Reduced susceptibility to vancomycin and biofilm formation in methicillin-resistant *Staphylococcus epidermidis* isolated from blood cultures

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This study aimed to correlate the presence of ica *genes, biofilm formation and antimicrobial resistance in 107 strains of* Staphylococcus epidermidis *isolated from blood cultures. The isolates were analysed to determine their methicillin resistance, staphylococcal cassette chromosome* mec *(SCC*mec*) type,* ica *genes and biofilm formation and the vancomycin minimum inhibitory concentration (MIC) was measured for isolates and subpopulations growing on vancomycin screen agar. The* mecA *gene was detected in 81.3% of the* S. epidermidis *isolated and 48.2% carried SCC*mec *type III. The complete* icaADBC *operon was observed in 38.3% of the isolates; of these, 58.5% produced a biofilm. Furthermore, 47.7% of the isolates grew on vancomycin screen agar, with an increase in the MIC in 75.9% of the isolates. Determination of the MIC of subpopulations revealed that 64.7% had an MIC ≥ 4 μg mL^{-1}, including 15.7% with an MIC of 8 μg mL^{-1} and 2% with an MIC of 16 μg mL^{-1}. The presence of the* icaADBC *operon, biofilm production and reduced susceptibility to vancomycin were associated with methicillin resistance. This study reveals a high level of methicillin resistance, biofilm formation and reduced susceptibility to vancomycin in subpopulations of* S. epidermidis*. These findings may explain the selection of multidrug-resistant isolates in hospital settings and the consequent failure of antimicrobial treatment.*

Key words: *Staphylococcus epidermidis* - *icaADBC* - *mecA* - SCC*mec* - vancomycin - MIC

Staphylococcus epidermidis, a member of the coagulase-negative staphylococci (CoNS) group, is the main bacterium found on human skin and causative agent of medical device-associated infections. The contamination of prostheses and intravenous devices with this pathogen is related to the production of a virulence factor, which represents an important pathogenic mechanism of implant infections. This substance, known as slime or biofilm, permits microorganisms to adhere to different materials (Costerton et al. 1995).

Biofilm formation is a process that involves microbial adhesion to and colonisation of a surface, cell proliferation and accumulation in multilayers, maturation and, finally, biofilm detachment and the release of cells (Houston et al. 2011). In addition to non-specific interactions such as electrostatic and hydrophobic interactions, specific adhesins, including two staphylococcal surface proteins (SSP-1 and SSP-2), are involved in the initial attachment to the polymer surface. When a certain material is implanted into an individual, the components of body fluids, such as serum proteins and platelets, start to cover the catheter or implant, modifying its surface properties and facilitating bacterial adhesion (Patti et al. 1994, von Eiff et al. 2002). *S. epidermidis* and *Staphylococcus aureus* express dozens of proteins on their surfaces. These proteins, called microbial surface components recognising adhesive matrix molecules, specifically bind to extracellular matrix proteins of the host, such as fibrinogen, collagen, fibronectin and vitronectin (Patti et al. 1994, von Eiff et al. 2002).

One of the main steps of biofilm formation is the production of polysaccharide intercellular adhesin (PIA), which is responsible for intercellular adhesion and the accumulation of cells in multilayers. The production of PIA is mediated by the products of the chromosomal intercellular adhesion genes (*ica*), which are organised into an operon that contains *icaADBC* genes, responsible for biosynthesis, and the *icaR* gene, which exerts a regulatory function (Otto 2008). PIA is synthesised from UDP-N-acetylglucosamine by N-acetylglucosamine transferase, an enzyme encoded by *icaA* and *icaD* genes, particularly *icaA*. The simultaneous expression of *icaA* and *icaD* promotes a significant increase in N-acetylglucosamine transferase (McCann et al. 2008, Otto 2008). Another possibility is that the protein IcaA (Mack et al. 1999) requires IcaD to assume an active conformation. The *icaC* gene, when expressed concomitantly with *icaA* and *icaD*, induces the synthesis of longer oligomers. After export, PIA is deacetylated by the protein IcaB, which introduces positive charges that are crucial for the superficial localisation and biological function of PIA (Otto 2008).

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In addition to PIA, adhesive proteins such as accumulation-associated protein and biofilm-associated protein (Bhp in *S. epidermidis* and Bap in *S. aureus*) may contribute to biofilm formation (Rupp et al. 2001, Von Eiff et al. 2002), even in the absence of the *icaADCB* operon. However, catheter-related and other nosocomial infections caused by biofilm-producing *S. epidermidis* are related to the presence of the *ica* operon, which is the main factor responsible for biofilm formation in this species (Cafiso et al. 2004).

The bacteria present inside a biofilm are protected against the action of the host immune system and antimicrobial drugs, thus permitting their survival (Mah & O'Toole 2001, Donlan & Costerton 2002). Biofilm-associated bacteria are usually less susceptible to antibiotics than planktonic bacteria; this can be explained by different mechanisms, such as the binding of antibiotics to biofilm components, reduced penetration of the antibiotic, slower growth of the microorganisms in the biofilm, high bacterial density and altered gene expression in the bacteria present in the biofilm (Stewart & Costerton 2001, Singh et al. 2010).

Oxacillin, one of the antibiotics most commonly used in Brazil for the treatment of staphylococcal infections, is no longer effective because of the high prevalence of resistant strains. Resistance to methicillin is generally conferred by the *mecA* gene, which produces a penicillin-binding protein (PBP2a or PBP 2') with reduced affinity for beta-lactam antibiotics when compared to other PBPs (Chambers et al. 1985).

The *mecA* gene is carried by a mobile genetic element, staphylococcal cassette chromosome *mec* (SCC*mec*). Eleven different types of SCC*mec* have been identified in *S. aureus* (I-XI) (sccmec.org); however, types VI, VII, IX-XI have not yet been described in CoNS (Zong et al. 2011, Vitali et al. 2014). SCC*mec* elements are more diverse in methicillin-resistant-CoNS (MR-CoNS) and new variants of the *ccr* genes continue to be identified, which cannot be typed with the currently available schemes. Thus, classification schemes of SCC*mec* in MR-CoNS are needed (Zong et al. 2011). SCC*mec* types III-V are prevalent in MR-CoNS (Zong et al. 2011). Type IV is the smallest, which reduces the cost of transfer between strains and selectively favours this type (Ito et al. 2001).

The high prevalence of methicillin resistance has led to the use of toxic antibiotics, such as the glycopeptide vancomycin for the treatment of Gram-positive infections. However, intermediate resistance to this drug was described in *Staphylococcus haemolyticus* in the 1980s (Schwalbe et al. 1987) and two strains (1 each of *S. epidermidis* and *S. haemolyticus*) with intermediate vancomycin resistance were found in Brazil in 1996 (Del'Alamo et al. 1999). This is a matter of concern because few other options are currently available for the treatment of staphylococcal infections.

In view of the importance of biofilm formation and antimicrobial resistance in infections caused by *S. epidermidis*, the objective of the present study was to characterise *S. epidermidis* strains isolated from blood cultures of patients hospitalised at a Brazilian teaching hospital with regard to their methicillin resistance, SCC*mec* type, reduced susceptibility to vancomycin, presence of the *ica* genes and biofilm formation.

TABLE I

General data of the patients included in the study and year of isolation of *Staphylococcus epidermidis* from blood cultures

Unit		ER	Neo-ICU	Nursery	ER-ICU	Paed	Dialysis	Central-ICU	IM	Paed-ICU	Cardio	Cor-ICU	IPD	No data
Patients (n)		33	23	15	9	5	4	3	4	2	2	1	1	2
Mean age		53 years	6 days	12.3 days	64.7 years	120 days	NA	NA	37 years	NA	59	NA	NA	NA
Gender	Male	19	19	0	6	3	2	0	1	2	1	0	1	0
(n)	Female	13	3	14	9	2	2	3	3	0	1	3	0	0
	NA	1	1	1	0	0	0	0	0	0	0	0	0	2
Year	2005 (17)	1	7	0	1	1	0	1	1	1	1	0	1	0
(n)	2006 (18)	3	4	1	4	3	0	1	1	1	0	0	0	0
	2007 (18)	0	10	1	0	0	4	1	1	0	0	0	0	1
	2008 (19)	9	2	4	3	0	0	0	0	0	0	0	0	0
	2009 (20)	6	0	9	1	1	0	0	1	0	1	1	0	0
	2010 (15)	14	0	0	0	0	0	0	0	0	0	0	0	1

cardio: cardiology ward; cor-ICU: coronary intensive care unit; ER: emergency room; IM: internal medicine; IPD: infectious and parasitic diseases; NA: not available; neo: neonatal; paed: paediatric.

SUBJECTS, MATERIALS AND METHODS

A total of 107 *S. epidermidis* strains isolated from blood cultures of patients hospitalised at a teaching hospital at Botucatu Medical School (FMB), São Paulo State University (UNESP), state of São Paulo, Brazil, between 2005-2010 were studied. The University Hospital of FMB provides tertiary care and possesses 385 beds, including 52 in intensive care units (ICUs) (30 adult, 15 neonatal and 7 paediatric ICU beds). The isolates are stored in the Culture Collection of the Department of Microbiology and Immunology, Botucatu Institute of Biosciences, UNESP, and were obtained from adult and paediatric patients of both genders hospitalised in different units of the hospital. Table I describes the characteristics of the patients and the year of *S. epidermidis* isolation. The criteria proposed by the Centers for Disease Control and Prevention/ National Healthcare Safety Network (CDC/NHSN 2014) were used to determine the inclusion of blood cultures.

Species of the genus *Staphylococcus* were isolated and identified as described by Baker (1984) and Koneman et al. (1997). The simplified method proposed by Cunha et al. (2004) was used for strain identification. DNA was extracted from isolates identified as *S. epidermidis* using the Illustra® kit (GE Healthcare). Amplification of the internal transcribed spacer region-polymerase chain reaction (PCR), as described by Barry et al. (1991) and Couto et al. (2001), was used to confirm that the isolates belonged to the species *S. epidermidis*.

The disk diffusion test employing oxacillin (1 µg) and cefoxitin (30 µg) disks was used for the phenotypic detection of methicillin resistance according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI 2007, 2012). For genotypic analysis, PCR was used to detect the *mecA* gene (Murakami et al. 1991). International reference strains were included in all reactions as positive (*S. aureus* ATCC 33591) and negative (*S. aureus* ATCC 25923) controls, according to the CLSI (2012). The strains that were positive for the *mecA* gene were subjected to SCC*mec* typing by multiplex PCR, according to Machado et al. (2007).

The presence of the *icaA*, *icaD*, *icaB* and *icaC* genes was determined by PCR (Arciola et al. 2001, 2005) and the results were validated using international reference strains as positive (*S. epidermidis* ATCC 35983) and negative (*S. epidermidis* ATCC 12228) controls.

Biofilm production by the isolates that were positive for *icaADBC* was detected by the polystyrene plate method described by Christensen et al. (1985) and modified by Oliveira and Cunha (2010). All isolates were screened for reduced susceptibility to vancomycin by growth on agar containing 4 and 6 µg mL^{-1} of the antibiotic, as described by Hiramatsu (2001) and CLSI (2012), but using an inoculum size of 2.0 McFarland standards. International reference strains (*Enterococcus faecalis* ATCC 29212 and ATCC 51299) were used as negative and positive controls, respectively. The presence of the *vanA* and *vanB* genes was determined by PCR (Clark et al. 1993) and the results were validated using international reference strains as positive (*E. faecalis* ATCC 51299) and negative (*E. faecalis* ATCC 29212) controls.

Vancomycin minimum inhibitory concentrations (MICs) were determined by a standardised broth microdilution method according to CLSI recommendations (CLSI 2012) and using panels prepared in-house. The following vancomycin concentrations were prepared with cation-adjusted Mueller-Hinton broth: 16, 8, 4, 2, 1, 0.5, 0.25 and 0.125 µg mL^{-1}. After inoculation, each well contained approximately 5 x 10^5 colony-forming unit mL^{-1}. The MIC was defined as the concentration that completely inhibited bacterial growth after 24 h of incubation at 35ºC. However, for comparison, the plates were also analysed after 48 h. The following international reference strains were used to validate the results: *E. faecalis* ATCC 51299, *E. faecalis* ATCC 29212 and *S. aureus* ATCC 29213.

The results were compared by the chi-squared test, adopting a level of significance of < 0.05 (Curi 1997).

RESULTS

Seventy-nine (73.8%) of the 107 *S. epidermidis* isolates studied were resistant to methicillin based on the disk diffusion method using cefoxitin or oxacillin disks; 87 (81.3%) of the isolates were positive for the *mecA* gene. The sensitivity and specificity of the two phenotypic methods were 87.3% and 85%, respectively.

The *mecA* gene-positive isolates were subjected to SCC*mec* typing. Twenty-one (24.1%) of the 87 isolates were classified as SCC*mec* type I, one (1.1%) as type II, 42 (48.2%) as type III and 18 (20.7%) as type IV; five (5.7%) could not be typed by this technique. A decrease in strains carrying SCC*mec* type III was observed over the period from 2005-2007 (56.8%) and from 2008-2010 (39.5%), whereas the prevalence of type IV increased almost threefold (from 11.3 to 30.3%).

Vancomycin susceptibility testing showed that 51 (47.7%) isolates were able to grow on agar plates containing 4 µg mL^{-1} of the antibiotic, whereas three (2.8%) isolates were able to grow on agar containing 6 µg mL^{-1}. The colonies were confirmed to be CoNS by Gram staining as well as catalase and coagulase tests to rule out the possibility of contamination. DNA was extracted from the colonies to determine the presence of the *vanA* and *vanB* genes and all of them were negative for these genes.

The vancomycin MICs obtained for subpopulations that grew on vancomycin screen agar and for the original populations are shown in Table II. The MIC was ≥ 4 µg mL^{-1} in 64.7% of the isolates that grew on agar plates containing 4 µg mL^{-1} vancomycin, with an MIC of 8 µg mL^{-1} in 15.7% of these isolates and an MIC of 16 µg mL^{-1} in one isolate (2%). MICs of 8 and 4 µg mL^{-1} were observed in two (66.6%) and one of the three strains that grew on agar plates with 6 µg mL^{-1} vancomycin, respectively. A comparison of MICs between the original isolates and the subpopulations that grew on vancomycin screen agar showed an increase in MIC in 75.9% of the isolates after 24 h of incubation. A two, four or eight-fold increase was observed in 96.3% of the strains after 48 h.

Regarding the presence of *ica* genes, at least one *ica* gene was detected in 96 (89.7%) of the isolates. Forty-one (38.3%) *S. epidermidis* isolates carried the complete *ica* operon, 43 (40%) carried the *icaA+icaD+icaC* genes, 47

TABLE II

Vancomycin minimum inhibitory concentrations (MICs) obtained after 24 h and 48 h of incubation of *Staphylococcus epidermidis* strains and subpopulations that grew on vancomycin screen agar

	Strains n (%)[a]		Subpopulation on vancomycin agar n (%)[a]			
			4 μg mL^{-1}		6 μg mL^{-1}	
MIC (μg mL^{-1})	24 h	48 h	24 h	48 h	24 h	48 h
1	17 (32.7)	14 (26.9)	0 (0)	0 (0)	0 (0)	0 (0)
2	34 (65.4)	30 (57.7)	19 (37.3)	4 (7.8)	0 (0)	0 (0)
4	1 (1.9)	8 (15.4)	24 (47)	31 (60.8)	1 (33.3)	0 (0)
8	0 (0)	0 (0)	8 (15.7)	16 (31.4)	2 (66.6)	3 (100)
16	0 (0)	0 (0)	1 (2)	1 (2)	0 (0)	0 (0)
Total	52 (100)	52 (100)	51 (100)	51 (100)	3 (100)	3 (100)

a: parental strains.

TABLE III

Frequency of the *mecA* and *ica* genes, biofilm formation, reduced susceptibility to vancomycin, increased vancomycin minimum inhibitory concentrations (MICs) on screen agar and associations found

	Isolates (n)	*ica*[a] (96)	*icaA+icaD* (47)	*icaA+icaD+icaC* (43)	*icaADBC* (41)	*icaADBC*/ biofilm production (24)	Reduced susceptibility to vancomycin (52)	Subpopulation with increased vancomycin MIC (39)
MRSE	Total (87)	79	45[b]	41[b]	39[b]	24[b]	44	33
	I (21)	17	6	6	6	3	7	3
	II (1)	1	1	1	1	1	1	1
	III (42)	39	24[c]	20	19	14	28	24[c]
	IV (18)	17	11	11	10	6	8	5
	NT (5)	5	3	3	3	0	0	0
MSSE (20)		17	2	2	2	0	8	6

MRSE: methicillin-resistant *Staphylococcus epidermidis* based on the presence of the *mecA* gene; MSSE: methicillin-sensitive *S. epidermidis*; NT: no typed; *a*: at least one *ica* gene; *b*: significantly associated with *mecA* gene positivity ($p < 0.05$); *c*: significantly associated with the presence of staphylococcal cassette chromosome *mec* type III ($p < 0.05$).

(43.9%) concomitantly carried the *icaA* and *icaD* genes and 11 (10.9%) were negative for all genes of the operon. All *icaA* gene-positive isolates were positive for *icaD* (Table III). Twenty-four (58.5%) of the isolates carrying the complete *ica* operon produced a biofilm; of these, 15 (36.6%) were classified as weakly adherent and nine (22%) as strongly adherent.

Eight (33.3%) of the isolates that produced a biofilm and carried the *icaADBC* operon exhibited vancomycin MICs ≥ 8 μg mL^{-1}, whereas this level of resistance was observed in only nine (8.4%) of all isolates studied. The only isolate that exhibited a vancomycin MIC equal to 16 μg mL^{-1} produced a strongly adherent biofilm.

Comparisons of the isolates that exhibited an increase in the vancomycin MIC after 24 h of incubation (n = 39; 36.4%) with those that did not grow (n = 55; 51.4%) and those that did grow, but maintained the same MIC (n = 13; 12.1%), revealed that the increase in vancomycin MIC was positively associated with methicillin resistance using oxacillin or cefoxitin disks ($p = 0.02$) and the presence of SCC*mec* type III ($p = 0.0003$).

An association was observed between the presence of the *mecA* gene and the presence of the *icaA+icaD* genes ($p = 0.001$): most isolates carrying the *mecA* gene were also positive for the *icaA+icaD+icaC* genes ($p = 0.002$) and also for the complete *icaADBC* operon ($p = 0.004$). In ad-

dition, the presence of *icaA* and *icaD* was associated with SCC*mec* type III (p = 0.030) (Table III). Furthermore, the presence of the complete *ica* operon and biofilm production were significantly associated with the presence of the *mecA* gene (100%; p = 0.006) and with growth on vancomycin screen agar (66.7%; p = 0.05) when compared to isolates that did not carry the complete operon or that did carry the operon, but did not produce a biofilm.

DISCUSSION

Formerly considered to be an innocuous commensal bacterium of human skin, *S. epidermidis* is now recognised as an important opportunistic pathogen. This microorganism is one of the most common causative agents of medical device-related infections (Sievert et al. 2013). We isolated 107 *S. epidermidis* strains from blood cultures of patients seen at a Brazilian teaching hospital and analysed antimicrobial resistance patterns, the presence of *ica* operon genes and biofilm formation. The results showed a high prevalence of methicillin resistance as detected by both phenotypic and genotypic methods (73.8% and 81.3%, respectively). This finding was expected because the prevalence of methicillin resistance is high (70-80%) among CoNS in Latin America (Diekema et al. 2001).

The prevalence of SCC*mec* types III (48.2%) and IV (20.7%) was high, which is a finding that was also reported by Wisplinghoff et al. (2003). SCC*mec* type IV is smaller and the metabolic cost of its transfer is thus lower; as a consequence, its prevalence is expected to increase over time. Indeed, in the present study, the proportion of SCC*mec* type IV increased three-fold over a period of six years. Conversely, the prevalence of type III decreased during the same period, although its prevalence continues to be high. This high proportion of SCC*mec* type III might be related to the selection of this type in the hospital environment due to the multidrug resistance of these strains. These facts should be considered in infection control policies.

SCC*mec* type II was detected in only one isolate. A similar frequency was reported by Machado et al. (2007), suggesting that MR *S. epidermidis* (MRSE) carrying this SCC*mec* type is not prevalent in Brazil. SCC*mec* type I, a hospital-associated type, was frequent (24.1%); this finding stands in contrast to previous studies in which type I was found to be rare (Ibrahem et al. 2009, Barbier et al. 2010, Zong et al. 2011), but is similar to the findings reported in other Brazilian studies (Machado et al. 2007, Pereira & Cunha 2013, Ternes et al. 2013). These findings might be related to the local epidemiology of MRSE, indicating a higher frequency of SCC*mec* type I in Brazilian hospitals. These data indicate the existence of a vast reservoir of SCC*mec* among *S. epidermidis* strains (Wisplinghoff et al. 2003). Although these similarities prevail, the distribution of SCC*mec* types depends on several factors, including geographic location and the use of antimicrobial agents.

The incidence of the *ica* genes was high in the present study, with 90.6% of the isolates being positive for at least one *ica* gene and 38.3% being positive for all genes. Biofilm production was detected by the phenotypic method in 58.5% of the isolates carrying the complete *ica* operon. Similarly, Oliveira and Cunha (2010) observed that 56.6% of strongly adherent CoNS carried the *icaA*+*icaC*+*icaD* genes. The regulation of the *ica* operon is complex and the expression of the *ica* genes is variable: it can be activated or deactivated according to in vivo conditions. Some events, such as the addition of the insertion sequence IS*256*, appear to be associated with this phenomenon (Ziebuhr et al. 1999).

In the present study, all isolates that were positive for the *icaA* gene were also positive for *icaD*, in agreement with Arciola et al. (2001) and Gad et al. (2009). Cafiso et al. (2004) showed that the *icaD* gene was always expressed in *S. epidermidis*, but that phenotypic biofilm production only occurred when *icaA* was expressed simultaneously. This relationship might be explained by the selective pressure exerted by biofilm production because, when expressed, the *icaD* gene alone does not induce transferase activity and *icaA* induces little activity; in contrast, the combined expression of *icaA* and *icaD* produces large amounts of PIA (Gerke et al. 1998). Although the *icaA* and *icaD* genes overlap and are co-transcribed, the fact that the isolates were more frequently positive for *icaD* than *icaA*, *icaB* and *icaC* in the present study and in previous studies (Zhou et al. 2013) may be related to the loss or mutation of one of these genes.

The *mecA* gene was found to be associated with the presence of the *icaADBC* operon and biofilm production ($p < 0.05$). In addition, the concomitant presence of *icaA*+*icaD* was associated with SCC*mec* type III. Zhou et al. (2013) also detected *icaD* more frequently than *icaA* and observed a significant association between *icaD* and the *mecA* gene in *S. epidermidis*. Furthermore, other studies have demonstrated that methicillin resistance is correlated with biofilm production and the presence of *ica* genes (Alcaráz et al. 2003, Abassi et al. 2008, Koksal et al. 2009). These associations might be a consequence of the intimate contact between biofilm bacteria, facilitating the horizontal transfer of genetic material such as antimicrobial resistance genes (Klingenberg et al. 2005), which would explain the horizontal transfer of *mecA* to *ica* gene-positive strains (Kozitskaya et al. 2004). The present results suggest the presence of the *icaADBC* operon in conjunction with biofilm production and the presence of the *mecA* gene to be an important marker of nosocomial strains with the potential to cause infection. These markers are therefore useful for evaluating the clinical significance of CoNS.

The indiscriminate use of antibiotics has led to the selection of bacterial populations that contain cells with distinct levels of susceptibility to antimicrobial agents, including resistant cells. Intermediate and complete resistance to vancomycin in *S. epidermidis* has been reported (Palazzo et al. 2005) and biofilm formation is known to significantly reduce the effects of this antibiotic on bacteria (Raad et al. 2007). In the present study, 47.7% and 2.8% of the *S. epidermidis* isolates grew colonies on screen agar plates containing 4 and 6 $\mu g\ mL^{-1}$ vancomycin, respectively. One-third of the isolates that were biofilm producers also exhibited vancomycin MICs $\geq 8\ \mu g\ mL^{-1}$ after growing on vancomycin screen agar, characteristics that can lead to treatment failure and may be a precursor of glycopeptide resistance.

The term vancomycin heteroresistance refers to strains that contain a subpopulation of cells with different levels of susceptibility to this antibiotic, including resistant cells and cells with intermediate susceptibility (Raad et al. 2007). Our analysis of colonies grown on vancomycin screen agar showed an increase in MICs in 75.9% of the isolates, with an increase from 1 to ≥ 4 μg mL^{-1} in 64.7% of the isolates. MIC increases were also observed in 100% of the colonies that grew on agar with 6 μg mL^{-1} vancomycin. Using isolates that grew on screen agar with 4 μg mL^{-1} vancomycin, Ma et al. (2011) reported slightly lower rates: 64.7% of strains showed increased MICs and 35.3% showed MICs ≥ 4 μg mL^{-1}. MICs ≥ 4 μg mL^{-1} were observed in 85.7% of the subpopulations that grew on screen agar with 6 μg mL^{-1} vancomycin. In our study, increased MICs were detected in 96.3% of the isolates after 48 h of incubation. This finding confirms the existence of heteroresistant isolates.

The existence of vancomycin heteroresistance in nosocomial *S. epidermidis* is an alarming finding. Furthermore, the observation of subpopulations with MICs > 4 μg mL^{-1} (64.7%), as well as MICs of 8 μg mL^{-1} (15.7%) and 16 μg mL^{-1} (2%), classifying the isolates as intermediate resistant, indicates that infections caused by these microorganisms will be difficult to treat. Indeed, reports have shown higher mortality among patients infected with heteroresistant bacteria and strains with higher MICs compared to those infected with susceptible strains (Wong et al. 1999). However, therapeutic alternatives for infections caused by staphylococci with reduced vancomycin resistance have been approved by the US Food and Drug Administration and include linezolide, daptomycin, tigecycline and quinupristin/dalfopristin (Micek 2007). Two next-generation cephalosporins, ceftobiprole and ceftaroline fosamil, are currently available for the treatment of patients carrying MR and vancomycin-intermediate staphylococci (Saravolatz et al. 2011, Lovering et al. 2012).

In the present study, reduced susceptibility to vancomycin was significantly associated with methicillin resistance detected by phenotypic methods ($p < 0.05$). Other studies (Wong et al. 1999, Loomba et al. 2010) have shown that the isolation of MR staphylococci is significantly more common among patients with bacteraemia due to vancomycin-heteroresistant staphylococci. Although the mechanism underlying the association between methicillin resistance and reduced vancomycin susceptibility remains unclear, it might be related to the treatment of infections caused by MR bacteria with vancomycin, thereby selecting heterogeneous populations and strains with low susceptibility.

The proportion of strains with reduced susceptibility to vancomycin was higher among the isolates harbouring SCC*mec* type III. Differing results have been reported by Moise et al. (2007), who found that higher vancomycin MICs were significantly associated with SCC*mec* type II in *S. aureus*, whereas lower MICs were related to SCC*mec* type IV. The association between reduced vancomycin susceptibility and SCC*mec* type III observed in the present study may be due to the high prevalence of SCC*mec* type III in the hospital environment and the frequent use of vancomycin.

The presence of subpopulations with higher vancomycin MICs and intermediate resistance demonstrates the heterogeneity of *S. epidermidis* strains in terms of susceptibility to this antibiotic. Because the main genes responsible for vancomycin resistance (*vanA* and *vanB*) were not detected, this reduced susceptibility may be related to a thickening of the bacterial cell wall that prevents uptake of the antibiotic by the microorganism (Billot-Klein et al. 1996). However, further studies are needed to elucidate how these events occur in staphylococci.

The present study demonstrated an association between the presence of the *icaADBC* operon, biofilm formation and antimicrobial resistance in *S. epidermidis* strains isolated from blood cultures. Furthermore, the detection of *S. epidermidis* subpopulations with intermediate vancomycin resistance associated with methicillin resistance highlights the role of this species as an important multidrug-resistant microorganism and the consequent need to implement measures for the control of antibiotic use.

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