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Rapid Discrimination of *Mycobacterium tuberculosis* Complex Strains by Ligation-Mediated PCR Fingerprint Analysis

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A ligation-mediated PCR (LMPCR) method for the amplification of sequences flanking the IS*6110* of the *Mycobacterium tuberculosis* complex has been developed. The method uses one primer specific for IS*6110* and a second specific for a linker ligated to *Sal*I-restricted genomic DNA. LMPCR is a rapid screening method, valuable for the fingerprinting of *M. tuberculosis* complex strains.

The development of molecular methods for *Mycobacterium tuberculosis* complex strain typing has greatly advanced the study of tuberculosis epidemiology. Restriction fragment length polymorphism (RFLP) analysis, based on the detection of the IS*6110* element, facilitates tracing of epidemiologically related cases, identification of nosocomial infections, differentiation of new infections from relapses, and identification of laboratory contamination (15). Standard RFLP typing is laborious and slow and involves many technical steps. Most alternative PCR methods amplify IS*6110*-flanking regions to display polymorphism (2, 4, 5, 8–13). None of these methods has replaced the standard RFLP technique.

We describe here a ligation-mediated PCR (LMPCR) procedure to amplify the flanking sequence located on the 5′ side of IS*6110*. The value of this method for strain discrimination was compared to that of the standard IS*6110* RFLP technique on a set of 112 *M. tuberculosis* complex strains.

The oligonucleotides used as linkers and primers are described in Table 1. An asymmetrical, double-stranded linker was constructed by annealing two nonphosphorylated oligonucleotides, Salpt and Salgd. These were designed to ligate to the *Sal*I cohesive ends, thus eliminating the *Sal*I restriction site after ligation. The primers were directed outward from the element they recognize. Primers IS1 and IS2 are located in the IS*6110* element (13). Primer IR1 is the complementary sequence of the 3′ side inverted repeat of IS*6110*, 30 bp outward from the sequence recognized by IS2. DRa recognizes the downstream part and DRb recognizes the upstream part of the 36-bp direct repeat (DR), and they allow the amplification of the spacer regions between the DRs (1).

The LMPCR process is summarized in a schematic flowchart (Fig. 1). Aliquots of genomic DNA were digested by *Sal*I (Boehringer Mannheim GmbH, Mannheim, Germany) and ligated to the linker. After ligation, the T4 DNA ligase was heat inactivated. The samples were then digested with *Sal*I to cleave any remaining restriction sites resulting from partial genomic digestion or regeneration through ligation. Template DNA (5 μl of a 1/10 dilution) was added to the PCR mixture, which contained 1 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), deoxynucleoside triphosphates (200 μM each), and 5 μl of dimethyl sulfoxide in 1× PCR buffer. The Salgd and IS2 primers (1 μM each) were added, and the DNAs were denatured by incubation of the mixture at 95°C for 9 min. Amplification was achieved by using 35 cycles of PCR (95°C for 30 s, 55°C for 30 s, 72°C for 90 s) followed by a final extension at 72°C for 10 min. The amplified products were separated in a 1.5% agarose gel.

Under these conditions, the linker was ligated by its long Salgd strand to the 5′ phosphate cohesive ends (TCGA) of *Sal*I-digested genomic DNA. Salgd-Salpt duplexes are stable under ligation conditions but not at PCR temperature. The Salpt strand was not ligated during the tag procedure and was separated from the DNA matrix during the heat denaturation step of the PCR. The PCR performed with the IS*6110*-specific primer (IS2) and the linker primer (Salgd) amplified only DNA-tagged fragments containing the IS*6110*-flanking sequence on the 5′ side. In our hands, the linker was stable for 2 months at −20°C and five freeze-thaw cycles did not affect stability.

For heminested and nested amplifications, the PCR product diluted 1/200 was added to the reaction mixture (as described above). Oligonucleotide primers (1 μM each) were IR1 and Salgd for heminested PCR and IR1 and DRa or IR1 and DRb for nested PCR. The PCR conditions were as described above.

For sequencing, the PCR product was purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany). Sequencing was performed on an ABI 373 DNA sequencer with the *Taq* DyeDeoxy terminator cycle sequencing kit (Perkin-Elmer).

To demonstrate the specificity of LMPCR, a set of 14 strains (5 *M. tuberculosis* and 9 *Mycobacterium bovis* strains) with IS*6110* single-band RFLP patterns was further investigated. In such strains, the single IS*6110* element is usually located in the

TABLE 1. Oligonucleotides used in this study

Primer[a]	Sequence (5′ to 3′)[b]	Reference
Salgd	TAG CTT ATT CCT CAA GGC ACG AGC	This study
Salpt	**TCG** AGC TCG TGC	This study
IS1	GGC TGA GGT CTC AGA TCA G	16
IS2	ACC CCA TCC TTT CCA AGA AC	16
IR1	GAG TCT CCG GAC ATG CCG GGG CGG TTC A	17
DRa	CCG AGA GGG GAC GGA AAC	1
DRb	GGT TTT GGG TCT GAC GAC	1

[a] Salgd and Salpt, linker primers. IS1, IS2, and IR1, IS*6110* primers. DRa and DRb, DR primers.
[b] The *Sal*I restriction site is indicated in boldface, and the linker complementary sequences are underlined.

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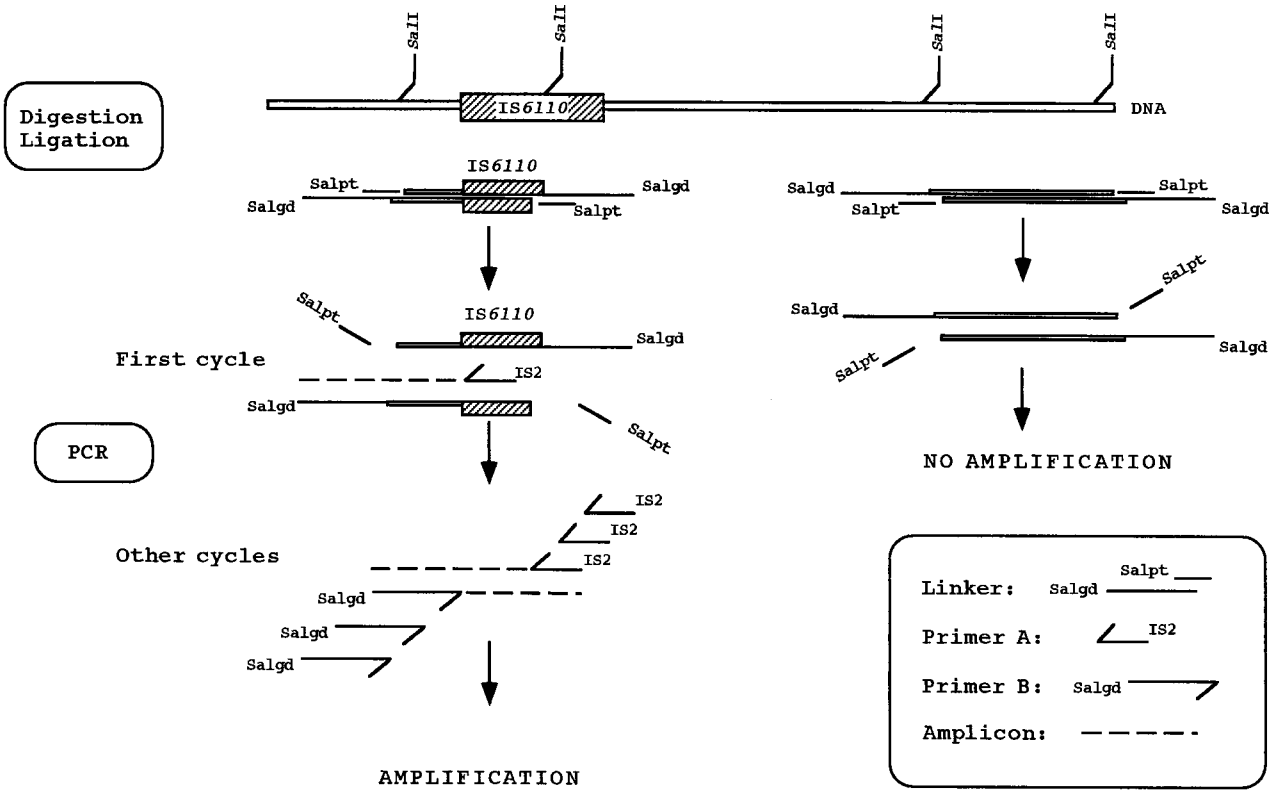

FIG. 1. LMPCR strategy. PCR specifically amplifies the upstream IS*6110*-flanking region with primers IS2 and Salgd.

DR region of the chromosome (6). All 14 strains tested gave one LMPCR-amplified fragment either 800 or 350 bp in length (Fig. 2A). The specificity of the LMPCR fragment was confirmed by heminested PCR, using Salgd and IR1 primers, performed on five strains (three producing 350-bp amplicons and two producing 800-bp amplicons). The amplicons produced by heminested PCR were of the expected molecular size (Fig. 2A). The nature of the flanking region was analyzed by nested PCR performed on diluted LMPCR product with IR1 and DRa primers (Fig. 2B). As expected, several amplified products (corresponding to the DR copy number) were obtained from the region between IS*6110* and the first *Sal*I restriction site (Fig. 2A). No amplified product was obtained from nested PCR with DRb, a DR oligonucleotide primer in reverse orientation (data not shown).

In addition, the 350-bp LMPCR product was sequenced. The sequence analysis showed that there were four identical DR regions and four spacers (SP26 to SP30 according to the Hermans et al. [6] nomenclature). The DR30 region was interrupted by the sequence of IS*6110*. The spacer SP26 was truncated at the *Sal*I restriction site and ligated to the linker sequence. The amplicons produced by nested PCR (Fig. 2A) resulted from amplification of sequences between DR27 and DR29 and of the IR1 inverted sequence (Fig. 2B).

The LMPCR procedure and the standard IS*6110* RFLP method (15) were performed on a total of 98 *M. tuberculosis* complex isolates with IS*6110* multiple-band RFLP patterns. The RFLP patterns contained up to 20 bands (median, 9), whereas LMPCR produced between 2 and 11 bands (median, 6). The molecular size of the amplicons was between 100 and 2,000 bp.

Fifty-eight strains had unique RFLP patterns. They gave 48

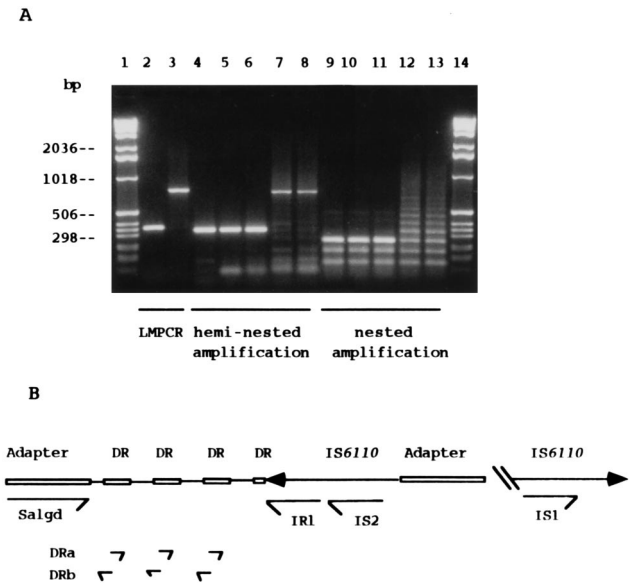

FIG. 2. Investigation of IS*6110*-flanking regions of strains with a single RFLP band. (A) Lanes 1 and 14, 1-kbp ladders; lane 2, 350-bp amplicon obtained by LMPCR; lane 3, 800-bp amplicon obtained by LMPCR; lanes 4 to 6, heminested PCR with primers IR1 and Salgd for three strains with 350-bp LMPCR amplicons; lanes 7 and 8, heminested PCR with primers IR1 and Salgd for two strains with 800-bp LMPCR amplicons; lanes 9 to 11, nested PCR with primers DRa and IR1 for the three strains shown in lanes 4 to 6 with 350-bp LMPCR amplicons; lanes 12 and 13, nested PCR with primers DRa and IR1 for the two strains with 800-bp LMPCR amplicons. (B) Schematic illustration of the IS*6110* element, DR flanking region, and oligonucleotide primers used in LMPCR, heminested PCR, and nested PCR.

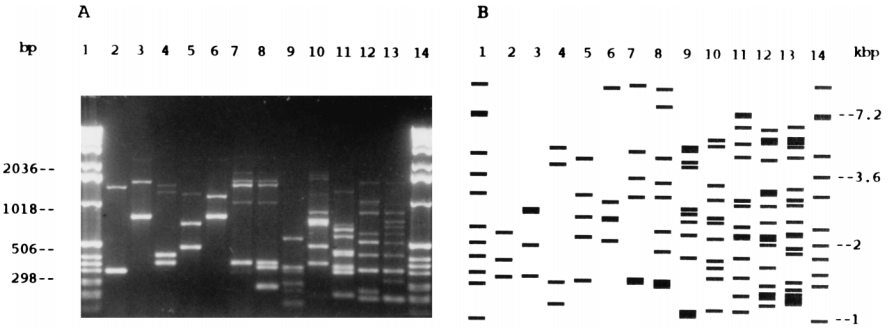

FIG. 3. LMPCR analysis and RFLP fingerprints of 12 *M. tuberculosis* isolates with unique RFLP patterns. (A) Lanes 1 and 14, 1-kbp ladders; lanes 2 to 13, LMPCR patterns of 12 *M. tuberculosis* isolates. (B) Schematic representation of RFLP patterns. Lanes 1 and 14, reference strain Mt 14323 (15). Lanes 2 to 13, RFLP patterns of the same 12 isolates shown in panel A.

LMPCR patterns (Fig. 3), as 10 strains had similar, but not identical, LMPCR patterns. These latter patterns differed by a single band, due either to the absence of one band or to the presence of an additional band or one of a different molecular size. We concluded that these strains could not be reliably differentiated by LMPCR.

LMPCR was also used to examine 40 isolates, grouped into 13 clusters based on RFLP banding patterns. The LMPCR patterns were identical within each cluster. We further investigated the largest cluster, which consisted of eight strains (Fig. 4). The RFLP patterns were identical for all strains in this cluster except one which had an additional insertion element band (Fig. 4C, lane 9). The LMPCR profiles were identical for strains with identical RFLP patterns (Fig. 4A and C, lanes 2 to 8) and similar, but not identical, for the strain in lane 9. Amplification of the downstream flanking region of IS*6110* with the IS1 primer produced a similar pattern: again there was a faint additional band (by bp 1018) for the strain in lane 9 (Fig. 4B).

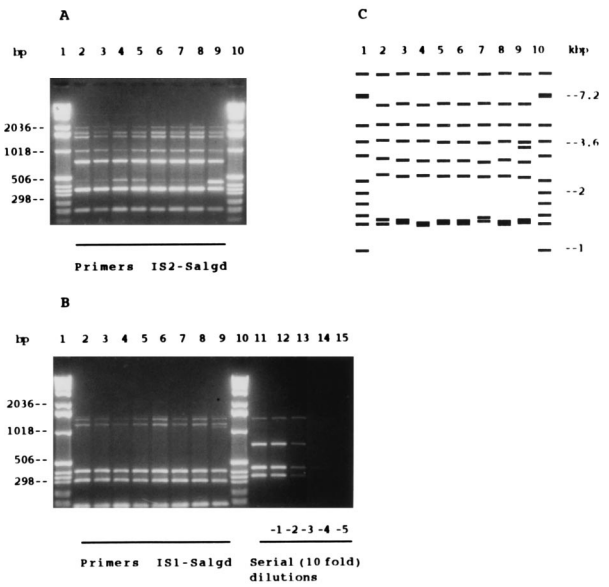

FIG. 4. LMPCR of eight isolates from a single RFLP cluster. (A) Lanes 1 and 10, 1-kbp ladders; lanes 2 to 9, fingerprints generated by LMPCR with primers IS2 and Salgd. (B) Lanes 1 and 10, 1-kbp ladders; lanes 2 to 9, fingerprints generated by LMPCR with primers IS1 and Salgd of the same strains shown in panel A; lanes 11 to 15, LMPCR patterns from serial (10-fold) dilutions of a *M. tuberculosis* DNA matrix. (C) Schematic representation of RFLP patterns. Lanes 1 and 10, reference strain Mt 14323 (15); lanes 2 to 9, RFLP patterns of the same strains shown in panel A.

The sensitivity of the LMPCR method was assessed by using serial dilutions of template DNA (Fig. 4B). LMPCR was able to generate identical fingerprint patterns with as little as 2 ng of chromosomal DNA. The method was robust, as use of different DNA concentrations diluted up to log 2 did not affect the LMPCR pattern.

The method described here can be completed in 8 to 10 h after DNA extraction, thus shortening the time required for strain typing. In order to fit well with the work flow of clinical laboratories, the LMPCR can be performed on two consecutive days, with preparation of the linker-tagged DNA on the first day and amplification and electrophoresis on the following day. We did not test LMPCR on direct specimens. However, it has to be stressed that LMPCR requires DNA of high quality, a condition which may prevent tentative applications of LMPCR on clinical samples.

LMPCR specifically amplified DNA sequences from the IS*6110*-flanking region on the 5′ side. Nonspecific amplicons were prevented by use of AmpliTaq Gold. All tagged restriction fragments were amplified when standard *Taq* polymerase was used, whereas only the IS2-Salgd fragments were amplified with AmpliTaq Gold (data not shown). Moreover, the design of the asymmetrical linker contributed to minimizing undesirable extension due to annealing of the short strand during PCR cycles. Compared to other PCR techniques developed for *M. tuberculosis* typing, heminested inverse PCR has been claimed to overcome several drawbacks of PCR techniques involving ligation (e.g., low efficiency of intermolecular ligation and the presence of unused linkers) (10). In our hands, however, the ligation procedure was reliable and sensitive. The interference of unused linkers was minimized by dilution of the linker-tagged DNA matrix. Moreover, the linker primer Salgd and the IS2 primer (13) were specially designed to avoid mispriming. Their use produced well-resolved bands, whereas important smears due to single-stranded amplification were observed in heminested inverse PCR tests (10). The method described here shows that a single PCR amplification without heminested or nested PCR may be used to obtain polymorphic patterns along with reducing both the time required for strain typing and the risk of PCR contamination.

The results demonstrate the potential of the LMPCR method for identifying and monitoring outbreaks. For strains with distinct RFLP patterns, most of the LMPCR patterns were unique. However, some strains could not be reliably differentiated by LMPCR, as their patterns differed by only one band. Numerous studies based on IS*6110* RFLP analysis have shown on the one hand that epidemiologically related strains may present RFLP patterns differing by one band and on the other hand that no epidemiological conclusions may be drawn

from identical patterns with less than 5 bands (3, 7, 14, 16, 17). These difficulties are expected to be more frequent with LMPCR, as the number of amplicons is equal to or lower than the IS*6110* copy number. For strains with identical patterns or similar patterns differing by one band, the results of LMPCR screening should be checked by using the "gold standard" RFLP technique.

The LMPCR method is technically simple and reproducible and requires much less DNA than the standard RFLP typing method. Due to its speed, the technique may be useful as a screening method for contact-tracing investigation or identification of cross-contamination in clinical mycobacteriology laboratories.

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REFERENCES

1. **Aranaz, A., E. Liébana, A. Mateos, L. Dominguez, D. Vidal, M. Domingo, O. Gonzolez, E. F. Rodriguez-Ferri, A. E. Bunschoten, J. D. A. Van Embden, and D. Cousins.** 1996. Spacer oligonucleotide typing of *Mycobacterium bovis* strains from cattle and other animals: a tool for studying epidemiology of tuberculosis. J. Clin. Microbiol. **34:**2734–2740.
2. **Butler, W. R., W. H. Haas, and J. T. Crawford.** 1996. Automated DNA fingerprinting analysis of *Mycobacterium tuberculosis* using fluorescent detection of PCR products. J. Clin. Microbiol. **34:**1801–1803.
3. **Chaves, F., Z. Yang, H. El Hajj, M. Alonso, W. J. Burman, K. D. Eisenach, F. Dronda, J. H. Bates, and M. D. Cave.** 1996. Usefulness of the secondary probe pTBN12 in DNA fingerprinting of *Mycobacterium tuberculosis*. J. Clin. Microbiol. **34:**1118–1123.
4. **Friedman, C. R., M. Y. Stoeckle, W. D. Johnson, Jr., and L. W. Riley.** 1995. Double-repetitive-element PCR method for subtyping *Mycobacterium tuberculosis* clinical isolates. J. Clin. Microbiol. **33:**1383–1384.
5. **Haas, W. H., W. R. Butler, C. L. Woodley, and J. T. Crawford.** 1993. Mixed-linker polymerase chain reaction: a new method for rapid fingerprinting of isolates of the *Mycobacterium tuberculosis* complex. J. Clin. Microbiol. **31:** 1293–1298.
6. **Hermans, P. W. M., D. van Soolingen, E. M. Bik, P. E. W. de Haas, J. W. Dale, and J. D. A. van Embden.** 1991. Insertion element IS*987* from *Mycobacterium bovis* BCG is located in a hot-spot integration region for insertion elements in *Mycobacterium tuberculosis* complex strains. Infect. Immun. **59:** 2695–2705.
7. **Hermans, P. W. M., D. van Soolingen, J. W. Dale, A. R. J. Schuitema, R. A. McAdam, D. Catty, and J. D. A. van Embden.** 1990. Insertion element IS*986* from *Mycobacterium tuberculosis*: a useful tool for diagnosis and epidemiology of tuberculosis. J. Clin. Microbiol. **28:**2051–2058.
8. **Mazurek, G. H., V. Reddy, B. J. Marston, W. H. Haas, and J. T. Crawford.** 1996. DNA fingerprinting by infrequent-restriction-site amplification. J. Clin. Microbiol. **34:**2386–2390.
9. **Otal, I., S. Samper, M. P. Asensio, M. A. Vitoria, M. C. Rubio, R. Gomez-Lus, and C. Martin.** 1997. Use of a PCR method based on IS*6110* polymorphism for typing *Mycobacterium tuberculosis* strains from BACTEC cultures. J. Clin. Microbiol. **35:**273–277.
10. **Palittapongarnpim, P., S. Chomyc, A. Fanning, and D. Kunimoto.** 1993. DNA fingerprinting of *Mycobacterium tuberculosis* isolates by ligation-mediated polymerase chain reaction. Nucleic Acids Res. **21:**761–762.
11. **Patel, S., S. Wall, and N. A. Saunders.** 1996. Heminested inverse PCR for IS*6110* fingerprinting of *Mycobacterium tuberculosis* strains. J. Clin. Microbiol. **34:**1686–1690.
12. **Plikaytis, B. B., J. T. Crawford, C. L. Woodley, W. R. Butler, K. D. Eisenach, M. D. Cave, and T. M. Shinnick.** 1993. Rapid amplification-based fingerprinting of *Mycobacterium tuberculosis*. J. Gen. Microbiol. **139:**1537–1542.
13. **Ross, B. C., and B. Dwyer.** 1993. Rapid, simple method for typing isolates of *Mycobacterium tuberculosis* by using the polymerase chain reaction. J. Clin. Microbiol. **31:**329–334.
14. **Torrea, G., C. Offredo, M. Simonet, B. Gicquel, P. Berche, and C. Pierre-Audigier.** 1996. Evaluation of tuberculosis transmission in a community by 1 year of systematic typing of *Mycobacterium tuberculosis* clinical isolates. J. Clin. Microbiol. **34:**1043–1049.
15. **van Embden, J. D. A., M. D. Cave, J. T. Crawford, J. W. Dale, K. D. Eisenach, B. Gicquel, P. Hermans, C. Martin, R. McAdam, T. M. Shinnick, and P. M. Small.** 1993. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. J. Clin. Microbiol. **31:**406–409.
16. **Warren, R., M. Richardson, S. Sampson, J. H. Hauman, N. Beyers, P. R. Donald, and P. D. van Helden.** 1996. Genotyping of *Mycobacterium tuberculosis* with additional markers enhances accuracy in epidemiological studies. J. Clin. Microbiol. **34:**2219–2224.
17. **Yang, Z., F. Chaves, P. F. Barnes, W. J. Burman, J. Koehler, K. D. Eisenach, J. H. Bates, and M. D. Cave.** 1996. Evaluation of method for secondary DNA typing of *Mycobacterium tuberculosis* with pTBN12 in epidemiologic study of tuberculosis. J. Clin. Microbiol. **34:**3044–3048.