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# Identification and Differentiation of *Mycobacterium avium* and *M. intracellulare* by PCR

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**Known DNA sequences coding for the 16S rRNAs of 14 slowly growing *Mycobacterium* species were analyzed. Three sets of primers were synthesized: MAV and MIN, for *M. avium* and *M. intracellulare*, respectively, and MYCOB, for the slowly growing mycobacteria. Whole-cell DNAs of 14 reference species were extracted and amplified by PCR with the MYCOB, MAV, and MIN primers. The MYCOB primer amplified a 0.9-kb segment from the DNAs of all 14 species. The MAV and MIN primers each amplified one highly specific 1.3-kb segment from the homologous DNA, respectively. DNAs from each of 10 clinical isolates of *M. avium* and *M. intracellulare* identified by conventional methods were amplified with the MYCOB as well as the MAV and MIN primers; 9 of 10 isolates of each species were identified with their respective primers. One isolate of *M. intracellulare* was subsequently found to have been mislabeled. One isolate designated *M. avium* reacted only with the MYCOB primer. The hypervariable region of this strain was shown by DNA sequence analysis to be distinct from all known 16S rRNA sequences of *Mycobacterium* spp. Our data indicate that the currently identified *M. avium-M. intracellulare* complex includes strains genetically diverse from *M. avium* and *M. intracellulare.***

The slowly growing species *Mycobacterium avium* and *M. intracellulare* have overlapping phenotypic properties that make their identification difficult by conventional procedures. Because of this, strains are frequently identified as the *M. avium-M. intracellulare* complex (MAC) (3). MAC strains appear to be distributed widely in the environment, have been isolated from water, soil, and dairy products, and may also be carried by birds (8, 19). MAC strains occasionally cause human disease that is indistinguishable from tuberculosis (12, 13). The organisms infect lung, lymph nodes, skin, bones, and gastrointestinal and genitourinary tracts (5). Although MAC strains cause only occasional infections in the general population, the incidence of MAC infection among AIDS patients may range as high as 30 to 80% (1, 14). Molecular probes have been used for the identification of *M. tuberculosis*, *M. kansasii*, and MAC strains (4, 6, 7, 10, 11, 18). In recent years, GenProbe Inc. (San Diego, Calif.) advertised probes specific for *M. avium* and *M. intracellulare.*

Kulski et al. (9) reported the use of a multiplex PCR to detect and identify *M. avium*, *M. intracellulare*, and *M. tuberculosis* in blood culture fluids from AIDS patients. They used multiple sets of primers in a single PCR tube. Five of the 55 confirmed *M. avium* isolates were identified as *M. intracellulare* by their multiplex PCR. In their system, 2 of 32 isolates of *M. intracellulare* were identified as *M. avium* by PCR. In their blood culture testing, one of their strains identified as *M. intracellulare* by a routine method was found to be *M. avium* (9).

We have analyzed the published DNA sequences coding for the 16S rRNAs of *M. avium*, *M. intracellulare*, and 12 other slowly growing mycobacteria. Three sets of primers were designed: two were specific for *M. avium* and *M. intracellulare*, respectively, and the third was a universal primer set that should amplify DNAs from all slowly growing mycobacteria. These three primer sets were evaluated for their specificities for 14 reference strains via PCR amplification and electrophoresis. The primers for *M. avium* and *M. intracellulare* were evaluated further with 10 strains each of preidentified *M. avium* and *M. intracellulare* organisms.

Cultures were obtained from the Centers for Disease Control and Prevention (CDC) and Gillis W. Long Hansen's Disease Center (GWLHDC) (Table 1). Mycobacteria from GWLHDC were grown on Middlebrook 7H10 agar plates containing 550 ng of cycloheximide per ml and 2 mg of lincomycin per liter. The cultures were incubated at 37°C for up to 6 weeks. The additional 20 clinical isolates of *M. avium* and *M. intracellulare* were obtained from the culture collection at CDC and were identified by conventional as well as high-performance liquid chromatography (HPLC) procedures. Six of the 10 isolates of *M. avium* (including strain 18) and 2 of the 10 isolates of *M. intracellulare* (excluding strain 2) were probed with their homologous probes (GenProbe). These cultures, all isolated from AIDS patients, were grown either on Löwenstein-Jensen slants for about 3 weeks or in 7H9 broth for 1 week.

DNA sequences coding for the 16S rRNAs of 14 reference strains were obtained from GenBank. The 16S rRNA sequence of each *Mycobacterium* species was aligned and compared with those of *M. avium* and *M. intracellulare* by using the PCGene software package (release 6.01; IntelliGenetics, Inc.). The regions that contained the most mismatched bases (hypervariable fragments) were used for the synthesis of primers (Table 2). The "universal" MYCOB primer sets designed for the detection of all slowly growing mycobacteria was based on the conserved region of the 16S rRNA.

DNA was extracted by the minibead beating procedure (15). In brief, a 200-μl suspension of heat-killed mycobacteria was mixed with an equal volume of chloroform in a 2-ml beveled-bottom, screw-cap sterile tube with siliconized glass beads. The mixture was agitated for 2 min at the maximum setting on a Mickle apparatus (Mickle Laboratory, Gomshall, United Kingdom). The chloroform layer was separated in a centrifuge, and

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TABLE 1. *Mycobacterium* reference strains and descriptions

| Species | Code no. | Validation[a] |
|---|---|---|
| *M. avium* | CDC 86-8880 | a, b, c |
| *M. bovis* | ATCC 35724 | a, b |
| *M. gastri* | ATCC 15754 | a |
| *M. gordonae* | CDC 87-513 | a, b, c |
| *M. intracellulare* | ATCC 13950 | a, b |
| *M. kansasii* | ATCC 12478 | a |
| *M. malmoense* | CDC 1661 | a |
| *M. marinum* | CDC 84-842A | a |
| *M. scrofulaceum* | CDC 88-1216 | a, b |
| *M. simiae* | CDC 85-797 | a |
| *M. szulgai* | CDC 82-824 | a, b |
| *M. terrae* | CDC TI-285 | a, b |
| *M. tuberculosis* | ATCC 27294 | a, b |
| *M. xenopi* | ATCC 19250 | a |

[a] a, identified by biochemical tests; b, confirmed by the HPLC; c, probed by GenProbe system.

the upper aqueous layer containing DNA was collected and stored at −20°C for PCR.

The PCR Reagent Kit with Native *Taq* DNA polymerase (GeneAmp; Perkin-Elmer) was used for the amplification of the DNA according to the manufacturer's instructions. The total volume was 100 μl; 10 μl of each template DNA was used. The thermal cycler (MiniCycler; MJ Research, Inc.) conditions were set as follows: denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 6 min (2). A 15-min extension period at 72°C was added after 40 cycles. Negative controls, with the exclusion of the template DNA, as well as positive controls were included. The PCR products were revealed by electrophoresis and ethidium bromide staining.

As demonstrated by others (2, 16), our DNA computer analysis showed a high degree of homology among the 14 reference strains. The base identities between *M. avium*, *M. intracellulare*, and the other species ranged from 93.7 to 99.7%. The MYCOB primer set (sense, 5′-ATG CAA GTC GAA CGG AAA GG-3′ [positions 18 to 37 bp]; antisense, 5′-TGC ACA CAG GCC ACA AGG GA-3′ [positions 976 to 995 bp]) was designed to contain sequences that appear in all of the slowly growing species studied. The MAV primer set, specific for *M. avium* (sense, 5′-CCT CAA GAC GCA TGT CTT CT-3′ [positions 141 to 160 bp]; antisense, 5′-ACA GCT CCC TCC CAA AAG GG-3′ [positions 1,400 to 1,419 bp]), and the MIN primer set, specific for *M. intracellulare* (sense, 5′-CCT TTA GGC GCA TGT CTT TA-3′ [positions 130 to 149 bp]; antisense, 5′-GCA CAG CTC CCT CCC AAG GG-3′ [positions 1,389 to 1,408 bp]) were designed to correspond to the two regions of the 16S rRNA sequences which contain the largest number of mismatched bases (Table 2). The nucleotide position 1 in the numbering given above refers to *Escherichia coli* 16S rRNA position 161 (2, 16).

TABLE 2. Mismatched bases in two hypervariable fragments between *M. avium* and *M. intracellulare* and other mycobacteria

| Organism | No. of mismatched bases | | | |
|---|---|---|---|---|
| | *M. avium* | | *M. intracellulare* | |
| | 140–159 bp | 1,400–1,419 bp | 140–159 bp | 1,400–1,419 bp |
| *M. avium* | —[a] | — | 5 | 2 |
| *M. bovis* | 5 | 2 | 6 | 0 |
| *M. gastri* | 6 | 3 | 6 | 1 |
| *M. gordonae* | 5 | 2 | 9 | 1 |
| *M. intracellulare* | 5 | 2 | — | — |
| *M. kansasii* | 6 | 3 | 6 | 1 |
| *M. malmoense* | 6 | 3 | 6 | 1 |
| *M. marinum* | 7 | 2 | 10 | 2 |
| *M. scrofulaceum* | 6 | 3 | 5 | 0 |
| *M. simiae* | 6 | 3 | 6 | 3 |
| *M. szulgai* | 6 | 2 | 6 | 0 |
| *M. terrae* | 8 | 3 | 8 | 1 |
| *M. tuberculosis* | 5 | 3 | 8 | 1 |
| *M. xenopi* | 10 | 5 | 8 | 7 |

[a] —, homologous sequences.

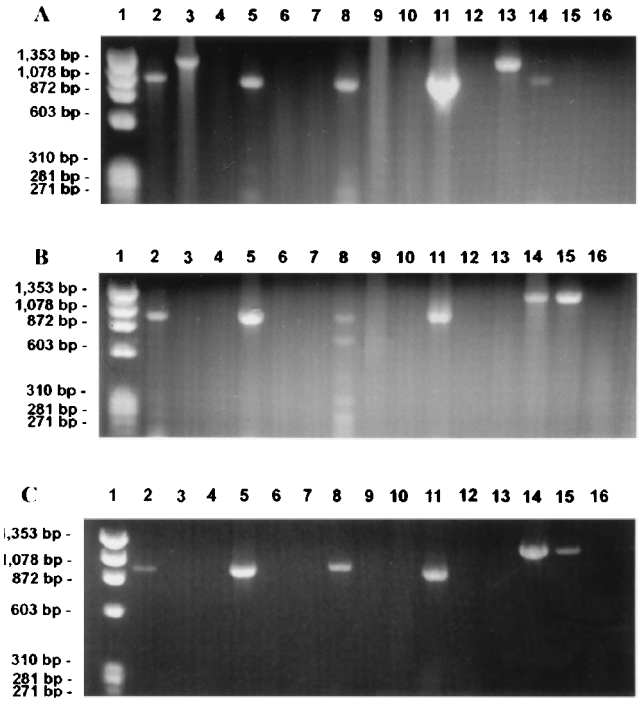


FIG. 1. PCR products of 14 strains of slowly growing mycobacteria amplified by MYCOB, MAV, and MIN. (A) Lane 1, DNA marker (ϕX174, *Hae*III digested); lanes 2 to 4, *M. avium* DNA; lanes 5 to 7, *M. gastri* DNA; lanes 8 to 10, *M. gordonae* DNA; lanes 11 to 13, *M. intracellulare* DNA; lanes 14 to 16, *M. kansasii* DNA. (B) Lane 1, DNA marker (ϕX174, *Hae*III digested); lanes 2 to 4, *M. marinum* DNA; lanes 5 to 7, *M. scrofulacem* DNA; lanes 8 to 10, *M. szulgai* DNA; lanes 11 to 13, *M. terrae* DNA; lanes 14 to 15, positive controls for MAV and MIN; lane 16, negative control. (C) Lane 1, DNA marker (ϕX174, *Hae*III digested); lanes 2 to 4, *M. tuberculosis* DNA; lanes 5 to 7, *M. xenopi* DNA; lanes 8 to 10, *M. malmoense* DNA; lanes 11 to 13, *M. bovis* DNA; lanes 14 to 15, positive controls for MAV and MIN, respectively; lane 16, negative control.

The PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin-Elmer) was used for DNA sequencing on an ABI model 373A DNA Sequencer (Applied Biosystems, Inc.). The PCR products amplified with the MYCOB primer set were sequenced. The primer used for sequencing was the sense fragment of MYCOB. Another primer located within the MYCOB amplification product was synthesized for sequencing to verify the sequencing results from the opposite direction.

DNAs from all 14 *Mycobacterium* reference species were amplified by the MYCOB primer set and produced strong bands approximately 1 kb in size (978 bp, as from the sequence; Fig. 1), along with weak bands of various sizes. The search of repeat sequences of the primers (both sense and antisense) on the 16S rRNA sequences of those species did not reveal any additional sequences on the sense or antisense strand corresponding to the MYCOB primer set (data not

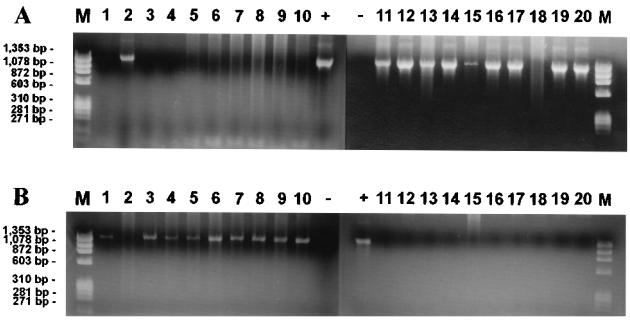


FIG. 2. DNAs from 20 clinical isolates amplified with MAV and MIN. Lane M, DNA marker (ϕX174, *Hae*III digested); lanes + and −, positive and negative controls for MAV and MIN, respectively; lanes 1 to 10, *M. intracellulare* strains; lanes 11 to 20: *M. avium* strains. (A) DNA was amplified by MAV. (B) DNA was amplified by MIN.

shown). Nonspecific amplification probably produced the shorter (less than 900-bp) bands. Alternatively, some sequences complementary to the MYCOB primer set may be located outside of the 16S rRNA gene.

The MAV primer set amplified DNAs from *M. avium* strains only and produced a band of approximately 1.3 kb (1,279 bp, on the basis of the sequence). DNA was not amplified from any of the other 13 species tested (Fig. 1). The MIN primer amplified DNAs from *M. intracellulare* strains only and produced an approximately 1.3-kb band (1,279 bp, on the basis of the sequence) (Fig. 1).

The DNAs of all 20 clinical MAC isolates were amplified efficiently by the MYCOB primers (data not shown), indicating that each sample contained sufficient DNA for detection by this procedure. DNAs from 9 of 10 isolates labeled *M. intracellulare* were identified with the MIN primer (Fig. 2). The strain that was not identified by the MIN primer was amplified by the MAV primer (strain 2). The DNA from this strain was sequenced, and its hypervariable fragments were found to be identical to the published *M. avium* sequence (Table 3). On the basis of the direct sequence evidence, it was concluded that strain 2 was mislabeled. As a control, another clinical isolate, *M. avium* CDC 88-1354, was also sequenced, and its hypervariable fragment was shown to be identical to the published sequence (*M. avium* DSM 43216, obtained from GenBank).

The MAV primer amplified DNAs from 9 of 10 *M. avium* isolates (Fig. 2). The DNA from one putative *M. avium* strain (strain 18; Fig. 2) was not amplified by the MAV or MIN primer. DNA from this strain was sequenced, and the hypervariable fragment was found to exhibit sequence differences from both *M. avium* (3 of 20 mismatches) and *M. intracellulare* (2 of 20 mismatches) (Table 3). Additionally, the sequence of the hypervariable fragment of strain 18 was found to be unique when it was compared with all published 16S rRNA sequences of mycobacteria (available in GenBank, including fast growing and "newer" discovered mycobacteria). Soini et al. (17), during the review process for this report, reported genetic diversity for six strains of the *M. avium* complex that were positive with AccuProbe. The 16S rRNA genes of these strains were also partially sequenced, but specific sequences were not presented. Apparently, in the hypervariable fragment, as few as 2 bp can distinguish *M. avium* and *M. intracellulare* from other *Mycobacterium* species.

TABLE 3. DNA sequences of hypervariable fragments in 16S rRNA

| Strain | Code no. | Hypervariable fragment sequence |
|---|---|---|
| *M. avium*[a] | DSM 43216 | 5′-CCTCAAGACGCATGTCTTCT-3′ |
| *M. avium*[b] | CDC 88-1354 | 5′-CCTCAAGACGCATGTCTTCT-3′ |
| Strain 2[b] | CDC 89-587 | 5′-CCTCAAGACGCATGTCTTCT-3′ |
| Strain 18[b] | CDC 87-830 | 5′-CCTTTAGACGCATGTCTTTT-3′ |
| *M. intracellulare*[a] | DSM 15985 | 5′-CCTTTAGGCGCATGTCTTTA-3′ |

[a] DNA sequences from GenBank, submitted by Rogall et al. (16).
[b] From CDC culture collections, derived from clinical isolates; strain 2 was labeled *M. intracellulare*; strain 18 was labeled *M. avium.*

In total, 35 strains of slowly growing mycobacteria, including 11 strains of *M. avium* and 11 strains of *M. intracellulare*, were tested with three primer sets. No false-positive or false-negative results were observed. In those cases in which the amplification results did not confirm the previously assigned strain designations (strains 2 and 18; Fig. 2), subsequent sequence analysis verified the specificity of the PCR results.

Our data support the findings of Kulski et al. (9) that *M. avium* and *M. intracellulare* may be differentiated by specific 16S rRNA primers and PCR technology. Additionally, our data indicate that the currently identified MAC includes strains genetically diverse from *M. avium* and *M. intracellulare*.

## REFERENCES

1. **Bermudez, L. E., M. Petrofshy, P. Kolonoski, and L. S. Young.** 1992. An animal model of *Mycobacterium avium* complex disseminated infection after colonization of the intestinal tract. J. Infect. Dis. **165:**75–79.
2. **Boddinghaus, B., T. Rogall, T. Flohr, H. Blocker, and E. C. Bottger.** 1990. Detection and identification of mycobacteria by amplification of rRNA. J. Clin. Microbiol. **28:**1751–1759.
3. **Good, R. C.** 1992. The genus *Mycobacterium*—medical, p. 1238–1270. *In* A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The prokaryotes—a handbook on the biology of bacteria: ecophysiology, isolation, identification, applications, vol. II. Springer-Verlag, New York.
4. **Huang, Z. H., B. C. Ross, and B. Dwyer.** 1991. Identification of *Mycobacterium kansasii* by DNA hybridization. J. Clin. Microbiol. **29:**2125–2129.
5. **Iseman, M. D., R. F. Corp, and R. J. O'Brien.** 1985. Disease due to *Mycobacterium avium-intracellulare*. Chest **87**(Suppl.)**:**139–149.
6. **Jonas, V., M. J. Alden, J. I. Curry, K. Kamisango, C. A. Knott, R. Lankford, J. M. Wolfe, and D. F. Moore.** 1993. Detection and identification of *Mycobacterium tuberculosis* directly from sputum sediments by amplification of rRNA. J. Clin. Microbiol. **31:**2410–2416.
7. **Jurtshuk, R. J., M. Blick, J. Bresser, G. E. Fox, and P. Jurtshuk, Jr.** 1992. Rapid in situ hybridization technique using 16S rRNA segments for detecting and differentiating the closely related gram-positive organisms *Bacillus polymyxa* and *Bacillus macerans*. Appl. Environ. Microbiol. **58:**2571–2578.
8. **Kazda, J. F.** 1982. The principles of the ecology of mycobacteria, p. 323–338. *In* J. L. Stanford (ed.), The biology of mycobacteria, vol. 2. Academic Press, London.
9. **Kulski, J. K., C. Khinsoe, T. Pryce, and K. Christiansen.** 1995. Use of myltiplex PCR to detect and identify *Mycobacterium avium* and *M. intracellulare* in blood culture fluids of AIDS patients. J. Clin. Microbiol. **33:**668–674.
10. **Lebrun, L., F. Espinasse, J. D. Poveda, and V. Vincent-Levy-Frebault.** 1992. Evaluation of nonradioactive DNA probes for identification of mycobacteria. J. Clin. Microbiol. **30:**2476–2478.
11. **Lim, S. D., J. Todd, J. Lopez, E. Ford, and J. M. Janda.** 1991. Genotypic identification of pathogenic *Mycobacterium* species by using a nonradioactive oligonucleotide probe. J. Clin. Microbiol. **29:**1276–1278.
12. **Meissner, G. and W. Anz.** 1977. Sources of *Mycobacterium avium* complex infection resulting in human disease. Am. Rev. Respir. Dis. **116:**1057–1064.
13. **Merchx, J. J., E. H. Soule, and A. G. Kailson.** 1964. The histopathology of lesions caused by infection with unclassified acid-fast bacteria in man. Am. J. Clin. Pathol. **41:**244–255.
14. **Nightingale, S. D., L. T. Brid, P. M. Southern, J. D. Jockusch, S. X. Cal, and B. A. Wynne.** 1992. Incidence of *Mycobacterium avium-intracellulare* complex bacteria in human immunodeficiency virus-positive patients. J. Infect. Dis. **164:**1082–1085.
15. **Plikaytis, B. B., R. H. Gelber, and T. M. Shinnick.** 1990. Rapid and sensitive detection of *Mycobacterium leprae* using a nested primer gene amplification assay. J. Clin. Microbiol. **28:**1913–1917.
16. **Rogall, T., J. Wolters, T. Flohr, and E. Bottger.** 1990. Towards a phylogeny and definition of species at the molecular level within the genus *Mycobacterium*. Int. J. Syst. Bacteriol. **40:**323–330.
17. **Soini, H., E. Eerola, and M. K. Viljanen.** 1996. Genetic diversity among *Mycobacterium avium* complex AccuProbe-positive isolates. J. Clin. Microbiol. **34:**55–57.
18. **Tomioka, H., K. Sato, H. Saito, and H. Tasaka.** 1991. Identification of *M. avium* and *M. intracellulare* using three DNA probe tests, and their distributions in Japan. Kekkaku **66:**739–764.
19. **Wolfgang, K. J.** 1992. Mycobacteria, p. 512–515. *In* K. J. Wolfgang (ed.), Zinsser microbiology. Appleton & Lange, Norwalk, Conn.