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

Lida Bimanand 1, Morovat Taherikalani 1, Farid Azizi Jalilian 1, Nourkhoda Sadeghifard 1, Sobhan Ghaforian 1,2, Zahra Mahdavi 1, Sattar Mohamadi 1, Kouroes Sayehmehr 1, Ali Hematian 2, Iraj Pakzad 1,2*

1 Clinical Microbiology Research Center, Ilam University of Medical Sciences, Ilam, Iran
2 Department of Microbiology, Faculty of Medicine, Ilam University of Medical Sciences, Ilam, Iran
3 Department of Biostatistic, Faculty of Medicine, Ilam University of Medical Sciences, Ilam, Iran

**A R T I C L E  I N F O**

**Article type:**
Original article

**Article history:**
Received: Oct 3, 2016
Accepted: Sep 28, 2017

**Keywords:**
Antiseptic
Biofilm
MRSA
Resistance genes
**qacA/B**

**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, respectively. 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 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.

**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 ica 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

---

*Corresponding author: Iraj Pakzad. Department of Microbiology, Faculty of Medicine and Clinical Microbiology Research Center, Ilam University of Medical Sciences, Ilam, Iran; Tel: +98-8412227120; Fax: +98-8412227120; Email: pakzad_i2006@yahoo.com*
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

Suscettibility 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), cincircide (15 μg), tigecyclin (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.

Antibiotic susceptibility testing

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

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

| Percent of adhesion genes | cna | clf A | fnb A | ica D | ica C | ica B | ica A |
|---------------------------|-----|-------|-------|-------|-------|-------|-------|
| Degree of biofilm formation | S (%) | R (%) | S (%) | R (%) | S (%) | R (%) | S (%) | R (%) |
| 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 | 32.1 | 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

### Table 4. Drug resistance pattern in biofilm former and non-biofilm former of *Staphylococcus aureus*

| Antibiotic | Non biofilm former (N=6) | Biofilm former (N=92) | Total (N=96) |
|-----------|---------------------------|-----------------------|-------------|
| | S (%) | R (%) | 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 |
| Penicillin | 0 | 100 | 0 | 100 | 0 | 100 |
| Synecid | 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 |

### 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 fnb A genes is related.
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.

Acknowledgment
The authors would like to thank Deputy of Research and Technology of Ilam University of Medical Sciences, Iran, for financial support. The results described in this paper were part of student thesis.

References
1. Hoyle BD, JW. Costerton. Bacterial resistance to antibiotics: the role of biofilms. Prog Drug Res 1991; 37: 91–105.
2. Yazdani R, Oshaghi M, Havayi A, Salehi R, Sadeghizadeh M, Forooshesh H. Detection of icaAD gene and biofilm formation in Staphylococcus aureus isolates from wound infections. Iranian J Publ Health 2006; 35: 25-28.
3. Ammendolia MG, Rosa RD, Montanaro L, Arciola CR and Baldassarri L. Slime production and expression of slime-associated antigen by staphylococcal clinical isolates. J Clin Microbiol 1999; 37: 3235-3238.
4. OGara JP, Humphreys H. Staphylococcus epidermidis biofilms importance and implications. H Med Microbiol 2001; 50: 582-587.
5. Nathan KA, Mark JM, Costerton JV, Leid JG, Powers ME, Shirtliff ME. Staphylococcus aureus biofilms. Virulence 2011; 2: 445-459.
6. Cucarella C, Tormo MA, Ubeda C, Trotonda MP, Monzón M, Peris C. Role of biofilm-associated protein bap in the pathogenesis of bovine Staphylococcus aureus. Infect. Immun 2004; 72: 2177–2185.
7. Clinical and Laboratory Standards Institute (CLSI). CLSI document M100-S24. Wayne, PA: Clinical and Laboratory Standards Institute 2015.
8. Francois P, Vaudaux P, Foster TJ, Lew DP. Host-bacteria interactions in foreign body infections. Infect Control Hosp Epidemiol 1996;17: 514–520.
9. Montanaro L, Arciola CR, Borsetti E, Brigotti M, Baldassarri L. A polymerase chain reaction (PCR) method for the identification of collagen adhesin gene (cna) in Staphylococcus-induced prosthesis infections. New Microbiol 1998;21: 359–363.
10. Cramaton, SE, Gerke C, Gotz F. In-vitro method to study Staphylococcal biofilm formation. Methods Enzymol 2001; 336: 239-55.
11. Arciola, CR, Baldassarri L, Montanaro L. Presence of icaA and icaD genes and slime production in a collection of Staphylococcal strains from catheterassociated infections. J Clin Microbiol 2001;39: 2151 –2156.
12. Serray B, Oufrid S, Hannaoui I, Bourjilate E, Soraa N, Mliji M, et al. Genes encoding adhesion factors and biofilm formation in methicillin-resistant Staphylococcus aureus in Morocco. J Infect Dev Ctries 2016; 10: 863-9.
13. Halebeedu Prakash P, Rajan V, Gopal S. Predominance of SCCmec types IV and V among biofilm producing device-associated Staphylococcus aureus strains isolated from tertiary care hospitals in Mysuru, India. Enferm Infecc Microbiol Clin 2017; 35: 229-235.