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PCR-based ribotyping of *Staphylococcus aureus*

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Abstract

Genotyping techniques are valuable tools for the epidemiologic study of *Staphylococcus aureus* infections in the hospital setting. Pulsed-field gel electrophoresis (PFGE) is the current method of choice for *S. aureus* strain typing. However, the method is laborious and requires expensive equipment. In the present study, we evaluated the natural polymorphism of the genomic 16S-23S rRNA region for genotyping purpose, by PCR-based ribotyping. Three primer pairs were tested to determine the size of amplicons produced and to obtain better discrimination with agar gel electrophoresis and ethidium bromide staining. The resolution of the typing system was determined using sets of bacteria obtained from clinical specimens from a large tertiary care hospital. These included DNA from three samples obtained from a bacteremic patient, six strains with known and diverse PGFE patterns, and 88 strains collected over a 3-month period in the same hospital. Amplification patterns obtained from samples from the same patient were identical, and PFGE from samples known to be different produced three genotypes. Amplification of DNA from 61 methicillin-resistant isolates produced only one pattern. Methicillin-sensitive strains yielded a diversity of patterns, pointing to a true polyclonal distribution throughout the hospital (22 unique patterns from 27 strains). Computer-based software can be used to differentiate among identifiable strains, given the low number of bands and good characterization of PCR products. PCR-based ribotyping can be a useful technique for genotyping methicillin-sensitive *S. aureus* strains, but is of limited value for methicillin-resistant strains.

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- Genotyping
- Methicillin-resistant *Staphylococcus aureus*

Introduction

Staphylococcus aureus is a major pathogen in both community and hospital settings (1). Phenotyping and genotyping techniques are important for tracing the epidemiology of *S. aureus* infections. Phenotyping techniques, such as antibiotic susceptibility and phage typing, are based on unstable characteristics and can show poor reproducibility. Genotyping techniques are based on chromosomal or plasmid material and can provide better results in terms of reproducibility and strain differentiation.

Pulsed-field gel electrophoresis (PFGE) is considered the genotyping technique of choice by reference laboratories such as the CDC (2), but this methodology is labor-intensive, time-consuming, and requires expensive equipment. In contrast, PCR-based genotyping technologies are simple and fast. The primers used in the reactions can be arbitrary, i.e., not complementary to any specific region (RAPD), or can be complementary to known sequences of the organism's genome (REP-PCR).

The amplification of the genomic 16S-23S rRNA spacer region was first described as a reliable technique for typing *Pseudomonas cepacia*, *Staphylococcus aureus*, *Enterococcus faecium*, *Escherichia coli*, and *Enterobacter* sp (3). In the present study we explored the natural polymorphism of the genomic 16S-23S rRNA region from *S. aureus* as a genotyping tool. Three primer pairs were tested to check for the size of amplicons produced, and best visualization upon agar gel electrophoresis and ethidium bromide staining, typeability, and discriminatory power were determined using a collection of bacteria obtained from clinical specimens from Hospital Municipal Dr. Mário Gatti, a 200-bed tertiary care hospital in Campinas, São Paulo State, Brazil, during a 3-month period.

Material and Methods

Strains

The following strains were used: a) isolates (N = 3) grown on blood cultures collected on different days from a patient with acute endocarditis, designated A1, A2, and A3; b) ATCC 25923 isolates subcultured overnight for 1, 10, and 30 times, designated B1, B2, and B3; c) six strains with known and diverse PFGE patterns, designated C1 to C6 (4), and d) 88 isolates collected from several sources (clinical specimens) during a 3-month period (June through September, 2000) in the hospital. Isolates were stored at -18ºC in 10% glycerol-supplemented BHI broth.

PCR

S. aureus template DNA was obtained from overnight grown cultures using standard extraction protocols (3). Three 1 primer pairs targeting the 16S-23S rRNA region were used. Primer pair A: 5'-TTG TAC ACA CCG CCC GTC-3' and 5'-TGC CAA GGC ATC CAC CGT-3'; primer pair B: 5'-TTG TAC ACA CCG CCC GTC-3' and 5'-GGT ACC TTA GAT GTT TCA GTT C-3', and primer pair C: 5'-GAA GTC GTA ACA AGG-3' and 5'-CAA GGC ATC CAC CGT-3' (3,5,6). The reactions were performed in a final volume of 50 µl at the following reagent concentrations: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 200 µM dNTP, 0.5 µM of each primer, 3 mM $MgCl_2$, 2.5 U Taq polymerase, and 200 ng of template DNA. Amplification mixtures using primers A and B were submitted to 40 cycles: 94ºC for 1 min, 55ºC for 1 min, 72ºC for 2 min, and a final extension at 72ºC for 7 min; with primers C, the mixture was submitted to 25 cycles: 94ºC for 1 min, 55ºC for 7 min, 72ºC for 2 min, and a final extension at 72ºC for 12 min.

Susceptibility tests

Methicillin resistance was determined by the NCCLS protocol (7,8) in terms of the width of the inhibition zone produced by a 5 µg oxacillin disk on a 5% NaCl-supplemented Mueller-Hinton agar plate.

Electrophoresis

PCR products were run on 2% agarose gels (85 V at room temperature). Molecular sizes were determined based on a 100-bp ladder molecular mass marker. Kodak Digital Science 1D Image Analysis software was used to estimate sizes and hierarchical analyses were performed using an appropriate statistical software (SPSS 7.5).

Results

Amplification profiles of three isolates taken randomly using the three sets of primer pairs (A,B,C) are shown in Figure 1 (lanes 1 to 9). The amplicons obtained with primer pair C (6) corresponded to the smallest fragments which were best resolved under the electrophoretic conditions used. This primer pair was subsequently used in all reactions.

Isolates from the same patient with acute endocarditis (A1, A2, A3) produced a single pattern that can be seen in Figure 1 (lanes 10 to 12). Initial and subcultured ATCC isolates, designated B1, B2, and B3 (1st, 10th and 30th passage), yielded the same amplification profile, as can be seen in Figure 2. This figure also shows the amplicons ob-

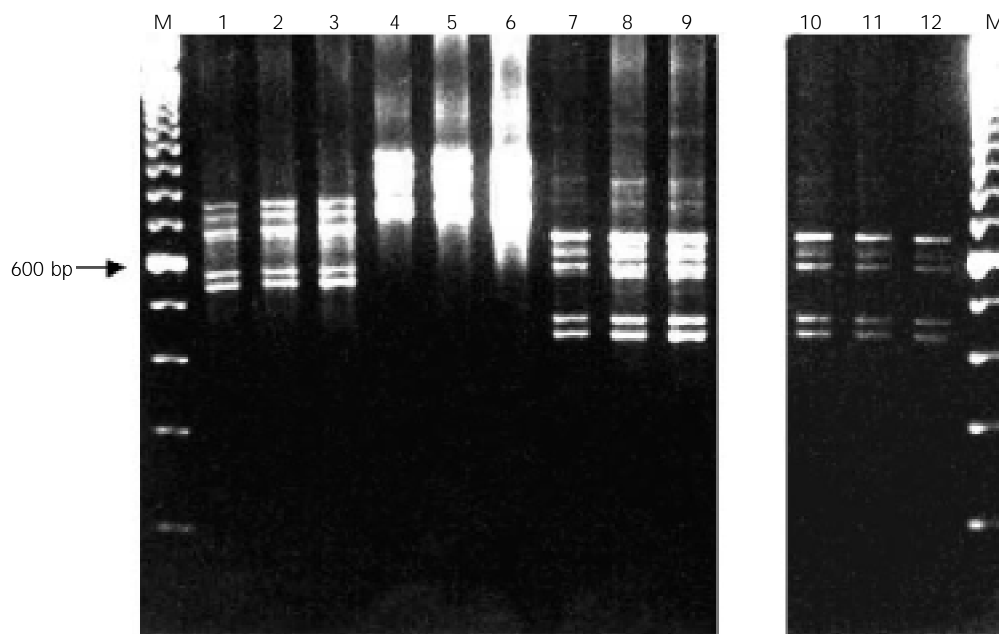

Figure 1. Electrophoretic patterns of PCR ribotypes of three S. aureus strains obtained with the three different primer pairs: lanes 1 to 3 with primer pair A, described by Gürtler and Stanisich (5), lanes 4 to 6 with primers B, described by Kostman et al. (3), and lanes 7 to 9 with primers C, described by Jensen et al. (6). Lanes 10 to 12 show PCR ribotypes from three S. aureus isolates collected from the same patient on different days. Lane M: 100-bp ladder.

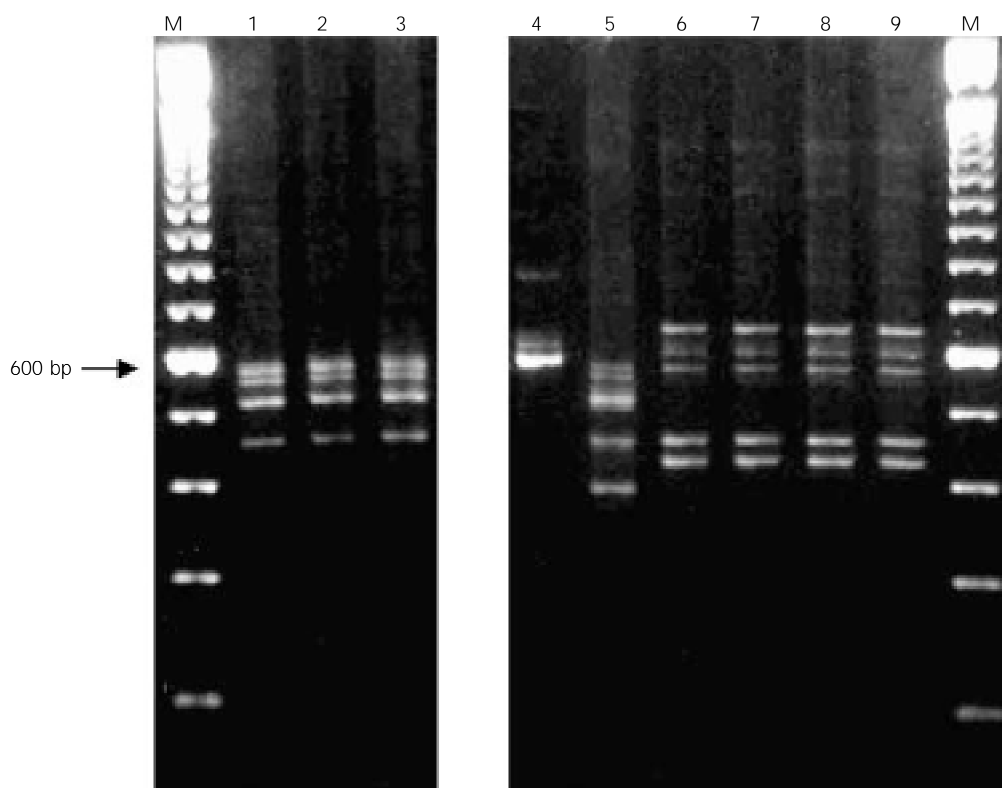

Figure 2. Electrophoretic patterns of PCR ribotypes of the ATCC 25923 isolate subcultured 1, 10 and 30 times; lanes 1 to 3, respectively. Lanes 4 to 9 show the PCR ribotypes of six different PFGE S. aureus isolates. Lane M: 100-bp ladder.

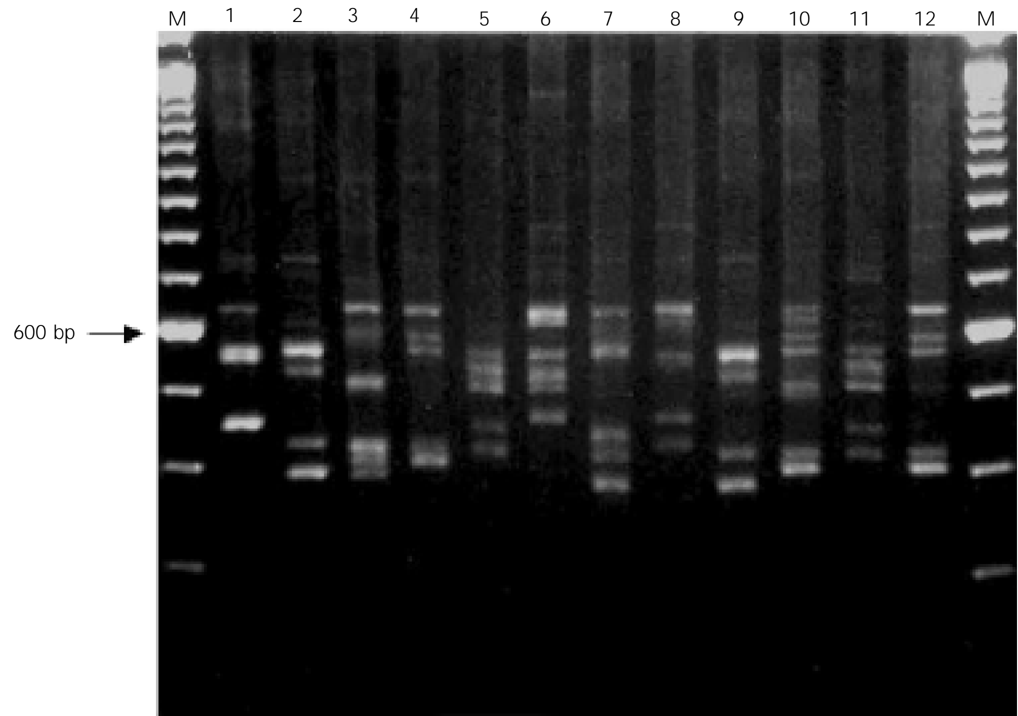

Figure 3. Electrophoretic patterns of PCR ribotypes of 12 isolates of methicillin-sensitive S. aureus (lanes 1 to 12). Lane M: 100-bp ladder.

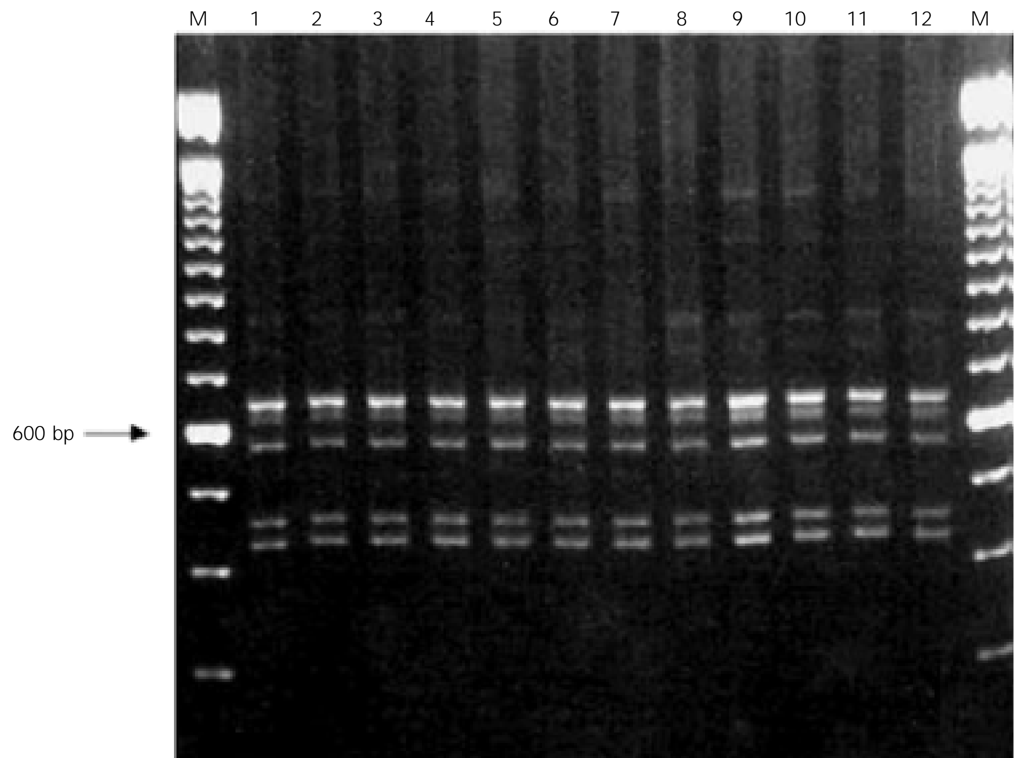

Figure 4. Electrophoretic patterns of PCR ribotypes of 12 methicillin-resistant S. aureus isolates (lanes 1 to 12). Lane M: 100-bp ladder.

tained from six isolates with different known PFGE profiles (C1 to C6). Three distinct amplification patterns were found.

Twenty-seven of 88 isolates (37%) collected in the hospital were methicillin-sensitive. DNA amplification of these isolates yielded three to nine bands per isolate with molecular weights ranging from 360 to 733 bp. All isolates were typeable. Methicillin-sensitive isolates showed 22 different patterns, some of which are illustrated in Figure 3. Hierarchical analysis showed a similarity ranging from 45 to 100% (simple matching).

The amplification of the methicillin-resistant *S. aureus* (MRSA) isolates produced the same band pattern as shown in Figure 4. The molecular weights ranged from 431 to 644 bp. All isolates were also typeable.

Discussion

Staphylococcus aureus is a major nosocomial pathogen that causes a range of diseases, including endocarditis, osteomyelitis, pneumonia, toxic-shock syndrome, food poisoning, carbuncles, and boils. In the early 1950's, acquisition and spread of beta-lactamase-producing plasmids thwarted the effectiveness of penicillin for treating *S. aureus* infections. In 1959, methicillin, a synthetic penicillin, was introduced. However, by 1960, MRSA strains were identified as a direct result of *S. aureus* acquiring the *mec*A gene, which encodes for an altered penicillin-binding protein gene (PBP2a).

Bacterial strain typing, or subspeciation, has become an important clinical tool to investigate suspected outbreaks and to evaluate nosocomial transmission.

Previous studies have shown that MRSA strains are the result of a recent evolutionary change linked to the acquisition of the *mec*A gene (9). Several typing methods have been used to confirm this hypothesis, such as *mec*A RFLP and Tn554 blot-hybridization.

PCR ribotyping is a fast and reliable typing method with good typeability and reproducibility for several bacterial species, such as *P. cepacia*, *E. coli* and *Enterobacter* sp. This approach targets the 16S-23S rRNA region which is polymorphic and repetitive in the genome of such bacteria. Earlier reports have shown that *S. aureus* can be typed with the use of this technique. In the present study, we evaluated the basic characteristics of this methodology, i.e., typeability, reproducibility and discriminatory power using isolates collected from a general tertiary care hospital.

When typing methicillin-sensitive *S. aureus* this technique yielded amplification patterns consistent with good attributes such as typeability, reproducibility, and discriminatory power. However, amplification of DNA from MRSA produced a pattern with low discriminatory power. In fact, strains from different PFGE groups could not be distinguished with the use of PCR ribotyping. The short interval (3 months) for the collection of isolates in this study could explain these findings. Another possibility is the existence of just one clone of SAMR largely disseminated in the hospital.

Otherwise, these findings corroborate others that point to a recent evolutionary change of this microorganism dating back to the early 1960's, when they probably acquired genetic material that encodes for resistance to methicillin (*mec*A gene). This acquisition had a major role in the complexity of the *S. aureus* genome, since many integration and deletion events occur in this resistance island. The recent description of the whole genome sequences of MRSA and vancomycin-resistant *S. aureus* by Japanese researchers certainly contributed to this hypothesis (10).

MRSA genotyping still poses a challenge to several research teams. The use of a single method such as PFGE, Southern blot ribotyping, Tn554 and *mec*A hybridization, and others recently described, like spa- and coa-typing, which are based on sequencing, has yielded disappointing results (11). The com-

bination of two or more of these methods seems to be the best approach to establish the clonal nature of organisms from hospital settings (12).

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