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The *mtp40* Gene Is Not Present in All Strains of *Mycobacterium tuberculosis*

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A multiplex PCR-based assay that targets IS*6110* and the *mtp40* gene was evaluated for the rapid differentiation of *Mycobacterium bovis* and *M. tuberculosis*, two of the causative agents of tuberculosis. The IS*6110* target is present in both species, whereas the *mtp40* gene was thought to be specific for *M. tuberculosis* (P. Del Portillo, L. A. Murillo, and M. E. Patarroyo, J. Clin. Microbiol. 29:2163–2168, 1991). However, the *mtp-40* gene is not present in all *M. tuberculosis* strains and, hence, is not useful for differentiating *M. tuberculosis* and *M. bovis*.

Although *Mycobacterium tuberculosis* causes the vast majority of cases of tuberculosis in the United States, the contributions of *M. bovis* and *M. africanum* infections to the tuberculosis burden of the United States have not been well characterized because in most diagnostic laboratories, the biochemical tests necessary to separate these three closely related species are not performed (4). A rapid method of differentiating *M. tuberculosis* and *M. bovis* would be helpful clinically because of the intrinsic resistance of *M. bovis* to pyrazinamide and epidemiologically because risk factors for *M. bovis* infections may be different from those for *M. tuberculosis* infections (4).

In 1991, Parra et al. (10) isolated and characterized a gene, designated *mtp40*, that appeared to be present in *M. tuberculosis* strains and absent in *M. bovis* strains. This gene encodes a 13,800-Da protein and was isolated by screening an *M. tuberculosis* recombinant DNA library with a rabbit polyclonal antiserum against *M. tuberculosis* proteins. Southern hybridization studies and PCR amplifications with two *M. tuberculosis* strains (H37Rv and H37Ra), one *M. bovis* strain, one *M. bovis* bacillus Calmette-Guérin (BCG) strain, and single strains of nine other *Mycobacterium* species revealed that the *mtp40* gene is present only in the two *M. tuberculosis* strains. On the basis of these results, Del Portillo et al. (5) proposed that PCR amplification of *mtp40* could be a diagnostic tool for detecting *M. tuberculosis* infections and for differentiating them from *M. bovis* infections. While our studies were in progress, Del Portillo et al. reported using a multiprimer PCR system which simultaneously targets the *mtp40*, IS*6110*, and 32-kDa antigen genes of *M. tuberculosis* to differentiate mycobacterial species (6). With a limited number of isolates (e.g., nine of *M. tuberculosis*), the technique allowed discrimination of *M. tuberculosis*, *M. bovis*, and other, nontuberculous mycobacteria.

Building on early published studies (5, 10), we also designed and evaluated a multiplex PCR-based assay targeting *mtp40* and IS*6110* to differentiate *M. tuberculosis* and *M. bovis*. The amplification of IS*6110*, an insertion sequence that is found in essentially all members of the *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, and *M. africanum*) (12), serves as an internal amplification control and indicates the presence of a member of the *M. tuberculosis* complex. The amplification of *mtp40* would distinguish *M. tuberculosis* and *M. bovis* strains because *M. tuberculosis* strains should produce both the IS*6110* and *mtp40* amplicons, whereas *M. bovis* strains should produce only the IS*6110* amplicon. In evaluating the multiplex PCR with 99 isolates of *M. tuberculosis*, we discovered that the *mtp40* gene is not present in all strains of *M. tuberculosis.*

Mycobacterial strains and DNA preparation. The mycobacterial strains were from the collection of the Mycobacteriology Laboratory of the Centers for Disease Control and Prevention. A total of 99 *M. tuberculosis* strains were studied, including H37Rv, H37Ra, 63 isolates from the April 1991 survey of drug resistance in New York City (8), and 34 other clinical isolates. *M. bovis* strains included TMC401, TMC404, TMC405, TMC407, TMC409, TMC410, TMC412, TMC605, TMC606, and TMC609 from the Trudeau Mycobacterial Culture Collection and eight clinical isolates. *M. bovis* BCG strains included TMC1002, TMC1009, TMC1010, TMC1012, TMC1019, TMC1020, TMC1021, TMC1022, TMC1024, TMC1025, TMC1028, TMC1029, TMC1030, TMC1103, and TMC1108 and four isolates submitted for identification. DNAs from the cultured bacteria were purified as described previously (13), or crude lysates were used as template DNA in the amplification reactions (11).

Oligonucleotide primers and PCR. Primers corresponding to portions of the *M. tuberculosis* IS*6110* and *mtp40* sequences were synthesized on a 381A DNA synthesizer (Applied Biosystems, Foster City, Calif.) at the Biotechnology Core Facility, National Center for Infectious Diseases, Centers for Disease Control and Prevention. The IS*6110* primers were IS52 (5′-TCAGGTCGAGTACGCCTTCT-3′, complement of bases 987 to 968) (12) and IS57 (5′-GGTCGCAGAGATCCGCGGTC-3′, bases 114 to 133) (12). The *mtp40* primers were PT3 (5′-GGTTCCCAACACCACGTTAG-3′, bases 173 to 192) (10) and PT5 (5′-CCAACATCGACGCAGTACC-3′, complement of bases 504 to 486) (10).

The amplification reactions contained 5 μl of template DNA and 45 μl of a reaction mixture (200 μM [each] deoxynucleoside triphosphates, 1.0 μM [each] primers, 1.25 U of *Taq* polymerase, 10 mM Tris hydrochloride [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin), as recommended by the *Taq* polymerase supplier (Perkin-Elmer Cetus, Norwalk, Conn.). The samples were amplified through 30 cycles in a programmable

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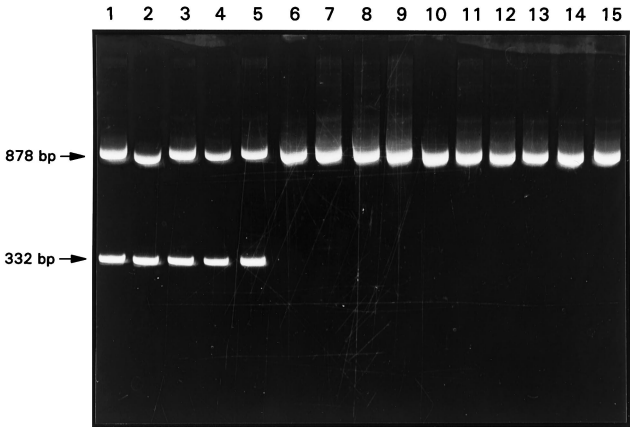

FIG. 1. Amplification products from *mtp40* multiplex PCR. Arrows indicate the 878-bp IS*6110* amplicon and the 332-bp *mtp40* amplicon. DNA templates were from *M. tuberculosis* H37Rv (lane 1); four clinical isolates (lanes 2 to 5); *M. bovis* TMC609 (lane 6), TMC407 (lane 7), TMC606 (lane 8), TMC404 (lane 9), and TMC401 (lane 10); and *M. bovis* BCG TMC1019 (lane 11), TMC1024 (lane 12), TMC1103 (lane 13), TMC1009 (lane 14), and TMC1022 (lane 15).

thermal cycler (Perkin-Elmer Cetus) with a three-step cycle of denaturation for 1.5 min at 94°C, annealing for 1.75 min at 60°C, and extension for 2.5 min at 72°C. The amplification products were analyzed by electrophoresis through 1.5% agarose or 6% polyacrylamide gels with a Tris-borate-EDTA buffer system and visualized by ethidium bromide fluorescence. The molecular sizes of the products were estimated by comparing the migration distances with those of a 100-bp ladder (GIBCO BRL, Gaithersburg, Md.).

Southern blotting and hybridization. About 1 μg of genomic DNA was digested with *Bam*HI (GIBCO BRL) for 2 h at 37°C and then electrophoresed through a 1.0% agarose–Tris–borate–EDTA gel for 17 h at 40 V. The DNA was denatured, neutralized, and transferred by capillary blotting to a Hybond-N^+ membrane (Amersham, Arlington Heights, Ill.) as recommended by the manufacturer of the membrane. DNA was bound to the membrane by using a Stratalinker UV Cross-Linker (Stratagene, La Jolla, Calif.). The samples were hybridized with the 332-bp *mtp40* amplicon, which had been labeled by using the ECL Direct Nucleic Acid Labeling System (Amersham), in the hybridization buffer supplied by Amersham containing 0.1 M NaCl. After overnight incubation at 42°C, the blot was washed stringently at 42°C in 7.5 mM sodium citrate–75 mM sodium chloride–0.4% sodium dodecyl sulfate–6 M urea. After development with the ECL detection solutions, the signals were detected by using X-Omat AR autoradiography film (Eastman Kodak, Rochester, N.Y.).

In preliminary studies (data not shown), primer concentrations and amplification conditions were adjusted to generate strong amplification of an 878-bp fragment of IS*6110* and a 332-bp fragment of the *mtp40* gene from *M. tuberculosis* H37Rv, which contains 1 copy of the *mtp40* gene and 15 copies of the IS*6110* element. Next, 33 *M. tuberculosis* isolates, 19 *M. bovis* isolates, and 19 *M. bovis* BCG isolates were assayed by using the multiplex PCR-based assay. As expected, all 71 isolates produced the 878-bp IS*6110* amplicon. Of the 33 *M. tuberculosis* isolates tested, 25 produced both the 878-bp IS*6110* amplicon and the 332-bp *mtp40* amplicon (Fig. 1, lanes 2 to 5) and 8 produced only the IS*6110* amplicon. Of the 19 *M. bovis* isolates tested, 17 produced only the IS*6110* fragment (Fig. 1, lanes 6 to 10) and 2 produced both fragments. Each of the 19 *M. bovis* BCG isolates tested produced only the IS*6110* fragment (Fig. 1, lanes 11 to 15).

To evaluate further the performance of the multiplex PCR assay, we tested DNA samples from 63 *M. tuberculosis* isolates collected during April 1991 from tuberculosis patients in New York City (8). These 63 strains represented 38 unique IS*6110* restriction fragment length polymorphism (RFLP) types (3). Of the 63 samples, 47 produced the expected two-band pattern, 15 produced only the IS*6110* amplicon, and 1 produced only the *mtp40* amplicon. Of the strains with unique RFLP types, 30 produced the characteristic two-band pattern, 1 produced only the *mtp40* fragment, and 7 produced only the IS*6110* fragment. The strain that produced only the *mtp40* amplicon in the multiplex assay carries two copies of the IS*6110* element and did produce the IS*6110* amplicon when amplified with only the IS*6110* primers (data not shown). This raises the possibility that the failure to detect the IS*6110* amplicon was due to competition from amplification of the *mtp40* gene.

Of the seven IS*6110* RFLP types that did not yield the *mtp40* amplicon, two were the strain W type and a variant of strain W (1), three others were variants of another type (type 16-A) that is common in New York City, one was type 17-U, and one was type 12-T. The eight strains in the preliminary screen that failed to amplify *mtp40* were also either the strain W or 16-A RFLP type.

To determine if the *mtp40* gene is present in these strains, Southern blot analysis with the 332-bp *mtp40* amplicon as the hybridization probe was performed. *Bam*HI digests of genomic DNAs from eight *M. tuberculosis* strains representing eight unique IS*6110* RFLP types, which produced both bands in the multiplex PCR assay, contained a fragment of approximately 3,000 bp that hybridized with the 332-bp probe (Fig. 2, lanes 1 to 8). This fragment was absent in digests from four strains with the strain W or strain W variant fingerprints, three strains with the 16-A or variant fingerprints, and one strain with the 17-U fingerprint (Fig. 2, lanes 9 to 16).

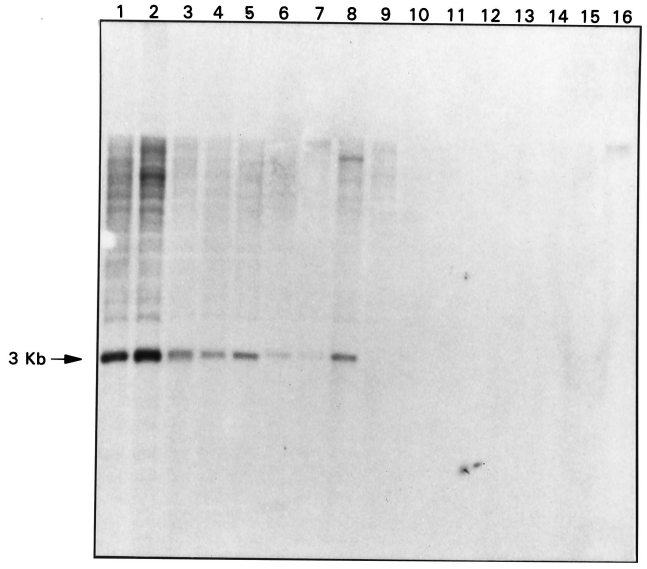

FIG. 2. Analysis of eight *M. tuberculosis* strains which produced the *mtp40* amplified products and eight which were negative for the *mtp40* amplified product. Southern blot of *Bam*HI-digested genomic DNAs from *mtp40* PCR-positive strain H37Rv (lane 1) and clinical isolates (lanes 2 to 8) and *mtp40* PCR-negative clinical isolates (lanes 9 to 16). The 3,000-bp fragment which hybridized with a probe specific for *mtp40* is indicated.

Interestingly, all of the strains that failed to produce the *mtp40* amplicon are multidrug resistant. Strains with the strain W and strain W variant RFLP patterns were associated with outbreaks of multidrug-resistant tuberculosis in hospital C, in a state correctional facility, and subsequently in four other New York City hospitals (2, 3). Over 200 isolates of this strain have been obtained from New York City patients, and this strain has also been isolated from patients in six other cities in the United States (1). The 16-A and 16-A variant RFLP patterns were also associated with clusters of multidrug-resistant tuberculosis among hospitalized patients (7). The significance of the absence of the *mtp40* gene in these multidrug-resistant strains is unclear, although it must be noted that other multidrug-resistant strains do contain an *mtp40* gene.

Overall, our data indicate that the *mtp40* gene is present in most, but not all, *M. tuberculosis* strains and is absent in most, but not all, *M. bovis* strains. The variability in the presence of this gene is somewhat surprising because *M. tuberculosis* strains generally exhibit very little sequence variation in the absence of selective pressure (9). Finally, the absence of the *mtp40* gene in these four important multidrug-resistant *M. tuberculosis* strains and its presence in two strains of *M. bovis* negate the potential use of this gene as a target of gene amplification tests for detection of *M. tuberculosis* or differentiation of *M. tuberculosis* and *M. bovis.*

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