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Original Article

Comparison of HRM analysis and three REP-PCR genomic fingerprint methods for rapid typing of MRSA at a Brazilian hospital

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Abstract

Introduction: Infections caused by multidrug-resistant bacteria are increasingly common and represent a serious problem for public health. *Staphylococcus aureus* is one of the major agents of infections, and methicillin-resistant *S. aureus* (MRSA) has spread worldwide. The aim of this study was to phenotypically and genotypically characterize 55 MRSAs isolated in the University Hospital of Londrina, Paraná, Brazil, during 2010.

Methodology: Bacterial isolates were characterized based on their antimicrobial susceptibility profile, biofilm production capacity, and staphylococcal chromosome cassette *mec* (SCC*mec*) type. Determination of clonal groups was performed by polymerase chain reaction using the RW3A, JB1, and BOX A1R primers and high-resolution melting (HRM) analysis.

Results: The majority of isolates harbored SCC*mec* type II. SCC*mec* III, characteristic of the Brazilian endemic clone, was observed in four strains. Only two isolates harbored SCC*mec* type IV, which is common in community-acquired MRSA strains. Most isolates also showed resistance to more than four of the tested antimicrobials, and 30 isolates exhibited the ability to produce biofilm. DNA polymorphism analysis showed a higher discriminatory power for the JB1 primer, but RW3A revealed several clonal groups of MRSA with similar genotypic and phenotypic characteristics. HRM analysis showed eight different sequence types.

Conclusions: These results are important for epidemiological studies involving MRSA infections.

Key words: antimicrobial; MRSA; genotypic profile; *Staphylococcus aureus*

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Introduction

Staphylococcus aureus is a major cause of infections in both healthcare facilities and the community, causing pneumonia, bacteremia, endocarditis, osteomyelitis, abscesses, and septic arthritis.

Since the introduction of methicillin in 1960, outbreaks due to different clones of methicillin-resistant *Staphylococcus aureus* (MRSA) have occurred in different regions [1-7], contributing to the persistence of MRSA as an important pathogen worldwide [8].

The Centers for Disease Control and Prevention (CDC) of the United States reported that more than 80,000 illnesses and 11,000 deaths in the hospital setting were caused by MRSA during 2011. Compared to earlier periods, there was a 31% decline in global rates of invasive MRSA infections [2]. The largest decrease (54%) was observed in infections in patients during hospitalization; this was due to preventive measures, which are important for controlling outbreaks [2].

SCC*mec* elements are highly diverse in their organizational structure and gene content, allowing their discrimination into types and subtypes. SCC*mec* types I to XI have been described [9-17]. The SCC*mec* elements vary in size from 20 kb to 67 kb, but the *mec*A region (2 kb) represents only a small proportion of the SCC*mec* chromosomal cassette [16,18].

Both healthcare-associated MRSA (HA-MRSA) and community-acquired MRSA (CA-MRSA) exhibit resistance to beta-lactam antimicrobials. However, CA-MRSA strains are typically less resistant to other

classes of antimicrobials. Oxacillin resistance is encoded by the *mec*A gene, which is located on the staphylococcal chromosome cassette *mec* (SCC*mec*). Overall, HA-MRSA strains are classified as SCC*mec* I, II, or III, whereas CA-MRSA strains containing SCC*mec* types are generally IV, V, and VI. CA-MRSA strains may produce Panton-Valentine leukocidin (PVL) toxin, which has been associated with skin abscesses and necrotizing pneumonia [1].

The genetic relatedness of MRSA isolates has been analyzed by different methodologies. The gold standards for this analysis are pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and *spa* typing, but these techniques are expensive, time consuming, laborious, and not accessible to all research laboratories [19-21].

Polymerase chain reaction (PCR)-based methods for analyzing the genetic relatedness of MRSA strains offer the advantage of being faster and easier to perform [22]. In general, repetitive sequences with unknown function are present in genomic DNA from different bacterial species [23], and these regions have been used to determine the DNA genetic similarity between several bacterial species, including MRSA [24-26].

The RW3A, JB1, and BOX primers were initially used to evaluate the clonality of some bacteria. The RW3A primer was derived from a *Mycoplasma pneumoniae* repetitive sequence (RepMP3) [27] and has been applied in studies with MRSA [25,26]. The JB1 primer was sequenced from *Enterococcus faecium* and found to be useful for differentiating *E. faecalis* strains [28], another bacteria that can transfer resistance encoding genes to *S. aureus.* BOX elements contain sub-sequences that are differentially conserved, representing the first repeating element from *Streptococcus pneumoniae* [24], and have been used to characterize different species of *Staphylococcus* [29].

High-resolution melting (HRM) analysis of single-nucleotide polymorphisms (SNPs) of small fragments derived from the MLST database has been also used for genotyping MRSA. This technique was described by Lilliebridge *et al.* [30], performed with real-time PCR with small fragments derived from MLST and is capable of verifying the MRSA sequence type [31].

Some advantages of the techniques used in these experiments are their low cost, rapidity, and use of the instrumentation available in laboratories.

The aim of this study was to characterize MRSA isolates from patients seen at the University Hospital of Londrina, Paraná, Brazil, during 2010 using phenotypic and genotypic methods. All isolates were tested for antimicrobial susceptibility, SCC*mec* typing, and biofilm-forming capacity. In addition, the genetic diversity between the isolates was analyzed by PCR-based methods using SNP DNA as the target, and HRM analysis.

Methodology

Bacteria

MRSA isolates were isolated from patients hospitalized at the University Hospital of Londrina between January and September 2010. The bacteria were deposited in the bacterial collection of the Department of Pathology, Clinical and Toxicological Analysis from University Hospital of Universidade Estadual de Londrina (UH/UEL). A total of 55 isolates were obtained from blood (n = 15), urine (n = 4), tracheal aspirates (n = 9), and general discharge (n = 27), such as tissue secretion, bone fragment, and peritoneal fluid. MRSA BEC 9393 [32] and N315 [33] isolates were provided by Dr. Agnes Marie Sá Figueiredo (Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil) and Dr. Elsa Masae Mamizuka (Universidade de São Paulo, São Paulo, Brazil), respectively. Methicillin-sensitive *S. aureus* (MSSA) ATCC 25923 and 29213 were used as reference strains. These reference strains were provided by Dr. Marcelo Brocchi (Universidade Estadual de Campinas, Campinas, SP, Brazil). All bacteria were stored at -80°C in brain-heart infusion (BHI) (Difco, Detroit, USA) plus 25% glycerol (Sigma, Poole, UK).

The protocols of this study were in accordance with the National Research Council and were approved by the ethics and human research of UEL (protocol 186/09 CEP/UEL).

Species identification and antimicrobial susceptibility profile

Species identification and antimicrobial susceptibility profiling were performed using an automated method with the MicroScan system (Siemens, Frimley, Camberley, UK), except for vancomycin (VAN), which was evaluated using minimum inhibitory concentration (MIC) by the broth-dilution method according to the Clinical Laboratory Standards Institute (CLSI, 2013) [34]. MicroScan system shown breakpoints that were reported based on CLSI 2013 breakpoints. Species identification was confirmed by PCR using specific primers for the *coa* (coagulase) gene (*coa* F 5'GGGATAACAAAGCAGATGCGATAG 3' and *coa* R 5' ACGTTGATTCAGTACCTTGTGG 3') according to Tiwari *et al*. [35]. The following antimicrobials were tested: erythromycin (ERY),

sulfamethoxazole/trimethoprim (SXT), clindamycin (CLI), ciprofloxacin (CIP), gentamicin (GEN), tetracycline (TET), rifampin (RIF), and linezolid (LNZ); cefoxitin (FOX) was used to define methicillin-resistant *S. aureus* (MRSA). All antimicrobial testing was performed in the MicroScan system (Siemens, Frimley, Camberley, UK).

For urine isolates, CLI and ERY were not evaluated because they are not used for the treatment of MRSA urinary tract infections.

These tests were in accordance with the criteria established by the CLSI [34]. *S. aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 51299 were used as controls.

MRSA typing

SCC*mec* typing and the *mec*A gene of all MRSA isolates was performed by multiplex PCR assay, as described by Milheiriço *et al.* [36]. 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 controls. These control strains were provided by Dr. Elsa Masae Mamizuka (Universidade de São Paulo, São Paulo, Brazil).

DNA extraction

Total DNA was extracted using alkaline lysis. Bacteria were grown in 3 mL BHI (Difco, Detroit, USA) and incubated at 37°C for 24 hours. The bacterial pellets obtained after centrifugation at 10,000 × g for 5 minutes were resuspended in TE buffer (Tris-EDTA) (Sigma, Poole, UK). Reagents used for genomic DNA extraction were TE buffer (Sigma, Poole, UK), 10 µg/mL lysozyme (Invitrogen, Carlsbad, USA), 10% (w/v) sodium dodesyl sulfate (SDS) (Sigma, Poole, UK), 20 mg/L proteinase K (Invitrogen, Carlsbad, USA), 5M NaCl (Sigma, Poole, UK), CTAB (Sigma, Poole, UK)/NaCl solution, 25:24:1 phenol/chloroform/isoamyl alcohol (Invitrogen, Carlsbad, USA), ethanol (Sigma, Poole, UK) and 70% ethanol according to the procedures described in Sambrook and Russell [37], and were stored at -20°C until use.

Repetitive DNA sequence genotyping

The genetic diversity of the MRSA isolates was analyzed by PCR using RW3A (5'TCGCTCAAAACAACGACACC3') [25], JB1 (5'GATTTTATGGCCGTCCGC3') [28], and BOX A1R (5' CTACGGCAAGGCGACGCTGACG3') [24] primers, as described previously. These primers amplify repetitive DNA found in different regions of the bacterial genome.

The PCR products and a molecular DNA weight marker X (0.07–12.2 kb) (Roche, Indianapolis, USA) were separated by electrophoresis in 2% agarose gel (Invitrogen, Carlsbad, USA) at 75 V and visualized on a UV transilluminator after staining with Gel Red (Biotium, Hayward, USA).

The cluster analysis was performed using BioNumerics version 4.6 (Applied Mathematics, Kortrijk, Belgium) using the UPGMA algorithm and the Jaccard coefficient (J) [38] with a tolerance of 3%.

High-resolution melting PCR method

The isolates used for this analysis were chosen based on different genotypes obtained from RW3A and JB1 primers analysis. One to three samples from each clonal group were selected. The real-time PCR reaction and HRM analysis were done according to Lilliebridge *et al*. [30]. The SNPs of fragments derived from *arcC*78/210, *aroE*88/155, *gmk*286, *pta*294, *tpi*36, and *tpi*241/243 were analyzed following the methodology described by Lilliebridge *et al*. [30]. The HRM analysis was performed using the Rotor-Gene Q 5Plex HRM System (Qiagen, West Sussex, UK) using a Type-it HRM PCR Kit (Qiagen, West Sussex, UK). For each separate run, three control strains with known ST type (ST239, ST5, and ST243) were included as melting curve standards. Reactions were carried out in duplicate.

The melting curves of all samples were generated automatically by the Rotor-Gene Q software, version 2.1.0.9 (Qiagen, West Sussex, UK) and normalized using the default setting. HRM difference plot was also generated by the software, which could give a better comparison of melting temperatures among different MRSA ST types.

Biofilm test on a polystyrene surface

The MRSA isolates were tested for biofilm production on a polystyrene surface stained with crystal violet according to Stepanovic *et al*. [39]. MRSA BEC 9393 and culture medium alone were used as the positive and negative controls, respectively. The optical density (OD) of each well was measured at 570 nm using an automated plate reader (Synergy HT, Biotek, Winooski, USA).

The mean value of the OD of the negative control (ODc) was used for comparison with those obtained from the isolates (ODi). The ODi of each isolate was obtained by the mean minus the ODc. The isolates were

classified as non-biofilm producers when ODi ≤ ODc and as biofilm-producers when ODi > ODc.

Statistical analysis

Significant differences among the results obtained were examined using the Fisher method. Differences were considered significant at $p < 0.05$ using BioEstat version 5.0 software.

Results

Patients, antimicrobial susceptibility profile, and species identification

The age of the patients enrolled in this study ranged from three months to 79 years (median of 48 years), and the majority of them were men (n = 43; 78.18%).

All MRSA isolates in this study showed the presence of *coa* and *mec*A genes, FOX resistance, and LNZ susceptibility. Among the other antimicrobial agents tested, resistance to CIP (94.5%), ERY (94.1%), and CLI (92.2%) was most prevalent in these isolates. However, the percentages of SXT (29.1%), RIF (23.6%), and TET (16.4%) resistance were lower than that of the other antimicrobials.

Based on the antimicrobial resistance profiles (number and type of antimicrobials), seven antibiotypes (A, B, C, D, E, F, and G) were established for these isolates. Antibiotype A was resistant to CIP, CLI, ERY, GEN, SXT, TET; antibiotype B was resistant to CIP, CLI, ERY, GEN, SXT; antibiotype C was resistant to CIP, CLI, ERY, GEN, RIF; antibiotype D was resistant to CIP, CLI, ERY, GEN; antibiotype E was resistant to CIP, CLI, ERY, RIF; antibiotype F was resistant to CIP, CLI, ERY; antibiotype G was resistant to ERY; and antibiotype O was resistant to CIP and some others.

Antibiotype A, representing the more resistant isolates (resistant to seven antimicrobials), was observed in 14.5%; antibiotype G, the less resistant isolate (two antimicrobials), was observed in 3.7%. Uncharacteristic profiles that were not grouped into any of the seven antibiotypes were designated by the letter O (Table 1). Other antibiotypes and their percentages were determined: B (9.1%), C (5.5%), D (21.8%), E (16.4%), F (14.5%), and O (14.5%).

Most isolates were susceptible to vancomycin (42/55; 76.36%), and among them, 3.63% (2/55), 1.81% (1/55), 34.54% (19/55), and 36.36% (20/55) had MIC values of 0.25, 0.5, 1.0, and 2.0 µg/mL, respectively. Of 55 isolates, 13 were intermediately resistant to vancomycin, based on CLSI 2013 criteria. Among these, 92.30% (12/13) and 7.69% (1/13) of isolates showed MIC values of 4 and 8 µg/mL, respectively.

SCCmec typing

SCC*mec* type II was the most prevalent, being present in 24 isolates, followed by SCC*mec* type I, which was found in 19 isolates. SCC*mec* types III and IV were found in 4 and 2 isolates obtained from general discharges, respectively (Table 1). Four isolates (MRSA 102, 109, 119, and 122) showed SCC*mec* type III, equivalent to MRSA BEC9393, and these isolates showed the A antibiotype. Six isolates were not typeable by the technique used here.

Clustering of genotypes of SNPs

Applying HRM analysis, eight different STs between 21 isolates were found. ST5 was the most frequent and was present in 10 isolates, displaying the same ST of *S. aureus* N315. STs from control were based on reference strains. Results of all isolates are shown in Table 2.

Although few isolates had been selected from each clonal group detected in the RW3A-JB1 methodology, there was no correlation with the clonal groups in the HRM methodology.

Biofilm

Among the 55 isolates, 30 produced biofilm on the polystyrene surface, and none of the seven catheter isolates showed biofilm production (Table 1). Some isolates produced more biofilm than others, with results that were four times greater than ODc, being the strongest biofilm producers. MRSA 112 produced the largest amount of biofilm (data not shown). All strains with antibiotypes A (except MRSA 122), B, and C produced biofilm; however, MRSA 118 strain with antibiotype E showed biofilm production. All SCC*mec* NT isolates produced biofilm (Table 1).

Analysis of genetic relatedness

Genetic relatedness between the MRSA isolates was analyzed by three PCR methods using complementary primers to repetitive DNA sequences in the bacterial genome. Using a cutoff value of 80% similarity, primer JB1 showed the highest discriminatory power compared with the other primers. Fourteen genotypes were discriminated by JB1, eleven by RW3A, and nine by BOX A1R. Comparing primers RW3A and JB1 using the same cutoff value, eleven genotypes were discriminated, with agreement for a few isolates (Figure 1 and Table 1).

Figure 1. Profile of similarity among isolates of methicillin-resistant *Staphylococcus* aureus (MRSA) using a combination of the RW3A and JB1 primers.

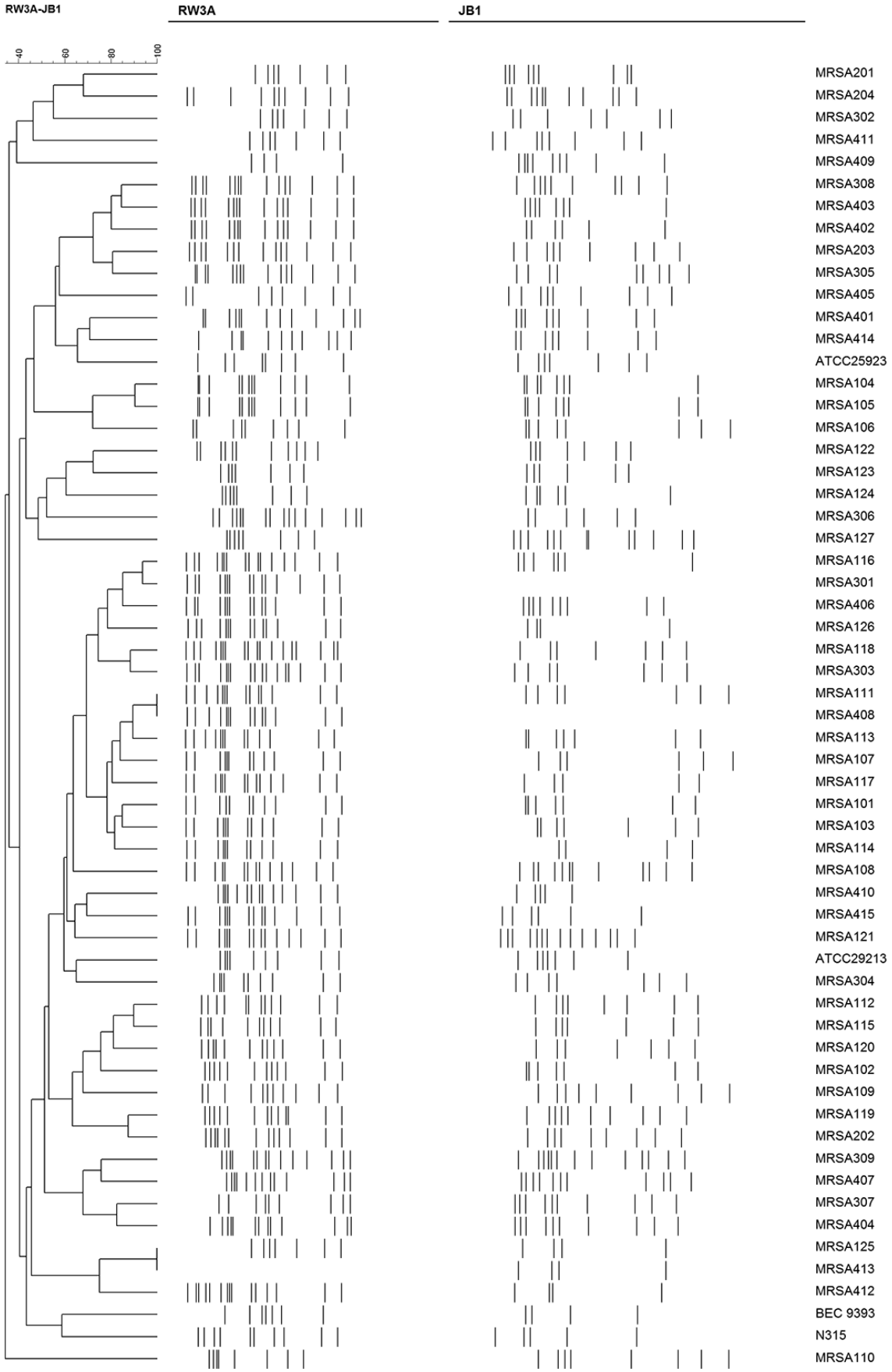

Table 1. Characteristics of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from patients seen at the University Hospital of Londrina-PR: SCC*mec* type, antibiotype, genotype, and biofilm production.

								GENOTYPE				
MRSA	**Age**	**Gender**	**Isolation date**	**SCC *mec***	**Source**	**Antibiotype**	**Vancomycin (µg/mL)**	**RW3A**	**JB1**	**BOX A1R**	**RW3A-JB1**	**Biofilm production**
MRSA414	48	M	08/05	I	Blood	B	1.0	SI	2G	3I	SI	+
MRSA107	22	M	02/10	I	General discharge	B	8.0	1B	2B	SI	4F	+
MRSA126	35	M	07/28	I	General discharge	B	1.0	1B	SI	3I	4D	+
MRSA402	46	M	03/08	I	Blood	C	2.0	1H	SI	3I	4A	+
MRSA309	55	F	01/18	I	Tracheal aspirates	C	1.0	1F	2I	3F	SI	+
MRSA401	25	M	02/03	I	Blood	D	2.0	SI	2G	3F	SI	-
MRSA406	75	M	06/28	I	Blood	D	2.0	1B	2E	3I	4D	-
MRSA413	48	M	08/09	I	Blood	D	4.0	∞	2K	∞	4K	-
MRSA103	59	F	01/30	I	General discharge	D	1.0	1B	SI	SI	4G	+
MRSA125	34	M	06/30	I	General discharge	D	2.0	1I	2K	3I	4K	-
MRSA127	16	F	09/16	I	General discharge	D	2.0	1K	SI	3I	SI	+
MRSA104	16	M	01/27	I	General discharge	D	1.0	1G	2F	3B	4C	-
MRSA116	26	M	04/01	I	General discharge	D	0.5	1C	2F	SI	4D	-
MRSA121	58	M	05/21	I	General discharge	D	0.25	1C	2N	SI	SI	-
MRSA403	47	M	04/27	I	Blood	D	2.0	1H	2E	3E	4A	-
MRSA123	26	M	06/04	I	General discharge	D	4.0	SI	2L	3G	SI	+
MRSA404	79	M	05/24	I	Blood	O	2.0	1F	2G	3D	4J	-
MRSA117	47	M	04/09	I	General discharge	O	1.0	1C	SI	SI	4F	-
MRSA203	58	M	03/03	I	Urine	O	2.0	1H	2G	3G	4B	+
MRSA120	1	F	04/19	II	General discharge	A	1.0	1D	SI	SI	4H	+
MRSA302	69	M	05/03	II	Tracheal aspirates	C	2.0	SI	SI	3I	SI	+
MRSA405	72	M	07/05	II	Blood	E	4.0	1J	2G	3H	SI	-
MRSA408	67	M	07/29	II	Blood	E	4.0	1B	∞	3D	4F	-
MRSA415	67	M	08/02	II	Blood	E	1.0	1C	2M	3I	SI	-
MRSA409	67	M	07/29	II	Blood	E	1.0	SI	2E	3D	SI	-
MRSA407	72	M	07/09	II	Blood	E	1.0	1F	SI	3H	4J	-
MRSA113	30	M	03/01	II	General discharge	E	4.0	1B	2A	3A	4F	-
MRSA114	26	M	03/09	II	General discharge	E	2.0	1B	SI	3A	4G	-
MRSA118	36	M	05/13	II	General discharge	E	4.0	1C	SI	SI	4E	+
MRSA411	NA	M	07/08	II	Blood	E	1.0	1I	SI	3I	SI	-
MRSA412	69	F	08/09	II	Blood	F	1.0	1B	2K	∞	SI	-
MRSA101	59	F	01/18	II	General discharge	F	4.0	1B	2A	SI	4G	+
MRSA105	35	M	02/02	II	General discharge	F	4.0	1G	2A	3A	4C	+
MRSA106	3m	M	02/06	II	General discharge	F	0.25	SI	2B	SI	SI	-
MRSA111	54	F	02/26	II	General discharge	F	4.0	1B	2B	SI	4F	-
MRSA124	46	M	06/14	II	General discharge	F	4.0	1K	2A	3G	SI	-
MRSA108	59	F	02/08	II	General discharge	F	1.0	1C	2I	3B	SI	-
MRSA307	45	M	08/16	II	Tracheal aspirates	F	1.0	SI	2G	3G	4J	+
MRSA304	51	F	05/04	II	Tracheal aspirates	O	2.0	1A	2J	3I	SI	+

Table 1 (continued). Characteristics of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from patients seen at the University Hospital of Londrina-PR: SCC*mec* type, antibiotype, genotype, and biofilm production.

MRSA	Age	Gender	Isolation date	SCC *mec*	Source	Antibiotype	Vancomycin (µg/mL)	GENOTYPE				Biofilm production
								RW3A	JB1	BOX A1R	RW3A-JB1	
MRSA301	54	F	02/04	II	Tracheal aspirates	O	4.0	1C	∞	3I	4D	+
MRSA202	37	M	02/19	II	Urine	O	2.0	1D	2H	3I	4I	+
MRSA204	77	M	04/21	II	Urine	O	2.0	1J	2N	3I	SI	+
MRSA201	69	M	01/21	II	Urine	O	2.0	1I	SI	3I	SI	+
MRSA122	59	M	05/21	III	General discharge	A	4.0	SI	2L	3C	SI	-
MRSA119	31	M	04/19	III	General discharge	A	1.0	1D	2H	SI	4I	+
MRSA102	63	M	01/28	III	General discharge	A	1.0	1D	2A	SI	SI	+
MRSA109	31	M	02/25	III	General discharge	A	1.0	1D	2D	3C	SI	+
MRSA410	1	M	05/17	IV	Blood	G	2.0	SI	SI	3I	SI	-
MRSA308	20	F	09/17	IV	Tracheal aspirates	G	1.0	1H	SI	3G	4A	+
MRSA112	37	M	03/03	NT	General discharge	A	2.0	1E	2C	SI	4H	+
MRSA303	75	F	03/01	NT	Tracheal aspirates	A	2.0	1C	2J	3I	4E	+
MRSA306	77	M	06/28	NT	Tracheal aspirates	A	2.0	SI	2L	3I	SI	+
MRSA110	31	M	02/25	NT	General discharge	B	2.0	SI	2D	SI	SI	+
MRSA115	67	M	03/09	NT	General discharge	B	1.0	1E	2C	3C	4H	+
MRSA305	79	M	05/24	NT	Tracheal aspirates	D	2.0	1H	2J	3I	4B	+

M: male; F: female; NA: not available; NT: not typeable; Antibiotype A (resistant to CIP, CLI, ERY, GEN, SXT, TET); antibiotype B (resistant to CIP, CLI, ERY, GEN, SXT); antibiotype C (resistant to CIP, CLI, ERY, GEN, RIF); antibiotype D (resistant to CIP, CLI, ERY, GEN); antibiotype E (resistant to CIP, CLI, ERY, RIF); antibiotype F (resistant to CIP, CLI, ERY); antibiotype G (resistant to ERY); antibiotype O (resistant to CIP and some others); Vancomycin: ≤ 2 = susceptible; 4–8 = intermediately; ≥ 16 = resistant (CLSI, 2013); RW3A genotype: 1A, 1B, 1C, 1D, 1E, 1F, 1G, 1H, 1I, 1J, 1K; JB1 genotype: 2A, 2B, 2C, 2D, 2E, 2F, 2G, 2H, 2I, 2J, 2K, 2L, 2M, 2N; BOX A1R genotype: 3A, 3B, 3C, 3D, 3E, 3F, 3G, 3H, 3I; RW3A-JB1 genotype: 4A, 4B, 4C, 4D, 4E, 4F, 4G, 4H, 4I, 4J, 4K; Single isolate (SI): unique clonal isolate. Strain not amplified (∞); Biofilm non-producer (-); Biofilm producer (+).

The genotypes obtained with the RW3A primer were designated 1A–1K. Seven isolates were 100% similar; thirteen clonal groups with only one isolate showing similarity of more than 80% were designated as single isolates (SIs). The genotypes obtained by JB1 were designated 2A–2N. Eleven isolates were 100% similar, and eighteen isolates were considered SIs. The genotypes obtained using BOX A1R were designated 3A–3I. Thirty-one strains were 100% similar, and thirteen isolates were considered SIs. Based on the RW3A-JB1 dendrogram, the genotypes obtained were designated by 4A–4K. Eleven isolates were 100% similar, and eighteen isolates were considered SIs. Most isolates within a given clonal group showed different phenotypes and SCC*mec* types.

MRSA BEC 9393 showed 45% similarity with 30 isolates using the RW3A primer. Based on the RW3A and JB1 primers, MRSA BEC 9393 and N315 showed 50% similarity with 33 isolates.

There was no relationship between the genotyping groups based on the three primers with the SCC*mec* types; the presence of certain types of SCC*mec* was not characteristic of a specific clonal group.

Discussion

MRSA is commonly characterized as having multiple resistances to different antibiotics.

Studies conducted by SENTRY during 2005 to 2008 showed that MRSA strains were resistant to CIP (93%), CLI (89%), ERY (94%), SXT (68%), and TET (47%), with a high susceptibility to antimicrobial LNZ (99%) and vancomycin (100%) [40].

Currently, MRSA is considered the most common multidrug-resistant microorganism in hospitals [41]. In Brazil, the prevalence of MRSA isolates ranges from 40% to 80% [40]. A study conducted in the city of São Paulo showed the variation in susceptibility profiles, with MRSA isolates showing susceptibility to CIP (42%), CLI (55%), ERY (15%), SXT (55%), GEN (37%), RIF (78%), and TET (64%) [42]. As these results are different from those of our study, the therapeutic approach in relation to the use of

antimicrobials in hospitals may vary based on location (city, state, and country).

In our study, we also found high resistance to CIP, CLI, and ERY and susceptibility to LNZ. Conversely, resistance to SXT, RIF, and TET was lower. The predominant antibiotic used to treat MRSA infections at the Hospital UH/UEL is vancomycin. We observed that the majority of MRSA strains from blood (66.7%) were antibiotype D (26.7 %) or E (40,0 %) (Table 1). All isolates from urine were antibiotype O (Table 1). All MRSA strains with antibiotype D had SCC*mec* I, except for one that had non-typeable SCC*mec*. All MRSA strains with SCC*mec* III and IV were antibiotype A and G, respectively (Table 1). We did not find correlation of antibiotypes with patients' ages.

Although the Brazilian endemic clone (BEC) with SCC*mec* type III has been shown to be the clone that is spread in Brazil, this was not the SCC*mec* type found most frequently in our isolates; indeed, SCC*mec* type II was most prevalent, according to another study described by Oliveira *et al*. [43]. Furthermore, the analysis of DNA polymorphism performed in our study showed a low genetic similarity (45%) of strain BEC9393 with most of the strains studied. Researchers from southern Brazil analyzed isolates from a hospital in Porto Alegre and found the presence of SCC*mec* type I, which is related to the Cordoba/Chilean clone, warning of its possible spread, replacing BEC [44]. A study with blood strains collected from patients at the Hospital das Clínicas in São Paulo, Brazil, showed predominantly SCC*mec* type II [45]. Our results and previous research show that epidemiological studies involving clonality of MRSA should be more common because new clones, including different antimicrobial resistance profiles, may arise within a period of years.

Two isolates (MRSA 410 and MRSA 308) showed SCC*mec* type IV, a type that is not common in healthcare-associated infections and is characteristic of

Table 2. High-resolution melting (HRM) analysis profile of single-nucleotide polymorphisms of methicillin-resistant *Staphylococcus aureus*.

	HRM fragments						
S. aureus	***arcC78/210***	***aroE88/155***	***gmk286***	***pta294***	***tpi36***	***tpi241/243***	**ST**
ATCC29213	53	23	12	43	66	43	5
N315	53	23	12	43	66	43	5
MRSA 101	53	23	12	43	66	43	5
MRSA 105	53	23	12	43	66	43	5
MRSA 118	53	23	12	43	66	43	5
MRSA 203	53	23	12	43	66	43	5
MRSA 301	53	23	12	43	66	43	5
MRSA 308	53	23	12	43	66	43	5
MRSA 404	53	23	12	43	66	43	5
MRSA 406	53	23	12	43	66	43	5
MRSA 408	53	23	12	43	66	43	5
MRSA 410	53	23	12	43	66	43	5
MRSA 125	52	23	13	43	65	42	27
MRSA 115	51	23	13	44	65	43	128
MRSA 120	51	23	13	44	65	43	128
BEC9393	51	23	13	44	65	43	239
ATCC25923	51	24.5	12	45	65	44	243
MRSA 202	51	23	13	44	65	42	368
MRSA 102	52	23	13	45	65	43	486
MRSA 107	53	23	12	43	66	42	835
MRSA 403	53	23	12	43	66	42	835
MRSA 110	51	24.5	13	45	65	43	863
MRSA 112	51	24.5	13	45	65	43	863
MRSA 119	51	24.5	13	45	65	43	933
MRSA 122	51	24.5	13	45	65	43	933

ST: sequence type.

CA-MRSA. In addition, these isolates showed resistance to ERY. The presence of these isolates in this hospital suggests that the spread of MRSA may occur between the two environments (community and hospital).

Most isolates showed the ability to produce biofilm, which might represent a greater difficulty in controlling MRSA infection. Six strains with *mec*A but different SCC*mec* types, I to IV (NT), all produced biofilm, showing that this important feature would be present in strains or new MRSA clones.

According to the dendrograms evaluating the similarity among the isolates, RW3A revealed the presence of 17 isolates (30.9%) with 100% similarity distributed among different clonal groups. However, the strains did not exhibit the same phenotypes, being resistant to different classes of antimicrobials according to another study [26]. This result indicates the existence of identical or similar genetic profiles, but we cannot confirm that there is a clonal spread. DNA polymorphism techniques have been recommended for identifying MRSA clonality, which is extremely important in the epidemiology of the disease, and for determining the type of treatment of these infections. Using the RW3A primer, Del Velchio *et al.* [25] showed eight different fingerprint patterns in MRSA strains from different sources. Our study showed a higher number of DNA bands (4 to 16 bands), and some isolates did not produce a high-intensity amplicon of approximately 325 bp. This difference in DNA fingerprint profile can be due to different MRSA strains with respect to geographic distribution and antibiotic treatment.

The isolates showed a genotypic similarity profile of over 35% with the RW3A and JB1 primers, slightly higher than that reported by other researchers (20%) [26], though the previously reported similarity using BOX A1R was greater than 20%.

Genotypic analysis is an important tool for clinical practice because it provides reliable data for determining the epidemiology and pathogenesis of infectious processes. The PCR-based methods used in these studies showed good results for evaluating the similarity between *S. aureus* strains isolated from different locations [26,46]. This method using the RW3A primer has been demonstrated to be an interesting tool for distinguishing among different genotypic profiles. In our study, the 1N clonal group (using RW3A) revealed three isolates (75%) with SCC*mec* type III and identical characteristics (antibiotype A and biofilm production); these three isolates showed different genotypes with JB1 and BOX. Another isolate with SCC*mec* type III was designated as SI and was not able to produce biofilm on a polystyrene surface. This phenotypic characteristic was important for discriminating the SCC*mec* type III isolates using the RW3A primer.

The JB1 primer, which was described for *Enterococcus faecium* [28], exhibited desirable discriminatory power, suggesting that this primer could be used for purposes including the phylogenetic analysis of MRSA isolates.

In our study, the RW3A primer proved to be the best, and the clonal groups became more distinct with respect to genotypic and phenotypic characteristics. A previous study concluded that the correlation between antimicrobial resistance and the dissemination of clonal groups of *S. aureus* is an important tool during outbreaks or in endemic areas [47].

We recommend not using these three primers in combination because primer BOX A1R did not show a good discriminatory power and, in fact, interfered with the results obtained using the other primers.

Few studies have been performed using the RW3A primer for *S. aureus* typing. Some researchers analyzed *S. aureus* susceptibility and resistance to oxacillin and identified no correlation between resistance and the percentage of similarity between strains. In our study, there was no relationship between the clonal groups with particular SCC*mec* types and resistance profiles.

The HRM method has been introduced for *Clostridium difficile* ribotyping, *Enterococcus* species typing, and MRSA typing [48-50]. A study developed by Chen *et al*. [50] using the HRM technique applied to 55 MRSA collected from Hong Kong hospital during 2011 showed 12 different types of *spa* in concordance with 100% of the *spa* type method. The authors confirmed the cost effectiveness of HRM; the method required less time to be developed and the reagent cost was one-fifth of the cost of the conventional method. Studies confirmed that HRM typing methodology could be useful for MRSA community transmission monitoring and hospital outbreak control. Our study demonstrated the practical application of this low-cost HRM method for MRSA typing on clinical isolates.

Among the 21 isolates on which were performed HRM, this methodology showed eight clonal groups and RW3A method showed nine groups, thus, both techniques did not present any clonality between MRSA isolates. However, the clonal groups obtained by the techniques were not concordant. In clonality studies in MRSA isolates, both methodologies would be interesting because they are rapid, low cost, and use the

instruments available in some laboratories, as compared to MLST.

The ST generated did not represent the same groups generated by primer RW3A, as there were samples with the same ST grouped in different clonal groups (based on the primer RW3A). In two samples (MRSA 308 and MRSA 404) with the same ST5 at HRM (Table 2), there was only 40% similarity using the primer RW3A- JB1.

These results show that the RW3A primers, JB1 and BOX, are not good tools for pre-selection of samples for more discriminatory techniques such as MLST, PFGE, and *spa*.

For our study, the HRM technique was an important tool to verify the genotypic variability in our sample.

Although antimicrobial susceptibility tests provide information for routine surveillance, genetic typing studies with greater power of discrimination must also be performed. The correlation between clonality and antimicrobial susceptibility profile is an important tool for assessing the stage of an outbreak and characterizing the epidemiology of hospital isolates. Our results showed that the HRM technique used in our study can quickly provide important epidemiological information for MRSA with low operating costs.

Conclusions

Antimicrobial susceptibility tests provide information for routine surveillance, and genetic studies with greater discriminatory power for typing can also be performed. Correlation between the clonality of strains and antimicrobial susceptibility profiling are important tools to assess the stage of outbreaks and characterize the epidemiology of nosocomial isolates. The findings presented here indicate that the HRM technique utilized may provide important information regarding the epidemiology of MRSA, with faster results and lower operating costs.

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