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Novel Polymorphic Region of the *rpoB* Gene Containing *Mycobacterium* Species-Specific Sequences and Its Use in Identification of Mycobacteria

Hyeyoung Lee,[1] Hye-Eun Bang,[2] Gill-Han Bai,[3] and Sang-Nae Cho[2,4]*

Department of Biomedical Laboratory Science, College of Health Sciences, Yonsei University, Wonju 220-710,[1] and Department of Microbiology and Brain Korea 21 Project for Medical Sciences, Yonsei University College of Medicine, Seoul 120-752,[2] and Department of Microbiology, Korean Institute of Tuberculosis, Seoul 137-140,[3] and The International Vaccine Institute, Seoul 151-600,[4] Republic of Korea

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Sequence analysis of a specific region of the mycobacterium *rpoB* gene in 35 mycobacterial strains representing 26 different mycobacterial species of clinical importance showed that there exists a highly polymorphic region. Based on the sequences of the polymorphic region, the oligonucleotide probes of 14 mycobacterial species with relatively high clinical importance were designed and shown to be specific to their corresponding mycobacterial species by dot blot hybridization. The results showed that the probes designed in this study are highly specific to each mycobacterial species, which suggests that these sequences may be useful for the species identification of mycobacteria.

Tuberculosis (TB) is still a major public health problem in the world, with about 8 million new cases and over 2 million deaths reported annually. With the recent dissemination of human immunodeficiency virus infection throughout the world, infections with nontuberculous mycobacteria as well as TB have increased in many parts of the world over the last decade. For example, *Mycobacterium avium* infections accounted for almost 50% of mycobacterial infections among AIDS patients in certain geographical areas (4, 7). In order to provide proper drug regimens to patients with mycobacterial infections, it is important that species be identified correctly and rapidly, because drug regimens for TB may differ from those for other mycobacterial infections. In addition, with the wide use of liquid culture systems such as the BACTEC system (Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.) and the MB/BacT system (Organon Teknika Corp., Boxtel, The Netherlands), the rapid identification of mycobacterial species has become even more important (3, 17).

Although biochemical tests are available for *Mycobacterium* species identification, it has proven to be difficult to use these tests because of time-consuming and often incorrect identification. In order to overcome such difficulties, high-performance liquid chromatography has been widely used for species identification based on mycolic acid analysis (1, 14, 25). In addition, with recent developments in molecular techniques and the availability of genome sequencing data, several molecular tests have been developed and are used in clinical mycobacterial laboratories. rRNA sequences, notably that of 16S rRNA, have been most widely used for mycobacterium species identification (18), and commercial kits based on such sequences are available (AccuProbe; Gen-Probe Inc., LiPA, Innogenetics N. V., Zwijnaarde, Szinjdrecht, Belgium). In addition, the *hsp65* gene (8, 15, 16, 23), the intergenic region between 16S and 23S rRNA (19), and the *rpoB* gene (9, 10, 13) are among the targets for molecular technique-based species identification. Sequencing (11, 15, 18, 20–22), DNA hybridization (6, 12), PCR-restriction fragment length polymorphism analysis (RFLP) (8, 9, 13, 16, 23), and microarray technology have also been employed to differentiate *Mycobacterium* species (5, 24).

Among the target genes, we were particularly interested in the *rpoB* gene. PCR sequence analysis of a region of the *rpoB* gene was suggested as a possible means of differentiating 44 species (9, 10, 13). We have also reported a new RFLP method (13) based on a different region of the *rpoB* gene, which is located between the first variable region (V1) and the second conserved region (C2), as determined using the genetic information of the *Escherichia coli rpoB* gene. The 360-bp region of the *rpoB* gene (bases 902 to 1261 and codons 302 to 420 of the *rpoB* gene of *M. tuberculosis*; GenBank accession number P47766) was found to be useful in the differentiation of more than 50 *Mycobacterium* species by a simple RFLP using two restriction enzymes. This clearly indicates that this 360-bp region of the *rpoB* gene contains highly informative sequences. In the present study, we analyzed sequences of this *rpoB* region of 35 mycobacterial strains representing 26 different mycobacterial species and prepared DNA probes that can be used in simple DNA hybridization tests for the identification of *Mycobacterium* species.

A total of 48 mycobacterial reference strains representing 39 *Mycobacterium* species were used for the PCR amplification of the 360-bp region of the *rpoB* gene in the present study (Table 1). Among them, 39 mycobacterial strains were obtained from the Korean Institute of Tuberculosis (KIT), Seoul, Korea, and three species were obtained from the Korean Collection for Type Cultures (KCTC) at the Korean Research Institute of Bioscience and Biotechnology (KRIBB). *M. abscessus*, which

* Corresponding author. Mailing address: Department of Microbiology, Yonsei University College of Medicine, 134 Shinchon-dong, Seoul 120-752, Republic of Korea. Phone: 822-361-5282. Fax: 822-392-9310. E-mail: raycho@yumc.yonsei.ac.kr.

TABLE 1. Bacterial strains used in this study

Species	Strain	Source[a]
M. africanum	ATCC 25420	KIT
M. scrofulaceum	ATCC 19981	KIT
M. gilvum	ATCC 43909	KIT
M. gastri	ATCC 15754	KIT
M. asiaticum	ATCC 25276	KIT
M. aurum	ATCC 23366	KIT
M. avium	ATCC 25291	KIT
M. moriokaense	ATCC 43059	KRIBB
M. abscessus	Pettenkofer Institute	YUMC
M. celatum type I	ATCC 51130	KIT
M. celatum type II	ATCC 51131	KIT
M. chelonae	ATCC 35749	KIT
M. bovis	ATCC 19210	KIT
M. flavescens	ATCC 14474	KIT
M. fortuitum type I	ATCC 6841	KIT
M. fortuitum type II	ATCC 49404	KIT
M. gallinarum	ATCC 19710	KRIBB
M. genavense	ATCC 51233	KIT
M. microti	ATCC 19422	KIT
M. gordonae type I	ATCC 14470	KIT
M. gordonae type II		KIT
M. gordonae type III		KIT
M. gordonae type IV		KIT
M. haemophilum	ATCC 29548	KIT
M. intracellulare	ATCC 13950	KIT
M. interjectum	ATCC 51457	KIT
M. intermedium	ATCC 51848	KIT
M. kansasii type I		Pasteur Institute
M. kansasii type II		Pasteur Institute
M. kansasii type III		Pasteur Institute
M. kansasii type IV		Pasteur Institute
M. kansasii type V		Pasteur Institute
M. mucogenicum	ATCC 49650	KIT
M. neoaurum	ATCC 25795	KIT
M. nonchromogenicum	ATCC 19530	KIT
M. parafortuitum	ATCC 19686	KIT
M. peregrinum	ATCC 14467	KIT
M. phlei	ATCC 11758	KIT
M. pulveris	ATCC 35154	KRIBB
M. malmoense	ATCC 29571	KIT
M. marinum	ATCC 927	KIT
M. szulgai	ATCC 35799	KIT
M. terrae	ATCC 15755	KIT
M. thermoresistibile	ATCC 19527	KIT
M. triviale	ATCC 23292	KIT
M. ulcerans	ATCC 19423	KIT
M. vaccae	ATCC 15483	KIT
M. xenopi	ATCC 19250	KIT
M. tuberculosis H37Rv	ATCC 27294	KIT

was recently separated from *M. chelonae* as an independent new species, was obtained from Department of Clinical Pathology at Yonsei University Medical Center (YUMC). Finally, five subtypes of *M. kansasii* were generously provided by V. Vincent at the Laboratoire de Référence des Mycobactéries, Institut Pasteur, Paris, France. Clinical isolates that were subjected to dot blot hybridization to evaluate the specificity of each mycobacterial species-specific probes were obtained from the KIT. All clinical isolates used in this study were identified on the basis of conventional tests that included microbiological characterization and biochemical tests and an *rpoB*-based RFLP method (13) to precisely identify the clinical isolates.

The primer sets used to amplify the target *rpoB* gene were 5′-TCAAGGAGAAGCGCTACGA-3′ (RPO5′) and 5′-GGATGTTGATCAGGGTCTGC-3′ (RPO3′), which resulted in a 360-bp PCR product (13). PCR was carried out in a final volume of 50 μl with 10 μl of DNA supernatant containing approximately 10 ng of genomic DNA, 10 pmol of each primer, 2 mM $MgCl_2$, 200 μM concentrations of deoxynucleoside triphosphates, and 1 U of DyNAzymeII DNA polymerase (Finnzymes, Espoo, Finland). DNA samples were first denatured completely by incubation at 94°C for 5 min and then amplified using 35 cycles of (i) denaturation at 94°C for 1 min, (ii) primer annealing at 58°C for 1 min, and (iii) elongation at 72°C in a thermocycler (Perkin-Elmer model 9600; Applied Biosystems, Foster City, Calif.). After the last amplification cycle, the samples were incubated further at 72°C for 7 min to obtain complete elongation of the final PCR products. Positive and negative controls were always included in each PCR. The positive control was the PCR mix with the DNA of the reference strain, *M. bovis*, and the negative control was a PCR mix without any DNA. After the PCR, the amplification results were visualized using 1.5% agarose gel electrophoresis and ethidium bromide staining.

For sequencing, PCR products were purified using the GeneClean III kit (Bio 101, Vista, Calif.) and cloned into a PCR-TOPO vector in the TOPO TA cloning kit (Invitrogen Co., Carlsbad, Calif.). The TOPO vectors containing PCR products were used for transformation of TOP10 competent cells (Invitrogen Co.). Plasmids containing inserts were purified from broth cultures with a Qiagen (Valencia, Calif.) plasmid kit and sequenced with the AutoRead sequencing kit and ALF DNA sequencer (Pharmacia Biotech, Uppsala, Sweden). Sequences were aligned using the Multialign program developed by F. Corpet (2).

In order to characterize the genetic nature of the 360-bp region of the *rpoB* gene, a total of 35 reference strains representing 26 different mycobacterial species were sequenced. Some species such as *M. gordonae*, *M. kansasii*, *M. celatum*, and *M. fortuitum* are known to have several subspecies, and thus these subspecies were also included in the sequence analysis. Among the 360-bp region sequenced in this study, sequences of 216 bp that have not been reported elsewhere are shown in Fig. 1, which shows that there exists a highly polymorphic region (black letters) flanked by highly conserved regions (red letters). Interestingly, the highly polymorphic region seemed to be suitable for the differentiation of mycobacterial species. For example, species differentiation between *M. kansasii* and *M. gastri* was possible since the sequences of *M. kansasii* are different from those of *M. gastri*, whose differentiation is not possible by 16S rRNA sequence analysis (18). Moreover, these polymorphic sequences were different even between highly closely related species, such as *M. abscessus* and *M. chelonae* or *M. fortuitum* and *M. peregrinum*, whose exact species identification has been extremely difficult by conventional culture-based microbiological and biochemical tests. In addition, the sequences of this polymorphic region in subspecies of *M. kansasii*, *M. fortuitum*, and *M. gordonae* were also differentiable, suggesting that this region of the *rpoB* gene may be used as a molecular signature for the differentiation of mycobacteria to the species or even to the subspecies level. However, there was no sequence difference in this region among species of the *M. tuberculosis* complex, including *M. tuberculosis*, *M. bovis*, *M. microti*, and *M. africanum.*

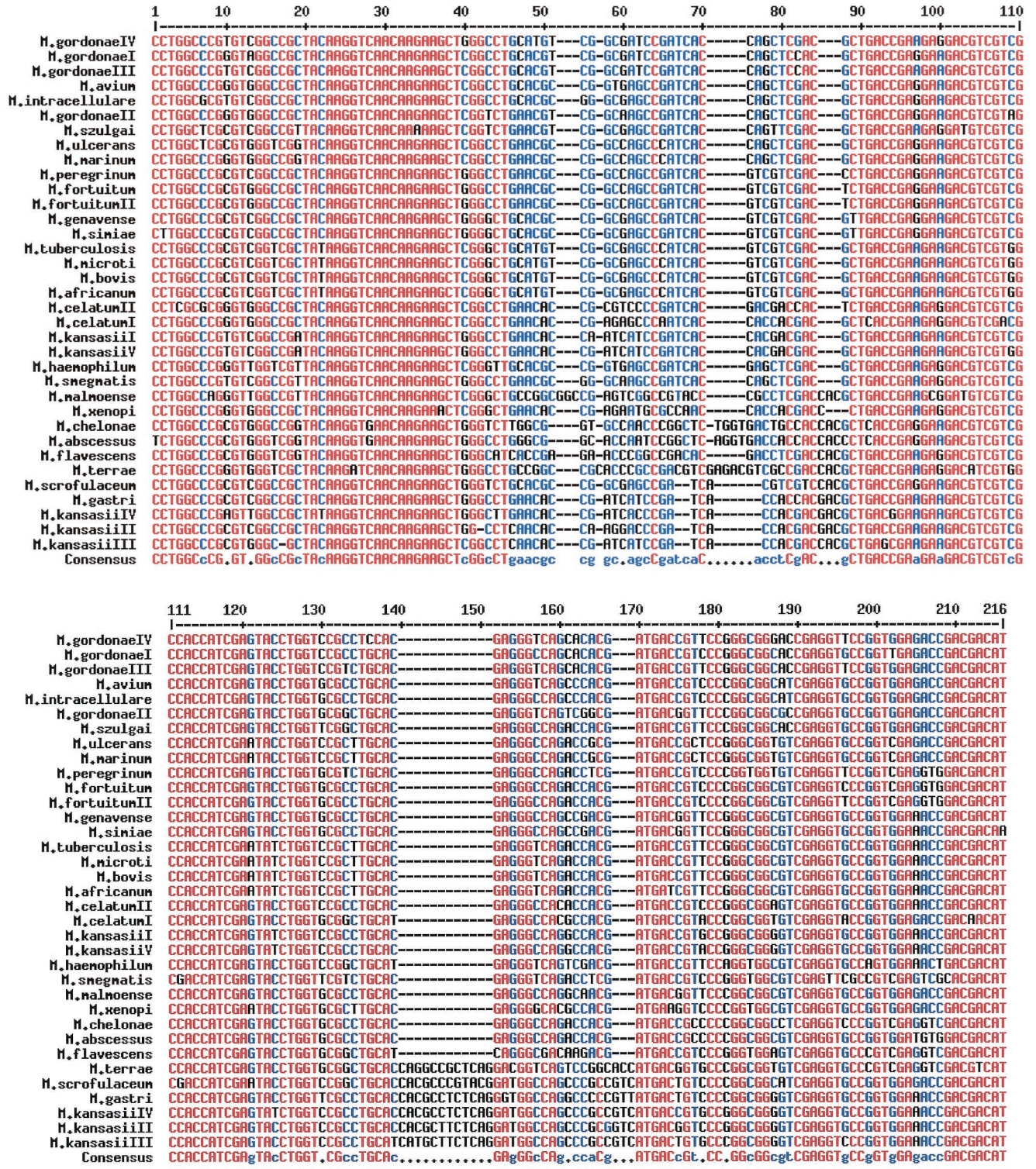

FIG. 1. Multialignment sequences identified in the *rpoB* region of 35 strains of 26 mycobacterial species of clinical importance. The software used for the alignment of multiple sequences was obtained from reference 2. Sequences with high consensus are shown as red letters, low consensus as blue letters, and neutral consensus as black letters. Among the 360-bp sequences identified in this study, only the 216-bp region of the upstream sequences is shown, since the downstream sequences have been reported elsewhere. The sequences shown here have been submitted to GenBank.

TABLE 2. Mycobacterial species-specific oligonucleotide probes designed and confirmed for their specificity by dot blot hybridization in this study

Name of oligonucleotide	Sequence	Target organism(s)
MYC	GACGTCGTCGCCACCATCGA	All mycobacterial species
MTB	CATGTCGGCGAGCCC	*M. tuberculosis* complex
AVI	CGGTGAGCCGATCACC	*M. avium*
INT	CCTGCACGCGGGCGA	*M. intracellulare*
SCR	CGTACGGATGGCCAGC	*M. scrofulaceum*
KAN-I	GGCCACGATGACCGTG	*M. kansasii* types I and V
KAN-II	TCTCAGGATGGCCAGC	*M. kansasii* types II, III, and IV
GAS	TCTCAGGGTGGCCAGG	*M. gastri*
FOR-C	CCTGAACGCCGGCCAG	*M. fortuitum* complex
PER	GTTCCGGTCGAGGTGG	*M. peregrinum*
CHE	TGGTGACTGCCACCACG	*M. chelonae*
ABS	GGTGACCACCACCACC	*M. abscessus*
ULC	GGCCAGCCCATCACC	*M. ulcerans*
GEN/SIM	CCAGCCGACGATGACG	*M. genavense-M. simiae*
GOR-I	GTCGGCGATCCGATCA	*M. gordonae* types I, III, and IV
GOR-II	CGTCGGCAAGCCGA	*M. gordonae* type II
SZU	TCTGAACGTCGGCGAG	*M. szulgai*

Based on the sequence information, we designed 15- to 20-mer oligonucleotide molecules as species- and subtype-specific probes for 16 strains of 14 mycobacterial species of clinical importance and one for all the mycobacterial species (Table 2). Subsequently, in order to determine the specificity of the probes for the targeted mycobacterial species, dot blot hybridization was carried out using each probe molecule. For these experiments, the target region of the *rpoB* gene from 48 different mycobacterial strains, representing 39 species listed in Table 1, was amplified by PCR, and the PCR products were then dot blotted on membranes.

To prepare the DNA dot blot, precut membrane (Hybond-N^+ [10 by 10 cm]; Amersham Pharmacia Biotech Korea Ltd., Seoul, Korea) was immersed into the denaturing solution (0.4 N NaOH, 25 mM EDTA; pH 8.0) for 1 min. After excess denaturing solution was allowed to drip from the membrane, it was placed on Whatman 3MM filter paper, and 1 to 2 μl of the PCR product was blotted onto the membrane. The membrane was then air dried for 5 min, rinsed with denaturing solution for another 1 min, placed between two sheets of 3MM filter paper, and baked for 2 h at 80°C. Oligonucleotide probes were labeled using an enhanced chemiluminescence kit for 3′ oligolabeling and detection (Amersham Pharmacia Biotech Korea Ltd.). Subsequent processing, including hybridizing the oligonucleotide probes to the membrane (42°C for 1 h), membrane washing (52°C for 15 min), and signal detection, was carried out using the method recommended by the manufacturer. Each species-specific probe was tested separately using the membrane onto which all PCR products were blotted, and there was no difference in dot blot hybridization conditions for all 16 probes analyzed in this study.

Figure 2 showed dot blot hybridization results using the *M. tuberculosis* complex probe and *M. gastri* probe as examples. Each probe hybridized only to the corresponding mycobacterial species, indicating the specificity of molecular probes to each mycobacterial species. The rest of the probes in Table 2 also showed species-specific hybridization to the corresponding species (data not shown).

Finally, in order to determine if all clinical isolates that belong to each mycobacterial species can be detected using its specific probe, dot blot hybridization using the *M. avium*-probe molecule was carried out using 36 clinical isolates, including 6 isolates of *M. avium*. As shown in Fig. 3, all six *M. avium* isolates were identified correctly by the *M. avium*-specific probe in a dot blot hybridization assay, while the probe did not bind to any clinical isolates of other mycobacterial species.

This study demonstrates that the *rpoB* gene of *Mycobacterium* species contains a highly polymorphic region whose DNA sequences can be used for species identification. Sequence analysis of the region showed clearly the difference in nucleotide sequences among 26 *Mycobacterium* species and subtypes of four species examined in this study. The results also supported clearly our previous report on species identification of mycobacteria by RFLP of the polymorphic region (13). In addition, we showed that the oligonucleotide probes based on the sequences of the region were specific to each *Mycobacterium* species and useful for species identification of mycobacteria in a dot blot hybridization.

The *rpoB* gene encodes the β subunit of RNA polymerase, which produces RNA molecules in cells. Thus, *rpoB* is one of the very critical housekeeping genes that are closely related to cellular vitality and thus becomes the target for rifampin, the major bactericidal drug for *M. tuberculosis* and *M. leprae*. It is, therefore, reasonable to assume that the genetic structure of the *rpoB* gene is highly conserved within the same species. However, unlike 16S rRNA or any other rRNA whose primary structure is functionally critical, the *rpoB* gene seems to tolerate a more diverse sequence alteration without causing any changes in protein function. In particular, the DNA region that is not involved in the active site of the protein seems to be more polymorphic and does not cause major functional defects. Based on these relationships, it is easily understood that there exist highly conserved DNA regions and relatively variable DNA regions in the *rpoB* gene. The tolerable sequence variation in the *rpoB* gene becomes a useful clue for species identification of mycobacteria as reported previously (9, 10, 13). However, sequence analysis of the 360-bp region of the *rpoB* that we reported herein clearly revealed more extensive variation than expected, leading to develop mycobacterial species-specific probe molecules.

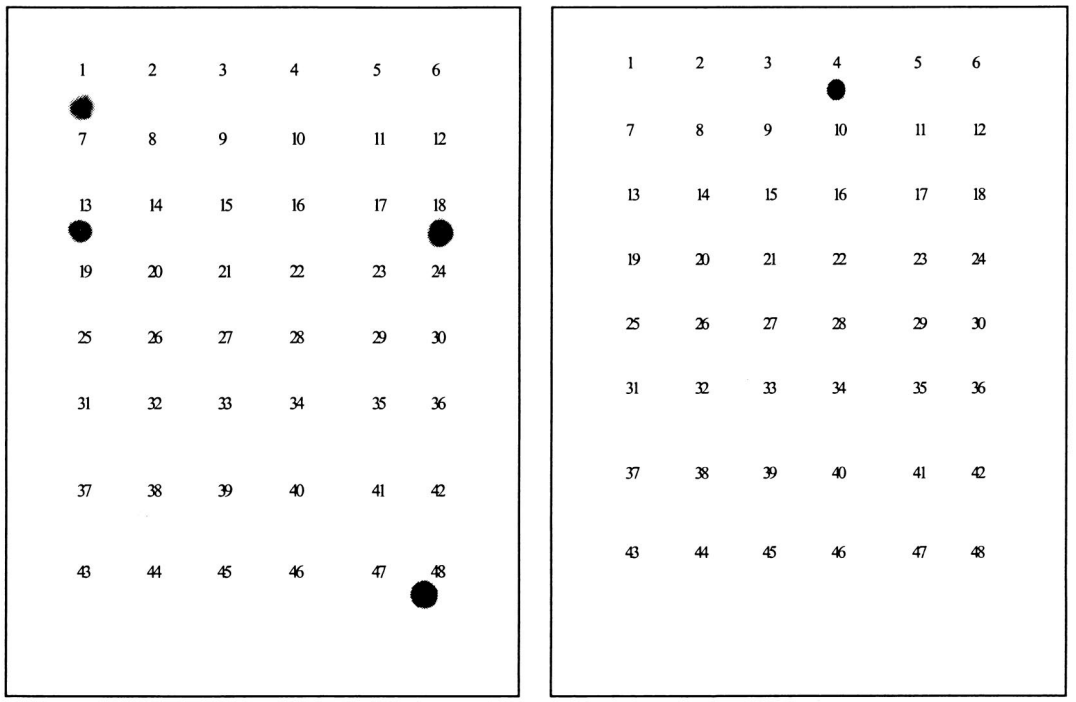

FIG. 2. The results of the dot blot hybridizations using each mycobacterial species-specific oligomer probe derived from the novel region of the *rpoB* genes of species. Dot blot hybridizations were conducted using probes specific for *M. tuberculosis* complex and *M. gastri*. The PCR-amplified products from 48 mycobacterial species were blotted on the membrane, and this was followed by hybridization with probes. The identification numbers in the membranes are matched with numbers and species names in Table 1.

The oligonucleotide probes designed on the basis of this polymorphic region were useful in *Mycobacterium* species identification by dot blot hybridization assay. No cross-reactive hybridization was found between the 16 mycobacterial species-specific probes (Table 2) and 48 strains of 39 *Mycobacterium* species (Fig. 2). For example, the *M. gastri*-specific probe did not hybridize with five subtypes of *M. kansasii*, although the two species could not be differentiated by 16 rRNA sequence analysis (18). One of our concerns was sequence variation in the 360-bp region among clinical isolates of each *Mycobacterium* species. In this study, however, there seems to be no variation in the nucleotides of the region among *M. avium* clinical isolates, because the probe hybridized with all *M. avium* clinical isolates, as shown in Fig. 3. This was also supported by our previous study in which no variation was found in RFLP enzyme restriction sites among 40 clinical isolates of *M. tuberculosis*, 40 clinical isolates of *M. avium*, 50 clinical isolates of *M. intracellulare*, and 25 clinical isolates of *M. gordonae*, etc. (13). Although other probes still need to be confirmed for their sensitivity and specificity using multiple clinical isolates of each *Mycobacterium* species, the probes will be useful in developing a reverse blot hybridization assay by which many isolates can be analyzed for their species identification at the same time. In addition, since the 360-bp region is located near the *rpoB* mutation sites, which are associated with resistance to rifampin, one can develop an assay in the future which can simultaneously provide information about mycobacterial species identity and rifampin resistance.

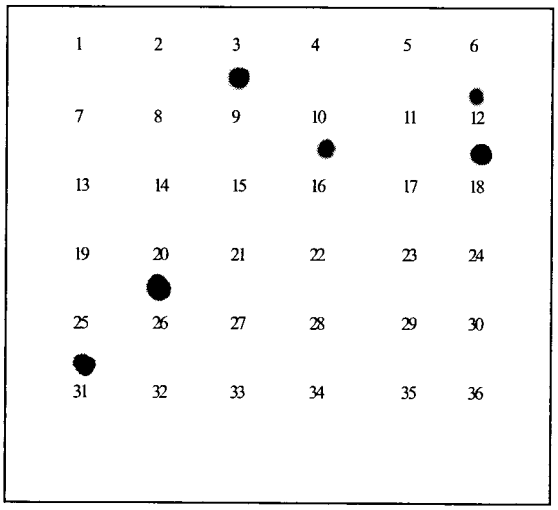

FIG. 3. Dot blot hybridizations using the *M. avium* specific oligomer probe. The membrane contained PCR products amplified from 36 clinical isolates of mycobacteria, including six isolates of *M. avium* species. The clinical isolates were identified by conventional culture and biochemical tests at the KIT.

Nucleotide sequence accession numbers. The nucleotide sequences listed in Table 2 have been submitted to the EMBL database and have been given the following accession num-

bers: AY271315 for *M. microti*, AY271316 for *M. terrae*, AY271317 for *M. scrofulaceum*, AY271318 for *M. marinum*, AY271319 for *M. szulgai*, AY271320 for *M. gastri*, AY271321 for *M. malmoense*, AY271322 for *M. avium*, AY271323 for *M. bovis*, AY271324 for *M. peregrinum*, AY271325 for *M. fortuitum*I, AY271326 for *M. celatum* type II, AY271327 for *M. flavescens*, AY271328 for *M. intracellulare*, AY271329 for *M. abscessus*, AY271330 for *M. africanum*, AY271331 for *M. haemophilum*, AY271332 for *M. xenopi*, AY271333 for *M. kansasii* type I, AY271334 for *M. kasasii* type II, AY271335 for *M. kansaii* type IV, AY271336 for *M. kansasii* type IV, AY271336 for *M kansaii* AY271337 *M. celatum* type I, AY271338 for *M. genavense*, AY271339 for *M. simiae*, AY271340 for *M. fortuitum* type II, AY271341 for *M. gordonae* type IV, AY271342 for *M. gordonae* type I, AY271343 for *M. gordonae* type II, AY271344 for *M. gordonae* type III, and AY271345 for *M. smegmatis*.

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