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Association between biofilm production, adhesion genes and drugs resistance in different SCCmec types of methicillin resistant *Staphylococcus aureus* strains isolated from several major hospitals of Iran

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ABSTRACT

Objective(s): The ability of bacteria to produce biofilm and adhesion makes them more resistant to antibiotics. The current study aims to evaluate the biofilm formation by Staphylococcus aureus and to determine the prevalence of adhesion genes, also their correlation with drug resistance.

Materials and Methods: A total of 96 MRSA were collected from hospitals of Iran's western provinces during 2012 to 2013. The presence of *ica A, B, C, D, clfA, cna, fnbA, mecA* genes were determined by PCR technique. Biofilm formation was studied by microtiter plate assay, the clonal relations of the strains were examined by SCCmec and Spa typing.

Results: The results demonstrated that 96 % of isolates were biofilm producers. The distributions of biofilm formation between isolates were 4.2%, 54.2%, 35.4% as high, moderate and weak, respectivelly. The highest biofilm production was observed from blood culture isolates. All virulent genes *icaA ,B, C, D, clfA, cna, fnbA* were observed in moderate and weak biofilm formation isolates. Among high biofilm formation isolates, icaB and cna genes were not seen. Statistical analysis showed that there was a significant correlation between *ica*, *fnbA* and the biofilm production, but there was not a significant correlation between the type of samples and drug resistance, spa type and SCCmec type with biofilm production (P>0.05). Frequency of All virulent genes in type III SCCmec was higher than other types.

Conclusion: The majority of MRSA isolates were biofilm producers and blood isolates ranked as the great biofilm producer. In these isolates *ica D* and *fnbA* genes are correlated with biofilm production.

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Introduction

The surface adhering of bacteria to implantable medical devices is due to biofilm formation. Device associated infections distinctly impact patient morbidity and mortality (1). One of the most frequent causes of biofilm-associated infections is *Staphylococcus aureus* (2). Biofilm matrix and change of phenotypic characteristics of bacteria in biofilm related to infection result to resistance to antimicrobial drugs. In healthcare center, where there is an antibiotic abuse, a key survival mechanism factor of *S. aureus* is capacity to biofilm formation on implanted medical devices and damaged host tissues. Almost the majority of studies reported the *icaA, D, B, C* operon, produced polysaccharide intercellular adhesion (PIA) (3), as main mechanism for biofilm formation. But another mechanism on biofilm formation independent of the ica operon has also been reported in *S. aureus*. The four ica operon biosynthesis genes are *icaA, icaD, icaB*, and *icaC* and a transcribed repressor, *icaR* (4). A study revealed mutation in the ica genes of *S. aureus* diminished biofilm development and PIA making (5). Another mechanism of ica independent biofilm formation mediated by the biofilm associated protein (Bap), this mechanism is sensitive to proteinase k (6). This study aims to evaluate the drug resistance, biofilm formation and prevalence of adhesion gene in *S. aureus* collected from four major hospitals of western provinces of Iran.

Materials and Methods

Bacterial strains

Ninety six methicillin resistant *S. aureus* were collected from four major hospitals including Ilam, Kermanshah, Hamadan, and Khoramabad hospitals in Iran during 2012 to 2013. The presence of *mecA* gene confirmed by PCR. MRSA strains were stored at −80 ˚C in the appropriate media with 15% (v/v) glycerol until use.

Microtiter plate assay for assessment of biofilm formation

MRSA clinical isolates were grown overnight in Muller Hinton broth (MH; GibcoBRL) supplemented with 0.25% glucose. Cultures were then diluted 1:200 and incubated overnight in 96 microtiter plates at 35 ˚C. Microtiter wells were washed twice with phosphate-buffered saline, dried in an inverted position, and

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Table 1. The PCR conditions and specific primers used in the present study

Gene	Primer sequence	PCR product (bp)	Tm annealing (◦C)
mecA	F: GTG GAA GTT AGA TTG GGA TCA TAG R: GTC AAC GAT TGT GAC ACG ATA GC	544	67
Ica A	F: GAC CTC GAA GTC AAT AGA GGT R: CCC AGT ATA ACG TTG GTA ACC	188	57
Ica B	F: ATC GCT TAA AGC ACA CGA CGC R: TAT CGG CAT CTG GTG TGA CAG	900	57
Ica C	F: ATA AAC TTG AAT TGA TGT ATT R: ATA TAT AAA ACT CTC TTA ACA	900-1100	53
Ica D	F: AGC CAA TAT CCA ACG GTA CAG R: GTC ACG ACC TTT CTT ATA CGC	250	53
cna	F: AGG ATC AGA TTC AAG GTG ACA R: GAG TGC CTT TCC CAA CCT GAC	600	53
clfA	F: CGA TTG GGC GTG GCt TTC AGC R: GCC AGT AGC CCA ATG TCA CGC	300	57
fnbA	F: GCG GAG ATC AAA GAC ATA TCA R: CCA TCT ATA GCT GTG TGG TCC	110	53

Table 2. Distribution of 96 biofilm forming strains of *Staphylococcus aureus* according to clinical samples

Clinical sample (No)	No of strains according to degree of biofilm formation								*P*-value
	High		Moderate		Weak		Non biofilm		
	No	%	No	%	No	%	No	%	
Urine 41	2	2.1	16	16.7	19	19.8	4	4.2	<0.05
Blood culture 34	2	2.1	21	21.9	11	11.5	0	0	<0.05
Ulcer 15	0	0	12	12.5	3	3.1	0	0	<0.05
Sputum 6	0	0	5	5.2	1	1	0	0	<0.05
Total 96	4	4.2	54	56.2	34	35.4	4	4.2	<0.05

stained with 0.1% crystal violet (2). Then, plates were incubated at room temperature for 15 minutes, and after 2 times washing, solubilized in 200 µl of 95% ethanol and then read with ELISA reader at 570 nm wavelength (Jenway, England).

Antibiotic susceptibility testing

Susceptibility testing of isolates was done using the Kirby Bauer disc diffusion method according to the references of the Clinical and Laboratory Standards Institute (CLSI) (7). Antimicrobial susceptibility testing to the following antibiotics was accomplished by using the disc diffusion method: oxacillin (1 µg), amikacin (30 µg), gentamycin (10 µg), ciprofloxacin (30 µg), cefotaxime (Ce, 30 µg), vancomycin (30 µg), tetracycline (30 µg), rifampin (5 µg), linezolid (30 µg), cinercide (15 µg), tigecycllin (5 µg), clindamycin (10 µg), erythromycin (15 µg), and imipenem(10 µg)(Mast, Engeland).

Detection of virulence genes

DNA was extracted using a Genomic DNA purification kit (Fermentas, USA) according to the manufacturer's instruction. The *icaA, icaB, icaC, icaD, clfA, cna, fnbA*, and *mecA* genes in *S. aureus* were investigated by polymerase chain reaction. All primer sequences and PCR condition are shown in Table 1. The PCR reactions were performed using Bio-Rad Thermocycle (Bio-Rad ,USA). In the PCR, each reaction contained 1 µl (10 pmol) of forward primer, 1 µl (10 pmol) of reverse primer for each primer, 12,5 µl master mix, 4 µl of DNA extract and final volume was 25 µl by adding sterile water. PCR of *icaC*, *icaD*, *can* and *fnbA* genes were performed using an initial denaturation step 95 ˚C for 5 min, followed by 35 cycles of 95 ˚C for 30 sec, 53 ˚C for 45 sec, 72 ˚C for 30 sec, and a final extension step at 72 ˚C for 5 min. While the cycling conditions for *ica A*, *ica B* and *clfA* genes were as follows: DNA denaturation at 95 ˚C for 5 min, 35 cycles of 95 ˚C for 60 sec, 57 ˚C for 57 sec, 72 ˚C for 60 sec, and a final extension step at 72 ˚C for 5 min. The cycling conditions for *mecA* gene was similar with *ica C* ,but annealing temperature was 67 ˚C for 45 sec. The PCR products were stained with DNA safe and electrophoresed in 1% agarose gel at 80 V for 30 min.

Statistical analysis

The data on production of biofilms by the strains of *S. aureus* was analyzed by the statistical software SPSS version 19.0. *P*-value were calculated using the Chi square test. $P<0.05$ was considered to be statistically significant.

Results

Out of 96 isolates of *S. aureus* examined, 92 (95.8%) produced biofilms. The power and distribution of biofilm production according to the source of isolates are shown in Table 2. The distribution of virulence genes is presented in Table 3 and 5. The results demonstrated that 96 % of isolates were biofilm producers. The distributions of biofilm formation between isolates were 4.2%, 54.2%, 35.4% as high, moderate and weak, respectivelly. The highest biofilm production was observed from blood culture isolates. All virulent genes *icaA, B/C/D, clfA, cna, fnbA* were observed in moderate and weak biofilm formation isolates. Among high biofilm formation isolates, *icaB* and *cna* genes were not seen. Statistical analysis showed that there was a significant correlation between *icaD* and *fnbA* and the biofilm production (Figure 1), but there was not a significant correlation between the type of samples and drug resistance or spa type and SCCmec type with biofilm production ($P>0.05$). Frequency of all virulent genes in type III SCCmec was higher than other types. Table 4 demonstrates the antibiotic susceptibility results.

Table 3. Frequency of adhesion genes in *Staphylococcus aureus* isolates according to sample sources

Percent of adhesion genes Degree of biofilm formation	*cna*	*clf A*	*fnb A*	*ica D*	*ica C*	*ica B*	*ica A*
High	4.2	0	1	3.1	3.1	4.2	0
Moderate	32.2	9.4	6.3	44.8	37.6	34.4	11.4
Weak	29.2	5.2	4.2	18.8	15.5	28.1	4.2
Non biofilm	2.1	0	0	2.1	2.1	2.1	0
Total	67.7	14.6	11.5	68.8	58.3	68.8	15.6
P value	>0.05	>0.05	<0.05	>0.05	<0.05	>0.05	>0.05

The intercellular adhesion (ica) locus, *ica ABCD*, Collagen adhesion-encoding gene (*can*), Clumping factor A gene (*clf A*), Fibronectin binding protein A gene (*fnb A*)

Table 4. drug resistance pattern in biofilm former and non-biofilm former of staphylococcus aureus

Antibiotic	Non biofilm former(N=4)		Biofilm former(N=92)		Total(N=96)	
	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)
Tetracyclin	75	25	76.1	23.9	76	24
Gentamicin	75	25	69.6	30.4	69.8	30.2
Oxacillin	0	100	23.9	76.1	22.9	77.1
Erythromycin	100	0	66.3	33.7	67.7	32.3
Vancomycin	100	0	100	0	100	0
Penicilln	0	100	0	100	0	100
Synercid	100	0	100	0	100	0
Amikacin	100	0	83.7	16.3	84.4	15.6
Imipenem	100	0	97.9	2.1	97.9	2.1
Linezolid	100	0	100	0	100	0
Tigecyclin	100	0	100	0	100	0
Ciprofloxacin	75	25	71.8	28.2	71.9	28.1
Clindamycin	100	0	71.8	28.2	72.9	27.1
Rifampin	100	0	87	13	87.5	12.5

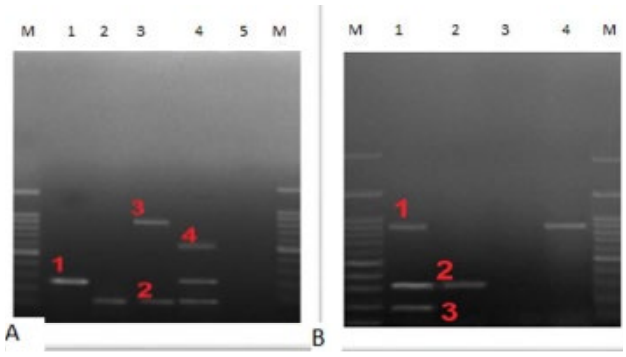

Figure 1. Electrophoresis of PCR products for identification of adhesion genes of *S. aureus*: A, M (100 bp marker), *ica C* (lane 1), *ica D* (lane 2), *fnb A* (lane 2), *cna* (lane 3) genes. B *ica A* (band 3), *ica B*(band 1) , *clf A*(band 2)

Discussion

The ability of bacteria to produce biofilm and adhesion makes them more resistant to antibiotics. Bacterial adhesion factor is considered as a virulence factor that plays an important role in infections associated with catheters and other indwelling medical devices (8). The ability of *S. aureus* to colonize in artificial material is associated with two main mechanisms; production of polysaccharide slime, and adhesions for the host matrix proteins that are adsorbed onto the biomaterial surface (9). When the biofilm formed, it would be easy to escape from immune systems and to cause chronic infections (10). Although PIA is important for biofilm formation by *S. aureus*, this study found that only one gene of ica operon i.e *icaD* and *fnbA* genes is related

Table 5. Frequency of Adhesion genes in deferent SCCmec types of *Staphylococcus aureus*

SCCmec Types	Frequency of adhesion genes (%)						
	ica A	*ica B*	*ica C*	*ica D*	*fnb A*	*clf A*	*cna*
Non type,	20.8	5.2	2.1	15.6	15.6	20.8	1
III	27.1	6.3	3.1	31.3	22.9	26	6.2
IVc	7.3	2.1	4.2	11.5	11.5	9.4	6.2
IVd	9.4	1	2.1	9.4	6.2	10.5	2.1
V	3.1	0	0	1	2.1	2.1	0
Total	67.7	14.6	11.5	68.8	58.3	68.8	15.6
P-Value	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05

Classification of Staphylococcal cassette chromosome mec (SCCmec),The intercellular adhesion (*ica*) locus, ica ABCD, Collagen adhesion-encoding gene (*can*), Clumping factor A gene (*clf A*), Fibronectin binding protein A gene (*fnb A*)

with biofilm production in this isolates. Our analysis demonstrated that the blood culture isolates were more biofilm producers. So that, we suggest more study should be done to determine why the biofilm formation in different conditions of body is not equal. The only reason we focused on is immune system and escaping of bacteria via biofilm production. Although, many genes and conditions are responsible to biofilm production but our results demonstrated that *icaD* and *fnbA* genes has a critical role in biofilm formation. Study by Arciola *et al* (11). demonstrated that all *S. aureus* biofilm positive strains possess *icaD* genes that required for full slime synthesis. Another study results confirmed that there is a relationship only between *icaD* and biofilm production in MRSA strains (12); this is consistent with our results that showed the main role of *icaD* for biofilm formation. In this study, in spite of this fact that SCCmec type III is the most prevalent in MRSA strains, but SCCmec non-type able strains are more prevalent than SCCmec type III among biofilm producers. This indicates that biofilm producers' strains are mainly community acquired. Another study showed that MRSA strains which had the ability to produce biofilm were SCCmec type III and V (13).

Conclusion

The majority of MRSA isolates were biofilm producers and blood isolates placed as the great biofilm producer. Only *icaD* and *fnbA* genes are correlated with biofilm production, But there was not a significant correlation between the drug resistance or spa type and SCCmec type with biofilm production.

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