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RESEARCH ARTICLE OPEN ACCESS

HIGH PREVALENCE OF MULTIDRUG-RESISTANCE AND TOXIN-ENCODING GENES IN STAPHYLOCOCCUS AUREUS ISOLATED FROM CHILDREN ATTENDING THE EMERGENCY DEPARTMENT OF A UNIVERSITY HOSPITAL IN SOUTHERN BRAZIL

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ABSTRACT

Staphylococcus aureus can be found as a member of human microbiota, but it is also a successful pathogen capable of causing a wide variety of infections. Herein, the prevalence of colonization by *S. aureus* in children attended in a university hospital was investigated. Furthermore, phenotypic and genotypic characteristics of the bacterial isolates were analyzed. *S. aureus* isolates were characterized according to their antimicrobial susceptibility, presence of *mecA* and three virulence-encoding genes. SCC*mec* typing and genetic relatedness of methicillin-resistant *S. aureus* (MRSA) were analyzed. Of 197 children, 31.0% were colonized by *S. aureus*. Among the isolates, 40.0% were classified as MRSA. Three isolates were susceptible to cefoxitin and harbored the *mecA* gene. The *mecA*-harboring isolates displayed the SCC*mec* types I (14.3%), II (9.5%), IV (47.6%), V (4.8%) and 23.8% of the isolates were *non*-typeable. All *S. aureus* isolates were susceptible to vancomycin and rifampicin and high rates of resistance were observed for penicillin (93.4%), erythromycin (63.9%) and clindamycin (42.6%). Thirty-two isolates were classified as multidrug-resistant. Most isolates (90.2%) harbored at least one virulence-encoding gene and the prevalence was *icaA*, 85.2%; *lukS-PV/lukF-PV*, 44.2%; and *tst*, 24.6%. rep-PCR analysis identified high genetic diversity among most MRSAs. Most MRSA SCC*mec* IV belonged to two of the major clonal complexes, CC5 and CC30. A high prevalence of *tst* and *lukS-PV/lukR-PV* genes in multi-drug resistant *S. aureus* colonizing children was detected, highlighting the importance of continuous monitoring of *S. aureus* colonization as a measure to control staphylococcal infections in this population.

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INTRODUCTION

Staphylococcus aureus is one of the most common human pathogens worldwide. This Gram-positive bacterial species can cause a variety of infections, ranging from superficial, such as skin and soft tissue injuries, to invasive and potentially fatal diseases, such as sepsis and necrotizing pneumonia (Tong *et al*., 2015). A substantial proportion of these infections are caused by methicillin-resistant *S. aureus* (MRSA), which can also present resistance to most antimicrobials used for the treatment of staphylococcal infections, representing a significant therapeutic challenge (de Oliveira *et al*., 2015; Duarte *et al*., 2018; Lakhundi; Zhang, 2018; Rossato *et al*., 2020).

MRSA was first detected in the1960s, in clinical specimens from patients attending a hospital in the United Kingdom (Jevons, 1961). In the decades following this first description, MRSA caused outbreaks in several hospitals in many countries (Chambers; Deleo, 2009), and nowadays it remains a leading cause of infections in healthcare-associated environments (Healthcare-Acquired MRSA – HA-MRSA) all over the world (Duarte *et al*., 2018; Andrade *et al*., 2020; Weiner-Lastinger *et al*., 2020). In the 1990s, MRSA emerged in the community (Community-Acquired MRSA– CA-MRSA) in individuals who had no prior history of hospitalization, especially among indigenous population in Australia (Udo *et al*., 1993) and healthy children in the United States (CDC, 1999).

S. aureus colonization represents a major risk of infection in humans (Young *et al*., 2017; Thomsen *et al*., 2019). As observed in adults, persistent colonization may increase the risk of subsequent staphylococcal infection in children (Cavalcante *et al*., 2015; Thomsen *et al*., 2019). In fact, around 20-30% of the human population can persistently harbor this bacterium in the anterior nares, although it can be found in several extra-nasal sites (Wertheim *et al*., 2005). Colonization by *S. aureus* in newborns may occur in the first days of life and, in general, a colonized mother seems to be the major source of bacterial transmission (Regev-Yochay*et al*., 2009; Reiss-Mandel *et al*., 2019). A high rate of *S. aureus* nasal carriage around birth is followed by a rapid decline after three months. By the second and third year of life, the rates of *S. aureus* carriage rise again, and becomes similar to the ones observed in adults (Regev-Yochay*et al*., 2009; Carvalho *et al*., 2017). Notably, early acquisition of *S. aureus* is an important determinant of persistent nasal colonization by this bacterium in infancy (Reiss-Mandel *et al*., 2019).

S. aureus, including MRSA, remains a leading cause of bacteremia and invasive diseases that include pulmonary, skin and soft tissues, as well as musculoskeletal infections in pediatric population (Leung *et al*., 2018; Ensinck *et al*., 2021). Therefore, the surveillance of *S. aureus* carriage in children should be performed, so that information can be gathered in order to assist in the clinical management of staphylococcal infections in this population. The aim of the present study was to determine the prevalence of *S. aureus,* especially regarding MRSA carriage, in healthy children attended in a university hospital in southern Brazil. The antibacterial susceptibility profile, genetic relatedness and occurrence of *icaA* (encoding N-acetylglucosaminyl transferase of the intercellular adhesion locus), *lukS-PV/lukF-PV* (encoding the β-pore-forming Panton-Valentine leukocidin) and *tst* (encoding toxic shock syndrome toxin) genes were also evaluated.

MATERIALS AND METHODS

Study population, specimen collection and processing: A total of 197 children seen at the emergency department of a university hospital in southern Brazil, from May 2018 to April 2019, were enrolled in this study. Five descriptive characteristics (gender, age, antimicrobial use and/or hospitalization in the last six months, and direct contact with healthcare professionals) were collected from all participants. One sample (from nasal, oral, axillary and inguinal sites) was collected from each participant using the Stuart collection device (COPAN Diagnostic, Italy). The samples were inoculated into Tryptone Soya Broth (TSB, Oxoid, Brazil) supplemented with 6.5% sodium chloride, and incubated at 35°C for 24 h. Each sample was subsequently cultured on Mannitol Salt Agar (Oxoid, Brazil), at 35°C for 24 h. Suggestive colonies of staphylococci were subjected to phenotypic identification through standard tests (Becker *et al*., 2015), and species identification was confirmed by multiplex-PCR targeting the *nuc* gene (encoding thermonuclease) (Hirotaki *et al*., 2011). Bacteria were stored at -80°C in TSB containing 30% glycerol.

Antimicrobial susceptibility testing: The isolates were tested for antimicrobial susceptibility to cefoxitin (30 μg), penicillin (10 U), erythromycin (15 μg), clindamycin (2 μg), gentamicin (10 μg), ciprofloxacin (5 μg), sulfamethoxazole-trimethoprim (23.75/1.25 μg), rifampicin (5 μg), linezolid (10 μg) and tigecycline (15μg) by using the disk-diffusion method according to the Clinical Laboratory Standards Institute (CLSI, 2019) guidelines. Cefoxitin disk and/or the presence of *mecA* gene, detected as described below, were used to define MRSA. The susceptibility breakpoints were those recommended by CLSI (2019), except tigecycline, which was interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2019). *Enterococcus faecalis* ATCC 29212 and *S. aureus* ATCC 25923 were used as quality controls.

DNA purification: One colony of each bacterium was cultured in 3 mL TSB at 35ºC for 24 h. The cells were harvested by centrifugation (10,000 x *g* for 5 min), washed once with sterile 0.15 M phosphate-buffered saline (PBS) pH 7.2, and resuspended in 300 μL lysis solution (10 mM Tris-HCl, 1 mM EDTA pH8.0, 1.0 mg/mL lysozyme). DNA was extracted as described in Ausubel *et al*. (1999), and 2 μL were used in all amplification reactions.

Detection of mecA and virulence-encoding genes by PCR: The gene *mecA* (encoding PBP2a) was detected as described by Milheiriço *et al*. (2007); the genes *icaA, lukS-PV/lukF-PV* and *tst* were detected as described by Campbell *et al*. (2008). *S. aureus* BEC 9393 (nuc^+, $mecA^+$, $icaA^+$, tst^+), and *S. aureus* ATCC 25923 (nuc^+, coa^+, *lukS-PV/lukF-*PV^+) were used as controls.

MRSA typing: The genetic relatedness of all MRSA isolates was analyzed by repetitive element sequence based-PCR (rep-PCR), according to Del Vecchio *et al*. (1995). Finger printings containing more than one band differing in size were considered different rep-PCR types (van der Zee *et al*., 1999). Banding patterns were categorized using the UPGMA algorithm and Jaccard coefficient (Sneath; Sokal, 1973) of the Bionumerics v.6.5 software (Applied Mathematics, Kortrijk, Belgium), with the band tolerance set at 3% and the threshold cutoff value set at 85% (de Oliveira *et al*., 2015). SCC*mec* typing was performed by multiplex-PCR as described previously (Milheiriço *et al*., 2007). Non-typeable isolates were designated NT. *S. aureus* NCTC10442 (type I), N315 (type II), 85/2082 (type III), 81/108 (type IV), WIS [WBG8318] (type V), and HDE288 (type VI) strains were used as controls. The High-Resolution Melting (HRM) analysis of Single Nucleotide Polymorphisms (SNPs), as described by Lilliebridge *et al*. (2011), was performed to further investigate the clonal relatedness among SCC*mec*-IV MRSAs.

Statistical analysis: The statistical analysis was performed by using the software Statistical Package for the Social Sciences (SPSS - IBM Corp., New York, USA), version 20.0 for Windows. Categorical variables were expressed as absolute number (*n*) and percentage (%), and continuous variables were expressed as mean ± standard deviation. Categorical variables were analyzed using the Chi-square test or Fisher's exact test, when appropriate. Variables with p value $\leq$ 0.05 were considered statistically significant.

Ethics: All children's parents signed an informed consent form allowing them to participate in this study, agreeing to the publication of this report. The study protocol was approved by the Ethics Committee of UEL (CEP/UEL) under the number CAAE 86708018.4.0000.523.

RESULTS AND DISCUSSION

Prevalence of* Staphylococcus aureus *colonization in children seen at the emergency department of a university hospital in southern Brazil: In the present study, multiple body sites of children attended at the emergency department of a university hospital were screened for *S. aureus* carriage. The limitation of this study is that one single sampling was performed on each participant, which does not allow the identification of persistent carriers. A total of 197 children were enrolled in this study. Of these, 90 were males and 107 were females, and their mean age was 4.6 ± 3.4 years (ranging from 1 month to 12 years old). *S. aureus* was isolated from 61 (31.0%)

Table 1. Antimicrobial resistance profile of methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* isolated from healthy children attended in a University Hospital in southern Brazil

Antibiotype	Resistance profile	MSSA* *n*=36 (%)	MRSA *n*=25 (%)		Total *N*=61 (%)
			mecA$^+$	*mecA*$^-$	
AT1	P	7 (19.4)	1 (4.0)	-	8 (13.1)
AT2	P,E	7 (19.4)	-	-	7 (11.5)
AT3	P,SXT	2 (5.6)	-	-	2 (3.3)
AT4	P,TE	2 (5.6)	-	-	2 (3.3)
AT5	P,E,DA	11 (30.6)	-	-	11 (18.0)
AT6	P,E,DA,SXT	3 (8.3)	-	-	3 (4.9)
AT7	P,E,CN,SXT	1 (2.8)	-	-	1 (1.6)
AT8	P,DA,SXT	-	1 (4.0)	-	1 (1.6)
AT9	P,E,SXT,TGC	-	1 (4.0)	-	1 (1.6)
AT10	FOX	-	1 (4.0)	-	1 (1.6)
AT11	FOX,P	-	3 (12.0)	-	3 (4.9)
AT12	FOX,P,E	-	2 (8.0)	-	2 (3.3)
AT13	FOX,P,SXT	-	1 (4.0)	-	1 (1.6)
AT14	FOX,P,E,DA	-	2 (8.0)	1 (4.0)	3 (4.9)
AT15	FOX,P,E,TE	-	1 (4.0)	-	1 (1.6)
AT16	FOX,P,SXT,TE	-	1 (4.0)	-	1 (1.6)
AT17	FOX,P,E,DA,SXT	-	-	2 (8.0)	2 (3.3)
AT18	FOX,P,E,DA,TE	-	2 (8.0)	-	2 (3.3)
AT19	FOX,P,E,CN,TE	-	1 (4.0)	-	1 (1.6)
AT20	FOX,P,E,DA,LZD,TGC	-	1 (4.0)	-	1 (1.6)
AT21	FOX,P,E,DA,SXT,TE	-	-	1 (4.0)	1 (1.6)
AT22	FOX,P,E,DA,CN,SXT,TE	-	1 (4.0)	-	1 (1.6)
AT23	FOX,P,E,CIP,CN,SXT,TE	-	1 (4.0)	-	1 (1.6)
AT24	FOX,P,E,DA,LZD,SXT,TGC	-	1 (4.0)	-	1 (1.6)

*Antimicrobial suceptibility profile determined by disk-diffusion (CLSI, 2019) and interpreted according to the CLSI (2019) and EUCAST (2019). Three isolates were susceptible to all antimicrobials tested. P: penicillin (10 U); FOX: cefoxitin (30 µg); E: erythromycin (15 µg); DA: clindamycin (2 µg); CN: gentamicin (10 µg); CIP: ciprofloxacin (5 µg); LZD: linezolid (30 µg); STX: sulfamethoxazole/trimethoprim (23.75/1.25 µg); TE: tetracycline (30 µg); TGC: tigecycline (15 µg). -: not detected.

Table 2: Virulence-encoding genes profile in methicillin-susceptible (MSSA) and methicillin-resistant (MRSA) *Staphylococcus aureus* isolated from children attended in the emergency department of the University Hospital of Londrina, Paraná, Brazil

Virulence marker	Number of isolates		
	MSSA *n* = 36 (%)	MRSA *n* = 25 (%)	Total *N* = 61 (%)
icaA	28 (77.8)	23 (92.0)	51 (83.6)
lukS-PV/lukF-PV	18 (50.0)	9 (36.0)	27 (44.3)
tst	6 (16.7)	9 (36.0)	15 (24.6)
Virulence profile			
icaA	12 (33.3)	8 (32.0)	20 (32.8)
lukS-PV/lukF-PV	2 (5.6)	-	2 (3.3)
tst	1 (2.8)	-	1 (1.6)
icaA, lukS-PV/lukF-PV	12 (33.3)	6 (24.0)	18 (29.5)
icaA, tst	1 (2.8)	6 (24.0)	7 (11.5)
icaA, lukS-PV/lukF-PV, tst	4 (11.1)	3 (12.0)	7 (11.5)

The *icaA* (encoding *N*-acetylglucosaminyltransferase of the intercellular adhesion *locus*), *lukS-PV/lukF-PV* (encoding Panton-Valentine leukocidin - PVL) and *tst* (encoding toxic shock syndrome toxin-TSST-1) genes were detected as described by Campbell *et al*. (2008).

Table 3. Sequence types and clonal complexes of MRSA *mecA*$^+$/SCC*mec* type IV isolates exhibiting different antimicrobial susceptibility, virulence-encoding gene and rep-PCR fingerprinting profiles

MRSA	Variable[a]	Antibiotype[b]	rep-PCR[c]	Virulence genes profile[d]	ST[e]	CC[e]
C69	-	FOX,P,E,CN,TE	L	*icaA,tst*	328	5
C70	-	FOX,P	L	*icaA, lukS-PV/lukF-PV, tst*	30	30
C84	-	FOX,P,E,DA,TGC,LDZ	D	*icaA*	4	45
C85	H	FOX,P,E,DA,CN,TE,SXT	J	*icaA,tst*	30	30
C121	-	P,E,TGC,SXT	K	*icaA, lukS-PV/lukF-PV*	6	5
C122	H	FOX,P,E,DA,TGC,LZD,SXT	K	*icaA, lukS-PV/lukF-PV*	30	30
C148	-	P	G	*icaA*	873	873

[a]History of hospitalization (H) in the last six months. [b]Antimicrobial suceptibility profile determined by disk-diffusion (CLSI, 2019) and interpreted according to the CLSI (2019) and EUCAST (2019): P: penicillin; FOX: cefoxitin; E: erythromycin; DA: clindamycin; CN: gentamicin; LZD: linezolide; STX: sulfamethoxazole/ trimethoprim; TE: tetracycline; TGC: tigecycline. [c]rep-PCR fingerprinting was determined according to del Vecchio *et al*. (1995). [d]The*icaA* (encoding *N*-acetylglucosaminyltransferase of the intercellular adhesion *locus*), *lukS-PV/lukF-PV* (encoding Panton-Valentine leukocidin-PVL) and *tst* (encoding toxic shock syndrome toxin-TSST-1) genes were detected as described by Campbell *et al*. (2008). [e]Sequence Type (ST) and Clonal Complex (CC) were determined as described by Lilliebridge *et al.* (2011).

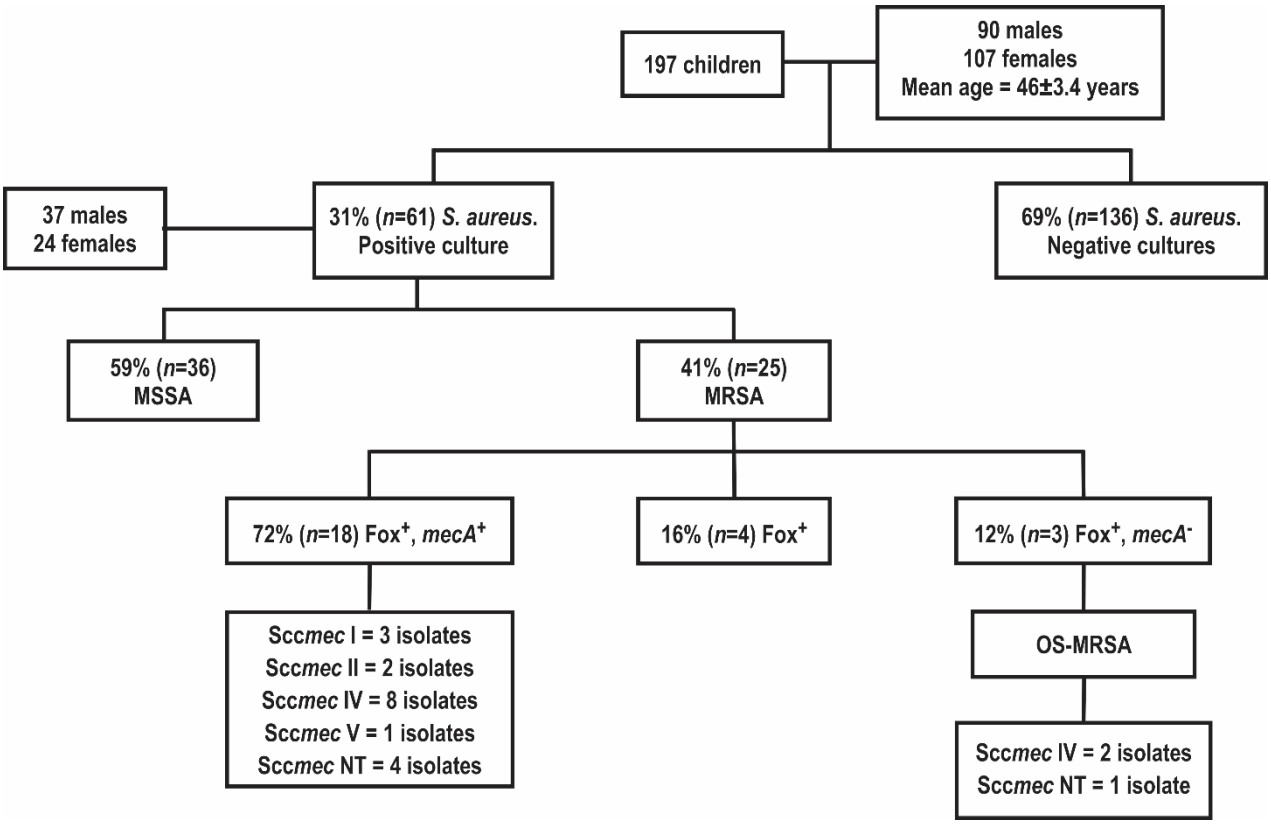
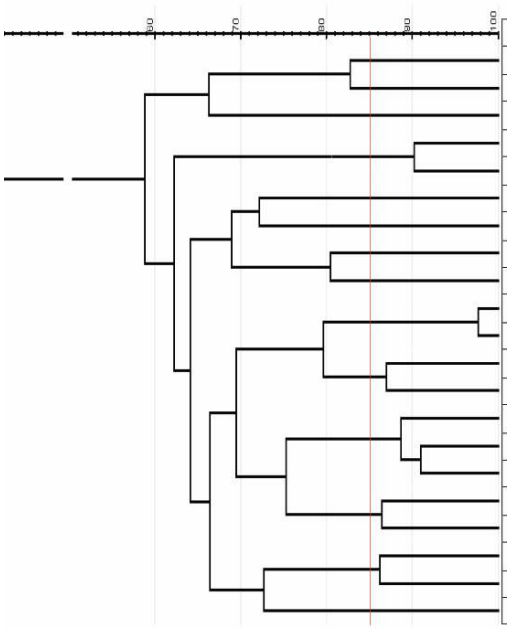


Figure 1: Flowchart of the results for general swabs obtained from children attending the emergency department of the University Hospital of Londrina, Paraná, Brazil. MSSA: methicillin-susceptible *Staphylococcus aureus*; MRSA: methicillin-resistant *S. aureus*; OS-MRSA: oxacillin-susceptible *mecA*-positive *S. aureus*; Fox$^+$: cefoxitin resistance was determined and interpreted according to the CLSI (2019); *mecA*$^+$ and SCC*mec* typing were determined by multiplex PCR assay (Milheiriço *et al*., 2007). NT: non-typeable; *mecA*$^-$: the gene was not detected



S. aureus	rep-PCR	SCC*mec*	Antibiotype	Virulence
C66	A	I	FOX, P, E, DA, TE	-
C62	B	NT	FOX, P, SXT, TE	*icaA, tst*
C53	C	V	FOX, P, E, TE	-
C84	D	IV	FOX, P, E, DA, LZD, TGC	*icaA*
C141	D	NT	FOX, P, E, DA	*icaA*
C140	E	NT	FOX, P, E, DA	*icaA, tst*
C130	F	II	FOX, P, E	*icaA, lukS-PV/lukF-PV, tst*
C148	G	IV	P	*icaA*
C87	H	IV	FOX, P	*icaA, lukS-PV/lukF-PV*
C113	I	NT	FOX, P, SXT	*icaA, lukS-PV/lukF-PV*
C101	I	I	FOX, P, E, CIP, CN, SXT, TE	*icaA, lukS-PV/lukF-PV*
C172	J	IV	FOX, P	*icaA*
C85	J	IV	FOX, P, E, DA, CN, SXT, TE	*icaA, tst*
C122	K	IV	FOX, P, E, DA, LZD, SXT, TGC	*icaA, lukS-PV/lukF-PV*
C127	K	II	FOX, P, E	*icaA, lukS-PV/lukF-PV, tst*
C121	K	IV	P, E, SXT, TGC	*icaA, lukS-PV/lukF-PV*
C70	L	IV	FOX, P	*icaA, lukS-PV/lukF-PV, tst*
C69	L	IV	FOX, P, E, CN, TE	*icaA, tst*
C192	M	NT	P, DA, SXT	*icaA*
C128	M	IV	FOX, P, E, DA, TE	*icaA*
C44	N	I	FOX	*icaA*

Figure 2: Characteristics of methicillin-resistant *Staphylococcus aureus* (*mecA*$^+$) isolated from colonized children attending the emergency department of the University Hospital of Londrina. rep-PCR (Del Vecchio *et al.*, 1995) cluster analysis was performed using UPGMA algorithm and Jaccard coefficient of the Bionumerics v. 6.5 software, with band tolerance set at 3% and threshold cut-off value set at 85% (de Oliveira *et al*., 2015). SCC*mec* typing was performed by multiplex PCR assay (Milheiriço *et al*., 2007). Antimicrobial resistance profile (antibiotype) was determined by disk-diffusion method (CLSI, 2019) and interpreted according to the CLSI (2019) and EUCAST (2019). The *icaA* (encoding N-acetylglucosaminyltransferase of the intercellular adhesion locus), *lukS-PV/lukF-PV* (encoding Panton-Valentine leukocidin - PVL) and *tst* (encoding toxic shock syndrome toxin-TSST-1) genes were detected as described by Campbell *et al*. (2008). NT, non-typeable; FOX, cefoxitin; P, penicillin; CIP, ciprofloxacin; CN, gentamycin; DA, clindamycin; E, erythromycin; LZD, linezolid; SXT, sulphamethoxazole-trimethoprim; TE, tetracycline; TGC, tigecycline.

Supplementary Information: High prevalence of multidrug-resistance and toxin-encoding genes in *Staphylococcus aureus* isolated from children attending the emergency department of a university hospital in southern Brazil

Table S1: Variables of children seen at the emergency department of the University Hospital of Londrina, Paraná, Brazil and association with *Staphylococcus aureus* colonization

Variable	Child		*P*value*
	Colonized (*n*=61)	Non-colonized (*n*=136)	
Gender			
Male	24	66	0.2793
Female	37	70	
Antimicrobial use			
Yes	11	26	>0.9999
No	50	110	
Hospitalization in the last six months			
Yes	7	13	0.7990
No	54	123	
Direct contact with healthcare professionals			
Yes	6	11	0.7850
No	55	124	

* Fisher's exact test. Variables with *P* value ≤ 0.05 were considered statistically significant.

Table S2: Association of virulence-encoding genes and MSSA and MRSA isolated from colonized children seen at the emergency department of the University Hospital of Londrina, Paraná, Brazil

Gene	MSSA, *n*=36 (%)	MRSA, *n*=25 (%)	*P*value*
icaA			
Negative	8 (22.2)	2 (8.0)	0.2862
Positive	28 (77.8)	23 (92.0)	
lukS-PV/lukF-PV			
Negative	18 (50.0)	16 (64.0)	0.3073
Positive	18 (50.0)	9 (36.0)	
tst			
Negative	30 (83.3)	16 (64.0)	0.1302
Positive	6 (16.7)	9 (36.0)	

The *icaA* (encoding *N*-acetylglucosaminyltransferase of the intercellular adhesion *locus*), *lukS-PV*/*lukF-PV* (encoding Panton-Valentine leukocidin - PVL) and *tst* (encoding toxic shock syndrome toxin-TSST-1) genes were detected as described by Campbell *et al*. (2008) *Fisher's exact test. Variables with *p* value ≤ 0.05 were considered statistically significant.

children, being 37 (60.6%) female and 24 (39.4%) male. Among the isolates, 41.0% (25/61) were identified as MRSA, representing 12.7% (25/197) of all participants. Of the 25 MRSA isolates: a) 18 (72.0%) were resistant to cefoxitin and harbored the *mecA* gene; b) 4 (16.0%) were resistant to cefoxitin and did not harbor the *mecA* gene. Homologs of *mecA* have been identified; the *mec* gene can be classified in five classes (A to E) according to the position of insertion sequences and regulatory sequences located upstream and downstream of this gene (Lakhundi; Zhang, 2018), and they were not screened in this study, which may explain this result; c) 3 (12.0%) were susceptible to cefoxitin and harbored the *mecA* gene and these isolates were classified as oxacillin-susceptible MRSA (OS-MRSA) isolates (Figure 1). OS-MRSA has been increasingly isolated from different sources worldwide, including human (Duarte *et al*., 2019; Danelli *et al*., 2020; Ma *et al*., 2021), food (Quijada *et al*., 2019) and animals (Fabri *et al*., 2021). Importantly, *in vitro* and *in vivo* reversions of methicillin susceptibility to methicillin resistance in OS-MRSA on exposure to antimicrobial agents have been reported (Prouxl *et al*., 2016), reinforcing the need for additional tests to accurately distinguish the various methicillin-susceptibility profiles of *S. aureus*. The mechanism by which OS-MRSA exhibit susceptibility to oxacillin is still unclear. Recent studies based on comparative genome analysis have shown different mutations in OS-MRSA that may be associated with the oxacillin sensitivity phenotype. Studies in the literature have reported a correlation between *S. aureus* (particularly MRSA) colonization in children with several factors including gender, age, day care attendance, use of antimicrobial agents and presence of underlying chronic diseases (Lamaro-Cardoso *et al*., 2009; Lin *et al*., 2016; Neto *et al*., 2020; del Rosal *et al*., 2020; Dayie *et al*., 2021).

Conversely, no characteristics of the participants (gender, age, antimicrobial use and/or hospitalization in the last six months, and direct contact with healthcare professionals) was positively associated with *S. aureus* colonization in children of the present study (Chi-square test or Fisher's exact test). (Supplementary File Table S1).

Prevalence of *S. aureus* and/or MRSA colonization can varygeographically. For instance, the meta-analysis carried out by Lin *et al*. (2016), during January 2005 to December 2015, revealed a prevalence of 3.8% of MRSA nasal carriage in healthy Chinese children. Kateete*et al*. (2020) reported a prevalence of 25.3% of *S. aureus* carriers among healthy children under five years of age living in rural Eastern Uganda, and MRSA was identified in 28.5% of the colonized subjects. The study of Dayie *et al*. (2021) reported a prevalence of 23.2% in children under five colonized by *S. aureus,* and of these, 0.49% were colonized by MRSA, in Accra, Ghana. A prevalence of 21.0% and 3.0% of *S. aureus* and MRSA carriers, respectively, were detected in children and adolescents (mean age 7.5 years, range 0 to 18 years) within 48 hours of admission to a hospital in Texas, United States (Okoye *et al*., 2019). Few Brazilian studies analyzed the prevalence of both *S. aureus* and MRSA in healthy children. Lamaro-Cardoso *et al*. (2009) carried out a cross-sectional study with healthy children under five seen at day care centers in Goiânia, Goiás, and a prevalence of 31.0% and 1.2% of *S. aureus* and MRSA carriers were observed, respectively. A study carried out in São Paulo revealed a prevalence of 65.0% and 5.1% of *S. aureus* and MRSA carriage in children (Mimica; Bádue-Pereira, 2014). Carvalho *et al*. (2017) reported a prevalence of 47.3% and 7.4% of *S. aureus* and MRSA carriers, respectively, in healthy children attending day care centers in Vitoria da Conquista, Bahia. Neves *et al*. (2019) described a prevalence of 37.0% and 8.2% of *S. aureus* and MRSA

Table S3: Variables in children[a] colonized by *Staphylococcus aureus*, and phenotypic and genotypic characteristics of bacterial isolates

Sample	Age (years)	Gender	Hospitalization[b]	Antimicrobial use (Type)[c]	Contact[d]	*S. aureus*[e]	Oxacillin[f]	Antibiotype[f]	*mecA*[g]	SCC*mec*[g]	Virulence[h]
C11	10	F	Y	N	N	MSSA	S	P, E	-		-
C116	5	F	N	N	N	MSSA	S	P, E, DA, SXT	-		*lukS-PV/lukF-PV*
C117	10	M	N	N	N	MSSA	S	P, E, DA, SXT	-		*icaA, lukS-PV/lukF-PV*
C13	8	M	N	Y	N	MSSA	S	P, E	-		*lukS-PV/lukF-PV*
C118	2	F	N	N	N	MRSA	R	FOX, P, E, DA, SXT	-		*icaA, lukS-PV/lukF-PV*
C135	8	M	N	Y (AMC)	Y	MRSA	R	FOX, P, E, DA, SXT	-		*icaA*
C138	4	F	N	Y (AZI)	Y	MSSA	S	*	-		*icaA*
C14	6	F	N	N	N	MSSA	S	P, E, DA	-		*icaA, lukS-PV/lukF-PV*
C140	4	F	N	N	N	MRSA	R	FOX, P, E, DA	+	NT	*icaA, tst*
C141	3	F	N	N	N	MRSA	R	FOX, P, E, DA	+	NT	*icaA*
C144	1	F	N	N	Y	MSSA	S	P, E, DA	-		*icaA*
C145	12	F	N	Y (LVX)	N	MSSA	S	P, E, DA	-		*icaA*
C146	4	M	N	Y (LEX)	N	MSSA	S	P, E, DA	-		*icaA*
C148	6	M	N	N	N	OS-MRSA	S	P	+	IV	*icaA*
C150	12	M	N	N	N	MSSA	S	P, E	-		*icaA*
C154	10	F	N	N	N	MSSA	S	P	-		*icaA*
C163	1	F	N	N	N	MSSA	S	P, E, DA	-		-
C164	10	M	Y	N	N	MSSA	S	P, SXT	-		*icaA*
C165	11	F	N	N	N	MSSA	S	P, E	-		*icaA, lukS-PV/lukF-PV*
C166	6	F	N	N	N	MSSA	S	P, E	-		*icaA, lukS-PV/lukF-PV*
C136	6	M	N	N	N	MRSA	R	FOX, P, E, DA, SXT, TE	-		*icaA, tst*
C172	7	F	N	N	Y	MRSA	R	FOX, P	+	IV	*icaA*

Continue …

Sample	Age (years)	Gender	Hospitalization[b]	Antimicrobial use (Type)[c]	Contact[d]	*S. aureus*[e]	Oxacillin[f]	Antibiotype[f]	*mecA*[g]	SCC*mec*[g]	Virulence[h]
C173	4	F	N	Y (AMX)	N	MSSA	S	P	-		*icaA, lukS-PV/lukF-PV, tst*
C174	6	M	N	Y (AMX)	N	MSSA	S	P, E, DA, SXT	-		*icaA, lukS-PV/lukF-PV, tst*
C182	8	F	N	N	N	MSSA	S	P, TE	-		*icaA*
C128	5	F	N	Y (AMC)	N	MRSA	R	FOX, P, E, DA, TE	+	IV	*icaA*
C171	11	F	N	N	Y	MSSA	S	P	-		*icaA, lukS-PV/lukF-PV, tst*
C192	0.08	F	Y	N	N	OS-MRSA	S	P, DA, SXT	+	NT	*icaA*
C196	10	M	Y	N	N	MSSA	S	P, E, DA	-		*icaA, tst*
C2	5	M	N	N	N	MSSA	S	P	-		*tst*
C20	5	M	N	N	N	MSSA	S	P, E, DA	-		*icaA, lukS-PV/lukF-PV*
C30	11	F	N	N	N	MSSA	S	P, E, DA	-		*icaA, lukS-PV/lukF-PV*
C43	11	F	N	N	N	MSSA	S	P, TE	-		*icaA, lukS-PV/lukF-PV*
C45	9	F	N	N	N	MSSA	S	P, E	-		*icaA, lukS-PV/lukF-PV*
C186	6	F	N	Y (AMC)	N	MSSA	S	P, E, DA	-		*icaA*
C5	4	M	N	N	N	MSSA	S	P, E, DA	-		-
C56	9	F	N	N	Y	MSSA	S	*	-		*icaA, lukS-PV/lukF-PV*
C58	5	F	N	N	N	MSSA	S	P	-		*icaA, lukS-PV/lukF-PV*
C59	0.6	M	N	N	N	MSSA	S	P, E	-		*icaA*
C87	8	F	N	N	N	MRSA	R	FOX, P	+	IV	*icaA, lukS-PV/lukF-PV*
C85	8	F	Y	N	N	MRSA	R	FOX, P, E, DA, CN, SXT, TE	+	IV	*icaA, tst*
C84	2	F	N	N	N	MRSA	R	FOX, P, E, DA, LZD, TGC	+	IV	*icaA*
C78	7	M	N	N	N	MSSA	S	P	-		*icaA, lukS-PV/lukF-PV*
C7	5	F	N	Y	N	MSSA	S	P, E, DA	-		-
C72	4	M	N	N	N	MSSA	S	P, SXT	-		*icaA*
C70	1	M	N	N	N	MRSA	R	FOX, P	+	IV	*icaA, lukS-PV/lukF-PV, tst*
C74	5	M	N	N	N	MSSA	S	P	-		*icaA, lukS-PV/lukF-PV, tst*

Sample	Age (years)	Gender	Hospitalization[b]	Antimicrobial use (Type)[c]	Contact[d]	*S. aureus*[e]	Oxacillin[f]	Antibiotype[f]	*mecA*[g]	SCC*mec*[g]	Virulence[h]
C69	1	F	N	N	N	MRSA	R	FOX, P, E, CN, TE	+	IV	*icaA, tst*
C76	6	F	N	N	N	MSSA	S	*	-		*icaA, lukS-PV/lukF-PV*
C66	2	M	Y	Y (AMX/LEX)	N	MRSA	R	FOX, P, E, DA, TE	+	I	-
C63	1	M	N	N	N	MRSA	R	FOX, P, E, DA	-		*icaA, tst*
C62	2	F	N	N	N	MRSA	R	FOX, P, SXT, TE	+	NT	*icaA, tst*
C53	7	F	N	N	N	MRSA	R	FOX, P, E, TE	+	V	**
C44	7	F	N	N	N	MRSA	R	FOX	+	I	*icaA*
C88	6	M	N	N	N	MSSA	S	P, E, CN, SXT	-		*icaA*
C130	2	F	N	N	N	MRSA	R	FOX, P, E	+	II	*icaA, lukS-PV/lukF-PV, tst*
C127	5	F	N	N	N	MRSA	R	FOX, P, E	+	II	*icaA, lukS-PV/lukF-PV, tst*
C122	9	M	Y	N	N	MRSA	R	FOX, P, E, DA, LZD, SXT, TGC	+	IV	*icaA, lukS-PV/lukF-PV*
C121	8	M	N	N	N	OS-MRSA	S	P, E, SXT, TGC	+	IV	*icaA, lukS-PV/lukF-PV*
C113	4	F	N	N	N	MRSA	R	FOX, P, SXT	+	NT	*icaA, lukS-PV/lukF-PV*
C101	1	M	N	N	N	MRSA	R	FOX, P, E, CIP, CN, SXT, TE	+	I	*icaA, lukS-PV/lukF-PV*

[a]Children were attended in the emergency department of the University Hospital of Londrina, Paraná, Brazil. [b]History of hospitalization in the last 6 months. [c]Use of antimicrobials in the last 6 months. [d]Direct contact with health professionals. [e]Classification of *S. aureus* according to the methicillin susceptibility and the presence of *mecA* gene. [f]Antimicrobialsuceptibility profile determined by disk-diffusion (CLSI, 2019) and interpreted according to the CLSI (2019) and EUCAST (2019): Three isolates were susceptible to all antimicrobials tested. P: penicillin (10 U); FOX: cefoxitin (30 µg); E: erythromycin (15 µg); DA: clindamycin (2 µg); CN: gentamicin (10 µg); CIP: ciprofloxacin (5 µg); LZD: linezolid (30 µg); STX: sulfamethoxazole/trimethoprim (23.75/1.25 µg); TE: tetracycline (30 µg); TGC: tigecycline (15 µg). [g]The gene *mecA* and SCC*mec* types were detected as described by Milheiriço*et al*. (2007). [h]The*icaA* (encoding N-acetylglucosaminyltransferase of the intercellular adhesion locus), *lukS-PV/lukF-PV* (encoding Panton-Valentine leukocidin - PVL) and *tst* (encoding toxic shock syndrome toxin-TSST-1) genes were detected as described by Campbell *et al*. (2008). F: female; M: male; N: no; Y: yes; AMC: amoxicillin *plus* clavulanic acid; AZI: azithromycin; LVX: levofloxacin; LEX: cephalexin; AMX: amoxacillin; S: sensitive; R: resistant; MSSA: methicillin-susceptible *S. aureus*; MRSA: methicillin-resistant *S. aureus*; OS-MRSA: oxacillin-susceptible MRSA; *Isolate was susceptible to all antimicrobials tested; -: not detected; +detecte

carriers, respectively, in children attending private and public clinics for routine preventive care in Niteroi, Rio de Janeiro.These authors observed that children living in a low-income community and also the number of households were variables significantly associated with MRSA colonization. Finally, the study of Neto *et al*. (2020)carried out with children attending day care centers, an outpatient clinic of a university hospital, and inpatient clinics of public hospital in Niteroi, Rio de Janeiro, detected a prevalence of 51.2% of *S. aureus* carriers, and among them, 19.5% were identified as MRSA carriers.

High prevalence of multidrug-resistant Staphylococcus aureus carriage was detected in children seen at the emergency department of a university hospital in southern Brazil: According to the disk-diffusion results, all isolates were susceptible to rifampicin and three isolates were susceptible to all other antimicrobial agents. Most isolates were also susceptible to linezolid (59/61, 96.7%), tigecycline (58/61, 95.1%), gentamycin (57/61, 93.4%), ciprofloxacin (60/61, 98.3%), tetracycline (51/61, 83.6%) and sulfamethoxazole/ trimethoprim (45/61, 73.7%). In contrast, a high rate of resistance was observed for penicillin (57/61, 93.4%), erythromycin (39/61, 63.9%), and clindamycin (26/61, 42.6%).The rates of resistance to penicillin and erythromycin were consistent with previous Brazilian studies, however, unlike what was observed in the present study, no resistance to clindamycin was observed (Carvalho *et al*., 2017; Neves *et al*., 2019).

A substantial number of isolates (33/61, 54.1%) presented resistance to at least three antimicrobial classes and they were classified as multidrug-resistant according to Magiorakos*et al*. (2012) criteria. Overall, all *S. aureus* isolates were classified into 24 groups (Table 1) according to theirantimicrobial resistance profile [named as antibiotype (AT)AT1 to AT24]. Seven antibiotypes (AT1-AT7) were observed among MSSA isolates; and co-resistance to penicillin, erythromycin, and clindamycin (AT5) was the most frequent (11/36, 30.6%). On the other hand, 18 antibiotypes (AT1, AT8-AT24) were identified among MRSA isolates; and 13 of themconsisted of a single isolate. The most frequent antibiotypes among MRSA isolates were AT11 (co-resistance to cefoxitin and penicillin) and AT14 (co-resistance to cefoxitin, penicillin, erythromycin and clindamycin), consisting of three isolates each. Notably, the antibacterial susceptibility profile of *S. aureus* isolates from children was similar to that detected in adults who were hospitalized (de Oliveira *et al*., 2015; Duarte *et al*., 2018) or in healthcare workers and students attending (Danelli *et al*., 2020) the same university hospital of the present study.

Approximately 25.0% of *S. aureus* genome is composed by mobile genetic elements, where antimicrobial resistance, virulence and host immune evasion determinants are commonly found. This accessory genome, which can be acquired by horizontal transfer, plays a vital role in bacterial adaptability in different environmental conditions and contributes to the emergence of successful new strains that can adversely affect human healthcare (Lindsay; Holden, 2004). The use of antimicrobial agents in multiple sectors (human and veterinary care, andagriculture) is an important driver of antimicrobial resistance, contributing to the selection and dissemination of resistant microbial species within/between these sectors and also globally (McEwen; Collignon, 2018). The carriage of multidrug-resistant *S. aureus* in absence of potential risk factors associated with host colonization may reflect the strong selective pressure exerted by the environments. These data highlight the importance of effective strategies for the appropriate use of antimicrobials in different sectors, as well as maintaining continuous surveillance of the mechanisms of antimicrobial resistance circulating in these environments.

Detection of virulence-encoding genes: Notably, a high prevalence of *S. aureus* carrying the virulence-encoding genes investigated in this study was identified, and the overall prevalence was as follows:*icaA*, 83.6% (51/61); *lukS-PV/lukF-PV*, 44.2% (27/61); and *tst*, 24.6% (15/61).Of the 61 isolates, six (9.8%) did not harbor any of the virulence-encoding genes analyzed in this study; for the others, the following combination was observed: 18 (29.5%)*icaA* and *lukS-PV/lukF-PV* genes; 7 (11.5%) *icaA* and *tst* genes; and 7 (11.5%) *icaA*, *tst* and *lukS-PV/lukF-PV* genes. Twenty isolates (32.8%) harbored only the *icaA* gene, and two (3.3%) and one (1.6%) isolates harbored only the *lukS-PV/lukF-PV* and *tst* genes, respectively. There was no significant difference ($p< 0.05$) between MSSA and MRSA isolates, regarding their profile of the virulence-encoding genes analyzed in this study (Table2 and Supplementary File Table S2). Data from *S. aureus* carrying the *icaA*, *lukS-PV/lukF-PV*and *tst*genes in Brazilian children are sparse, andin general, the surveys were carried out in MRSA isolates.

The study of Neves *et al*. (2019) reported a prevalence of 20.4% (10/49) of MRSA carrying the *lukS-PV/lukF-PV* genes colonizing children attending public or private pediatric clinics for primary care in Niteroi, Rio de Janeiro.Another study conducted in the same city, reported a prevalence of 26.4% (38/144 isolates) of MRSA carrying the *lukS-PV/lukF-PV* genes colonizing children and adolescents attending the daycare centers, outpatient clinics and hospitals. Additionally, these MRSA strains were more frequently isolated from children at hospitals, compared to the other healthcare settings (Neto *et al*., 2020). High prevalence of *S. aureus* carrying the *icaA*, *lukS-PV/lukF-PV* and*tst* genes is a matter of concern as *S. aureus* carriers are at risk of subsequent endogenous staphylococcal infections (Cavalcante *et al*., 2015; Young *et al*., 2017; Thomsen *et al*., 2019). Moreover, infections caused by S. aureus carrying these genes have been associated with poor outcome (Otto, 2018; Gillet *et al*., 2018; Kim *et al*., 2019). The *icaA* gene is a member of the *icaADBC* operon that encodes the biosynthesis of the polysaccharide intercellular adhesin (PIA),also called poly-N-acetylglucosamine (PNAG) (Cramton *et al*., 1999). This adhesinparticipates in the biofilm maturation step, promoting intercellular aggregation and adhesion to abiotic surfaces (Rohde *et al*., 2010). However, *ica*-independent biofilm formation mechanism by *S. aureus* has also been described (Fitzpatrick *et al*., 2005). Biofilm formation on biotic or abiotic (such as implanted medical devices) surfaces contributes to the success of *S. aureus* as a colonizer or a pathogen. This mode of growth confers some advantages to bacteria, such as protection against defense molecules of the immune system, and resistance to antimicrobial agents. Therefore, infections associated with biofilm formation are difficult to treat, contributing to the bacterial persistence in the host (Otto, 2018), increasing both the treatment costs and mortality rates (Song *et al*., 2010; Yousif *et al*., 2015).

Besides biofilm formation,which contributes to evasion of immune defenses, *S. aureus*produces toxins that kill phagocytes, avoiding being eliminated by these cells. The *lukS-PV/lukF-PV* genes play an important role in this process; these genes encode the bi-component pore-forming toxin Panton-Valentine Leukocidin (PVL) that targets and lysis polymorphonuclear leukocytes, monocytes, and macrophages (Kaneko; Kamio, 2004). The study of Jenkins *et al*. (2015) showed that the gene *lukF-PV* was upregulated in a murine model of bacteremia compared to a nasal colonization model, indicating an active mechanism of bacterial evasion of the host immunedefense. Moreover, *S. aureus* carrying the *lukS-PV/lukF-PV* geneshas been associated with severe necrotizing pneumonia (Gillet *et al*., 2018). Importantly, these genes are carried by lysogenic bacteriophages, and thus can be disseminated to different *S. aureus*strains by horizontal transfer(Kaneko; Kamio, 2004). The gene *tst* encodes the toxic shock syndrome toxin (TSST-1), a potent exotoxin of the superantigen family. This toxin activates T lymphocytes, inducing the overproduction of proinflammatory cytokines that causes systemic inflammation and shock (Xu; McCormick, 2012). Infections caused by *S. aureus*carrying the *tst* gene are also potentially fatal (Kim *et al*., 2019). This gene is located on a pathogenicity island, and can be transferred among different strains by a helper bacteriophage (Novick; Ram, 2017).

MRSA isolates typing: The genetic relatedness of *mecA*-positive MRSA isolates was analyzed by rep-PCR, and a high genetic diversity among them was observed. The banding patterns were automatically sized and all isolates with the same type were recognized to be identical. The level of similarity between the rep-PCR fingerprinting of the MRSA isolates ranged from 85%. Clusters

analysis, by using a cutoff value of 85% (de Oliveira *et al*., 2015), revealed that most of the MRSA isolates were distributed into minor groups. Eight isolates exhibited unique fingerprinting patterns. Clusters D, I, J, L and M harbored two isolates each, and cluster K harbored three isolates (Figure 2). According to the SCC*mec* typing, the 21 *mecA*-positive MRSA isolates were distributed into four types as follows (Figures 1 and 2): 10 (47.6%) harbored the SCC*mec*type IV; three (14.3%), two (9.5%) and one (4.8%) isolates harbored the types I, II and V, respectively; five (23.8%) isolates were classified as NT.

The SCC*mec* type IVhas been frequently identified in MRSA isolated from colonized healthy children (Lamaro-Cardoso *et al*., 2009; Carvalho *et al*., 2017; Neves *et al*., 2019; Neto *et al*., 2020). Compared to the other SCC*mec* types, the type IV has the smallest cassette structure, combining a class B *mec* gene complex with a type 2 *ccr* gene complex, and the transposon Tn*4001* into the J3 region (Ma *et al*., 2002). Due to its smaller size, no antimicrobial resistance marker other than *mecA* was identified in SCC*mec* IV (Ma *et al*., 2002), which is consistent with low rates of resistance to several *non*-beta-lactam antimicrobials observed in MRSA harboring this cassette. However, among the 10 MRSA SCC*mec* type IV of the present study, six were classified as multidrug-resistant (Figure 2): three were isolated from children with history of hospitalization (*n*=2) and consumption of antimicrobial agents (*n*=1) in the last six months; the others were isolated from children with no descriptive variables analyzed in the present study (Supplementary file Table S3).

To gain insight concerning the sequence type (ST) and the clonal complex (CC) of MRSA *mecA*-positive SCC*mec* IV, which was the most prevalent characteristic among MRSA isolates observed in the present study, seven were selected according to their antimicrobial susceptibility, virulence-encoding genes and rep-PCR fingerprinting profiles (Table 3). According to the HRM analysis of SNPs (Lilliebridge*et al*.,2011), five ST belonging to four CC were detected, which include one MRSA isolate each of ST4/CC45, ST6/CC5, ST328/CC5 and ST873/CC873 MRSA. Three MRSAs harbored the ST30 and belonged to CC30 (Table 3).Neto *et al*. (2020) reported that SCC*mec* IV/ST5/CC5 and SCC*mec* IV/ST30/CC30 were the predominant MRSA strains colonizing children and adolescents attending the daycare centers, outpatient clinics and hospitals in Niteroi, Rio de Janeiro.

Although a limited number of MRSA isolates were analyzed in this study, most MRSASCC*mec* IV(Table 3) belonged to two of the major CC [CC5 (two isolates) and CC30 (three isolates)] frequently detected worldwide (Lakhundi; Zhang, 2018). Importantly, theseMRSAswere classified as multidrug-resistant, except the C70 isolate that was resistant only to beta-lactam antimicrobials.Besides, all five isolates harbored the *icaA* gene in combination with *lukS-PV*/*lukF-PV* and/or *tst* genes. Indeed, CC5 MRSA has already been detected in patients with bacteremia admitted to the hospital of the present study (Duarte *et al*., 2018). However, CC30 MRSA was first detected in our community. As SCC*mec* IV-ST30, which is typically associated with community-associated MRSA (CA-MRSA)Mediavilla*et al*., 2012), has also been detected in healthcare-associated infections (Song *et al*., 2011), we must be alert to the possibility of this strain “invading” our hospital environment.

In fact, CA-MRSA isolates were initially distinguished through particular features that differed from those of HA-MRSA isolates, including low rate of resistance to various *non*-beta-lactam antimicrobials to which HA-MRSA was commonly resistant; predominance of SCC*mec* types IV and V; and a number of virulence factors and toxins, especially the Panton-Valentine leukocidin (PVL), which is consistent with the increased virulence in animal models (Li *et al*., 2010;Otter; French, 2012). However, CA-MRSA isolates have emerged in hospitals, posing a major concern due to the severity of infections and the acquisition of multidrug-resistance (Lakhundi; Zhang, 2018). These characteristics were observed in MRSA isolated from children of the present study, reinforcing the importance of continuous monitoring *S. aureus* carriage in these subjects.

CONCLUSION

A high prevalence of multidrug-resistant and potentially virulent *S. aureus*colonizing children was detected in this study. In addition, OS-MRSA carriers were also identified among this population. Altogether, these findings provide important new data for the development of strategies to control staphylococcal infections in this population and their spread within the community and the hospital environments.

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