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Evidence for the Geographic Spread of a Methicillin-Resistant *Staphylococcus aureus* Clone between Portugal and Spain

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Methicillin-resistant *Staphylococcus aureus* (MRSA) isolates collected during a 7-month period in 1992 and 1993 at Hospital Pulido Valente (340 beds), Lisbon, Portugal, were characterized by a combination of genotypic and phenotypic methods. Clonal identities were determined by probing *Cla*I digests (i) with a *mecA* probe and (ii) with a Tn*554* probe and (iii) by pulsed-field gel electrophoresis (PFGE) patterns of chromosomal *Sma*I digests. *mecA-Cla*I type I was predominant among these isolates (38 of 43). Most of these (37 of 38 [97.4%]) were associated with a single Tn*554* pattern, pattern E, and the majority (23 of 38 [61%]) also showed a relatively uniform chromosomal background, as indicated by PFGE (PFGE pattern A). The major clone (*mecA-Cla*I type I::Tn*554* type E and PFGE pattern A) at Hospital Pulido Valente was indistinguishable by these molecular typing criteria from the dominant clone that had been identified in two major current outbreaks of MRSA disease in Spain (Barcelona and Madrid). The Portuguese and Spanish clones also had a common heterogeneous class 3 phenotype and identical multidrug resistance patterns. The data presented in this work support the notion that MRSA clones can spread across considerable geographic distances.

The central genetic element of methicillin resistance, the *mecA* gene, shows only minimal sequence variation in methicillin-resistant *Staphylococcus aureus* (MRSA) isolates. On the other hand, testing the vicinity of *mecA* (through hybridization of *Cla*I-digested chromosomal DNA with a *mecA*-specific probe) has shown sufficient strain-to-strain variation to provide useful fingerprints for strains through the particular *mecA* polymorphs they carried. These studies have already resulted in the identification of five new *mecA* polymorphs (6), in addition to the previously identified six (9), and several new Tn*554* types.

We previously used this technique in combination with other tests to probe the general chromosomal backgrounds of staphylococcal isolates in order to define MRSA clones in molecular epidemiological investigations of MRSA outbreaks at several hospitals in the United States and Europe (2, 3, 6, 7). This molecular fingerprinting technique allowed us to identify from the Hospital Pulido Valente, Lisbon, Portugal, the same MRSA clone that was responsible for recent outbreaks at several hospitals in Spain (6).

MATERIALS AND METHODS

Hospital. Hospital Pulido Valente is a small (340-bed) hospital in Lisbon, Portugal, which was built in 1910, with medical and surgical care for adult patients, excluding obstetrics and burn units. It has three intensive care units of cardiology, pulmonology, and surgery and ambulatory services for dermatology and gastroenterology. During 1993, 9,100 patients were admitted. The percentage of *S. aureus* isolates among all gram-positive bacteria isolated at this hospital was 59%, and the percentage of MRSA among *S. aureus* isolates was 45%.

This hospital has no active MRSA infection control program in the sense that patients colonized or infected with MRSA are not isolated, and mupirocin is not used for eradication of MRSA in colonized patients or health care workers. There is, however, reinforcement of careful handwashing and educational programs throughout the hospital, although in several wards there are not enough facilities to implement these programs.

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Bacterial strains. The 43 MRSA clinical isolates were obtained during a 7-month period (from August 1992 to February 1993). They were given the designation HPV (for Hospital Pulido Valente) and are listed in Table 1. Isolates were recovered from a variety of infection sites, pus (21%), bronchial secretion (19%), sputum (16%), hemoculture (16%), pleural liquid (21%), vaginal exudate (2%), and nasal exudate (5%). PER34 is a representative strain of one of the dominant clones responsible for current outbreaks of MRSA disease in Spain (Barcelona and Madrid) and was obtained from the Rockefeller University collection (6). CPS23 and CPS62 are representative strains of the dominant clone responsible for an outbreak of MRSA disease at another Lisbon hospital, Hospital Dona Estefânia (3).

DNA probes. The DNA probes used were a 1.196-kb *Pst*I-*Xba*I internal fragment of the *mecA* gene from plasmid pMF13 (3, 10, 11) and a 5.5-kb fragment of transposon Tn*554* that had been cloned in a pBluescript II vector (7, 9).

Antimicrobial susceptibilities. Antimicrobial susceptibilities were determined by disk diffusion methods (13). The following antibiotics were tested: ampicillin, amikacin, amoxicillin-clavulanate, ciprofloxacin, cephalothin, cephalosporin (narrow spectrum), cefoxitin, cefuroxime, ceftazidime, doxycycline, dicloxacycline, erythromycin, fusidic acid, fosfomycin, gentamicin, imipenem, kanamycin, lincomycin, methicillin, nitrofurantoin, netilmicin, norfloxacin, oxacillin, penicillin, pefloxacin, piperacillin, pristinamycin, rifampin, tetracycline, teicoplanin, tobramycin, trimethoprim, trimethoprim-sulfamethoxazole, and vancomycin.

Assays for methicillin resistance and population analysis profiles. All strains were assayed for their methicillin resistance phenotypes by using cultures grown overnight in tryptic soy broth. Methicillin resistance levels were first assessed in a preliminary test with disks that contained 1 mg of methicillin (5). Four reference strains, CDC1, NYHB3, BM79, and COL (Rockefeller University collection), were used as controls. On the basis of the diameters of inhibition halos, the strains were assigned to tentative phenotypic expression classes (17). Then preliminary classifications were confirmed by population analysis profiles, as previously described (3, 4, 17).

DNA electrophoresis. Preparation of chromosomal DNAs for conventional and pulsed-field gel electrophoresis (PFGE), endonuclease digestion, conventional gel electrophoresis, and PFGE were all carried out as previously described (3).

DNA-DNA hybridization. DNA transfer was performed by standard methodology (16). Probe labeling and hybridization were done by using the ECL nonradioactive labeling kit RPN3000 (Amersham, Amersham, United Kingdom) according to the manufacturer's instructions.

RESULTS

Genetic and physiological characterizations of isolates. (i) *Cla*I-*mecA* pattern. Ten *mecA* polymorphs (I to X) have been described so far for *S. aureus* isolates (6, 9). Chromosomal DNA preparations of all the MRSA clinical isolates were di-

TABLE 1. Genotypic characterizations of the 43 MRSA clinical isolates obtained from patients (95%) and carriage (5%) at Hospital Pulido Valente (Lisbon, Portugal) during August 1992 to February 1993

HPV isolate(s)	Clonal type (*Cla*I-*mecA*::Tn*554*)[a]	PFGE pattern	No. of isolates	Clonal type (*mecA-Cla*I::Tn*554*::PFGE)
37, 105, 107	I::E	A1	3	I::E::A
63, 64, 66, 72, 78, 79, 80, 106	I::E	A2	8	I::E::A
36, 48, 54, 55, 56, 77, 93	I::E	A3	7	I::E::A
3	I::E	A4	1	I::E::A
39	I::E	A5	1	I::E::A
92	I::E	A6	1	I::E::A
34, 38	I::E	A7	2	I::E::A
26, 40, 67	I::E	B	3	I::E::B
14, 25, 44, 73, 82, 91	I::E	C	6	I::E::C
2, 60, 62	I::E	D	3	I::E::D
68	I::E	E1	1	I::E::E
76	I::E	E2	1	I::E::E
17	I::NH	F1	1	I::NH::F
99	II::NH	F2	1	II::NH::F
11, 12, 24	II::J	G	3	II::J::G
15	III::α	H	1	III::α::H

[a] NH, absence of homology with transposon Tn*554*.

gested with restriction endonuclease *Cla*I and hybridized with the *mecA* probe. The majority were *Cla*I-*mecA* pattern I strains (38 of 43), four strains were found to be *Cla*I-*mecA* pattern II (HPV11, HPV12, HPV24, and HPV99), and only one belonged to polymorph *Cla*I-*mecA* pattern III (HPV15). Representative hybridizations of *Cla*I restriction digests with the *mecA* probe are shown in Fig. 1.

(ii) *Cla*I::Tn*554* pattern. The gels used for *Cla*I-*mecA* pattern determinations were rehybridized with a Tn*554*-specific probe (7, 9). The presence of this transposon was also assessed by plating strains on tryptic soy agar with 500 μg of spectinomycin ml^{-1}, since resistance to this concentration of spectinomycin in *S. aureus* is specifically associated with the presence of Tn*554* (9).

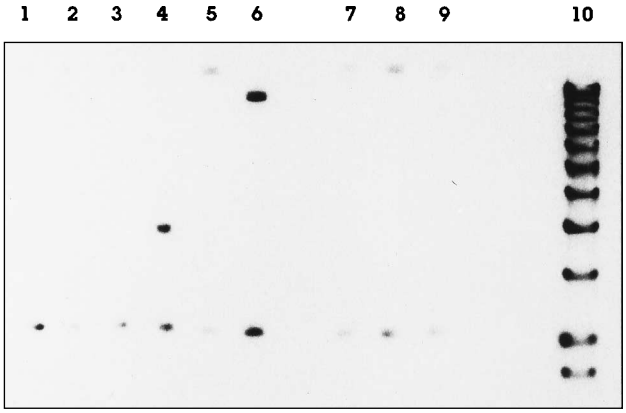

FIG. 1. Different *Cla*I-*mecA* patterns among the MRSA clinical isolates from Hospital Pulido Valente. *Cla*I-*mecA* pattern I, strains HPV82 (lane 1), HPV91 (lane 2), HPV92 (lane 3), HPV93 (lane 5), HPV105 (lane 7), HPV106 (lane 8), and HPV107 (lane 9); *Cla*I-*mecA* pattern III, strain HPV15 (lane 4); *Cla*I-*mecA* pattern II, strain HPV99 (lane 6). Lane 10, DNA molecular size markers (1-kb DNA ladder [Bethesda Research Laboratories]).

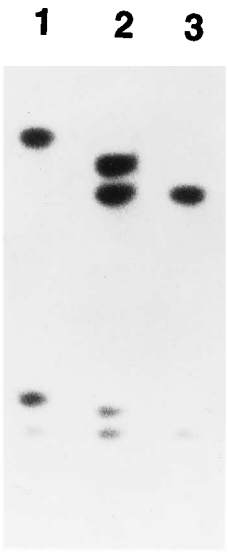

FIG. 2. *mecA-Cla*I::Tn*554* typing of MRSA clinical isolates from Hospital Pulido Valente. Lane 1, HPV15 (*mecA-Cla*I type III::Tn*554* type α); lane 2, HPV12 (*mecA-Cla*I type II::Tn*554* type J); lane 3, HPV107 (*mecA-Cla*I type I::Tn*554* type E).

*Cla*I-*mecA* pattern I was most frequently (37 of 38 strains) associated with a single *Cla*I::Tn*554* pattern, already described as Tn*554* pattern E (9), which was also identified in Spanish isolates as pattern a (6) (in fact, it is the same as pattern E). The remaining *Cla*I-*mecA* polymorph I strain had no Tn*554* insertions (HPV17). Three of the four *Cla*I-*mecA* pattern II strains identified (HPV11, HPV12, and HPV24) were associated with a single Tn*554* pattern, already described as Tn*554* pattern J (9). The fourth isolate (strain HPV99) was Tn*554* negative. The only *Cla*I-*mecA* type III strain (HPV15) detected among these 43 isolates showed a new Tn*554* pattern, designated α, which was recently identified in isolates from two Portuguese hospitals (2, 3). Figure 2 shows representatives of the Tn*554* patterns found among the clinical MRSA isolates of Hospital Pulido Valente.

(iii) PFGE. Chromosomal DNAs restricted with *Sma*I were separated by PFGE with an LKB/Pharmacia Gene Navigator apparatus. The different patterns were defined by a variation in migration of at least three fragments between strains (8). By these criteria, eight profiles (among the 43 strains studied), types A through H, were identified. Figure 3 shows the various PFGE types and subtypes that were defined in this study.

Six different PFGE patterns (A through F) were associated with the *mecA-Cla*I type I polymorph, while PFGE pattern F was also seen in association with a *mecA-Cla*I type II polymorph. The majority of *mecA-Cla*I type I (23 of 38) and Tn*554* type E isolates belonged to PFGE pattern A. PFGE type A was subdivided into seven subgroups (A1 to A7). PFGE type B included three clinical isolates. PFGE types C and D included six and three isolates, respectively. PFGE type E strains exhibited minor differences in their restriction profiles and were therefore divided into two subgroups (E1 and E2). All of the isolates that belonged to PFGE patterns B through E had a

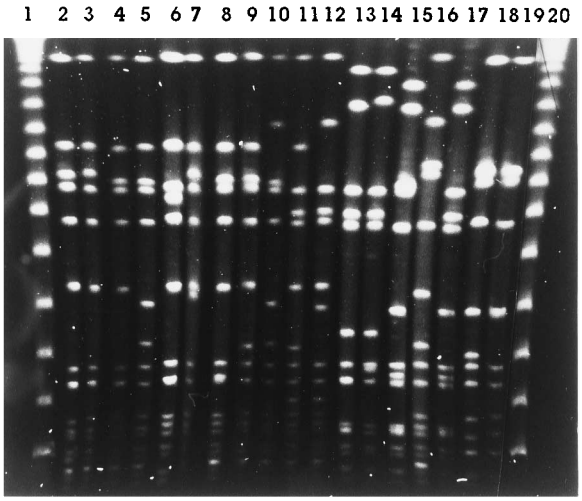

FIG. 3. PFGE patterns of MRSA isolates from Hospital Pulido Valente. *Sma*I restriction patterns and subtypes (a total of 16 representatives) were identified at the hospital from August 1992 to February 1993. Strains that were representative of major MRSA outbreaks at Hospital de Bellvitge Princeps d'Espanya (Barcelona, Spain), strain PER34 (6), and Hospital Dona Estefânia (Lisbon, Portugal), strain CPS23 (3), were also included on the gel. The positions of various PFGE patterns on the gel are as follows: lanes 1 and 20, lambda ladder; lane 2, PER34, PFGE pattern A (6); lane 3, HPV107, PFGE pattern A1; lane 4, HPV78, PFGE pattern A2; lane 5, HPV77, PFGE pattern A3; lane 6, HPV3, PFGE pattern A4; lane 7, HPV39, PFGE pattern A5; lane 8, HPV92, PFGE pattern A6; lane 9, HPV34, PFGE pattern A7; lane 10, HPV40, PFGE pattern B; lane 11, HPV14, PFGE pattern C; lane 12, HPV60, PFGE pattern D; lane 13, HPV68, PFGE pattern E1; lane 14, HPV76, PFGE pattern E2; lane 15, HPV17, PFGE pattern F1; lane 16, HPV24, PFGE pattern G; lane 17, HPV99, PFGE pattern F2; lane 18, HPV15, PFGE pattern H; lane 19, CPS23, PFGE pattern A (3).

common Tn*554* pattern, type E. One *mecA-Cla*I type I strain, free of Tn*554*, was included in PFGE type F (subtype F1).

*mecA-Cla*I type II::Tn*554* type J (HPV11, HPV12, and HPV24) strains showed yet another PFGE pattern, type G. Strain HPV99, *mecA-Cla*I type II but without Tn*554*, showed a PFGE subtype, F2, that was very similar to the PFGE profile of strain HPV17 (*mecA-Cla*I type I and Tn*554* free).

A single strain (HPV15) that carried the *mecA-Cla*I type III::Tn*554* α polymorph showed PFGE pattern H. The properties of this strain were of the same clonal type as those of the dominant clone found previously at another Lisbon hospital (3).

Table 1 summarizes the clonally related properties of these 43 MRSA isolates.

(iv) Methicillin resistance phenotypes. The majority of strains (37 of 43) belonged to class 3, four strains belonged to class 2, and two strains belonged to class 1.

(v) Resistance to other antibiotics. Half of the MRSA isolates that represented the major clonal types (*mecA-Cla*I type I::Tn*554* type E::PFGE subtype A2 [I::E::A2 or I::E::A3]) were tested for their susceptibilities against a panel of antibiotics. All of these strains were resistant to ampicillin, amoxicillin-clavulanate, ciprofloxacin, cephalothin, doxycycline, gentamicin, erythromycin, imipenem, methicillin, penicillin, and tetracycline and were susceptible to vancomycin. Also, MRSA strains HPV105 and HPV107 (clonal type I::E::A1), which are identical to PER34 (representative of the Spanish clone), were uniformly resistant to methicillin, oxacillin, ampicillin, gentamicin, erythromycin, penicillin, and tetracycline and susceptible to teicoplanin and vancomycin. The other antibiotics listed in Materials and Methods were not tested in these comparisons.

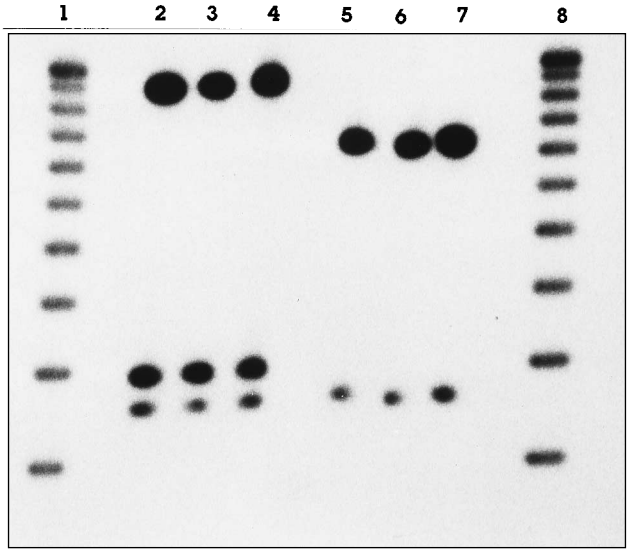

FIG. 4. Hybridization of *Cla*I restriction digests with the Tn*554* probe. Comparison of isolates from Hospital Pulido Valente, Lisbon, Portugal (HPV strains); Hospital Dona Estefânia, Lisbon, Portugal (CPS strains); and Hospital de Bellvitge Princeps d'Espanya, Barcelona, Spain (strain PER34). Lanes: 1 and 8, molecular weight ladders (1-kb DNA ladder [Bethesda Research Laboratories]); 2 and 4, strains CPS23 and CPS62, respectively (*mecA-Cla*I type III::Tn*554* type α [2]); 3, strain HPV15 (*mecA-Cla*I type III::Tn*554* type α); 5 and 7, strains HPV37 and HPV107, respectively (*mecA-Cla*I type I::Tn*554* type E); 6, strain PER34 (*mecA-Cla*I type I::Tn*554* type E, previously named *mecA-Cla*I type I::Tn*554* type a [6]).

Geographic spread of MRSA clones. The molecular typing criteria discussed above were used to compare several MRSA clinical strains of Hospital Pulido Valente with strain PER34, which is representative of the dominant clone responsible for a current major outbreak at a hospital in Barcelona, Spain (6). The strains were compared by *Cla*I-*mecA* patterns, Tn*554* insertion sites, PFGE restriction profiles, and population analysis profiles. By all these criteria, the Barcelona strain, PER34 (6), and isolates HPV37, HPV105, and HPV107, which are representative of the major MRSA clone at Hospital Pulido Valente, were closely related. They all belonged to clonal type I::E::A1, according to our nomenclature, and had a common heterogeneous class 3 phenotype. Strains HPV105, HPV107, and PER34 also had identical multidrug resistance patterns.

Figures 3 and 4 show chromosomal PFGE patterns (after *Sma*I digestion) and Tn*554* hybridization patterns of PER34 and several representatives of the dominant MRSA clone from Hospital Pulido Valente. Also included in these two figures are fingerprints of strain HPV15 and strains CPS23 and CPS62 (3). All of these strains carry the same *mecA-Cla*I polymorph (*mecA-Cla*I type III::Tn*554* α) and PFGE pattern H. While HPV15 was a solitary isolate at Hospital Pulido Valente, CPS23 and CPS62 represent the dominant MRSA clone at Hospital Dona Estefânia (3).

DISCUSSION

The most frequent clonal type of MRSA that was identified at Hospital Pulido Valente by the investigation described here carried the *mecA-Cla*I type I polymorph in combination with Tn*554* type E and a common genetic background, defined here as PFGE type A. Interestingly, this is the same MRSA clone already identified as dominant in several hospital outbreaks in Barcelona and Madrid, Spain (6). The same Portuguese and

Spanish MRSA isolates also shared multidrug resistance patterns and class 3 methicillin phenotypes. The *Cla*I-*mecA* type I polymorph (particularly in combination with Tn*554* type E) was also the one most frequently found in a sample of over 450 MRSA isolates from a variety of geographic sources (9).

Interestingly, a single strain among the 43 MRSA isolates from Hospital Pulido Valente had a unique clonal type (*mecA*-*Cla*I type III::Tn*554* α::PFGE type H), which corresponds to the clonal type of a dominant MRSA already described at another Lisbon hospital (3). Transmission of this latter clone may have occurred through transfer of patients or health care personnel between hospitals.

Among the MRSA isolates described here, the great majority (37 of 43) carried the *mecA*-*Cla*I type I polymorph in a uniform background that was characterized by one Tn*554* pattern, while the PFGE patterns of the same isolates showed somewhat more varied chromosomal backgrounds, with 23 strains belonging to subtypes of PFGE pattern A and the other 14 strains belonging to PFGE patterns B, C, D, and E. It has been demonstrated that *Cla*I and Tn*554* fingerprints of MRSA isolates remain unaltered during extensive in vitro cultivation (9), and the same has recently been shown for PFGE types (2a). The stability of these markers was also demonstrated in serial sampling of infected patients and/or MRSA carriers over extended periods (3, 7). Therefore, the changes observed in DNA fingerprints of MRSA isolates must register either genuine evolutionary distance or the occurrence of molecular events of sufficient magnitude (e.g., loss or gain of transposons and/or prophages and/or consequent chromosomal rearrangements) to alter these fingerprint patterns. At present, there is insufficient evidence to allow an interpretation of differences in the DNA fingerprint patterns seen among MRSA isolates from Hospital Pulido Valente in such molecular terms.

Elucidation of the mechanism of geographic spread of drug-resistant staphylococci has been greatly facilitated by the techniques of molecular epidemiology. The introduction of molecular typing methods has already demonstrated the widespread geographic distribution of both *mecA* polymorphs and distinct MRSA genetic lineages, for instance, by PFGE (1, 12, 14). High incidences of MRSA among Portuguese hospital isolates of *S. aureus* have been reported since the mid-1970s. In contrast, the first MRSA outbreaks in Spain were reported only at the beginning of 1989 (6, 15). The frequent occurrence of clonal type I::E::A among MRSA isolates in both Lisbon and Barcelona (1,200 km apart) suggests that transmission of an epidemic Iberian MRSA clone may occur via healthy carriers, implying more frequent colonization of healthy carriers by MRSA than is currently assumed. Experiments are in progress to test this proposition.

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