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Nine-Year Surveillance of Methicillin-Resistant *Staphylococcus aureus* in a Hospital Suggests Instability of *mecA* DNA Region in an Epidemic Strain

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The distributions of the antibiotic resistance patterns in a population of *Staphylococcus aureus* isolates from a teaching hospital were studied over a 9-year period. The results indicate the existence of successive major epidemic methicillin-resistant strains and the emergence of a methicillin-susceptible strain with an unusual resistance pattern. Our findings suggest that this methicillin-susceptible *S. aureus* strain could be derived from the dominant gentamicin-susceptible methicillin-resistant *S. aureus* strain with the loss of a 40-kb DNA fragment.

Methicillin-resistant *Staphylococcus aureus* (MRSA) has long been known to spread through hospitals. Most epidemiological studies of MRSA are not longitudinal studies but are cross-sectional studies and often involve several hospitals (14). There are a few reports of long-term surveys of MRSA in one hospital (8). We report on the computerized surveillance of MRSA in a French teaching hospital over a 9-year period and provide arguments for the reason for the instability of methicillin resistance in one of the most epidemic clones of MRSA in France.

The University Hospital of Rennes is a 1,800-bed teaching hospital with five facilities. For the 9-year period from 1992 to 2000, the rate of methicillin resistance among *S. aureus* isolates was 36.5%. During this period, the Committee for Nosocomial Infections Control had published general recommendations for avoidance of the spread of multidrug-resistant bacteria, but policies relating specifically to the prevention of transmission of MRSA were not in place.

Between 1992 and 2000, 13,321 *S. aureus* isolates were recovered from samples from patients hospitalized in four facilities. Isolates were identified by production of acid on Chapman agar and the presence of catalase and coagulase. Antimicrobial susceptibility was tested by the agar diffusion method (Diagnostics Pasteur, Marnes-la-Coquette, France) on Mueller-Hinton agar (Oxoid, Dardilly, France), according to the recommendations of the Antibiogram Committee of the French Microbiology Society, except that isolates with fosfomycin inhibition zone diameters of >14 and ≤23 mm were categorized as intermediate to this antibiotic. Patient information (sex, age, sample) and antibiogram results were collected from the Laboratory Information System and stored in a specific database. If several isolates with the same antibiograms were recovered at different times from a patient, only the first one was retained. Consequently, 3,350 MRSA isolates have been retained for further analysis and have been grouped by resistance pattern by use of EPILOG software (Saric International, Nanterre, France). A resistance pattern was characterized by combinations of results (susceptible and intermediate or resistant) for oxacillin and the following antibiotics: tobramycin, gentamicin, erythromycin, lincomycin, sulfamethoxazole, pefloxacin, rifampin, and fosfomycin. The combinations of patterns of resistance to all these antibiotics except the glycopeptides were assumed to reflect the patterns of resistance to the most important antibiotics used for the treatment of staphylococcal infections (Table 1). The finding of a resistance pattern in 10 or more oxacillin-resistant *S. aureus* (MRSA) isolates for 1 year was suspected to represent an epidemic.

The clonal nature of epidemic MRSA strains was assessed for randomly selected isolates with each major pattern by two typing methods: the Euclidian distances method and DNA polymorphism after *Sma*I restriction and pulsed-field gel electrophoresis (PFGE). The diameters of the zones of inhibition for seven antibiotics (tobramycin, gentamicin, erythromycin, lincomycin, sulfamethoxazole, rifampin, and fosfomycin) were collected and the Euclidian distances were calculated by use of ITCF software (Institut Technique des Céréales et Fourrages, Paris, France). As described by Blanc et al. (1), the Euclidian distance method is a means of multivariate analysis of inhibition zone diameters (x) around discs of n antibiotics in which the similarity between two strains, j and k, is calculated by the formula

$$E_{jk} = \sqrt{\sum_{i=1}^{n} (x_{ij} - x_{ik})^2}$$

where E is Euclidian distance and i is rank of test (from 1 to n).

For PFGE, *S. aureus* DNA preparation and *Sma*I restriction were done as described previously (3). For epidemiological

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TABLE 1. Descriptions of the five major epidemic MRSA patterns and the MSSA ΔVII pattern

Pattern	Resistance to[a]:
I	OXA, TOB, GEN, LIN, SUL, PEF, RIF, FOF
II	OXA, TOB, GEN, ERY, LIN, PEF
III	OXA, TOB, GEN, ERY, PEF
VI	OXA, TOB, GEN, ERY, LIN, SUL, PEF, RIF
VII	OXA, TOB, ERY, LIN, PEF, FOF
ΔVII	ERY, LIN, PEF, FOF

[a] OXA, oxacillin; TOB, tobramycin; GEN, gentamicin; LIN, lincomycin; ERY, erythromycin; SUL, sulfamethoxazol; PEF, pefloxacin, RIF, rifampin; FOF, fosfomycin.

surveillance, PFGE was performed with a Gene Navigator apparatus (Amersham Pharmacia, Orsay, France), and for comparison of MRSA and methicillin-susceptible *S. aureus* (MSSA) strains, PFGE was performed with a CHEF-II apparatus (Bio-Rad, Ivry-sur-Seine, France). After electrophoresis, the gels were stained with ethidium bromide and the DNA fragments were visualized with a UV light box. Gel Compar software (Applied Maths, Sint-Martens-Latem, Belgium) was used to calculate the Dice similarity indices and to perform cluster analysis by unweighted pair group matching analysis (tolerance, 2.0%).

PCR was used to amplify the sequences from two different target sites: the *mecA* gene encoding PBP 2a and the *coa* gene encoding staphylococcal coagulase. The two sets of primers used have been described previously (9, 19). Positive and negative controls (an MRSA isolate and *S. aureus* ATCC 25923, respectively) were included with each run for *mecA* amplification. The thermocycling conditions were as follows: 94°C for 5 min for 1 cycle and then 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min for 35 cycles on a Perkin-Elmer thermocycler. After electrophoresis, the gel was stained with ethidium bromide and the amplicons were visualized. Amplicons produced by *coa* amplification were restricted with *Alu*I, and the restriction fragments were resolved by 3% agarose gel electrophoresis at 110 V for 45 min. The presence of PBP 2a was investigated by a slide agglutination test with latex particles coated with a monoclonal antibody directed toward the protein (22). The test was performed according to the manufacturer's recommendations (Servibio, Meudon, France) with an MRSA isolate as a positive control and *S. aureus* ATCC 25923 as a negative control.

We were able to distinguish 106 different MRSA resistance patterns for the period from 1992 to 2000, and among these, 12 fit the definition of an epidemic pattern (>10 isolates with a single pattern recovered in 1 year). Five of them (designated patterns I, II, III, VI, and VII) included more than 70 strains, and we considered strains with these patterns to be major epidemic MRSA strains, with the number of isolates per year ranging from 0 to 243 (Table 1 and Fig. 1). The seven remaining patterns (patterns IV, V, VIII, IX, X, XI, and XII) included less than 40 strains, and we considered strains with these patterns to be minor epidemic MRSA strains. The isolates which belonged to a minor or a major epidemic pattern accounted for 77.56% ± 4.25% of all MRSA isolates by year.

The distributions of the different major epidemic MRSA strains argue strongly for clonal dissemination (Fig. 1). One or two dominant strains could be identified in each year: isolates

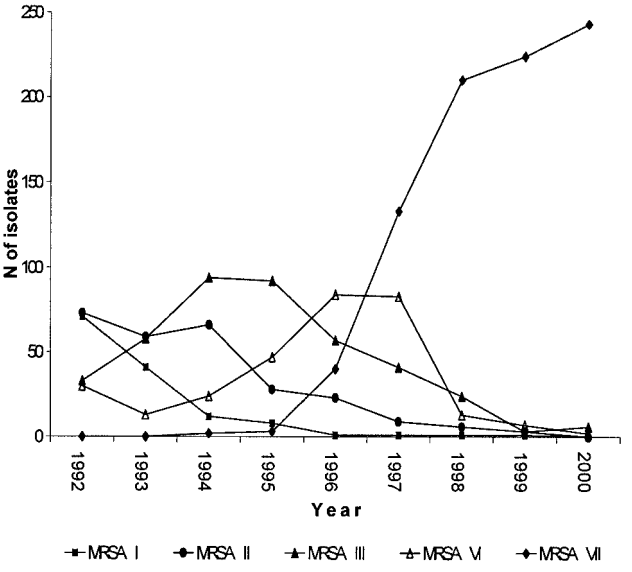

FIG. 1. Distributions of major epidemic MRSA isolates between 1992 and 2000.

with patterns I and II in 1992; isolates with patterns II and III in 1993; isolates with pattern III in 1994 and 1995; isolates with pattern VI in 1996; and isolates with pattern VII in 1997, 1998, 1999, and 2000. The more striking feature is the emergence of MRSA isolates with pattern VII, which consisted of gentamicin-susceptible strains which had replaced almost all the previous gentamicin-resistant, major epidemic MRSA strains. Before 1996 the successive epidemic strains were dominant for more than 1 year (MRSA pattern I isolates have been epidemic in our hospital since 1989), and replacement of one dominant strain by another one was progressive. Conversely, MRSA pattern VI isolates, the next to the last epidemic strains, were dominant only in 1996, and their numbers decreased dramatically in 1998. Thereafter, as previous major epidemic MRSA strains (those with patterns I to VI) have disappeared and no new major epidemic strain has emerged, about 60% of MRSA strains isolated between 1998 and 2000 have MRSA pattern VII (Fig. 1).

By the Euclidian distances method and by use of a cutoff value of 18 mm for the inhibition zone diameter, the five major epidemic MRSA patterns were clearly separated from each other (data not shown). Antibiotyping has proved to be a powerful method for the typing of MRSA strains when a quantitative method was used (1, 21). In this study, for a well-defined period and in one hospital, qualitative antibiotyping has worked as well as a quantitative method; this is likely due to the small number of epidemic strains with distinct resistance patterns.

After *Sma*I restriction and PFGE, all the major epidemic MRSA grouped into two well-separated clones: those with patterns I, VI, and VII and those with patterns II and III (Fig. 2). Each genomic clone was homogeneous, with a high degree of internal similarity among the clone with patterns II and III (index = 94%) but with a rather low degree of similarity among the clone with patterns I, VI, and VII (index = 87.5%). Most gentamicin-susceptible MRSA strains remained resistant

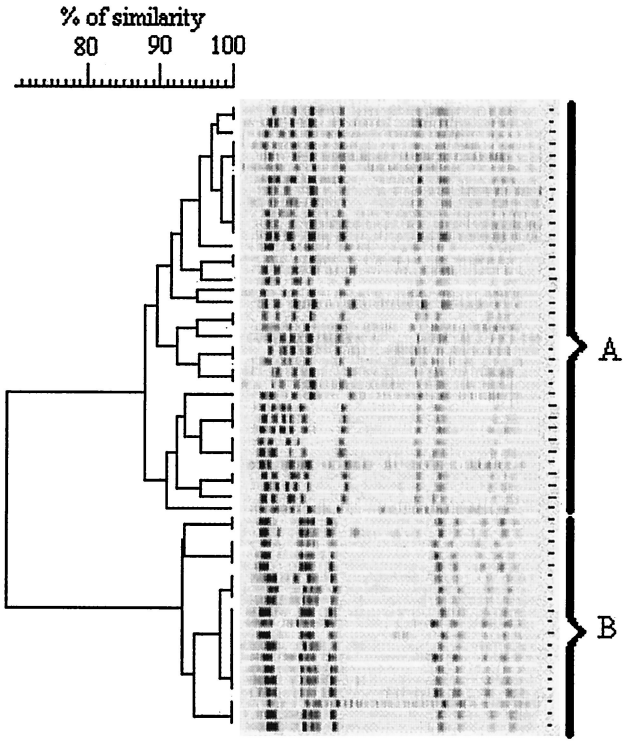

FIG. 2. Classification of PFGE restriction patterns of MRSA pattern I, II, III, VI, and VII isolates (55 isolates) by using the Dice coefficient and unweighted pair grouping matching analysis.

to tobramycin by the production of the nucleotidyltransferase ANT4′ but did not produce the bifunctional enzyme APH6′-AAC2″, which inactivates gentamicin and netilmicin (6, 7). These strains have spread widely in French hospitals since 1992, and their epidemiological and molecular features have been well described in recent years (2, 7, 17, 18). Our results fully agree with previous findings: all the isolates have the same resistance pattern (pattern 1) and most of them have the same pulsotype (pulsotype A1) as those reported by Lemaître et al. (18) (Fig. 2). More recently, Laurent and colleagues (15) have suggested that French epidemic strains of MRSA (including an MRSA pattern VII strain from our hospital) have a competitive advantage over gentamicin-resistant clones but are also genetically related to some of them. On the basis of this relatedness, some investigators have suggested that gentamicin-sensitive MRSA clones could have emerged from gentamicin-resistant clones (2, 7, 17). Therefore, we cannot prove an exogenous origin of the MRSA pattern VII clone rather than the local emergence from a gentamicin-resistant MRSA strain, but its presence in many hospitals in the context of the nationwide spread argues well for the first hypothesis. Epidemic MRSA strains have been described for a long time, and there have been many reports of very large outbreaks that are due to a clone and that involve hospitals in a region, a country, or a continent (5, 14).

MSSA strains are usually susceptible to most antibiotics except benzylpenicillin (to which 88% of isolates are resistant) and, at lower rates, macrolides-lincosamides-streptogramin B (MLS_B) (rate of inducible resistance to MLS_B, 14.5%) and fluoroquinolones (to which 4.5% of isolates are resistant). Constitutive resistance to MLS_B is rare in MSSA isolates (2.5%) but frequent in MRSA isolates (77%). Since 1997 we have identified MSSA isolates with a surprising resistance pattern that consists of constitutive MLS_B resistance, resistance to fluoroquinolones, and resistance to fosfomycin. Moreover, these isolates were resistant to spectinomycin, an aminoglycoside antibiotic which we used as a resistance marker for some MRSA strains, especially MRSA strains with pattern VII. Since this resistance pattern was the same except for a lack of resistance to oxacillin and tobramycin, we have assumed that this strain was derived from an MRSA pattern VII strain and have designated it MSSA pattern ΔVII. The number of MSSA pattern ΔVII isolates has progressively increased over 5 years: in 1997, 2 isolates; in 1998, 5 isolates; in 1999, 14 isolates; in 2000, 22 isolates; and from 1 January to 30 June 2001, 24 isolates. Five patients were first infected with an MRSA pattern VII strain and then with a MSSA pattern ΔVII strain a few months later (Table 2).

TABLE 2. Samples and dates of recovery of major epidemic MRSA pattern VII and MSSA pattern ΔVII isolates from five patients

Patient	MRSA pattern VII isolates		MSSA pattern ΔVII isolates	
	Sample	Recovery date (day.mo.yr)	Sample	Recovery date (day.mo.yr)
1	Surgical wound	10.10.1998	Blood culture	16.02.1999
2	Abdominal drainage	27.03.1999	Abdominal abcess	24.04.1999
3	Urine	07.01.2000	Abcess	23.02.2000
4	Urine	04.08.2000	Urine	30.05.2001
5	Urine	18.01.2001	Urine	18.05.2001

Methicillin susceptibility was confirmed by the absence of expression of PBP 2a (agglutination test negative) and the absence of the *mecA* gene (PCR negative) in all MSSA pattern ΔVII isolates tested. The *Alu*I polymorphisms of the *coa* PCR products showed that MRSA pattern VII and MSSA pattern ΔVII strains had identical restriction patterns (data not shown). The PFGE pattern of MSSA pattern ΔVII strains differs from that of major epidemic MRSA pattern VII strains by only one band, corresponding to the loss of a 40-kb fragment from the 200-kb DNA band (Fig. 3). Phenotypic as well as genotypic markers indicated that MSSA pattern ΔVII strains could be derived from major epidemic MRSA strains by the loss of the *mecA* region. Previous reports have suggested that some MRSA strains possess an unstable *mecA* DNA region. The instability of *mecA* was first described in vitro (10, 11) and more recently has been described in clinical situations (4, 12, 16, 20, 23). The role of recently discovered cassette chromosomal recombinases CCRA and CCRB has been underlined by the induction of the precise excision of the *mecA* DNA region (13). The deletion of the *Staphylococcus* chromosome cassette (SCC) is responsible for a new resistance phenotype (resistance to erythromycin and spectinomycin only) in MRSA strain N315 (13). Similarly, methicillin susceptibility in MSSA pattern ΔVII strains should be due to the loss of *mecA*. As the genes *mecA* and *aad* (the gene encoding for ANT4′) are localized on the same 185- to 215-kb *Sma*I restriction fragments in French gentamicin-sensitive MRSA clones (17) and the unique difference between the MRSA pattern VII and the

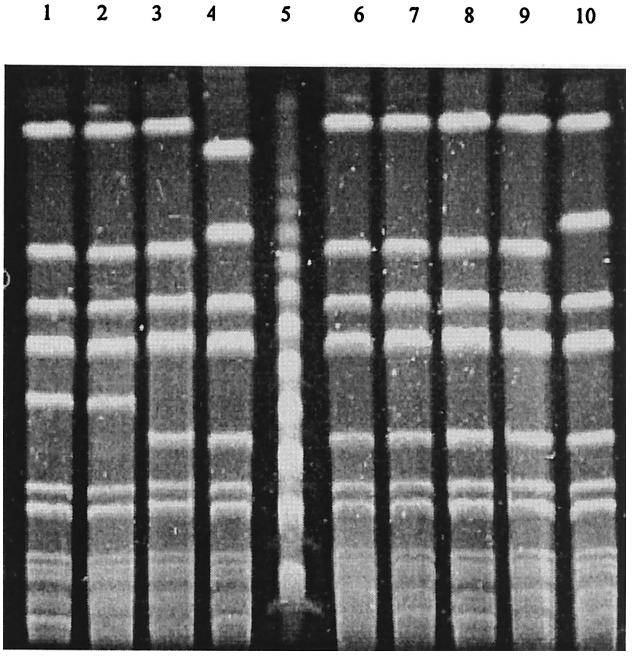

FIG. 3. PFGE restriction patterns of MRSA pattern VII and MSSA pattern ΔVII isolates. Lane 1, MRSA pattern VII strain from patient 1; lane 2, MRSA pattern VII strain from patient 2; lane 3, MSSA pattern ΔVII strain from patient 1; lane 5, DNA ladder ($n \times$ 48.5-kb fragments); lane 6, MSSA pattern ΔVII strain from patient 2; lanes 4, 7, 8, 9, and 10, MSSA pattern ΔVII strains from other patients

MSSA pattern ΔVII *Sma*I restriction patterns is found on these fragments, tobramycin susceptibility is likely due to the loss of *aad*. Excision of SCC implies the deletion of Tn *554*, which is localized upstream of *mecA*, and then the loss of genes for resistance to erythromycin and spectinomycin. According to Katayama and collaborators (13), we assume that the remaining resistance to these two antibiotics is due to the presence of multiple copies of Tn *554*.

Wagenvoort and colleagues (23) have indicated that the loss of epidemicity relies on the loss of *mecA*. On the one hand, the recovery of MSSA pattern ΔVII strains from patients formerly infected with a major epidemic MRSA pattern VII strain suggests that most of these strains are directly derived from MRSA strains. On the other hand, we have recovered MSSA pattern ΔVII strains from premature twins who were hospitalized in the same room of a neonatology intensive care unit and who were infected with such strains but who had not previously had staphylococcal infections. Nose carriage of MSSA pattern ΔVII strains has been detected in medical staff members during investigations of two outbreaks due to major epidemic MRSA pattern VII strains. Moreover, the increasing number of MSSA pattern ΔVII isolates might indicate that such strains are potentially transmissible from person to person, independent of the deletion of *mecA*.

We have described the evolution of dominant MRSA strains in our hospital and collected data which indicate that the *mecA* region is unstable in the most epidemic strain. It remains unclear if insertion of *mecA* and reacquisition of methicillin resistance could occur in such strains. Conversely, it would be of interest to know if the loss of *mecA* could occur in other epidemic MRSA strains and contribute to the temporal evolution of MRSA in our hospital.

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