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Characteristics related to antimicrobial resistance and biofilm formation of widespread methicillin-resistant *Staphylococcus epidermidis* ST2 and ST23 lineages in Rio de Janeiro hospitals, Brazil☆

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Abstract

Staphylococcus epidermidis is a leading cause of hospital-acquired infections, mostly associated with the use of medical devices in seriously ill or immunocompromised patients. Currently, the characteristics of methicillin-resistant *S. epidermidis* (MRSE) isolates from Rio de Janeiro hospitals are unknown. In this study, staphylococcal chromosomal cassette *mec* (SCC*mec*) types, antimicrobial susceptibility profiles, biofilm formation genes, and multilocus sequence types (MLST) were investigated in 35 MRSE clinical isolates. The collection of isolates was previously well characterized by pulsed-field gel electrophoresis (PFGE) into 2 main genotypes (A and B, 22 isolates) and 10 sporadic genotypes (13 isolates). MLST revealed a total of 8 different sequence types (STs), but ST2 and ST23, which were *icaAB*-positive, represented the majority (71.4%) of MRSE isolates tested. Almost all isolates (91.4%) belonged to clonal complex 2. SCC*mec* types III and IV were identified among 71.4% of the isolates, while the remaining was nontypeable. The predominant MRSE genotypes were defined as SCC*mec* type III/ST2 (PFGE type A) and SCC*mec* type IV/ST23 (PFGE type B) isolates, which were both associated with high antimicrobial resistance and presence of biofilm-related genes.

Keywords: Methicillin-resistant *Staphylococcus epidermidis*; PFGE; SCC*mec*; MLST; Biofilm formation genes

1. Introduction

Staphylococcus epidermidis is a ubiquitous coagulase-negative staphylococci (CNS) of the human skin and mucosal surfaces and a major cause of indwelling medical device infections (Otto, 2009). Biofilm formation is an important factor for the establishment of *S. epidermidis* as a nosocomial pathogen (Götz, 2002). Although De Araujo et al. (2006) had reported that biofilm production capacity was common among commensal isolates, the biofilm and associated genes have been suggested as markers for clinical significance (Iorio et al., 2011a; Růzicka et al., 2004). The process of biofilm production has not been completely clarified yet, but it seems to occur in 2 steps: i) adherence to an inert surface and ii) biofilm accumulation. The *ica* operon, constituted by the *icaR* (regulatory gene) and *icaADBC* genes, encodes the synthesis of the polysaccharide intercellular adhesin (PIA) (Götz, 2002).

In addition, 2 proteins involved in PIA-independent biofilm formation have been identified in *S. epidermidis*: the accumulation-associated protein (Aap) (Hussain et al., 1997)

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and the Bap homologue protein (Bhp) (Gill et al., 2005; Tormo et al., 2005). The Aap, encoded by the *aap* gene, is a 140-kDa intercellular adhesin that leads to biofilm accumulation. It has been proposed also that Aap plays a role in anchoring PIA to the *S. epidermidis* cell surface since a mutant, reportedly deficient in Aap expression, reduced biofilm formation (Hussain et al., 1997). The Bhp, encoded by the *bhp* gene, is a cell wall surface anchor protein, also named *S. epidermidis* surface D (SesD) protein (Gill et al., 2005). It was first posted by Tormo et al. (2005) as the *Staphylococcus aureus* Bap (biofilm associated protein) homologue from *S. epidermidis* RP62A, proposed to promote primary attachment to abiotic surfaces, as well as intercellular adhesion during biofilm formation (Cucarella et al., 2001). Aap and Bhp were found to be important during biofilm formation, suggesting that these proteins probably mediate cellular accumulation and intercellular adhesion by association with the PIA (Bowden et al., 2005).

Methicillin resistance is an additional important factor in the establishment of *S. epidermidis* as a nosocomial pathogen. The *mecA* gene, which encodes a penicillin-binding protein with low affinity for beta-lactam antibiotics (PBP2A), is carried by a genetic mobile element called the staphylococcal chromosomal cassette *mec* (SCC*mec*) (Chambers, 1997). Eleven types (I to XI) of SCC*mec* have been assigned for *S. aureus* based on the classes of the *mec* gene complex and the types of the *ccr* gene complex (IWG-SCC, 2011). In CNS, these elements are diverse and many isolates are defined as nontypeable (Garza-González et al., 2010; Mombach Pinheiro Machado et al., 2007; Ruppé et al., 2009). However, the most frequently detected SCC*mec* types in MRSE isolates have been IV (Jamaluddin et al., 2008; 29) and III (Li et al., 2009; Mombach Pinheiro Machado et al., 2007; Ruppé et al., 2009).

Although *S. epidermidis* has a high genetic diversity, a few major methicillin-resistant *S. epidermidis* (MRSE) genotypes have been defined by PFGE (Miragaia et al., 2002; Nunes et al., 2005; Widerström et al., 2009). The success of these PFGE types could be explained by colonization advantages, such as biofilm production and synthesis of extracellular proteins, or by factors involved in interactions with host defense mechanisms (Otto, 2009). However, it is not clear whether these genotypes disseminate clonally or whether virulence genes are transferred horizontally among the strains. Multilocus sequence typing (MLST) has been used successfully to infer a population structure for *S. epidermidis* (Miragaia et al., 2007). By using MLST (Thomas et al., 2007), some authors verified that sequence type (ST) 2 was the most found in nosocomial *S. epidermidis* isolates (Li et al., 2009; Miragaia et al., 2007). Indeed, Otto (2009) proposed that strains from this ST are potentially most invasive.

Characteristics associated with biofilm formation, antimicrobial resistance, and clonal lineages of MRSE isolates from hospitals in Rio de Janeiro have not been described yet. Moreover, studies that characterize widespread MRSE genotypes in hospitals are rare. The present study aimed to analyze in a deeper level 35 MRSE isolates that were previously distinguished into PFGE profiles by our group (Nunes et al., 2005).

2. Materials and methods

2.1. MRSE Strains

A collection of 35 MRSE isolates, which were previously well characterized by PFGE (Nunes et al., 2005) in 2 dominant genotypes (A and B, 22 isolates) and 10 sporadic (C to M, 13 isolates), were analyzed in order to understand the reasons for their spread in Rio de Janeiro hospitals. The 35 MRSE isolates were isolated from blood (14 isolates), nares (7), surgical site (4), catheter tip (2), and throat (2), and 1 isolate of each source as follows: umbilical secretion, tracheal aspirate, ocular secretion, liquor, sputum, and peritoneal fluid. All isolates were confirmed as *S. epidermidis* by phenotypic tests (Iorio et al, 2007) and by polymerase chain reaction (PCR) for *recN* gene (Iorio et al, 2011b) and as methicillin resistant by the proofs described below.

2.2. Disk diffusion test

The susceptibilities of the isolates to 15 antimicrobial agents were determined by the disk diffusion method, according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2009a, 2010), except for mupirocin (Fuchs et al., 1990) and tigecycline (EUCAST, 2011). The antimicrobial agents tested included ciprofloxacin, clindamycin, chloramphenicol, erythromycin, gentamicin, oxacillin, rifampin, trimethoprim-sulfamethoxazole, teicoplanin, tetracycline, vancomycin (CECON, São Paulo, Brazil), cefoxitin, linezolid, mupirocin, and tigecycline (Oxoid, Basingstoke, England).

2.3. MIC of oxacillin and vancomycin

The MICs of oxacillin and vancomycin (Sigma, St. Louis, MO, USA) were determined by the agar dilution method (CLSI, 2009b, 2010). Briefly, bacterial suspensions were adjusted to a 0.5 McFarland standard, diluted 1:10, and inoculated (10^4 colony-forming units) onto Mueller-Hinton agar (Difco Laboratories, Detroit, MI, USA) plates. For oxacillin, 2% (wt/vol) NaCl was added to the medium. The concentrations ranged from 0.5 to 256 μg/mL for oxacillin and from 0.25 to 32 μg/mL for vancomycin. The plates were incubated at 35 °C for 24 h.

2.4. Screening of vancomycin heteroresistance

Vancomycin heteroresistance was determined by the vancomycin agar screen method, according to Nunes et al. (2007). Bacterial inoculum was prepared from a suspension in saline adjusted to 0.5 McFarland standard (~10^8 CFU/mL). Isolates were inoculated onto Brain Heart Infusion agar (Becton, Dickinson and Company, Sparks, MD, USA) plates containing 4 μg/mL of vancomycin. Cultures were incubated at 35 °C for 48 h, and the cell growth was inspected at 24 and 48 h. If at least 2 colonies were apparent within 48 h, the isolate was

designated as a possible vancomycin heteroresistant isolate. *S. aureus* Mu50 (donated K. Hiramatsu, Juntendo University, Tokyo, Japan) and ATCC 29213 were used as control strains.

2.5. SCCmec typing

S. epidermidis isolates were characterized for the 2 central elements of the staphylococcal cassette chromosome *mec* (SCC*mec*), namely, the *ccr* complex encoding for recombinases and the *mec* complex encoding for broad-spectrum beta-lactam resistance. The multiplex PCR strategy, M-PCR 1, was used to identify the 5 types of *ccr* gene complex, and M-PCR 2 to identify class A to class C *mec* complex, as previously described (Kondo et al., 2007). The following *S. aureus* reference strains and clinical isolates were used as positive controls for SCC*mec* typing: *S. aureus* Mu50 (SCC*mec* II) (Hiramatsu et al., 1997), HU25 (SCC*mec* III) (Vivoni et al., 2006), and 527 (SCC*mec* IV) (Schuenck et al., 2009).

2.6. Phenotypic detection of biofilm formation and related genes

Biofilm formation was detected according to Stepanović et al. (2001), with modifications. Isolates were cultured onto blood agar (Plast Labor, Rio de Janeiro, Brazil) and incubated aerobically at 35 °C for 24 h. The grown cultures were used for preparation of bacterial suspensions in sterile distilled water with densities adjusted to 0.5 McFarland standard. The wells of a sterile 96-well flat-bottomed plastic tissue culture plate with a lid (TPP, Trasadingen, Switzerland) were filled with 180 μL of tryptic soy broth (Becton, Dickinson and Company). Aliquots of 20 μL of bacterial suspension were added into each well. Isolates were tested in triplicate, at least 2 times. The plates were incubated aerobically for 24 h at 35 °C; the content of each well was then removed and the wells washed 4 times with 200 μL of sterile distilled water. The plates were emptied, let to air dry at room temperature for 15 min, and stained with 200 μL per well of 2% crystal violet for 5 min. Overload stain was rinsed off under running tap water. After air drying, the dye was resolubilized with 200 μL of 95% ethanol. The optical density (OD) of each well was measured at 570 nm with an ELISA Auto Reader (model 550, Bio-Rad Microplate Reader, Bio-Rad Laboratories, Hercules, CA, USA). *S. epidermidis* RP62A strain and its mutant HAM 892, which lacks the ability to produce biofilm (Christensen et al., 1990) (donated by L. Baldassarri, Instituto Superiore di Sanità, Rome, Italy) were used as positive and negative controls, respectively. All isolates were classified into the following categories: strong (+++), moderate (++), weak (+), and nonproducer of biofilm (−). The comparative analyses were performed based upon the ODs of bacterial films according to Stepanović et al. (2000), using the wells inoculated with the HAM 892 strain as negative controls.

The *icaAB* (Frebourg et al., 2000), *aap* (Vandecasteele et al., 2003), and *bhp* (Bowden et al, 2005) genes were detected by PCR. *S. epidermidis* ATCC 35984 was used as positive control and *S. epidermidis* ATCC 12228 as negative control to both genes, *icaAB* and *bhp*.

2.7. Multilocus sequence typing

MLST was performed using previously published primer sequences and conditions for the PCR amplification of the 7 housekeeping genes *arcC*, *aroE*, *gtr*, *mutS*, *pyr*, *tpi*, and *yqiL* (Thomas et al., 2007). The PCR products were sequenced bi-directionally at the Multidisciplinary Genomic Unit of Carlos Chagas Filho Biophysics Institute of the Federal University of Rio de Janeiro. STs were determined using the MLST database (http://www.mlst.net) and characterized as singletons or members of a clonal complex (CC)

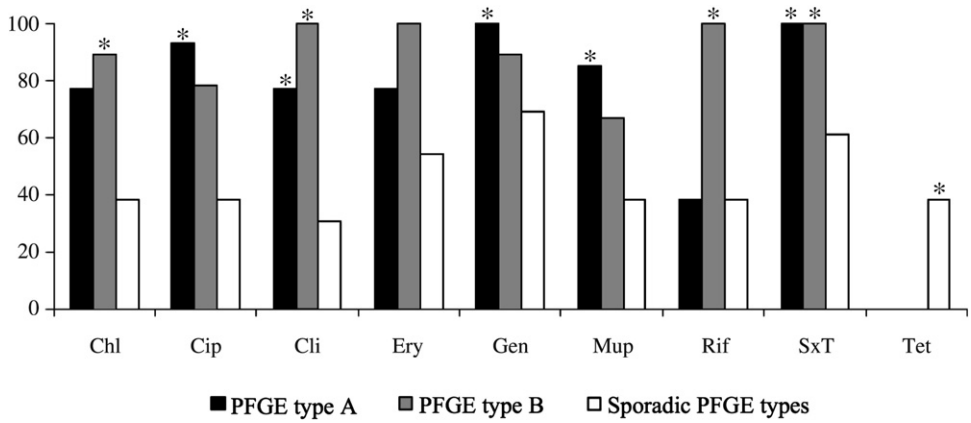


Fig. 1. Antimicrobial resistance patterns of MRSE isolates clustered according to predominant and sporadic genotypes. ∗, Significant differences ($P < 0.05$) between PFGE type A or PFGE type B in relation to sporadic PFGE types; Chl = chloramphenicol; Cip = ciprofloxacin; Cli = clindamycin; Ery = erythromycin; Gen = gentamicin; Mup = mupirocin; Rif = rifampin; SxT = trimethoprim–sulfamethoxazole; Tet = tetracycline. All strains were susceptible to the following 4 antibiotics: linezolid, teicoplanin, tigecycline and vancomycin. (Resistance data are represented as percentages of PFGE type A, PFGE type B, and sporadic PFGE types).

Table 1
Characteristics of 35 MRSE isolates clustered according to prevalent and sporadic PFGE types

Isolate	Isolation source	Hospital[a]	PFGE	Complex		SCC*mec* Types[c]	MLST			MIC (μg/mL)		Resistance profile other than beta-lactam[e]
				ccr	*mec*[b]		Allelic profile	ST	CC[d]	Oxacillin	Vancomycin	
Predominant PFGE types												
13	Surgical site	H2	A1	3	A	III	7-1-2-2-4-1-1	2	2	64	1	Chl, Cip, Cli, Ery, Gen, SxT
25	Catheter tip	H6	A1	3	A	III	7-1-2-2-4-1-1	2	2	64	1	Chl, Cip, Cli, Ery, Gen, Mup, SxT
27	Blood	H2	A1	3	A	III	7-1-2-2-4-1-1	2	2	64	1	Chl, Cip, Cli, Ery, Gen, Mup, SxT
29	Blood	H2	A1	3	A	III	7-1-2-2-4-1-1	2	2	64	1	Chl, Cip, Cli, Ery, Gen, Mup, SxT
117	Nares	H3	A2	3	A	III	7-1-2-2-4-1-1	2	2	64	1	Chl, Cip, Cli, Ery, Gen, Mup, SxT
63	Throat	H5	A2	3	A	III	7-1-2-2-4-1-1	2	2	128	1	Cip, Cli, Ery, Gen, Mup, Rif, SxT
161	Tracheal aspirate	H7	A3	3	A	III	7-1-2-2-4-1-1	2	2	256	1	Cip, Gen, Mup, Rif, SxT
181	Surgical site	H2	A3	3	A	III	7-1-2-2-4-1-1	2	2	256	1	Chl, Cip, Gen, Mup, Rif, SxT
142	Umbilical secretion	H1	A4	3	A	III	7-1-2-2-4-1-1	2	2	256	0.5	Chl, Cip, Cli, Ery, Gen, Mup, Rif, SxT
214	Throat	H5	A5	3	A	III	7-1-2-2-4-1-1	2	2	256	1	Chl, Cip, Gen, Mup, Rif, SxT
24	Surgical site	H2	A6	3	A	III	7-1-2-2-4-1-1	2	2	32	1	Chl, Cip, Cli, Ery, Gen, SxT
72	Blood	H1	A7	2-3	A	nt	7-1-2-2-4-1-1	2	2	64	1	Cip, Cli, Ery, Gen, Mup, SxT
16	Surgical site	H2	A8	3-4-5	A	nt	7-1-2-2-4-1-1	2	2	64	1	Chl, Cli, Ery, Gen, Mup, SxT
127	Nares	H3	B1	2	B	IV	7-1-2-1-3-3-1	23	2	32	0.5	Chl, Cip, Cli, Ery, Gen, Mup, Rif, SxT
147	Blood	H1	B1	2	B	IV	7-1-2-1-3-3-1	23	2	16	1	Chl, Cip, Cli, Ery, Gen, Mup, Rif, SxT
162	Nares	H4	B1	2	B	IV	7-1-2-1-3-3-1	23	2	16	1	Chl, Cip, Cli, Ery, Gen, Mup, Rif, SxT
172	Nares	H4	B1	2	B	IV	7-1-2-1-3-3-1	23	2	8	1	Chl, Cip, Cli, Ery, Gen, Mup, Rif, SxT
86	Blood	H1	B1	2	B	IV	7-1-2-1-3-3-1	23	2	16	1	Chl, Cip, Cli, Ery, Gen, Mup, Rif, SxT
94	Blood	H1	B2	2	B	IV	7-1-2-26-3-3-1	231	2	8	0.5	Chl, Cip, Cli, Ery, Gen, Rif, SxT
54	Blood	H3	B3	2	-	nt	7-1-2-1-3-3-1	23	2	32	0.5	Chl, Cip, Cli, Ery, Gen, Mup, Rif, SxT
20	Ocular secretion	H6	B4	2-5	C	nt	7-1-2-1-3-3-1	23	2	32	1	Cli, Ery, Gen, Rif, SxT
32	Blood	H2	B5	2	B	IV	7-1-2-1-3-3-1	23	2	64	1	Chl, Cli, Ery, Gen, Rif, SxT
Sporadic PFGE types												
148	Blood	H1	C1	2-4-5		nt	2-1-1-1-2-1-1	59	2	2	0.5	Ery, Gen, Mup, Tet
96	Blood	H1	C2	2-4	B	nt	2-1-1-1-2-1-1	59	2	2	0.5	Chl, Gen, Tet
140	Peritoneal secretion	H1	D1	2-4	B	nt	2-17-1-1-2-1-1	81	2	2	0.5	Gen, Tet
65	Sputum	H5	D2	2-4	B	nt	2-17-1-1-2-1-1	81	2	4	0.5	Ery, Gen, Tet
165	Nares	H4	E	2	B	IV	7-1-2-1-3-3-1	23	2	16	0.5	Chl, Cip, Cli, Ery, Mup, Rif, SxT
169	Nares	H4	F1	3	A	III	7-1-2-2-4-7-1	22	2	64	1	Cli, Cip, Ery, Gen, Mup
28	Liquor	H2	F2	3	A	III	7-1-2-2-4-16-1	237	2	128	1	Cli, Cip, Ery, Gen, Mup, Rif, SxT
4	Nares	H2	G	3	A	III	7-1-2-2-4-1-1	2	2	0.5	1	Chl, Cip, Rif, SxT
81	Blood	H1	H	5	-	nt	7-1-2-2-4-1-1	2	2	256	1	Chl, Gen, Rif, SxT
56	Blood	H3	I	3-4-5	A	nt	7-1-2-2-4-1-1	2	2	>256	0.5	Chl, Cip, Cli, Ery, Gen, Mup, SxT
103	Catheter tip	H1	J	2	B	IV	3-1-16-5-11-4-4	263	NPF	2	0.5	Ery, SxT
228	Blood	H1	K	2	B	IV	3-1-5-5-11-4-11	53	11	32	0.5	Rif, SxT
189	Blood	H2	L	2	B	IV	3-1-5-5-11-4-11	53	11	32	0.5	SxT, Tet

PFGE = Pulsed-field gel electrophoresis; SCC*mec* = staphylococcal chromosomal cassette *mec*; MLST = multilocus sequence typing; ST = sequence type; CC = clonal complex.

[a] H1 = Hospital Naval Marcílio Dias; H2 = Hospital Universitário Clementino Fraga Filho; H3 = Hospital Beneficência Portuguesa; H4 = Clínica Bambina; H5 = Hospital Cardoso Rodrigues; H6 = Hospital Universitário Antônio Pedro; H7 = Policlínica de Botafogo.

[b] − = No detectable *mec* complex.

[c] nt = Nontypeable.

[d] NPF = No predicted founder.

[e] Chl = chloramphenicol; Cip = ciprofloxacin; Cli = clindamycin; Ery = erythromycin; Gen = gentamicin; Mup = mupirocin; Rif = rifampin; SxT = trimethoprim-sulfamethoxazole; Tet = tetracycline. All strains were susceptible to the following 4 antibiotics: linezolid, teicoplanin, tigecycline, and vancomycin.

by the eBURST algorithm (accessible at http://eburst.mlst.net). Numbers for new alleles and STs reported here were assigned by the *S. epidermidis* MLST database curator.

2.8. Statistical methods

All comparisons were performed using the χ^2 test or the Fischer's exact test. Differences were considered statistically significant when values of $P < 0.05$ were obtained.

3. Results

All 35 isolates were confirmed as MRSE. Isolates of the main PFGE types were resistant to a mean of 7 of the 13 non–beta-lactam antimicrobial agents tested, while isolates of the sporadic genotypes were resistant to only 4 agents. The resistance rates to ciprofloxacin, chloramphenicol, clindamycin, gentamicin, mupirocin, rifampin, and trimethoprim–sulfamethoxazole were higher in predominant genotypes than in sporadic ones ($P < 0.05$) (Fig. 1). All MRSE isolates were susceptible to linezolid, teicoplanin, tigecycline, and vancomycin.

Oxacillin MICs ranged from 0.5 to >256 μg/mL for the 35 MRSE isolates (Table 1). The isolates from PFGE types A and B showed higher MICs (100%, MIC ≥8 μg/mL) than sporadic isolates (53.8%, MIC ≥8 μg/mL) ($P < 0.05$). For vancomycin, all isolates had MICs ≤1 μg/mL. SCC*mec* type III was detected in 85% of genotype A isolates, while 78% of isolates from genotype B harbored the SCC*mec* type IV. Almost 50% of sporadic genotypes were nontypeable, which possessed more than 1 *ccr* allotype or were *mecA* positive with no *mec* complex detectable. Isolates carrying SCC*mec* III and nontypeable (complex A to *mec* gene associated with *ccr* 3) presented higher oxacillin MICs and were more resistant to ciprofloxacin, gentamicin, and mupirocin ($P < 0.05$).

All isolates from the main genotypes carried *icaAB* genes, whereas only 6 (46.1%) isolates from sporadic genotypes harbored these genes ($P < 0.05$) (Fig. 2). The *aap* gene was detected in the majority (84.6%) of the isolates from PFGE type A, while only 2 (22.2%) isolates of type B were positive for this gene. On the other hand, the *bhp* gene was mainly found in isolates from PFGE type B. Thus, a significant correlation between the main genotypes and detection of at least 2 biofilm-related genes was observed ($P < 0.05$). The microtiter-plate adherence technique to detect biofilm formation showed 13 MRSE-positive isolates, 5 from PFGE type A, 2 from type B, and 6 from sporadic genotypes. Biofilm production was not observed in isolates without any biofilm-related genes. No significant difference ($P > 0.05$) was observed for biofilm formation between isolates from predominant and sporadic genotypes.

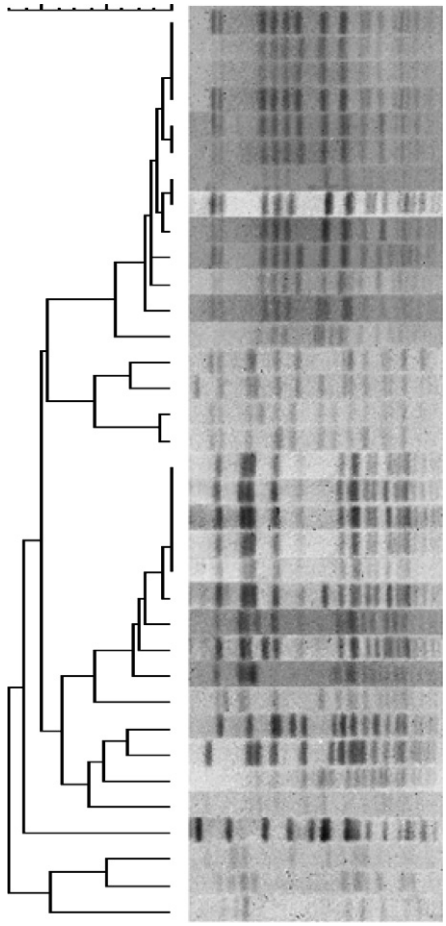
MLST revealed a total of 9 different STs among the 35 MRSE isolates, but the majority of them were clustered in ST2 (45.7%) and ST23 (25.7%) (Table 1). A total of 4 of these 9 STs corresponded to single isolates, while 3 STs included 2 isolates each. The majority of ST2 isolates (87.5%) had oxacillin MICs ≥64 μg/mL and almost all (93.8%) harboured the SCC*mec* III or were nontypeable. The biofilm-related genes, *icaAB* and *aap*, were detected respectively in 100% and 87.5% from ST2 isolates. The ST23 isolates showed that oxacillin MICs ranged from 8 to 64 μg/mL and that the majority of them (77.8%) harboured the SCC*mec* IV. Although only 2 isolates carried the *aap* gene, 100% and 88.9% were *icaAB* and *bhp* gene positive.

Isolates from PFGE types A and B, classified as ST2 and ST23, were included into CC2, the major CC of *S. epidermidis* (Table 1). One isolate from genotype B was classified as ST231/CC2, a new ST described in the present study (Fig. 3), which differs from ST2 by a single nucleotide into mutS allele. Sequencing of the mutS allele (isolate 94) was repeated 2 times bidirectionally to ensure reproducibility, and a new mutS allele type 26 and ST231 were assigned (http://www.mlst.net). Sporadic genotypes were included into 8 STs (2, 22, 23, 53, 59, 81, 237, and 263) and 2 CCs (2 and 11). The CC2 was associated with the majority (76.9%) of them. For another new ST assigned in this study, (ST263, isolate 103) (http://www.mlst.net), there is no predicted founder for the CC.

4. Discussion

Well-characterized studies in *S. epidermidis* have been limited historically, due to the fact that CNS isolates are often considered to be contaminants. In this study, we evaluated 35 MRSE isolates obtained previously by our group (Nunes et al., 2005) to understand why some genotypes were spread in Rio de Janeiro hospitals. We observed that the 2 most prevalent PFGE types were associated with 2 STs, high antimicrobial resistance, and presence of biofilm-related genes.

A few studies have shown the predominant genotypes of MRSE as a common cause of infections in hospitals (Klingenberg et al., 2007; Miragaia et al., 2002; Widerström et al., 2009). Miragaia et al. (2002) analyzed 230 European clinical MRSE isolates, being 94 from an Icelandic hospital and 136 from 5 Danish hospitals in greater Copenhagen. Considering both collections, 5 predominant MRSE genotypes, comprising 58.3% of isolates, were spread in different services of a single hospital and among different hospitals. The authors emphasized the importance of the spread of a few genotypes in those countries and a multidrug resistance for these specific PFGE types, but they did not evaluate other characteristics associated with these genotypes to explain their prevalence. In another study, a collection of 173 MRSE isolates from patients in 11 hospitals in northern Europe revealed 2 dominating PFGE types, which included 54.3% of isolates. The majority were resistant to 4 antimicrobials tested, while only 19% of isolates from 3 minor PFGE types and 47% of "noncluster" isolates were resistant to these drugs. The authors detected ST2 in 59.1% of randomly selected isolates. Although there was agreement with our susceptibility and ST data, here the authors did not perform,



Isolate	Hospital	PFGE	SCC*mec*	ST	MPA	genes *ica*	genes *aap*	genes *bhp*
13	H2	A1	III	2	(-)	+	+	-
25	H6	A1	III	2	(-)	+	+	-
27	H2	A1	III	2	(-)	+	+	-
29	H2	A1	III	2	(+)	+	+	-
117	H3	A2	III	2	(-)	+	+	-
63	H5	A2	III	2	(+)	+	+	-
161	H7	A3	III	2	(-)	+	-	-
181	H2	A3	III	2	(-)	+	+	-
142	H1	A4	III	2	(-)	+	-	-
214	H5	A5	III	2	(+)	+	+	-
24	H2	A6	III	2	(+++)	+	+	-
72	H1	A7	nt	2	(-)	+	+	-
16	H2	A8	nt	2	(+)	+	+	-
148	H1	C1	nt	59	(+)	-	+	-
96	H1	C2	nt	59	(-)	-	+	-
140	H1	D1	nt	81	(+)	-	+	-
65	H5	D2	nt	81	(+)	-	+	-
127	H3	B1	IV	23	(-)	+	-	+
147	H1	B1	IV	23	(-)	+	-	+
162	H4	B1	IV	23	(-)	+	-	+
172	H4	B1	IV	23	(-)	+	+	+
86	H1	B1	IV	23	(-)	+	-	+
94	H1	B2	IV	231	(+)	+	-	+
54	H3	B3	nt	23	(-)	+	+	-
20	H6	B4	nt	23	(+++)	+	-	+
32	H2	B5	IV	23	(-)	+	-	+
165	H4	E	IV	23	(-)	+	-	+
169	H4	F1	III	22	(-)	+	+	-
28	H2	F2	III	237	(++)	+	+	-
4	H2	G	III	2	(+++)	+	+	-
81	H1	H	nt	2	(+++)	+	+	-
56	H3	I	nt	2	(-)	+	+	-
103	H1	J	IV	263	(-)	-	-	-
228	H1	K	IV	53	(-)	-	-	-
189	H2	L	IV	53	(-)	-	-	-

Fig. 2. Dendrogram of the pulsed-field gel electrophoresis (PFGE) profile of *Sma*I-digested genomic DNA of 35 MRSE isolates and their associated characteristics. Similarities percentage is identified on a dendrogram derived from the unweighted pair group method using arithmetic averages and based on Dice coefficients. Isolates showing a similarity coefficient ≥80% were considered genetically PFGE related. SCC*mec* = Staphylococcal chromosomal cassette *mec*; ST = sequence type; MPA = microtiter-plate adherence; H1 = Hospital Naval Marcílio Dias; H2 = Hospital Universitário Clementino Fraga Filho; H3 = Hospital Beneficência Portuguesa; H4 = Clínica Bambina; H5 = Hospital Cardoso Rodrigues; H6 = Hospital Universitário Antônio Pedro; H7 = Policlínica de Botafogo; nt = nontypeable; (+++) = strong; (++) = moderate; (+) = weak, and (−) = nonproducer of biofilm producer; + = positive; − = negative.

however, a more accurate analysis of the MRSE lineages (Widerström et al., 2009).

In 2007, Klingenberg et al. (2007) analyzed aspects related to biofilm formation and antimicrobial resistance in 128 *S. epidermidis* isolates from a neonatal intensive care unit in Norway and detected 45 PFGE types among them. The main genotypes showed higher rates of resistance to methicillin, gentamicin, and macrolides. Biofilm formation and *ica* operon genes were also more detected among them, although the other biofilm-related genes, like *aap* and *bhp*, were shown in similar rates for all isolates.

Our study showed that the majority of MRSE isolates comprising 2 PFGE types that spread in 7 Rio de Janeiro hospitals carried mainly SCC*mec* types III (84.6% of genotype A) and IV (77.8% of genotype B) and presented resistance rates higher than sporadic genotypes ($P < 0.05$), which were nontypeable in almost 50%. Moreover, although the oxacillin MICs for type B isolates were lower than type A isolates, these predominant types showed higher oxacillin MICs than sporadic isolates ($P < 0.05$), suggesting that these high MICs can be associated with its prevalence in the hospitals evaluated. Additionally, the *ica* genes were detected preferentially in predominant genotypes ($P < 0.05$). Moreover, the *aap* gene was detected in the majority of type A isolates, while the *bhp* gene was found in type B isolates. Thus, although we did not find any difference in the "in vitro" biofilm expression between the MRSE groups analyzed (dominant and sporadic genotypes isolates), the presence of biofilm-related genes might represent a benefit for particular *S. epidermidis* genotypes, enabling the bacteria to colonize inert surfaces of medical devices and to resist a wide range of external conditions (Ziebuhr et al., 2006).

An international MLST database has been established for *S. epidermidis* by using a set of genes, some of which were different from those used for *S. aureus*, chosen for their

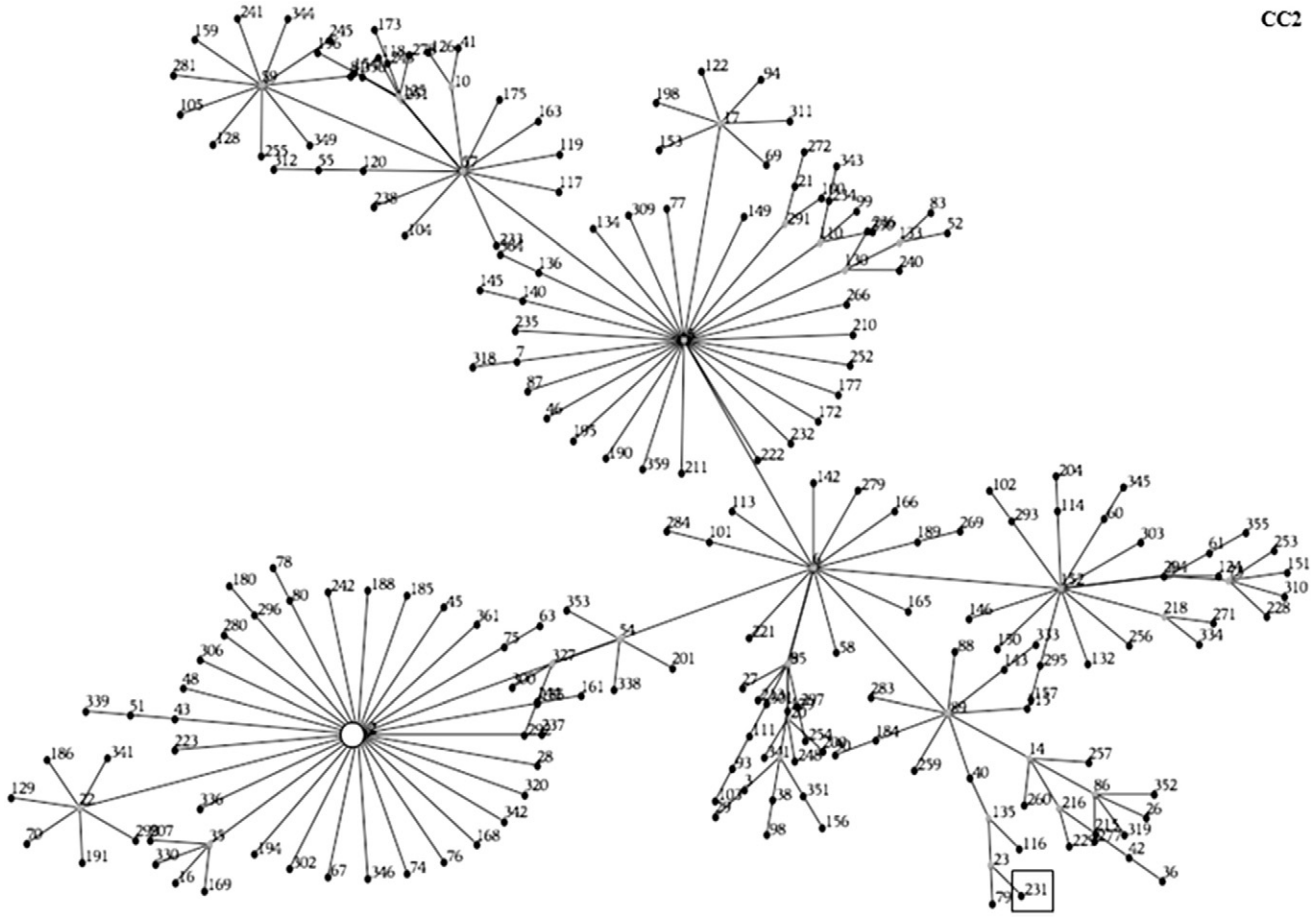


Fig. 3. *S. epidermidis* clonal complex (CC) 2 identified by eBURST analysis. Each ST is represented by a black dot. White and gray dots correspond to group and subgroup founders, respectively. Single-locus variants are linked by lines, and the CC or eBURST group corresponds to the group of connected STs. Square represents a new ST described in this work.

random genomic distribution (Thomas et al., 2007). In this study, the MLST results showed that the most found ST was ST2. To our knowledge, this is the first report that evaluates the PFGE types of MRSE isolates by MLST in Brazilian *S. epidermidis* isolates. The ST2 and ST23 lineages were associated with genotypes A and B, respectively, and were spread in hospitals from Rio de Janeiro. eBURST analysis found all these isolates into the highly CC2 group. Among all analyzed isolates, 91.4% fell into this CC, as had been reported previously by Li et al. (2009), who found the majority of ST within CC2.

ST2 has been reported to be the most widely disseminated hospital-associated ST type (Li et al, 2009; Miragaia et al., 2007; Widerström et al., 2009). A study using 217 *S. epidermidis* isolates from 17 countries around the world revealed 30.9% of the isolates as ST2, while ST23 was the second most disease-associated ST (Miragaia et al., 2007). The same occurred in a study performed by Li et al. (2009), who detected the ST2 in 31.3% of *S. epidermidis* isolates from a teaching hospital in Shanghai, China. This ST was also described for 4 of the 5 most frequent MRSE genotype isolates from patients in 11 hospitals in northern Europe (Widerström et al., 2009). According to Li et al. (2009), the successful spread of ST2 may be associated with the fact that all ST2 isolates analyzed by their group carried biofilm formation–related sequences (*ica* and IS*256*). They hypothesized that, by recombination, ST2 generates novel phenotypic and genotypic variants, such as *ica* genes and IS*256*-positive isolates, which makes ST2 isolates easily able to spread in the hospital environment.

In the present study, we observed 10 nontypeable isolates that had *mec–ccr* combinations, which did not fit into the current classification of the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC, 2011). Some of them had already been observed for this CNS species (Garza-González et al., 2010; Ruppé et al., 2009). Among them, we found 5 isolates harboring 2 distinct *ccr* allotypes and 3 isolates with 3 *ccr* allotypes. No class *mec* complex was detected in 3 *mecA* gene–positive isolates. According to Ruppé et al. (2009), the presence of *mec* complex combined with 1, 2, or 3 different *ccr* allotypes shows that *mec–ccr* combinations are much

more diverse than was previously thought, confirming the ability of the CNS isolates to act as an important reservoir of resistance genes.

In summary, we showed here that the occurrence of 2 genotypes of MRSE that spread in hospitals located in Rio de Janeiro may be associated with advantage and/or evolutionary factors, which permits its persistence and potential dissemination. PFGE types A and B, which were identified as SCC*mec* type III/ST2 and SCC*mec* type IV/ST23, from CC2 represented the majority of the evaluated isolates and were associated with high antimicrobial resistance and presence of *icaAB* and *aap* or *bhp* genes, characteristics that can make easy their survival and spread in a nosocomial environment.

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References

Bowden MG, Chen W, Singvall J, Xu Y, Peacock SJ, Valtulina V, Speziale P, Höök M (2005) Identification and preliminary characterization of cell-wall-anchored proteins of *Staphylococcus epidermidis*. *Microbiology* 151:1453–1464.

Chambers HF (1997) Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications. *Clin Microbiol Rev* 10:781–791.

Christensen GD, Barker LP, Mawhinney TP, Baddour LM, Simpson WA (1990) Identification of an antigenic marker of slime production for *Staphylococcus epidermidis*. *Infect Immun* 58:2906–2911.

Clinical and Laboratory Standards Institute (CLSI) (2009a) Performance standards for antimicrobial disk susceptibility tests; approved standard. 10th ed. Wayne, PA: M02-A10 CLSI.

Clinical and Laboratory Standards Institute (CLSI) (2009b) Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard. eighth ed. M07-A8 Wayne, PA: CLSI.

Clinical and Laboratory Standards Institute (CLSI) (2010) Performance standards for antimicrobial susceptibility testing; 20th informational supplement. M100-S20 Wayne, PA: CLSI.

Cucarella C, Solano C, Valle J, Amorena B, Lasa I, Penadés JR (2001) Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. *J Bacteriol* 183:2888–2896.

De Araujo GL, Coelho LR, de Carvalho CB, Maciel RM, Coronado AZ, Rozenbaum R, Ferreira-Carvalho BT, Figueiredo AM, Teixeira LA (2006) Commensal isolates of methicillin-resistant *Staphylococcus epidermidis* are also well equipped to produce biofilm on polystyrene surfaces. *J Antimicrob Chemother* 57:855–864.

EUCAST (2011) Breakpoint tables for interpretation of MICs and zone diameters. Version 1.3, January European Committee on Antimicrobial Susceptibility Testing. Available at: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/EUCAST_breakpoints_v1.3.xls.

Frebourg NB, Lefebvre S, Baert S, Lemeland JF (2000) PCR-Based assay for discrimination between invasive and contaminating *Staphylococcus epidermidis* strains. *J Clin Microbiol* 38:877–880.

Fuchs PC, Jones RN, Barry AL (1990) Interpretive criteria for disk diffusion susceptibility testing of mupirocin, a topical antibiotic. *J Clin Microbiol* 28:608–609.

Garza-González E, Morfín-Otero R, Llaca-Díaz JM, Rodriguez-Noriega E (2010) Staphylococcal cassette chromosome *mec* (SCC*mec*) in methicillin-resistant coagulase-negative staphylococci. A review and the experience in a tertiary-care setting. *Epidemiol Infect* 138:645–654.

Gill SR, Fouts DE, Archer GL, Mongodin EF, Deboy RT, Ravel J, Paulsen IT, Kolonay JF, Brinkac L, Beanan M, Dodson RJ, Daugherty SC, Madupu R, Angiuoli SV, Durkin AS, Haft DH, Vamathevan J, Khouri H, Utterback T, Lee C, Dimitrov G, Jiang L, Qin H, Weidman J, Tran K, Kang K, Hance IR, Nelson KE, Fraser CM (2005) Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. *J Bacteriol* 187:2426–2438.

Götz F (2002) *Staphylococcus* and biofilms. *Mol Microbiol* 43:1367–1378.

Hiramatsu K, Aritaka N, Hanaki H, Kawasaki S, Hosoda Y, Hori S, Fukuchi Y, Kobayashi I (1997) Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. *Lancet* 350:1670–1673.

Hussain M, Herrmann M, von Eiff C, Perdreau-Remington F, Peters G (1997) A 140-kilodalton extracellular protein is essential for the accumulation of *Staphylococcus epidermidis* strains on surfaces. *Infect Immun* 65:519–524.

International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC) (2011) Currently identified SCCmec types in strains. Available at: http://www.sccmec.org/Pages/SCC_TypesEN.html.

Iorio NL, Azevedo MB, Frazão VH, Barcellos AG, Barros EM, Pereira EM, De Mattos CS, Dos Santos KR (2011b) Methicillin-resistant *Staphylococcus epidermidis* carrying biofilm formation genes: detection of clinical isolates by multiplex PCR. *Int Microbiol* 14:13–17.

Iorio NL, Ferreira RB, Schuenck RP, Malvar KL, Brilhante AP, Nunes AP, Bastos CC, Dos Santos KR (2007) Simplified and reliable scheme for species-level identification of *Staphylococcus* clinical isolates. *J Clin Microbiol* 45:2564–2569.

Iorio NL, Lopes AP, Schuenck RP, Barcellos AG, Olendzki AN, Lopez GL, dos Santos KR (2011a) A combination of methods to evaluate biofilm production may help to determine the clinical relevance of *Staphylococcus* in blood cultures. *Microbiol Immunol* 55:28–33.

Jamaluddin TZ, Kuwahara-Arai K, Hisata K, Terasawa M, Cui L, Baba T, Sotozono C, Kinoshita S, Ito T, Hiramatsu K (2008) Extreme genetic diversity of methicillin-resistant *Staphylococcus epidermidis* strains disseminated among healthy Japanese children. *J Clin Microbiol* 46:3778–3783.

Klingenberg C, Rønnestad A, Anderson AS, Abrahamsen TG, Zorman J, Villaruz A, Flaegstad T, Otto M, Sollid JE (2007) Persistent strains of coagulase-negative staphylococci in a neonatal intensive care unit: virulence factors and invasiveness. *Clin Microbiol Infect* 13:1100–1111.

Kondo Y, Ito T, Ma XX, Watanabe S, Kreiswirth BN, Etienne J, Hiramatsu K (2007) Combination of multiplex PCRs for staphylococcal cassette chromosome *mec* type assignment: rapid identification system for *mec*, *ccr*, and major differences in junkyard regions. *Antimicrob Agents Chemother* 51:264–274.

Li M, Wang X, Gao Q, Lu Y (2009) Molecular characterization of *Staphylococcus epidermidis* strains isolated from a teaching hospital in Shanghai, China. *J Med Microbiol* 58:456–461.

Miragaia M, Couto I, Pereira SF, Kristinsson KG, Westh H, Jarløv JO, Carriço J, Almeida J, Santos-Sanches I, de Lencastre H (2002) Molecular characterization of methicillin-resistant *Staphylococcus epidermidis* clones: evidence of geographic dissemination. *J Clin Microbiol* 40:430–438.

Miragaia M, Thomas JC, Couto I, Enright MC, de Lencastre H (2007) Inferring a population structure for *Staphylococcus epidermidis* from multilocus sequence typing data. *J Bacteriol* 189:2540–2552.

Mombach Pinheiro Machado AB, Reiter KC, Paiva RM, Barth AL (2007) Distribution of staphylococcal cassette chromosome *mec* (SCC*mec*) types I, II, III and IV in coagulase-negative staphylococci from patients attending a tertiary hospital in southern Brazil. *J Med Microbiol* 56:1328–1333.

Nunes AP, Schuenck RP, Bastos CC, Magnanini MM, Long JB, Iorio NL, Santos KR (2007) Heterogeneous resistance to vancomycin and

teicoplanin among *Staphylococcus* spp. isolated from bacteremia. *Braz J Infect Dis* 11:345–350.

Nunes AP, Teixeira LM, Bastos CC, Silva MG, Ferreira RB, Fonseca LS, Santos KR (2005) Genomic characterization of oxacillin-resistant *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* isolated from Brazilian medical centres. *J Hosp Infect* 59:19–26.

Otto M (2009) *Staphylococcus epidermidis*—the 'accidental' pathogen. *Nat Rev Microbiol* 7:555–567.

Ruppé E, Barbier F, Mesli Y, Maiga A, Cojocaru R, Benkhalfat M, Benchouk S, Hassaine H, Maiga I, Diallo A, Koumaré AK, Ouattara K, Soumaré S, Dufourcq JB, Nareth C, Sarthou JL, Andremont A, Ruimy R (2009) Diversity of staphylococcal cassette chromosome *mec* structures in methicillin-resistant *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* strains among outpatients from four countries. *Antimicrob Agents Chemother* 53:442–449.

Růzicka F, Holá V, Votava M, Tejkalová R, Horvát R, Heroldová M, Woznicová V (2004) Biofilm detection and the clinical significance of *Staphylococcus epidermidis* isolates. *Folia Microbiol (Praha)* 49:596–600.

Schuenck RP, Nouér SA, Winter Cde O, Cavalcante FS, Scotti TD, Ferreira AL, Giambiagi-de Marval M, dos Santos KR (2009) Polyclonal presence of non-multiresistant methicillin-resistant *Staphylococcus aureus* isolates carrying SCC*mec* IV in health care-associated infections in a hospital in Rio de Janeiro, Brazil. *Diagn Microbiol Infect Dis* 64:434–441.

Stepanović S, Vuković D, Dakić I, Savić B, Svabic-Vlahović M (2000) A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *J Microbiol Methods* 40:175–179.

Stepanović S, Vuković D, Jezek P, Pavlović M, Svabic-Vlahović M (2001) Influence of dynamic conditions on biofilm formation by staphylococci. *Eur J Clin Microbiol Infect Dis* 20:502–504.

Thomas JC, Vargas MR, Miragaia M, Peacock SJ, Archer GL, Enright MC (2007) Improved multilocus sequence typing scheme for *Staphylococcus epidermidis*. *J Clin Microbiol* 45:616–619.

Tormo MA, Knecht E, Götz F, Lasa I, Penadés JR (2005) Bap-dependent biofilm formation by pathogenic species of *Staphylococcus*: evidence of horizontal gene transfer? *Microbiology* 151:2465–2475.

Vandecasteele SJ, Peetermans WE, Merckx RR, Rijnders BJ, Van Eldere J (2003) Reliability of the *ica*, *aap* and *atlE* genes in the discrimination between invasive, colonizing and contaminant *Staphylococcus epidermidis* isolates in the diagnosis of catheter-related infections. *Clin Microbiol Infect* 9:114–119.

Vivoni AM, Diep BA, de Gouveia Magalhães AC, Santos KR, Riley LW, Sensabaugh GF, Moreira BM (2006) Clonal composition of *Staphylococcus aureus* isolates at a Brazilian university hospital: identification of international circulating lineages. *J Clin Microbiol* 44: 1686–1691.

Widerström M, Monsen T, Karlsson C, Edebro H, Johansson A, Wiström J (2009) Clonality among multidrug-resistant hospital-associated *Staphylococcus epidermidis* in northern Europe. *Scand J Infect Dis* 41:642–649.

Ziebuhr W, Hennig S, Eckart M, Kränzler H, Batzilla C, Kozitskaya S (2006) Nosocomial infections by *Staphylococcus epidermidis*: how a commensal bacterium turns into a pathogen. *Int J Antimicrob Agents* 28 (suppl):14–20.