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Major Article

Differences in resistance profiles and virulence genes among methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* of different lineages at a public tertiary hospital

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Abstract

Introduction: *Staphylococcus aureus* is a major nosocomial pathogen that is associated with high virulence and the rapid development of drug resistance. **Methods**: We analyzed and compared the antimicrobial resistance, virulence profiles, and molecular epidemiology of 67 *S. aureus* strains, including 36 methicillin-sensitive (MSSA) and 31 methicillin-resistant (MRSA) strains recovered from a public hospital located in south-eastern Brazil. **Results**: The clones circulating in this hospital presented a great diversity, and the majority of the strains were related to clones responsible for causing worldwide epidemics: these included USA100 (New York/Japan clone), USA300, and USA600. The 31 MRSA (22 SCC*mec*II and 9 SCC*mec*IV) and 36 MSSA strains exhibited low resistance against gentamicin and trimethoprim/sulfamethoxazole. No MRSA strain showed resistance to tetracycline. Virulence gene carriage was more diverse and abundant in MSSA than in MRSA. Of the evaluated adhesion-related genes, *ebp*S was the most prevalent in both MSSA and MRSA strains. The genes *bbp* and *cna* showed a strong association with MSSA strains. **Conclusions**: Our findings reinforce the idea that MSSA and MRSA strains should be carefully monitored, owing to their high pathogenic potential.

Keywords: *Staphylococcus aureus*. Epidemiology. SCC*mec*. Resistance. Virulence.

INTRODUCTION

Staphylococcus aureus is a major nosocomial pathogen associated with high virulence and rapid drug resistance development worldwide[1]. The prevalence of methicillin-resistant *S. aureus* (MRSA) has necessitated the implementation of specific therapeutic and expensive prevention measures in hospital settings[2]. However, a recent study revealed the presence of a broad spectrum of virulence genes in the genomes of methicillin-susceptible *S. aureus* (MSSA) strains that could act as a potential source of infection. Thus, MSSA should be given the same attention as MRSA strains[3].

A study performed by Jiménez *et al*.[4] using *S. aureus* strains isolated from a pediatric population showed that MSSA lineages harbor a lot more virulence genes as compared to MRSA, and this difference was thought to be related to the fitness cost associated with methicillin resistance. The relationship between virulence and resistance was also noted by Seidl *et al*.[5], wherein the authors showed that the intrinsic virulence of MRSA strains is similar, or even less than that of MSSA, and that the increase in virulence is associated with the decrease in methicillin resistance levels.

The dynamics of the prevalence of *S. aureus* clones, including MRSA and MSSA strains, have been recently investigated[6]. As a consequence, the changes in the epidemiological overview

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have been observed worldwide, revealing the emergence of new clones replacing the previously established ones[7,8]. As monitoring resistance and virulence profiles is important to establish control strategies, here we aimed to analyze and compare the antimicrobial resistance patterns, virulence profiles, and molecular epidemiology of *S. aureus* strains isolated from a public hospital located in south-eastern Brazil.

METHODS

Bacterial strains, settings, and ethic statement

We evaluated 67 *S. aureus* strains isolated from healthcare-associated infections (31 MRSA and 36 MSSA) obtained from various clinical sources, including blood (35), surgical wounds (18), catheters (8), urine (3), ascitic fluid (2), and tissue fragments (1), of various patients who presented *S. aureus* infections at the University Hospital Cassiano Antônio de Moraes (HUCAM) between April 2011 and February 2012. HUCAM is a tertiary-care teaching hospital affiliated to the Federal University of Espírito Santo, Vitória city, Brazil; it is considered to be the largest hospital in the public health network of Espírito Santo, considering the high volume of services, especially those with high complexity.

The strains were identified as *S. aureus* using the MicroScan® system (Siemens Healthcare Diagnostics Inc., USA). Bacteria were stored in brain heart infusion medium (Merck, Germany) with 20% glycerol at -20°C. The present research was approved by the Human Research Ethics Committee of the Federal University of Espírito Santo under number 247/2011.

Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) for oxacillin and vancomycin were determined by the Etest® method (BioMérieux, France). All strains were tested for antibiotic resistance with the disk diffusion method, as per the guidelines recommended by the Clinical and Laboratory Standard Institute (CLSI)[9]. Resistance was evaluated using the following antibiotics (Oxoid, United Kingdom): erythromycin (15 µg), ciprofloxacin (5 µg), norfloxacin (10 µg), clindamycin (2 µg), rifampicin (5 µg), chloramphenicol (30 µg), gentamicin (10 µg), trimethoprim/sulfamethoxazole (23/75 µg), tetracycline (30 µg), and linezolid (30 µg). *S. aureus* ATCC 25923 was used as the control strain.

DNA isolation and SCC*mec* typing

Genomic DNA from *S. aureus* was extracted following the method described by Schuenck *et al*.[8], and used as a template for polymerase chain reaction (PCR). The expression of the gene *mecA* was evaluated in all the 67 strains included in the study, and SCC*mec* typing was performed for the samples deemed positive for *mecA*[10].

Molecular typing

Pulsed-field gel electrophoresis (PFGE) was performed for all strains after the analysis of genomic DNA macrorestriction with *Sma*I enzyme in a CHEF-DRIII system (Bio-Rad, USA), as previously described[11]. Band patterns were analyzed with BioNumerics v6.5 (Applied Maths, Belgium) using the Unweighted Pair Group Method (UPGMA) with the arithmetic mean based on Dice coefficients. Strains were considered to belong to the same pulsotype upon sharing at least 80% similarity in the banding patterns or same subtype upon showing identical banding patterns. The clonality of the strains was obtained by comparison with a previously published research[12].

One strain of each pulsotype of MRSA and two strains of two main pulsotypes of MSSA were further characterized using multi-locus sequence typing (MLST) with internal fragments of seven housekeeping genes (*arcC*, *aroE*, *glpF*, *gmK*, *pta*, *tpi*, and *yqiL*) amplified using specific primers as per the recommendations described in *S. aureus* MLST database (http://saureus.mlst.net/). All fragments amplified were purified using the Wizard SV gel and PCR Clean-up System (Promega, EUA) and sequenced using an ABI PRISM® 3130XL Genetic Analyzer (Applied Biosystems, EUA). An allelic number corresponding to a sequence that was already present in the database was assigned to each sequenced housekeeping gene. Sequence types (ST) and clonal complexes (CC) were assigned according to their allelic profiles.

Detection of virulence genes

The presence of five adhesin genes, namely, *cna* (collagen-binding protein), *bbp* (bone sialo-binding protein), *ebpS* (elastin-binding protein), *fnbA* (fibronectin-binding protein A), and *fnbB* (fibronectin-binding protein B) was evaluated with PCR. The detection of *cna*, *bbp*, *ebpS*, and *fnbB* was performed according to the methods described by Tristan *et al*.[13], while *fnbA* was detected as per the method described by Peacock *et al*.[14] *lukS/F* genes encoding Panton-Valentine leukocidin (PVL) were also investigated[15].

Statistical analysis

All statistical analyses were performed with the chi-square and Fisher's test using the BioEstat® software 5.3 version (Mamiraua, Brazil). The significance level was set at 0.05.

RESULTS

Antimicrobial susceptibility and SCC*mec* typing

Thirty-one (46%) *S. aureus* strains were found to be resistant (MIC_{50}: 128 µg/mL; MIC_{90}: 256 µg/mL) to oxacillin and 36 (54%) were found to be susceptible to oxacillin (MIC_{90}: 0.5 µg/mL). All strains showed susceptibility to vancomycin with an MIC_{90} value of 1 µg/mL.

The MRSA group included 22 SCC*mec* type II and nine SCC*mec* type IV strains. Most of the strains were susceptible to gentamicin (97% of strains from both groups) and trimethoprim/sulfamethoxazole (92% of MSSA strains and 100% of MRSA strains) (**Table 1**). The percentage of MSSA strains resistant to tetracycline was significantly higher than the percentage of MRSA strains resistant to tetracycline (28.0% versus 0%; P = 0.0001). All 22 MRSA SCC*mec* type II strains were resistant to ciprofloxacin and norfloxacin, while MRSA SCC*mec* type IV strains showed a significantly reduced resistance to these antibiotics (P = 0.0007). Furthermore, 17 (77.3%) MRSA SCC*mec* type II strains showed resistance to rifampicin, and

TABLE 1: Resistance profile of 67 *Staphylococcus aureus* strains.

Antibiotic	No. (%) of resistant strains		
	SCC*mec* II (n = 22)	SCC*mec* IV (n = 09)	MSSA (n = 36)
Erythromycin	22 (100)	08 (89)	07 (19)
Ciprofloxacin	22 (100)	04 (44)	03 (8)
Norfloxacin	22 (100)	04 (44)	03 (8)
Clindamycin	22 (100)	01 (11)	03 (8)
Rifampicin	17 (77)	0	0
Chloramphenicol	05 (23)	01 (11)	01 (3)
Gentamicin	0	01 (11)	01 (3)
Trimethoprim/Sulfamethoxazole	0	0	03 (8)
Tetracycline	0	0	10 (28)
Linezolid	0	0	0

significantly differed from the other groups, which were sensitive to this antibiotic (P = 0.00001).

Molecular typing of *S. aureus* strains

Based on the results of PFGE, we grouped the 67 strains into 16 pulsotypes (A to Q), and 31 MRSA strains were classified into five pulsotypes (A to E) and 12 subtypes (**Table 2**). Pulsotype A (n = 22) comprised all MRSA SCC*mec* type II strains and was distributed into five different subtypes belonging to ST5 (similar to the USA100/New York-Japan clone). The other MRSA strains (n = 9) were presented as type SCC*mec* type IV with four different pulsotypes (B-D). ST8 was described in pulsotype B (n = 4), which presented a PFGE pattern similar to that of the USA300 clone. Pulsotypes C and D were categorized into ST5 and pulsotype E, similar to the USA600 clone, and were presented as ST45.

The 36 MSSA strains showed high genetic variability and were distributed in 11 pulsotypes (F–Q). One strain of the two predominant pulsotypes (F and G) was selected for MLST analysis. The strain ST1635 (ST5-related) in the pulsotype F and ST30 in the pulsotype G corresponded to the main MSSA genotypes identified in the present study (**Table 3**).

Virulence genes and *S. aureus* lineages

The most prevalent virulence genes identified in the 67 strains were *ebpS* (82%) and *fnbA* (51%). We failed to observe differences in the distribution of both genes between MRSA and MSSA strains (P = 0.5 and P = 0.1, respectively) (**Figure 1**). However, MSSA strains harboring the adhesin genes *cna* (47% versus 3%) and *bbp* (31% versus 0%) (P = 0.0002 and P = 0.0024, respectively) were detected. The gene *fnbB* encoding fibronectin-binding protein was not detected in SCC*mec* type II strains but was highly prevalent in SCC*mec* type IV strains (44%, **Table 2**). Moreover, PVL-encoding genes were detected in 11 strains, including one MRSA SCC*mec* type II, four MRSA SCC*mec* type IV, and six MSSA.

TABLE 2: Virulence profile and epidemiologic characteristics of methicillin-resistant *Staphylococcus aureus* strains.

ST	CC	Pulsotype/Clonality	No. of subtypes	SCC*mec* type	Virulence profile						No. of strains
					cna	*bbp*	*ebpS*	*fnbA*	*fnbB*	*lukS/F*	
5	5	A/USA100 (NY/J)	5	II	-	-	+	-	-	-	13
					-	-	+	+	-	-	8
					-	-	+	-	-	+	1
8	8	B/USA300	3	IV	-	-	-	+	+	+	3
					-	-	-	-	+	+	1
5	5	C*	2	IV	-	-	+	-	-	-	2
5	5	D*	1	IV	-	-	+	-	-	-	1
					-	-	+	+	-	-	1
45	45	E/USA600	1	IV	+	-	+	-	-	-	1

*not determined.

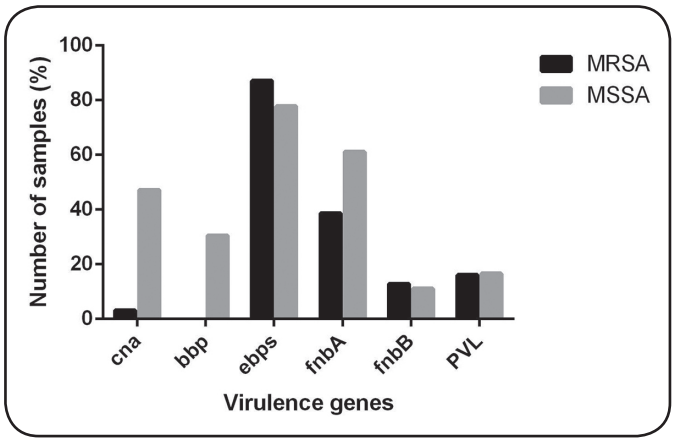

FIGURE 1: Presence of virulence genes in 67 *Staphylococcus aureus* strains.

In MRSA strains, the *ebpS* gene was observed in most pulsotypes except pulsotype B (ST8/USA300), which included *fnbB* and *lukS/F* as the most prevalent genes (**Table 2**).

The most prevalent gene detected in MSSA strains was *epbS* (78%), followed by *fnbA* (61%). Some virulence genes were associated with specific molecular types (**Table 3**), i.e., *cna* and *bbp* were predominantly detected in the strains of pulsotypes G and H, respectively.

DISCUSSION

The present study provides information about the molecular epidemiology of *S. aureus* clinical strains from a hospital located in south-eastern Brazil and reveals important findings on the distribution of virulence genes and antimicrobial resistance among MSSA and MRSA strains.

The results of molecular typing of *S. aureus* strains demonstrate a great diversity in clones circulating in this hospital environment, as all sequence types identified were associated with the important clonal complexes circulating in the American continent (i.e., CC5, CC8, CC45, and CC30). Furthermore, the majority of the strains isolated were related to worldwide epidemic clones such as USA100 (NY/J), USA300, and USA600[16,17].

The predominant MSSA strains characterized in the present study (ST1635-CC5 and ST30) were frequently detected in epidemiologic studies in Brazil[17,18]. Among MRSA strains, those with SCC*mec* type II related to worldwide epidemic clones such as USA100 (NY/J) were prevalent. This observation is consistent with the results of the study published by Caiaffa-filho *et al*.[19], wherein MRSA strains isolated from blood samples from Brazilian patients were studied.

The predominance of SCC*mec* type II (34/52, 65.4%) was also observed among the MRSA strains isolated from patients with bloodstream and respiratory tract infections during 2015-2016 in the University Hospital of Londrina in the Parana State, Brazil[20]. Interestingly, the predominance of MRSA strains harboring SCC*mec* II elements was also observed in a study during 2010 to 2013 in the same hospital[21]. These data suggest the shift in the MRSA population and emphasizes on the substitution of the strains harboring SCC*mec* III with those carrying SCC*mec* II that is becoming prevalent in some areas[19,20].

MRSA SCC*mec* type III strains related to the Brazilian Epidemic Clone (BEC)/ST239 were surprisingly not observed in the present study. This was the main lineage found in Brazilian hospitals in the past several decades. Thus, our findings may

TABLE 3: Virulence profiles and epidemiologic characteristics of methicillin-susceptible *Staphylococcus aureus*.

ST*	CC#	Pulsotype	No. of subtypes	Virulence profile						No. of isolates
				cna	*bbp*	*ebpS*	*fnbA*	*fnbB*	*lukS/F*	
1635	5	F	5	-	-	+	+	-	-	9
				-	-	+	-	-	-	2
				-	-	-	-	+	-	1
30	30	G	3	+	+	+	+	-	-	3
				+	+	+	+	-	+	3
				+	+	+	-	-	+	2
		H	3	+	+	+	+	-	-	3
				+	-	+	-	-	-	2
				+	-	+	+	-	-	1
		I	1	+	-	-	-	-	-	2
		J	1	-	-	-	-	+	-	2
		L	1	-	-	-	-	-	-	1
		M	1	-	-	+	+	-	-	1
		N	1	-	-	-	+	+	-	1
		O	1	-	-	+	-	-	-	1
		P	1	-	-	-	-	-	+	1
		Q	1	+	-	+	+	-	-	1

***ST:** sequence type (ST) analysis was performed only for pulsotypes F and G; #Clonal Complex.

reflect the changes in the prevalence of MRSA clones involved with nosocomial infections in Brazil. This epidemiologic change has been observed in other national studies, wherein the prevalence of new clones has become increasingly common[18-20].

The largest cassettes (I, II, and III) enhance the survival of MRSA in a hospital environment. However, smaller cassettes such as cassette IV are thought to promote evolutionary advantages through the horizontal transfer of this element[17].

The high degree of diversity in the genotype of MRSA SCC*mec* type IV distributed in four different genotypes indicates the polyclonal origin of these strains in the hospital investigated. The same high genotypic diversity observed for MRSA SCC*mec* type IV was also reported in other similar national studies[8,22]. In addition, many of these strains were similar to the USA300 (ST8) clone, which is a non-multidrug-resistant clone that predominates in community-onset infections[23].

The MRSA and MSSA strains exhibited low resistance to gentamicin and trimethoprim/sulfamethoxazole. Low resistance to these antibiotics was also observed in *S. aureus* strains in southern Brazil by Silveira *et al*.[24], supporting the potential applicability of gentamicin and trimethoprim/sulfamethoxazole as empiric agents against *S. aureu*s infections in Brazil. MSSA strains showed a significant resistance to tetracycline, while MRSA strains were deemed sensitive to this antibiotic. Cavalcante *et al*.[25] observed similar results for tetracycline resistance in MRSA SCC*mec* type IV, and these authors proposed that such low resistance can be a possible marker of SCC*mec* type IV. In the present study, rifampicin resistance was high in the USA100 (NY/J) SCC*mec* type II (77%), contradicting the profile observed for SCC*mec* type IV (0%) and MSSA strains (0%).

The present study has drawn attention to the occurrence of MSSA strains harboring a broader spectrum of virulence genes as compared to MRSA strains. The occurrence of PVL-encoding genes was similar between MRSA and MSSA strains. Although this exoprotein is traditionally seen in community-acquired MRSA such as the USA300 strain, it has also been identified in MSSA and hospital-acquired MRSA strains[18]. The spread of this gene is a matter of concern, as the clones that produce PVL are generally associated with high mortality rates all over the world[26].

Among the five adhesion molecules evaluated herein, *ebp*S was the most prevalent in both MSSA and MRSA strains. The high incidence of *ebp*S in multiple strains was observed in previous studies, consistent with the ubiquitous distribution of these genes in different *S. aureus* lineages[27]. Among the microbial surface components that recognize adhesive matrix molecules, FnbA and FnbB play important roles in *S. aureus* pathogenicity. These proteins promote bacterial attachment to fibrinogen, elastin, and fibronectin and participate in the initiation of the integrin-mediated intracellular uptake of bacteria via epithelial and endothelial cells[28]. In the present study, the distribution of *fnbA* gene was heterogeneous for both MRSA and MSSA. On the other hand, *fnbB* gene had heterogeneous distribution only among the MSSA strains; the only positive MRSA strains for *fnbB* expression were the SCC*mec* type IV, which were related to the USA300 lineage.

The *cna* gene was widespread among MSSA strains. However, only one strain, an MRSA-related USA600 clone (Berlin) CC45, presented *cna* gene. The low prevalence or absence of this gene in MRSA strains is well documented[1]. The role of *cna* gene product in the pathogenesis of bone infections and in the development of endovascular complications is well documented; however, it may play a less defined role in other infections[29]. In addition, consistent with our observation, the presence of *cna* was previously related to CC30 and CC45 lineages[29].

The gene encoding bone sialoprotein binding protein (Bbp) was specifically expressed in MSSA strains, all of which belong to the CC30 lineage. However, in accordance with the findings of a previous study, no MRSA strain was found to be positive for *bbp* positive[30]. The presence of *bbp* gene has been associated with osteomyelitis and arthritis in humans[13]. *S. aureus* ST30 lineages containing the *bbp* gene have been detected in an orthopedic hospital in Brazil[16]. The relationship of CC30 lineages with the *bbp* gene has been noted in previous studies, emphasizing the potential role of this gene as a molecular marker for CC30 identification[30,31].

The acquisition of antibiotic resistance in *S. aureus* is thought to involve changes in the virulence profile owing to the fitness costs associated with resistance genes[32]. In MRSA strains, this balance between virulence and resistance genes was closely associated with the size of SCC*mec* element possessed by the bacterium; strains with larger cassettes, such as SCC*mec* type II, have reduced numbers of virulence factors, while the presence of smaller cassettes, such as that observed in SCC*mec* type IV, is associated with a greater number of virulence genes, consistent with the observation reported in MSSA strains[32].

Although this study evaluated a limited number of strains from a single center between April 2011 and February 2012, it showed that the virulence gene carriage was more diverse and abundant in MSSA than in MRSA strains and that the distribution of some of these genes correlated with the specific *S. aureus* lineages. In addition, tetracycline resistance was related to MSSA strains. Our findings support the hypothesis that MSSA may be potentially more pathogenic, although further studies are warranted to identify the clinical relevance of this phenomenon. As the clinical outcome of *S. aureus* infections is influenced by both antimicrobial resistance and virulence factors, both these factors should be considered for a better understanding of the development and dynamics of the pathogen.

Conflict of interest

The authors declare that there is no conflict of interest.

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