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Staphylococcus epidermidis and *Staphylococcus haemolyticus*: detection of biofilm genes and biofilm formation in blood culture isolates from patients in a Brazilian teaching hospital

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ABSTRACT

Infections with coagulase-negative staphylococci are often related to biofilm formation. This study aimed to detect biofilm formation and biofilm-associated genes in blood culture isolates of *Staphylococcus epidermidis* and *S. haemolyticus*. Half (50.6%) of the 85 *S. epidermidis* isolates carried the *icaAD* genes and 15.3% the *bhp* gene, while these numbers were 42.9% and 0 for *S. haemolyticus*, respectively. According to the plate test, 30 *S. epidermidis* isolates were biofilm producers and 40% of them were strongly adherent, while only one (6%) of the 17 *S. haemolyticus* biofilm-producing isolates exhibited a strongly adherent biofilm. The concomitant presence of *icaA* and *icaD* was significantly associated with the plate and tube test results ($P \leq 0.0004$). The higher frequency of *icaA* in *S. epidermidis* and of *icaD* in *S. haemolyticus* is correlated with the higher biofilm-producing capacity of the former since, in contrast to IcaD, IcaA activity is sufficient to produce small amounts of polysaccharide. Although this study emphasizes the importance of *icaAD* and *bhp* for biofilm formation in *S. epidermidis*, other mechanisms seem to be involved in *S. haemolyticus*.

1. Background

Coagulase-negative staphylococci (CoNS), particularly *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*, are considered important nosocomial agents of medical device-associated infections (Vuong and Otto, 2002). Biofilm production is one of the main factors involved in the pathogenesis of these infections (Huebner and Goldmann, 1999). The biofilm permits the adherence and persistence of bacteria in foreign materials. Furthermore, bacteria organized in biofilms are protected from antimicrobials and from the host immune system (Mack et al., 2007).

The steps of biofilm production in *S. epidermidis* include adherence, in which adhesive proteins such as autolysin and adhesin (AtlE and Aae), Fbe/Sdrg, Embp, and lipase GehD play important roles (Mack et al., 2007). Polysaccharide intercellular adhesin (PIA), encoded by the *icaADBC* locus, is the main component of the accumulation step(Mack et al., 2007).The glycosyltransferase activity of PIA is increased when the *icaD* gene is co-transcribed with the *icaA* gene. Protein IcaC permits the production of complete oligomers and IcaB plays a role in the deacetylation of the exported carbohydrate, facilitating intercellular adhesion. In *ica*-negative strains, biofilm production is mediated by accumulation-associated protein (Aap) or by biofilm-associated protein (Bap) and the Bap homologue protein (Bhp) found in *S. epidermidis* (Ziebuhr et al., 2006). These molecules are involved in intercellular aggregation, in which Aap is associated with the secretion of protein-based biofilms, while Bap and Bhp are involved in the detachment of biofilm cells (Rohde et al., 2005; Tormo et al., 2007). However, the similarities and differences in the biofilm produced by *S. epidermidis* and *S. haemolyticus* remain unclear.

Therefore, the aim of this study was to characterize blood culture isolates of *S. epidermidis* and *S. haemolyticus* regarding the presence of the biofilm genes *icaA*, *icaD* and *bhp* and biofilm formation evaluated by two phenotypic methods.

2. Material and methods

2.1. Isolates

The isolates were obtained from blood cultures of inpatients admitted to the University Hospital of the Botucatu Medical School (Hospital das Clínicas, Faculdade de Medicina de Botucatu – HC-FMB), Paulista

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Table 1
Primer sequence, TaqMan® probe, and amplicon size.

Name	Product	Sequence	Reference	Amplicon size (bp)
icaA-1	IcaA	5′-AAAGATGTAGGTTATTGGGATACTGACA-3′	10	-
icaA-2		5′-CATAGAGCACGTGGTTCGTACTTAA-3′		
icaA probe TaqMan®		**FAM-5**′-TGCTGTTTCATGGAAACTCCATCTTTTTGATTA- **3**′-**TAMRA**		
icaD-1	IcaD	5′-ATCGTTGTGATGATTGTTTA-3′	11	-
icaD-2		5′- GATATGTCACGACCTTTCTT-3′		
bhp-1	Bhp	5′-ATGAAAAATAAACAAGGATTTC-3′	12	1278
bhp-2		5′-GCCTAAGCTAGATAATGTTTG-3′		

State University (Universidade Estadual Paulista – UNESP), Botucatu Campus, between 2000 and 2011. Only one isolate per patient was included in the study. The isolates were isolated as described by Koneman et al. (1997).

2.2. Species identification

The genus *Staphylococcus* was identified as described by Koneman et al. (1997). *Staphylococcus epidermidis* and *S. haemolyticus* were identified by the simplified method proposed by Cunha et al. (2004). Species identification was genetically confirmed by PCR amplification of the 16S-23S internal transcribed spacer (ITS) region as described by Couto et al. (2001) after DNA extraction with the Illustra kit (GE Healthcare, Little Chalfont, UK). The following international reference strains were used as controls: *S. epidermidis* (ATCC 12228), *S. epidermidis* (ATCC 35983), and *S. haemolyticus* (ATCC 29970).

2.3. Detection of the biofilm-associated genes icaA and icaD

The protocol proposed by Vandecasteele et al (Vandecasteele et al., 2003) was used for detection of the *icaA* gene by real-time PCR in the StepOnePlus® (Life) system. The reaction mixture contained 2 μL nucleic acids, 12.5 μL of 2× TaqMan® Fast Advanced Master Mix (PE Applied Biosystems), 900 nmol/L of each primer, and 200 nmol/L of the probe in a final volume of 25 μL. Parameters included initial holding at 50 °C for 2 min, denaturation for 20 s at 95 °C, 40 cycles of 1 s at 95 °C, and 20 s at 60 °C. For *icaD*, the primers described by Tan et al., (2012) were used in a reaction mixture containing 4 μL DNA, 0.3 μM of each primer, and 10 μL of 2× Fast Syber Green® Master Mix in a final volume of 20 μL. After initial denaturation at 95 °C for 20 s, 40 cycles at 95 °C for 3 s and annealing and extension at 60 °C for 30 s were performed. After amplification, the dissociation curve was analyzed to verify the specificity of the reactions (*icaD*: Tm = 69 ± 2 °C). The primer sequences and TaqMan® probe are shown in Table 1.

2.4. Detection of the biofilm-associated gene bhp

The reactions for detection of the *bhp* gene were performed according to Qin et al (Qin et al., 2007). The primers are described in Table 1.

2.5. Investigation of biofilm production by adherence to borosilicate test tubes

Biofilm production was evaluated using the tube adherence test proposed by Christensen et al (Christensen et al., 1982). Blood agar colonies were inoculated into tryptic soy broth (TSB) containing 2% glucose. Trypan blue (Sigma) was used for staining. The presence of a layer of stained material adhered to the inner wall of the tubes was defined as a positive result. The exclusive presence of a stained ring at the liquid-air interface was not classified as positive.

2.6. Investigation of biofilm production by adherence to polystyrene plates (Christensen et al., 1985)

Biofilm production was evaluated on polystyrene plates as proposed by Christensen et al (Christensen et al., 1985) and modified by Oliveira and Cunha (Oliveira and Cunha, 2010), using optical density readings of the adherent material produced by bacteria. Three to five colonies of each isolate were cultured for 22 h in TSB plus 2% glucose, adjusted to a 0.5 McFarland standard (corresponding to 1.5×10^8 CFU/mL), and diluted 1:1 in TSB-2% glucose. This suspension was transferred to polystyrene plates and incubated for 24 h at 37 °C. The plates were washed with phosphate-buffered saline, dried, and stained with crystal violet. The cutoff was calculated according to the formula of Christensen et al. (Qin et al., 2007) using a 540-nm filter. The isolates were classified into 3 categories: non-adherent, optical density ≤0.111; weakly adherent, optical density >0.111 or ≤0.222; strongly adherent, optical density >0.222.

2.7. Statistical analysis

The chi-square test was used to verify the association between variables, adopting a level of significance of <0.05. kappa statistic was used to evaluate agreement between methods.

3. Results

A total of 169 isolates were analyzed, including 85 *S. epidermidis* and 84 *S. haemolyticus*. Table 2 shows the results of biofilm gene detection (*ica* and *bhp*) and biofilm formation evaluated by the adherence tests.

Table 2
Positivity of *Staphylococcus epidermidis* and *S. haemolyticus* isolates for *icaAD* and *bhp* and phenotypic biofilm production evaluated by adherence to polystyrene plates and borosilicate tubes.

Isolates (n)	Biofilm genes								Biofilm production							
	Polysaccharide genes						Protein gene		Plate test						Tube test	
	*icaA**		*icaD***		*icaAD*		*bhp*		SA		WA		NA		Positive	Negative
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n %	n %
S. epidermidis (85)	24	28.2	0	0	43	50.6	13	15.3	12	14.1	18	21.2	55	64.7	30 35.3	55 64.7
S. haemolyticus (84)	2	2.4	35	45.7	36	42.9	0	0	1	1.2	16	19	67	79.8	18 21.5	66 78.6
Total (169)	26	13.8	35	20.7	79	46.7	13	7.7	13	7.7	34	20.1	122	72.2	48 28.4	121 71.6

SA = strongly adherent; WA = weakly adherent; NA = non-adherent.
*icaA** = *icaA* alone.
*icaD*** = *icaD* alone.