The Effect of Iatrogenic *Staphylococcus epidermidis* Intercellular Adhesion Operon on the Formation of Bacterial Biofilm on Polyvinyl Chloride Surfaces

Ye Lianhua,¹ Huang Yunchao,¹ Zhao Guangqiang,¹ Yang Kun,¹ Liu Xing,² and Guo Fengli³

Abstract

Background: The intercellular adhesion gene (*ica*) of *Staphylococcus epidermidis* is a key factor for bacterial aggregation. This study explored the effect of *ica* on the formation of bacterial biofilm on polyvinyl chloride (PVC) surfaces.

Methods: Genes related to bacterial biofilm formation, including 16S rRNA, autolysin (*atlE*), fibrinogen binding protein gene (*fbe*), and *ica* were identified and sequenced from 112 clinical isolates of iatrogenic *S. epidermidis* by polymerase chain reaction (PCR) and gene sequencing. Based on the sequencing result, *ica* operon-positive (*icaADB*+/*atlE*+/*fbe*+) and *ica* operon-negative (*icaADB*-/*atlE*+/*fbe*+) strains were separated and co-cultivated with PVC material. After 6, 12, 18, 24, and 30 h of co-culture, the thickness of the bacterial biofilm and quantity of bacterial colony on the PVC surface were measured under the confocal laser scanning microscope and scanning electron microscope.

Results: The positive rate of *S. epidermidis*-specific 16SrRNA in 112 iatrogenic strains was 100% (112/112). The genotype of *ica*-positive (*icaADB*+/*atlE*+/*fbe*+) strains accounted for 57.1% (64/112), and genotype of *ica*-negative (*icaADB*-/*atlE*+/*fbe*+) strains accounted for 37.5% (42/112). During 30 h of co-culture, no obvious bacterial biofilm formed on the surface of PVC in the *ica*-positive group, however, mature bacterial biofilm structure formed after 24 h. For all time points, thickness of bacterial biofilm and quantity of bacterial colony on PVC surfaces in the *ica* operon-positive group were significantly higher than those in *ica* operon-negative group ($p < 0.01$).

Conclusions: Iatrogenic *S. epidermidis* can be categorized into *ica* operon-negative and *ica* operon-positive strains. The *ica* operon plays an important role in bacterial biofilm formation and bacterial multiplication on PVC material.

STAPHYLOCOCCUS EPIDERMIDIS is one of the most common pathogens in reparative and reconstructive surgery. It invades the human body through the implantation of medical devices, adhering to the surface to form bacterial biofilm, which leads to recurrent post-operative infection that is resistant to treatment [1]. Studies have shown that the autolysin gene (*atlE*) and fibrinogen-binding protein gene (*fbe*) are two major factors in the adhesion stage during the formation of *S. epidermidis* biofilm, whereas the intercellular adhesion operon gene (*ica*) is the key factor in the aggregation stage [2–4]. In this study, *S. epidermidis* was isolated from infected biologic implants. The role of *ica* operon of iatrogenic *S. epidermidis* in bacterial biofilm formation on the surface

of polyvinyl chloride (PVC) material was investigated. We believe this provides useful information for preventing bacterial biofilm infections caused by biologic material implantation.

Patients and Methods

Main materials, reagents, and instruments

Polyvinyl chloride material (Dongguan Kewei Medical Apparatus Co., Ltd. Guangdong Province, China) was cut into 1 cm × 1 cm pieces, then fumigated and sterilized by ethylene oxide for preservation. Puregene Yeast/Bact Kit (Qiagen Inc., Alameda, CA); Live/Dead[®] BacLight[™] Bacterial

¹Department of Thoracic Surgery, ²Department of Molecular Biology, ³Department of Clinical Bacteriology Laboratory, The Third Affiliated Hospital of Kunming Medical University, Kunming, Yunnan, China.

Viability Kit (Invitrogen Inc., Carlsbad, CA); API test strips (bioMérieux, Marcy d'Etoile, France) were used. MRC-1024ES confocal laser microscope (Bio-Rad Inc., Hercules, CA) and XL30ESEM scanning electron microscope (Philips Inc., Best, The Netherlands) were used.

Experiment strains

Standard strains of *S. epidermidis* RP62A (control strains of *atlE*, *fbe*, *ica* genetic testing-positive) and ATCC 12228 (control strains of *ica* genetic testing negative) [5] were all purchased from Institute of Microbiology, Chinese Academy of Sciences, Beijing, China. Clinical specimens were obtained from First Affiliated Hospital, Second Affiliated Hospital, Third Affiliated Hospital of Kunming Medical University and Kunming General Hospital of Chengdu Military Region from January 2005 to September 2009. One hundred twelve clinical isolates of iatrogenic *S. epidermidis* were isolated and cultivated from infections associated with biologic material implantation. All isolates were identified as *S. epidermidis* by the API kit. They were stored and preserved in 40% fetal calf serum glycerol at -80°C before the experiment. Among them, eight strains were isolated from infective endocarditis, 12 from implanted foreign body cultivation, 16 from blood samples of patients infected by biologic material implantation, 16 from chest drainage tubes, 30 from central venous catheters, 18 from endotracheal tubes, and 12 from other sources.

Target gene amplification and detection

Specific primers of *icaADB*, *atlE*, *fbe*, and 16S rRNA were designed according to the protocol by Arciola et al. [6], and were synthesized by a commercial company (TaKaRa Biotechnology (Dalian) Co., Ltd., Liaoning Province, China). The sequence of primers is shown in Table 1. Bacterial genomic DNA of iatrogenic *S. epidermidis* and standard strains RP62A, ATCC12228 were extracted using the Puregene Yeast/Bact Kit. With the genomic DNA as a template, *atlE*, *fbe*, and *icaADB* genes were amplified. To ensure that all iatrogenic clinical isolates were *S. epidermidis*, species-specific 16SrRNA was used as internal control. Standard strain RP62A, ATCC 12228 were selected as positive and negative control in the genetic testing. Polymerase chain reaction (PCR) conditions were as follows: 4 min pre-denaturation at 94°C , 30 sec denaturation at 94°C , 40 sec renaturation at specific temperature, 1 min extension at 72°C , 30 cycles of amplification, and finally 7 min extension at 72°C . The PCR reaction renaturation temperature is shown in

Table 1. The PCR results were confirmed through the gel imaging analysis system.

Sequencing of Target Genes

Based on the result of PCR amplification, three positive PCR products, namely 16S rRNA, *atlE*, *fbe*, and *icaADB*, were selected for gene sequencing. Purification and sequencing of PCR products were all conducted by TaKaRa Biotechnology (Dalian) Co., Ltd. Sequencing results were then compared with the corresponding gene sequences in GenBank using Blast software (U.S. National Laboratory of Medicine, Bethesda, MD).

Experiment of Bacterial Biofilm Formation on PVC Surface Preparation of Bacterial Suspension

A small amount of iatrogenic *S. epidermidis* was removed and inoculated into the sterile broth medium. After 16 h of cultivation at 37°C , it was then inoculated in LB plates. Bacterial colonies were obtained after 24 h cultivation at 37°C . A single colony was chosen and inoculated into the broth medium. After 12–16 h cultivation at 37°C , purified bacterial suspension was used for the study. We used the pour-plate method, the concentration of bacterial suspension was adjusted at 1×10^5 colony-forming units per milliliter (CFU/mL) for the experiment.

Experimental Groups

Based on the results of gene sequencing, 106 strains of the obtained iatrogenic *S. epidermidis* were divided into two groups: *ica* operon-positive group (*icaADB*+/*atlE*+/*fbe*+; 64 strains) and *ica* operon-negative group (*icaADB*-/*atlE*+/*fbe*+; 42 strains) for bacterial biofilm formation test. Another 6 strains with the genotype of *icaADB*-/*atlE*-/*fbe*- or *icaADB*+/*atlE*-/*fbe*- were not included in the study. Five microliters of prepared bacterial suspension was added into a sterile bottle, together with 20 mL of broth medium and 10 pieces of PVC material. They were placed in 37°C water bath for cultivation. Two pieces of PVC material were removed from the culture bottle after 6, 12, 18, 24, and 30 h of cultivation, with one piece observed under the confocal laser microscopy and the other piece observed under the scanning electron microscopy.

Detection Index

Observation under the confocal laser microscope. Fluorescent dye was prepared in accordance with Live/Dead BacLight Bacterial Viability Kit manual. PVC pieces were

TABLE 1. PRIMER SEQUENCE AND TEMPERATION OF TARGET GENES OF *STAPHYLOCOCCUS EPIDERMIDIS* STRAINS

Target genes	Sequence of prime	Length of target gene fragment (bp)	Temperament of renaturation ($^{\circ}\text{C}$)
16SrRNA	Upstream 5'-GGCGACTTTCTGGTCTGTAAC-3' Downstream 5'-CTAGAGGGGTCAGAGGATGTCA-3'	285	60
<i>atlE</i>	Upstream 5'-CAACTGCTCAACCGAGAACA-3' Downstream 5'-TTTGTTAGATGTTGTGCCCCA-3'	682	56
<i>fbe</i>	Upstream 5'-TAAACACCGACGATAATAACCAAA-3' Downstream 5'-GGTCTAGCCTTATTTTCATATTCA-3'	495	55
<i>icaADB</i>	Upstream 5'-TTATCAATGCCGCGAGTTGTC-3' Downstream 5'-GTTTAACGCGAGTGCGCTAT-3'	515	57

TABLE 2. COMPARISON OF THE QUANTITY OF BIOFILM ON POLYVINYL CHLORIDE SURFACES BETWEEN THE *ica*-POSITIVE AND *ica*-NEGATIVE GROUP

Group	n	6 h	12 h	18 h	24 h	30 h
<i>ica</i> -negative	42	5.95 ± 1.56	7.81 ± 1.91	9.29 ± 1.76	8.95 ± 1.77	7.23 ± 2.02
<i>ica</i> -positive	64	9.16 ± 1.87	12.00 ± 1.22	17.34 ± 2.51	16.84 ± 2.29	15.72 ± 1.80
Statistical value	p = 0.000 (<i>F</i> = 435.985)					

In this study, 112 genes of *Staphylococcus epidermidis* were detected. A total of 106 were found, including the iatrogenic *S. epidermidis* of positive *icaADB*, *atlE*, *fbe* genotype (64 strains, *ica* operon-positive group) and *icaADB* negative, *atlE* and *fbe* positive (42 strains, *ica* operon-negative group). Another six that included *icaADB* negative, *atlE* and *fbe* negative and *icaADB* positive, *atlE* and *fbe* negative were not included in the statistical analysis.

washed three times with distilled water, and then stained with bacteria fluorescent dye for 20 min at room temperature before being put under the confocal laser microscope (argon laser [514/488 nm]) for observation. For each PVC plate one visual field was randomly selected to observe the number of bacterial colony. Then one bacterial colony was selected randomly to scan layer by layer from inside out and measure their thickness (bacterial biofilm thickness).

Observation under the scanning electron microscope

The PVC pieces were washed three times with sulfuric acid hydroxyethyl piperazine buffer solution (pH = 7), then fixed on the loading platform of the scanning electron microscope. After CO₂ supercritical drying, the fixed coating of ion sputtering surface gave the PVC plate golden-brown color. The surface structure of the bacterial biofilm was observed under the microscope.

Statistical Method

The SPSS 17.0 statistical software package (IBM, Inc., Armonk, NY) was used for analysis. Data are shown as mean ± standard deviation. Analysis of variance for repeated-measurement was used for comparison between two groups, and *p* < 0.05 was set as the significant value.

Results

Target gene test of clinical isolate strains of iatrogenic *S. epidermidis*

Results of agarose gel electrophoresis showed that positive rate of *S. epidermidis*-specific 16S rRNA was 100% (112/112) in 112 clinical isolates, indicating that all strains were *S. epidermidis*. Existence of *atlE* and *fbe* gene was widespread, with a positive rate of 94.6% (106/112), among which genotype of *icaADB*+/ *atlE*+/*fbe*+ accounted for 57.1%

(64/112) and genotype of *icaADB*-/*atlE*+/*fbe*+ accounted for 37.5% (42/112).

Target gene sequencing of clinical isolate strains of iatrogenic *S. epidermidis*

Sequencing results showed that sequences of target genes 16S rRNA, *atlE*, *fbe*, and *icaADB* were consistent with gene sequences in GenBank.

Observation under the confocal laser microscope

The observation under the confocal laser microscopy showed that the number of bacterial colony and the bacterial biofilm thickness per unit area of the PVC surface in *ica* operon-positive group were significantly higher than those of *ica* operon-negative group. The differences were statistically significant (*p* < 0.01; Table 2 and Table 3).

Observation under the scanning electron microscopy

Observation under the scanning electron microscopy indicated that during 30 h of cultivation there was no bacterial biofilm formation on PVC surface in the *ica* operon-negative group, nor did any mature bacterial biofilm structure appear. In the *ica* operon-positive group, the number of bacterial colony gradually increased with time on the PVC surface. Bacterial clusters were gradually wrapped by the mucus-like substance, and the volume of bacterial biofilm was also enlarged. The mature form of bacterial biofilm structure at 24 h appeared as compact, highly organized multi-cellular groups. After 30 h, the volume of bacterial biofilm tended to stabilize; some bacterial biofilm began pyknosis and collapsed (Fig. 1).

Discussion

In recent years, with the extensive application of biologic materials, *S. epidermidis* has become the major opportunistic pathogen in reparative and reconstructive surgery. Studies

TABLE 3. COMPARISON OF THE THICKNESS OF BIOFILM ON POLYVINYL SURFACES BETWEEN THE *ica*-POSITIVE AND *ica*-NEGATIVE GROUP

Group	n	6 h	12 h	18 h	24 h	30 h
<i>ica</i> -negative	42	4.15 ± 0.79	5.87 ± 0.87	7.30 ± 1.05	9.60 ± 1.13	9.91 ± 1.74
<i>ica</i> -positive	64	13.23 ± 2.32	21.84 ± 2.75	39.56 ± 3.54	58.45 ± 5.13	55.09 ± 5.43
Statistical value	p = 0.000 (<i>F</i> = 6714.395)					

In this study, 112 genes of *Staphylococcus epidermidis* were detected. A total of 106 were found, including the iatrogenic *S. epidermidis* of positive *icaADB*, *atlE*, *fbe* genotype (64 strains, *ica* operon-positive group) and *icaADB* negative, *atlE*, and *fbe* positive (42 strains, *ica* operon-negative group). Another six that included *icaADB* negative, *atlE* and *fbe* negative, and *icaADB* positive, *atlE* and *fbe* negative were not included in the statistical analysis.

FIG. 1. The image of the two group strains by scanning electron microscope examination at different times. (A) *ica*-negative group at 6 h. (B) *ica*-negative group at 12 h. (C) *ica*-negative group at 24 h. (D) *ica*-negative group at 30 h. (E) *ica*-positive group at 6 h. (F) *ica*-positive group at 12 h. (G) *ica*-positive group at 24 h. (H) *ica*-positive group at 30 h.

have shown that 40%–50% of endocarditis in patients after artificial heart valve surgery was caused by *S. epidermidis*. In patients who underwent peritoneal dialysis, *S. epidermidis* accounted for 40% of catheter contamination-related infections [6–8]. *Staphylococcus epidermidis* strains from different sources exhibit genetic differences. It is reported that *S. epidermidis* isolated from infections associated with clinical catheters differ from those isolated from normal human skin [6–9]. The positive rate of bacterial biofilm-related genes, such as *fbe*, *atlE*, and *icaADB*, varies with different sources as well. The results of our study were consistent with the above observations. The *fbe* and *atlE* genes existed in 112 clinical isolates of *S. epidermidis* in our study, accounting for 94.6% (106/112). The positive rate of *icaADB* gene was 57.1% (64/112). *Staphylococcus epidermidis* with different genetic background often showed different biologic characteristics, and whether *S. epidermidis* can form biofilm is the major criterion to distinguish between symbiotic and invasive *S. epidermidis* [3,9].

Studies on the biofilm formation of *S. epidermidis* showed that the enzyme responsible for biofilm formation is encoded by *ica* operon, which include *icaR* (regulatory gene), *icaA*, *icaD*, *icaB*, and *icaC* genes existing in series connection. The *icaA* gene alone shows low N-acetyl glucosamine transferase activity, but co-expression of *icaA* and *icaD* can increase the enzyme activity greatly, synthesizing the largest chain with up to 20 residues of oligomers. Four genes of *icaADBC* together can complete the synthesis of polysaccharide intercellular adhesion (PIA) [4,6], the necessary material to mediate the aggregation of *S. epidermidis* biofilm formation [2,6,8,9]. Studies on the biofilm formation of *S. epidermidis* based on planktonic conditions indicated that *S. epidermidis* lacking the *ica* operons has difficulty forming the mature bacterial biofilm, and *S. epidermidis* strains that can form bacterial biofilm, were shown to be mostly *ica* operon-positive, suggesting that biofilm formation by *S. epidermidis* is related to the existence of *ica* operons. However, for the infections associated with biologic material implantation, the relationship between the *ica* operon of iatrogenic *S. epidermidis* and the bacterial biofilm formation on the

surface of biologic material needs to be addressed further. Few relevant studies have been reported.

Our preliminary results showed that the biofilm formation of *S. epidermidis* is a dynamic process, with the initial adhesion to the biologic materials by the hydrophobic proteins or extracellular polysaccharides on the bacterial surface. Then the bacterium, with PIA as the main medium, aggregates with each other to form a bacterial biofilm. The number of bacterial colonies and the thickness of biofilm on biologic material surfaces are the indicators that reflect the phenotype of bacterial biofilms on the biologic material surface. The highly organized multi-cellular structure of bacterial biofilm on biologic material surface could be the source of clinical implantation-associated infections that are difficult to control. Thus, observation of bacterial biofilm structure in living conditions constitutes an important part of bacterial biofilm research [2,3].

In this study, PVC material, which is used commonly in reparative and reconstructive surgery, was used as the carrier of bacterial adhesion, and the role of *ica* operon of iatrogenic *S. epidermidis* on the bacterial biofilm formation on PVC surface was studied through the laser scanning technique. The results indicated that the number of bacterial colonies and the bacterial biofilm thickness of iatrogenic *S. epidermidis* with *ica* operon-positive on PVC surfaces were significantly higher than those with the *ica* operon-negative group. After further observation by scanning electron microscopy, it was found that after 24 h of cultivation, the *ica* operon-positive group can form the compact, highly organized bacterial biofilm structure on PVC surfaces, whereas the *ica* operon-negative group showed only part of the bacterial biofilm adhering to the PVC surface and no formation of any mature bacterial biofilm structure. One explanation could be that the latter contains the initial adhesion-related genes (*atlE*, *fbe*), which showed the initial adhesion function, but lack of *ica* operons results in failure of bacterial biofilm aggregation, therefore, they could not form a mature bacterial biofilm. This indicates that the *ica* operon of iatrogenic *S. epidermidis* plays an important role in the formation of bacterial biofilm on PVC surface.

There are many other factors that can influence the biofilm formation of *S. epidermidis*, such as pH value, and concentrations of glucose and NaCl in the culture medium [10–19]. Hypertonic saline solution (NaCl), ethanol, glucose, and the environmental pH value can change the expression of *ica* operon, regulating the biofilm formation of *S. epidermidis* [10–15]. Moreover, different factors regulate the biofilm formation of *S. epidermidis* via different pathways. Ethanol can directly or indirectly inhibit the repressor gene *icaR* transcription, leading to the increased *icaADBC* transcription, therefore promoting bacterial biofilm formation. Sodium chloride, on the other hand, works together with RsbU protein to activate SigB, the latter then promotes *icaADBC* transcription and bacterial biofilm formation [16–19]. However, current studies relevant to *ica* operon and the bacterial biofilm of *S. epidermidis* are based mostly on the planktonic growth pattern, and limited by the laboratory mutant bacterial strains. Also, for infections associated with biologic material implantation, the process of biofilm formation and organizational structure of the biofilm are different than those caused by planktonic infections [20–21]. Therefore, to characterize the genetic feature and biologic behavior of *S. epidermidis* on PVC surfaces would provide useful information for clinical treatment. By suppressing the expression of iatrogenic *S. epidermidis* *ica* operon, thus to effectively inhibit the bacterial biofilm formation on the implant surface, chances of implant-associated infections would be reduced. Our study therefore provides some insight for targeted therapy.

In summary, the presence of *ica* operon of iatrogenic *S. epidermidis* can increase the number of bacterial colonies and the bacterial biofilm thickness on the PVC surface. It plays a role in the bacterial biofilm formation for medical implant-associated infection, and may serve as the genetic basis to distinguish between symbiotic and invasive *S. epidermidis*. Studies in the related area could benefit clinical work by examining *S. epidermidis* phenotype (e.g., *icaADB*, *atlE*, *fbe* genotype) of biomaterial implant infection to determine the initial bacterial biofilm-forming ability.

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Dr. Ye Lianhua

Department of Thoracic Surgery

The Third Affiliated Hospital

of Kunming Medical University

No. 519 Kun-zhou Rd.

Kunming City

Yunnan

China

E-mail: Lhye1204@aliyun.com