Identification of non-tuberculous mycobacteria by real-time PCR coupled with a high-resolution melting system

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Non-tuberculous mycobacteria (NTM) are increasingly important opportunistic pathogens responsible for a variety of clinical diseases. The aim of this study was to evaluate a novel technique, real-time PCR coupled with high-resolution melting analysis (real-time PCR-HRMA), for NTM identification. Two pairs of unique primers targeted to the 16S rRNA gene and the 16S–23S internal transcribed spacer region were selected for further evaluation. A total of 149 mycobacterial clinical isolates were subjected to analysis using the real-time PCR-HRMA system. Overall, 134 NTM identified by the 16S rRNA full-gene sequencing method were categorized into four major groups: Mycobacterium avium complex, Mycobacterium chelonae group, Mycobacterium gordonae and Mycobacterium fortuitum group. Of the 134 prevalent mycobacterial isolates, 101 mycobacteria (75.4 %) could be identified correctly by the real-time PCR-HRMA system. The individual sensitivities for the M. avium complex, M. chelonae group, M. gordonae and M. fortuitum groups were 90.9, 89.1, 100 and 36.8 %, respectively. The specificity of identifying these groups varied from 96.4 to 100 %. When identification failed, mostly it was attributable to various species in the M. fortuitum group. The real-time PCR-HRMA system is therefore a rapid and sensitive method for identifying prevalent NTM in a clinical laboratory.

INTRODUCTION

Mycobacteria, except for Mycobacterium tuberculosis complex and Mycobacterium leprae, are collectively referred to as non-tuberculous mycobacteria (NTM) (Gopinath & Singh, 2010; Tully et al., 2011). NTM are typical environmental organisms that can act as opportunistic pathogens in humans (Gopinath & Singh, 2010). It usually takes 6–8 weeks for primary culture and identification of slowly growing NTM on solid media (Lu et al., 2011). The introduction of a liquid-based system shortens the time for the detection of positive cultures. Previous studies have described the detection and identification of NTM by PCR-based methods (Leung et al., 2009; Li et al., 2009; Lin et al., 2011; Sun et al., 2009). Nevertheless, PCR-based methods, either multiplex PCR or PCR coupled with other techniques (e.g. sequencing, restriction enzyme digestion, microarray analysis and mass spectrometry) for rapid NTM identification are still time-consuming and require many cumbersome procedures to differentiate the NTM species (Foongladda et al., 2009; Li et al., 2009; Lim et al., 2008; Wang et al., 2010; Wu et al., 2008). There are several commercial systems using various PCR-based technologies for rapid identification of NTM but these are too expensive for most clinical laboratories (Lebrun et al., 2005; Padilla et al., 2004; Quezel-Guerraz et al., 2010; Said et al., 2011).

High-resolution melting analysis (HRMA) is a novel PCR-based method for detection of DNA sequence variation by demonstrating fluorescence changes in the melting process of the PCR amplicon with various saturation dyes such as LC Green, SYTO9 and Eva Green (Erali et al., 2008). Previous studies have combined HRMA with real-time
PCR assays for rapid detection and identification of clinically important bacteria (Ajitkumar et al., 2012; Chen et al., 2011; Cheng et al., 2006, Ricchi et al., 2011; Choi et al., 2010; Douarre et al., 2012; Pang et al., 2011; Pietzka et al., 2009; Tortoli, 2009; Won et al., 2010; Yang et al., 2009). The literature suggests that a combined real-time PCR-HRMA assay is a novel and rapid technique suitable for the genotyping of drug-resistant isolates of M. tuberculosis (Chen et al., 2011; Pietzka et al., 2009; Ramirez et al., 2010).

Accordingly, the objective of this study was to establish a combined real-time PCR-HRMA assay for common clinical NTM identification. We searched for and selected mycobacteria-specific primers from the literature and evaluated their discriminatory power among the common clinical NTMs. The performance of the combined real-time PCR-HRMA assay was compared with that of the 16S rRNA gene sequencing method.

**METHODS**

**Reference strains.** All reference strains used in this study were collected from the American Type Culture Collection (ATCC) and comprised: M. tuberculosis ATCC 27294, Mycobacterium fortuitum ATCC 6481, Mycobacterium kansasi ATCC 12478, Mycobacterium abscessus ATCC 19977, Mycobacterium avium ATCC 25291, Staphylococcus aureus ATCC 29213, Staphylococcus epidermidis ATCC 12228, Staphylococcus saprophyticus ATCC 15305, Streptococcus pneumoniae ATCC 49619, Streptococcus agalactiae ATCC 13813, Streptococcus pyogenes ATCC 19015, Klebsiella pneumoniae ATCC 13883, Klebsiella oxytoca ATCC 700324, Acinetobacter baumannii ATCC 15151, Acinetobacter lwoffii ATCC 17925, Pseudomonas aeruginosa ATCC 27853, Enterococcus faecalis ATCC 29212, Enterococcus faecium ATCC 19434, Enterococcus casseliflavus ATCC 700327, Enterococcus avium ATCC 14025, Enterobacter aerogenes ATCC 13048, Shigella sonne ATCC 25931, Stenotrophomonas maltophilia ATCC 15099, Salmonella arizonae ATCC 13314, Salmonella typhi ATCC 6539, Proteus mirabilis ATCC 10005, Burkholderia cepacia ATCC 25416, Enterobacter coli ATCC 25922 and Enterobacter cloacae ATCC 13047.

**Specimen processing and NTM identification.** All specimens were processed and decontaminated within 24 h after collection, as described previously (Sun et al., 2009). A mycobacteria growth indicator tube (MGIT) culture medium that yielded a positive culture signal using the MGIT 960 system (Becton Dickinson) was considered positive for mycobacteria as confirmed by acid-fast bacteria (AFB) staining. All of the AFB-positive MGIT culture broths in this study were identified as M. tuberculosis complex using an MGIT Tbc Identification Test (Tbc ID; Becton Dickinson), as described by the manufacturer. An aliquot of the positive MGIT culture was also inoculated onto Lowenstein–Jensen (LJ) slants (Becton Dickinson) for further identification. From October 2010 to May 2011, the AFB-positive MGIT culture broths and corresponding mycobacterial isolate were immediately stored at −80 °C for further studies. Total nucleic acids were extracted from MGIT culture broths for evaluation of real-time PCR-HRMA and from mycobacterial colonies on LJ medium for full-length 16S rRNA gene sequencing, respectively. The clinical NTM isolates were identified to the species level by DNA sequencing of the full-length 16S rRNA gene, which is consistent with previous studies (Hall et al., 2003; Lane et al., 1985; Reischl et al., 1994; Sun et al., 2009).

**Literature review and primer pair selection.** Primers targeted to the 16S rRNA gene, RNA polymerase gene (rpoB), hsp65 gene or 16–23S internal transcribed spacer (ITS) region have been reported in relation to detecting mycobacteria by PCR in previous publications. As the HRMA assay could differentiate gene fragments of up to 300 bp, four pairs of genus-specific primers flanking gene fragments of different mycobacteria were selected from five previous studies (Foongladda et al., 2009; Kim et al., 2010; Richardson et al., 2009; Roth et al., 2000; Shrestha et al., 2003). The primer sequences are listed in Table S1 (available in JMM Online). We used BioEdit software to confirm the conservativeness and specificity of these primers for mycobacteria. Two primer pairs, 16s_320bpF (5′-ATGCAAGTGCGAAGGGA-3′) and 16s_213bpR (5′-TGTGTCGGCTTGGTGATG-3′), and NTM ITS_F2 (5′-ACCTGCTTCTTACGAGGCC-3′) and NTM ITS_Myc2R (5′-ATGCGGGCAACACTATYCA-3′), were used for the real-time PCR-HRMA.

**Real-time PCR-HRMA.** The primer sequences used are listed in Table S1. Real-time PCR was performed with a DyNaMo Flash Probe qPCR kit (Finnzymes) and LC Green dye (Idaho Technology) on an ABI PRISM 7500 Real-Time PCR system (Applied Biosystems). Each PCR analysis contained one primer pair. Cycling conditions were as follows: denaturation at 95 °C for 10 min, followed by 50 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 30 s, with a final cycle at 95 °C for 30 s and 28 °C for 30 s. Each real-time PCR amplicon was subjected to HRMA on a LightScanner-96 instrument and analysed using LightScanner software version 2.0 (Idaho Technology). Analysis of data was performed using the Expert scanning module as described by the manufacturer. Derivative plots were generated to assess the number of melting peaks. With the melting profile of M. tuberculosis as reference, difference plots of the various NTM were generated. Difference plots of similar curve shape were clustered together by the ‘auto grouping’ module. A unique letter code was assigned arbitrarily to each cluster sequentially. Combination of the letter codes of the variable regions was then representative of the signature code of each organism.

**RESULTS**

**Specificity of the primers using real-time PCR-HRMA.** The analytical specificity of the selected primer pairs was tested with a panel of mycobacterial and non-mycobacterial strains. These genus-specific primers detected all mycobacterial strains and did not react with any non-mycobacterial strains. We used the DNA of several common NTM reference strains (M. fortuitum, M. kansasi, M. abscessus and M. avium) and M. tuberculosis reference strain H37Rv as a template to study the applicability of these primer pairs in the combined real-time PCR-HRMA system. The four pairs of primers for the real-time PCR were able to generate the anticipated amplicons for all mycobacterial strains. Melting curves of the amplicons of five different species were compared by visual inspection. Only two of the four primer pairs (16s_320bpF/16s_213bpR and NTM ITS_F2/NTM ITS_Myc2R) generated amplicons that showed significantly different melting profiles among the five species (data not shown). Using the melting curve of H37Rv as the reference, the difference plot for each tested organism was generated by subtracting the reference curve from its specific curve. The consensus of the difference plot for each organism was then verified further.

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Clinical application in AFB-positive MGIT culture broth

During the 8-month study period, we obtained 506 AFB-positive MGIT broths from 9066 clinical specimens (5.6%). We identified AFB-positive MGIT cultures using the TBc ID test and standard biochemical tests. *M. tuberculosis* and NTM were identified in 274 (54.2%) and 232 (45.8%) isolates, respectively. A total of 149 samples from 137 individual patients were collected and analysed by full-length sequencing of the 16S rRNA gene. The DNA extracted from AFB-positive MGIT broth was subjected separately to two real-time PCRs for the ITS region and the 16S rRNA gene. All DNA extracts were

Fig. 1. Difference plots of the real-time PCR-HRMA system for the ITS region and 16S rRNA gene, classified into seven (codes a–g) and five (codes a–e) types, respectively.
analysed twice using the real-time PCR-HRMA system. The melting curves of the isolates were demonstrated to be reproducible from run to run. Of the 149 isolates analysed, the difference plots of the real-time PCR-HRMA for the ITS region and the 16S rRNA gene were classified into seven (code a–g) and five (code a–e) types, respectively (Fig. 1). The combinations of letter codes for the ITS region and 16S rRNA genes were representative of unique signature codes. These signature codes for identifying the 149 NTM clinical isolates are summarized in Table 1. Of the 149 isolates, the HRMA system generated 18 different signature codes, including 13 clusters and five unique codes. The largest cluster was code b-a for 58 isolates (38.9 %, 58/149), followed by codes a-a, a-b and c-e for 22, 18 and ten isolates, respectively. In these four major clusters, 57 isolates (57/58, 98.3%) of code b-a were Mycobacterium chelonae group, 20 isolates (20/22, 90.9%) of code a-a were M. avium complex, 14 isolates (14/18, 77.8%) of code a-b were M. fortuitum group and ten isolates (10/10, 100%) of code c-e were Mycobacterium gordonae.

Table 1. Comparison of identification of 149 NTM isolates by real-time PCR-HRMA and 16S rRNA gene full-length sequencing

<table>
<thead>
<tr>
<th>ITS region</th>
<th>16S rRNA gene</th>
<th>Signature code (no. of isolates)</th>
<th>Group or species by 16S rRNA gene sequencing</th>
<th>No. (% of isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>a</td>
<td>a-a (22)</td>
<td>M. avium complex</td>
<td>20 (90.9)</td>
</tr>
<tr>
<td>a</td>
<td>b</td>
<td>a-b (18)</td>
<td>M. fortuitum group</td>
<td>2 (9.1)</td>
</tr>
<tr>
<td>a</td>
<td>c</td>
<td>a-c (1)</td>
<td>M. fortuitum group</td>
<td>2 (9.1)</td>
</tr>
<tr>
<td>a</td>
<td>e</td>
<td>a-e (3)</td>
<td>M. fortuitum group</td>
<td>2 (9.1)</td>
</tr>
<tr>
<td>b</td>
<td>a</td>
<td>b-a (58)</td>
<td>M. chelonae group</td>
<td>57 (98.3)</td>
</tr>
<tr>
<td>b</td>
<td>c</td>
<td>b-c (1)</td>
<td>M. avium complex</td>
<td>1 (1.7)</td>
</tr>
<tr>
<td>b</td>
<td>d</td>
<td>b-d (3)</td>
<td>M. avium complex</td>
<td>3 (100)</td>
</tr>
<tr>
<td>c</td>
<td>a</td>
<td>c-a (1)</td>
<td>M. avium complex</td>
<td>1 (100)</td>
</tr>
<tr>
<td>c</td>
<td>b</td>
<td>c-b (5)</td>
<td>M. avium complex</td>
<td>1 (100)</td>
</tr>
<tr>
<td>c</td>
<td>e</td>
<td>c-e (10)</td>
<td>M. fortuitum group</td>
<td>1 (100)</td>
</tr>
<tr>
<td>d</td>
<td>b</td>
<td>d-b (2)</td>
<td>M. fortuitum group</td>
<td>1 (100)</td>
</tr>
<tr>
<td>e</td>
<td>a</td>
<td>e-a (3)</td>
<td>M. fortuitum group</td>
<td>3 (100)</td>
</tr>
<tr>
<td>e</td>
<td>b</td>
<td>e-b (2)</td>
<td>M. fortuitum group</td>
<td>2 (100)</td>
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<tr>
<td>e</td>
<td>c</td>
<td>e-c (1)</td>
<td>M. fortuitum group</td>
<td>1 (100)</td>
</tr>
<tr>
<td>f</td>
<td>a</td>
<td>f-a (9)</td>
<td>M. fortuitum group</td>
<td>6 (66.7)</td>
</tr>
<tr>
<td>f</td>
<td>b</td>
<td>f-b (7)</td>
<td>M. fortuitum group</td>
<td>7 (100)</td>
</tr>
<tr>
<td>g</td>
<td>a</td>
<td>g-a (1)</td>
<td>M. fortuitum group</td>
<td>1 (100)</td>
</tr>
<tr>
<td>g</td>
<td>b</td>
<td>g-b (2)</td>
<td>M. fortuitum group</td>
<td>1 (100)</td>
</tr>
</tbody>
</table>

Sensitivity and specificity

The 149 clinical NTM isolates were identified in terms of species, group or complex level by full 16S rRNA gene sequencing as a standard method (Table 2). Overall, 134 of the 149 NTM (90.0 %) were successfully identified into four groups or species, comprising M. avium complex (22), M. chelonae group (64), M. gordonae (10) and M. fortuitum group (38). Of these 134 prevalent mycobacterial isolates, 101 mycobacteria (75.4 %) could be identified correctly by real-time PCR-HRMA. The individual sensitivities for M. avium complex (code a-a), M. chelonae group (code b-a), M. gordonae (code c-e) and M. fortuitum group (code a-b) were 90.9, 89.1, 100 and 36.8 %, respectively (Table 3). The individual specificities for M. avium complex (code a-a), M. chelonae group (code b-a), M. gordonae (code c-e) and M. fortuitum group (code a-b) were 98.4, 98.8, 100 and 96.4 %, respectively (Table 3). All of the isolates with misidentification at the species level by real-time PCR-HRMA were analysed further by sequencing of the PCR products of the 16S rRNA gene and ITS region. Nucleotide
polymorphisms were found in the sequences of these isolates comprising between one and four nucleotide differences.

**DISCUSSION**

The melting profile of a PCR product depends on its G+C content, length, sequence and heterozygosity. Melting of the PCR product is also influenced by various factors, such as salt in the buffer solution, DNA quality, DNA concentration and DNA extraction methods, along with other factors that can influence the behaviour of the
melting curves. A previous study suggested another two-step HRMA technique that alters the shape of the melting transition to increase the sensitivity and specificity by forming heteroduplexes (Hoek et al., 2008). This two-step technique may in fact cause cross-contamination and may be cumbersome. Another strategy is to analyse more than one target gene to get higher discriminatory power. As the culture medium may contain other bacteria, we selected Mycobacterium genus-specific primers to avoid the impact of the DNA from other bacteria. We analysed the melting profiles generated from two hypervariable regions in the 16S rRNA gene and ITS region of Mycobacterium species. The melting curve of M. tuberculosis was used as the reference curve, and the melting curves of clinical strains were compared with the reference curve. The difference plots were generated by subtracting these curves from the reference curve. Compared with the primary melting curve, the difference plot minimizes the effect of interfering factors and can easily be differentiated by visual inspection in a clinical laboratory (Won et al., 2010; Yang et al., 2009).

Previous studies have shown that HRMA is capable of distinguishing different sequence types with the same G+C content (Slinger et al., 2007) or discriminating single-nucleotide differences between closely related species (Yang et al., 2009). Despite its theoretically high discrimination power, we found that amplicons of different NTM species could generate similar melting curves (e.g. both M. kansasi and M. fortuitum had code a-b), whereas the closely related NTM species could generate different melting curves (e.g. between members of the M. fortuitum group) (Table 1). Similar findings have been reported in the literature (Yang et al., 2009).

These unsatisfactory observations may have resulted from: (i) multiple different species in the M. fortuitum group, as discriminated by 16S rRNA gene sequencing, (ii) nucleotide polymorphisms of the 16S rRNA gene and ITS region within the species (e.g. M. kansasi), and (iii) mutations in the ITS region that are not lethal to the organism itself.

M. cheloneae group, M. avium complex, M. fortuitum group and M. gordonae are the four most prevalent NTM species in Taiwan (Chen et al., 2011). In our study, 90.6% (87/96) of the isolates belonging to the prevalent NTM groups, except for the M. fortuitum group, were correctly identified by real-time PCR-HRMA. Of the 38 isolates of the M. fortuitum group, 13 isolates were correctly identified as M. fortuitum species, and 85% (11/13) of the M. fortuitum species could be correctly identified as the representative code a-b by the real-time PCR-HRMA. The sensitivity for identifying other species of the M. fortuitum group was relatively low because 22 isolates of the M. fortuitum group were found to be false negative for code a-b in the real-time PCR-HRMA.

There are several novel real-time PCR instruments capable of processing both thermal cycling and HRMA (Rotorgene 6000, LightScanner 32, AB 7300 and LightCycler 480) (Herrmann et al., 2007). The real-time PCR-HRMA system employed in these instruments is usually a closed-tube system, which not only reduces the risk of contamination but also increases throughput, as there is no requirement for physical separation of DNA molecules (Chen et al., 2011). Various HRMA results for the same PCR product may occur in different instruments (Herrmann et al., 2007). Because of the limited NTM species in our study, we were not able to demonstrate the complete patterns of difference plots for all NTM. Moreover, we noted that the discriminatory power of the primer pairs (for the 16S rRNA and ITS genes) may not be enough for NTM identification.

Misidentification of rapid and slow-growing mycobacteria is a crucial issue from a clinical point of view. Accuracy should always be the primary concern in clinical laboratories. The real-time PCR-HRMA system can identify the prevalent NTM species in a simple, rapid and cost-effective manner, and the specificit is >90%. Despite this, more NTM species should be included in future studies to find better mycobacteria-specific targets to improve the discrimination power, sensitivity and specificity of this technique.

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REFERENCES


