Reemergence of Gentamicin-Susceptible Strains of Methicillin-Resistant Staphylococcus aureus: Roles of an Infection Control Program and Changes in Aminoglycoside Use

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The spread of methicillin-resistant Staphylococcus aureus (MRSA) in our hospital in the 1980s correlated with increasing acquisition of resistance to antibiotics including gentamicin, rifampin, and fluoroquinolones. During the period 1993-1995, there was a major change in clinical MRSA isolates: the percentage of aminoglycoside-resistant MRSA isolates decreased from 75% to 52%, while the proportion of heterogeneous MRSA strains susceptible to gentamicin, rifampin, and tetracycline increased gradually from 4.9% to 27.5%. We used five epidemiological markers (i.e., antibiotyping, phage typing, pulsed-field gel electrophoresis, and restriction analysis of PCR amplified coagulase and protein A genes) to characterize recent isolates. With use of these techniques, we confirmed the persistence of the aminoglycoside-resistant MRSA clone and identified a clone of erythromycin-susceptible strains among the gentamicin-susceptible isolates and found that the remaining strains were diverse. These changes were due to the introduction of various MRSA strains from outside the hospital, while implementation of infection control measures in 1991 could have led to reduced transmission of the aminoglycoside-resistant MRSA strain. Changes in antibiotic prescribing patterns that resulted in reduced selective pressure from gentamicin may have contributed to the spread of gentamicin-susceptible MRSA strains.

Methicillin-resistant Staphylococcus aureus (MRSA), which was first reported in 1962, has emerged as a major cause of nosocomial infections [1]. MRSA strains are efficient colonizers of patients and can cause outbreaks of serious infections that are difficult to control [1–3]. In addition, MRSA strains have demonstrated a remarkable ability to acquire resistance to other antibiotics. The introduction of erythromycin, tetracycline, gentamicin, and the fluoroquinolones was followed in many countries by the emergence of resistance to these drugs among MRSA strains [1, 2, 4]. As a consequence, accumulation of resistance traits was observed in MRSA strains recently isolated in Europe [5] and worldwide [6]. High rates of resistance to many antibiotics can be observed in hospital isolates of S. aureus, mostly related to the spread of epidemic strains.

A similar pattern of evolution toward multiresistance was observed in MRSA strains isolated at our hospital. During the period 1985–1992, 60%–80% of MRSA strains from our hospital were coresistant to fluoroquinolones, gentamicin, macrolides, rifampin, and tetracycline and expressed homogeneous resistance to methicillin [7]. Because of the high incidence (reaching nearly 40% in 1991–1992) of homogeneous methicillin- and aminoglycoside-resistant S. aureus isolates, an infection control program was implemented and was based on a “search and destroy” strategy [8]. Two years later, new MRSA phenotypes emerged that were characterized by heterogeneous resistance to methicillin and susceptibility to gentamicin, rifampin, tetracycline, and, often, to erythromycin.

In the present study, we investigated selected recent MRSA isolates by using pulsed-field gel electrophoresis, polymorphism of the 3’ ends of the coagulase gene (coa) and of the protein A gene (spa), and phenotypic methods. The coa- and spa-gene typing method is based on the variability of the 3’ coding regions of the coa and spa genes, which contain a series of 81-bp or 24-bp repeats, respectively; the number of repeats differs between strains. In addition, individual repeats differ in point mutations and, as a consequence, in the presence or absence of certain restriction sites [9–11]. The overall comparison between these markers showed the emergence of diverse clones of heterogeneous MRSA strains, whereas the incidence of the endemic aminoglycoside-resistant MRSA strains decreased in the hospital. We also analyzed antibiotic consumption as a putative cause for this epidemiological change.

Materials and Methods

Antibiotic resistance survey. Henri Mondor Hospital is a 1,080-bed University-affiliated hospital located in the suburbs of Paris; there are 125 medical and surgical-intensive-care-unit
beds. Since the hospital opened in 1969, our laboratory has routinely determined the antibiotic susceptibility of 33,263 S. aureus isolates with use of the disk-agar diffusion technique, according to the annual recommendations of the Committee for Antimicrobial Testing of the French Society for Microbiology [12]. Methicillin resistance was screened by testing methicillin disks from 1969 to 1977 and oxacillin disks since 1978. Screening was performed with Mueller-Hinton (MH) medium supplemented with 5% NaCl from 1969 to 1982, un-supplemented MH incubated at 30°C from 1983 to 1992, and both techniques since 1993. Data on isolates recovered from surveillance cultures for detection of MRSA carriage were not included in the antibiotic resistance data reported. In 1977 and since 1980, bacteriophage typing of all MRSA isolates has been performed according to the method of Blair and Williams [13] by using the international set of typing phages. The phages were used at concentrations of routine test dilution (RTD) and 100 RTD. Only reactions showing major lysis were considered.

Infection control measures. In mid-1991, the infection control committee at our hospital made the control of multidrug-resistant pathogens, including MRSA, a major goal. An MRSA control program was progressively implemented, first in high-risk departments and, since 1994, in the whole hospital. Detection of new cases of MRSA by the microbiology laboratory was immediately reported to the physicians caring for MRSA-infected patients. Since mid-1992, surveillance cultures of specimens obtained from the nose, axilla, and groin have been performed to detect MRSA colonization in patients who were considered at risk. MRSA-positive patients were to be kept in separate rooms or cohorted, and special barrier precautions were instituted, including the use of disposable gowns and gloves, handwashing with an antiseptic soap, and strict environmental hygiene measures. Each patient with MRSA was classified as having a hospital-acquired or imported infection, according to definitions published by the Centers for Disease Control [14].

Bacterial strains. Thirty-three MRSA strains isolated from clinical samples obtained from January 1990 to April 1996 were selected for further molecular studies. These strains were isolated from patients hospitalized at different times in different wards, and no link could be established between the patients, whose infections were considered epidemiologically unrelated. The isolates included 26 heterogeneous MRSA strains that were susceptible to gentamicin, rifampin, and tetracycline and resistant (eight strains) or susceptible (18 strains) to erythromycin; these strains were isolated during the period 1993 (the beginning of the emergence of this phenotype)–1996. Seven homogeneous aminoglycoside-resistant MRSA isolated during the period 1990–1994 were also included. The aminoglycoside-resistant MRSA strain KB5 from our collection was used as a control for molecular analysis [11].

Pulsed-field gel electrophoresis (PFGE) analysis. Genomic DNA was prepared in agarose plugs, as described previously [11]. Two low-frequency cleaving enzymes, Sma I (5’-CCGGG-3’) (Pharmacia, Saint Quentin en Yvelines, France) and Csp I (5’-CGGTCCG-3’) (Promega Biotech, Madison, WI), were used for macrorestriction analysis; both of these enzymes cleave the S. aureus chromosome at fewer than 15 sites. Each portion of agarose plug was incubated for 6 hours with 24 IU/mL of Sma I or Csp I at 25°C or 30°C, respectively. Restriction fragments were separated by PFGE with CHEF DRII (Bio-rad, Ivry-sur-Seine, France) in 1.2% agarose gels run at 200 V in 0.5X Tris-Borate-EDTA buffer with use of a 1–45-second pulse-time linear gradient for 24 hours. All strains were tested by two independent electrophoretic runs. Strains were assigned to the same macrorestriction genotype when they shared common electrophoretic restriction patterns that differed by three or fewer fragments and displayed a coefficient of similarity (CS) ≥0.85 [15]. The CS was calculated as follows: CS = twice the number of matching bands/the total number of bands in both strains [16].

Restriction analysis of the 3’ ends of the coa gene and of the spa gene. DNA was prepared as follows. For each strain, a colony from an overnight culture on heart infusion agar was resuspended in 400 µL of autoclaved water and boiled for 15 minutes at 100°C. The 3’-end region of the coa gene was amplified by using the primers COA2 5’-AGCGAGACC-AAGATTCAACAAGCCA-3’ and COA5 5’-CCATATGTAGCAGTACCATCTGCATGT-3’ [11]. Ten µL of extracted DNA were added to a PCR mixture containing 20 pmol of forward and reverse primers, 10 µL of 10-fold concentrated PCR buffer (200 mM Tris-HCL, pH 8.3; 10 mM MgCl2; 500 mM KCl, 0.1% gelatin; and 0.5% Tween 20), 50 µM of each deoxynucleotide triphosphate, and 2.5 U of Taq polymerase in a final volume of 100 µL of deionized water.

Each sample was subjected to 35 cycles consisting of 30 seconds at 94°C, 30 seconds at 60°C (annealing for the coa gene), and 30 seconds at 72°C in a Perkin-Elmer 9600 thermocycler (Foster City, CA). Amplimers (10 µL) of the coa gene were digested 30 minutes with 6 IU of restriction endonuclease Hae III (Pharmacia). Both PCR products and restriction digest fragments were separated by electrophoresis at 130 V for 1 hour in 3% agarose gel and visualized on gel stained with ethidium bromide.

The spa gene was amplified, following the same procedure, by using forward primer 5’-GCTAAAAAGCTAAACGATGC-3’ and reverse primer 5’-CCACCAAATACAGTTGTACC-3’ whose infections were considered epidemiologically unrelated. The annealing temperature was 47°C, and the profiles were obtained after digestion with restriction endonuclease Rsa I (Pharmacia) [9].

Statistical methods. In the analysis of data, differences in proportions were evaluated with use of either Fisher’s exact test or the χ2 test. Computations were performed by using Epi-Info Version 5.1 (Centers for Disease Control and Prevention).

Results

Evolution of methicillin resistance in S. aureus. From 1969 to 1995, 25 antibiotics were tested against ~1,000–2,100 non-
repetitive (unique patient isolates) *S. aureus* strains isolated each year from clinical samples at Henri Mondor hospital. Although methicillin resistance was first reported in France in 1962 [17], its incidence was already estimated at 39% in our hospital in 1969. The incidence of methicillin resistance decreased to 11% in 1975 and subsequently increased and stabilized between 1980 and 1990 at ~25%–30%. During the early 1990s, a further increase in the incidence of methicillin resistance was observed, reaching 40% in 1992.

Since 1991, percentages of MRSA isolates recovered in high-risk departments at our hospital ranged from 50% to 60%. Forty percent of the MRSA isolates were causing infections in patients. Forty-eight percent of MRSA isolates were acquired by the patients in our hospital. Twenty-eight percent were acquired in other hospitals and imported in our hospital. The remaining 24% of MRSA isolates were detected on admission of the patients, and the origin of these isolates and the date of colonization were not known.

After implementation of infection control measures, the incidence of methicillin resistance began to decrease to 35% over 3 years. During the 1991–1995 period, the incidence of MRSA infections per admission in our medical and surgical intensive care units decreased from 7.9 per 100 admissions to 3.0 per 100 admissions; there were nearly 4,000 admissions per year ($\chi^2 = 73; P < .0001$).

The total number of episodes of bacteremia increased from 845 in 1991 to 1,492 in 1995, reflecting an increase in the incidence of gram-negative bacilli. The number of *S. aureus* strains recovered remained stable, whereas the percentage of isolates decreased from 22.8% to 12%. The proportion of MRSA recovered from patients with *S. aureus* bacteremia remained stable at ~27%. Overall, the relative proportion of MRSA strains recovered from patients with bacteremia decreased from 5.5% in 1992 to 3.7% in 1994 and to 3.4% in 1995 ($\chi^2 = 5.8; P = .02$).

**Evolution of antibiotic-resistant phenotypes.** Since 1969, MRSA strains have displayed dramatic increases in resistance to many other antistaphylococcal agents including rifampin, pefloxacin (a fluoroquinolone), and tetracycline, as shown in figure 1. Resistance to chloramphenicol, which decreased from 70% in 1971 to <10% after 1985, was an exception. Gentamicin resistance emerged in 1975 and reached an incidence of 90% in 1980; only since 1993 did this resistance begin to decrease gradually, for an incidence of 63.7% in 1995. A similar evolution of resistance was recorded for tetracycline and rifampin.

During the 1969–1976 period, diverse resistance phenotypes coexisted among strains of MRSA. In 1977, phenotypes with gentamicin resistance emerged, and the major phenotype associated with resistance to methicillin, streptomycin, gentamicin, tetracycline, erythromycin, and lincomycin represented 40%–60% of MRSA strains recovered until 1980 (figure 2). Sixty-three percent of strains belonging to this phenotype were lysed by phages of the 47/54/75/85 complex. New phenotypes emerged that were characterized by the successive emergence of rifampin resistance and fluoroquinolone (pefloxacin) resistance in 1980 and 1984, respectively. Since 1985, the latter phenotype was largely predominant (60%–80% of MRSA strains).

Spread of fluoroquinolone resistance appeared to be related to the emergence of a new phage-type, 77, representing 56% of the fluoroquinolone-resistant aminoglycoside-resistant MRSA strains, while the remaining strains were nontypeable. Fluoroquinolone resistant strains then became increasingly nontypeable, and the incidence of phage type 77 decreased from 25%
in 1989 to 1% of MRSA in 1992, whereas the incidence of nontypeable strains increased from 50% to 80%.

Nearly all MRSA strains isolated between 1969 and 1975 were heterogeneously resistant to methicillin, whereas most strains isolated after 1977 expressed homogeneous resistance. However, strains expressing heterogeneous methicillin resistance recently reemerged in our hospital, and their incidence increased from <1% in 1992 to 5.1% (29 of 567 strains) in 1993 and 29.9% (111 of 371 strains) of MRSA in 1995.

The heterogeneous strains that emerged in 1993 markedly differed from the previous aminoglycoside-resistant MRSA in their susceptibilities to gentamicin, rifampin, and tetracycline. Most heterogeneous MRSA strains could be grouped into two resistance phenotypes, A and B, according to susceptibility or cross-resistance to erythromycin and lincomycin, respectively. Both types were resistant to kanamycin and tobramycin, probably by the synthesis of a 4'-4'' aminoglycoside nucleotidyltransferase, and to fluoroquinolones in addition to methicillin. Whereas the erythromycin-resistant phenotype B was predominant in 1993 among heterogeneous MRSA strains, the incidence of phenotype A rose from 9% of heterogeneous strains in 1993 to 50% of heterogeneous strains in 1995 ($P = .0001$) (figure 3). Most phenotype A strains were lysed by phages 85 and/or 53 at 100-fold the routine test dilution, or they were nontypeable.

The epidemiological analysis of 475 patients colonized or infected with MRSA in 1994–1996 showed that the homogeneous strains were significantly more often acquired in our hospital (RR = 1.3; $P = .011$), whereas the heterogeneous strains ($n = 180$) as a whole were mostly imported into our hospital. A major source for importation of the strains was transfer of patients from other institutions or readmission of patients. Further analysis showed that the type A strains were significantly more often acquired in the hospital than the type B strains ($P = .027$).

**Analysis of the clonality of MRSA strains by molecular techniques.** For 26 heterogeneous MRSA and seven aminoglycoside-resistant MRSA strains, genomic DNA analysis by PFGE allowed the distinction of eight different patterns after digestion with $Sma$ I and $Csp$ I restriction enzymes. Fifteen (83%) of 18 phenotype A MRSA strains were grouped into a single pulse type numbered I (figure 4). Digestion with $Csp$ I restriction endonuclease confirmed the homogeneity of this group (CS $\geq 0.95$) (data not shown). The three remaining phenotype A MRSA strains displayed other pulse types (II, III, and IV) distantly related to pulse type I, with a CS ranging from 0.3 to 0.5 and from 0.2 to 0.4 after digestion with $Sma$ I and $Csp$ I, respectively. Only one of the eight phenotype B MRSA strains shared pulse type I, whereas the seven remaining phenotype B strains were distantly related (CS, from 0.5 to 0.7 with use of the $Sma$ I restriction enzyme and from 0.2 to 0.4 with the $Csp$ I restriction enzyme) and diverse (figure 4). The patterns of these strains were as distant from pulse type I as was the unique pulse type, designated as V, of the seven aminoglycoside-resistant MRSA strains (CS = 0.2 with use of the $Sma$ I and $Csp$ I restriction enzymes) (data not shown). This latter profile was identical to that of the aminoglycoside-resistant MRSA strain KB5, considered as a representative of the endemic clone that was homogeneously resistant to methicillin.

The PCR amplifications of the 3' ends of the $spa$ gene and of the $coa$ gene were intended to differentiate the strains on the basis of the size of PCR products and of the patterns restriction digest with $Rsa$ I and $Hae$ III, respectively.

PCR amplification of the $spa$ gene of 26 heterogeneous MRSA strains (18 strains of phenotype A and eight strains of phenotype B) yielded a single fragment equal to 523 bp for 24 strains or different in size for two strains. The PCR products were cleaved in two $Rsa$ I fragments of 214 bp and 19 bp and in a third $Rsa$ I fragment, the size of which differed according to the number of 24-bp repeats. Five $Rsa$ I patterns could be distinguished (patterns PI to PV). Twenty two of 26 strains were clustered into the major pattern PI (19 bp, 214 bp, and 291 bp), including 16 of 18 strains with phenotype A and six of eight strains with phenotype B. Thus, this technique appeared less discriminative when we compared it with PFGE. The homogeneous aminoglycoside-resistant MRSA displayed a different pattern PVI (19 bp, 214 bp, and 339 bp).

For 23 of 26 heterogeneous MRSA strains (15 phenotype A and eight phenotype B strains) and the eight aminoglycoside-resistant MRSA strains, PCR amplification of the $coa$ gene yielded a 402-bp PCR product, which, after digestion with $Hae$ III, generated three fragments of 176 bp, 146 bp, and 81 bp composing the profile called CI. The three remaining heterogeneous MRSA strains, which displayed phenotype A and pulse types II, III, and IV, had a PCR product of 300 bp (one strain) or >402 bp (two
strains). Their restriction patterns also differed from Cl. This PCR technique had the lower discriminatory power.

Antibiotic consumption. Aminoglycoside consumption in our hospital from 1979 to 1995 is shown in figure 5. The major change was in the consumption of amikacin, which doubled between 1983 and 1987, whereas that of gentamicin and sisomycin was markedly reduced. We also observed a remarkable decrease in consumption of macrolides. In particular, prescriptions for erythromycin per year decreased from 32 kg in 1986 to 7 kg in 1991 and 3.5 kg in 1995 and were not replaced by prescriptions for other macrolides.

Discussion

The evolution of MRSA isolates toward multiple resistance seemed to be unrelenting in our hospital during the past years. In particular, gentamicin resistance, which was due to the synthesis of a bifunctional 2′ aminoglycoside phosphotransferase-6′ aminoglycoside acetyltransferase in the first strains isolated in our hospital in 1975 [18], was expressed by nearly all strains isolated from 1977 to 1992. The multiple resistance to aminoglycosides, macrolides, and quinolones became increasingly common between 1985 and 1992. The high prevalence of this resistance phenotype appeared to be related mainly to the spread of a clonal phage type 77 strain, which has also become endemic in other French hospitals [19]. The reemergence of heterogeneous MRSA phenotypes that are susceptible to gentamicin and other antibiotics was therefore surprising.

Phage typing also showed the changes in staphylococcal strains, although the high number of nontypeable strains rendered the method less useful, as has been shown in previous studies [2, 20, 21]. PFGE confirmed the clonality of phenotype A strains but demonstrated that the phenotype B strains were more diverse than initially believed on the basis of susceptibil-
increase in gentamicin resistance in 1975–1980 was also ob-

changes in the use of aminoglycosides. An analysis of the spread of the epidemic strains could help define the current relative diversity of MRSA strains in our hospital. Under these conditions, a reduced selective pressure by gentamicin could make possible the spread of gentamicin-susceptible MRSA. However, we cannot exclude the possibility that this evolution reflects the natural history of the MRSA strains in our hospital. Similar changes were reported at a hospital in Cincinnati, where an epidemic caused by a gentamicin- and tetracycline-resistant MRSA strain began in 1977 and persisted until 1981 [27]. Although the opening of a new burn unit correlated with the resolution of the epidemic, the MRSA clone was replaced by multiple tetracycline-susceptible MRSA strains that became the major problem at the hospital. The authors [27] suggested that the diversity of MRSA strains in the hospital might have resulted from increased transmissions in the community, followed by introduction of the strains in the hospital. However, these investigators did not analyze the use of antibiotics in their hospital.

The overall analysis of the typing results of recent MRSA strains showed that the endemic aminoglycoside-resistant MRSA strain was still present in our institution but was gradually replaced by various heterogeneous MRSA strains, including a new epidemic clone. Frénay et al. [9] have recently suggested that the number of 24-bp repeats in the 3’ variable region of the spa gene could be related to the epidemic character of MRSA strains. This C-terminus region is required for cell-wall attachment. A longer region could allow a better exposition of the Fc-binding region of the protein A at the cell surface, thereby facilitating adhesion to the skin and dissemination. We could not confirm this hypothesis since the number of repeats (n = 10) in the 26 heterogeneous MRSA strains studied was the same, whether the strains were epidemic or not. The endemic aminoglycoside-resistant MRSA displayed a larger number of repeats.

A reason for the emergence of gentamicin-susceptible MRSA could be changes in the use of aminoglycosides. An increase in gentamicin resistance in 1975–1980 was also observed in other French hospitals and was attributed to the frequent use of gentamicin for treatment of severe nosocomial gram-negative infections that predominated during these years [25]. Since then, gentamicin consumption decreased progressively in our hospital, and this antibiotic was replaced for the most part by amikacin. However, it is difficult to conceive that these modifications, which occurred in 1983–1987, could account for the emergence of gentamicin-susceptible clones 10 years later. Emergence of gentamicin-susceptible MRSA has also been reported recently from other French hospitals [26], and it would be interesting to analyze the evolution of aminoglycoside consumption in these institutions.

Implementation of control measures, which likely resulted in reduced transmission of the predominant endemic organisms, combined with importation of unrelated strains from various institutions or from the community could explain the current relative diversity of MRSA strains in our hospital. Under these conditions, a reduced selective pressure by gentamicin could make possible the spread of gentamicin-susceptible MRSA. However, we cannot exclude the possibility that this evolution reflects the natural history of the MRSA strains in our hospital. Similar changes were reported at a hospital in Cincinnati, where an epidemic caused by a gentamicin- and tetracycline-resistant MRSA strain began in 1977 and persisted until 1981 [27]. Although the opening of a new burn unit correlated with the resolution of the epidemic, the MRSA clone was replaced by multiple tetracycline-susceptible MRSA strains that became the major problem at the hospital. The authors [27] suggested that the diversity of MRSA strains in the hospital might have resulted from increased transmissions in the community, followed by introduction of the strains in the hospital. However, these investigators did not analyze the use of antibiotics in their hospital.

An important issue is the control of MRSA in large hospitals, where the incidence of these organisms is already high and where resident MRSA strains have become established [28]. Our results reflected a complex epidemiological situation where the endemic-epidemic aminoglycoside-resistant MRSA strain persisted while new clones were introduced in the hospital that in turn became epidemic. In our recent experience, the control measures appeared to be only partially effective in decreasing the incidence of MRSA infections over 3 years. However, the infection control program had been implemented too recently in our hospital to draw any firm conclusion. The effectiveness of this program should be evaluated again in several years.

A major source of MRSA now is the importation of strains via healthy carriers or via infected patients who are readmitted or transferred from other institutions. These findings are similar to those reported in a recent study [29]. A more thorough analysis of the spread of the epidemic strains could help define more precisely the interrelation and exchanges between community and hospital strains.

References


