Emergence and Spread in French Hospitals of Methicillin-Resistant Staphylococcus aureus with Increasing Susceptibility to Gentamicin and Other Antibiotics

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Oxacillin (methicillin) resistance in methicillin-resistant Staphylococcus aureus (MRSA) is associated with an increased incidence of resistance to other antibiotics, which has increased since it was first reported in 1969. In 1992 a new phenotype of MRSA arose in France; this was characterized by a heterogeneous expression of resistance to oxacillin and susceptibility to various antibiotics, including gentamicin but also tetracycline, minocycline, lincomycin, pristinamycin, co-trimoxazole, rifampin, and fusidic acid. In French hospitals a longitudinal nationwide surveillance of antibiotic resistance in S. aureus has allowed for the detection of changes in antibiotic susceptibility profiles. Seven French clinical laboratories (six from the mainland and one from the West Indies) reported the results of susceptibility testing of 57,347 S. aureus strains isolated in their institutes between 1992 and 1998. Over a 7-year period the incidence of isolation of gentamicin-susceptible MRSA (GS-MRSA) strains has steadily increased to represent, in 1998, 46.8 to 94.4% of the MRSA strains, irrespective of the overall incidence of MRSA. Two predominant types recognized by pulsed-field gel electrophoresis (PFGE) accounted for the majority of the GS-MRSA in different mainland hospitals, both differing from the predominant type observed in the French West Indies. Some GS-MRSA and gentamicin-resistant MRSA (GR-MRSA) strains had closely related PFGE profiles, and hybridization studies confirmed the lack in GS-MRSA of the aac6’-aph2’ gene, which confers resistance to all aminoglycosides, with conservation of the ant4’ gene, which confers resistance to kanamycin, tobramycin, and amikacin. Thus, it is likely that certain GS-MRSA strains could have emerged from GR-MRSA strains by excision or deletion of the aac6’-aph2’ gene.

In many countries oxacillin (methicillin)-resistant Staphylococcus aureus (MRSA) has become a significant nosocomial pathogen. MRSA was first reported in the United Kingdom in 1961, soon after the introduction of methicillin, and by the mid-1970s had become endemic in many countries (26). Some strains of MRSA have been designated epidemic strains; these are associated with a higher prevalence and have been shown to have spread within hospitals, between hospitals, and between countries (1, 10, 16, 17, 21). The first MRSA isolates expressed so-called heterogeneous phenotypic resistance to oxacillin, meaning that the oxacillin MICs for only subpopulations of isolates are high. Progressively, the heterogeneous oxacillin-resistant phenotype was replaced by the homogeneous oxacillin-resistant phenotype, which is characterized by the expression of oxacillin resistance by all populations. Initially, early isolates were also resistant to various other drugs, including penicillin, tetracycline, and, usually, streptomycin (some strains were also resistant to erythromycin, lincomycin, neomycin, kanamycin, and novobiocin). In 1969, the first clinical gentamicin-resistant MRSA (GR-MRSA) strain was isolated (11), and by the 1980s GR-MRSA had become epidemic in Australia, the United States, and Europe (5). Such GR-MRSA strains were usually resistant to a broad number of other antibiotics, including trimethoprim and, more recently, ciprofloxacin and mupirocin. In addition to increasing multi-antibiotic drug resistance, the overall incidence of MRSA isolation has gradually increased in many countries to present levels of around 30% in Spain, France, and Italy (26) and up to 54% in Japan (14). The emergence of new epidemic MRSA strains more susceptible to antibiotics has been recently reported by two French hospitals (2, 12). These strains were characterized mainly by the unexpected reappearance of heterogeneous resistance to oxacillin, susceptibility to gentamicin, and variable resistance to macrolides, lincosamides, and streptogramin type B antibiotics; they remained resistant to tobramycin, which was associated with the presence of the aminoglycoside nucleotidyltransferase ANT(4’)(2, 12). A marked decrease in the use of gentamicin was suspected to be a factor contributing to the emergence of gentamicin-susceptible MRSA (GS-MRSA) from predominantly GR-MRSA populations (2, 12).

The aim of the present study was to investigate whether the previously reported evolution of antibiotic resistance of MRSA in two hospitals in the Paris area could be relevant at the nationwide level, by collecting antibiotic susceptibility data.
from seven French clinical laboratories between 1992 and 1998. Subsets of isolates were retrieved and studied in more detail in an attempt to better understand the molecular basis of increasing gentamicin susceptibility.

**MATERIALS AND METHODS**

Source of bacterial strains and analysis of antibiotic susceptibility. Clinical laboratories from seven hospitals dispersed throughout French territory (hospitals A, B, and C in Paris; hospital D in Lille [northern France]; hospital E in Lyon [central France]; hospital F in Bordeaux [southwestern France]; and hospital G in Fort-de-France, French West Indies) reported data for susceptibility to oxacillin and gentamicin of 57,347 *S. aureus* isolated between 1992 and 1998, after omission of consecutive isolates from the same patients. In addition, hospitals E and G were selected as representative hospitals for exhaustive antibiotic susceptibility analysis of their respective strains. All reported antibiotic susceptibility data were derived from the routine clinical laboratory databases of the participating hospitals and were determined according to the guidelines set by the Committee for Antimicrobial Testing of the French Society for Microbiology (6).

Hospitals A to C and E to G used the agar diffusion technique as the antibiotic resistance detection method, whereas hospital D used the ATB expression system (bioMérieux, Marcy l’Etoile, France). Strains were considered resistant to oxacillin if the MIC was $\geq 2\, \mu g/\text{ml}$ or if there was a diameter of inhibition of $\leq 20\, \text{mm}$ around a 5-$\mu g$ oxacillin disk (6). The disk diffusion assay with oxacillin disks was performed either on Mueller-Hinton agar plates incubated for 24 h at 30°C or on Mueller-Hinton agar plates supplemented with 2% NaCl and incubated for 24 h at 37°C. Susceptibility testing with the ATB expression system was performed according to the instructions of the manufacturer (bioMérieux). Population analysis, in order to determine oxacillin resistance classes of referred isolates, was performed as described by Tomasz et al. (25). Briefly, stationary-phase culture ($10^9$ to $10^{10} \,\text{CFU/ml}$) were plated at seven dilutions ($10^{-1}$ to $10^{-7}$) on a series of agar plates containing serial twofold dilutions of oxacillin at concentrations ranging from 0 to 1,000 $\mu g/\text{ml}$. The plates were incubated at 37°C for 48 h before the colonies were counted. The number of bacteria capable of

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**FIG. 1.** Percentage of *S. aureus* strains resistant to oxacillin (●), and percentages of GS-MRSA (■) and GR-MRSA (□) among MRSA. Mean numbers of *S. aureus* strains isolated per year: hospital A, 548; hospital B, 1,839; hospital C, 1,321; hospital D, 952; hospital E, 1,188, hospital F, 2,425; hospital G, 612. Data for 1992 and 1993 are not available for hospital F. Data were obtained from a total of 57,347 *S. aureus* isolates.
form. colonies were plotted against the concentration of oxacillin, producing population analysis profiles. Statistical analysis was performed with Epi-Info software (version 5; Centers for Disease Control and Prevention, Atlanta, Ga.). One hundred eighty-three MRSA isolates (145 GS-MRSA and 38 GR-MRSA) originating from the seven participating hospitals were randomly selected for molecular typing by pulsed-field gel electrophoresis (PFGE). All strains were kept frozen at −80°C until used.

PFGE. Smal macrorestriction patterns were obtained by using a contour-clamped homogeneous electric field system with a CHEF DR-2 (Bio-Rad, Richmond, Calif.) apparatus as previously described (9, 13). Comparisons of resolved macrorestriction patterns were based on the recommendations of Tenover et al. (23). Strains differing in up to three fragments only were deemed clonally related and were described as subtypes of a given clonal type. In the case of no differences between banding patterns, strains were considered identical. When they differed by four or more fragments, strains were considered separate types. Letters were used to denote major genotypes, and each variant subtype was indicated by a numerical suffix.

Hybridization studies. Smal macrorestriction profiles were vacuum transferred onto nylon membranes (19). Probe labeling and hybridization were carried out by using the nonradioactive digoxigenin DNA labeling and detection kit (Boehringer Mannheim). The GenBank data for the methicillin resistance gene (mecA) (accession no. X52933); the 4′-4′-aminoglycoside nucleotidytranferase gene (ant4′), responsible for tobramycin resistance (M19465); and the bifunctional 2′-amino-2′-deoxyadenosine phosphotransferase-6′-aminoglycoside acetyltransferase gene (aac6′-aph2′), responsible for amikacin, tobramycin, and gentamicin cross-resistance (M18886) were used to design PCR primers allowing the synthesis of gene-specific DNA probes. S. aureus ATCC 6538P was used as a negative control strain for the mecA, ant4′, and aac6′-aph2′ genes. S. aureus CIP6525, BM3002, and FK422 were used as positive control strains for the mecA, ant4′, and aac6′-aph2′ genes, respectively.

RESULTS

In 1992, the frequency of GS-MRSA within the total population of MRSA was below 7.4% in all of the study hospitals (data were not available for hospital F) (Fig. 1). From 1992 to 1998, this rate progressively increased to reach between 46.8% (hospital D) and 94.4% (hospital G) (Fig. 1). Since the overall incidence of isolation of MRSA remained stable (hospitals A and E), increased slightly (hospitals F and G), or decreased slightly (hospitals B, C, and D) (Fig. 1), this strongly suggested that GS-MRSA strains were supplanting the GR-MRSA population. In the cases of hospitals F and G, the slight increase in the overall incidence of MRSA population arose partly in addition to the endogenous GR-MRSA population.

For practical reasons, hospitals E and G were selected as a model for studying the evolution of the antimicrobial resistance associated with oxacillin resistance, and the susceptibility data from these hospitals were further analyzed for all MRSA isolates collected in 1996 after omission of consecutive isolates from the same patients. All GS-MRSA strains from hospital E expressed heterogeneous resistance to oxacillin in that, after incubation at 30°C, they showed a clear zone of inhibition around the oxacillin disk and the presence of colonies near the disk, in contrast to all GR-MRSA strains, which showed homogeneous resistance with confluent growth right up to the oxacillin disk (Table 1). Population analysis showed class 1 phenotype expression for selected GS-MRSA strains in that only a very low proportion (10−7 to 10−8) of bacteria could form colonies in the presence of oxacillin at up to 25 μg/ml or more (data not shown) (25). GS-MRSA strains were significantly more frequently susceptible to kanamycin, tobramycin, lincomycin, pristinamycin, tetracycline, minocycline, co-trimoxazole, rifampin, and fusidic acid, and were more frequently resistant to chloramphenicol, than GR-MRSA strains (Table 1). No significant differences between GR- and GS-MRSA in resistance to erythromycin, ofloxacin, and fosfomycin were observed. For hospital G, the analysis of antibiotic susceptibility patterns, while not including exactly the same antibiotics, showed susceptibility profiles similar to those for the isolates from hospital E, except for fusidic acid (Table 1).

Molecular typing analysis using PFGE was performed for 184 randomly selected isolates from the seven participating hospitals. For each isolate, macrorestriction profiles generated by Smal cleavage comprised 15 to 17 fragments varying in size from <80 to 700 kb (Fig. 2). PFGE profiles of GS-MRSA strains were mostly clustered into three major PFGE types: types A and B, comprising 42% (48 of 114 isolates) and 41% (47 of 114 isolates), respectively, of GS-MRSA strains from the six mainland hospitals, and type C, representing the dominant GS-MRSA type (64% of GS-MRSA strains) in the French
West Indies hospital (Table 2). Type A and B strains were seldom detected among isolates derived from the French West Indies, and conversely, type C strains were not detected among isolates derived from mainland hospitals.

GR-MRSA isolates available from hospitals E and G comprised several types (including type A) and subtypes (Table 2). In several cases, the PFGE profiles of GS- and GR-MRSA strains of the same type differed by two bands only (Table 2 and Fig. 2). Moreover, some other GS-MRSA strains that were resistant to kanamycin and tobramycin had PFGE types strictly identical to those of MRSA strains that were susceptible to all aminoglycosides (Fig. 2). Southern blot hybridizations with the meca-specific and ant4-specific probes were positive for the 19 representative isolates of PFGE types A and B, on the same ~185- to 215-kb Smal fragment, except for one isolate which was susceptible to all aminoglycosides and showed negative hybridization with the ant4-specific probe (Table 3). Hybridization studies confirmed the loss of the meca-aph2 gene in the GS-MRSA derivatives.

Spontaneous GS-MRSA derivatives (with no changes in any other antibiotic resistance) were obtained after long-term storage (2 years) at room temperature from two GR-MRSA strains (A960651 and A960649). In contrast, no change from the original GR-MRSA phenotype was observed in strains maintained for the same time period at −80°C. The PFGE types of the parent GR-MRSA strains and revertant GS-MRSA strains were indistinguishable (Fig. 2, lanes 5 to 8). Hybridization studies confirmed the loss of the meca-aph2 gene in the GS-MRSA derivatives.

**DISCUSSION**

*S. aureus*, and in particular MRSA, has long been one of the more serious and problematic nosocomial pathogens, repeatedly responding to the challenge of new antistaphylococcal antibiotics by acquiring new resistance (3). A more prudent use of antibiotics, in addition to the implementation of better infection control and hygiene measures for reducing nosocomial infections, has been encouraged in many countries and may play a role in reducing the incidence of multiply resistant organisms. Although these implemented measures may have been responsible for the major decline in the incidence of MRSA in Denmark from 15 to 0.2% between 1971 and 1984 (18), they have been relatively unsuccessful in other, larger countries, where the prevalence of MRSA seems to be still increasing (22, 26). It is clear that in most French hospitals, not only hospitals nationwide within the mainland but also a geographically distinct hospital situated in the French West Indies, distinct MRSA clones that are more susceptible to multiple antibiotics, particularly gentamicin, are increasing in incidence, often replacing the endogenous classical MRSA clones, while the overall prevalence of oxacillin resistance among *S. aureus* isolates remains stable or varies slightly (Fig. 1). This observation confirms the recent reports from two French hospitals (one of which is included in the present study) (2, 12) of the emergence of new epidemic MRSA strains that are more susceptible to antibiotics.

We showed that the same two GS-MRSA PFGE types, types A and B, were predominant in each of the mainland hospitals (A to F) included in the study and that another distinct PFGE type C was predominant in a hospital in the French West Indies (G) (Table 2). It is clear that in most French hospitals, not only hospitals nationwide within the mainland but also a geographically distinct hospital situated in the French West Indies, distinct MRSA clones that are more susceptible to multiple antibiotics, particularly gentamicin, are increasing in incidence, often replacing the endogenous classical MRSA clones, while the overall prevalence of oxacillin resistance among *S. aureus* isolates remains stable or varies slightly (Fig. 1). This observation confirms the recent reports from two French hospitals (one of which is included in the present study) (2, 12) of the emergence of new epidemic MRSA strains that are more susceptible to antibiotics.

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**TABLE 2.** PFGE types and subtypes of GS- and GR-MRSA) from strains from mainland hospitals A to F and French West Indies hospital G

<table>
<thead>
<tr>
<th>Type</th>
<th>Subtype(s)</th>
<th>A to D and F, GS-MRSA (n = 55)</th>
<th>E, GS-MRSA (n = 59)</th>
<th>GR-MRSA (n = 18)</th>
<th>G, GS-MRSA (n = 31)</th>
<th>GR-MRSA (n = 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>A.1</td>
<td>5</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>A.2 to A.8</td>
<td>12</td>
<td>16</td>
<td>7</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>B.1</td>
<td>21</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>B.2 to B.7</td>
<td>9</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
<td>C.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>C.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td>8</td>
<td>11</td>
<td>11</td>
<td>10</td>
<td>19</td>
</tr>
</tbody>
</table>

* Hospital A, 8 isolates; B, 16 isolates; C, 14 isolates; D, 5 isolates; E, 12 isolates.

* Each type includes fewer than six isolates. In these other types, none of the GS-MRSA types were identical to GR-MRSA types.
respective. However, data documenting changes in the level
presented 4.9 and 27.5% of the MRSA isolates in 1993 and 1995
factor of two (2); in this hospital GS-MRSA isolates repre-
1987 was reported, whereas that of amikacin increased by a

mecA ant4 aac6' -aph2'

AAC-6' aminoglycoside selective pressure over a period of 5 years or
GR-MRSA isolates, supports this hypothesis. It is possible that
GR- and GS-MRSA strains have closely related PFGE types,
GR-MRSA strains at room temperature. The fact that both
non that we have reproduced in vitro by long-term storage of
frequently carried by transposon Tn
aac6
(aac6' -aph2')

A960451 GR-MRSA, type A.7

A980157 GS-MRSA, type A.3

A980147 MRSA strain susceptible to all aminoglycosides, type A.3

A980651 GR-MRSA, type B.1

A980400 GS-MRSA, type B.1, derivative of A960651

A980649 GR-MRSA, type B.1

A90849 GS-MRSA, type B.1, derivative of A960649

NH* NH NH

≈185–215 NH NH

≈185–215 ≈185–215 NH

≈185–215 ≈185–215 >700

≈185–215 ≈185–215 >700

≈185–215 ≈185–215 >700

≈185–215

* NH, negative hybridization.

type, type C, predominated in hospital G located in the French
West Indies. This is probably due to the frequent exchange of
patients between hospitals A to F, while exchange between these
mainland hospitals and hospital G in the French West
Indies is uncommon, explaining the quasianabsence of common

... GS-MRSA types. Using the definition of a distinct PFGE

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MRSA WITH INCREASING ANTIBIOTIC SUSCEPTIBILITY


