Diversity of staphylococcal cassette chromosome *mec* structures in coagulase-negative staphylococci and relationship to drug resistance

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The objective of this study was to determine the distribution of staphylococcal cassette chromosome *mec* (SCC*mec*) elements in methicillin-resistant coagulase-negative staphylococci (MR-CoNS) isolated from a tertiary-care hospital in Mexico and to examine the relationship to drug resistance. Fifty selected MR-CoNS isolates collected from catheters ($n=15$), blood ($n=15$), bone ($n=9$), bronchial lavage ($n=2$) and urine ($n=2$) and one isolate each from an abscess, cerebrospinal fluid, eye, pleural effusion, synovial fluid, tracheal aspirate and wound secretion were examined. Susceptibility testing was performed by the broth microdilution method. SCC*mec* types were determined by multiplex PCR and PFGE was carried out as described previously for *Staphylococcus aureus*. Among the MR-CoNS strains studied, the most frequently isolated species were *Staphylococcus epidermidis* ($n=26$) and *Staphylococcus haemolyticus* ($n=13$). *Staphylococcus cohnii* ($n=5$), *Staphylococcus hominis* ($n=3$), *Staphylococcus sciuri* ($n=1$), *Staphylococcus pasteurii* ($n=1$) and the recently described species *Staphylococcus pettenkoferi* ($n=1$) were also identified. The most frequent MR-CoNS genotype identified was SCC*mec* type IVa in *S. epidermidis* isolates, which also showed a high diversity in their PFGE patterns. A clone was found that amplified both SCC*mec* III and V elements in five isolates examined. The single MR *S. pettenkoferi* isolate harboured SCC*mec* type IVd and the single MR *S. pasteurii* isolate harboured SCC*mec* type I. The carriage of SCC*mec* type III was associated with resistance or intermediate resistance to meropenem ($P<0.05$). These results confirm the high prevalence of *S. epidermidis* SCC*mec* IVa and the high genetic diversity among MR-CoNS strains. As far as is known, this is the first report describing the newly identified *S. pettenkoferi* possessing SCC*mec* IVd and *S. pasteurii* harbouring SCC*mec* type I. MR-CoNS harbouring SCC*mec* type III were found to be more resistant to meropenem.

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INTRODUCTION

In recent years, coagulase-negative staphylococci (CoNS) have emerged as important causative agents of illness,

Abbreviations: CoNS, coagulase-negative staphylococci; MR, methicillin-resistant; MRSA, methicillin-resistant *Staphylococcus aureus*; MRSE, methicillin-resistant *Staphylococcus epidermidis*; SCC*mec*, staphylococcal cassette chromosome *mec*.

The GenBank/EMBL/DDJB accession numbers for the partial DNA sequences of the 16S rRNA gene and SCC*mec* type IVd of *Staphylococcus pettenkoferi* are GQ145596 and GQ145595, respectively.

frequently involved in nosocomial infections related to medical devices and prostheses in immunocompromised patients (Curtis & Shetty, 2008; Falcone *et al.*, 2007; Viale & Stefani, 2006). This scenario has worsened because of drug-resistant CoNS, such as methicillin-resistant (MR)-CoNS (Lyytikäinen *et al.*, 2002).

Methicillin resistance in staphylococci is conferred by the penicillin-binding protein PBP2a (PBP2') encoded by the *mecA* gene and located on the staphylococcal cassette chromosome *mec* (SCC*mec*) (Katayama *et al.*, 2000). Eight different SCC*mec* types (I–VIII) have been identified and

Table 1. Distribution of SCCmec cassettes among MR staphylococcal isolates

Isolate	Species	Patient age (years)	Patient gender (male/female)	Specimen source	ccr	mec complex	SCCmec type	MIC ($\mu\text{g ml}^{-1}$)*							
								AMX	TZP	LVX	CRO	AMP	MIN	TIG	MEM
1492	<i>S. epidermidis</i>	17	M	Catheter	NT	A	III	>8/4	>16/4	32	64	>16	4	2	>16
395	<i>S. epidermidis</i>	1	M	Catheter	NT	A	III	2/1	2/4	8	32	>16	<0.25	0.06	4
4982	<i>S. epidermidis</i>	77	M	Catheter	NT	A	III	4/2	2/4	>32	32	8	8	2	8
1237	<i>S. epidermidis</i>	78	M	Bone	NT	NT	III and IVa	4/2	8/4	>32	>64	>16	0.5	0.25	>16
2390	<i>S. epidermidis</i>	0	F	Catheter	NT	A + B	III and IVa	8/4	>16/4	4	>64	>16	<0.25	0.125	>16
1085	<i>S. epidermidis</i>	35	M	Blood	NT	B	III and IVa	>8/4	>16/4	>32	>64	>16	0.25	0.125	>16
118	<i>S. epidermidis</i>	89	F	Tracheal aspirate	2	B	III and IVa	8/4	16/4	>32	>64	>16	<0.25	0.06	>16
488	<i>S. epidermidis</i>	50	M	Urine	2	B	III and IVa	8/4	8/4	>32	32	>16	<0.25	0.06	>16
965	<i>S. epidermidis</i>	0	F	Blood	3	A	III and V	>8/4	16/4	8	>64	16	0.5	0.25	16
1439	<i>S. epidermidis</i>	1	M	Catheter	3	A	III and V	>8/4	16/4	8	>64	16	0.5	0.5	16
1543	<i>S. epidermidis</i>	0	M	Catheter	3	A	III and V	8/4	8/4	4	>64	8	<0.25	0.125	8
1955	<i>S. epidermidis</i>	2	F	Catheter	3	A	III and V	8/4	8/4	4	>64	8	0.5	0.125	8
1981	<i>S. epidermidis</i>	17	M	Catheter	3	A	III and V	8/4	8/4	4	>64	8	0.5	0.5	8
1086	<i>S. epidermidis</i>	35	M	Bone	2	NT	IVa	1/0.5	1/4	>32	16	>16	<0.25	0.125	2
1458	<i>S. epidermidis</i>	0	F	CSF	2	NT	IVa	1/0.5	2/4	0.12	16	>16	>8	0.25	4
1102	<i>S. epidermidis</i>	60	M	Blood	2	B	IVa	1/0.5	8/4	0.12	32	8	0.5	0.25	2
778	<i>S. epidermidis</i>	35	M	Catheter	2	B	IVa	2/1	2/4	0.12	16	>16	1	0.5	2
960	<i>S. epidermidis</i>	72	F	Blood	2	NT	IVa	2/1	2/4	4	16	>16	0.5	0.25	4
1006	<i>S. epidermidis</i>	23	M	Bone	2	NT	IVa	2/1	4/4	0.12	16	>16	0.5	0.125	2
4981	<i>S. epidermidis</i>	70	F	Catheter	2	NT	IVa	2/1	4/4	4	32	8	<0.25	0.06	4
2078	<i>S. epidermidis</i>	80	M	Synovial	2	B	IVa	1/0.5	>16/4	4	16	>16	8	2	2
1083	<i>S. epidermidis</i>	32	M	Bone	NT	A	V	1/0.5	4/4	0.12	8	8	0.5	0.25	2
848	<i>S. epidermidis</i>	54	F	Blood	NT	A	NT	>8/4	>16/4	2	>64	>16	0.5	0.25	>16
1739	<i>S. epidermidis</i>	50	M	Pleural effusion	2	B	NT	0.5/0.25	>16/4	>32	16	16	0.5	0.25	1
353	<i>S. epidermidis</i>	0	F	Blood	2 and 5	A	NT	1/0.5	>16/4	>32	8	>16	>8	0.25	2
1089	<i>S. epidermidis</i>	0	F	Bone	NT	B	NT	1/0.5	2/4	0.06	16	>16	0.5	0.25	2
1460	<i>S. haemolyticus</i>	54	F	Blood	NT	NT	II	>8/4	>16/4	>32	>64	16	>8	>16	>16
2165	<i>S. haemolyticus</i>	80	M	Catheter	1	NT	II	>8/4	>16/4	>32	>64	>16	0.5	0.25	>16
1628	<i>S. haemolyticus</i>	66	F	Blood	5	NT	II and V	0.5/0.25	2/4	4	16	4	<0.25	0.125	0.5
6196	<i>S. haemolyticus</i>	27	M	Bronchial lavage	5	NT	II and V	2/1	4/4	4	32	4	1	0.06	1
12	<i>S. haemolyticus</i>	88	M	Abscess	5	NT	II and V	4/2	16/4	4	32	16	<0.25	0.125	2
28	<i>S. haemolyticus</i>	66	M	Blood	NT	NT	II and V	4/2	8/4	>32	32	>16	<0.25	0.06	2
2215	<i>S. haemolyticus</i>	1	M	Catheter	3	NT	III and V	0.12/0.06	>16/4	4	8	2	<0.25	0.5	<0.12
1879	<i>S. haemolyticus</i>	37	F	Wound	NT	NT	V	8/4	>16/4	2	>64	>16	<0.25	0.25	8
1069	<i>S. haemolyticus</i>	62	F	Urine	NT	NT	V	>8/4	>16/4	>32	>64	>16	<0.25	0.25	>16
286	<i>S. haemolyticus</i>	47	M	Bronchial lavage	NT	NT	NT	>8/4	>16/4	>32	>64	>16	<0.25	0.125	>16
1222	<i>S. haemolyticus</i>	2	M	Blood	NT	NT	NT	>8/4	>16/4	>32	>64	>16	0.5	0.5	>16
988	<i>S. haemolyticus</i>	0	F	Eye	NT	NT	NT	>8/4	>16/4	4	>64	>16	0.5	0.25	>16

Table 1. cont.

Isolate	Species	Patient age (years)	Patient gender (male/female)	Specimen source	ccr	mec complex	SCCmec type	MIC ($\mu\text{g ml}^{-1}$)*							
								AMX	TZP	LVX	CRO	AMP	MIN	TIG	MEM
2452	<i>S. haemolyticus</i>	7	M	Blood	1	NT	NT	>8/4	>16/4	>32	>64	>16	0.5	0.25	>16
663	<i>S. hominis</i>	27	M	Bone	NT	NT	NT	0.25/0.12	>16/4	2	2	4	0.5	0.125	0.12
1494	<i>S. hominis</i>	36	M	Bone	NT	NT	NT	0.25/0.12	1/4	8	4	>16	>8	2	1
397	<i>S. hominis</i>	0	F	Blood	1 and 5	A	III	>8/4	>16/4	>32	32	16	>8	2	>16
57	<i>S. cohnii</i>	NA	M	Blood	NT	NT	NT	>8/4	>16/4	>32	>64	>16	>8	>16	>16
2074	<i>S. cohnii</i>	52	F	Catheter	NT	NT	NT	>8/4	8/4	>32	>64	8	<0.25	0.5	2
349	<i>S. cohnii</i>	53	M	Catheter	NT	NT	NT	8/4	>16/4	>32	>64	4	>8	2	2
3281	<i>S. cohnii</i>	0	F	Blood	NT	NT	NT	2/1	8/4	0.25	>64	1	>8	0.125	0.25
849	<i>S. cohnii</i>	20	F	Catheter	NT	NT	NT	4/2	8/4	>32	32	1	1	0.25	0.5
2658	<i>S. pettenkoferi</i>	0	M	Blood	NT	NT	IVd	>8/4	>16/4	>32	>64	16	<0.25	0.25	>16
2247	<i>S. sciuri</i>	26	M	Bone	NT	NT	NT	4/2	16/4	0.5	32	2	<0.25	0.25	1
3223	<i>S. pasteurii</i>	0	F	Bone	NT	NT	I	≤ 2	8/4	4	4	>8	<0.25	0.25	1

NA, Data not available; NT, non-typable.

*AMX, amoxicillin/clavulanic acid; TZP, piperacillin/tazobactam; LVX, levofloxacin; CRO, ceftriaxone; AMP, ampicillin; MIN, minocycline; TIG, tigecycline; MEM, meropenem.

are categorized by different sets of chromosome cassette recombinase (*ccr*) genes and differences in the *mec* gene complex (Chongtrakool *et al.*, 2006; Ito *et al.*, 2001; Ma *et al.*, 2002; Zhang *et al.*, 2009). Although the *mec* origin remains unknown, it has been suggested that SCCmec can be transferred between staphylococci and that *mecA*-positive CoNS may act as potential SCCmec donors, accounting for the rise in new MR *Staphylococcus aureus* (MRSA) clones (Hanssen & Ericson Sollid, 2006; Musser & Kapur, 1992). In relation to the origin of the SCCmec complex, it has been suggested that *Macroccoccus caseolyticus* may be closely associated with the origin of the meticillin resistance gene complex in *S. aureus* (Baba *et al.*, 2009).

The objective of this study was to determine the distribution of SCCmec elements in MR-CoNS isolated from a tertiary-care hospital in Mexico and to examine its relationship to drug resistance.

METHODS

Bacterial cultures. A total of 215 CoNS isolates collected between 15 May 2006 and 15 November 2006 were included in this study, and all collection and study protocols were approved by the Ethics Committee of the Universidad Autónoma de Nuevo León, Mexico. Isolates were identified by conventional biochemical tests (Bannerman & Peacock, 2007) and via the use of API galleries (bioMérieux). All strains met the laboratory criteria for a significant pathogen, and were considered probable causative agents of a hospital-acquired infection. For all isolates, resistance to meticillin was determined by a cefoxitin disc test with the interpretative criteria ≤ 24 (resistant) and ≥ 25 (susceptible) (CLSI, 2009), as well as by PCR (Vannuffel *et al.*, 1995).

Fifty selected MR-CoNS strains isolated from catheters ($n=15$), blood ($n=15$), bone ($n=9$), two each from bronchial lavage and urine, and one each from an abscess, cerebrospinal fluid, eye, pleural effusion, synovial fluid, tracheal aspirate and wound secretion were studied further. The selected clinical isolates were collected from patients between 0 and 89 years of age, including nine newborns. MR-CoNS with uncertain biochemical patterns defined by API galleries analysis were examined further by amplification and sequencing of the 16S rRNA gene using primers described previously (Pei *et al.*, 2004). All sequencing was performed at the Instituto de Biotecnología de la Universidad Nacional Autónoma de México. Bacterial isolates were stored in *Brucella* broth containing 15% glycerol at -70°C . Only one isolate per patient was included.

Antimicrobial susceptibility assays. Susceptibility testing was performed by broth microdilution using panels from Sensititre (TREK Diagnostic Systems) as described by the manufacturer. For all assays, the assay control organism (*S. aureus* ATCC 29213) and the susceptibility breakpoints were those recommended by the Clinical and Laboratory Standards Institute (CLSI, 2009). The interpretive breakpoint used for tigecycline was $\leq 0.5\text{ }\mu\text{g ml}^{-1}$ (susceptible) (Andrews & Working Party for the British Society for Antimicrobial Chemotherapy, 2009).

SCCmec typing and PFGE. Preparation of template DNA and SCCmec, *ccr* and *mec* class typing were performed as described previously (Zhang *et al.*, 2005). All SCCmec typing was performed twice. PFGE was carried out as described previously for *S. aureus* (Murchan *et al.*, 2003) using a CHEF-DRIII instrument (Bio-Rad

Laboratories) on *Staphylococcus epidermidis* clinical isolates. Patterns were analysed and further interpreted according to the criteria of Tenover *et al.* (1995).

Statistical analysis. The significance between antibiotic profiles and SCCmec profiles was determined using a χ^2 test. A value of $P < 0.05$ was considered significant. Epi Info version 3.2.2 software (Centers for Disease Control and Prevention, GA, USA) was used for statistical analysis.

RESULTS AND DISCUSSION

Staphylococci identified

We evaluated the distribution of SCCmec types in CoNS isolated from patients at the Hospital Universitario Dr José Eleuterio Gonzalez over a 6-month period. From 215 clinical isolates included in the study, 149 were MR-CoNS and we randomly selected 50 isolates for further studies. Among the 50 selected MR-CoNS clinical isolates, as reported by others in similar studies, *S. epidermidis* was the most common species identified ($n=26$) followed by *Staphylococcus haemolyticus* ($n=13$) (Mombach Pinheiro Machado *et al.*, 2007; Ruppé *et al.*, 2009) (Table 1). Species identification of 17 of the isolates, including some *Staphylococcus haemolyticus*, and all *Staphylococcus cohnii*, *Staphylococcus hominis* and the single *Staphylococcus sciuri*, *Staphylococcus pasteurii* and *Staphylococcus pettenkoferi* isolates was confirmed by PCR and partial sequencing of the 16S rRNA gene. The fragments obtained were compared with GenBank sequence entries using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov/BLAST>).

For the recently described *S. pettenkoferi*, the sequence gave 99% identity with *S. pettenkoferi* strains SNUBH406 (GenBank accession no. FJ222447.1), TPL06 (EU373375.1), K6999 (AM265622.1), 229 (DQ538517.1), 230 (DQ538518.1) and 231 (DQ538519.1) and the A6664 (DQ538520) strain previously described by Trülsch *et al.* (2002, 2007). Using the API Staph V4.0 identification system (bioMérieux), this strain was originally identified as *Kocuria varians* with an identification probability of 94.4%.

The single *S. pasteurii* isolate was identified as *S. aureus* using the API Staph V4.0 identification system, with an identification probability of 96.9%. The coagulase result was confirmed as negative. For the *S. pasteurii* isolate, the sequence gave 100% identity with *S. pasteurii* strains D4027 (FJ161258.1), JS10 (FJ205745.1) and NQ20 (EU919211.1).

SCCmec types identified

Among the MR *S. epidermidis* (MRSE) isolates examined, three harboured SCCmec type III, five had both III and IVa elements and eight harboured SCCmec type IVa (Table 1). The most frequent SCCmec element for *S. epidermidis* or any other species examined was IVa, which was recently described as the predominant type among *S. epidermidis* clinical isolates by Jamaluddin *et al.* (2008) in a Japanese population. In addition, in a long-term care facility in

Finland, the distribution of SCCmec types in *S. epidermidis* was diverse, with the majority belonging to type IV (33 %), followed by type V (18 %) (Ibrahim *et al.*, 2009). A report that included 44 strains isolated between 1973 and 1983 from blood showed that 16 strains (36 %) harboured SCCmec type IV (Wisplinghoff *et al.*, 2003). In addition, Ruppé *et al.* (2009) described 75 MRSE strains from four countries, 34 of which were non-typable for SCCmec. Among the typable strains, type IV was the most common (27/75), followed by type V (13/75) (Ruppé *et al.*, 2009). In contrast, Mombach Pinheiro Machado *et al.* (2007) found only 1/87 MRSE strains harbouring SCCmec type IV, with the most frequent MRSE SCCmec type being type III–IV (30/87). In our study, only 15 % of *S. epidermidis* strains were untypable for SCCmec. Furthermore, the isolation of strains that amplified two types of SCCmec [III and IVa (five isolates), III and V (six isolates), and II and V (four isolates)] strongly suggests that new variants may be present in CoNS and may have a different impact on drug resistance. The typing of isolates that amplified two SCCmec types was confirmed by using single primers for each locus.

Evidence has suggested horizontal transfer of SCCmec from MR *S. haemolyticus* to meticillin-susceptible *S. aureus* strains, resulting in the creation of a new MRSA clone that could result in a potential outbreak (Berglund & Söderquist, 2008). This concern underlines the importance of studying the prevalence of SCCmec in all CoNS strains.

In this study, we obtained only one isolate each of *S. sciuri*, *S. pasteuri* and *S. pettenkoferi*. The MR *S. pettenkoferi* isolate harboured SCCmec type IVd. An alignment of the DNA sequence of type IVd SCCmec of *S. pettenkoferi* and *S. aureus* type IV.4 (IVd) (GenBank accession no. AB097677.1) showed 100 % sequence identity (identities = 848/848), as did the alignment with *S. aureus* type IVd cassette chromosome *mec* (GenBank accession no. EF634484.1) (identities = 746/746). To our knowledge, this is the first description of an *S. pettenkoferi* *mecA*-positive isolate possessing SCCmec-like type IVd and this subtype had 100 % homology to SCCmec IVd from *S. aureus* in the region sequenced. *S. pettenkoferi* was

described in 2007 (Trülsch *et al.*, 2007) and has been reported as a causative agent of osteomyelitis (Loiez *et al.*, 2007) and bloodstream and wound infections (Song *et al.*, 2009; Trülsch *et al.*, 2002). The *S. pettenkoferi* strain reported here was isolated from a prematurely born (male) neonate (gestation age 30.1 weeks) who presented with surfactant deficiency, respiratory distress syndrome and venoclysis as predisposing factors. This strain is susceptible only to tigecycline and minocycline.

The single *S. pasteuri* isolate was isolated from bone in a newborn female with trisomy 21 and interauricular and interventricular communication, who had had multiple transfusions of blood and platelets. Interestingly, this particular species has been isolated from a leukaemic patient with bacteraemia (Savini *et al.*, 2009a) and as a contaminant of platelet units (Savini *et al.*, 2008, 2009b). To our knowledge, this is the first description of the presence of SCCmec in *S. pasteuri* and of the isolation of this species from bone.

The single MR *S. pasteuri* isolate harboured SCCmec type I. The alignment of type I SCCmec of *S. pasteuri* and *S. aureus* NCTC 10442 showed 100 % sequence identity.

PFGE genotyping and genotype cluster distribution

Evaluation of banding patterns revealed an *S. epidermidis* clone with 100 % homology in the PFGE patterns that amplified for both SCCmec III and V in samples 965, 1439, 1543, 1955 and 1981 (Fig. 1). This clone was isolated over a period slightly longer than 3 months (30 June 2006 to 4 October 2006). It is not likely that this clone could transmit these genetic elements to *S. aureus* isolates because it has been shown that the type 3 *ccr* has lost the function of excising and integrating SCCmec into the staphylococcal chromosome. Instead, MRSE carrying SCCmec type IV is probably a reservoir of SCCmec that can be transferred to other species. The most frequent species and genotype of MR-CoNS isolated in our hospital was *S. epidermidis* SCCmec type IVa and these isolates showed high diversity. In Fig. 1, we have shown the only isolates with the same

Fig. 1. PFGE dendrogram comparing representative *S. epidermidis* clones from the Hospital Universitario Dr José Eleuterio Gonzalez. The SCCmec type is included. The bar represents similarity. Similarity coefficients were generated from a similarity matrix calculated with the Jaccard coefficient using SPSS 10.0 software.

PFGE pattern and some examples of the diversity found in the SCCmec IVa. All other PFGE patterns were completely different and no major cluster was detected among these strains.

Antibiotic susceptibility testing

The results of susceptibility testing are shown in Table 1. In this study, we detected the previously described high resistance to antibiotics associated with the presence of SCCmec type III (Ito *et al.*, 2001), although the only statistically significant association found was the carriage of SCCmec type III with resistance or intermediate resistance to meropenem ($P < 0.05$). Interestingly, three out of four strains that amplified only SCCmec III were resistant to tigecycline and the strains that amplified SCCmec type III plus another type (III and IVa, or III and V) were all susceptible to this drug.

In conclusion, we confirmed the high genetic diversity among the MR-CoNS isolates studied. This is the first report describing *S. pettenkoferi* and *S. pasteurii* carrying SCCmec elements and the first description of an *S. epidermidis* clone that amplified both SCCmec type III and V elements. These data are important not only for defining hospital control strategies, but also for shedding light on the heterogeneous pool of SCCmec types present in CoNS strains, which may act as a source of virulence factors for more pathogenic *S. aureus* strains.

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