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Epidemiologic Import of Tuberculosis Cases Whose Isolates Have Similar but Not Identical IS*6110* Restriction Fragment Length Polymorphism Patterns

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Isolates of *Mycobacterium tuberculosis* from patients with epidemiologic links frequently demonstrate identical IS*6110* restriction fragment length polymorphism (RFLP) patterns (i.e., RFLP clustering) because they are infected with the same strain. Uncertainty arises with isolates that differ from one another by a few IS*6110* hybridizing bands. During the period from 1 January 1996 to 31 December 1999, isolates from 585 tuberculosis (TB) cases were analyzed by RFLP, representing 98.2% of the 596 culture-positive TB cases reported in Arkansas during the study period. Of the 585 cases for which RFLP was available, 419 (71.6%) had an RFLP pattern with more than five copies of IS*6110*. Of the total 74 clusters, 48 comprised isolates with more than five copies of IS*6110* and included 164 cases. Sixty-nine isolates with more than five copies of IS*6110* comprising 16 clusters and 60 unique isolates were found to be similar to at least 1 other isolate (differing from it by one or two hybridizing bands). Among the 129 cases whose isolates were similar to other clustered or unique isolates, 16 cases were discovered with epidemiologic links: 14 (15.2%) were among the 92 cases with IS*6110* RFLP patterns similar to those in clusters, and 2 (5.2%) were among the 37 unique cases that were similar to another unique case. The isolates from the epidemiologically linked patients shared common spoligotypes; all except one case shared common polymorphic GC-rich sequence (PGRS) patterns. Of the 129 patients whose isolates differed from another by one or two hybridizing IS*6110* bands, 101 (78.3%) shared common spoligotypes and 87 (67.4%) shared common PGRS RFLP patterns.

DNA fingerprinting of *Mycobacterium tuberculosis* (MTB) strains based on the insertion element IS*6110* is an important tool in differentiating strains and studying the epidemiology of tuberculosis (TB) (1, 3, 15). The major limitation of the technique is its low discriminating power for isolates with ≤5 copies of IS*6110*. Secondary typing with the polymorphic GC-rich sequence (PGRS) and spoligotyping of isolates with ≤5 copies of IS*6110* has been the subject of previous studies (2, 4), and each has proven to be useful in identifying linked cases.

Another source of uncertainty occurs with isolates having more than five copies of IS*6110* that differ from one another by only one or two hybridizing bands. IS*6110* is an insertion element; it undergoes transposition to other sites in the genome, causing minor changes in the restriction fragment length polymorphism (RFLP) pattern. Single-nucleotide mutations in a restriction site or insertion-deletion events in DNA flanking IS*6110* can cause similar changes. Therefore, it is not surprising that serial isolates taken from a patient over time (5) or isolation of MTB from different body sites of the same patient can result in isolates in which the IS*6110* RFLP patterns differ by one or two hybridizing bands (i.e., the bands are similar but not identical). Moreover, such differences have been noted in the RFLP patterns of isolates from patients that are linked in a transmission chain (10). In the present study, the epidemiologic import of isolates with similar but not identical IS*6110* RFLP patterns (i.e., differing from one another by one or two hybridizing bands) was assessed. Moreover, secondary typing methods using pTBN12 RFLP of the PGRS and spoligotyping of the direct repeat (DR) locus were employed to determine the usefulness of these techniques in identifying patients with epidemiologic links whose MTB isolates have similar IS*6110* RFLP patterns.

MATERIALS AND METHODS

IS6110 RFLP analyses. The study period extended from 1 January 1996 to 31 December 1999. During this period, at least one isolate from each patient cultured by the mycobacteriology laboratory of the Arkansas Department of Health was analyzed by IS*6110* RFLP.

IS*6110* RFLP was performed according to the standard procedure with a PCR product complementary to the sequence on the right side of the PvuII site within IS*6110*, extending from bp 568 to 1089 (16, 17). Autoradiographs were studied by computer-assisted analysis with Whole Band Analyzer software, version 3.4 (Bioimage, Ann Arbor, Mich.). Isolates with identical RFLP patterns were a 100% match at a band deviation of 2.5%. Isolates were determined to have similar IS*6110* patterns if their RFLP patterns contained more than five hybridizing bands and differed only by one or two bands. To confirm that the cases identified by bioimage analysis were indeed similar, a second restriction digestion of DNA from each isolate having similar IS*6110* RFLP patterns was electrophoresed in adjacent lanes of an agarose gel, and the RFLP patterns were compared by visual inspection.

Defining the study group. All patients whose isolates had more than five IS*6110* hybridizing bands comprised the study group. IS*6110* RFLP patterns were defined as follows: identical (clustered), when the IS*6110* RFLP pattern was

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indistinguishable from that of another case; unique, when the IS*6110* RFLP pattern was not identical to that of any other case; or similar, when a clustered or unique IS*6110* RFLP pattern differed from that of another case by only one or two IS*6110* hybridizing bands.

Secondary typing. PGRS fingerprinting was carried out on AluI-restricted MTB DNA probed with a 3.4-kb insert of a copy of the PGRS in recombinant plasmid pTBN12, as described previously (6). PGRS analysis was performed by visual inspection of PGRS patterns in the region above 1.6 kb. Isolates with similar IS*6110* RFLP patterns were electrophoresed in adjacent lanes. Patterns that were indistinguishable by visual inspection were considered identical.

Spoligotyping the DR locus was carried out as described previously (11) with spoligotype membranes supplied by the Centers for Disease Control and Prevention and primers complementary to the ends of the 36-bp DR. Spoligotyping results were analyzed by visual inspection. Spoligotypes that matched exactly were considered identical.

Epidemiologic investigation. Detailed data on patient demographics, social history, clinical characteristics, and risk factors related to TB transmission and disease were obtained by review of the medical and public health records for all culture-confirmed TB cases. Patient contact investigation records were reviewed. All patients with culture-confirmed disease who had isolates with identical, similar, or unique RFLP patterns were interviewed with an extensive standardized questionnaire. Patients with epidemiologic links lived in the same household or shared the same indoor airspace when at least one of the patients was judged to be infectious.

RESULTS

Demographics of study population. During the study period (1 January 1996 to 31 December 1999), 772 TB cases were reported in Arkansas. A total of 685 (88.7%) patients had pulmonary TB (including pleural and miliary TB) and 224 (29.0%) had acid-fast bacillus-positive organisms in their sputum. Cavitary disease was present in 185 (23.9%) cases. The average age of the patients was 56 years; 470 (60.8%) of patients were male. Fifty-one percent of patients were white, 35.7% were black, 5.8% were Asian or Pacific Islanders, 0.4% were Native Americans, and 6.9% were Hispanic. Persons born outside the United States accounted for 72 (9.3%) of the cases. Being homeless, having resided in a nursing home, or having resided in a correctional facility accounted for 2.2, 9.5, and 6.0% of cases, respectively.

***IS6110* RFLP analysis.** Of the 772 TB cases, 596 (77.2%) were confirmed by culture. DNA from 585 (98.2%) culture-positive cases was analyzed by IS*6110* RFLP and spoligotyping. Computer-assisted analysis of the RFLP patterns identified 416 RFLP patterns. Among these 416 patterns, 342 (82.2%) were found in only one patient, and 74 (17.7%) were shared by more than one patient. Strains having more than five copies of IS*6110* accounted for 419 (71.6%) of the cases. Of the 74 clusters, 48 had more than five copies of IS*6110* and included 164 (39.1%) of those cases. Isolates in 16 clusters and 60 unique isolates having more than five copies of IS*6110* were found to be similar to an isolate from at least one other patient (differing from that isolate by one or two hybridizing bands) (Tables 1 and 2). Among the 16 clusters were isolates from 69 patients that matched those of other patients in the same cluster (Table 2). There were 23 cases whose isolates had a unique RFLP that was similar to the cases in these clusters (Table 2). There were also 37 patients with samples with unique IS*6110* RFLP patterns similar to those of another patient (Table 1).

In Tables 1 and 2, each pattern is given a number (IS*6110* RFLP patterns 1 to 31). Similar patterns are distinguished by a letter (e.g., patterns 31, 31a to 31f) (Table 2 and Fig. 1). The IS*6110* band number and RFLP pattern relationship show how samples with similar patterns are related; for example, pattern 31 has 12 bands, and pattern 31a has 13 bands and differs from pattern 31 by a shift in size of one band and the addition of one band, shown as RFLP pattern relationship 31 (+1, S1). The number of cases with an RFLP pattern indicates the number of cases whose isolates had that RFLP pattern (e.g., there were four patients who had isolates showing RFLP pattern 31 and one patient with isolates showing pattern 31a). The presence of an identical PGRS indicates the number of cases whose isolates had a matching PGRS pattern; for example, the PGRS patterns of none of the four cases with IS*6110* RFLP pattern 31 were identical; the PGRS pattern of the case with pattern 31a did not match that of the case with pattern 31. An identical spoligotype indicates the number of isolates that had a matching spoligotype; for example, two of the four cases with pattern 31 matched by spoligotype and two did not. Cases having epidemiologic links to other cases with pattern 31 were not discovered (cases with epidemiologic links to identical RFLP)

TABLE 1. Cases with unique IS*6110* RFLP patterns similar to that of another case with a unique IS*6110* RFLP pattern

RFLP pattern	No. of IS*6110* bands	RFLP pattern relationship[a]	Identical PGRS[b]	Identical spoligotypes[b]	Epidemiologic Link[b]
1	8				
1a	6	1 (−2)	N	Y	N
2	8				
2a	10	2 (+2)	N	N	N
3	9				
3a	7	3 (−2)	N	Y	N
4	10				
4a	10	4 (S1)	Y	Y	N
5	10				
5a	11	5 (+1, S1)	N	Y	Y
6	11				
6a	9	6 (−2)	Y	N	N
7	11				
7a	10	7 (−1)	N	N	N
8	11				
8a	11	8 (S1)	Y	Y	N
9	11				
9a	12	9 (S1, +1)	N	Y	N
10	11				
10a	12	10 (+1)	N	N	N
11	12				
11a	10	11 (−2)	N	N	N
12	12				
12a	11	12 (−1)	Y	N	N
13	13				
13a	13	13 (S2)	N	N	N
14	13				
14a	14	14 (+1)	N	N	N
15	14				
15a	13	15 (−1)	Y	Y	N
16	14				
16a	14	16 (S1)	N	Y	N
17	16				
17a	15	17 (−1, S1)	Y	Y	N
18	10		Y	N	N
18a	9	18 (−1)	Y	N	N
18b	11	18a (+2)	Y	N	N

[a] Pattern relationship is the number of bands (*n*) in the RFLP pattern that differ from another pattern by more IS*6110* bands (+*n*), fewer bands (−*n*), or changes in size (S*n*) of bands.
[b] Y, yes; N, no.

TABLE 2. Cases with IS*6110* RFLP patterns similar to those of cases with clustered IS*6110* RFLP patterns

RFLP pattern	No. of IS*6110* bands	RFLP pattern relationship[a]	No. of cases with RFLP pattern	Identical PGRS[b]	Identical spoligotypes[b]	Cases with similar RFLP[b]	Epidemiologic links found (identical RFLP)[b]
19	9		2	Y	Y	N	N
19a	8	19 (S1, −1)	1	N	Y	N	NA
19b	9	19 (S2)	1	N	N	N	NA
20	10		3	Y	Y	N2, Y1	N
20a	9	20 (−1, S1)	1	Y	Y	Y	NA
21	10		6	Y	Y	N	N4, Y2
21a	11	21 (+1)	1	Y	Y	N	NA
21b	9	21 (−1, S1)	1	Y	Y	N	NA
22	10		11	Y	Y	N7, Y2, U2	Y7, N2, U2
22a	11	22 (+1)	3	Y	Y	N1, Y2	N1, Y2
23	11		14	Y11, N3	Y	Y2, N12	Y9, N5
23a	12	23 (+1, S1)	1	Y	Y	Y	NA
23b	12	23 (+1)	1	Y	Y	Y	NA
24	12		4	Y3, N1	Y	N	Y2, N2
24a	11	24 (−1)	1	N	N	N	NA
25	13		2	Y	Y	Y1, N1	N
25a	13	25 (S1)	1	Y	Y	Y	NA
26	13		3	Y	Y	Y1, N2	Y2, N1
26a	12	26 (−1)	2	Y1, N1	Y	Y1, N1	Y1, N1
26b	13	26a (+1)	1	Y	Y	N	NA
26c	13	26 (S1)	1	Y	Y	N	NA
27	13		2	Y	Y	N	N
27a	14	27 (+1)	1	N	Y	N	NA
28	14		2	Y	Y	N	N
28a	14	28 (S1)	1	Y	N	N	NA
28b	14	28 (S1)	1	N	N	N	NA
28c	13	28 (−1)	2	Y	Y	N, U	NA
29	15		2	Y	Y	N	N
29a	14	29 (−1)	1	Y	Y	N	NA
30	15		7	Y	Y	N	Y2, N5
30a	15	30 (S1)	1	Y	Y	N	NA
30b	16	30a (+1, S1)	1	Y	N	N	NA
31	12		4	4N	Y2, N2	N	N
31a	13	31 (+1, S1)	1	N	N	N	NA
31b	13	31 (+1)	1	N	Y	N	NA
31c	13	31 (+1, S1)	1	N	Y	N	NA
31d	13	31 (+1)	1	N	N	N	NA
31e	13	31 (+1, S1)	1	N	Y	N	NA
31f	13	31 (+1)	1	N	Y	N	NA

[a] Pattern relationship is the number of bands (*n*) in the RFLP pattern that differ from another pattern by more IS*6110* bands (+*n*), fewer bands (−*n*), or change in size (S*n*) of bands.
[b] Y, yes; N, no; U, unknown; NA, not applicable (only one case with that pattern).

nor were links found to any cases having RFLP patterns 31a to 31f (cases with epidemiologic links to similar RFLPs). The case with RFLP pattern 31a was not linked to pattern 31 (no epidemiologic links to similar RFLP). Since there was only one case with pattern 31a, epidemiologic links to those with an identical RFLP pattern did not apply.

Epidemiologic links. Among the 164 cases with identical RFLP patterns having more than five copies of IS*6110* that were in 48 RFLP clusters, interviews with 151 (92%) patients were completed; epidemiologic links were found among 60 (39.7%) of these cases. Among the 69 isolates that were part of the 16 clusters to which other isolates had similar RFLP patterns, patient interviews were completed with 67 (97%) patients, and 27 (40.2%) were linked epidemiologically to patients whose isolates had an identical IS*6110* RFLP pattern. Among the 129 patients whose isolates were similar to others, interviews were completed for 124 (96.1%) patients, and an epidemiologic link was found in 16 (12.9%) cases, the isolates of which shared similar RFLP patterns. Epidemiologic links were discovered among 14 of the 92 (15.2%) patients with IS*6110* RFLP patterns that were similar to cases in clusters (Table 2). There were six clusters in which 23 patients had an RFLP pattern similar to that of another cluster. Epidemiologic links were discovered between 6 (26%) cases with similar RFLP patterns belonging to four of these clusters. Among the 23 isolates with unique RFLP patterns that were similar to those of 69 cases in 16 clusters, 8 (34.7%) had epidemiologic links to cases in clusters to which their RFLP patterns were similar.

There were 37 patients who had unique IS*6110* RFLP patterns that were similar to that of another patient with a unique pattern and which formed 18 groups of isolates with similar IS*6110* patterns (Table 1). Seventeen of these groups were made up of pairs of patients with similar patterns. One group was made up of three patients with unique isolates with similar patterns (Fig. 1). Among these 37 patients, epidemiologic links were discovered between 2 (5.4%) patients with similar RFLP patterns.

Secondary typing of isolates. Among the 69 clustered isolates that had an RFLP pattern similar to another isolate, 60

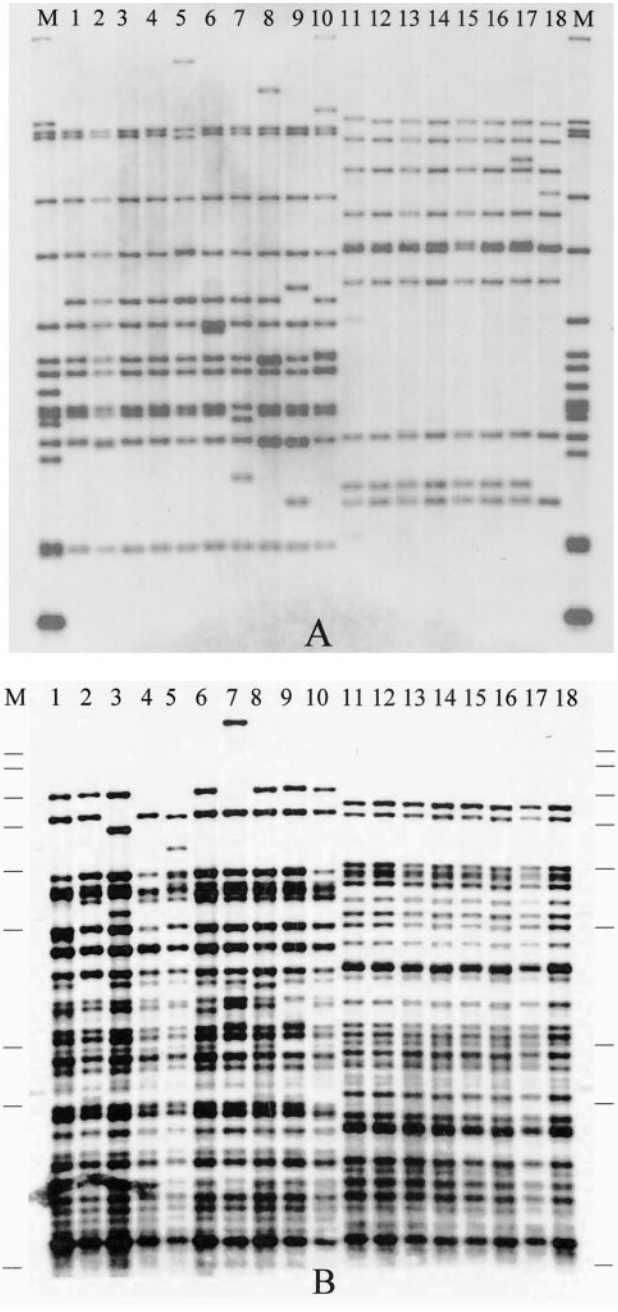

FIG. 1. Comparison of isolates from cases having identical and similar IS*6110* RFLP patterns. (A) IS*6110* RFLP; (B) PGRS RFLP. Lanes M, molecular size standards, H_{37}Rv DNA(A) or 1-kb ladder (B); lanes 1 to 4, cases with pattern 31; lane 5, pattern 31a; lane 6, pattern 31b; lane 7, pattern 31c; lane 8, pattern 31d; lane 9, pattern 31e; lane 10, pattern 31f; lanes 11 to 16, cases with pattern 21; lane 17, pattern 21a; and lane 18, pattern 21b. The spoligotypes of isolates having pattern 31 and those to which it is similar are 000777777760771, 757777777760771, 076777777760771, 777777777760731, and 777777777760771. The spoligotype of isolates having pattern 21 and those to which it is similar is 000000000003771.

(86.9%) had a PGRS pattern that was indistinguishable from the isolates to which they were similar or clustered, 67 (97%) shared spoligotypes with the isolates to which they were similar or clustered, and 60 (86.9%) matched by spoligotype and PGRS (Fig. 2). Among those isolates having different PGRS patterns, three isolates with IS*6110* pattern 23 showed a weak band that was not present in the isolates of other patients belonging to that same cluster. Distinctively different PGRS patterns were found in six isolates with indistinguishable IS*6110* RFLP patterns. Four isolates with RFLP pattern 31 each had different PGRS patterns (Table 2; Fig. 1).

Among the 60 cases with unique IS*6110* RFLP patterns that were similar to other cases, the spoligotype and PGRS matched that of the case(s) to which it was similar in 18 (30%) of the cases (Fig. 2). Among the total 129 patients with similar and identical IS*6110* RFLP patterns, 87 (67.4%) were matched by PGRS, 101 (78.2%) matched by spoligotype, and 76 (5,8.9%) matched by both spoligotype and PGRS (Fig. 2). Epidemiologic links were found in 16 patients. In 14 (87.5%) of these cases, the isolates had a PGRS pattern and a spoligotype identical to that of the related case(s) (Fig. 2). Among the 108 patients whose isolates had a similar RFLP pattern but in which no epidemiologic links were discovered, 62 (57.4%) shared an identical PGRS pattern and spoligotype (Fig. 2).

DISCUSSION

Previous studies have demonstrated the inability of RFLP to discriminate strains of MTB with fewer than five copies of IS*6110* (4, 6). The sites of IS*6110* insertion are highly conserved among strains with less than five copies of IS*6110* (8). Moreover, in studies of serial isolates collected from patients over a period of time, changes in IS*6110* RFLP pattern were significantly associated with strains having more than five copies of IS*6110* but not in low-copy-number isolates (19). To identify patients who were infected with closely related organisms more accurately, the present study was restricted to MTB strains with more than five copies of IS*6110*.

Several factors are known to account for changes in the size of restriction fragments. These include single-nucleotide mutations that create a new site or that lead to loss of a preexisting site and insertions, duplications, inversions, or deletions that cause changes in the size of restriction fragments. In regard to IS*6110* RFLP pattern, changes can also be accounted for by transposition of the insertion sequence itself. Although the IS*6110* RFLP pattern is sufficiently stable to enable inferences to be drawn concerning linking of patients in a transmission chain, minor changes in the IS*6110* pattern have been observed.

Minor changes in the IS*6110* RFLP pattern, such as the addition or loss of a fragment containing a copy of the insertion element or a change in the size of a fragment, occur very rarely during serial cultivation of MTB (18); commonly encountered laboratory strains like H37Rv do show minor differences among laboratories (12). IS*6110* RFLP patterns of *Mycobacterium bovis* BCG strains Brazil, Russia, and Japan have two copies of IS*6110*, while those of Denmark and most other BCG strains have a single copy of IS*6110* (9). Moreover, isolates of MTB isolated from different body sites of the same patient or multiple isolates from the same patient over a period of time may demonstrate minor changes in the IS*6110* RFLP pattern (5).

Several studies have estimated the rate of change in IS*6110* RFLP pattern. Changes in IS*6110* RFLP were observed in

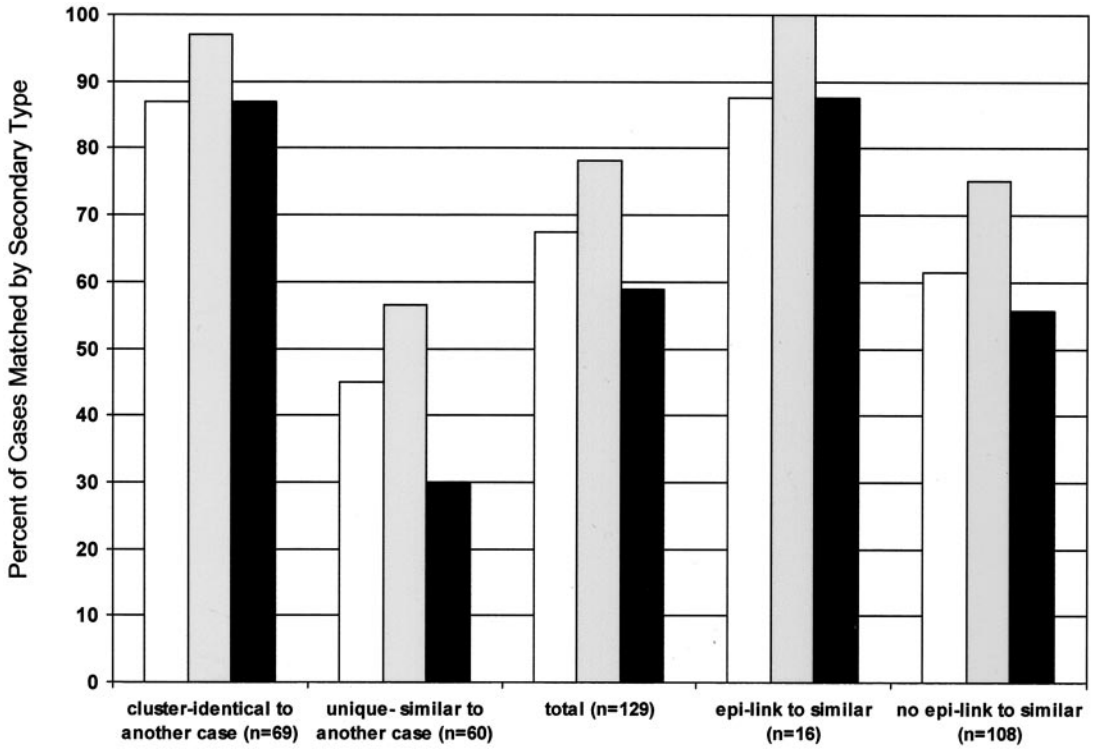

FIG. 2. Secondary typing of cases (n = 129) with similar IS*6110* RFLP patterns. The percentage of cases with a similar IS*6110* RFLP pattern that had an identical PGRS pattern (white bars), an identical spoligotype (gray bars), or matched by both PGRS and spoligotype (black bars) is indicated. epi-link, epidemiologic link.

sequential isolates from 12 of 49 (24%) patients (21). The collection dates for the sputum cultures were separated by at least 90 days. The changes were found in isolates containing 8 to 14 copies of IS*6110*. In another study, sequential isolates from 56 patients were analyzed, and isolates from 5 (9%) patients underwent change (13). In a larger study, follow-up isolates differed from initial isolates in 25 of 544 (4.6%) cases, and the half-life of an IS*6110* RFLP pattern was estimated to be approximately 3.2 years (7). Other studies of IS*6110* transposition in serial isolates from patients, estimate the half-life for a typical strain of *M tuberculosis* with 10 copies of IS*6110* to be 2.1 years (14).

Changes in IS*6110* patterns were observed in serial isolates from patients in 4% of the cases in an area with a high incidence of TB (20). A half-life of 8.74 years was estimated. This estimate may be composed of a high rate of change in early phase of disease in which the estimate was 0.57 years and a low rate of change in late disease when the half-life was estimated to be approximately 10.69 years. The differences in rate may be explained by active bacterial replication prior to therapy and a much slower rate of replication during or after therapy (20).

To estimate the rate of change during transmission of disease, changes in IS*6110* genotype were estimated in epidemiologically linked patients. The minimum rate of appearance of variant strains was estimated to be 0.14 variants per source case (20). Although not quantified, incidents of transmission of MTB within clusters of patients who shared similar but not identical IS*6110* RFLP patterns have been used to discover epidemiologic links missed during routine TB contact investigations (10).

In conclusion, one increases the number of meaningful epidemiologic links among cases by approximately 50% when one considers cases whose IS*6110* RFLP patterns are similar but not identical to those in clusters. The lower frequency of epidemiologic links among patients with similar patterns may be related to the fact that the half-life of the IS*6110* RFLP pattern is estimated to be 2 to 3 years, so that transmission between patients having similar RFLP patterns is more likely to have been remote rather than recent. It follows that epidemiologic links are more readily discovered among cases that recently transmitted disease and have identical RFLP patterns than among those who were linked in the remote past and whose RFLP patterns may have begun to diverge. Epidemiologic links are also to be discovered among cases with similar unique IS*6110* RFLP patterns, but the yield of linked cases is much less than that among those similar to clustered isolates (5.2 versus 15.2%). Among all patients with isolates that are similar to unique or clustered cases, including some that share identical IS*6110* RFLP patterns, less than 60% matched by both spoligotype and PGRS. In cases that have similar IS*6110* RFLP patterns and in which epidemiological links have been found approximately 90% matched by spoligotype and PGRS RFLP, indicating that secondary typing with both procedures can be useful in restricting the number of cases with similar IS*6110* RFLP patterns that need be investigated for epidemiologic links.

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