Detection of *ica*A/*ica*D Genes and Biofilm Formation among Clinical Isolates of *Staphylococcus aureus* from Shiraz, Iran

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ABSTRACT

***Introduction*:** Biofilm producing *Staphylococcus aureus* is known as one of the major causative agents of infections, failure of implanted devices and persistent infection among hospitalized patients**.** The aim of the present study was to determine the frequency of biofilm producing *S. aureus* isolates amongst the clinical specimens.

Methods: This cross-sectional study was conducted during 2012 to 2013 in two teaching hospitals in Shiraz, southwest of Iran. Totally, 345 *S. aureus* isolates from various clinical specimens were included. Biofilm producing isolates were phenotypically detected using Congo Red Agar (CRA) and genotypically by PCR assay for the *ica*A and *ica*D genes.

Results: Of the 345 *S. aureus* isolates, 42.3% were methicillin-resistant *S. aureus* (MRSA) and subsequently 57.7% were methicillin susceptible isolates. The results of CRA plates showed that 77 (52.7%) and 74 (37.2%) of MRSA and MSSA were biofilm producing isolates. The frequency of *ica*A/D genes among MRSA and MSSA isolates was 127 (87%) and 167 (83.9%), respectively.

Conclusion: Such a high rate of *ica*A/D harboring *S. aureus* among clinical isolates suggest the risk for establishing persistent infections in the hospital settings.

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Introduction

There is strong evidence on the role of S*taphylococcus aureus* in increased risk of nosocomial infections (1). *S. aureus* is responsible for various ranges of infections from mild superficial skin to severe systemic infections (2). A variety of virulent factors are produced by *S. aureus* which facilitate progress of infections (3). Biofilm produced by *S. aureus* is known as a virulence factor which plays an important role in a range of chronic infections and infections associated with indwelling medical devices (1). The biofilm formation and adherence ability of *S. aureus* are involved in several bacterial and external factors (4). The most substantial role in *S. aureus* biofilm formation is synthesis of the polysaccharide intercellular adhesion (PIA) which mediates aggregation of bacteria cell to each other (1, 5). The PIA production is directly influenced by *ica* operon located on bacterial chromosome (6). The *ica* operon contains the *ica* ADBC genes and *ica*R gene which play a regulatory function (7). It has been shown that the *ica*A and *ica*D genes have a significant role in PIA production and subsequently biofilm formation in *S. aureus* (7). Biofilm producing *S. aureus* is known as one of the major causative agents of infection and failure of indwelling medical devices, such as intravenous catheters, ear and central nervous system shunts, cosmetic surgical implants and many other implanted devices (1, 8). Implant infections represent a serious clinical problem, given that the colonized devices may become the cause of persistent infections and subsequently localized and generalized infections (2, 9). The risk of nosocomial infections of biofilm producing *S. aureus* is not limited to implanted devices, since it has been implicated that *S. aureus* is the most commonly isolated bacteria from chronic wound infections (1). The eradication of biofilm formation bacteria is usually associated with challenges because of greater resistance to antibiotics chemotherapy, host defenses and even disinfectants (1). Emergence of methicillin-resistant *S. aureus* (MRSA) which is commonly resistant to a wide range of antimicrobials has made eradication of biofilm formation bacteria more challenging (9). Due to the role of biofilm producing *S. aureus* in the infection of indwelling medical devices and their inherent resistance which limits therapeutic options, the early detection and management of such potential pathogens can be useful toward decreased rate of morbidity and mortality in the related patients and reduction in health service costs (10). The aim of the present study was to determine the prevalence of biofilm producing MRSA isolates from clinical specimens comparing to that for methicillin-susceptible *S. aureus* (MSSA) isolates.

Materials and Methods

Study design and Specimens

This cross-sectional study was conducted during 2012 to 2013 in two hospitals, Nemazee and Faghihi, in Shiraz, a major city in the Southwest of Iran. Nemazee and Faghihi Hospitals are two major tertiary care hospitals with 1000 beds, affiliated to Shiraz University of Medical Science. Totally, 345 *S. aureus* isolates were recovered from various clinical specimens including blood, pus, wound, urine, etc. Specimens were collected from different wards in our hospitals. Duplicated isolates and specimens labeled as outpatient were not included.

Bacterial identification

The isolates were recognized as *S. aureus* using conventional microbiologic procedures (colony morphology, Gram stain, catalase activity, growth on mannitol salt agar, DNase test and tube coagulase). Primary MRSA and MSSA isolates were screened based on resistance to cefoxitin (30µg) discs (MAST, UK) by disc diffusion method according to Clinical

and Laboratory Standards Institute (CLSI) guidelines (11). *S. aureus* ATCC 25923 which is a MSSA was used in this study as the control strain in antibacterial susceptibility testing. Confirmed isolates were stored at -70°C until subsequent studies.

Phenotypically biofilm assay

Biofilm producing MRSA and MSSA isolates were detected using Congo red agar (CRA). Congo red stain (PML, Canada) was made as a strong aqueous solution by dissolving 37 g of the stain in 50 mL of distilled water which was sterilized (121°C for 15 minutes) separately from 15g brain heart infusion agar (BHI) (Merck, Germany). The Congo red solution was supplemented to the agar when the temperature reached 55-50°C. Finally 37g sucrose was filtered through 0.45 µm filter and added to the rest of components. After bacterial inoculation and incubation for 24 hours at 37°C, biofilm producing isolates appeared as black colonies, while the non-producing isolates remained red (Figure 1).

DNA extraction and molecular biofilm assay

Genomic DNA was extracted from *S. aureus* isolates by using the small-scale phenol-chloroform extraction method and used as polymerase chain reaction (PCR) templates (12). DNA concentration was determined by spectrophotometer at A_{260} based on µg/ml concentration. In this study, the quantity of DNA samples used ranged from 10 to 1000 ng. Extracted DNA samples were preserved at -20 °C. The phenotypically confirmed MRSA isolates were subsequently tested for presence of *mecA* gene by a set of primers previously described by Zhang *et al.*(13). All MRSA and MSSA isolates were assayed for the presence of the *ica*A *and ica*D gene by using previously described primers (14). MRSA reference strain subspecies COL was served as the positive *mecA*, *ica*A and *ica*D gene production which was kindly provided by Professor Alborzi Clinical Microbiology Research Center, Shiraz, Iran. PCR amplifications were performed in a DNA Thermal Cycler 5530 (Ependrof master, Germany). PCR products were mixed with 1 µl loading buffer solution and loaded into the wells of agarose gel (1.5%) carefully and electrophoresed at 75V for 90 min. The gel was then stained with ethidium bromide (Merck, Germany) solution for 15 min and observed under the UV trans-illuminator (Figure 2). Statistical analysis was performed using SPSS^{TM} software, version 19.0. Chi–square or Fisher's exact tests were performed to analyze the data. P <0.05 was regarded as significant.

Results

Of the 345 *S. aureus* isolates included in this study, 42.3% were found to be MRSA by cefoxitin screen and presence of *mecA* and subsequently 57.7% were methicillin susceptible isolates. Results of colony phenotype on CRA plates showed that of 146 MRSA isolates, 77 (52.7%) isolates produced black colonies and were subsequently considered as biofilm producing isolates. Of 199 MSSA isolates, 74 (37.2%) isolates were able to produce biofilm. The differences in frequencies of biofilm formation ability between MRSA and MSSA isolates by CRA method were statistically significant (P< 0.006). PCR assays for the *ica*A and *ica*D genes, revealed that the presence of these genes in all positive isolates were associated with each other. Biofilm producing genes was confirmed by presence of *ica*A/D genes in 127 (87%) MRSA isolates. Frequency of *ica*A/D genes among MSSA isolates was 167 (83.9%). The rate of *ica*A/D genes detection among MRSA isolates was higher than MSSA isolates however, the difference was not statically significant (P= 0.523).The distribution of biofilm producing isolates among different clinical specimens based on methicillin-resistance pattern is presented in Table 1.

Table 1. Distribution of biofilm producing isolates among different clinical specimens based on the susceptibility to methicillin

Isolates / Sample	MRSA		MSSA	
	CBA+ Positive /Total	icaA/D+ Positive /Total	CBA+ Positive /Total	icaA/D+ Positive /Total
Sputum	29/58	54/58	15/35	31/35
Blood	9/20	14/20	5/26	16/26
Wound	15/18	18/18	17/23	23/23
Urine	7/14	13/14	11/37	32/37
Skin	5/8	8/8	8/17	15/17
ETT	6/6	6/6	3/3	3/3
Throat	1/4	3/4	3/8	8/8
Body fluids	2/4	2/4	1/4	3/4
Nose	1/3	3/3	5/24	22/24
Eye	0/3	3/3	0/6	4/6
CSF	1/2	1/2	0/3	1/3
Ear	NS[a]	NS	0/2	2/2
Axillary	1/2	1/2	NS	NS
Plural	0/1	0/1	NS	NS
TIPS	NS	NS	2/2	2/2
Other	0/3	1/3	4/9	5/9
Total No. (%)	77 (52.7)	127 (87)	74 (37.2)	167 (83.9)

[a] NS: No sample

Only for the samples obtained from implanted devices; trans jugular intrahepatic portosystemic shunt (TIPS) and endotracheal tube (ETT), both results of CRA method and molecular assays were the same. In both methods, all the recovered isolates from TIPS (2/2) and ETT (9/9) specimens were identified as the biofilm producers.

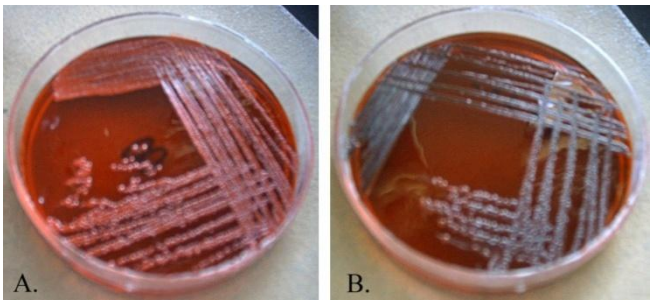

Figure 1. Detection of biofilm formation by CRA plates; A: non-biofilm producing isolates as red bacterial colonies; B: biofilm producing isolates which were appeared as black colonies

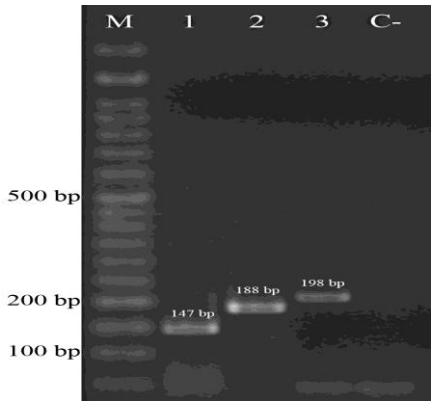

Figure 2. Representative gel image of molecular biofilm assay by PCR; M: 50bp DNA ladder; lane 1: positive control for *mecA* (147bp), lane 2: positive control for *icaA* (188bp), lane 3: positive control for *icaD* (198bp), C-: negative control (distilled water)

Discussion

Attachment and accumulation via biofilm formation to implanted medical devices and different sites of the host, such as wounds, were considered as one of the important pathogenesis factors in *S. aureus* (5). Pathogenesis of biofilm producing *S. aureus* even in conjunctivitis was described previously (15). Usually a phenotypic methods along with the detection of the *ica* operon is used to measure the capacity of biofilm formation *in vitro* for *S. aureus* isolates (5, 7, 16). In the present study we used CRA for phenotypic detection of biofilm formation among our recovered *S. aureus* isolates. The CRA method is the most favorable phenotypic test used for detection of biofilm formation among clinical isolates of *S. aureus* and is a practical tool for comparing the adherence of different strains. CRA test is rather easy, takes a short time and has a good sensitivity for screening biofilm formation of *S. aureus* (17). The results of CRA assays in our *in vitro* study revealed that the majority (52.7%) of MRSA isolates produce biofilm. Biofilm formation in MSSA isolates (37.2%) was significantly less than MRSA isolates. Previously, closest to our findings, Eftekhar *et al.* in a hospital survey from Tehran showed that 53.3% of MRSA isolates were potential biofilm producers by CRA method (5). Rezaei *et al.* from Kashan reported a frequency of 69.2% of biofilm formation in MRSA isolates recovered from the nasal carriers (18). While Jain *et al.* from India documented a rate of up to 70% biofilm production in *S. aureus* isolates (17), Arciola *et al.* from Italy and Nasr *et al.* form Egypt reported the rates of 60.8% and 46%, respectively (7, 19). In our study, MRSA isolates that were recovered from ETT, wound and skin specimens were three major sources of biofilm producing isolates with frequencies of 100%, 83.3% and 62.5%, respectively. It seemed that the favorite sites of colonization of biofilm producing MSSA isolates in our study were the same as MRSA isolates, but with the different rates for ETT and TIPS (100%), wound (73.9%) and skin (47.1%) samples.

High rates of biofilm producing *Staphylococcus* isolation, especially *S. aureus* from indwelling medical devices, have been also noted in several studies (6, 9, 20). Also, the importance of wounds and skin as the colonization sites for biofilm producing *S. aureus* has been demonstrated in several other studies. Yazdani *et al.* from Isfahan reported biofilm formation frequency of 54% among *S. aureus* isolates recovered from wounds (16). Previously, Taj *et al.* In Pakistan showed biofilm formation with a high frequency among the isolates obtained from catheters (57.9%) and wound (42.1%) samples (21). In a hospital survey from Scotland, *S. aureus* isolates that were originated from the skin had a significantly greater capacity than the isolates from the other body sites for biofilm production (22). The undeniable role of the *ica* operon in staphylococcal biofilm formation through the regulation and production of PIA has already been shown (1, 4). Our results indicated that the presence of *ica*A and *ica*D genes in all positive isolates were associated with each other, it was in agreement with some other studies (9,23). The frequency of *ica*A/D genes presence among our MRSA isolates was higher than MSSA isolates (87 vs. 83.9%); however, the difference was not statistically significant. These high rates of *ica*A/D genes detection among *S. aureus* isolates are not uncommon, since in some studies detection rates up to 100% were reported (16, 23, 24). Eftekhar *et al.* from Iran and Diemond-Hernández *et al.* from Mexico separately showed that approximately 80% of *S. aureus* isolates carried the *ica* operon (5, 6). Despite the important role of *ica*A/D genes, it has been shown that biofilm formation has not always been associated with these genes. In fact, some alternative mechanisms, independent of PIA were involved for biofilm formation in staphylococcus (5, 7, 22). Anyhow, in our finding, similar to several other studies, there was no association between phenotypic and genotypic method for detection of biofilm formation *in vitro* (7, 16, 23). While the majority of our isolates carried *ica*A/D genes, phenotypic biofilm formation was not detected in all of the isolates which may be attributed to the reliability of the phenotypic tests (14), suggesting the need for establishment of a good *in vivo* model associate infections with the biofilm formation (8).

Conclusion

The presence of high rate of *icaA/D* genes among clinical *S. aureus* isolates in the present study, suggest their potential ability for establishing a persistent infection in hospitalized patients and the necessity of paying attention to infection control policies. In addition, our findings provide useful background for epidemiological studies and future attempts to find a comprehensive solution for eradication of biofilm producing *S. aureus* for reducing their risk of serious nosocomial infections.

Conflict of interest

None declared conflicts of interest.

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