Novel methicillin-resistant coagulase-negative *Staphylococcus* clone isolated from patients with haematological diseases at the Blood Bank Centre of Amazon, Brazil

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Methicillin-resistant Staphylococcus *remains a severe public health problem worldwide. This research was intended to identify the presence of methicillin-resistant coagulase-negative staphylococci clones and their staphylococcal cassette chromosome mec (SCC*mec*)-type isolate from patients with haematologic diseases presenting bacterial infections who were treated at the Blood Bank of the state of Amazonas in Brazil. Phenotypic and genotypic tests, such as SCC*mec *types and multilocus sequence typing (MLST), were developed to detect and characterise methicillin-resistant isolates. A total of 26 Gram-positive bacteria were isolated, such as:* Staphylococcus epidermidis *(8/27),* Staphylococcus intermedius *(4/27) and* Staphylococcus aureus *(4/27). Ten methicillin-resistant staphylococcal isolates were identified. MLST revealed three different sequence types:* S. aureus *ST243,* S. epidermidis *ST2 and a new clone of* S. epidermidis*, ST365. These findings reinforce the potential of dissemination presented by multi-resistant* Staphylococcus *and they suggest the introduction of monitoring actions to reduce the spread of pathogenic clonal lineages of* S. aureus *and* S. epidermidis *to avoid hospital infections and mortality risks.*

Key words: SCC*mec* - ST clones - coagulase-negative *Staphylococcus*

Methicillin-resistant staphylococci stand as main pathogens responsible for high levels of infectious processes worldwide. Methicillin resistance is encoded by the *mecA* gene, located at the staphylococcal cassette chromosome *mec* (SCC*mec*). In *Staphylococcus aureus,* types I, VII and XI are recognised, whereas types IV and V are found in *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* (Strandén et al. 2009, Iorio et al. 2012). The genotype distributions of staphylococci differ across different geographical regions. The USA300 community-acquired methicillin-resistant *S. aureus* clone sequence typing ST8 is dominant in the United States, as the ST80 and ST30 clones are in Europe, Kuwait, Singapore, the Southwest Pacific islands and New Zealand (Udo et al. 2008), and Brazilian epidemic clone ST239 that is predominant in different hospitals (Carvalho et al. 2010, Almeida et al. 2012). Regarding *S. epidermidis*, the ST69 clone is predominant in Greece, ST57 and ST88 in Portugal, ST2 in China, ST2, ST22, ST61 and ST71 in Mexico and ST63 in Argentina (Miragaia et al. 2009, Liakopoulos et al. 2010).

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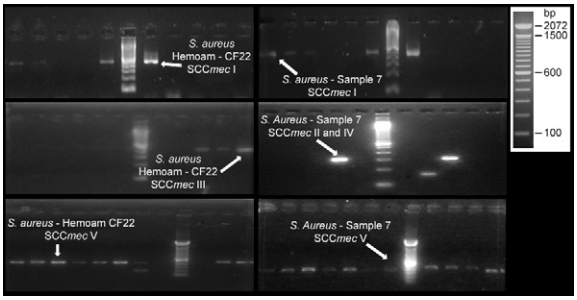
The risk for immunocompromised patients can be significant if they become infected by those species while in hospitals. The presence of immunocompromised patients at the hospital and blood bank where this research was undertaken was common, as the patients were receiving chemotherapy treatments against leukaemia, myeloma, haemolytic anaemia, haemophilia etc. These treatments can increase morbimortality risks due to prolonged hospitalisation, the spread of multi-resistant strains and rises in treatment costs. Given these clinically diagnosed infectious processes in immunocompromised patients, in this study, we studied the resistance of these pathogens to the antibiotics used in these treatments and the pathogens' genotypes for a broader epidemiological view of these staphylococci. Being aware of geographic distributions and transmission mechanisms - factors that contribute to the appearance and spread of these genotypes - will help us to control hospital infection outbreaks and dissemination and to implement programs to supervise the resistance presented by these pathogens.

From July 2007-August 2008, we conducted a study including haematology patients of both sexes admitted to the Haematology and Haemotherapy Foundation (HEMOAM) presenting clinical signs and symptoms suggestive of acute bacterial infection. The HEMOAM is located in Manaus, the capital city of the state of Amazonas. It is a public blood bank that provides health care to patients with haematological disorders and provides blood and blood derivatives to the residents of the entire city. The biological samples provided include urine, blood, sputum, catheter tips, bone marrow aspirate and abscess fluid, along with oropharyngeal, ocular, peria-

nal, nasopharyngeal, open wound and skin lesion secretions. Phenotypic tests were performed on all isolates using standard biochemical tests, including Gram staining, colonial morphology on 5% sheep's blood, mannitol and Mueller-Hinton agar plates (Himedia Hexasystens-Mumbai, India), catalase, coagulase (Newprov), glucose, maltose, sucrose and lactose fermentation tests, urease (Himedia Hexasystens-Mumbai, India), susceptibility testing with 5 µg of novobiocin (Laborclin) and 0.04 UI bacitracin (Newprov), 300 polymyxin B (Newprov), PYR testing (Probac, Brazil) and nucleotide sequencing of the 16S rRNA gene region using the bacterial primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Miyoshi et al. 2005). The E-test® and reference values for sensitivity, reduced sensitivity or resistance to antibiotics were set according to the Clinical and Laboratory Standards Institute (CLSI 2010) (Wayne, Pennsylvania, USA) manual. ATCC 25923 *S. aureus* was used for quality control of the culture media and for susceptibility tests. PBP 2a tests (Oxoid, Cambridge, UK) were performed on all isolates to identify methicillin resistance. Genomic DNA extraction from all *Staphylococcus* strains was performed using an Kit Easy DNA (Invitrogen, Carlsbad, CA, USA) and a polymerase chain reaction (PCR) protocol for the identification of the *mec*A gene, SCC*mec,* toxin genes (*seh*, *arc*A, *etd*) and the Panton-Valentine leukocidin gene, according to Jonas et al. (2002), Milheiriço et al. (2007) and Strommenger et al. (2008). *S. aureus* ATCC strains 25923, 29213 and 33591 and *S. epidermidis* ATCC 12228 were used as control strains. Multilocus sequence typing (MLST) was used to identify the genetic backgrounds of the isolates. MLST was performed according to the methods published for *S. aureus* and *S. epidermidis*. Internal fragments of the seven housekeeping genes were amplified by PCR and both strands were sequenced. Alleles and STs were determined from the *S. aureus* and *S. epidermidis* MLST databases (mlst.net). At the end of this research, the MLST website did not yet have proposal protocols for the genotypes of other coagulase-negative *Staphylococcus* species.

One hundred forty-six clinical samples were obtained from 69 patients over a period of 13 months. Among the collected samples, we were able to isolate 44 bacterial strains, 26 of which were Gram-positive. With regard to sex, 35 of 69 patients were male and 34 of 69 were female, with an average age of 24 years. The most frequent haematological diseases identified in patients were acute lymphocytic leukaemia (36/69) and acute myeloid leukaemia (6/69). Biochemical tests and 16S rDNA sequencing detected *S. epidermidis* (8/26), *S. intermedius* (4/26) and *S. aureus* (4/26). Other isolated species included *Staphylococcus hyicus* (1/26), *S. haemolyticus* (1/26), *Staphylococcus simulans* (1/26), *Staphylococcus lugdunensis* (2/26) and *Staphylococcus* spp (5/26). Of the 26 staphylococci strains, 10 were methicillin-resistant. PBP 2a tests were performed on those 10 isolates, confirming the presence of the *mec*A gene and the absence of the *luk*PV gene. Only one sample of *Staphylococcus* sp. harboured the *Seh* gene for enterotoxin H. The MLST analysis revealed three different STs among these isolates: *S. aureus* ST243, *S. epidermidis* ST2 and two new isolates of the *S. epidermidis* ST365 clone, which was not found among isolates in the MLST scheme of Miragaia et al. (2009) in our hospital environment. The ST243 strain harboured SCC*mec* types I, III and V (Figure), the ST2 strain harboured SCC*mec* types II, III and V and the ST365 strained harboured SCC-mec types I, III and V (Table I). The other coagulase-negative *Staphylococcus* species were nontypeable. The DNA sequences of each allele at the seven *loci* in this study have been deposited in GenBank under accessions JN398483-JN398507. They can be downloaded from the aforementioned website.



Polymerase chain reaction staphylococcal cassette chromosome *mec* (SCC*mec*) typing of *Staphylococcus aureus* strains. HEMOAM: Haematology and Haemotherapy Foundation.

The *S. epidermidis* ST 365 novel pathogenic clone was isolated from the peripheral blood of two patients: a boy (9 years old) and a girl (2 years old) presenting with acute lymphocytic leukaemia. Susceptibility tests indicated the resistance of these strains to antibiotics (Table II). The detection of this resistant methicillin isolate belonging to this pathogenic clonal lineage in hospitalised - mainly immunocompromised - patients can increase the risk for morbimortality if these patients acquire infections in hospitals caused by those species.

The *S. epidermidis* ST2 clone was isolated from a blood culture sample from a 24-year-old patient with chronic myeloid leukaemia presenting resistance to antibiotics, such as amikacin, ceftazidime and cefepime (Table II). The identification of these strains in the hospital might have been due to the following causes: (a) colonised patient (reservoir) circulation, (ii) contaminated healthcare worker-to-patient transmission, (iii) antibiotic selective pressure of the isolate (Sousa et al. 2009, Barbier et al. 2010), (iv) high biofilm formation - recognised as a critical factor for the colonisation of medical devices (Botelho et al. 2012), (v) high level of genetic flexibility including heterogeneous expression of the gene, (vi) the presence of SSC*mec* in disease-associated isolates, (vii) the *ica* gene cluster and (viii) insertion sequence element IS256. These causes reflect great capacity to adapt through genetic exchange to environmental changes and antibiotic therapy (Weisser et al. 2010, Widerström et al. 2012).

TABLE I

Phenotype/genotype profiles of the methicillin-resistant staphylococcal strains isolated from patients with haematologic diseases

Strains	Phenotype	Genotype									
	PBP 2a	*mec*A[a]	SCC*mec*	*arcC*	*aroE*	*gtR*	*mutS*	*pyR*	*tpiA*	*ygiL*	ST
Staphylococcus epidermidis (HEMOAM CF16 strain)	POS	POS	I, III, V	3	25	5	5	3	4	4	365
S. epidermidis (HEMOAM CF36 strain)	POS	POS	I, III, V	3	25	5	5	3	4	4	365
S. epidermidis (HEMOAM CF40 strain)	POS	POS	II, III, V	7	1	2	2	4	1	1	2
Staphylococcus aureus (HEMOAM CF22 strain)	POS	POS	I, III, V	2	2	5	2	6	3	2	243

a: gold standard; HEMOAM: Haematology and Haemotherapy Foundation; POS: positive; SCC*mec*: staphylococcal cassette chromosome *mec*; ST: sequence type.

ST2, founder of the CC2 clonal complex, is considered the prevalent international cause of most *S. epidermidis* hospital infections; it has also been detected as an epidemic hospital pathogen worldwide and in different European hospitals (Weisser et al. 2010, Widerström et al. 2012). In Brazil, it was identified in a hospital in Rio de Janeiro (Iorio et al. 2012). The detection of methicillin-resistant strains belonging to this virulent clonal lineage in hospitalised - mainly immunocompromised - patients points to possible hospital infections, as these patients had been hospitalised for more than 72 h, they did not come from other hospitals, they received no prolonged antibiotic therapy at the moment of collection and they presented with frequent use of catheters because of their treatments. Therefore, studies monitoring the resistance of these strains to antibiotics are essential in light of the reports of coagulase-negative staphylococci species resistant to linezolid therapy, a therapeutic option for multidrug-resistant Gram-positive pathogens, including methicillin-resistant *S. aureus* and vancomycin-resistant enterococci (Almeida et al. 2012).

The *S. aureus* ST243 clone, isolated from a 30-year-old haemophilic patient with a blood infection, demonstrated resistance to antibiotics, such as ceftazidime, chloramphenicol and tetracycline (Table II). Despite in vitro sensitivity against imipenem and cefepime, CLSI M-100 S20 (CLSI 2010) recommendations suggest that they should be reported as resistant due to low treatment effectiveness. The presence of ST243 in our hospital could has come from patient-to-patient or colonised patient-to-contaminated healthcare worker transmission or from prolonged antibiotic therapy. Other risk factors that can be considered for haematogenous infection are nasal carriers and skin/soft tissue infections (Carvalho et al. 2010, Yamamoto et al. 2010, Widerström et al. 2012). This clone has spread across the United States, Thailand and Japan (data available from mlst.net), although only the Thai clone is methicillin-resistant.

The presence of the SCC*mec* III cassette in *S. epidermidis*, considered with no clinical importance in infections must be revised (Tables I, III), because the horizontal transfer of a large number of resistance genes among species opens the way to methicillin resistance (Sousa et al. 2009, Barbier et al. 2010, Iorio et al. 2012), which induces the use of different antibiotics as therapeutic options (mainly vancomycin). Another observation was the presence of SCC*mec* type V in *S. epidermidis* (Table III). Types IV and V are small, structurally similar elements that carry the *mec*A gene as a unique antibiotic resistance encoder. As these types have high competitive transfer capacity, this fact can explain the presence of SCC*mec* type V in relation to type IV (Ito et al. 2004, Barbier et al. 2010). Another important factor detected was the existence of various SCC*mec* types (Figure, Tables I, III). Other *Staphylococcus* strains, such as *S. intermedius* and *Staphylococcus* sp., presented SCC*mec* types I, III and V. *S.hyicus* presented SCC*mec* types I, II and IV and *S. lugdunensis* SCC*mec* type III. The structural diversity of SCC*mec* in coagulase-negative *Staphylococcus* has been described in other studies (Ruppé et al. 2009 Barbier et al. 2010, Carvalho et al. 2010). The presence of these different SCC*mec* types might suggest that multiple introductions are occurring and their presence in the same ST could suggest possible horizontal transfer among species (Enright et al. 2000, Barbier et al. 2010) or diversity in the combination of the *mec* and *ccr* allotypes (Ruppé et al. 2009).

Horizontal transfer of the *mec* gene might be occurring, favouring the appearance of methicillin resistance among species. These findings serve as an alert for hospitals and possibly for the region to introduce actions to reduce the spread of the pathogenic clonal lineages of *S. aureus* and *S. epidermidis*. Microbial resistance monitoring programs are important to avoid hospital infection outbreaks and the spread of pathogenic clonal lines. Further research in regional hospitals and the community are required for a more accurate view of the regional epidemiological distribution of these clones and of the new ST365 clone.

TABLE II

Phenotype/genotype profiles of the staphylococcal strains isolated from patients with haematologic diseases

Strains	ST	Susceptibility tests/MIC/antibiotics (µg/mL)									
		Oxacillin	Penicillin	Amikacin	Vancomycin	Tetracycline	Chloramphenicol	Ceftazidime	Ciprofloxacin	Imipenen	Cefepime
Staphylococcus epidermidis (HEMOAM CF16)	365	R (1)	R (> 32)	S (2)	S (1.5)	R (24)	S (1)	RS (16)	S (0.19)	S (0.25)	S (1)
S. epidermidis (HEMOAM CF36)	365	R (1.5)	R (3)	S (1)	S (2)	R (64)	S (8)	RS (16)	S (0.25)	S (0.094)	S (1)
S. epidermidis (HEMOAM CF40)	2	R (256)	R (> 32)	R (256)	S (2)	R (16)	RS (16)	R (256)	S (0.19)	S (0.094)	R (256)
*Staphylococcus aureus (*HEMOAM CF22)	243	R (1)	R (6)	S (4)	S (0.5)	R (256)	R (32)	R (6)	S (0.50)	S (0.064)	S (1.5)
S. epidermidis - sample 1	ND	S (0.190)	S (0.016)	S (1)	S (1)	S (2)	S (8)	S (4)	S (0.38)	S (0.32)	S (0.75)
S. epidermidis - sample 2	ND	S (0.125)	S (0.125)	S (0.125)	S (3)	S (0.50)	S (3)	S (8)	S (0.064)	S (0.012)	S (2)
S. epidermidis - sample 6	ND	S (0.190)	R (1)	RS (48)	S (3)	R (64)	S (8)	S (8)	S (0.25)	S (0.125)	S (1)
S. epidermidis - sample 8	ND	S (0.125)	S (0.064)	S (2)	S (3)	S (0.50)	S (0.125)	S (4)	S (0.125)	S (0.19)	S (1.5)
S. epidermidis - sample 9	ND	S (0.190)	S (0.032)	S (1)	S (3)	S (0.75)	R (4)	S (4)	S (0.94)	S (0.012)	S (1)
S. aureus - sample 7	ND	S (0.50)	S (0.032)	S (1)	S (1)	S (2)	RS (16)	S (6)	S (0.38)	S (0.32)	S (1.5)
S. aureus - sample 27	ND	S (0.50)	S (0.50)	S (0.125)	S (0.75)	R (24)	S (6)	S (6)	S (0.064)	S (0.064)	S (1.5)
S. aureus - sample 29	ND	S (0.75)	S (0.125)	S (6)	S (1.5)	R (64)	S (6)	S (6)	S (0.25)	S (0.25)	S (1.5)
S. intermedius - sample 5	ND	R (0.75)	S (0.032)	S (0.125)	S (1.5)	R (24)	RS (16)	S (8)	RS (3)	S (0.047)	S (0.50)
S. intermedius - sample 11	ND	R (256)	S (0.125)	S (2)	S (0.50)	R (256)	S (4)	RS (12)	R (32)	S (0.019)	S (0.047)
Staphylococcus intermedius - sample 28	ND	R (1)	R (32)	S (2)	S (0.75)	R (256)	S (0.75)	S (6)	S (0.032)	S (0.064)	S (0.75)
S. intermedius - sample 33	ND	S (0.250)	S (0.064)	S (3)	S (1.5)	S (1.5)	S (6)	S (6)	S (0.125)	S (0.032)	S (1)
Staphylococcus lugdunensis - sample 24	ND	S (2)	R (1)	S (1.5)	S (1.5)	R (32)	S (2)	S (6)	S (0.125)	S (0.094)	S (1)
S. lugdunensis - sample 25	ND	R (8)	R (2)	S (1.5)	S (1.5)	R (24)	RS (12)	S (6)	S (1)	S (0.047)	S (1.5)
Staphylococcus sp. - sample 20	ND	R (0.50)	S (0.094)	S (0.75)	S (3)	S (2)	RS (16)	S (6)	S (0.19)	S (0.064)	S(0.75)
Staphylococcus sp. - sample 23	ND	R (0.75)	S (0.094)	S (2)	S (1.5)	S (2)	S (6)	S (8)	S (0.19)	S (0.047)	S (0.75)
Staphylococcus sp. - sample 30	ND	S (0.094)	R (3)	S (2)	S (1.5)	R (64)	S (8)	RS (24)	S (0.50)	S (0.064)	S (2)
Staphylococcus sp. - sample 42	ND	R (1)	R (24)	S (4)	S (2)	R (64)	S (8)	S (8)	S (0.38)	S (0.064)	S (2)
Staphylococcus sp. - sample 44	ND	R (6)	R (32)	RS (42)	S (4)	S (3)	R (256)	RS (16)	R (12)	S (0.064)	R (32)
Staphylococcus haemolyticus - sample 31	ND	S (0.094)	S (0.094)	S (1.5)	S (0.25)	S (2)	S (2)	S (3)	S (1)	S (0.094)	S (0.047)
Staphylococcus simulans - sample 35	ND	S (0.250)	R (4)	S (0.25)	S (1.5)	R (24)	S (8)	S (6)	S (0.19)	S (0.094)	S (0.75)
Staphylococcus hyicus - sample 43	ND	R (6)	R (32)	RS (24)	S (2)	S (3)	R (256)	RS (24)	R (4)	S (0.064)	R (256)

HEMOAM: Haematology and Haemotherapy Foundation; MIC: minimum inhibitory concentration; ND: not done; R: resistant; RS: reduced susceptibility; S: susceptible; ST: sequence type.

TABLE III

Phenotype/genotype profile of the staphylococcal species isolated from patients with haematologic diseases

	Phenotype		Genotype
Strains	PBP 2a	*mec*A[a]	SCC*mec*
Staphylococcus aureus - sample 7	NEG	NEG	I, II or IV[b], V
Staphylococcus intermedius - sample 28	POS	POS	I, III, V
Staphylococcus sp. - sample 20	POS	POS	I, III, V
Staphylococcus sp. - sample 23	NEG	POS	III, V
Staphylococcus epidermidis - sample 8	NEG	NEG	I,
S. epidermidis - sample 9	NEG	NEG	I
Staphylococcus sp. - sample 42	NEG	NEG	I
Staphylococcus sp. - sample 44	POS	POS	I
Staphylococcus hyicus- sample 43	POS	POS	I, II or IV[b]
Staphylococcus lugdunensis - sample 24	POS	POS	III
S. epidermidis - sample 2	NEG	NEG	NT
S. epidermidis - sample 6	NEG	NEG	NT
S. aureus - sample 27	NEG	NEG	NT
S. aureus - sample 29	NEG	NEG	NT
S. intermedius - sample 5	NEG	NEG	NT
S. intermedius - sample 11	NEG	NEG	NT
S. intermedius - sample 33	NEG	NEG	NT
S. epidermidis - sample 1	NEG	NEG	NT
S. lugdunensis - sample 25	NEG	NEG	NT
Staphylococcus sp. - sample 30	NEG	NEG	NT
Staphylococcus haemolyticus - sample 31	NEG	NEG	NT
Staphylococcus simulans - sample 35	NEG	NEG	NT

a: gold standard; *b*: primers amplifies both staphylococcal cassette chromosome *mec* (SCC*mec*) II or IV; NEG: negative; NT: nontypeable; POS: positive.

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