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Detection of Biofilm Production Capability and *icaA/D* Genes Among Staphylococci Isolates from Shiraz, Iran

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Abstract

Background: Biofilm formation capacity is recognized as an important virulence factor in staphylococci that makes the organisms more resistant to antibiotics and host defenses.

Objectives: This study aimed to determine the biofilm producing ability and presence of *icaA/D* genes in staphylococcal isolates obtained from different clinical specimens.

Methods: This cross-sectional study was performed on a total of 151 staphylococcal isolates (79 *Staphylococcus aureus* and 72 *S. epidermidis*) obtained from different clinical specimens from February to August 2013 in Shiraz, Southwest of Iran. Slime production ability was evaluated using the both phenotypic (by cultivation of staphylococcal isolates on Congo red agar (CRA)) and genotypic (detection of the presence of *icaA/D* genes by PCR) methods.

Results: Overall, of the 79 *S. aureus* isolates tested with CRA method, 64.7% of methicillin-resistant *S. aureus* (MRSA) isolates, and 46.7% of methicillin-sensitive *S. aureus* (MSSA) isolates were able to produce biofilm. The relative frequency of biofilm producing *S. epidermidis* isolates was 70.8% that was significantly higher than that of *S. aureus* isolates. The most common source of biofilm producing isolates in both *S. aureus* and *S. epidermidis* isolates was endotracheal tube (ETT) with 100% biofilm formation. Moreover, the presence of *icaA/D* genes was detected in 63.3% and 81.9% of *S. aureus* and *S. epidermidis* isolates, respectively.

Conclusions: The remarkable rates of biofilm production ability among clinically isolated staphylococci emphasize the necessity of more effective infections control policies to prevent biofilm formation on medical devices and hospital environmental surfaces.

Keywords: MRSA, Biofilm, Antibiotic Resistance, *Staphylococcus*

1. Background

Staphylococci are well known as the normal flora of the skin and mucous membranes of animals and human (1-3). However, staphylococcal infections are often caused by strains that have colonized in parts of the human body and make the colonized persons a reservoir for the spread of the organisms (4, 5). Due to the biofilm producing ability of staphylococci, they are mostly associated with chronic and implanted medical devices infections (6, 7). *Staphylococcus epidermidis* was the first species identified as biofilm producer; however, the same capacity was subsequently implicated in *S. aureus* and other coagulase negative Staphylococci (CoNS) (8, 9).

Biofilm structure contains bacteria in which cells adhere to each other on a surface and are surrounded in exopolysaccharide matrix (10). The bacteria enclosed in a biofilm make them intrinsically resistant to many antimicrobial drugs and host defenses (9, 11). The slime production in CoNS isolates occurs in three steps: attachment of bacteria to a biomaterial or artificial surface, formation of an extracellular slime such as polysaccharide intercellular adhesin (PIA) which mediates cell to cell adhesion and disassembly of the biofilm, followed by community expansion (12, 13).

It has been shown that both *S. aureus* and *S. epidermidis* contain the intercellular adhesion (ica) operon responsible for biofilm formation (14). This operon contains the *icaA*, *icaD*, *icaB* and *icaC* genes which are regulated through the product of *icaR* gene (15). The ica operon (*ica*ADBC gene cluster) encodes the PIA; thus, there is a correlation between the presence of this operon and slime production in staphylococci, especially CoNS (13, 16).

2. Objectives

Identifying and controlling biofilm-forming staphylococci can be one of the essential steps for prevention and management of nosocomial infections. This study aimed to determine the biofilm producing ability and the presence of *icaA/D* genes among staphylococcal clinical isolates and their probable association with their antibiotic resistance profile.

3. Methods

3.1. Clinical Samples

This cross-sectional study was conducted between February and August 2013 at two Teaching hospitals (Nemazee and Faghihi) of Shiraz, a major city in the Southwest of Iran. Totally, 151 staphylococcal isolates (79 *S. aureus* isolates and 72 *S. epidermidis* isolates) were obtained from different clinical specimens and various wards of the studied hospitals. The clinical samples were blood, pus, wound, urine, endotracheal tube (ETT), sputum, cerebrospinal fluid (CSF), skin, etc.

3.2. Identification of Isolates

The isolates were recognized as *S. aureus* and *S. epidermidis* using the conventional microbiological methods, including colony morphology, Gram stain, catalase activity, growth on mannitol salt agar, DNase and tube coagulase tests, and susceptibility to novobiocin and polymyxin B. The preliminary differentiation of methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) isolates was based on resistance to cefoxitin (30 μg) (MAST, UK) using the disc diffusion assay according to Clinical and laboratory standards institute (CLSI) recommendations (17). The confirmed isolates were stored at -70°C for further analysis.

3.3. Biofilm Production Assay

Biofilm producing isolates were detected using the Congo red agar (CRA) method. Congo red stain (PML, Canada) was prepared as a strong aqueous solution by dissolving 37 g of the stain in 50 mL of distilled water which was sterilized (at 121°C for 15 minutes) separately from 15 g brain heart infusion agar (BHI) (Merck, Germany) in 450 mL distilled water. Congo red solution was added to the agar at the temperature of 50 - 55°C. Finally, 37 g sucrose was filtered using 0.45 μm Millipore filter (Sigma, UK) and added to the culture medium. After plating of staphylococci and overnight incubation at 37°C, biofilm producing isolates appeared as black crusty colonies, while non-biofilm producing isolates developed pink or red colonies.

3.4. Antimicrobial Susceptibility Testing

Antibiotic susceptibility against ampicillin, clindamycin, gentamicin, ciprofloxacin, trimethoprim-sulfamethoxazole, erythromycin, tetracycline, rifampin, linezolid, and vancomycin was tested on Muller-Hinton agar (Oxoid, UK) using the disk diffusion method according to CLSI recommendations (17). All the discs were obtained from Rosco Co., Denmark. *S. aureus* ATCC 25923 (a methicillin-sensitive strain) was used as control strain in antibacterial susceptibility tests. The diameters of the inhibition zones were interpreted according to the guidelines of CLSI (17).

3.5. DNA Extraction and PCR Assay

Genomic DNA was extracted from individual isolates using the small-scale phenol-chloroform extraction method and subjected to polymerase chain reaction (PCR) (18). For molecular confirmation of MRSA isolates, the presence of *mecA* gene was sought by a set of previously described primers (19). All staphylococcal isolates were analyzed for the presence of *icaA* and *icaD* genes by the primers described by Arciola et al. (20). MRSA reference strain subspecies COL, which was applied as *mecA*, *icaA*, and *icaD* positive genes, was kindly provided by the professor Alborzi clinical microbiology research center, Shiraz, Iran.

PCR was performed in a final volume of 25 μL, containing 3 μL DNA template, 2.5 μL PCR buffer (1X), 1 μL deoxyribonucleotide triphosphates solution (dNTPs, 200 μM), 1.5 μL $MgCl_2$ (1.5 mM), 0.25 μL Taq DNA polymerase (1 Unit) and 1 μL each specific primer (1 μM) (Table 1). PCR tests were carried out in a thermocycler 5530 (Ependrof master, Germany) as follows: 5 minutes initial denaturation at 94°C, 30 cycles of denaturation at 94°C for 45 seconds, annealing (for *icaA* at 50°C for 45 seconds and for *icaD* at 56°C for 45 seconds), extension at 72°C for 1 minute, and final extension at 72°C for 10 minutes. All the reagents were obtained from Ampliqon Co., Denmark. In each run of PCR products, one negative (DNase-free water) and one positive control were included in agarose gels. Then, PCR products were loaded into 1.5% agarose gel and stained with 1% ethidium bromide (Merck, Germany) and visualized under UV transilluminator.

3.6. Statistical Analysis

Analysis was performed by using SPSS™ software, version 16.0 (Chicago, IL, USA). The results are presented using descriptive statistics in terms of relative frequency. Values were expressed as the percentages of the group (for categorical variables). Chi-square test was used to determine any statistical association. Statistical significance was regarded as P values < 0.05.

4. Results

4.1. Detection of Slime Production in Staphylococci by the CRA Assay

Of 79 *S. aureus* isolates, 34 (43%) were identified as MRSA and 45 (57%) as MSSA. The distribution of biofilm producer staphylococci according to the source of isolates is presented in Table 2. Among *S. aureus* isolates examined with

Table 1. The List of Used Primers in the Present Study

Primer	Oligonucleotide Sequence (5′ - 3′)	Product Size, bp	Reference
***mecA*-F**	GTGAAGATATACCAAGTGATT	147	19
***mecA*-R**	ATG CGCTATAGATTGAAAGGAT		
***icaA*-F**	TCTCTTGCAGGAGCAATCAA	188	20
***icaA*-R**	TCAGGCACTAACATCCAGCA		
***icaD*-F**	ATGGTCAAGCCCAGACAGAG	198	20
***icaD*-R**	CGTGTTTTCAACATTTAATGCAA		

CRA assay, 43 (54.4%) showed the ability to produce biofilm; of the MRSA and MSSA isolates, 22 (64.7%) and 21 (46.7%) isolates produced black colonies, respectively, and this difference was not statistically significant (P = 0.17). On the other hand, out of 72 clinical isolates of *S. epidermidis*, 51 (71%) were biofilm producers. The relative frequency of biofilm producing *S. epidermidis* isolates (70.8%) was significantly higher than that of *S. aureus* isolates (54.4%) (P < 0.038). The most common source of biofilm producing isolates in both *S. aureus* and *S. epidermidis* isolates was ETT with the rate of 100% biofilm formation. The results of antibiotic resistance pattern of biofilm producing staphylococci are shown in Table 3. Overall, none of *S. aureus* and *S. epidermidis* isolates were resistant to linezolid and vancomycin. Interestingly, most of biofilm producing isolates exhibited higher rates of antibiotic resistance compared to non-biofilm producing isolates.

4.2. Detection of Slime Production in Staphylococci by PCR

The results of PCR on *icaA* and *icaD* genes revealed that the presence of each gene in staphylococcal isolates was related to the presence of the other one. Slime production was confirmed by the presence of *icaA/D* genes in 50 (63.3%) *S. aureus* isolates. Among *S. epidermidis* isolates, 59 (81.9%) carried the *icaA/D* genes. The *icaA/D* genes were more prevalent among *S. epidermidis* isolates than *S. aureus* ones and the difference was statistically significant (P < 0.011). The presence of the *icaA* (188 bp) and *icaD* (198 bp) genes in all the investigated staphylococci was shown by the amplification of the corresponding fragments (Figure 1).

5. Discussion

The biofilm formation on mucosal and inanimate surfaces such as invasive medical devices has been considered as a major virulence determinant in staphylococci (13). In view of large number of infections caused by biofilm- producing bacteria, it seems that early detection and elimination of these bacteria are necessary.

To evaluate slime production among staphylococci, usually a phenotypic method along with ica operon detection is used. The CRA method due to its easiness and good sensitivity has been widely used for phenotypic detection of biofilm production in different bacteria (21). In the present study, 54.4% and 70.8% of *S. aureus* and *S. epidermidis* isolates were recognized as biofilm producer by CRA method, respectively. As indicated by Osman et al. CRA is a reliable and suitable method for routine evaluation of slime production (7). Several studies previously applied CRA phonotypical method to assay biofilm formation among staphylococci isolates in Iran and obtained various results based on the source of isolation, type of species, and geographic region. In the survey of Eftekhar et al. in Tehran, 53.3% of MRSA isolates were assessed to be potential biofilm producers (22). Solati et al. in a multicenter hospital study reported a rate of 50% biofilm formation by *S. epidermidis* isolates (11). Ohadian Moghadam et al. showed high rates of biofilm production (more than 80%) among *S. aureus* isolates obtained from bourn wound samples in Tehran, North of Iran (23). In another study from Kashan, all MRSA isolates from nasal carries were able to form biofilms (24). These variations in frequency of biofilm producing staphylococci are documented in other parts of the world, as well. The rates of biofilm production in staphylococci isolates have been reported in the range of approximately 30% to more than 70% in different Asian, African, and European countries (20, 25, 26).

In the present study, three major sources of biofilm producing isolates were ETT, wound, and blood samples. In accordance with our findings, medical instruments were suggested as a common source of biofilm producing staphylococci (27, 28). However, such high rates of biofilm producing isolates from wound and blood samples in our study are not uncommon, since several authors have shown high rates of biofilm formation among staphylococci obtained from these settings (29-31).

Our results demonstrate that biofilm forming isolates exhibited remarkable rates of antibiotic resistance com-

Table 2. Results of Biofilm Production in Staphylococci Isolates Based on the Source of Isolation

Specimen	*S. aureus*		*S. epidermidis*	
	CRA + Positive/Total	*icaA/D* + Positive/Total	*CRA* + Positive/Total	*icaA/D* + Positive/Total
ETT	5/5	5/5	8/8	8/8
Wound	10/16	13/16	9/12	11/12
Urine	8/14	8/14	5/7	6/7
Sputum	5/11	7/11	5/8	5/8
Blood	8/10	10/10	13/15	15/15
CSF	3/7	3/7	4/7	5/7
Skin	1/4	1/4	3/7	3/7
Other	3/12	3/12	4/8	6/8
Total, No. (%)	43 (54.4)[a]	50 (63.3)[b]	51 (70.8)[a]	59 (81.9)[b]

Abbreviations: CRA, Congo red agar; CSF, Cerebrospinal fluid; ETT, Endotracheal tube.
[a]The CRA differences were statistically significant ($P < 0.038$).
[b]The *icaA/D* genes differences were statistically significant ($P < 0.011$).

Table 3. Results of Antibiotic Resistance Pattern of Staphylococci Isolates Based on the Ability of Biofilm Production[a]

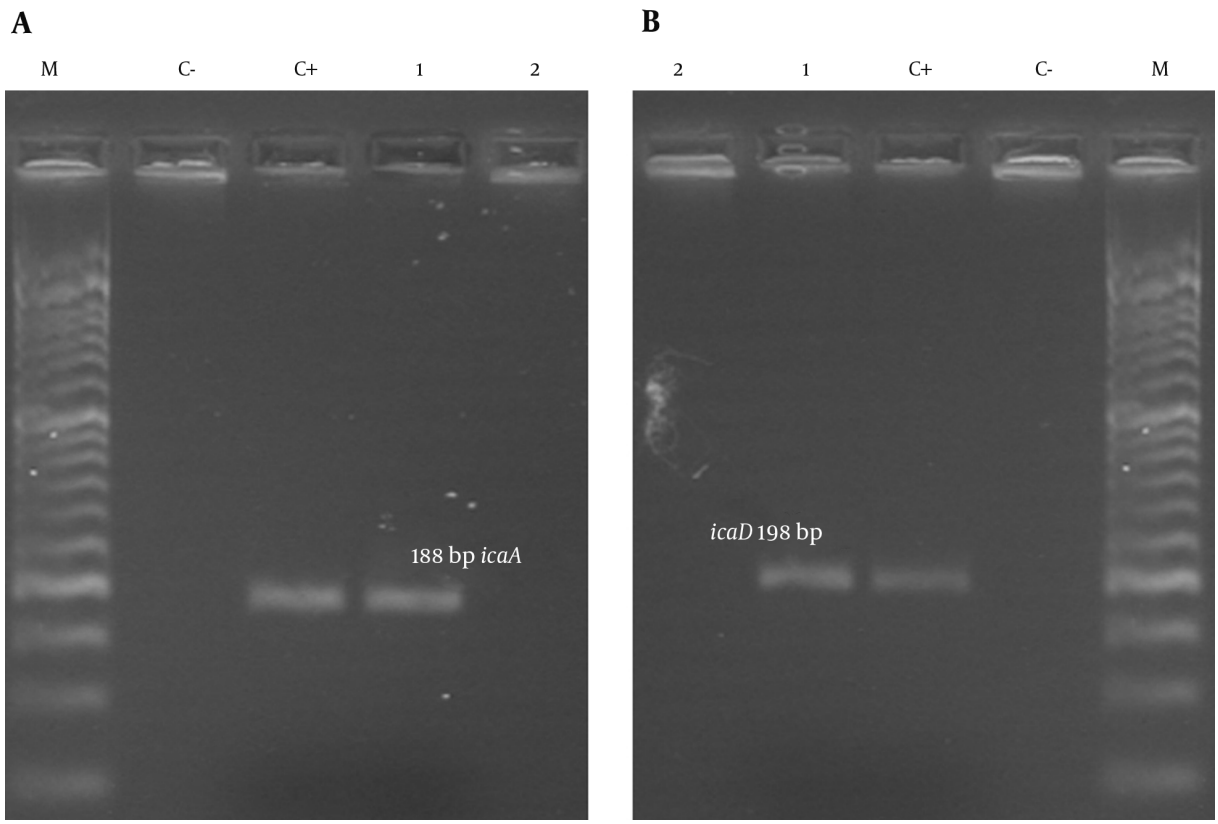
Antibiotic	*S. aureus*		*S. epidermidis*	
	Total (N = 79)	CRA + (N = 43)	Total (N = 72)	CRA + (N = 51)
Ampicillin	76 (96.2)[b]	43 (100)	63 (87.5)	48 (94.1)[c]
Tetracycline	45 (57)	29 (67.4)[c]	41 (56.9)	25 (40)[c]
Ciprofloxacin	32 (40.5)	24 (55.8)[c]	37 (51.4)	28 (54.9)
Erythromycin	45 (57)	27 (62.8)	50 (69.4)	35 (68.6)
Clindamycin	40 (50.6)[b]	23 (53.5)	48 (66.7)	34 (66.7)
Gentamycin	29 (36.7)	19 (44.2)	26 (36.1)	20 (39.2)
Rifampin	20 (25.3)	11 (25.6)	9 (12.5)	5 (9.8)
Co-trimoxazole	8 (10.1)	6 (13.9)	49 (68.1)	37 (72.5)
Linezolid	0	0	0	0

Abbreviations: CRA, Congo red agar;
[a]Values are expressed as No. (%).
[b]Differences between *S. aureus* and *S. epidermidis* isolates were statistically significant ($P < 0.05$).
[c]The differences were statistically significant compared to non-biofilm producing isolates ($P < 0.05$).

pared to non-biofilm producing isolates. Antibiotic resistance is a growing health concern, especially in developing countries (32). Among biofilm producing *S. aureus* isolates, the resistance rate against tetracycline and ciprofloxacin was significantly higher compared to non-biofilm producing isolates. On the other hand, *S. epidermidis* biofilm producers revealed higher resistance to ampicillin and tetracycline compared to non-biofilm producing isolates ($P < 0.05$). In two Iranian studies, similar to our findings, most of the biofilm forming staphylococci isolates had higher antibiotic resistance and multiple drug resistance (MDR) rates (23, 24). The same findings have been reported by Sahal et al. in Turkey, indicating that majority of strong biofilm forming *S. epidermidis* strains were β-lactams resistant, and notably 100% of them were MDR (33).

The fundamental role of co-expression of the *icaA* and *icaD* genes in biofilm formation among *S. aureus* and *S. epidermidis* causing catheter-associated and nosocomial infections through the regulation and production of PIA has already been emphasized (8). In the present investigation, the *icaA* and *icaD* were evaluated because the detection of these genes implies the biofilm formation by staphylococcal isolates. In addition, these genes regulate the slime production (7, 13). In our study, the presence of *icaA* gene

Figure 1. PCR Results for A, *icaA* and B, *icaD* Genes



M, Molecular size marker (50 bp); C-, (negative control); C+, (positive control); lanes 1 and 2, clinical isolates.

in all tested isolates was associated with the presence of *icaD* gene and vice versa, which has also been mentioned by other authors (28, 34). The frequency of *icaA/D* genes among our staphylococci isolates was relatively high; however, such remarkable rates of ica operon among staphylococci isolates are not unexpected, since the same finding has been implicated in several studies (19, 34, 35).

The results of this study and previous studies indicate a high prevalence of the *icaA/D* genes among staphylococci isolates, and that their presence is not necessarily associated with in-vitro formation of biofilm (36). In the current study, the proportion of *S. epidermidis* isolates that were positive for *icaA/D* genes in PCR method was higher than the proportion of those that were positive in CRA method. This discrepancy can be attributed to the presence of other molecules involved in the biofilm formation such as ClpP, BHP, and Aae among CoNS isolates (13). Moreover, different studies have demonstrated that the expression of the *ica* locus is considerably variable and can be affected by many factors, which, in turn, results in the increase or decrease of biofilm production (7). The elucidation of the adhesive processes among clinical isolates might be a solution for developing anti-adhesive approaches to fight with infections due to opportunistic bacteria.

There are some limitations in our study. First, we did not perform other phenotypic methods used in the confirmation of biofilm formation. Second, other genes involved in the slime production process were not evaluated in this study.

5.1. Conclusions

CRA method is a practicable procedure that is in agreement with genotypic methods, particularly among *S. aureus* isolates. The remarkable rate of *icaA/D* genes and higher rate of antibiotic resistance among biofilm producing staphylococci isolates found in the present study suggest their potential risk for establishing persistent infection and therapeutic failure in hospitalized patients. However, further studies are needed to evaluate the role of slime production in nosocomial infections.

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Footnotes

Conflict of Interest: None declared.

Authors' Contribution: Study concept and design: Reza Khashei; acquisition of data and sampling: Mehrdad Zalipour; analysis and interpretation of data: Mehrdad Zalipour; drafting of the manuscript: Reza Khashei, Hadi Sedigh Ebrahim-Saraie; critical revision of the manuscript for important intellectual content: Reza Khashei; study supervision: Reza Khashei, Jamal Sarvari.

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