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Rapid Differentiation of *Mycobacterium tuberculosis* and *M. bovis* by High-Resolution Melt Curve Analysis▽

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Identification and characterization of the *Mycobacterium tuberculosis* strains are important for clinical and therapeutic management of tuberculosis. Real-time PCR with a high-resolution melt assay was found to improve the diagnostic process. The assay includes differentiation between *M. tuberculosis* and *Mycobacterium bovis* based on one single-nucleotide polymorphism (SNP) in the *narGHJI* and *oxyR* genes and determination of *M. bovis* based on the region of differences 1 (RD1). This assay correctly identified the 7 tested *Mycobacterium* reference strains and 52 clinical samples with a sensitivity of 2 pg DNA. This assay will help in prescribing adequate treatment and monitoring disease dynamics.

The *Mycobacterium tuberculosis* complex (MTC) includes *Mycobacterium tuberculosis* and *Mycobacterium africanum*, both of which are considered human pathogens, and *Mycobacterium microti* and *Mycobacterium bovis*, which are usually associated with animal infections. Although *M. tuberculosis* is the main cause of human tuberculosis (TB), it has been estimated that *M. bovis* is responsible for 10 to 15% of new human TB cases in the developing countries (7). The attenuated tuberculosis vaccine strain *M. bovis* bacillus Calmette-Guérin (BCG) can also cause human TB, especially in patients diagnosed with cellular immunodeficiency (15) or among neonates and children in regions of endemicity who have been vaccinated (5, 7). In 2003, a Palestinian study described an outbreak of BCG complications in the Gaza strip of the Palestinian territories. It affected 225 infants (average age, 4 months), with a complication rate of 36.61 per 1,000 vaccinations (2). Thus, differentiation and identification of *M. tuberculosis* from other members of the MTC should improve the clinical and therapeutic management of TB. Moreover, differentiation of *Mycobacterium* species contributes to the understanding of TB epidemiology. Several molecular methods have been reported for genotyping these two pathogens, i.e., multiplex PCR, PCR restriction analysis, allele-specific PCR, and real-time PCR using fluorescent resonance energy transfer (FRET) probes (3, 8, 10, 11, 13). However, these methods are time-consuming, expensive, and complicated. Comparative genome analysis has shown that *M. bovis* has numerous single-nucleotide polymorphisms (SNPs) in comparison to *M. tuberculosis* (4). Of these SNPs, the C-to-T transition at position −215 upstream of the GTG start codon in the promoter region of the *narGHJI* genes was hypothesized to be responsible for the differing nitrate reductase activities between *M. tuberculosis* and *M. bovis* (14). Another polymorphic nucleotide was identified in the *oxyR* gene (11, 12) and was found to be specific for *M. bovis*. In this study, we describe the use of high-resolution melt curve analysis (HRM) to differentiate between *M. bovis* and *M. tuberculosis* by two stepwise reactions. The first reaction is based on the T-to-C transition at position −215 in the promoter region of the *narGHJI* genes which differentiates between *M. tuberculosis* and all other members of the MTC (*M. africanum*, *M. microti*, and *M. bovis*) (11). The second reaction is based on the A-to-G polymorphism in the *oxyR* gene which is specific to *M. bovis* and which therefore differentiates between *M. bovis* and *M. africanum* or *M. microti*. Such definitive differentiation is essential, even if the organisms are confined to different restricted geographical areas and to different host species. In addition, all *M. bovis* strains were further identified by targeting the region of differences 1 (RD1; a 9,650-bp deletion which appears as a specific marker for *M. bovis* BCG) using the HRM assay. The present study was aimed at developing a rapid assay for differentiation between *M. bovis* and *M. tuberculosis* with minimal requirements of cost and time.

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The study included 52 samples: 7 were previously identified as *M. tuberculosis* (6) and kindly provided by the Austrian Agency for Health and Food Safety, Vienna, Austria; 15 clinical isolates were recovered from sputum samples at the central laboratories of the Palestinian Ministry of Health; and 30 DNA samples were extracted from Ziehl-Neelsen-stained sputum smears (during the years 2005 to 2009). All archival sam-

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TABLE 1. Primers used in this study

PCR	Reaction	Gene name	Primer	Primer sequence	Product size (bp)	Reference	Product (T_m [°C])[a]
Preliminary	1	*narGHJI*	LC66	5′AACCGACGGTGTGGTTGAC′3	155	Stermann et al. (13)	
			LC67	5′ATCTCGATGGATGGGCGTC′3			
	2	*oxyR*	LC90	5′CGGGTGCCGCTGACCGCG′3	200	Stermann et al. (13)	
			LC91	5′CCAGCCGGCTTCGCGTGG′3			
Real-time	1	*narGHJI*	narF	5′CGCCGTCAACTTGGTTAGA′3			*M. tuberculosis* (84.26 ± 0.09)
			narR	5′GTCCTGCCCGGAAGTTGT′3	108		*M. bovis* (85.04 ± 0.02)
	2	*oxyR*	oxyF	5′ACACTGATTCCGCAGACC′3			*M. bovis* (91.9 ± 0.03)
			oxyR	5′AAAGTCAGCTCTGACAGCGC′3	151		*M. bovis* (91.27 ± 0.04)

[a] The product melting temperatures (T_m) are given as means ± standard errors.

ples and isolates had been previously identified by IS*6110*-based PCR to the complex level. Purified DNA from the reference strains *M. tuberculosis* (H37Rv), *M. bovis*, and *M. bovis* BCG and from nontuberculosis mycobacteria (NTM) (*M. phlei*, *M. avium*, *M. intracellulare*, and *M. kansasii*) were generously provided by the Hebrew University-Hadassah Medical School, Jerusalem, Israel.

Ten samples from TB-negative sputum smears (confirmed negative by IS*6110*- based PCR) were included as negative controls in the study.

The Ziehl-Neelsen-stained material was scraped off the microscopic slides after the addition of 200 μl of tissue lysis buffer and then processed by proteinase K digestion, followed by extraction according to manufacturer's instructions (High-Pure PCR template preparation; High-Pure, Mannheim, Germany).

A two-reaction approach for differentiating between *M. bovis* and *M. tuberculosis* was carried out using two real-time PCR assays followed by HRM analysis. Assay 1 consisted of the identification of the *Mycobacterium* species based on one SNP, a T-to-C transition, within the *narGHJI* promoter. Assay 2 consisted of the identification of the *Mycobacterium* species based on one SNP, an A-to-G transition, of the *oxyR* gene. The primers used in the two assays were designed to amplify short fragments covering these transitions in both targeted genes. Primer selection was facilitated by Primer3 (http://frodo.wi.mit.edu/). A homology search in GenBank revealed 100% specificity of the primers used for the MTC strains. The primer sequences are shown in Table 1.

Each reaction mixture contained 10 μl of 2× Thermo-Start PCR master mix (Thermo Scientific) and 1.5 μM SYTO 9 (Invitrogen); primer mixes were used in final concentrations of 250 μM. DNA from the clinical isolates and controls was added in 2-μl volumes in total reaction mixtures of 20 μl. The amplification reaction for both amplicons (108 and 151 bp) was done as follows: the temperature was held at 95°C for 15 min for the hot start reaction and then 40 cycles of 5 s of denaturation, 10 s of annealing, and an extension at 55°C with a temperature increase of 0.2°C done were for each step. The melt domain was between 75 and 95°C. Reactions were carried out using the Rotor-Gene 6000 real-time thermal analyzer (Corbett Life Science).

To obtain sufficient DNA for HRM analysis using 14 DNA extracts from Ziehl-Neelsen-stained sputum smears with a low bacterial load, fragments of 200 and 155 bp from the targeted genes were amplified using the previously published primers (13) (Table 1) and then 0.5-μl volumes from the PCR products were subjected to the real-time PCR HRM analysis as described above. In all amplification reactions, we used the negative-control samples to rule out the possibility of PCR contamination. Moreover, each PCR assay included DNA from the reference strains *M. tuberculosis* (H37Rv), *M. bovis*, and *M. bovis* BCG as positive controls to validate the characterization of the tested samples. In addition to the reference strains, seven clinical isolates identified as *M. tuberculosis* were added to the PCR assays for confirmation. All reactions were performed in duplicate.

The first step was to optimize the assay, which was carried out on the seven reference samples. The results of the real-time PCR HRM assay based on the *narGHJI* and *oxyR* SNPs distinguished between *M. tuberculosis* isolates and *M. bovis*, as shown in the normalized melt curves (Fig. 1A and B). The melting temperatures of the PCR product for *M. tuberculosis* were significantly different from those for *M. bovis* ($P < 0.05$) (Table 1). Confirmation of the amplicon size was carried out by agarose gel electrophoresis (data not shown).

The analytical sensitivity of the HRM assay was clarified by performing 10-fold dilutions using 2 ng of purified DNA for both strains (*M. bovis* and *M. tuberculosis*). The melting curve was shown as the DNA level reached 2 pg/reaction. The two reactions showed similar sensitivities. HRM specificity was demonstrated by the absence of melting curves with NTM DNA samples in both reactions.

The second step was to use the optimized assay on the 15 clinical isolates. Fourteen isolates were identified as *M. tuberculosis* and one isolate as *M. bovis* using the *narGHJI* and *oxyR* SNP assays. The *M. bovis* isolate was further identified as *M. bovis* BCG by targeting the RD1 region (13) and applying the HRM assay (Fig. 1C). This isolate was obtained from a 2-year-old female child. The results for the 30 DNA samples extracted from Ziehl-Neelsen-stained sputum smears with unknown *Mycobacterium* strain identification indicated that 29 samples were *M. tuberculosis* (their normalized melting curve were identical to those of control strains) and one sample (3%) was *M. bovis*, which was also obtained from a 3-year-old female child. It is important to note that among the *M. tuberculosis* strains identified, one was obtained from a nonlocal resident originating from Indonesia, where TB is endemic (imported case), and who was

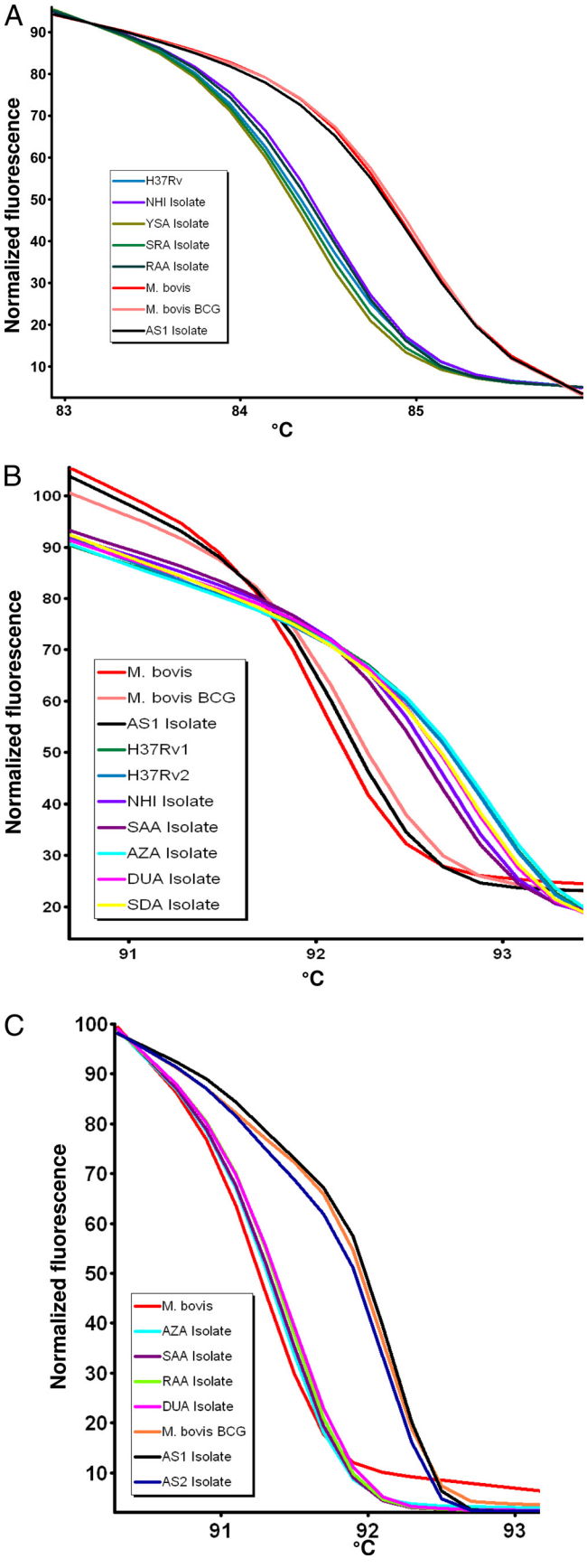

FIG. 1. Characterization of *M. tuberculosis* and *M. bovis* based on real-time PCR with high-resolution melt curve analysis identified *M. tuberculosis* based on the T-to-C transition in the *narGHJI* genes (A), *M. bovis* based on the A-to-G transition in the *oxyR* gene (B), and *M. bovis* BCG based on the RD1 region (C). For each HRM graph, the *x* axis shows the temperature in degrees (°C) and the *y* axis represents the fluorescence signals.

undergoing treatment at the time of sampling (smear positive and culture negative).

The real-time PCR HRM results were confirmed by direct sequencing. The two *M. bovis* sequences obtained in this study were found to be identical to each other and to the published sequences of *M. bovis* and *M. bovis* BCG (GenBank accession no. BX248342.1 and AP010918.1). The sequences of all samples positive for *M. tuberculosis* (isolates and reference strains) were also found to be 100% identical to each other and to the published *M. tuberculosis* sequence (GenBank accession no. BX842579.1). Representative DNA sequences (*oxyR* gene and *narGHJI* promoter) from patients were deposited in GenBank. The multiple alignment of these DNA sequences is shown in (Fig. 2A and B).

The identification of *M. bovis* among the clinical cases studied is very important, especially for health management. Given that all *M. bovis* strains are naturally resistant to pyrazinamide (PZA), a first-line antituberculosis agent (1, 7, 9), and that all patients newly diagnosed with TB are placed on a four-drug regimen that includes PZA, definitive differentiation between *M. tuberculosis* and *M. bovis* allows for appropriate treatment and reduces the emergence of drug-resistant strains.

The results of this study support the use of a real-time PCR HRM assay on DNA extracted directly from positive Ziehl-Neelsen-stained sputum smear slides. The enrichment of the DNA by PCR prior to the real-time PCR HRM assay was found to be crucial for samples with a low bacterial load. Establishment of this method is of great interest, especially in patients with negative cultures or when the patient is on antitubercular treatment. Determination of the exact strain of *Mycobacterium* prior to confirmation by culture, which can take up to 3 weeks or more, will improve the treatment by applying the most appropriate drugs. In addition, the possibility of cross-reactivity of the designed primers with other NTM strains was excluded, as none of the tested NTM strains showed any melting curve. The real-time PCR HRM assay developed in this study is less complicated than the assay developed by Stermann and colleagues (13) as it does not require flourescence-labeled probes or combined primers and probes. The simplicity of the assay and its low cost make it applicable for diagnostic laboratories. An advantage of performing HRM analysis on a real-time PCR machine with HRM capability is that the PCR amplification and HRM analysis are performed in the same run and the results are available for assessment of amplification of all samples earlier than if HRM analysis done solely as a quality control measure. Therefore, we recommend this method for the rapid differentiation of *M. tuberculosis* from *M. bovis.* This differentiation will aid in choosing an efficient and appro-

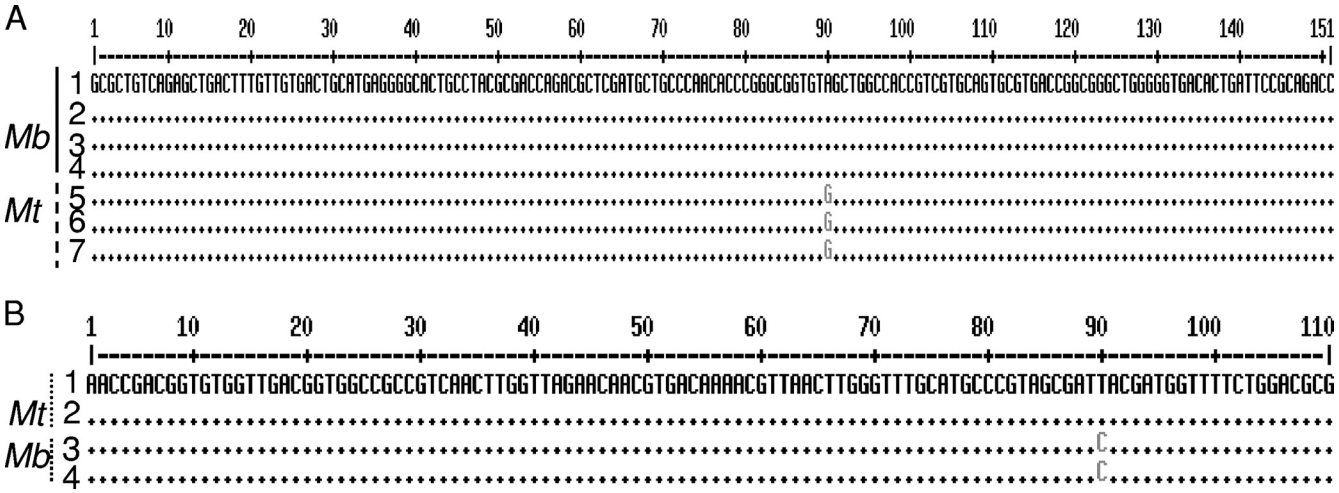

FIG. 2. (A) Multiple alignment of DNA sequences for the *oxyR* gene. 1 to 3, sequences belonging to *M. bovis* (*Mb*); 4 to 6, sequences belonging to *M. tuberculosis* (*Mt*). 1, *M. bovis*; 2, *M. bovis* BCG; 3, CaseTBA isolate; 4, CaseASA isolate (accession no. HM135443); 5, *M. tuberculosis* (H37Rv); 6, CaseSAA isolate (accession no. HM135444); 7, CaseNHI isolate (accession no. HM135445). (B) Multiple alignment of DNA sequences for the *narGHJI* genes. 1 and 2, sequences belonging to *M. tuberculosis* (*Mt*); 3 and 4, sequences belonging to *M. bovis* (*Mb*). 1, *M. tuberculosis* H37Rv; 2: CaseSAA isolate; 3, CaseASA isolate (accession no. HM135442); 4, *M. bovis* BCG.

priate treatment for TB patients, will reduce the transmission of the disease, and may prevent further outbreaks.

Nucleotide sequence accession numbers. Sequences identified in the present study have been deposited in GenBank under accession numbers HM135442, HM135443, HM135444, and HM135445.

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ERRATUM

Rapid Differentiation of *Mycobacterium tuberculosis* and *M. bovis* by High-Resolution Melt Curve Analysis

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Vol. 48, no. 11, p. 4269-4272, 2010. Page 4270, Table 1, column 8, line 7: "*M. bovis* (91.9 ±0.03)" should read "*M. tuberculosis* (91.9 ±0.03)."