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 is important for clinical and therapeutic management of tuberculosis. Real time PCR with high-resolution melt assay were found to improve the diagnostic process. The assay includes differentiation between *M. tuberculosis* and *M. bovis* based on one SNP in the *narGHJI* and *oxyR* genes, and determination of *M. bovis* based on the *RD1* region. This assay correctly identified the tested seven *Mycobacterium* reference strains and fifty two clinical samples with a sensitivity of 2 pg DNA. This will help in prescribing adequate treatment and monitoring disease dynamics.
*Mycobacterium tuberculosis* complex (MTC) includes *M. tuberculosis, M. africanum* both of which are considered human pathogens while *M. microti* and *M. bovis* usually are associated with animal infections. Though *M. tuberculosis* (MTB) is the main cause of human tuberculosis (TB), it has been estimated that *M. bovis* responsible for 10 to 15% of new human TB in the developing countries (1). The attenuated tuberculosis vaccine strain, Bacilli Calotte Guerin (BCG), can also cause human TB especially in cases diagnosed with cellular immunodeficiency (2) or among neonates and children in endemic regions that were vaccinated (1, 3). 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 was 4 months) with a complication rate of 36.61 per 1000 vaccinations (4). Thus, differentiation and identification of the MTB from other members of the MTC should improve the clinical and the therapeutic management of TB. Moreover, it contributes to the understanding of the TB epidemiology. Several molecular methods have been reported for genotyping these two pathogens, e.g., Multiplex PCR, PCR restriction analysis, alleles specific PCR and real-time PCR using FRET probes (5, 6, 7, 8, 9). However, these methods are time consuming, expensive and complicated. Comparative genome analysis has shown that *M. bovis* have numerous single nucleotide polymorphisms (SNPs) compared to MTB (10). Of these SNPs, the C/T transition at position -215 upstream of the GTG start codon in the promotor region of the *narGHJI* gene was hypothesized to be responsible for the differential Nitrate Reductase activity between MTB and *M. bovis* (11). Another polymorphic nucleotide was identified in the *oxyR* gene (6, 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/C transition at position -215 in the promoter region of the *narGHJI* gene which differentiates between MTB and all other members of the MTC (*M. africanum, M. microti* and *M. bovis*) (6). The second reaction is based on the A/G polymorphism in the *oxyR* gene which is
specific to *M. bovis* and therefore, differentiates between *M. bovis* and *M. africanum* or *M. microti*. Such definitive differentiation is essential, even if they are confined to different restricted geographical areas and to different host species. In addition, all *M. bovis* strains were further identified 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.

The study included 52 samples: seven were previously identified as *M. tuberculosis* (13) and kindly provided from the Austrian Agency for Health and food safety, Vienna, Austria, fifteen clinical isolates recovered from sputum samples at the central laboratories of the Palestinian Ministry of Health and 30 DNA samples extracted from Ziehl-Neelsen stained sputum smears (during the years 2005-2009). All archival samples and isolates had previously identified by IS6110-based PCR to the complex level. Purified DNA from the reference strains *M. tuberculosis* (H37Rv), *M. bovis*, *M. bovis* BCG and from non-tuberculosis mycobacteria (NTM) (*M. phlei*, *M. avium*, *M. intracellulare* and *M. kansasii*) were generously provided from The Hebrew University-Hadassah Medical School, Jerusalem, Israel.

Ten samples from TB negative sputum smears (confirmed negatives by IS6110- based PCR) were included as negative controls in the study.

The Ziehl-Neelsen stained material was scraped off from the microscopic slides after addition of 200 µl of tissue lysis buffer, and then processed by proteinase k digestion, followed by extraction according to manufactures instruction (High pure PCR template preparation, Mannheim, Germany).

A two reactions approach was used to differentiate between *M. bovis* and MTB was carried out using two real-time PCR assays followed by HRM analysis:
Assay 1: identification of *Mycobacterium* based on one SNP, T/C transition, within the *narGHJI* promoter. Assay 2: identification of *Mycobacterium* based on one SNP, A/G transition, of the *oxyR* gene. The primers used in both assays were designed to amplify short fragments covering these transitions in the both targeted genes. Primer selection was facilitated by primer3 (http://frodo.wi.mit.edu/). A homology search in the GenBank revealed 100% specificity of the used primers for MTC strains. The primers sequences are shown in Table 1.

Each reaction mixture contained: 10 µl of 2X Thermo-start PCR Master Mix (Thermo scientific), 1.5 µM SYTO 9 (Invitrogen), primers mixes were used as 250 µM final concentrations. DNA from the clinical isolates and controls were added in 2 µl volume in total reaction of 20 µl. Amplification reaction for both amplicons (108 and 151 bp) was done as followed: hold at 95 °C for 15 min for hot start reaction, then 40 cycles of 5 s denaturation, 10 s annealing and extension at 55 °C with temperature rising of 0.2 °C was done for each step. The melt domain was between 75- 95 °C. Reactions were carried out using Rotor-Gene 6000 Real-Time Thermal Analyzer (Corbett Life Science).

To obtain a sufficient DNA for HRM analysis using fourteen DNA extracts from Ziehl-Neelsen stained sputum smears with low bacterial load, a fragment of 200 and 155 bp from the targeted genes were amplified using the previously published primers (9) (Table 1), then 0.5 µl from 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 of 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 also added to the PCR assays for confirmation. All reactions were performed in duplicate.
The first attempt 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 SNP’s 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 MTB were significantly different from those of the *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 10 fold dilutions using 2 ng of purified DNA for both strains (*M. bovis* and *M. tuberculosis*). The melting curve was shown as DNA level reached 2 pg/reaction. Both reactions showed similar sensitivity. HRM specificity was demonstrated by the lack of the 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 assay. The *M. bovis* isolate was further identified as *M. bovis* BCG targeting the RD1 region (9) and applying the HRM assay (Fig. 1C). This isolate was obtained from 2 years old female child. The results of the 30 DNA samples extracted from Ziehl-Neelsen stained sputum smears with unknown *Mycobacterium* strain identification indicated that 29 samples were of *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 3 years old female child. It is important to note that among the *M. tuberculosis* identifications one was obtained from a non local resident originating from Indonesia, where TB is endemic (imported case) and being under treatment at 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 (BX248342.1; AP010918.1). The sequences of all positive samples for *M. tuberculosis* (isolates and reference strains) were also found to be 100%
identical to each other and to the published *M. tuberculosis* sequence (BX842579.1). Representative DNA sequences (*oxyR* gene and *narGHJI* promoter) from patients were deposited in the Genbank. The multiple alignment of these DNA sequences was 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 resistance to Pyrazinamide (PZA), a first-line anti tuberculosis agent (1, 14, 15) and all patients newly diagnosed with TB are placed on a four drug regimen including PZA, thus definitive differentiation between MTB and *M. bovis* allows for appropriate treatment and reducing the emergence of drug resistance strains.

The results of this study support the use of real-time PCR HRM assay on DNA extracted directly from positive Ziehl-Neelsen stained sputum smear slides. The enrichment of the DNA by PCR reaction 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 anti-tubercular treatment. Determination of the exact strain of *Mycobacterium* prior to confirmation by culture that can last up to 3 weeks or more will improve the treatment by applying the appropriate drugs. The cross reactivity of the designed primers with other NTM strains was also 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 as compared to the assay developed by Stermann and colleagues (9) as it does not require fluorescent-labeled probes or combined primers and probes. This simplicity of the assay and its low cost makes it applicable for diagnostic laboratories. An advantage of performing HRM analysis on a real time PCR machine with its 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 for all samples before HRM analysis in which be used as a quality control.
measure. Therefore, we recommend this method for rapid differentiation of MTB from *M. bovis*.

This differentiation will aid in choosing an efficient and appropriate treatment to the TB patients and will reduce the transmission of the disease and may prevent further outbreaks.

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REFERENCES


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<tr>
<th>Preliminary PCR</th>
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<th>Primer sequences</th>
<th>Product size (bp)</th>
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<tr>
<td>Reaction 1</td>
<td>narGHJI</td>
<td>LC66: 5'AACCGACGGTGTGGTTGAC'3</td>
<td>155</td>
<td>Stermann et al., 2003</td>
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<tr>
<td></td>
<td></td>
<td>LC67: 5'ATCTCGATGGATGGGCGTC'3</td>
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<td>Reaction 2</td>
<td>oxyR</td>
<td>LC90: 5'CGGGTGCGGCTGACCGCG'3</td>
<td>200</td>
<td>Stermann et al., 2003</td>
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<td></td>
<td></td>
<td>LC91: 5'CCAGCGGGCTTGCCG'3</td>
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**Real-time PCR**

<table>
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<td></td>
<td>narGHJI</td>
<td>narF: 5'CGCCGCTCAACCTGGTTGAGA'3</td>
<td>MTB: 84.26 ± 0.09</td>
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<td></td>
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<td>narR: 5'GTCTCGGCGGGAAGTTGGT'3</td>
<td>M. bovis: 85.04 ± 0.02</td>
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<tr>
<td>Reaction 2</td>
<td>oxyR</td>
<td>oxyF: 5'GACACTGATTCCGCAGACC'3</td>
<td>MTB: 91.9± 0.03</td>
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<tr>
<td></td>
<td></td>
<td>oxyR: 5'AAAGTCAGCTCTGACAGGC'3</td>
<td>M. bovis: 91.27± 0.04</td>
</tr>
</tbody>
</table>

**FIGURE LEGENDS**

**FIG. 1.** Characterization of *M. tuberculosis* and *M. bovis* based on real-time PCR with High - Resolution Melt curve analysis (A) Identified *M. tuberculosis* based on the T/C transition in the narGHJI gene; (B) Identified *M. bovis* based on the A/G transition in the oxyR gene; (C) Identification of *M. bovis* BCG based on RD1 region. For each HRM graph, the x axis shows the temperature in degrees (°C) and the y axis represents the fluorescence signals.

**FIG. 2.** Multiple alignment of DNA sequences for A: oxyR gene: 1-3 are belonged to *M. bovis* (Mb); 4-6 are belonged to *M. tuberculosis* (Mt). 1: *M. bovis*, 2: *M. bovis* BCG, 3: TBA Isolate (patient), 4: ASA Isolate (patient) (accession number HM135443), 5: *M. tuberculosis* (H37Rv), 6: SAA Isolate (patient) (accession number HM135445); B: narGHJI gene: 1-2 are belonged to Mt; 3-4 are belonged to Mb. 1: *M. tuberculosis* (H37Rv), 2: SAA Isolate(patient), 3: ASA Isolate (patient) (accession number HM135442), 4: *M. bovis* BCG.
FIG 1.A

Normalized fluorescence

°C