Prevalence of IS256 among Ica-Positive and Biofilm Non-Producing Staphylococcus epidermidis Clinical Isolates

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**ABSTRACT**

**Background:** S. epidermidis is one of predominant members of human normal microflora, however it may be the main cause of nosocomial infections related to medical devices put into the body and thus the biofilm formation is a main route for pathogenesis which is affected by icaADBC operon. In this study, the prevalence of IS256 sequence among ica-positive and biofilm non-producer clinical isolates of S. epidermidis was investigated.

**Methods:** In this study, 100 clinical isolates of S. epidermidis were collected from different infections. The IS256 sequence, icaADBC operon and biofilm formation by microtiter plate assay were evaluated among them. The antibiotic susceptibility of these isolates was done with disc diffusion by using cefoxitin, ciprofloxacin, erythromycin, gentamycin, oxacillin and tetracycline discs.

**Results:** Of 100 isolates, 18 (18%) were ica operon-positive from which 18%, 14%, 16% and 17% contained icaA, icaD, icaB and icaC genes, respectively. Moreover, 14 of 18 (77.77%) ica-positive isolates amplified the IS256 gene. The biofilm formation by microtiter plate assay showed that 18 (18%) isolates were strong biofilm producers, 21 (21%) produced intermediate level biofilm and 14 (14%) and 47 (47%) isolates were weak and non-biofilm producers, respectively. In the antibiotic susceptibility test, the majority of isolates were resistant to oxacillin and lowest resistance was against ciprofloxacin.

**Conclusion:** The statistical analysis with p<0.05 exhibited that there was a reverse relation between biofilm production and the insertion of IS256, and in fact the higher prevalence of IS256 among isolates, the biofilm formation declined. Data showed that amongst most of ica-positive isolates, the IS256 was detected and therefore other genetic factors affect the expression of this operon.


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Introduction

Staphylococcus epidermidis is the most important member of coagulase negative staphylococci and the most prevalent species on the human skin and mucosa. The bacterium causes the infection through intravenous catheters and graft prostheses into the cardiac valves and joints. Furthermore, it is the predominant agent of nosocomial infections, especially with clinical devices put into the body. Biofilm formation is one of effective agents in the pathogenesis of this bacterium. Biofilm formation causes the resistance of S. epidermidis to the antibiotics and body defense system which play a key role in developing of nosocomial infections. This bacterium progresses the nosocomial infections by biofilm formation on the surface of intravenous and urinary tract catheters and artificial prostheses. Biofilms are structures formed by multiple bacterial species which are produced by them and surrounded in it.

Biofilm formation is initiated when bacterial cells are attached to a surface and secrete a thin layer of glue-like sticky materials between cells named polysaccharide intercellular adhesion (PIA) which is produced by icaADBC operon. The ica operon includes icaA, icaB, icaC and icaD genes. This polysaccharide is a polymer from glycosaminoglycan units with beta 1-6 bounds and a smaller part of non-acetylated glycosaminyl containing phosphate and succinate esters which is synthesized by N-acetyl glucose amyl transferase. The icaA and icaD genes encode N-acetyl glucosamine transferase and icaC is responsible for long chains while icaB deacetylates the poly-N-acetyl glucosamine.

The ica operon can be regulated by IS256 insertion sequence. IS256 is responsible for genetic rearrangement in S. epidermidis as its host which results in phenotypic alterations. Biofilm formation as a great factor for pathogenesis is affected by IS256. IS256 insertion leads to the change in phase of biofilm genes expression mainly by inhibition of icaADBC operon which encodes enzymes responsible for biofilm biosynthesis. Recently, the role of ica operon in biofilm formation and the effect of IS256 on the expression of this operon has been interested for research. The aim this study was assessment of prevalence of IS256 sequence and the relation between the presence of the ica operon and IS256 as the regulator of this operon and inhibition of biofilm formation.

Material and method

Bacterial isolates

A total of One-hundred isolates of S. epidermidis were collected during one year from two hospitals of Boroujerd (East of Iran). One-hundred isolates of S. epidermidis were collected from hospitalize patients. All the isolates were identified with diagnostic tests including gram-staining, catalase, coagulase, DNase, mannitol fermentation, and novobiocin and polymixin B susceptibility.

Antibiotic susceptibility testing

The antibiotic susceptibility of isolates was performed by using 6 antibiotics (Rosco, Denmark) oxacillin (10 μg), tetracycline (30 μg), ciprofloxacin (5 μg), erythromycin (15 μg), gentamicin (10 μg) and cefoxitin (30 μg) according to Kirby Bauer and Clinical and laboratory Standards Institute (CLSI) guidelines. Briefly, a half McFarland of each bacterium was prepared and lawn on Mueller Hinton Agar (MHA) with sterile swab and then the disks were put on the medium. After 18-24 h incubation, the zone of no-growth for each disk was measured.

Biofilm formation

Ability of biofilm production by isolates was done by microtiter plate assay (MTP). 180 microlitre of trypticase soy broth with 1% of each glucose and NaCl was added into microtiter plate wells. Next, 20 ul of bacterial suspension equal to half MacFarland in sterile saline was added in each well (triple test was considered for each isolate).
The plate was placed at 37°C for 48 h. After incubation, the wells were washed by saline phosphate buffer three times for washing those isolates without attaching. Next, bacterial biofilms were fixed with 150 ul of methanol for 20 min. Then the methanol was discarded and the wells were stained with 1% safranin for 15 min and washed completely with water and dried. By addition of 150 ul of 95% ethanol for 30 min, the stains were solubilized from bacteria and the OD of them was read in 490 nm using ELISA reader. The ability for biofilm formation was measured based on absorbance of safranin attached to cells in biofilm and the cut-off was detected as depicted in table 1.

**PCR detection of icaADBC and IS256 genes**

For DNA extraction from *S. epidermidis* isolates, some colonies of each bacteria was prepared in 20 ul of lysis buffer (0.25% SDS and 0.05% NaOH) and placed in 95°C for 7 min. Then the suspension was centrifuged at 16000 g for 2 min, and next 180 ul of sterile water was added and centrifuged 2 times for 5 min and the supernatant was used as template DNA.

IS256 and icaABCD genes detection was performed as described previously (12-15). The PCR was done in 25 ul total volume and by application of Biorad Thermal-cycler. Two microliter of DNA template, 12.5 ul Red Master Mix, and 1 ul of each primers and 8.5 ul ddH2O were mixed and reached to 25 ul.

After PCR, electrophoresis was done in 1.5% agarose gel for 40 min and 80 V. The 100 bp DNA ladder was used.

**Results**

The antibiotic susceptibility test of 100 clinical isolates of *S. epidermidis* showed that 71 (71%) were resistant to cefoxitin, 31 (31%) to ciprofloxacin, 62 (62%) to erythromycin, 32 (32%) to gentamicin, 50 (50%) to tetracycline and 81 (81%) to oxacillin, and the rate of susceptibility, resistance and intermediate level has been exhibited in figure1. Furthermore, the highest rate of resistance was against oxacillin and the lowest was to ciprofloxacin.

The results of phenotypic biofilm formation by microplate (MTP) assay showed that 47 (47%) isolates did not produced biofilm, while 18 (18%) isolates produced biofilm strongly, 21 (21%) isolates produced intermediate level and 14 (14%) produced weak biofilm.

The statistical analysis with p value <0.05 showed a reverse relationship between IS256 presence and reduced biofilm formation.

Results of PCR showed that 76 (76%) isolates carried IS256 and the prevalence of icaA, icaB, icaC and icaD was 18 (18%), 16 (16%), 17 (17%) and 14 (14%), respectively. On the other hand, prevalence of icaADBC operon was 13%, icaA operon was 1%, icaABC was 3% and icaD was 1%.

Among 100 isolates, 14 (14%) contained IS256 and icaADBC together, 4 (4%) had not IS256, but had ica genes, 34 (34%) contained IS256 and produced biofilm and 13 (13%) were ica negative and produced biofilm. The figure 2 shows the IS256 PCR product for several isolates.

**Discussion**

During recent years, *S. epidermidis* has been known as a major nosocomial pathogen which attaches to catheters and external body devices by biofilm production. Mechanisms for sustained biofilm formation are multiple and may be different for antibiotics or body defense system. On the other, several studies have shown that there is no correlation between presence of ica operon and biofilm formation in clinical isolates of *S. epidermidis*. Results of biofilm showed that 18 isolates among 18 ica positive isolates, approximately 60% were biofilm producers. Data analysis p value<0.05 exhibited that there was a direct relationship between ica operon and biofilm formation, meaning ica operon expression in most of isolates led to biofilm production. Of 100 isolates, 76 (76%) showed the amplified IS256 gene. Among 18 ica-positive isolates, 14 (77.7%) were IS256
positive. In 24% of isolates, the IS256 was not detected and of 24 IS256-negative isolates, 7 isolates produced no biofilm, 3, 9 and 4 of them produced weak, intermediate and strong biofilms, respectively. Analysis of correlation between biofilm formation and IS256 showed that there is a converse relationship between them, and with higher IS256 prevalence, biofilm formation reduced. A study by Koskeles showed that 16/32 (50%) of S. epidermidis isolates from artificial cartilage infections carried ica operon and 26 (81%) of them had IS256, which only 1 isolate contained IS256 despite commensal isolates. In another study by Arciola, of 120 S. epidermidis clinical isolates, 51 (43%) were ica-positive and 69 (57%) were negative, and the rate of IS256 among ica-positive and ica-negative isolates was 8/69 and 34/51, respectively. Liduma findings exhibited that icaA and aap have important role in biofilm formation and the presence of these 2 genes is not sufficient, because of presence of biofilm negative isolates with icaA+/aap+ genotype, suggesting that various factors have the role for the phenotypic and genotypic changes. The expression of icaADBC operon is a variable factor which is affected by regulatory and genetic mechanisms such as phase variation and genetic rearrangements. One of effective factors on the expression of adhesive intercellular polysaccharide and therefore biofilm formation is the IS256. The icaC gene has a site for IS256 insertion which this leads to the inactivation of biofilm expression in polymer dependent infections due to S. epidermidis in bacterial biologic conditions. The phase variation is also influencing on biofilm formation which is done by substitution of IS256 that has inhibitory effect on biofilm formation and it was confirmed in this study. The data showed that IS256 role is control of virulence factors in bacterial pathogens. The results demonstrated that IS256 is considerably active in S. epidermidis and regulates the icaADBC operon. IS256 plays a role in S. epidermidis genome which is also effective on biofilm formation and expression of aminoglycoside resistance genes. In this study, the resistance of isolates with IS256 was assessed and the results depicted that among 76 isolates containing IS256, 46 isolates had resistance to cefoxitin, 20 isolates to ciprofloxacin, 39 isolates to erythromycin, 18 isolates to gentamicin, 40 isolates to tetracycline and 57 isolates to oxacillin. A high rate of isolates was resistant to cefoxitin, erythromycin and oxacillin. In the present study, 23.6% of IS256-positive isolates were resistant to gentamicin which, therefore, can be used for detection of resistant strains and for proper treatment.

### Table 1. Biofilm formation ability by microtiter plate assay.

<table>
<thead>
<tr>
<th>Biofilm formation ability</th>
<th>Cut-off value detection</th>
<th>OD results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strong</td>
<td>OD &gt; 4×ODc&lt;sup&gt;2&lt;/sup&gt;</td>
<td>OD &gt; 0.30152</td>
</tr>
<tr>
<td>Moderate</td>
<td>2×ODc &lt; OD ≤ 4×ODc</td>
<td>0.15076 &lt; OD ≤ 0.30152</td>
</tr>
<tr>
<td>Weak</td>
<td>ODc &lt; OD ≤ 2×ODc</td>
<td>0.07538 &lt; OD ≤ 0.15076</td>
</tr>
<tr>
<td>No attachment</td>
<td>OD &lt; ODc</td>
<td>OD &lt; 0.07538</td>
</tr>
</tbody>
</table>

<sup>1</sup>Optical density  
<sup>2</sup>ODc = average of OD negative control + (3×SD of negative control)
**Figure 1.** The rate of susceptibility, resistance and intermediate level in this study.

**Figure 2.** Electrophoresis of *IS256* gene product among *S. epidermidis* isolates. Well 1: ladder, well 2: *IS256* negative isolate, well 3: control positive, wells 4-14: isolates positive for *IS256* gene with 1103 bp, well 15: negative control.
Conclusion

In this study, the prevalence of IS256 was detected among \textit{S. epidermidis} isolates and also the relation between \textit{ica} operon and IS256 inhibitory factor was uncovered. Considering that IS256 separation from \textit{ica} operon causes the return of biofilm formation and enhance of resistance, the assessment of the operon among positive isolates which do not produce biofilms is helpful for better antibiotic treatment of patients and during the research in this field.

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Ethical Considerations

Our research proposal was approved by The Ethics and Research Committee of Islamic Azad University, Boroujerd Branch (proposal number: 432440).

Conflict of interest

The authors declare no conflicts of interest.

Financial disclosure

None declared.

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