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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 aminyl transferase. The *icaA* and *icaD* genes encode N-acetyl glucosamine transferase and *icaC* is responsible for long chains while *icaB* de-acetylates 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 (10ug), tetracycline (30 ug), ciprofloxacin (5 ug), erythromycin (15 ug), gentamicin (10 ug) and cefoxitin (30 ug) 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 30min, 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 of 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 20ul of lysis buffer (0.25% SDS and 0.05% NaOH) and placed in 95 ºC 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 1ul 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-postive 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.

Biofilm formation ability	Cut-off value detection	OD results
Strong	OD > 4×ODc[2]	OD > 0.30152
Moderate	2×ODc < OD ≤ 4×ODc	0.15076< OD ≤0.30152
Weak	ODc < OD ≤ 2×ODc	0.07538< OD ≤ 0.15076
No attachment	OD ≤ ODc	OD ≤ 0.07538

[1]Optical density

[2]ODc = average of OD negative control + (3×SD of negative control)

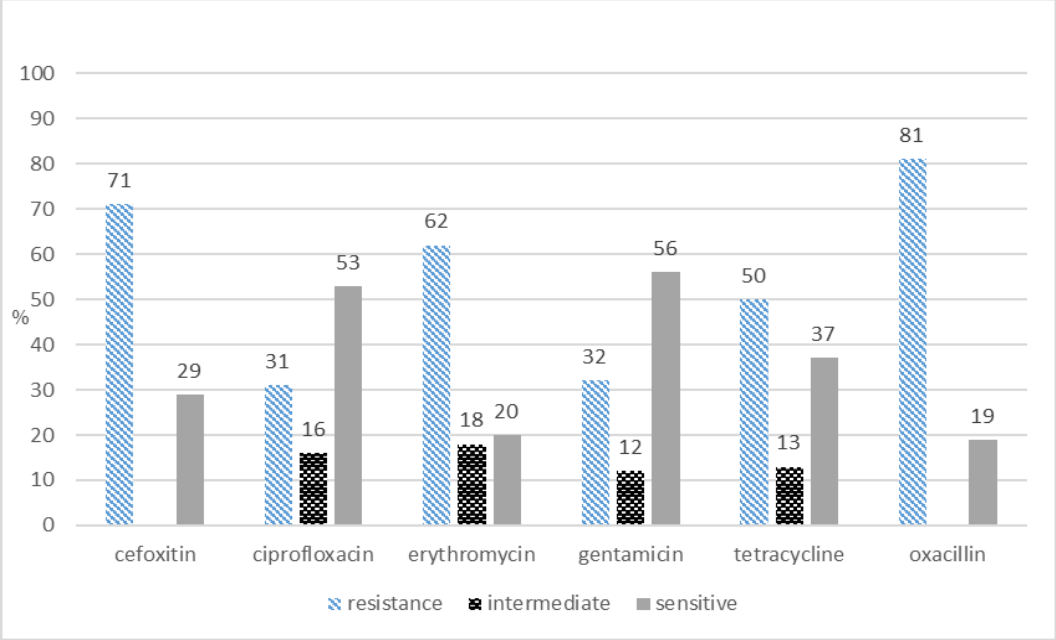

Figure 1. The rate of susceptibility, resistance and intermediate level in this study.

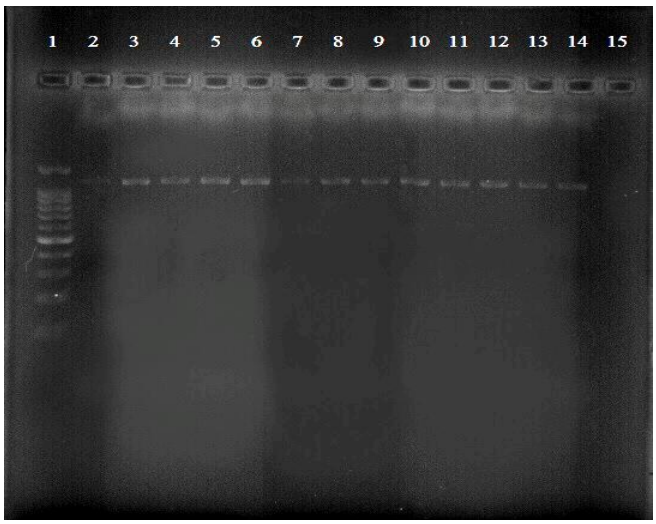

Figure 2. Electrophoresis of *IS256* gene product among *S. epidermidis* isolates. Well 1: ladder, well 2: *IS256* negative isolate, well3: control positive, wells 4-14: isolates positive for *IS256* gene with 1103 bp, well 15: negative control.

Conclusion

In this study, the prevalence of *IS256* was detected among *S. epidermidis* isolates and also the relation between *ica* operon and *IS256* inhibitory factor was uncovered. Considering that *IS256* separation from *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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