Use of PCR and Reverse Line Blot Hybridization Macroarray Based on 16S-23S rRNA Gene Internal Transcribed Spacer Sequences for Rapid Identification of 34 Mycobacterium Species

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The aim of this study was to develop a PCR and reverse line blot hybridization (PCR-RLB) macroarray assay based on 16S-23S rRNA gene internal transcribed spacer sequences for the identification and differentiation of 34 mycobacterial species or subspecies. The performance of the PCR-RLB assay was assessed and validated by using 78 reference strains belonging to 55 Mycobacterium species, 219 clinical isolates which had been identified as mycobacteria by high-performance liquid chromatography or gas chromatography, three skin biopsy specimens from patients with suspected leprosy which had been shown to contain acid-fast bacilli, and isolates of 14 nonmycobacterial species. All mycobacteria were amplified in the PCR and hybridized with a genus-specific probe (probe MYC). The 34 species-specific probes designed in this study hybridized only with the corresponding Mycobacterium species. The mycobacterial PCR-RLB assay is an efficient tool for the identification of clinical isolates of mycobacteria; it can reliably identify mixed mycobacterial cultures and M. leprae in skin biopsy specimens.
<table>
<thead>
<tr>
<th>Species</th>
<th>Total no. of isolates</th>
<th>Identification method(^a) (no. of isolates)</th>
<th>PCR result</th>
<th>RLB result with probe MvC(^a) (no. of isolates)</th>
<th>Species-specific probe used for RLB (no. of isolates)</th>
<th>ITS sequencing result (no. of isolates)</th>
</tr>
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<tbody>
<tr>
<td><em>M. abscessus</em></td>
<td>12</td>
<td>GC (8)</td>
<td>+</td>
<td>+</td>
<td>ABS (12)</td>
<td>ND(^d)</td>
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<tr>
<td><em>M. chelonae complex</em></td>
<td>28</td>
<td>GC (28)</td>
<td>+</td>
<td>+</td>
<td>AB (26)</td>
<td>ND</td>
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<td><em>M. abscessus</em></td>
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<td><em>M. chelonae a/b</em></td>
<td>4</td>
<td>HPLC (4)</td>
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<td>+</td>
<td>CHEI (2)</td>
<td>ND</td>
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<tr>
<td><em>M. chelonae</em></td>
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<td></td>
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</tr>
<tr>
<td><em>M. chelonae a/b</em></td>
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<td></td>
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<tr>
<td><em>M. chelonae c</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>MAC</td>
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<td>+</td>
<td>+</td>
<td>INT (3)</td>
<td>ND</td>
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<td><em>M. avium</em></td>
<td>23</td>
<td>HPLC (23)</td>
<td>+</td>
<td>+</td>
<td>AVI (11), INT (10), AVI and KANI (2)</td>
<td>ND</td>
</tr>
<tr>
<td><em>M. fortuitum complex</em></td>
<td>29</td>
<td>GC (22)</td>
<td>+</td>
<td>+</td>
<td>FOR1 (12), FOR2 and FOR3 (1); FOR1, FOR2, and FOR3 (9)</td>
<td>Uninterpretable(^b) (1)</td>
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<tr>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>UNINTERPRETABLE (2)</td>
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<tr>
<td><em>M. gordonae</em></td>
<td>28</td>
<td>GC (20)</td>
<td>+</td>
<td>+</td>
<td>GOR (8)</td>
<td>ND</td>
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<td><em>M. intracellulare</em></td>
<td>2</td>
<td>HPLC (8)</td>
<td>+</td>
<td>+</td>
<td>AVI (1), INT (1)</td>
<td><em>M. avium</em> (1), <em>M. intracellulare</em> (1)</td>
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<tr>
<td><em>M. kansasii</em></td>
<td>3</td>
<td>GC (1)</td>
<td>+</td>
<td>+</td>
<td>KANI (1)</td>
<td>ND</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>ND</td>
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<td>2</td>
<td>HPLC (2)</td>
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<td>+</td>
<td>LEN (2)</td>
<td><em>M. lentiflavum</em> (2)</td>
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<td></td>
<td><em>M. lentiflavum</em> (2)</td>
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<tr>
<td><em>M. leprae</em></td>
<td>3(^f)</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>LEP (2), MTBC and INT (1)</td>
<td><em>M. leprae</em> (2), uninterpretable (1)</td>
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<td><em>M. malmoense</em></td>
<td>1</td>
<td>HPLC (1)</td>
<td>+</td>
<td>+</td>
<td>MAL (1)</td>
<td>ND</td>
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<tr>
<td><em>M. nonchromogenicum</em></td>
<td>11</td>
<td>GC (11)</td>
<td>+</td>
<td>+</td>
<td>− (10), FOR1 (1)</td>
<td><em>M. fortuitum</em> a (1)</td>
</tr>
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<td><em>M. phlei a</em></td>
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<td>GC (12)</td>
<td>+</td>
<td>+</td>
<td>PHL1 (12)</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td><em>M. scrofulaceum</em></td>
<td>15</td>
<td>GC (8)</td>
<td>+</td>
<td>+</td>
<td>SCR (7), SCR and INT (1)</td>
<td>Uninterpretable (1)</td>
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<tr>
<td><em>M. szulgai</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ND</td>
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<td><em>M. smegmatis</em></td>
<td>2</td>
<td>HPLC (2)</td>
<td>+</td>
<td>+</td>
<td>SME (2)</td>
<td>ND</td>
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<tr>
<td><em>M. suilai</em></td>
<td>1</td>
<td>GC (1)</td>
<td>+</td>
<td>+</td>
<td>SZU (1)</td>
<td>ND</td>
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<tr>
<td><em>M. triple</em></td>
<td>2</td>
<td>HPLC (2)</td>
<td>+</td>
<td>+</td>
<td>TRI (2)</td>
<td><em>M. triple</em> (2)</td>
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<tr>
<td><em>M. tuberculosis complex</em></td>
<td>39</td>
<td>GC (20)</td>
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<td>+</td>
<td>MTBC (39)</td>
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<tr>
<td><em>M. ulcerans/M. marinum</em></td>
<td>1</td>
<td>HPLC (1)</td>
<td>+</td>
<td>+</td>
<td>ULC/MAR (1)</td>
<td>ND</td>
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<tr>
<td><em>M. xenopi</em></td>
<td></td>
<td>HPLC (1)</td>
<td>+</td>
<td>+</td>
<td>XEN (1)</td>
<td>ND</td>
</tr>
</tbody>
</table>

**Total:** 222\(^b\)

\(^a\) GC was performed with isolates obtained from Shenzhen Chronic Disease Hospital and Guangzhou Thoracic Hospital, China; HPLC was performed with isolates from the Mycobacterium Reference Laboratory, Centre for Infectious Diseases and Microbiology, ICPMR, Westmead, Australia.

\(^b\) MvC, Mycobacterium genus-specific probe; +, positive hybridization.

\(^c\) For the specificities of the probes on RLB, see Table 2.

\(^d\) ND, not done; sequencing was not performed if the HPLC or GC and PCR-RLB assay results were the same.

\(^e\) All cultures in which mixed species were present, as identified by the PCR-RLB assay, produced uninterpretable sequencing results.

\(^f\) Three clinical biopsy specimens with acid-fast bacilli on microscopy presumed to be *M. leprae*.

\(^g\) No probes specific for *M. nonchromogenicum* were included in the RLB.

\(^h\) The total includes 219 clinical isolates and three skin biopsy specimens.

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**Nonmycobacterial reference strains.** Reference strains of the following nonmycobacterial species were included: Corynebacterium pyocamumholobum ATCC 8368, Klebsiella pneumoniae subsp. pneumoniae ATCC 25304, Legionella pneumophila subsp. pneumophila ATCC 33153, Mycoplasma pneumoniae ATCC 29342, Nocardia asteroides ATCC 19247, Nocardia brasiliensis ATCC 19296, Nocardia farcinica ATCC 3308, Nocardia transvalensis ATCC 6886, Rhodococcus equi ATCC 4939, Rhodococcus erythropolis ATCC 25344, Rhodococcus rhodochrous ATCC 13808, Rhodococcus equi ATCC 29627, Rhodococcus weisflaviensis ATCC 51786, and Streptococcus pneumoniae SSIP pn2L.

**Clinical isolates.** A total of 219 mycobacterial isolates were obtained from the following sources: Center for Infectious Disease and Microbiology (CIDM), Institute of Clinical Pathology and Medical Research, Westmead, Australia (n = 85); the Institute of Tuberculosis, Shenzhen Chronic Disease Hospital, China (n = 64); and the Clinical Laboratory, Guangzhou Thoracic Hospital, China (n = 70) (Table 1). They included isolates of the *M. tuberculosis* complex and the following nontuberculous Mycobacterium species: *M. abscessus*, *M. avium*, *M. chelonae*, *M. fortuitum*, *M. gordonae*, *M. intracellulare*, *M. kansasii*, *M. lentiflavum*, *M. malmoense*, *M. nonchromogenicum*, *M. phlei*, *M. scrofulaceum*, *M. smegmatis*, *M. szulgai*, *M. triplex*, *M. ulcerans*, and *M. xenopi*. All isolates were from different
DNA extraction and PCR amplification. The extraction of DNA was carried out with a commercially available kit (InstaGene Matrix; Bio-Rad Laboratories), according to the manufacturer’s specifications. Amplification of an ITS segment was performed with primers Sp1 and Sp2 in a 25-µl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 200 µM (each) deoxynucleoside triphosphate (dATP, dGTP, dCTP, and dUTP), 25 pmol of each primer, 0.75 U of Taq DNA polymerase (all reagents were from Qiagen, Pty. Ltd., Australia), and 5 µl of DNA. The thermal profile involved initial denaturation for 5 min at 96°C and 35 cycles with the following steps: 30 s of denaturation at 94°C, 30 s of annealing at 60°C, and 1 min of extension at 72°C. The final extension was for 10 min at 72°C. The PCR products were analyzed with SRBR Safe DNA gel stain (Molecular Probes Europe BV, The Netherlands).

Nucleotide sequencing. The purified PCR products were directly sequenced with forward primers Sp1 and Sp2 by using an Applied Biosystems model 373A DNA sequencer (Perkin-Elmer Applied Biosystems) and a BigDye Terminator cycle sequencing kit. Sequences were identified by using the FastA program group accessed through WebANGIS.

RLB assay. The RLB assay was performed by using a system which has previously been described in detail (28, 31). Briefly, the slots in a Miniblotter 45 apparatus (Amersham) were filled with 150 µl of optimized concentrations of probe solution. Each PCR product was denatured and immediately chilled on ice. Hybridization was performed at 60°C for 40 min. The washed membrane was incubated in peroxidase-labeled streptavidin conjugate (Roche, Mannheim, Germany) at 42°C for 40 min. The washed membrane was then incubated in chemiluminescence blotting substrate (ECL Direct system; Roche) for 1 min, covered with Hyperfilm X-ray film (Amersham) for detection of chemiluminescence, and with autoradiography film for 7 min.

Sensitivities of species-specific PCR and RIS-PCR assay. The RLB assay was performed with forward primers Sp1 and Sp2 by using an Applied Biosystems model 373A DNA sequencer (Perkin-Elmer Applied Biosystems) and a BigDye Terminator cycle sequencing kit. Sequences were identified by using the FastA program group accessed through WebANGIS.

patients. Skin biopsy specimens from three patients with presumed leprosy which had been shown to contain acid-fast bacilli by microscopy were included in the study.

Mycobacteria were grown on Löfftner-Jensen (L-J) slants with weekly inspection until visible growth was detected or in 7H12 broth (MGIT liquid medium; Becton Dickinson) until a positive growth index was signaled by the automated system. Colonies were scraped from the L-J slants and suspended in 1 ml Tris-HCl buffer (10 mM; pH 7.5). The bacteria were killed by heating them to 100°C for 30 min and then stored at 4°C. The clinical isolates used had been identified to the species level by standard biochemical procedures, and their identities were confirmed by HPLC (CIDM isolates) or GC (isolates from both Chinese laboratories) (2).

DNA extraction and PCR amplification. The extraction of DNA was carried out with a commercially available kit (InstaGene Matrix; Bio-Rad Laboratories), according to the manufacturer’s specifications. Amplification of an ITS segment was performed with primers Sp1 and Sp2 in a 25-µl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 200 µM (each) deoxynucleoside triphosphate (dATP, dGTP, dCTP, and dUTP), 25 pmol of each primer, 0.75 U of Taq DNA polymerase (all reagents were from Qiagen, Pty. Ltd., Australia), and 5 µl of DNA. The thermal profile involved initial denaturation for 5 min at 96°C and 35 cycles with the following steps: 30 s of denaturation at 94°C, 30 s of annealing at 60°C, and 1 min of extension at 72°C. The final extension was for 10 min at 72°C. The PCR products were analyzed with SRBR Safe DNA gel stain (Molecular Probes Europe BV, The Netherlands).

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determined by amplification and hybridization of the DNA of each species serially diluted from 5 ng/μl to 500 fg/μl with molecular biology-grade water and tested in parallel.

**RESULTS**

**Sensitivities of PCR-RLB assay.** The detection limits of the species-specific PCRs for both *M. tuberculosis* H37Rv and *M. abscessus* DNA were 5 pg/μl (data not shown). The PCR-RLB assay was 10 times more sensitive than the species-specific PCR assays for both *M. tuberculosis* H37Rv (Fig. 1) and *M. abscessus* DNA (data not shown); i.e., it was able to detect 500 fg/μl. The MYC (genus-specific) probe was more sensitive (50 fg/μl) than the MTBC (species-specific) probe for the detection of *M. tuberculosis* H37Rv (Fig. 1) but was less sensitive (500 fg/μl) than the ABS probe (50 fg/μl) for the detection of *M. abscessus* (data not shown).

**Specificity of species-specific mycobacterial PCR-RLB assay.** PCR amplification with primers Sp1 and Sp2 of DNA from all 78 mycobacterium reference strains produced single bands ranging in length from 200 to 320 bp. The amplicons from the slowly growing *Mycobacterium* species were smaller (200 to 250 bp) than those from the rapidly growing species (250 to 320 bp). *M. xenopi* produced the smallest product (~200 bp), and *M. gilvum* produced the largest product (~320 bp).

There were cross-reactions between the *Mycobacterium* genus-specific primers (Sp1 and Sp2) and DNA extracts from *Nocardia* spp. and *Rhodococcus* spp., which produced two to three copies of the amplicon (Fig. 2). However the genus-specific probe (MYC) hybridized only with *Mycobacterium* spp., and with a few exceptions, all species-specific probes hybridized only with the corresponding species. The exceptions were as follows: the probe for *M. marinum* cross-reacted with...
were confirmed to be *M. lentiflavum* isolates presumptively identified as *M. fortuitum* by HPLC were shown by the PCR-RLB assay and Two clinical isolates that had been reported as resembling *M. triplex* sequencing to be complex by GC, 26 were identified as *M. chelonae-M. abscessus*, and one was identified as *M. avium* (ATCC 35796) or have very similar 16S rRNA gene sequences (e.g., *M. malmoense* and *M. szulgai*) (24).

The 16S-23S rRNA gene ITS region has been identified as a potentially suitable target for probes that can differentiate closely related species. It contains both conserved and highly variable signatures and is rather small. The 16S-23S rRNA gene ITS-based PCR produces a relatively small PCR product (200 to 350 bp) (Fig. 2). Roth et al. reported that PCR based on the 16S-23S rRNA gene ITS and restriction fragment length polymorphism (RFLP) analysis is a promising method with advantages over the previously used *hsp65* gene-based method for the reliable and easy identification of mycobacteria (19, 20). However, it has been impeded by difficulties, such as minor differences in band sizes between some species and the occurrence of new patterns not previously reported (17, 27). Moreover, RFLP analysis cannot reliably detect mixtures of

DNA from two of the three biopsy specimens from patients with clinical leprosy hybridized with the LEP probe and were confirmed by sequencing to contain *M. leprae*. The other hybridized with both the MTB and the INT probes, but the sequencing result was uninterpretable, suggesting that both *M. tuberculosis* and *M. intracellulare* were present. In addition to this clinical specimen, the PCR-RLB assay identified mixed species in 14 separate clinical isolates and the sequencing results were uninterpretable.

**DISCUSSION**

In comparison to conventional biochemical methods, GC and HPLC provide rapid results. However, both require expensive instrumentation, reproducible patterns depend on standardized conditions of growth, and interpretation of the results is not always easy. The PCR-RLB assay does not require standardized growth conditions, and it is more accurate and objective than either GC or HPLC. In our study, two clinical isolates which had presumptively been identified as *M. fortuitum* by HPLC were identified by the PCR-RLB assay as *M. triplex*, and this result was confirmed by sequencing. The PCR-RLB assay identified mixed mycobacterial cultures which had not been recognized by HPLC or GC and which gave uninterpretable sequencing results. Some previously unidentified species were potentially clinically significant. The finding of four mixed cultures initially identified as containing only *M. gordonae* indicates that this contaminant *Mycobacterium* species can mask potential pathogens. It is not clear whether the high proportion of mixed subtypes of *M. fortuitum* indicates that cross-reactions between probes occur or that mixtures of subtypes commonly occur in nature.

In addition, the PCR-RLB assay was able to discriminate between *M. chelonae* and *M. abscessus*, which could not be distinguished by GC, and between *M. avium* and *M. intracellularum*, which could not be distinguished by HPLC (Table 1).

Sequencing of the 16S rRNA gene is often regarded as the “gold standard” for the identification of mycobacteria (5), but it has a demonstrated lower variability with several examples of species difficult to separate from the genus *Mycobacterium* (10, 25). Some *Mycobacterium* species share the same 16S rRNA gene sequence (*M. kansasi* and *M. gastri*, *M. senegalense* and *M. farcinogenes*) or have very similar 16S rRNA gene sequences (e.g., *M. malmoense* and *M. szulgai*) (24).

The 16S-23S rRNA gene ITS region has been identified as a potentially suitable target for probes that can differentiate closely related species. It contains both conserved and highly variable signatures and is rather small. The 16S-23S rRNA gene ITS-based PCR produces a relatively small PCR product (200 to 350 bp) (Fig. 2). Roth et al. reported that PCR based on the 16S-23S rRNA gene ITS and restriction fragment length polymorphism (RFLP) analysis is a promising method with advantages over the previously used *hsp65* gene-based method for the reliable and easy identification of mycobacteria (19, 20). However, it has been impeded by difficulties, such as minor differences in band sizes between some species and the occurrence of new patterns not previously reported (17, 27). Moreover, RFLP analysis cannot reliably detect mixtures of

![FIG. 2. PCR results for *Mycobacterium* species and other bacteria obtained by using primers Sp1 and Sp2 (based on the 16S-23S rRNA gene ITS).](image-url)
species within samples. Others have also found that the presence of more than one species in a culture or a clinical sample makes it difficult or impossible to identify the species present by sequencing or RFLP analysis of 16S-23S rRNA gene ITS PCR products (10).

In our study, from the analysis of 78 reference strains, 219 clinical isolates, and three skin biopsy specimens, the specificity of our PCR-RLB assay, based on the 16S-23S rRNA gene ITS region, was close to 100% (except that M. senegalense reference strain ATCC 35796 reacted with the FOR1 probe). Several other systems that are similar to our PCR-RLB assay system are available: the INNO-LiPA strip (version 2; Immuno-technologies) (15, 23, 26), the GenoType Mycobacterium system (Hain Life Science) (13), and an oligonucleotide array (16) can identify 16, 13, and 19 Mycobacterium species, respectively. Our PCR-RLB assay based on the 16S-23S rRNA gene ITS can differentiate 34 mycobacterial species and subspecies by the use of 35 probes and has a high degree of specificity.

Our assay is as simple as other reverse hybridization methods, but it is more sensitive because it uses a nonprecipitating enhanced chemiluminescent substrate and Hyperfilm X-ray film. It successfully identified mycobacteria directly in clinical specimens and has the potential to be used for the direct identification of Mycobacterium species from other types of clinical specimens. In addition, it has the advantage that the nylon filters can be stripped and reused at least 30 times and therefore is relatively inexpensive.

In conclusion, the PCR-RLB assay method described here is faster than conventional biochemical tests, is more accurate than HPLC or GC, is less expensive than sequencing, and does not require specialized instrumentation. It is more discriminatory than methods based on 16S rRNA gene-specific PCR or PCR-RFLP analysis of other genes (e.g., rpoB) and identifies more species than other PCR-RLB assays based on the 16S-23S rRNA gene ITS. It has the potential to be used to identify Mycobacterium species directly in clinical specimens.

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