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New multilocus sequence typing of MRSA in São Paulo, Brazil

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Abstract

An increased incidence of nosocomial and community-acquired infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) has been observed worldwide. The molecular characterization of MRSA has played an important role in demonstrating the existence of internationally disseminated clones. The use of molecular biology methods in the surveillance programs has enabled the tracking of MRSA spread within and among hospitals. These data are useful to alert nosocomial infection control programs about the potential introduction of these epidemic clones in their areas. Four MRSA blood culture isolates from patients hospitalized at two hospitals in the city of São Paulo, Brazil, were analyzed; one of them was community acquired. The isolates were characterized as SCC*mec*, *mecA* and *PVL* by PCR, pulsed-field gel electrophoresis (PFGE) profile and molecular sequence typing (MLST) genotyping. The isolates presented type IV SCC*mec*, and none proved to be positive for *PVL*. The isolates showed a PFGE profile similar to the pediatric clone. MLST genotyping demonstrated that the isolates belonged to clonal complex 5 (CC5), showing a new *yqiL* allele gene, resulting in a new sequence typing (ST) (1176). Our results showed that strains of MRSA carrying a new ST are emerging in community and nosocomial infections, including bacteremia, in São Paulo, Brazil.

Key words: Methicillin-resistant *Staphylococcus aureus*; Molecular sequence typing; Resistance; Molecular typing

Introduction

Staphylococcus aureus has been the most important pathogen related to nosocomial infections in Brazil and remains the main agent isolated from community-acquired infections in most countries (1). Among species of *Staphylococcus*, they account for 30 to 50% of nosocomial infections and this number is on the rise (2,3). After the description of the Brazilian epidemic clone (BEC) in 1992 (4), other studies further demonstrated the persistence of this microorganism in Brazilian hospitals (3). In these studies, the molecular characterization of methicillin-resistant *S. aureus* (MRSA) has been conducted primarily by pulsed-field gel electrophoresis (PFGE) and plasmid analysis, which showed the existence of internationally disseminated clones, reflecting epidemiological and geographical relatedness (5). The ability to correctly identify the MRSA strains is important for epidemiological understanding and decision making in public health (6).

Genetic structure models of bacterial populations were

demonstrated in 1998 when the multilocus sequence typing (MLST) technique was introduced (<http://www.mlst.net/>). Molecular biology methods in monitoring programs have permitted the determination of inter- and intra-hospital spread of MRSA. Moreover, the MLST data obtained can be used to answer basic questions about the evolution and biology of the population of bacterial species (7). Such information can be of help, for example, in the development of more efficient vaccines against pathogenic microorganisms or for the planning and implementation of epidemiological measures. The generated data can be useful to alert the committees of nosocomial infection control about the introduction of potentially epidemic clones in their areas. The aim of the present study was to detect MRSA gene rearrangements with subsequent acquisition or loss of genetic determinants through different molecular typing techniques, including SCC*mec* typing, PFGE, and MLST.

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Material and Methods

The study was carried out using selected bloodstream MRSA isolates obtained from a patient with community-acquired pneumonia admitted to a private general hospital in 2006 (A26151), from 2 patients with nosocomial infection admitted to a public university hospital in 2004 (A13357 and A14711), and from a patient with nosocomial infection admitted to a public general hospital in 2007 (A30988). The identification of *S. aureus* was confirmed by positive DNase and tube coagulase tests. Resistance to methicillin was determined according to Clinical and Laboratory Standard Institute guidelines (8). All strains were tested for the presence of the nuclease (*nuc*) and *mecA* genes. DNA was isolated from the reference strain by the method of van Soolingen et al. (9). *SCCmec* was determined by the multiplex PCR protocol developed by Oliveira and de Lencastre (10). All samples previously classified as *SCCmec* type IV were subjected to PCR for the detection of the *lukF-LUKS-PV* and *PV* genes encoding the virulence factor PVL (Panton-Valentine leukocidin) (11). PFGE of total DNA was performed as described elsewhere (3). Global and local representative MRSA clones were included in the experiments. BEC, *SCCmec* type I (NCTC 10422), *SCCmec* type II (N315), *SCCmec* type III (85/2082), *SCCmec* type IVa (JCSC 1968), WB72 (USA300/ST8), WB49 (OSPC/ST30), and 481 (USA400/ST1) were used as control isolates.

PFGE patterns were compared using the BioNumerics version 5.0 software (Applied Maths, Belgium). The PFGE profiles of MRSA were classified as clone "A" with subtypes

"A1" and "A2", sharing more than 80% of Dice coefficient similarity between them. The dendrogram was constructed using the unweighted pair-group with arithmetic mean (UPGMA) method algorithm for phylogenetic analysis. The molecular typing by MLST was based on the sequence of seven housekeeping genes according to procedures previously described (12). The resulting sequences were analyzed and compared with those deposited in the database at the <http://saureus.mlst.net/site>. Using the START program and eBURSTv2 at <http://pubmlst.org/software/analysis/start>, a dendrogram on sequence typing (ST) was built based on the data bank of each sample to illustrate the sample relationship and also to separate it into groups, representing clonal complexes, when available (13).

Results and Discussion

The MRSA samples studied here were resistant to oxacillin and showed the presence of the *nuc* and *mecA* genes. *SCCmec* determination showed that the A14711, A13357 and A26151 samples were *SCCmec* type IVa, and the A30988 sample was *SCCmec* type II.

All samples classified as *SCCmec* type IV were examined for the detection of the PVL virulence factor by PCR and were shown to be negative (data not shown).

The A13357 and 14711 isolates showed 85.7% similarity despite a difference in the *yqiL* allele and both had 82.9% similarity compared to the A26151 isolate. These isolates showed 76.7% similarity to the pediatric clone (Figure 1). The dendrogram constructed using the isolates character-

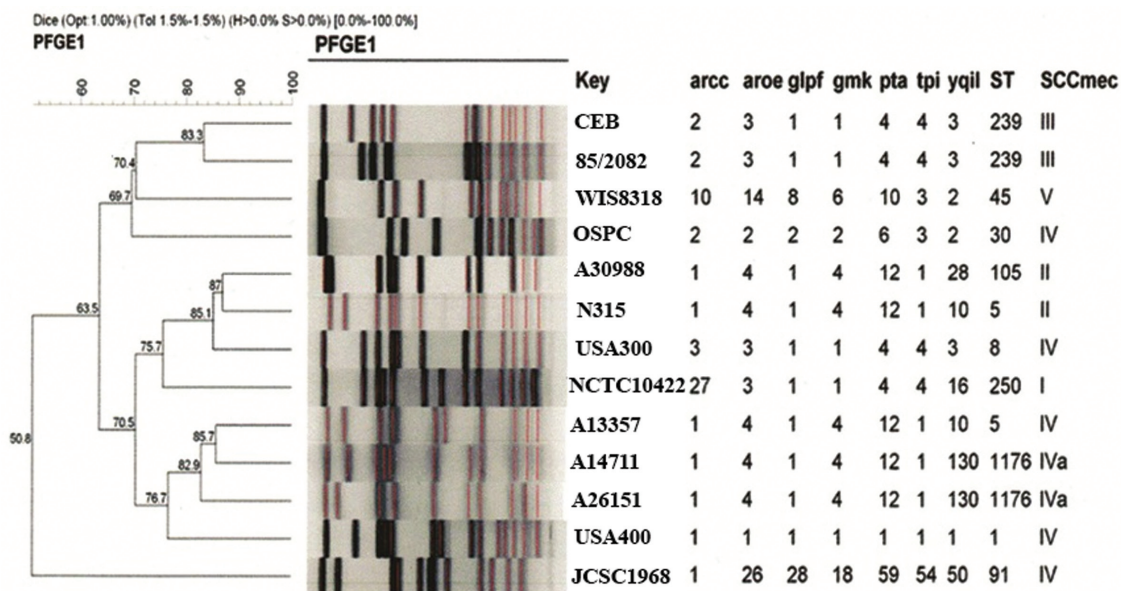


Figure 1. Pulsed-field gel electrophoresis (PFGE) dendrograms representing genetic relatedness using the Dice coefficient similarity and unweighted pair-group with arithmetic mean (UPGMA) method. Also included are the international methicillin-resistant *Staphylococcus aureus* (MRSA) clones. The right dendrogram columns indicate the number of alleles, sequence typing (ST) and *SCCmec* type, respectively.

ized in the present study revealed more than 82% similarity between the A13357, A26151, and A14711 samples. When these samples were compared to the A30988 isolate the similarity was 70.5% and to the BEC 50.8%.

MLST was identified by the PCR amplification of the *S. aureus* housekeeping genes in genomic DNA and resulted in approximately 500-bp fragments. For each isolate, the sequences obtained at each locus were compared to the database, and the alleles were numbered consecutively. Sequences were assigned as distinct alleles even though they differed at a single nucleotide site. Based on the numbers of the seven alleles identified for each sample, they received a number representing the ST. The evolutionary origins were examined by the eBURST algorithm in order to group the received allelic profiles into clonal complexes. The grouping was based on the similarities between sequence types at seven loci and was defined as a set sharing seven loci with any other in the data set.

The genetic similarity among isolates was also analyzed by the neighbor joining method. In this case, the allele nucleotide sequences of the seven genes were grouped into a single 3298-bp nucleotide sequence for each isolate. The sequences of different isolates were compared by determining the different nucleotide number between each sequence, for example in the genetic distance between isolates. These distances are represented in the gene tree branches constructed by this method (Figure 2). It was observed that the A30988 isolate was located in the same genogroup with a genetic distance of 86.4% showing similarity of 85.7% to A26151, A14711, A13357, and the pediatric clone. There is a clear bifurcation dividing the isolates belonging to the Brazilian epidemic clone, located at the bottom of the tree showing 86.1% similarity.

We found 4 different alleles for the *glpF* and *tpi* genes, 5 alleles for the *arcC* and *gmk* genes, 6 alleles for the *aroE* and *pta* genes, and 8 alleles for the *yqiL* gene. The polymorphic sites between nucleotides ranged from 7 (*tpi* gene) to 12 (*yqiL* gene). Analysis of the eBURSTv2 allelic frequency of the *S. aureus* samples studied showed that the most common alleles were number 1 for the *arcC*, *glpF*, *gmk*, and *tpi* genes (50, 78.6, 35.7, and 50%, respectively), number 4 for the *aroE* gene (42.9%), number 12 for the *pta* gene (42.9%), and numbers 3 and 10 for the *yqiL* gene (21.4% each). The genes of the *S. aureus* isolates were relatively uniform and polymorphic site distribution apparently occurred in conserved regions.

The number of polymorphic sites among the 7 loci in the test samples ranged from 9 (*arcC*) to 2 (*pta* and *tpi*).

There were 12 ST between 9 and 5 control strains and the clinical isolates. The ST5 (ID: 19922) for A13357 SCCmec type IVa and ST105 (ID: 2184) for A30988, SCCmec type II samples were found among our isolates. Furthermore, we observed a new allele of the *yqiL* gene sequences for A14711 (ID: 1990) and A26151 (ID: 1991) samples. As a result, we describe a new ST belonging to clonal complex 5 (CC5) (ST1176) carrying SCCmec type IVa.

In an attempt to determine the molecular epidemiology of MRSA strains in São Paulo, Brazil, from 2006 to 2007, four isolates (A13357, A14711, A26151, and A30988) were characterized by different molecular typing methods. Two isolates (A13357 and A14711) were characterized as ST1176 in the present study, belonging to CC5 with other Brazilian isolates (ST641, 744, and 1204). The BEC from São Paulo belongs to CC8, together with other Brazilian isolates (ST8, 239, 644, and 250).

MRSA have become a global concern. The dissemination of the MRSA clone probably reflects their ability to cause infection and to persist and spread from one region to another, including different continents (5). The predominance of BEC (SCCmec III) in Brazilian hospitals has been well documented (3,4,14). Recent changes in the profile of clonal MRSA isolated in hospitals have been observed, which have increased the number of MRSA isolates belonging to SCCmec type IV (pediatric clone), as shown in the study by Trindade et al. (15), who reported a 13% rate of occurrence of MRSA isolates in patients carrying this SCCmec admitted to a São Paulo hospital.

Different typing methods are necessary to determine the molecular epidemiology of MRSA strains and their genetic diversity. Enright et al. (16), by analyzing MRSA samples

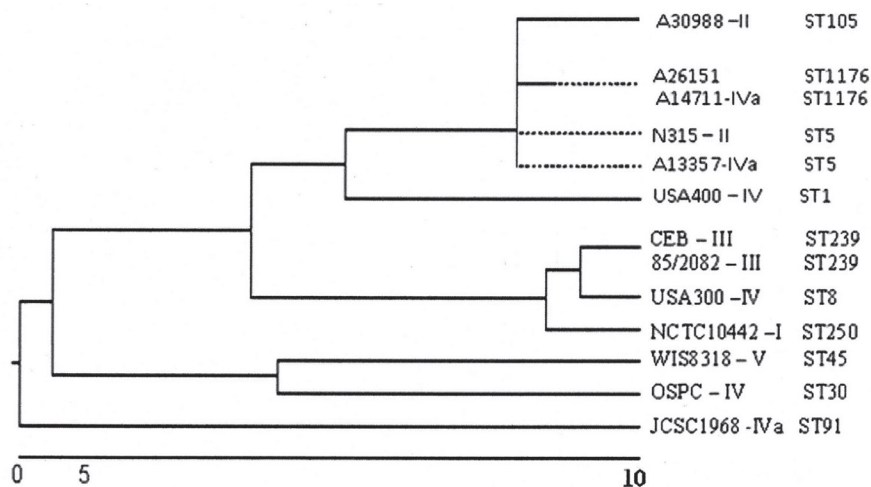


Figure 2. Dendrogram showing the neighbor joining phylogenetic tree, based on the sequence substitution number of 3298 nucleotides. The scale bar below the tree indicates the branch size equivalent to 10 substitutions.

isolated from hospitals and from the community in different countries using MLST and SCC*mec* typing, discovered the existence of CCs, allowing the classification of *S. aureus* into evolutionary lineages, resulting in evolutionary models for each lineage (11). These CCs were composed of *S. aureus* samples with the same ST. The comparison of nucleotide sequences of the housekeeping genes between the MRSA samples allowed the establishment of a common ancestor (a methicillin-sensitive *Staphylococcus aureus*, MSSA). Therefore, samples of MRSA originated from an MSSA by different events of *mec* acquisition, corroborating the hypothesis of horizontal transmission of the *mecA* gene (17).

Among the five major MRSA CCs described, the CC5 drift consisted mainly of strains found among the pediatric clones (ST5, SCC*mec* VI) and NY/J (ST5, SCC*mec* II). Strains showing ST8, ST239, ST247, and ST250 are part of CC8, which are found in the Archaic and Iberian clones (SCC*mec* I) and BEC (SCC*mec* III) (11). Our results showed that, according to PFGE and MLST, the studied isolates belong to CC5. This CC represents diverse MRSA strains and contains current pandemic clones (11).

Since ST strains have different SCC*mec* types it is possible that this type was acquired several times, showing

different genetic compositions in different *S. aureus* strains (11). Thus, we believe that our non-BEC belonging isolates are descendants of a common ancestor, the pediatric clone, first described in Portugal, Poland, Argentina, Colombia, and in New York hospitals' pediatric patients (18, 19) or from the pediatric clone NY/Japan that appeared and disseminated in Tokyo and USA hospitals (19). Although in Brazil there was a predominance of BEC in hospitals in several states (3, 4, 14, 20) we found that there was no similarity between this clone and the isolates investigated in the present study. The ST that showed a closely related pattern also had a similar PFGE profile. The present study provides new information about MRSA samples prevalent in São Paulo. The discovery of new strains with allele ST1176 suggests that these bacteria have suffered recent mutations.

Pediatric clone samples seem to be associated with nosocomial infections in other Brazilian cities like Rio de Janeiro, Recife, and in Rio Grande do Sul (14) and São Paulo (15). More studies are needed to characterize MRSA clones not belonging to BEC in Brazil genotypically.

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References

- Rezende EM, Couto BR, Starling CE, Modena CM. Prevalence of nosocomial infections in general hospitals in Belo Horizonte. *Infect Control Hosp Epidemiol* 1998; 19: 872-876.
- Richards MJ, Edwards JR, Culver DH, Gaynes RP. Nosocomial infections in medical intensive care units in the United States. National Nosocomial Infections Surveillance System. *Crit Care Med* 1999; 27: 887-892.
- Teixeira LA, Resende CA, Ormonde LR, Rosenbaum R, Figueiredo AM, de Lencastre H, et al. Geographic spread of epidemic multiresistant *Staphylococcus aureus* clone in Brazil. *J Clin Microbiol* 1995; 33: 2400-2404.
- Sader HS, Pignatari AC, Hollis RJ, Jones RN. Evaluation of interhospital spread of methicillin-resistant *Staphylococcus aureus* in São Paulo, Brazil, using pulsed-field gel electrophoresis of chromosomal DNA. *Infect Control Hosp Epidemiol* 1994; 15: 320-323.
- Oliveira DC, Tomasz A, de Lencastre H. Secrets of success of a human pathogen: molecular evolution of pandemic clones of methicillin-resistant *Staphylococcus aureus*. *Lancet Infect Dis* 2002; 2: 180-189.
- Vogel U, Morelli G, Zurth K, Claus H, Kriener E, Achtman M, et al. Necessity of molecular techniques to distinguish between *Neisseria meningitidis* strains isolated from patients with meningococcal disease and from their healthy contacts. *J Clin Microbiol* 1998; 36: 2465-2470.
- Feil EJ, Spratt BG. Recombination and the population structures of bacterial pathogens. *Annu Rev Microbiol* 2001; 55: 561-590.
- Clinical and Laboratories Standard Institute (CLSI). *Performance standards for antimicrobial disk susceptibility tests*. 16th Informational supplement. Approved standard M100-S17; 9th edn. Wayne: Clinical and Laboratories Standard Institute, 2007.
- van Soolingen D, Hermans PW, de Haas PE, Soll DR, van Embden JD. Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. *J Clin Microbiol* 1991; 29: 2578-2586.
- Oliveira DC, de Lencastre H. Multiplex PCR strategy for rapid identification of structural types and variants of the *mec* element in methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2002; 46: 2155-2161.
- Robinson DA, Enright MC. Evolutionary models of the emergence of methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2003; 47: 3926-3934.
- Enright MC, Day NP, Davies CE, Peacock SJ, Spratt BG. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *J Clin Microbiol* 2000; 38: 1008-1015.
- Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J Bacteriol* 2004; 186: 1518-1530.
- Vivoni AM, Diep BA, de Gouveia Magalhaes AC, Santos KR, Riley LW, Sensabaugh GF, et al. Clonal composition of *Staphylococcus aureus* isolates at a Brazilian university hospital: identification of international circulating lineages. *J Clin Microbiol* 2006; 44: 1686-1691.

15. Trindade de A, Pacheco RL, Costa SF, Rossi F, Barone AA, Mamizuka EM, et al. Prevalence of SCCmec type IV in nosocomial bloodstream isolates of methicillin-resistant *Staphylococcus aureus*. *J Clin Microbiol* 2005; 43: 3435-3437.
16. Enright MC, Robinson DA, Randle G, Feil EJ, Grundmann H, Spratt BG. The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proc Natl Acad Sci U S A* 2002; 99: 7687-7692.
17. Musser JM, Kapur V. Clonal analysis of methicillin-resistant *Staphylococcus aureus* strains from intercontinental sources: association of the mec gene with divergent phylogenetic lineages implies dissemination by horizontal transfer and recombination. *J Clin Microbiol* 1992; 30: 2058-2063.
18. Sa-Leao R, Santos Sanches I, Dias D, Peres I, Barros RM, de Lencastre H. Detection of an archaic clone of *Staphylococcus aureus* with low-level resistance to methicillin in a pediatric hospital in Portugal and in international samples: relics of a formerly widely disseminated strain? *J Clin Microbiol* 1999; 37: 1913-1920.
19. Aires SM, De Lencastre H, Sanches IS, Kikuchi K, Totsuka K, Tomasz A. Similarity of antibiotic resistance patterns and molecular typing properties of methicillin-resistant *Staphylococcus aureus* isolates widely spread in hospitals in New York City and in a hospital in Tokyo. *Japan Microb Drug Resist* 2000; 6: 253-258.
20. de Miranda OP, Silva-Carvalho MC, Ribeiro A, Portela F, Cordeiro RP, Caetano N, et al. Emergence in Brazil of methicillin-resistant *Staphylococcus aureus* isolates carrying SCCmecIV that are related genetically to the USA800 clone. *Clin Microbiol Infect* 2007; 13: 1165-1172.