



Detection of Intracellular Adhesion (*ica*) Gene and Biofilm Formation *Staphylococcus aureus* Isolates from Clinical Blood Cultures

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ARTICLE INFO

Article type:
Original Article

Article history:

Received: 13 Feb 2014

Revised: 03 Mar 2014

Accepted: 11 Mar 2014

Keywords:

Staphylococcus aureus
Biofilm
ica locus

ABSTRACT

Background: In fact the biofilms are composed of bacterial cells living in multicellular structures such as tissues and organs embedded within a self-produced matrix of extracellular polymeric substance (EPS). Ability to attach and biofilm formation are the most important virulence factors *Staphylococcus aureus* isolates. The aims of this study were to detect intracellular adhesion (*ica*) locus and its relation to the biofilm formation phenotype in clinical isolates of *S. aureus* isolated from blood cultures.

Methods: A total of 31 clinical *S. aureus* isolates were collected from Loghman Hospital of Tehran, Iran. In vitro biofilm formation ability was determined by microliter tissue culture plates. All clinical isolates were examined for determination the *ica* locus by using PCR method.

Results: Twelve (38.7%) of the isolates were strong biofilm producers. The results showed that 18(80.6%) of the isolates carried *icaD* gene, whereas the prevalence of *icaA*, *icaB* and *icaC* were 51.6%, 45.1% and 77.4% respectively.

Conclusions: *S. aureus* clinical isolates have different ability to form biofilm. This may be caused by the differences in the expression of biofilm related genes, genetic make-up and physiological conditions.

- **Please cite this paper as:** Mirzaee M, Najar-Peerayeh SH, Behmanesh M, Forouzandeh Moghadam M, Ghasemian AM. Detection of intracellular adhesion (*ica*) gene and biofilm formation *Staphylococcus aureus* isolates from clinical blood cultures. *J Med Bacteriol.* 2014; **3**(1, 2): pp.1-7.

Introduction

Staphylococcus aureus is frequently implicated as the causative organism of acute and chronic infections contributing significantly to patient mortality, as well as enhanced health care costs associated with treatment (1). Several studies have indicated that many of human infections are caused by the ability of bacteria to develop biofilms. Biofilms are the population of bacteria growing on the different surfaces and enclosed within a self-produced extracellular matrix exopolysaccharide (EPS), several proteins and DNA (2). Biofilm formation requires the adhesion of bacteria to a surface followed by cell-cell adhesion, forming the several layers of the biofilm (2, 3). The intercellular adhesion (*ica*) locus, *icaABCD*, was identified and shown to mediate cell-cell adhesion and polysaccharide intercellular adhesin (PIA) production (4). The *icaA* gene is encoding the N-acetylglucosamyltransferase. Co-expression of the *icaD* gene with this gene increases the activity of this enzyme. The *icaB* is the deacetylase responsible for the de-acetylation of PIA. The *icaC* encodes the transmembrane protein, which hypothetically plays a role in secretion and elongation of the growing extracellular polysaccharide (5). Several studies have shown that isolates producing PIA are able to form thick biofilms on polystyrene microtiter plates (6). Therefore, there is a necessity to evaluate a simple valid phenotypic method for detection of biofilm formation ability (7). Not all *ica*-positive isolates of *S. aureus* produce biofilms. This has been explained by spontaneous insertion of IS 256 in the *ica* operon and by strict regulation of the *ica* genes (8). Primary detection of biofilm formation ability in *S. aureus* infections can be one of the essential steps in prevention and management of device-associated nosocomial infections (9). The aim of the this study was to determine the biofilm formation ability and the presence of the *icaABCD* gene in *S.*

aureus isolated from different specimens collected from hospitalized children.

Material and method

Bacterial Isolates

During six months August 2012 to March 2013, a total 31 isolates of *S. aureus* were collected from blood samples of hospitalized patients with different clinical infections, in Lohman hospital of Tehran. All isolates were identified by conventional bacteriological tests. The bacterial isolates were kept frozen at -70° C before tested.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed as recommended by the Clinical Laboratory Standards Institute (CLSI) using disk diffusion method. Antimicrobial disks (Mast.UK) tested included oxacilin (1 µg), gentamycin (10 µg), amikacin (30 µg), ciprofloxacin (5 µg), tetracycline (30 µg), Co-trimoxazol (1.25 + 23.75 µg), erythromycin (15 µg), rifampin (5 µg), and clindamycin (2 µg) and linezolid (30µg). *Staphylococcus aureus* ATCC 25923 was used as a control strain (10).

Microtiter plate (MTP) method

This method was followed as previously described. Briefly, the wells of microtiter plate were filled with 180 µl trypticase soy broth (TSB) supplemented with 1% glucose. 20 µl of the bacterial culture with turbidity equal to 0.5 McFarland standards was added to individual wells of sterile, polystyrene, 96-well, flat-bottomed tissue culture plates. After 24 h incubation at 35°C, the cells were decanted and each well was washed three times with sterile phosphate buffered saline. Biofilms formed by adherent fixed by methanol for 20 min, then 150 µl of safranin (0.1%) was added to each well. After 15 min, the excess stain was rinsed off by decantation and the plate was washed and left to dry. The safranin dye bound to the adherent cells

was dissolved with 1mL of 95% ethanol per well, and the optical densities (OD) of plates were read at 490nm (A490) by using a microtiter-plate reader. Each assay was performed in triplicate. As a negative control, TSB medium was used to determine background OD. Optical density cut-off (ODc) was determined. It was determined as average OD of negative control + 3× standard deviation (SD) of negative control. ODc value was calculated for each microtiter plate separately. When a negative value was obtained, it was presented as zero, while any positive value indicated biofilm production (6). For interpretation of the results, strains were divided into the different categories according to table 1.

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 assay

All isolates were evaluated for molecular screening of *icaABCD* genes using PCR method. PCR amplification was performed with an Eppendorf thermal cycler (Master cycler® gradient). Amplification program for *icaA*, *icaC* and *icaD* 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. 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 (11). The PCR products were analyzed by electrophoresis in a 1.5% agarose gel and stained

with gel red. The primers and sizes of the expected amplification product for PCR amplification are listed in table 3.

Result

Antibiotic susceptibility testing

The disk diffusion test using 11 antibiotics for 31 clinical isolates of *S. aureus* isolated from blood culture was shown in table 3. All the isolates were susceptible to vancomycin, and linezolid in the disk diffusion test. The highest resistance of isolates were observed for amoxicillin²³ (74.2%), followed by oxacilin, erythromycin and tetracyclin 14 (54.8%), ciprofloxacin 11 (35.6%) clindamycin 10 (32.3%), gentamycin 9 (29.1%), co-trimoxazole (16.1%) and rifampin 6 (19.4%).

Table 1. Classification of biofilm formation abilities by MTP method

Cut-off value calculation	Biofilm formation abilities
$OD > 4 \times ODc$	Strong
$2 \times ODc < OD \leq 4 \times ODc$	Moderate
$ODc < OD \leq 2 \times ODc$	Weak
$OD \leq ODc$	None

Microtiter tissue culture plates

The microtiter plates assay results showed that all *S. aureus* isolates tested were attached at different amount. Attachment abilities in 12 (38.7%) strains were strong, 11 (35.5%) strains were moderate, 8 (25.8%) strains were weak and none of them had any attachment.

Table 2. List of primers used in this study

gene	Primer designation	Sequences	Product size	Ref.
<i>icaA</i>	<i>icaA</i> -F	5'-ACACTTGCTGGCGCAGTCAA -3'	188bp	10
	<i>icaA</i> -R	5'-TCTGGAACCAACATCCAACA -3'		
<i>icaB</i>	<i>icaB</i> -F	5'- TCCTTATGGCTTGATGAATGACG -3'	190 bp	This work
	<i>icaB</i> -R	5'- CTAATCTTTTTTCATGGAATCCGTCC -3'		
<i>icaC</i>	<i>icaC</i> -F	5'- ATGGGTTATAACTACGAACGTG -3'	192bp	This work
	<i>icaC</i> -R	5'- CGTGCAAATACCCAAGATAAC -3'		
<i>icaD</i>	<i>icaD</i> -F	5'- ATGGTCAAGCCCAGACAGAG -3'	198bp	10
	<i>icaD</i> -R	5'- AGTATTTTCAATGTTTAAAGCAA -3'		

Identification of *ica* ABCD genes

All the primers used in the study showed specificity with a single band. The four *ica* genes were identified in *S. aureus* isolates by PCR method. The prevalence of *icaA*, *icaB*, *icaC* and *icaD* in *S. aureus* isolates was 51.6%, 45.1%, 77.4% and 80.6% respectively. In 12 (38.7%) of our isolates all *ica* genes were positive. In these isolates 8 (66.7%) strains were strongly, 3 (25%) strains were moderate and only 1 (8.3%) strain was weakly biofilm formation. No correlation was observed between infection types and source of infection.

Discussion

Biofilm producing bacteria cause a broad spectrum of human infections. Bacterial biofilm can be a critical health problem for patients need to use catheterization (12). Biofilm causes bacterial resistance to unsuitable conditions such as encounter to antibiotics and host immune response and having the suitable condition for growing slowly. *S. aureus* one of the most important pathogen that cause various infections and can be resistant to several antibiotics (13, 14). Additionally, biofilm producer *S. aureus* isolates that make them resistance to antibiotic agents are capable of causing chronic infections (2).

Table 3.Rate of Resistance to Various Antibiotics by the Disk Diffusion Method

Antibiotics	Susceptible N (%)	Resistant N (%)
Oxacillin	17 (54.8)	14 (45.2)
Ciprofloxacin	20 (64.5)	11 (35.6)
Erythromycin	17 (54.8)	14(45.2)
Tetracyclin	17 (54.8)	14(45.2)
Amoxicillin	8 (25.8)	23(74.2)
Gentamycin	22 (70.9)	9 (29.1)
Clindamycin	21 (67.7)	10(32.3)
Co-trimoxazole	26 (83.9)	7(16.1)
Rifampin	25 (80.6)	6(19.4)
Linezolid	31(100)	0(0)

In present study, microtiter tissue culture plate was selected for assessing biofilm formation ability of *S. aureus*. There are several methods such as, Congo red agar plate test, tube method and microtiter plate test used to evaluate the ability of bacteria for biofilm formation (15). The MTP assay is the most widely used and has been considered as a standard test for detection of biofilm formation (6). On the other hand, biofilm formation ability of the bacteria is very complex and dependent to several factors such as environmental condition, genetic background and different regulation systems. In our study, the result of microtitre plate assay showed that all of *S. aureus* isolates have the ability of biofilm formation, and 38.7% of them were able to form biofilm strongly. In the study of Mathur *et al* 57.8% of staphylococcal clinical isolates displayed a biofilm-positive phenotype and 14.47% exhibited high biofilm formation (16). A higher rate of biofilm formation was reported by Gad *et al* where 83.3% of *S. aureus* isolated from urinary tract catheterized patients produced biofilm by the MTP assay (17). This difference between various studies might be due to heterogeneity in the origins of bacteria such as genetics characterization, source of isolation and environmental condition.

Several studies have shown that biofilm formation ability in *S. aureus* isolates is associated with the presence of *ica ABCD* genes. All of the *ica ABCD* genes were detected in 12 (38.7%) *S. aureus* isolates in the present study. Similar to our study, several studies have shown that the *ica* genes were detected in all *S. aureus* isolates (18, 19). Interestingly, one of the *S. aureus* included in our study was negative for all of *ica* genes but still produced biofilm as shown by MTP method, suggesting that the difference between the phenotypic and the genotypic characterization of the strain may be explained by an alternative PIA-independent mechanism for biofilm formation in this isolate. On the other hand, inability of biofilm formation in some staphylococcal strains, despite the presence of *ica* genes can be caused by insertion of a 1332-bp insertion element (IS256), in *icaA* gene and causing its inactivation (20). Several reports have been conducted about antimicrobial resistance pattern of *S. aureus* clinical isolates. The high level of resistance to amoxicillin, erythromycin, tetracycline, oxacillin and ciprofloxacin were observed in the current study indicates that antimicrobial options for *S. aureus* strains are limited. Rifampin and gentamycin displayed antibacterial effects; linezolid was the most effective antibiotic against all strains. Comparable results have also been shown previously (21, 22).

Conclusion

All of the *S. aureus* isolates included in this study were capable of forming biofilm; however, the presence of *icaABCD* genes was not always associated with in vitro formation of biofilm. Additionally, the biofilm formation ability of one isolate in the absence of *icaABCD* genes highlights the significance of further genetic researches of *ica* independent biofilm formation mechanisms.

Acknowledgment

This study was supported by the Tarbiat Modares University and conducted in the Department of Bacteriology, Faculty of Medical Sciences as part of Ph.D thesis.

Conflict of interest

None declared conflicts of interest.

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