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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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 ≤ 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 icaA, 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 icaA/icaD 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 deacytlation 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...
2.4. Detection of the biofilm sequences and TaqMan® probe are shown in Table 1.

After amplification for 3 s and annealing and extension at 60 °C for 30 s were performed.

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 ica gene by real-time PCR in the StepOnePlus® (Life) system. The reaction mixture contained 2 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 denaturation 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 evaluation by the adherence tests.

<table>
<thead>
<tr>
<th>Isolates (n)</th>
<th>Biofilm genes</th>
<th>Biofilm production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polysaccharide genes</td>
<td>icaA*</td>
<td>icaA**</td>
</tr>
<tr>
<td>n %</td>
<td>n %</td>
<td>n %</td>
</tr>
<tr>
<td>S. epidermidis (85)</td>
<td>24 28.2</td>
<td>0 0</td>
</tr>
<tr>
<td>S. haemolyticus (84)</td>
<td>2 2.4</td>
<td>35 45.7</td>
</tr>
<tr>
<td>Total (169)</td>
<td>26 13.8</td>
<td>35 20.7</td>
</tr>
</tbody>
</table>

SA = strongly adherent; WA = weakly adherent; NA = non-adherent.

icaA* = icaA alone.
icaA** = icaA alone.
icaD* = icaD alone.
Table 3

<table>
<thead>
<tr>
<th></th>
<th>SA</th>
<th>WA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>icaAD icA* icA** bhp</td>
<td>icaAD icA* icA** bhp</td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>10 1 0 1</td>
<td>13 3 0 2</td>
</tr>
<tr>
<td>S. haemolyticus</td>
<td>1 0 0 0</td>
<td>9 0 0 0</td>
</tr>
<tr>
<td>Total</td>
<td>11 1 0 1</td>
<td>22 3 6 2</td>
</tr>
</tbody>
</table>

SA = strongly adherent; WA = weakly adherent. icA* = icA alone. icA** = icA 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 icA gene (78.8%), while 84.5% of S. haemolyticus carried icAD. The exclusive presence of icA 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 icA also carried icAD, and icA was present in all icA-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%).

Only 4 isolates (1 S. haemolyticus and 3 S. epidermidis) were biofilm producers, but icA or icAD negative. Forty-six isolates (26 S. haemolyticus and 20 S. epidermidis) were positive for icA and icAD and biofilm negative. Among all isolates classified as strongly adherent (n = 13, 7.7%), only one (0.6%) did not carry icA or icAD 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 icA and icAD was significantly associated with adherence, in the polystyrene plate test (P = 0.0004) and borosilicate tube test (P = 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 icA 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 icAD positivity. Other studies found icAD in 77% of CoNS and icA in 27.8%, most of them S. epidermidis (Hernández et al., 2010).

Although exhibiting weak N-acetylmuramoylalanine 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 icAD. 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 icA and icAD 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 icA 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 <i>S. epidermidis</i> seems to be associated with the presence of the ica<sup>a</sup> 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 <i>S. haemolyticus</i>, although the ica<sup>d</sup> gene was found at a high frequency in that species. Biofilm production is an indicator of the potential persistence of clinical strains of <i>S. epidermidis</i> and <i>S. haemolyticus</i>.

Conflicts of interest

None to declare.

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References


