Iranian Journal of Medical Microbiology | ISSN:2345-4342

Evaluation of Genotypic and Phenotypic Biofilm Formation by *Staphylococcus aureus* Isolated from Clinical Samples and Their Association with Antimicrobial Resistance

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10.30699/ijmm.14.5.441

ABSTRACT

Background: *Staphylococcus aureus* is one of the most important bacteria causes nosocomial infections, which by the biofilm formation can be effective in the creation of chronic diseases, and the creation and strengthening of drug resistance. The present study aimed to evaluate the genotypic and phenotypic biofilm formation by *S. aureus* isolated from clinical samples and their association with antimicrobial resistance.

Materials & Methods: In this descriptive cross-sectional study from Dec 2019 to Sep 2019, 200 clinical samples were obtained from AJA hospitals in Tehran. All samples were analyzed using blood agar, Baird-Parker Agar, mannitol salt agar and catalase, OF and coagulase assays. Antimicrobial resistance pattern of isolates was determined by the disc diffusion method. Multiplex PCR method was used to identify biofilm formation genes, includes *icaA*, *icaB*, *icaC*, and *icaD* genes. Data analyzed using SPSS 20 and the X^2 test.

Results: Out of 200 cultivated samples, 83 (41.5%) cases were confirmed as *S. aureus*. The highest resistance was observed to Penicillin (94%), Tetracycline (72%), Ampicillin (54%), and Cefoxitin (51%), respectively. Phenotypic biofilm formation ability reported in 65% of isolates. The frequency of presence of *icaA*, *icaB*, *icaC*, and *icaD* genes was estimated at 67.4%, 60.2%, 61.4%, and 62.6%, respectively. Eighty-seven percent of biofilm producing strains were multidrug-resistant, while all the biofilm negative strains were non- multiple drug resistance ($P< 0/05$).

Conclusion: According to the results, Biofilm-positive strains have a very high propensity to demonstrate antimicrobial resistance, multidrug resistance and resistance to methicillin.

Keywords: *Staphylococcus aureus*, Biofilm, Antimicrobial Resistance, MRSA

Received: 2020/05/14; **Accepted**: 2020/08/31; **Published Online:** 2020/09/26

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Introduction

Biofilm is the aggregation of bacteria embedded in an extracellular matrix that can create on both abiotic and biotic surfaces **(1)**. This layer provides ideal conditions for bacteria to grow in unstable environments. The most important features of biofilm are bacterial survival in extreme environmental

conditions, role in pathogenicity and creating chronic diseases, strengthening drug resistance through antibiotic impermeability in the polymeric matrix, facilitate gene transfer through conjugation and increase genetic mutations due to these bacterial connections, development of new genotypic strains by mutation within the biofilm and activation of genes that they are responsible for bacterial virulence **(2, 3)**. *S. aureus* is one of the pathogens that can cause disease in humans and animals **(4)**. This bacterium is the cause of many diseases in humans, included skin infections, acute and invasive infections such as pneumonia, soft tissue and bone infections, heart valve infections and lethal sepsis. It is one of the most important causes of bacteremia and is responsible for 20-40% of deaths **(5, 6)**. In addition to resistance, this bacterium has the ability to produce various pathogenic factors such as toxins, surface antigens, extracellular enzymes and biofilm production **(6, 7)**.

In *S. aureus*, the pathogenicity of the biofilm is very important. The presence of this layer is encoded by a structural operon called intra cellular adhesin (*ica*), which has different gene locus including *icaA*, *icaD*, *icaB* and *icaD* **(8,9, 10)**. The *icaA* gene is a primary inducer for start biofilm production. It is activated by the presence of UDP-N-acetyl glucosamine and is the only *ica* gene that has transferase properties. The IcaD protein is a messenger (chaperone) for other genes in this locus that helps the *icaA* gene, activates certain enzymes to express the *icaC* and *icaD* genes. When the *icaA* and *icaD* genes start cooperation, biofilm production increases 20 times **(9, 10).** The *icaC* gene communicates between the inside and outside of the bacterial cytoplasmic membrane, that it communicates the IcaD on the inside of the cytoplasmic membrane to the IcaB on the outside. The IcaB is the extracellular protein and cause the superficial association of the bacterium with intercellular adhesion polysaccharide (IAP). One of the factors that increases the expression of this operon is the relationship between IcaB and IPA, that more correlation leads to more biofilm production **(11).**

Biofilms in hospital environments, that are considered as a reservoir of infection transmission, responsible for causing 65% of nosocomial infections **(12)**. Biofilm binds bacteria to surfaces and other hospital instruments and in this way, to provide the ground for infection in hospitalized people and users of these medical devices **(13)**. In order to prevent the formation of biofilm and subsequent microbial resistance, increased hospitalization, increased costs, and increased mortality (especially in antibiotic-resistant *S. aureus*); proper use of antibiotics (under the supervision) as well as arbitrarily use of drugs and antimicrobial compounds, regular evaluation of resistance and expression of related genes at the hospital can greatly reduce the incidence of these problems.

Due to the high prevalence of nosocomial infections caused by *S. aureus* and also the spread of factors that increase antibiotic resistance, the present study aimed to evaluate the genotypic and phenotypic biofilm formation by *S. aureus* isolated from clinical samples and their association with antimicrobial resistance.

Materials and Methods

This descriptive cross-sectional study from Dec 2019 to Sep 2019 was conducted in the School of Paramedical Sciences, AJA University of Medical Sciences. The present study investigated by the ethics committee of the AJA University of Medical Sciences and approved with the code IR.AJAUMS.REC.1397.087. Using the formula for calculating the sample size and according to the results of similar studies **(3, 10)**, 200 samples were calculated.

A total of 200 clinical samples from 63 patients admitted to different wards of AJA hospitals in Tehran were collected. Samples included blood (63 samples), urine (63), catheter (23 samples), discharge (17), ulcers (16 samples), sputum (12 samples), and nasal swabs (6 samples) which were referred to the paramedical faculty of the AJA University of Medical Sciences under sterile conditions. Inclusion criteria to study were included hospitalization in different wards of the hospital and long-term treatment with various antibiotics, and exclusion criteria were included outpatient treatment (no hospitalization) and no long-term use of antibiotics.

The samples were first cultured on blood Agar medium and incubated for 24h at 37°C. After observing the colonies, standard biochemical tests were performed to confirm the isolates including catalase test, Gram-staining, and then bacterial culture on mannitol salt agar media (mannitol fermentation test), Baird-Parker agar (formation of black colonies with clear and opaque halos), DNase and coagulase tests **(14)**.

Antimicrobial Susceptibility Test

This test performed by Müller–Hinton agar (MHA) using the standard antibiotic disk including penicillin (10 IU), ciprofloxacin (5 µg), chloramphenicol (30 µg), gentamicin (10 µg), doxycycline (30 µg), Tetracycline (30 µg), clindamycin (2 µg), cotrimoxazole (25 µg), ampicillin (15 µg), erythromycin (15 µg), cephalothin (30 µg) and cefoxitin (30 µg), purchased from HiMedia company (Mumbai, India). **(10, 14)**. The isolates were further screened for methicillin resistance by the cefoxitin disk **(15)**. In these tests, *S. aureus* ATCC 33591 was used as a positive control. The results exegesis conducted using Clinical and Laboratory Standards Institute guidelines **(16)**.

Phenotypic Evaluation of Biofilm Producing Strains

Congo red and a modified microtiter plate (MTP) assay were used to identify biofilm-producing strains. Confirmed isolates were cultured on brain heart infusion (BHI) agar medium containing 0.8 g/L Congo red and 36 g/L sucrose. After 24h of incubation at 37°C, black colonies as strong biofilms, dark red colonies as weak biofilms and light red colonies as negative biofilm strains were considered **(17)**.

To quantitatively evaluate the production of biofilm by MTP assay, from the samples enriched on trypticase soy broth (TSB) medium, turbidity equivalent to 0.5 McFarland was prepared and then 200 µL of each suspension was transferred to wells of 96-well polyester microplate and incubated for 20h at 37°C. Then, the wells washed (4 times) using phosphate buffered saline (PBS) and dried completely. Next, the wells were stained with crystal violet dye (1%) for 15 minutes and to remove the dye from the bacterial wall, 100 µL of a mixture of isopropyl alcohol 10% and ethanol 70% was added to each well. Finally, the light absorption of each well at 570 nm was investigated using an ELISA reader. Finally, the optical density (OD) of the wells were measured using an ELISA reader at a wavelength of 570 nm. The test was conducted in triplicate. Exegesis of results were performed as per the criteria explained **(18)** and the bacteria were divided into weak (non-producer), moderate and strong biofilm producers. *S. aureus* ATCC 25923 was used as positive control, for the biofilm assay.

DNA Extraction

DNA extraction was performed by the boiling technique using lysis buffer (1% Triton x100, 0.5% Tween 20, Tris 10 mmol with pH: 8 and EDTA 1 mmol) **(19)**.

Molecular Confirmation of *S. aureus* Isolates

Amplifying the thermonuclease gene by polymerase chain reaction (PCR) was used for molecular confirmation of isolates (Table 1). For this test, distilled water and *S. aureus* ATCC 33591 were utilized as negative and positive controls, respectively. The final volume of each reaction was considered to be 20 µL and the PCR reaction temperature program is shown in table 2. The products of PCR were evaluated using 1% agarose gel and UV transilluminator. Samples with 279 bp band were considered as *S. aureus* **(20)**.

Molecular Identification of Biofilm-Producing Strains

Amplification of *icaABCD* operon genes (with *icaA*, *icaD*, *icaC* and *icaB* genes) was used to identify biofilm-producing strains by PCR (Table 1). Distilled water and *S. aureus* ATCC 25923 were used as negative and positive controls, respectively, for this test. The final volume of each reaction was considered to be 20 µL and the PCR reaction temperature program of this operon is shown in Table 2. The products of PCR were evaluated using 1% agarose gel and UV transilluminator. The size of products of the genes are shown in Table 1 **(21).**

Statistical Analysis

Data are analyzed by SPSS 20 (SPSS Inc., Chicago, IL., USA). X^2 (Chi-square) test was utilized for data analysis. P-value<0.05 was considered statistically significant.

Table 1. Primers used in the study

Gene	Product size (bp)	Primer sequence	Reference
nuc	279	F: GCGATTGATGGTGATACGGTT R: AGCCAAGCCTTGACGAACTAAAGC	20
icaA	188	F: ACACTTGCTGGCGCAGTCAA R:TCTGGAACCAACATCCAACA	21
icaB	880	F: AGAATCGTGAAGTATAGAAAATT R: TCTAATCTTTTTCATGGAATCCGT	21
icaC	1066	F: ATGGGACGGATTCCATGAAAAAGA R: TAATAAGCATTAATGTTCAATT	21
icaD	198	F: ATGGTCAAGCCCAGACAGAG R: AGTATTTTCAATGTTTAAAGCAA	21

Table 2. PCR reaction program (30 cycles)

Steps, temperature and time of PCR reaction					
Gene	Primary denaturation	Denaturation	Annealing	Extension	Final extension
nuc	94°C, 5 min	94°C, 30 sec	55°C, 55 sec	72°C, 60 sec	72°C, 10 min
icaA	94°C, 5 min	94°C, 60 sec	55°C, 60 sec	72°C, 60 sec	72°C, 10 min
icaB	94°C, 5 min	94°C, 60 sec	52°C, 30 sec	72°C, 90 sec	72°C, 10 min
icaC	94°C, 5 min	94°C, 60 sec	55°C, 30 sec	72°C, 30 sec	72°C, 10 min
icaD	94°C, 5 min	94°C, 0 sec	55°C, 30 sec	72°C, 60 sec	72°C, 10 min

Results

Out of 200 cultured clinical specimens, 83 (41.5%) cases were phenotypically identified as *S. aureus*; 23 isolates (27.71%) from urine, 17 isolates (20.48%) from catheter, 15 isolates (18.07%) from blood, 12 isolates (14.45%) from wound, 9 isolates (10.84%) from secretions, 5 isolates (6.02%) from nasal swabs and 2 isolates (2.40%) from sputum were isolated, that all phenotypically confirmed isolates had *nuc* gene by molecular tests (Figure 1).

Antimicrobial Resistance Patterns

The results of the antimicrobial resistance test are shown in Table 3.

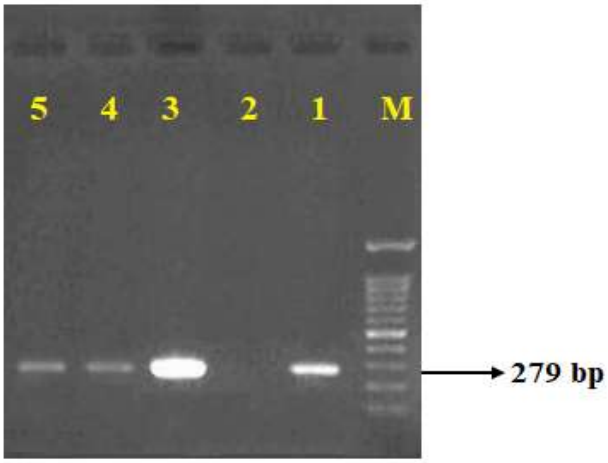

Figure 1. Electrophoresis of *nuc* gene PCR product lane M -marker 100 bp, lane 1- Positive control (*S. aureus* ATCC33591), lane 2- Negative control (distilled water), lanes 3 to 5- Study samples

Table 3. Antibiogram test results of *Staphylococcus aureus* isolates

Antibiotic	Resistant/ n (%)	Intermediate/ n (%)	Sensitive/ n (%)
Penicillin (10 IU)	78 (94*)	-	5 (6)
Ciprofloxacin (5 µg)	35 (43)	2 (2)	46 (55)
Tetracycline (30 µg)	60 (72)	10 (12)	13 (16)
Chloramphenicol (30 µg)	36 (43)	24 (29)	23 (28)
Cefoxitin (30 µg)	42 (51)	-	41 (49)
Gentamicin (10 µg)	37 (45)	1 (1)	45 (54)
Erythromycin (15 µg)	36 (43)	3 (4)	44 (53)
Cephalothin (30 µg)	27 (33)	12 (14)	44 (53)
Ampicillin (15 µg)	45 (54)	9 (11)	29 (35)
Clindamycin (2 µg)	23 (28)	-	60 (72)
Cotrimoxazole (25 µg)	32 (39)	4 (5)	47 (56)
Doxycycline (30 µg)	3 (4)	5 (6)	75 (90)

* Percentages are rounded

The highest resistance of isolates was to penicillin (94%), tetracycline (72%), ampicillin (54%) and cefoxitin (51%), respectively, and the highest susceptibility of isolates was reported to doxycycline (90%), clindamycin (72%), cotrimoxazole (56%) and ciprofloxacin (55%), respectively. Based on the results, 51% (42 cases) of isolates were considered as Methicillin-resistant *S. aureus* (MRSA) strains.

Biofilm Formation

According to the results of the Congo red test, out of 83 isolates, 23 (27.70%) isolates had a black colony (strong biofilm), 31 (37.30%) isolates had a dark red colony (weak biofilm) and 29 (35%) isolates with bright red colonies (lacking the ability to produce biofilm). The results of quantitative biofilm formation test (MTP) showed that 29 (35%) isolates had strong biofilm, 25 (30%) isolates had weak biofilm and 29 (35%) isolates lacked the ability to produce biofilm. Overall, 54 (65%) isolates were considered as positive biofilm strains.

Based on the results of table 4, a statistically significant difference was observed between qualitative results (Congo red) and quantitative results (MTP) of biofilm production by chi-square test (P<0.05, 0.001, X^2 = 54.9, df = 4).

As shown in Table 5, there is a statistically significant difference between the microbial resistance of positive and negative biofilm of *S. aureus* strains to the antibiotics of ciprofloxacin, cefoxitin, gentamicin, erythromycin, cephalothin and cotrimoxazole (P <0.05).

Table 4. Comparison of quantitative (Congo red) and qualitative (MTP) test results of biofilm production

MTP test / Congo red test	Strong adherence	weak adherence	No adhesion	Total
Black colony	17	6	0	23
Dark red colony	12	19	0	31
Bright red colonies	0	0	29	29
Total	29	25	29	83

Table 5. Comparison of antimicrobial resistance pattern in biofilm-negative and biofilm- positive isolates

Antibiotic	BP[1] (n= 54)/ n (%)	BN[2] (n= 29)/ n (%)	P-value
Penicillin	54 (100*)	24 (83)	0.465
Ciprofloxacin	33 (61)	2 (7)	0.026
Tetracycline	42 (78)	18 (62)	0.185
Chloramphenicol	25 (46)	11 (38)	0.505
Cefoxitin	40 (74)	2 (7)	0.007
Gentamicin	37 (100)	-	0.002
Erythromycin	36 (100)	-	0.002
Cephalothin	27 (100)	-	0.002
Ampicillin	31 (57)	14 (48)	0.427
Clindamycin	17 (31)	6 (21)	0.315
Cotrimoxazole	32 (100)	-	0.002
Doxycycline	2 (3)	1 (3)	-

1. Biofilm producer
2. Biofilm non-producer

Percentages are rounded

Of the total isolates, 47 (57%) were multidrug-resistant that included all biofilm-positive isolates,47 (57%), while 7 (13%) were non-MDR. All the biofilm negative isolates were non-MDR isolates (P<0.05; Table 6). Also, 24 (29%) of the isolates were MRSA. It is worth noting, 44% of biofilm positive isolates were MRSA, while all biofilm negative isolates were methicillin-sensitive *S. aureus* (MSSA) (P<0.05; Table 6). There was a statistically significant difference between the type of strain and biofilm production by X^2 test (P<0.05 0.001, X^2 = 108.1, df = 3).

Table 6. Comparison of MDR and resistance to methicillin in biofilm-negative and biofilm- positive isolates

Isolate type	MDR[1], n (%)	Non-MDR, n (%)	MRSA, n (%)	MSSA[2], n (%)
Biofilm producer (n= 54)	47 (87*)	7 (13)	24 (44)	30 (56)
Biofilm non-producer (n= 29)	0 (-)	29 (100)	0 (-)	29 (100)
Total (n= 83)	47 (57)	36 (43)	24 (29)	59 (71)

1- Multidrug-resistant
2- Methicillin-sensitive *S. aureus*
* Percentages are rounded

Molecular Evaluation of ica*ABCD* Operon

In the Molecular evaluation of ica*ABCD* operon, the frequency of *icaA*, *icaB*, *icaC* and *icaD* genes were 67.4% (56 isolates), 60.2% (50 isolates), 61.4% (51 isolates) and 62.6% (52 isolates), respectively. Accordingly, the most common gene encoding biofilm production in the studied isolates are *icaA* and *icaD* genes. Out of 83 isolates, 50 isolates (60%) had both *icaA* and *icaD* genes and 34 isolates (41%) had all *ica* operon genes. There was not statistically significant difference between the presence of ica operon genes in *S. aureus* isolates.

Figure 2. Electrophoresis of ica operon genes PCR products
lane M -marker 100 bp, lanes 1 and 2- positive samples for *icaA* gene (188 bp), lanes 3 to 5- positive samples with *icaD* gene (198 bp), lanes 6 and 7- positive samples for *icaC* gene (1066 bp), lanes 8 and 9- positive samples for *icaB* gene (880 bp), lane 10- Negative control (distilled water)

Discussion

According to the results of this study, the prevalence of *S. aureus* in clinical specimens was 41.5%. The results of various studies have shown similarities and differences in this field, including: Neopane *et al.* (2018) in Nepal reported that the prevalence of *S. aureus* in clinical specimens is 28.66% **(11)**. In another study in India (2018), Manandhar et al reported a prevalence 43% of this bacterium in clinical specimens **(23)**. In a similar study in Tehran that it conducted by Mashaiekhi and Amini (2018), the prevalence of *S. aureus* was calculated to be 17.09% (**24**). The difference in the prevalence of *S. aureus* in clinical specimens may be related to differences in specimen type, geographical location, sampling time, sampling location, etc.

Based on the results of antibiogram test in the present study, the highest resistance of isolates was to penicillin (94%), tetracycline (72%), ampicillin (54%), cefoxitin (51%) and gentamicin (37%), respectively. The results of Nourbakhsh and Momtaz's study (2016) showed that the highest resistance of *S. aureus* isolates in clinical samples was related to penicillin (100%) and tetracycline (76%), which is consistent with the results of the present study **(25)**. In the study of Sadri *et al.*, resistance to tetracycline was reported to be 42% (26). In the study of Ahmadi *et al.*, 20% resistance to gentamicin was reported in Kermanshah, also, the resistance to ampicillin was reported to be 55% **(27)**. 23% resistance of *S. aureus* strains to gentamicin was also observed in the study of Hauschild *et al.* **(28)**. A study by Mirzaee *et al.* in Tehran showed that more than 80% of *S. aureus* isolates are ampicillin-resistant **(29)**. The present study shows the high prevalence of MRSA strains (51%) in different wards of AJA hospitals that comparing these results with the results of other studies in this field, showed many differences and similarities. Abdollahi *et al.* (2011) in Fars reported thet the rate of methicillin-resistance in *S. aureus* isolates was 47.56% (30). Also, several studies were conducted on the prevalence of MRSA islolates, which 43.5%, 50%, 12.6% and 30% of isolates in the study of Abu-Shady *et al.* **(31)**, Hassanzadeh *et al.* (34), Tabaei *et al.* **(32)** and Rahimi *et al.* **(33)**, were resistant to methicillin, respectively. The resistance of *S. aureus* isolates to methicillin can be due to overuse of antimicrobial compounds for disinfecting hospital environments, treatment of infections, transfer of patients colonized with these strains from one hospital to another, etc **(22)**. As a result, it is necessary to monitor the use of drugs and disinfectant compounds, teach the correct methods of using antibiotics and infection control proceedings in all wards of hospitals.

Numerous studies on the pattern of antibiotic resistance of *S.aureus* isolates have been published from different wards of hospitals in different regions that are consistent or inconsistent with the results of our study. In the study of Ahmadi *et al.* (2014), the highest antibiotic resistance was reported to penicillin, tetracycline, methicillin and ampicillin **(27)**. Also, in the study of Hatefizade *et al.* in Tehran (2016), the highest resistance to penicillin and ampicillin was observed **(35)**, which these results were consistent with the results of the present study. In 2017, a study was conducted by Motamedi *et al.* in Hamedan. The results of this study showed that the highest antibiotic resistance is to erythromycin **(36)**, which is in contrary to the results of the present study. These differences in the results can be due differences in a geographical area, the hygienic status of hospital wards **(34)** as well as creating chromosomal resistance during generation or transmission of resistance factors between bacterial species **(36)**.

In the present study, in addition to emphasizing the spread of multiple drug resistance in clinical specimens, the ability to produce biofilm as a

phenotype was reported to be 65%; This was consistent with the results of studies by Namvar *et al.* (2013) **(13)** and Croes *et al.* (2009) **(37)**. In a similar study by Gad *et al.* (2009), the ability to produce biofilm was reported in 83% of isolates (38). In another study in Nepal conducted by Neopane *et al.* (2018), biofilm-producing strains were reported about 70% **(10)**. Various factors can contribute to biofilm formation, including environmental factors (such as sugars, or proteases in the growth medium), nutrient availability, geographical origin, sample type, area surface (rough or smooth), porosity, Environmental stresses (such as antibiotic exposure), surface adhesion characteristics, and bacterial genetic arrangement. Furthermore, mutations in the *ica* operon genes and the regulatory genes of this operon are associated with a reduction in the ability of *S. aureus* to produce biofilms **(10)**.

In the present study, the frequency of *icaA*, *icaB*, *icaC* and *icaD* genes were 67.4% (56 isolates), 60.2% (50 isolates), 61.4% (51 isolates) and 62.6% (52 isolates), respectively, which is consistent with the results of Nourbakhsh and Momtaz's study in 2016 **(25)**. In a study by Eftekhar *et al.* (2011), 73% of the isolates contained the *icaA* and *icaB* genes **(22)**. Also, in the study of Namvar *et al.*, all isolates have had *icaC* gene **(13)**. In the present study, 60% of the isolates had both *icaA* and *icaD* genes and 41% of the isolates had all the ica operon genes, while strong biofilm was observed in only 35% of the isolates. In the study of Mirzaee *et al.* (2014) in Tehran, it was found that about 28% of the isolates had all the *ica* operon genes, while only half of these isolates were able to form a strong biofilm **(29)**. In the Mashaiekhi and Amini's study (2016), 75% of *S. aureus* isolates have had both *icaA* and *icaD* genes **(24)**. In the study of the presence of genes and phenotypic biofilm formation, differences were observed that depending on the factors mentioned earlier. Therefore, the presence or absence of a gene alone can't play a major role in biofilm formation. In the present study, there were two genes (*icaA* and *icaD*) in 60% of isolates and there were in 41% of isolates all of *ica* operon genes. The Isolates were placed between strong and weak spectra in biofilm formation, but none of these isolates were seen with the inability to form biofilms.

Conclusion

Pursuant to the results of this study, *S. aureus* isolates have had high resistance to most of the studied antibiotics (especially methicillin). Also, the significant abundance of biofilm-producing genes in these isolates can be effective in increasing the multiple drug resistance, persistence of bacteria in the environment (especially in hospital environments). In the present study, there was a statistically significant relationship between biofilm formation and antibiotic resistance ($P<0.05$). The presence of biofilm-producing genes and their role in antibiotic resistance can have consequences such as prolonged hospitalization, increased costs, and increased mortality (especially those admitted to the burn ward, immunosuppressed patients, and those undergoing aggressive treatments such as the use of artificial implants).

Acknowledgment

The authors thank the Research Assistance of AJA University of Medical Sciences and the staff of AJA hospitals in Tehran for their assistance in conducting this study.

Conflict of Interest

Authors declared no conflict of interests.