JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1994, p. 2081–2087
0095-1137/94/$04.00+0

Vol. 32, No. 9

Spread and Maintenance of a Dominant Methicillin-Resistant *Staphylococcus aureus* (MRSA) Clone during an Outbreak of MRSA Disease in a Spanish Hospital

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Received 14 April 1994/Returned for modification 31 May 1994/Accepted 15 June 1994

It was not until November 1989 that the 1,000-bed University-affiliated Hospital de Bellvitge "Princeps d'Espanya" in Barcelona first acquired methicillin-resistant *Staphylococcus aureus* (MRSA). Since that time, the outbreak of MRSA disease has continued. We have analyzed by genomic DNA fingerprinting 189 MRSA isolates collected between late 1989 and the end of 1993. The isolates include both invasive and colonizing strains as well as isolates from health-care workers and environmental sources. In addition, 52 clinical isolates of methicillin-susceptible *S. aureus* (MSSA) collected in the same hospital were also analyzed. Isolates were classified into clonal types on the basis of molecular typing techniques. A single MRSA clone (I::B::a) belonging to *Cla*I type I, pulsed-field gel electrophoretic pattern B, and Tn*554* pattern *a* was responsible for the great majority of infections (73% of blood cultures and 79% of specimens from other clinical sources). This clone appeared at the very beginning of the outbreak, spread throughout the hospital wards, and was also carried by inpatients and health-care workers and on environmental surfaces. In contrast, no dominant lineage was apparent among MSSA isolates (33 distinct pulsed-field gel electrophoretic patterns among 52 isolates). Two MSSA isolates seem to have originated from the dominant clone by deletion of the *mecA* gene and some additional DNA. In several isolates, different *mecA* polymorphs were present in identical chromosomal backgrounds or cells with distinct chromosomal backgrounds carried the same *mecA* polymorph, suggesting horizontal transfer of the *mecA* gene.

Methicillin-resistant *Staphylococcus aureus* (MRSA) was not a major problem in Spain until 1989. However, at the end of that year, three nosocomial outbreaks were detected in Madrid, Barcelona, and Valencia. Three years later, 24 more MRSA outbreaks from different geographic areas in Spain were reported to the National Reference Laboratory (Centro Nacional de Microbiologia, Instituto de Salud Carlos III, Madrid, Spain) (19).

It is a common experience that once MRSA has settled in a hospital, it is very difficult to eradicate it. Accurate epidemiologic typing has become of primary importance for the identification of MRSA clones resident in a hospital and for the reduction and control of their spread (2, 13).

The reasons for the tenacity of MRSA persistence in a hospital are not understood. It is also unclear to what extent horizontal transmission of the resistance determinant contributes to the epidemiology of MRSA. Some mobility of the *mecA* gene is implied by the association of this genetic determinant with distinct chromosomal lineages (8). Although a number of typing schemes have been employed to study the spread of MRSA strains in epidemiologic investigations, the exact number of chromosomal genotypes that commonly harbor the *mecA* gene is unknown (11).

We report here the use of a combination of four genomic DNA fingerprinting techniques for the analysis of MRSA as well as methicillin-susceptible *Staphylococcus aureus* (MSSA) clinical isolates in a single nosocomial environment, in order to (i) describe the molecular epidemiology and the clones involved in the outbreak and (ii) analyze the chromosomal background of the *mecA* gene.

MATERIALS AND METHODS

Hospital. Hospital Bellvitge "Princeps d'Espanya" is a 1,000-bed university-affiliated hospital with medical and surgical care for adult patients, excluding obstetrics and burns. It has five intensive care units (ICUs) and an active organ transplant program. The yearly admittance rate is about 22,000 patients.

An active MRSA infection control program was introduced in 1990 after the detection of initial MRSA isolates. The program includes identification and isolation of all patients with MRSA colonization or infection, use of mupirocin for health-care workers and patients, and reinforcement of careful handwashing and educational programs throughout the hospital (15).

Bacterial isolates and plasmids. Since the beginning of the outbreak in 1989, one isolate has been stored (frozen in skim milk at −20°C) from each new MRSA case (carrier or infected) detected. From this collection, 189 MRSA isolates have been selected for molecular typing: a single isolate collected in 1988, prior to the outbreak, and the rest collected from infection or colonization sites in medical and surgical wards and ICUs between November 1989 and July 1993. Of these isolates, 161 were obtained from 156 different inpatients: 79

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TABLE 1. MRSA isolate sources and year of isolation

Source of MRSA isolate	No. of MRSA isolates in:						Total
	1988	1989	1990	1991	1992	1993	
Inpatients	1	2	23	53	48	34	161
CHCW[a]		1	4	13	4		22
Environment				1	5		6
Total	1	3	27	68	57	34	189

[a] CHCW, colonized health-care workers.

were from blood cultures, 23 were from other infected clinical sources (such as tracheal aspirate, exudate, and osteomyelitis or peritonitic fluid), and 59 were from mucocutaneous sites (colonizing strains). An additional 22 isolates came from different carriers among the health-care workers (nasal swabs), and 6 strains were isolated from environmental sources located in ICUs in which the incidence of MRSA was particularly high (bedside tables, floor, sheets, and gloves) (Table 1).

Fifty-two MSSA isolates were also collected from individual patients among nosocomial isolates: 29 were from 1987, epidemiologically unrelated to the outbreak; 23 were isolates from 1992, 10 of which were isolates recovered from patients carrying MRSA and the remaining 13 of which were from inpatients and health-care workers located in the same hospital wards in which MRSA strains were found.

The reference strains for the *Cla*I-*mecA* patterns and *Cla*I::Tn*554* patterns, obtained from B. Kreiswirth of the Public Health Research Institute (New York, N.Y.), and the *Escherichia coli* strains harboring the plasmids containing the *mec* gene and the transposon Tn*554* are listed in Table 2.

Antimicrobial susceptibility was determined by disk diffusion methods (12).

Phage typing. Phage susceptibility was determined in 133 of the MRSA isolates according to standard procedures (1) at the National Reference Laboratory (Centro Nacional de Microbiologia, Instituto de Salud Carlos III). Isolates were typed by the international set of typing phages, at both the routine test dilution and 100× this dilution of the phage suspensions.

Preparation of chromosomal DNA. For conventional and pulsed-field gel electrophoresis (PFGE), the preparation of chromosomal DNA has been described in detail previously (5). DNA restriction was done with *Sma*I and *Cla*I endonucleases, according to the manufacturer's recommendations (New England BioLabs). Conventional gel electrophoresis was carried out in 1% agarose in 1× TAE buffer (5) for 16 h at 30 to 35. PFGE was run either in an LKB 2015 Pulsaphor System (Pharmacia) or in a CHEF-DR II apparatus (Bio-Rad), under conditions described previously (5). DNA was transferred to nitrocellulose membranes by vacuum blotting from both conventional electrophoresis and PFGE. In the latter case, some modifications to the manufacturer's instructions were introduced as described by de Lencastre et al. (5).

Hybridization. Two probes, one bearing the *mecA* gene and the other bearing the Tn*554* transposon (Table 2), were used. Standard methods were followed for ^{32}P labeling of the probes by nick translation, prehybridization, and hybridization (16), which was performed at 42°C in 50% formaldehyde.

Analysis of reproducibility and similarity. In order to assure accuracy in the assignment of restriction patterns, each new pattern was included in subsequent PFGE tests for comparison.

Similarity of PFGE patterns was assessed by using the Dice coefficient [(2 × number of shared fragments/total number of fragments in the two samples) × 100] for each pair of isolates of macrorestriction patterns (17).

Population analysis profiles. Aerobically grown overnight cultures were plated at four dilutions (10^{-1}, 10^{-2}, 10^{-4}, and 10^{-6}) on plates containing serial (twofold) dilutions of methicillin at concentrations of 0 and 0.75 to 800 μg/ml. Colonies were counted after incubation for 48 h at 37°C. Interpretation of results was carried out as previously described: MRSA isolates were grouped into four expression classes on the basis of the MIC for the majority of cells (>99.9%) in the culture (18).

RESULTS

Antibiotic resistance phenotype and phage type. Of the 189 MRSA isolates, 178 (93.2%) shared a common antibiotype: resistance to penicillin, oxacillin, cephalothin, clindamycin, tetracycline, rifampin, erythromycin, gentamicin, and ciprofloxacin and susceptibility to trimethoprim-sulfamethoxazole, vancomycin, mupirocin, fosfomycin, and fusidic acid. Of the remaining 11 isolates, 3 were also resistant to trimethoprim-sulfamethoxazole and 8 represented a miscellaneous group of bacteria with variable antibiotypes.

Of the 133 MRSA isolates tested for phage susceptibility, 101 (75.9%) were nontypeable by the international set of phages (1) and 9 strains (6.8%) showed a mixed lytic pattern of groups I and III (29/77/85) after heat treatment. The remaining 23 MRSA isolates (17.3%) belonged to group III (6/42E/47/54/75/77/84/85).

PFGE pattern analysis. PFGE after restriction with *Sma*I resolved chromosomal DNA of 189 MRSA isolates into 11 main distinct PFGE patterns (A to K). Every PFGE profile was

TABLE 2. Plasmids and reference strains

Strain	Relevant genotype	Source (reference[s])
S. aureus		
RN 7178	*Cla*I-*mecA* type I	B. Kreiswirth (9)
RN 158	*Cla*I-*mecA* type II	B. Kreiswirth (9)
BK 788	*Cla*I-*mecA* type III	B. Kreiswirth (9)
BK 499	*Cla*I-*mecA* type IV	B. Kreiswirth (9)
RN 6322	*Cla*I-*mecA* type V	B. Kreiswirth (9)
BK 793	*Cla*I-*mecA* type VI	B. Kreiswirth (9)
E. coli		
MC1061.1(pKMF13)	pMF13 contains a 1.196-kb *Pst*I-*Xba*I fragment from the *mecA* gene cloned in pTZ19	P. R. Matthews (5, 9)
RN 7951	Harbors a plasmid containing a 4.5-kb *Eco*RV fragment of Tn*554* cloned in a pBluescript II vector	B. Kreiswirth (9, 5)

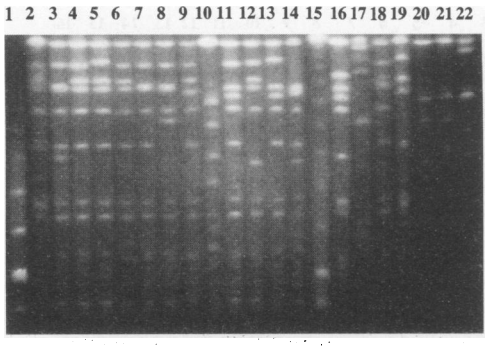

FIG. 1. *Sma*I PFGE patterns of MRSA isolates from the outbreak in Hospital "Princeps d'Espanya." The positions of the various PFGE patterns and subtypes (a total of 18 representatives) are shown. Lanes: 2 to 9, PFGE patterns A1, A2, B1, E, and B2 through B5, respectively; 10, a coagulase-negative isolate; 11 to 14, PFGE patterns B7, B8, C, and D, respectively; 16, PFGE pattern F; 17, a coagulase-negative staphylococcal isolate; 18 to 22, PFGE patterns G, H, I, J, and K, respectively. Lanes 1 and 15 contain molecular size markers (lambda ladder).

found to be stable during numerous passages in vitro. Plasmids did not interfere with the patterns obtained since the multiple dialysis steps used in the sample preparation leads to the release of small plasmid DNA (14).

Assuming that a single base mutation in the chromosomal DNA could introduce maximally a three-fragment difference in the restriction pattern (17), strains showing more than three-fragment variations were assumed to represent major patterns (assignment of capital letters), while one- to three-fragment differences were considered to represent subtypes (capital letter with numerical subcode) (Fig. 1).

PFGE type B contained 157 strains (83.1%), which could be further classified into eight subtypes (B.1 to B.8), of which subtype B.1 represented the majority (136 isolates [71.9%]). PFGE type A had two subtypes (A.1 and A.2) and appeared in 10 strains (5.3%). The remaining PFGE patterns had no subtypes and, except PFGE types C, D, and E (containing 8, 3, and 5 isolates, respectively), were found only in single isolates (Table 3). The Dice coefficient of similarity among major patterns had an average value of 68%. The subtype variants showed similarity values that were always higher than 88%.

The 52 MSSA isolates tested for PFGE yielded 33 distinct patterns (Fig. 2). It is noteworthy that two MSSA isolates from the period of the MRSA outbreak (Fig. 3, lanes 3 and 8) were closely related to PFGE type B (subtype B.1), representing the most frequent MRSA clone. Most interestingly, one of these MSSA isolates was detected in the patient who was also colonized at the same time with an MRSA of clonal type B.1. The PFGE patterns of these two MSSA isolates differed from the PFGE pattern B.1 only in the size of the *Sma*I fragments carrying *mecA*: the 215-kb fragments carrying the *mecA* gene were replaced by fragments of 147 and 181 kb. This observation strongly suggests the deletion of a 68-kb piece of DNA in one MSSA isolate and a 34-kb deletion in the other.

Analysis of the *mec* region. The chromosomal location and vicinity of the *mecA* gene in the different isolates were investigated with a DNA probe internal to the *mecA* gene (i) after *Sma*I digestion of genomic DNA and PFGE and (ii) after *Cla*I restriction and conventional electrophoresis.

(i) *Sma*I digestion and PFGE. PFGE of *Sma*I digests were transferred and hybridized with the *mecA* DNA probe (Fig. 3B). In all the strains with PFGE patterns A and B (except those with subtypes B.4 and B.8), *mecA* was located in a 215-kb band. This was also the location of *mecA* in isolates belonging to PFGE types C, D, and E. In the remaining isolates belonging to PFGE types F to K and in subtype B.8, *mecA* was in smaller fragments (102 to 200 kb). Only subtype B.4 bore the gene in a larger fragment (254 kb).

(ii) *Cla*I restriction and conventional electrophoresis. Six different *mecA* polymorphs (I to VI) have been described

TABLE 3. MRSA PFGE major patterns and subtypes

PFGE major pattern	PFGE subtype	No. of strains (%)
A	A.1	9 (4.8)
	A.2	1 (0.5)
B	B.1	136 (71.9)
	B.2	2 (1.1)
	B.3	3 (1.6)
	B.4	1 (0.5)
	B.5	1 (0.5)
	B.6	3 (1.6)
	B.7	10 (5.3)
	B.8	1 (0.5)
C		8 (4.2)
D		3 (1.6)
E		5 (2.6)
F		1 (0.5)
G		1 (0.5)
H		1 (0.5)
I		1 (0.5)
J		1 (0.5)
K		1 (0.5)
TOTAL		189

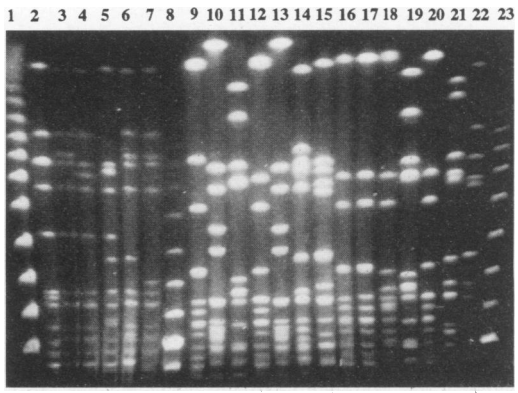

FIG. 2. *Sma*I restriction fragments generated from chromosomal DNAs of 14 MSSA isolates were examined by PFGE. Representatives of the major MRSA PFGE patterns A1, B1, B7, D, and B8 were also included (lanes 2 to 6, respectively) for comparison. Lanes 1, 8, and 23 contain molecular size markers (lambda ladder).

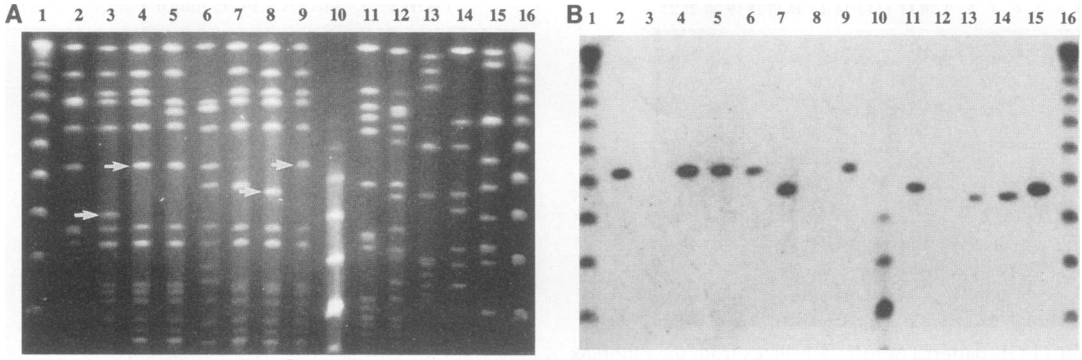

FIG. 3. Evidence for the apparent deletion of *mecA* in two clinical strains isolated during the MRSA outbreak. The PFGE results (A) were also tested with a *mecA* DNA probe (B). Note that the *S. aureus* isolates in lanes 3 and 8 were *mecA* probe negative but their PFGE patterns closely resembled the PFGE pattern B1 of the MRSA isolates shown in lanes 4 and 9. Lanes: 1, 10, and 16, molecular size markers (lambda ladder); 12, MSSA isolate (*mecA* probe negative); 13, coagulase-negative (*mecA* probe-positive) isolate; 2, 5, 6, 9, 11, 14, and 15, other MRSA isolates with unique PFGE patterns.

previously after chromosomal *Cla*I restriction (9). Of these, *Cla*I type I was found in 175 of our MRSA isolates (92.6% of the total). The type II *mecA* polymorph was found in only two isolates (1.1%). The remaining 12 isolates belonged to five distinct and novel (as-yet-undescribed) *mecA* polymorphs, which we named VII (in one strain [n = 1]), VIII (n = 6), IX (n = 1) X (n = 3), and XI (n = 1) (Fig. 4). The type I polymorph was most frequently associated with PFGE pattern B, but it was also found in association with PFGE patterns A, C, E, F, and I. *Cla*I type II was identified in PFGE patterns K and G. Of the new *Cla*I patterns, type VII was associated with PFGE pattern J, types VIII and IX were associated with PFGE pattern B, type X was associated with PFGE pattern D, and type XI was associated with PFGE pattern H (Table 4).

***Cla*I::Tn*554* pattern.** Given the small number of *mecA* hybridization patterns, a Tn*554* probe was used to provide a higher degree of resolution on the same gels obtained after *Cla*I restriction (19). All (n = 185) but four MRSA isolates carried Tn*554* in eight different attachment site patterns: a to h. The single most frequent Tn*554* profile, pattern a, was found in 151 isolates, of which 145 belonged to type I *mecA* polymorph, 5 belonged to *Cla*I type VIII, and 1 belonged to *Cla*I type IX. Both patterns d (21 isolates) and b (5 isolates) were associated with *Cla*I type I. Pattern e was found in three isolates which were *Cla*I type X. The patterns c, f, g, and h were represented each by a single isolate (Table 4).

Clones involved in the outbreak. All MRSA isolates were defined as clones on the basis of *Cla*I::PFGE::Tn*554* profiles. Together, the results of the molecular study revealed 18 clones involved in the nosocomial outbreak in the Barcelona hospital since 1989 (Table 5). However, the representation of these 18 clones was strikingly different throughout the whole period.

I::B::a was the most frequent endemic lineage. It appeared at the beginning of the outbreak and has been found persistently in the subsequent years. It is also noteworthy that this clone was able to spread in the hospital into ICUs, surgical wards, and even medical wards. It was found carried by

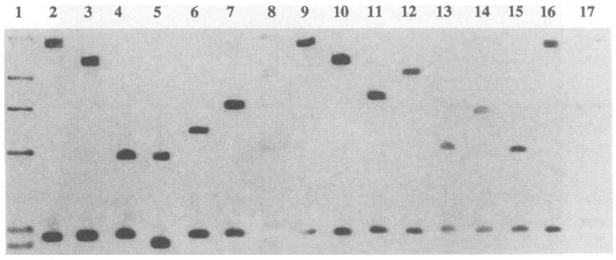

FIG. 4. Different *mecA* polymorphs in the MRSA isolates from Hospital "Princeps d'Espanya." *mecA* *Cla*I patterns of reference strains with patterns I through VI (9) are shown in lanes 2 to 7. Lanes: 9 and 16, *Cla*I pattern most frequently seen for MRSA isolates from this hospital outbreak (pattern I); 10, pattern II; 11 to 15, novel *Cla*I patterns identified among MRSA isolates of this hospital outbreak (patterns VII through IX); 1, 8, and 17, molecular weight markers (lambda ladder).

TABLE 4. MRSA clones involved in outbreak

*Cla*I Polymorph type (n)[a]	Pattern type (n)		Clone	No. of strains (%)
	PFG	Tn*554*		
I (175)	A (10)	a (9)	I::A::a	9 (4.8)
		d (1)	I::A::d	1 (0.5)
		ϕ[b] (1)	I::B::ϕ	1 (0.5)
	B (149)	a (123)	I::B::a	123 (65.1)
		b (5)	I::B::b	5 (2.6)
		d (21)	I::B::d	21 (11.1)
	C (8)	a (8)	I::C::a	8 (4.2)
	E (5)	a (5)	I::E::a	5 (2.6)
	F (1)	ϕ (1)	I::F::ϕ	1 (0.5)
	I (1)	ϕ (1)	I::I::ϕ	1 (0.5)
II (2)	G (1)	ϕ (1)	II::G::ϕ	1 (0.5)
	K (1)	f (1)	II::K::f	1 (0.5)
VII (2)	J (1)	h (1)	VII::J::h	1 (0.5)
VIII (6)	B (6)	a (5)	VIII::B::a	5 (2.6)
		c (1)	VIII::B::c	1 (0.5)
IX (1)	B (1)	a (1)	IX::B::a	1 (0.5)
X (3)	D (3)	e (3)	X::D::e	3 (1.6)
XI (1)	H (1)	g (1)	XI::H::g	1 (0.5)

[a] n, number of isolates.
[b] ϕ, Tn*554* negative.

TABLE 5. Evolution of MRSA clones by year

Clone	No. of isolates (%) isolated in:						Total
	1988	1989	1990	1991	1992	1993	
I::A::a		1 (33.3)	6 (21.4)	1 (1.5)	1 (1.8)		9
I::A::d		1 (33.3)					1
I::B::ϕ[a]			1 (3.6)				1
I::B::a		1 (33.3)	18 (64.3)	61 (91)	36 (64.3)	7 (20.6)	123
I::B::b				3 (4.5)	2 (3.6)		5
I::B::d					4 (7.1)	17 (50)	21
I::C::a						8 (23.5)	8
I::E::a					5 (8.9)		5
I::F::ϕ					1 (1.8)		1
I::I::ϕ	1 (100)						1
II::G::ϕ			1 (3.6)				1
II::K::f					1 (1.8)		1
VII::J::h						1 (2.9)	1
VIII:B:a				1 (1.5)	4 (7.1)		5
VIII:B:c					1 (1.8)		1
IX::B::a					1 (1.8)		1
X::D::e			2 (7.1)	1 (1.5)			3
XI::H::g						1 (2.9)	1
Total	1	3	28	67	56	34	189

[a] ϕ, Tn*554* negative.

inpatients and health-care workers and on environmental surfaces (Fig. 5). Clone I::B::a as an infectious agent was recovered in 73.4% of the blood cultures and 79.2% of the specimens from other clinical sources. By 1993, I::B::d emerged as the major clone and clone I::C::a also appeared among the isolates analyzed. Both clones are likely to represent divergent evolutionary lineages from the same ancestral clone, I::B::a (I::B::d by changing the Tn*554* profile; I::C::a by a small change in the PFGE pattern), rather than a replacement of the endemic clone.

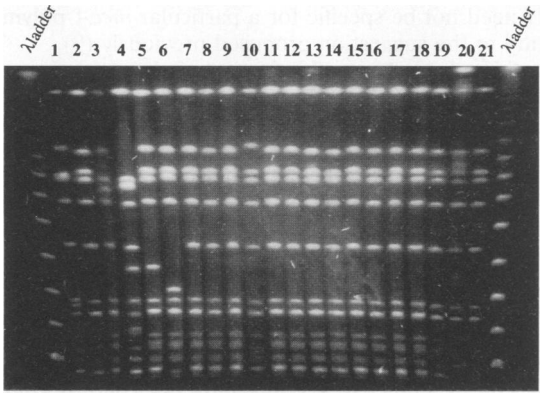

FIG. 5. Predominant MRSA clones among colonizing and invasive isolates. *Sma*I fragmentation patterns from MRSA isolates representing the major clones (identified by a combination of PFGE and other DNA-level fingerprinting methods) are shown. Lanes: 1 to 5, representative PFGE patterns A1, B1, B7, D, and B8, respectively; 6, a methicillin-susceptible staphylococcal isolate; all other lanes (except 10 and 20), isolates sharing the common PFGE pattern of the major MRSA clone. Strains isolated from the same ICU ward and during 1992 are shown in the following lanes: lanes 7, 8, 16, and 18, isolates from colonizing sources in patients; lanes 13 to 15, isolates from colonizing sites from health-care personnel; 11 and 17, invasive isolates; lanes 9 and 12, isolates from environmental sources. Lanes 10 and 19 to 21 are isolates from other wards.

There were also other well-defined minor clones. I::A::a appeared together with I::B::a at the very beginning of the outbreak. However, its frequency decreased, and none of the 1993 isolates belonged to this clone. Another clone, I::E::a, was represented by five isolates (one nasal isolate carried by a health-care worker and blood cultures from four patients), all obtained in May 1992 from the same ICU. In retrospect, these five MRSA isolates may be considered a micro-outbreak that would have gone undetected by classical typing methods (antibiotype and phage type).

Some strains from the general outbreak showed resistance against trimethoprim-sulfamethoxazole. Three of these isolates were tested by molecular typing methods, confirming their clonal origin (X::D::e), which was already suspected on the basis of their distinct antibiotype. Finally, there were six transient clones, the PFGE patterns of which (i.e., F to K) were quite different from those in the most frequent clonal types (i.e., A to E). These six transient MRSA clones were represented by single isolates: three were unrelated to the outbreak (the patient may have acquired the strain in another hospital, or the isolate may have been present prior to the outbreak), two were from colonized sites, and only one (II::K::f) caused infectious disease in the outbreak context.

Phenotypic expression of methicillin resistance. Virtually all (181 of 189) MRSA isolates showed a high level of methicillin resistance (phenotypic class 3 or 4). Eight isolates, of which

one belonged to the major clone, showed more heterogeneous resistance (phenotypic class 1 or 2).

DISCUSSION

Epidemiological features. The clinical characteristics of this nosocomial outbreak in the "Princeps d'Espanya" hospital have been analyzed recently by Pujol et al. (15). The outbreak started at the end of 1989 in one ICU and spread rapidly to the other units and some surgical wards. Few medical wards have been affected. Currently, the outbreak has been partially controlled, although MRSA has become endemic in two ICUs. As stated in the study mentioned above, MRSA arrived at the "Princeps d'Espanya" hospital as a new nosocomial pathogen already resistant to many antibiotics, including ciprofloxacin. Similarly, MRSA did not replace but became superimposed on MSSA disease that was already endemic in the hospital in 1989, according to observations made previously (3, 20).

Most isolates were untypeable by the international set of phages, and therefore, this method was of little use for strain characterization.

The molecular tools applied here provided us with an approach to examine the epidemiology of this nosocomial environment. Of the two main clones detected at the beginning, one (I::A::a) decreased gradually while the other clone, I::B::a, has become established as the endemic lineage (Table 5). An observation of primary concern is the ability of this major clone to spread uniformly and persistently throughout the hospital during the entire period. The environmental samples of MRSA (Fig. 4, lanes 9 and 12) originated in the same ICU in which patients with both invasive and colonizing MRSA isolates of the same clonal type were identified. This clone was also identified as colonizing health-care personnel servicing this ICU. These data suggest the importance of health-care personnel in transmitting MRSA. Another example suggesting transmission through colonized health-care personnel is the micro-outbreak by clone I::E::a described in Results. Whether environmental and colonizing sites serve as long-range reservoirs for these bacteria is not clear from our data.

The capacity of the dominant clone (I:B::a) for geographic spread is also demonstrated by its detection 600 km away in an MRSA outbreak in a hospital in Madrid (4a). This clone may carry some superior virulence-related trait as well. Other clones with PFGE patterns F to K in our collection were associated with colonization rather than infection; of these six isolates, three were unrelated to the outbreak, two were carried nasally, and only one was a pathogen (isolated from bacteremia). In addition, these strains also had variable antibiotic resistance patterns and did not show the multidrug resistance pattern common to the major clones.

PFGE patterns A, B, and C appear to be closely related and may represent evolutionary divergence, since the majority of isolates belonging to PFGE patterns A, B, and C carry Tn*554* in pattern a and belong to *Cla*I type I.

In sharp contrast to the dominance of a single MRSA clone among the multidrug-resistant staphylococci, the PFGE patterns of MSSA showed a wide range of variability in spite of the fact that both the MRSA and MSSA isolates were derived from the same nosocomial field. The reason for this may be, in part, the limited variation in the chromosomal backgrounds of MRSA, compared with the highly variable genetic backgrounds of MSSA.

Molecular features. *Cla*I restriction was shown to be a useful molecular tool to study the *mecA* gene vicinity. Until recently, only relatively few hybridization patterns (*mecA* polymorphs) have been described among several hundred MRSA isolates examined by this method (9). In addition, these *mecA* polymorphs also tended to be associated with distinct, unique genetic backgrounds. Our findings demonstrating the same *mecA* polymorph in two different PFGE backgrounds suggest horizontal transfer of the *mecA* gene. This could also explain the parallel observation of the presence of two different *mecA* polymorphs in identical chromosomal backgrounds.

Tn*554* was present in most (97.9%) of the MRSA isolates studied. Chromosomal DNA hybridization with a Tn*554* specific probe provided a further resolution for typing the isolates. Tn*554* pattern *a* was most frequent in our isolates carrying the *mecA* polymorphs I and VIII and was also present in the single type IX polymorph. These findings indicate that the location of Tn*554* need not be specific for a particular *mecA* polymorph, contrary to the suggestion proposed previously (9).

Another observation of interest was the detection of two MSSA homologs of MRSA strains, showing only slight differences in the chromosomal PFGE restriction pattern, which involved only the fragment carrying the *mec* gene. This finding suggests deletion of the *mec* region, which may occur not only under laboratory conditions but also in the clinical environment (8). Most interestingly, the $MecA^+$ and $MecA^-$ homologs were isolated from the same patient (Fig. 3, lanes 8 and 9). An alternative explanation, namely the acquisition of *mecA* by susceptible strains with the appropriate genetic backgrounds, is less likely but cannot be rigorously excluded (8).

Phenotypic expression of methicillin resistance. Population analysis profiles of MRSA strains have been shown to provide stable and reproducible phenotypic fingerprints (18). The identification of the phenotypic properties of MRSA clones involved with a nosocomial outbreak is of crucial importance for the clinical management of the infected patients, as well as for an accurate and correct microbiological identification. A high degree of error (18%) in the detection of MRSA by traditional techniques (e.g., disk diffusion or microbroth dilution) was reported (6) when the majority of the isolates (75%) in a hospital belonged to expression class 1 or 2, following the criteria set up by Tomasz et al. (18). In our case, most strains (97.5%) belonged to phenotypic class 3 or 4 (Table 6), and therefore the routine Kirby-Bauer test was an adequate tool

TABLE 6. Phenotypic expression classes[a]

Clone	No. of isolates (%) in class				Total
	1	2	3	4	
I::A			2 (20)	8 (80)	10
I::B	1 (0.6)		10 (6.7)	139 (92.7)	150
I::C				8 (100)	8
I::E				5 (100)	5
I::F		1 (100)			1
I::I		1 (100)			1
II::G		1 (100)			1
II::K		1 (100)			1
VII::J	1 (100)				1
VIII:B				6 (100)	6
IX::B				1 (100)	1
X::D		3 (100)			3
XI::H			1 (100)		1
Total	2	7	13	167	189

[a] The phenotypic expression classes 1 to 4 were defined according to Tomasz et al. (18): the methicillin MICs for the majority of the cells was 3 to 6 μg/ml for class 1, 12 to 25 μg/ml for class 2, 50 to 200 μg/ml for class 3, and >400 μg/ml for class 4.

for the detection of methicillin resistance. The single strain misidentified as methicillin sensitive (it gave a positive signal after hybridization with the *mecA* gene) showed a heterogeneous population structure and belonged to phenotypic class 1.

Some of the MRSA isolates with a common genetic background (same *mecA* polymorph and PFGE pattern) nevertheless exhibited different resistance phenotypes (Table 6). This was particularly striking in the case of a single isolate which showed a class 1 phenotype in spite of the fact that it belonged to the majority clone I::B::a, all other isolates of which shared a common class 3 or 4 phenotype. Since all these isolates carried the same *mec* region, variation in the phenotypic expression of resistance must be controlled by determinants that are not in the vicinity of the *mecA* gene. Likely candidates for such determinants would be the auxiliary genes identified by transposon mutagenesis experiments at various chromosomal sites (7, 10), some of which appear to be cell wall structural genes (4). Such natural auxiliary mutants may also exist among clinical MRSA isolates.

ACKNOWLEDGMENT

M.A.D. was supported by grant no. 93/5178 from the Fondo de Investigaciones Sanitarias de la Seguridad Social, National Health Service, Madrid, Spain.

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