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Chromosomal DNA Fingerprint Patterns Produced with IS*6110* as Strain-Specific Markers for Epidemiologic Study of Tuberculosis

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Mycobacterium tuberculosis **isolates were studied by comparing chromosomal DNA fingerprint patterns produced by digestion of chromosomal DNA with *Bam*HI, followed by agarose electrophoresis and hybridization with radiolabeled probes of insertion sequence IS*6110*. DNA fingerprints of 14 isolates from separate members of five households or closely associated individuals were compared. Marked differences were observed when unrelated isolates were compared. There were no or minimal differences in the restriction fragment patterns generated from isolates of any one household or associated group. Among related isolates, the only noticeable difference was an additional fragment of IS*6110* in the fingerprint pattern of one isolate. Insertional activity was also suggested when restriction fragment patterns of H37Rv DNA isolated in 1987 and 1990 were compared. In a similar manner, *M. tuberculosis* reference strain Erdman was compared to a clinical isolate from an individual working with that strain. These isolates had identical DNA fingerprints which were distinct from all other isolates, verifying laboratory-acquired infection. Chromosomal DNA fingerprint patterns produced with IS*6110* are excellent strain-specific markers for the epidemiologic study of tuberculosis.**

Epidemiological methods used to study tuberculosis have been limited in effectiveness. Tracing a particular strain of *Mycobacterium tuberculosis* as it moves through a susceptible population group has been almost impossible, since in most cases the strain of interest cannot be distinguished from other strains. The earliest methods of strain comparison relied upon antibiotic sensitivity patterns, but this approach is very limited since so many strains show identical sensitivity patterns. Bacteriophage typing has also been used, but this technique is not definitive since most strains are grouped into only a few distinct phage types.

With advances in molecular biology, strain-specific epidemiologic studies of tuberculosis are becoming available. Recently, Cave et al. described a DNA fingerprinting technique which uses probes of the insertion sequence IS*6110* to detect differences in genomic digests of *M. tuberculosis* strains (1). IS*6110* was conserved in all of the *M. tuberculosis* strains studied and was usually present in high copy numbers. The chromosomal fingerprint patterns were obtained by digesting genomic DNA with *Bam*HI, separating the fragments by agarose gel electrophoresis, transferring the DNA fragments to a nylon membrane, and hybridizing the membrane with cloned DNA segments representing two different portions of IS*6110*. The IS*6110* probes hybridized to all of the *M. tuberculosis* strains studied (six clinical isolates and two reference strains). The number and size of restriction fragments hybridizing with the IS*6110* probes varied such that no two strains analyzed produced identical patterns. Hermans et al. used similar methods to compare restriction digests of clinical *M. tuberculosis* isolates (6). One epidemiologically related group of isolates produced identical DNA fingerprints, while unrelated strains had dissimilar patterns. Otal et al. reported that the DNA fingerprints from *M. tuberculosis* isolates obtained after relapse of tuberculosis were identical to those of the originally cultured organism (7).

The purpose of our study was to examine extensively the use of chromosomal DNA fingerprint patterns as strain-specific markers and then demonstrate their usefulness in the epidemiologic study of tuberculosis.

MATERIALS AND METHODS

Bacterial isolates. Clinical isolates were obtained from the microbiology laboratories of the University of Texas Health Science Center at Tyler, the Alabama State Health Department, and the Arkansas State Health Department. These isolates were cultured from members of five households or closely associated individuals. Clinical and epidemiologic information about these isolates (designated Tb-1 through Tb-14) are summarized in Table 1. Each isolate was received on a Lowenstein-Jenson slant, grown in Proskauer-Beck broth supplemented with Dubos oleic albumin complex, and then stored at −70°C until studied. Additionally, *M. tuberculosis* reference strain Erdman and a clinical isolate (Tb-15) recovered from an individual working with this strain were obtained from a reference laboratory. *M. tuberculosis* H37Rv was originally obtained from the Trudeau collection and grown continuously in broth culture. DNA was isolated in 1987 and again in 1990 from samples of this continuously growing culture.

Isolation of mycobacterial DNA. Mycobacteria were cultured in Proskauer-Beck broth supplemented with Dubos oleic albumin complex. When the optical density exceeded 1, 1 mg of D-cycloserine per ml and 0.1 mg of ampicillin per ml were added to assist in lysis (4). After 16 h, the cells were harvested by centrifugation and lysed with lysozyme and sodium dodecyl sulfate at 55°C. The chromosomal DNA was isolated and concentrated on cesium chloride gradients containing a small amount of ethidium bromide. After extraction with *n*-butanol and exhaustive dialysis, the DNA

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TABLE 1. Clinical and epidemiologic information

Isolate	Cluster code	Original culture date	Age/sex[a]	Relationship[b]	X-ray finding
Tb-1	A	Sept. 1989	10 mo/F	Daughter of [Tb-2]	Noncavitary
Tb-2	A	Sept. 1989	33 yr/F	Mother of [Tb-1]	Cavitary
Tb-3	B	July 1989	36 yr/M	Boyfriend of mother of [Tb-4]	Cavitary
Tb-4	B	July 1989	17 yr/F	Daughter of girlfriend of [Tb-3]	Noncavitary
Tb-5	B	July 1989	16 mo/F	Daughter of [Tb-4]	Miliary
Tb-6	B	July 1989	4 yr/F	Niece of [Tb-4]	Noncavitary
Tb-7	B	July 1989	35 yr/M	Friend of [Tb-3]	Cavitary
Tb-8	B	Aug. 1989	48 yr/F	Friend of [Tb-3]	Noncavitary
Tb-9	C	Nov. 1989	59 yr/F	Wife of [Tb-10]	Noncavitary
Tb-10	C	Dec. 1989	59 yr/M	Husband of [Tb-9]	Cavitary
Tb-11	D	April 1989	44 yr/M	Brother of [Tb-12]	Cavitary
Tb-12	D	May 1989	38 yr/M	Brother of [Tb-11]	Cavitary
Tb-13	E	April 1989	28 yr/M	Friend of [Tb-14]	Cavitary
Tb-14	E	April 1989	23 yr/M	Friend of [Tb-13]	Noncavitary

[a] F, female; M, male.
[b] Brackets indicate the person from whom the isolate in parentheses was cultured.

was precipitated with ethanol, suspended in TE buffer (0.01 M Tris-HCl, 0.001 M EDTA, pH 8), and stored at 4°C.

Restriction analysis. Mycobacterial DNA was digested with restriction enzymes under conditions specified by the manufacturer. Five to 10 U of enzyme per μg of DNA was incubated at 37°C overnight. Digested DNA was electrophoresed on 0.8% agarose gels containing ethidium bromide. DNA fragments were transferred from the gel to GeneScreen Plus membranes (Dupont, NEN Products, Boston, Mass.) by alkaline capillary transfer (2). The membranes were hybridized overnight with labeled probe in 1 M NaCl–1% sodium dodecyl sulfate at 68°C. They were washed twice with 2× SSC (0.3 M sodium chloride, 0.03 M sodium citrate) at room temperature for 5 min, twice with 2× SSC–0.1% sodium dodecyl sulfate at 68°C for 30 min, and twice with 0.1× SSC at room temperature for 30 min. Autoradiographs were prepared by exposing the hybridized blots for various times at −70°C to Kodak X-Omat AR film and Cronex Lightning-Plus intensifying screens.

DNA probes. Two DNA probes were used to produce chromosomal DNA fingerprint patterns. These probes have been described previously (1) and are referred to as the *Sst*II fragment of pDC51 and the *Bam*HI-*Sal*I fragment of pDC73. They recognize IS*6110* on either side of its single *Bam*HI restriction site. The probes were labeled with [^{32}P]dCTP (3,000 mCi/mmol) by the random primer method of Feinberg and Vogelstein (5).

RESULTS

Six distinct DNA fingerprint patterns were produced by each IS*6110* probe when the 16 *M. tuberculosis* isolates were studied (Fig. 1A and B). Tb-1 was isolated from a 10-month-old child with a 2-month history of low-grade fever and chest congestion. Her chest X-ray showed right middle- and lower-lobe collapse without cavitation. Tb-2 was subsequently recovered from this child's mother, a 33 year old who had bilateral cavitary pulmonary infiltrates. Tb-1 and Tb-2 produced identical DNA fingerprints (lanes 1 and 2).

Tb-3 through Tb-8 produced a second group of identical fingerprints (lanes 3 through 8), with only three fragments which hybridized to IS*6110*. Attention to this outbreak arose in July 1989, when an 18-month-old male was hospitalized with tuberculous meningitis. Although isolates from this child were not available, isolates from multiple members of his household (Tb-3 through Tb-7) and an individual with close contact (Tb-8) were recovered and submitted for DNA fingerprint comparison. Tb-3 was recovered from the boyfriend of this child's grandmother. He had extensive bilateral cavitary pulmonary disease and was felt to be the source. Tb-4 was recovered from the daughter of the source's girlfriend. Tb-5 and Tb-6 were recovered from granddaughters of the source's girlfriend. Tb-7 was recovered from a friend of the source. All of these people were close household contacts. Tb-8 was recovered from a friend and close contact of the source.

Tb-9 was recovered from the sputum of a 59-year-old woman with abdominal cramps, diarrhea, fever, and shortness of breath. Tb-10 was recovered from her asymptomatic husband, who had cavitary pulmonary infiltrates. When isolates Tb-9 and Tb-10 were restricted with *Bam*HI and hybridized with the *Sst*II fragment of pDC51, eight hybridizing fragments identical in size were seen (Fig. 1A, lanes 9 and 10). However, Tb-10 had an additional band not seen in Tb-9 (arrow). Similarly, an additional band was seen in the *Bam*HI digests of Tb-10 when it was hybridized with the *Bam*HI-*Sal*I fragment of pDC73 (Fig. 1B, lanes 9 and 10, arrow). When DNAs from these isolates were digested with *Kpn*I, an enzyme which does not cleave IS*6110*, an extra band was apparent in the Tb-10 digest (Fig. 2, lanes 1 and 2, arrow). When these DNAs were restricted with *Sst*II or *Sal*I, enzymes which cleave IS*6110* at two sites, a single band was seen in the Tb-9 digests, as expected (lanes 3 and 5). However, when restricted with the same enzymes, Tb-10 digests had an additional fragment which hybridized weakly to the probes (lanes 4 and 6, arrows). The weak hybridization of the additional fragment in the *Bam*HI digest of Tb-10 suggested that this isolate has an additional partial copy of IS*6110*. This is supported by the fact that the additional copy lacked at least one of the *Sal*I and *Sst*II recognition sites usually present in IS*6110*.

To investigate the insertional activity of IS*6110* further, DNA fingerprints of H37Rv DNA isolated in 1987 and 1990 from a continuously growing culture were compared (Fig. 3). Minor differences were encountered. Although each batch of H37Rv DNA had numerous bands identical in size, four bands were different (Fig. 3, arrows).

A fourth pattern was obtained from Tb-11 and Tb-12 (Fig. 1A and B, lanes 11 and 12), each of which demonstrated nine hybridizing bands. These isolates were obtained from a 44-year-old man and his 38-year-old brother. Both individu-

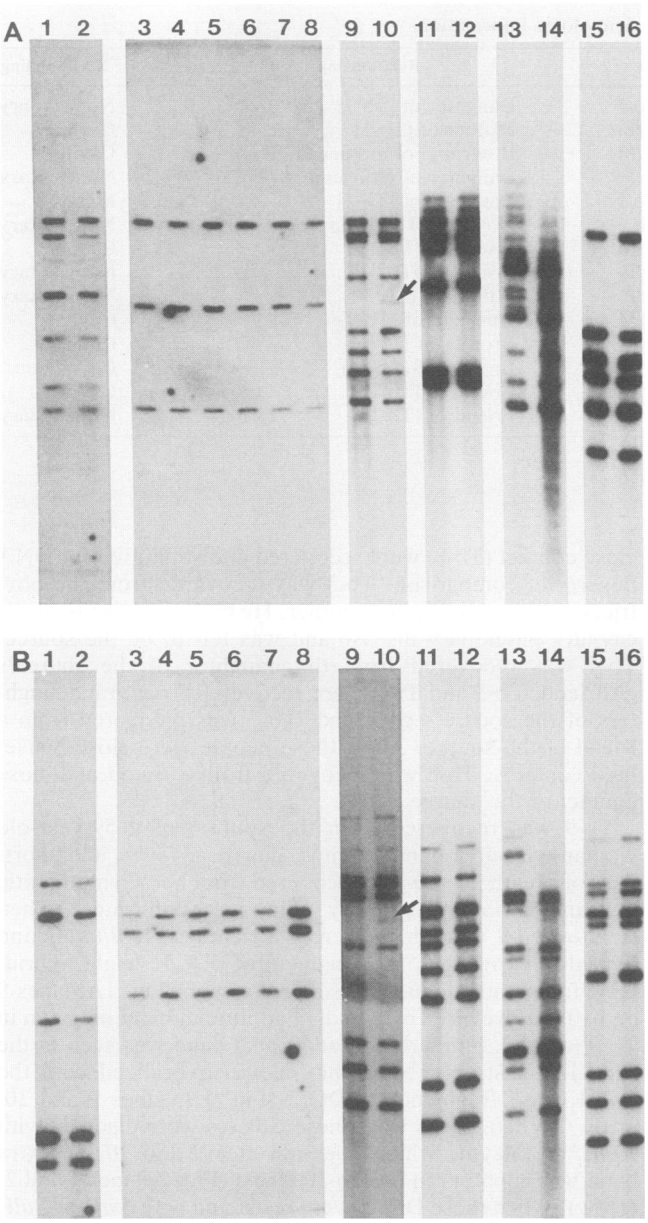

FIG. 1. Autoradiographs of Southern blots of *Bam*HI-restricted DNAs from Tb-1 and Tb-2 (lanes 1 and 2), Tb-3 through Tb-8 (lanes 3 through 8), Tb-9 and Tb-10 (lanes 9 and 10), Tb-11 and Tb-12 (lanes 11 and 12), Tb-13 and Tb-14 (lanes 13 and 14), Tb-15 (lane 15), and reference strain Erdman (lane 16) hybridized with the [^{32}P]CTP-labeled *Sst*II fragment of pDC51 (A) or the *Bam*HI-*Sal*I fragment of pDC73 (B). The arrows indicate an additional band in Tb-10 not present in Tb-9.

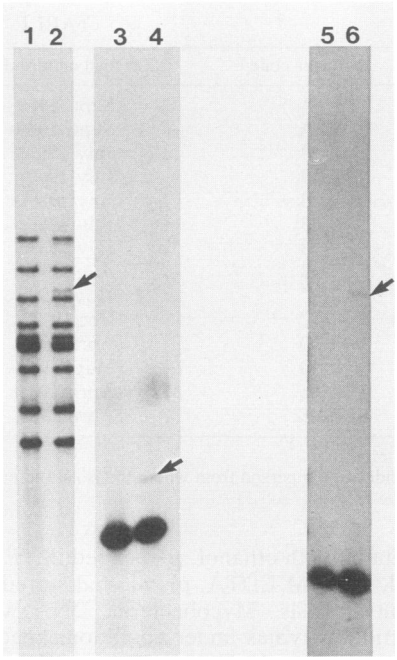

FIG. 2. Autoradiograph of Southern blots of DNAs from isolates Tb-9 (odd-numbered lanes) and Tb-10 (even-numbered lanes) restricted with *Kpn*I (lanes 1 and 2), *Sst*II (lanes 3 and 4), and *Sal*I (lanes 5 and 6) and hybridized with the [^{32}P]CTP-labeled *Sst*II fragment of pDC51 (lanes 1 through 4) or the *Bam*HI-*Sal*I fragment of pDC73 (lanes 5 and 6). The arrows indicate an additional band in Tb-10 not present in Tb-9.

als had pulmonary symptoms and cavitary pulmonary infiltrates. The 38-year-old man had been diagnosed with sarcoidosis 1 year earlier and had chronic renal insufficiency and diabetes mellitus.

Tb-13 was recovered from a 28-year-old Vietnamese man during contact screening after his child was diagnosed with primary tuberculosis. His chest X-ray showed a right-sided cavitary infiltrate. Tb-14 was recovered from a close friend of the family and frequent visitor. His chest X-ray revealed a left noncavitary infiltrate and adenopathy. A fifth unique DNA fingerprint pattern was produced by Tb-13 and Tb-14 (lanes 13 and 14).

Tuberculosis reference strain Erdman and clinical isolate Tb-15 produced a sixth distinctive DNA fingerprint pattern (lanes 15 and 16). Tb-15 was cultured from a individual who worked with *M. tuberculosis* reference strain Erdman in a laboratory.

DISCUSSION

In this study, we demonstrated the use of chromosomal fingerprint patterns as strain-specific markers in the epidemiologic study of tuberculosis. We studied 14 *M. tuberculosis* isolates from five groups of closely associated individuals. Each IS*6110* probe produced five distinct sets of fingerprint patterns, each differing in the number and size of hybridizing fragments (bands). *M. tuberculosis* isolates recovered from closely associated individuals showed identical or nearly identical DNA fingerprint patterns. Epidemiologically unrelated isolates were markedly different.

The possibility of simple geographic clustering of strains with specific fingerprint patterns is considered unlikely. The *M. tuberculosis* isolates from clusters A, B, and C were recovered from individuals living within a 150-mile (242-km) radius, yet each cluster had a distinct fingerprint pattern. The four isolates from clusters D and E were collected within a 20-mile (32-km) radius of each other and also had distinct fingerprint patterns. No two random tuberculosis isolates from these areas had identical DNA fingerprint patterns. Additionally, investigators previously have demonstrated extensive differences in the DNA fingerprint patterns of isolates from specific geographic locales (1, 6).

The presence of only three hybridizing bands in the

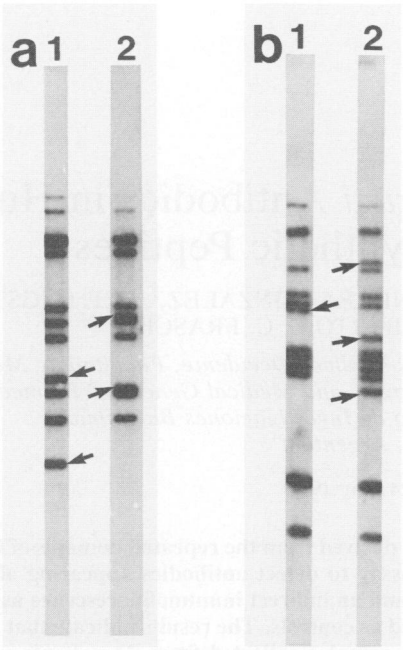

FIG. 3. Autoradiograph of Southern blots of H37Rv DNA isolated in 1987 (lanes 1) and 1990 (lanes 2) hybridized with the [^{32}P]CTP-labeled *Sst*II fragment of pDC51 (a) or the *Bam*HI-*Sal*I fragment of pDC73 (b). The arrows indicate bands that differed between the isolates.

fingerprint of the second group of *M. tuberculosis* isolates is unusual. In our experience, this is more typical of *M. bovis*. Repeated biochemical studies in multiple laboratories confirmed that these isolates were *M. tuberculosis*.

We applied the experience gained from studying these 14 *M. tuberculosis* isolates to the documentation of laboratory-acquired infection. The DNA fingerprint pattern of an isolate recovered from a laboratory worker was identical to that obtained from the reference strain with which he was working. These isolates had a DNA fingerprint which was distinct from all others, thus verifying laboratory-acquired infection.

Of interest is the pair of isolates from a woman and her husband which have identical DNA fingerprints except for a single band. This band appears to represent an additional fragment of IS*6110*, suggesting an insertional event. Although IS*6110* has characteristics of a functional transposable element, rearrangements associated with IS*6110* have not been previously described. Further support for insertional activity of IS*6110* is provided by comparing the DNA fingerprints of H37Rv DNA isolated in 1987 and 1990. These fingerprints are similar, with conservation of most of the hybridizing fragments, yet four fragments were different.

For chromosomal DNA fingerprinting to be useful in epidemiologic studies of tuberculosis, a certain rate of genetic alteration is necessary. If there were no or only minimal alteration in the mycobacterial genome, as is suggested for *M. leprae* (3, 8), the DNA fingerprint patterns of all strains would be identical. Obviously, this is not the case with *M. tuberculosis*. Significant differences in restriction fragment patterns of unrelated *M. tuberculosis* isolates have been reported and were observed by us (1, 6). Additionally, the rate of genetic alteration must not be excessive. If it were, DNA fingerprints of closely related (clonal) isolates would be different. Our results suggest that genetic alterations do occur in *M. tuberculosis* but not at an excessive rate in that DNA fingerprint patterns of clonal *M. tuberculosis* isolates are identical or, at most, minimally different.

In summary, IS*6110* appears to be a functional insertion sequence which is stable enough to allow precise epidemiologic investigation. Chromosomal DNA fingerprint patterns generated with probes of IS*6110* show great promise as epidemiologic tools in the study of tuberculosis.

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