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Epidemiologic Typing and Delineation of Genetic Relatedness of Methicillin-Resistant *Staphylococcus aureus* by Macrorestriction Analysis of Genomic DNA by Using Pulsed-Field Gel Electrophoresis

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To evaluate the usefulness of phenotypic and genotypic analyses for the epidemiologic typing of methicillin-resistant *Staphylococcus aureus* (MRSA), we characterized 64 epidemic MRSA isolates and 10 sporadic methicillin-susceptible *S. aureus* isolates from a university hospital and 18 MRSA isolates from hospitals in different geographical areas. Chromosomal DNA macrorestriction analysis with *Sst*II was resolved by pulsed-field gel electrophoresis and compared with antibiotype analysis, phage type analysis, and standard genomic DNA restriction analysis with *Bgl*II. Indices of the discriminatory ability of these methods were 0.982, 0.959, 0.947, and 0.959, respectively. Macrorestriction patterns of 94% of MRSA isolates from patients, personnel, and the environment associated with a nosocomial outbreak were closely related (similarity coefficient, 85 to 100%). In contrast, methicillin-susceptible *S. aureus* isolates showed a marked diversity of macrorestriction patterns (median similarity, 41%). MRSA isolates from other geographical areas showed diverse macrorestriction patterns, with the exception of four isolates displaying identical or closely related patterns; these isolates were associated with concurrent outbreaks in four other Belgian hospitals. A concordance of genomic DNA macrorestriction typing with phenotypic methods was observed for 60 to 65% of MRSA isolates, and a concordance with standard DNA restriction analysis was found for 79 to 98% of these isolates. In conclusion, genomic DNA macrorestriction analysis was a useful complement to phenotypic methods for delineating epidemic isolates of MRSA, for identifying their nosocomial reservoirs, and for tracing their intra- and interhospital spread. The genetic relatedness of MRSA isolates, as estimated by this technique, appeared to correlate with their space-time clustering.

Endemic and epidemic nosocomial infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) represent an increasing problem in many parts of the world (3, 6, 25). Epidemic strains of MRSA are associated with excess morbidity and mortality; therefore, specific surveillance and control programs are required to target cost-effective control efforts (25). For identifying the sources and monitoring the spread of epidemic MRSA strains, a number of epidemiologic markers have been useful, including antibiotype, biotype, phage type, plasmid profile, structural protein or enzyme electropherotype, and restriction analyses of chromosomal DNA by classical electrophoresis, pulsed-field gel electrophoresis (PFGE), or Southern hybridization with various probes (6–9, 11, 12, 14, 15, 17–21). However, phenotypic systems have limitations in typing ability and stability, and many typing systems have a low discriminatory ability (7, 20). Among genotypic analysis techniques under active investigation, PFGE of macrorestriction fragments appears to be a highly sensitive method that may detect subtle genetic variations among phylogenetically and epidemiologically related isolates of various bacterial species, including *S. aureus* (1, 14, 21, 23).

We describe here a typing system for MRSA based on contour-clamped homogeneous electrical field (CHEF) analysis of genomic DNA macrorestriction fragments generated by low-frequency-cleaving endonucleases (1). This technique was compared with standard restriction analysis of total DNA, antibiotype analysis, and phage type analysis for the characterization of nosocomial reservoirs and patterns of transmission of epidemic MRSA strains in a university hospital. In addition, CHEF macrorestriction analysis was used to estimate the genetic relatedness of these epidemic strains with other epidemic MRSA strains associated with outbreaks in six Belgian hospitals during the past decade.

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MATERIALS AND METHODS

Bacterial isolates. Study isolates (n = 92) were of three origins: Erasme Hospital, other Belgian hospitals, and an international collection. Erasme Hospital isolates were collected by storage at −70°C in glycerol during an outbreak associated with over 300 MRSA infections occurring among patients admitted to 22 departments of the hospital during the period from November 1988 to May 1991. Blood isolates selected for study included MRSA isolates (n = 28) from every other consecutive episode of MRSA bacteremia during the period from November 1988 to May 1991. A control group of methicillin-susceptible *S. aureus* isolates (n = 10) was selected randomly from blood isolates recovered during the period from January 1988 to May 1990. MRSA isolates (n = 11) from patients' wounds and nasal swabs were collected in a cross-sectional fashion from patients in different wards

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during June and July 1990, at the peak of the epidemic. MRSA isolates were from 39 patients admitted to 13 departments, whereas methicillin-susceptible *S. aureus* isolates were from 10 patients in 5 departments. MRSA isolates from colonized hospital personnel included isolates from nasal carriers (n = 15) and hand carriers (n = 3) working in eight departments of the hospital. Environmental MRSA isolates (n = 7) were recovered from contaminated surfaces of the rooms occupied by MRSA-colonized patients in three departments.

Representative isolates (n = 8) from seven outbreaks in six other Belgian hospitals were selected as the predominant phage type(s) associated with each outbreak. Three of these outbreaks occurred between 1981 and 1984, and four occurred between 1990 and 1991. These isolates were maintained freeze-dried in the collection of the Institut Pasteur du Brabant, Brussels, Belgium. In addition, the following unrelated MRSA isolates (n = 10) of diverse geographical origins (8, 20) and collected between 1960 and 1987 were kindly provided by R. Marples from the collection of the Central Public Health Laboratory, London, England: E2, E03/M307, 85/2147, 85/5484, 84/9580, 85/4670, 85/1836, and 85/1774. Strains NCTC 10442 and NCTC 11940 were purchased from the National Collection of Type Cultures, Central Public Health Laboratory.

PFGE of macrorestriction fragments. For each culture, 5 ml of overnight growth in brain heart infusion broth was pelleted by centrifugation and washed with EET buffer [100 mM EDTA, 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 10 mM Tris hydrochloride (pH 8.0)]. Washed cells were resuspended in EET buffer and adjusted to a density of 10^9 CFU/ml by nephelometry. The bacterial suspension was mixed with an equal volume of low-melting-temperature agarose (InCert Agarose; FMC Bioproducts, Rockland, Maine), 2% (wt/vol) in EET buffer. After solidification, agarose plugs containing the bacteria were incubated in buffer containing 1 mg of lysostaphin per ml and 1 mg of lysozyme per ml for 18 h at 30°C. The plugs were incubated in EET buffer containing 1 mg of proteinase K per ml and 1% (wt/vol) sodium dodecyl sulfate for 48 h at 50°C. Protein digestion products were removed by washing of the agarose plugs two times in buffer containing 10 mM Tris hydrochloride (pH 8.0), 0.1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride for 1 h at 37°C and three times in Tris-EDTA for 1 h at 21°C. Macrorestriction of genomic DNA was carried out with plugs equilibrated in the appropriate restriction buffer for 30 min at 37°C by the addition of 150 μl of restriction buffer containing 50 U of *Sst*II (GIBCO-BRL, Ghent, Belgium) and incubation for 18 h at 37°C. The digestion reaction was stopped by the addition of 0.5 M EDTA.

The DNA restriction fragments were separated by PFGE in a CHEF-DR II system (Bio-Rad Laboratories, Nazareth, Belgium). Plugs containing the restricted DNA were inserted into 1% (wt/vol) agarose gels (molecular biology certified agarose; Bio-Rad). Electrophoresis was carried out at 200 V in a buffer containing 44.5 mM Tris hydrochloride (pH 8.0), 44.5 mM boric acid, and 1 mM EDTA with alternating pulses at a 120° angle in a 2- to 8-s pulse time gradient for 12 h and then a 10- to 15-s gradient for 12 h. A temperature of 14 to 16°C was maintained during the electrophoresis by buffer circulation through a counter current refrigerating coil connected to running cold water. A lambda DNA polymer (Bio-Rad) was used as a molecular size marker. The gels were stained with ethidium bromide, rinsed, and photographed under UV light.

Analysis of DNA relatedness. Dice coefficients of similarity [(number of shared fragments × 2 × 100)/(total number of fragments in the two samples)] were determined for each pair of isolates by visual comparison of macrorestriction patterns (12). Similarity coefficients were calculated as the mean of duplicate coefficients measured by two independent observers. Only gels showing reproducible mobilities of internal controls, including a lambda polymer and restricted DNA from a study strain of MRSA (EH-SAM 44), were included in intergel comparisons. Strains were clustered on the basis of their level of genetic similarity by the method of unweighted pair-group average linkage by use of a previously described program (16). The median genetic similarity values for groups of MRSA strains were compared by the Mann-Whitney test with the Stat-Pac Gold statistical package for an IBM-compatible microcomputer (Malonick, Minneapolis, Minn.).

REA. For restriction endonuclease analysis (REA) of total DNA, genomic DNA was extracted and prepared as described previously (11). Purified DNA was digested with *Bgl*II in accordance with the recommendations of the manufacturer (GIBCO-BRL). Restriction fragments were separated by electrophoresis at 50 V for 18 h in 0.7% agarose gels containing 0.5 μg of ethidium bromide per ml and photographed under UV light. A linear DNA ladder (GIBCO-BRL) was used as a molecular size marker.

Antibiotype. The susceptibilities of the isolates to the 20 antimicrobial agents listed in Table 1 were tested by a standard disk diffusion method with an inoculum of 1.5×10^8 CFU/ml adjusted by nephelometry, Mueller-Hinton II Agar Gold Label (Becton Dickinson, Erembodegem, Belgium), and Neo-Sensitabs tablets (Rosco, Taastrup, Denmark). Incubation was carried out for 24 h at 30°C for oxacillin testing and at 35°C for other antimicrobial agent testing. Isolates showing a resistant or an intermediate level of susceptibility to at least one agent were considered of an antibiotype distinct from that of isolates fully susceptible to this agent, except for isolates with intermediate zone sizes for rifampin, chloramphenicol, and teicoplanin, which were not considered different from isolates susceptible to these agents.

Phage type. Phage typing was performed at the routine test dilution (RTD) and 100 times the RTD with the International Basic Set at the Service de Lysotypie et de Génétique Bactérienne, Institut Pasteur du Brabant.

Determination of reproducibility and discriminatory ability. To assess the reproducibility of typing, we typed pairs of duplicate blood culture MRSA isolates from six patients in the same run. Reproducibility was defined as the percentage of pairs with concordant types. The index of discriminatory ability was calculated as previously described (13) on the basis of the type distribution among the 18 control MRSA strains from different hospitals. Because a single base mutation in the chromosomal DNA is sufficient to introduce a difference of three fragments in its restriction pattern, isolates with restriction patterns showing a one- to three-fragment difference were considered to belong to a common major restriction type.

RESULTS

PFGE of macrorestriction fragments. *S. aureus* DNA digested with *Sst*II produced 8 to 22 well resolved fragments of 20 to 500 kb (Fig. 1). Only fragments delimited by molecular weight markers (50 to 500 kb) were analyzed for the com-

TABLE 1. Distribution of MRSA isolates by macrorestriction type (PFGE), standard restriction type (REA), antibiotype (AB), phage type (PT), and geographical origin

Type				No. of isolates from:		
PFGE[a]	REA[b]	AB[c]	PT[d]	Erasme Hospital	Other Belgial hospitals	Other countries
1a	a1	PMAcEClGKC	I	24	2	0
			II	2	0	0
			IV	4	0	0
			V	1	0	0
		PMAcEClGKCCh	I	3	0	0
		PMAcEClGKCRfCh	IV	1	0	0
		PMAcEClGKCRfChSxt	I	1	0	0
		PMAcEClGKCMu	II	1	0	0
		PMAcEClGKChRf	I	1	0	0
			XII	1	0	0
			VI	1	0	0
			NT	1	0	0
	a2	PMAcEClGKCMu	XII	1	0	0
	a3	PMAcEClGKC	II	1	0	0
		PMAcEClGKCRf	II	1	0	0
		PMAcEClGKCCh	VII	1	0	0
	a4	PMAcEClGKC	VI	1	0	0
	c	PMAcEClGKCMu	I	1	0	0
1b	a1	PMAcEClGKC	VII	1	0	0
	a3	PMAcEClGKC	I	1	0	0
		PMAcEClGKCRf	I	1	0	0
1c	a1	PMAcEClGKC	VII	1	0	0
	a3	PMAcEClGKC	VII	3	0	0
		PMAcEClGKCRfMu	VII	1	0	0
		PMAcEClGKCRf	VII	1	0	0
1d	a1	PMAcEClGKC	I	1	0	0
		PMAcEClGKC	II	1	0	0
		PMAcEClGKCRf	I	2	0	0
1e	a5	PMAcEClGKC	VII	0	1	0
1f	a1	PMAcEGKC	IX	0	1	0
2	a4	PMAcEClGK	III	1	0	0
	b	PMAcEClGK	VIII	1	0	0
3	b	PMAcEClGK	VIII	1	0	0
5	d	PMAcEClGKCRf	III	1	0	0
6	e	PMAcEClGKCh	X	0	1	0
7	f	PMAcEGK	VIII	0	1	0
8	a6	PMAcEClGK	XV	0	1	0
9	g	PMAcEGKF	XIV	0	1	0
10a	h	PMAcEClGKChD	XIX	0	0	1
10b	h	PMEClSxt	XIX	0	0	1
11	i	PMAcEGK	XVI	0	0	1
12	j	PMAcEKCh	XVII	0	0	1
13	k	PMAcEClGKD	NT	0	0	1
14	l	PMAcEClGKChDMi	XVIII	0	0	1
15	m	PMAcEGK	NT	0	0	1
16	n	PMAcKD	XIII	0	0	1
17	o	PMAcEGKD	XI	0	0	1
18	p	PMAcEClGKDMi	NT	0	0	1

[a] Electropherotype of macrorestriction fragments obtained with *Sst*II.
[b] Standard restriction endonuclease electropherotype obtained with *Bgl*II.
[c] Antibiotype, expressed as the resistance profile observed with the following antimicrobial agents: penicillin (P), methicillin (M), amoxicillin-clavulanic acid (Ac), erythromycin (E), clindamycin (Cl), gentamicin (G), kanamycin (K), ciprofloxacin (C), rifampin (Rf), chloramphenicol (Ch), mupirocin (Mu), trimethoprim-sulfamethoxazole (Sxt), fosfomycin (F), doxycycline (D), minocycline (Mi), fusidic acid, vancomycin, teicoplanin, virginiamycin, and novobiocin.
[d] Determined at 100 times the RTD and coded as follows: NT, nontypeable; I, 47/54/75/77/84/85; II, 29/47/54/75/77/83A/84/85; III, 6/47/54/75/84/85; IV, 77/(84); V, 55; VI, 47/54/75; VII, 29/42E/47/53/54/75/77/83A/84/85/81; VIII, 6/42E/47/54/75/85/(81)/95; IX, 29/42E/47/53/54/75/77/84/85; X, 29/6/42E/47/53/54/75/77/83A/84/85/81/95; XI, 75/85; XII, 47/77; XIII, 42E/47/53/54/75/77/84/85/81; XIV, 6/47/53/54/75/77/84/85; XV, 6/47/53/54/75/85; XVI, 75/83A/85; XVII, 79/80/77/84/95; XVIII, 84; and XIX, 85. Parentheses denote variable reactions.

parison of banding patterns. The intragel reproducibility of banding patterns was 100% for six pairs of duplicate MRSA isolates. When a one-band difference was used to distinguish PFGE types, a total of 23 types were found among all MRSA isolates (Table 1) and the index of discriminatory ability was 0.982. Nine major types were found among Erasme Hospital and other Belgian hospital MRSA isolates, and another nine major types were found among MRSA isolates from other countries.

Analysis of DNA relatedness. Methicillin-susceptible *S. aureus* blood isolates from Erasme Hospital patients showed markedly distinct patterns (median similarity value, 41%; range, 17 to 90%; Fig. 1 and 2). In contrast, MRSA blood isolates from this patient population clustered in major type

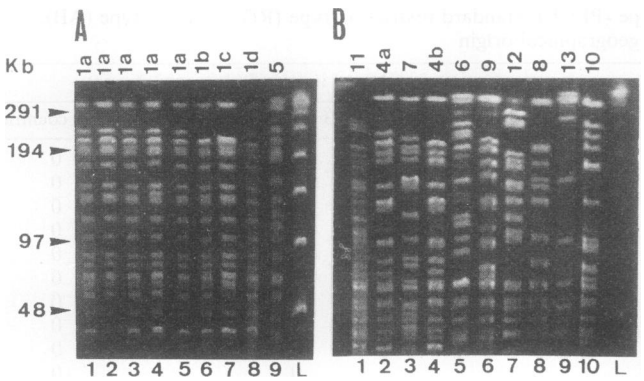

FIG. 1. PFGE separation of *Sst*II macrorestriction fragments of *S. aureus* genomic DNA. Fragments were from blood isolates associated with nosocomial sepsis in distinct patients at Erasme Hospital between 1988 and 1990. (A) MRSA isolates (lanes 1 to 9). (B) Methicillin-susceptible *S. aureus* isolates (lanes 1 to 10). Lanes L, molecular size marker (lambda concatemer). PFGE types are labeled as in Table 1.

1, with four variant patterns (1a through 1d). These variants differed by one to three fragments only and showed similarity values above 85% (Fig. 1 and 2). In addition, concordant epidemic DNA macrorestriction types were found among the isolates from infected patients, colonized hospital personnel, and contaminated environmental surfaces (Fig. 2). Three MRSA isolates from Erasme Hospital were of distinct major PFGE types: MRSA type 2 was recovered from the nares of a surgeon and a nurse in the same department during the same week, MRSA type 3 was isolated from a postoperative wound infection in a patient who had been transferred 2 weeks earlier from another hospital at which surgery had been performed, and MRSA type 5 was associated with catheter-related bacteremia in a trauma patient who had been admitted 10 days earlier after prehospitalization emergency care.

Macrorestriction analysis demonstrated that the type 1a MRSA isolates associated with the Erasme Hospital outbreak caused concurrent epidemics in hospitals B and C

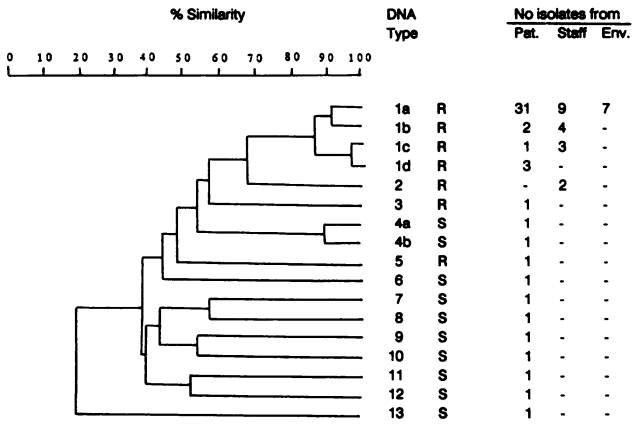

FIG. 2. Dendrogram of the percent similarity between macrorestriction (*Sst*II) DNA types of isolates of *S. aureus* from patients (Pat.), colonized hospital personnel (Staff), and the environment (Env.) of Erasme Hospital (1988 to 1991). Isolates are categorized on the basis of methicillin susceptibility (S) or resistance (R) and the source of isolation.

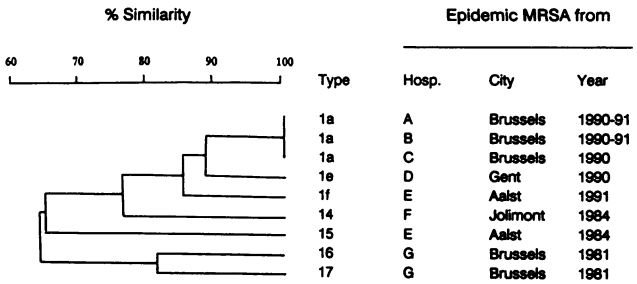

FIG. 3. Dendrogram of the percent similarity between macrorestriction (*Sst*II) DNA types found in MRSA isolates associated with outbreaks in Belgian hospitals (Hosp.) during the periods from 1981 to 1984 and 1990 to 1991. Hospital A is Erasme Hospital.

(Fig. 3 and 4). Transfers of staff and patients between these hospitals, which are located in Brussels and are affiliated with the same university, are common. Furthermore, type 1e and 1f epidemic MRSA isolates associated with outbreaks in hospitals D and E in northern Belgium during this period were 85 to 88% related to the type 1a isolates. The median similarity value for macrorestriction patterns, relative to type 1a, of Belgian MRSA isolates for the period from 1990 to 1991 was 94%, whereas that for the period from 1981 to 1984 was 64% ($P < 0.05$; Mann-Whitney test). The latter isolates were associated with outbreaks in hospitals located in southern, central, and northern Belgium.

REA. Restriction fragments of 2.5 to 6 kb were well resolved; therefore, only these fragments were compared to determine the REA types. Seven restriction patterns were found in MRSA isolates from Erasme Hospital (Table 1). However, 46 (72%) of these isolates belonged to predominant type a1. Another 14 (22%) isolates differed by one to three fragments only and were considered variants of the main type (a2 through a4) (Table 1). The reproducibility of REA was 100%, and its index of discriminatory ability was 0.959. There were seven major types among MRSA isolates from all Belgian hospitals and nine major types among MRSA isolates from other countries.

Antibiotype. Eight antibiotypes were found in the 64 MRSA isolates from Erasme Hospital (Table 1). The predominant type accounted for 41 (64%) isolates, and another 20 (31%) isolates differed by a single additional resistance. Among MRSA isolates of other origins, this predominant

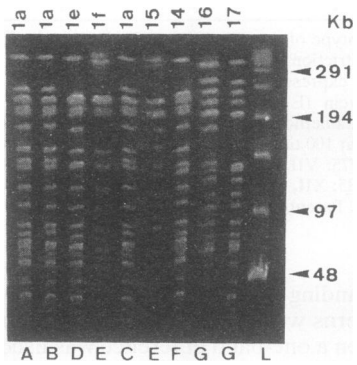

FIG. 4. PFGE separation of *Sst*II macrorestriction fragments of epidemic MRSA strains from the seven Belgian hospitals listed in Fig. 3. PFGE types and hospitals are labeled as shown in Fig. 3. Lane L, lambda concatemer.

antibiotype was found only in strains isolated in the same period from hospitals B and C in Brussels and from hospital D in Flanders. The antibiotype had an index of discriminatory power of 0.959 and a reproducibility of 83%.

Phage type. At the RTD, 46 (50%) MRSA isolates were typeable. Typeable isolates from Belgium were lysed by group III phages, particularly phages 77 and 84. At 100 times the RTD, 86 (93%) isolates were typeable and showed extended lytic patterns with group III phages and phage 29 (Table 1). MRSA isolates from Erasme Hospital were distributed in six to nine phage types on the basis of the working phage dilution. However, 35 (55%) of these isolates clustered in a predominant phage type that was also found in epidemic MRSA isolates from hospitals B and C in Brussels (Table 1). The reproducibilities of phage typing were 100% at 100 times the RTD and 34% at the RTD, owing to inconsistent typing at the latter dilution. The index of discriminatory power was 0.947 at 100 times the RTD.

Concordance between typing methods. In comparison with classification by macrorestriction analysis, concordant classification into minor types was observed for 65% of MRSA isolates by antibiotyping, 31 to 60% isolates by phage typing at the RTD and 100 times the RTD, respectively, and 79% by REA. The last technique showed a 98% concordance with macrorestriction analysis for the classification of MRSA into major restriction types, distinguished by more than three different fragments. When a single fragment difference was taken into account, MRSA isolates that belonged to the predominant major restriction type (type 1, as determined by macrorestriction analysis, and type a, as determined by REA) could be further subdivided into 11 types by the combined restriction profiles.

Additional profile variants detected by the combination of macrorestriction analysis and conventional REA were useful for distinguishing between relapse or a possible new infection or colonization episode, both in patients and in chronic carriers among patient care personnel. Isolates showing minor genotypic differences from the prototype epidemic isolates were not recovered more frequently in any particular time period during the course of the outbreak. Several of these genotypic variants were closely associated epidemiologically. MRSA genotype 1c/a3, for example, was recovered on the same day from the hands of two nurses in the same ward as well as from two patients in another ward over a 2-week period. Likewise, MRSA genotype 1d/a1 was isolated from the nares of two nurses in yet another ward over a 1-week period.

In contrast, antibiotype or phage type variants of the prototype isolates (genotype 1a/a1) showed no obvious epidemiologic relevance to this outbreak investigation. No space-time clustering could be observed, and these variants appeared randomly distributed across wards and weeks of study. Moreover, for seven environmental isolates, concordance of type with that of isolates from patients in these wards was noted in seven pairs by PFGE, five pairs by REA, six pairs by phage type, but only one pair by antibiotype.

DISCUSSION

MRSA strains tend to be similar in many phenotypic traits and appear to belong to an evolutionarily restricted subpopulation within the species *S. aureus* (6–8, 17, 18, 20, 21). As a result, discrimination between MRSA strains by classical typing methods, such as antibiotyping or phage typing, has often been difficult (6, 7, 18). The majority of MRSA strains worldwide have become resistant to multiple antibiotics, including beta-lactams, aminoglycosides, macrolides-lincosamides and, more recently, fluoroquinolones (3, 6, 11, 18). Similar resistance profiles were found for the Belgian MRSA strains examined in this study and a previous study (24). It is likely that these resistance characteristics provide MRSA strains with a selective advantage for nosocomial colonization and subsequent spread. Additional resistance, e.g., to chloramphenicol, occasionally has been useful in distinguishing between MRSA strains, but such a characteristic is often plasmid borne and therefore can prove to be unstable during the course of an epidemic (6, 7, 18). In this outbreak investigation, variations in antimicrobial resistance pattern from that of the prototype strains did not appear epidemiologically relevant when they were dissociated from observable genotypic variations. These findings suggest that in vivo or in vitro phenotypic instability may occasionally hamper reproducibility rather than increase the discriminatory power of these combined typing methods. Nevertheless, resistance typing showed a high discriminatory ability for strains of unrelated geographical origins and a good correlation with DNA fingerprinting, suggesting that it may be a useful primary typing tool for MRSA. In fact, only 13 of the 20 antimicrobial agents tested contributed to the overall discrimination, since all MRSA strains were resistant to beta-lactams and, conversely, all were susceptible to glycopeptides, novobiocin, fusidic acid, and virginiamycin.

Bacteriophage typing has been the reference epidemiologic typing method for *S. aureus* despite problems in reproducibility (7, 18). However, some MRSA strains are poorly typeable with the standard set of phages, and typeable strains tend to react with similar group III phages irrespective of their epidemiologic origins (6, 7, 18). In this study, the discriminatory ability of phage typing was biased towards an overestimate, since control strains were selected as the predominant phage types associated with distinct hospital outbreaks. Nevertheless, we found that phage typing at 100 times the RTD was a sensitive method that allowed discrimination between unrelated strains and confirmed the epidemic dissemination of predominant phage types among patients and personnel in our institution.

Molecular techniques have proved to be additional useful tools in recent epidemiologic studies of MRSA (4–9, 11, 14, 15, 17–21). Plasmid analysis has enabled tracing of hospital transmission of MRSA strains, although problems related to plasmid instability or to poor discrimination among the few conserved plasmids commonly found in MRSA are significant limitations of this marker system (7, 14, 15, 18). Additional discrimination may be achieved by REA of plasmid DNA (18). Multilocus enzyme electrophoresis has shown limited discriminatory ability for MRSA from French hospitals (4). Electrophoretic profiling of staphylococcal proteins resolved by direct staining or immunoblotting has displayed variable discriminatory capacity (8, 18). Strict standardization of the latter techniques is required to obtain reproducible results, and numerical analysis of densitometric profiles appears necessary to study the relatedness of MRSA strains determined by these complex electropherotypes (8). REA of genomic DNA can detect nucleotide sequence variations randomly distributed along the bacterial chromosome, and this approach has been found superior to conventional typing in studies of many bacterial species, including *S. aureus* (1, 6, 9, 10, 12, 14, 15, 18–21, 23). A problem arises with high-frequency-cleaving enzymes, because hundreds of restriction fragments are obtained, often leading to poorly resolved patterns in conventional electrophoresis. For overcoming the difficulty of comparing these

complex patterns, densitometric analysis has been proposed (2) or DNA fragments have been subjected to Southern hybridization with rRNA gene probes (9, 11, 20), random chromosomal DNA probes (19), or aminoglycoside resistance gene probes (17), the last showing superior discriminatory power.

PFGE can resolve the large DNA fragments obtained from chromosomal DNA digested with low-frequency-cleaving enzymes, thereby allowing macrorestriction analysis of genomic DNA of various bacterial species (1, 10, 23), including MRSA strains (9, 14, 21). Recent data suggest that this technique is the most discriminating of the currently available genotypic methods (1, 18, 23). Because it allows the detection of minor genomic rearrangements, recent evolutionary changes can be observed in isolates obtained serially from patients with chronic infections (10). The definition of clonality should therefore be a relative one that is empirically adapted to the discriminatory power of the analysis and to the time frame and population under study (1, 18). In the present investigation, we considered that single-enzyme DNA macrorestriction profiles differing by up to three fragments were more likely to represent genotypic variants of the same epidemic MRSA clone than a chance convergence of highly related chromosomal DNA sequences. Indeed, a single point mutation in the bacterial chromosome could introduce such a three-fragment difference in a restriction pattern. This difference corresponds to a profile similarity of 85% or higher. With this definition of clonality, macrorestriction analysis showed that 95% of clinical isolates and 92% of personnel and environmental isolates of MRSA recovered over the course of a 3-year outbreak in our hospital belonged to the epidemic clone. This finding prompted us to consider both carriers among hospital personnel and the contaminated environment as potential reservoirs of epidemic MRSA. A significant reduction in the nosocomial acquisition of MRSA was documented following mupirocin treatment of nasal carriers of the epidemic clone among patients and personnel (22), suggesting that this reservoir was indeed a source of MRSA transmission in our setting.

The present comparison of epidemiologic typing systems for MRSA indicated a good correlation between phenotypic and genotypic methods, with 60 to 65% concordance in the type classification of strains. Antibiotype and phage type variants were observed among MRSA strains belonging to a single genotype, as defined by both standard and macro DNA restriction analysis. These phenotypic subtypes within genotypes had no clear epidemiologic relevance in tracing transmission in the hospital, suggesting that they may reflect in vitro artifacts. Whether these phenotypic variants actually reflect changes in prophage or plasmid DNA, technical variability, or phenotypic instability should be clarified before this combination of phenotypic and genotypic techniques can be proposed to increase the discriminatory power of either standard or macro DNA restriction analysis, as previously suggested (12, 15, 21).

When a single fragment difference was used to distinguish types, standard genomic DNA restriction analysis with the enzyme *Bgl*II showed a 78% concordance with *Sst*II macrorestriction analysis of MRSA strains. When a clone was defined on the basis of a maximal divergence of three restriction fragments, an almost complete agreement was found between these genotypic techniques. However, subdivision of the epidemic MRSA clone into 11 genotypic variants was obtained by the combined restriction analyses. Because we compared low-molecular-weight *Bgl*II restriction fragments, it is possible that some variant patterns were related to a distinct plasmid content (2). In contrast, plasmid DNA does not appear to interfere with macrorestriction profiles (21). However, compared with macrorestriction analysis, standard restriction analysis had the advantage of being a more simple and rapid technique. We found it best suited for practical purposes, such as the epidemiologic classification of chronic MRSA carriers considered for systemic eradication therapy.

Macrorestriction analysis appeared more suitable for the determination of genetic relatedness among MRSA strains of various origins because of its well resolved, reproducible, easily compared restriction fragment patterns. Other techniques have been used to assess the relatedness of MRSA strains from diverse geographical areas. By sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of total protein, epidemic MRSA strains of British and Australian origins were found to cluster at similarity levels above 80% (8). By ribotyping, the same strains were found to cluster at similarity levels above 90% (20). In the present study, epidemic MRSA strains of Belgian and worldwide origins were well delineated by macrorestriction analysis. Similarity levels were as low as 62% for unrelated MRSA strains from Belgium and even lower for sporadic methicillin-susceptible *S. aureus* strains. Of particular interest was the observation that the genetic relatedness of MRSA strains, as approximated by visual analysis of macrorestriction patterns, appeared to correlate with their respective distance in place and time of isolation. If confirmed in larger studies, this observation would suggest that the measured similarity of the macrorestriction banding patterns is proportional to the conservation of the distribution of restriction sites along the chromosome and thus to sequence homology, despite potential interference by other factors, such as the restriction-modification system. Collaborative work is in progress to define better the degree of DNA relatedness among MRSA strains of worldwide origins by a variety of macrorestriction approaches and standardized gel analysis (5). Macrorestriction analysis also established that a "new clone" of MRSA was associated with a multihospital epidemic in Belgian hospitals. This finding has prompted a national survey to determine the extent of MRSA strain dissemination in this country and to explore potential links to epidemics in neighboring countries. Further standardization of macrorestriction techniques, including computerized pattern recognition analysis, should allow more accurate intergel and interlaboratory reproducibilities (5). This standardization is a prerequisite to transforming these techniques into a library typing system that may be suitable for the large-scale study of the epidemiology and evolution of MRSA.

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REFERENCES

1. **Arbeit, R. D., M. Arthur, R. D. Dunn, C. Kim, R. K. Selander, and R. Goldstein.** 1990. Resolution of recent evolutionary divergence among *Escherichia coli* from related lineages: the application of pulsed field electrophoresis to molecular epidemiology. J. Infect. Dis. **161**:230–235.

2. **Bialkowska-Hobrazanska, H., D. Jaskot, and O. Hammerberg.** 1990. Evaluation of restriction endonuclease fingerprinting of chromosomal DNA and plasmid profile analysis for characterization of multiresistant coagulase-negative staphylococci in bacteremic neonates. J. Clin. Microbiol. **28**:269–275.
3. **Boyce, J. M.** 1990. Increasing prevalence of methicillin-resistant *Staphylococcus aureus* in the United States. Infect. Control Hosp. Epidemiol. **11**:639–642.
4. **Branger, C., P. Goullet, A. Boutonnier, and J. M. Fournier.** 1990. Correlation between esterase electrophoretic types and capsular polysaccharide types 5 and 8 among methicillin-susceptible and methicillin-resistant strains of *Staphylococcus aureus*. J. Clin. Microbiol. **28**:150–151.
5. **Cookson, B., P. Aparicio, A. Deplano, R. Goering, R. Marples, and M. J. Struelens.** 1992. An inter-laboratory comparison of pulsing techniques for typing MRSA, abstr. 67. Abstr. VIIth Int. Symp. Staphylococci Staphylococcal Infections.
6. **Cookson, B., and I. Phillips.** 1988. Epidemic methicillin-resistant *Staphylococcus aureus*. J. Antimicrob. Chemother. **21**(Suppl. C):57–65.
7. **Cookson, B., H. Talsania, J. Naidoo, and I. Phillips.** 1986. Strategies for typing and properties of epidemic methicillin-resistant *Staphylococcus aureus*. Eur. J. Clin. Microbiol. **5**:702–709.
8. **Costas, M., B. D. Cookson, H. G. Talsania, and R. J. Owen.** 1989. Numerical analysis of electrophoretic protein patterns of methicillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. **27**:2574–2581.
9. **Goering, R. V., and T. D. Duensing.** 1990. Rapid field inversion gel electrophoresis in combination with an rRNA gene probe in the epidemiological evaluation of staphylococci. J. Clin. Microbiol. **28**:426–429.
10. **Grothues, D., V. Koopmann, H. van der Horst, and B. Tümmler.** 1988. Genome fingerprinting of *Pseudomonas aeruginosa* indicates colonization of cystic fibrosis siblings with closely related strains. J. Clin. Microbiol. **26**:1973–1977.
11. **Hadorn, K., W. Lenz, F. H. Kayser, I. Shalit, and C. Krasemann.** 1990. Use of a ribosomal RNA gene probe for the epidemiological study of methicillin and ciprofloxacin resistant *Staphylococcus aureus*. Eur. J. Clin. Microbiol. Infect. Dis. **9**:649–653.
12. **Hall, L. M. C., J. Z. Jordens, and F. Wang.** 1989. Methicillin-resistant *Staphylococcus aureus* from China characterized by digestion of total DNA with restriction enzymes. Epidemiol. Infect. **103**:183–192.
13. **Hunter, P. R.** 1990. Reproducibility and indices of discriminatory power of microbial typing methods. J. Clin. Microbiol. **28**:1903–1905.
14. **Ichiyama, S., M. Ohta, K. Shimokata, N. Kato, and J. Takeuchi.** 1991. Genomic DNA fingerprinting by pulsed-field gel electrophoresis as an epidemiological marker for study of nosocomial infections caused by methicillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. **29**:2690–2695.
15. **Jordens, J. Z., and L. M. C. Hall.** 1988. Characterization of methicillin-resistant *Staphylococcus aureus* isolates by restriction endonuclease digestion of chromosomal DNA. J. Med. Microbiol. **27**:117–123.
16. **Li, W. H.** 1981. Simple method for constructing phylogenetic trees from distance matrices. Proc. Natl. Acad. Sci. USA **78**:1085–1089.
17. **Monzon-Moreno, C., S. Aubert, A. Morvan, and N. El Solh.** 1991. Usefulness of three probes in typing isolates of methicillin-resistant *Staphylococcus aureus* (MRSA). J. Med. Microbiol. **35**:80–88.
18. **Mulligan, M. E., and R. D. Arbeit.** 1991. Epidemiologic and clinical utility of typing systems for differentiating among strains of methicillin-resistant *Staphylococcus aureus*. Infect. Control Hosp. Epidemiol. **12**:20–28.
19. **Mulvey, M., J. P. Arbuthnott, and C. Coleman.** 1986. Molecular typing of methicillin and gentamicin resistant *Staphylococcus aureus* in Dublin. Eur. J. Clin. Microbiol. **5**:719–725.
20. **Preheim, L., D. Pitcher, R. Owen, and B. Cookson.** 1991. Typing of methicillin resistant and susceptible *Staphylococcus aureus* strains by ribosomal RNA gene restriction patterns using a biotinylated probe. Eur. J. Clin. Microbiol. Infect. Dis. **10**:428–436.
21. **Prévost, G., B. Pottecher, M. Dahlet, M. Bientz, J. M. Mantz, and Y. Piemont.** 1991. Pulsed field gel electrophoresis as a new epidemiological tool for monitoring methicillin-resistant *Staphylococcus aureus* in an intensive care unit. J. Hosp. Infect. **17**:255–269.
22. **Struelens, M. J., A. Maas, F. Rost, A. Deplano, and E. Serruys.** 1991. Efficacy of mupirocin treatment of carriers in preventing nosocomial transmission of methicillin-resistant *Staphylococcus aureus*. Program Abstr. 31st Intersci. Conf. Antimicrob. Agents Chemother., abstr. 32.
23. **Struelens, M. J., N. Maes, F. Rost, A. Deplano, F. Jacobs, C. Liesnard, N. Bornstein, F. Grimont, S. Lauwers, M. P. McIntyre, and E. Serruys.** 1992. Genotypic and phenotypic methods for the investigation of a nosocomial *Legionella pneumophila* outbreak and efficacy of control measures. J. Infect. Dis. **166**:22–30.
24. **Van der Auwera, P., C. Godard, C. Denis, S. De Maeyer, and R. Vanhoof.** 1990. In vitro activities of new antimicrobial agents against multiresistant *Staphylococcus aureus* isolated from septicemic patients during a Belgian national survey from 1983 to 1985. Antimicrob. Agents Chemother. **34**:2260–2262.
25. **Wenzel, R. P., M. D. Nettleman, R. N. Jones, and M. A. Pfaller.** 1991. Methicillin-resistant *Staphylococcus aureus*: implications for the 1990s and effective control measures. Am. J. Med. **91**(Suppl. 3B):221S–227S.