Characterization of the Brazilian Endemic Clone of Methicillin-Resistant *Staphylococcus aureus* (MRSA) from Hospitals Throughout Brazil

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The objective of this study was to characterize patterns of the Brazilian endemic clone of methicillin-resistant *Staphylococcus aureus* (MRSA) from hospitals throughout Brazil. We studied 83 MRSA strains isolated from patients hospitalized in 27 public and private hospitals in 19 cities located in 14 Brazilian states from September, 1995, to June, 1997. The MRSA strains were typed using antibiograms, bacteriophage typing and pulsed field gel electrophoresis (PFGE). The analysis of genomic DNA by PFGE showed that 65 isolates presented the same PFGE pattern. This pattern was present in all of the hospitals studied indicating the presence of an endemic MRSA clone widely disseminated throughout Brazilian hospitals (BEC). All isolates belonging to the BEC proved to be resistant to ciprofloxacin, erythromycin, lincomycin, trimethoprim-sulphamethoxazole, and tetracycline. Variable susceptibility to these drugs was found only in isolates belonging to clones other than the BEC. The results show that, among MRSA, the BEC is common in Brazil. The best method for mapping changes in the frequency of this clone among MRSA is pulsed field gel electrophoresis. Use of molecular mapping is an important tool for monitoring the spread of potentially dangerous microbes.

**Key Words:** *Staphylococcus aureus*, MRSA, epidemiology, Brazilian endemic clone.

Methicillin-resistant *Staphylococcus aureus* (MRSA) has become one of the most important nosocomial pathogens throughout the world, capable of causing a wide range of hospital infections [1-3]. In order to better understand the epidemiology of these microorganisms, a number of systems have been used for typing *S. aureus*. These include antibiograms, bacteriophage typing, plasmid typing, ribotyping, techniques based on PCR and Pulsed Field Gel Electrophoresis (PFGE). Due to the genetic similarities presented by these microorganisms, the association of more than 1 typing method has been indicated [4-6]. In this study, we used antibiograms, bacteriophage typing, and PFGE; the latter still remains as the gold standard for typing MRSA [4-6].

In Brazil, *Staphylococcus aureus* is the microorganism most frequently isolated from nosocomial infections, and the prevalence of the isolation of MRSA strains varies from 40% to 80% in most Brazilian hospitals [7-9]. These isolates are generally resistant to aminoglycosides, chloramphenicol, lincosamides, macrolides, quinolones, sulphamethoxazole-trimethoprim, and tetracycline, with greater susceptibility only to rifampin [10-12].

The dissemination of the Brazilian endemic MRSA clone (BEC) in several hospitals in Brazil, South America, and Europe has been described [3, 12-21], but a study of national scope had not been carried out in Brazil prior to our study. The purpose of this study was establish the extent of the spread of the BEC in Brazilian hospitals, and to elucidate the epidemiology of infections caused by MRSA in Brazil.
Materials and Methods

Isolates

Because the objective of this study was to evaluate the dissemination of the BEC within Brazil, samples of MRSA isolates were not collected consecutively or selected randomly. In this study, 83 of 238 MRSA strains that had been collected from a multicenter study carried out in several Brazilian cities were selected. The 83 selected strains were obtained from 27 public and private hospitals with more than 250 beds each, located in 19 cities situated in 14 different Brazilian states. When selecting participant hospitals, the need to represent all of the Brazilian territory, in accordance with the data obtained from the Brazilian State Department of Health was carefully considered. On average, we selected three MRSA isolates from each hospital. The 83 strains were chosen based on previously obtained bacteriophage typing and antibiogram results. Most selected strains presented similar phage types and different antibiogram profiles.

These isolates were recovered from patients hospitalized from September, 1995, to June, 1997, from human clinical samples such as blood, pleural fluid, cerebrospinal fluid, dialysis fluids, burn wounds, abscesses, surgical wounds, and catheter tips.

Bacterial Identification

The Staphylococcus aureus strains were identified by Gram’s staining, catalase test, coagulase test [22], and biochemical tests performed by the automated MicroScan-Baxter System making use of the Pos Combo Type 6 Panel (Dade Behring Inc., MicroScan Division, West Sacramento, California).

Antimicrobial Susceptibility Testing

Microdilution susceptibility testing was carried out using the automated MicroScan-Baxter System Inc. (Pos Combo Type 6 Panel), according to the manufacturer’s recommendations. The disk diffusion test was carried out according to the standards of the National Committee for Clinical Laboratory Standards - NCCLS [23].

The antimicrobial drugs tested by the disk diffusion method were: ampicillin-sulbactam 10-10 mg, clindamycin 2 mg, chloramphenicol 30 mg, ciprofloxacin 5 mg, erythromycin 15 mg, gentamicin 10 mg, rifampin 5 mg, sulphamethoxazole-trimethoprim 1.25-23.75 mg, and vancomycin 30 mg. Screening for oxacillin resistance was performed according to the NCCLS recommendations [23].

Bacteriophage typing

Bacteriophage typing was performed as previously described using the international bacteriophage typing set of 23 phages (group I, 29, 52, 52A, 79 and 80; group II, 3A, 3C, 55 and 71; group III, 6, 42E, 47, 53, 54, 75, 77, 83A, 84 and 85; group V, 94 and 96; not classified, 81 and 95). Six additional experimental phages (89, 90, HK2, D11, 83C and 932) were also tested. Isolates were typed both at the routine test dilution (RTD) and 100x RTD. Phage types that differed by the presence or absence of 1 phage were considered related. Differences by the presence or absence of 2 or more phages were considered to be unrelated strains [24].

Pulsed Field Gel Electrophoresis (PFGE)

The use of PFGE as the ‘gold standard’ in molecular epidemiology has been previously described by other authors [4-6]. Preparation of cells and restriction enzyme digestion of their genomic DNA with SmaI was carried out as previously described [25]. Electrophoresis was performed using the CHEF DRIII electrophoresis system (Bio-Rad, Melville, N.Y.). In order to improve the analysis of similarity, 2 different switch times were used. One pulse time with 2 consecutive ramps of 12h each, the first with a pulse increase from 1s to 5s, the second from 15s to 30s, and another was ramped from 10s to 90s by 24h. The voltage was 6 volts/cm, the angle of 120°, and the temperature of 14° C. The gels were stained with ethidium bromide and photographed. Interpretation was done according to previously determined standards [26].
Results

The analysis of genomic DNA by PFGE performed in 83 MRSA isolates showed that 65 (78.3%) presented the same PFGE pattern (genotype A). This pattern was present in all hospitals studied indicating the presence of an endemic MRSA clone widely disseminated in Brazilian hospitals (Brazilian Endemic Clone – BEC). PFGE demonstrated the presence of 13 different profiles, identified from A to M (Figure 1). Among the MRSA strains belonging to the BEC, 12 (18.5%) were susceptible exclusively to vancomycin and 43 (66.2%) were susceptible to only 2 drugs, including vancomycin. These isolates demonstrated variable susceptibility to chloramphenicol, netilmicin and rifampin (Table 1). Susceptibility to ciprofloxacin, lincomycin, sulfamethoxazole-trimethoprim and tetracyclin was found only in isolates belonging to clones different from BEC (genotypes B to M).

Among the 65 isolates belonging to BEC, 21 were characterized as “non-typeable” by bacteriophage typing, and 39 were lysed exclusively by group lytic III and experimental phages. One isolate was lysed by group lytic III and NC; 2 by EP and NC; 1 by group lytic III, EP and NC; and 1 was lysed exclusively by group lytic II. The results of bacteriophage typing of the PFGE patterns named from B to M showed that 13 isolates were lysed by phages of group lytic III and EP, 2 were non-typeable, 1 isolate was lysed by group lytic II, 1 by group lytic I and EP, 1 lysed by group lictics I, II, III, EP and NC.

The 65 isolates belonging to the BEC genotype showed more than 20 distinct phagetypes and 8 different susceptibility profiles to antibiotics. In addition, the BEC identified in the present study showed the same PFGE pattern as other Brazilian epidemic MRSA clones previously described [12-16].

Discussion

The large variety of virulence factors produced by Staphylococcus aureus associated with its enhanced capacity to acquire resistance to antibiotics, has made it one of the microorganisms responsible for higher rates of morbidity and mortality in hospitals. In Brazil, S. aureus is the most commonly isolated microorganism from nosocomial infections [7-9, 11].

Previous studies have documented the appearance of 2 widely disseminated multiresistant MRSA clones [17-21]. One of them is known as the Iberian MRSA clone [18], and the other, known as the Brazilian MRSA clone, has been shown to be disseminated in some Brazilian cities [12-16]. However, a study of national scope involving many medical centers had not been carried out until this study. Our study shows the wide dissemination of the BEC throughout Brazilian hospitals, having been isolated in 19 cities situated in 14 different Brazilian states. The genotype A proved to be indistinguishable from the Brazilian epidemic MRSA clone previously described [12-16]. A comparison of our results with those of other studies led us to consider that the BEC might be widely disseminated in 24 of the main urban centers in Brazil (Figure 2). Another factor that supports this theory is the absent reporting of another MRSA epidemic clone in Brazil.

With the current use of PFGE, bacteriophage typing is no longer considered to be the reference method for typing Staphylococcus aureus [4, 5]. Phagetypes represent phenotypical characteristics that are usually accompanied by low stability and reproducibility. Nevertheless, bacteriophage typing is still invaluable in the characterization of a large number of samples because of its low cost when compared to PFGE [4, 5]. In this work, using bacteriophage typing, we were able to subdivide the genotype “A” into more than 20 different phagetypes. Thus, the use of bacteriophage typing allowed for the differentiation of non-related endemic strains isolated from different regions, that would have been indistinguishable by PFGE. Among the strains characterized as non-typeable by bacteriophage typing, 91.3% (21 of 23) presented the same PFGE pattern, suggesting an elevated genetic similarity between these strains. The majority of strains belonging to the BEC were lysed by group lytic III and experimental phages [27, 28].

We found that bacteriophage typing was a sensitive method that allowed discrimination between unrelated strains. However, phagetype variants were observed
Figure 1. Results of PFGE showing genotypes identified from A to M using the pulse time ramped from 1s to 30s by 24h. Line 1: genotype A (BEC), lines 2-14: genotypes B to M, and line 15: molecular size standard (48.5 Kb).

Figure 2. Map of Brazil showing the cities in which the BEC is disseminated.

· = cities in which the BEC was found to be disseminated in this study.
□ = cities in which the BEC was found to be disseminated in other studies [12-16].
among MRSA strains belonging to a single genotype. On other occasions, typeable MRSA strains tend to react with similar group III and experimental phages, irrespective of their epidemiological origins. Here, the question arises whether this differentiation into subtypes represents true strain differences. Nevertheless, it should be pointed out that phenotypical methods can be helpful when used properly in a small-scale epidemiological study, such as surveillance of MRSA strains within a hospital over a relatively short span of time [29]. Consequently, when analyzing our results, it is important to consider the long length of time and large area over which the bacterial isolated were collected. Therefore, these findings suggest that \textit{in vivo} or \textit{in vitro} phenotypic instability may occasionally hamper reproducibility, rather than increase the discriminatory power of the bacteriophage typing [28], and that the phenotypic subtypes found within genotypes did not appear to be epidemiologically relevant [4, 27, 28].

Table 1. Percentage of different susceptibility profiles and results of bacteriophage typing based on group litic presented by Genotype A (BEC)

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Percentage of susceptibility profiles (Number of strains)</th>
<th>Phage groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAN</td>
<td>18.5% (12)</td>
<td>II, III, EP, NC, NT</td>
</tr>
<tr>
<td>CHL, VAN</td>
<td>3.1% (2)</td>
<td>III, EP, NT</td>
</tr>
<tr>
<td>NET, VAN</td>
<td>1.5% (1)</td>
<td>NT</td>
</tr>
<tr>
<td>RIF, VAN</td>
<td>43.1% (28)</td>
<td>III, V, EP, NC, NT</td>
</tr>
<tr>
<td>CHL, NET, VAN</td>
<td>3.1% (2)</td>
<td>III, NT</td>
</tr>
<tr>
<td>NET, RIF, VAN</td>
<td>16.9% (11)</td>
<td>III, EP, NT</td>
</tr>
<tr>
<td>CHL, RIF, VAN</td>
<td>9.2% (6)</td>
<td>III, NT</td>
</tr>
<tr>
<td>CHL, NET, RIF, VAN</td>
<td>4.6% (3)</td>
<td>III, EP</td>
</tr>
</tbody>
</table>


The BEC multiresistant profile has been emphasized by several authors [12-16]. Our study demonstrated that all strains belonging to the BEC proved to be resistant to ciprofloxacin, erythromycin, lincomycin, tetracycline and trimethoprim-sulphamethoxazole (Table 1). On the other hand, susceptibility to some of these antibiotics was found only among clones other than the BEC. As the overuse of antibiotics facilitates the emergence of resistance, the accumulation of additional resistance and, consequently, the selection of multiresistant strains [8, 30], it could be inferred that the use of these aforementioned antibiotics might have promoted the slow selection of a multiresistant MRSA clone in Brazilian hospitals over the years [30, 31]. Although evidence has been found to support the existence of a relationship between MRSA and antimicrobial use [30-34], we could not confirm that the use of the antibiotics is the unique factor that favors the spread of MRSA and even more of the BEC in Brazilian hospitals.

MRSA infections are a growing problem, especially in regions where the rates of resistance to anti-staphylococcal antibiotics are high [35], as in Brazil...
Resistance to multiple antibiotics increases the ability of bacteria to survive the antimicrobial selective pressure in hospitals [35]. We can anticipate that the large scale use of antibiotics in some Brazilian hospitals [8] could increase even more the prevalence of MRSA infections and the spread of the BEC. Therefore, interventions aimed at promoting more rational prescribing patterns and the control of antimicrobial use should be supported for preventing and controlling MRSA infections. However, the relationships between antimicrobial use and MRSA are complex [30, 31], and the role of antimicrobial selective pressure in Brazilian hospitals requires more investigation.

Other factors, such as the capacity to colonize, multiply, and invade the mucoid and epithelial surface of the host, and the ability to survive in hospital environments could also contribute to the widespread colonization and dissemination of these endemic bacteria [36-42]. In the same way, several other conditions facilitate the spread of the BEC or other multidrug-resistant bacteria from one hospital to another and between cities or states in Brazil. Among these are 1) overcrowding in hospitals; 2) transferring patients between hospitals, sometimes in different cities; and 3) a lack of effective hospital infection control committees and control of the use of broad-spectrum antibiotics [7, 8, 10].

The isolation of the BEC in other Latin American countries such as Argentina and Uruguay, has been reported [17, 19, 21]. In various hospitals in Argentina, about 50% to 60% of isolated MRSA belonged to the BEC [19, 21]. In Europe, Souza, et al. [20], have also described the dissemination of the BEC in many Portuguese hospitals. The isolation and dissemination of the BEC has also been reported in the Czech Republic [2]. These data suggest the potential for increased spread of the BEC, even in hospitals where other MRSA epidemic clones already existed. If measures to control the dissemination of this clone are not taken [1], other countries or continents may also experience the wide dissemination of this multidrug-resistant MRSA clone.

In Brazil, the emergence of heterogeneous resistance to vancomycin [43] and vancomycin-intermediate Staphylococcus aureus (VISA) [44] among isolates belonging to the BEC has been reported. One could, thus, anticipate that the emergence of VISA strains among the BEC could bring about a very serious problem. The overuse or incorrect use of vancomycin in some Brazilian hospitals increases the potential risk for the emergence of VISA [45]. Whether the BEC presents a higher probability of developing resistance to vancomycin, is still unclear. With this in mind, Brazilian hospitals should test nosocomial staphylococcal isolates for vancomycin susceptibility and must encourage more prudent use of vancomycin.

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