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Two International Methicillin-Resistant *Staphylococcus aureus* Clones Endemic in a University Hospital in Patras, Greece

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Pulsed-field gel electrophoresis (PFGE) of *Sma*I macrofragments and hybridization of *Cla*I digests with the *mecA*- and Tn*554*-specific DNA probes were used to define the endemic clones of methicillin-resistant *Staphylococcus aureus* (MRSA) among strains collected in 1993 and 1998 to 2000 at the University Hospital of Patras, Patras, Greece. Representatives of each clonal type were analyzed by *spaA* typing, multilocus sequence typing (MLST), and staphylococcal chromosomal cassette *mec* (SCC*mec*) typing. The results indicated the existence of two successive international MRSA clones: (i) a clonal type with PFGE type A, sequence type (ST) 30 (ST30), and SCC*mec* type IV, which was very similar to a clone widely spread in the United Kingdom, Mexico, and Finland, and (ii) a clonal type with PFGE type B, ST239, and SCC*mec* III, which was related to the Brazilian clone. Both clones seem to be widespread in Greece as well. A novel MRSA clone is also described and is characterized by a new MLST type (ST80) associated with SCC*mec* type IV and with the presence of Panton-Valentine leukocidin genes.

In Greece, as in many other countries, methicillin-resistant *Staphylococcus aureus* (MRSA) remains a major cause of nosocomial infection. Kosmidis et al. (16) reported an MRSA frequency of 32% (range, 17 to 60%) in 1986 in a study that included 12 hospitals in Athens. Ten years later, the prevalence of MRSA was estimated to be 12 to 50% among 28 hospitals (39), and in 1997 the prevalence was 41% in a study that included 7 hospitals in the Athens area (15). Therefore, the prevalence of MRSA in Greece seems to be among the highest in Europe at present (12). Reports from Greece documenting the clonality of MRSA isolates are still scarce, the hospitals studied were mostly from the Athens and Thessaloniki areas, and molecular typing was mainly based on pulsed-field gel electrophoresis (PFGE) alone. Tassios et al. (36) reported two major MRSA clones, identified by PFGE, among isolates collected in 1990 in a large University Hospital in Athens, where the rate of MRSA infections was estimated to be 25%. In a more recent study involving strains collected during 1999 and 2000 in the five major hospitals of the district of Thessaly, PFGE distributed the MRSA isolates into three pulsotypes (28).

Multiple DNA-based methods have been introduced to genetically type *S. aureus* strains and to track the dissemination of MRSA clones. During the last decade, five major internationally spread MRSA clones, the Iberian (8, 34), Brazilian (37), Hungarian (7, 23), New York-Japan (1, 31), and pediatric (32) clones, were identified by using the combination of *Cla*I-*mecA* polymorphisms, Tn*554* insertion patterns, and PFGE. Multilocus sequence typing (MLST) has recently been proven to be the most adequate method both for long-term and global epidemiologic studies and for population genetic studies (10, 27), whereas for short-term or local epidemiologic studies of *S. aureus*, PFGE continues to be the method of choice (27). MLST combined with staphylococcal cassette chromosome *mec* (SCC*mec*) typing established that there are relatively few major epidemic MRSA clones (11, 26).

The aim of this study was to define the MRSA clonal types and their evolution over time in a teaching hospital in Patras (southwestern Greece) by different molecular typing methods, including *Cla*I-*mecA* polymorphism analysis, Tn*554* insertion pattern analysis, PFGE, *spaA* typing, MLST, and SCC*mec* typing. A comparison with representatives of two major clones previously identified in Greece was also done.

MATERIALS AND METHODS

Hospital. The University Hospital of Patras is a 750-bed tertiary-care teaching hospital that receives patients from the southwestern part of Greece. The frequency of MRSA infections in the hospital decreased significantly from 1993 to 2000 (38.0% in 1993, 17 to 18% in 1998 and 1999, and 14.7% in 2000), probably due to the implementation of several infection control policies, such as a decrease in the inappropriate use of broad-spectrum antibiotics, together with an emphasis on hand washing.

Bacterial isolates. One hundred eighteen MRSA isolates collected from single patients during 1998 (40 isolates), 1999 (31 isolates), and 2000 (37 isolates) as well as 10 isolates collected in the first trimester of 1993 were analyzed. The isolates were from patients on a range of wards and comprised 45 isolates from blood cultures, 25 isolates from catheter-related infections, and 48 isolates from wound samples. The isolates were confirmed to be MRSA by hybridization with the *mecA* probe (6). The following reference strains were used in this study: A3680 (36), HPV107 (34), HU25 (37), MCO58 (2), EMRSA-16 (Harmony Project [www.phls.org.uk/International/Harmony/Harmony.htm]), and 75916-Helsinki V (Harmony Project). The strains belong to the collection of the Molecular Genetics Laboratory, Instituto de Tecnologia Química e Biológica da Universidade Nova de Lisboa.

Susceptibility tests. Susceptibility tests were performed by the standard disk diffusion method with the computer software SIRSCAN 2000 (Becton Dickin-

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TABLE 1. Phenotypic and genotypic properties of MRSA strains from different inpatients at the University Hospital of Patras, Patras, Greece

Clonal type[a] (*Cla*I-*mecA* type:: Tn*554* type:: PFGE type)	Antibiotic resistance[b]	Oxacillin MIC (mg/liter)	Yr of isolation	No. (%) of isolates	*spaA* type	Allelic profile[d]	ST	SCC*mec* type
VII′::NH::A	Amp, Oxa, Amc, Ipm	4–256	1993	7	WGKAKAOMQQQ			IV variant[e]
	Amp, Oxa, Amc, Ipm	64–1,024	1998	14	WGKAKAOMQQQ	2-2-2-2-6-3-2	30	IV variant[e]
	Amp, Oxa, Amc, Ipm	64–1,024	1999	8	WGKAKAOMQQQQ	2-2-2-2-6-3-2	30	IV variant[e]
	Amp, Oxa, Amc, Ipm	≥1,024	2000	5	WGKAKAOMQQQ			IV variant[e]
Total PFGE type A				34 (29)				
X′::KK::B	Amp, Oxa, Amc, Ipm, Ery, Cip, Fd, Sxt	≥128	1998	13	WGKAOMQ	2-3-1-1-4-4-3	239	IIIA
	Amp, Oxa, Amc, Ipm, Ery, Cip, Fd, Sxt	>1,024	1999	12	XKAOMQ	2-3-1-1-4-4-3	239	IIIA
	Amp, Oxa, Amc, Ipm, Ery, Cip, Fd, Sxt	≥1,024	2000	16	XKAOMQ			IIIA
III′::KK::B	Amp, Oxa, Amc, Ipm, Ery, Cip, Fd, Sxt	≥128	1998	11	WGKAOMQ	2-3-1-1-4-4-3	239	III
	Amp, Oxa, Amc, Ipm, Ery, Cip, Fd, Sxt	≥1,024	1999	9	WGKAOMQ			III
	Amp, Oxa, Amc, Ipm, Ery, Cip, Fd, Sxt	>1,024	2000	3	ND[f]			III
III::KK::B	Amp, Oxa, Amc, Ipm, Cip, Fd, Sxt, Amk	128–256	1993	2	WGKAOMQ			III
	Amp, Oxa, Amc, Ipm, Ery, Cip, Fd, Sxt[c]	≥1,024	1999	2	WGKAOMQ			III
	Amp, Oxa, Amc, Ipm, Ery, Cip, Fd, Sxt	>1,024	2000	3	WGKAOMQ			III
Total PFGE type B				71 (60)				
II::NH::C	Amp, Oxa, Amc, Ipm, Ery	64	1998	1	UJGBBPB	1-3-1-14-11-51-10	80	IV
	Amp, Oxa, Amc, Ipm, Fd	≥32	2000	10	UJGBBPB	1-3-1-14-11-51-10	80	IV
Total PFGE type C				11 (9)				
II::E::D	Amp, Oxa, Amc, Ipm, Ery, Sxt, Spc	8	1993	1	YGFMBQBLPO	3-3-1-1-4-4-3	8	IV
III::B::E	Amp, Oxa, Amc, Ipm, Ery, Sxt	16	1998	1	XKAOMMQ	2-3-1-1-4-4-3	239	III

[a] The *Cla*I-*mecA*::Tn554 polymorphs molecular sizes of the respective hybridization fragments were as follows: II, III (17), and III′ (2.2 and 4.05 kb); VII′ (2.2 and 7.6 kb); and X′ (2.2 and 5.9 kb). The Tn*554* polymorphs were as follows: NH, no homology (lack of transposon); B and E (17); and KK, novel pattern (7.4 kb).
[b] The panel of antimicrobials included ampicillin (Amp), oxacillin (Oxa), amoxicillin clavulanic acid (Amc), imipenem (Ipm), erythromycin (Ery), ciprofloxacin (Cip), fusidic acid (Fd), sulfamethoxazole-trimethoprim (Sxt), amikacin (Amk), and spectinomycin (Spc).
[c] One isolate was susceptible to erythromycin, ciprofloxacin, fusidic acid, and sulfamethoxazole-trimethoprim.
[d] Allelic profile assignment (*arcC-aroE-glpF-gmk-pta-tpi-yqiL*) (10).
[e] The SCC*mec* type was determined by PCR amplification of the *ccr* gene (13, 21) and the *mec* gene complex (21) when the SCC*mec* type could not be inferred by the multiplex PCR strategy (24).
[f] ND, not determined.

son, Aalst, Belgium), according to National Committee for Clinical Laboratory Standards guidelines (19), with ampicillin, oxacillin, amoxicillin-clavulanic acid, imipenem, amikacin, netilmicin, erythromycin, ciprofloxacin, fusidic acid, and sulfamethoxazole-trimethoprim (Oxoid, Basingstoke, England). The MICs of oxacillin and vancomycin (Sigma Chemical Co, Steinheim, Germany) were determined by the agar dilution method (18). Resistance to 500 mg of spectinomycin per liter was evaluated as described previously (1).

β-Lactamase assay. The production of β-lactamase was tested with nitrocefin disks (Difco Laboratories, Detroit, Mich.) according to the instructions of the manufacturer.

PVL detection. Panton-Valentine leukocidin (PVL) genes were detected by PCR, as described elsewhere (9).

Molecular typing. Southern blot hybridization of *Cla*I digests with *mecA*- and Tn*554*-specific DNA probes (6), PFGE of *Sma*I digests of chromosomal DNAs (6), *spaA* typing (35), and MLST (10) were performed as described previously. When the PFGE results were interpreted, one to six band differences defined a PFGE subtype and seven or more band differences defined a distinct PFGE type (38). The primers used for *spaA* typing were as follows: primer *spaAF1* (GAC GAT CCT TCG GTG AGC; nucleotides 1096 to 1113) and primer *spaAR1* (CAG CAG TAG TGC CGT TTG C; nucleotides 1534 to 1516) (GenBank accession number J01786) (23). Previously described primers were adopted for MLST (10); the exception was primer *arcCF2* (CCT TTA TTT GAT TCA CCA GCG; the forward primer for the *arcC* gene) (5). The SCC*mec* types were determined by a multiplex PCR strategy (24) or by PCR amplification of the *ccr* (cassette chromosome recombinase) gene (13, 21) and the *mec* gene complex (21) when at least one of the structural features shown to be typical of a particular SCC*mec* type was not identified by the multiplex PCR strategy.

RESULTS AND DISCUSSION

The antimicrobial resistance patterns varied among the isolates and were related to the PFGE types (Table 1). Strains of PFGE type B (see below) were mainly multiresistant, expressing resistance to more than three different classes of antibiotics, including quinolones and fusidic acid. Strains belonging to PFGE types A and C (see below) were resistant to β-lactams only, and almost all isolates of PFGE type C expressed, in addition, resistance to fusidic acid. For most isolates resistant to β-lactams only, oxacillin MICs seemed to be much lower than those for the multiresistant isolates, which had already been observed for isolates belonging to the pediatric MRSA clone (32). All isolates were resistant to ampicillin, and β-lactamase production was detected in 113 of 118 (96%) MRSA isolates. Among the strains carrying Tn*554*, only the one with Tn*554*::E expressed resistance to 500 mg of spectinomycin per liter; similarly to what was described before for strains with Tn*554*::B (3, 22), the strains with Tn*554*::KK were spectinomycin susceptible. All isolates were susceptible to netilmicin and vancomycin.

While the 118 MRSA isolates could be separated into five *Cla*I-*mecA* polymorphs, the majority of the isolates were char-

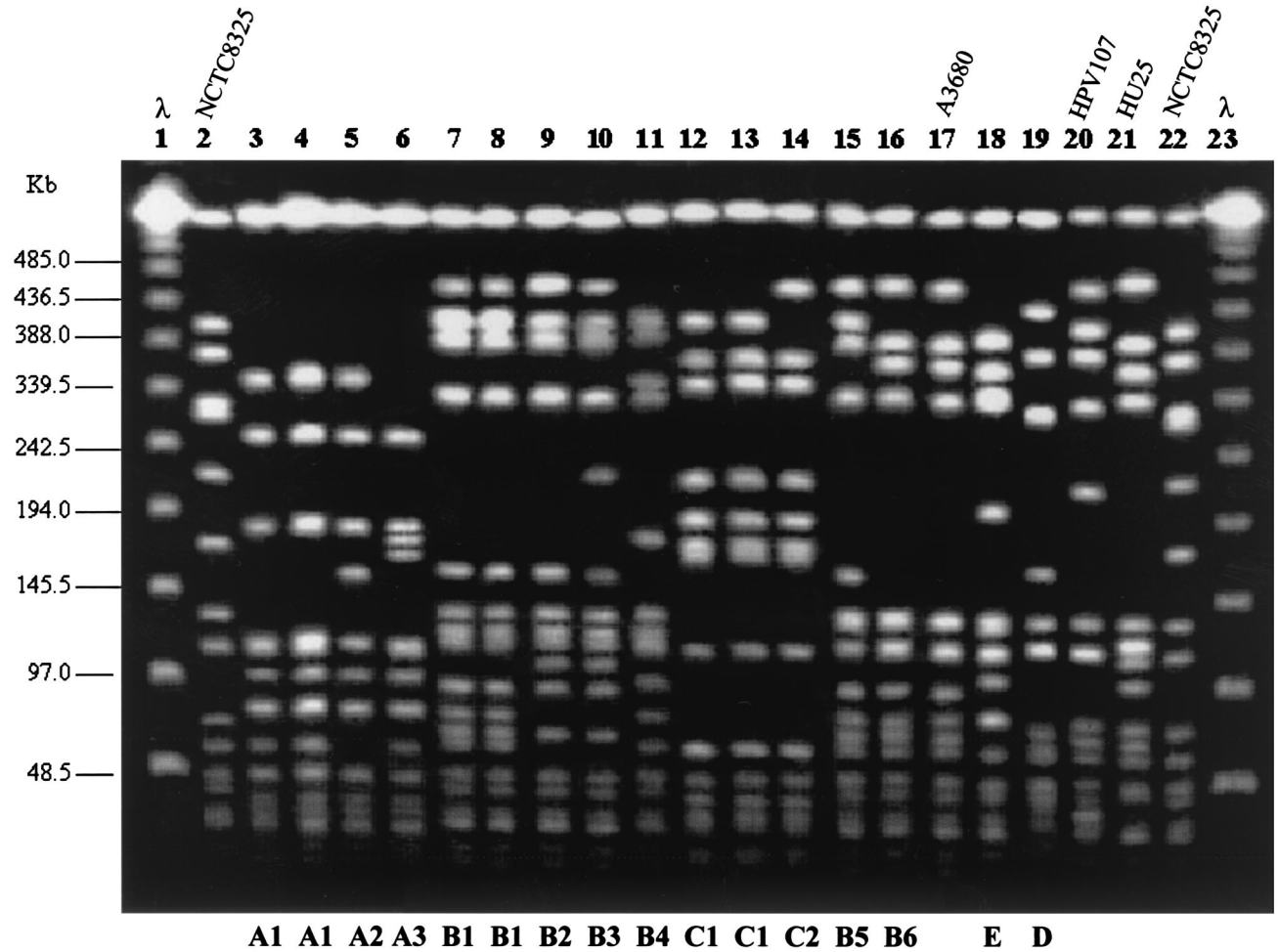

FIG. 1. PFGE of *Sma*I macrorestriction fragments of MRSA clinical isolates. Lanes 1 and 23, molecular size standards (bacteriophage lambda oligomers); lanes 2 and 22, NCTC 8325; lane 17, strain A3680, representative of the Athens MRSA clone (36); lanes 20 and 21, strains HPV107 and HU25, representatives of the Iberian (34) and the Brazilian (37) clones, respectively; lane 3, GRE112; lane 4, GRE145; lane 5, GRE104; lane 6, GRE7; lane 7, GRE3; lane 8, GRE151; lane 9, GRE159; lane 10, GRE2; lane 11, GRE328; lane 12, GRE413; lane 13, GRE365; lane 14, GRE398; lane 15, GRE163; lane 16, GRE109; lane 18, GRE108; lane 19, GRE120. The letters at the bottom indicate PFGE types or subtypes.

acterized by *Cla*I-*mecA* types X′ (n = 41; 35%) or VII′ (n = 34; 29%), which are small variations of previously described patterns X and VII, respectively (8). The isolates were classified into three *Cla*I-Tn*554* polymorphs, but the great majority had no homology (NH) with the transposon (n = 45; 38%) or shared polymorph KK (n = 71; 60%); polymorph KK is described for the first time in this study and showed a single hybridization band of approximately 7.4 kb (Table 1).

PFGE analysis grouped the 118 MRSA strains into five types (Table 1 and Fig. 1), of which 29% had pattern A, 60% had pattern B, and 9% had pattern C. Patterns D and E were represented by single isolates only. Among the isolates with pattern B there were 23 different subtypes, and among the isolates with pattern A there were 10 different subtypes.

The combination of the three molecular typing methods classified the 118 isolates into seven clonal types (*Cla*I-*mecA* type::*Cla*I-Tn*554* type::PFGE type). However, two clones were found to be dominant: VII′::NH::A (n = 34; 29%) and X′::KK::B (n = 41; 35%) (Table 1).

Representatives of the two major types were compared to strains belonging to previously characterized MRSA clones (Fig. 1 and 2). Clone VII′::NH::A showed a high degree of similarity with the dominant and unique clone from a pediatric hospital in Mexico, although the clone is characterized by *Cla*I-*mecA* polymorph I (2). In addition, PFGE type A is very similar to the PFGE type characteristic of a major MRSA clone (EMRSA-16) that has been shown to be disseminated in hospitals in the United Kingdom (4) and that has also been found in Finland (33) (Fig. 2). Clonal type X′::KK::B is related to the widely spread Brazilian clone (Fig. 1). However, the Brazilian MRSA clone is characterized by *Cla*I-*mecA* type XI and *Cla*I-Tn*554* type B (2, 37). *Cla*I-*mecA* type XI differed from type X′ in the molecular size of one of the hybridization bands, which was larger in type X′ (4.3 kb for type XI and 5.9 kb for type X′), and *Cla*I-Tn*554* type B showed three Tn*554* insertions (five hybridization bands of 2.8, 3.6, 4.1, 4.9, and 8.4 kb) instead of the single one for pattern KK (a single hybridization band of 7.4 kb).

Several selected isolates representing the two major clonal lineages (PFGE types A and B) were examined by *spaA* typing

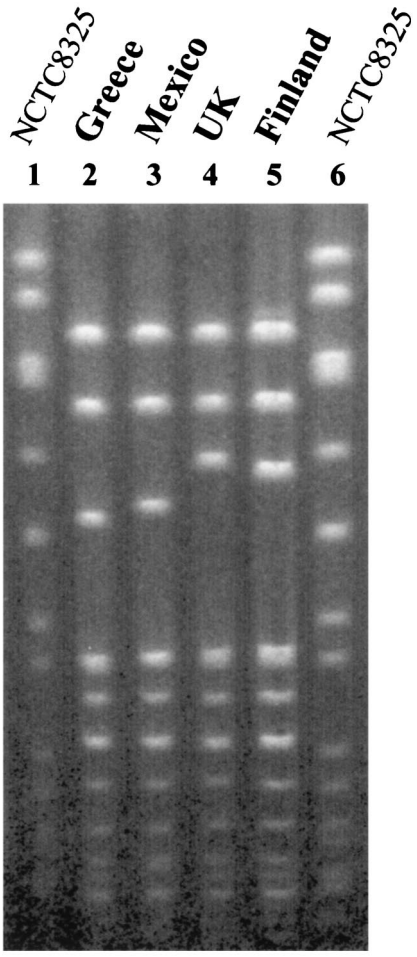

FIG. 2. Comparison of PFGE type A with international samples. Lanes 1 and 6, NCTC 8325; lane 2, GRE5 (from Greece; this study); lane 3, MCO58 (from Mexico) (2); lane 4, EMRSA-16 (from the United Kingdom) (Harmony Project); lane 5, 75916-Helsinki V (from Finland) (Harmony Project).

(16 isolates), MLST (five isolates), and SCC*mec* typing (18 isolates) (Table 1). Isolates of PFGE types A and B presented similar *spaA* repeat codes: WGKAKAOMQQQ and WGKA OMQ, respectively. Isolates of PFGE type A share the WGKA KAOMQQQ repeat, and strains of type B share the WGKA OMQ repeat or, less frequently, the XKAOMQ repeat. The last two repeats were very similar to the one originally described for the Hungarian and Brazilian clones (23, 25). The two PFGE types could also be distinguished by their MLST and SCC*mec* types: isolates of PFGE type A were characterized by sequence type (ST) 30 (ST30) and SCC*mec* type IV, whereas isolates of PFGE type B were characterized by ST239 and SCC*mec* type III or IIIA, identical to the ones described for the Hungarian and the Brazilian clones (25). Strains of ST30 were associated with a SCC*mec* type IV variant, since the locus internal to the *dcs* region, a structural feature shown to be present in SCC*mec* types I, II, and IV (25), was not amplified by the multiplex strategy (24); however, the SCC*mec* type IV variant strains shared two of the elements characteristic of SCC*mec* type IV, namely, *ccr* type 2 and the class B *mec* gene complex (21).

Clone ST30-IV had previously been found among only a few MRSA isolates in Sweden, Germany (11), Spain (24), and Argentina (M. Aires de Sousa et al., unpublished results). International clone EMRSA-16 is characterized by SCC*mec* type II and ST36, the latter of which is a single-locus variant of ST30 (11): ST36 possesses allele 2 at *pta*, whereas ST30 possesses allele 6.

Strains with PFGE pattern A also seem to represent the predominant clone among isolates collected during 1999 and 2000 from the five major hospitals of the district of Thessaly (in central Greece) (28). In addition, two recent studies conducted in 1999 and 2000 in Hippokration General Hospital, Thessaloniki, Greece, reported the spread of an MRSA clone that was susceptible to all non-β-lactam antibiotics and that had a PFGE pattern very similar to, if not identical to, PFGE type A (29, 30). In our study, all isolates belonging to PFGE type A were also resistant to β-lactams only, but contrary to the study of Pournaras et al. (30), in the hospital that we studied, this clone seems to have been replaced by multiple-resistant isolates characterized by PFGE type B. Three isolates (G78, G122, and G185) representing the dominant clone in Hippokration General Hospital (29, 30) were confirmed to belong to a clonal type characterized by PFGE type A, as they share the *spaA* motif WGKAKAOMQQQ (including the three final Q repeats) and ST30 (data not shown), suggesting the circulation of this clone, clone ST30-IV, in different regions of Greece.

PFGE type B, which was found to be the PFGE type for 60% of the isolates collected in the University of Patras during the study period (1993 and 1998 to 2000), is identical to the main clone found in Athens hospitals (36) and was also detected in hospitals of the district of Thessaly (28), confirming its capacity to spread extensively. In 1999, Kantzanou et al. (14) reported on the emergence of an MRSA isolate that displayed heterogeneous expression of reduced vancomycin susceptibility in a hospital in Athens and that had the same PFGE pattern, PFGE type B, and was described as one of the major Greek MRSA clones.

The characterization by *spaA* typing, MLST, and SCC*mec* typing of isolates belonging to minor clone II::NH::C or to sporadic isolates (II::E::D and III::B::E) showed that II::NH::C and II::E::D isolates were not related to the two major lineages (PFGE types A and B), contrary to clone III::B::E, which is related to the lineage associated with PFGE type B, and II::NH::C and II::E::D isolates might have had as a common ancestor the Brazilian clone (Table 1). Isolate II::E::D had *spaA* type YGFMBQBLPO, ST8, and SCC*mec* type IV, defined by amplification of the *dcs* region by multiplex PCR (24) and the presence of *ccr* type 2 and the *mec* gene complex type B (21). Therefore, isolate II::E::D was ST8-IV, a clone that is also referred to in the literature as EMRSA-2 and -6 and that was previously identified in several European countries (the United Kingdom, Finland, France, Germany, Ireland, The Netherlands) and the United States (11). Clone II::NH::C was very different in genotype, being characterized by a distinct *spaA* type (UJGBBPB) and a new ST, ST80, which differs from the closest STs described so far (ST12, ST13, and ST63) at three of the loci used in MLST, and by the presence of SCC-IV, defined not only by the amplification of the *dcs* region by multiplex PCR (24) but also by the presence of *ccr* type 2 and

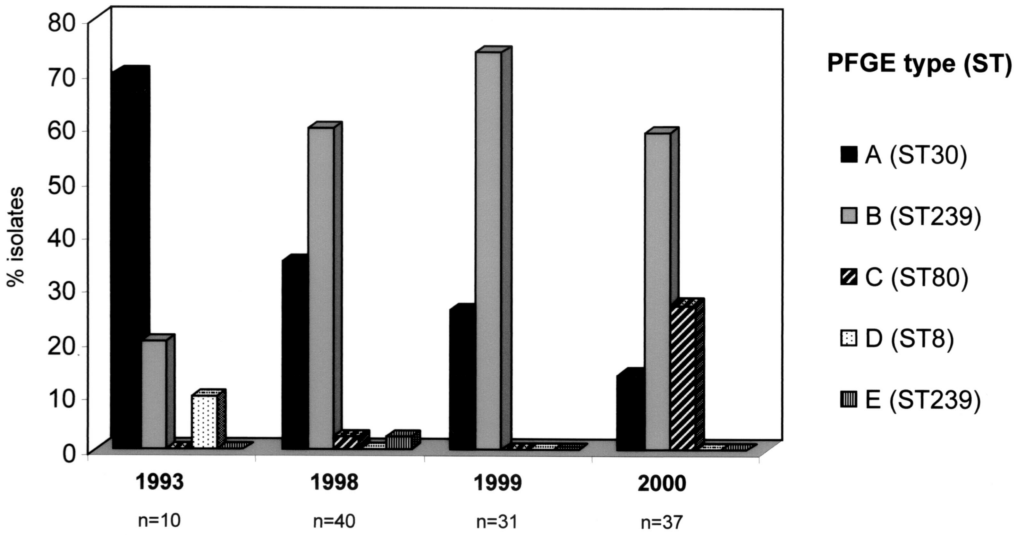

FIG. 3. Variations of MRSA clonal types over time in the University Hospital of Patras, Patras, Greece.

mec gene complex type B (21). All isolates of this clone harbored the PVL genes. The first strain (which was recovered in 1998) was recovered from the catheter of a premature baby 1 week after birth. This baby had never been discharged from the hospital and was kept on the neonatal intensive care unit, providing evidence that the MRSA infection with the ST80-IV strain was acquired in the hospital, possibly through contact with health care workers. The strains of clonal type ST80-IV recovered in 2000 were isolated from 10 additional patients suffering from traumatic skin infections, bacteremia, renal failure, arthritis, diabetes mellitus, and cirrhosis. These isolates were recovered from patients who had been hospitalized for 3 to 20 days, suggesting again that these MRSA strains were acquired in the hospital. However, since none of the patients was screened for MRSA colonization at the time of hospital admission, we cannot completely exclude the possibility that the isolates were already carried by the patients before admission.

There is a strong association between the community-acquired *S. aureus* strains that cause primary skin infections and severe necrotizing pneumonia and the presence of PVL genes in those strains, and PVL genes have never been detected in MRSA isolates associated with hospital-acquired infections (9). Clone ST80-IV shows features characteristic of community-acquired MRSA strains, namely, the SCC*mec* type, the lack of resistance to most non-β-lactam antibiotics (21), and the presence of PVL genes (9). An Australian study reported that a hospital outbreak was due to the introduction of a strain that originated in the community into the hospital setting (20). At present, the origin of clone ST80-IV is not clear.

From 1993 to 2000, we observed a variation of the clonal types present in the University Hospital of Patras, namely, a progressive decrease in PFGE clone A (or ST30-IV), an increase in clone B (or ST239-III/IIIA), and the spread of clone C (ST80-IV) in 2000 (Fig. 3). Clones A and B seem to have spread in different areas of Greece and show high degrees of similarity by MLST, *spaA* typing, and PFGE to the international EMRSA-16 and Brazilian MRSA clones, respectively. It has already been proved that the Brazilian clone has a strong capacity to cause epidemics and the capacity to spread. When it was introduced in Portugal, it progressively replaced an existing major MRSA clone with a strong capacity to cause epidemics (3), as happened in the hospital in Patras. Clone C, which is relatively sensitive to antimicrobials but which contains the PVL genes, seems to have the capacity to cause epidemics. The large number of PFGE subtypes, as well as the variations in *mecA* polymorphisms and Tn*554* insertion patterns, found in MRSA isolates with PFGE patterns A and B, suggest the continuous evolution of these clones.

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M.A.D.S. and C.B. contributed equally to this work.

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