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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*.

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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.

Table 3
ica and *bhp* gene profile of *Staphylococcus epidermidis* and *S. haemolyticus* isolates that produced a strongly and weakly adherent biofilm.

	SA				WA			
	icaAD	*icaA**	*icaD***	*bhp*	*icaAD*	*icaA**	*icaD***	*bhp*
S. epidermidis	10	1	0	1	13	3	0	2
S. haemolyticus	1	0	0	0	9	0	6	0
Total	11	1	0	1	22	3	6	2

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

A strongly adherent biofilm was detected in only one *S. haemolyticus* isolate, while 12 (14.1%) *S. epidermidis* isolates produced a strongly adherent biofilm in the plate test. In the borosilicate tube test, the prevalence of biofilm formation was 35.3% in *S. epidermidis* and 21.5% in *S. haemolyticus*.

Most *S. epidermidis* isolates carried the *icaA* gene (78.8%), while 84.5% of *S. haemolyticus* carried *icaD*. The exclusive presence of *icaA* was observed in 28.2% and 2.4% of the *S. epidermidis* and *S. haemolyticus* isolates, respectively. These frequencies were 0% and 45.7% for *icaD*.

It was observed that 94.9% of the *S. haemolyticus* isolates carrying *icaA* also carried *icaD*, and *icaA* was present in all *icaD*-positive *S. epidermidis* isolates. Eleven of the 84 *S. haemolyticus* isolates and 18 of the 85 *S. epidermidis* isolates were *ica* negative, corresponding to 29 (15.5%) isolates that were negative for these genes. The *bhp* gene was detected only in *S. epidermidis* isolates (15.3%).

Only 4 isolates (1 *S. haemolyticus* and 3 *S. epidermidis*) were biofilm producers, but *icaA* or *icaD* negative. Forty-six isolates (26 *S. haemolyticus* and 20 *S. epidermidis*) were positive for *icaAD* and biofilm negative. Among all isolates classified as strongly adherent ($n = 13$, 7.7%), only one (0.6%) did not carry *icaA* or *icaD* and one (0.6%) carried only *icaA* but was *bhp* positive. Table 3 shows the gene profile of isolates that were strongly and weakly adherent in the plate test.

The concomitant presence of *icaA* and *icaD* was significantly associated with adherence. in the polystyrene plate test ($P = 0.0004$) and borosilicate tube test ($P \leq 0.0001$). This association was also observed for *S. epidermidis* ($P = 0.0015$ and $P = 0.0004$, respectively) when the two species were analyzed separately, but not for *S. haemolyticus*.

kappa agreement between the phenotypic methods was 0.9854 for all isolates, 1.0 for *S. epidermidis*, and 0.9639 for *S. haemolyticus*.

4. Discussion

The pathogenesis of CoNS infections in medical devices is mainly due to the ability of these microorganisms to produce a biofilm. In the present study, biofilm formation was found in 35.3% of the *S. epidermidis* isolates. Higher (Oliveira and Cunha, 2010; Ninin et al., 2006) and lower (de Silva et al., 2002) rates of biofilm formation have been reported for that species depending on the origin and localization of the isolates.

The *bhp* gene was detected in 13 (15.3%) of the *S. epidermidis* isolates. Bowden et al., (2005) detected this gene in only 9%, 0%, and 13% of bacteremia, blood culture contaminants, and skin isolates, respectively. Studies have shown that Bhp is important for the formation of a strongly adherent biofilm (Bowden et al., 2005). However, in the present study, only three of the 13 isolates carrying *bhp* were able to produce a detectable biofilm. Other molecules may play a more important role in strongly adherent biofilms of blood culture isolates. Further studies are needed to determine the function of Bhp in the establishment of a mature and functional biofilm.

The frequency of *S. haemolyticus* biofilm producers was 21.5%. In contrast, Fredheim et al. (2009), using the same phenotypic method, detected 74% of biofilm producers. According to these authors, *S. haemolyticus* mainly produces a PIA-independent biofilm. Our results confirm this hypothesis since the *ica* genes were significantly associated with biofilm formation in *S. epidermidis*, but not in *S. haemolyticus*. As in *S. haemolyticus*, PIA-independent biofilms have also been described in *S. epidermidis*, *S. aureus*, and *S. lugdunensis* (Qin et al., 2007; Fitzpatrick et al., 2005; Frank and Patel, 2007). The *bhp* gene was also not detected in any of the *S. haemolyticus* isolates, suggesting that this gene does not contribute relevantly to the protein components of *S. haemolyticus* biofilms, different from S. *epidermidis*.

It is assumed that staphylococci isolated from infected sites must be able to form a biofilm, produce PIA, and contain the *ica* operon. However, Fitzpatrick et al. (2005) suggested that the presence of the *ica* locus alone is not sufficient for biofilm formation. We found 62.1% of isolates carrying *icaA*, including 78.8% of *S. epidermidis* and 45.2% of *S. haemolyticus* isolates, while the *icaD* gene was detected in 67.5% of CoNS, including 50.6% of *S. epidermidis* and 84.5% of *S. haemolyticus* isolates. In contrast, other authors described only four of 72 *S. haemolyticus* isolates carrying *icaD* (Fredheim et al., 2009). Both *icaA* and *icaD* were detected in 50.6% of *S. epidermidis* and 42.9% of *S. haemolyticus* isolates, respectively. Similar results have been reported by Oliveira and Cunha (Oliveira and Cunha, 2010) who observed 40% of *icaAD* positivity. Other studies found *icaD* in 77% of CoNS and *icaA* in 27.8%, most of them *S. epidermidis* (Hernández et al., 2010).

Although exhibiting weak *N*-acetylglucosaminyl transferase activity, the IcaA protein plays an important role in the synthesis of PIA since IcaD and the other components of the Ica system do not have exclusive transferase activity (Gerke et al., 1998). We may suggest the presence of *icaA* in most of the *S. epidermidis* isolates, in spite of *S. haemolyticus*, to be associated with the larger number of biofilm-producing *S. epidermidis* strains, although this association was not statistically significant. The role of *icaD* in *S. haemolyticus* isolates should be investigated since this gene apparently showed no correlation with the *S. haemolyticus* biofilm, although it was detected at a high frequency in that species.

Eleven of the 13 isolates classified as strongly adherent carried *icaAD*. De Silva et al (de Silva et al., 2002) described an association between the *ica* operon and quantitative biofilm formation, while other authors could not find such association in clinical isolates of *S. epidermidis* (Ninin et al., 2006; de Silva et al., 2002). In the present study, the concomitant presence of *icaAD* was significantly associated with biofilm production, highlighting the important role of these genes in biofilm formation. Furthermore, genes involved in biofilm formation have been suggested as potential markers of clinically relevant isolates. It is believed that isolates carrying the *ica* genes possess a competitive advantage in medical device colonization, especially in the case of long-term catheters (Ninin et al., 2006).

In our study, four isolates did not produce a biofilm, regardless of the concomitant presence of the *icaA* and *icaD* genes. The expression of *ica* mRNA has been demonstrated in non-biofilm-producing *S. epidermidis* and in isolates that do not produce a biofilm or produce an undetectable biofilm under experimental conditions (Dobinsky et al., 2003). Apparently, PIA production is subjected to the on/off activity of its coding genes that may be involved in a phase variation in *S. epidermidis*, conditions that facilitate bacterial survival and growth under changing environmental conditions *in vivo* (Ziebuhr et al., 1999). Moreover, evidence supports the hypothesis of dissemination of new clones referred to as biofilm negative, but positive for both *icaA* and *icaD* (Arciola et al., 2001).

The kappa agreement between the polystyrene plate method and the borosilicate tube test was 0.9854, similar to the value reported by Oliveira and Cunha (Oliveira and Cunha, 2010).The tube test has shown low sensitivity in the detection of biofilm formation in other studies (Hassan et al., 2011). However, its routine use is justified by its high efficacy in detecting biofilms, its easy and practical application, and good results obtained in this and in other studies (Cunha et al., 2006). The addition of glucose to the TSB medium is indicated since it increases the sensitivity of detection (Kim, 2001).

Differences in biofilm formation related to the presence of associated genes and even between phenotypic methods may be due to the influence of different culture media, pH, temperature, and osmotic pressure.

Possible differences between *in vitro* and *in vivo* biofilm formation may be related to factors such as stress and molecules released by the host immune system.

5. Conclusions

The biofilm of *S. epidermidis* seems to be associated with the presence of the *icaA* gene and, to a lesser extent, with *bhp*. The *ica* and *bhp* genes do not seem to play an important role in biofilm production by *S. haemolyticus*, although the *icaD* gene was found at a high frequency in that species. Biofilm production is an indicator of the potential persistence of clinical strains of *S. epidermidis* and *S. haemolyticus*.

Conflicts of interest

None to declare.

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