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Original Article

Detection of *icaABCD* Genes and Biofilm Formation in Clinical Isolates of Methicillin Resistant *Staphylococcus aureus*

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ABSTRACT

Background & Objectives: **Methicillin resistance *Staphylococcus aureus* (MRSA) is one of the most important pathogens that causes several nosocomial and community infections. Adhesion to surfaces and biofilm formation is considered main step in staphylococcal infection. The aims of this study were to determine presence of *icaABCD* genes and relation to the biofilm formation in of MRSA isolates.**

Methods: **Of the 63 MRSA clinical isolates collected from selected hospitals in Tehran, Iran,quantitative biofilm formation was determined by microtiter tissue culture plates (Mtp). All MRSA isolates were examined for determination the *icaABCD* genes by using PCR method.**

Results: twenty nine (46%) of the isolates were strong produced biofilm on Mtp. All of the MRSA isolates carried *icaD* and *icaC* genes, whereas, the prevalence of *icaA* and *icaB* was 60.3% and 51% respectively.

***Conclusions: S. aureus* clinical isolates have different capacity to production biofilm and adhesion. This may be caused by a different in the expression of biofilm genes and hetrogenicity in genetic origins.**

***Keywords: Staphylococcus aureus*, Biofilm, *icaABCD*, MRSA**

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Introduction

Staphylococcus aureus is one of the major causes of nosocomial infections; especially pneumonia, surgical site infections and blood stream infections continue to be a major cause of community-acquired infections. The development of high levels of penicillin resistance followed by the spread of strains resistant to the semisynthetic derivative of penicillin (e.g. methicillin) and other antibiotics has made the therapy of staphylococcal infection international challenge (1). Finding methicillin resistant *Staphylococcus aureus* (MRSA) strains; just two years after the clinical use of penicillinase-resistant penicillin, put specialists into difficulty with treating these infections (2). Biofilms pose a serious problem for public health because of the increased resistance of biofilm-associated bacteria to antibiotics and host defenses.

Biofilm formation is affected by several factors such as, environmental condition (osmolality, nutrition, implicated device). One of the most important factors was ability to synthesis of the polysaccharide intercellular adhesion (PIA) by this organism (3). The intracellular adhesion (*ica*) locus consist of the four genes *icaABCD*, these genes encodes proteins mediating the synthesis of the PIA (4). The *icaA* gene is encoding the N-acetylglucosamyltransferase. This enzyme is not very active *in vitro*, but co-expression of the *icaD* gene due to increases the activity. *icaB* is the deacetylase responsible for the de-acetylation of mature PIA and the transmembrane protein. *icaC* encodes the transmembrane protein, which is hypothetically involved in to secretion and elongation of the growing polysaccharide (5). Expression of this operon increased under anaerobic internal environment of biofilm, other conditions for the expression of these genes are including glucose, ethanol, osmolarity, temperature, and antibiotics such as tetracycline (6).

Biofilm formation organisms are essentially different from populations of planktonic cells. Bacteria within biofilm show increased resistance to antibiotics. Additionally, the importance of biofilm is well recognized in medicine (7). A number of methods have been created for cultivation and quantification of biofilm formation ability. Currently several different methods are used, such as tube test, microtiter plate test, radiolabeling, microscopy, tube method and Congo red agar (CRA) plate test (8, 9). However, the microtiter plate method remains among the most frequently used assays for investigation of biofilm, and a number of modifications have been expanded for the in vitro cultivation and quantification of bacterial biofilms formation ability. Additionally, in several studies, the Mtp is a quantitative and reliable method to detect biofilm forming bacteria. Compared to tube and CRA methods, it can be recommended as a general screening method for detection of biofilm producing bacteria in laboratories (10, 11).

The present study was carried out to determine the biofilm producing ability by quantitative microtitre plate assay as well as the presence of *icaABCD* operon genes in clinical isolates of methicillin resistance *S.aureus* as well as correlation between biofilm formation and presence of this operon.

Material and Methods

Bacterial Isolates

A total 63 isolates of MRSA were chosen from a collection of *S. aureus* isolates during six months (Aug 2012 to March 2013), from two university hospitals (Loghman and Children Medical Center) of Tehran. All isolates were identified by conventional bacteriological tests. The bacterial isolates were kept frozen at -70ºC before tested.

Biofilm Formation Assay

A modified microtiter plate method was followed as previously described (7). Briefly, the wells of microtiter plate were filled with 180 µl of trypticase soy broth (TSB) supplemented with 1% glucose. Then, a 20 µl quantity of previously prepared

bacterial suspensions with turbidity equal to 0.5 Macfarland standards was added to each well. The negative control wells contained 200 µl of TSB supplemented with 1% glucose. Incubation was carried out at 37°C for 24 h before removal of the cultures. Then, the cells were decanted, and each well was washed 3-times with sterile phosphate buffered saline, fixed by methanol for 20 min, dried at room temperature and finally strained with 0.1% saferanin. The safranin dye bound to the adherent cells was dissolved with 1mL of 95% ethanol per well, and the plates were read at 490nm (A_{490}) using ELISA reader. Optical density cut-off (ODc) was determined. It is defined as average OD of negative control + 3× standard deviation (SD) of negative control. Formation of biofilm by isolates was analyzed and categorized relying on the absorbance of the safranin-stained attached cells (Table 1) (12). . Biofilm-producing *S. aureus* ATCC 35556 strain was used for strongly biofilm producing control, while *S. epidermidis* ATCC 12228 strains were used asthe negative control.

Table 1-Classification of biofilm formation abilities by Mtp method

Cut-off value calculation	Mean of OD values results	Biofilm formation abilities
OD > 4×ODc	OD > 0.33296	Strong
2×ODc < OD ≤4×ODc	0.16648 < OD ≤0.33296	Moderate
ODc< OD ≤ 2×ODc	0.08324 < OD ≤ 0.16648	Weak
OD ≤ 0.08324	OD ≤ 0.08324	None

Genomic DNA Extraction

For extraction of genomic DNA of *S. aureus* isolates several colonies of each isolate were suspended in 20 µl of a lysis buffer (0.25% SDS, 0.05 N NaOH) and heated at 95°C for 7 min, cooled on ice, centrifuged briefly at 16000 g for 2 min and diluted by adding 180 µl of distilled water. Therefore, a centrifugation for 5 min at 16000 g was performed to remove cell debris. The supernatant was used as the source of template for DNA amplification.

PCR

Methicillin resistance isolates were selected for molecular screening for *icaABCD* genes using PCR.

***icaA* gene detection**

PCR amplification was performed with an Eppendorf thermal cycler (Mastercycler® gradient). Amplification program for *icaA* consisted of initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 60 sec, annealing at 55 °C for 60 sec and extension at 72 °C for 60 sec with a final step of 72 °C for 10 min. The PCR products were analyzed by electrophoresis in a 1.4% agarose gel and stained with gel red (13).

***icaB*gene detection**

Amplification program for *icaB* consisted of initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 60 sec, annealing at 52 °C for 30 sec and extension at 72 °C for 90 sec with a final step of 72 °C for 10 min (14).

***icaC*gene detection**

Amplification program for *icaC* consisted of initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 60 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 30 sec with a final step of 72 °C for 10 min (14).

***icaD*gene detection**

Amplification program for *icaD* consisted of initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 sec, annealing at

55 °C for 30 sec and extension at 72 °C for 60 sec with a final step of 72 °C for 10 min. The primers and sizes of the expected amplification product for PCR amplification are listed in Table 2(13).

Table 2- List of primers used in this study

Target (s)	Primer	Sequence	Product Size (bp)	Reference
icaA	F	5-ACACTTGCTGGCGCAGTCAA-3	188	(8)
	R	5-TCTGGAACCAACATCCAACA-3		
icaB	F	5-AGAATCGTGAAGTATAGAAAATT-3	900	(9)
	R	5-TCTAATCTTTTTCATGGAATCCGT-3		
icaC	F	5-ATGGGACGGATTCCATGAAAAAGA-3	1100	(8)
	R	5-TAATAAGCATTAATGTTCAATT-3		
icaD	F	5-ATGGTCAAGCCCAGACAGAG-3	198	(9)
	R	5-AGTATTTTCAATGTTTAAAGCAA-3		

Results

Clinical Bacterial Strains

Of the 63 MRSA clinical isolates included in this study, 40 (63.4%)were isolated from trachea, 9 (14.2%) from wounds, 4 (6.5%) from blood culture, 3 (4.8%) from urine culture, 2 (3.25%) from bronchoalveolar lavage, 2 (3.25%) from drainage and other specimen with frequency equal to oneisolated from sputum, catheter, eye and biopsy.

Microtiter Tissue Culture Plates

The microtiter plates assay results showed that all MRSA strains tested were attached at different amount (Table 3). Attachment abilities in 29 (46%) strains were strong, 23 (36.5%) strains were moderate, 11 (17.5%) strains were weak and none of them had no attachment.

Table 3- Biofilm formation by microtiter tissue culture plates method

Method	Adhesion formation	MRSA (n=60)	Percentage (%)
Microtiter tissue culture plates	Strong	29	46
	Moderate	23	36.5
	Weak	11	17.5
	None	0	0

Identification of *icaABCD* genes

All the primers used in the study showed specificity with a single band. The 4 biofilm related genes were identified in MRSA strains by PCR method. The prevalence of *icaA*, *icaB*, *icaC* and *icaD* in MRSA strains was 60.3%, 34.2%, 100% and 100% respectively. In 20 (31.7%) MRSA strains all *ica* genes were positive.In these strains 11 (55%) strains were strongly, 8 (40%) strains were moderate and only 1 (5%) strain was weakly biofilm formation. No relation was observed between source of bacteria such as wards and type of specimen, and potential of biofilm formation in MRSA strains. On the other hand, correlation between presence of *ica* genes in MRSA strains and biofilm formation was observed.

Discussion

The attachment and biofilm formation on abiotic surfaces such as, catheters and implanted devices is one of the major virulence factors in *S. aureus*. Biofilm causes bacterial resistance to inappropriate conditions such as, antibiotics, stress, host phagocytosis and immune response (resistance to oxygen radicals and proteases) (15, 16). The study of adhesion and biofilm formation and genetics characteristics of biofilm genes in dif-

ferent isolates of *S. aureus* may allow a better understanding of the complex process of biofilm formation and infections caused by this microorganism (6).

In this study, microtiter tissue culture plates were selected for assay biofilm formation and quantify attachment. Yet, presence and expression of biofilm genes should be confirmed by genotypic characterization methods. There are several reports concerning prevalence rate of *ica* genes in MRSA strains from different Countries (17-19). In the present study, we found that 29 (46%) of MRSA strains were capable of producing strongly biofilm formation.

Our findings were similar to the observation by Atshan *et al.* who analyzed a 60 different clone of clinical MSSA and MRSA at selective hospital in Putra Malaysia (20). Many reports have been published on correlation between phenotypic methods such as microtiter plates and presence of *ica* genes in clinical isolates in different parts of the world (12, 17, 20).

The present study indicates a high prevalence of *ica* genes among MRSA isolates, but the presence of the biofilm related genes did not always correlate with biofilm production. Thus, the difference between phenotypic and genotypic characterization may be due to the heterogeneity in the genetic origins, and not because of the presence or absence of genes required for the biofilm formation. In our study, all of the MRSA isolates harbored *icaD* and *icaC* genes.

By contrast, Yazdani *et al.* observed that *icaA* was present in all *S.aureus* strains studied (21). These results indicated the high prevalence of *icaDC* genes among MRSA strains; however, the presence of *icaDC* genes was not always associated with in vitro biofilm formation (21). These data indicated biofilm formation regulated by several factors such as, environmental condition.

Conclusion

These results suggest that biofilm formation in vitro presses is very complex and independent to onlypresence of *ica* genes harbor in MRSA strains. Thus, genetics methods for detection of genes involved in biofilm formation is not appropriate methods for the actual biofilm phenotype under *in vitro* conditions.

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