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Diversity and Stability of Restriction Enzyme Profiles of Plasmid DNA from Methicillin-Resistant *Staphylococcus aureus*

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Nosocomial infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) are a significant epidemiological problem. Detecting the sources of epidemic strains and preventing their access to patients, however, depend upon the availability of techniques to reliably distinguish among MRSA strains. We evaluated restriction enzyme analysis of plasmid DNA for use as an epidemiological marker of MRSA strains. The diversity of plasmid types was assessed by examining 120 clinical and environmental MRSA isolates from five southern California hospitals and from the American Type Culture Collection. Thirty-seven distinctive *Eco*RI digestion patterns were observed. We characterized each strain by the number of plasmids it contained and the sizes of the fragments that were generated by *Eco*RI. Very few of the isolates (4.2%) lacked plasmids, and some (6.7%) contained DNA that was not digested by *Eco*RI. Several isolates (12.5%) contained two or more plasmids. We were able to assess the stability of MRSA plasmid types by tracking epidemic strains over a 2-year period. We also examined successive isolates from 10 individual patients during their hospitalization. In all but one case, the patient's plasmid profiles remained unchanged. We conclude that the diversity and stability of MRSA plasmid types make them excellent epidemiological markers. In support of this conclusion, we found that our data provided significant epidemiological insights. Two epidemic strains, accounting for more than half of the infections, were identified in the five hospitals. The remaining cases were sporadic, caused by MRSA strains that appeared very infrequently and that may have originated from sources outside the hospitals.

There has been a steady increase in the incidence of nosocomial infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) in recent years (2, 17). These infections complicate the treatment of patients, prolong hospitalization, increase the cost of medical care, and are sometimes life threatening (10). Reducing the number of MRSA infections by detecting and eradicating the sources of the organisms or by interrupting their path to the patient are important goals. Since MRSA often colonize hospital personnel (2, 5, 12), attaining these goals depends, in turn, upon techniques for characterizing epidemic MRSA and distinguishing them from resident strains.

Antibiograms and phage typing are commonly used to characterize MRSA in hospital epidemiologic studies. However, antibiograms are frequently inadequate to accomplish the differentiation, and phage typing, which requires an inventory of bacteriophages unavailable at most hospitals, is often uninformative or too slow for epidemiological work (1, 10). Furthermore, recent reports indicate that plasmid analysis is more useful and reproducible than phage typing, antibiograms, or detection of aminoglycoside-inactivating enzymes in identifying both methicillin-susceptible *S. aureus* and MRSA and in defining outbreaks (1, 6, 8, 13, 15). Consequently, plasmid DNA analysis has emerged as a tool that appears to be valuable in tracing MRSA strains in nosocomial outbreaks.

In this study, we extended the evaluation of plasmid DNA analysis by characterizing a large number of MRSA strains isolated in hospital settings. Our primary aim was to determine whether the number of distinctive plasmids or plasmid combinations is sufficiently large to distinguish between isolates from many apparently independent infections. In addition, we wished to characterize the plasmids systematically and to report the data in a form that would permit other workers to recognize the same plasmids.

Our preliminary work and the reports of others (7) showed that intact plasmids from many strains of this species may differ only slightly in their electrophoretic mobilities. Therefore, to attain our goals we used restriction endonuclease digestion of the plasmid DNA to increase discrimination between molecules of similar size (16).

To obtain a broad representation of MRSA plasmid types, we examined 90 isolates from Loma Linda University Medical Center (LLUMC) and compared the restriction patterns of their plasmids with those of 53 samples from other sources, including four Los Angeles-Riverside area hospitals.

(A preliminary report of this work was presented previously [I. Roy, A. Zuccarelli, G. Harding, and J. Couperus, Program Abstr. 28th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 985, 1988].)

MATERIALS AND METHODS

MRSA strains. The numbers of MRSA strains analyzed from each source are listed in Table 1. All of the strains, except those obtained from the American Type Culture Collection and those identified as "environmental," were isolated from patient specimens. LLUMC environmental samples were isolated from inanimate objects in hospital rooms occupied by patients with MRSA infections and before cleaning the rooms after the patients had been discharged. LLUMC clinical strains were obtained during two MRSA outbreaks, one occurring in 1986 and a second occurring between September 1987 and June 1988. Strains from all sources were tested for coagulase reaction and methicillin and oxacillin susceptibilities before their use in the study. The antibiotic susceptibilities were determined by the Kirby-Bauer disk diffusion method (3). LLUMC strains

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TABLE 1. Sources of MRSA isolates

Source[a]	No. of isolates
ATCC	6
LLUMC	
1987–1988	68[b]
1986	6
Environmental	16
Hospital B	10
Hospital C	8
Hospital D	11
Hospital E	18

[a] Hospitals B and C are 0.5 and 10 miles (1 mile equals ca. 1.6 km) from LLUMC, respectively. The MRSA populations at LLUMC and hospitals B and C are probably not independent of each other since these institutions regularly exchange personnel and patients. MRSA outbreaks were occurring at LLUMC and hospital C when the samples were isolated. Hospitals D and E are about 70 and 80 miles from LLUMC, respectively, and they are not known to have personnel or patients in common with LLUMC.

[b] This number includes repeated isolations (37 samples in all) from 10 patients.

were also tested for latex agglutination (Staphaurex; Wellcome Diagnostics, Dartford, England).

Plasmid isolation. Plasmid DNA was isolated from the cells by a modification of the alkaline lysis method (4). Cells were grown in 30 ml of L broth (9) overnight in an incubated shaker at 37°C, harvested, and washed once with 1.0 ml of TE buffer (10 mM Tris [pH 7.5], 0.1 mM disodium EDTA). All subsequent manipulations were performed in 2.0-ml microcentrifuge tubes. The cells were suspended in 0.2 ml of TE buffer containing 50 μg of lysostaphin (Sigma Chemical Co., St. Louis, Mo.) per ml and incubated for 30 min at 37°C. To this, 0.4 ml of 0.2 M NaOH–1% (wt/vol) sodium dodecyl sulfate was added. After 10 min on ice, 0.3 ml of 3 M potassium–5 M acetate was added, and the lysate was cooled for another 10 min on ice. The mixture was centrifuged at 12,000× *g* for 10 min at 0°C, and the supernatant was extracted once with buffer-saturated phenol and twice with ether. The preparation was incubated with 2 μl of heat-treated pancreatic RNase A (10 mg/ml; Worthington Diagnostics, Freehold, N.J.) for 15 min at 37°C. After an extraction with 1.0 ml of phenol-chloroform (1:1), the DNA was precipitated with 3 volumes of ethanol. The precipitate was collected by centrifugation, washed once with cold 70% (vol/vol) ethanol, desiccated under vacuum, and dissolved in 40 μl of *Eco*RI buffer (New England BioLabs, Beverly, Mass.).

Restriction enzyme digestion. Half of each plasmid preparation was digested with 10 U of *Eco*RI or *Hin*dIII (New England BioLabs) for 3 h at 37°C. Digested and undigested samples were applied to adjacent wells on horizontal, 10- by 15-cm, 0.8% (wt/vol) agarose gels (GTG agarose; FMC Corp., Rockland, Mass.) in TBE buffer (89 mM Tris hydroxide, 89 mM boric acid [pH 8.3], 2 mM EDTA). DNA markers were applied to other wells in each gel: 1-kilobase (kb) DNA ladder, *Hin*dIII digest of lambda phage DNA, and supercoiled plasmids (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Electrophoresis was at 120 V for 3 to 4 h at room temperature. Gels were stained for 30 min with 0.5 μg of ethidium bromide per ml in TBE buffer and photographed on a 302-nm-wavelength transilluminator onto Polaroid type 665 film through an orange filter. The negatives were projected to prepare ×10 enlargements, from which measurements were made. For each gel, the Curve Fitter-PC program (Interactive Microware, Inc., State College, Pa.), running on an IBM-XT computer, was used to construct a standard curve relating the electrophoretic mobilities of the marker molecules to the logarithm of their size and to estimate the sizes of MRSA plasmid fragments from the standard curve.

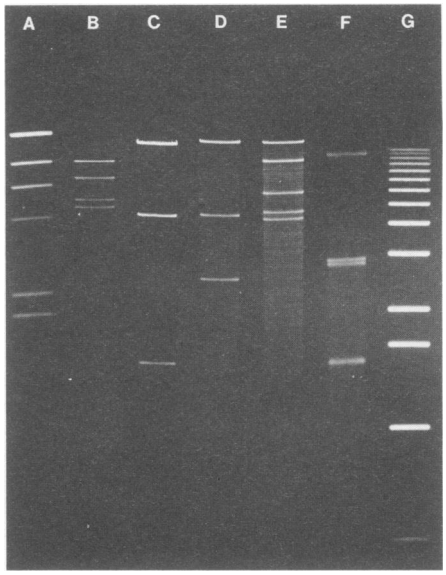


FIG. 1. Gel electrophoresis of plasmid DNA from five MRSA strains after digestion with *Eco*RI. Lanes a and g contain marker DNA fragments (*Hin*dIII digest of phage λ DNA and 1-kb ladder, respectively). Lanes b through f show the plasmid digestion patterns of isolates exhibiting profiles 1, 2, 3, 10, and 24, respectively.

RESULTS

We observed 37 different *Eco*RI fragment profiles of plasmid DNA in 120 independently isolated MRSA strains. Figure 1 shows five patterns of fragments obtained from MRSA strains after *Eco*RI digestion and electrophoresis.

The number of independent isolates that displayed each digestion profile and the estimated sizes of the *Eco*RI fragments are summarized in Table 2. Most of the isolates (89.1%) were assigned to one of the 37 profiles on the basis of the sizes of fragments that appeared after enzyme digestion. A few strains (4.2%) contained no plasmid DNA (profile 0). Eight strains (6.7%), each containing one plasmid, were arbitrarily placed in a single group (profile 50) because their DNA was not digested by *Eco*RI. Judging from slight differences in the mobilities of their intact plasmids, the individuals in this group may include at least four different plasmid types. It is likely that these strains could be reliably distinguished from each other by digesting their plasmid DNA with a different restriction endonuclease.

The assignments of individual isolates to particular fragment profiles were initially verified by direct comparison. Strains analyzed on different gels that appeared to produce similar fragment patterns were run together on a single gel to clarify their relationships. Selected representatives of profile 1, originally detected in preparations run on several different gels, were subsequently examined together after digestion with *Eco*RI or *Hin*dIII. This comparison confirmed their classification. At a later stage, the Curve Fitter-PC program was used to generate a representation of all the fragment patterns as they might appear after electrophoresis on a single gel (Fig. 2). As the number of different patterns

TABLE 2. Digestion profiles of MRSA plasmids

Fragment profile	No. of isolates[a]	Sizes of *Eco*RI fragments (kb)
0	5	No plasmids
1[b]	54	10.0, 7.6, 5.6, 5.0
2	9	18.2, 4.8, 1.58
3	1	18.0, 4.8, 2.8
4	1	13.3, 10.3, 4.6, 3.2, 2.6, 1.40, 0.96
5	1	12.5, 11.3, 9.0, 6.4, 5.0, 1.48
6	1	10.9, 9.6, 9.2, 6.1, 3.4, 3.0, 1.85, 1.46, 1.29
7	1	10.5, 9.2, 8.0, 6.1, 5.0, 2.9, 2.4, 1.47
8	1	7.0, 4.8, 4.3, 3.6, 2.9, 2.5, 1.6, 0.85
9	1	10.4, 6.1, 4.8, 2.3
10	1	20.3, 10.8, 6.6, 5.1, 4.7
11	1	4.1, 1.67
12	1	11.9, 7.5, 5.5, 5.3
14	1	21.9, 15.6, 8.9, 5.4, 4.9, 3.0, 2.2, 1.63, 1.56, 1.03
15	3	20.3, 11.8, 5.2, 3.0
16	1	11.3, 7.3, 3.0, 2.6, 1.45
18	3	12.5, 2.9
20	1	( )[c], 11.0, 8.7, 2.8, 1.49
21	1	7.8, 1.61
22	1	24.0, 10.5, 2.8
23	1	16.7, 8.8, 3.6, 2.4
24	1	11.0, 2.9, 2.8, 1.48
25	1	13.7, 11.0, 9.7, 5.6, 4.6, 3.3, 3.1, 2.1, 1.56, 1.47, 1.30, 1.19, 1.00
26	1	( ), 20.5, 6.3, 4.5, 3.5, 3.4, 3.1, 2.1, 1.87, 1.66, 1.40, 1.03, 0.86
27	1	9.0, 6.2, 2.2, 1.46
28	3	9.8, 7.2
32	1	10.9, 7.0, 2.6, 2.3, 1.56
33	1	11.4, 8.6, 7.0, 4.6, 2.7, 2.4, 1.19
35	1	12.4, 8.0, 5.4, 3.9, 3.1, 2.5, 1.51, 0.83
36	1	( ), 8.6, 5.6, 2.6, 2.0
37	1	17.2, 5.2, 1.95
38	1	9.5, 8.5
39	1	30.0, 24.1, 21.1, 17.8, 9.8, 6.7, 5.2, 2.3
41	1	( ), 10.8, 8.9, 7.8, 6.4, 2.8, 2.4
44	4	17.3, 8.9, 3.1, 1.51
46	1	3.0, 1.43
47	1	9.9, 5.0, 3.2
48	1	17.0, 9.8, 2.9
50	8	Undigested plasmids

[a] Repeated isolations from individual patients are generally excluded from this table because they do not represent independent isolations. Only the first sample from each patient is recorded, except for cases in which the plasmid characteristics of MRSA from a patient changed.

[b] The *Hin*dIII fragments of this plasmid are 9.9, 8.6, 7.6, and 1.80 kb.

[c] ( ), Presence of a band whose size could not be estimated readily because it had lower mobility than any of the markers fragments.

increased, we found that the classification of new isolates was facilitated by visually comparing a standardized computer representation of the new pattern with those observed earlier.

Fragment size estimates. The reliability of the fragment size estimates was 5% of the values, up to 15 kb. This appraisal was derived from the standard deviations of the sizes of fragments in patterns that were estimated repeatedly (e.g., for plasmid profile 1: 10.00 ± 0.52, 7.56 ± 0.39, 5.56 ± 0.20, and 4.99 ± 0.15 kb [$n = 52$]). Above 15 kb the estimates were reliable within ±15% of the value. The sizes of a few large fragments could not be estimated readily, because they migrated more slowly than any of the molecules in our DNA marker sets. Fragments smaller than 1.0 kb were not reliably detected.

Multiple plasmids. More than one plasmid species was detected in some isolates. The number of different types of plasmid molecules in each MRSA strain was assessed from the bands observed after 10 μl of extracted DNA was run on an agarose gel without *Eco*RI digestion. These gels were often difficult to interpret, because single plasmid species frequently appeared in several topoisomeric forms, each having a different mobility and producing a distinct band. In later analyses this problem was avoided by exposing the intact plasmid DNA to 40 U of S1 nuclease (Sigma) for 3 h at 37°C to convert all of the molecules to full-length linear duplexes before electrophoresis (18).

The members of 23 of the 39 profile categories defined in Table 2 and 87% of the individual isolates contained only one plasmid each (Table 3). Although the occurrence of multiple plasmids was low (9.2%) among clinical MRSA isolates, 33% of the ATCC cultures and 25% of the environmental samples contained more than one plasmid.

Repeated isolations. Multiple samples (a total of 37) were taken from 10 LLUMC patients at various times during their hospitalization. In nine of the patients, the digestion profile of successive isolates remained constant. However, the MRSA strains obtained at various times from one patient displayed three distinctly different digestion patterns (profiles 38, 12, and 1, in succession; Table 2). Each strain differed from the next in one antibiotic susceptibility (profile 38: cefamandole resistant, chloramphenicol susceptible; profile 12, cefamandole susceptible, chloramphenicol susceptible; profile 1, cefamandole susceptible, chloramphenicol resistant). Their responses to eight other antibiotics were identical (susceptible to gentamicin, imipenem, rifampin, novobiocin, and cephalothin; resistant to tobramycin, erythromycin, and amikacin). The present data are insufficient to determine whether the patient was infected successively with different MRSA strains or whether the plasmid of a single strain experienced two successive reorganizations during the sampling period.

Digestion profiles observed at different hospitals. Figure 3 displays the relative occurrences of various digestion profiles in each of the five hospitals. The MRSA strain that produced digestion profile 1 occurred in all of the hospitals that were sampled, and it was the most common isolate in four of them. Only one other plasmid (profile 2) appeared at more than one location. All of the other profiles were restricted to individual hospitals. The frequency with which particular plasmids appeared was distinctive for individual sites. The occurrence of profiles 1 and 2 at LLUMC (62.2 and 4.4%, respectively), for instance, was significantly different from their appearance at hospital E (22.2 and 38.9%, respectively) ($\chi^2 = 14.84$, df = 2, $P = 0.0006$).

DISCUSSION

The potential usefulness of plasmid digestion profiles to bacterial epidemiology depends upon the degree to which four requirements are satisfied. We have provided data to evaluate several of these factors which were not specifically addressed in earlier work. (i) The bacterial strains under study must contain plasmids. (ii) Plasmids must be sufficiently diverse so that independent isolates, not recently derived from a common progenitor, are likely to carry distinctly different plasmid DNA. (iii) Differences between plasmids must be distinguishable by the fragments that appear after restriction endonuclease digestion. Since type 2 restriction enzymes sample, in effect, the nucleotide sequence by the specificity of their cleavage, this requirement

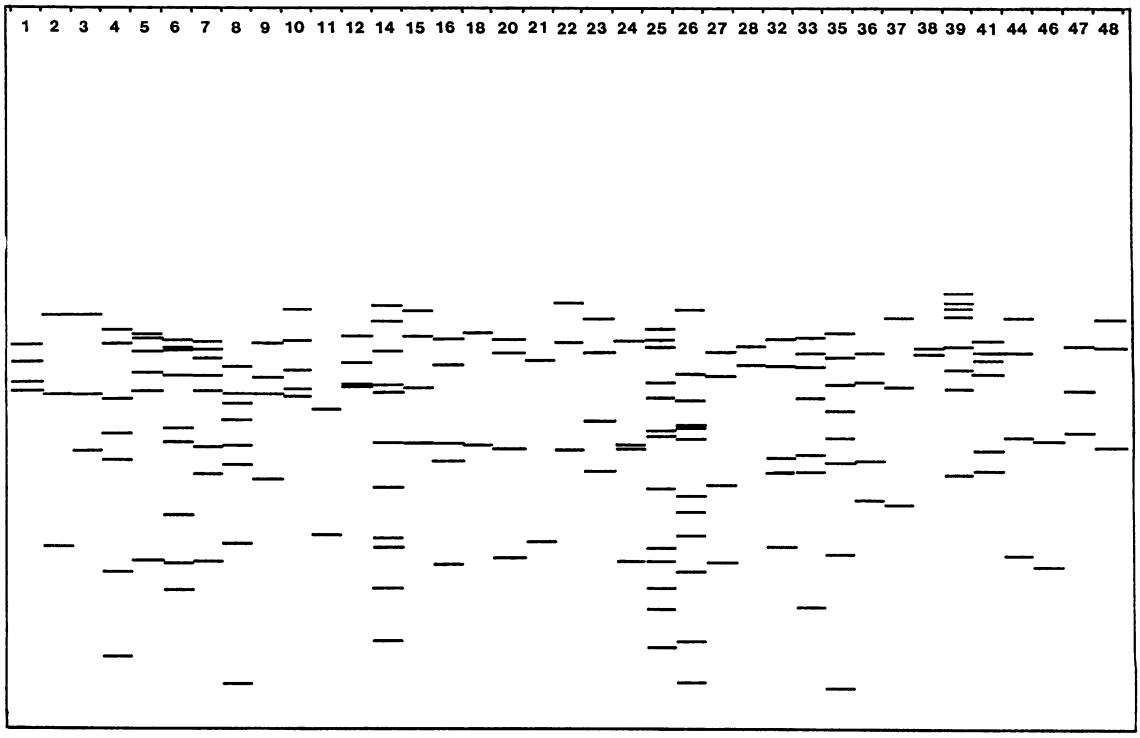


FIG. 2. DNA fragments of the 37 *Eco*RI profiles listed in Table 2 represented schematically as they might appear after electrophoresis on a single gel as described in Materials and Methods. The position of each fragment was calculated from its size by using the equation of a standard curve from a typical electrophoretic gel. The curve, relating gel mobility to the logarithm of fragment size (kilobases), took the form of a fourth-degree polynomial with the following coefficients (over the range of 0.53 to 47 kb): *a*, 10.08197; *b*, -1.295659; *c*, 6.628919×10^{-2}; *d*, -1.554084×10^{-3}; *e*, 1.360895×10^{-5}.

can be satisfied by the judicious selection of enzymes. (iv) Methods for plasmid profiling must be rapid, reproducible, inexpensive, and accessible to clinical laboratories.

In agreement with earlier work (11), we found that very few (4.2%) of the MRSA strains lacked plasmids entirely. This observation assures the wide applicability of plasmid profiling to MRSA epidemiology.

We identified 37 different digestion profiles in 120 independent MRSA isolates by restriction enzyme analysis of their plasmid DNA (23 additional samples, representing repeated isolations from individual patients, would increase the total to 143). Two additional categories were identified: strains without plasmids and strains with single, *Eco*RI-resistant plasmids. DNA molecules in the latter group were not identical and may be classified more precisely after digestion with a different enzyme. Restriction endonuclease digestion was essential to the analysis, since a majority of the strains (83.3%) carried only one plasmid each, and these had electrophoretic mobilities that were frequently indistinguishable. Only a small number of isolates (12.5%) contained two or more plasmid species, and many of these were environmental samples or type cultures.

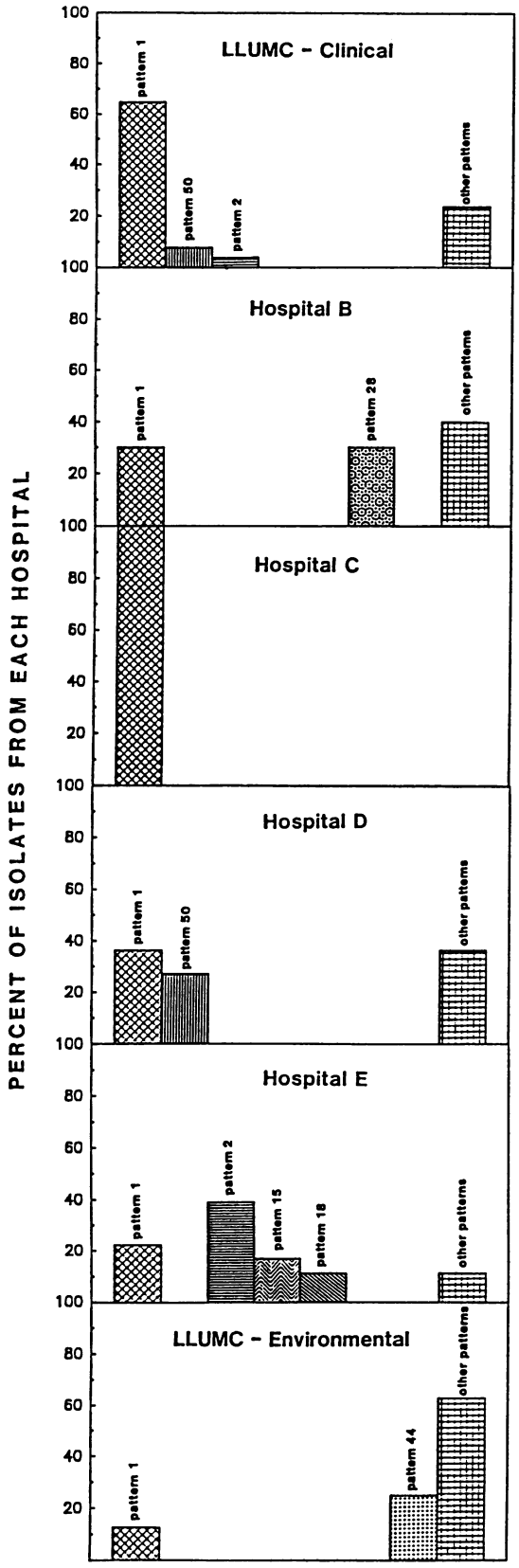
These observations suggest that the number of different plasmid and plasmid combinations that occur in clinical MRSA strains is vast—much greater than the 37 that we have identified. Such diversity endows the technique with the exceptional resolving power needed for epidemiological studies.

The rich diversity of plasmids among MRSA suggests that there are mechanisms at work, perhaps involving conjugation, phage-mediated contact, transduction, transposition, and site-specific or homologous recombination (10, 11, 14), that are continuously generating new plasmid forms. The rate at which these DNA reorganizations occur has a bearing upon the usefulness of plasmid profiling to epidemiology. This application requires that the plasmid composition of pathogenic MRSA strains remain stable at least for the period of a typical infection and, hopefully, for the duration of an MRSA outbreak. We observed that plasmid digestion profiles of MRSA isolated repeatedly from nine patients over periods of up to 3 months remained unchanged. This suggests that plasmid rearrangements do not regularly occur within the time scale of individual infections. We also observed that specific digestion profiles were persistently detected in hospitals over a period of more than 2 years (see below) (1).

TABLE 3. Number of plasmids in MRSA isolates

No. of different plasmids in isolates	Fragment profile no.
0	0[a]
1	1, 2, 3, 4, 5, 7, 8, 9, 10, 12, 15, 16, 18, 24, 25, 27, 28, 32, 35, 38, 44, 47, 50[b]
2	21, 39, 46
3	6, 11, 14, 33, 37, 48
≥4	20, 22, 23, 26, 36, 41

[a] Five individual isolates that lacked plasmids were assigned to profile 0.
[b] Fragment profile 50 is an artificial group of isolates each containing a plasmid that is not cleaved by *Eco*RI.



In one case, three distinctly different digestion profiles were obtained from a patient at different times. The patient was housed in six different wards during a 2-month hospitalization. The last change coincided with the appearance of a new antibiotic resistance, a reduction in plasmid size, and the appearance of profile 1, the most common plasmid in our population. None of these changes conclusively discriminates between the alternative hypotheses of successive infection and plasmid reorganization. We are currently using DNA hybridization to determine the relationships among all of the plasmids. These studies may reveal whether these three molecules share a common ancestry.

Plasmid DNA can be extracted routinely from bacteria by using a variety of simple protocols (16). Many samples can be analyzed concurrently. With the manual protocol we describe, 24 samples were conveniently prepared for electrophoresis in 1 day. Automated equipment, now becoming available, could greatly accelerate the extraction of plasmid DNA and make it practical for many clinical laboratories. Convenient methods for data reduction and pattern matching are necessary, since we found it much more laborious to quantitate and correlate the profiles than to generate them.

Epidemiologic conclusions. Although our primary aims were to assess the diversity and stability of plasmid types in MRSA isolates, the data we have accumulated suggest specific epidemiological conclusions.

Plasmid profile 1 appeared in 45% of the isolates; it was the only plasmid detected in every clinical setting that we sampled and was usually the most common representative at each hospital. Plasmid profile 1 was found in both the 1986 and 1987-to-1988 episodes at LLUMC, and it was the only clinical profile also to appear in an environmental sample. The extraordinarily high occurrence of this plasmid was in significant contrast to the infrequent appearance of the other 36 profiles (see below). In our subsequent work, we have found profile 1 frequently among samples from widely dispersed hospitals throughout the continental United States. We conclude that plasmid profile 1 marks an endemic MRSA strain at all five institutions. Plasmid profile 2 was the most common representative at one hospital and was the only other strain to appear at multiple locations. It may represent an endemic strain at those institutions.

The data allow for the interpretation that these two strains are transmitted between patients or between personnel and patients. Surveillance of the personnel at each facility may identify individual carriers. There exists the intriguing possibility that these two plasmids may carry genetic determinants that contribute to the virulence of the organism.

Many of the other digestion profiles occurred uniquely in individual patients (Table 2). Profiles 3 through 50 accounted for 43% of the infections, yet they were each detected in a mean of 1.24 patients. The data suggest that these sporadic MRSA strains were not contracted from a common source nor were they transmitted between patients. Since these cases had no detected antecedents in the hospitals, they may represent infections by strains resident in the patients before hospitalization.

Thirty-five of the 37 digestion profiles were confined to

FIG. 3. Occurrence of various digestion profiles displayed as a percentage of the isolates from each hospital. Plasmids that appeared in more than one hospital or in more than one patient are represented by vertical bars. Isolates that appeared only once in the entire sample population are grouped together in the bar labeled "other patterns."

individual hospitals. Each facility appeared to have a distinctive MRSA flora composed of strains appearing regularly and those detected at a low frequency (identified as sporadic; Fig. 3). The two plasmids that were detected regularly at several locations had significantly different frequencies in specific hospitals.

We conclude that restriction analysis of MRSA plasmids has all the attributes of a powerful epidemiological marker. The diversity and stability of plasmid profiles provide an effective means for discriminating between strains. Rapid methods for DNA extraction and data handling may make it suitable and cost effective for hospital clinical laboratories.

ACKNOWLEDGMENTS

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