BACTERIAL EPIDEMIOLOGY AND TYPING

Analysis of different molecular methods for typing methicillin-resistant *Staphylococcus aureus* isolates belonging to the Brazilian epidemic clone

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The extensive geographic spread of MRSA isolates belonging to the Brazilian epidemic clone (BEC) limited the value of pulsed-field gel electrophoresis (PFGE) in epidemiological studies of outbreaks caused by these strains. Thus, the discriminatory power of eight different molecular methods was evaluated in an attempt to establish a methodology for genotyping BEC isolates involved in intra-hospital outbreaks. BEC isolates from five hospitals in Teresina City, Piauí State were genotyped by conventional electrophoresis or PFGE of *Cla*I- or *Sma*I-digested genomic DNA hybridised with specific labelled *mecA*, Tn554, IS257 and IS256 probes. The combination of PFGE with *Cla*I/*mecA*, *Cla*I/Tn554, *Cla*I/IS257, *Sma*I/*mecA* and *Sma*I/IS257 probe-fingerprinting techniques provided a very poor discriminatory power for BEC strains. Although *Cla*I/IS256 fingerprinting discriminated 17 different polymorphisms among the isolates displaying PFGE A₁ pattern, this strategy was not reproducible. In contrast, the combination of PFGE and *Sma*I/IS256 polymorphisms differentiated BEC isolates into nine stable polymorphisms. Thus combination of PFGE and hybridisation with IS256 probe may be recommended as a useful means of typing BEC strains involved in intra-hospital infections.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a common hospital pathogen world-wide and may be a cause of severe infections in which its multiple antibiotic resistance is a serious complication [1–3].

MRSA strains were first reported in the UK in 1961, soon after the introduction of β -lactamase-resistant penicillin in clinical therapy [4]. Epidemics caused by MRSA were described in Australian hospitals in the early 1980s by Pavillard and co-workers [5]. Later, it

was demonstrated that the Australian clone had also appeared in different regions of the UK [6, 7]. Because of their ability to spread quickly, these MRSA isolates were called epidemic methicillin-resistant *Staphylococcus aureus*-1 (EMRSA-1) [8].

The extensive geographic spread of specific clones of MRSA has also been reported by others [9–21]. The Iberian clone, a well-characterised epidemic MRSA, was initially detected in an outbreak in the Bellvitge Hospital in Barcelona, Spain, in 1989 [9]. Later, this MRSA clone was detected in at least eight Portuguese hospitals, as well as in Scotland, Italy, Belgium, Germany and New York [11–13, 16, 17]. In 1999, Sá-Leão and co-workers described the intercontinental spread of another unique clone defined and seen as characteristic of infections in paediatric patients in Portugal, Poland, Argentina, Colombia and New York [18]. The predominance of specific MRSA clones was also observed in Turkey, Spain, France, Germany,

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Belgium and Japan [22–24]. Recently, Da Silva Coimbra *et al.* described the spread and predominance of two unique MRSA clones in hospitals located in different cities on the coast of the USA [21].

In Brazil, a unique multiresistant *S. aureus* clone (Brazilian epidemic clone, BEC) is disseminated in different hospitals from the north to the south of the country [10, 25–27]. BEC has also been detected as the cause of invasive infections in other South American countries (Argentina and Uruguay) and in Europe (Portugal and the Czech Republic) [14, 15, 19, 20]. More recently, the analysis of a small collection of MRSA, obtained from a Canadian hospital in the early 1990s, provided evidence that a cluster of strains all resembling BEC isolates or related genotype was spread in that hospital [28].

Besides the social and economic problems caused by the epidemic spread of specific clones of MRSA in hospitals, the extensive dissemination of a unique clone type limits the analysis of outbreaks, as all isolates involved have the same or very similar pulsed-field gel electrophoresis (PFGE) patterns. Thus, the discriminatory power of PFGE for genotyping MRSA strains displaying the same or very similar profiles became reduced [29].

Kreiswirth *et al.*, in 1993, studied the clonal origin of the *mecA* gene by analysis of a collection of MRSA isolates from different time periods and geographic locations. By digesting the DNA of these strains with the endonuclease *ClaI* and hybridising the fragments obtained with *mecA* or Tn554 DNA probes, they distinguished six *ClaI/mecA* polymorphisms (I–VI) and 29 *ClaI/Tn554* patterns based on the location of the *mecA* gene and the Tn554 transposon insertions in the bacterial genome, respectively [30]. Since the publication of these studies, other workers have used *ClaI/mecA*, *ClaI/Tn554* and *SmaI/mecA* polymorphisms to characterise the genomic diversity of MRSA hospital isolates [10, 12, 17, 19].

The use of insertion sequence (IS)-based probes is becoming an increasingly important tool in genetic discrimination of bacterial strains [23, 31–37]. Insertion sequences are small mobile elements (*c.* 0.7–1.5 kb), usually composed of a single gene encoding a transposase, flanked by short inverted repeated sequences [38]. Two IS sequences (IS257 and IS256) are well characterised IS elements in *S. aureus* [39, 40]. More recently, the combination of IS257 or IS256 probe-fingerprinting with PFGE has been used to further discriminate *S. aureus* isolates [23, 34].

This study evaluated the discriminatory power of eight different DNA methods, commonly used for MRSA genotyping, in an attempt to establish a methodology that would help in studies of molecular epidemiology of outbreaks involving BEC isolates.

Materials and methods

Bacterial isolates

A total of 84 MRSA clinical isolates was obtained during 1996–1998, from five hospitals located in Teresina City, Pi. The isolates were collected from different infection sites of patients hospitalised in several wards. Only one isolate was obtained from each patient presenting with hospital infection, except for seven patients, from whom two or three isolates were collected. Three further BEC isolates obtained from our laboratory collection were used to evaluate the reproducibility of the methods.

PFGE and conventional electrophoresis (CE)

Agarose inserted genomic DNA was prepared as described previously [41]. PFGE of *SmaI*-digested DNA was also performed as described previously [41]. In another approach, the agarose inserted DNA was digested with *ClaI* (Amersham Pharmacia, Piscataway, NJ, USA) and CE was run at 38 V for 14 h in $1 \times$ TAE buffer.

Probe preparation

The *Escherichia coli* plasmids, which contain the DNA fragments used as probes, were prepared with a Flex-Prep Kit (Amersham Pharmacia) as recommended by the manufacturer. The DNA fragments of *E. coli* plasmids were purified electrophoretically from an agarose 0.8% gel with a Sephaglas Band-Prep kit (Amersham Pharmacia) according to the manufacturer's recommendations. The procedures to obtain non-radioactive labelled probes by the ECL direct nucleic acid labelling and detection system (RPN3000; Amersham Pharmacia) were also performed according to the manufacturer's instructions. The *mecA* DNA probe used was a 1196-bp *PstI-XbaI* fragment of the *mecA* gene cloned into pTZ19 [42]. A 5.5-kb *EcoRV* transposon-specific fragment was used to detect the Tn554 [30]. The IS257 probe was a 630-bp *HindIII* fragment cloned in pIB1644 and the IS256 DNA probe was the 468-bp fragment obtained by PCR, as described above [43].

Primers 1 (5'- CAGAACAGCTGGATCCTATGG-3') and 2 (5'- GTCGACTTTTAGCCTCACGCG-3') were purchased from Gibco/BRL (Life Technologies, Rockville, USA). These primers correspond to bp 523–543 and 990–970 of the IS256 sequence and lead to the amplification of a 468-bp fragment from within ORF390 of the IS256 [31, 44]. DNA amplification was performed in 50 μ l of reaction mixture which contained 200 ng of the template DNA; 400 μ M each of dATP, dCTP, dGTP and dTTP; 0.4 μ M of each primer and $1 \times$ PCR buffer (Amersham Pharmacia). A volume corresponding to 2.5 U of Taq DNA polymerase (Amersham Pharmacia) was added only after the pre-denaturation step. Cycling was performed in a GeneAmp PCR System 9700 (Perkin-Elmer Applied

Biosystems, Foster City, CA, USA) with the following programme: pre-denaturation at 94°C for 5 min and 25 cycles of 30 s at 94°C, 30 s at 60°C and 1 min at 72°C, and with a final 5 min extension step at 72°C. A 5- μ l sample of the PCR product was run in an agarose 1.5% gel electrophoresis in 1 $\times$ TAE buffer. The 468-bp PCR product was purified with the Sephaglas Band-Prep Kit, as recommended by the manufacturer.

Blotting and hybridisation experiments

DNA fragments from PFGE and CE were transferred to Hybond N+ nylon membrane (Amersham Pharmacia) by vacuum transfer of DNA (VacuGene XL; Amersham-Pharmacia) in an alkaline solution (0.5 M NaOH, 1.5 M NaCl, final concentrations), according to the manufacturer's instructions. The DNA was fixed by baking at 80°C for 2 h. After hybridisation with the *mecA* probe, the DNA was re-probed (after being stripped by boiling) with Tn554, IS256 and IS257 probes. The hybridisation procedures were performed following the methodology described in the ECL protocol.

Criteria for interpreting PFGE and probe-fingerprinting techniques

Strains showing the same PFGE pattern or differing by one-to-three bands were grouped in a type (clone) and assigned the same capital letter. Numeric subcodes were used to represent differences (subtypes or sub-clones). The prevalent isolate of a type was assigned the capital letter and subcode 1 and used as the standard strain for analysing band differences. Isolates that differed by four-to-six PFGE fragments were grouped in a different but closely related type that was assigned to a different capital letter. Finally, MRSA isolates that differed by seven or more PFGE fragments were considered to be a different and unrelated type and were also assigned a different capital letter [10].

Isolates displaying the same *ClaI*/IS256 polymorphisms were assigned the same capital letter and numerical index. Strains differing by only one-to-three bands from the dominant type (A¹) were assigned a different numerical index, to facilitate the analysis and presentation of the data. Thus, MRSA isolates with patterns that differed in up to three bands were classified in subtypes. Finally, isolates differing by four or more bands were assigned a different capital letter.

The *SmaI*/IS256 polymorphisms of isolates that displayed the same hybridisation pattern were grouped in the same genotype and identified by the same Roman numeral. The patterns that displayed one or more different bands were grouped in a different genotype using different numerals. The *SmaI* endonuclease enzyme has a relatively small number of

restriction sites in the *S. aureus* genome. Because it would be very rare to detect a genetic event involving these sites during the course of a hospital outbreak, the criterion of one band difference was chosen to define the *SmaI*/IS256 genotype. To facilitate the visual analysis of the patterns only hybridised fragments ≥ 48.5 kb were considered.

Reproducibility assay

To investigate the frequency of genetic changes in the *S. aureus* DNA, three MRSA isolates displaying different but similar PFGE patterns were submitted to 30 serial passages on Tryptic Soya Agar. Another three pairs of MRSA isolates obtained from three different patients, during a period of about 1 week, 1 month or 2 months, respectively, were also analysed. These pairs formed by the strains 109 and 117; 86 and 107; and 11 and 56 were chosen because each strain of a pair displayed the same PFGE pattern and *SmaI*/*mecA*, *SmaI*/IS257, *ClaI*/*mecA*, *ClaI*/IS257 and *ClaI*/Tn554 polymorphisms, as well.

Results

PFGE

A total of 78 MRSA resembling BEC strains was obtained from the collection of 84 MRSA isolates associated with hospital infections, in patients from five hospitals located in Teresina City, Pi (Table 1). The isolates were collected from March 1996 to July 1998. All these 78 MRSA isolates had very similar PFGE patterns, referred to here as pattern A (Fig. 1). Fifty-seven (c. 70%) of the MRSA strains shared the same predominant PFGE pattern A₁ (Table 1), previously described among the BEC isolates from Brazil [10]. Twenty-one MRSA strains differed from the BEC A₁ pattern by only one, two or three PFGE bands. Thus, besides pattern A₁, six other subtypes (A₂–A₇, Fig. 1) were detected. Among these variants, subtype A₂ was the most frequent (13 strains), followed by A₄ (four strains). The absolute predominance of BEC strains in Brazil and the wide spread of this clone type to other countries limited the discriminatory power of PFGE for genotyping these MRSA isolates [10, 14, 15, 19, 20, 25–27].

*Characterisation of the MRSA isolates by traditional DNA fingerprinting methods (*ClaI*/*mecA*, *ClaI*/Tn554 and *SmaI*/*mecA* polymorphisms) for typing of MRSA isolates*

This study tested the 78 BEC isolates by the methodologies described by Kreiswirth *et al.* [30]. The different patterns obtained are listed in Table 1.

The great majority of the BEC isolates studied (71 of 78) had the same *ClaI*/*mecA* polymorphism (here called pattern III₁), except seven that gave pattern III.

Table 1. Genotypic characterisation of 78 MRSA isolates from five hospitals in Teresina City, Pi

Strain no.	PFGE	Genomic patterns*						
		<i>Sma</i> I <i>mecA</i>	<i>Sma</i> I IS257	<i>Cla</i> I <i>mecA</i>	<i>Cla</i> I IS257	<i>Cla</i> I Tn554	<i>Sma</i> I IS256	<i>Cla</i> I IS256
01,14	A ₁	G	a ₁	III	α	B ₁	I	A ¹⁴
02	A ₁	G	a ₁	III ₁	α	B	IV	A ¹⁶
03	A ₄	G	a ₁	III ₁	α	B ₁	XI	A ²
04,06,10,11,24,51,56,74,84,87,93,98	A ₁	G	a ₁	III ₁	α	B ₁	I	A ¹
08	A ₄	G	a ₁	III	α	B ₁	XIV	ND
09,13,49,53,54	A ₁	G	a ₁	III ₁	α	B ₁	V	A ¹⁰
12	A ₁	G	a ₁	III ₁	α	B	IV	E
16	A ₁	G	a ₁	III ₁	α	B ₁	Viii	A ¹⁰
17,39,88	A ₁	G	a ₁	III ₁	α	B ₁	III	A ¹
20	A ₁	G	a ₁	III ₁	α	B	III	G
21,64,66,72,73,109,117	A ₁	G	a ₁	III ₁	α	B ₁	VIII	A ²
48	A ₁	G	a ₁	III	α	B ₁	III	A ¹⁴
50	A ₁	G	a ₁	III	α	B ₁	I	A ¹
57,113	A ₁	G	a ₁	III ₁	α	B ₁	V	A ¹
61	A ₁	G	a ₁	III ₁	α	B ₁	I	A ¹⁵
62	A ₄	G	a ₁	III	α	B ₁	XI	A ²
65	A ₁	G	a ₁	III ₁	α	B ₁	VIII	A ¹³
69,94	A ₁	G	a ₁	III ₁	α	B ₁	I	A ₁₂
78	A ₃	G	a ₁	III ₁	α	B ₁	III	A ¹⁸
83	A ₁	G	a ₁	III ₁	α	B ₁	III	A ¹²
85,89,102,105	A ₂	G	a ₂	III ₁	α	B ₁	I	A ¹⁰
86	A ₂	G	a ₂	III ₁	α	B ₁	I	A ³
90	A ₅	G	a ₁	III ₁	α	B ₁	XII	B
91,92	A ₁	G	a ₁	III ₁	α	B ₁	II	A ¹⁷
100	A ₁	H	a ₂	III ₁	α	B ₂	I	C
107	A ₂	G	a ₂	III ₁	α	B ₁	I	A ¹
111	A ₁	G	a ₁	III ₁	α	B ₁	V	A ⁷
112	A ₁	GJ	a ₁	III ₁	α	B ₁	VI	A ¹
121	A ₁	G	a ₁	III ₁	α	B ₁	VI	A ²
122	A ₁	G	a ₁	III ₁	α	B	I	F
128	A ₁	G	a ₁	III ₁	α	B	VII	F
130	A ₁	G	a ₁	III ₁	α	B ₁	V	A ²
131	A ₁	G	a ₁	III ₁	α	B ₁	VII	A ¹¹
135	A ₂	G	a ₂	III ₁	α	B	I	A ⁵
136	A ₆	CG	a ₃	III ₁	α	B ₂	XIII	A ⁵
140	A ₂	G	a ₂	III ₁	α	B ₁	I	A ⁶
143,149	A ₂	G	a ₂	III ₁	α	B ₁	I	A ²
144,148	A ₂	H	a ₂	III ₁	α	B ₃	I	A ²
150,152	A ₁	G	a ₁	III ₁	α	B ₁	I	A ⁴
151	A ₄	G	a ₁	III ₁	α	B ₁	X	A ⁴
153	A ₁	G	a ₁	III ₁	α	B ₁	VII	A ¹
154,155	A ₁	G	a ₁	III ₁	α	B ₁	I	A ⁹
170	A ₇	G	a ₁	III	α	B	XV	A ⁸
184	A ₁	G	a ₁	III ₁	α	B	IX	D

ND, not done.

*Chromosomal DNA was digested with *Sma*I or *Cla*I endonucleases and the fragments were separated by PFGE or conventional electrophoresis, respectively. After blotting and fixing on to nylon membranes, the digested genomic DNA was hybridised with *mecA*, IS257, Tn554 and IS256 specific probes (see *Materials and methods*).

Pattern III₁ is very similar to the pattern displayed by the standard type III, strain RN7164, a Canadian isolate recently identified as belonging to BEC clone type [28]. Also, all BEC isolates from Teresina had an identical or very similar *Cla*I/Tn554 pattern B to that observed for the standard type strain RN7164 [30]. The types B₁, B₂ and B₃ displayed by BEC isolates probably differed from the standard type B by only one genetic event (data not shown). Pattern B₁ was the most frequent among these BEC isolates (66 strains). Another eight isolates had pattern B. The other strains were distributed among patterns B₂ and B₃.

The great majority of BEC isolates (73 strains) had the *mecA* gene located in the same *Sma*I PFGE fragment (band G of 115 kb).

The combination of PFGE pattern with the probe-fingerprinting techniques, described by Kreiswirth *et al.* [30], provided a total of 12 genotypes. However, 47 of 57 PFGE A₁ isolates had the same genotype, displaying PFGE pattern A₁, *Cla*I/*mecA* polymorphism III₁ and Tn554 insertion pattern B₁ (III₁::B₁). The other 10 A₁ isolates were distributed in two other patterns (Table 2).

Analysis of the discriminatory power of IS257 for differentiating MRSA isolates belonging to a unique clone type

All 78 BEC strains were grouped in a very similar *Sma*I/IS257 genotype. Sixty-four isolates gave the same *Sma*I/IS257 pattern a₁, 13 displayed pattern a₂

Fig. 1. *Sma*I PFGE patterns of MRSA isolates belonging to the BEC obtained from different hospitals in Teresina City, Pi. Lanes 1 and 10, λ ladder; 2–8, PFGE patterns predominant in this study (patterns A₁–A₇, respectively); 9, pattern displayed by the strain BMB9393, a representative BEC isolate.

Table 2. Distribution of the molecular types displayed by the 57 MRSA isolates belonging to PFGE pattern A₁

<i>Cla</i> I/ <i>mecA</i> :: <i>Cla</i> I/Tn554*		<i>Sma</i> I/IS256*	
Pattern	Number of A ₁ isolates	Pattern	Number of A ₁ isolates
III ₁ ::B	6	I	23
III ₁ ::B ₁	47	II	2
III ₁ ::B ₁	4	III	6
...	...	IV	2
...	...	V	9
...	...	VI	2
...	...	VII	3
...	...	VIII	9
...	...	IX	1

*Chromosomal DNA was digested with *Sma*I or *Cla*I endonucleases. The fragments were separated by PFGE or conventional electrophoresis, respectively. After blotting and fixing on nylon membranes the digested genomic DNA was hybridised with *mecA*, Tn554 and IS256 specific probes (see *Materials and methods*).

and one pattern a₃. Patterns a₂ and a₃ were verified among the isolates that had, respectively, PFGE patterns A₂ and A₆ (Table 1). Neither of these had PFGE

band I (90 kb), displayed by BEC A₁ isolates (Fig. 1), where one insertion of the IS257 is commonly located in the BEC genome (data not shown). Thus, the changes observed in the *Sma*I/IS257 polymorphism were probably due to one genetic event involving the *Sma*I restriction site. Moreover, all the 78 isolates studied had exactly the same *Cla*I/IS257 polymorphism (pattern α , Table 1). Thus, the use of either *Sma*I/IS257 or *Cla*I/IS257 genotyping, with very rare exceptions, did not discriminate BEC isolates.

Genotyping of BEC isolates with IS256 probe

The number of bands (9–15) obtained for BEC isolates with the IS256 probe indicated that the IS256 was present in numerous copies. Although the nucleotide sequence of IS256 has one *Cla*I restriction site located at nt 1005–1010, the probe used in the present study (nt 523–990) does not include this site [44]. Furthermore, *Sma*I sites are not present in the IS256 sequence. Thus, if insertion, deletion or genome re-arrangement, modifying or creating a *Sma*I or *Cla*I restriction site,

does not occur, a distinct hybridisation band will correspond to one IS256 copy.

The discrimination of BEC isolates could be increased when the IS256 probe was hybridised with *Sma*I-digested genomic DNA (Table 1). Analysis of the *Sma*I/IS256 polymorphisms with the criterion of one band difference, gave 15 different patterns (I–XV, Fig 2) for the BEC isolates. These criteria allowed the differentiation of the PFGE subtype A₁ into nine patterns (I–IX, Fig. 2). The combination of PFGE and *Sma*I/IS256 techniques had a specificity of *c.* 85%, when *Sma*I/IS256 polymorphism was compared with the association of PFGE with *mecA*, Tn554 and IS257 probe fingerprinting.

The frequency with which these IS256 patterns occurred among the 57 A₁PFGE isolates is given in Table 2. The association of IS256 polymorphism with the other techniques tested did not further improve the subtyping of the 23 A₁:I isolates, even when *Cla*I/IS256 polymorphism was used. Although 24 different *Cla*I/IS256 patterns were obtained for the 78 BEC isolates (Table 1), the methodology was not stable after in-vitro or in-vivo passages. Variations in the hybridisation patterns up to three band differences were verified for a unique isolate with this technique, as shown below.

Stability of IS256 after in-vivo or in-vitro passages

Because IS elements inherently have the potential to cause genetic re-arrangements, the stability of the methodologies was tested with IS256 [38]. For this purpose, the reproducibility was evaluated using six pairs of strains obtained before and after in-vivo or in-vitro passages. The study verified that the PFGE pattern (Fig. 3a) and *Sma*I/IS256 polymorphism (Fig. 3b) did not show any alteration after these passages. As well as the stability of these techniques, slight changes in the *Cla*I/IS256 polymorphisms were verified for three pairs that were submitted to in-vitro passages (BVTP1/AVTP1 and BVTP2/AVTP2) or in-vivo passages (BVVP2/AVVP2). However, these changes never involved more than three bands and they were probably due to one or two genetic events after the in-vitro (about 2000 generations) or in-vivo passages (Fig. 3c).

Discussion

DNA technology has been intensively applied to the study of the molecular epidemiology of outbreaks. Various methods has been suggested for the characterisation of many different pathogenic bacteria [45–47]. Chromosomal DNA fingerprinting is a technique of

Fig. 2. Different *Sma*I/IS256 polymorphisms obtained for BEC strains. Lanes 1 and 19, λ ladder; 2–10, patterns I–IX identified among the strains displaying PFGE pattern A₁; 11–18, patterns I, III and X–XV displayed by isolates with PFGE patterns A₂–A₇, respectively.

increasing interest to epidemiologists evaluating and controlling nosocomial and community infections. This strategy is highly sensitive to minor genomic differences. Although various molecular techniques have been used to type MRSA strains, PFGE is considered discriminatory, reproducible and applicable to epidemiological studies involving MRSA [13, 22, 23, 32, 34]. Currently, there is a consensus among molecular epidemiologists that PFGE is the method of choice for genotyping bacteria associated with community- or hospital-associated outbreaks [32, 47, 48]. In the present study, the great majority of MRSA isolates obtained from the period 1996–1998 from Teresina city, Pi, displayed identical or very similar PFGE patterns, limiting the use of this technique as a powerful tool for bacterial genotyping.

Clonal spread of MRSA and the epidemic behaviour of some specific MRSA clones have been documented in several countries [9–24, 30]. The BEC is an MRSA clone well adapted not only to cause hospital-associated diseases in many countries, but also to support acquisition of new genetic traits without affecting its virulence or extraordinary ability to spread [33, 49, 50]. The wide spread of BEC isolates in the hospitals studied and in many other hospitals in Brazil, as well as in other countries, prompted an investigation of the value of different typing methods in detecting minor genomic variations among BEC isolates, in order to establish a methodology that would help in the studies of molecular epidemiology of outbreaks involving BEC isolates.

Other useful typing approaches, apart from PFGE, are chromosomal probe-fingerprinting techniques. In these methodologies the fragmented genomic DNA is transferred by blotting on to nylon or nitrocellulose membranes and the location of a subset of fragments containing a particular nucleotide sequence can be identified by hybridisation with the specific labelled DNA or RNA probe [45–47].

mecA or Tn554 probes, in combination with PFGE, have been used extensively for characterisation of MRSA [9–19, 32, 41]. The present study verified that the association of *ClaI/mecA* and *ClaI/Tn554* polymorphism generated only three types among the isolates displaying PFGE pattern A₁ studied: patterns A₁:III₁::B₁, A₁:III₁::B₁ and A₁:III₁::B. Furthermore, 47 of 57 BEC strains displaying PFGE A₁ pattern were typed as A₁:III₁::B₁. Moreover, a unique physical location (band G of 115 kb) was observed when the *mecA* probe was hybridised with *SmaI*-digested genomic DNA for the great majority of the isolates studied. The lower discriminatory power of these methodologies showed that the degree of variability in the genome regions flanking these target sequences is very low among BEC isolates. Thus, the genomic diversity detected by these probes was very limited for genotyping these strains. Similar results were obtained

by Tenover *et al.* when testing different epidemic MRSA clones [32].

The combination of IS257 or IS256 probe-fingerprinting with PFGE and conventional gel electrophoresis (CE) was also used to further discriminate BEC isolates. The IS257 probe-fingerprinting techniques did not discriminate BEC isolates when *ClaI*-digested DNA was used. All isolates displayed equal patterns (polymorphism α). Similarly, when *SmaI*-cut DNA was tested, only three profiles (a₁, a₂ and a₃) were obtained. In contrast, Yoshida *et al.* distinguished MRSA isolates sharing an identical PFGE type into eight distinct genotypes by IS257 typing [34]. This lack of agreement is probably due to the fact that all BEC isolates so far analysed in this laboratory from 1992 to the present displayed a small and constant number of IS257 copies inserted in the genome of BEC strains (two copies). In contrast, the strains analysed by Yoshida and co-workers, isolated from Malaya University Hospital, Japan, in the period 1987–1989, had a variable number of IS257 copies (two-to-five copies).

Some researchers have proposed the use of IS256 for genotyping MRSA strains [23, 35–37]. Morvan *et al.*, in 1997, verified that *SmaI*/IS256 polymorphism improved RFLP analysis by PFGE for discriminating between the intra-hospital, outbreak-related, phage type 77 MRSA strains and endemic strains disseminated in various cities and countries [23]. Similarly, in the present study, the 78 MRSA isolates belonging to BEC clone type were discriminated into 15 different *SmaI*/IS256 patterns. Among them, the 57 PFGE A₁ isolates gave nine distinct *SmaI*/IS256 polymorphisms.

The discrimination of BEC isolates could not be improved when the IS256 probe was hybridised with *ClaI*-digested DNA, separated by conventional DNA gel electrophoresis. Although this typing method differentiated BEC strains into 24 different patterns, it was not sufficiently stable for the analysis of BEC strains involved in hospital outbreaks. The method showed poor reproducibility after in-vivo or in-vitro passages of these MRSA isolates. The fact that *ClaI* restriction endonuclease cuts the genomic DNA into numerous fragments, associated with the high number of IS256 copies verified in the genome of BEC isolates (9–15 copies), could explain the poor reproducibility of this method. Moreover, IS256 can mediate genomic re-arrangements and these changes can be detected after in-vitro manipulation when enzymes that cut DNA frequently are used [30, 36].

In conclusion, *ClaI/mecA*, *ClaI/Tn554*, *ClaI*/IS257, *SmaI/mecA* and *SmaI*/IS257 polymorphisms do not efficiently discriminate BEC isolates; therefore, the use of these techniques as molecular strategies is not recommended for genotyping these strains.

It is of concern that when infections caused by the

Fig. 3. (a) PFGE patterns obtained for the strains belonging to BEC clone type before or after in-vitro or in-vivo passages. Lanes 1, 8 and 15, λ ladder; 2–7, pairs 1, 2 and 3 before and after in-vitro passages (BVTP and AVTP, respectively); 8–14, pairs 11/56, 86/107 and 109/117 collected from three different patients before (BVVP) and after (AVVP) periods of 1 week to 2 months. (b) *Sma*I/IS256 polymorphisms displayed by pairs of strains obtained before or after passages. Lanes 1, 8 and 15, λ ladder; 2–7, patterns displayed by pairs of strains (1, 2 and 3) before or after in-vitro passages (BVTP, AVTP, respectively); 8–14, patterns displayed by pairs of strains (11/56, 86/107 and 109/117) collected from three different patients before (BVVP) or after (AVVP) periods of 1 week to 2 months. (c) *Cla*I/IS256 polymorphisms presented by pairs of strains obtained before or after passages. Lanes 1, 8 and 15, λ HindIII; 2–7, patterns displayed by pairs of strains (1, 2 and 3) before or after in-vitro passages (BVTP, AVTP, respectively); 8–14, patterns displayed by pairs of strains (11/56, 86/107 and 109/117) obtained from three different patients before (BVVP) or after (AVVP) periods of 1 week to 2 months.

same bacterial species affect different patients in a unit, infection control measures could be improved by making use of the molecular epidemiological analysis of the strains involved [47]. On the basis of the results of the present study, it is concluded that the inclusion of *Sma*I/IS256 probe-fingerprinting as an approach to detect variants of BEC displaying PFGE A₁ pattern enhances the ability to distinguish BEC strains associated with intra-hospital infections. Thus, the use of this probe fingerprinting in epidemiological studies involving strains from BEC clone type may be recommended. Also, it would appear that the use of IS probes in combination with PFGE could be a useful

strategy to further characterise other clusters of MRSA isolates.

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