Determination of the ability to form biofilms and the presence of effective genes in adhesion (*ica* A, *ica* B, *ica* C and *ica* D) by *Staphylococcus aureus* isolated from clinical samples

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Abstract
Background & Objective: *Staphylococcus aureus* especially methicillin-resistant *S. aureus* (MRSA) strains are a major cause of nosocomial and community acquired infections with high morbidity and mortality rates worldwide. The role of Biofilm is unclear in the spread of antibiotic resistance genes among gram-positive, such as *S. aureus* when compared with Gram-negative. The aims of this study were to determine Staphylococcal adhesion genes and multi-drug resistant (MDR) isolates and detect the relative between their ability among clinical isolates of *S.aureus*.
Methods: Sixty clinical specimens of *Staphylococcus aureus* were collected from clinical samples. Antibiotic susceptibility of *S. aureus* to 9 different antibiotics was investigated using disk diffusion method based on CLSI table. DNA extraction of all isolates was performed by boiling method and PCR was performed. Biofilm formation ability was investigated using microtiter method. Then, by using the PCR method, the presence of the operons, *ica*A and *ica*B, *icaC* and *ica*D genes in all isolates was evaluated. Also, to find out the relationship between the binding genes in the specimens and the ability to produce biofilms and the presence of *ica*A and *ica*B target genes, was investigated. In the most isolated isolates of *S. aureus*, neither genes were found simultaneously. No significant correlation was found between the presence of fnbA binding gene and phenotypic antibiotic resistance tests (P value> 0.05).
Conclusion: The target genes of *icaA* and *icaB* were found in most of the isolates of this study. The high presence of *icaA* and *icaB* simultaneously among a number of isolates should be considered as an important concern in the resistance phenomenon and biofilm formation.
Key Words: Staphylococcus aureus, MRSA, multi-drug resistance, icaA and icaB.

Introduction

Staphylococcus aureus is one of the major causes of infection from the community and the hospital (1). The bacterium is a member of the human microbe that is capable of producing several toxins such as enterotoxin, Panton–Valentine, exfoliative toxin, which is responsible for a wide range of infections such as food poisoning, subcutaneous abscess, scab, skin skeletal syndrome, toxic shock syndrome, sepsis and pneumonia (1-5). The first methicillin was marketed in 1961. Following the introduction of methicillin, some Methicillin -Resistant strains emerged (4). The first reports of Methicillin-resistant strains from the British Hospital, which quickly spread to the world. One of the important factors in increasing the pathogenicity and resistance to antimicrobial agents is the ability to form biofilms (3-4). The emergence of methicillin-resistant strains (MRSA) and its potential role in the emergence of biofilms can be of clinical significance for the treatment of *Staphylococcus aureus* infections (6). This bacterium has undergone a lot of genetic changes. Since the bacterium has a genome of flexibility, the pathogenic and resistant strains of the drug have expanded. In recent years, the role of *S. aureus* in the development of hospital infections and the community has led to an increase in research on this bacterium (6-8). Before the mid-1990s, these resistant strains were confined to health centers and hospitals and were called Healthcare Associated (HA-MRSA), but gradually a number of MRSA

infections among those who did not have contact with health centers(8). It was also reported that these new strains of MRSA were named Community Associated MRSA (CA-MRSA)(8). CA-MRSA strains were spread rapidly among a large population of people, and patients who had no contact with health centers (9). Treatment for infections caused by biofilm production is often difficult because the biofilm matrix and the phenotypic characteristics of the bacterium cause resistance to host immune response and the function of antimicrobial drugs (11). The researchers have shown that *S. aureus* is the first stage to infect these bacteria to levels such as medical devices, host tissues, and others (13). This step involves the intercellular sticky polysaccharide (PIA), which interferes with the interactions between the cells of ica A, ica B, and ica D in the production process. This collection is located on an opera. This polyester polysaccharide from glucose-amino glycan units with beta-binding is from 6 (80 to 85 percent) and a smaller portion of glycosides, nonionic anionic amines containing phosphate and suction-ester (20 to 25 percent) Acetylglucosaminyltransferase. The expression of these enzymes is made by Lucas *ica*. The synthesis of this polysaccharide is followed by the expression of the enzyme by *ica* (15). The participation of *ica* D in this locus increases the synthesis of polysaccharides and the creation of phenotypes (17). In a study conducted in May 2014 by Mirzaei et al. in order to isolate *ica* gene and biofilm formation in *S. aureus* isolates, from 31 clinical specimens of it's collected from Loghman Hospital in Tehran, 12 samples (38.7%) produced a strong biofilm. The results showed that 18 of the samples (6.80%) had *ica*D gene.The role of the *ica* B gene is to acetylate the polysaccharide from binding to the cell membrane and the Ica gene encodes a membrane protein that helps elongate and release the polysaccharide from the cell (16). The expression and function of ADBC *ica* are increased by regulatory systems such as Sar A and Sigma B (SigB) (18). On the other hand, *ica* R as a potent negative controller by connecting the promoter region of ica can reduce the expression of the genes present in this operon. Research has shown that mutation in SarA inhibits the production of biofilms in clinical strains of MRSA. SarA is a known repressor for the 4 extracellular protease enzymes called SsPA, SSpB, Aar, and ScpA. Also, the removal of genes in the agr regulatory system strengthens the ability to form biofilms in MRSA strains, while they have no significant effect on the ability to form biofilms of MSSA strains (19). In a study conducted by Shojaei and colleagues in 2014 to investigate the distribution of biofilm producers in *S. aureus* strains isolated from raw milk delivered in Sanandaj, 120 samples of raw milk were sampled, 49 strains were tested by experiments Standard biochemical and the proliferation of specific species of trichomonucleosis gene (nuc) as *S. aureus* were identified. Based on the results of multiplex PCR, biofilm producers, *fnb*A(38.68%), *clfa*B(32.6%), *ica*D (38/77%) and *ica*A(18.59%) were detected in strains (20).

Materials and methods

Bacterial isolates: The present study was a cross-sectional descriptive study. In 2017s, 60 strains of *S. aureus* from inpatients and outpatients of Rasht hospitals and the burn center of the Guilan province were isolated. Isolates by standard microbiological methods such as Gram staining , catalase and coagulase tests, mannitol sugar fermentation, Novobiocin disc sensitivity, DNase test (Merck Company, Germany) were identified. In this study, *S. aureus* ATCC35556 was used as a positive control of the strong biofilm producer and *Staphylococcus epidermidis* as a negative control.

Assessment of phenotypic and genotypic antibiotic susceptibility: Kirby –Bauer method was used to evaluate antibiotic susceptibility. A bacterial suspension of equivalent half-McFarland tube was prepared. Suspensions were cultured using a sterile swab on the Mueller Hinton Agar. Then antibiogram test of each isolate for antibiotic disks including standard concentrations of vancomycin (30 µg), ciprofloxacin (5 µg), sulfamethoxazole (2.5 µg), tetracycline (10 µg), erythromycin (15 µg), clindamycin (2 µg), rifampicin (5 µg), oxacillin (30 µg), amoxicillin (30

µg) was conducted. Determination of antibiotic resistance pattern and sensitivity of *S. aureus* isolates were evaluated in accordance with the CLSI (Clinical and Laboratory Standards Institute) guidelines, and based on the diameter of growth inhibition around the disks, they were expressed as sensitive, intermediate and resistant (12).

Detection of Biofilm formation by phenotypic method: In this study, for detection of biofilm production, *S. aureus* was cultured on the TSB (Tryptic Soy Broth) (Merck, Germany) medium. For micro plate titration, the quaternized enriched samples were prepared equivalent to half McFarland, and then 200 µl of each suspension was transferred to a 96-well polystyrene micro plate wells and heated to 37 ° C for 20 hours (14). The wells were washed four times with phosphate buffer saline (PBS) and then completely dried. In the next step, staining should be done to stained the wells with crystal violet color for 15 minutes. Then, the color of each well was washed using ordinary water and to release the stain in the wall biofilm producer, 100 µl of isopropyl alcohol 10% plus 70% ethanol were added to each well (15, 16). Finally, the color released in each well at a wavelength of 570 nm was measured using a sample reader. ELISA negative control in this method for biofilm formation was TSB medium contained 1% glucose. To ensure the correctness of the work for the isolates studied, three times the absorbance of each isolate was investigated. The method for calculating the quotient amount for each group is presented in Table 1.

Table 1: Classification of the ability to form biofilms by microtiter plate method

The ability to form biofilms	Calculation of quorum rate	Results of the mean maximum optical absorption (OD
Strongly	$OD > 4 \times ODC^2$	$\mathbf{OD > 0.332}$
Moderately	$2 \times ODC < 4 \times ODC$	$\mathbf{0.166 < OD \leq 0.332}$
Weakly	$ODC < OD \leq 2 \times ODC$	$\mathbf{0.083 < OD \leq 0.166}$
Non	$OD \leq 0.083$	$\mathbf{OD \leq 0.083}$

OD= Optical Density

Genotypic evaluation to detect *ica*ABCD genes: In order to detect the presence of *ica*A, *ica*B, *ica*C and *ica*D genes in isolates, PCR was used using the primers shown in Table 2 (17).

Table 2: Primers used to detect the presence of *ica*A, *ica*B, *ica*C and *ica*D genes

Primer	Genes size	Target gene
F: 5'-ACACTTGCTGGCGCAGTCAA-3' R: 5'-TCTGGAACCAACATCCAACA-3'	188	*ica* A
F: 5'-TCCTTATGGCTTGATGAATGACG-3' R: 5'-CTAATCTTTTTCATGGAATCCGTCC-3"	900	*ica* B
F: 5'-ATGGGTTATAACTACGAACGTG-3' R: 5'-CGTGCAAATACCCAAGATAAC-3'	1100	*ica* C
F: 5'-ATGGTCAAGCCCAGACAGAG-3' R: 5'-AGTATTTTCAATGTTTAAAGCAA-3'	198	*ica* D

DNA extraction steps using the boiling method were used from fresh colony cultures of isolated *S. aureus*, 3 to 5 colonies of each sample were dissolved in a 1.5 ml volumetric flask containing 200 µl of distilled sterilized water, and the samples were completely dissolved. The vials were placed in boiling water (100 ° C) for 10-15 minutes (so that the boiling water level was 2/3 vial in boiling water). The vials were then centrifuged at 14000 rpm for 10 to 5 minutes, and supernatant vials containing DNA were transferred to the sterilized micro vials for the PCR reaction. Thermo

cycler (SensoQuest GmbH, Germany) was used to carry out PCR. The heat plan used was given according to Table 3.

Table 3: Thermo cycler program for PCR

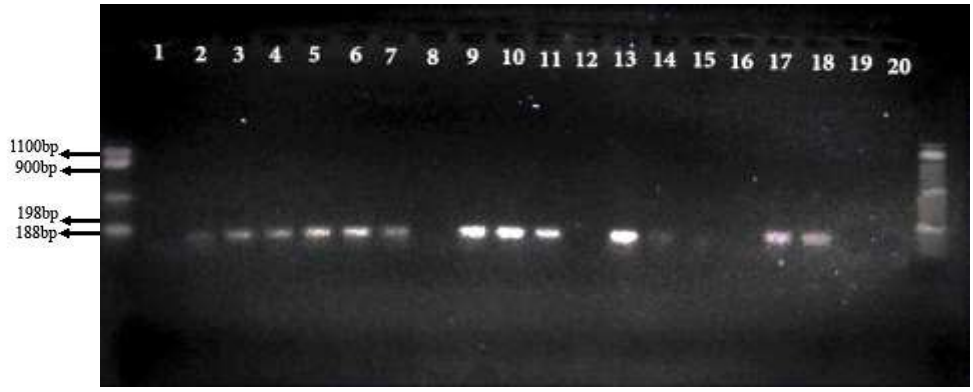
Process	Temperature	Time	Number of cycles
Initial denaturation	95ºC	3 minutes	1 cycle
Denaturation	95ºC	30 sec	35 cycle
Annealing	55 ºC	45 sec	35 cycle
Extension	72 ºC	1 minutes	35 cycle
Final extension	72 ºC	5 minutes	1 cycle
Stop	4 ºC		∞

Statistical analysis: Data were analyzed by SPSS software version 16 and chi-square test at 95% confidence level (0.05). The statistical relationship between biofilm formation capacity in *S. aureus* isolates and pattern antibiotic resistance of isolates was determined.

Results

In the present study, 60 isolates of *S. aureus* from infectious diseases of Rasht Hospital (Iran) of burns and injuries, which included ulcers (20%) and urine (88.3%), blood (10%) and chips (1.7%) were investigated. All isolates were able to produce biofilms in a variety of phenotypes (micro plate titration). In the final investigation of phenotypic biofilm isolates, 2% of isolates had strong ability to connect, 34% of them had medium power binding, and 20% of isolated isolates had weak linking ability and the rest are classified as suspicious specimens in biofilm production. The frequency of presence of *icaA* gene was 33.33%, *icaB* gene 68.66%, 13.33% *icaC* gene and 6.66% *icaD* gene. The frequency of presence of the biofilm generating genes and its related bands is shown in Fig. 1.

A



B

C

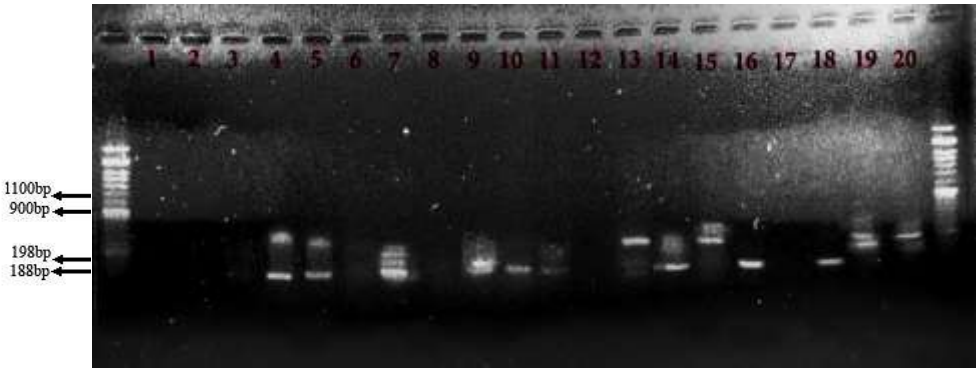


Figure 1. The presence of biofilm genes (a: *ica*A, b: *ica*B, c: *ica*C and *ica*D genes)

To determine the genotypic potential of biofilm production in isolates, *ica* genes were detected by PCR method. In the meantime, *ica*B gene is the most common gene encoding biofilm production in *S. aureus* isolates. However, there was no statistically significant difference between the presence of these four genes in *S. aureus* (p = 0.79). However, in statistical analysis of the data, there was a statistically significant difference between biofilm formation capacity in *S. aureus* that isolated from urine samples with this factor in isolates from wound and blood samples at 95% confidence level (p = 0.49) was observed with chi-square test. The phenotypic evaluation of the antibiotic resistance pattern of *S. aureus* strains indicated that the highest antibiotic resistance was observed for the antibiotics of oxacillin (78%), erythromycin (38%), tetracycline (38%), clindamycin (35%), ciprofloxacin (25%) and rifampicin (13%), with the least resistance to amoxicillin and vancomycin antibiotics. Statistical analysis of antibiotic resistance data with chi-square test showed a significant difference between the antibiotic resistance of *S. aureus* isolates to oxacillin, erythromycin, ciprofloxacin, penicillin and tetracycline antibiotics with resistance to antibiotics vancomycin, amoxicillin and rifampicin were found at 95% confidence level (p = 0.15). The antibiotic resistance patterns of S. *aureus* are presented in Table 4.

Table 4: Pattern of antibiotic resistance of *Staphylococcus aureus* isolates

Antibiotics	Resistance	intermediate	Sensitive
Erythromycin	38%	27%	35%
Sulfamethoxazole	3.54%	10.46%	86%
Tetracycline	38%	4%	58%
Amoxicillin	10%	2%	88%
Rifampicin	14%	38%	48%
Vancomycin	1%	1%	98%
Clindamycin	35%	0%	65%
Ciprofloxacin	25%	0%	75%
Oxacillin	78%	0%	22%

Discussion

The purpose of this study was to investigate the antibiotic resistance pattern and to study the biofilm production potential of isolates by phenotypic and genotypic methods. The results of this study showed that the ability to form biofilms using some phenotypic and genotypic indices is associated with the development of antibiotic resistance in infectious specimens. The expansion of clinical samples of biofilm with multiple antibiotic resistances is considered

as a serious risk for patients and can increase the mortality rate in hospitals. Regarding the structure and specific mechanisms in biofilm, in addition to the influence of antibacterial agents, the contamination of therapeutic tools can also be effective in creating critical conditions and ways of transmission of infection (27). Regarding the formation of multilayer units in biofilm structures, biofilm specimens can be considered as a key stage in increasing infections and also the development of antibiotic resistance (18). Considering the differences in the production of biofilm in *S. aureus* isolates and taking into account different etiologic conditions in biofilm production, extensive studies have been carried out in this field in our country. In 2011, a study by Professor colleagues was conducted on *S. aureus* isolates, of which 73% of the infectious isolates had *ica*A and *ica*B genes. Also, in a study by Namwar et al in 2013, of 60 isolates of *S. aureus*, all of the samples had *ica*C gene, and 65% of the isolates were phenotypically productive of biofilm (22). In the present study, all isolates were able to produce biofilms at different levels, of which 3.3% had strong interconnection, 56.7% medium connectivity and 33.3% poor coupling ability for biofilm production Showed. In a study by Wang and colleagues in Germany in 2000, 78% of infectious *S. aureus* isolates were reported as biofilm generators, of which 73.5% of the isolates were able to attach strong, 33.5% medium power and 15.4% of isolates showed poor binding ability in biofilm production. The results of this study are similar to the statistics provided by Wang et al., Which indicates the prevalence of biofilm production of *S. aureus* (21). In the present study, the lowest antibiotic resistance was reported with 2% resistance to vancomycin. And the highest antibiotic resistance to Oxacillin has been reported with 78% resistance. The results are similar to those of Ahmadi and his colleagues in 2014 on isolated clinical specimens from Kermanshah health centers. In this study, the lowest antibiotic resistance was reported for Nitrofurantoin (8%) and vancomycin (14%) (23). In Rahimi's study of healthy individuals in 1995 to produce biofilm from *S. aureus* strains, 79 isolates from *S. aureus* isolated from healthy individuals revealed 53 strains as biofilm and 26 isolates They were also identified as negative biofilms. In all 53 strains, the two genes *ica*A and *ica*D were identified. All 79 strains were resistant to penicillin and showed the highest resistance to erythromycin, ciprofloxacin, amikacin, kanamycin, tobramycin, tetracycline and clindamycin antibiotics. None of the strains showed resistance to vancomycin and linezolid (26).

Also, in the present study, the highest percentage of urine specimens (68.3%) and their least wounds (1.7%) were found that there was no significant relationship between biofilm formation and sampling site. Also, in the present study, the presence of *ica*A, *ica*B, *ica*C and *ica*D genes and their association with the ability of biofilm formation were investigated. There was no significant relationship between biofilm formation and *ica*A, *ica*C and *ica*D genes. The only significant relationship was related to the frequency of *icaB* gene. Increasing antibiotic resistance can be very effective in increasing the ability of the bacterium to form biofilms. Due to the specific structure and mechanisms in the formation of biofilms, in addition to the inactivation of antibacterial agents, contamination of medical and medical devices can also be effective in creating critical conditions and ways of transmission of infection.

Considering that in this study a high percentage of samples showed resistance to oxacillin (78%). which was consistent with the study of Mirzai and his colleagues in 2013? In the study, a high level of resistance to ampicillin and a moderate level of resistance to ciprofloxacin, tetracycline and clindamycin were observed. Meanwhile, more than 28% of the samples were resistant to oxacillin, and vancomycin and linezolid were effective antibiotics in all of the samples. Biofilm formation in the 26 strains (36.1%) was strong in 30 strains (41.6%) moderately, in 16 strains (63.68%) containing icaC gene and 50 strains (5.69%) containing icaD gene.

Conclusion

Regarding the importance of *Staphylococcus aureus* as a hospital pathogen and with increasing antibiotic resistance in clinical isolates in order to prevent the formation of biofilm and colonize the bacteria in the individuals and the environment of the hospital, the appropriate sterilization in the therapeutic tools relative to the patient, it is recommended to prevent transmission of infection and also the formation of biofilm.

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