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Quantitative Antibiogram Typing Using Inhibition Zone Diameters Compared with Ribotyping for Epidemiological Typing of Methicillin-Resistant *Staphylococcus aureus*

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Antibiogram typing of methicillin-resistant Staphylococcus aureus with selected antibiotics was evaluated as a primary epidemiological typing tool and compared with ribotyping. Antibiograms were derived with the Kirby-Bauer disk diffusion method by using erythromycin, clindamycin, cotrimoxazole, gentamicin, and ciprofloxacin. For typing, antibiogram data were analyzed by similarity analysis of disk zone diameters (quantitative antibiogram typing). One hundred seventy-two isolates were typed. Reproducibility reached 98% for the quantitative antibiogram and 100% for ribotyping. With three selected restriction enzymes (*Eco*RV, *Hin*dIII, and *Kpn*I), 40 epidemiologically unrelated isolates could be classified into 21 ribotypes, whereas quantitative antibiogram typing classified these isolates into 19 groups. To evaluate the discriminatory power of the methods, we calculated an index of discrimination from data obtained with these 40 isolates. This index takes into consideration both the number of types defined by the typing method and their relative frequencies. With both ribotyping and quantitative antibiogram typing, high discrimination indices (0.972 and 0.954, respectively) were obtained. When epidemiological links between patients (ward, period of hospitalization, and contacts between staff and patients) were compared with the results of ribotyping or the quantitative antibiogram typing method, it appeared that both methods were able to discriminate epidemiological clusters, with only a few discrepancies. In conclusion, quantitative antibiogram typing, although not necessarily based on genomic markers, is a simple method which enables a reliable workup of methicillin-resistant *S. aureus* epidemic when sophisticated molecular typing methods are not available.

Patterns of susceptibility to antimicrobial agents are used for typing because they are readily available, easy to determine, and relatively inexpensive. However, an inherent weakness of this method is that changes in antimicrobial susceptibility are often related to environmental factors or plasmids. Furthermore, the method is not always reproducible when the same strain is repeatedly tested and is thought to have poor discriminatory power. To enhance the value of this method as a tool for nosocomial infection surveillance, Giacca et al. (6) proposed a similarity analysis of susceptibility testing results, taking into consideration the diameters of growth inhibition zones in disk diffusion tests. However, neither the discriminatory power nor the epidemiological value of this method has been evaluated. Therefore, we assessed the value of antibiogram typing of methicillin-resistant *Staphylococcus aureus* (MRSA) as a primary epidemiological tool and compared it with ribotyping, an established molecular typing method. The evaluation and comparison criteria were reproducibility, discriminatory power, and value in an epidemiological setting.

MATERIALS AND METHODS

Bacterial strains. One hundred seventy-two isolates were used in this study. Forty of them were considered to be epidemiologically unrelated either because they originated from different geographic areas around the world (22 isolates) or because they had been isolated from recently (<48 h) admitted patients with no link to other known MRSA carriers in the hospital (18 isolates). The other isolates were collected in our hospital over a 4-year period (1989 to 1992) (10).

Antibiograms. Antibiogram typing was performed by the conventional Kirby-Bauer disk diffusion test on Mueller-Hinton agar with 24 h of incubation at 35°C. The antibiotics tested were erythromycin, clindamycin, co-trimoxazole, gentamicin, and ciprofloxacin. The criteria by which antibiotics were chosen were (i) variable and independent resistance among MRSA isolates and/or (ii) chromosomal resistance and/or (iii) relatively low frequency of use in the hospital. Diameters of growth inhibition were measured with a caliper, and antibiograms were interpreted in accordance with National Committee for Clinical Laboratory Standards recommendations (11). With antibiogram data, typing was performed by one qualitative method, i.e., resistance profiles, and one quantitative method, i.e., similarity analysis of inhibition zone diameters (see below).

Ribotyping. Ribotyping was performed as already described (1, 2, 7, 8). Briefly, whole-cell DNA was digested with a restriction enzyme (RE). DNA fragments were separated by horizontal agarose gel electrophoresis and transferred to a nylon membrane. Hybridization was performed with plasmid pKK3535, containing an rRDNA operon of *Escherichia coli* (4). The DNA probe was labeled with biotin-7-dATP by using a nick translation kit (Gibco-BRL, Life Technologies). Hybrids were revealed on the membrane by using the BluGene (Gibco-BRL, Life Technologies) nonradioactive nucleic acid detection system. To select REs giving a high level of discrimination

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	A	B	C
AB1	7	12	15
AB2	17	20	25
AB3	16	15	11

FIG. 1. Diameters (in millimeters) of inhibition zones created by three antibiotics for three strains.

between isolates, ribotyping was initially performed on 20 unrelated isolates with the following REs: *Apa*I, *Bam*HI, *Cfo*I, *Cla*I, *Dra*I, *Eco*RI, *Eco*RV, *Hae*I, *Hin*dIII, *Kpn*I, *Pst*I, *Sfu*I, *Sma*I, *Xba*I, and *Xho*I. A combination of three enzymes (*Eco*RV, *Hin*dIII, and *Kpn*I) gave the greatest number of types and was used to type the remaining isolates.

Similarity analysis. Similarity analysis was performed for antibiogram typing and ribotyping. For antibiogram data (diameters of inhibition zones), we choose the Euclidean distance as a similarity coefficient, given by the following formula:

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

where x_{ij} and x_{ik} are the values of the i^{th} character of the j^{th} and k^{th} organisms, respectively. The greater the distance between two organisms, the smaller is the resemblance between them. As an example, Fig. 1 shows data for three strains (organisms), A, B, and C, for which the diameters of inhibition zones created by three antibiotics, AB1, AB2, and AB3, were measured.

The Euclidean distance between A and B is $E_{AB} = \sqrt{(7-12)^2 + (17-20)^2 + (16-15)^2} = 5.9$. Similarly, Euclidean distances between other pairs of organisms were computed, and the data may be presented as a triangular matrix. A similarity matrix for the data in Fig. 1 is shown in Fig. 2.

A certain variation of inhibition zone diameters can be observed when the same strain is repeatedly tested. To define a cutoff distance below which discrepancies are due to casual variability, the antibiograms of several isolates were determined twice on different days and similarities between the first and the second determinations were analyzed. The cutoff distance was set up such that >95% of the distances between the first and the second determinations would be smaller than the cutoff. Therefore, the reproducibility of the method would be >95%. Thus, two isolates were considered to be similar when the Euclidean distance between them was lower than the cutoff value.

For ribotyping data, the presence or absence of bands was considered and the mismatch coefficient was used to evaluate the similarity between isolates (2) on the basis of the formula $S = (a + d)/n$, where a is the number of bands present in both isolates, d is the number of bands not present in both isolates, and n is the total number of bands.

	A	B	C
A	0		
B	5.9	0	
C	12.4	7.1	0

FIG. 2. Similarity matrix based on the data in Fig. 1.

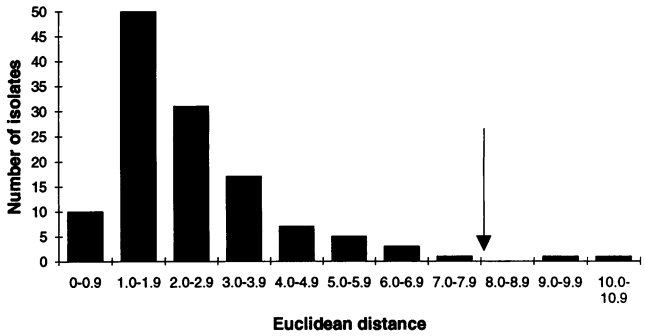

FIG. 3. Distribution of Euclidean distances calculated between the first and second antibiograms of 126 MRSA isolates. The arrow indicates the cutoff distance.

The algorithm used to produce a dendrogram for both antibiogram typing and ribotyping was the unweighted pair group method of analysis (14).

Discriminatory power. To compare the discriminatory powers of the typing methods, we used the index of discrimination (D) proposed by Hunter (9) for the situation in which strains cannot be placed into mutually exclusive groups. D is defined as follows:

$$D = 1 - [1/N(N-1)] \sum_{j=1}^{s} a_j,$$

where a_j is the number of strains in the population which are indistinguishable from the j^{th} strain and N is the total number of strains. Each strain is, in turn, compared with all of the other strains to determine how many other strains are similar from it to give a_j.

RESULTS

Among the MRSA isolates from 122 patients at our hospital from 1989 to 1992, 88% were resistant to erythromycin, 68% were resistant to clindamycin, 5% were resistant to co-trimoxazole, 87% were resistant to gentamicin, and 79% were resistant to ciprofloxacin.

Quantitative antibiogram typing. The reproducibility of zone diameter measurement was assessed by repeated antibiogram typing of 126 isolates on different days. The Euclidean distances calculated between the first and the second tests are plotted in Fig. 3. The greatest Euclidean distance was 10.8, and 98.4% of the same isolates had a distance of less than 8.0. Therefore, a cutoff at a distance of 8.0 was defined below which discrepancies were due to casual variability. The reproducibility of the method was therefore 98.4%. Thus, two isolates with a distance of less than 8.0 were considered similar. Discriminatory power was assessed by analyzing the data on the 40 epidemiologically unrelated isolates. The dendrogram based on quantitative antibiogram typing classified the 40 isolates into 19 groups (Fig. 4), and a D of 0.972 was obtained.

Resistance profiles. The reproducibility of the resistance profiles was assessed by using the same data. Two isolates (1.6%) had a different resistance profile at the second test (reproducibility, 98.4%). Twelve resistance profiles were ob-

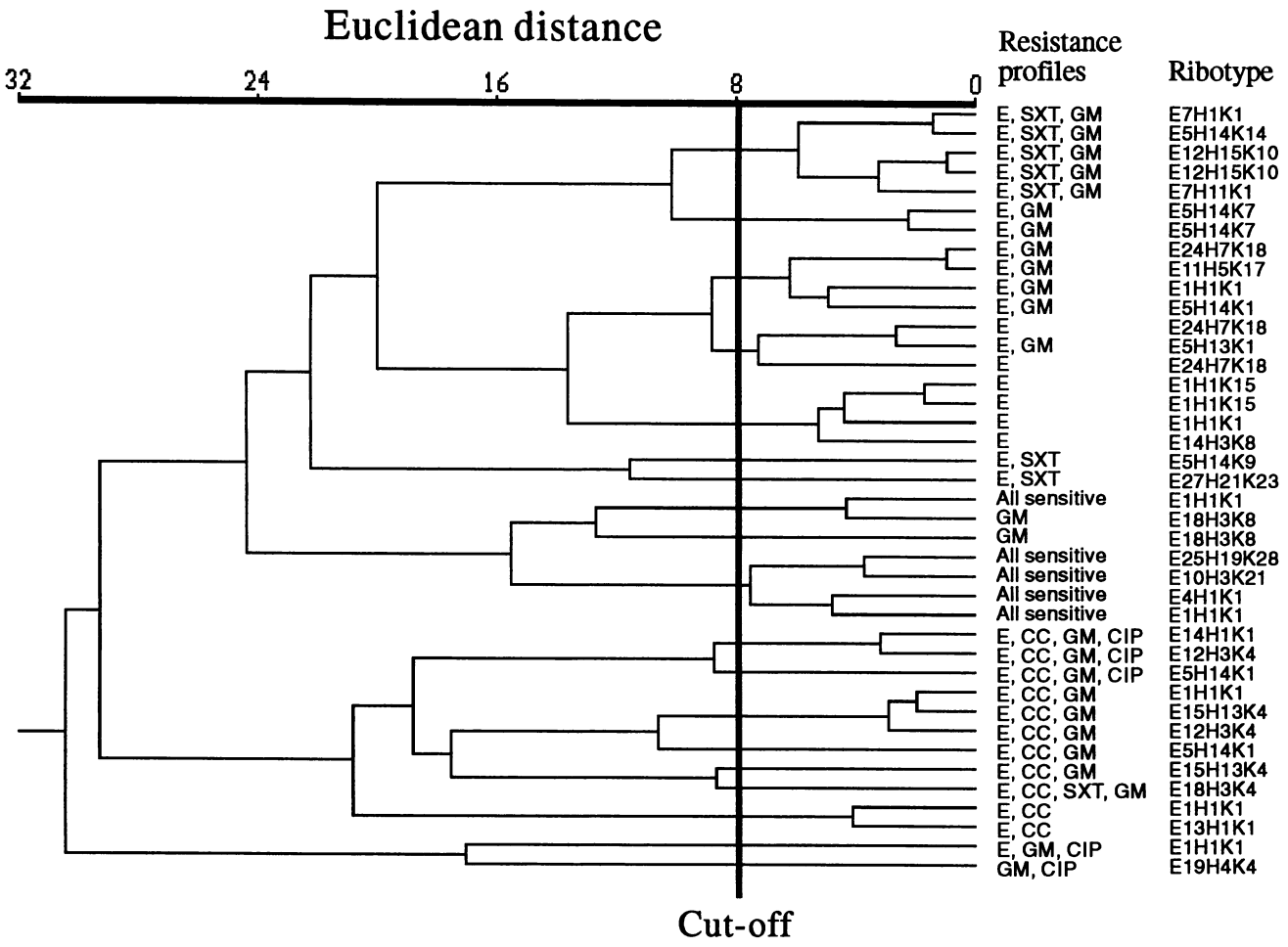

FIG. 4. Dendrogram of epidemiologically unrelated MRSA strains obtained by antibiogram cluster analysis (quantitative antibiogram typing), resistance profiles, and results of ribotyping.

tained from the 40 unrelated isolates, and a *D* of 0.908 was obtained. As this value was lower than the *D* based on quantitative antibiogram typing, resistance profiles were used for further comparison with ribotyping data in the epidemiological evaluation of MRSA.

Ribotyping. More than 20 strains were typed several times, and identical banding patterns were observed for each isolate with all three REs, giving a reproducibility of 100%. Among the 172 isolates, 27 different ribotyping patterns (labeled E1 to E27) were obtained with *Eco*RV, 21 (labeled H1 to H21) were obtained with *Hin*dIII, and 29 (labeled K1 to K29) were obtained with *Kpn*I. Isolates were considered to belong to the same ribotype when they shared the same patterns for all three REs. Thus, all 172 isolates were distributed into 45 different ribotypes (Fig. 5 and 6) and a *D* of 0.954 was calculated from the data obtained with the 40 epidemiologically unrelated strains (Fig. 4).

Stability. During the 4 years of the survey in our hospital, strains of the same ribotypes were isolated during epidemiological clusters lasting up to 12 months, which is indicative of the relative stability of ribotype expression in a natural environment. The same stability was observed for antibiograms. However, 20 months after the first isolation of MRSA, one patient had an isolate belonging to a different ribotype (E8H1K1) which was not encountered elsewhere and which differed by only one band from the previous isolates (E1H1K1), whereas they were considered to be similar on the basis of quantitative antibiogram typing. Furthermore, during a small epidemic lasting 7 months in a burn unit, all six patients had isolates belonging to the same ribotype and sharing the same quantitative antibiogram, except for one, which differed only in

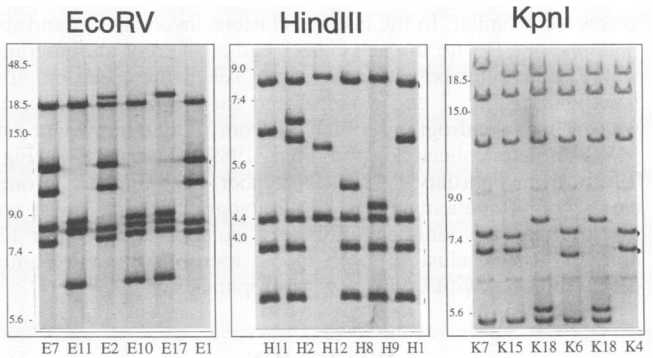

FIG. 5. Examples of *Eco*RV, *Hin*dIII, and *Kpn*I RE rRNA patterns of MRSA. The numbers to the left of each panel are molecular sizes in kilobase pairs.

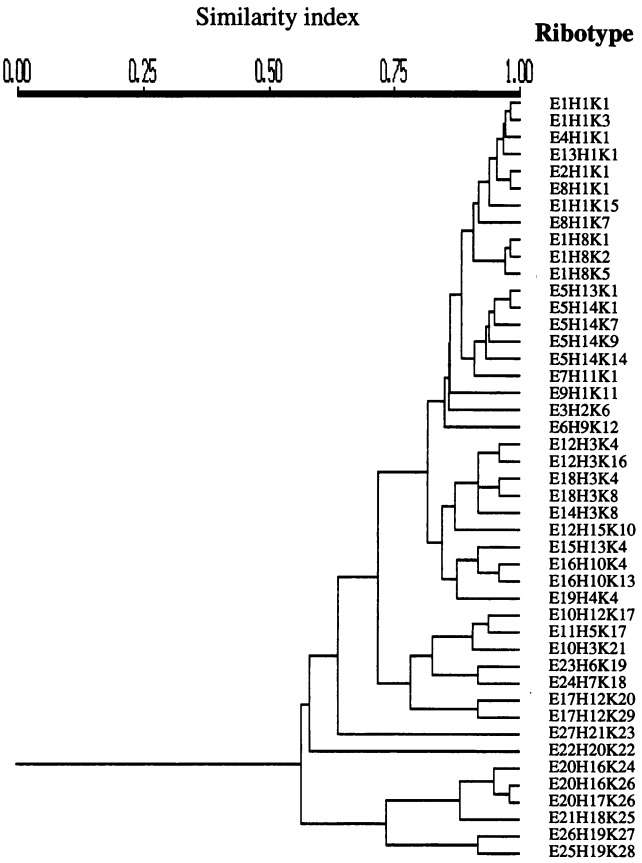

FIG. 6. Dendrogram of similarities (obtained by the mismatch coefficient based on the presence or absence of bands) among 45 ribotypes obtained from 172 isolates.

the susceptibility result of one antibiotic. In both cases, the minor ribotype or antibiogram changes observed were most likely due to a genetic change in the strain.

Epidemiological evaluation. The values of quantitative antibiogram typing and ribotyping were compared in the epidemiological setting of a 4-year period study of MRSA in our hospital (10). From 1989 to 1992, 13 epidemic clusters were suspected on the basis of epidemiological data. In nine clusters, involving two to nine patients, all isolates within the clusters belonged to the same ribotypes and had similar antibiograms. In two clusters, involving 10 and 18 patients, six and one isolates, respectively, belonged to different ribotypes whereas the antibiograms were similar. In the last two clusters, involving five and six patients, one isolate in each cluster had a different antibiogram whereas the ribotypes were the same. All of these clusters are illustrated in Fig. 7 and 8 (separated for graphical purposes), which show dendrograms obtained from antibiogram data on MRSA isolates collected from January 1989 to January 1991 (Fig. 7) and from February 1991 to December 1992 (Fig. 8). In one cluster of 10 patients (Fig. 8), antibiogram typing failed to discriminate five genetically different isolates. In the other 12 epidemiological clusters, both typing methods had the same discrimination, with only a few discrepancies.

DISCUSSION

Epidemiologists frequently rely on laboratory identification and typing of nosocomial pathogens to provide evidence of the

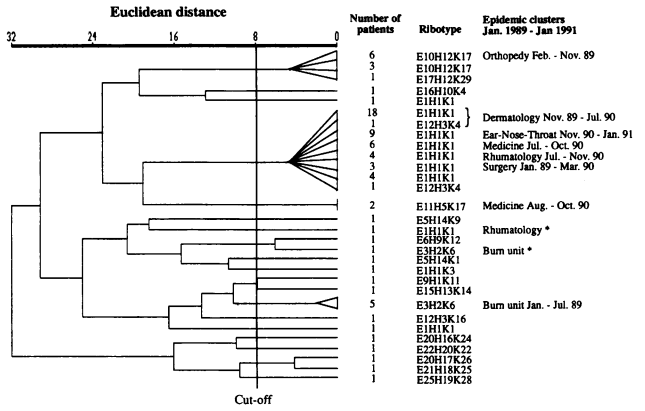

FIG. 7. Dendrogram obtained from antibiogram data for all MRSA isolates collected from January 1989 to January 1991 (one isolate per patient). Epidemic clusters are displayed in front of the corresponding antibiogram clusters. The dates indicate the duration each of epidemic, and an asterisk indicates a patient who was included in an epidemic cluster (same ribotype) but had a different antibiogram.

relatedness of organisms. However, while two isolates with different types are likely to be unrelated, it is more difficult to assess whether two organisms with similar properties genuinely belong to the same strain. Therefore, it is important to use typing methods with high powers of discrimination and good reproducibility to decrease the risk that two unrelated isolates will appear to belong to the same type. Ideally, a typing method recognizes each unrelated isolate as unique. In practice, the method may be considered statistically useful if this risk is $<5\%$, which means that the D of Hunter (9) is >0.95.

The results of our study suggest that antibiogram data might be of epidemiological interest provided that they are analyzed quantitatively. Indeed, the discriminatory power of quantitative antibiogram typing of MRSA strains which were epidemiologically unrelated was equivalent to that of ribotyping (D = 0.972 and 0.954, respectively). In addition, when links between patients, based on epidemiological data (e.g., wards, periods of hospitalization, or contacts between staff and patients), were compared with quantitative antibiogram typing and ribotyping results, it appeared that both methods were able to confirm epidemiological clusters, with only a few discrepancies. In contrast, the resistance profile method, with a D of 0.907, would not be statistically useful for epidemiological purposes.

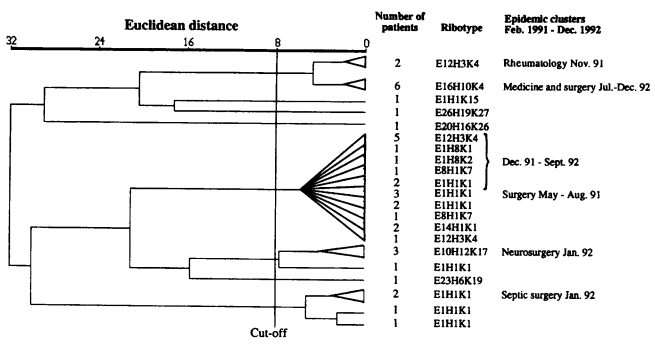

FIG. 8. Dendrogram obtained from antibiogram data for MRSA isolates collected from February 1991 to December 1992 (one isolate per patient). For details, see the legend to Fig. 7.

This indicates that this method is less reliable than the quantitative antibiogram method.

In epidemiological investigations, antibiogram typing is a traditional typing method used to distinguish between individual strains. It is readily available, easy to perform, and relatively inexpensive. However, one drawback of the method is that markers of antibiotic resistance are often carried by labile or movable genetic elements (e.g., plasmids or transposons) whose selection of expression may depend on environmental conditions. Moreover, since the advance of DNA-based typing methods, it has been repeatedly shown that MRSA isolates which were indistinguishable by antibiotic susceptibility tests could be discriminated on the basis of their genotypes (3, 5, 12, 13, 15, 16). Thus, antibiogram typing is considered to have poor discriminatory power and is used by microbiologists only in the first instance for rapid screening of the similarities between different clinical isolates.

The good results of antibiogram typing in our study were probably due to the following reasons: (i) the usual qualitative antibiogram analysis was refined by adding quantitative measurement of inhibition zones around antibiotic disks, (ii) the five antibiotics used (i.e., erythromycin, clindamycin, co-trimoxazole, gentamicin, and ciprofloxacin) had been selected because they were mostly chromosomal markers which were unlinked and varied among the MRSA strains isolated in our hospital; and (iii) the analysis was restricted to isolates of a well-defined epidemiological setting. Although some genetically unrelated strains showed great similarity by antibiogram typing, this appeared to be relatively infrequent and may be offset by the speed, availability, and the relatively high discriminatory power of this technique. The method could easily be adapted to the epidemiological setting of other institutions by changing or adding antibiotics and redefining the cutoff value to fit the particular hospital and laboratory setting.

The stability of characters of a bacterial clone in a natural environment is another important feature to consider when typing methods are used for epidemiological purposes. Moreover, methods with very high discriminatory power may become so sensitive that they detect epidemiologically irrelevant differences within a single strain, such as point mutations or DNA rearrangements. In our study, we observed only a few such changes in both antibiogram typing and ribotyping. For antibiogram typing, the change appeared during an outbreak lasting 7 months, whereas for ribotyping the change occurred in one patient 20 months after the first isolation of MRSA. We presume that these values represent a preliminary estimation of the minimal duration of strain stability for each method. This may suggest that ribotyping is more stable than antibiogram typing, which is not unexpected. Indeed, susceptibility to antibiotics may be under significant selective pressure and thus more likely than essential chromosomal loci, such as ribosomal operons, to undergo genetic rearrangements such as gene duplications, deletions, or transfers among strains.

Conclusion. Ribotyping and quantitative antibiogram typing are powerful methods for epidemiological investigation of MRSA outbreaks. While ribotyping is a sophisticated technique which may not be routinely used in most clinical microbiology laboratories, quantitative antibiogram typing as described above may be a valuable tool which allows rapid epidemiological investigations with minimal investment. Ribotyping analysis, or another molecular typing method, may be reserved for situations in which epidemiological and antibiogram typing data conflict or are inconclusive.

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