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DNA Fingerprinting of *Mycobacterium tuberculosis* Complex Culture Isolates Collected in Brazil and Spotted onto Filter Paper

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The usefulness of filter paper for preservation of bacterial cells was shown by mixed-linker DNA fingerprint analysis of *Mycobacterium tuberculosis* isolates from 77 Brazilian patients. DNA fingerprints of samples spotted onto filter paper and conventional culture material were identical. Thus, filter paper specimens analyzed by an amplification-based typing method provide a new resource for epidemiological studies of infectious diseases.

The standard method recommended for DNA fingerprinting of *Mycobacterium tuberculosis* is the restriction fragment length polymorphism, based on the variability in copy number and sites of the IS*6110* insertion within the genome (8, 17, 18). However, its application in large epidemiological studies is often hampered by difficulties in preservation and transport of mycobacterial cultures and the need for a large quantity (≥2 μg) of mycobacterial DNA (1, 7, 17). Amplification-based typing methods like the mixed-linker (ML) PCR have the advantage of being applicable to nonviable cells and requiring only a small amount (≥1 pg) of genomic DNA (7). In this report, we describe the adaptation of a filter paper method for preservation of mycobacterial cells isolated by culture and demonstrate its usefulness for amplification-based DNA fingerprinting by analysis of *M. tuberculosis* complex strains isolated in Paraná, southern Brazil.

The state of Paraná has a population of approximately 9 million people and an incidence of tuberculosis of about 28/100,000 inhabitants (5). Among 1,580 samples submitted for mycobacterial culture at the Central Laboratory (LACEN) of Paraná over a period of 2 years (between April 1994 and April 1996), a total of 249 (15.8%) positive cultures on Loewenstein-Jensen (LJ) medium were identified as *M. tuberculosis* complex. Antimicrobial susceptibility testing and DNA fingerprinting were performed for 78 (31.3%) isolates. These isolates were obtained from 77 patients living in 10 different cities of the state of Paraná, 57 (74%) of whom resided in the capital, Curitiba. The age of the patients varied from <1 to 76 years (41.5% between 30 and 44 years), with a male/female ratio of 1.8:1. Eleven patients (14.3%) were known to be human immunodeficiency virus positive. Species identification of the mycobacterial isolates and drug susceptibility testing were performed at LACEN according to standard procedures (2).

Colonies grown on LJ medium were transferred with sterile cotton swabs onto filter paper cards (Whatman, Maidstone, United Kingdom) and allowed to dry suspended horizontally in a biosafety cabinet at room temperature. Bacteria were heat killed by incubation of the filter paper for 1 h at 80°C. The cards were kept in individual protective plastic envelopes. Before analysis, one fragment of about 3-mm diameter from each filter paper spot was cut out with a disposable blade and rehydrated in 0.5 ml of sterile water. The viability of the mycobacterial cells was tested by inoculation of 0.3 ml of this solution onto LJ medium and incubation for 8 weeks at 37°C. Parallel samples were prepared from 55 of the 78 cultures by suspension of one loop of colonies in 1 ml of 0.9% NaCl. All 55 paired specimens were stored at room temperature for 6 to 12 months. For DNA isolation of the filter paper and control strains, freeze-thawing and sonication methods were compared. Freeze-thawing methods consisted of three cycles, 30 min each, at 25°C and then incubation on dry ice or at 80°C and then incubation on dry ice. Sonication was performed with 25 μl of glass beads (Sigma, Deisenhofen, Germany) in an ultrasound water bath (Bandelin Electronic, Berlin, Germany) for 10 min. All methods were equally effective in releasing a sufficient amount of mycobacterial DNA from the rehydrated filter paper samples. In this study, the mycobacterial cells were lyzed by freeze-thawing at 80°C and incubation on dry ice. This lysis method required minimal handling of the specimens to avoid the risk of contamination.

DNA fingerprinting was performed using a 2-μl aliquot of the lysate without further purification by the ML PCR method as described previously (7). Hybridization analysis with an IS*6110*-specific probe was carried out at the stringent temperature (58°C) as described elsewhere (7). The produced patterns were analyzed with GelCompar software, version 3.1b (Applied Math's BVBA, Kortrijk, Belgium).

An ML PCR DNA fingerprint suitable for computer analysis was produced for all specimens (Fig. 1). Fingerprint patterns of 55 of the 78 filter paper specimens were compared with fingerprints of the respective cultures that were kept in suspension (Fig. 2). They were identical for 54 of these paired isolates, while a single additional band was found for the filter paper specimen of strain 6. This extra band was confirmed by hybridization of the patterns with an IS*6110*-specific probe (Fig. 3, strain 6b).

The IS*6110* copy number of the 78 analyzed strains varied from 1 to 17, the majority displaying 6 to 14 fragments with sizes ranging between 150 and 500 bp. Five clusters were found

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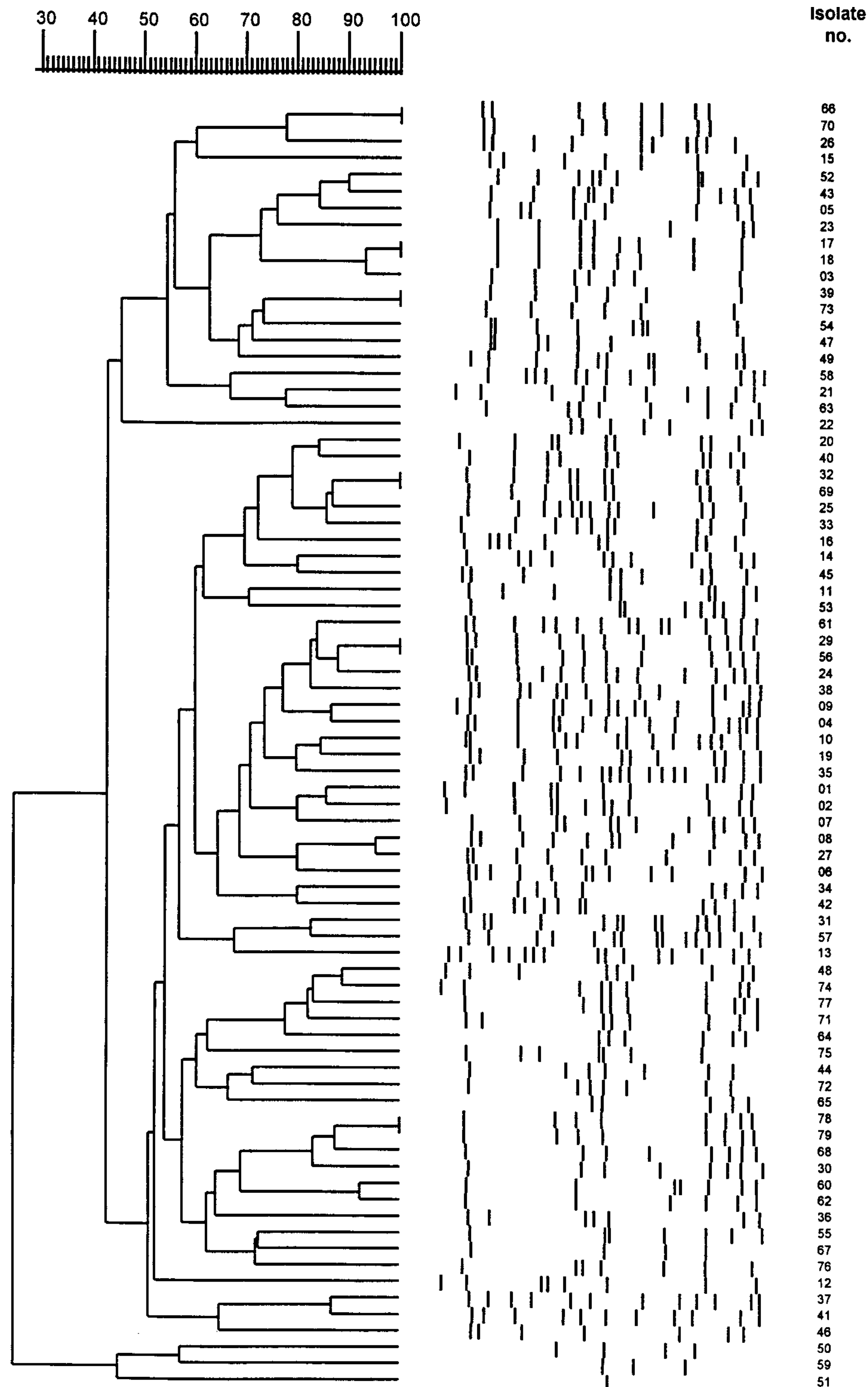

FIG. 1. Dendrogram illustrating the epidemiological relationships between 78 *M. tuberculosis* complex isolates from 77 different patients from southern Brazil. The similarity (percent) of DNA fingerprint patterns is indicated above the dendrogram and was calculated by the unweighted pair group method arithmetic averages and Dice similarity coefficient by the program GelCompar 3.1b. Matching bands were identified by using a position tolerance of 1.0%. The calculation is based upon electrophoretic patterns of DNA fragments obtained by ML PCR fingerprinting analysis.

for isolates from 10 individual patients (Fig. 1). Each cluster contained two isolates with identical patterns. Fingerprint patterns of seven isolates differed from that of another isolate by one IS*6110* fragment. In contrast, the DNA fingerprint patterns of the remaining 48 strains (61.5%) were highly polymorphic. No correlation was observed between fingerprint clusters and the 13 drug-resistant isolates (4 of which were multidrug resistant).

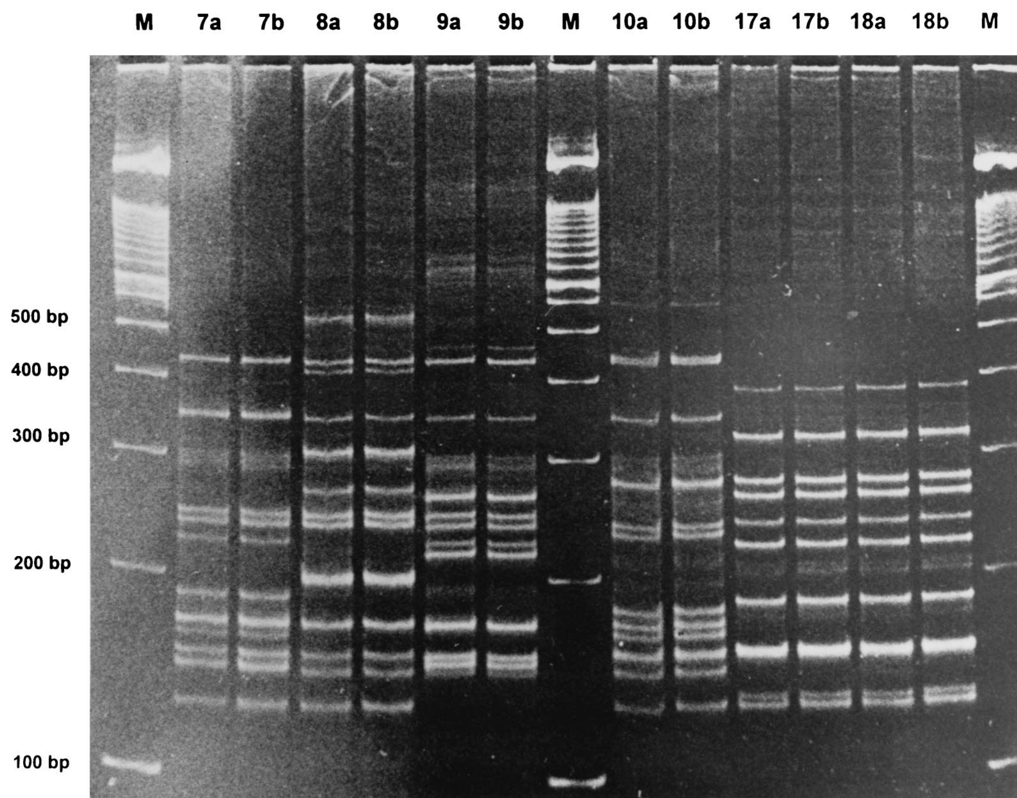

FIG. 2. DNA fingerprints of six paired *M. tuberculosis* isolates. Lanes a, conventional culture samples; lanes b, filter paper samples; lanes M, size markers (100-bp DNA ladder; Gibco-BRL, Eggenstein, Germany) in base pairs.

To our knowledge, this is the first study reporting the usefulness of filter paper specimens for molecular-epidemiological analysis of tuberculosis. Previous studies have described the use of dried blood spots on filter paper for diagnosis of viral diseases such as measles, hepatitis B, and human immunodeficiency virus infection (6, 10). Using ML PCR, we have shown that a small aliquot of each culture (20 to 30 colonies) spotted onto filter paper provided sufficient mycobacterial DNA to produce complete fingerprint patterns for all samples.

The analysis of 55 paired specimens showed no difference in the resulting fingerprint patterns between the filter paper method and conventional samples of cultured cells (Fig. 2). Thus, dehydration seemed to have no negative influence on DNA stability. Single-band differences as observed for strain 6 (Fig. 3) due to transposition of IS*6110* have also been described for standard IS*6110* restriction fragment length polymorphism typing and seem to be independent from the filter paper method (4, 12). The finding of identical fingerprint patterns of two samples from the same patient (Fig. 1, strains 32 and 69) collected at different times confirmed the reproducibility of the ML PCR results.

Most of the fingerprint patterns found in this retrospective analysis of 78 Brazilian *M. tuberculosis* isolates were unique, and only 10 isolates were grouped into five small clusters, suggesting a predominance of reactivated disease. In addition, the identification of seven strains that varied from other isolates only by a single band might suggest endemic transmission of specific strains over a long time (1). However, the sample has been too small to correctly estimate the rate of transmission.

DNA stability has been described for dried filter paper blood spots stored at room temperature (25°C) for as long as 12 years and suggested that they could be kept indefinitely (13, 19, 20). In our study, the *M. tuberculosis* DNA was stable in the filter paper specimens for up to 1 year. In mycobacterial samples the thick cell wall might provide an additional barrier to protect the DNA (21). The filter cards cannot be broken or split like culture vessels, are light weight and cost effective, and require minimal storage space (14). Heat-treated filter paper specimens showed no growth on LJ culture, further reducing the biohazard risk. In addition, the filter paper specimens can be easily obtained even from geographically isolated populations and may be shipped by mail in an envelope to a central laboratory for molecular diagnostics (ease of transport). However, caution must be taken to avoid cross-contamination between specimens during sampling and handling. For DNA isolation the filter paper spot was punched out with a disposable blade. Alternatively, a cleaning step for the blade in depurinating solution could be introduced (9, 16).

In conclusion, filter paper specimens are a valuable source of mycobacterial DNA for amplification-based methods that facilitate collection and transport and allow long storage at room temperature (3, 11, 15). The ML PCR fingerprinting of mycobacterial cells spotted onto filter paper has proven, in our experience, to be an effective, reliable, reproducible, and rapid method for typing of strains, facilitating the study of molecular epidemiology of tuberculosis. The ability to use bacterial culture isolates spotted onto filter paper for DNA analysis may provide a generally applicable tool for epide-

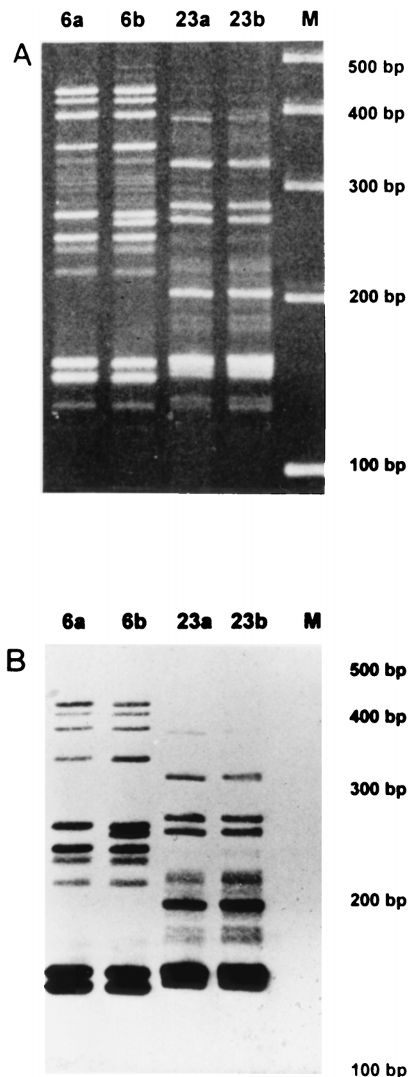

FIG. 3. Reproducibility of ML PCR by gel electrophoresis and hybridization. (A) An 8% Polyacrylamide gel with PCR fragments visualized under UV light after staining with ethidium bromide; (B) chemiluminescent detection of PCR fragments after hybridization with an IS*6110*-specific oligonucleotide. Lanes a, conventional culture samples; lanes b, filter paper samples; lanes M, size markers (100-bp DNA ladder; Gibco-BRL) in base pairs.

miological studies of infectious diseases using molecular genetic techniques.

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