Original Article

J Babol Univ Med Sci
Vol 20, Issu 6; June 2018. P:27-35

Detection of Intercellular Adhesion (*ica*) Genes Involved in Biofilm and Slime Formation in Clinical Isolates of Staphylococcus Aureus Harboring *mecA* Gene

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J Babol Univ Med Sci; 20(6); June 2018; PP: 27-35
Received: Jun 26th 2017, Revised: Oct 17th 2017, Accepted: Apr 4th 2018.

ABSTRACT

BACKGROUND AND OBJECTIVE: Prevalence of methicillin resistant *Staphylococcus aureus* (MRSA) strains is one of the most important health care problems and life-threatening in worldwide. The methicillin resistant *S. aureus* strains producing biofilm and slime have potential to colonize and transmit. The present study was conducted to detect intercellular adhesion (*ica*) genes involved in biofilm and slime formation in clinical isolates of methicillin resistant *S. aureus* harboring *mecA* gene.

METHODS: In this cross-sectional study, a total of 85 bacterial isolates suspected to *S. aureus* were prepared from clinical samples. The antibiotic susceptibility testing of bacteria to the penicillin, gentamicin, oxacillin, ciprofloxacin, ofloxacin and vancomycin was carried out based on disk diffusion agar method. Biofilm and slime formation of bacteria were examined by tissue culture polystyrene plate (TCP) and Congo red agar (CRA). The presence and frequency of *icaA*, *icaD* and *mecA* genes were detected by multiplex PCR.

FINDINGS: 45 out of 85 (52.94%) *S. aureus* isolates were resistant to the methicillin. All of methicillin resistant *S. aureus* were able to produce biofilm and slime. Consumedly surface hydrophobicity was seen in 55.55% and 100% of strains producing strong biofilm and slime, respectively. The *icaA*, *icaD* and *mecA* genes were present in all biofilm and slime producing isolates.

CONCLUSION: Our results showed that the all methicillin resistant *S. aureus* isolates with some abilities, including polysaccharide intercellular adhesion, bacterial attachment, biofilm and slime production were positive for *icaA* and *icaD* genes.

KEY WORDS: *Staphylococcus aureus*, Biofilm, Slime, *icaA*, *icaD*.

Please cite this article as follows:

Yousefi Nojookambari N, Yazdansetad S, Ardebili A, Saki M, Najjari E. Detection of Intercellular Adhesion (*ica*) Genes Involved in Biofilm and Slime Formation in Clinical Isolates of Staphylococcus Aureus Harboring *mecA* Gene. J Babol Univ Med Sci. 2018;20(6):27-35.

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Introduction

Methicillin-Resistant Staphylococcus aureus is one of the most important causes of hospital-acquired infections throughout the world. This organism is a life-threatening factor with colonization in hospitalized patients and in cases such as dialysis, surgery, catheter, and artificial limbs. Today, the emergence of Staphylococcus aureus strains resistant to several antibiotics, the treatment of infections caused by them has become a major challenge (1). All of the methicillin resistant Staphylococcus aureus strains carry the large SCCmec chromosome cassette that codes for the mecA gene. The approximate size of the Kb2 gene is mecA. This gene is located in a 25- Kbg region of the chromosome called the mec region, which does not have a similar allele in the methicillin-sensitive strains. The mec region carries transposons attachment portions and at least one of the IS257 sequences needed to obtain antibiotic-resistant plasmids.

The structure and function of the mec region indicates that it is an abnormal transposon with its own locus. The mecA gene sequence has been well maintained in methicillin-resistant Staphylococcus aureus strains and negative methicillin-resistant coagulase-negative strains (2, 1). Methicillin is the first semi-synthetic penicillin introduced in 1960. A year later, methicillin resistant species quickly appeared. The mechanism of resistance is due to the increased transcription of the mecA gene and the point mutation in the promoter of the gene. methicillin resistant Staphylococcus aureus strains, produce a new penicillin-linked protein called PBP2a. PBP2a has a slight tendency to bind to methicillin and other beta-lactam antibiotics. However, the synthesis of peptidoglycan cells from the cell wall of the bacterium does not stop and continue with these antibiotics (2). In 1993, Boudewijn proved that the addition of methicillin, even at low concentrations, to the methicillin-resistant Staphylococcus aureus environment results in the synthesis of an abnormal peptidoglycan, which is a PBP2a product. (3). Biofilm formation is one of the most important causes of pathogenicity and multi-drug resistance of the bacterium. The bacteria, by producing capsular polysaccharide / adhesin connects to the surfaces and with the polysaccharide intercellular adhesin, as well as the synthesis of polysuccinyl glucosamine, cause thickening of the biofilm layers. These steps are controlled by the icaADBC operon, especially the icaA and icaD genes. The icaA gene codes N-acetyl-glucosaminyl transferase enzyme that plays a role in the synthesis of N-acetyl-glucosamine oligomers from UDP-N-acetyl-glucosamine. IcaD gene plays an important role in increasing the expression of the N-acetylglucoseamine aminyl transferase enzyme, which leads to an increase in the expression of the polysaccharide capsule (5, 4).

Hydrophobicity of the bacterial surface facilitates the formation of biofilms as another pathogenic factor (6). The Hydrophobicity nature of the external surface of the bacteria is important in its non-specific binding to the plastic surfaces, binding to phagocytes and other mammalian cells, as well as in the growth of cells on hydrophobic insoluble layers such as hydrocarbons (7). The formation of Slime by bacteria is another pathogenic agent. The slime consists of glucose aminyl glycan units with a glycosylated β [1-6] bond. This extracellular polysaccharide, such as cement matrix, plays an important role in binding of bacteria to surfaces and facilitating the formation of biofilms. Studies have shown that slime synthesis is also controlled by ica. Therefore, ica plays an important role in the pathogenesis of bacteria (8, 2). Staphylococcus aureus strains with the ability to form biofilms and slimes have the potential for colonization, prevalence and transmission (9).

New strategies for controlling infection require the study of molecular mechanisms and the correlation of the genes involved in the pathogenicity of the organism. The present study was conducted to investigate the presence of icaA and icaD genes associated with biofilm and slam formation in clinical isolates of methicillin-resistant Staphylococcus aureus carrying mecA gene.

Methods

Sample collection: This cross-sectional study was carried out after approval at the Ethics Committee of Golestan University of Medical Sciences with the code ir.goums.rec.1394.172 on 85 suspected Staphylococcus aureus bacteria were isolated from clinical specimens: blood, sputum, abscess, bed sore, skin ulcers and surgical wounds of hospitalized patients in Azar, Shahid Sayyad Shirazi and Taleghani hospitals of Gorgan from February 2015 to May 2016.

Culturing and purification of bacteria: Staphylococcus aureus isolates were first cultured on a medium of Mueller Hinton Agar and then on a medium of Blood Agar. The bacterial colonies were evaluated macroscopically, microscopically and warm reaction was performed. Identification of isolates was confirmed using conventional biochemical tests such as catalase, coagulase, mannitol sugar fermentation and DNase test.

Antibiotic susceptibility test: Determination of sensitivity of bacteria to antibiotics was performed using modified Kirby & Bauer agar disc diffusion method according to CLSI (Clinical and Laboratory Standards Institute) guidelines. A fresh strain of bacteria cultured in a biotype triplet culture medium (Biolife, Italy) was prepared as a standard suspension of half-McFarland (Barium Diphosphoric acid 1.175% and Sulfuric acid 1%). The bacterial suspension was densly cultured on a Mueller Hinton Agar (Biolife, Italy) medium. Antibiotic discs (MAST, UK) include penicillin (10 µg), gentamicin (10 µg), oxalicin (1Mg), ciprofloxacin (5 µg), ofloxacin (5 µg) and vancomycin (30 µg) were placed on the culture medium of bacteria according to the standard principles.

Staphylococcus aureus ATCC 25923 was used as a control sample. Plates were heated at 37 ° C for 24 hours. After 24 hours, the diameter of the non-growth of bacteria was measured with a special stainless steel strap and was evaluated according to the standard table prepared by the manufacturer of antibiotype discs in accordance with CLSI standards. In order to determine the minimum inhibitory concentration, the E-test Oxacillin (Liofilchem, Italy) strips were used in accordance with the manufacturer's instructions. After 24 hours of heating at 37 ° C, strains with a minimum inhibitory concentration equal to or less than 2 µg / ml as methicillin-susceptible strains and at minimum inhibitory concentration equal to or greater than 4 µg / ml were considered as methicillin resistant strains (10).

Evaluation of biofilm formation: The ability of bacteria to form biofilms was investigated by Tissue Culture Plate method on a 96-nm polyester micro titer plate. For this purpose, the bacteria were inoculated into Tryptic Soy Broth medium with 1% glucose and heated to 37 ° C for 24 hours. The bacterial suspension was prepared at a dilution of 1: 100, and 200 µl was loaded into the wells of a micro titer plate. One of the wells was considered as control and loaded with 200 µl of Tryptic Soy Broth medium. The micro titer plate was heated to 37 ° C for 24 hours. Then, the wells of the plate were empty and washed 3 times with sterile physiology serum. To ensure complete removal of unwanted and unbound bacteria into the wells, the micro titer plate was stirred vigorously several times. In order to fix the binding of bacteria, 200 µl 96% ethanol was added to the wells. After 15 minutes, the wells were drained and dried at the laboratory temperature. The wells were stained with 200 µl 2% Crystal Violet for 5 minutes. The wells were washed with distilled water and loaded with 200 µl acetic acid solvent (33%) and heated at 37 ° C for 15 minutes. Optical density of wells stained with crystal violet was read by the ELISA reader (HumaReader HS, Germany) at 492 nm (4). Quantitative study on the production of biofilms by bacteria was carried out using a conventional formula in accordance with Table 1 (11).

Table 1. Calculation of biofilm production by bacteria based on optical absorption at 492 nm

Formula	Strong	Moderate	Weak	Negative
BF*= AB**-CW***	≥0.300	0.200–0.299	0.100–0.199	<0.100

* Biofilm Formation; ** Stained attached Bacteria; *** Stained Control Wells

Investigating the production of slime and hydrophobicity of bacteria: To test the ability of bacterial isolates to bind to the surface, slime production test was performed according to the standard method. For this purpose, bacterial isolates and standard strain of Staphylococcus aureus (ATCC 25923) were prepared. The bacterial colonies were cultured in brain heart infusion agar (BHI) with Congo red color and 10% sucrose and heated to 37 ° C for 24 h. Then, in order to complete the slime production, the plates were placed at the laboratory temperature for 72 hours (5, 4).

To determine the hydrophobicity of bacterial cell surface, bacterial binding to microbial adhesion to hydrocarbon was used as a binding surface. The bacterial cell deposition was dissolved in a phosphate buffer (PBS 0.01 M) at pH 7.2 and supplemented with standard half-MacFarland. Optical absorbance of the suspension was measured at 640 nm and recorded as initial light absorption (A). In the next step, octane hydrocarbon was added to the suspension and mixed well. For the separation of the aqueous-organic phase, the sample was settled for 10 minutes and absorbed by optical wave-wavelength at 640 nm for 10 minutes was recorded as secondary optical absorption (B). Finally, the percentage of hydrophobicity and the ratio of bacterial cells binding to octane hydrocarbons were calculated as follows (12, 7).

$$\text{Hydrophobicity} = \left(\frac{A-B}{B}\right)\times 100$$

A: Primary optical absorption at 640 nm, B: Secondary optical absorption at 640 nm

DNA extraction of bacteria: DNA of bacteria was extracted by using the Kado and Liu method with a few changes and using phenol-chloroform solution. To the cellular deposition of bacteria, 150 µl of Tris-acetate and sodium-EDTA buffer (Tris-Acetate 40mM, Sodium EDTA 2mM, pH 7.9) and 3 µl of lysozyme (20 mg / ml) and 200 µl of a lysing buffer (pH 12.6, SDS 3%, 50mM Tris) was added. Then, bacteria lysate with 900 µL of phenol-chloroform solution were extracted. DNA was precipitated from the aqueous phase by addition of isopropanol and after washing with ethanol, genomic DNA was dissolved in 50 µl distilled water (13).

Molecular identification of mecA, icaA, icaD genes: Specific primers for icaA, icaD and mecA gene fragments were developed (Table 2). Multiple PCR reactions of mecA, icaA, icaD genes with 25 µl final volume containing 10 ng / µl of template DNA, 0.4 µm of each forward primer (F) and reverse primer (R), 0.2 µM dNTP, 2 µM MgCl2, 2.5 µl buffer 10X and 1 unit of Taq DNA Polymerase (GENET BIO, Korea) enzyme.

The PCR reaction was performed on Thermal Cycler PeQLab Primus 25-United Kingdom with primary annealing temprature of 94 ° C for 4 minutes followed by 35 cycles, including annealing at 94 ° C for 45 seconds, the connection at 45 ° C for 30 seconds, amplification at 72 ° C for 60 seconds, and ultimately final amplification at 72 ° C for 7 minutes. The reaction products were electrophoresed on agarose gel 1% and electrophoresed for 40 minutes at 80 volts (14).

Table 2. characteristics of specific primers for gene fragments

gene	Sequence	Product siz(bp)	reference
icaA	F: 5'-CCTAACTAACGAAAGGTAG-3' R: 5'-AAGATATAGCGATAA GTGC-3'	1315	15
icaD	F: 5'-AAACGTAAGAGAGGTGG-3' R: 5'-GGCAATATGATCAAG ATAC-3'	381	15
mecA	F: 5'-TGGCTATCGTGTCACAATCG-3' R: 5'-CTGGAACTTGTTGAGCAGAG-3'	533	16

Statistical analysis: The experiment was performed with three replications and the data obtained from the results were analyzed by SPSS software version 22 using chi-square test and $p < 0.05$ was considered significant.

Results

Microscopic observation and biochemical tests: Microscopic study of bacterial isolates by gram staining method confirmed the presence of gram-positive cocci with irregular cluster arrangement. Key tests of catalase, coagulase and DNase were positive for isolates. Mannitol consumption was also confirmed by isolates by changing the color of the environment from red to yellow.

Antibiotic susceptibility test: There were 45 isolates resistant to oxacillin from 85 (52.94%), 25 of 85 isolates (29.41%) were semi-susceptible to oxacillin and 15 of 85 isolates (17.65%) were susceptible to oxacillin; 80 out of 85 isolates (94.11%) were resistant to penicillin, 0 of 85 (0%) isolates were semi-sensitive to penicillin and 5 of 85 isolates (5.89%) were sensitive to penicillin; 28 of 85 isolates (32.94%) were resistant to ofloxacin, 4 of 85 isolates (70% 4) were semi-susceptible to ofloxacin and 53 of 85 (62.35%) were susceptible to ofloxacin; 21 out of 85 isolates (24.70%) were resistant to ciprofloxacin, 9 out of 85 isolates (10.6%) were semi-susceptible to ciprofloxacin and 55 of 85 isolates (70.64%) were susceptible to ciprofloxacin; 19 of 85 isolates (35 / 22%) were resistant to gentamicin, 6 out of 85 isolates (6%) were semi-susceptible to gentamicin and 60 of 85 isolates (70.58%) were susceptible to gentamicin; 1 out of 85 isolates (1.17%) was resistant to vancomycin, 1 out of 85 isolates (1.18%) was semi-sensitive to vancomycin and 83 of 85 isolates (97.64%) were sensitive to vancomycin (Fig. 1). In the review of the minimum inhibitory concentration for oxacillin antibiotics, 45 of 85 isolates (52.94%) were resistant.

Investigating biofilm formation by microtiter plate: The ability of attachment of the methicilin-resistant Staphylococcus aureus isolates to the poly styrene surface and biofilm formation with Microtiter Plate method was investigated (Fig. 1). Optical absorption (OD) at 492 nm showed that 66.6% of the bacteria had a strong binding and 33.3% of the bacteria had a

relatively strong (moderate) binding to the poly styrene, and in fact all the isolates had the ability to form a biofilm. The results of this study showed that there was no significant difference in the binding of bacteria to the surface of poly styrene and biofilm formation ($p < 0.05$).

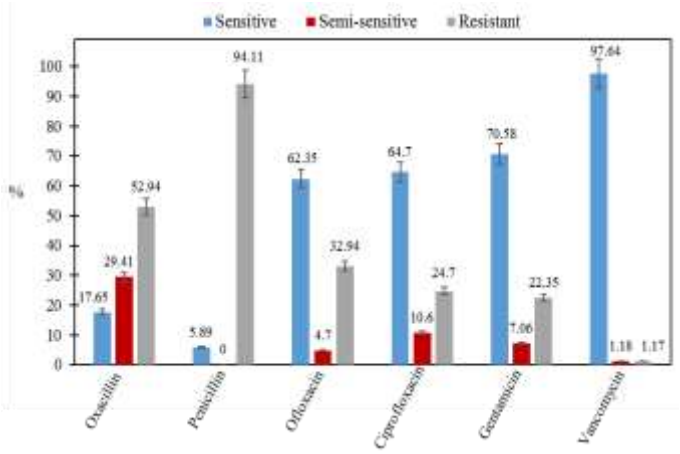

Chart 1. Percentage of antibiotic resistance-sensitivity distribution of Staphylococcus aureus isolates. (The test is performed with three repetitions and the error bars are based on relative error.)

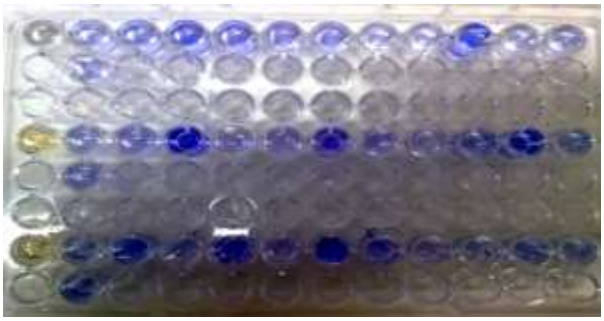

Figure 1. Biofilm composed of bacteria in the poly styrene surface in a microtiter plate (a well with a bold blue color: a strong biofilm, a well with ordinary blue: medium biofilm, and a Bluish white color well: weak biofilm)

Slime production in the Congo red agar medium: All methicilin-resistant Staphylococcus aureus isolates which were cultured in the congo red agar media, by creating rough, dry colonies and with black, gray and brown, and the color changes from pink to blackish brown were able to produce slime (Fig. 2). Slime production at 24, 48 and 72 hours was investigated from bacterial isolates. The bacteria that created black colonies in the environment had a strong slime, bacteria with gray colonies had medium slime, and bacteria with brown colonies had weak slime (Fig. 2).

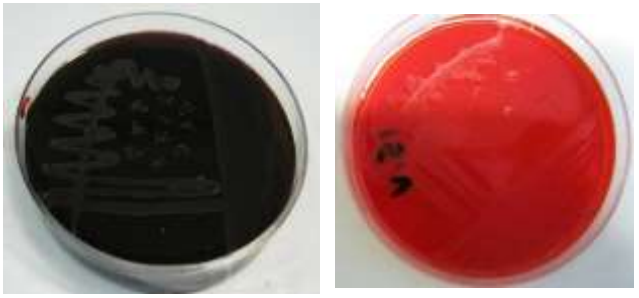

Figure. No slime production by isolates in Congo red agar medium (right), slime production by isolates in Congo red agar medium (left)

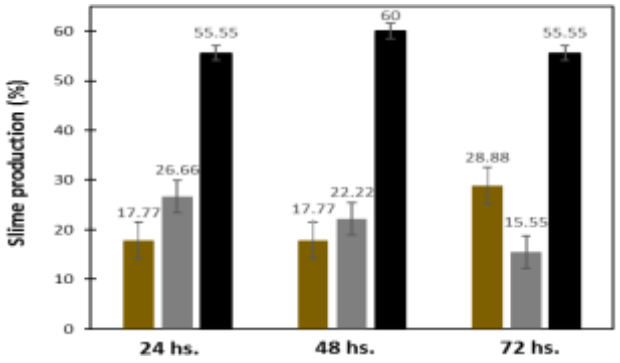

Figure 2. The percentage of slime production by isolates by creating black, gray, brown colonies in the Congo red agar medium after 24, 48 and 72 hours. (The experiment was performed with three repetitions and the error bars are based on relative error.)

Hydrophobicity study and its relationship with biofilm and slime: By examining hydrophobicity and attachment of bacterial cells to octane hydrocarbons, it was determined that 60% of methicillin-resistant Staphylococcus aureus isolates had a strong hydrophobic property, 17.77% had moderate hydrophobicity and 22 / 22% had poor hydrophobicity. Investigating the relationship between hydrophobicity and biofilms showed that 55.55% of bacteria that formed a strong biofilm also had a strong hydrophobic property. Investigating the relationship between hydrophobicity and slime showed that all bacteria that had high ability to produce slime also had a strong hydrophobic property. Investigating the relationship between Biofilm and Slime also showed that the formation of biofilm with Slime has a direct relationship.

Identification of mecA, icaA, icaD genes by multiplex PCR: multiple proliferation of icaA, mecA and icaD genes revealed the existence of these three genes in the methicillin-resistant clinical isolates of

Staphylococcus aureus, with the ability to form biofilms and slimes (Figure 3).

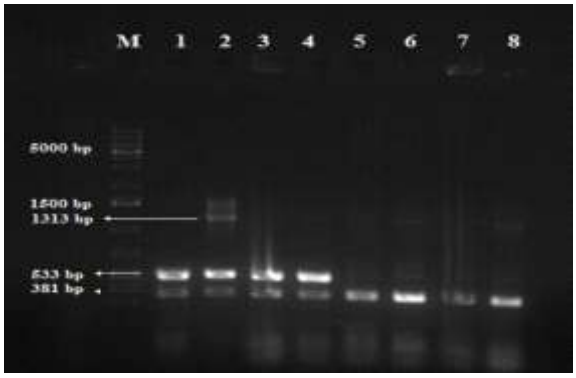

Figure 3: Column M: size marker (O, Gene Ruller 1kb plus-Fermentas), Columns 1-8: Multiple replication of icaA (1313 bp), mecA (533 bp) and icaD (381 bp) genes of Staphylococcus aureus isolates

Discussion

In the current study, the prevalence of methicillin resistant Staphylococcus aureus was 52.94%. According to studies conducted in several parts of the world and the studied communities, the highest prevalence of methicillin -resistant Staphylococcus aureus has been reported in the United States, Asia, and the Malta Island at a rate of 50% and with an average frequency of 25-50% in Africa, China and Europe.

However, in some regions of Europe, the prevalence of methicillin resistant Staphylococcus aureus is less than 50% (17). In an epidemiological study by Askari et al. regarding the prevalence of mecA gene in methicillin-resistant Staphylococcus aureus strains in the cities of Ahvaz, Falavarjan, Fasa, Gorgan, Hamedan, Isfahan, Kashan, Mashhad, Sanandaj, Shahrekord, Shiraz, Tabriz, Tehran and Tonekabon, about 52.7% of the strains contained the mecA gene. The highest frequency of methicillin resistant Staphylococcus aureus was reported from Tehran 90% and the lowest frequency was reported from Isfahan with 20.48% (18).

One of the causes of antibiotic resistance to methicillin-resistant Staphylococcus aureus and the lack of response of the organism to treatment is the formation of biofilms. The formation of biofilms by bacteria causes stable and chronic infection (4). There are several reports of the prevalence of ica genes in methicillin-resistant Staphylococcus aureus strains in different countries (19). The icaA and icaD genes are the most important genes involved in biofilm formation and bacterial infection intensification (6, 4). In the present study, 45 isolates resistant to methicillin containing the mecA gene were selected, and the ability to form biofilms was confirmed by 100% isolates, which showed that most isolates (66.6%) had a strong ability to form biofilms. The icaA and icaD genes were present in all isolates of methicillin-resistant Staphylococcus aureus. Ohadian Moghadam et al. reported a high prevalence (54.61%) of mecA gene in isolated methicillin-resistant Staphylococcus aureus from patients hospitalized in the burn ward of Motahari Hospital in Tehran. 97.5% of isolates were able to produce slime and biofilms in their study, and all Staphylococcus aureus isolates were resistant to methicillin, as well as all isolated with ability of biofilm formation contained both icaA and icaD genes (20) Our study was consistent with the recent study that 100% of methicillin-resistant Staphylococcus aureus isolates in our study had the ability to produce slime and biofilms. According to Ohadian Moghadam et al., icaA and icaD genes are required for intercellular polysaccharide adhesion and bacterial binding and biofilm formation (20).

Sanchez et al. reported that methicillin-resistant Staphylococcus aureus strains, with resistance to several antibiotics, usually form a stronger biofilm (12). Investigating the relationship between hydrophobicity and biofilm in our study showed that 55.55% of Staphylococcus aureus strains that had strong biofilms also had strong hydrophobicity. Mafu et al reported that Staphylococcus aureus has a moderate hydrophobic property for binding to the poly styrene (21). Pagedar et al. also showed that the adhesion of the bacterium to the surfaces is directly related to the hydrophobicity of the cell, and as the cell is more hydrophilic, adhesion to the surface will also be greater (22). All strains of methicillin-resistant Staphylococcus aureus in our study were able to produce Slime. Investigating the relationship between hydrophobicity and slime in our study also showed that bacteria with strong hydrophobicity produced more slime. Oliveria et al., using the Congo Red Agar method, reported slime production in methicillin-resistant Staphylococcus aureus strains 73%. All strains of S. aureus producing slime in their study carried both icaA and icaD genes (23). Based on the findings of Türkyilmaz et al., slime production may contribute to the resistance of strains of Staphylococcus aureus to antibiotics (24). Ciftci and colleagues also found similar results in a study on methicillin-resistant Staphylococcus aureus isolates, and showed an increase in antibiotic resistance in

Staphylococcus aureus strains due to slime production (2). Satorres et al. examined the production of slime and biofilm in Staphylococcus aureus strains, and 35.2% of these strains were evaluated positive for the presence of both icaA and icaD genes (25). In our study, regarding the relationship between the formation of biofilm and the production of slime, it was found that there is a direct relationship between these two cases, and the icaA and icaD genes were present in all isolates with the ability to form biofilm and slime. This requires more research and study of a large size population. In the present study, due to the time constraints, it was not possible to study the higher number of isolates. In addition, two other genes of ica (icaB and icaC) were not included in our research.

Slime production is the same as biofilm under the control of ica operon (26). The study by Kara Terki et al. indicated that ica genes were considered as pathogenic markers in Staphylococcus species. This association with biofilm-forming strains shows that the expression of icaA and icaD genes play an important role in the mechanisms of organism damage (27). El-Mahallawy et al. showed that there is a strong and meaningful relationship between the presence of ica genes and the production of slime and biofilm (28). Yazdani et al. also showed that the production of slime and biofilm occurs in the presence of icaA and icaD genes [5]. Nuryastuti et al. also stated that slime production among strains of Staphylococcus aureus has a significant relationship with the formation of strong biofilm in these strains (29). Fowler et al. indicated that icaA and icaD genes exist in all strains of Staphylococcus aureus with the ability to form biofilm (30). Nasra et al., using the two methods of Congo-Red Agar and Microtiter Plate in Staphylococcus strains, reported a biofilm production of 46% and reported the presence of icaA and icaD genes of 32%. According to findings of Nasra et al., despite the presence of icaA and icaD genes in Staphylococcus strains, there was no significant relationship between biofilm formation in vitro and some strains of Staphylococcus strains with the ability to form biofilms have not icaA and icaD genes (31). Fitzpatrick et al. also acknowledged that biofilm formation in clinical isolates of Staphylococcus aureus was studied in vitro independently of the presence of icaADBC genes (32). Eftekhar et al. obtained similar results in this regard, and the production of biofilms in Staphylococcus aureus was independent of the ica genes (33). Biofilm formation in Staphylococcus strains is dependent on environmental conditions and is influenced by environmental signals that can respond to external stress and inhibitory concentrations of antibiotics (34). Anaerobic conditions and low iron concentrations also help to form biofilms (27). Failure to form biofilms despite the presence of ica genes can be due to the inactivation of erythrocytes by activating the icaR repressor or under the influence of post-transcriptional processes (35). This requires more genetic studies and iInvestigate the molecular mechanisms independent of the ica operon.

Based on the results of this study, icaA and icaD genes for intercellular polysaccharide adhesion, bacterial bonding and biofilm and slime formation in Staphylococcus aureus isolates are considered essential. Understanding this requires more molecular studies of ica operon and related mechanisms.

Acknowledgment

We would like to thank Vice chancellor for Research and Technology of Golestan University of Medical Sciences.

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