

# DNA Amplification and Reverse Dot Blot Hybridization for Detection and Identification of Mycobacteria to the Species Level in the Clinical Laboratory

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A method incorporating DNA amplification and reverse dot blot hybridization for the detection and identification of mycobacteria to the species level is described. The amplification procedure allowed for the incorporation of digoxigenin-labeled UTP, which was detected by chemiluminescence, removing the need for radioactivity. Using a set of primers and probes from the gene for the 65-kDa heat shock protein of mycobacteria, previously reported in the literature, the reverse dot blot method correctly identified 12 of the 12 *M. tuberculosis* isolates and 45 of the 50 *M. avium* complex isolates. Two of the nonhybridizing *M. avium* complex isolates were reidentified as *M. xenopi*. The other three nonhybridizing *M. avium* complex isolates, which were identified as *M. intracellulare*, hybridized with the probe for *M. tuberculosis*, as did two ATCC strains of *M. intracellulare*. The amplified DNA of *M. intracellulare* was sequenced, and the sequence was compared with the sequence from *M. tuberculosis*. The sequence for *M. avium* differed from *M. tuberculosis* by 5 of 20 bases. The sequence for *M. intracellulare* differed from *M. tuberculosis* by 2 of 20 bases, but this difference did not result in sufficient thermal instability to affect hybridization. The use of chemiluminescence allowed as few as 10<sup>2</sup> CFU to be detected. The format of the assay is readily applicable for implementation in the clinical laboratory.

The mycobacteria are aerobic rod-shaped organisms characterized by being acid fast and by their very slow growth rate. Mycobacteria are of great importance in human and veterinary medicine and have reemerged as a serious health problem worldwide. There are at least 25 *Mycobacterium* species, with *M. tuberculosis* and species within the *M. avium* complex being the most common isolates in clinical laboratories in the United States (5, 8). In developing countries, tuberculosis remains a major problem, causing an estimated annual death rate of greater than 2,500,000 (2). The detection and identification of mycobacteria to the species level have been fraught with both low sensitivity and a long turnaround time, leading to significant delays in diagnosis. To improve upon the detection of mycobacteria, methods utilizing the amplification of mycobacterial DNA by the polymerase chain reaction (PCR) coupled with Southern blotting have been developed (1, 3, 4, 6, 7, 9, 10). However, these methods are not yet suitable for routine use in the clinical laboratory because of the rigorous methods required and the need to use radioactive isotopes.

We sought to develop a method which used the sensitivity of DNA amplification, did not require radioisotopes, and was amenable to use in a clinical laboratory. This paper describes such a method using nonradioactive reverse dot blot hybridization, adapted from the method originally described for the detection of human genetic polymorphisms (13). Primers were chosen to amplify and label DNA from all species of mycobacteria. Internal probes designed to differentiate the species were fixed to a nylon membrane support. The amplified product was used for the hybridization reaction with the strips containing the spotted series of species-specific probes.

## MATERIALS AND METHODS

**Mycobacterial cultures and isolates.** Clinical isolates of *M. tuberculosis*, *M. avium* complex, and other *Mycobacterium* species were collected from the Microbiology Section, Clinical Laboratory, University of California Medical Center, San Francisco. All clinical isolates were identified to the species level by using conventional methods by the staff of the San Francisco Department of Public Health. The species and number of isolates tested were as follows: *M. tuberculosis*, 12; initial identification *M. avium* complex, 50; *M. kansasii*, 1; *M. xenopi*, 1; *M. gordonae*, 2; *M. scrofulaceum*, 2; and *Rhodococcus* spp., 1. In addition, American Type Culture Collection (ATCC) strains were used: *M. tuberculosis* ATCC 25177, *M. avium* ATCC 25291, *M. intracellulare* ATCC 13950 and ATCC 35770, *M. fortuitum* ATCC 6841, and *M. bovis* BCG ATCC 19015. Mycobacteria were grown on Lowenstein-Jensen solid medium (Remel, Lenexa, Kans.).

**DNA isolation and purification.** A single mycobacterium colony, large enough to cover a 1- $\mu$ l loop, on the Lowenstein-Jensen solid medium was lysed in 0.1 ml of 0.1 M NaOH-2 M NaCl-0.5% sodium dodecyl sulfate (SDS) at 95°C for 15 min (6). The solution was then extracted with an equal volume of chloroform-isoamyl alcohol (24:1, vol/vol), and the DNA from the resulting aqueous phase was purified with GeneClean (Bio 101, La Jolla, Calif.) according to the manufacturer's protocol and eluted with 50  $\mu$ l of sterile distilled water.

**Method for seeding and testing sputum.** An *M. avium* complex clinical isolate was serially diluted in sterile water to a concentration of 10<sup>5</sup> CFU/ml and spiked into sputum from known uninfected patient samples, which had been stored 1 month at -20°C, yielding final concentrations of 10<sup>4</sup> and 10<sup>3</sup> CFU/ml. The 1.0-ml samples were then centrifuged at 12,000  $\times$  g in a Beckman Microfuge (Beckman Instru-

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ments, Inc., Palo Alto, Calif.) for a total of 5 min, lysed in 0.1 ml of 0.1 M NaOH–2 M NaCl–0.5% SDS at 95°C for 15 min, and purified as described above.

**Selection of probes and primers.** The primers TB1 and TB2 and probes TB4 (probe for *M. tuberculosis*) and TB5 (probe for *M. avium*) used in this study were previously described by Hance et al. (6) based on the sequence of a region of the 65-kDa heat shock protein in mycobacteria (15). The 65-kDa heat shock protein is a major mycobacterial antigen which is highly conserved. The sequences of the primers and probes are as follows: TB1, 5'GAGATCGAGCTGGAGGATCC; TB2, 5'AGCTGCAGCCCAAAGGTGTT; TB4, 5'CGAAATCGCTGCGGTGGCCG; and TB5, 5'CTGCCACCGCGGC CATCTCC.

**Amplification of DNA by PCR.** Aliquots (10  $\mu$ l) of DNA samples were amplified, as previously described (9, 12), in 50- $\mu$ l reaction mixtures that contained 50 mM KCl; 10 mM Tris (pH 8.3); 0.01% gelatin; 2 mM MgCl<sub>2</sub>; 200  $\mu$ M each dATP, dCTP, and dGTP; 180  $\mu$ M dTTP; 20  $\mu$ M digoxigenin-11-dUTP (Boehringer Mannheim Biochemicals, Indianapolis, Ind.); 30 pmol of each primer TB1 and TB2; and 2.5 U of Amplitaq (Perkin-Elmer/Cetus, Norwalk, Conn.). Amplification in a programmable heat block (DNA Thermal Cycler; Perkin-Elmer/Cetus) was for 35 cycles of 94°C for 1 min (denaturation), 55°C for 1 min (annealing), and 72°C for 2 min (extension). Positive and negative controls were included in each set of specimens tested. After amplification, one-fifth of the reaction volumes was loaded onto a 2% agarose gel and electrophoresed; the gel was then stained with ethidium bromide and photographed under UV illumination.

**Southern hybridization.** The amplified products were fractionated by electrophoresis in a 2% agarose gel, denatured, neutralized, and transferred to a nylon membrane (Zeta-probe; Bio-Rad Laboratories, Richmond, Calif.). The blot was hybridized with either probe TB4 or probe TB5. The oligonucleotide probes were labeled by tailing their 3' ends with digoxigenin-11-dUTP and terminal transferase. Hybridization was performed as specified by the Genius kit (Boehringer Mannheim) except that the colorimetric substrate in the kit was substituted with a 0.24 mM concentration of the chemiluminescent substrate AMPPD (Tropix, Bedford, Mass.) in 0.1 M diethanolamine–1 mM MgCl<sub>2</sub>–0.02% sodium azide.

**Preparation of the membranes for reverse dot blots.** Oligonucleotides were synthesized at the Biomolecular Resource Center (University of California, San Francisco, Calif.); 100 pmol of each oligonucleotide was tailed with dTTP at 37°C for 45 min in a reaction containing 100  $\mu$ l of terminal transferase buffer (Boehringer Mannheim), 1 mM CoCl<sub>2</sub>, 2.5 mM dTTP, and 1.5  $\mu$ l of terminal transferase (25 U/ $\mu$ l). To further extend the length of the poly(T) tail, an additional 1  $\mu$ l of terminal transferase was added, and the incubation was continued for an additional 45 min. The reaction was stopped by the addition of 2  $\mu$ l of 0.5 M EDTA, pH 8.0.

Nylon membranes (Zeta Probe; Bio-Rad Laboratories) which had been presoaked in 10 $\times$  SSC (20 $\times$  SSC contained 3 M NaCl and 0.3 M sodium citrate, pH 7.0) were cut into strips (1 by 3 cm). Each strip was spotted with 2 pmol of tailed oligonucleotide probes TB4 and TB5. The membranes were UV fixed at 50 J (Stratalinker; Stratagene, La Jolla, Calif.), and the irradiated membranes were washed in 5 $\times$  SSPE (20 $\times$  SSPE contained 3 M NaCl, 0.2 M sodium phosphate, and 20 mM EDTA, pH 7.4) with 0.5% SDS for 30 min at 50°C to remove unbound probes, rinsed briefly with distilled water, air dried, and stored at –20°C until used.

**Reverse dot blots.** The nylon membranes were prehybrid-

ized in 5 $\times$  SSPE–0.5% SDS–0.5% dextran sulfate at 60°C for 5 min. Twenty microliters of amplified product was denatured by heating at 95°C for 10 min, added to the nylon membranes containing the prehybridization mix, and hybridized at 60°C for 1 h in Whirl Pak bags (Nasco Corp., Fort Atkinson, Wis.). The membranes were washed in 2 $\times$  SSPE–0.1% SDS twice for 5 min at room temperature and once for 10 min at 60°C and then rinsed briefly in Buffer 1 (0.1 M Tris [pH 7.5], 0.15 M NaCl). For chemiluminescent detection, the membranes were blocked with 1% Genius block in Buffer 1 for 30 min at room temperature, incubated with antidigoxigenin antibody conjugated with alkaline phosphatase (1:5,000 dilution in Buffer 1) for 30 min, and washed with Buffer 1 for 15 min. The filters were incubated with 0.24 mM AMPPD in 0.1 M diethanolamine–1 mM MgCl<sub>2</sub>–0.02% sodium azide for 5 min, wrapped in plastic wrap, exposed to Hyperfilm-ECL (Amersham Corp., Arlington Heights, Ill.) for 20 min, and then developed.

The detection limit of the reverse dot blot method was determined by serially diluting a clinical isolate of *M. avium* complex, determining the number of viable organisms from each sample, and isolating the DNA from samples containing 10<sup>4</sup> down to 10<sup>1</sup> CFU/ml. A 1/5 volume of each resulting sample was amplified with TB1 and TB2 and detected by reverse dot blot hybridization as described above. A positive control, DNA from an *M. avium* clinical isolate, and a negative control, no added DNA, were always included in the procedure.

**Sequencing by dideoxynucleotide chain termination.** Single-stranded DNA was generated by asymmetric PCR and sequenced with the chain termination method (14) by using the Sequenase kit (United States Biochemical Corp., Cleveland, Ohio) and either TB1 or TB2.

**Species identification of mycobacteria by using a commercially available DNA probe test.** The SNAP hybridization assay (Syngene, San Diego, Calif.) for the species identification of members of the *M. avium* complex was performed according to the manufacturer's instructions. Briefly, the target DNA was fixed to a nylon membrane and hybridized to alkaline phosphatase-conjugated DNA probes directed against the *M. avium* complex. After hybridization, the spots were detected with a colorimetric alkaline phosphatase substrate.

## RESULTS

**PCR amplification of DNA with the primers TB1 and TB2.** Primers TB1 and TB2 amplified a 383-bp DNA product from the coding sequence of the mycobacterial 65-kDa heat shock protein (Fig. 1A). Southern hybridization with digoxigenin-labeled DNA probes TB4 and TB5 yielded bands confirming the identity of *M. tuberculosis* and the *M. avium* complex (data not shown). Reverse dot blot analysis with probes TB4 and TB5 yielded results similar to those obtained with Southern hybridization (Fig. 1B).

**Detection limit of the reverse dot blot method.** Serial dilutions of a clinical isolate of the *M. avium* complex were tested by using the amplification and reverse dot blot method. The 383-bp amplification product could be visualized with ethidium bromide staining in samples containing as few as 10<sup>3</sup> CFU of mycobacteria. However, reverse dot blot hybridization resulted in a 10-fold increase in sensitivity over gel electrophoresis, and as few as 10<sup>2</sup> CFUs could consistently be detected. Dilutions of the same *M. avium* complex isolate put into sputum also resulted in a detection limit of 10<sup>2</sup> CFU by reverse dot blot.

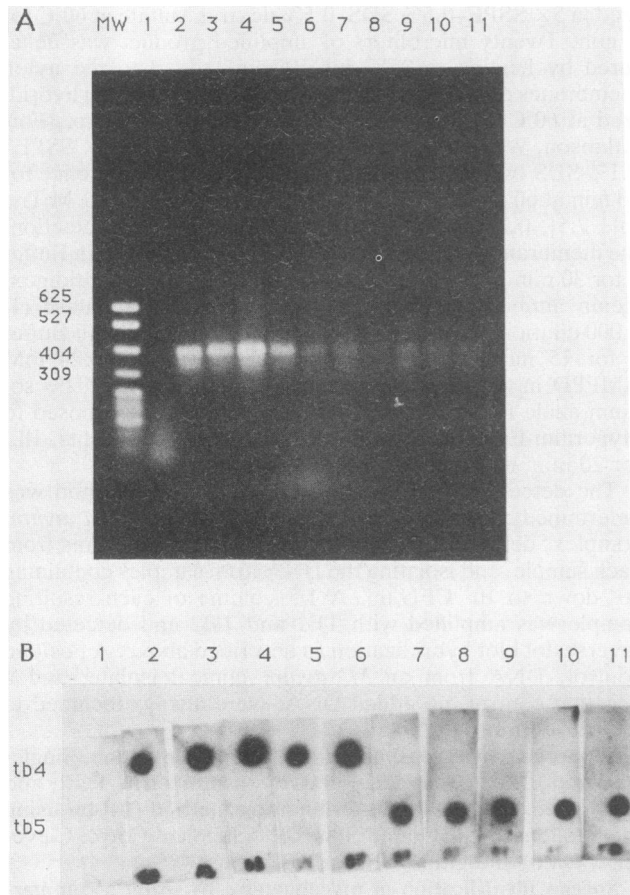


FIG. 1. Amplification of mycobacterial DNA. (A) Ethidium bromide-stained agarose gel electrophoresis of amplified DNA from five *M. tuberculosis* isolates (lanes 2 to 6) and five *M. avium* complex isolates (lanes 7 to 11); lane MW, molecular weight standard, pBR322 *MspI* digest; lane 1, negative control for the DNA amplification. (B) Reverse dot blot of the amplified samples from panel A. The strips were spotted with probes TB4 (for *M. tuberculosis*) and TB5 (for the *M. avium* complex), followed by hybridization with the digoxigenin-labeled amplification product, washing, and detection as specified in Materials and Methods. The autoradiogram for the reverse dot blots was exposed for 20 min. Strip 1, negative control; strips 2 to 6, *M. tuberculosis* isolates; strips 7 to 11, *M. avium* complex isolates.

**Specificity of the reverse dot blot method.** A preliminary screen of isolates from clinical specimens of various species was performed to assess the specificity of this method. The TB4 probe hybridized to *M. tuberculosis* and *M. bovis* BCG but not to the *M. avium* complex, *M. kansasii*, *M. xenopi*, *M. gordonae*, *M. scrofulaceum*, or *Rhodococcus* spp. The TB5 probe hybridized to *M. avium* but not to *M. tuberculosis*, *M. bovis* BCG, *M. kansasii*, *M. xenopi*, *M. gordonae*, or *Rhodococcus* spp. In addition, probe TB5 cross-hybridized with *M. scrofulaceum* (Fig. 2B, strips 6 and 7). These *M. scrofulaceum* isolates did not hybridize with the commercially available SNAP *M. avium* DNA probe test, indicating that TB5 sequences were shared between *M. avium* and *M. scrofulaceum*.

A total of 12 *M. tuberculosis* isolates and 50 *M. avium* complex isolates were tested. All *M. tuberculosis* isolates hybridized exclusively with TB4, whereas only 45 *M. avium*

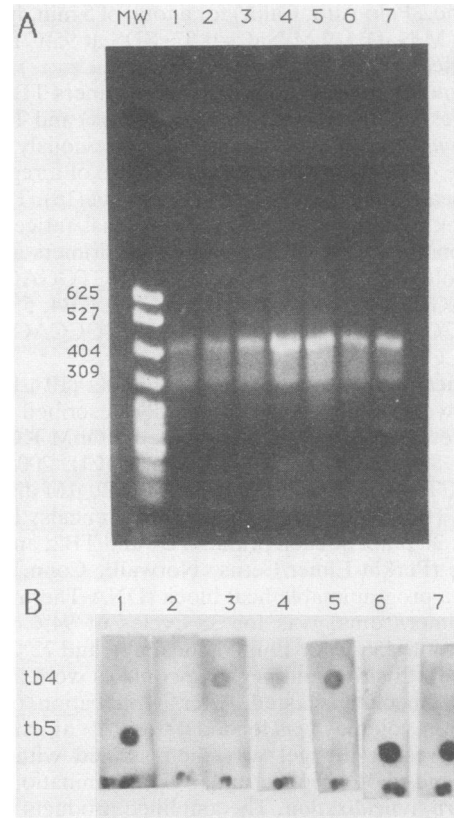


FIG. 2. Amplification of mycobacterial DNA. (A) Ethidium bromide-stained agarose gel electrophoresis of amplified DNA from isolates: lane 1, *M. avium*; lane 2, *M. xenopi*; lanes 3 to 5, *M. intracellulare*; lanes 6 and 7, *M. scrofulaceum*; lane MW, molecular weight standard, pBR322 *MspI* digest. (B) Reverse dot blot of the amplified samples from panel A. The strips were spotted with probes TB4 (for *M. tuberculosis*) and TB5 (for the *M. avium* complex), followed by hybridization with the digoxigenin-labeled amplification product, washing, and detection as specified in Materials and Methods. The autoradiogram for the reverse dot blots was exposed for 20 min. Strips correspond to lanes in panel A.

complex isolates were detected exclusively by TB5. Of the remaining five *M. avium* complex isolates, three hybridized exclusively with TB4 (Fig. 2B, strips 3 to 5) and two hybridized with neither probe (Fig. 2B, strip 2, one of the two isolates). Increasing the stringency of the washing step after hybridization did not eliminate the cross-hybridization of TB4 with the three *M. avium* complex isolates. The two *M. avium* complex isolates which did not hybridize with either probe were also not detected with the SNAP probe for the *M. avium* complex. These isolates were further examined by biochemical methods and identified as *M. xenopi*.

The hybridization results of the three *M. avium* complex isolates that reacted with TB4 were further examined. DNA from *M. avium* ATCC 25291, *M. intracellulare* ATCC 13950 and ATCC 35770, and *M. tuberculosis* ATCC 25177 were amplified and tested by reverse dot blot. Whereas the *M. avium* DNA hybridized with TB5 only and the *M. tuberculosis* DNA hybridized with TB4 only, the two *M. intracellulare* DNAs hybridized with TB4 and not with TB5. This suggested that the three *M. avium* complex clinical isolates which hybridized with the *M. tuberculosis* probe were in fact *M. intracellulare* and that TB4 cross-hybridized with

M. b/tb	CTC	AAA	CGC	GCC	ATC	GAA	AAG	GCC	GTG	GAG	AAG	GTC	ACC	GAG	ACC	196
M. avium	---	--G	---	---	---	--G	---	---	--C	---	---	---	---	---	---	
M. intrac	---	--G	---	---	---	--G	---	---	--C	---	---	---	---	---	---	
M. b/tb	CTG	CTC	AAG	GCC	GCC	AAG	GAG	GTC	GAG	ACC	AAG	GAG	CAG	ATT	GCG	241
M. avium	---	---	---	TCG	---	---	---	---	---	---	---	---	--C	---	--T	
M. intrac	---	---	---	TCG	---	---	---	---	---	---	---	---	--C	---	--T	
M. b/tb	GCC	ACC	GCA	GCG	ATT	TCG	GCG	GGT	GAC	CAG	TCC	ATC	GGT	GAC	CTG	286
M. avium	---	---	--G	--C	--C	--C	---	---	---	---	--G	---	--C	---	---	
M. intrac	---	---	--G	---	--C	---	---	---	---	---	--G	---	---	---	---	
M. b/tb	ATC	GCC	GAG	GCG	ATG	GAC	AAG	GTG								310
M. avium	---	---	---	---	---	---	---	--C								
M. intrac	---	---	---	---	---	---	---	---								

FIG. 3. Comparative sequences of the mycobacterial amplified products. Residues that are identical to *M. bovis* are indicated by dashes. *M. bovis* and *M. tuberculosis* (*M. b/tb*) sequences are identical. *M. intrac*, *M. intracellulare*. Sequences for *M. bovis* and *M. avium* are from Hance et al. (6). Probes TB4 and TB5 hybridize with bases 240 to 259.

*M. intracellulare*. To verify this possibility, additional primers and probes were constructed by using 16S rRNA sequences which were specific for *M. avium*, *M. intracellulare*, and *M. tuberculosis*; the three *M. avium* complex isolates which hybridized with probe TB4 hybridized with the probe for the 16S rRNA sequence of *M. intracellulare* (3a).

To further investigate the cross-reactivity of the *M. intracellulare* isolates with TB4, the DNA sequences of the amplified products were determined from *M. intracellulare* ATCC 13950 and *M. tuberculosis* ATCC 25177 (Fig. 3). In the region of TB4 and TB5 hybridization, the sequence for *M. avium* differed from *M. tuberculosis* by 5 out of 20 nucleotides, whereas the sequence for *M. intracellulare* differed from *M. tuberculosis* by 2 nucleotides.

### DISCUSSION

A method is described for the rapid identification of an array of mycobacterial species by PCR and the chemiluminescent detection of species-specific DNA sequences with reverse dot blot hybridization. The format of the assay was readily applicable for implementation in the clinical laboratory. The assay required no radioactivity and had a fast turnaround time of 1 to 2 days as compared with approximately 2 weeks to as long as 8 weeks for routine or radiometric culture methods. The use of premade PCR reaction mixtures and DNA fixed membrane strips could increase the speed of the assay. We routinely isolated the DNA in less than 2 h, performed the amplification overnight, and performed reverse dot blot hybridization in 4 h the following day.

Use of the chemiluminescent method enabled as few as 10<sup>2</sup> CFU to be detected in a sample. These results were in agreement with earlier reports which utilized the same set of primers (11); however, we have removed the need for radioisotopes, which should simplify the transfer of the assay from research to clinical laboratories.

The choice of primers and probes was critical for this method to be successful. In this report, we used primers and probes previously reported by another laboratory to detect *M. tuberculosis* and *M. avium* in specimens from patients. However, the testing of a large number of isolates was not reported. We, therefore, felt it was necessary to ascertain the sensitivity and specificity of these probes. The 50 *M. avium* complex isolates tested represented a random sam-

pling of *M. avium* complex isolates encountered in our laboratory. We were fortunate that the sampling included a few isolates of *M. intracellulare*, promoting the observation that probe TB4, but not probe TB5, hybridized to the *M. intracellulare* ATCC strains and the *M. intracellulare* clinical isolates.

PCR sequencing of the amplified 383-bp product from *M. tuberculosis* and *M. intracellulare* revealed nucleotide substitutions encompassing the region of both the TB4 and TB5 probes. The probes on the reverse dot blot strip were designed to discriminate single-base-pair changes. Although DNA sequencing revealed that the TB4 probe differed by two nucleotides from the *M. intracellulare* sequence, the hybridization was not affected because the substitution that occurred at the 3' end of the probe did not result in sufficient thermal instability to affect hybridization. The second substitution at position 250 (A to G) was stabilized by the nature of the mismatch (G:T) which could form stable hydrogen bonding between the two nucleotides. Such mismatches are stable and can often result in cross-hybridization.

There were two additional unexpected results from this study. The first was that the probe for *M. avium*, TB5, hybridized with the two *M. scrofulaceum* isolates tested, suggesting that TB5 lacked adequate specificity. The second unexpected finding was that the two isolates which did not hybridize with either the probe for *M. tuberculosis* or the probe for *M. avium* were both misidentified. These isolates had been initially identified as *M. avium* complex isolates but were reidentified with additional biochemical testing as *M. xenopi*. *M. xenopi* and *M. avium* are difficult to distinguish by biochemical testing, leading to the misidentification of *M. xenopi*. It is possible that other *M. avium* complex isolates originally reported to be nonhybridizing in commercial DNA probe tests were in fact *M. xenopi*.

This study examined the identification of mycobacteria to the species level on the basis of reverse dot blot analysis with amplified mycobacterial DNA. This technique will be useful in the identification of mycobacteria to the species level. Since both probes TB4 and TB5 were designed from the sequencing of single isolates, it was important to test the sensitivity of these probes with multiple isolates. In future studies it will be important to design probes for hybridization after sequencing appropriate regions of DNA from a large number of isolates from multiple species, thus eliminating the possibility of cross-hybridization or low sensitivity.

The ultimate goal for application of the reverse dot blot technology with amplified mycobacterial DNA is the direct detection of mycobacterial infection, providing rapid diagnosis and circumventing the need for culture. The data in our paper have demonstrated the feasibility of this application.

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