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A Simple and Efficient Multiplex PCR Assay for the Identification of *Mycobacterium* Genus and *Mycobacterium tuberculosis* Complex to the Species Level

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Purpose: The *Mycobacterium tuberculosis* complex comprises *M. tuberculosis*, *M. bovis*, *M. bovis* bacillus Calmette-Guérin (BCG) and *M. africanum*, and causes tuberculosis in humans and animals. Identification of *Mycobacterium* spp. and *M. tuberculosis* complex to the species level is important for practical use in microbiological laboratories, in addition to optimal treatment and public health. **Materials and Methods:** A novel multiplex PCR assay targeting a conserved *rpo*B sequence in *Mycobacteria* spp., as well as regions of difference (RD) 1 and RD8, was developed and evaluated using 37 reference strains and 178 clinical isolates. **Results:** All mycobacterial strains produced a 518-bp product (*rpo*B), while other bacteria produced no product. Virulent *M. tuberculosis* complex strains, *M. tuberculosis*, *M. bovis* and *M. africanum*, produced a 254-bp product (RD1), while *M. bovis* BCG, *M. microti* and nontuberculous mycobacteria produced no RD1 region product. Additionally, *M. tuberculosis* and *M. africanum* produced a 150-bp product (RD8), while *M. bovis* and *M. bovis* BCG produced a 360-bp product (deleted form of RD8). *M. microti* and nontuberculous mycobacteria produced no RD8 region product. This assay identified all *Mycobacterium* spp. and all *M. tuberculosis* complex strains to the species level. **Conclusion:** The multiplex PCR assay of the present study could be implemented as a routine test in microbiology laboratories, and may contribute to more effective treatment and surveillance of tuberculosis stemming from the *M. tuberculosis* complex.

Key Words: *M. tuberculosis* complex, multiplex PCR, *rpo*B, RD1, RD8

INTRODUCTION

Tuberculosis is a global health problem, with approximately 8.8 million new cases and 1.4 million deaths reported in 2010.[1] The disease is caused by members of the *Mycobacterium tuberculosis* (*M. tuberculosis*) complex, a group of closely related species and subspecies, which include *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. bovis* bacillus Calmette-Guérin (BCG), *M. microti*, *M. canettii*, *M. pinnipedii*, and

M. mungi.[2] *M. tuberculosis* is the most common cause of tuberculosis in humans. *M. bovis* causes tuberculosis in humans and animals, and is responsible for 10 to 15% of new human tuberculosis cases in developing countries.[3] *M. bovis* BCG, a live attenuated strain of *M. bovis*, is a widely used anti-tuberculosis vaccine, and is used for the treatment of bladder cancer.[4] However, *M. bovis* BCG is known to lead to complications, such as osteomyelitis, abscesses, lymphadenitis, and dissemination of *M. bovis* BCG, most severely in human immunodeficiency virus-infected neonates and children. In *M. bovis* BCG-treated patients, complications such as pneumonitis, hepatitis and noncaseating granulomas have been reported.[5]

Identification of the *M. tuberculosis* complex to the species level is important for public health, epidemiology and treatment.[6] In the laboratory, tuberculosis is diagnosed by acid-fast bacilli (Ziehl-Neelsen) staining and culturing of mycobacteria, followed by identification of the cultured mycobacteria. For more rapid identification, several polymerase chain reaction (PCR)-based methods have been developed, and PCR assay that targets *M. tuberculosis*-specific sequences, including IS*6110* and the MPT64 gene, has been widely applied.[7] However, while this assay can differentiate the *M. tuberculosis* complex from nontuberculous mycobacteria, it cannot identify the *M. tuberculosis* complex to the species level.[7] For rapid and efficient identification of the *M. tuberculosis* complex, several PCR-based methods have been developed.[8-10] However, these are rarely used as a routine test in diagnostic laboratories as they require two rounds of PCR reaction and electrophoresis to distinguish the *M. tuberculosis* complex from other mycobacteria and to identify *M. tuberculosis* complex to the species level.[11,12] Moreover, identification of nontuberculous mycobacteria to the species level is of greater importance due to the increased incidence of infection with nontuberculous mycobacteria. Thus, more rapid and efficient methods of identification are needed.

A well-validated probe for the *Mycobacterium* genus targets the conserved sequence of the *rpo*B gene, which encodes the β subunit of RNA polymerase, and not only facilitates identification of all *Mycobacterium* spp., but also distinguishes *Mycobacterium* spp. at the species level by restriction enzyme analysis.[13] In a comparative genomics study of *M. tuberculosis* H37Rv, 16 regions of the genome (regions of difference, RD) were shown to be deleted in *M. bovis* and *M. bovis* BCG.[10] One of them, RD1, is present in virulent *M. tuberculosis* complex strains, including *M. tuberculosis* and *M. bovis*, but is absent in *M. bovis* BCG, allowing for *M. tuberculosis* and *M. bovis* to be differentiated from *M. bovis* BCG.[11,12] Additionally, a novel RD8 is present in *M. tuberculosis* and *M. africanum* that is deleted in *M. bovis* and *M. bovis* BCG and absent in *M. microti* and nontuberculous mycobacteria.[14]

In this study, we developed a rapid and efficient multiplex PCR assay that enables not only the identification of the genus *Mycobacterium* using the *rpo*B sequence conserved in all mycobacteria, but also the identification of the *M. tuberculosis* complex to the species level, using RD1 and RD8 sequences. The assay was shown to be simple and efficient in identifying *Mycobacterium* genus and distinguishing the *M. tuberculosis* complex to the species level.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains used in this study are listed in Table 1. Seventy-six clinical *M. tuberculosis* isolates from humans and 52 clinical *M. bovis* isolates from cattle were provided kindly from Yonsei University College of Medicine, Seoul, Korea. Twenty-seven clinical *M. avium* isolates and 23 clinical *M. intracellulare* isolates from humans were provided by the Korea Institute of Tuberculosis, Osong, Korea.

Culture media and DNA extraction

Bacterial culture and chromosomal DNA isolation were performed as previously described.[13] Briefly, bacteria were cultured on Middlebrook 7H9 media (Difco, Detroit, MI, USA) supplemented with 10% Middlebrook OADC Enrichment media (BBL, Sparks, MD, USA) at 37°C for 2 to 4 weeks. Bacterial cells were collected by centrifugation at 10000×g, resuspended in distilled water, and boiled for 10 min. Cell debris was sedimented by centrifugation for 2 min at 10000×g, and the resultant DNA concentration was measured using a spectrophotometer (260 nm, Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). DNA was stored at -20°C prior to use.

PCR primer design

The primers used in this study are listed in Table 2. The genome sequence of *M. tuberculosis* H37Rv was imported into the Vector NTI® Software (Life technologies, Grand Island, NY, USA), and *rpo*B, RD1 and RD8 were created according to the genome address. Primers were selected by

Table 1. Bacterial Strains Used in This Study

Species (no. of strains)	Reference no./Source
M. tuberculosis H37Rv	ATCC27294
M. tuberculosis Erdman	ATCC35801
M. tuberculosis (76)	YUMC
M. bovis AN5	ATCC35726
M. bovis (52)	YUMC
M. bovis BCG Pasteur	YUMC
M. bovis BCG Danish	YUMC
M. bovis BCG Tokyo	YUMC
M. bovis BCG Tice	YUMC
M. abscessus	ATCC19977
M. africanum	ATCC25420
M. avium	ATCC25291
M. avium	ATCC35719
M. avium (27)	KIT
M. celatum	ATCC51130
M. celatum	ATCC51131
M. chelonae	ATCC19237
M. fortuitum	ATCC49403
M. fortuitum	ATCC49404
M. gilvum	ATCC43909
M. gordonae	ATCC14470
M. intracellulare	ATCC13950
M. intracellulare (23)	KIT
M. kansasii	ATCC12478
M. marinum	ATCC927
M. microtii	ATCC19422
M. mucogenicum	ATCC49649
M. peregrinum	ATCC14467
M. senegalense	ATCC35796
M. septicum	ATCC700731
M. smegmatis	ATCC19420
M. szulgai	ATCC 35799
M. terrae	ATCC15755
M. ulcerans	ATCC19423
Enterococcus faecalis	ATCC29212
E. faecium	ATCC35667
Escherichia coli	ATCC25922
Haemophilus infulenza	ATCC49247
Micrococcus lutens	ATCC49732
Pseudomonas aeroginosa	ATCC27853
Staphylococcus aureus	ATCC29213

YUMC, Yonsei University College of Medicine, Seoul, Korea; KIT, Korea Institute of Tuberculosis, Osong, Korea; BCG, bacillus Calmette-Guérin.

analysis of the appropriate genomic regions using Primer 3 software (http://frodo.wi.mit.edu/) (Fig. 1, Table 2).

PCR amplification

The amplification reaction mixture consisted of 10 mM Tri-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each primer, 1.5 units of *Taq* DNA polymerase (Bioneer, Daejeon, Korea), and 20 ng of genomic DNA as the template. PCR amplification was performed in an automated thermal cycler (PTC-100 Programmable Thermal Controller, MJ Research, Inc., Waltham, MA, USA). The cycling parameters were 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 68°C for 30 s and a final extension at 72°C for 7 min. Sterile distilled water was used as a negative control. Analysis of PCR products was performed by electrophoresis in ethidium bromide (0.5 μg/mL)-stained 1.7 % (w/v) agarose gels.

RESULTS

Validation of the PCR method

PCR conditions were optimized with four sets of primer pairs using *M. tuberculosis* H37Rv, *M. bovis* AN5 and *M. bovis* BCG Pasteur as representative strains (Figs. 1 and 2), and confirmed using all bacterial strains by gel electrophoresis. The PCR products for each target sequence from each set of primer pairs were also confirmed. All reference mycobacterial strains consistently yielded a single 518-bp product with primers rpoB-F and rpoB-R, but other bacterial strains yielded no products, indicating that all mycobacterial strains contained the specific *rpo*B gene (Table 3). *M. tuberculosis* H37Rv, *M. africanum* and *M. bovis* AN5 consistently yielded a single 254-bp product with RD1-F and RD1-R primers, but other bacterial strains, including all *M. bovis* BCG strains and *M. microti*, yielded no product with RD1-F and RD1-R primers, indicating the RD1 sequence is present in virulent *M. tuberculosis* complex strains. *M. tuberculosis* and *M. africanum* type strains consistently yielded a single 150-bp product with RD8-F and RD8-T-R primers, but yielded no product with RD8-F and RD8-T-R primers, indicating the presence of the RD8 sequence. Conversely, *M. bovis* and all *M. bovis* BCG strains consistently yielded a single 360-bp product with RD8-F and RD8-B-R primers, but yielded no product with RD8-F and RD8-T-R primers, indicating a deletion of the RD8 sequence in *M. bovis*, including *M. bovis* BCG (Fig. 1, Table 3). However, other bacteria, including nontuberculous mycobacteria and *M. microti*, yielded no product with either RD8-F and RD8-T-R or RD8-F and RD8-B-R primers.

Multiplex PCR on reference strains and clinical isolates

PCR products using the four sets of primer pairs were per-

Table 2. Primers Used in This Study

Description of target	Product length (bp)	Primer names	Primer sequences
rpoB	518	rpoB-F	GCTGGACATCTACCGCAAGCTGC
		rpoB-R	CAGCGGGTTGTTCTGGTCCATG
RD1 (ESAT-6)	254	RD1-F	CGAGGGGAAGCAGTCCCTGA
		RD1-R	AGGTCGAACTCGCCCGATCC
RD8 present	150	RD8-F	GTCGAAGCGGGGCGCTCT
		RD8-T-R	GCGCAACGGATTTCCATCGT
RD8 deleted	360	RD8-F	GTCGAAGCGGGGCGCTCT
		RD8-B-R	GGTTCTTGGCGTCTTGGAAGG

RD, regions of difference.

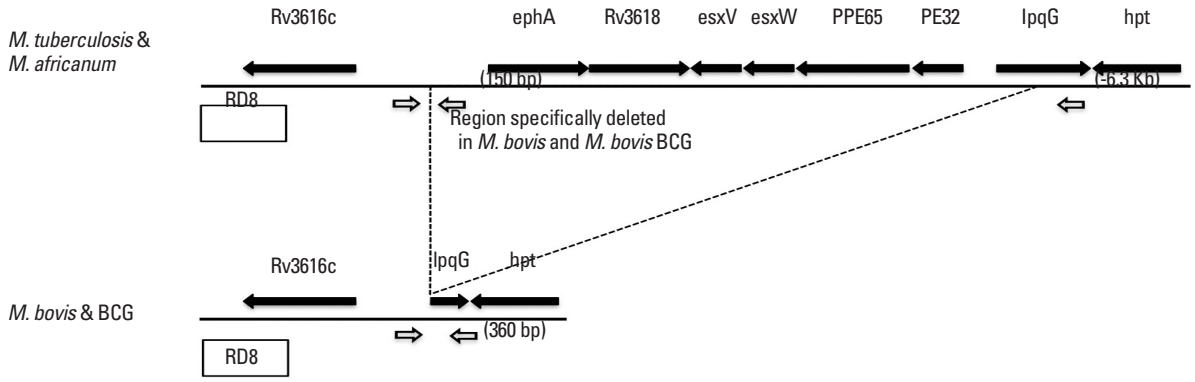

Fig. 1. Schematic diagram showing regions flanking the direct repeat region in *M. tuberculosis* H37Rv, *M. bovis* and *M. bovis* bacillus Calmette-Guérin (BCG). This region is specifically deleted in *M. bovis* and *M. bovis* BCG, indicated by dotted lines. Primer positions are indicated by small arrows. RD, regions of difference.

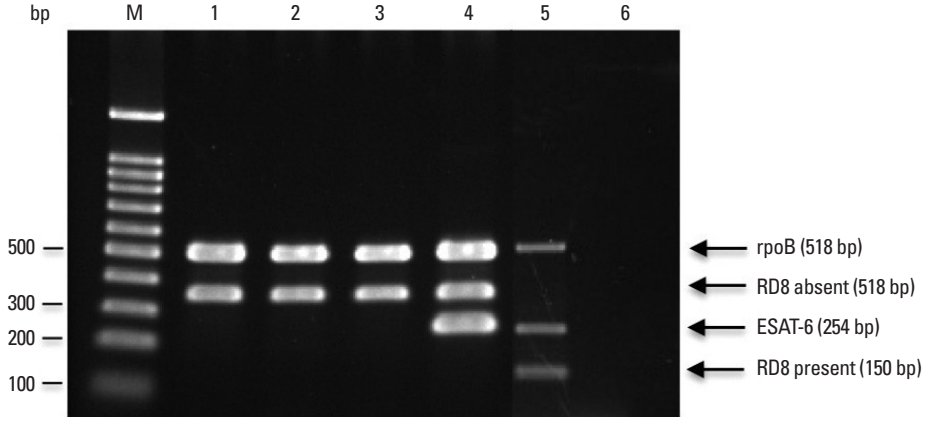

Fig. 2. Multiplex PCR for *M. tuberculosis*, *M. bovis* and *M. bovis* bacillus Calmette-Guérin (BCG) strains. PCR products were separated by electrophoresis and visualized. Lanes M: molecular size marker (100-bp ladder); 1: *M. bovis* BCG Pasteur; 2: *M. bovis* BCG Tice; 3: *M. bovis* BCG Tokyo; 4: *M. bovis* AN5; 5: *M. tuberculosis* H37Rv; 6: negative control. Expected sizes of PCR products are shown. RD, regions of difference; PCR, polymerase chain reaction; ESAT-6, early secreted antigenic target 6 kDa protein.

formed on 30 reference strains and 178 clinical isolates (Figs. 3, 4 and 5). Multiplex PCR of *M. tuberculosis* H37Rv and *M. tuberculosis* Erdman yielded three 518-, 254- and 150-bp products, indicating the presence of *rpo*B, RD1 and RD8, respectively (Fig. 4). Multiplex PCR of 76 clinical *M. tuberculosis* isolates provided results identical to the *M. tuberculosis* reference strain (Fig. 4). Multiplex PCR of *M. africanum*, a member of the *M. tuberculosis* complex, yielded results identical to *M. tuberculosis* (Fig. 3). However, multiplex PCR of *M. microti* yielded only a 518-bp product, similar to nontuberculous mycobacteria.

Multiplex PCR on *M. bovis* AN5 and 52 clinical *M. bovis* isolates yielded 518-, 360- and 254-bp products, indicating the presence of *rpo*B and RD1, as well as the deletion of RD8 (Fig. 5). All four *M. bovis* BCG strains yielded 518- and 360-bp products, indicating the presence of *rpo*B, the

absence of RD1 and the deletion of RD8. Multiplex PCR of 23 nontuberculous mycobacteria reference strains and 50 clinical nontuberculous mycobacteria isolates yielded only a 518-bp product, indicating the absence of RD1 and RD8. Multiplex PCR of other non-mycobacterial bacteria yielded no positive results. The data were consistently reproducible, and complete concordance between independent researchers was confirmed.

Table 3. Signature Patterns of Positive and Negative Multiplex PCR Used to Discriminate Mycobacterial Strains

Organism	PCR result for target			
	*rpo*B	RD1	RD8 present	RD8 deleted
M. tuberculosis	+	+	+	-
M. bovis	+	+	-	+
M. bovis BCG	+	-	-	+
M. africanum	+	+	+	-
M. microti	+	-	-	-
NTM	+	-	-	-
Other bacteria	-	-	-	-

RD, regions of difference; BCG, bacillus Calmette-Guérin; PCR, polymerase chain reaction; NTM, non-tuberculous Mycobacteria.

DISCUSSION

We developed a simple and effective multiplex PCR assay that enabled the differentiation of *Mycobacteria* spp. from other bacteria and identified the *M. tuberculosis* complex to the species level. Our analysis using reference strains and clinical isolates suggested that the multiplex PCR assay is specific for *M. tuberculosis*, *M. bovis* and *M. bovis* BCG, as well as nontuberculosis mycobacteria. The conserved *rpo*B sequence of the mycobacteria was used to differentiate the genus *Mycobacterium* from other bacteria, resulting in data specific for all *Mycobacteria* spp. tested.[13] Moreover, the fragment length polymorphism of the 518-bp PCR products targeting *rpo*B were effective for species identification (data not shown). This sequence offers advantages over other target sequences, including IS*6110* and 16S RNA. IS*6110* can be used for the detection of *M. tuberculosis* complex, but cannot differentiate members of the *M. tuberculosis* complex.[7] 16S RNA has been used for the identification of *Mycobacteria* spp., but is unable to provide identification of

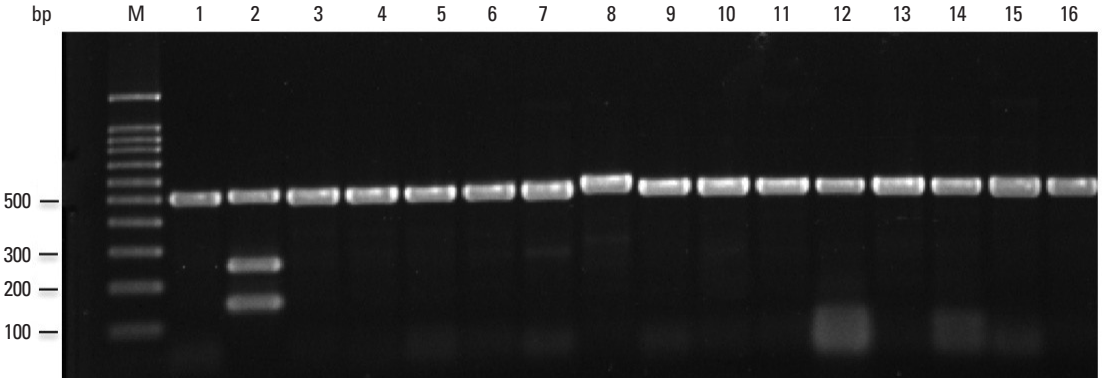

Fig. 3. Multiplex PCR for the *Mycobacterium* species. PCR products were separated by electrophoresis and visualized. Lanes M: molecular size marker (100-bp ladder); 1: *M. avium*; 2: *M. africanum*; 3: *M. abscesses*; 4: *M. celatum*; 5: *M. chelonae*; 6: *M. fortuitum*; 7: *M. gordonae*; 8: *M. intracellulare*; 9: *M. kansasii*; 10: *M. marinum*; 11: *M. microtii*; 12: *M. phlei*; 13: *M. septicum*; 14: *M. szulgai*; 15: *M. terrae*; 16: *M. ulcerans*; PCR, polymerase chain reaction.

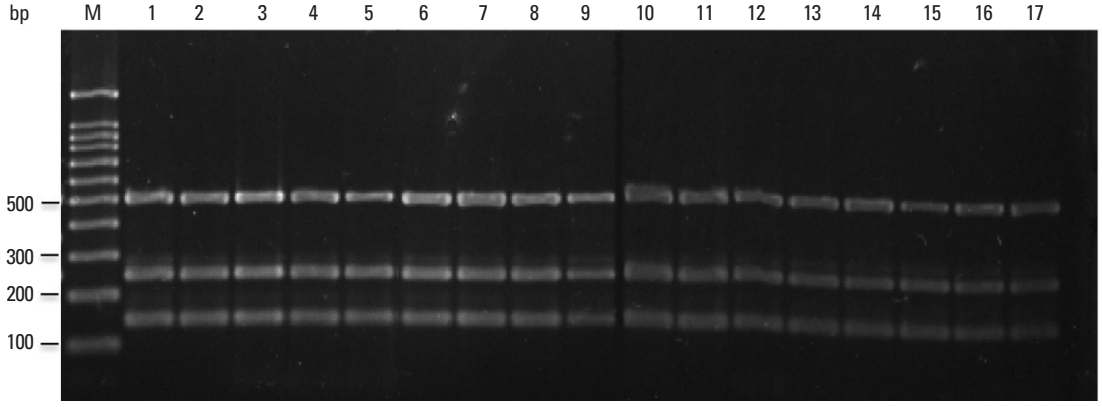

Fig. 4. Multiplex PCR for clinical *M. tuberculosis* isolates. PCR products were separated by electrophoresis and visualized. Lanes M: molecular size marker (100-bp ladder); 1-15: *M. tuberculosis* isolates; 16: *M. tuberculosis* H37Rv; 17: *M. tuberculosis* Erdman; PCR, polymerase chain reaction.

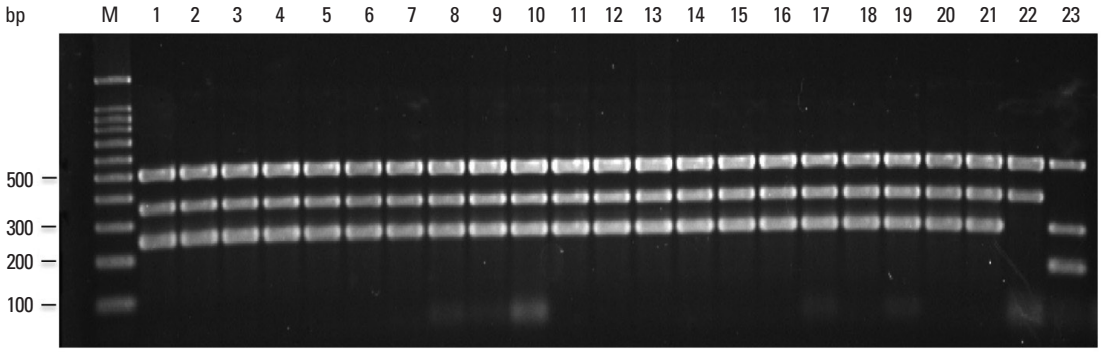

Fig. 5. Multiplex PCR for clinical *M. bovis* isolates. PCR products were separated by electrophoresis and visualized. Lanes M: molecular size marker (100-bp ladder); 1-20: *M. bovis* isolates; 21: *M. bovis* AN5; 22: *M. bovis* BCG Pasteur; 23: *M. tuberculosis* H37Rv. BCG, bacillus Calmette-Guérin; PCR, polymerase chain reaction.

M. tuberculosis complex to the species level, due to their high similarity.[15] The present assay was based on the detection of RD1 and RD8 in order to distinguish members of the *M. tuberculosis* complex. Using this assay, RD1 was detected in all *M. tuberculosis* complex strains, including *M. tuberculosis*, *M. bovis* and *M. africanum*, but not *M. bovis* BCG or *M. microti*, in accordance with previous reports.[11,12] RD8 was detected in *M. tuberculosis* and *M. africanum*, but a deleted form of RD8 was detected in *M. bovis* and *M. bovis* BCG. Interestingly, this sequence was absent in *M. microti* even though *M. microti* belongs to the *M. tuberculosis* complex. This result was in agreement with previous reports that demonstrated the presence of RD8 in *M. tuberculosis*, but not *M. microti*.[16] In our study, the *M. africanum* reference strain (ATCC 25420) contained RD8; however, investigation using other *M. africanum* strains would be necessary since Vasconcellos, et al.[17] reported RD8 to be present in the West African-1 lineage, but absent from the West African-2 lineage. Our assay using RD8 possessed advantages over other RDs, including RD4 and RD9, since RD8 can distinguish mycobacteria to three groups, *M. tuberculosis* and *M. africanum*, *M. bovis* and *M. bovis* BCG, and *M. microti* and nontuberculous mycobacteria, by determining the presence of RD8, the deletion of RD8 or the absence of RD8, respectively. Conversely, RD4 and RD9 can distinguish between *M. tuberculosis* and *M. bovis*, including *M. bovis* BCG, but not between *M. africanum* and *M. microtii*.[11] Multiplex PCR assays that target RD8 combined with RD1 effectively distinguish the *M. tuberculosis* complex to the species level.

Several methods of identifying the *M. tuberculosis* complex currently exist, including high-performance liquid chromatography,[18] restriction fragment length polymorphism,[7,8] and spoligotyping.[19] However, these assays are generally not used routinely in clinical microbiology laboratories as they are time consuming and costly. Recently, multiplex PCR assays have been proposed as a diagnostic method due to a single reaction and multiple targets.[7,11,12] The present assay is the first multiplex PCR assay shown to identify the *Mycobacterium* genus and distinguish the *M. tuberculosis* complex to the species level by targeting the *rpo*B sequence, as well as RD1 and RD8 sequences, respectively.

This assay can be used as a routine diagnostic test in microbiology laboratories and may contribute to determination of an optimal treatment regimen, improve the surveillance of tuberculous disease due to members of the *M. tuberculosis* complex, and aid in the identification of the *M. tuberculosis* complex and nontuberculous mycobacteria.

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REFERENCES

1. Raviglione M, Marais B, Floyd K, Lönnroth K, Getahun H, Migliori GB, et al. Scaling up interventions to achieve global tuberculosis control: progress and new developments. Lancet 2012;379:1902-13.
2. Ernst JD, Trevejo-Nuñez G, Banaiee N. Genomics and the evolution, pathogenesis, and diagnosis of tuberculosis. J Clin Invest 2007;117:1738-45.
3. de la Rua-Domenech R. Human Mycobacterium bovis infection in the United Kingdom: incidence, risks, control measures and review of the zoonotic aspects of bovine tuberculosis. Tuberculosis

(Edinb) 2006;86:77-109.
4. Lamm DL, Stogdill VD, Stogdill BJ, Crispen RG. Complications of bacillus Calmette-Guerin immunotherapy in 1,278 patients with bladder cancer. J Urol 1986;135:272-4.
5. Kamphuis JT, Buiting AG, Miseré JF, van Berge Henegouwen DP, van Soolingen D, Rensma PL. BCG immunotherapy: be cautious of granulomas. Disseminated BCG infection and mycotic aneurysm as late complications of intravesical BCG instillations. Neth J Med 2001;58:71-5.
6. O'Reilly LM, Daborn CJ. The epidemiology of Mycobacterium bovis infections in animals and man: a review. Tuber Lung Dis 1995;76 Suppl 1:1-46.
7. Sankar S, Ramamurthy M, Nandagopal B, Sridharan G. An appraisal of PCR-based technology in the detection of Mycobacterium tuberculosis. Mol Diagn Ther 2011;15:1-11.
8. Frothingham R. Differentiation of strains in Mycobacterium tuberculosis complex by DNA sequence polymorphisms, including rapid identification of M. bovis BCG. J Clin Microbiol 1995;33:840-4.
9. Kjeldsen MK, Bek D, Rasmussen EM, Priemé A, Thomsen VØ. Line probe assay for differentiation within Mycobacterium tuberculosis complex. Evaluation on clinical specimens and isolates including Mycobacterium pinnipedii. Scand J Infect Dis 2009;41:635-41.
10. Parsons LM, Brosch R, Cole ST, Somoskövi A, Loder A, Bretzel G, et al. Rapid and simple approach for identification of Mycobacterium tuberculosis complex isolates by PCR-based genomic deletion analysis. J Clin Microbiol 2002;40:2339-45.
11. Halse TA, Escuyer VE, Musser KA. Evaluation of a single-tube multiplex real-time PCR for differentiation of members of the Mycobacterium tuberculosis complex in clinical specimens. J Clin Microbiol 2011;49:2562-7.
12. Pinsky BA, Banaei N. Multiplex real-time PCR assay for rapid identification of Mycobacterium tuberculosis complex members to the species level. J Clin Microbiol 2008;46:2241-6.
13. Lee H, Park HJ, Cho SN, Bai GH, Kim SJ. Species identification of mycobacteria by PCR-restriction fragment length polymorphism of the rpoB gene. J Clin Microbiol 2000;38:2966-71.
14. Gordon SV, Eiglmeier K, Garnier T, Brosch R, Parkhill J, Barrell B, et al. Genomics of Mycobacterium bovis. Tuberculosis (Edinb) 2001;81:157-63.
15. Dobner P, Feldmann K, Rifai M, Löscher T, Rinder H. Rapid identification of mycobacterial species by PCR amplification of hypervariable 16S rRNA gene promoter region. J Clin Microbiol 1996;34:866-9.
16. Frota CC, Hunt DM, Buxton RS, Rickman L, Hinds J, Kremer K, et al. Genome structure in the vole bacillus, Mycobacterium microti, a member of the Mycobacterium tuberculosis complex with a low virulence for humans. Microbiology 2004;150(Pt 5):1519-27.
17. Vasconcellos SE, Huard RC, Niemann S, Kremer K, Santos AR, Suffys PN, et al. Distinct genotypic profiles of the two major clades of Mycobacterium africanum. BMC Infect Dis 2010;10:80.
18. Butler WR, Jost KC Jr, Kilburn JO. Identification of mycobacteria by high-performance liquid chromatography. J Clin Microbiol 1991;29:2468-72.
19. Streicher EM, Victor TC, van der Spuy G, Sola C, Rastogi N, van Helden PD, et al. Spoligotype signatures in the Mycobacterium tuberculosis complex. J Clin Microbiol 2007;45:237-40.