

## Major Article

# Coagulase-negative staphylococci in Southern Brazil: looking toward its high diversity

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## Abstract

**Introduction:** Coagulase-negative staphylococci (CoNS) are the most prevalent pathogens in nosocomial infections and may serve as a reservoir of mobile genetic elements such as the staphylococcal cassette chromosome *mec* (SCC*mec*) encoding methicillin resistance. Molecular characterization of SCC*mec* types combined with advanced molecular typing techniques may provide essential information for understanding the evolution and epidemiology of CoNS infections. We therefore aimed to investigate the SCC*mec* distribution, multidrug-resistance (MDR), and biofilm formation in CoNS blood culture isolates from a hospital in Southern Brazil. **Methods:** We analyzed 136 CoNS blood culture isolates obtained during 2002-2004 from patients admitted to a tertiary care hospital in Brazil. SCC*mec* types I to V were determined using multiplex PCR. The clonal relationship of *Staphylococcus epidermidis* was determined using pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). Molecular epidemiological data were interpreted along with data on biofilm formation, presence of the *icaD* gene, and MDR. **Results:** The most prevalent species were *S. epidermidis*, *Staphylococcus haemolyticus*, and *Staphylococcus hominis* harboring mainly SCC*mec* types II, III, and V. Overall, the presence of multiple SCC*mec* was associated with non-MDR, except for *S. epidermidis*. *S. epidermidis* isolates showed a high prevalence of *icaD*, but had low phenotypic biofilm formation. PFGE and MLST revealed high genetic diversity in the *S. epidermidis* population. **Conclusions:** Our results suggest a major shift in SCC*mec* types within a short period and reveal a different behavior of *S. epidermidis* with regard to the association between the presence of multiple SCC*mec* types and MDR profile.

**Keywords:** Coagulase-negative staphylococci. SCC*mec*. Multidrug-resistance. Biofilm. Molecular typing.

## INTRODUCTION

Coagulase-negative staphylococci (CoNS) are currently the most prevalent microorganisms responsible for nosocomial infections related to indwelling medical devices<sup>(1)</sup>. In prospective surveillance studies, CoNS were identified as the most common pathogens in nosocomial bloodstream infections of pediatric patients<sup>(2) (3)</sup>. Among CoNS, the main isolated species from nosocomial infections is *Staphylococcus epidermidis*, in particular in relation with indwelling devices<sup>(1)</sup>.

Coagulase-negative staphylococci infections are often difficult to treat due to multidrug resistance (MDR) and biofilm

formation<sup>(4) (5) (6)</sup>. This may result in infections with limited therapeutic options, increased risk of treatment failure, and high cost<sup>(6)</sup>. Moreover, their high genetic diversity and constant presence on the human body makes CoNS a permanent reservoir of genetic material for more virulent staphylococcal species including *Staphylococcus aureus*<sup>(7)</sup>.

The staphylococcal cassette chromosome (SCC) is a family of mobile genetic elements found in the genus *Staphylococcus*. SCC*mec* harbors the *mec* genes that encode for resistance to methicillin and almost all  $\beta$ -lactam antibiotics<sup>(8)</sup>. Furthermore, the SCC*mec* element frequently harbors integrated insertion sequences, plasmids, and transposons that often encode additional resistance determinants<sup>(9)</sup>. According to the current SCC*mec* classification scheme in methicillin-resistant *Staphylococcus aureus* (MRSA) (<http://www.sccmec.org>), a high number of non-typable and new previously unidentified SCC*mec* types in *S. aureus* have been detected in CoNS<sup>(9) (10) (11) (12) (13)</sup>. The high

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genetic diversity within SCC*mec* elements carried by CoNS makes the identification of SCC*mec* types in CoNS challenging and reflects a high degree of genetic flexibility<sup>(11)(14)</sup>.

In addition to SCC*mec* classification, other molecular techniques are currently used to characterize CoNS. Pulsed field gel electrophoresis (PFGE) is considered the most discriminatory method to explore local epidemiological outbreaks<sup>(10)(15)(16)</sup>, and multilocus sequence typing (MLST) is a more robust tool to identify population structure and global epidemiology<sup>(17)(18)(19)</sup>. To date, there are few studies evaluating the molecular epidemiology of CoNS from Brazilian hospitals<sup>(16)(20)(21)</sup>.

The aim of this study was to investigate the SCC*mec* distribution, MDR, and biofilm formation in CoNS blood culture isolates from a hospital in Southern Brazil. We also compared the SCC*mec* distribution with data from other studies from the same institution in order to observe possible modifications in the prevalence of this mobile genetic element. Furthermore, for *S. epidermidis*, we investigated the molecular epidemiology to reveal the possible spread of successful clones.

## METHODS

### Setting

We used a collection of 136 CoNS isolated from the blood culture of patients admitted at *Santa Casa de Misericórdia de Porto Alegre* Hospital, a tertiary care hospital in South Brazil, during the period of 2002 to 2004. The isolates were stored at -80°C on skimmed milk (Difco Skim Milk, Becton Dickinson), and for this study, each isolate was grown on blood agar 24 hours prior to each test procedure.

### Multidrug-resistant definition criteria

The antimicrobial susceptibility profile of each isolate was determined as described before.<sup>(22)</sup> MDR was defined as a resistant phenotype to at least one agent in three or more antimicrobial categories<sup>(23)</sup>.

### Biofilm formation in *Staphylococcus epidermidis*

Semi-quantitative determination of biofilm formation for *S. epidermidis* was performed as described previously<sup>(4)(24)</sup>. All the *S. epidermidis* isolates were tested in polystyrene microtiter plates with three parallel runs using tryptic soy broth (TSB) with 1% glucose to induce biofilm formation. We determined the optical density (OD) of the crystal violet-stained adherent biofilm using a spectrophotometer (model MR580; Dynatech Laboratories, Inc.) at 570nm. For each parallel, the highest and the lowest values of OD were removed to exclude outliers, and the remaining values were averaged. The isolates were considered biofilm producers if they had an OD  $\geq 0.12$ . The cutoff value was chosen to distinguish between isolates that produced significant amounts of biofilm and those that did not, taking into account the OD values for the negative controls included in each experiment. The presence of the *icaD* gene was determined by polymerase chain reaction (PCR) as described previously<sup>(4)</sup>. In both assays, *S. epidermidis* RP62A was used as a positive control and *S. epidermidis* ATCC 12228 and *Staphylococcus haemolyticus* 51-03 were included as negative controls.

## Determination of the SCC*mec* types among all the CoNS

Deoxyribonucleic acid (DNA) for PCR was extracted using the QIAamp DNA mini kit (Qiagen) as described by Oliveira et al.<sup>(25)</sup>. The SCC*mec* types and subtypes I, II, III, IVa, IVb, IVc, IVd, and V and *mecA* were investigated using the multiplex PCR designed by Zhang et al.<sup>(26)</sup> with the following modifications: the PCR reaction mixtures contained 1.5mM MgCl<sub>2</sub> and 1.15 unit of Platinum *Taq* DNA polymerase (Invitrogen Inc., Carlsbad, CA). The final concentrations of each pair of primers were I (0.096μM), II (0.064μM), III (0.08μM), IVa (0.208μM), IVb (0.184μM), IVc (0.156μM), IVd (0.56μM), and V (0.12μM). The positive control strain for each gene was as follows: type I (NCTC10442), type II (N315), type III (85/2082), type IVa (CA05), type IVb (8/6-3P), type IVc (MR108), type IVd (JCSC4469), and type V [WIS (WBG8318)-JCSC3624]<sup>(5)</sup>. Methicillin-resistant isolates that did not produce PCR products using any of the primers tested were considered non-typable.

### Pulsed field gel electrophoresis of the *Staphylococcus epidermidis* isolates

Agarose plugs (Certified Megabase Agarose, BioRad) containing chromosomal DNA were prepared as previously described<sup>(10)</sup>; Lysozyme (Omega) and Lysostaphin (Sigma) were used in the lysis step. Restriction digestion of chromosomal DNA was performed using *Sma*I (New England Biolabs), and the fragments were separated using PFGE employing a CHEF-DRIII device (Bio-Rad) as previously described<sup>(10)</sup>. The PFGE types were defined after analysis using the BioNumerics software version 7.1 (Applied Maths). Clustering was performed using the Dice similarity coefficient and the unweighted pair group method with arithmetic means (UPGMA) with 1.3% of tolerance and 0.8% optimization. The PFGE types were automatically assigned using a cutoff similarity value of 79%; the types obtained were represented by numbers.

### Multilocus sequence typing of the *Staphylococcus epidermidis* isolates

Fragments of seven housekeeping genes were amplified by conventional PCR using the MLST scheme published by Thomas et al.<sup>(27)</sup>. The alleles and sequence type (ST) numbers were assigned using the *S. epidermidis* MLST database (<http://sepidermidis.mlst.net>). The most likely patterns of evolutionary descent in the collection were assessed using the eBurst algorithm (<http://eburst.mlst.net>).

### Statistical analysis

Comparisons between the prevalence of independent samples were performed using the binomial test. The associations of different variables were assessed using the Pearson Chi-square test, and when necessary, Fischer's exact test. Eventually adjusted standardized residual analysis<sup>(28)</sup> was included to identify the associated variables. The significance level used was 5%.

## RESULTS

### Staphylococcal cassette chromosome *mec*

The species and SCC*mec* distribution among all the methicillin-resistant CoNS (MR-CoNS) isolates are presented in **Table 1**. The 10 methicillin-sensitive isolates were all *S. epidermidis* species. One hundred (79%) of the 126 methicillin-resistant isolates harbored at least one of the SCC*mec* types tested in this study.

Overall, MDR was seen among 106/136 (78%) isolates. There was a significant association between the presence of SCC*mec* and MDR ( $p = 0.005$ ). However, an association between individual SCC*mec* types and positive MDR was significant only for type III ( $p < 0.001$ ), and type V ( $p = 0.011$ ). As the frequency of some SCC*mec* types were too low to perform solid statistical tests, and since we observed several combinations of SCC*mec* types in our isolates (**Table 1**), the types were divided into three categories: single, multiple, and non-typable. The association between the different categories and MDR is presented in **Table 2**. For CoNS as a whole and *S. haemolyticus* as single species, there was an association between the presence of single SCC*mec* and MDR, while *S. epidermidis* showed similar results but without significance. For *Staphylococcus hominis* and other less prevalent species, there were not enough isolates for reliable statistical calculation.

### Biofilm formation

Twenty-eight (45%) of the 62 *S. epidermidis* isolates had an OD of  $\geq 0.12$  and were considered biofilm producers. Of these, 26 (93%) were *icaD* positive and 2 (7%) were *icaD* negative. On the other hand, of the 34 non-biofilm producers, 24 (71%) were *icaD* positive and 10 (29%) were *icaD* negative. The overall prevalence of the *icaD* gene was 81% (50/62) and that of biofilm formation was 45% (28/62). No significant associations were found between biofilm production or the presence of *icaD* and the MDR phenotype ( $p = 0.494$  and  $p = 0.389$ , respectively).

### Pulsed field gel electrophoresis

PFGE analysis of 62 *S. epidermidis* isolates clustered 40 (64%) isolates in 14 groups. The remaining 22 (36%) isolates were considered sporadic strains (**Figure 1**). The largest cluster (PFGE type 11;  $n = 7$ ) showed a positive association with MDR ( $p = 0.037$ ); 4 of these 7 isolates harbored SCC*mec* type III and were identified as ST6. The second largest cluster (PFGE type 7;  $n = 4$ ) showed a positive association between biofilm formation and the presence of *icaD* (both  $p = 0.034$ ). For the remaining PFGE types, there was no difference between the clustered and sporadic strains with regard to MDR ( $p = 0.055$ ), biofilm formation ( $p = 0.223$ ), or the presence of *icaD* ( $p = 0.741$ ). PFGE types 5, 6, 8, and 12 were triplets, and the remaining 8 types found (1, 2, 3, 4, 9, 10, 13, 14) comprised only 2 isolates. Two pairs of isolates showed identical banding patterns (isolates BRA526/575 and BRA246/267).

### Multilocus sequence typing

All the 62 *S. epidermidis* isolates were typed using MLST. In total, 29 different sequence types (ST) were identified, of which 12 are new (STs 499 to 510). **Figure 2** illustrates the distribution of our isolates in *S. epidermidis* clonal complex 2

TABLE 1 - Distribution of SCC*mec*\* types in MDR-CoNS isolated from a hospital in Southern Brazil from 2002 to 2004.

Species	Number	SCC <i>mec</i> type											II + III + V	III + IVd	III + IVa	II + V	I + III	I + V	Non-typable
		I	II	III	IVa	IVb	IVc	IVd	V										
<i>Staphylococcus epidermidis</i>	52	-	-	20	4	-	-	6	4	4	-	1	1	5	3	1	1	7	
<i>Staphylococcus haemolyticus</i>	41	-	5	16	-	-	-	-	6	-	-	-	9	-	-	1	-	4	
<i>Staphylococcus hominis</i>	19	-	-	14	-	-	-	-	-	-	-	-	1	-	-	-	-	4	
<i>Staphylococcus warneri</i>	6	-	-	1	-	-	-	-	-	-	-	-	1	-	-	-	-	4	
<i>Staphylococcus saprophyticus</i>	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4	
<i>Staphylococcus xylosum</i>	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	
<i>Staphylococcus cohnii</i>	1	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
<b>Total</b>	<b>126</b>	<b>-</b>	<b>5</b>	<b>52</b>	<b>4</b>	<b>-</b>	<b>-</b>	<b>6</b>	<b>10</b>	<b>-</b>	<b>-</b>	<b>6</b>	<b>12</b>	<b>5</b>	<b>3</b>	<b>2</b>	<b>26</b>		
Percentage (%)	100.0	-	4.0	41.2	3.2	-	-	4.8	7.9	-	-	4.8	9.5	4.0	2.4	1.6	20.6		

SCC*mec*: Staphylococcal cassette chromosome *mec*; MDR-CoNS: multidrug-resistant coagulase-negative staphylococci. \*SCC*mec* type was determined using multiplex PCR as described by Zhang et al. (2005).

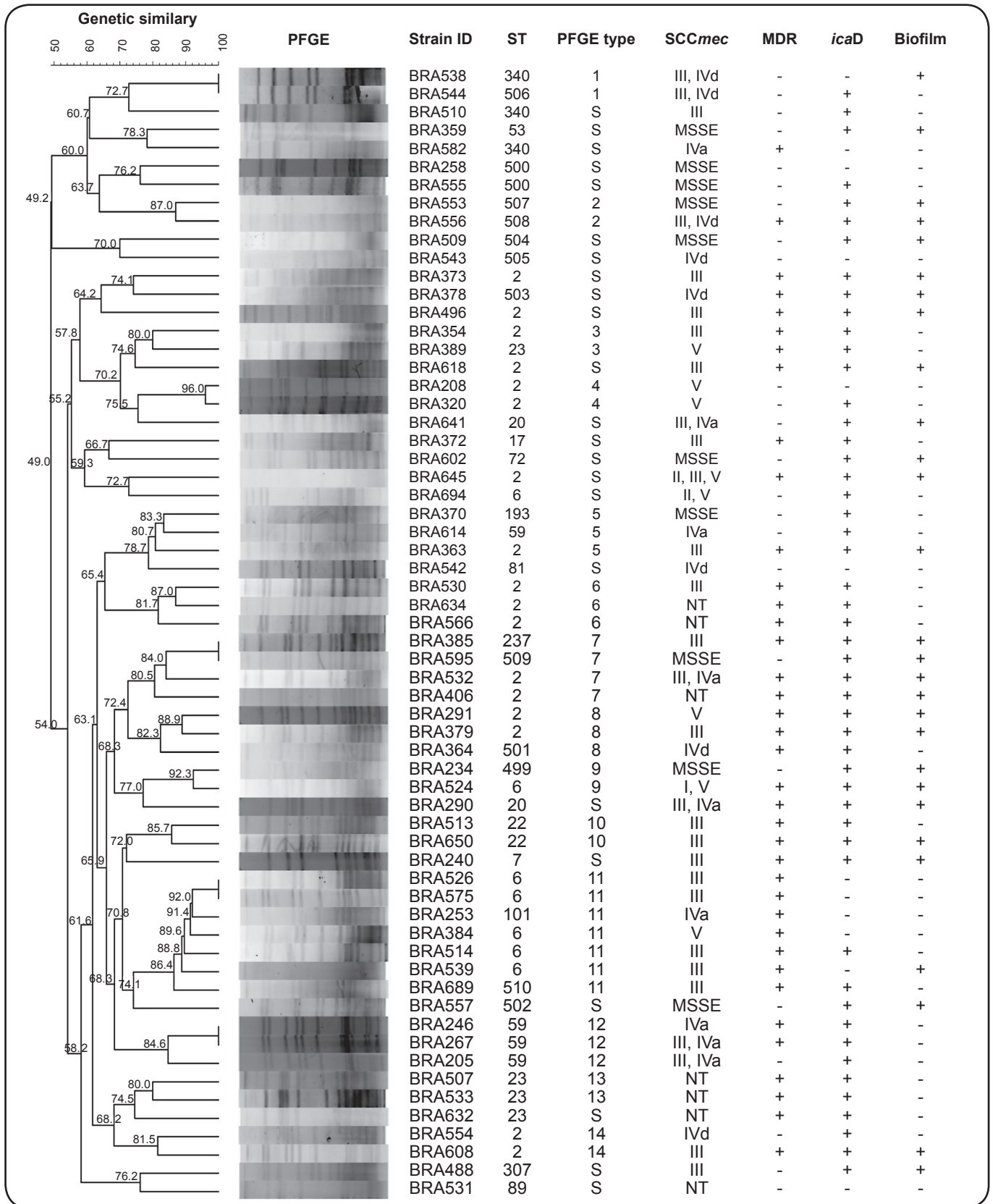


FIGURE 1 - Dendrogram of the PFGE profiles and main characteristics of the 62 *Staphylococcus epidermidis* isolates from Southern Brazil. Numbers in the horizontal upper bar and connection lines indicate similarity (percent). Isolates showing similarity  $\geq 79\%$  were considered genetically related. PFGE: pulsed-field gel electrophoresis; ID: identification; ST: sporadic strain; SCCmec: staphylococcal cassette chromosome mec; MDR: multidrug-resistant; IcaD: icaD gene; NT: non-typable; MSSE: methicillin susceptible *Staphylococcus epidermidis*.

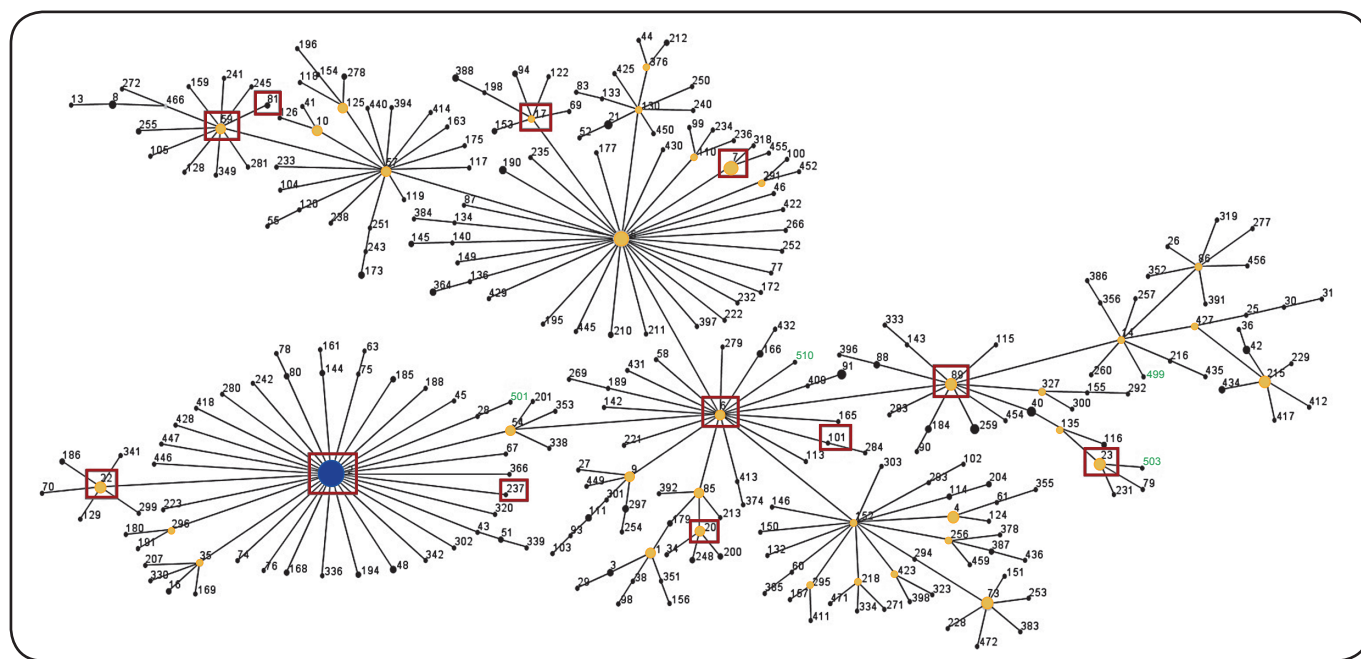


FIGURE 2 - eBurst V3 analysis of *Staphylococcus epidermidis* CC2 after adding our MLST data to all the isolates available in the MLST database on April 2016. Each ST is represented by a dot, and lines connect single locus variants. The blue dot represents the founder of CC2, and the yellow dots represent subgroup founders. Green numbers represent new STs found in this study. Red squares represent STs reported in previous studies that were also found in this study. eBurst V3: algorithm eBurst version 3; CC2: clonal complex 2; MLST: multilocus sequence typing; ST: sequence type.

TABLE 2 - Association between MDR and SCCmec category in Brazilian CoNS nosocomial isolates from 2004.

Species	MDR (n)	SCCmec									p value
		single			multiple			non-typable			
		n	%	AR	n	%	AR	n	%	AR	
<i>Staphylococcus epidermidis</i>	Pos (39)	27	69.2	1.0	6	15.4	-1.8	6	15.4	0.7	0.198
	Neg (13)	7	53.8	-1.0	5	38.5	1.8	1	7.7	-0.7	
<i>Staphylococcus haemolyticus</i>	Pos (36)	26	72.3	2.3	6	16.5	-3.1	4	11.2	0.8	0.008
	Neg (5)	1	20.0	-2.3	4	80.0	3.1	0	0.0	-0.8	
<i>Staphylococcus hominis</i>	Pos (13)	9	69.3	-*	1	7.7	-*	3	23.0	-*	-*
	Neg (6)	4	66.6	-*	1	16.7	-*	1	16.7	-*	
Other species	Pos (8)	1	12.5	-*	1	12.5	-*	6	75.0	-*	-*
	Neg (6)	1	16.7	-*	0	0.0	-*	5	83.3	-*	
<b>Total</b>	<b>Pos (96)</b>	<b>63</b>	<b>(65.6)</b>	<b>2.1</b>	<b>14</b>	<b>(14.6)</b>	<b>-2.3</b>	<b>19</b>	<b>(19.8)</b>	<b>-0.3</b>	<b>0.045</b>
	<b>Neg (30)</b>	<b>13</b>	<b>(43.3)</b>	<b>-2.1</b>	<b>10</b>	<b>(33.4)</b>	<b>2.3</b>	<b>7</b>	<b>(23.3)</b>	<b>0.3</b>	

MDR: multidrug-resistant; SCCmec: staphylococcal cassette chromosome mec; CoNS: coagulase-negative staphylococci; AR: adjusted residual; Pos: positive; Neg: negative. AR value  $\geq [2.0]$  was considered statistically significant; \*Unreliable results due to low n.

(CC2). Fifty-two percent (32/62) of the isolates were identified as ST2 (n = 17), ST6 (n = 7), ST59 (n = 4), and ST23 (n = 4). The majority of these most frequent STs were MDR (84%), *icaD* positive (87%), and biofilm negative phenotypically (59%). STs 2 and 6 were associated with SCCmec type III, and ST59 was found to be associated with SCCmec III and IVa (p < 0.05). The remaining isolates were assigned as singletons, pairs, or triplets

of different STs. The new STs were mainly positive for *icaD* and biofilm formation, but not for MDR (Figure 1).

## DISCUSSION

In this study, we determined the SCCmec characteristics of an old CoNS collection and compared it with a new one from the same institution but collected with a 6-8-year interval<sup>(5)</sup>.

We found that the types and combinations of SCCmec within the CoNS population had changed dramatically in this period. Upon comparing the results from the different periods, we observed an increase in the number of different SCCmec types and non-typable isolates and a decrease in the presence of multiple cassettes in the same isolate. Typing of the *S. epidermidis* isolates revealed a great genetic diversity, but some similarities in ST distribution were observed when compared with *S. epidermidis* isolates from other institutions in Brazil and around the world.

We conducted the same SCCmec multiplex PCR as that used in a former study<sup>(5)</sup> to enable comparisons between CoNS isolates collected from the same hospital. Statistically significant differences were found in the prevalence of all the SCCmec types except for the subtype IVd. Overall, we observed a decreasing prevalence of SCCmec types II, III, and V over the years ( $p < 0.0001$ ) and an increasing prevalence of SCCmec types I and IV ( $p < 0.0001$ ). It is noteworthy that just one isolate harbored SCCmec type I and none of the isolates harbored types IVb and IVc (**Table 1**). A previous study in the same institution<sup>(5)</sup> reported the emergence of 2 and 4 isolates harboring SCCmec types IVb and IVc, respectively, and the presence of 14 isolates harboring type I. We consider the appearance of new SCCmec types that were previously not found in this hospital especially concerning because this reflects the ongoing horizontal gene transfer between species. Unfortunately, the SCCmec typing scheme used failed to classify 26 out of the 126 (20.6%) isolates, which is a large proportion of the CoNS isolates.

The acquisition and accumulation of resistance genes through mobile elements like SCCmec is enabled by integration into regions of the SCCmec element called the joining (J) regions<sup>(29)</sup>. Antibiotic resistance determinants like *tet* (tetracycline), *aacA-aphD* (aminoglycosides), or *ermA* (erythromycin) genes may be carried within J regions originating in MDR strains<sup>(14)(30)</sup>. In our isolates, there was a strong association between the presence of SCCmec and MDR. However, when we grouped our results to analyze the presence of more than one SCCmec type in the same isolate (multiple), we observed that the presence of multiple SCCmec types is associated with non-MDR and the presence of a single SCCmec type is associated with MDR. Interestingly, no such associations were found when the *S. epidermidis* isolates were analyzed separately (**Table 2**).

The ability to form biofilms is considered the most important pathogenic factor for *S. epidermidis*, and the *icaD* genes are recognized as one of the most important genes involved in biofilm formation<sup>(31)</sup>. In our *S. epidermidis* isolates, despite the high prevalence of *icaD* (81%), we did not find a high prevalence of *in vitro* biofilm formation (45%). In addition, there was no statistical association between *icaD* presence and biofilm formation (data not shown). As suggested by Mertens and Ghebremedhin<sup>(32)</sup>, the natural occurrence of insertion sequence elements like IS256 might be one of the reasons for this divergence. Likewise, the presence of two isolates producing biofilms in absence of the *icaD* gene reinforces the fact that although *icaD* is important for the development of biofilm, other factors<sup>(31)</sup> and genes like *aap* and *bhp*<sup>(30)</sup> could be involved in the process.

As reported in other studies<sup>(15)(17)</sup>, analysis of our *S. epidermidis* strains using two reliable typing methods revealed a high degree of genetic diversity; our isolates showed high Simpson's indexes of diversity (SID) in PFGE (SID = 97.7%), and MLST (SID = 91.1%) (data not shown). PFGE analysis revealed several small clusters as well as a considerable number of sporadic strains, and MLST presented a range of different STs and several new alleles and STs (**Figure 1** and **Figure 2**). The proportion of different STs observed in this study (29 STs among 62 isolates) was higher than that previously reported by Mendes et al.<sup>(19)</sup> in 2012 (27 STs among 71 isolates) and Miragaia et al.<sup>(17)</sup> in 2007 (74 STs among 217 isolates). SCCmec typing also amplified all the SCCmec types searched and some different combinations of SCCmec types in individual strains, thus confirming the high variability of SCCmec types in *S. epidermidis*<sup>(10)(17)</sup>. Finally, despite the presence of 4 closely related isolates revealed by PFGE, no large cluster indicating an epidemic outbreak was detected.

The only two major clusters found in our study were assigned by MLST and match two very common MLST types detected worldwide: ST2 ( $n = 17$ ) and ST6 ( $n = 6$ ). Furthermore, our MLST results agree with the increasing high prevalence of STs 20, 22, 23, and 89 detected in several countries<sup>(10)(17)(19)(32)(34)(35)</sup>. Interestingly, we did not find any ST5 isolates, which is a very frequent type worldwide<sup>(17)(36)</sup>, but we found two ST5 single locus variants (ST7 and ST17) (**Figure 2**). However, the second most common ST worldwide (ST23)<sup>(17)</sup> was among the four most prevalent ST in our collection. As suggested previously, the genetic diversity of *S. epidermidis* nosocomial isolates may be caused by the need to adapt to different environments in hospital settings, leading to increased frequency of horizontal gene transfer and dissemination of mobile genetic elements<sup>(12)</sup>.

A comparison of the overall results generated by PFGE with the results of MDR, biofilm formation, and *icaD* presence revealed no difference between the clustered and sporadic strains. The high diversity of our isolates might be the reason for this result, since other studies have shown cluster isolates identified by PFGE with higher rates of antibiotic resistance and biofilm formation than non-cluster isolates<sup>(18)(32)</sup>. Finally, there is currently a lack of data concerning the epidemiology of both nosocomial and community *S. epidermidis* isolates from Brazil. Further studies need to be conducted in order to determine if there exist two different populations in these two settings, as previously reported for *S. epidermidis* in Europe<sup>(33)(34)</sup>.

This study has limitations. The multiplex PCR employed is easy to use, but the detection of only a single locus of each SCCmec type gives less discriminatory power to this assay when compared with that of the current recommended methodology (<http://www.sccmec.org>). Moreover, the isolates were not stratified according to the place of origin or period, and it is not possible to perform comparison between hospital wards. Novel typing studies with more recent isolates from the same institution and/or from the local community could be very useful in the elucidation of several epidemiological aspects of CoNS from South Brazil.

In conclusion, this study confirms that CoNS isolates have high genetic diversity despite being isolated from the same

institution. As a novelty, we observed a different behavior of *S. epidermidis* with regard to the association between the presence of multiple SCCmec types and a MDR profile when this specie was compared with other CoNS. In addition, we did not find an epidemic clone of this species using well-established molecular tools. A rapid shift in the prevalence of the SCCmec types from our study and to a previous study from the same hospital<sup>(6)</sup> performed 6-8 years after indicate a high degree of horizontal gene transfers, which confirm the hypothesis that CoNS is a permanent reservoir of genetic material that can be exchanged within and between Staphylococcal species.

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#### Conflicts of Interest

The authors declare that there is no conflict of interest.

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