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Original Article

Correlation between staphylococcal biofilm formation *in vitro* and potential for catheter-related infections

Salwa Oufrid[1,2], Zineb Ghazlane[2], Loubna Jamali[2], Fatima El Otmani[3], Mustapha Talmi[3], Naima Elmdaghri[2], Khalid Zerouali[1,4], Mohammed Timinouni[2]

[1] *Laboratory of Microbiology, Faculty of Medecine and Pharmacy, Hassan II University, Casablanca, Morocco*
[2] *Molecular Bacteriology Laboratories, Pasteur Institute of Morocco, Casablanca, Morocco*
[3] *Microbiology, Health and Environment Team, Department of Biology, School of Sciences,Chouaib Doukkali University, EL Jadida, Morocco*
[4] *Department of Microbiology, University Hospital Center Ibn Rochd, Casablanca, Morocco*

Abstract

Introduction: The present study evaluated biofilm-forming capacity and the presence of both *icaA* and *icaD* genes among staphylococcal strains isolated from catheter-related infections and blood culture.

Methodology: Ninety staphylococcal isolates, which included 45 strains of catheter infection origin and 45 strains of blood culture origin, were tested for their ability to produce biofilm using microtiter test plates and a catheter test. The presence of *icaA* and *icaD* genes was determined by polymerase chain reaction (PCR).

Results: Of the 45 strains of catheter infection origin, 22 (48.88%) formed biofilm. In comparison, only 10 (22.22%) of the 45 strains of blood culture origin formed biofilms. Similar results were obtained from both the microplate test and catheter test. In the 32 strains that were able to form biofilm, 30 were positive for *icaA* and *icaD* genes, and the remaining 2 strains were negative for both genes. Fifteen staphylococcal strains of all origins presented only the *icaA* locus and did not form biofilm. In 88 of 90 tested strains (97.77%), there was a positive correlation between biofilm production and presence of *icaA* and *icaD* genes, and between no biofilm production and absence of both or only one of the tested genes.

Conclusions: The ability of staphylococcal isolates to form biofilm *in vitro* appears to be an indication of a virulence trait that enhances the ability of isolates to cause catheter-related infections. In addition, our results indicate an important role of *ica* genes and phenotypic variability of biofilm production as virulence factors in staphylococcal infections.

Key words: catheter; biofilm; *ica* operon; *Staphylococcus.*

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Introduction

Staphylococci are recognized as the most common species of bacteria that are responsible for causing biofilm-associated infections [1,2]. These bacteria are generally associated with chronic infections related to catheters and other indwelling medical devices [3]. The predominant species isolated in these infections are *Staphylococcus epidermidis* and *Staphylococcus aureus*. The major pathogenic factor in *Staphylococcus* is their ability to form biofilm on polymeric surfaces to which they adhere and colonize artificial materials [4].

Formation of a biofilm can represent a persistent source of infection by microorganisms that in turn enhanced antimicrobial resistance as well as increased protection from host defenses [5].

Molecular studies haves shown that staphylococcal biofilm formation is mediated by polysaccharide intercellular adhesion (PIA), synthesized by products of the *icaADBC* operon [6,7]. Once this operon is activated, four proteins are transcribed, IcaA, IcaD, IcaB, and IcaC. The *icaA* gene encodes the enzyme N-acetylglucosaminyl transferase, which catalyzes the synthesis of poly-N-acetylglucosamine polymer. The expression of the *icaA* gene generates only a low enzymatic activity. However, when *icaA* is co-expressed with *icaD*, the N-acetylglucosaminyl transferase activity increases significantly [8,9]. *IcaB* is the deacetylase responsible for the deacetylation of mature PIA. In addition, the transmembrane protein IcaC seems to be involved in externalization and elongation of the growing polysaccharide [10].

The aim of this study was to compare the biofilm-forming characteristics of blood culture isolates and the catheter-related infection isolates in order to determine whether this trait may contribute to staphylococcal virulence. We further evaluated the correlation between biofilm production by clinical isolates and the presence of *icaA* and *icaD* genes.

Methodology

Clinical strains

The present study focused on 90 *Staphylococcus* spp. obtained from the university hospital center (CHU) Ibn Rochd of Casablanca. These included 45 strains isolated from catheter-related infections and 45 strains isolated from blood cultures.

Identification

The microbiological analysis of the removed catheter was performed using the Brun-Buisson technique as described by Brun-Buisson *et al*. [11], which consists of rinsing the catheter lumen with sterile water and vortexing its intravesical end before cultivation on Chapman agar medium, which allows the selection of staphylococci.

Moreover, all isolates (catheter infection origin and blood culture origin) were identified by classic microbiological methods including colony morphology, Gram staining, catalase test, coagulase test, and the API Staph test.

Microtiter polystyrene plate test

The capacity to form biofilms was assayed in 96-well microtiter polystyrene plates as described by O'Toole *et al*. [12]. Briefly, cells were grown on liquid LB media in 96-well microplates, and incubated at 37°C. Then, the content of each well was aspirated, and each well was washed three times with water. The plates were vigorously shaken in order to remove all non-adherent bacteria. The remaining attached bacteria were fixed with 0.1% crystal violet for 20 minutes. The excess crystal violet dye was washed by rinsing the wells with water, the amount of biofilm formed was estimated by solubilization of the dye with 95% ethanol, and the optical density (OD) was determined at 540 nm using a microplate reader. All isolates were tested in triplicate. Biofilm production was considered high, moderate, or non/weak, as shown in Table 1.

Catheter test

In vitro experiments assessed the ability of staphylococcal isolates to adhere to catheter tubing.

Starting from an overnight liquid culture, dilutions containing approximately 10^7-10^8 CFU/mL were made. One-centimeter pieces of sterile catheter were inoculated with 200 µL of these dilutions in a microtiter plate. The plate was covered and incubated at 37°C for 24 hours. Then, the content of each well was aspirated, and unattached bacterial cells were removed by recurrent washing with water. The adherent cells were stained with 220 µL of 0.1% crystal violet solution for 20 minutes. After the incubation period, wells that contained catheters were washed with sterile distilled water to remove any loosely associated or planktonic bacteria. The plates were air-dried, and each catheter was transferred to a new polystyrene microtiter dish. Quantitative assessment of biofilm formation was obtained by extracting the crystal violet with 220 µL of 95% ethanol; this dissolved the bound crystal violet and produced a violet-colored solution in each well. The intensity of coloration was determined by measuring the absorbance at 540 nm using a microplate reader. All isolates were tested in triplicate.

Bacterial DNA extraction

DNA templates for the polymerase chain reaction (PCR) process were generated by suspending an overnight staphylococcal culture derived from five colonies each of clinical isolates growing on Luria Bertani agar (Bio-Rad, Marnes-la-Coquette, France) in 500 µL of DNase and RNase-free water (Invitrogen, Carlsbad, USA). The suspension was boiled at 100°C for 10 minutes in a thermal block (Polystat 5, Illkirch-Graffenstaden, France), centrifuged at 15,000 rpm for 5 minutes. An aliquot of 1 µL of the supernatant was used as a DNA template for PCR.

Detection of icaA and icaD loci

The presence of *icaA* and *icaD* DNA were detected by PCR using forward and reverse primers

Table 1. Classification of bacterial adherence by microtiter plates method

Biofilm formation	Adherence	Mean OD values
Non/weak	Non/weak	< 0.120
Moderate	Moderately	0.120–0.240
High	Strong	> 0.240

for *icaA* and *icaD*. For *icaA*, the forward primer (corresponding to nucleotides 1337–1356) had the sequence 5'-TCT CTT GCA GGA GCA ATC AA-3'; the reverse primer (corresponding to nucleotides 1505–1524) had the sequence 5'-TCA GGC ACT AAC ATC CAG CA-3'. The primer sequences for *icaD* were: forward (nucleotides 1963–1982), 5'-ATG GTC AAG CCC AGA CAG AG- 3'; reverse (nucleotides 2138–2160), 5'-CGT GTT TTC AAC ATT TAA TGC AA-3'. The PCR reaction volume was in 25 uL, containing the above-mentioned primers (1 μM each), 150 ng of the extracted DNA, 100 uM each of dATP, dCTP, dGTP, and dTTP, 1 U of *Taq* DNA polymerase, and buffer (10 mM Tris-HCl [pH 9.0], 50 mM KCl, 0.1% Triton X-100, and 2.5 mM $MgCl_2$). The PCR amplification protocol for both *icaA* and *icaD* was as follows: incubation at 94°C for 5 minutes, followed by 50 cycles at 94°C for 30 seconds (denaturation), 55.5°C for 30 seconds (annealing), 72°C for 30 seconds (extension), and 72°C for 1 minute after the conclusion of the 50 cycles. After the first 30 cycles, a further 1 U of *Taq* DNA polymerase was added.

Results

Detection of biofilm formation

Phenotypic production of biofilm by all strains under study was assessed by a catheter test and microtiter plate test. Table 2 shows data related to phenotypic characteristics of the 90 tested strains. Thirty-two strains were positive both to the catheter test and microtiter plate test. The remaining 58 strains were non-biofilm forming. The results on biofilm formation for catheters showed good correlation with those obtained for microtiter plate. Results of comparison between the two infection types suggest that catheter-related infection isolates (22 strains, 48.88%) produced significantly more biofilm than did blood culture isolates (10 strains, 22.22%).

PCR detection of icaA *and* icaD *loci*

The PCR technique was applied to the 90 staphylococcal strains. The *icaA* and *icaD* genes were detected in 30 of 90 (33.33%) strains, giving a 188 base pair band for the *icaA* gene and a 198 base pair band for the *icaD* gene. Twenty-two strains (48.80%) of catheter-related infection origin were positive for *icaA* and *icaD* genes, compared to 8 strains (17.77%) of blood culture origin (Table 3). Moreover, 15 staphylococcal strains of all origin presented only the *icaA* loci.

Relationships between presence of the ica operon and phenotype

Thirty-two staphylococcal strains showed the ability to form biofilm (Table 3). Among these, 30 were positive for *icaA* and *icaD* genes. The remaining two strains were *icaA* and *icaD* negative. Fifteen isolates that were *icaA* positive and *icaD* negative were unable to produce biofilm. Two isolates showed biofilm production, even though *icaD* and *icaA* genes were not present. In 88 of 90 tested strains (97.77%),

Table 2. Biofilm production among clinical isolates using microtiter plate method and catheter method

Strain origin	Strain	Catheter test			Microtiter plate test		
		High	Moderate	Weak/Non	High	Moderate	Weak/Non
Catheter-related infections (45 strains)	*S. epidermidis* (32 strains)	5	7	20	4	8	20
	S. aureus (13 strains)	6	4	3	3	7	3
Blood culture (45 strains)	*S. epidermidis* (38 strains)	3	4	31	1	6	31
	S. aureus (7 strains)	2	1	2	1	2	2

Table 3. Correlation between biofilm production and the presence of *icaA* and *icaD* genes

Strain origin	Strain	Biofilm producer (32 strains)				Non-biofilm producer (58 strains)			
		icaA +	*icaA* -	*icaD*+	*icaD*-	*icaA* +	*icaA* -	*icaD*+	*icaD*-
Catheter-related infections	*S. epidermidis*	12	0	12	0	5	15	0	20
	S. aureus	10	0	10	0	1	2	0	3
Blood culture	*S. epidermidis*	7	0	7	0	5	26	0	31
	S. aureus	1	2	1	2	4	0	0	4

there was a correlation between biofilm production and presence of *icaA* and *icaD* genes, and between no biofilm production and absence of both or only one of the tested genes.

Discussion

Catheter-related infections are frequent complications among hospitalized patients; in many cases, these infections are caused by staphylococci. The ability of these pathogens to form biofilm on medical devices is one of their main virulence traits and plays a crucial role in the induction of severe nosocomial infections in hospitals [13].

Almost 36% of the tested strains were biofilm producers. Results of a comparison between the two infection types showed that the isolates from catheter-related infections produced significantly more biofilm than did blood culture isolates. Gad *et al*. [14] reported that staphylococci isolated from catheter segments showed a higher extent of biofilm production than did the strains isolated from urine samples.

Among the tested clinical strains, 97.77% showed a correlation between biofilm production and presence of *icaA* and *icaD* genes, and between no biofilm production and absence of both or only one of the tested genes. This is in agreement with Liberto *et al*. [15], who reported a similar correlation in their study.

However, the presence of *icaA/D* genes was not always associated with *in vitro* formation of biofilm. Ruzicka *et al*. demonstrated that 20% of strains with *ica* genes did not express phenotype [16].

Two clinical strains of *S. aureus* were found to be biofilm producers but negative for *icaA* and *icaD* genes. The detection of biofilm production, despite the absence of the *ica* genes, could be attributed to the existence of alternative mechanisms to induce biofilm development. One of the findings in recent studies on *S. aureus* biofilms was that a number of surface proteins are able to induce biofilm development/accumulation in the absence of exopolysaccharides [17-19] such as SasG [20]. It has been described to exert its action during the biofilm accumulation phase. A further *S. aureus* protein factor having a role in cell aggregation and biofilm production is SasC, probably implicated in the infection pathogenesis during the colonization phase [21]. The biofilm-forming ability of some isolates in the absence of *icaA/D* genes highlights the importance of further genetic investigations of *ica*-independent biofilm formation mechanisms.

Fifteen isolates that were *icaA* positive and *icaD* negative were unable to produce biofilm. An important contribution to cell-cell adhesion and biofilm formation is the production of polysaccharide intercellular adhesin (PIA), whose synthesis is mediated by the intercellular adhesion (*icaADBC*) locus [6]. Expression of only *icaA* induces a low enzyme activity, while *icaA* and *icaD* co-expression leads to a significant increase in enzyme activity [22,23]. Several studies have shown that formation of biofilm in staphylococci causing catheter-associated infections is associated with the presence of both *icaA* and *icaD* genes [23,24].

Conclusions

The ability of staphylococcal isolates to form biofilm *in vitro* appears to be a marker of a virulence trait that enhances the ability of isolates to cause catheter-related infections. Moreover, these results also indicate an important role of *ica* genes and phenotypic variability of biofilm production as virulence factors in staphylococcal infection. We suggest that a study of the presence and expression of *ica* genes may help in clarifying the relevance of the different adhesion mechanisms in the pathogenesis of staphylococcal infections.

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Corresponding author

Mohammed Timinouni
Molecular Bacteriology Laboratory, Pasteur Institute of Morocco
1 Place Louis Pasteur
Casablanca (20360), Morocco
Phone: + (212) 05 22 43 44 50
Fax: + (212) 05 22 26 09 57
Email: mohammed.timinouni@pasteur.ma

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