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Usefulness of the Secondary Probe pTBN12 in DNA Fingerprinting of *Mycobacterium tuberculosis*

FERNANDO CHAVES,[1,2,3] ZHENHUA YANG,[1,2] HIYAM EL HAJJ,[1,2] MERCEDES ALONSO,[3] WILLIAM J. BURMAN,[4] KATHLEEN D. EISENACH,[1,2] FERNANDO DRONDA,[3] JOSEPH H. BATES,[1,5] AND M. DONALD CAVE[1,6]*

Mycobacteriology Research Laboratory[1] and Departments of Pathology,[2] Medicine,[5] and Anatomy,[6] University of Arkansas for Medical Sciences and John L. McClellan Memorial Veterans Hospital, Little Rock, Arkansas 72205; Department of Clinical Microbiology and Infectious Diseases, Hospital General Penitenciario, 28044 Madrid, Spain[3]; and the Department of Public Health, Denver Health and Hospitals and the Department of Medicine, University of Colorado Health Sciences Center, Denver, Colorado[4]

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A comparison was made between DNA fingerprints of *Mycobacterium tuberculosis* produced with the insertion sequence IS*6110* and those produced with the polymorphic GC-rich repetitive sequence contained in the plasmid pTBN12. A total of 302 *M. tuberculosis* isolates from the prison system in Madrid, Spain, and the Denver Public Health Department (Denver, Colo.) were analyzed with the two probes. Both probes identified the same isolates in the same clusters when the fingerprints had six or more copies of IS*6110*. Analysis of isolates with unique IS*6110* fingerprints demonstrated that they were unique with pTBN12. The pTBN12 probe had greater discriminating power in isolates having five or fewer copies of IS*6110*. Forty-seven isolates from Denver having fewer than five copies of IS*6110* which were grouped in 11 clusters with identical fingerprint patterns were subdivided into 35 different patterns by pTBN12. Isolates with IS*6110* fingerprints with more than six copies of IS*6110* that differed from one another by only one or two hybridizing bands were analyzed with pTBN12. Most of these sets of isolates demonstrated identical patterns with pTBN12. However, some exceptions were observed, suggesting that those having nearly identical IS*6110* patterns should not necessarily be included in the same cluster. Since IS*6110* provides more polymorphism in the fingerprint, it is most useful in identifying isolates with unique fingerprint patterns and those in clusters in which the isolates contain six or more copies of the insertion. However, it is necessary to employ a secondary probe, such as pTBN12, to discriminate isolates with five or fewer copies of IS*6110* and those with similar but not identical IS*6110* patterns.

Analysis of restriction fragment length polymorphism (RFLP) based on the insertion sequence IS*6110* is a powerful epidemiologic tool for differentiating strains of *Mycobacterium tuberculosis* (2, 9, 14). In most strains, the insertion sequence is present in multiple copies which are distributed throughout the *M. tuberculosis* genome. Restriction of purified *M. tuberculosis* DNA with an appropriate restriction enzyme produces fragments of various lengths which are separated by electrophoresis and hybridized to a segment of the insertion element to produce strain-specific fingerprint patterns. Widespread application of a standardized *M. tuberculosis* DNA fingerprinting procedure has led to important advances in the understanding of the epidemiology of tuberculosis (1, 8, 16, 18, 19). However, isolates of *M. tuberculosis* which possess one or two copies of IS*6110* do not generate sufficient polymorphism to be readily distinguished by this technique (25). Furthermore, a few strains of *M. tuberculosis* lack IS*6110* and therefore cannot be fingerprinted by this technique (24, 26).

Another unanswered question concerns strains of *M. tuberculosis* which have more than five copies of IS*6110* with almost identical fingerprint patterns, except for one or two hybridizing bands. Are such strains closely related, and, if so, should they be grouped in the same cluster or should they be considered unique when they are compared for epidemiologic purposes? In some cases, epidemiologic links among patients infected with such strains have been established (20). Failure to discriminate among such strains would lead to misinterpretation of the extent of transmission of infection in a community.

A second probe, which is referred to as pTBN12, has been found to be useful for fingerprinting mycobacteria (17). This recombinant plasmid contains an insert characterized as a polymorphic GC-rich repetitive sequence. The polymorphic GC-rich repetitive sequence is a short sequence which is repeated multiple times in the genomes of *M. tuberculosis* and other mycobacteria. It has proven useful in distinguishing strains of *Mycobacterium bovis* which usually contain only a few copies of IS*6110* (6).

The purpose of this study was to determine the extent of correlation among isolates of *M. tuberculosis* fingerprinted with IS*6110* and pTBN12. We analyzed isolates of *M. tuberculosis* having from 1 to 20 copies of IS*6110* with both probes and determined the minimum number of copies of IS*6110* necessary for maximum discrimination among strains. In addition, we investigated the usefulness of pTBN12 for distinguishing isolates of *M. tuberculosis* which differed by only one or two IS*6110* hybridizing bands.

MATERIALS AND METHODS

***M. tuberculosis* strains.** *M. tuberculosis* isolates from the Department of Public Health (Denver, Colo.) isolated between 1988 and 1994 and from the prison system in Madrid, Spain, isolated between 1993 and 1994 were fingerprinted. All isolates were identified as *M. tuberculosis* in the originating laboratories and were submitted for DNA fingerprinting analysis.

RFLP analysis. The isolates were grown in Dubos medium with albumin for 2 or 3 weeks, inactivated by heating (80°C for 30 min), and harvested by centrifugation, and the bacilli were lysed with lysozyme and sodium dodecyl sulfate

* Corresponding author. Mailing address: VA Hospital, Room GB-126, 4300 West 7th St., Little Rock, AR 72205. Phone: (501) 661-1202, ext. 2981. Fax: (501) 664-6748.

(SDS). The lysates were purified with cetyltrimethylammonium bromide, and genomic DNA was recovered by ethanol precipitation (15).

DNA fingerprinting with IS*6110* was performed according to a standardized procedure (23), in which 1 μg of DNA was restricted with *Pvu*II and hybridized to a ^{32}P-labeled 522-bp IS*6110* fragment generated by PCR (22). *Pvu*II-digested chromosomal DNA of *M. tuberculosis* H37Rv containing 14 copies of IS*6110* was used as a standard marker. To cover a wider range of molecular sizes, two additional DNA fragments of 13.5 kb and 690 bp were added to the standard marker. The 13.5-kb fragment is a linearized plasmid, pJC110 (provided by Jack Crawford, the Centers for Disease Control), containing the IS*6110* sequence, and the 690-bp fragment is PCR derived and contains a part of IS*6110*.

For RFLP analysis with pTBN12, DNA was restricted with *Alu*I and electrophoresed for 21.5 h at 50 V on a 0.7% agarose gel. A 1-kb DNA ladder (Gibco, Gaithersburg, Md.) or λDNA restricted with *Hin*dIII was used as a marker. The plasmid pTBN12 was a gift of Bruce Ross (Fairfield Infectious Disease Hospital, Fairfield, Australia). The probes pTBN12 and the 1-kb ladder or λDNA were labeled with [α-^{32}P]dCTP according to the random primer method (10). The membranes were hybridized overnight in 5 M NaCl–10% SDS at 68°C, and washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature for 5 min, twice with 2× SSC–1% SDS at 68°C for 30 min, and twice with 0.1× SSC at room temperature for 30 min. Autoradiographs were obtained by exposing the hybridized blots at −70°C to Kodak X-Omat AR film.

Analyses of RFLP patterns. Membranes hybridized with the IS*6110* probe were exposed on a PhosphorImager cassette and scanned with Image Quant software (Molecular Dynamics, Sunnyvale, Calif.). The fingerprint patterns of the isolates were analyzed and compared by computer-assisted analyses with Whole Band Analyzer version 3.3 software (Bioimage, Ann Arbor, Mich.). The sizes of hybridizing bands on each image were determined against three lanes of standard reference ($H37R_v$ DNA) on the same image. Patterns were matched by the average linkage clustering method. A deviation of 2.5% was used in the matching of all of the lanes. Patterns that had a 100% match were defined as identical.

To facilitate the visual comparison of pTBN12 fingerprints, samples were electrophoresed together on the same gel according to results previously obtained with IS*6110*. When necessary, a computer-assisted analysis was performed to compare results from different membranes. For pTBN12 fingerprinting, only hybridizing fragments of greater than 1.6 kb were analyzed.

With both probes, patterns were considered identical when they had the same numbers and sizes of bands. Isolates were placed in a cluster when they had identical fingerprints.

RESULTS AND DISCUSSION

The samples used in this study originated from two different epidemiologic environments. One set originated from the city of Denver, which has a low prevalence of tuberculosis (approximately 3 cases per 100,000 [4]), and the other originated from the prison population of Madrid, Spain, which has a much higher prevalence of tuberculosis (1,170 per 100,000) (5). Among persons in Denver, one would expect the range of patient contacts to be quite broad, while among the prisoners the range of contacts would be very restricted. Thus, the two populations provide different epidemiologic paradigms.

pTBN12 fingerprinting of strains with identical IS*6110* patterns. Eighty-two isolates of *M. tuberculosis* from Denver which were grouped in 22 clusters according to their IS*6110* fingerprint patterns were fingerprinted with the pTBN12 probe, and the results are recorded in Table 1. Strains within clusters having four or fewer copies of IS*6110* could be readily distinguished with pTBN12 (Fig. 1). Eleven clusters with 4 or fewer copies of IS*6110* were divided into 35 clusters with the pTBN12 fingerprint (Table 1; Fig. 1), whereas strains in 11 clusters having 6 to 20 copies of IS*6110* demonstrated no additional subdivision after fingerprinting with pTBN12.

A total of 142 *M. tuberculosis* isolates from the Madrid prison system which segregated into 23 different clusters on the basis of their IS*6110* patterns demonstrated 25 pTBN12 patterns (Table 2). A cluster of isolates containing five copies of IS*6110* was subdivided into three pTBN12 patterns. Clusters with six or more copies of IS*6110* failed to show any further subdivision after fingerprinting with pTBN12 (Table 2; Fig. 2).

Previous studies have reported that *M. tuberculosis* strains with a single IS*6110* copy of the same size were indistinguishable with the IS*6110* probe (24). In these cases, the pTBN12 and the direct repeat probes allowed differentiation of these strains. Hermans et al. (13) have shown that 16 Ethiopian isolates with one IS*6110* copy were differentiated into 12 pTBN12 and 8 direct repeat types, and 17 isolates with a two-copy IS*6110* pattern were divided into 5 pTBN12 and 3 direct repeat types. The lack of polymorphism among isolates with one or two IS*6110* copies was thought to be due to a site-specific preference for insertion of the insertion element (12). In the present study, 29 isolates with one or two copies of IS*6110* grouped into 22 pTBN12 patterns. Furthermore, *M. tuberculosis* isolates showing seven different patterns with three to five IS*6110* copies yielded 16 different pTBN12 types (Tables 1 and 2). The decrease in IS*6110* polymorphism among these isolates can be explained by the findings of Fomukong et al., which suggest that the sites of IS*6110* insertion are highly conserved in strains having only a few copies of IS*6110* (11). This lack of polymorphism can be misleading. Templeton et al. (21) demonstrated a lack of epidemiologic links among patients in clusters having fewer than five IS*6110* copies compared with patients in clusters with isolates having more than five copies, many of whom had epidemiologic links. In Denver, isolates with five or fewer copies of IS*6110* constituted 42% of the isolates from cases having identical IS*6110* patterns (Table 1) or 31% of the 188 cases with unique and clustered fingerprints collected over a 5-year period (data not shown).

TABLE 1. pTBN12 fingerprint patterns of *M. tuberculosis* isolates from Denver having identical IS*6110* patterns

No. of IS*6110* copies	No. of isolates	No. of IS*6110* patterns	No. of pTBN12 patterns
1	12	3	10
2	17	2	12
3	13	4	8
4	5	2	5
6	13	1	1
9	4	2	2
10	4	2	2
12	4	2	2
13	3	1	1
17	2	1	1
19	3	1	1
20	2	1	1
Total	82	22	46

These results have important implications for future epidemiologic studies, since isolates with five or fewer copies showing the same IS*6110* pattern should not be grouped in a cluster when there is a lack of strong epidemiologic support. For these isolates, the pTBN12 probe demonstrates sufficient polymorphism to be used for secondary fingerprinting. When the fingerprints had six or more copies of IS*6110*, the pTBN12 probe demonstrated identical groupings of patterns. This observation reinforces the usefulness of the IS*6110* probe with these isolates and adds weight to the interpretation that these isolates are a true cluster, with consequent epidemiologic implications.

pTBN12 fingerprinting of strains with similar IS*6110* patterns. Thirty-three isolates with six or more copies of IS*6110* were analyzed to determine to what extent isolates with similar but not identical IS*6110* patterns were related by pTBN12. These isolates were from the same number of patients with tuberculosis incarcerated in seven prisons of Madrid. The isolates were divided into three groups according to their IS*6110* banding patterns. In some cases, some isolates were included in more than one group. Group A was composed of isolates with identical fingerprints except for the addition of a single

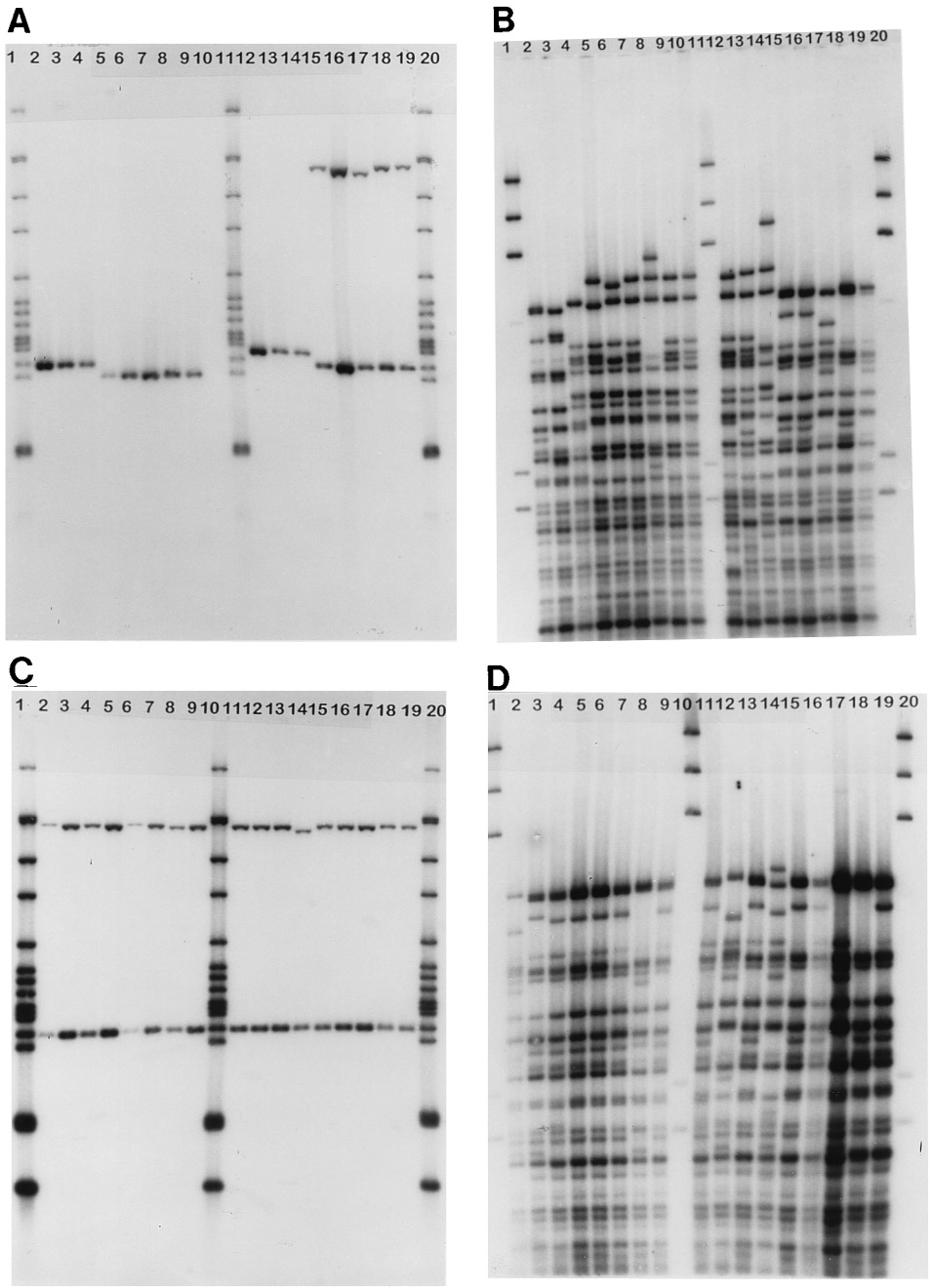

FIG. 1. Genomic blots of *M. tuberculosis* DNA isolates from Denver with one or two copies of IS*6110*. (A and C) *Pvu*II-restricted DNA probed with [α-^{32}P]dCTP-labeled IS*6110*; (B and D) *Alu*I-restricted DNA from the same isolates probed with [α-^{32}P]dCTP-labeled pTBN12. Lanes 2 to 10 and 12 to 14 (A) show isolates having one copy of IS*6110*. These isolates are resolved into 10 subgroups with pTBN12 (B). Lanes 2 to 9 and 11 to 19 (C) show 17 isolates containing two copies of IS*6110*. These isolates are resolved into 9 subgroups with pTBN12 (D). Lanes 1, 11, and 20 (A and B) and lanes 1, 10, and 20 (C and D) contain DNA markers. In panels A and C, *Pvu*II-restricted H37Rv DNA probed with [α-^{32}P]dCTP-labeled IS*6110* gave the following sizes (top to bottom): 13.5, 5, 4.8, 3.5, 2.8, 2.2, 1.9, 1.8, 1.7, 1.61, 1.58, 1.54, 1.4, 1.3, 0.9, and 0.7 kb; in panels B and D, *Hin*dIII-restricted bacteriophage λDNA probed with [α-^{32}P]dCTP-labeled bacteriophage λ gave the following sizes (top to bottom): 23, 9.4, 6.5, 2.3, and 2 kb.

hybridizing fragment. There were 25 such strains in 14 combinations of pairs having 6 to 17 copies of IS*6110*. The sizes of the additional band ranged from 1 to 4.7 kb. RFLP analysis with pTBN12 yielded identical patterns for each pair, with the exception of two isolates having 9 to 10 hybridizing IS*6110* bands, respectively. Group B was composed of isolates with identical fingerprints except for a change in the size of a single hybridizing fragment. There were 10 such isolates with 7 to 11 copies of IS*6110*. To analyze these isolates, they were grouped in seven pairs. Five of the seven pairs showed identical pTBN12 patterns for each paired isolate. In the remaining two pairs, each with eight copies of IS*6110*, the pTBN12 patterns were unique for the isolate in each pair. Group C was composed of isolates with identical fingerprints except for the addition of two IS*6110* bands. This group was composed of 14 isolates with 10 different IS*6110* fingerprint patterns. These

TABLE 2. pTBN12 fingerprint patterns of *M. tuberculosis* isolates obtained from Spain having identical IS*6110* patterns

No. of IS*6110* copies	No. of isolates	No. of IS*6110* patterns	No. of pTBN12 patterns
5	6	1	3
6	2	1	1
7	4	2	2
8	21	1	1
9	15	2	2
10	25	2	2
11	21	1	1
12	10	4	4
13	6	2	2
14	13	2	2
15	13	2	2
16	2	1	1
17	4	2	2
Total	142	23	25

patterns differed either by the addition of two bands, a change in the sizes of two bands, or the addition of one band a shift in the size of another. In all cases, DNA fingerprinting with pTBN12 gave the same patterns for each pair.

Most *M. tuberculosis* isolates containing six or more copies of IS*6110* whose fingerprints were very similar but not identical to each other (differing by one or two hybridizing bands) demonstrated identical pTBN12 fingerprints. Figure 3 shows the correlation between IS*6110* typing and pTBN12 typing. Figures 2A (lanes 9 to 11 and 16 to 18) and 3A (lanes 2 to 4 and 5 to 11) show isolates with IS*6110* fingerprints which differ by one or two hybridizing fragments. The same isolates probed with pTBN12 demonstrate identical fingerprints (Fig. 2B, lanes 9 to 11 and 16 to 18, and Fig. 3B, lanes 2 to 4 and 5 to 11). However, there are some exceptions, as demonstrated in Fig. 3C and D. IS*6110* patterns differing by one band, as shown in lanes 3 and 4 in Fig. 3C, show different patterns with pTBN12 (Fig. 3D, lanes 3 and 4).

These results show that the *M. tuberculosis* isolates with very similar but nonidentical IS*6110* fingerprints which originated from patients having epidemiologic links in the prison environment have the same pTBN12 fingerprint in most instances. Previous reports have shown minor alterations in the IS*6110* banding patterns of *M. tuberculosis* strains isolated from patients in clusters who had been well-studied epidemiologically (3, 13, 18). After evaluation of a cluster of tuberculosis cases among homeless patients during a 7-year period, Dwyer et al. (7) suggested that pTBN12 fingerprints are more stable than those of IS*6110*. This point was based on the observation that the isolates from these patients had identical pTBN12 patterns over a 7-year period, whereas the IS*6110* fingerprints of 4 of 27 isolates showed differences in a single hybridizing band. In general, these minor alterations might be explained by a mutation creating or eliminating a restriction site or the insertion of IS*6110* into a new site. It is interesting to note that in a large epidemiologic study, failure to confirm these minor differences might interfere with tracing specific strains. In these cases, the pTBN12 probe might be useful for confirming the IS*6110* clusters. Further studies which correlate epidemiologic data with the fingerprinting data are necessary to confirm epidemiologic links. Another question which could be addressed with such information would be whether these two probes are useful for predicting a temporal association regarding the spread of tuberculosis in a cluster and when transmission of *M. tuberculosis* takes place.

pTBN12 fingerprinting of strains which have unique IS*6110* patterns. Forty-five *M. tuberculosis* isolates from patients in the

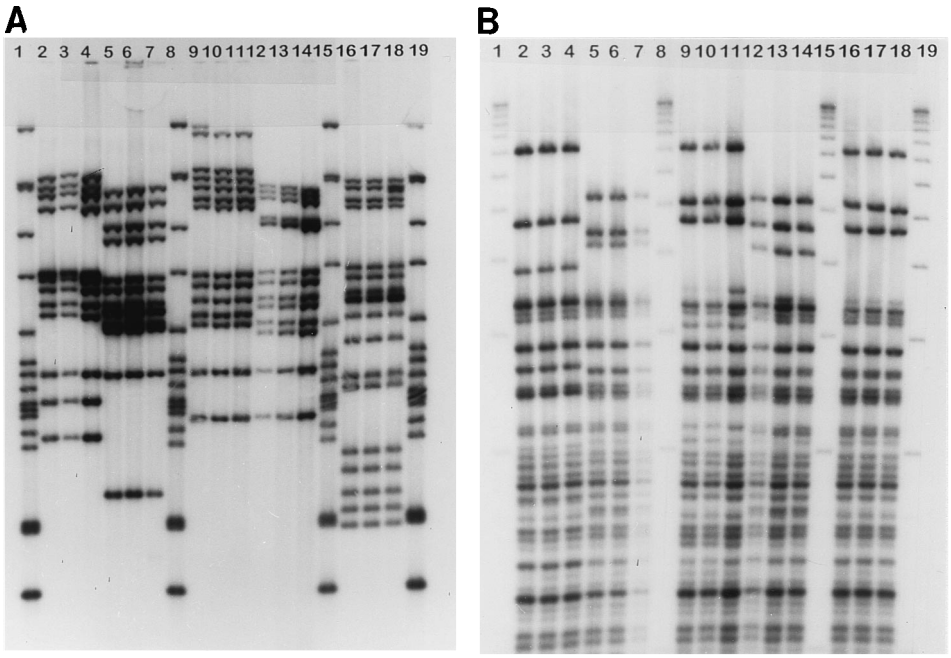

FIG. 2. Genomic blots of *M. tuberculosis* DNA isolates from Madrid with high copy numbers of IS*6110*. Lanes 2 to 4, 5 to 7, 9 to 11, 12 to 14, and 16 to 18 show *Pvu*II-restricted DNA from *M. tuberculosis* isolates hybridized with IS*6110* (A) and restricted with *Alu*I and hybridized with pTBN12 (B). Fifteen strains grouped into five IS*6110* clusters (A) were resolved into the same clusters with pTBN12 (B). Note that the isolates in lanes 9 and 18 have an additional IS*6110* hybridizing band with regard to lanes 10 and 11 and lanes 16 and 17, respectively. Lanes 1, 8, 15, and 19 contain marker H37Rv DNA probed with [α-^{32}P]dCTP-labeled IS*6110* (A) or a 1-kb DNA ladder probed with a [α-^{32}P]dCTP-labeled 1-kb DNA ladder (B).

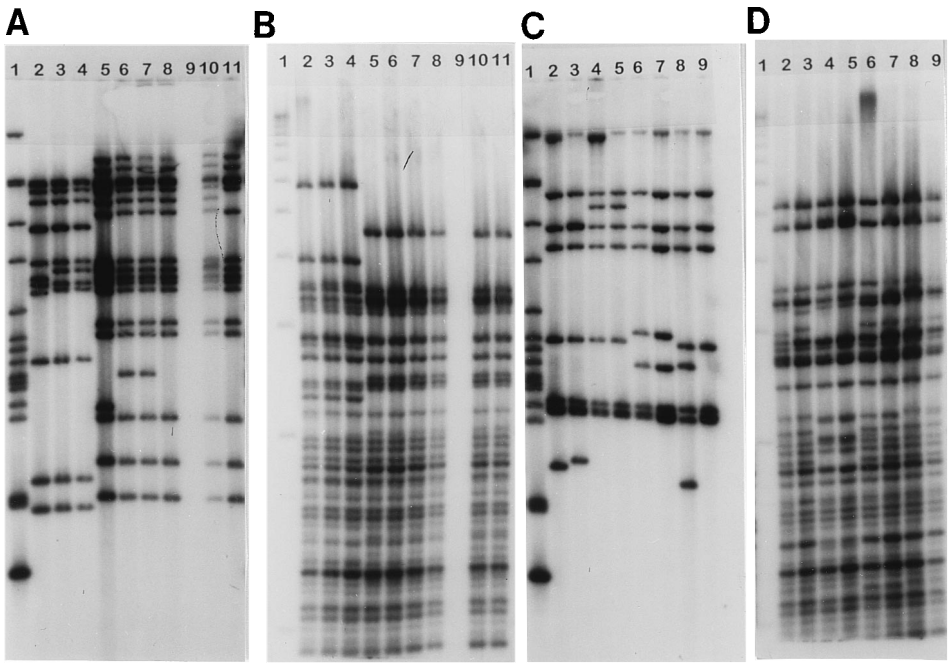

FIG. 3. Genomic blots of *M. tuberculosis* isolates from Madrid with similar IS*6110* fingerprint patterns. (A and C) *Pvu*II-restricted DNA probed with [α-^{32}P]dCTP-labeled IS*6110*; (B and D) *Alu*I-restricted DNA from the same isolates probed with [α-^{32}P]dCTP-labeled pTBN12. Lanes 2 to 4 and 5 to 11 (A) show that two groups of isolates with similar IS*6110* fingerprints give two identical fingerprints with pTBN12 (B) (lanes 9 in panels A and B contain no DNA). Lanes 2 to 9 (C) show that *M. tuberculosis* isolates having IS*6110* patterns differing in the size and number of one or two hybridizing bands are resolved into two groups with pTBN12 (D). Lane 1 contains marker DNA, H37Rv DNA probed with [α-^{32}P]dCTP-labeled IS*6110* (A and C) or a 1-kb DNA ladder probed with [α-^{32}P]dCTP-labeled IS*6110* (B and D).

Madrid prison system showed unique IS*6110* patterns, and each also showed unique pTBN12 patterns. Although the extent of polymorphism with IS*6110* and pTBN12 was not directly measured, the fingerprints appear to be more polymorphic with IS*6110* than with pTBN12. There was no correlation between the numbers of different bands when either probe was used. In some isolates, the IS*6110* patterns differed by four or five hybridizing fragments, while for the same strains the pTBN12 fingerprints showed a difference in the size of only a single band. In general, *M. tuberculosis* isolates which demonstrated different IS*6110* fingerprint patterns showed different patterns with pTBN12, although we observed less variability in the pTBN12 patterns.

Stability of pTBN12. The stability of pTBN12 fingerprinting in comparison with that obtained with IS*6110* was analyzed for four *M. tuberculosis* isolates obtained from a single patient over a period of 3.5 years at intervals of 12, 18, and 42 months after diagnosis. All isolates from this patient were resistant to isoniazid and rifampin. Fingerprinting with pTBN12 and IS*6110* confirmed the same pattern in all samples without any change in the number or size of any hybridizing fragment. This observation and the fact that strains having six or more bands with IS*6110* that are identical will almost always show identical pTBN12 fingerprints indicate that the stability of the pTBN12 fingerprint is equal to that of IS*6110* and is sufficiently stable for use in epidemiologic studies. Previous observation with the *M. tuberculosis* reference strains H37Rv and H37Ra revealed fewer changes with the pTBN12 fingerprints than with the IS*6110* fingerprints (24).

In general, isolates with identical IS*6110* fingerprint patterns that have only a few copies of IS*6110* can be subdivided by pTBN12. Conversely, pTBN12 groups *M. tuberculosis* isolates with closely related IS*6110* fingerprints containing many copies of IS*6110* into fewer clusters.

Overall, IS*6110* and pTBN12 are useful markers for differentiating *M. tuberculosis* strains, and both probes show a good correlation when they are used for RFLP analysis. Since IS*6110* provides greater polymorphism in fingerprinting, it is convenient to type isolates first with IS*6110* to identify isolates which have identical or nearly identical fingerprints. Subsequently, isolates which demonstrate identical fingerprints and have five or fewer copies of IS*6110* and isolates having more than five copies of IS*6110* with similar but not identical fingerprints should be analyzed with a second probe, such as pTBN12, to identify more accurately isolates that may have an epidemiologic link.

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