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Molecular epidemiology of coagulase-negative bloodstream isolates: detection of *Staphylococcus epidermidis* ST2, ST7 and linezolid-resistant ST23

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ABSTRACT

The mechanisms contributing to persistence of coagulase-negative staphylococci are diverse; to better understanding of their dynamics, the characterization of nosocomial isolates is needed. Our aim was to characterize phenotypic and molecular characteristics of *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* human blood isolates from two tertiary care hospitals in Mexico, the Hospital Universitario in Monterrey and the Hospital Civil in Guadalajara.

Antimicrobial susceptibility was determined. Biofilm formation was assessed by crystal violet staining. Detection of the *ica* operon and Staphylococcal Cassette Chromosome *mec* typing were performed by PCR. Clonal relatedness was determined by Pulsed-fiel gel electrophoresis and Multi locus sequence typing.

Methicillin-resistance was 85.5% and 93.2% for *S. epidermidis* and *S. haemolyticus*, respectively. Both species showed resistance >70% to norfloxacin, clindamycin, levofloxacin, trimethoprim/sulfamethoxazole, and erythromycin. Three *S. epidermidis* and two *S. haemolyticus* isolates were linezolid-resistant (one isolate of each species was *cfr*+). Most isolates of both species were strong biofilm producers (92.8% of *S. epidermidis* and 72.9% of

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S. haemolyticus). The *ica* operon was amplified in 36 (43.4%) *S. epidermidis* isolates. SCC*mec* type IV was found in 47.2% of the *S. epidermidis* isolates and SCC*mec* type V in 14.5% of *S. haemolyticus* isolates. No clonal relatedness was found in either species. Resistance to clindamycin, levofloxacin, erythromycin, oxacillin, and cefoxitin was associated with biofilm production for both species ($p < 0.05$). A G2576T mutation in 23S rRNA gene was detected in an *S. haemolyticus* linezolid-resistant isolate. All linezolid-resistant *S. epidermidis* isolates belonged to ST23; isolate with SCC*mec* type IV belonged to ST7, and isolate with SCC*mec* type III belonged to ST2. This is the first report of ST7 in Mexico.

There was a high genetic diversity in both species, though both species shared characteristics that may contibute to virulence.

Introduction

Coagulase-negative staphylococci (CoNS) are among the main causative agents of bacteremia.[1] *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* are the CoNS species most frequently isolated from blood.[2] These species are often associated with infections in immunocompromised patients who have medical device implants.[3] These species persist on medical devices because they can form biofilms, bacterial clusters that attach to materials such as plastics. Biofilm formation has been associated with the presence of the *ica* operon that encodes for the production of a polysaccharide intercellular adhesin (PIA). This operon contains the *icaA*, *icaB*, *icaC*, *icaD*, and *icaR* genes; expression of these genes have been found to be involved in biofilm formation.[4] Furthermore, biofilm production has been associated with an increased resistance to antibiotics.[5] CoNS strains may present a high proportion of resistance to antibiotics,[6,7] particularly methicillin resistance, thus complicating the management of these infections.

Methicillin resistance in *Staphylococcus aureus* was first reported in 1961.[8] Methicillin-resistant *S. aureus* strains produce a penicillin-binding protein, known as PBP2a or PBP2′, that has low binding affinity for β-lactams. PBP2a is encoded by *mecA*, which is contained within the Staphylococcal Cassette Chromosome *mec* (SCC*mec*).[9] Also, methicillin-resistance has been found in CoNS species more often than in *S. aureus* species isolated from clinical samples.[10] To date, 11 types of SCC*mec* have been described in *S. aureus* (http://www.sccmec.org/), and evidence suggests that SCC*mec* structures may be more diverse in CoNS. These various structures may contain combinations of *mec* and *ccr* complexes not described for *S. aureus* or may contain multiple *ccr* complexes.[11] Since methicillin-resistance is more frequent in CoNS, methicillin-resistant CoNS (MR-CoNS) may serve as a large reservoir of SCC*mec* and may contribute to the formation of methicillin-resistant *S. aureus* (MRSA) strains.[10]

Since CoNS are components of the human skin microbiota, often endogenous strains are capable of causing infections in immunocompromised individuals. However, there are reports of persisting strains in hospital wards.[12,13] The genetic relatedness between these strains has been determined by Pulsed-Field Gel Electrophoresis (PFGE); a technique that has been widely used for molecular typing of nosocomial pathogens. Nevertheless, Multilocus Sequence Typing (MLST), based on sequencing of conserved housekeeping genes, is proving to be the most appropriate tool for the study of the global epidemiology, allowing the comparison of isolates from different countries and the naming of international clones. To date, a widely acceptable MLST scheme and database have been developed for *S. epidermidis* only but not for *S. haemolyticus*.[14]

The persistence of strains of these species may be due to their increasing high resistance to antimicrobials, which may enhance their fitness to hospital environments, including linezolid-resistance. At the same time, the production of biofilm has been associated with antibiotic resistance and the persistence of the strains in medical devices. Furthermore, the presence of SCC*mec* worsens the scenario, since horizontal transference of these elements may contribute to antibiotic resistance. Finally, the information of genetic relatedness would allow us to know the dynamics of transmission of these infections. We hypothesized that the presence of strains with traits such as biofilm formation, high resistance to antibiotics and diverse SCC*mec* elements may contribute to the persistence of these strains. Thus, we aimed to characterize phenotypic and molecular characteristics of *S. epidermidis* and *S. haemolyticus* blood isolates from two Mexican tertiary-care hospitals, the Hospital Universitario in Monterrey and the Hospital Civil in Guadalajara, in order to determine distribution of SCC*mec* elements, antibiotic resistance, genetic relatedness, and biofilm formation, as well as to examine its relationship to drug resistance. Both settings are teaching hospitals that offer a variety of services, educational programs, a space for clinical research and health-related community services working as a reference facility for two of the major metropolitan areas in Mexico.

Materials and methods

Hospital setting and identification of clinical isolates

This study was performed at the Hospital Civil de Guadalajara "Fray Antonio Alcalde", a 1000-bed tertiary care teaching hospital, with approximately 30,000 admissions annually, located in Guadalajara, Jalisco, and at the Hospital Universitario "Dr. José Eleuterio González", a 450-bed tertiary-care teaching

hospital with an average hospital admittance rate of 26,500 patients yearly, located in Monterrey, Nuevo León.

Isolates of *S. epidermidis* ($n = 83$) and *S. haemolyticus* ($n = 59$) were collected from the two tertiary care hospitals in Mexico from 2006 to 2013. Isolates were obtained from blood cultures. Only one isolate per patient was included. Regarding the unit ward of precedence, 93/142 (65.5%) of isolates proceeded from intensive care units and 90/142 (63.4%) of patients were male.

Blood cultures were cultivated using Versa TREK REDOX bottles and processed using the Versa TREK blood culture system (Thermo Scientific, Oakwood Village, OH, USA). Cultures were incubated at 37 °C and the bottles were monitored for seven days before being discarded as negative. When positive, the bottle was subcultured in blood agar and incubated at 37 °C up to 72 h. Isolates were identified by biochemical and molecular methods. For the biochemical method, Sensititre Panels (TREK Diagnostic Systems Inc., Cleveland, OH, USA) were used according to the manufacturer's instructions. For the molecular method, DNA was obtained by phenol-chloroform extraction and species were identified by multiplex PCR of the *nuc* gene fragment as described previously.[15] *S. epidermidis* ATCC 14990 and *S. haemolyticus* ATCC 29970 were used as the reference strains. All isolates were stored in *Brucella* broth containing 15% glycerol at −70 °C.

Methicillin-resistance and antimicrobial susceptibility testing

Methicillin-resistance was evaluated using the cefoxitin disk test described in the M02-A11 document of the Clinical and Laboratory Standards Institute (CLSI). Minimum inhibitory concentration (MIC) ranges, MIC_{50} and MIC_{90} were determined using the broth microdilution method. Panels from Sensititre (TREK Diagnostic Systems Inc.) were used according to the manufacturer's instructions. The antimicrobial agents tested were oxacillin, vancomycin, erythromycin, tetracycline, levofloxacin, clindamycin, trimethoprim-sulfamethoxazole, and linezolid. Methicillin-resistance and antimicrobial susceptibility test results were interpreted according to the M100-S24 document of the CLSI. *S. aureus* ATCC 29213 and *S. aureus* BAA-44 were used as quality controls.

Sequencing of 23S RNA gene and detection of cfr

Domain V of 23S rRNA gene was amplified to identify possible mutations in linezolid-resistant isolates, using the primers previously described.[16] PCR products were purified by using a precipitation protocol with 3 M sodium acetate and absolute ethanol, at −20 °C. The precipitate was recovered by centrifugation and washed with 70% ethanol. The precipitate was dissolved in sterile nuclease-free water. The products were then sequenced (Macrogen, Korea) and aligned with the corresponding nucleotide sequence from reference strains of *S. aureus* and *Escherichia coli* (GenBank accession numbers X68425 and AF053966, respectively). Presence or absence of the *cfr* gene in the linezolid-resistant isolates was determined as described previously.[17] Extraction of plasmid DNA was performed using an alkaline lysis protocol.

The biofilm formation assay and amplification of the ica operon

Biofilm formation was evaluated by crystal violet staining. Overnight cultures grown in tryptic soy broth were diluted 1:100 in tryptic soy broth supplemented with 1% glucose or tryptic soy broth supplemented with 3% NaCl. Each dilution was dispensed into four wells of flat-bottom polystyrene plates (Falcon, Franklin Lakes, NJ). After incubation for 24 h at 37 °C, the absorbance of planktonic cells was determined at 595 nm. The broth was eliminated and the wells were washed twice with sterile PBS (pH 7.3). Biofilms were stained with Hucker's crystal violet for 15 min; the stain was removed, and the wells were washed with sterile deionized water. The stained biofilms were dissolved in 200 μL of 30% acetic acid, and optical densities (OD) were measured at 595 nm. Biofilm formation was evaluated using the cut-off value suggested by Christensen et al.[18] If the OD were less than or equal to 0.120, we classified the strain as non-biofilm producer. If the OD was above 0.240, the strain was classified as strong biofilm producer. Strains with OD greater than 0.120 but less than or equal to 0.240 were classified as weak biofilm producers. The biofilm index (biofilm absorbance/planktonic cell absorbance) was also calculated, which normalizes for differences in growth rates.

Genes in the *ica* operon were detected by multiplex PCR as described by Arciola et al.[19] All five *ica* genes, *icaA*, *icaD*, *icaB*, *icaC*, and *icaR*, were tested, and PCR products were visualized on a 2.5% agarose gel stained with 1 mg/mL of ethidium bromide.

Detection of mecA and SCCmec typing

Amplification of *mecA* and SCC*mec* typing were performed by multiplex PCR as described by Zhang et al.[20] and Kondo et al.[21] The multiplex PCR assay by Kondo et al. contained a modification to the primers used to amplify *mec* complex class C and *ccr* complex type 4 and type 5 as described by Ruppé et al.[11]

Clonal relatedness

Clonal relatedness of isolates was determined by PFGE as described previously,[10] but with modifications for CoNS in running conditions: the pulse times were 1–35 s for 24 h at 200 V. DNA was digested with the restriction enzyme SmaI, on a CHEF-DRIII instrument (Bio-Rad Laboratories, Hercules, CA) electrophoresis was performed. Gels were stained with ethidium bromide and images were obtained using the Labworks 4.5 software package. Banding patterns were analyzed visually, a database was created, and statistical analysis was performed using the statistical software package SPSS 20.0 (IBM Corporation, Somers, NY).

Multi locus sequence typing of S. epidermidis isolates

Primers and conditions were obtained from a previously described scheme.[14] Determination of alleles and sequence type (ST) were performed using the *S. epidermidis* MLST database (http://sepidermidis.mlst.net/).[22]

Table 1 – Antimicrobial susceptibility of *S. epidermidis* and *S. haemolyticus* isolates.

Antimicrobial	*S. epidermidis*				*S. haemolyticus*			
	MIC_{50}[a] (μg/mL)	MIC_{90}[b] (μg/mL)	Resistant *n* (%)	Susceptible *n* (%)	MIC_{50}[a] (μg/mL)	MIC_{90}[b] (μg/mL)	Resistant *n* (%)	Susceptible *n* (%)
Oxacillin	>0.5	>0.5	72 (86.7)	11 (13.3)	>0.5	>0.5	55 (93.2)	4 (6.8)
Vancomycin	2	4	0 (0)	83 (100)	2	2	0 (0)	59 (100)
Erythromycin	>4	>4	66 (79.5)	16 (19.3)	>4	>4	53 (89.8)	6 (10.2)
Tetracycline	≤2	>8	10 (12)	71 (85.6)	≤2	>8	13 (22.0)	44 (74.6)
Levofloxacin	>2	>2	62 (74.7)	18 (21.7)	>2	>2	54 (91.5)	4 (6.8)
Norfloxacin	>8	>8	64 (77.1)	18 (21.7)	>8	>8	53 (89.8)	6 (10.2)
Clindamycin	>2	>2	68 (81.9)	12 (14.5)	>2	>2	53 (89.8)	5 (8.5)
Trimethoprim/ sulfamethoxazole	>2/38	>2/38	60 (72.3)	23 (27.7)	>2/38	>2/38	45 (76.3)	14 (23.7)
Linezolid	≤2	4	3 (3.6)	80 (96.4)	≤2	≤2	2 (3.4)	57 (96.6)

Minimum inhibitory concentration (MIC) ranges, MIC_{50} and MIC_{90} were determined using the broth microdilution method. Panels from Sensititre (TEK Diagnostic Systems Inc.) were used according to the manufacturer's instructions.

[a] MIC_{50}, minimal inhibitory concentration for 50% of the isolates.

[b] MIC_{90}, minimum inhibitory concentration for 90% of the isolates.

Statistical analysis

The correlation between drug resistance and biofilm production was analyzed using a chi-square test and OpenEpi software version 3.03 (Rollins School of Public Health, Emory University). A p-value <0.05 was considered significant.

Results

Methicillin-resistance and antimicrobial susceptibility profiles

Of the *S. epidermidis* isolates tested, 85.5% (71/83) were methicillin-resistant, whereas 93.2% (55/59) of the *S. haemolyticus* isolates were methicillin-resistant, both by the cefoxitin disk test. In *S. epidermidis*, all methicillin-resistant isolates were also resistant to oxacillin and contained *mecA*; however, a methicillin-susceptible isolate was resistant to oxacillin and contained *mecA*. In *S. haemolyticus*, all methicillin-resistant isolates were also resistant to oxacillin and contained *mecA*.

Resistance to oxacillin, erythromycin, levofloxacin, clindamycin, and trimethoprim/sulfamethoxazole was >70% for both species (Table 1). Twelve percent of the *S. epidermidis* isolates were tetracycline-resistant, whereas 22% of the *S. haemolyticus* isolates were tetracycline-resistant. Three (3.6%) of the *S. epidermidis* and two (3.4%) of the *S. haemolyticus* isolates were resistant to linezolid.

Sequencing of 23S RNA gene and detection of cfr

Analysis of domain V of 23S rRNA in linezolid-resistant isolates showed a G to T mutation at position 2576 (*E. coli* numbering) in a *S. haemolyticus* isolate (isolate 2975). Remaining isolates were negative for mutations in domain V. The *cfr* gene was detected in one linezolid-resistant *S. epidermidis* isolate (isolate 14565) and one linezolid-resistant *S. haemolyticus* isolate (isolate 9976) (Table 2). Also, plasmid DNA extraction was performed in *cfr*-negative isolates in order to determine the presence of the *cfr* gene in plasmids. The *cfr* gene was not amplified in this isolates.

All linezolid-resistant isolates, except one *S. haemolyticus* isolate, were also resistant to methicillin. None of the isolates was resistant to vancomycin.

Biofilm production and presence of the ica operon

Of the *S. epidermidis* isolates, 77 (92.8%) were strong biofilm producers, 5 (6%) were weak biofilm producers, and 1 (1.2%) did not form biofilms in glucose-supplemented broth. In NaCl-supplemented broth, 72 (86.7%) of the isolates were strong biofilm producers, 7 (8.4%) were weak biofilm producers, and 4 (4.8%) did not form biofilms.

Similar results were found with the *S. haemolyticus* isolates; 43 (72.9%) were strong biofilm producers, 12 (20.3%) were weak biofilm producers, and 4 (6.8%) did not form biofilms in glucose-supplemented broth. In NaCl-supplemented broth, 30 (50.8%) were strong biofilm producers, 7 (11.9%) were weak biofilm producers, and 22 (37.3%) did not form biofilms.

Genes within the *ica* operon were present in 36 (43.4%) of the *S. epidermidis* isolates but were not found in any of the *S. haemolyticus* isolates. All the *ica*-positive isolates contained *icaA*, *icaD*, *icaC*, *icaB*, and *icaR*. All but one of the *ica*-positive isolates produced strong biofilms in the glucose-supplemented broth. Thirty-two of the 36 *ica*-positive isolates produced strong biofilms in the NaCl-supplemented broth. For both *S. epidermidis* and *S. haemolyticus*, an association was found between strong biofilm production and resistance to norfloxacin, clindamycin, levofloxacin, erythromycin, oxacillin, and cefoxitin ($p < 0.05$) when testing biofilm production in the glucose-supplemented broth.

Detection of mecA and SCCmec typing

The *mecA* gene was found in 72/83 (86.7%) of *S. epidermidis* isolates and 55/59 (93.2%) of *S. haemolyticus* isolates. Among the *S. epidermidis* isolates containing *mecA*, 5 (6.9%) were classified as SCC*mec* type III (*ccr* type 3, class A *mec*) and 34 (47.2%) were classified as type IV (*ccr* type 2, class B *mec*). Also, 22 (64.7%)

Table 2 – SCC*mec* typing, susceptibility profile and biofilm production of linezolid-resistant isolates.

Isolate	Species	SCC*mec* typing			Susceptibility profile[a]			Linezolid resistance[b]		Biofilm production		
		mecA	*mec* class	*ccr* type	FOX	OX	TET	MIC (μg/mL)	*cfr*	Glucose	NaCl	*ica* operon
14583	*S. epidermidis*	+	A	3+5	R	R	S	8	Negative	Strong	Strong	Negative
14565	*S. epidermidis*	+	A	3+5	R	R	I	>32	Positive	Strong	Strong	Positive
12701	*S. epidermidis*	+	A	3	R	R	S	8	Negative	Strong	Strong	Positive
9976	*S. haemolyticus*	–	NA	NA	S	S	S	8	Positive	NP	NP	Negative
2975	*S. haemolyticus*	+	NT	NT	R	R	R	32	Negative	Strong	NP	Negative

NA, not applicable; NT, not typable; FOX, cefoxitin; OX, oxacillin; TET, tetracycline; MIC, minimum inhibitory concentration; NP, non-producer. Methicillin-resistance was evaluated using the cefoxitin disk test, minimum inhibitory concentration was determined using the broth microdilution method. Biofilm formation was assessed by crystal violet staining. Amplification of the *ica* operon, *mecA* and SCC*mec* typing were performed by multiplex PCR.

[a] All isolates were resistant to norfloxacin, clindamycin, levofloxacin, trimethoprim/sulfamethoxazole, and erythromycin. All isolates were susceptible to vancomycin.

[b] Mutation G2576T was found in isolate 2975.

Table 3 – SCC*mec* typing, resistance profile, and biofilm production of *S. epidermidis* isolates.

No. of isolates	SCC*mec* typing			Resistance profile[a] *n* (%)							Biofilm production[b] *n* (%)	
	mec class	*ccr* type	SCC*mec*	NOR	CLI	TET	LEV	LZD	SXT	ERY	Glucose	NaCl
34	B	2	IV	30 (88)	30 (88)	3 (8.8)	30 (88)	0 (0)	25 (74)	30 (88)	33 (97)	30 (88)
5	A	3	III	5 (100)	4 (80)	0 (0)	5 (100)	1 (20)	4 (80)	4 (80)	5 (100)	4 (80)
3	A	1	New	3 (100)	3 (100)	2 (67)	3 (100)	0 (0)	2 (67)	3 (100)	3 (100)	2 (67)
1	A	5	New	1 (100)	1 (100)	0 (0)	1 (100)	0 (0)	1 (100)	1 (100)	1 (100)	1 (100)
1	A	1+2	Variant	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	1 (100)	0 (0)
5	B	1+2	Variant	5 (100)	5 (100)	1 (20)	5 (100)	0 (0)	4 (80)	5 (100)	5 (100)	4 (80)
1	A	2+3	Variant	1 (100)	1 (100)	0 (0)	1 (100)	0 (0)	1 (100)	1 (100)	1 (100)	1 (100)
7	A	3+5	Variant	7 (100)	7 (100)	1 (14)	7 (100)	2 (29)	7 (100)	7 (100)	7 (100)	7 (100)
5	B	2+5	Variant	3 (60)	5 (100)	1 (20)	2 (40)	0 (0)	3 (60)	4 (80)	5 (100)	5 (100)
2	A	2+4	Variant	2 (100)	2 (100)	0 (0)	2 (100)	0 (0)	2 (100)	2 (100)	2 (100)	2 (100)
1	A	3+4	Variant	1 (100)	1 (100)	0 (0)	1 (100)	0 (0)	1 (100)	1 (100)	0 (0)	1 (100)
1	B	2+4	Variant	1 (100)	1 (100)	0 (0)	1 (100)	0 (0)	1 (100)	1 (100)	1 (100)	1 (100)
2	A	1+4+5	Variant	0 (0)	2 (100)	0 (0)	0 (0)	0 (0)	2 (100)	2 (100)	2 (100)	1 (50)
1	NT	2+5	NT	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)	1 (100)	1 (100)
1	NT	2	NT	1 (100)	1 (100)	0 (0)	1 (100)	0 (0)	1 (100)	1 (100)	1 (100)	1 (100)
2	A	NT	NT	2 (100)	2 (100)	1 (50)	1 (50)	0 (0)	2 (100)	2 (100)	2 (100)	2 (100)

NT, not typeable; CLI, clindamycin; TET, tetracycline; LEV, levofloxacin; LZD, linezolid; SXT, trimethoprim/sulfamethoxazole; ERY, erythromycin. Methicillin-resistance was evaluated using the cefoxitin disk test, minimum inhibitory concentration was determined using the broth microdilution method. Biofilm formation was assessed by crystal violet staining. Amplification of the *ica* operon and SCC*mec* typing were performed by multiplex PCR.

[a] All isolates were resistant to cefoxitin and oxacillin. All isolates were susceptible to vancomycin.

[b] Strong producers.

of the SCC*mec* type IV isolates were subtyped as SCC*mec* IVa and one (2.9%) was subtyped as SCC*mec* IVb. The remaining isolates could not be subtyped to SCC*mec* IVc or IVd. *S. epidermidis* with SCC*mec* type IV were more likely to be resistant to norfloxacin and erythromycin. The five SCC*mec* type III isolates were subtyped as subtype III.1. Among the remaining isolates, more diverse SCC*mec* combinations were found; four isolates (5.6%) had *ccr* and *mec* combinations not included in the classification scheme for *S. aureus*, and 23 (31.9%) isolates amplified for more than one *ccr* complex. Four (5.6%) of the isolates could not be typed since *mec* or *ccr* complexes were not amplified in these strains (Table 3).

Of the *S. haemolyticus* isolates containing *mecA*, only 8 (14.5%) isolates could be typed and they were classified as SCC*mec* type V (*ccr* complex type 5, *mec* complex class C2). SCC*mec* subtype V.1 was not detected. Four (7.3%) isolates contained more than one *ccr* complex. 43 (78.1%) of the isolates did not contain *mec* complex, but 13 (26.6%) of them contained *ccr*. More than half of the isolates (30/55, 54.5%) did not contain *ccr* or *mec* complexes (Table 4).

Clonal diversity

SmaI restriction digestion of 76 *S. epidermidis* isolates generated 7–17 fragments. For seven of the isolates, three or fewer fragments were generated. Therefore, these isolates were not considered for further analysis. A total of 75 different restriction patterns with similarities ranging from 0 to 95% were obtained. Only two of the isolates (isolates 11566 and 11567) were 95% homologous and were considered as a clone (Fig. 1). Both strains were classified as SCC*mec* type III by the methodology described by Zhang et al., but were classified as variants

Table 4 – SCC*mec* typing, resistance profile, and biofilm production of *S. haemolyticus* isolates.

No. of isolates	SCC*mec* typing			Resistance profile[a]				Biofilm production[b]	
	mec class	*ccr* type	SCC*mec*	CLI	TET	LZD	ERY	Glucose	NaCl
8	C2	5	V	7 (88)	3 (38)	0 (0)	7 (88)	7 (88)	4 (50)
1	C2	1+2+5	Variant	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)
3	C2	1+4+5	Variant	3 (100)	3 (100)	0 (0)	3 (100)	3 (100)	2 (67)
12	NT	4	NT	10 (83)	3 (25)	0 (0)	11 (92)	8 (67)	5 (42)
1	NT	5	NT	1 (100)	1 (100)	0 (0)	1 (100)	1 (100)	0 (0)
30	NT	NT	NT	30 (100)	3 (10)	1 (3)	30 (100)	23 (77)	18 (60)

NT, not typeable; CLI, clindamycin; TET, tetracycline; LZD, linezolid; ERY, erythromycin.
Methicillin-resistance was evaluated using the cefoxitin disk test, minimum inhibitory concentration was determined using the broth microdilution method. Biofilm formation was evaluated by crystal violet staining. Amplification of the *ica* operon and SCC*mec* typing were performed by multiplex PCR.

[a] All isolates were resistant to cefoxitin, oxacillin, norfloxacin, levofloxacin and trimethoprim/sulfamethoxazole. All isolates were susceptible to vancomycin.

[b] Strong producers.

by Kondo et al. methodology, since they also amplified *ccrC*. Both were resistant to norfloxacin, clindamycin, levofloxacin, trimethoprim/sulfamethoxazole, erythromycin, oxacillin, and cefoxitin, but susceptible to linezolid and vancomycin. Also, both strains were classified as strong biofilm producers by both supplemented broths and proceed from intensive care units.

SmaI restriction digestion of the *S. haemolyticus* isolates generated 7–15 fragments, and 57 different patterns were produced. Isolates 9990 and 9982 were considered clone A, and isolates 14425 and 14162 were considered clone B (Fig. 2). Both clones had 95% of similarity. The remaining isolates had similarities of 60% or less.

Isolates 9990 and 9982 were classified as non-typable SCC*mec*. Both were resistant to clindamycin, levofloxacin, trimethoprim/sulfamethoxazole, erythromycin, oxacillin, and cefoxitin. Also, both strains were classified as strong biofilm producers by NaCl-supplemented broths and proceed from intensive care units. Isolates 14425 and 14162 were classified as SCC*mec* type V. Both were resistant to norfloxacin, clindamycin, levofloxacin, trimethoprim/sulfamethoxazole, erythromycin, oxacillin, and cefoxitin. Also, both strains were classified as strong biofilm producers by both supplemented broths and proceed from intensive care units.

MLST typing

Five *S. epidermidis* isolates were chosen to be analyzed by MLST: the linezolid-resistant isolates, an isolate representative of the group harboring SCC*mec* type IV, and an isolate representative of the group harboring SCC*mec* type III. MLST analysis showed that all the linezolid-resistant *S. epidermidis* isolates ($n = 3$) belonged to ST23 while the isolate with SCC*mec* type IV and the isolate with SCC*mec* type III belonged to ST7 and ST2, respectively.

Discussion

CoNS infections are associated with high antimicrobial resistance, which makes them difficult to treat. In this study, we analyzed *S. epidermidis* and *S. haemolyticus* human blood isolates for biofilm formation, antimicrobial resistance, SCC*mec* type, and clonal diversity.

We identified three linezolid-resistant *S. epidermidis* and two linezolid-resistant *S. haemolyticus* isolates. Four of these were also methicillin-resistant. Linezolid is a synthetic oxazolidinone that inhibits protein synthesis by binding to ribosomal peptidyl transferase, and it is recommended to treat multidrug-resistant Gram-positive infections. Although linezolid resistance is rare (<1% in *S. aureus* and <2% in CoNS), the emergence of linezolid-resistant strains is a serious healthcare concern.[23] In the Hospital Civil in Guadalajara, linezolid is the most commonly used antibiotic for nosocomial pneumonia, surgical wound infections, and is also used for bloodstream infections not associated with catheter. In order to reduce the spreading of linezolid-resistant strains a stewardship antibiotic consumption program limiting linezolid use has been implemented.

In two of the isolates, linezolid-resistance was associated with the presence of the *cfr* gene, which encodes for an adenine methyltransferase that modifies the adenosine at position 2503 in *23S rRNA*. *cfr* also confers resistance to phenicol compounds, lincosamide, oxazolidinone, pleuromutilin, and streptogramin A.[24] *cfr* has primarily been found on plasmids in *S. epidermidis* and *S. haemolyticus* isolates,[25,26] but can also be found on the chromosome as a result of the activities of transposons and insertion sequences.[27] The presence of *cfr* on mobile genetic elements, which may carry additional resistance genes, probably facilitates *cfr* dissemination. The two *cfr*-containing isolates had different MIC values for linezolid; isolate 9976 had an MIC of 8 μg/mL, whereas isolate 14565 had an MIC > 32 μg/mL. These differences suggested the presence of additional resistance mutations in isolate 14565, so we sought for mutations in domain V of *23S rRNA* in all linezolid-resistant isolates. However, only isolate 2975 harbored the G2576T mutation, which is the most frequently reported.[28] This isolate had an MIC of 32 μg/mL and no *cfr* gene. Linezolid-resistance can also be conferred by mutations in ribosomal proteins L3 and/or L4.[29] Our linezolid-resistant isolates should be analyzed further to identify additional mutations explaining the differences in MIC.

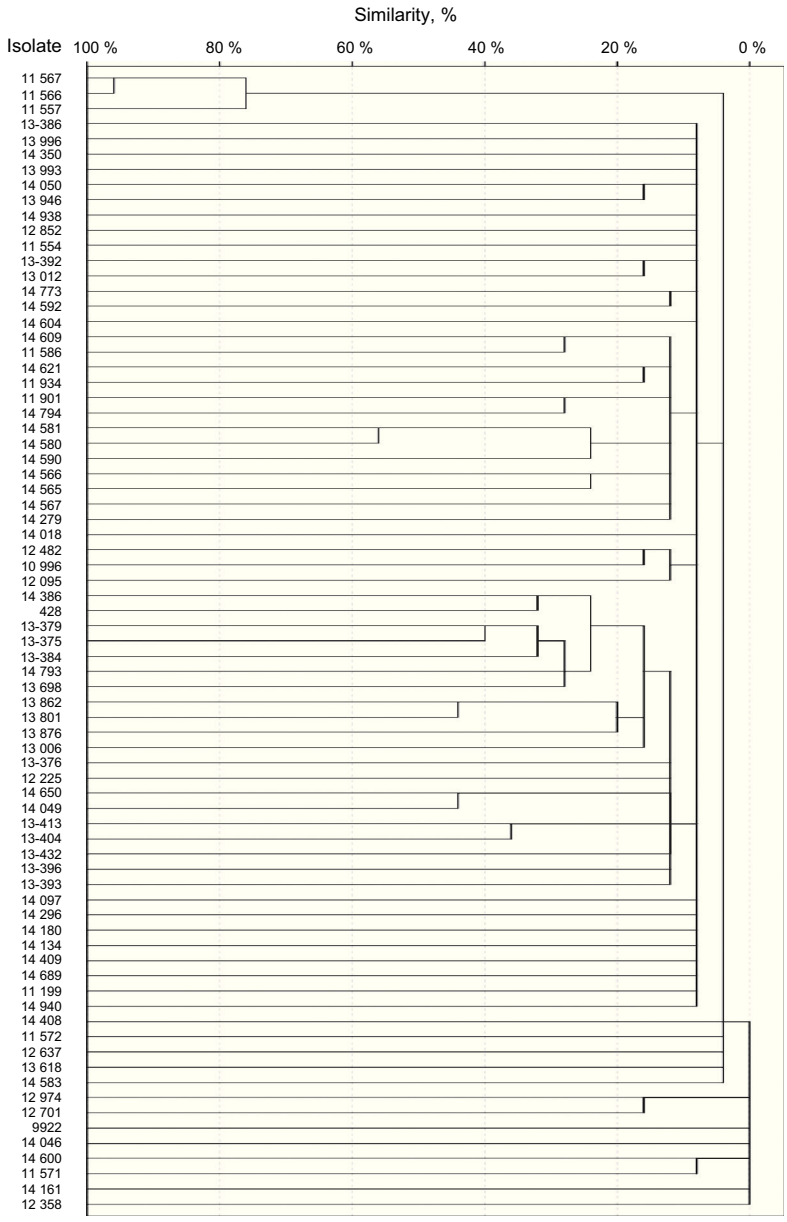

Fig. 1 – Dendrogram of *S. epidermidis* isolates.

In Mexico, *S. epidermidis* STs 2, 23, 46, 61, 71, and 82 have been reported.[30] The three linezolid-resistant *S. epidermidis* isolates analyzed belonged to ST23. Linezolid-resistance has been reported in the ST23 clone. In a study from Italy, clinical isolates from blood and cerebrospinal fluid were analyzed. The most frequently ST found in linezolid-resistant strains were ST23; also, almost the half of the ST23 strains were *cfr* positive (22/50), which suggested an association between the genetic background with the *cfr* gene.[31] ST23 has also been reported in Argentina, Germany, Greece, Hungary, Iceland, Poland, Portugal, United States and Uruguay (http://sepidermidis.mlst.net).

One of our isolates was ST2. Linezolid-resistant strains belonging to ST2 have been described in a Brazilian tertiary-care hospital in isolates from skull, blood, and catheter cultures.[32] ST2 has also been described in Germany, Argentina, Italy, Poland, Spain, Mexico, Cape Verde, Denmark, Greece, Hungary, Uruguay; Bulgaria and Colombia, Japan, Netherlands, and United States. (http://sepidermidis.mlst.net). In Mexico, Juarez-Verdayes et al. analyzed isolates from healthy skin, healthy conjunctiva, and ocular infections; ST2 lineage was the most frequent among the isolates (50% for healthy skin, 25% for healthy conjunctiva and 46.5% for ocular infections).[33] Similarly, Flores-Paez et al. reported ST2 and ST23 from isolates from ocular infections.[34] None of ST23 or ST2 has been previously reported from sterile sites in Mexico. To the best of our knowledge *S. epidermidis* ST7 has not been reported in Mexico. ST7 has been reported in isolates from catheter-related bloodstream infections[35] and prosthetic valve endocarditis.[14]

Both *S. epidermidis* and *S. haemolyticus* have been shown to be strong biofilm producers, and the *ica* operon has been associated with biofilm production in *S. epidermidis*.[36] We found

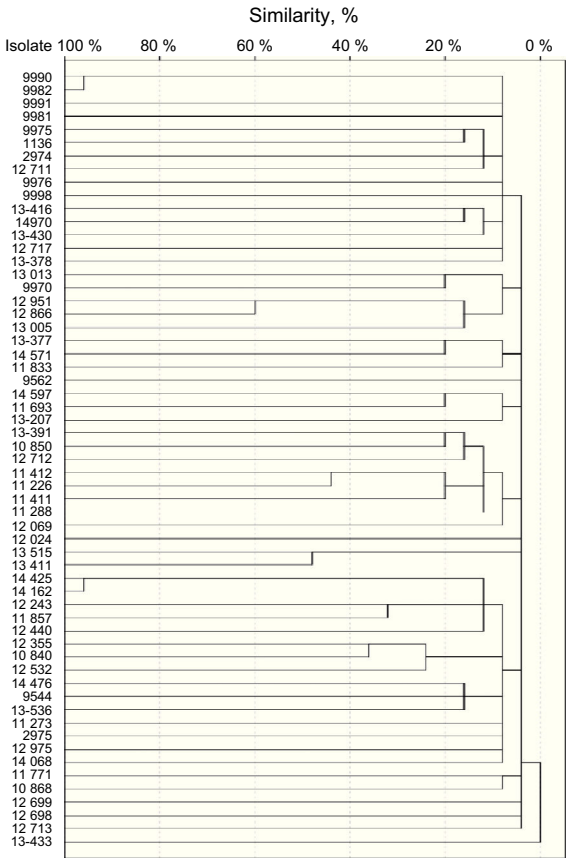

Fig. 2 – Dendrogram of *S. haemolyticus* isolates.

the *ica* operon to be present in 36 of the *S. epidermidis* isolates, and 35 of these isolates were strong biofilm producers. On the other hand, the *ica* operon was not found in any of the *S. haemolyticus* isolates; this is consistent with a report by Fredheim et al. in which nearly all of their *S. haemolyticus* isolates were *ica*-negative.[37] Although *S. haemolyticus* has been associated with *ica*-independent biofilm formation, a report by Pereira et al. showed that 58% of their isolates harbored *icaA*.[38] Genes such as *aap*, *bap*, *bhp* have been shown to be involved in *ica*-independent biofilm formation.[4] Moreover, studies on biofilm detachment have shown that PIA is not a major component of *S. haemolyticus* biofilms.[37]

All three linezolid-resistant *S. epidermidis* isolates were strong biofilm producers, and two were positive for the *ica* operon. Biofilm formation has long been associated with antibiotic resistance, and increased MICs have been linked to the presence of biofilms.[39] One *S. haemolyticus* linezolid-resistant isolate was a strong biofilm producer, but neither *S. haemolyticus* linezolid-resistant isolate contained the *ica* operon. Resistance to norfloxacin, clindamycin, levofloxacin, erythromycin, cefoxitin, and oxacillin were associated with strong biofilm production ($p < 0.05$) for both the *S. epidermidis* and *S. haemolyticus* isolates.

As previously described, SCC*mec* type IV, especially SCC*mec* type IVa, was frequently found (47.2%) among the *S. epidermidis* isolates. This frequency has been found among isolates from both inpatients and carriers. In a study that analyzed 44 blood isolates, SCC*mec* type IV was found at a frequency of 36%,[40] whereas SCC*mec* type IVa was found at a frequency of 65% among healthy subjects.[41] Wisplinghoff et al. suggested that genetic information was transmitted between *S. epidermidis* and *S. aureus* since SCC*mec* IV sequences from both species were >98% homologous.[40] Transmission of SCC*mec* between these species may lead to an increase in beta-lactam antibiotic resistance in *S. aureus*; currently, methicillin-resistance in *S. aureus* is not as high as in CoNS.

In this study, we found several combinations of *mec* and *ccr* complexes in *S. epidermidis*. Only two SCC*mec* types (IV and III) were identified. The other *S. epidermidis* isolates had *mec* and *ccr* combinations distinct from the 11 SCC*mec* types reported to date or could not be typed. A problem with amplification of more than one *ccr* complex is that it is unknown whether the amplified *ccr* complexes are located within the same SCC*mec*.[11,42]

In *S. haemolyticus* isolates the only SCC*mec* found was SCC*mec* type V, and it has been found at frequencies as high as 55%.[6,11] Similar to the transfer of SCC*mec* type IV from *S. epidermidis* to *S. aureus*, SCC*mec* type V may be transferred from *S. haemolyticus* to *S. aureus*. Notably, we found that most of the *S. haemolyticus* isolates could not be typed. Likewise, Barros et al.[6] reported that 43% (24 isolates) of their *S. haemolyticus* isolates could not be typed. There appears to be great SCC*mec* diversity among *S. haemolyticus* isolates.

Despite being commensals, both *S. epidermidis*, and *S. haemolyticus* have been found to be clonal, particularly *S. haemolyticus*.[7] However, only a clone composed of two *S. epidermidis* isolates was found. In addition, two clones with two strains or *S. haemolyticus* each one were detected in this study. Thus, the hypothesis of clonal transmission at the two source hospitals is discarded. Since both species are components of the normal microbiota, it is likely that the infections were endogenous.

The actual knowledge of the molecular epidemiology of both species includes an extremely high genetic diversity and recombination. PFGE is mostly useful for an outbreak or short-term epidemiological investigations and may not detect clonal relatedness in isolates recovered through the years. On the contrary, MLST is more useful, particularly for the *S. epidermidis* isolates since it allows to determine their genetic backgrounds and to compare to those previously described from other countries. Due to the relevance of the linezolid-resistant isolates, MLST analysis was considered for these isolates.

In conclusion, this study is the first performed in Mexico that characterizes biofilm production, antimicrobial susceptibility, SCC*mec* and clonal relatedness of *S. epidermidis* and *S. haemolyticus* blood isolates. Both species were found to be highly resistant to antibiotics. We also detected linezolid-resistance, which is a concern for infection control practices.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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