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Multiplex Real-Time PCR Assay for Rapid Identification of *Mycobacterium tuberculosis* Complex Members to the Species Level[▽]

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The species identification of members of the *Mycobacterium tuberculosis* complex is critical to the timely initiation of both appropriate antibiotic therapy and proper public health control measures. However, the current commercially available molecular assays identify mycobacteria only to the complex level and are unable to differentiate *M. tuberculosis* from the closely related *M. bovis* and *M. bovis* BCG. We describe here a rapid and robust two-step, multiplex, real-time PCR assay based on genomic deletions to definitively identify *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, and other members of the complex. When tested against a panel of well-characterized mycobacterial reference strains, the assay was both sensitive and specific, correctly identifying all strains. We applied this assay to 60 clinical isolates previously identified as *M. tuberculosis* complex and found 57 *M. tuberculosis* isolates and 3 *M. bovis* BCG isolates from patients who had received intravesical BCG. Furthermore, analysis of 15 clinical specimens previously identified as *M. bovis* by spoligotyping revealed an isolate of *M. tuberculosis* that had been misidentified. We propose that this assay will allow the routine identification of *M. tuberculosis* complex members in the clinical laboratory.

Tuberculosis is a global health epidemic resulting in significant morbidity and the deaths of ~2 million people per year (28). This devastating disease is caused by members of the *Mycobacterium tuberculosis* complex (MTC), a group of closely related species and subspecies that includes *M. tuberculosis*, *M. bovis*, the causative agent of bovine tuberculosis, and *M. bovis* BCG, the live, attenuated tuberculosis vaccine strain (15).

It is important to identify isolates of the MTC to the species level for epidemiologic and public health considerations and to optimize treatment (26). Though *M. tuberculosis* is the most common cause of tuberculosis in humans, *M. bovis* accounts for 0.5 to 7.2% of human tuberculosis cases in industrialized nations and is estimated to be responsible for 10 to 15% of new cases in the developing world (13). Consistent with these estimates, *M. bovis* accounts for ~11% of pediatric tuberculosis cases along the California-Mexico border (11). In addition, there is an emerging problem of disseminated *M. bovis* BCG due to vaccination of neonates and children in endemic regions with high rates of vertical transmission of human immunodeficiency virus (HIV) (1, 18). Finally, ~1% of intravesical *M. bovis* BCG immunotherapy for bladder cancer results in disseminated disease (21). Thus, from a public health perspective, the natural history and control measures for disease differ substantially between complex members. For example, a diagnosis of *M. bovis* should prompt questions of zoonotic exposure or investigation into contamination of dairy products, the primary routes of human infection (16, 26). Therapeutically, both *M. bovis* and BCG are intrinsically resistant to pyrazinamide

(PZA), a first-line antituberculosis agent, making their identification critical to timely initiation of appropriate antibiotic therapy (31).

Despite the clinical implications of MTC identification, the routine identification of mycobacteria in the clinical microbiology laboratory is currently done only to the complex level, and no rapid and simple commercial assays are available in the United States to further identify the MTC species. Though there are several assays commonly used in reference laboratories that can differentiate members of the MTC, these assays either are unable to definitively identify some of the clinically relevant species or are limited by a complexity that exceeds the technical resources of most clinical laboratories. For example, mycolic acid analysis via high-performance liquid chromatography can identify *M. bovis* BCG as distinct from the rest of the MTC but is unable to distinguish *M. tuberculosis* from *M. bovis* (7). In contrast, spacer oligotype analysis, or spoligotyping, can identify key members of the MTC, but the methodology is challenging, interpretation requires an advanced nomenclature accessible only to experts in the field, and an easily searchable database is not publicly available (6, 10, 20).

The lack of rapid and simple commercial assays for the species identification of MTC members is partly due to the observation that members of the complex are 99.9% similar at the nucleotide level and have identical 16S rRNA sequences, the most commonly used molecular marker in microbiology for species identification (34). However, comparative genomic analysis showed that members of MTC evolved from a common ancestor through sequential DNA deletions with precise genomic locations (5, 23). To address the deficiency in diagnostics, we developed a rapid and robust real-time PCR assay based on genomic deletion analysis to distinguish between members of the MTC. This assay uses melting-curve analysis in two PCRs to detect the presence or absence of regions of

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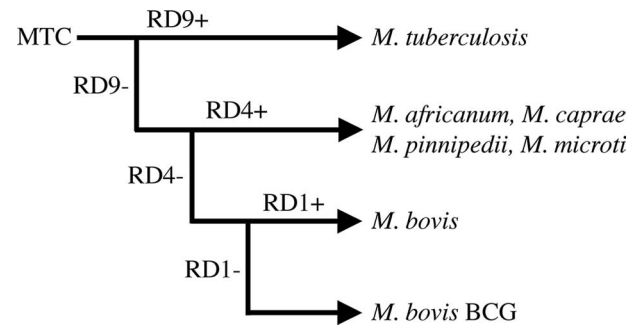


FIG. 1. A simplified evolutionary scenario for the MTC illustrates the presence (+) or deletion (–) of the conserved RD sequences, RD9, RD4, and RD1.

difference (RD) RD9, RD4, and RD1 (Fig. 1), allowing for definitive identification of *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG, respectively, from cultures identified as MTC. Though PCR-based genotyping of the MTC based on genomic deletions has been proposed previously (3, 8, 19, 22, 25, 27, 35, 36), this assay is the first to combine RD analysis with both real-time PCR and a multiplexed primer approach, thus eliminating postamplification handling and the potential to introduce amplicon contaminants. The assay was validated with a series of ATCC strains and then applied to a collection of clinical specimens that had been previously identified to the complex or species level.

MATERIALS AND METHODS

Mycobacterial strains. A list of ATCC strains used in this study is shown in Table 1. Sixty clinical MTC isolates were recovered from patient specimens at the Stanford Hospital Clinical Microbiology Laboratory (Stanford, CA) during 2005 and 2006. These isolates were sent to Focus Diagnostics (Cypress, CA), where they were identified to the complex level by using the AccuProbe assay (Gen-Probe, San Diego, CA). In addition, Ed Desmond and Grace Lin (California Department of Health Services, Richmond, CA) graciously provided DNA from 15 clinical isolates of *M. bovis* and 5 clinical isolates of *M. africanum*.

Culture media and DNA extraction. Bacteria were cultured on Middlebrook 7H11/Middlebrook 7H11 selective biplates (Hardy Diagnostics, Santa Maria, CA) and incubated at 37°C for 2 to 4 weeks. One loopful (1-μl loop) of bacteria was resuspended in 500 μl of molecular-grade water (Sigma, St. Louis, MO) and then boiled for 10 min. Cellular debris was sedimented by centrifugation for 2 min at 11,000 × g, and PCR was performed on the supernatant.

PCR primer design. The primers used in this study are listed in Table 2. The *M. tuberculosis* H37Rv conserved sequence (9) was imported into the software program Clone Manager Professional Suite (Science and Educational Software, Cary, NC), and RD1, RD4, and RD9 were created according to their genomic addresses (5). Primer selection was facilitated by analyzing the appropriate genomic regions with Primer3 (<http://frodo.wi.mit.edu/>).

Real-time PCR. Each 25-μl reaction mixture contained 2.5 μM of each primer, 1× FastStart Sybr green master mix (Roche Diagnostics, Indianapolis, IN), and 7.5 μl of extracted DNA. Reaction 1 had primers to detect the presence of RD9 (RD9 Present Forward and RD9 Present Reverse) or the absence of RD1 (RD1 Deleted Forward and RD1 Deleted Reverse). In addition, reaction 1 included primers to a region of the 16S rRNA gene that is common to all mycobacteria (Genus Control Forward and Genus Control Reverse). Reaction 2 had primers to detect the presence or absence of RD4 (RD4 Common Forward, RD4 Present Reverse, and RD4 Deleted Reverse). The reactions were carried out in a Smart-Cycler PCR system (Cepheid, Mountain View, CA), and Sybr green fluorescence was monitored in real time. After initial denaturation at 95°C for 5 min, the reactions underwent 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The final step involved a 60°C-to-95°C temperature ramp at a rate of 0.2°C/s to generate the melting curve. The product sizes and melting temperatures are shown in Table 2. During development of the assay, amplicon size was confirmed by agarose gel electrophoresis (data not shown).

RESULTS

Elegant comparative genomic analyses previously demonstrated that the evolution of the tubercle bacillus involved characteristic and precise genomic deletions, thereby providing a molecular signature that conclusively identifies isolates of the MTC to the species level (5, 23) (Fig. 1). To apply these findings to the setting of the clinical microbiology laboratory, we designed a series of primers for use in a multiplex, real-time assay for RD analysis. The primer targets included RD9, present in all isolates of *M. tuberculosis*, and RD1, absent in all isolates of BCG (Fig. 1). In addition, we targeted RD4, a region not found in *M. bovis* but present in complex members associated with other ecotypes: *M. africanum*, *M. caprae*, *M. pinnipedii*, and *M. microti* (32).

Primer sets designed to identify the presence of RD9, the absence of RD1, and both the presence and absence of RD4 (Table 2) were tested individually against DNA extracts from *M. tuberculosis* H37Rv and *M. bovis* BCG Pasteur. Amplification was monitored by observing the increase in Sybr green fluorescence, and melting-curve analysis identified the expected amplicons (data not shown). As anticipated, we confirmed the known RD patterns for each reference strain.

We next combined primer sets into two reaction mixtures to minimize the number of reactions needed to identify MTC species (Fig. 2A). Reaction 1 contained primer sets to detect the presence of RD9 and the absence of RD1, thus allowing the identification of both *M. tuberculosis* and *M. bovis* BCG, respectively. To this reaction we also added primers to a region of the 16S rRNA gene that is conserved in all mycobacteria. If nucleic acid from any mycobacterial species is present, this primer set should amplify a specific product, thus controlling for extraction efficiency and reaction inhibitors. Critically, if the specimen is neither *M. tuberculosis* nor *M. bovis* BCG, this genus control primer set distinguishes between a failed reaction and an alternative MTC isolate. Reaction 2 contained primer sets to detect the presence or absence of RD4. This reaction allowed us to distinguish *M. bovis* from other MTC members that also have the same RD9 (absent) and RD1 (present) patterns.

Reactions 1 and 2 were then validated against the well-characterized MTC reference strains: *M. tuberculosis* H37Rv, *M. africanum* ATCC 25420, *M. bovis* Ravenel, and *M. bovis*

TABLE 1. Bacterial strains used in this study

Organism	ATCC no.
<i>M. abscessus</i>	19977
<i>M. africanum</i>	25420
<i>M. avium</i> subsp. <i>avium</i>	25291
<i>M. bovis</i> Ravenel	35720
<i>M. bovis</i> BCG Pasteur	35734
<i>M. chelonae</i>	35752
<i>M. fortuitum</i> subsp. <i>fortuitum</i>	06841
<i>M. intracellulare</i>	13950
<i>M. kansasii</i>	12478
<i>M. parafortuitum</i>	19686
<i>M. phlei</i>	10142
<i>M. scrofulaceum</i>	19981
<i>M. tuberculosis</i> H37Rv	27294
<i>M. xenopi</i>	19250

TABLE 2. Primers used in this study

Description of target	Product length (bp)	Product <i>T_m</i> (°C) ^a	Primer names	Primer sequences
RD9 present	51	76.3 ± 0.1	RD9 Present Forward	TTTCGAGCCGTAAATTACTGTG
			RD9 Present Reverse	GAGCATTCTCGCTCCGAAT
RD1 deleted	226	86.2 ± 0.6	RD1 Deleted Forward	GGATTTGACGTCGTGCTTCT
			RD1 Deleted Reverse	TTCAACGGGTTACTGCGAAT
RD4 present	55	77.8 ± 0.2	RD4 Common Forward	AGAAGCGCAACACTCTTGGA
			RD4 Present Reverse	CATGCGCCCTATTGATCTC
RD4 deleted	94	83.0 ± 0.2	RD4 Common Forward	AGAAGCGCAACACTCTTGGA
			RD4 Deleted Reverse	TTGCTGAAAAATGGCTATTGA
Mycobacterial 16S rRNA	78	79.3 ± 0.1	Genus Control Forward	CAACGCGAAGAACCTTACCT
			Genus Control Reverse	TGCACACAGGCCACAAGGGA

^a The product melting temperatures (*T_m*) show the mean ± the 95% confidence interval.

BCG Pasteur (Fig. 2B to F). Figure 2B shows a characteristic melting curve in reaction 1 for the identification of *M. tuberculosis* using template DNA from H37Rv. A peak at 76.4°C indicates that RD9 is present, and a peak at 79.4°C confirms the mycobacterial genus. Because RD1 is present in *M. tuberculosis*, no peak is apparent at ~86°C, the melting temperature for the RD1 deletion amplicon. In contrast, Fig. 2C shows a representative melting curve in reaction 1 for the identification of *M. bovis* BCG using template DNA from BCG Pasteur. A specific peak at 86.5°C demonstrates the deletion of RD1, along with the expected peak at 79.0°C for the genus control. Given that RD9 is deleted in *M. bovis* BCG, there is no corresponding RD9 peak at ~76°C. Figure 2D shows the peak in reaction 1 when performed on template DNA from *M. africanum* or *M. bovis* Ravenel. Only the genus-specific peak is present, since both of these strains lack RD9 and contain RD1. Figures 2E and F show characteristic melting curves in reaction 2 when it was performed on template DNA from *M. africanum* and *M. bovis* Ravenel, respectively. Whereas *M. africanum* showed a specific peak at 77.5°C, indicating the presence of RD4 (Fig. 2E), *M. bovis* lacks this region and displayed a specific RD4-deleted peak at 83.2°C (Fig. 2F). These results demonstrate that the multiplex reactions 1 and 2 for analysis of RD9, RD1, and RD4 can differentiate *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG from other MTC species. We therefore propose a paradigm for the stepwise, two-reaction approach to the identification of members of the MTC (Fig. 2A).

To further assess the specificity of these primer sets, we ran both multiplex reactions (1 and 2) using DNA extracted from 10 other mycobacterial reference strains (see Table 1), including *M. kansasii*, an opportunistic mycobacterium that also contains an RD1-like region (2). While the genus control product was amplified from all isolates, no RD-specific amplicons were observed for any of these ATCC strains (data not shown). These findings indicate that these two multiplex reactions can also be utilized as primary tools for MTC and species identification from mycobacterial cultures.

To assess the clinical utility of this stepwise, two-reaction approach to the identification of members of the MTC and to monitor the performance of our novel multiplex, real-time assay, we analyzed 60 clinical isolates cultured at the Stanford Clinical Microbiology Lab that had previously been identified only to the MTC level. We found that 95% (57/60) of the Stanford isolates were indeed *M. tuberculosis*. Strikingly, we

identified the remaining 3 isolates as *M. bovis* BCG (5%; 3/60). Investigation into the clinical circumstances of specimen submission revealed that all three patients had histories of intravesical BCG treatment for bladder cancer and that all three BCG cultures were isolated from urine specimens submitted for acid-fast bacillus culture. While monoresistance to PZA is often used as a criterion for the identification of *M. bovis* and *M. bovis* BCG, we found that one of our BCG isolates showed resistance to both PZA and ethionamide, a second-line anti-tuberculosis agent. Conversely, two of the *M. tuberculosis* isolates demonstrated PZA monoresistance. Consistent with previous work, these results suggest that the pattern of antimicrobial resistance does not reliably distinguish between members of the MTC (12).

To further characterize the assay, we analyzed an additional 15 clinical isolates representing 15 distinct spoligotypes commonly associated with strains of *M. bovis*, as well as 5 clinical isolates of *M. africanum* (14). Whereas all 5 of the *M. africanum* isolates had the expected RD pattern, surprisingly, we found that our assay identified 13% (2/15) of these putative *M. bovis* isolates as MTC members other than classical *M. bovis*. Of the two discrepant specimens, one had the RD pattern of *M. tuberculosis* while the other lacked RD1 and was identified as *M. bovis* BCG. The *M. bovis* BCG isolate had been intentionally included in this sample set and had been previously identified by mycolic-acid high-performance liquid chromatography profiling (Grace Lin, personal communication). Interestingly, the initial misidentification of the *M. tuberculosis* strain was likely due to the presence of a spoligotype (477777770000600 [octal code]) that is only rarely associated with *M. tuberculosis* (Grace Lin, personal communication) and is not found in the 4th International Spoligotyping Database (6). These results highlight the limitations and complexity of the use of spoligotyping to distinguish between members of the MTC and suggest that our multiplex, real-time assay may provide a simple alternative that is superior to the currently available techniques.

DISCUSSION

Though mycobacteria belonging to the MTC are commonly isolated from clinical specimens, these isolates are not routinely identified to the species level. Because human infections with members of this complex require distinct treatment regi-

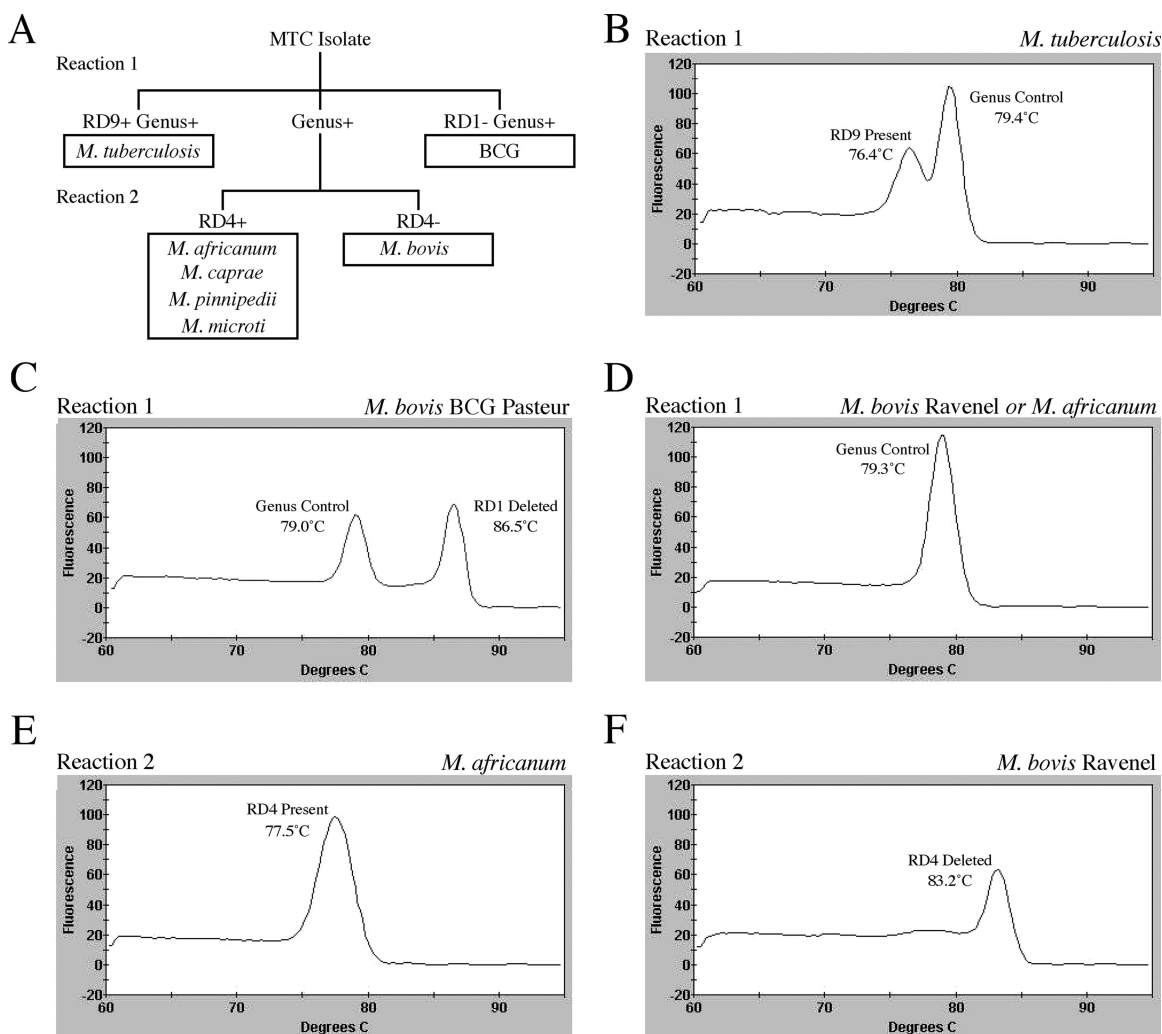


FIG. 2. RD analysis using multiplex, real-time PCR and melting-curve assessment allows the identification of members of the MTC. (A) The stepwise, two-reaction paradigm for MTC species identification. Reaction 1 contains primers that detect the presence of RD9 and the absence of RD1 to identify *M. tuberculosis* and *M. bovis* BCG, respectively, as well as genus control primers that amplify a conserved region of the 16S rRNA gene. Reaction 2 contains primers that detect the presence or absence of RD4 to distinguish *M. bovis* from *M. africanum* and other members with the RD9-absent, RD1-present pattern. (B, C, and D) Representative reaction 1 melting curve for *M. tuberculosis* (B), *M. bovis* BCG (C), *M. bovis* Ravenel, or *M. africanum* (D). (E and F) Representative reaction 2 melting curve for *M. africanum* (E) or *M. bovis* Ravenel (F). For each melting curve, the x axis shows the temperature in degrees C and the y axis is labeled with arbitrary fluorescence units depicting the negative change in fluorescence over the change in time.

mens and public health control measures, it is important that assays capable of distinguishing between these mycobacteria be readily accessible for routine use in the clinical mycobacterial laboratory. We describe here such an assay: a rapid and simple, multiplex, real-time PCR assay that allows the identification of *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG.

This two-step assay is based on the detection of stable genomic deletions that have accumulated over the evolution of these closely related mycobacteria (5, 23). The presence or absence of these RD provides a molecular signature that clearly distinguishes among members of the complex. Our analysis of a panel of well-characterized reference mycobacterial strains, including members of the MTC as well as a variety of pathogenic nontuberculous mycobacteria, suggests that the assay is both sensitive and specific for *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG. Future work with additional mycobacterial

strains and direct comparison with reference methods of MTC identification will be needed to confirm these promising performance characteristics.

When we applied this assay to 60 clinical specimens previously identified to the complex level, we found three cultures positive for *M. bovis* BCG in isolates from patients with histories of intravesical BCG treatment for transitional cell carcinoma of the bladder. In patients receiving BCG, it is important to distinguish treatment-related complications from *M. tuberculosis*, either primary infection or reactivation of latent disease, because of the intrinsic resistance of *M. bovis* BCG to PZA (17). Though there are no prospective trials evaluating the optimal treatment regimen for infection with *M. bovis* and *M. bovis* BCG, the American Thoracic Society/Centers for Disease Control and Prevention/Infectious Disease Society of America guidelines indicate that patients with PZA-monore-

sistant isolates should receive a 2-month course of three-drug therapy with isoniazid, rifampin, and ethambutol, followed by 7 months of isoniazid and rifampin (4). Given that patients newly diagnosed with tuberculosis are initially placed on a four-drug regimen including PZA, definitive species identification of *M. bovis* or *M. bovis* BCG using our assay will facilitate the rapid discontinuation of this drug, thus reducing adverse medication effects and preventing the emergence of drug resistance.

Though PCR-based genotyping of the MTC targeting genomic deletions has been proposed previously (3, 8, 19, 22, 25, 27, 35, 36), this assay is the first to combine RD analysis with both real-time detection and a multiplexed primer approach. This minimizes the number of reactions, reduces turnaround time, and eliminates the need for postamplification specimen handling. In addition, the use of multiplexing and melting-curve analysis provides cost savings compared to single-tube conventional methods of PCR product detection. We estimate the reagent cost at \$1 per assay. To date, the assay has been examined only for its ability to identify MTC species from positive culture. However, future studies will characterize its direct application to primary patient specimens to further decrease the time to diagnosis.

One competing methodology for MTC species identification is the GenoType MTBC DNA strip assay (29, 30). This assay requires an initial PCR amplification step followed by hybridization of the amplified products to a strip containing immobilized nucleic acid probes. Though recent work suggests that the GenoType MTBC is as accurate as traditional PCR-based deletion analysis (33), the multiple steps required for this protocol constitute a distinct disadvantage compared with our 80-min assay that combines amplification and detection in a single tube. Each additional step allows the introduction of error and adds to the overall turnaround time. It is estimated that the GenoType MTBC amplification step alone takes 3 h, suggesting that the entire procedure requires significantly more time than our assay (24). While this test is not currently available in the United States, it is an option for MTC identification in other parts of the world.

Another approach used primarily in public health reference laboratories to distinguish between members of the MTC is spoligotyping. This technique involves molecular deletion analysis of the direct repeat locus, a region containing a series of well-conserved repeats interspersed with nonrepetitive spacer sequences. For example, *M. bovis* strains typically lack spacers 39 to 43, whereas these sequences are usually present in *M. tuberculosis* isolates (20). To further challenge our assay, we investigated a collection of putative *M. bovis* strains from the California Tuberculosis Control Branch that shared this common absence of spacers 39 to 43 but otherwise had distinct spoligotypes. Strikingly, our analysis identified one isolate that was clearly *M. tuberculosis* based on the presence of RD9. This isolate had an unusual spoligotype (477777770000600 [octal code]) not described in the 4th International Spoligotyping Database (6). Given that the loss of RD are thought to be irreversible genetic events, it is very unlikely that this is an *M. bovis* strain that has reacquired regions normally found in *M. tuberculosis*. However, the series of direct-repeat deletions that lead to this spoligotype is not clear, since this pattern cannot be easily explained by a single deletion event from *M. tuberculosis*

isolates with known spoligotypes. It should be noted that new spoligotypes are continuously being identified and that 7% of known spoligotypes remain unclassified (6). Taken together, these observations suggest that while spoligotyping may be well suited for MTC epidemiological investigations, the stability of the targets and the simplicity of our assay make our approach to MTC species identification more appropriate for routine diagnosis in the clinical laboratory.

At present, the worldwide incidence of tuberculous disease due to *M. bovis* and other non-*M. tuberculosis* members of the MTC is not known. As species identification is integrated into the normal evaluation of mycobacterial isolates, it is likely that assays such as the one described here will reveal the true spectrum of disease caused by these MTC members.

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