JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2000, p. 2966–2971 Vol. 38, No. 8
0095-1137/00/$04.00+0

Species Identification of Mycobacteria by PCR-Restriction Fragment Length Polymorphism of the *rpoB* Gene

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Received 18 February 2000/Returned for modification 30 April 2000/Accepted 29 May 2000

PCR-restriction fragment length polymorphism analysis (PRA) using the novel region of the *rpoB* gene was developed for rapid and precise identification of mycobacteria to the species level. A total of 50 mycobacterial reference strains and 3 related bacterial strains were used to amplify the 360-bp region of *rpoB*, and the amplified DNAs were subsequently digested with restriction enzymes such as *Msp*I and *Hae*III. The results from this study clearly show that most of the mycobacterial species were easily differentiated at the species level by this PRA method. In addition, species with several subtypes, such as *Mycobacterium gordonae*, *M. kansasii*, *M. celatum*, and *M. fortuitum*, were also differentiated by this PRA method. Subsequently, an algorithm was constructed based on the results, and a blinded test was carried out with more than 260 clinical isolates that had been identified on the basis of conventional tests. Comparison of these two sets of results clearly indicates that this new PRA method based on the *rpoB* gene is more simple, more rapid, and more accurate than conventional procedures for differentiating mycobacterial species.

Since the early 1980s there has been an increase in disease caused by organisms called nontuberculous mycobacteria (NTM), which is the generic name for mycobacteria other than *Mycobacterium tuberculosis* and *M. leprae*. They affect both immune-competent and immune-compromised persons, and patients with the human immunodeficiency virus are known to be especially vulnerable. The NTM most frequently involved in disease cases are *M. avium* complex, *M. kansasii*, *M. chelonae*, *M. abscessus*, *M. xenopi*, *M. malmoense*, *M. scrofulaceum*, *M. marinum*, *M. ulcerans*, and *M. haemophilum* (28). Clinical diagnosis and treatment of NTM infections are an increasingly frequent challenge to clinicians.

Currently, identification of clinical isolates of mycobacteria to the species level is primarily based on cultural characteristics and biochemical tests. These conventional tests take several weeks, and they sometimes fail to provide precise identification. The procedures for these tests are complex and laborious, and they are usually impeded by the slow growth of mycobacteria in clinical laboratories. Additional methods, such as high-performance liquid chromatography, gas-liquid chromatography, and thin-layer chromatography (5, 21, 36) as well as DNA sequence analysis (3, 4, 15–17, 19, 26, 31, 32), can differentiate mycobacteria to the species level, but these techniques are labor intensive and difficult to perform for routine use in clinical laboratories. Recently developed molecular techniques using amplified DNA and probe hybridization (6, 8–10, 18, 20, 23) are also useful for direct and rapid identification of NTM species, but their overall cost is high. In addition, currently available kits are limited to the identification of a few species. In contrast to the above-mentioned techniques, PCR-restriction fragment length polymorphism analysis (PRA) offers an easy, rapid, and inexpensive way to identify several mycobacterial species in a single experiment. PRA techniques have been developed to target mycobacterial genes which are present in all mycobacteria, such as *hsp65* (7, 11, 25, 29, 30, 34, 35), the 16S rRNA gene (2, 14, 37), and *dnaJ* (33). However, the previous PRA techniques are still cumbersome since they require several enzyme digestions for species identification, and the results are not easy to interpret for species identification due to the limited size differences of DNA fragments after digestion.

Therefore, the aim of this study was to develop a new PRA method that is easier to perform and more precise for mycobacterial species identification than currently available PRA techniques. We chose as a target for our new PRA method *rpoB*, a conserved gene that encodes the β subunit of RNA polymerase. The information-rich nature of the *rpoB* gene has been recently used in differentiation of mycobacteria by DNA hybridization array (10) and by DNA sequence (16) analyses. However, the *rpoB* region used in these previous studies has limited sequence differences, making it difficult to generate diverse restriction fragment length polymorphism (RFLP) profiles that can be used for species identification of mycobacteria. In the present study, we extended the genetic knowledge of the *rpoB* gene to a region that is relatively polymorphic and thus suitable for PRA. In addition, this region of the *rpoB* gene is flanked by conserved sequences, making it suitable for PCR amplification with the same set of PCR primers.

A 360-bp region of *rpoB* was amplified from the DNA of 50 mycobacterial reference strains, representing 44 species and a total of 260 clinical isolates, to evaluate this method for mycobacterial species identification. The results of this study show that this novel PRA method based on the *rpoB* genes of mycobacteria generates clear and distinctive results for easy, rapid, and precise identification of mycobacterial species.

MATERIALS AND METHODS

Mycobacterial samples. A total of 50 mycobacterial reference strains representing 44 mycobacterial species and 3 related species which belong to 2 different genera (Table 1) were used to develop the new PRA method in this study. Among them, 40 mycobacterial strains and 3 related species were obtained from the Korean Institute of Tuberculosis (KIT), the Korean National Tuberculosis Association in Seoul. Four species were obtained from the Korean Collection for Type Cultures at the Korean Research Institute of Bioscience and Biotechnology

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TABLE 1. Bacterial strains used in this study

Species	Strain	Source
M. abscessus	Pettenkofer Institute	YUMC[a]
M. africanum	ATCC 25420	KIT
M. arcinogenes	ATCC 35753	KIT
M. asiaticum	ATCC 25276	KIT
M. aurum	ATCC 23366	KIT
M. austroafricanum	ATCC 33464	KRIBB
M. avium	ATCC 25291	KIT
M. bovis	ATCC 19210	KIT
M. bovis BCG	French strain 1173P2	KIT
M. celatum type I/II	ATCC 51130/ATCC 51131	KIT
M. chelonae	ATCC 35749	KIT
M. chitae	ATCC 19627	KIT
M. fallax	ATCC 35219	KIT
M. fortuitum type I/II	ATCC 6841/ATCC 49404	KIT
M. gallinarum	ATCC 19710	KRIBB
M. gastri	ATCC 15754	KIT
M. genavense	ATCC 51233	KIT
M. gilvum	ATCC 43909	KIT
M. gordonae	ATCC 14470	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-V		Pasteur Institute
M. malmoense	ATCC 29571	KIT
M. marinum	ATCC 927	KIT
M. moriokaense	ATCC 43059	KRIBB
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. scrofulaceum	ATCC 19981	KIT
M. smegmatis	ATCC 19420	KIT
M. szulgai	ATCC 35799	KIT
M. terrae	ATCC 15755	KIT
M. thermoresistibile	ATCC 19527	KIT
M. triviale	ATCC 23292	KIT
M. tuberculosis H37Rv	ATCC 27294	KIT
M. ulcerans	ATCC 19423	KIT
M. vaccae	ATCC 15483	KIT
M. xenopi	ATCC 19250	KIT
Nocardia brasiliense	ATCC 19296	KIT
N. nova	ATCC 21197	KIT
Rhodococcus equi	ATCC 10146	KIT

[a] YUMC, Yonsei University Medical College.

(KRIBB), and *M. abscessus*, which was recently separated from *M. chelonae* as an independent new species, was obtained from the Department of Clinical Pathology at Yonsei University Medical College. Five subtypes of *M. kansasii* were generously given by V. Vincent of the Laboratoire de Référence des Mycobactéries, Institut Pasteur, Paris, France.

Clinical isolates subjected to PRA for evaluation of the new method were obtained from KIT. All clinical isolates used in this study were identified on the basis of conventional techniques that included microbiological characteristics and biochemical tests. In some cases, strains were subjected to the conventional PRA method, based on the *hsp65* gene (7, 35), to aid in their precise identification.

DNA preparation. To prepare a DNA sample for PCR amplification, a loopful of a bacterial colony was taken from a Lowenstein-Jensen medium culture and resuspended in 400 μl of distilled water in a screw-cap microcentrifuge tube. The sample was then boiled for 5 min prior to being centrifuged for 5 min to settle cell debris, and about 10 μl of supernatant, containing the genomic DNA, was used for subsequent PCR amplification.

PCR amplification. Amplification of the region of interest in *rpoB* with a primer set consisting of 5′-TCAAGGAGAAGCGCTACGA-3′ (RPO5′) and 5′-GGATGTTGATCAGGGTCTGC-3′ (RPO3′) resulted in 360-bp PCR products (bases 902 to 1261 and codons 302 to 420, based on the sequence of the *rpoB* gene of *M. tuberculosis* [GenBank accession no. P47766]). The primer sequences were selected from the region of the *rpoB* gene which have been previously identified from *M. tuberculosis*, *M. leprae*, and *M. smegmatis* (12, 13, 22). The primers amplified the region between the first variable region (V1) and second conserved region (C2) based on the genetic information for the *rpoB* gene of *Escherichia coli*. As a result, the PCR products included 171 bp of variable region and 189 bp of conserved region. The variable region was amplified in this experiment based on the theory that the polymorphic nature of this region might help in the clear distinction of each mycobacterial species by the new PRA method. The RPO5′ primer was derived from relatively conserved sequences found in the variable region of *rpoB*.

PCR was carried out in a final volume of 50 μl consisting of 10 μl of DNA supernatant containing approximately 10 ng of genomic DNA, 10 pmol of each primer, 2 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, and 1 U of DyNAzyme II DNA polymerase (FINNZYMESY, Espoo, Finland). DNA samples were first denatured completely by incubation at 94°C for 5 min before the amplification cycle; then DNA was amplified by subjecting it to 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 for 1 min, using a Thermocycler (model 9600, Perkin-Elmer). After the last amplification cycle, the samples were incubated further at 72°C for 7 min for complete elongation of the final PCR products. Positive and negative controls were always included in each PCR. The positive control was the PCR mixture with DNA of the reference strain, *M. bovis*, and the negative control was the PCR mixture without any DNA. After the PCR, the amplification results were visualized by performing 1.5% agarose gel electrophoresis and ethidium bromide staining.

RFLP. After successful amplification of the 360-bp PCR products was confirmed, the PCR products were subjected to restriction enzyme digestion. Most of the time, 10 to 16 μl of PCR product (approximately 1 to 1.5 μg of DNA) was digested in a 20-μl reaction volume containing 5 U of *Msp*I (Boehringer Mannheim Biochemicals, Mannheim, Germany) and 2 μl of the 10× reaction buffer supplied by manufacturer. Similarly, 10 to 16 μl of PCR product was digested in a 20-μl reaction volume containing 5 U of *Hae*III enzyme (Takara Shuzo Co., Ltd., Shiga, Japan) and the corresponding enzyme buffer. After 2 h of incubation at 37°C, 4 μl of gel loading buffer (0.25% bromophenol blue, 40% sucrose in water) was added, and the samples were loaded onto a 4% Metaphore agarose gel (FMC BioProducts, Rockland, Maine). Then, enzyme-digested fragments were visualized under UV light after ethidium bromide staining.

For the interpretation of the PRA profile generated by each species, a 50-bp ladder DNA size marker (Boehringer Mannheim) and the PRA profile of *M. bovis*, which generates restriction fragments of about 175, 80, 60, and 40 bp, were used as internal size markers. Using these markers, the sizes of the restricted fragments of each species were determined, and an algorithm was constructed based on this information.

RESULTS

To develop a more rapid and precise yet simple and easy-to-use PRA method for mycobacterial species identification, we chose one of the more highly conserved genes, *rpoB*. Since the genetic information for the *rpoB* genes of some mycobacteria is available, sequences were aligned and searched for regions suitable for PRA. As a result, a set of PCR primers was selected to amplify a 360-bp sequence of *rpoB* which contains a polymorphic region flanked by conserved regions.

A total of 50 mycobacterial reference strains and 3 related bacterial strains that belong to the same class as mycobacteria (*Actinomycetes*) were used to amplify the 360-bp region of the *rpoB* gene (Table 1). The results showed that a conserved *rpoB* gene present in all mycobacteria and in some other bacteria, such as *Nocardia* and *Rhodococcus* species, was amplified. Subsequently, PCR products were subjected to two sets of restriction enzyme digestion, with *Msp*I and *Hae*III being used individually. These two enzymes were selected on the basis of sequence information for the *rpoB* genes of *M. tuberculosis*, *M. leprae*, and *M. smegmatis* (12, 13, 22). Based on this information, PCR products were subsequently subjected to RFLP analysis (Fig. 1). In short, this analysis showed that the RFLP profiles of PCR products from each mycobacterial species were distinctive. *M. kansasii* can be easily differentiated from *M. gastri*, which has much in common with nonpigmented variants of *M. kansasii*. In addition, *M. abscessus*, which had been classified as a subgroup of *M. chelonae* and was not easily differentiated from it by conventional biochemical tests, was also differentiated from the latter. Furthermore, for some spe-

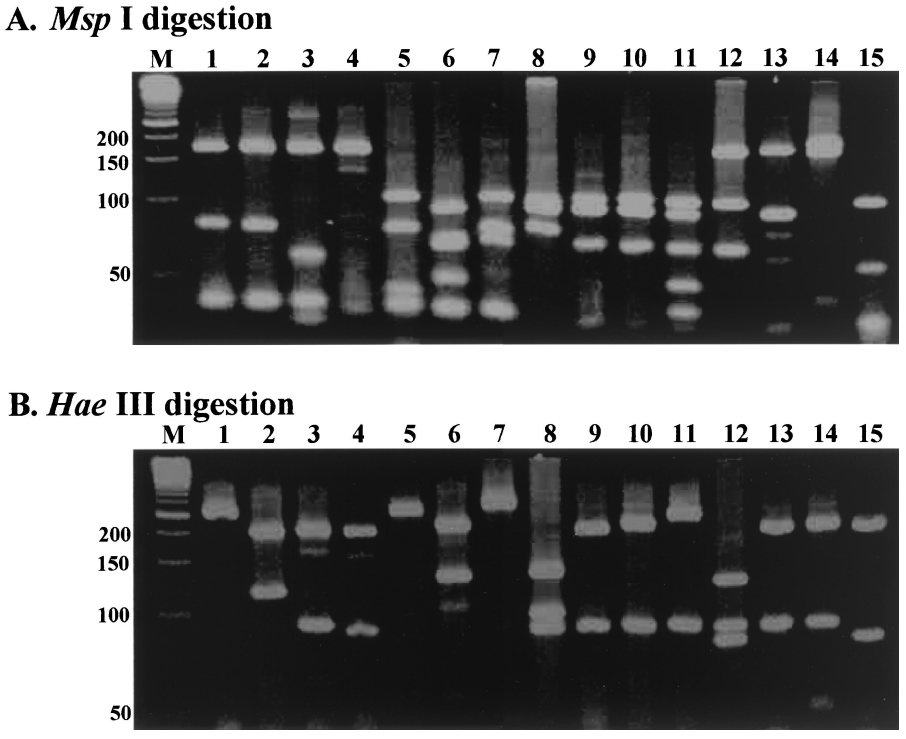

FIG. 1. Results of PRA for mycobacterial reference strains. Primer pair RPO5′ and RPO3′ was used. Amplified DNA was digested with *Msp*I (A) or *Hae*III (B) restriction enzyme and run on a 4% Metaphore agarose gel. Lanes: M, DNA size marker (50-bp ladder; positions are indicated on left [in base pairs]); 1, *M. gordonae* type IV; 2, *M. szulgai*; 3, *M. kansasii* type I; 4, *M. gallinarum*; 5, *M. avium*; 6, *M. scrofulaceum*; 7, *M. asiaticum*; 8, *M. chelonae*; 9, *M. moriokaese*; 10, *M. phlei*; 11, *M. pulveris*; 12, *M. fortuitum* type I; 13, *M. austroafricanum*; 14, *M. smegmatis*; 15, *M. marinum*.

cies, such as *M. fortuitum*, *M. celatum*, *M. gordonae*, and *M. kansasii*, that are known to contain several subtypes, each subtype generated distinctive restriction profiles. Therefore, the results clearly indicated that this new PRA method could differentiate mycobacteria at the species and even the subspecies level.

On the basis of the above PRA results, an algorithm was constructed (Fig. 2). In the algorithm, restriction fragments smaller than 40 bp were omitted in order to reduce confusion with primer-dimer bands. The different sizes of fragments were clearly separated from each other, making interpretation of results easier. In brief, the algorithm clearly shows that most mycobacterial species and other related bacterial species can be differentiated at the subspecies level by PRA using restriction enzymes *Msp*I and *Hae*III. In fact, except for a few mycobacterial species, most of these organisms can be identified by using a single enzyme, *Msp*I, thus making this new method more useful for mycobacterial species identification in the clinical laboratory.

For those strains that were not differentiated by two enzyme digestions, a third enzyme digestion was useful. For example, even though the members of *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, and *M. africanum*) were not differentiated by using *Msp*I and *Hae*III, a third enzyme, *Sau*3AI, could be used to differentiate *M. africanum* from other members of *M. tuberculosis* complex. As another example, *Hin*cII can be used to differentiate *M. gordonae* type I from *M. celatum* type I (Fig. 2).

The different RFLP profiles generated for the PCR products strongly suggested to us that the *rpoB* region amplified by PCR in this study has a polymorphic nature. The next question was whether these differing RFLP profiles were species and, possibly, strain specific. If strains of a certain species also have polymorphic RFLP profiles, the use of this method for mycobacterial species identification will be very complex. Therefore, in a blinded test, clinical isolates that had been identified on the basis of conventional tests were subjected to PRA to determine their species based on the algorithm constructed during this study. The results of this experiment clearly show that there are no differences in the profiles of the various among clinical isolates that belong to the same species (data not shown). Subsequently, a substantial number of clinical isolates that had been identified on the basis of conventional tests were subjected to PRA. In this experiment, a total of 260 clinical isolates were analyzed, including *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. fortuitum*, *M. chelonae*, *M. abscessus*, *M. terrae*, *M. gordonae*, and *M. szulgai* specimens (Table 2). For easy interpretation of the PRA profiles generated by each clinical isolate, a 50-bp ladder size marker was used as a standard size marker and the PRA profile of *M. bovis* was used as an internal size marker (Fig. 3). Results from the PRA of clinical isolates were evaluated with the help of an algorithm generated on the basis of PRA profiles of reference strains. Most of the PRA results were consistent with conventional test results, while the PRA profiles of a few strains were not present in the reference algorithm. Based on the results of conventional tests and on molecular biological sequence analysis, some of these strains were determined to be *M. terrae* complex.

DISCUSSION

Mycobacterial identification to the species level not only is of academic interest but also is important because it provides a great deal of useful information on the epidemiology and

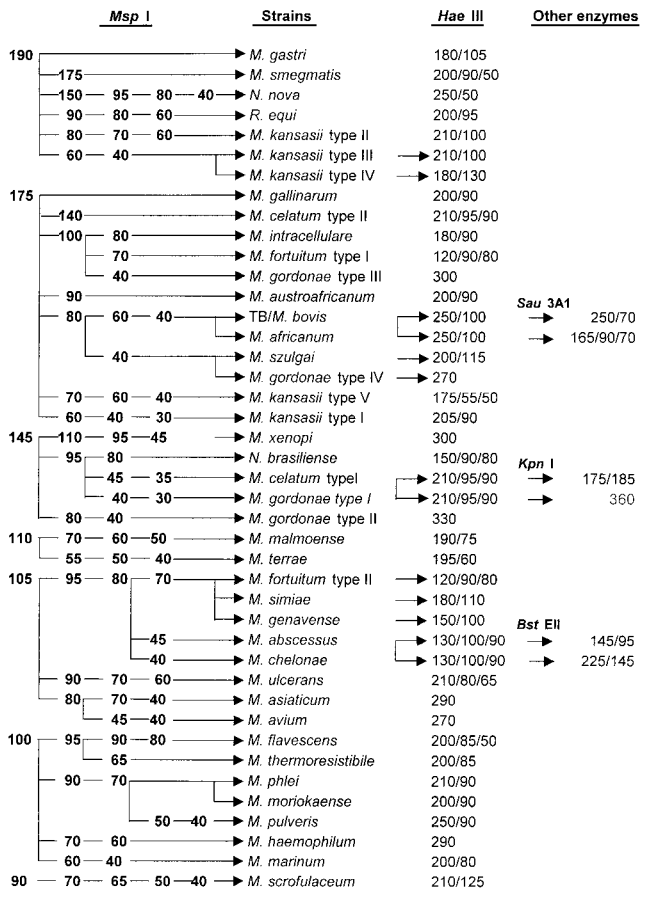

FIG. 2. An algorithm was constructed based on the results of PRA with 40 mycobacterial reference strains and 3 other related bacterial strains. For algorithm conciseness, the PRA results of 10 other mycobacterial reference strains are not listed in this figure. TB, *M. tuberculosis*. The numbers indicate sizes (in base pairs) of resulting fragments.

pathogenesis of the organism, suggesting potential intervention strategies, including successful treatment of patients on a clinical basis. It is therefore important to develop methods that are rapid and simple but yet precise and cost-effective for use in a wide variety of clinical laboratories around the world. Currently available methods for the differentiation of mycobacteria to the species level are time-consuming evaluations based on phenotypic and biochemical tests or laborious procedures requiring expensive equipment.

The PRA technique certainly fits these requirements better than other molecular methods. It is rapid and precise since it employs PCR, and it is simple and cost-effective since it does not require any expensive equipment or laborious processes and can differentiate numerous species of mycobacteria within a single experiment. Owing to these advantages, several PRA methods based on different genes of mycobacteria have been developed (2, 7, 11, 14, 25, 29, 30, 33–35, 37). However, most of those methods require the use of more than two enzymes to differentiate mycobacteria at the species level, as well as a computer-assisted software program to differentiate restriction fragments since the profiles of certain mycobacterial species are not distinctive enough for bare-eyed interpretation.

The new PRA method developed through this investigation has more advantages than the previous ones. As presented in Fig. 1, it is apparent that most of the species have unique PRA profiles. Unlike other PRA profiles, which may require computer-assisted analysis and interpretation of the gels, ours does not face problems in terms of resolution of all patterns obtained during the experiments. Furthermore, problems such as gel-to-gel variation and confusion with regard to the sizes of the restriction fragments were limited by the use of the 50-bp size marker and of the *M. bovis* PRA profile as an internal size marker.

The restriction analysis of a 360-bp fragment within this gene after single *Msp*I digestion is highly effective for differentiating most mycobacteria even at the species level. Only a few species require digestion with an additional enzyme, such as *Hae*III, *Sau*3AI, or *Hin*cII. For some species, such as *M. gordonae*, *M. kansasii*, *M. fortuitum*, and *M. celatum*, the discrimination was even to the subtype level. For *M. kansasii*, this subdivision was clearly linked to genetic divergence observed previously by other investigators (1, 24, 27). It is therefore possible that by using this PRA method, discrimination at the subgroup level for other species could be similarly linked to bacteriological and clinical specificities. Currently, we are investigating several clinical isolates, with PRA profiles distinct from those of any of our reference strains, which also exhibit distinctive microbiological and biochemical characteristics.

On the other hand, the four members of the *M. tuberculosis* complex, which are difficult to differentiate by methods such as sequence analysis and high-performance liquid chromatography of mycolic acids, were also undistinguishable by our PRA method, confirming that they are genetically similar. However, unlike other methods, this new PRA method can further differentiate *M. africanum* from the other *M. tuberculosis* complex members with the added step of *Sau*3AI digestion. Therefore, in cases in which a clinical isolate exhibits *M. tuberculosis* complex profiles, PCR products can be further processed with *Sau*3AI to differentiate *M. africanum* from other *M. tuberculosis* complex constituents. In addition, *M. tuberculosis* and *M. bovis* can be differentiated by PCR amplification using PCR primers derived from the *esat-6* gene, which is known to be present only in the genome of *M. tuberculosis*.

The fact that the species *M. terrae* contains strains of diverse genotype is consistent with previous observations by other investigators (35). Currently, we are investigating the genetic nature of these strains by sequence analysis and their phenotypic specificities by conventional tests. The data from these studies will be used for establishing a new mycobacterial taxonomy. In this regard, PRA seems to be a very effective method for identifying new mycobacterial species or subgroups of mycobacteria from clinical samples.

Currently, a substantial number of mycobacterial clinical

TABLE 2. Clinical isolates of mycobacteria subjected to species identification by the new PRA method

Species tested	No. of clinical isolates tested
M. tuberculosis	40
M. avium	40
M. intracellulare	50
M. gordonae	25
M. szulgai	10
M. fortuitum	25
M. chelonae	15
M. abscessus	15
M. kansasii	20
M. terrae	20
Total	260

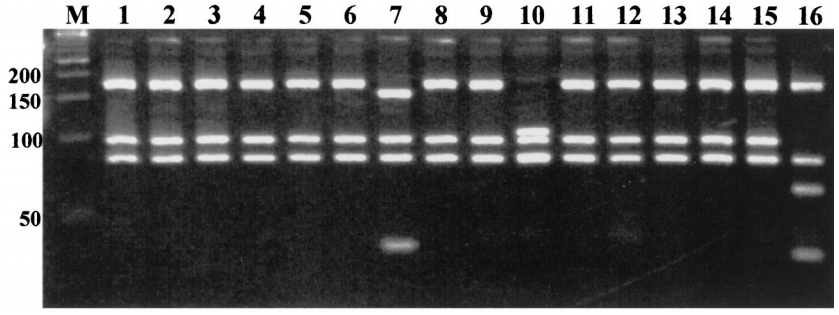

FIG. 3. Application of *rpoB*-based PRA for species identification of mycobacterial isolates in the clinical laboratory. Clinical isolates were amplified using primers RPO5′ and RPO3′, digested with *Msp*I, and run on a 4% Metaphore agarose gel. For each test, DNA size markers (lane M; 50-bp ladder (positions are indicated on the left; [in base pairs]) was employed, as were the PRA results for *M. bovis*, which were used as internal size markers (lane 16). Using the algorithm shown in Fig. 2, these clinical isolates were determined to be *M. intracellulare* (lanes 1 to 6, 8, 9, and 11 to 15), *M. gordonae* type II (lane 7), and *M. abscessus* (lane 10).

isolates in our laboratory have now been identified by our new PRA method in parallel with other reference methods, including conventional tests and molecular biological methods such as PRA based on the *hsp65* gene and sequence analysis based on the *rpoB* gene. From our experimental data it can be concluded that this new PRA is a rapid, cost-effective, and efficient method for the identification of mycobacteria in a clinical microbiology laboratory. The whole procedure can be done in 2 days when a culture is used. Successful PRA has been achieved with a loopful of culture taken from solid medium or 100 μl of a liquid culture such as MGIT for mycobacterial species identification. Both of the systems work well even when the genomic DNA is simply boiled for 5 min.

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