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In Vivo Stability and Discriminatory Power of Methicillin-Resistant *Staphylococcus aureus* Typing by Restriction Endonuclease Analysis of Plasmid DNA Compared with Those of Other Molecular Methods

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We evaluated test discriminatory power and DNA type alterations among methicillin-resistant *Staphylococcus aureus* strains by testing 199 sequential isolates from 39 patients collected over 30 to 228 days. Isolates were typed by one or three different methods (restriction endonuclease analysis of plasmid DNA [REAP] with or without pulsed-field gel electrophoresis of genomic DNA [PFGE] and immunoblotting [IB]). REAP was highly discriminatory compared with PFGE and IB. However, the initial isolates from 4 of the 39 patients lacked detectable plasmid DNA and could not be typed by REAP. Typing of individual patient isolates showed that a different REAP type was identified only once every 138 days. Among 25 comparisons, seven sequential isolate pairs demonstrating REAP differences were also different by PFGE and IB. This likely represented the presence of more than one strain. Eighteen other pairs with REAP differences were identical or related to one another by PFGE and IB typing, and 17 of these differences were likely caused by a single genetic alteration within the same strain or clone. The rate of PFGE differences explicable by single genetic alterations among sequential isolates identical by REAP was similar to the overall rate for REAP differences in the whole collection. We conclude that REAP and PFGE typing differences explicable by single genetic alterations are relatively infrequent but not rare. These isolates should be examined by alternative typing systems to further support or refute clonality.

Molecular typing of methicillin-resistant *Staphylococcus aureus* (MRSA) generally demonstrates improved discriminatory power compared with assays of phenotypic traits, and it is frequently preferred over other methods for this reason (13, 14, 18, 20). However, bacteria have a propensity to undergo genetic alterations over time (1, 6, 13, 20). This has led investigators to suggest that differences in types that are explicable by single genetic events should not be used to define isolates as different from one another (6, 13, 20). The frequency of genetic alterations among MRSA strains in vivo and whether plasmid DNA alterations are more common than genomic DNA alterations are essentially unknown; these issues are the main subject of this investigation.

We typed MRSA isolates from patients who had positive cultures for long periods by using one (restriction endonuclease analysis of plasmid DNA [REAP]) or more (pulsed-field gel electrophoresis of genomic DNA [PFGE] and immunoblotting [IB]) molecular methods. The discriminatory power was assessed, and the frequency of and likely etiology for DNA-based typing differences among serial isolates were analyzed.

(A preliminary report of this work was presented previously [18a].)

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MATERIALS AND METHODS

Patients and bacterial isolates. During a period of 294 days (21 November 1991 through 11 September 1992), all available patient isolates of MRSA, identified by conventional means (10, 17), from three hospitals (a university hospital, a pediatric hospital, and a public hospital, all on the campus of Indiana University Medical Center) and from their associated outpatient facilities were subcultured, inoculated into 10% skim milk (Difco Laboratories, Detroit, Mich.), and frozen at −20°C. From this collection, sequential isolates from patients with positive cultures for 30 days or more were typed.

Isolate typing. REAP was performed by previously described modifications (8) of the cetyltrimethylammonium bromide method of Townsend et al. (21). Isolates with plasmid DNA were subjected to *Hin*dIII restriction endonuclease enzyme digestion (New England Biolabs, Beverly, Mass.) according to the manufacturer's recommendations and then to overnight electrophoresis at 25 V.

Typing by PFGE was performed by previously described modifications (3) of the methods of Smith and Cantor (19). *Sma*I (New England Biolabs) was employed as the low-frequency-cleavage endonuclease enzyme. Electrophoresis was performed with the CHEF DR-II system (Bio-Rad, Richmond, Calif.). Run conditions were 13°C, 23 h, and 6 V/cm, with linear pulse ramping of 5 to 35 s.

Typing by IB was done as previously described (15). Samples of pooled human sera at a 1:50 dilution were used as the antibody source.

Order of isolate typing. All isolates were initially typed by REAP. Each patient's serial isolates were typed at the same time. REAP preparations were electrophoresed on the same gel whenever possible.

PFGE, IB, and repeat REAP typing were performed on selected isolates in a blinded manner. Selected isolates from each patient included the initial isolate, sequential isolates with a different REAP type, and the last isolate when different REAP types were not demonstrated. Subcultures of these isolates were coded by an author (A.I.H.) not responsible for assigning types and then were blindly processed. Molecular size standards (1-kb ladder [Bethesda Research Laboratories, Gaithersburg, Md.] for REAP, lambda DNA ladder [Bio-Rad] for PFGE, and a sodium dodecyl sulfate-polyacrylamide gel electrophoresis prestained standard [Bio-Rad] for IB) were added to each gel for the comparison and measurement of bands.

Discriminatory power of typing tests. Discriminatory power was assessed by

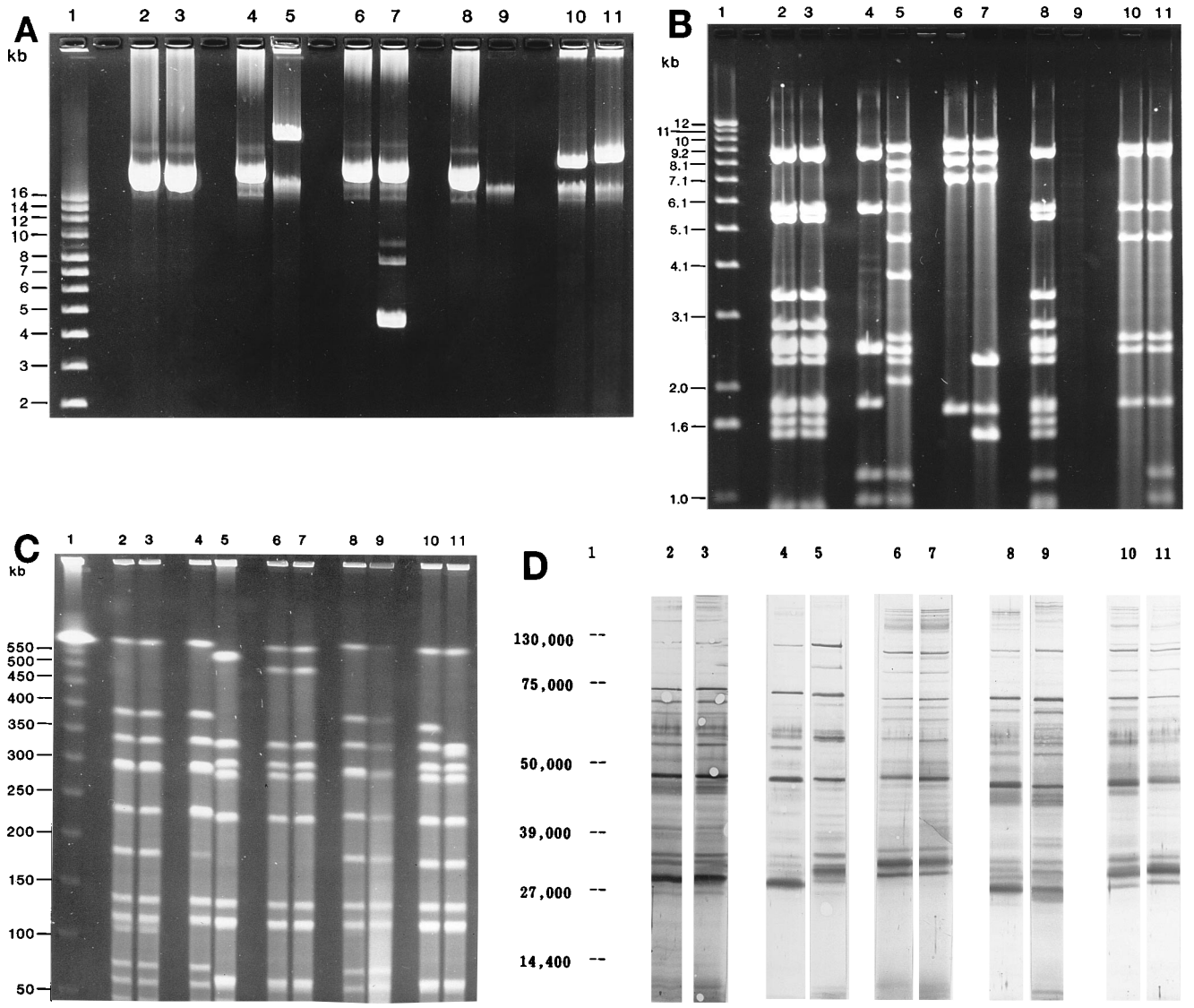

FIG. 1. Example of isolate comparisons as assessed by plasmid DNA-screening gel (A), REAP (B), PFGE (C), and IB (D). Lanes 1, molecular size markers. Lanes 2 and 3, isolates that are identical by REAP, PFGE, and IB. Lanes 4 and 5, isolates that are different by REAP, PFGE, and IB. Lanes 6 and 7, an apparent gain of plasmid DNA (as exhibited in lanes 7 compared with lanes 6 of panels A and B) in isolates that are identical by PFGE and IB. Lanes 8 and 9, an apparent loss of plasmid DNA (as exhibited in lanes 9 compared with lanes 8 of panels A and B) in isolates that are identical by PFGE and have related subtypes by IB. Lanes 10 and 11, isolates that are related by REAP (showing two different bands), related by PFGE (showing two different bands), and identical by IB. In panel D, numbers on the left are molecular sizes.

comparing the number and distribution of types among the patients' initial isolates. For this analysis, isolates with any differences in the banding pattern (by REAP, PFGE, or IB) were considered unique types or subtypes.

Comparative in vivo stabilities of PFGE and IB typing. Comparative in vivo stabilities of PFGE and IB typing were assessed by comparing the PFGE and IB types of the initial and last sequential isolates from each patient that demonstrated identity by REAP.

Analysis of REAP differences among sequential patient isolates. Differences in REAP typing results for each patient's sequential isolates were divided into categories that were based on assessment of the plasmid-screening and REAP gels. Categories included differences which could be explained by gain of a plasmid, by loss of a plasmid, by isolates belonging to related REAP types, or by more than one of these events. Related REAP types were defined as isolates demonstrating a coefficient of similarity (CS) equal to or exceeding 0.85. The CS was calculated as follows: CS = 2 × (number of matching bands)/total number of bands in both strains (4). REAP types considered to be related did not have new or absent bands on the plasmid-screening gels and had only one to three band differences on the REAP gels.

The sequential patient isolates with different REAP types were also distinguished according to their PFGE and IB types. PFGE types were categorized as identical (all bands matching by number and molecular size), related to one another (CS $\geq$ 0.85), or different from one another (CS $<$ 0.85). Related PFGE types had one to three band differences. By IB, isolates were considered different types if they had one or more major bands that were consistently different from those of all other types. Subtypes were assigned if there were minor or faint bands that were different but all major bands were the same. Bands used to identify major types were often of low molecular size ($<$60,000 kb), whereas bands indicative of subtypes were often of higher molecular size. Figure 1 illustrates the comparison of five pairs of isolates by plasmid DNA screening, REAP, PFGE, and IB.

Finally, we assumed that sequential patient isolates, regardless of the category for the REAP difference, which were different by both PFGE type and IB type or subtype were different strains. Those which differed by PFGE type or IB type but not by both were considered probably different strains. Those found to be identical by PFGE type and IB type or subtype were considered identical strains. Isolates with any other results from the comparison were judged possibly identical strains. This last group included isolates which were related (but not identical) by PFGE or which had similar (but not identical) subtypes by IB.

TABLE 1. Study patients and isolates

Feature	Value for patients with isolates:	
	Without REAP differences	With REAP differences
No. of patients	20	19
No. of isolates	98	101
Mean no. of isolates per patient (range)	4.9 (2–16)	5.3 (2–18)
Mean no. of days between positive cultures (range)	78 (30–228)	99 (31–179)
No. of patients with isolates over:		
30–60 days	10	7
61–90 days	3	3
91–120 days	4	3
121–150 days	1	2
>150 days	2	4
No. of patients with isolates from:		
1 site	7	7
2 sites	9	9
3 sites	4	2
4 sites		1
No. of patients with:		
1 REAP type	20	
2 REAP types		14
3 REAP types		4
4 REAP types		1

RESULTS

Patients and bacterial isolates. Thirty-nine patients with positive cultures for MRSA over 30 days or more were identified. Twenty-two, 5, and 12 patients received care at the university hospital, the pediatric hospital, and the public hospital, respectively. Twenty of the 39 patients were considered to have community acquisition. Among the 19 patients with possible nosocomial acquisition of MRSA, 13 were in the university hospital, 5 were in the public hospital, and 1 was in the pediatric hospital. Only 2 of these 19 patients were recognized to be associated with a cluster or outbreak of nosocomial MRSA colonization or infection.

Typing by REAP. A total of 199 isolates were typed by REAP (Table 1). Twenty patients with 98 sequential isolates did not have different types by REAP, whereas 19 patients with 101 sequential isolates had two or more REAP types. The mean numbers (4.9 versus 5.3) and ranges (2 to 16 versus 2 to 18) of isolates per patient, the distributions of patients by interval days between positive cultures (30 to 228 versus 31 to 179), and the numbers of culture-positive sites (usually one or two) for the two patient populations were similar. Patients with isolates different by REAP did have a longer mean duration (99 days) between the initial and last positive cultures than patients without isolates different by REAP (78 days). However, the mean duration between the initial culture and the sequential culture revealing the first isolate different by REAP in these patients was only 73 days.

There was substantial variation in the time when sequential isolates from a patient first demonstrated different REAP types. Five patients had different types during the first 30 days (including a patient with two isolates on day 1 which showed different REAP types), seven patients had different types during the subsequent 30-day interval, and four patients had different types first recognized after an interval of more than 150 days. Other patients had different REAP types first demonstrated on days 88, 103, and 134 of culture positivity, respectively.

TABLE 2. Initial isolate typing results

Patient no.	REAP type	PFGE type	IB type[a]
1	1	a	C6
2	1	a	C6
3	1	a	C3″
4	1	b	C3
5	1	b	C3
6	1	c	C3
7	1	d	C3
8	1	e	C3
9	1	f	C3‴
10	2	a	C4
11	2	a	C3
12	2	a	C3
13	2	g	C1
14	2	h	C3
15	3	i	B1
16	3	i	B2
17	3	i	B2
18	3	j	B3
19	4	h	C3
20	4	h	C3
21	4	h	C3
22	4	k	C3
23	5	h	C8
24	5	h	C3
25	5	h	C3
26	6	i	B2
27	6	i	B2
28	6	i	B1
29	7	f	C3
30	7	f	C3
31	8	h	C2
32	9	h	C3
33	10	f	C3
34	11	h	C3′
35	12	l	B2
36	NT[b]	d	C3
37	NT	a	C4
38	NT	h	C3
39	NT	m	C4

[a] Prime(s) indicates subtype of the indicated isolate.
[b] NT, not typeable by REAP because no plasmid DNA was present.

Discriminatory power and typeability. Typing results for the 39 initial patient isolates by all three tests are outlined in Table 2. Twelve types were discriminated by REAP and IB (including three IB subtypes), and 13 types were discriminated by PFGE. REAP demonstrated the fewest isolates within the most prevalent type (9 were type 1 by REAP, 11 were type h by PFGE, and 20 were type C3 by IB). Among isolates that were not among the most prevalent type, no more than five were identical to each other by REAP or by IB. By PFGE, sets of six isolates (type i) and seven isolates (type a) were identified as identical to one another.

All initial isolates were assigned a type or subtype by PFGE and IB. Three isolates were considered subtypes rather than a distinct type by IB. Each of these was identified as a subtype of the most prevalent IB type. Four of the 39 (10%) initial isolates did not have plasmid DNA detected and were classified as nontypeable by REAP. These four isolates had two different IB types and four different PFGE types.

Comparative in vivo stabilities of PFGE and IB typing. Sixteen of the 20 pairs with identical REAP types were identical to one another by PFGE. The other four pairs were

TABLE 3. Comparison of REAP differences versus PFGE and IB typing

REAP comparison	No. of strains as compared by PFGE and IB:			
	Different	Probably different	Identical	Possibly identical
Related			1	2
New plasmid present			6	2
Old plasmid gone	1		6	
More than one event	4	2	1	
Total	5	2	14	4

related types (all with a CS of >0.90) and had one or two band differences within the pair. Eighteen of the 20 pairs were identical types or subtypes by IB. The other two pairs were similar subtypes (each being a subtype of the other). The related PFGE types and similar IB subtypes were all identified in different pairs.

Analysis of REAP differences among sequential patient isolates. Twenty-five comparisons were done as part of analyzing the sequential isolates with different REAP types (two to four isolates from 19 patients) (Table 3). PFGE and IB typing suggested that 18 of the 25 REAP differences among sequential isolates represented plasmid DNA alterations within an identical or possibly identical strain. Seventeen of these 18 findings could be explained by a single event, the most common being either gain (8 events) or loss (6 events) of a plasmid. These different isolate types were identified at various times following the initially positive culture (31 to 179 days).

PFGE and IB typing suggested that seven of the REAP differences among sequential isolates represented the presence of different or probably different strains rather than plasmid DNA alterations within a previously identified strain. Six of these seven findings could not be explained by a single event (such as loss of a plasmid, gain of a plasmid, or an insertion, deletion or base mutation within the plasmid and leading to a related banding pattern by REAP). Isolates different by REAP were from cultures done on the day of through day 170 after the first positive culture.

DISCUSSION

The high degree of discrimination associated with typing of bacteria by DNA analyses is dependent upon an evolutionary genetic divergence arising from nonlethal mutations, acquisitions, or deletions of plasmid or chromosomal DNA (1, 13). If such events occur too frequently or rapidly in an isolate or strain, the applicability of DNA typing would be diminished. Indeed, investigators have suggested several criteria to classify strains as identical or related to, as well as different from, each other, because DNA alterations can be easily demonstrated during in vitro cultivation (3, 6, 13). Reservations about the value of plasmid-based DNA typing have been most frequent, because plasmids are nonessential DNA elements, potentially very mobile, and presumably prone to be gained or lost by a bacterial cell (18). However, concerns about chromosomal DNA alterations have also been raised because of readily demonstrable point mutations, insertions, or deletions (7, 13).

These issues are specially germane for MRSA typing. Typing to assess the probability of cross-transmission is commonly advised as part of epidemiologic investigations (2, 12, 14, 16, 18, 20, 22). However, MRSA isolates represent a relatively restricted subset of a species demonstrating limited evolutionary variations (11, 14). Because of this latter observation, the preferable use of very discriminatory typing tests is paramount for differentiating epidemiologically unrelated isolates from one another.

We addressed discriminatory power as well as the presence, frequency, and nature of DNA alterations among MRSA by typing a collection of sequential isolates from 39 patients with positive cultures over an interval of 30 to 228 days. The patients and likely time or place of organism acquisition were not highly associated with one another (i.e., there were three different hospitals and many patients with community acquisition, and only two of the nosocomial cases were identified as part of an outbreak).

On typing of the initial isolates by three methods, REAP was somewhat more discriminatory than PFGE or IB. Unlike with the two other systems, 10% of isolates could not be typed by REAP because of an absence of plasmid DNA. Each of the three systems revealed 12 or 13 types. IB identified 20 patients with a single type, and 6 to 11 patients had one of the three most prevalent types by PFGE. By comparison, only nine patients had the most common REAP type, and no more than five patients had any other REAP type.

The REAP type of sequential isolates from individual patients was quite stable. Only 25 type differences among 160 comparisons (199 isolates minus the initial 39 isolates), or 16%, occurred over a total culture-positive interval of 3,457 days. This was an average of one REAP difference being detected over each 138 days between positive cultures. REAP differences were sporadically identified between days 1 and 179 of sequential-culture positivity.

Typing some of the sequential isolates by PFGE and IB allowed us to determine the frequency and extent of other typing test differences among isolates identical by REAP and allowed us to make inferences about the likely cause or etiology for differences among isolates different by REAP. Compared isolates that were found to be identical by REAP always had identical (80%) or related (20%) PFGE types. As banding differences between related PFGE type designations were limited to one or two per pair, a single alteration in genomic DNA rather than a true strain difference seems to be the most plausible reason for these variations (6, 13, 20). Finding these compared isolates to be type identical (90%) or with only subtype differences (10%) by IB further supported this reasoning. Interestingly, the rate of PFGE differences (20%) among these putatively identical but genetically altered isolates was similar to the overall observed rate for sequential-isolate REAP differences among all patient isolates (16%).

Twenty-five sequential-isolate comparisons demonstrated REAP differences, and seven of these differences were inexplicable by a single genetic event (such as loss of a plasmid, gain of a plasmid, insertion or deletion of DNA within a plasmid, or a base mutation). Four of these seven compared pairs were different from each other by PFGE and IB, one was found to be related by PFGE but different by IB, and one was found to be different by PFGE but identical by IB. We believe that these six pairs most likely represented multiple strains or clones affecting these six patients. Such a phenomenon has been demonstrated previously among recurrently bacteremic or long-term-carrier patients with methicillin-susceptible strains of *S. aureus* typed by REAP, IB, or bacteriophage lysis pattern (9, 23).

Eighteen isolate comparisons showed REAP differences explicable by the occurrence of a single genetic event, and 17 of these demonstrated identical (14 pairs) or related (3 pairs) PFGE types and identical (16 pairs) IB types or similar (1 pair) IB subtypes. A loss or a gain of a plasmid (for 14 of the differences), or a single mutation, deletion, or insertion of

DNA within a plasmid already present (for the remaining 3 differences), occurring within the same MRSA strain or clone seemed to be the most logical explanation for these findings. However, REAP differences associated with plasmid loss or gain may create more difficulties than a limited number of band differences by PFGE or IB when comparing isolates.

In summary, the REAP, PFGE, and IB types of MRSA isolates are very stable in vivo. Unlike the case for PFGE and IB, about 10% of isolates cannot be typed by REAP. REAP is otherwise highly discriminatory. Isolates with REAP or PFGE type differences that are inexplicable by single genetic alterations are very likely to represent different strains or clones. REAP and PFGE differences that are explicable by a single genetic event do occur at apparently equivalent but low frequencies. The timing of such genetic changes or exchanges is not predictable. Consideration of this phenomena is appropriate whenever MRSA cross-infection is being assessed by either of these techniques. Typing of these isolates by a second system before assuming that they arose from different clones is advised. Because of limited genetic diversity within *S. aureus* in general and MRSA in particular, multiple typing tests may also be needed to definitively support the contention that epidemiologically related isolates are clonal and possibly cross-transmitted (3, 5, 11, 13, 14, 20).

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