the MDR and non-MDR clinical isolates of *S. aureus* is compared in Table 2. The results indicate higher antibiotic resistance of MDR strains than non-MDR strains. In this study, *fnbA* and *fnbB*genes were identified in91% and 17% clinical isolates of *S. aureus*, respectively. Of the isolates studied, 89 were able to produce biofilms (Table 3). Biofilm production ability was strong in 54 isolates (60.67%), moderate in 28 isolates (31.46%) and weak in 7 isolates (7.86%) (Table 4).The frequencies of *fnbA* and *fnbB* genes in biofilm-producing strains and non-biofilm producer ones are shown in Table 4. Significant differences were observed in the frequency of *fnbA* and *fnbB* genes between biofilm producer and non-biofilm producing isolates in this study (p <0.05).Also, there was a significant difference in the frequency of *fnbA* gene between MDR and non-MDR isolates, while there was no significant difference in*fnbB* gene frequency in these isolates (Table 5). The frequency of biofilm production in MDR and non-MDR strains was 100% and 83.82%, respectively.

Discussion

S. aureus is one of the most important bacteria causing nosocomial infections (17). The high ability of this bacterium in biofilm production has led to the occurrence of chronic infections and the emergence of multi-drug resistant *S. aureus* strains (17). The ability to produce biofilms is one of the most important virulence factors in *S. aureus* that allows the organism to bind to different levels and increase the antibiotic resistance level, which ultimately leads to increased mortality in hospitals.In the present study, the highest and lowest antibiotic resistance were reported for penicillin and vancomycin, respectively, which was almost similar to the results of another Iranian study conducted in 2016(1). In their study, 100 and 2% of the isolates were resistant to penicillin and vancomycin, respectively. Similar results were also

reported by Rahimi and his colleagues in 2013 from Iran, while their isolates were collected from the poultry sources, and all of the isolates were methicillin-resistant (18).The high resistance of the isolates to penicillin in Iran was expected to be similar to the results obtained by other Iranian researchers. This may be due to lack of attention to drug dosage, empirical treatment regardless to antibiogram results, incomplete treatment, overdose and over-the-counter use of penicillin family antibiotics, use of penicillins in aviculture, and high levels of beta-lactamase production by *S. aureus*(19).The evaluation of biofilm production ability in the present study showed that 89% of the isolates studied were capable to produce biofilms, while in other study conducted in Iran (1), all of their clinical isolates were biofilm producers. However, the clinical samples of the mentioned study were similar to our research. In a study conducted by Vuong et al. in Germany, 78% of *S. aureus* clinical isolates were biofilm producer (20). The percentage of biofilm production power varies in different studies but in most studies is high level indicating the important role of biofilm production ability in the pathogenicity of *S. aureus* in clinical practice. While in the present study a total of 91% of *S. aureus* isolates were positive for the presence of *fnbA* gene, in a study carried out by Nashev et al. on clinical isolates obtained from skin and nose in hospitals of Germany, 36.7% of the isolates carried the *fnbA* gene(21). Also, in a study conducted by Soltani and colleagues in Iran in 2019 on strains isolated from nasal swabs, 7.2% of the isolates contained *fnbA* gene (22). The reason for these differences may be related to the type of clinical samples, whereas the skin and nose isolates are normal flora and the virulence factors associated with the pathogenicity are less common in these bacteria.A study performed in the USA on clinical isolates reported the prevalence of 98.7% and 20.1% for *fnbA* and *fnbB* genes, respectively (23), which was much closed to the results of the present study. On the other hand, a study conducted in India also showed a high prevalence of both *fnbA* and *fnbB* genes (77.8% and 81%, respectively), which is consistent with the results of our study regarding *fnbA* gene (24). Interestingly, Arciola et al. exhibited that 98% of *Staphylococcus aureus* isolates associated with orthopedic infections contained *fnbA*&B genes, indicating the important role of these virulence factors in biofilm production in various infections, especially orthopedic ones(25).Abbas and his colleagues in Iraq, the border with Iran, reported 59% *fnbA* gene frequency(26), while in 2019, Azmi et al. reported the frequency of *fnbA*and *fnbB* genes in Palestine as 78.2% and 29%, respectively (27).These differences in the prevalence of *fnb* genes indicate the genetic diversity of clinical isolates of *S. aureus* in different regions of the world and the necessity to study this diversity in each region.Also, 32% of *Staphylococcus aureus* clinical isolates were MDR in the present study, which showed significantly higher resistance to ciprofloxacin, tetracycline, clindamycin and erythromycin than non-MDR strains.Two other studies conducted in Iran and Palestine also reported 46% and 26.6% prevalence of MDR clinical isolates of *S. aureus*, respectively (27, 28). However, the MDR rate between strongly positive biofilm-producing isolates in the Palestinian research was reported as 38.5%, while this rate in our study was 66.6%. It seems that strongly biofilm-production had a great impact on the development of antibiotic resistance in our research. On the other hand, we found that the frequency of *fnbA* and *fnbB* genes in MDR strains were 100% and 21.9%, respectively, and all of these isolates were capable of biofilm formation. However, in the study by Azmi et al., 26.6% of the isolates were MDR, while 50.7% and 20% of MDR isolates carried *fnbA* and *fnbB* genes, respectively, and all of which were detected as biofilm producer isolates (27).Today, with increasing use of antibiotics and increasing prevalence of methicillin and vancomycin resistant

Staphylococcus aureus strains and the high ability of this organism in biofilm production, the treatment of infections caused by it has become a major challenge in the world (3, 29).

Conclusions

In conclusion, based on our results, it can be argued that clinical isolates of *S. aureus* have a high ability to form biofilms and *fnb* genes with high diversity and high prevalence play an important role in biofilm construction. Also, given the high prevalence of the *fnbA* gene, it can be expected that the isolates containing this gene play a more effective role in pathogenesis. Although one of the major causes of infection emergence and drug resistance is attributed to biofilm production, and the presence of related genes, but by identifying and inhibiting the genes, sources, and pathways of infection transmission, we can prevent the biofilm formation and reduce the antibiotic pressure, and the selection of the most effective antibiotics for the treatment of the infections, and on the other hand, it can prevent the spread of resistance genes and the emergence of strains with multidrug resistance phenotype.

Declarations

Acknowledgements

This study was supported by the Mazandaran University of Medical Sciences, Sari, Iran with the Grant number: 1466. Also, we thank the laboratory staffs of Imam Khomeini and Zare hospitals for assist in the collection of the clinical isolates.

Conflict of interest

The authors declare that they have no conflict of interest.

Availability of data and materials

Data generated and/or analyzed during this study are included in this article.

Authors'Contributions

Conceptualization: [Hamid Reza Goli], Methodology: [Mohammad Ahanjan and Mehrdad Gholmi], Formal analysis and investigation: [Hossein Jafari Soghondicolaei and Bahman Mirzaei], Writing - original draft preparation: [Hossein Jafari Soghondicolaei andHamid Reza Goli]; Writing - review and editing: [Mehrdad Gholami and Hamid Reza Goli].All authors read and approved the final manuscript.

Ethics approval

This study was approved by the Ethics Committee of the Mazandaran University of Medical Sciences, Sari, Iran (Approved Number: IR.MAZUMS.REC.1397.1466) and Iran National Committee for Ethics in Biomedical Research (Approved Number: IR.MAZUMS.REC.1397.312). Although, we did not have a direct

connection with the patients, the informed written consent was obtained from patients.We only obtained the clinical samples of the patients without their names from the hospital laboratories and the data was kept secret by the authors.

Consent to Participate

All individual participantssigned an informed consent to participation in the work.

Consent for publication

Patients signed informed consent regarding publishing their data.

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Tables

Table 1
Antibiotic resistance pattern of 100 S. aureus clinical isolates

Antibiotics	Percentage of the isolates which were		
	Resistant	Intermediate	Susceptible
Ciprofloxacin	37	16	47
Penicillin	94	-	6
Tetracycline	50	40	10
Vancomycin	6	-	94
Clindamycin	35	16	49
Erythromycin	42	24	34

Table 2

Comparison of antibiotic resistance pattern of MDR and non-MDR S. aureus

Antibiotics	Resistance pattern			P-Value
		MDR isolates (n = 32)	Non-MDR isolates (n = 68)	
Ciprofloxacin	Resistant	32 (100)	5 (7.35)	0.01
	Intermediate Resistant	-	16 (23.52)	
	Susceptible	-	47 (69.11)	
Penicillin	Resistant	32 (100)	62 (91.17)	0.1
	Intermediate Resistant	-	-	
	Susceptible	-	6 (8.82)	
Tetracycline	Resistant	32 (100)	18 (26.47)	0.04
	Intermediate Resistant	-	40 (58.82)	
	Susceptible	-	10 (14.7)	
Vancomycin	Resistant	-	6 (8.82)	0.02
	Intermediate Resistant	-	-	
	Susceptible	32 (100)	62 (91.17)	
Clindamycin	Resistant	28 (87.5)	7 (10.29)	0.02
	Intermediate Resistant	3 (9.37)	13 (19.11)	
	Susceptible	1 (3.12)	48 (70.58)	
Erythromycin	Resistant	32 (100)	10 (14.7)	0.03
	Intermediate Resistant	-	24 (35.29)	
	Susceptible	-	34 (50)	

Table 3
Frequency of fnbA and fnbB genes in biofilm producer and non-biofilm producer S. aureus

Genes	Biofilm producer	Non-biofilm producer	P-value
fnbA Positive (n = 91)	83 (91.2%)	8 (8.79%)	0.025
fnbA Negative (n = 9)	6 (66.66%)	3 (33.33%)	NS
fnbB Positive (n = 17)	16 (94.11%)	1 (5.88%)	0.01
fnbB Negative (n = 83)	73 (87.95%)	10 (12.04%)	NS

NS, Not Statistically significant

Table 4
Distribution of biofilm production ability and its relation to the presence of fnbA and fnbB genes in S. aureus isolates

Strains with or without genes	The ability of biofilm production			
	Strong No. (%)	Moderate No. (%)	Weak No. (%)	No biofilm No. (%)
$fnbA^{+}$ (n = 91)	48 (52.74)	28 (30.76)	7 (7.69)	8 (8.79)
$fnbA^{-}$ (n = 9)	6 (66.66)	-	-	3 (33.33)
$fnbB^{+}$ (n = 17)	13 (76.47)	3 (17.64)	-	1 (5.88)
$fnbB^{-}$ (n = 83)	41 (49.39)	25 (30.12)	7 (8.43)	10 (12.04)
$fnbA^{+}\&B^{+}$ (n = 15)	13 (86.66)	2 (13.33)	-	-
$FnbA^{-}\&B^{-}$ (n = 6)	5 (83.33)	-	-	1 (16.66)

Table 5
Frequency of fnbA and fnbB genes in MDR and non-MDR S. aureus isolates

Genes	MDR (n = 32)	Non-MDR (n = 68)
fnbA Positive	32 (100%)	59 (86.76%)
fnbA Negative	-	9 (13.23%)
P-value	0.000	NS
fnbB Positive	7 (21.87%)	10 (14.7%)
fnbB Negative	25 (78.12%)	58 (85.29%)
P-value	NS	NS

NS, Not Statistically significant