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Original Article

Molecular and phenotypic characteristics of methicillin-resistant *Staphylococcus aureus* isolated from hospitalized patients

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Abstract

Introduction: Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the leading causes of infections acquired in both community and hospital settings. In this study, MRSA isolated from different sources of hospitalized patients was characterized by molecular and phenotypic methods.

Methodology: A total of 123 *S. aureus* isolates were characterized according to their genetic relatedness by repetitive element sequence based-PCR (REP-PCR), *in vitro* antimicrobial susceptibility profile, SCC*mec* typing and presence of seven virulence factor-encoding genes.

Results: REP-PCR fingerprinting showed low relatedness between the isolates, and the predominance of one specific lineage or clonal group was not observed. All isolates were susceptible to teicoplanin and linezolide. All isolates were resistant to cefoxitin and penicillin, and the majority were also resistant to one or more other antimicrobials. Fifty isolates (41.7%) were intermediately resistant to vancomycin. Most isolates harbored SCC*mec* type II (53.7%), followed by type I (22.8%), type IV (8.1%) and type III (1.6%). All isolates harbored at least two virulence factor-encoding genes, and the prevalence was as follows: *coa*, 100%; *icaA*, 100%; *hla*, 13.0%; *hlb*, 91.1%, *hld*, 91.1%; *luk*S-PV and *luk*F-PV, 2.4%; and *tst*, 34.1%. A positive association with the presence of *hla* and SCC*mec* type II, and *tst* and SCC*mec* type I was observed.

Conclusion: This study showed the high virulence potential of multidrug-resistant MRSA circulating in a teaching hospital. A high prevalence of MRSA showing intermediate vancomycin resistance was also observed, indicating the urgent need to improve strategies for controlling the use of antimicrobials for appropriate management of *S. aureus* infections.

Key words: antimicrobial resistance; intermediate vancomycin resistance; MRSA; SCC*mec* typing; virulence factors.

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Introduction

Staphylococcus aureus can be found as a harmless colonizer of skin and mucosa, mainly the anterior nares, in 20%-60% of the population [1-3]. Colonization by this bacterium is one important predisposing factor for staphylococcal infections [1]. In fact, as an opportunistic pathogen, *S. aureus* can cause diseases ranging from superficial skin and soft tissue infections to life-threatening disseminated infections [4]. Currently, *S. aureus* is one of the leading causes of healthcare-associated infection worldwide. Most importantly, a substantial proportion of staphylococcal infections are caused by methicillin-resistant *S. aureus* (MRSA), which also exhibits resistance to several other antimicrobials [5,6], contributing to its persistence as a human pathogen for decades.

The acquisition of the *mecA* gene, which encodes a penicillin-binding protein (PBP) with low affinity for the antimicrobial, called PBP2' or PBP2a, is the most common mechanism of methicillin-resistance. The gene *mecA* is inserted into a mobile genetic element (MGE) known as staphylococcal cassette chromosome *mec* (SCC*mec*), and currently, eleven types (SCC*mec* I to XI) have been described in MRSA strains isolated from various sources [7-10].

The virulence potential of *S. aureus* is extensive, with it being represented by both structural and secreted products, whose encoding genes are mostly located in MGEs, which also contribute to bacterial genome plasticity and evolution [11]. This diverse array of virulence factor-encoding genes facilitates the adhesion of bacterial cells to biotic or abiotic surfaces, resistance to host defenses, invasion and cell injury [12]. The differential expression of these genes may enhance *S. aureus* virulence, enabling the bacterium to cause specific clinical presentation [13]. Furthermore, the virulence of this bacterium may vary between isolates from different geographical regions [14].

Continuous efforts to understand the biological basis of MRSA antimicrobial resistance and virulence are therefore necessary. This knowledge may contribute not only to the adoption of effective measures to control the infection, but also to the development of new anti-infective drugs that inhibit bacterial growth plus control virulence [12,15].

In this study, MRSA strains isolated from different sources of patients seen at the University Hospital of Londrina, Paraná, Brazil were characterized by phenotypic and molecular methods. The *in vitro* antimicrobial susceptibility profile, genetic relatedness and occurrence of virulence genes *icaA* from the intercellular adhesion *locus* (encoding N-acetylglucosaminyl transferase), *hla*, *hlb* and *hld* (encoding α-, β- and δ-hemolysin, respectively), *lukS*-PV and *lukF*-PV (encoding the β-pore-forming Panton-Valentine leukocidin) and *tst* (encoding toxic shock syndrome toxin) were also evaluated.

Methodology

Microorganisms

The University Hospital of Londrina, Paraná, Brazil is a 353-bed tertiary care center that serves the city of Londrina, in addition to several localities in the states of Paraná, São Paulo, and Mato Grosso do Sul. This is the major referral center for the Sistema Único de Saúde (a governmental healthcare assistance program) of northern Paraná. A total of 123 non-duplicate *S. aureus* isolates from patients seen in this hospital between June 2010 and June 2013 were randomly taken from the bacterial collection of the Laboratory of Clinical Microbiology of Universidade Estadual de Londrina (UEL). The isolates were classified according to CDC definitions of healthcare-associated infections [16]. The study protocol was approved by the Ethics Committee (CAAE no. 3346.0.000.268.09/protocol 186/09 CEP-UEL) and all assays were carried out in the Laboratory of Molecular Biology of Microorganisms, Department of Microbiology, UEL. Bacterial isolates were identified to the species level by standard phenotypic methods on the basis of colony morphology, Gram staining, catalase, DNase and mannitol fermentation after growing in Columbia agar base (Oxoid, Basingstoke, UK) supplemented with 5% sheep blood at 37^{o}C for 24 hours. Bacteria were kept at -80^{o}C in tryptone soya broth (TSB, Oxoid) containing 30% glycerol. Species identification was also performed by a polymerase chain reaction (PCR)-based method using specific primers for *coa* gene (encoding coagulase) regions according to Tiwari *et al.* (2008) [17].

Antimicrobial susceptibility pattern

Bacterial isolates were tested for antimicrobial susceptibility to cefoxitin (30 μg), ciprofloxacin (5 μg), clindamycin (2 μg), erythromycin (15 μg), gentamicin (10 μg), linezolide (10 μg), oxacillin (1 μg), penicillin (10 U), rifampicin (5 μg), sulfamethoxazole-trimethoprim (23.75/1.25 μg), teicoplanin (30 μg) and tetracycline (30 μg) using the disk-diffusion assay. Cefoxitin and oxacillin were used to define methicillin-resistant *S. aureus* (MRSA) isolates. The minimal inhibitory concentration (MIC) for vancomycin was determined by the broth-dilution method. MIC was determined as 100% growth inhibition. Both methods were performed and interpreted according to the Clinical Laboratory Standards Institute (CLSI, 2013) [18]. *S. aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 51299 were used as controls.

DNA extraction

A single bacterial colony was transferred to 3 mL TSB and incubated at 37^{o}C for 24 hours. The bacterial pellets obtained after centrifugation at 10,000 x *g* for 5 minutes were washed once with sterile 0.15 M phosphate-buffered saline (PBS), pH 7.2, resuspended in 300 μL sterile solution (10 mM Tris-HCl and 1 mM EDTA, pH 8.0, and 1.0 mg/mL lysozyme). Genomic DNA was extracted following the procedure described by Ausubel *et al.* (1991) [19], and a 2-μL aliquot was used in all amplification reactions.

MRSA typing

The identification of SCC*mec* type of all MRSA isolates was performed by multiplex PCR assay as described by Milheiriço *et al.* (2007) [20]. Non-typeable isolates were designated NT.NCTC10442 (type I), N315 (type II), 85/2082 (type III) and 81/108 (type IV) strains were used as control. The genetic

relatedness of all MRSA was analyzed by repetitive element sequence based-PCR (REP-PCR) using the primer RW3A as described by Del Vecchio *et al.* (1995) [21]. Banding patterns were categorized using the UPGMA algorithm and Jaccard coefficient [22] of the Bionumerics v.4.6 software (Applied Mathematics, Kortrijk, Belgium), with the band tolerance set at 3% and the threshold cutoff value set at 85%.

Detection of virulence factor-encoding genes

The detection of nucleotide sequences corresponding to genes encoding virulence factors of *S. aureus* was performed by PCR. The genes *icaA*, *lukS*-PV and *lukF*-PV and *tst* were amplified as described by Campbell *et al.* (2008) [23]. The genes *hla, hlb* and *hld* were amplified as described by Jarraud *et al.* (2002) [24]. All PCRs were performed in a Veriti 96-well thermal cycler (Applied Biosystems, Waltham, Massachusetts, USA), and reactions without any template DNA were carried out simultaneously as negative control.

Statistical analysis

The categorical variables were compared with the chi-square test or Fisher's exact test using the BioEstat Software version 5.3. *P* values less than 0.05 were considered significant.

Results

Patients, MRSA identification and genotyping

The age of the patients enrolled in this study ranged from three months to 87 years (median of 50 years old), and the majority of them were men (n = 86, 70.0%). The isolates were recovered from various clinical sources as follows: blood (n = 43, 35.0%), tissue fragment (n = 21, 17.1%), general discharge (n = 18, 14.6%), tracheal aspirates (n = 15, 12.2%), central venous catheter line (n = 10, 8.1%), urine (n =7, 5.7%) and general swab (n =9, 7.3%). In 17 patients (13%), MRSA infection was identified less than 48 hours after hospital admission. All isolates harbored the gene *coa* and exhibited resistance to cefoxitin, although seven (5.7%) isolates were classified as susceptible to oxacillin.

Cluster analysis and visual observation of bands generated by REP-PCR typing revealed low relatedness between the isolates. By using a cutoff value of 95% and 85% similarity, a total of 94 and 62 different genotypes were respectively identified among the isolates, indicating their high diversity. To compare the REP-PCR pattern with other features of MRSA, those isolates showing 65% similarity were clustered in the same group, and the analysis of the dendrogram resulted in 17 different genotypes, named A to Q. The groups with similar REP-PCR profiles consisted of 50 (E, 40.7%), 16 (F, 13.0%), 14 (H, 11.4%), 13 (A, 10.6%), 8 (I, 6.5%), 5 (B, 4.1%) and 4 (G, 3.3%) isolates each. Three genotypes (D, M, N,

Table 1. Relationship between phenotypic antimicrobial resistance profile and SCC*mec* types of MRSA isolates.

Group	Antimicrobial resistance profile	SCC*mec* - Number of isolates (%*)					
		Type I	Type II	Type III	Type IV	NT	Total (%#)
I	FOX, P				2		2 (1.7)
II	FOX, OX, P				1		1 (0.8)
III	FOX, OX, P, E				2		2 (1.7)
IV	FOX, OX, P, SXT		1		1	1	3 (2.4)
V	FOX, P, CN, SXT		1				1 (0.8)
VI	FOX, P, CIP, DA, E		3				3 (2.4)
VII	FOX, OX, P, CIP, CN, E	1 (3.6)					1 (0.8)
VIII	FOX, OX, P, CIP, DA, E	3 (10.7)	47				50 (40.7)
IX	FOX, P, CIP, CN, DA, E				1		1 (0.8)
X	FOX, OX, P, CIP, CN, DA, E	22 (78.6)	3		3	2	30 (24.4)
XI	FOX, OX, P, CIP, DA, E, RD		8				8 (6.5)
XII	FOX, OX, P, CIP, DA, E, SXT		1				1 (0.8)
XIII	FOX, OX, P, CIP, DA, E, TE					1	1 (0.8)
XIV	FOX, OX, P, CIP, CN, DA, E, RD	2 (7.1)					2 (1.7)
XV	FOX, OX, P, CIP, CN, DA, E, STX					3	3 (2.4)
XVI	FOX, OX, P, CIP, DA, E, TE, STX			1			1 (0.8)
XVII	FOX, OX, P, CIP, CN, DA, E, TE, STX		2			8	10 (8.1)
XVIII	FOX, OX, P, CIP, DA, E, TE, SXT, RD			1		2	3 (2.4)
	Total (%#)	**28 (22.8)**	**66 (53.7)**	**2 (1.6)**	**10 (8.1)**	**17 (13.8)**	**123 (100)**

*Among the total isolates in each group; #Among all isolates analyzed. FOX, cefoxitin; OX, oxacillin; P, penicillin; E, erythromycin; STX, sulphamethoxazole-trimethoprim; CN, gentamycin; CIP, ciprofloxacin; DA, clindamycin; RD, rifampicin; TE, tetracyclin.

1.6% each) consisted of two isolates each. The other seven (5.6%) isolates had unique banding profiles.

Phenotypic and genotypic characterization of antimicrobial susceptibilities

The *in vitro* susceptibility patterns of MRSA to various antimicrobials are given in Table 1. All isolates were susceptible to teicoplanin and linezolide. Besides being resistant to cefoxitin, all isolates showed resistance to penicillin. MRSA isolates were distributed into four SCC*mec* types, according to the multiplex PCR used in this study, and type II (66/123, 53.7%) was the most frequent, followed by type I (28/123, 22.8%), type IV (10/123, 8.1%) and type III (2/123, 1.6%). Seventeen (13.8%) isolates were classified as NT (Table 1).

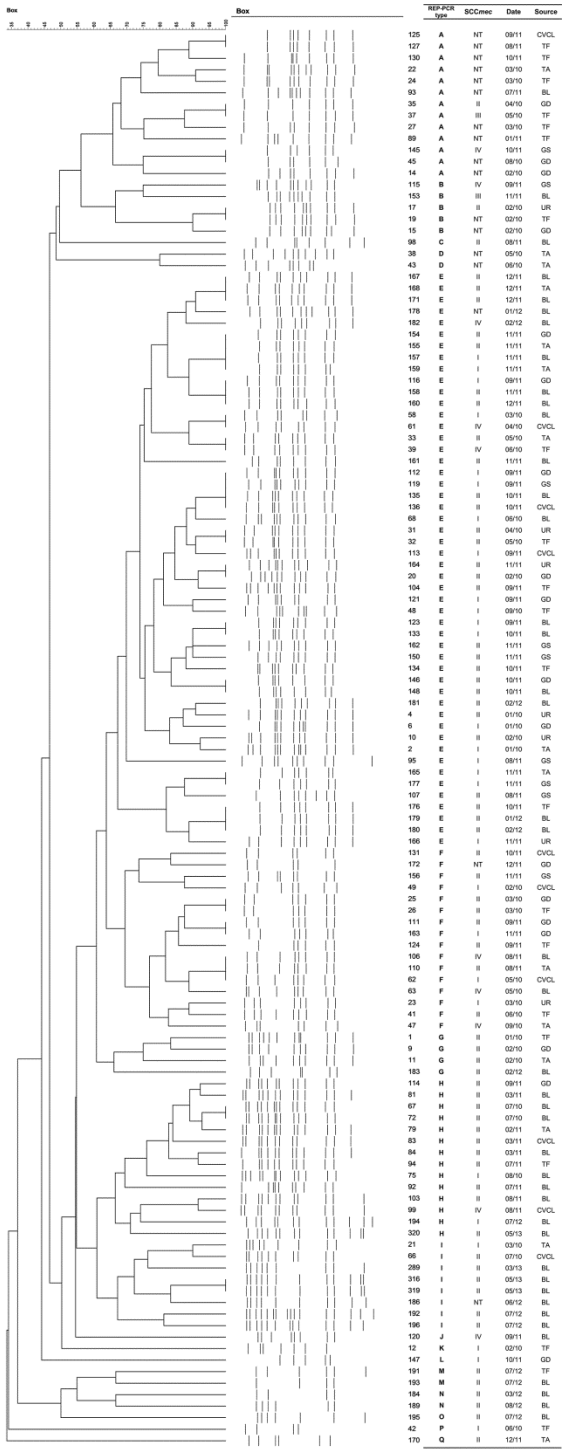
Concerning the phenotypic resistance profile, the MRSA isolates were classified into 18 groups (Table 1). Three isolates were resistant only to β-lactam antimicrobials, and harbored the SCC*mec* type IV. Most isolates (50/123, 40.7%) displayed the group VIII phenotypic antimicrobial resistance profile (cefoxitin, oxacillin, penicillin, ciprofloxacin, clindamycin, and erythromycin) and harbored the SCC*mec* type I (3/50) and type II (47/50). Thirty (24.4%) out of 123 isolates were clustered in group X (cefoxitin, oxacillin, penicillin, ciprofloxacin, gentamycin, clindamycin, and erythromycin) and harbored the SCC*mec* types I (22/30), II (3/30), IV (3/30) and NT (2/30). Most isolates classified as NT were those with resistance to a greater number of antimicrobials.

Most isolates were susceptible to vancomycin (73/123, 59.4%), and among them 1.4% (1/73), 13.7% (10/73) and 84.9% (62/73) had MIC values of 0.25, 1.0 and 2.0 μg/mL, respectively. For all isolates, the MIC50 and MIC90 values were 2.0 and 4.0 μg/mL, respectively. Fifty (40.7%) isolates out of 123 isolates were intermediately resistant to vancomycin, according to CLSI (2013) criteria. Among these, 92% (46/50) and 8% (4/50) of isolates showed MIC values of 4 and 8 μg/mL, respectively. However, patients with intermediate MIC values did not have higher in-hospital mortality than those with susceptible MIC values.

Relationship between SCCmec types and REP-PCR genotyping

Eighteen MRSA SCC*mec* type I (18/28, 64.3%) isolates were grouped in genotype E, and four (14.3%) and two (7.1%) in genotypes F and H, respectively. One isolate each (3.6%) was grouped in genotypes I,

Figure 1. REP-PCR fingerprinting pattern and SSC*mec* types obtained from 123 methicillin-resistant *Staphylococcus aureus* isolates from different sources of patients seen at the University Hospital of Londrina.



Cluster analysis was performed using UPGMA algorithm and Jaccard coefficient of the Bionumerics v. 4.6 software, with band tolerance set at 3% and threshold cutoff value set at 85%. SCC*mec* typing was performed by multiplex PCR assay as described by Milheiriço *et al.* (2007). All *S. aureus* isolates were resistant to cefoxitin. NT: non-typeable; BL: blood; TF: tissue fragment; GD: general discharge; TA: tracheal aspirates; CVCL: central venous catheter line; UR: urine; GS: general swab.

K, L, and P. The SCC*mec* type II isolates were mainly distributed into genotype E (28/66, 42.4%), followed by genotype H (11/66, 16.7%), F (8/66, 12.1%), I (6/66, 9.1%) and G (4/66, 6.0%). Two isolates each (3.0%) were grouped in genotypes M and N, and one isolate each (1.5%) in genotypes A, B, C, O and Q. One isolate each of SCC*mec* type III displayed the genotypes A and B. Three isolates each (30%) of SCC*mec* type IV were grouped in genotypes E and F, and one isolate each (10%) in genotypes A, B, H and J. Most NT-SCC*mec* (10/17, 58.8%) isolates were grouped in genotype A, and the other isolates were distributed into genotypes B (2/17, 11.8%), D (2/17, 11.8%), E (1/17, 5.9%), F (1/17, 5.9%) and I (1/17, 5.9%) (Figure 1).

Detection of virulence factor-encoding genes

The presence of the genes *coa*, *icaA*, *hla*, *hlb*, *hld*, *lukS*-PV, *lukF*-PV and *tst* in MRSA isolates was detected by PCR. The overall prevalence was as follows: *coa*, 100%; *icaA*, 100%; *hla*, 13.0%; *hlb*, 91.1%; *hld*, 91.1%; *lukS*-PV and *lukF*-PV, 2.4%; and *tst*, 34.1%, and no significant association with the origin of the isolates was observed (Table 2). All isolates harbored at least two virulence markers (Table 3), and a significant association with the presence of *hla* gene and the SCC*mec* type II ($p < 0.005$), and *tst* and SCC*mec* type I ($p < 0.001$) was observed. Although no statistical correlation was found, it was also observed that: a) most isolates (59/123, 48.0%) harbored the combination of *coa*, *ica*A, *hlb* and *hld* genes, which were distributed into SCC*mec* types II (46/66, 69.7%), III (2/2, 100%), IV (1/10, 10%) and NT (10/17, 58.8%); b) isolates with the gene *hla* harbored the SCC*mec* types I, II, IV and NT; c) the presence of PVL-encoding genes was detected only in isolates belonging to SCC*mec* type IV. A larger number of isolates may corroborate these findings.

Discussion

In this study, MRSA isolates harboring four SCC*mec* types (I, II, III and IV) were identified. The predominance of one specific lineage or clonal group (as assigned by REP-PCR fingerprinting) was not

Table 2. Distribution of virulence encoded genes according to the origin of 123 methicillin-resistant *Staphylococcus aureus* isolates from hospitalized patients of University Hospital of Londrina.

Clinical source	Number of isolates harboring the virulence encoding gene (%)						
	coa*	***icaA****	***hla****	***hlb****	***hld****	***tst****	**PVL***
Blood (n=43)	43 (100)	43 (100)	5 (11.6)	33 (76.7)	33 (76.7)	14 (32.6)	1 (2.3)
Tissue fragment (n=21)	21 (100)	21 (100)	3 (14.3)	20 (95.2)	20 (95.2)	4 (19)	-
General discharge (n=18)	18 (100)	18 (100)	3 (16.7)	18 (100)	18 (100)	7 (38.9)	-
Tracheal aspirates (n=15)	15 (100)	15 (100)	-	15 (100)	15 (100)	6 (40)	-
Venous central catheter line (n=10)	10 (100)	10 (100)	2 (20)	10 (100)	10 (100)	5 (50)	-
Urine (n=7)	7 (100)	7 (100)	2 (28.6)	7 (100)	7 (100)	1 (14.3)	-
General swab (n=9)	9 (100)	9 (100)	1 (11.1)	9 (100)	9 (100)	5 (55.6)	2 (22.2)
Total (%[#])	**123 (100)**	**123 (100)**	**16 (13)**	**112 (91.1)**	**112 (91.1)**	**42 (34.1)**	**3 (2.4)**

*Among the total isolates in each group; [#]Among all isolates analyzed. *coa*: coagulase; *icaA*: intercellular adhesion *locus*encoding N-acetylglucosaminyltransferase; *hla*: α-hemolysin, *hlb*: β-hemolysisn; *hld*: δ-hemolysin; *tst:* toxic shock syndrome toxin; PVL: *luk*S-PV and *luk*F-PV of the β-pore-forming Panton-Valentine leukocidin. -: absence

Table 3. Methicillin-resistant *Staphylococcus aureus* isolates harboring clusters of virulence encoded genes according to their SCC*mec* typing.

Virulence markers[¥]	Number of isolates (%*)					
	Type I	**Type II**	**Type III**	**Type IV**	**NT**	**Total (%[#])**
coa, *ica*A		10 (15.1)				10 (8.1)
coa, *ica*A, *hla*, *tst*	1 (3.6)					1 (0.8)
coa, *ica*A, *hlb*, *hld*		46 (69.7)	2 (100)	1 (10)	10 (58.8)	59 (48.0)
coa, *ica*A, *hlb*, *hld*, PVL				3 (30)		3 (2.4)
coa, *ica*A, *hla*, *hlb*, *hld*	1 (3.6)	3 (4.5)		1 (10)	4 (23.5)	9 (7.3)
coa, *ica*A, *hlb*, *hld*, *tst*	21 (75.0)	7 (10.7)		5 (50)	2 (11.8)	35 (28.5)
coa, *ica*A, *hla*, *hlb*, *hld*, *tst*	5 (17.8)				1 (5.9)	6 (4.9)
Total (%[#])	**28 (22.8)**	**66 (53.7)**	**2 (1.6)**	**10 (8.1)**	**17 (13.8)**	**123 (100)**

*Among the total isolates in each group; [#]Among all isolates analyzed.[¥]*coa*: coagulase; *icaA*: intercellular adhesion *locus*encoding N-acetylglucosaminyltransferase; *hla*: α-hemolysin, *hlb*: β-hemolysisn; *hld*: δ-hemolysin; *tst:* toxic shock syndrome toxin; PVL: *luk*S-PV and *luk*F-PV of the β-pore-forming Panton-Valentine leucocidin

observed, indicating that many different strains circulated in the hospital during the period analyzed here. These SCC*mec* types were also detected in MRSA surveys conducted in different Brazilian hospitals. However, the prevalence of SCC*mec* types varied between different regions [3,25-31]. The Brazilian epidemic clone (BEC) carrying the SCC*mec* type III has been the predominant clone in several hospitals in Brazil [28,29,32]. However, in this study, a prevalence of SCC*mec* type II was observed, as in Caiaffa-Filho *et al.* (2013) [30], who detected a high prevalence of this SCC*mec* type in a tertiary care teaching hospital in São Paulo, Brazil.

Not surprisingly, most MRSA strains harboring the SCC*mec* types I, II and III were resistant to more than three other non-β-lactam antimicrobial classes. Among SCC*mec* type IV isolates, most were susceptible to almost all non-β-lactam antimicrobials, which is a common feature of these strains. However, four isolates harboring SCC*mec* type IV showed resistance to more than three antimicrobial classes. This study also detected NT-SCC*mec* isolates, and except for one, all displayed resistance to a greater number of antimicrobial classes. Of note was a high proportion of MRSA strains showing intermediate resistance to vancomycin, one of the last therapeutic choices for the treatment of invasive infections. In fact, glycopeptide treatment failure and poor clinical outcomes have been reported in infections caused by vancomycin-intermediate *S. aureus* [33].

Besides being resistant to many antimicrobial agents, *S. aureus* possesses a number of virulence determinants, which makes it highly adaptive and versatile. The expression of the virulence factor-encoding genes seems likely to be dependent on the site of infection [34,35]. In this study, the genes *coa*, *icaA*, *hlb* and *hld* were highly conserved among MRSA isolates, independently from the clinical origin. Coagulase plays a role in intravascular coagulation, facilitating the aggregation of *S. aureus* in blood, which in turn promotes bacterial survival [35]. The gene *icaA* encodes the transmembrane protein *N*-acetylglucosaminyl transferase, which is involved in the biosynthesis of polysaccharide intercellular adhesin (PIA). This adhesin promotes cellular aggregation during the maturation stage of PIA-dependent biofilm formation by *S. aureus* [36]. The gene *hld* encodes the δ-Hemolysin (Hld or δ-toxin), a cytotoxic protein of phenol-soluble modulins family [37] that can also contribute in structuring and detachment stages of biofilm development [38]. The gene *hlb* encodes a magnesium-dependent sphingomyelin-specific phospholipase called β-hemolysin (Hlb), which can lyse erythrocytes, neutrophils and lymphocytes [39].

The pore-forming hemolysin-α (Hla or α-toxin), encoded by *hla* gene is virtually produced by all strains [34]. However, this gene was detected in only 13% of the isolates in this study, and its presence was strongly associated with the SCC*mec* type II-harboring isolates. The mechanism of cell lysis mediated by Hla is dependent on the initial interaction with a specific receptor on host membranes, often targeting a particular cell type [40]. It can damage epithelial cells, fibroblast, erythrocytes, lymphocytes, monocytes and macrophages, but not neutrophils [41]. The expression of Hla contributes to the pathogenesis of sepsis, skin infections and pneumonia in murine experimental infection [35,40,42]. Furthermore, the role of this exotoxin during biofilm formation *in vitro* and in a mucosal model of *S. aureus* infection has been shown elsewhere [43,44].

It has been previously shown that SCC*mec* type IV harboring the gene encoding the bi-component PVL was characteristic of community-acquired MRSA, which is a highly virulent strain [26,31]. However, PVL has also been detected in other SCC*mec* strains [45]. This pore-forming toxin, whose genes *lukF*-PV and *lukS*-PV are lysogenic bacteriophage-encoded (MGE), can lyse macrophages, monocytes and neutrophils and cause tissue necrosis [46]. PVL has been associated with *S. aureus* skin and soft tissue infections, and necrotizing pneumonia and septic shock [35,47]. In this study, the PVL-encoding genes were detected in three SCC*mec* type IV-harboring isolates, and except for one that showed resistance to erythromycin, the other two isolates were susceptible to all non-β-lactam antimicrobials analyzed here. In addition, in two patients who were outpatients, MRSA infection was identified less than 48 hours after hospital admission, and none was using medical devices during the time of cultures. Altogether, these data indicate that these two cases may be epidemiologically characterized as community-associated MRSA infection [10].

The *tst* gene, which encodes the pyrogenic toxic shock-syndrome toxin 1 (TSST-1), is present in only a small number of *S. aureus* strains, and this can be explained by its genomic location in a pathogenicity island (MGE). In this study, a low prevalence of *tst* gene was also observed among MRSA isolates, and its presence was associated with SCC*mec* type I. TSST-1, a non-cytolytic toxin, is a member of superantigen (SAgs) family, which induces a potent activation of T

cell and macrophages with large production of cytokines that can interfere with the host immune responses. In addition, the expression of *tst* or other SAgs has been associated with enhanced susceptibility to endotoxic shock syndrome development, with high mortality rates [48].

Limitations of this study, which may reduce generalization of the results, are a) the number of isolates of each clinical source; b) the evaluation of a single hospital; and c) the presence or absence of a particular virulence factor-encoding gene being evaluated only by PCR.

Conclusion

This study showed the high virulence potential of multidrug-resistant MRSA circulating in our hospital. Besides, a high prevalence of MRSA showing intermediate vancomycin resistance was observed. These results corroborate the importance of continuous monitoring of antimicrobial susceptibility profiles and potential virulence of *S. aureus*. In addition, they will contribute to improving strategies for controlling the use of antimicrobials for appropriate management of infections caused by this bacterium.

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References

1. Kluytmans J, van Belkum A, Verbrugh H (1997) Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. Clin Microbiol Rev 10: 505-520.
2. Prates KA, Torres AM, Garcia LB, Ogatta SF, Cardoso CL, Tognim MC (2010) Nasal carriage of methicillin-resistant *Staphylococcus aureus* in university students. Braz J Infect Dis 14: 316-318.
3. Pacheco RL, Lobo RD, Oliveira MS, Farina EF, Santos CR, Costa SF, Padoveze MC, Garcia CP, Trindade PA, Quitério LM, Rivitti EA, Mamizuka EM, Levin AS (2011) Methicillin-resistant *Staphylococcus aureus* (MRSA) carriage in a dermatology unit. Clinics (São Paulo) 66: 2071-2077.
4. Lowy FD (1998) *Staphylococcus aureus* infections. N Engl J Med 339: 520-532.
5. Carvalho KS, Mamizuka EM, Gontijo Filho PP (2010). Methicillin/Oxacillin-resistant *Staphylococcus aureus* as a hospital and public health threat in Brazil. Braz J Infect Dis 14: 71-76.
6. Sievert DM, Ricks P, Edwards JR, Schneider A, Patel J, Srinivasan A, Kallen A, Limbago B, Fridkin S, National Healthcare Safety Network (NHSN) Team and Participating NHSN Facilities (2013) Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009-2010. Infect Control Hosp Epidemiol 34: 1-14.
7. IWG-SCC (2009): international working group on the classification of staphylococcal cassette chromosome elements. Classification of Staphylococcal Cassette Chromosome *mec* (SCC*mec*): guidelines for reporting novel SCC*mec* elements. Antimicrob Agents Chemother 53: 4961-4967.
8. Shore AC, Deasy EC, Slickers P, Brennan G, O'Connell B, Monecke S, Ehricht R, Coleman DC (2011) Detection of staphylococcal cassette chromosome *mec* type XI carrying highly divergent *mecA*, *mecI*, *mecR1*, *blaZ* and *ccr* genes in human clinical isolates of clonal complex 130 methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 55: 3765-3773.
9. Hiramatsu K, Ito T, Tsubakishita S, Sasaki T, Takeuchi F, Morimoto Y, Katayama Y, Matsuo M, Kuwahara-Arai K, Hishinuma T, Baba T (2013) Genomic basis for methicillin resistance in *Staphylococcus aureus*. Infect Chemother 45: 117-136.
10. Figueiredo AM, Ferreira FA (2014) The multifaceted resources and microevolution of the successful human and animal pathogen methicillin-resistant *Staphylococcus aureus*. Mem Inst Oswaldo Cruz 109: 265-278.
11. Lindsay JA (2014) *Staphylococcus aureus* genomics and the impact of horizontal gene transfer. Int J Med Microbiol 304: 103-109.
12. Watkins RR, David MZ, Salata RA (2012) Current concepts on the virulence mechanisms of methicillin-resistant *Staphylococcus aureus*. J Med Microbiol 61: 1179-1193.
13. Li M, Cheung GY, Hu J, Wang D, Joo HS, Deleo FR, Otto M (2010) Comparative analysis of virulence and toxin expression of global community-associated methicillin-resistant *Staphylococcus aureus* strains. J Infect Dis 202: 1866-76.

14. Wu K, Zhang K, McClure J, Zhang J, Schrenzel J, Francois P, Harbarth S, Conly J (2013) A correlative analysis of epidemiologic and molecular characteristics of methicillin-resistant *Staphylococcus aureus* clones from diverse geographic locations with virulence measured by a *Caenorhabditis elegans* host model. Eur J Clin Microbiol Infect Dis 32: 33-42.
15. Koyama N, Inokoshi J, Tomoda H (2012) Anti-infectious agents against MRSA. Molecules 18: 204-224.
16. Horan TC, Andrus M, Mudeck MA (2008) CDC/NHSN surveillance definition of health care-associated infection and criteria for specific types of infections in the acute care setting. Am J Infect Control 36: 309-332.
17. Tiwari HK, Sapkota D, Sem MR (2008) Evaluation of different tests for detection of *Staphylococcus aureus* using coagulase (*coa*) gene PCR as the gold standard. Nepal Med Coll J 10: 129-131.
18. CLSI (2013) Clinical and Laboratory Standards Institute. M100-S22, v. 32, n. 3; M100-S21, v. 31, n. 1, 2013.
19. Ausubel FM, Kingston RE, Brent R, Moore DD, Seidman JG, Smith JA, Struhl K (1991) Current protocols in molecular biology. Greene Publishing Associates & Wiley Interscience: New York.
20. Milheiriço C, Oliveira DC, de Lencastre H (2007) Update to the multiplex PCR strategy for assignment of *mec* element types in *Staphylococcus aureus*. Antimicrob Agents Chenother 51: 3374-3377.
21. Del Vecchio VG, Petroziello JM, Gress MJ, McCleskey FK, Melcher GP, Crouch HK, Lupski JR (1995) Molecular genotyping of methicillin-resistant *Staphylococcus aureus* via fluorophore-enhanced repetitive-sequence PCR. J Clin Microbiol 33: 2141-2144.
22. Sneath PHA, Sokal RR (1973) Numerical Taxonomy. Freeman, San Francisco 573 p.
23. Campbell SJ, Deshmukh HS, Nelson CL, Bae IG, Stryjewski ME, Federspiel JJ, Tonthat GT, Rude TH, Barriere SL, Corey R, Fowler VG Jr (2008) Genotypic characteristics of *Staphylococcus aureus*isolates from a multinational trial of complicated skin and skin structure infections. J Clin Microbiol 46: 678-684.
24. Jarraud s, Mougel C, Thioulouse J, Lina G, Meugnier H, Forey F, Nesme X, Etienne J, Vandenesch F (2002) Relationships between *Staphylococcus aureus* genetic background, virulence factors, agr groups (alleles), and human disease. Infect Immun 70: 631-641.
25. Trindade A, Pacheco RL, Costa SF, Rossi F, Barone AA, Mamizuka EM, Levin AS (2005) Prevalence of SCC*mec* type IV in nosocomial bloodstream isolates of methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol 43: 3435-3437.
26. Ribeiro A, Coronado AZ, Silva-Carvalho MC, Ferreira-Carvalho BT, Dias C, Rozenbaum R, Del Peloso PF, da Costa Ferreira Leite C, Teixeira LA, Figueiredo AM (2007) Detection and characterization of international community-acquired infections by methicillin-resistant *Staphylococcus aureus* clones in Rio de Janeiro and Porto Alegre cities causing both community and hospital-associated diseases. Diagn Microbiol Infect Dis 59: 339-345.
27. Scribel LV, Silva-Carvalho MC, Souza RR, Superti SV, Kvitko CH, Figueiredo AM, Zavascki AP (2009) Clinical and molecular epidemiology of methicillin-resistant *Staphylococcus aureus* carrying SCC*mec*IV in a university hospital in Porto Alegre, Brazil. Diagn Microbiol Infect Dis 65: 457-461.
28. Becker AP, Santos O, Castrucci FM, Dias C, D'Azevedo PA (2012) First report of methicillin-resistant *Staphylococcus aureus* Cordobes/Chilean clone involved in nosocomial infections in Brazil. Epidemiol Infect 140: 1372-1375.
29. Caboclo RM, Cavalcante FS, Iorio NL, Schuenck RP, lendzki AN, Felix MJ, Chamon RC, dos Santos KR (2013) Methicillin-resistant *Staphylococcus aureus* in Rio de Janeiro hospitals: dissemination of the USA400/ST1 and USA800/ST5 SCC*mec* type IV and USA100/ST5 SCC*mec* type II lineages in a public institution and polyclonal presence in a private one. Am J Infect Control 41: 21-26.
30. Caiaffa-Filho HH, Trindade PA, Gabriela da Cunha P, Alencar CS, Prado GV, Rossi F, Levin AS (2013) Methicillin-resistant *Staphylococcus aureus* carrying SCC*mec* type II was more frequent than the Brazilian endemic clone as a cause of nosocomial bacteremia. Diagn Microbiol Infect Dis 76: 518-520.
31. Gelatti LC, Bonamigo RR, Inoue FM, Carmo MS, Becker AP, Castrucci FM, Pignatari AC, D' Azevedo PA (2013) Community-acquired methicillin-resistant *Staphylococcus aureus* carrying SCC*mec* type IV in southern Brazil. Rev Soc Bras Med Trop 46: 34-38.
32. Rodrigues MV, Fortaleza CM, Riboli DF, Rocha RS, Rocha C, da Cunha ML (2013) Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in a burn unit from Brazil. Burns 39: 1242-1249.
33. Howden BP, Ward PB, Charles PG, Korman TM, Fuller A, du Cros P, Grabsch EA, Roberts SA, Robson J, Read K, Bak N, Hurley J, Johnson PD, Morris AJ, Mayall BC, Grayson ML (2004) Treatment outcomes for serious infections caused by methicillin-resistant *Staphylococcus aureus* with reduced vancomycin susceptibility. Clin Infect Dis 38: 521-528.
34. Grumann D, Nübel U, Bröker BM (2014) *Staphylococcus aureus* toxins-their functions and genetics. Infect Genet Evol 21: 583-592.
35. Powers ME, Bubeck WJ (2014) Igniting the fire: *Staphylococcus aureus* virulence factors in the pathogenesis of sepsis. PLoS Pathog 10: e1003871.
36. Cramton SE, Gerke C, Schnell NF, Nichols WW, Götz F (1999) The intercellular adhesion (*ica*) locus is present in *Staphylococcus aureus* and is required for biofilm formation. Infect Immun 67: 5427-5433.
37. Wang R, Braughton KR, Kretschmer D, Bach TH, Queck SY, Li M, Kennedy AD, Dorward DW, Klebanoff SJ, Peschel A, DeLeo FR, Otto M (2007) Indentification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. Nat Med 13: 1510-1514.
38. Periasamy S, Joo HS, Duong AC, Bach TH, Tan VY, Chatterjee SS, Cheung GY, Otto M (2012) How *Staphylococcus aureus* biofilms develop their characteristics structure. Proc Natl Acad Sci USA 109: 1281-1286.
39. Marshall MJ, Bohach GA, Boehm DF (2000) Characterization of *Staphylococcus aureus* beta-toxin induced leukotoxicity. J Nat Toxins 9: 125-138.
40. Inoshima I, Inoshima N, Wilke GA, Powers ME, Frank KM, Wang Y, Bubeck WJ (2011) A *Staphylococcus aureus* pore-forming toxin subverts the activity of ADAM10 to cause lethal infection in mice. Nat Med 17: 1310-1314.
41. Valeva A, Walev I, Pinkernell M, Walker B, Bayley H, Palmer M, Bhakdi S (1997) Transmembrane beta-barrel of staphylococcal alpha-toxin forms in sensitive but not in resistant cells. Proc Natl Acad Sci USA 94: 11607-11611.

42. Kennedy AD, Bubeck Wardenburg J, Gardner DJ, Long D, Whitney AR, Braughton KR, Schneewind O, DeLeo FR (2010) Targeting of alpha-hemolysin by active or passive immunization decreases severity of USA300 skin infection in a mouse model. J Infect Dis 202: 1050-1058.
43. Caiazza NC, O'Toole GA (2003) Alpha-toxin is required for biofilm formation by *Staphylococcus aureus*. J Bacteriol 185: 3214-3217.
44. Anderson MJ, Lin Y-C, Gillman AN, Parks PJ, Schlievert PM, Peterson ML (2012) Alpha-Toxin Promotes *Staphylococcus aureus* Mucosal Biofilm Formation. Front Cell Infect Microbiol 2: 64.
45. Machuca MA, Sosa LM, González CI (2013) Molecular Typing and Virulence Characteristic of Methicillin-Resistant *Staphylococcus aureus* Isolates from Pediatric Patients in Bucaramanga, Colombia. PLoS One 8: e73434.
46. Kaneko J, Kamio Y (2004) Bacterial two-component and hetero-heptameric pore-forming cytolytic toxins: structures, pore-forming mechanism, and organization of the genes. Biosci Biotechnol Biochem 68: 981-1003.
47. Elisabeth P, Maria S, Irene G, Helen G, Apostolos A (2014) Success stories about evere pneumonia caused by Panton-Valentine leucocidina-producing *Staphylococcus aureus*. Braz J infect Dis 18: 341-345.
48. Novick RP (2003) Mobile genetic elements and bacterial toxinoses: the superantigen-encoding pathogenicity islands of *Staphylococcus aureus*. Plasmid 49: 93-105.

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