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

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Coagulase-negative staphylococci (CoNS) are now recognized as the aetiological agents of an important range of infections in humans. Most developed countries have reported an increase in CoNS infections in hospitalized patients that are resistant to meticillin and other antibiotics. Staphylococcal cassette chromosome *mec* (SCC*mec*) typing is essential for understanding the molecular epidemiology of meticillin-resistant *Staphylococcus* strains. SCC*mec* elements are currently classified into types I to VI based on the characteristics of the *mec* and *ccr* gene complexes and are further classified into subtypes according to their 'junkyard DNA' region. We evaluated the distribution of SCC*mec* types in CoNS from patients attending the Hospital de Clínicas de Porto Alegre over the period August 2004–December 2005. Among the 129 bloodstream isolates, 36 (27.9%) harboured SCC*mec* type I, 4 (3.0%) harboured SCC*mec* type II, 67 (52%) harboured SCC*mec* type III, 1 (0.8%) harboured SCC*mec* type IV and 4 (3.0%) harboured SCC*mec* types I and III. Seventeen isolates were not typable. Identification of CoNS at the species level indicated that *Staphylococcus epidermidis* was the most common species, with 87 isolates, followed by *Staphylococcus haemolyticus* (15), *Staphylococcus hominis* (13), *Staphylococcus capitis* (12) and *Staphylococcus sciuri* (1). SCC*mec* type III was the most prevalent among isolates of *S. epidermidis* (52%). Among these strains, 30 (23%) harboured a modified SCC*mec* type III which contained an additional *dcs* region in comparison with regular type III. SCC*mec* type III was also highly prevalent (75%) among *S. capitis* isolates. The predominant SCC*mec* type found among *S. haemolyticus* isolates was type I. However, all four isolates harbouring SCC*mec* type II belonged to *S. haemolyticus*. Our results indicate that SCC*mec* type III was the most prevalent among the CoNS. Isolates with SCC*mec* type III were more resistant to non- β -lactam antimicrobials than isolates harbouring SCC*mec* types I, II and IV, although the increase in resistance was statistically significant only for clindamycin ($P=0.021$), rifampicin ($P=0.010$) and levofloxacin ($P=0.005$).

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INTRODUCTION

Coagulase-negative staphylococci (CoNS), particularly *Staphylococcus epidermidis*, are considered as the main pathogens of nosocomial bacteraemia associated with catheter and neonatal sepsis (Krediet *et al.*, 2001, 2004). These infections are associated with high morbidity and mortality, mainly when CoNS are resistant to semi-synthetic β -lactam antibiotics such as meticillin. In fact, meticillin-resistant *Staphylococcus* strains are a common

cause of nosocomial infections worldwide (Wisplinghoff *et al.*, 2004; Favre *et al.*, 2005).

The resistance of CoNS to meticillin and other β -lactam antibiotics is mediated by a penicillin-binding protein which has reduced affinity for these antibiotics (PBP2a). This protein is encoded by the *mecA* gene (Chambers, 1988), inserted in a genomic island called staphylococcal cassette chromosome *mec* (SCC*mec*) (Ito *et al.*, 2003). SCC*mec* is a mobile genetic element which is a vehicle for exchanging resistance genes between *Staphylococcus* strains and is widely distributed among CoNS species and in *Staphylococcus aureus* (Katayama *et al.*, 2001; Ito *et al.*, 1999; Hiramatsu *et al.*, 2004; Ito *et al.*, 2003).

Abbreviations: CoNS, coagulase-negative staphylococci; MRSA, meticillin-resistant *Staphylococcus aureus*; SCC, staphylococcal cassette chromosome.

SCCmec was initially described as 'mec DNA', which was found only in methicillin-resistant *S. aureus* (MRSA) and absent in methicillin-sensitive *S. aureus* (MSSA) (Ito *et al.*, 1999; Katayama *et al.*, 2000). This genetic element is composed of different combinations between the *mec* complex, which encodes resistance to methicillin, and the *ccr* complex, which encodes recombinase enzymes responsible for the mobility of the genetic element. The *mec* complex is composed of IS431, *mecA* and intact or truncated sequences of *mecI* and *mecR1* regulatory genes. The *ccr* complex can be composed of recombinase genes *ccrA* and *ccrB* or *ccrC* (Ma *et al.*, 2002; Chongtrakool *et al.*, 2006; Ito *et al.*, 2004; Oliveira *et al.*, 2006; Kondo *et al.*, 2007). Based on such diversity, six types of SCCmec have been described in *S. aureus*, and five types (I–V) have also been described in CoNS. Moreover, community methicillin-resistant *S. epidermidis* (C-MRSE) have been found with SCCmec type IV (Hisata *et al.*, 2005; Wisplinghoff *et al.*, 2003), and SCCmec type V has already been discovered in CoNS, particularly in *Staphylococcus haemolyticus* (Ito *et al.*, 2004).

The structure and distribution of SCCmec types in *S. aureus* have been studied frequently (Chongtrakool *et al.*, 2006; Hisata *et al.*, 2005; Hiramatsu *et al.*, 2004; Ito *et al.*, 2001; Katayama *et al.*, 2001), but data related to CoNS are only comparative and have been obtained in studies the purpose of which was to determine whether there was transmission between species (Hanssen *et al.*, 2004; Hisata *et al.*, 2005). In this study, we evaluated the distribution of SCCmec types I, II, III and IV in methicillin-resistant CoNS obtained from patients at a tertiary hospital in southern Brazil.

METHODS

Bacterial isolates. A total of 129 clinical isolates of methicillin-resistant CoNS was obtained from blood cultures from 125 patients hospitalized at the Hospital de Clínicas de Porto Alegre between August 2004 and December 2005. The study included only one clinical isolate per patient, or more than one isolate when the second isolate was obtained after a 3 day interval. Blood cultures were processed using the BacT/Alert automated system (bioMérieux) and subcultured on trypticase soy agar containing 5% sheep blood (bioMérieux). Colony morphology, Gram stain reaction, the catalase test and the absence of coagulase were used to screen for CoNS.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was performed by the disc diffusion method on Mueller–Hinton agar (bioMérieux) according to the recommendations of the CLSI (2006), with the following antibiotics: oxacillin (1 µg), cefoxitin (30 µg), vancomycin (30 µg), gentamicin (10 µg), clindamycin (2 µg), chloramphenicol (30 µg), doxycycline (30 µg), erythromycin (15 µg), levofloxacin (5 µg), rifampicin (5 µg) and trimethoprim/sulfamethoxazole (co-trimoxazole) (25 µg). *S. aureus* ATCC 25923 was used for quality control.

DNA extraction and quantification. The isolates were cultured on Mueller–Hinton agar incubated at 35 °C for 18–24 h. A bacterial suspension equivalent to 1.0 McFarland standard was prepared in 500 µl TE (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). The suspension was homogenized by vortexing and heated at 100 °C for 10 min

(York *et al.*, 1996), frozen at –20 °C for 2 h and then centrifuged at 9000 g for 3 min. The DNA from non-typable isolates and 20 random isolates was quantified using the GeneQuant RNA/DNA calculator (Amersham Pharmacia). Non-typable isolates were submitted to thermal lysis and then extracted using a QIAamp kit for tissues (Qiagen) according to the manufacturer's instructions.

Species identification. Molecular identification of *S. epidermidis* was performed by PCR of the *tuf* gene with primers from a species-specific probe for identification (Martineau *et al.*, 1996). Isolates that were not identified as *S. epidermidis* were tested in the API ID 32 STAPH semi-automated system (bioMérieux).

Determination of the *mecA* gene. PCR for the *mecA* gene was performed according to the protocol described by Vannuffel *et al.* (1995). *S. epidermidis* ATCC 12228 and *S. aureus* ATCC 33591 were used as negative and positive controls, respectively.

Determination of SCCmec type. The SCCmec type was determined in a multiplex PCR which included four pairs of primers for loci A, B, D and E (Oliveira & de Lencastre, 2002). Locus A is exclusive to SCCmec type I and amplifies a region of the *pls* gene, locus B is exclusive to SCCmec type II and amplifies an internal region of the *kpd* operon, locus D is common to SCCmec types I, II and IV and amplifies a region of the *dcs* gene and locus E is exclusive to SCCmec type III and amplifies a region between integrated plasmid p1258 and transposon Tn554 (Fig. 1). In addition, a single PCR for each locus was standardized in order to check results for 30 isolates which amplified locus E and D and the results of the non-interpretable types obtained by multiplex PCR.

Multiplex PCR was performed in 50 µl reactions with 1 × enzyme buffer (JMR Holdings), 1.25 U *Taq* polymerase DNA (SuperTherm; JMR Holdings), 200 µM dNTPs (ABgene), 10 pmol each of primers CIF2 F2 and CIF2 R2, 6 pmol each of primers KDP F1 and KDP R1, 5 pmol each of primers DCS F2 and DCS R1 and 5 pmol each of primers RIF4 F3 and RIF4 R9 (Invitrogen) and 10 µl extracted DNA. The amplification process was performed using an MJ Research PTC 100 Thermocycler. Each single PCR was performed in a 25 µl reaction following the same protocol as for the multiplex PCR.

Cycle sequencing reactions were performed at 92 °C for 3 min followed by 30 cycles of 92 °C for 1 min, 56 °C for 1 min and 72 °C for 1.5 min for both protocols. PCR products were detected on a 2% agarose gel and stained with ethidium bromide.

Fig. 1. Multiplex PCR for SCCmec type from CoNS. Lanes: M, molecular size standard (100 bp ladder); 1 and 2, isolate 173 (*S. epidermidis*) harbouring SCCmec IV (342 bp); 3 and 4, isolate 110 (*S. capitis*) harbouring SCCmec III (243 bp); 5 and 6, isolate 109 (*S. haemolyticus*) harbouring SCCmec II (342 and 284 bp); 7 and 8, isolate 204 (*S. epidermidis*) harbouring SCCmec I (342 and 495 bp).

Sequencing and computer analysis of sequences. Using the DNA fragments amplified by single PCR, the nucleotide sequences of loci A, B, D and E and the *mecA* gene were determined. A sequence similarity search was performed with BLAST and Chromas programs and the DDBJ/EMBL/GeneBank databases.

Statistical analysis. The chi-squared test with a 0.05 significance level was used for statistical analysis. A descriptive statistical analysis was performed to evaluate the relationship between antimicrobial susceptibility and SCCmec type.

RESULTS AND DISCUSSION

A wide distribution of the *mecA* gene in CoNS species has been demonstrated in previous studies (Pierre *et al.*, 1990) and was confirmed in our study of CoNS clinical isolates belonging to five species. This study also described the distribution of the SCCmec types I, II, III and IV among a population of CoNS from blood cultures obtained from patients at a tertiary hospital.

The presence of the *mecA* gene was tested in all 129 isolates of meticillin-resistant CoNS by PCR using specific primers, and all isolates gave a positive result. Sequencing of the amplified fragment of the *mecA* gene from one clinical sample gave 100 % similarity with the *mecA* gene sequence for *S. aureus* strain N315 (GenBank accession no. D86934), demonstrating that this gene is well-conserved in *Staphylococcus* species (Ito *et al.*, 2001). This isolate was used as a positive control in all PCR tests.

There was agreement of 100 and 97 % in disc diffusion testing with cefoxitin and oxacillin, respectively, with the presence of the *mecA* gene, detected by PCR in all 129 isolates. The disc diffusion test with a cefoxitin substrate is preferred over the test with oxacillin to estimate resistance to oxacillin mediated by the *mecA* gene for both *S. aureus* and CoNS (Swenson *et al.*, 2005). This was also observed in our study.

Identification of CoNS species was performed in two stages. First, all 129 isolates were tested using a species-specific primer for the *tuf* gene, and 87 (67.4 %) of the isolates were identified as *S. epidermidis*. The remaining 42 isolates were identified using a semi-automated system, resulting in 15 (11.6 %) *S. haemolyticus*, 13 (10 %) *S.*

hominis, 12 (9.3 %) *S. capitis* and 1 (0.8 %) *S. sciuri* isolates and one isolate that could not be identified. These represent the meticillin-resistant CoNS species usually found in the clinical microbiology laboratory (Caierão *et al.*, 2006; York *et al.*, 1996; Silva *et al.*, 2002).

The multiplex PCR for the four SCCmec types (I, II, III and IV) identified 36 (28 %) isolates with SCCmec type I, 4 (3.0 %) with SCCmec type II, 67 (52 %) with SCCmec type III, of which 30 were positive for both SCCmec types III and IV, 1 (0.8 %) with SCCmec type IV and 4 (3.0 %) isolates positive for two SCCmec types, I and III (Fig. 1). Seventeen isolates (13 %) were not typable by the method employed (Table 1). These results are comparable to the distribution of SCCmec types among staphylococci in an earlier study (Chung *et al.*, 2004), but differed from another study where SCCmec type IV was the most prevalent in hospital isolates of *S. epidermidis* (Wisplinghoff *et al.*, 2003).

Non-typable CoNS were tested by PCR using single primers for the four loci, but we were not able to establish the SCCmec type of these isolates. Most (59 %) of the non-typable isolates were identified as *S. hominis*. The relatively large number of non-typable isolates may be due to the fact that a few variants of SCCmec type II do not have the *kpd* operon in the 'junkyard region' and, therefore, they cannot be identified as SCCmec type II using this multiplex PCR (Shore *et al.*, 2005). Moreover, Shore *et al.* (2005) described a variant of SCCmec type IV which did not present locus D of the *dcs* region and named it SCCmec type IV-*dcs*. It is possible that some of our non-typable strains could be SCCmec type IV-*dcs*. The variability in the identification of SCCmec might also be explained by the presence of novel structures or rearrangements and recombinations of the *mec* element (Chung *et al.*, 2004; Zhang *et al.*, 2005).

The distribution of SCCmec types among the *S. epidermidis* isolates indicated that 25 (19 %) isolates harboured SCCmec type I and 24 (18.6 %) harboured SCCmec type III. Interestingly, 30 (23 %) isolates harboured both SCCmec type III and locus D (type IV). All isolates with this profile were identified as *S. epidermidis*, and this may indicate the existence of a clonal relationship among these

Table 1. Distribution of SCCmec elements among meticillin-resistant CoNS

Values are numbers of isolates (percentages of total number of isolates).

SCCmec type	Total	<i>S. epidermidis</i>	<i>S. hominis</i>	<i>S. haemolyticus</i>	<i>S. capitis</i>	<i>S. sciuri</i>	Not identified
I	36	25 (19.4)	0 (0)	10 (7.8)	1 (0.8)	0 (0)	0 (0)
II	4	0 (0)	0 (0)	4 (3.1)	0 (0)	0 (0)	0 (0)
III	37	24 (18.6)	3 (2.3)	0 (0)	9 (7)	1 (0.8)	0 (0)
IV	1	1 (0.8)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
I and III	4	4 (3.1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
III and IV	30	30 (23.2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Non-typable	17	3 (2.3)	10 (7.8)	1 (0.8)	2 (1.5)	0 (0)	1 (0.8)

isolates. These results warrant further studies using another molecular typing technique. It was possible to identify only one (0.8 %) isolate with SCCmec type IV. On the other hand, four (3.1 %) isolates resulted in amplification of the loci for SCCmec types I and III and were considered as non-interpretable.

The 30 isolates of *S. epidermidis* that harboured SCCmec type III (locus E) and the *dcs* region (locus D) were tested again by single PCRs for locus D and locus E. These PCRs confirmed the amplification of both loci in all 30 isolates. The presence of locus D (*dcs* region) is not exclusive to SCCmec types I, II and IV; Chongtrakool *et al.* (2006) identified 39 isolates with locus D and SCCmec type III. This profile has also been found in other studies. In order to investigate the nature of these cassettes, genes of the *ccr* complex were characterized, and it was shown that a few isolates with typical SCCmec type III also gave an amplification product for locus D and contained the *ccrAB3* type (Aires de Sousa & de Lencastre, 2003; Budimir *et al.*, 2006; Qi *et al.*, 2005).

Among the 15 isolates of *S. haemolyticus*, 10 (7.8 %) harboured SCCmec type I, 4 (3.1 %) harboured SCCmec type II and 1 (0.8 %) isolate harboured an SCCmec type that could not be identified. Among the 13 isolates identified as *S. hominis*, SCCmec type identification could not be performed in 10 (7.8 %) isolates, and only 3 (2.3 %) were identified, as SCCmec type III. Among the 12 isolates classified as *S. capitis*, 1 (0.8 %) harboured SCCmec type I and 9 (7 %) harboured SCCmec type III, but it was not possible to identify the SCCmec type in 2 (1.5 %) isolates.

Molecular typing techniques such as multilocus sequence typing (MLST), SCCmec typing and others have shown that most nosocomial MRSA infections are caused by a few pandemic clones. Among the five representative MRSA pandemic types characterized by MLST is clonal complex 8 (CC8), which includes a Brazilian clone (ST 239) with SCCmec IIIA (Shore *et al.*, 2005; Robinson & Enright, 2003). The hypothesis of SCCmec transfer between species of staphylococci may provide a mechanism to explain the

detection of CoNS isolates of this study, often identified as SCCmec III, which are related to the presence of this MRSA pandemic clone in our institution (data not shown).

The hypothesis of SCCmec transfer between *S. epidermidis* and *S. aureus* is based on several pieces of evidence. Hanssen *et al.* (2004) found genetically unique MRSA containing variants of *ccrAB* genes that are identical to those found in methicillin-resistant CoNS. It seems that *S. aureus* and CoNS contain the same pool of genes, with resistance and recombinase genes; however, it is still necessary to define the resistance-transfer mechanisms and routes between staphylococci.

Sequencing of amplified loci to identify SCCmec types I, II, III and IV gave 100 % similarity. The differences were only in point mutations in one of the two strands. The amplicons were compared to sequences from GenBank with accession numbers AB033763 for SCCmec type I, D86934 for SCCmec type II, AB037671 for SCCmec type III and AB033763 for SCCmec type IV. These isolates were used as positive controls in the PCRs. The sequences of two selected isolates that amplified SCCmec type III and type IV (locus D) showed 100 % similarity to a type IVb sequence deposited in GenBank under accession number AB063173, but only 96 % similarity to SCCmec type III (AB037671).

A descriptive analysis of resistance was performed for the susceptibility profile of CoNS to non- β -lactam antimicrobials among SCCmec types (Table 2). According to the literature, isolates containing SCCmec type III contain a large number of resistance genes (Ito *et al.*, 1999, 2001). In this study, we also found isolates with SCCmec III to be more resistant to non- β -lactam antibiotics, but this difference was not statistically significant for most antibiotics (erythromycin, $P=0.171$; doxycycline, $P=0.982$; trimethoprim/sulfamethoxazole, $P=0.115$; gentamicin, $P=0.860$; and chloramphenicol, $P=0.358$), the exceptions being rifampicin ($P=0.010$), levofloxacin ($P=0.005$) and clindamycin ($P=0.012$).

CoNS are reservoirs of resistance genes and are probably able to transmit these genes to *S. aureus*. The similarity of

Table 2. Distribution of non- β -lactam antibiotic resistance among 129 isolates of methicillin-resistant CoNS by SCCmec type

Values are numbers of isolates (percentages of isolates of that type).

Antibiotic	I (n=36)	II (n=4)	III (n=67)	IV (n=1)	Non-typable (n=17)
Clindamycin	24 (66.6)	1 (25)	48 (73.8)	1 (100)	6 (37.5)
Chloramphenicol	18 (50)	0 (0)	25 (39.1)	0 (0)	5 (31.3)
Doxycycline	12 (33.3)	0 (0)	8 (12.5)	0 (0)	5 (31.3)
Erythromycin	28 (71.4)	1 (25)	49 (75.4)	1 (100)	10 (62.5)
Gentamicin	33 (91.7)	2 (50)	58 (89.2)	1 (100)	13 (81.3)
Levofloxacin	14 (38.9)	1 (25)	43 (70.5)	1 (100)	5 (37.5)
Rifampicin	10 (27.8)	0 (0)	21 (32.3)	1 (100)	3 (18.8)
Co-trimoxazole	24 (66.6)	1 (25)	51 (78.5)	1 (100)	13 (81.3)
Vancomycin	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

SCCmec regions among *S. aureus* and CoNS suggests horizontal transmission between species of *Staphylococcus*. In addition, the extensive rearrangement observed in *ccr* and *mec* complexes indicates the frequent exchange of genetic material (Hanssen & Ericson Sollid, 2006).

It is noteworthy that SCCmec type IV was first identified in *S. epidermidis* in 1970 and only described around 10 years later in *S. aureus* (Wisplinghoff *et al.*, 2003). One could consider, therefore, that the SCCmec type III found in the widely distributed MRSA in Brazil (Brazilian clone) may have originated from CoNS (Da Silva Coimbra *et al.*, 2000).

The most relevant point is that SCCmec type III encodes the largest number of resistance genes, and this information is epidemiologically important for institutional infection control. Traditionally, CoNS have been recognized as bacteria of minor clinical importance; however, they may now be considered one of the factors that determines the generation of new MRSA strains.

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