EXPERIMENTAL INVESTIGATIONS

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Phenotypic and Genetic Analysis of Biofilm Formation by *Staphylococcus epidermidis*

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***Key words**: Staphylococcus epidermidis; nosocomial infections; biofilm formation; virulence factors.*

***Summary.** Objective. The most important virulence factor of Staphylococcus epidermidis is their capability to form a biofilm on the surfaces of implanted medical devices. The accumulative phase of biofilm formation is linked to the production of intercellular adhesin encoded by the icaADBC operon and accumulation-associated protein by the aap gene.*

The aim of the study was to investigate biofilm formation phenotypically and genetically in clinical strains of S. epidermidis in comparison with commensal strains.

Material and Methods. The study was carried out in 4 hospitals in Riga, Latvia. In total, 105 clinical strains of Staphylococcus epidermidis isolated from patients' blood (n=67) and intravenous catheters (n=38) in a case of laboratory-confirmed bacteremia were studied. Moreover, 60 Staphylococcus epidermidis commensal strains isolated from nose epithelium of healthy people were included as a control group. Appearance of the icaA and aap genes was tested by polymerase chain reaction. The microtiter plate method was used.

Results. Biofilm formation was detected in 50 (47%) of Staphylococcus epidermidis isolates in the clinical group and 15 (25%) of isolates in the control group (P=0.0049). Among 50 biofilm-forming clinical isolates, 46 (92%) were positive for the icaA and/or aap genes. The icaA and aap genes were not found only in 4 strains.

Conclusions. The clinical isolates of Staphylococcus epidermidis were more likely to form biofilms than the commensal strains. The carriage of the icaA or aap gene alone, or their absence, is not applicable as a molecular marker for the discrimination invasive Staphylococcus epidermidis strains from contaminants.

Introduction

Coagulase-negative staphylococci and, in particular, *Staphylococcus epidermidis* (*S. epidermidis*), have emerged as major nosocomial pathogens associated with the infections of implanted medical devices.

S. epidermidis is primarily a normal inhabitant of a healthy human mucosa and skin. As a commensal bacterium, it has a low pathogenic potential, but during the last decades, some strains have transformed into highly adaptable human pathogens (1, 2). Since the early 1980s, these strains have emerged as important nosocomial pathogens and are considered as the ones of the most frequent health care-associated microorganisms, featuring prominently among blood culture isolates and, as mentioned above, especially causing infections associated with implanted devices, such as intravascular catheters and prosthetic implants (3, 4).

The virulence factors of coagulase-negative staphylococci, including *S. epidermidis*, have not been well defined yet.

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Biofilm formation is thought to be the major pathogenic factor of *S. epidermidis*. A study on the pathogenesis of *S. epidermidis* infections by Peters et al. indicated a key role of biofilm formation in a successful colonization of foreign bodies (5).

The first step in the interaction between microorganisms and a host is adherence to the substrate and then formation of mucoid slime, an extracellular polysaccharide referred to as a biofilm. The initial bacterial adhesion is nonspecific, which is followed by a specific adhesion, mediated by a specific receptor (6, 7). Microbial cells are embedded in a slime layer; there they interact among themselves and adhere one to another, thus, forming a tight layer. *S. epidermidis* biofilm is characterized by a low metabolism of cells, decreased transcription and translation, and a shift from aerobic production of energy to fermentation, resulting in a nonaggressive and protected mode of growth that is less sensitive to antibiotics and the host immune defense (8, 9).

Biofilm production is mediated by several factors, mainly by adhesion molecules and a possibility to produce the great amounts of slime (10). Ac-

cording to the recent studies of adhesion molecules, polysaccharide intercellular adhesin (PIA) is considered to be the most important. PIA is a β-1,6-linked N-acetylglucosamine, which is responsible for a cell-to-cell attachment (11, 12). PIA production in S. *epidermidis* is encoded by the *icaADBC* operon.

Other factor that mediates biofilm formation independently of PIA is accumulation-associated protein (AAP) encoded by the *aap* gene. AAP is essential for biofilm development and is involved in the second accumulation phase of biofilm formation (13). Both *icaA* and *aap* genes are used as the potential virulence markers.

Therefore, the leading researchers, such as Vandecasteele et al. (14), have reported the *icaADBC* operon and the *aap* gene to be the most important genetic determinants of biofilm production and virulence markers.

One of the major challenges of daily diagnostic practice in a clinical microbiology laboratory is to discriminate between the clinically significant invasive strains of *S. epidermidis* and skin contaminants. Finding the virulence markers allowing a rapid differentiation between virulent and nonvirulent microorganisms would be of great clinical importance.

The aim of this study was to investigate biofilm formation phenotypically and genetically in the clinical strains of *S. epidermidis* in comparison with the commensal strains.

Material and Methods

Bacterial Strains. In total, 105 clinical strains of *S. epidermidis* isolated from patients' blood (n=67) and intravenous catheters (n=38) in a case of bacteremia were studied. The patients' age ranged from 35 to 85 years (mean, 61 years).

The clinical definition of *S. epidermidis* bacteremia was based on the CDC criterion of $\geqslant$2 positive blood cultures within 48 hours along with the symptoms of infection (i.e., fever >38°C, chills, or hypotension) that were not related to another infection. The patients were considered to have true coagulase-negative staphylococcal (CoNS) bacteremia if they met the CDC clinical criteria and if identical species and strains were isolated from positive blood culture sets. The blood cultures were processed by using the BacT/Alert™ system (Biomerieux, France) for aerobic and anaerobic bacteria.

For the analysis, 38 *S. epidermidis* isolates were recovered from catheters (>1000 colony-forming units on a quantitative catheter culture) of the patients with suspected bacteremia. Besides, 60 *S. epidermidis* isolates from normal nose epithelium flora were included in the control group, which was represented by 20–22-year-old healthy people not involved in public health care.

S. epidermidis cultures, isolated from specimens, were identified on the basis of colony morphology, gram staining, and positive results of catalase and negative coagulase tests (Slidex Staph Plus, bioMérieux) and then identified up to a species level using the BBL™ Crystal™ identification system (Becton, Dickinson and Company, USA).

The following international reference strains were used as controls: the nonbiofilm producers *S. epidermidis* ATCC 12228 and the biofilm producers *S. epidermidis* ATCC 35984 (RP62A).

Tissue Culture Plate Method. The biofilm formation using a microtiter plate was carried out as described by Christensen et al. (15). Overnight-grown bacteria in trypticase soy broth (TSB) were diluted (1:100), and 200-mL portions were inoculated into sterile 96-well flat bottom polystyrene microtiter plates (Sarstedt, Inc, Newton, USA). The incubation was carried out at 36°C for 22–24 hours before the removal of the cultures. The wells were washed 3 times with phosphate-buffered saline (PBS, pH 7.2) and then air dried and stained with 0.4% safranin. The optical density of the wells was measured at 490 nm using an ELISA microplate reader (Biochrom Asys Expert Plus GmbH, Austria).

An optical density of 0.12 was chosen to distinguish biofilm producers from those that did not form a biofilm. Biofilm-positive and negative strains of *S. epidermidis* were included in each plate, and the medium was also added without bacteria.

Isolation of Genomic DNA. All the isolates were grown overnight in brain-heart infusion broth. The bacteria were pelleted, and the DNA isolation was performed with a genomic DNA purification kit (Fermentas Life Science, EU) according to the manufacturer's instructions. DNA concentration was measured using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, USA).

Detection of icaA and aap Genes. All the isolates were tested for the *icaA* and *aap* genes using the polymerase chain reaction (PCR) method. The primers were synthesized by Invitrogen Life Technologies (UK).

The primer sequences were as follows: *icaA*1, 5'-CAC GTG CTC TAT GGA TG-3'; *icaA*2, 5'-CCG TTG GAT ATT GCC TCT GT-3'; *aap*1, 5'-ATA CAA CTG GTG CAG ATG GTT G-3'; and *aap*2, 5'-GTA GCC GTC CAA GTT TTA CCA G-3'.

All the PCRs were performed on the GeneAmp PCR System 9700 (PE Applied Biosystems, Foster City, CA, USA). The cycling conditions were as follows: preheating for 5 minutes at 94°C, followed by 25 cycles of 30 seconds at 94°C, 60 seconds at 55°C, and 60 seconds at 72°C. In each PCR, a positive control (*S. epidermidis* ATCC 35984, RP62A) and a negative control (distilled water) were included. The amplified products were analyzed by agarose gel electrophoresis. The amplicon of *icaA* was 502 bp, and that of *aap*, 399 bp.

Statistical Analysis. The GraphPad Prism program version 5.0 was used. All *P* values were calculated with the Fisher exact test.

Results

Using the microtiter plate method for the detection of slime production of *S. epidermidis* strains, the following results were obtained.

Nearly half (n=50, 47.6%) of 105 *S. epidermidis* isolates of the clinical group were biofilm producers. In the other 55 clinical strains, the production of extracellular slime was not registered. In the control group, the number of biofilm-positive strains was lower: only 15 (25%) of the 60 cultures were slime producers (P=0.0049; OR, 2.727; 95% CI, 1.356–5.486).

Specific molecular characteristics, i.e., the presence of the *ica*A and *aap* genes, were studied separately in the biofilm-producing and nonproducing strains. The results are presented in Table 1.

Among 50 biofilm-forming clinical isolates, 46 (92%) were positive for *icaA* and/or *aap* genes. Only in 4 strains, the *icaA* and *aap* genes were not found. Thus, the association between molecular markers and biofilm production was observed.

The analysis of 55 clinical strains in which the biofilm production was not found revealed the following: 13 (23.6%) of the strains were negative for the *ica*/*aap* genes, and 32 were positive for the *icaA*/*aap*, but did not produce an extracellular polymer.

Of the 60 control isolates, 45 (75%) did not form biofilms, and slime production was not observed. In 19 (42.2%) of them, the genetic markers *ica*/*aap* were not found, but the other 26 were positive for one or both genes. In 15 biofilm-positive control cultures, 13 (86.6%) were positive for the *ica*/*aap* genes, and only 2 isolates were negative.

The main attention was focused on the pathogenic *S. epidermidis* strains. Therefore, the strains isolated from blood and catheters were separately analyzed (Table 2). The results showed that they corresponded to the previously described principles, i.e., about half of strains were capable of forming biofilms, and the main part of them was *ica*/*aap* positive. There were 26 (38.8%) biofilm-positive *S. epidermidis* isolates from blood cultures (n=67) and 21 (55.2%) biofilm-positive isolates from catheters (n=38).

The presence of the *aap*/*icaA* genes and biofilm formation was slightly more often observed in catheter-related isolates than blood culture isolates; however, this difference did not reach the statistical significance (P=0.152; OR, 0.513; 95% CI, 0.229–1.150).

Discussion

The main virulence factor of coagulase-negative staphylococci is considered to be their ability to form biofilms. The pathogenesis of staphylococcal diseases depends on their capability to adhere and then form a mucoid layer (slime layer), especially in intravenous catheter- or prosthetic device-related bacteremia. The elaboration of a biofilm is a complex process in which different proteins play a role, with adhesion molecules, accumulation-associated proteins, and factors responsible for cell-to-cell interactions among them.

The objective of the present work was to search for *S. epidermidis* virulence factors, applying phenotypic and genotypic methods, and to evaluate

Table 1. Biofilm Formation and Presence or Absence of *icaA* and *aap* Genes in Clinical and Commensal *S. epidermidis* Isolates

Biofilm Formation *icaA* and *aap* Genes	Clinical Group n=105	Control Group n=60	*P*	Odds Ratio	95% Confidence Interval
BF+/*aap*+/*icaA*+	27 (25.7)	5 (8.3)	0.0073	3.808	1.380–10.510
BF+/*aap*–/*icaA*–	4 (3.8)	2 (3.3)	1.0000	0.871	0.155–4.903
BF+/*aap*+ or *icaA*+	19 (18.1)	8 (13.3)	0.5148	1.436	0.587–3.515
BF–/*aap*+/*icaA*+	14 (11.4)	8 (13.3)	1.000	1.000	0.393–2.543
BF–/*aap*–/*icaA*–	13 (12.4)	19 (31.7)	0.0039	0.305	0.138–0.676
BF–/*aap*+ or *icaA*+	28 (26.7)	18 (30)	0.7189	0.849	0.421–1.711

Values are number (percentage). BF, biofilm formation using the tissue culture plate method.

Table 2. Biofilm Formation and Presence or Absence of *icaA* and *aap* Genes in *S. epidermidis* Strains Isolated from Blood and Catheters

Biofilm Formation *icaA* and *aap* Genes	Blood Cultures n=67	Catheters n=38	*P*	Odds Ratio	95% Confidence Interval
BF+/*aap*+/*icaA*+	10 (14.9)	10 (26.3)	0.2092	0.517	0.200–1.336
BF+/*aap*–/*icaA*–	5 (7.5)	3 (7.9)	1.000	0.812	0.183–3.586
BF+/*aap*+ or *icaA*+	11(16.4)	8 (21)	0.6015	0.692	0.256–1.871
BF–/*aap*+/*icaA*+	8 (11.9)	5 (13.1)	1.000	0.874	0.271–2.814
BF–/*aap*–/*icaA*–	8 (11.9)	3 (7.9)	0.5401	1.741	0.450–6.742
BF–/*aap*+ or *icaA*+	25 (37.3)	9 (23.7)	0.1944	1.918	0.782–4.704

Values are number (percentage). BF, biofilm formation using the tissue culture plate method.

the studied features as the possible criteria for the discrimination between the virulent clinical nosocomial and nonvirulent contaminant strains. The attention was focused mainly on the capability of *S. epidermidis* to produce biofilms and on the association between biofilm production and the genetic markers, *icaA* and *aap* genes.

Our data revealed that biofilm formation was more characteristic of the clinical strains of *S. epidermidis*. The difference comparing with the control strains was statistically significant. The present study showed that the percentage of the biofilm-producing strains was 47.6%. It is very close to the one found by Arciola et al. (16), i.e., 46%; in other studies, a higher percentage of biofilm-producing cells were documented Ziebuhr et al. (17) reported that 87% of clinical *S. epidermidis* isolates were biofilm producers. At the same time, other phenotypic study by Rohde et al. (18) showed contrary results: the authors failed to find an association between the clinical significance of *S. epidermidis* and the ability to form a biofilm in vitro.

In the present study, the association between biofilm formation and presence of the *icaA* and *aap* genes was positive: 92% of the clinical biofilm-producing strains and 86.6% of the commensal strains were positive for the *icaA/aap* genes.

Similar data were obtained by Eftekhar and Mirmohamadi (19), who concluded that *S. epidermidis* isolated from patients with symptomatic infections were more virulent, and the expression level of *icaADBC* and other regulatory factors independent of PIA synthesis was of importance.

In some *ica/aap*-positive strains, biofilm production was not observed; this can be explained by an extreme sensitivity of this feature to different environmental factors like antibiotic concentration in a patient's body, high temperature, glucose level, etc. Our findings are in line with those of Frebourg et al. (20).

In summary, we propose that in biofilm formation by coagulase-negative staphylococci, the *ica* operon and the *aap* gene play a crucial role, but they are not the only players in the big orchestra as the regulation of biofilm development is a complex process. Detection of biofilm-producing *icaA/aap*-negative strains indicates this.

Conclusions

The clinical isolates of *Staphylococcus epidermidis* were more likely to form biofilms than the commensal strains. The carriage of the *icaA* or *aap* gene alone, or their absence, is not applicable as a molecular marker for the discrimination invasive *Staphylococcus epidermidis* strains from contaminants.

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Statement of Conflict of Interest

The authors state no conflict of interest.

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