Biofilm-Forming Capacity of *Staphylococcus epidermidis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* from Ocular Infections

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Purpose. To investigate the biofilm-forming capacity of *Staphylococcus epidermidis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* from ocular infections.

Methods. *S. epidermidis* strains, *S. aureus* strains, and *P. aeruginosa* strains were isolated from patients with ocular infections between 2009 and 2011. The biofilm-forming capacity of these bacteria was examined using Congo red agar (CRA) and microtiter plate assays. The biofilm-forming related genes, *icaA*, of *S. epidermidis* and *S. aureus*, and *pslA*, of *P. aeruginosa*, were detected using PCR. Additionally, the morphology of biofilms was observed using a scanning electron microscopy (SEM).

Results. Of the isolated *S. epidermidis* strains, 34.38% were CRA positive, 28.13% were adherence positive using the microtiter plate assay, and 40.63% carried the *icaA* gene. Of the isolated *S. aureus* strains, 55.56% were CRA positive, 51.90% were adherence positive, and 11.11% carried the *icaA* gene. None of the *P. aeruginosa* strains were phenotypic positive using CRA and microtiter plate assays, whereas 31.03% contained the *pslA* gene. There were significant differences between the three species when the biofilm-forming capacity of the strains was compared using the CRA method ($P < 0.01$) and the microtiter plate assay method ($P < 0.01$).

Conclusions. Ophthalmic isolates of *S. epidermidis* and *S. aureus* could produce biofilms in vitro, whereas clinical strains of *P. aeruginosa* could not. The bacterial strains possess the genetic ability to produce biofilms, but that does not necessarily mean that biofilms will be produced. The knowledge of the process of bacterial adhesion suggests that a timely and appropriate intervention strategy be implemented in the early stages of biofilm-mediated infections. (*Invest Ophthalmol Vis Sci.* 2012;53:5624–5631) DOI:10.1167/iovs.11-9114

Bacterial biofilms are estimated to play a major role in more than 80% of bacterial infections.[1,2] Sixty percent of hospital-associated infections are ascribed to the formation of biofilms on implantable medical devices.[3] In addition, there are many chronic and refractory diseases associated with biofilms, such as native valve endocarditis, cystic fibrosis pneumonia, periodontitis, chronic rhinosinusitis, and otitis media.[4,5]

The eye harbors an abundance of bacterial nutrients, and, although the eye is protected by a number of natural defense mechanisms, many patients suffer from a number of infections caused by bacteria. Many ocular infections often occur when prosthetic devices come in contact with or are implanted in the eye.[6] Biofilms were observed on contact lenses (CL), IOLs, glaucoma tubes, stents, punctual plugs, corneal sutures, scleral buckle, or other ocular prostheses.[7,8] It is also widely accepted that some chronic infectious ocular diseases, such as infectious crystalline keratopathy, are due to the formation of bacterial biofilms.[9]

The formation of biofilms is an important strategy used by many bacteria to survive in natural environments.[10,11] Biofilms are heterogeneous mixtures of bacteria that are held together by a secreted matrix called extracellular polymeric substances (EPS).[6] Polysaccharide intercellular adhesion (PIA) and capsular polysaccharide/adhesin (PS/A) are the main components of EPS in *Staphylococcus aureus* and *Staphylococcus epidermidis*. Several studies showed that the intercellular adhesion (*ica*) locus, particularly the *icaA* gene, encodes production of both PS/A and PIA.[12,13] PCR amplification of the *icaA* gene demonstrated the inherent biofilm producing nature of the isolates. Some previous investigations suggested an essential role of the *psl* gene cluster in the initial formation of biofilms from *Pseudomonas aeruginosa*.[14] The *pslA* gene, which represents the first gene in the *psl* gene cluster, performs an essential function in biofilm formation of *P. aeruginosa*.[15]

Earlier studies prove that *S. epidermidis, S. aureus*, and *P. aeruginosa* are common pathogens involved in causing ocular infections.[16–18] To our knowledge, the prevalence of biofilm-forming strains of *S. epidermidis*, *S. aureus*, and *P. aeruginosa* isolated from ocular infections has not been systematically analyzed. We, therefore, sought to determine the prevalence of biofilm-forming *S. epidermidis*, *S. aureus*, and *P. aeruginosa* strains in ocular infections.

In this study, we investigated the biofilm-forming ability of 96 *S. epidermidis*, 27 *S. aureus*, and 29 *P. aeruginosa* clinical strains obtained from patients with ocular infections, while three American Type Culture Collection (ATCC) strains (*S. epidermidis* ATCC 35984, *S. epidermidis* ATCC 12228, and *P. aeruginosa* PAO1 [ATCC 15692]) served as reference controls. The ability to form biofilms was determined using Congo red agar (CRA) and microtiter plate assays. The genetic basis for biofilm formation from these strains was determined by PCR and the morphology was observed using scanning electron microscopy (SEM).

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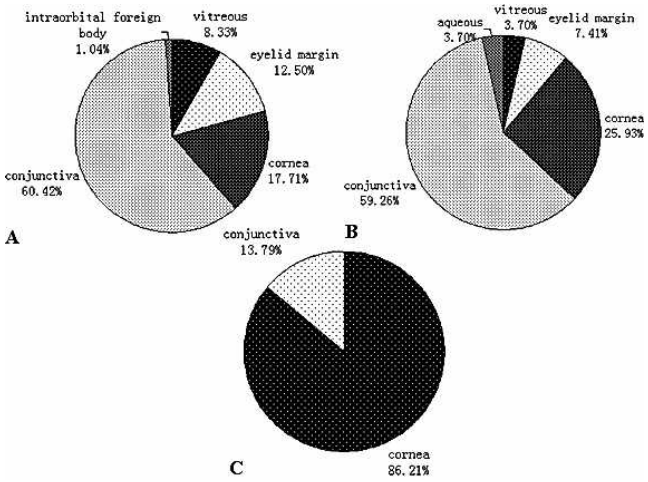

Figure 1. Isolation sites of the clinical isolates included in this study. (**A**) Isolation sites of *S. epidermidis* strains. (**B**) Isolation sites of *S. aureus* strains. (**C**) Isolation sites of *P. aeruginosa* strains.

Methods

Bacterial Strains

Ninety-six clinical isolates of *S. epidermidis*, 27 *S. aureus*, and 29 *P. aeruginosa* clinical strains were collected retrospectively over 30 months (2009–2011) from the Department of Ophthalmology in Tongren Hospital (Beijing, CHN) and submitted to the Microbial Laboratory of Beijing Institute of Ophthalmology (Beijing, CHN). The identification of strains was made according to their biochemical properties as determined using an automated system ATB Expression (bioMerieux S.A, Marcy-l'Étoile, France). Bacterial cultures were archived in glycerol stocks and were stored at −80°C. Two reference strains of *Staphylococcus* spp., the biofilm-forming strain, ATCC 35984, and the biofilm-negative strain, ATCC 12228, were used. The biofilm-forming strain, PAO1, was used as the reference strain of *P. aeruginosa*. The 152 isolates analyzed in this study were collected from the cornea, conjunctiva, eyelid margin, intraorbital foreign body, and vitreous and aqueous humors of patients who suffered from corneal ulcers, conjunctivitis, blepharritis, orbital cellulites, and/or endophthalmitis (Fig. 1).

Congo Red Agar (CRA) Method

For *Staphylococcus* spp., the CRA was composed of 37 g/L of brain–heart infusion broth (Becton Dickinson, Franklin Lakes, NJ), 36 g/L of sucrose (Sigma, St. Louis, MO), 15 g/L of agar (Becton Dickinson), and 0.8 g/L of Congo red (Sigma). The morphology of the colonies and their phenotypic changes were studied using CRA cultures as previously described.[19] Briefly, plates with Congo red medium were incubated aerobically for 24 hours at 37°C to obtain single bacterial colonies. CRA-positive strains appeared as black colonies, while CRA-negative strains remained red.

Microtiter Plate Assay

Biofilm production was detected using microtiter assay as previously described.[20–22] Briefly, *Staphylococcus* spp. were inoculated in 10 mL of tryptic soy broth (TSB; Becton Dickinson) with 0.25% glucose (Wako Pure Chemical Industries, Osaka, Japan) and incubated overnight with shaking at 37°C, while *P. aeruginosa* clinical strains were grown in lysogeny broth (LB). Next, the cultures were diluted 1:100, and 200 μL of the diluted cultures, per well, were inoculated into 96-well polystyrene microtiter plates (Costar 3599, Corning, Tissue Culture-Treated; Corning Inc., Corning, NY). After 24 hours incubation at 37°C under aerobic conditions, the plates were washed twice with sterile PBS (pH 7.2; Becton Dickinson), fixed in Bouin's fixation (Becton Dickinson) for 1 hour, and washed again with PBS. Subsequently, the plates were stained with 200 μL of 1% crystal violet, per well, for 10 minutes. Excess crystal violet was removed by gently washing the plate twice with distilled water. Finally, a volume of 250 μL of 95% ethanol solution, per well, was added to the plate and the optical density was measured at 570 nm. A well with sterile TSB or LB served as controls, whereby their ODs were subtracted from that of the experimental strains. The mean OD 570 nm value was determined using four replicates, and was considered to be adherence positive at OD 570 nm greater than or equal to 0.12 and adherence negative at OD 570 nm less than 0.12.

Genetic Techniques

In order to detect genes related to biofilm formation, bacterial cultures were lysed, DNA extracted, and gene specific primers were used to PCR amplify DNA fragments as previously described.[23] Genomic DNA was extracted using a NucleoSpin1 Tissue extraction kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's recommendations with slight modifications. The primer sequences and product length for *icaA* of *Staphylococcus* spp. and *pslA* of *P. aeruginosa* are presented in Table 1. The reaction volume was 50 μL containing PCR buffer 5 μL (10 mM), the forward and reverse primers (1 μL each), together with 1 μL of the extracted DNA, 1 μL of dNTP, 1 μL of *Taq* DNA polymerase, and 40 μL of double distilled H2O (ddH2O). A thermal step program was used, including the following parameters: incubation at 94°C for 10 minutes, followed by 30 cycles at 94°C for 1 minute (denaturation), 55°C for 30 seconds (annealing), and 72°C for 10 minutes after conclusion of the 30 cycles. Amplification products were analyzed using 2% agarose gel electrophoresis.

Scanning Electron Microscopy Analysis

Bacterial adherence was then investigated by SEM as previously described.[24] Biofilm-positive strains and biofilm-negative strains confirmed by all three methods listed above were selected to be cultured on cover slips in vitro. Briefly, strains were inoculated into 10 mL of TSB with 0.25% glucose and incubated overnight with shaking at 37°C in a water bath. Next, the solution was diluted 1:100 and inoculated into 24-well polystyrene microtiter plates, then, a cover slip was placed on the bottom of each well. After 1, 6, 24, and 72 hour incubations at 37°C under aerobic conditions, the cover slips were removed from wells with sterile forceps and gently rinsed by immersion in sterile PBS for 1 minute. For SEM analysis, the cover slips were fixed in 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2), postfixed in 1% osmium tetroxide in 0.2 M sodium cacodylate buffer (pH 7.2), and serially dehydrated in ethyl alcohol. After critical-point drying, they were coated with gold using a sputter-coating system (sputter-coater 5150A; Edwards High Vacuum International, Crawley, England) and then examined with a scanning electron microscope (DSM 962; Zeiss,

Table 1. The Primer Sequences and Product Length for *icaA* of *Staphylococcus* spp. and *pslA* of *aeruginosa*

PCR Product	PCR Sequences	Product Length
icaA	F: 5′-TCTCTTGCAGGAGCAATCAA-3′ R: 5′-TCAGGCACTAACATCCAGCA-3′	188 bp
pslA	F: 5′-CACTGGACGTCTACTCCGACGATAT-3′ R: 5′-GTTTCTTGATCTTGTGCAGGGTGTC-3′	1119 bp

Table 2. Results of CRA Test, Microtiter Plate Assay, and Gene Detection of *S. epidermidis*, *S. aureus*, and *P. aeruginosa*

Sample	CRA Positive, *n* (%)	Adherence Positive, *n* (%)	Gene Positive, *n* (%)
S. epidermidis	33/96 (34.38%)*	27/96 (28.13%)†	39/96 (40.63%)-*icaA*
S. aureus	15/27 (55.56%)*	14/27 (51.90%)†	3/27 (11.11%)-*icaA*
P. aeruginosa	0/29 (0%)*	0/29 (0%)†	9/29 (31.03%)-*pslA*

* $P < 0.01$.
† $P < 0.01$.

Oberkochen, Germany) operating at 20 kV. Images were digitized and stored as tagged image file format files in the microscope computer.

Statistical Analysis

Statistical analysis of biofilm formation was performed using SPSS software (IBM Corp., Armonk, NY). The number of biofilm-positive strains among *S. epidermidis*, *S. aureus*, and *P. aeruginosa* were compared, using the χ^2 test. The consistency test among the CRA method, the microtiter plate assay, and the gene detection method of *S. epidermidis* strains were performed by the χ^2 test. For the comparison of biofilm formation in different isolation sites, the χ^2 test was used to determine whether any of the groups exhibited a statistically significant different percentage of biofilm formation. For all tests, *P* less than 0.05 was considered significant.

Results

CRA Method

The CRA method was used on 96 *S. epidermidis*, 27 *S. aureus*, and 29 *P. aeruginosa* clinical strains. The colonies of the biofilm-positive strains, ATCC 35984 and PAO1, appeared black, while the colonies of the biofilm-negative strain, ATCC 12228, appeared red.

Thirty-three of 96 (34.38%) isolates of *S. epidermidis* formed black colonies, 15 of 27 (55.56%) *S. aureus* clinical strains formed black colonies, whereas no *P. aeruginosa* clinical strain formed black colonies (Table 2). Photographs of the isolates are shown in Figure 2. When the number of CRA-positive strains among *S. epidermidis*, *S. aureus*, and *P. aeruginosa* was compared, the difference was statistically significant ($P < 0.01$) (Table 2).

Microtiter Plate Assay

Using the microtiter plate assay, the biofilm-positive strains, ATCC 35984 and PAO1, had an OD greater than 0.12, while the biofilm-negative strain, ATCC 12228, had an OD less than 0.12. Twenty-seven of 96 (28.13%) *S. epidermidis* strains and 14 of 27 (51.90%) *S. aureus* strains were determined to be adherence-positive strains, while no *P. aeruginosa* strains were determined to be adherence positive. When the number of adherence-positive strains using the microtiter plate assay among *S. epidermidis*, *S. aureus*, and *P. aeruginosa* were compared, the difference was statistically significant (Table 2).

Genetic Techniques

Thirty-nine of 96 (40.63%) *S. epidermidis* strains carried the *icaA* gene, while only 3 of 27 (11.11%) *S. aureus* strains carried the *icaA* gene (Table 2). Eight strains of *S. epidermidis* carried the *icaA* gene, but were shown to be phenotypic negative using both the CRA method and the microtiter plate assay. Three phenotypic-positive strains using the CRA method and one adherence-positive strain using the microtiter plate assay, did not possess the *icaA* gene.

Twelve strains of *S. aureus* did not carry the *icaA* gene, but were determined to be phenotypic positive using both the CRA method and the microtiter plate assay. As expected, the *icaA* gene was detected in the biofilm-forming strain ATCC 35984, but not in the non–biofilm-forming strain ATCC 12228. The *pslA* gene was detected in the reference strain PAO1 and 9 of the 29 (31.03%) *P. aeruginosa* clinical strains (Table 2).

In a consistency test of *S. epidermidis* strains, consistency between the CRA method and the microtiter plate assay was 89.6% (86/96; $\kappa = 0.759$; $P < 0.01$) (Table 3). Consistency between the CRA method and the gene detection method was 87.5% (84/96; $\kappa = 0.734$; $P < 0.01$) (Table 3), and the consistency between the microtiter plate assay and the gene detection method was 85.4% (82/96; $\kappa = 0.682$; $P < 0.01$) (Table 3). Thus, these combined results showed correlations between the three methods.

Scanning Electron Microscopy Analysis

Our study was also designed to assess the morphologic features of strains that adhere to cover slips. For those biofilm-positive

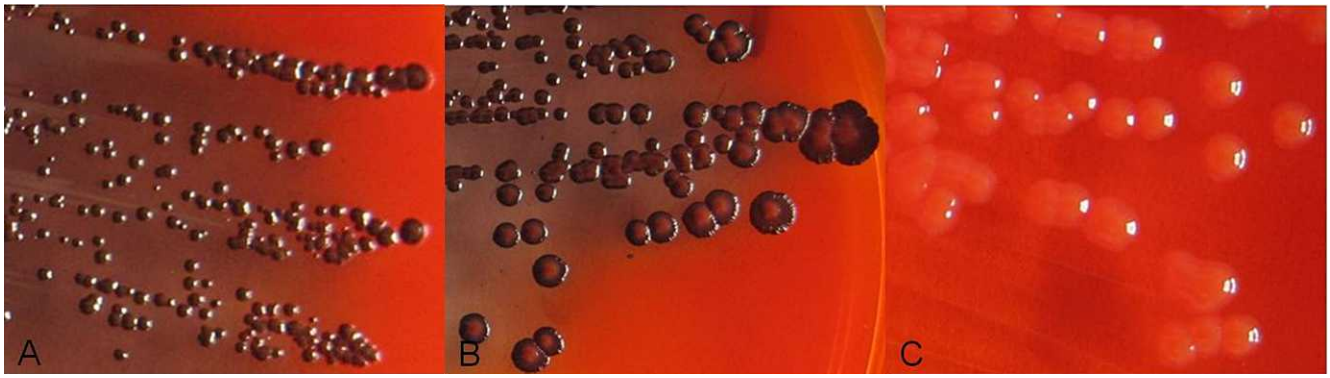

Figure 2. Results of the CRA method. (**A**) CRA-positive strain of *S. epidermidis* presented as black colonies. (**B**) CRA-positive strain of *S. aureus* presented as black colonies. (**C**) CRA-negative strain of *P. aeruginosa* presented as red colonies.

Table 3. The Consistency Test among the CRA Method, the Microtiter Plate Assay, and the Gene Detection Method of *S. epidermidis* Strains

CRA Microtiter Plate Crosstabulation*					CRA *icaA*-PCR Crosstabulation†					Microtiter Plate *icaA*-PCR Crosstabulation‡				
		–	+	Total			–	+	Total			–	+	Total
CRA	–	61	2	63	CRA	–	54	9	63	Microtiter	–	56	13	69
	+	8	25	33		+	3	30	33		+	1	26	27
Total		69	27	96	Total		57	39	96	Total		57	39	96

–, the number of negative strains; +, the number of positive strains.

* $\kappa = 0.759$; $P < 0.01$.

† $\kappa = 0.734$; $P < 0.01$.

‡ $\kappa = 0.682$; $P < 0.01$.

staphylococcal clinical strains and the ATCC 35984, after a 1 hour incubation, bacterial cells appeared to accumulate as small monolayer sheets. Thread-like appendages were found around the bacterial cells and projected from the bacterial cell wall toward the cover slip surface (Fig. 3A). After a 6 hour incubation, the previously small monolayer sheets developed into larger multilayer sheets, and the bacterial cells became encased in an mucoid material (Fig. 3B). After a 24 hour incubation, the three-dimensional (3D) biofilm structure was formed, and bacterial clusters appeared to be coated on all sides with a gelatinous material (Fig. 3C). After a 72 hour incubation, mature and 3D biofilm structures were formed. Water channels and thread-like appendages between subunits of bacterial colonies were distinctly observed (Fig. 3D). When the bacterial cells were removed during the course of sample preparation and, subsequently, observed using SEM, the 3D biofilm framework was clearly displayed (Fig. 3E). For those biofilm-negative *staphylococcal* clinical strains and the ATCC 12228, there were still a few bacterial cells scattering on the surface until after a 72 hour cultivation, without forming biofilm structures (Fig. 3F). For all *P. aeruginosa* clinical strains, until after a 72 hour cultivation, there were few bacterial cells scattering on the cover slip without accumulating together to form biofilms (Fig. 3G), whereas bacterial cells of PAO1 appeared to be accumulated together as big sheets, mucoid material was found around the bacterial cells, and the 3D biofilm structure was formed topically (Fig. 3H).

Comparison of the Biofilm-Forming Capacity between Collection Sites

The biofilm-forming capacity of *Staphylococcus* spp. strains that were obtained from different sites of ocular infections was determined using the microtiter plate assay and listed in Table 4. When the biofilm-forming capacity of *S. epidermidis* strains was compared with the sites from which they were collected, there was no significant difference among the four collection sites, which included the vitreous, the eyelid margin, the cornea, and the conjunctiva ($df = 3$; $P = 0.835$). The biofilm-forming capacity of *S. aureus* strains was compared with the different collection sites and showed significant differences between the cornea and conjunctiva ($P = 0.027$).

Discussion

Bacteria exist as free-floating planktonic cells or as biofilms. The formation of biofilms is an important strategy used by many bacteria to survive in natural environments.[10,11] Biofilms are defined as organized communities of collaborating bacteria that are attached to an inert or living surface and enclosed in a self-produced polymeric matrix primarily composed of exopolysaccharides, nucleic acids, and/or proteins.[25–28] Bacterial biofilms likely play a role in a variety of ocular infections. Previously, biofilms were observed on ocular abiotic materials such as CLs, IOLs, glaucoma tubes, stents, punctual plugs, corneal sutures, scleral buckle, and so on.[7,8] In addition, the presence of bacterial biofilms was shown to be associated with some chronic infections, such as diffuse lamellar keratitis (DLK) after LASIK and infectious crystalline keratopathy.[6–9] However, there are only a few studies analyzing large sample sizes to determine the prevalence of biofilm-forming capacity, and most of these studies only described the adherence of biofilm-positive reference strains or individual clinical isolates to ocular materials.[6] In this study, we investigated the biofilm-forming capacity of the most common pathogens in ophthalmology, which are *S. epidermidis*, *S. aureus*, and *P. aeruginosa* clinical strains obtained from different types of ocular infections.

Our phenotypic results indicated that 34.38% and 28.13% of *S. epidermidis* clinical strains were determined to be phenotypic positive using the CRA method and microtiter plate assay, respectively. The proportion of biofilm-positive strains in our study were lower than those found by Juárez-Verdayes et al.,[29] whose investigations showed that as much as 66% of the *S. epidermidis* strains obtained from patients with bacterial conjunctivitis, corneal ulcers, and endophthalmitis had the capacity of forming biofilms. In addition, Catalanotti et al.[17] also found that 74.1% of the *S. epidermidis* strains were slime producers. Although we are unable to explain the percentage differences between the studies, the CRA method, the microtiter plate assay, and the *icaA*-PCR analysis of *S.*

Table 4. Comparison of the Biofilm-Forming Capacity between Collection Sites of *Staphylococcus* spp.

	Biofilm	Vitreous	Eyelid Margin	Cornea	Conjunctiva	Intraorbital	Aqueous
S. epidermidis	Positive	2	2	5	17	1	0
	Negative	6	10	12	41	0	0
	Total	8	12	17	58	1	0
S. aureus	Positive	1	1	6	5	0	1
	Negative	0	1	1	11	0	0
	Total	1	2	7*	16*	0	1

* $df = 1$; $P = 0.027$.

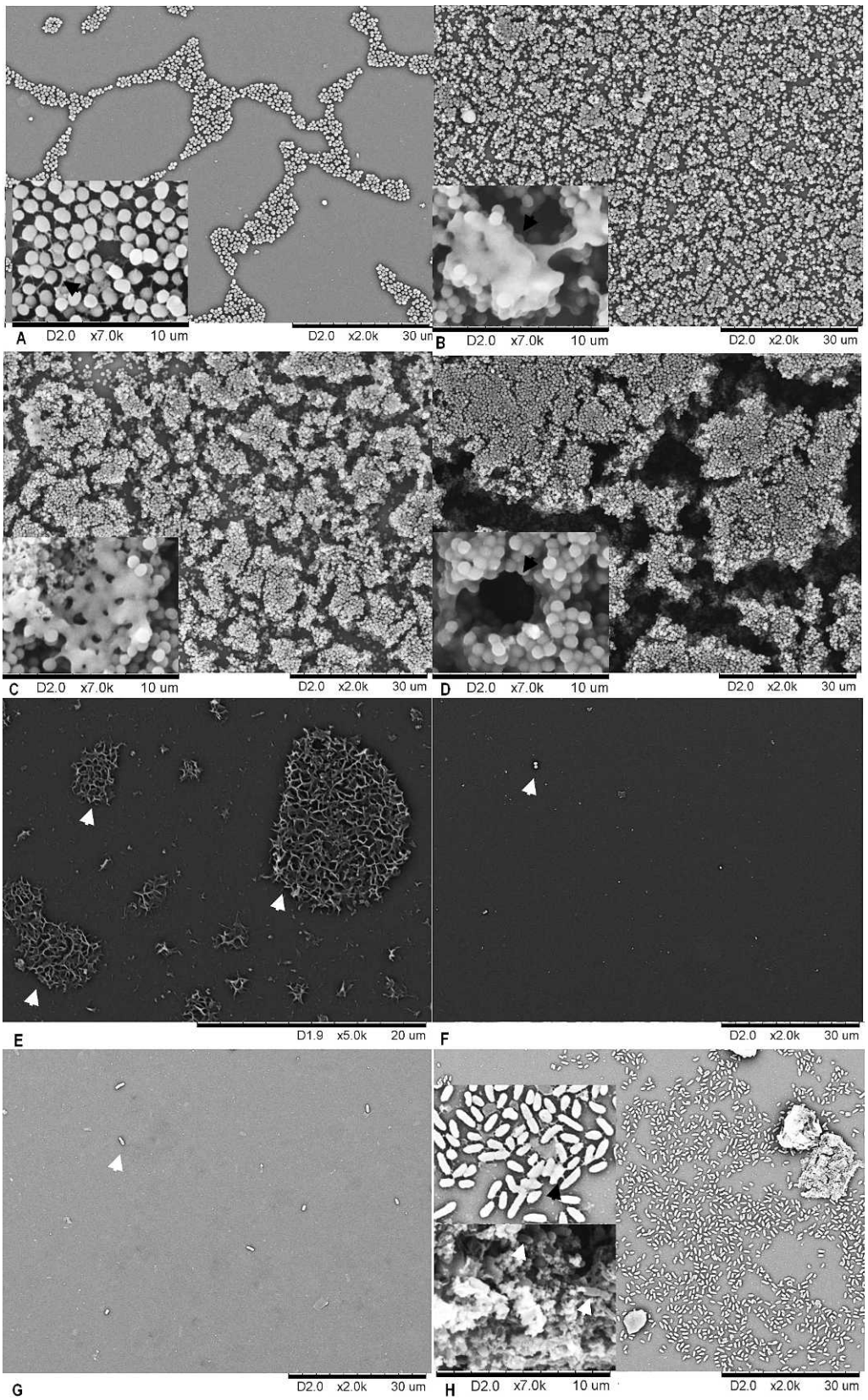

Figure 3. Results of scanning electron microscopy analysis. (**A**) After a 1 hour incubation, bacterial cells of biofilm-positive *staphylococcal* clinical strain appeared to accumulate as small monolayer sheets (2000×). Thread-like appendages (*arrow*) were found around the bacterial cells, and projected from the bacterial cell wall toward the cover slip surface (7000×). (**B**) For the biofilm-positive *staphylococcal* clinical strain, after a 6 hour incubation, the previously small monolayer sheets developed into larger multilayer sheets (2000×), and the bacterial cells became encased in an mucoid material (*arrow*) (7000×). (**C**) For the biofilm-positive *staphylococcal* clinical strain, after a 24 hour incubation, the 3D biofilm structure was formed (2000×), and bacterial clusters appeared to be coated on all sides with a gelatinous material (7000×). (**D**) For the biofilm-positive

staphylococcal clinical strain, after a 72 hour incubation, mature and 3D biofilm structures were formed (2000×). Water channels (*arrow*) and thread-like appendages between subunits of bacterial colonies were distinctly observed (7000×). (**E**) After a 24 hour incubation, when bacterial cells were removed during the course of sample preparation, and subsequently observed using SEM, the 3D biofilm framework of biofilm-positive *staphylococcal* clinical strain, was clearly displayed (*arrow*) (5000×). (**F**) For the biofilm-negative *staphylococcal* clinical strain, after a 72 hour cultivation, there were a few bacterical cells of scattering on the surface without forming biofilms (*arrow*) (2000×). (**G**) For the *P. aeruginosa* clinical strain, after a 72 hour cultivation, there were few bacterical cells scattering on the surface without forming biofilms (*arrow*) (2000×). (**H**) After a 72 hour cultivation, bacterial cells of the biofilm-forming strain PAO1 appeared to be accumulating together as big sheets, mucoid material was found around the bacterial cells (*black arrow*) (7000×). The 3D biofilm structure was formed topically (2000×), bacterial cells appeared to be coated on all sides with a gelatinous material (*white arrow*) (7000×).

epidermidis clinical strains showed statistical correlations. Therefore, we suggest that these phenotypic methods (the CRA method and the microtiter plate assay) could be used as putative screening methods for the detection of biofilm-positive strains of *S. epidermidis*, and may provide a convenient way for early diagnosis of biofilm-related infections. Eight strains of *S. epidermidis* carried the *icaA* gene but were shown to be phenotypic negative using both the CRA method and the microtiter plate assay, which indicates that although the bacterial strains possess the genetic ability to produce biofilms, that does not necessarily mean that biofilms will be formed. Further investigations will help us determine what suppresses the biofilm-forming capacity of these clinical strains. We found three phenotypic-positive strains using the CRA method and one phenotypic-positive strain using the microtiter plate assay, but none of these strains possessed the *icaA* gene. Our results differ from the findings of Takashi et al.,[30] who indicated that all black colony forming strains carried the *icaA* gene. However, Aparna et al.[16] found a strain from corneal scrapings that was biofilm-positive using the CRA method, yet this strain did not contain the *icaA* gene. These results indicate that the formation of biofilms requires a complicated network of factors and the *icaA* gene is likely just one of many factors that regulate biofilm-formation.

The *ica* locus, and particularly the *icaA* gene, of *S. epidermidis* was shown to encode PS/A and PIA, and is associated with the initial bacterial adherence, intercellular adhesion, and biofilm formation.[31–33] However, the role *icaA* plays in the biofilm-formation capacity of *S. aureus* is still a controversial subject. In *S. aureus* isolates from catheterized urinary tract patients, Gamal et al.,[34] found that all biofilm-producing strains were positive for the *icaA* gene, while the biofilm-negative strains were negative for the gene. In *S. aureus* isolates from bacteremic patients, Fowler et al.[35] found that although *icaA* was present in all 15 isolates, only one isolate weakly produced biofilm in vitro. Our data revealed that only 3 of 27 (11.11%) *S. aureus* clinical strains carried the *icaA* gene. As many as 12 strains of *S. aureus* did not carry the *icaA* gene, but were determined to be phenotypic positive using both the CRA method and the microtiter plate assay. These results suggest that *icaA* is not required for biofilm formation in some strains of *S. aureus* obtained from ocular infections. It is possible that biofilm formation could be influenced by other, yet unidentified, factor(s), which is in agreement with Beenken et al.[36]

Coban et al.[37] tested the biofilm-formation ability of *P. aeruginosa* isolates from patients with cystic fibrosis and found that biofilm production was detected in 33.3% (20/60) of the samples tested. Oncel et al.[38] found that 60% (6/10) of *P. aeruginosa* isolates from chronic rhinosinusitis produced bacterial biofilms. Kádár et al.[39] investigated the biofilm production of 60 *P. aeruginosa* strains isolated from clinical samples, and found that 23.3% of *P. aeruginosa* strains were biofilm positive. To our knowledge, there are no ophthalmic reports demonstrating the biofilm-forming capacity of *P. aeruginosa* clinical isolates from large sample size. Pinna et al.[40] showed a strong adherent ability of one corneal *P. aeruginosa* isolate to AcrySof IOLs in vitro, however, we were unable to identify a biofilm-positive strain of *P. aeruginosa*. Our results indicate ophthalmic isolates of *P. aeruginosa* could not produce biofilms in vitro. We analyzed the source of the strains and found that, unlike strains of *S. epidermidis* and *S. aureus*, which were collected from various locations of the eye, all the isolates of *P. aeruginosa* analyzed in this study were collected from the cornea and conjunctiva of patients who suffered from corneal ulcers and conjunctivitis. This may be one of the reasons that the isolates of *P. aeruginosa* in our study could not produce biofilms in vitro. So it deserves further study to explore the biofilm-forming capacity of the *P. aeruginosa* isolates collected from CL, IOL, glaucoma tubes, scleral buckle, or other ocular biomaterials, and vitreous and aqueous humors, or other different ocular tissues as well. On the other hand, our results are based on the experiments in vitro, so the estimation of the ability of *P. aeruginosa* to form biofilms in vivo may be different and requires further study. Previous reports suggested an essential role for the *psl* gene cluster in the initial step of *P. aeruginosa* biofilm formation,[15] so we focused on the functional assignment of the *pslA* gene. In our study, although none of the *P. aeruginosa* strains were biofilm-positive, 9 of 27 (31.03%) strains carried the *pslA* gene, which means that the expression of the *pslA* gene is influenced by other factors, or that the *pslA* gene is unnecessary for the formation of biofilms in bacterial strains taken from patients with ocular infections. In addition, there are reports that the *pel* gene clusters and *alg* gene clusters are also associated with biofilm formation.[41] Biofilm formation may be determined by the interaction of many gene clusters, some of which still have not been identified yet.

In our study of the morphologic features of biofilm-positive *staphylococci*, we found that after a 1 hour incubation, bacterial cells appeared to adhere to the surface, but after a 6 hour incubation, the bacteria covered the surface. These results indicate that it only takes a short time for biofilm-positive strains of *staphylococci* to complete their adherence to a surface. It's this adherence process that is the first and most critical step for biofilm formation. It also suggests that a timely and appropriate intervention strategy be implemented in the early stages of biofilm-mediated infections.

When the percentage of biofilm-positive strains obtained from patients with endophthalmitis, blepharitis, keratitis, and conjunctivitis were compared with different sites, there were no significant differences between the samples, suggesting no difference in the biofilm-forming capability of *S. epidermidis*, even when found at different locations of eye. For *S. aureus*, a higher percentage of keratitis isolates were biofilm positive, according to the microtiter plate test, than those from conjunctivitis isolates. We are unable to explain the percentage differences between the keratitis isolates and the conjunctivitis isolates. Further in vivo study and clinical research are needed to explain this observation.

Ophthalmic isolates of *S. epidermidis* and *S. aureus* could produce biofilms in vitro, whereas clinical strains of *P. aeruginosa* could not. The bacterial strains possess the genetic ability to produce biofilms, but that does not necessarily mean

that biofilms will be produced. On the other hand, the absence of biofilm-related genes could not exclude the phenotypic ability of the strains to develop biofilm in vitro. It only takes a short time for strains of *staphylococci* to complete their adherence to a surface, which suggests that a timely and appropriate intervention strategy be implemented in the early stages of biofilm-mediated infections.

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