Full Length Research Paper

Biofilm production by clinical isolates of *Staphylococcus epidermidis* and its relationship with genotypic profile, presence of virulence-related genes and antibiotic resistance

Renato G. Silva Filho¹,²*, Agostinho A. Lima e Silva², Carmen S. M. Saramago², Cleonice A. M. Bento², Isabel S. Souza², Maria José Souza³, Karyne Rangel Carvalho⁴ and Maria Helena S. Villas Bôas⁴

¹Programa de Pós-Graduação em Vigilância Sanitária, INCQS, FIOCRUZ, Rio de Janeiro, Brasil.
²Departamento de Microbiologia e Parasitologia, Instituto Biomédico, Universidade Federal do Estado do Rio de Janeiro, Brasil.
³Hospital Federal dos Servidores do Estado, Rio de Janeiro, Brasil.

Received 19 April, 2014; Accepted 23 February, 2015

*Staphylococcus epidermidis* is considered as the main infectious agent associated with implanted medical devices. This study determined biofilm production and composition, pulsed-field gel electrophoresis (PFGE) profile, antimicrobial susceptibility and presence of virulence-related genes (*ica* operon, *aap*, *bhp*, *embp*, *capB* and IS256 transposase) in 49 clinical isolates of *S. epidermidis*. Twenty-five isolates (51%) were classified as biofilm producers in microtiter plate (MTP) assay. In Congo red assay (CRA) test, 14 (29%) showed positive reaction and three (6%) had indeterminate reaction, all were biofilm-producers in MTP assay. Fourteen isolates with positive reaction in CRA test had the chemical nature of biofilm determined as polysaccharide, had the *ica* operon (PIA-dependent producers) and the majority was strong biofilm producer. Eight biofilm producer isolates showed negative reaction in CRA test and the chemical nature of their biofilm was proteinaceous (PIA-independent producers). Antimicrobial resistance rates were generally higher in biofilm producers and resistance to beta-lactams ranged from 82-96%, while 61% of the isolates were multidrug resistant (≥ 10 drugs). Resistance to daptomycin, quinupristin/dalfopristin, rifampin and trimethoprim/sulfamethoxazole was observed only in PIA-dependent isolates, while the resistance to gentamicin was present in all PIA-independent isolates and in just 53% of PIA-dependent of isolates. The most prevalent virulence-related genes were *capB* (80%) and *embp* (67%); the other genes were less frequent: *ica* operon (41%), *aap* (31%), IS256 transposase (22%) and *bhp* (10%). The presence of *ica* operon and IS256 transposase gene showed significant association with biofilm production and strong biofilm production. Moreover, these isolates presented significant higher resistance to levofloxacin, moxifloxacin, rifampin and trimethoprim/sulfamethoxazole. PFGE analysis showed 23 profiles, having the prevalent type 15 isolates. Of these, seven were PIA-independent biofilm producers and just one was PIA-dependent producer, unlike what was observed in other studies, where isolates of prevalent profiles were PIA-dependent biofilm producers.

**Key words:** *Staphylococcus epidermidis*, biofilm, *ica* operon, virulence-related genes, IS256, PFGE, multidrug resistant staphylococci.
INTRODUCTION

*Staphylococcus epidermidis* is primarily, a normal inhabitant of the healthy human skin and mucosal microbiota. In recent decades, this species has emerged as a common cause of numerous healthcare associated infections, preferentially affecting immunocompromised, long-term hospitalized and critically ill patients (Mack et al., 2013; Ziebuhr et al., 2006). These infections are often linked to the use of implanted medical devices and take a chronic or persistent course. Moreover, antimicrobial therapy is frequently unsuccessful and removal of the implanted device is often required (Mack et al., 2013). Unlike other pathogens, *S. epidermidis* has a limited set of virulence factors. Biofilm formation was the first one recognized and provides protection against mechanisms of immune defense and antimicrobials (Fey and Olson, 2010).

The most well-studied and prevalent molecule involved in *S. epidermidis* biofilm formation is polysaccharide intercellular adhesin (PIA), synthesized by enzymes encoded by the icaADBC operon. PIA is a homoglycan (Rohde et al., 2010) that can be specifically degraded by hexosaminidase dispersin B (DspB) (Kaplan et al., 2004) or undergo oxidation at linkages between glucosamine residues in the presence of metaperiodate (Wang et al., 2004). Although infrequent and poorly studied, biofilms constituted of proteins may be produced by clinically significant isolates of *S. epidermidis*. Accumulation associated protein (Aap) (Rohde et al., 2005), Bap homologue protein (Bhp) (Tormo et al., 2005) and extracellular matrix-binding protein (Embp) (Christner et al., 2010) have been described as involved in the formation of these PIA-independent biofilms. Mircrotiter plate (MTP) biofilm assay (Christensen et al., 1985) and Congo red assay (CRA) test (Freeman et al., 1989) are the most commonly used methods for detection of *in vitro* biofilm formation. MTP biofilm assay is used to quantitatively measure the optical density of stained bacterial films adhered to wells of a tissue culture plate, and CRA test employs a culture medium that qualitatively differentiates exopolysaccharide-forming isolates.

Another possible virulence factor in *S. epidermidis* is poly-γ-glutamic acid (PGA), an extracellular anionic polymer renowned for being responsible for the pathogenicity of *B. anthracis*. PGA contributes to the resistance of *S. epidermidis* to cationic antibacterial peptides and inhibits phagocytosis, playing a key role in the persistence of *S. epidermidis* during device-related infections (Kocianova et al., 2005).

Some studies have found a high frequency of the insertion sequence IS256 in clinically significant *S. epidermidis*, in comparison with commensal isolates, suggesting that this genetic element may be used to discriminate these isolates (Gu et al., 2005; Koskela et al., 2009; Kozitskaya et al., 2004). The reversible transposition of IS256 into genes of ica operon or their global regulators (rsbU and sarA) determines phase variation in the production of PIA-dependent biofilm (Ziebuhr et al., 1999). This possibility of variation in the expression of virulence associated surface factors is an effective strategy already observed in many pathogens. In addition, IS256 has the capacity to influence antibiotic resistance (Conlon et al., 2004; Hennig and Ziebuhr, 2010).

Pulsed-field gel electrophoresis (PFGE) analysis is a typing method known to provide reliable information on the short-term epidemiology of healthcare associated *S. epidermidis*, mainly when associated with resistance to antimicrobials and virulence markers (Nunes et al., 2005; Cherifi et al., 2014).

The aim of this study was to investigate in clinical isolates of *S. Epidermidis*, the biofilm production, the presence of the major virulence encoding genes (aap, bhp, embp and capB) and IS256 element, resistance to antimicrobials and genotypic profiles, and establish possible interrelationships between them.

MATERIALS AND METHODS

Bacterial isolates

Forty-nine clinical isolates of *S. epidermidis* (45 from blood cultures and four from central venous catheters) were obtained from infected patients, hospitalized between November 2011 and April 2012 at Hospital Federal dos Servidores do Estado (HFSE) in Rio de Janeiro city, Brazil. Blood cultures were processed using the BacT/Alert™ system (bioMérieux) for aerobic and anaerobic bacteria, and central venous catheters were processed by the semi-quantitative roll plate method (Maki et al., 1977). Biofilm non-producing *S. epidermidis* ATCC 12228 and the biofilm producer *S. epidermidis* ATCC 35844 were used as reference strains in phenotypic and genotypic methods. The Human Research Ethics Committee from HFSE approved this study with reference number 000.417.

Species identification and antimicrobial susceptibility analysis

The identification and determination of minimum inhibitory concentrations (MIC) of isolates was performed with MicroScan WalkAway-96 System (Dade Behring Inc.). The drugs tested were: amoxicillin/clavulanic acid (AMC), ampicillin/sulbactan (SAM), ampicillin (AMP), ceftriaxone (CRO), clindamycin (DA), daptomycin (DAP), erythromycin (E), gentamicin (GM), levofloxacin (LVX), linezolid (LZD), moxifloxacin (MXF), oxacillin (OX), penicillin (P), quinupristin/dalfopristin (SIN), rifampin (RD), tetracycline (TE), trimethoprim/sulfamethoxazole (SXT) and vancomycin (VA).

*Corresponding author. E-mail: renato.rgsf@gmail.com.*

Author(s) agree that this article remain permanently open access under the terms of the Creative Commons Attribution License 4.0 International License.
Microtiter plate biofilm assay

Biofilm production was quantified using the MTP assay as previously described (Stepanović et al., 2000). Briefly, an overnight culture grown in tryptone soya broth (TSB - HiMedia) was diluted to 1:100 in fresh medium and 200 μL per well were seeded in 96-well flat-bottomed polystyrene microtiter plate (Nunclon; Nunc A/S). These plates were incubated for 24 h at 35°C. After three washes with distilled water, wells were treated with 200 μL of methanol for 15 min, emptied and air-dried, then stained for 5 min with 200 μL of 2% Hucker's crystal violet solution. Plates were washed under running distilled water, air-dried and optical density (OD) at 570 nm was determined in a microplate reader (TP-Reader - Thermo Plate Devices). After extraction with 200 μL of 95% ethanol for 30 min, the OD of biofilm extract was measured. The average of OD values was determined for all isolates and negative control (S. epidermidis ATCC 12228), all tests being performed in triplicate and repeated three times. The OD cut-off value (ODc) was defined as three standard deviations (SD) above the mean OD of the negative control: ODc = OD average of negative control + (3 x SD of negative control). The OD average of biofilm extract of isolates was used to interpret results: OD ≤ ODc = no producer; ODc < OD ≤ 2xODc = weak producer; 2xODc < OD ≤ 4xODc = moderate producer; 4xODc < OD = strong producer (Stepanović et al., 2007).

Congo red agar test

Congo red agar (CRA) medium was composed of brain heart infusion broth (BHI – Oxoid) 37 g/L, sucrose (Merck) 50 g/L and agar (Agar No 1 – Oxoid) 10 g/L, and prepared as previously described by Freeman et al. (1989). The CRA plates were seeded with 10 μL of overnight TSB cultures by spot plate technique, incubated for 24 h at 35°C and overnight at room temperature. The tests were performed in triplicate and repeated three times. A positive result was considered when the spot was black, with a dry crystalline consistency and/or metallic sheen, negative if its color was light red and an indeterminate result was indicated by a darkening of the spot, but with the absence of a dry crystalline consistency (Freeman et al., 1989).

Biofilm detachment assay

The chemical nature of the biofilm matrix was determined by degradation in a test resembling MTP biofilm assay (Wang et al., 2004). Solutions of two degradation agents in 0.1 M PBS (pH 7.0) were used, 40 mM sodium metaperiodate (Vetec) for polysaccharide-dependent biofilm and 1 mg/mL proteinase K (Sigma) solutions for protein-dependent biofilm. The isolates were grown in a microtiter plate and the wells washed once with distilled water. Then, each well was treated with 200 μL of degrading agents or PBS (control) and the plates were incubated for 2 h at 35°C. The wells were washed twice with distilled water, and next steps follow as described in MTP biofilm assay. Tests were performed in triplicate and repeated three times. A reduction of over 50% in OD average, when compared to the control, of wells treated with degrading agents, indicated the chemical nature of the biofilm.

Detection of virulence-related genes

The DNA of all clinical isolates and reference strains was extracted using a boiling method (Ninini et al., 2006). Bacterial suspensions in distilled water, with a turbidity equivalent to 1.0 McFarland standard, were prepared from grown isolates in tryptone soya agar (TSA - HiMedia). Suspensions were boiled for 5 min, centrifuged at 12,000 xg for 5 min and supernatants were removed to be used as DNA template in polymerase chain reaction (PCR).

Identification of S. epidermidis was carried out using a PCR method to detect species-specific genomic fragment of 705-bp (Martineau et al., 1996). Others genes detected were: icaA, icaB and icaC (Ziebuhr et al., 1999); icaD (de Silva et al., 2002); icaR (Arciola et al., 2004); aap, bhp and embp (Rohde et al., 2004); capB (Kocianova et al., 2005) and IS256 transposase (Gu et al., 2005). PCR reactions were performed with Ampliqon Taq DNA Polymerase 2x Master Mix (1.5 mM MgCl2) (Ampliqon A/S, Denmark), according to the manufacturer’s directions, in a LifePro Thermal Cycler (Hangzhou Bioer Technology Co.). The amplified products were analyzed by agarose (1.5%) gel electrophoresis with GelRed™ and visualized using UV light. Their sizes were estimated by comparison with 100 bp DNA Ladder (Invitrogen - Life Technologies, Canada).

Pulsed field gel electrophoresis (PFGE) analysis

The preparation of agarose disks, containing chromosomal DNA for PFGE and Smal DNA restriction fragments separation, was performed as previously described (Chung et al., 2000). The interpretation of the band patterns obtained was based on the criteria described by Tenover et al. (1995) and the analyses were performed using BioNumerics® software version 5.0 (Applied Maths, Kortrijk, Belgium). A dendrogram of similarity was built using the unweighted pair-group method with arithmetic averages (UPGMA).

Statistical analysis

The degree of association between genes was measured by the significance of the Phi correlation coefficient. Results of antimicrobial susceptibility were compared using Chi-square or Fisher’s exact test. All tests were performed using BioEstat 5.3 (Instituto Mamirauá) with confidence level of 95% (α = 0.05).

RESULTS

Species identification

All isolates were identified as S. epidermidis by using MicroScan System and these results were confirmed by PCR analysis of species-specific 705-bp genomic fragment.

Microtiter plate biofilm assay

In the MTP biofilm assay, 21 (51%) of isolates were sorted as producers. Regarding the level of biofilm of these isolates, 14 were strong, five were moderate and six were weak biofilm producers (Table 1). In the same procedure, the OD before extraction of the dye bound to the biofilm with alcohol was also determined. This method is widely used in various studies to determine the biofilm production and the cut-off point, generally, fixed in 0.1 a 0.12 (Stepanović et al., 2007). With this method, only 35% of isolates were classified as biofilm producers. This difference was due to eight isolates classified as weak (5) and moderate (3) when the OD was used (data not show).
Table 1. Results of biofilm production in MTP biofilm assay, CRA test reaction and detection by PCR of virulence related genes in clinical isolates of S. epidermidis.

<table>
<thead>
<tr>
<th>Biofilm production in MTP assay</th>
<th>Number (%) of clinical isolates</th>
<th>CRA test</th>
<th>Detection by PCR of virulence related genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>positive</td>
<td>negative</td>
</tr>
<tr>
<td>Producer</td>
<td>25 (51)</td>
<td>14 (56)</td>
<td>8 (32)</td>
</tr>
<tr>
<td>Weak</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Level</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strong</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-producer</td>
<td>24 (49)</td>
<td>0</td>
<td>24 (100)</td>
</tr>
<tr>
<td>Total</td>
<td>49 (100)</td>
<td>14 (29)</td>
<td>32 (65)</td>
</tr>
</tbody>
</table>

*, Significant association in Phi correlation coefficient (p < 0.05) between the gene detected and biofilm production or biofilm production level a = Microtiter Plate biofilm assay; b = Congo Red Agar Test; c = IS256 transposase gene

**Congo red agar test**

In the CRA test, 14 (29%) isolates showed positive reaction, 32 (65%) were negative and three (6%) were indeterminate (Table 1). Of the 25 isolates classified as biofilm producers in MTP assay, 14 showed positive reaction in CRA test, eight were negative and three were indeterminate. Of these 14 isolates that had positive reaction in CRA test, 13 were strong biofilm producers and one was a moderate producer. The eight isolates with negative reaction in CRA test that were classified as producers in MTP assay, showed biofilm production levels weak (5) and moderate (3). In turn, each one of the three isolates that had indeterminate reaction in CRA test presented a different level of production in MTP assay.

**Biofilm detachment assay**

The determination of the chemical nature of the 25 biofilm producer isolates showed that 14 (56%) were polysaccharide-dependent and eight (32%) were protein-dependent. In three isolates, the treatment with the two degrading agents did not reach the minimal standard of OD reduction (50%). However, the results of degradation with sodium metaperiodate were very close to the standard. These results, supported by detection of transcription of icaA gene in real time PCR experiments (data not show), led us to assume that the biofilm of these isolates would be polysaccharide-dependent. Therewith, the number of isolates that produced this type of biofilm increased to 17 (68%). In MTP biofilm assay, 14 of these isolates were ranked as strong, two as moderate and one as weak. Furthermore, in CRA test, 14 of these isolates showed positive reaction and three were indeterminate. Regarding the eight protein-dependent biofilm producers, in MTP assay five were weak and three moderate producers, and all presented negative reaction in CRA test.

**Detection of virulence-related genes**

The most prevalent genes were capB (80%) and embp (67%) (Table 1). The icaA, icaB, icaC and icaD genes that form the ica operon, and the regulatory icaR gene were always detected together in 41% of isolates studied. Regarding the other genes searched, the aap gene was detected in 31% of isolates, IS256 transposase gene in 22% isolates and the bhp gene in only 10% of the isolates. The percentage of detection of all genes was higher in producer than in non-producer isolates, except bhp gene that was detect only in biofilm non-producer isolates.

The ica operon genes (ica+) showed a significant association with the presence of aap gene (p= 0.0058), biofilm production (p= 0.0003) and strong biofilm production (p= 0.0006). Likewise, the detection of IS256 transposase gene presented a significant correlation with biofilm production (p = 0.0479) and strong biofilm production (p = 0.0389). Furthermore, the IS256 transposase gene showed full association (p< 0.0001) with ica+ isolates.

Seventeen of the 20 isolates ica+ were biofilm producers including all that were strong (14), two moderate and one weak producers. All of these ica+/biofilm producer isolates had polysaccharide-dependent biofilms (PIA-dependent biofilm).

The eight biofilm producer isolates that did not have the ica operon, presented protein-dependent biofilm (PIA-independent). The search for other genes related to biofilm-production in these isolates detected embp (7) and aap (1) genes.

**Antimicrobial susceptibility analysis**

All isolates were susceptible to linezolid and vancomycin, and just one was resistant to daptomycin. The percentage of antimicrobial resistance was slightly higher in the biofilm producer isolates than in non-producers, except for quinupristin/dalfopristin and tetracycline where the resistance percentage for both antimicrobials was slight
higher in the biofilm non-producers (13 versus 12% and 17 versus 12%, respectively).

The resistance to beta-lactam antibiotics, including their combination with beta-lactamase inhibitors, ranged from 82 to 96% for all isolates, however being always higher in biofilm producers. Specifically in relation to oxacillin, resistance was observed in 84% of all isolates, and higher in biofilm producers (92%) than in non-producers (75%).

In PIA-dependent and independent biofilm producer isolates, the results of resistance to most antimicrobials tested were very close. However, resistance to daptomycin (6%), quinupristin/dalfopristin (18%), rifampin (35%) and trimethoprim/sulfamethoxazole (76%) was observed only in PIA-dependent isolates. On the other hand, resistance to gentamicin was present in all PIA-independent isolates, while resistance to this drug in PIA-dependent isolates was observed in 53% of isolates.

Resistance to 10 or more antimicrobials (multidrug resistant– MDR) was detected in 61% of isolates (Figure 1). Of these, 63% were biofilm producers and 37% of non-producers. The MDR isolates were resistant to all beta-lactam antibiotics. Resistance to erythromycin, gentamicin and levofloxacin was seen in all MDR biofilm non-producer isolates, while in MDR biofilm producers the frequencies were 95, 74 and 95%, respectively. The most expressive difference between these isolates was the resistance to gentamicin, which was lower (74%) in biofilm producers due to the small number of resistant isolates among PIA-dependent isolates (64%) in comparison with PIA-independent biofilm producers (100%). Another expressive difference between MDR biofilm producer isolates was the resistance to rifampin (43%) and trimethoprim/sulfamethoxazole (86%) only expressed by PIA-dependent.

All isolates that carried the IS256 transposase gene were resistant to beta-lactam antibiotics tested and levofloxacin (Figure 2), as well as 82% of them were MDR. Moreover, the resistance in isolates that had this genetic element was significantly higher than the quinolones tested, levofloxacin (p= 0.0068), moxifloxacin (p= 0.0327), as well as rifampin (p= 0.0364) and to the combination of trimethoprim/sulfamethoxazole (p= 0.0029).

PFGE analysis

The 49 isolates produced a broad range of restriction patterns, which were distributed into 23 different PFGE types. PFGE type A was the prevalent with 15 (31%) isolates. The other PFGE types, considered sporadic types, were B (4); H and O (3); C, D, E, I and V (all with 2), while the other types had a single isolate.

The PFGE type A profile was detected in almost equal proportions in biofilm producer (32%) and non-producer (29%) isolates. Among the eight biofilm producer isolates with this profile, seven were PIA-independent and just one was a PIA-dependent producer. In turn, sporadic PFGE types group was composed of equal numbers of biofilm producer (68%) and non-producer (71%) isolates. However, these represented 16 of 17 PIA-dependent biofilm producer isolates, and was detected in only one PIA-independent biofilm producer.

Regarding the virulence-related genes, none of PFGE type A isolates showed aap or bhp genes, and the percentages of detection of the embp (80%) and capB (87%) (Table 2) were slightly higher than sporadic PFGEtypes group (62% and 76%, respectively). isolates studied (67 and 80%, respectively). Moreover, the ica
Antimicrobial resistance patterns of clinical isolates of S. epidermidis according to the detection by PCR of the IS256 transposase gene. *, Significant differences (p < 0.05) between PFGE type A and sporadic PFGE types; amoxicillin/clavulanic acid (AMC), ampicillin/sulbactam (SAM), ampicillin (AMP), ceftaxim (CRO), clindamycin (DA), daptomycin (DAP), erythromycin (E), gentamicin (GM), levofoxacin (LVX), linezolid (LZD), moxifloxacin (MXF), oxacillin (OX), penicillin (P), quinupristin/dalfopristin (SYN), rifampin (RD), tetracycline (TE), trimethoprim/sulfamethoxazole (SXT), vancomycin (VA). (Resistance data are represented as percentages of IS256 positive and IS256 negative isolates).

In the isolates of sporadic PFGE types group, the percentages of detection of ica operon, aap and IS256 genes were higher than those in the set of isolates studied. However, except for a single isolate, all others that had ica operon and IS256 transposase genes belonged to sporadic PFGE types group.

The prevalent PFGE type showed higher resistance to beta-lactam antibiotics, clindamycin, erythromycin, gentamicin (p=0.0121), levofoxacin and moxifloxacin than isolates of sporadic PFGE types group (Figure 3). On the other hand, in the sporadic PFGE types group, the percentage of resistance to quinupristin/dalfopristin, rifampin and trimethoprim/sulfamethoxazole (p=0.0247) was higher than in isolates of the prevalent PFGE type. Moreover, resistance to daptomycin and tetracycline was present only in isolates of the sporadic PFGE types group.

**DISCUSSION**

*S. epidermidis* has great importance as the causative agent of healthcare associated infections, in particular, those linked to the use of implanted medical devices. Biofilm production has a known role in the pathogenesis of these infections, explaining its chronic or persistent course and also antimicrobial treatment failures (Mack et al., 2013).
Figure 3. Antimicrobial resistance patterns of clinical isolates of *S. epidermidis* clustered according to predominant and sporadic PFGE types. *, Significant differences (p < 0.05) between PFGE type A and sporadic PFGE types; amoxicillin/clavulanic acid (AMC), ampicillin/sulbactan (SAM), ampicillin (AMP), ceftriaxone (CRO), clindamycin (DA), daptomycin (DAP), erythromycin (E), gentamicin (GM), levofloxacin (LVX), linezolid (LZD), moxifloxacin (MXF), oxacillin (OX), penicillin (P), quinupristin/dalfopristin (SYN), rifampin (RD), tetracycline (TE), trimethoprim/sulfamethoxazole (SXT), vancomycin (VA). (Resistance data are represented as percentages of PFGE type A and sporadic PFGE types).

As reported in other similar studies (Arciola et al., 2006; García et al., 2004; Jain and Agarwal, 2009; Oliveira and Cunha, 2010), a higher positivity in the MTP biofilm assay than in CRA test was observed. The differences in these results may be explained by the mechanism of positive reactions in CRA medium, which depends on polysaccharide biofilm production (Freeman et al., 1989), while the MTP assay would be able to detect biofilm production, regardless of its composition.

In fact, all our isolates whose composition of biofilm matrix was identified as polysaccharide by degradation with sodium metaperiodate in detachment assay showed a positive CRA reaction. Conversely, the isolates that presented biofilm of proteinaceous composition had negative reaction in CRA test and isolates with reaction indeterminate did not have the chemical nature of their biofilm determined in detachment assay.

The originally described MTP biofilm assay technique measures directly the OD of stained bacterial films adherent to the bottom of microtiter plates (Christensen et al., 1985). Nevertheless, if the biofilm produced on bottom of the well is not homogeneous, an incorrect measure will occur, since the microplate reader measures the OD only at one point in the middle of the well. Therefore, to avoid this problem, solubilization of the dye attached to the biofilm cells with ethanol can be done, followed by measuring the OD of the biofilm extract (Stepanović et al., 2007).

In our study, the alcoholic biofilm extract OD value was used to evaluate and quantify biofilm production, and also the direct reading of the OD before alcoholic extraction was determined. Comparing the OD values before alcoholic extraction with the alcoholic biofilm extract, the evaluation of biofilm production of the isolates was concordant, except for the isolates characterized in biofilm production assay as protein-dependent biofilm producers. These isolates, classified as weak or moderate biofilm producers according to the alcoholic biofilm extract OD value, presented results of OD before alcoholic extraction very close or below the predefined values for the cut-off point. Therefore, if the results of OD before alcoholic extraction had been used to classify these isolates, they would have been misclassified as non-producers. These low levels of biofilm production in protein-dependent biofilm isolates, considerably smaller than that observed in the polysaccharide-dependent biofilm, also reported in others studies (Rohde et al., 2007), contribute to their low detection.

Another difficulty related to the detection of protein-dependent biofilm is that its formation can be impaired by the test conditions routinely employed in the MTP biofilm assay. This has already been described for the Aap protein that mediates biofilm formation after limited proteolysis by addition of trypsin, elastase or cathepsin G to the culture medium (Rohde et al., 2005). Similarly, the Embp protein is required for biofilm production, the
growth of the isolate in the presence of goat serum (50%) and prolonged (48 h) incubation (Christner et al., 2010).

The prevalence of detection of ica operon genes in our isolates was close to other studies with clinical S. epidermidis (Cafiso et al., 2004; Koskela et al., 2009; Li et al., 2009; Mekni et al., 2012). All genes (icaA, icaD, icaB and icaC) of the operon were always present, as well as accompanied by the regulatory gene icaR. The entire ica operon was also observed in other investigations (de Silva et al., 2002; Koskela et al., 2009; Ninin et al., 2006), together with the icaR when it was investigated (Arciola et al., 2005; Cafiso et al., 2004). However, some studies have reported the detection of isolated ica genes (Diemond-Hernández et al., 2010; Oliveira; Cunha, 2010; Paluch-Oleś et al., 2011), sometimes in the absence of icaR gene (Esteban et al., 2010).

Biofilm producer isolates that had ica operon genes (biofilm+/ica+) showed positive CRA reaction and had a polysaccharide-dependent biofilm, suggesting the PIA involvement in their biofilm. Almost all of these isolates expressed strong production, and a single isolate have a moderate production of biofilm. Therefore, the detection of IS256 transposase gene in this isolate could be one of the reasons for this decreased expression of ica operon (Conlon et al., 2004). Unlike these, three biofilm+/ica+ isolates could not be precisely characterized in CRA test and detachment assay. They were indeterminate in CRA test and the result of biofilm degradation with sodium metaperiodate was close to the 50% of degradation. Thus, the classification of these isolates as PIA-dependent was supported by the detection of the icaA gene transcription.

The presence of other substance(s) associated with the matrix of the biofilm, affecting the presentation of a typical positive reaction in these tests, could explain these results. Due to the relation of ica operon to PIA production, it would be expected that all ica+ isolates were biofilm producers, as observed in the majority of our ica+ isolates. However, three ica+ isolates were biofilm non-producers in the MTP assay, and in two of them, the presence of IS256 transposase gene was detected. Biofilm-/ica- isolates are not uncommon and have been reported in other studies (Arciola et al., 2006; Koskela et al., 2009; Līduma et al., 2012; Oliveira; Cunha, 2010; Rohde et al., 2007; Stevens et al., 2008). The presence of IS256 element has been identified as a possible reason for diminished or lack of ica operon expression in isolates with biofilm-/ica+ profile, since it can impair biofilm production when inserted in ica operon (Ziebuhr et al., 1999) or in the global regulatory genes, σB activator rsbU and sarA (Conlon et al., 2004).

The surface protein Aap has distinct roles in S. epidermidis biofilm, being able to determine the formation of PIA-independent biofilm when alone. In turn, it can increase the adhesion of PIA in PIA-dependent biofilm producers, by linking it to the Aap G5 domain (Otto, 2009). The prevalence of aap gene detection in our isolates was low as compared to other studies with clinical or commensal S. epidermidis (de Araujo et al., 2006; Petrelli et al., 2006; Pourmand et al., 2011; Rohde et al., 2004, 2007; Stevens et al., 2008; Vandecasteele et al., 2003). Although this gene has been most common in biofilm producer isolates, this finding may not be associated with the formation of a biofilm having Aap as a main structural component, since this gene was only detected in a single isolated protein-dependent biofilm producer. Nevertheless, the presence of aap gene showed significant correlation with the presence of ica operon genes, determining its prevalence in PIA-dependent isolates and in strong biofilm producers. Carriage of ica operon cannot serve as a general virulence marker in clinically relevant isolates (Līduma et al., 2012; Rohde et al., 2004, 2007). However, its association with strong biofilm (Mateo et al., 2008; Mekni et al., 2012) or aap gene (Stevens et al., 2008) has a better correlation with pathogenicity than these markers individually.

In relation to capB and embp genes, although they have been detected at high frequency, they cannot be considered as key determinants in biofilm formation, because they were found in equal proportions in biofilm producer and non-producer isolates. Conversely, the bhp gene was detected in only 10% of the isolates, being all biofilm non-producers.

Many authors have pointed out the IS256 as one of the best genetic markers correlated with S. epidermidis virulence. This statement was based in the high frequency of IS256 detection in clinical relevant isolates, which contrasts with its very low occurrence in commensal isolates (Gu et al., 2005; Koskela et al., 2009; Kozitskaya et al., 2004; Mekni et al., 2012). In our study, however, the prevalence of IS256 transposase gene was low. Despite this, its presence showed a significant correlation with biofilm production. Moreover, the ica operon was detected in all IS256-positive isolates. This association of IS256 with ica operon has already been observed and is pointed as a characteristic that, along with antimicrobial resistance, may indicate specific clones of S. epidermidis which are highly adapted to the hospital environment (Kozitskaya et al., 2005; Li et al., 2009).

High rates of resistance to beta-lactam antibiotics, and absence or low rates of resistance to linezolid, vancomycin or daptomycin were also observed in other studies (Cabrera-Contreras et al., 2013; Sader et al., 2009). Likewise, biofilm producing isolates were, generally, slightly more resistant than non-producing (Cabrera-Contreras et al., 2013). Among PIA-dependent and independent biofilm producer isolates, resistance to daptomycin, quinupristin/dalfopristin, rifampin and trimethoprim/sulfamethoxazole was observed only in PIA-dependent isolates and resistance to gentamicin was present in all PIA-independent isolates. The number of MDR isolates was higher in biofilm producing than in non-producing isolates, and the most remarkable differences were in gentamicin, rifampin and trimethoprim/sulfame-
thoxazole resistance. These differences were a result of higher resistance percentages to rifampin and trimethoprim/sulfamethoxazole presented by PIA-dependent isolates, and gentamicin by PIA-independent isolates.

Our results show that all IS256-positive isolates were resistant to the beta-lactams tested and to levofloxacin. Resistance to gentamicin in IS256-positive isolates would be expected due to the association of this element with Tn4001 transposon, implicated in the mediation of aminoglycoside resistance. Contrarily to this, resistance to gentamicin in IS256-positive isolates was minor than in IS256-negative isolates, and low if compared with that reported in other studies (Kozitskaya et al., 2004; Montanaro et al., 2007). A significant correlation of resistance to trimethoprim/sulfamethoxazole with the presence of IS256 was observed in our study, and all isolates that had this genetic element and were resistant to gentamicin also showed resistance to trimethoprim/sulfamethoxazole. The association of resistance to trimethoprim and gentamicin was described in S. epidermidis resistant to sulfamethoxazole by Totake et al. (1998) due to the formation of a composite transposon of IS256 with Tn4001 and Tn4003 transposons.

Such as was observed in our results, a large genetic variability in PFGE analysis of healthcare associated S. epidermidis has been reported in many studies (Nunes et al., 2005; Cherifi et al., 2013; Sani et al., 2014). In spite of the diversity observed in the PFGE analysis, multilocus sequence typing (MLST) studies of these isolates showed that the prevalent PFGE types generally belonged to few sequence types (ST) of clonal complex 2 (CC2); among them, ST2 and ST23 in Brazil (Iorio et al., 2012), ST2 and ST54 in Belgium (Cherifi et al., 2014) and ST2 in China (Li et al., 2009; Du et al., 2014). Isolates of these ST, most or all, had ica operon genes, and aap and/or bhp gene.

Conversely, in our study, just one isolate of the prevalent PFGE type had the ica operon, and none of them had aap or bhp genes. Despite the absence of major genes related to biofilm formation in the other isolates, seven isolates of this PFGE type produced protein-dependent biofilm. The frequency of detection (32%) of PIA-independent isolates and the fact that most of them belong to the prevalent PFGE type (A) gives them a highlight rarely observed in similar studies.

The second largest PFGE type (B) was included in sporadic PFGE type group because it was composed of just four isolates. All of them harbored the ica operon and aap gene, being also oxacillin resistant and MDR, features present in the most frequent ST of the worldwide healthcare associated with S. epidermidis of CC2 (Cherifi et al., 2014; Du et al., 2014; Iorio et al., 2012; Li et al., 2009).

**Conflict of interests**

The authors declare that they have no competing interests.

**ACKNOWLEDGEMENTS**

The authors are grateful to the Hospital Federal dos Servidores do Estado do Rio de Janeiro for providing the clinical isolates and Universidade Federal do Estado do Rio de Janeiro for supplying materials and laboratory equipment.

**REFERENCES**


de Araujo GL, Coelho LR, de Carvalho CB, Maciel RM, Coronado AZ, Rozenbaum R., Ferreira-Carvalho BT, Figueiredo AMS Teixeira LA


