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Multiplex Real-Time PCR Assay and Melting Curve Analysis for Identifying *Mycobacterium tuberculosis* Complex and Nontuberculous Mycobacteria

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A multiplex real-time PCR assay and melting curve analysis for identifying 23 mycobacterial species was developed and evaluated using 77 reference strains and 369 clinical isolates. Concordant results were obtained for all 189 (100%) isolates of the *Mycobacterium tuberculosis* complex and 169 (93.9%) isolates of nontuberculous mycobacteria. Our results showed that this multiplex real-time PCR assay is an effective tool for the mycobacterial identification from cultures.

In South Korea, tuberculosis remains a serious public health problem, although its incidence rate has been greatly reduced (33). Recently, the isolation of nontuberculous mycobacteria (NTM) from clinical specimens has increased, and NTM now comprises 10 to 30% of isolated mycobacteria (11, 14, 15, 17, 27, 29). Mycobacteria have a spectrum of virulence and different susceptibilities to antibiotics (5, 6, 7, 10, 32). Thus, it is very important to rapidly distinguish NTM from *Mycobacterium tuberculosis* and identify the species to administer the appropriate treatment (6, 28). Commercial kits that use various molecularly based technologies, like line probe hybridization and PCR-restriction fragment length polymorphisms, have been used to rapidly identify mycobacteria (4, 12, 16, 20, 24, 25, 26). Despite their simplicity, these tests require postamplification procedures and are prone to contamination. In addition, they are suited for small test volumes, but they are too costly for routine use in the clinical microbiology laboratory. Alternatively, the sequencing of 16S rRNA genes or other targets offers a rapid and reliable means of mycobacterial identification; however, this requires expensive instrumentation (2, 21, 34). In recent years, real-time PCR instruments have been employed in many clinical laboratories in South Korea. Real-time PCR technology does not require postamplification manipulation. Furthermore, amplicons can be detected by melting curve analysis without using costly fluorescent probes. To address the need for a simple, cost-effective molecular method, we developed a multiplex real-time PCR assay that uses a melting curve analysis without fluorescent probes. Here, we applied this method to routine identification of cultured mycobacterial isolates.

The efficacy of a multiplex real-time PCR assay was evaluated using 77 bacterial reference strains and 369 clinical isolates. The assay was initially validated with 69 mycobacterial reference strains and 8 reference strains closely related to mycobacteria (see Table S1 in the supplemental material). Then, we tested the 369 clinical isolates, including 189 *Mycobacterium tuberculosis* complex (MTC) isolates and 180 NTM isolates grown in Bactec MGIT 960 culture tubes (Becton Dickinson, Franklin Lakes, NJ) or on 3% Ogawa medium.

Template DNAs were prepared from different culture media. Several colonies growing on 3% Ogawa medium were harvested with a 1-μl loop and suspended in 0.5 ml of distilled water. MGIT culture medium (0.5 ml) was centrifuged at 13,000 $\times$ *g* for 3 min, the supernatant was discarded, and the pellet was resuspended in 0.5 ml of distilled water. Both sample types were heated in a boiling water bath for 10 min and centrifuged for 3 min. The supernatant was used in the real-time PCR, sequencing, and GenoType Mycobacterium assays.

All MTC isolates were identified with the BioSewoom Real Time PCR assay for tuberculosis (TB) cultures (BioSewoom, Seoul, South Korea). Of the 180 NTM isolates, 96 species were identified with the GenoType Mycobacterium assay (Hain Lifescience, Nehren, Germany), and 84 species were identified with 16S rRNA sequencing. The NTM species that had been discrepant between the GenoType assay or 16S rRNA sequencing and the real-time PCR assay were identified with the 16S-23S internal transcribed spacer (ITS) gene sequencing.

Sequencing of the 16S rRNA and ITS genes was performed as described previously (9). The sequences were identified by comparisons with genes in the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/index.html) and the Ribosomal Differentiation of Medical Microorganisms (RIDOM) database (http://rdna.ridom.de/). The GenoType Mycobacterium assay was conducted according to the manufacturer's instructions.

The 16S rRNA gene, the 16S-23S internal transcribed spacer (ITS), and the *hsp65* gene sequences were aligned with Sequencher 4.10 software (Gene Codes Co., Ann Arbor, MI). Based on sequence alignment, we identified regions of difference that would specifically amplify different targets of interest. The target gene for MTC was the insertion sequence IS*6110*. Primers were designed manually or with the Primer 3 program (http://frodo.wi.mit.edu/primer3/). The real-time PCR primers for the identification of mycobacterial species, their target genes, and their melting temperatures (T_m) are shown in Table 1.

PCRs were conducted with the Rotor-gene Q real-time PCR instrument (Qiagen Inc., Germantown, MD). The PCR mixture

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TABLE 1 Multiplex real-time PCR primers for the identification of mycobacteria

Primer mixture[a]	Primer target	Target gene[b]	Sequence(5′→3′)	T_m (°C)[c]
I	*M. gastri*	ITS	GGGCTTGTCTTGGACTCGT TGGTGGGACAACACTCTTGG	75.13 ± 0.11
	M. xenopi	ITS	GTTGGGCAGCAGGCAGTA GTTGCCTCAAAACCCAACAG	80.97 ± 0.43
	M. fortuitum	ITS	CCCGAGCCGTGAGGAAC CAATAGTGTGTCTGGCAGTCAAAA	81.65 ± 0.26
	MTC	IS*6110*	CGAACTCAAGGAGCACATCAG CAGGGTTAGCCACACTTTGC	84.47 ± 0.41
	NTM	16S rRNA	ATGTYTTSTGGKGSAAAGCTTT GTAGGAGTCTGGGCCGTA	88.14 ± 0.51
II	*M. abscessus*	ITS	ATGAACTAGGGAACATAAAGTATGCA AGGATTTACAAAACATATTCACCAAGT	72.72 ± 0.74
	M. intracellulare	16S rRNA	GGTCTAATACCGGATAGGACCTTTAG GCAAAAGCTTTCCACCAAA	75.38 ± 0.31
	M. szulgai	ITS	GGTCCTGAGGCAACACTCA CCAAGATGGTGGGACAACAG	80.00 ± 0.42
	M. terrae	ITS	GATTCCCCCGTACCTCACAT ACCACCGACCACCCACTAC	83.09 ± 0.33
III	*M. avium*	16S rRNA	GGGTCTAATACCGGATAGGACCT CGCAAAAGCTTTCCACCAGA	73.55 ± 0.34
	M. chelonae	ITS	TGTCCACCCCGTGGATA GTGCCAGCGTTTCAATTCTA	79.40 ± 0.25
	M. kansasii/M. gastri	16S rRNA	CGGAAAGGTCTCTTCGGAGAC TTTCCCAGGCTTATCCTGGT	81.42 ± 0.20
	M. gordonae	ITS	TGCAAGCCTTGAGTGGTCA GGGGACAGCACCAGAGG	84.04 ± 0.33
IV	*M. marinum/M. ulcerans*	ITS	GGGTCCTGAGGCAACATCT CAACATCCCGAAACCAACAG	74.34 ± 0.36
	M. simiae	ITS	CTCGGCCGACTTCGGTT AGATGGAGGGACACCACTTCA	76.34 ± 0.22
	M. ulcerans	*hsp65*	ACCGAGACCCTGCTCAAA GCTCCTTGGTCTCGACCTCT	79.03 ± 0.41
	M. septicum	ITS	ATGGCCTCGCACCTGTAG CCAATAGTGTGTCTGGCAGTTCTA	81.56 ± 0.25
	M. smegmatis	ITS	GAGCTGGAGCGCTGTAGTG GAAACAGCGTTTCCCACAC	84.28 ± 0.14
V	*M. scrofulaceum*	16S rRNA	ACCATCGACGAAGGCTCAC CACCTACCGTCAACCCACA	77.48 ± 0.41
	M. haemophilum	ITS	GCACAACAGCAAATGAATCG ACATGGGACAAGCCTGAGT	80.77 ± 0.06
	M. celatum	ITS	CACGAAAAACACTCCGCATC GCGATTTTTCCCATTTGTTG	85.85 ± 0.27
VI	*M. peregrinum*	ITS	ATTCGTTGGATGGCCTCAC CCACGCCAAGTTTGTTGAG	81.62 ± 0.07
	M. mucogenicum	ITS	CATTTACATGCCCTGATCCA CGACGACAATCCCAACCA	83.44 ± 0.29
	M. shimoidei	ITS	GAAGTCGAGCCGTGAGGA ACCACCAAAGATGAGGCAAC	86.87 ± 0.33

[a] We created six primer mixtures. Mixture I had primer sets specific for *M. gastri*, *M. xenopi*, *M. fortuitum*, MTC, and NTM. Mixture II had primer sets specific for *M. abscessus*, *M. intracellulare*, *M. szulgai*, and *M. terrae*. Mixture III had primer sets specific for *M. avium*, *M. chelonae*, *M. kansasii*, and *M. gordonae*. Mixture IV had primer sets specific for *M. marinum*, *M. simiae*, *M. ulcerans*, *M. septicum*, and *M. smegmatis*. Mixture V had primer sets specific for *M. scrofulaceum*, *M. haemophilum*, and *M. celatum*. Mixture VI had primer sets specific for *M. peregrinum*, *M. mucogenicum*, and *M. shimoidei*.

[b] ITS, 16S-23S internal transcribed spacer.

[c] The product T_m values represent means ± coefficient of variation.

TABLE 2 Comparison of multiplex real-time PCR results with 16S rRNA sequencing results for identification of 84 nontuberculous mycobacterium (NTM) isolates

Species identification by real-time PCR	No. of isolates identified by16S rRNA sequencing as:									
	M. abscessus/ M. chelonae	*M. avium*	*M. celatum*	*M. intracellulare*	*M. fortuitum*	*M. fortuitum/ M. farcinogenes/ M. senegalense*	*M. gordonae*	*M. kansasii/ M. gastri*	*M. septicum/ M. peregrinum*	*M. terrae*
M. abscessus	6									
M. avium		22		1[a]						
M. chelonae	1									
M. intracellulare		1		38						
M. fortuitum					1	1				
M. gordonae							1			
M. kansasii								2		
M. septicum						3			2	
M. peregrinum									1	
M. terrae										3
NTM species			1							

[a] This is a mixed culture of *M. avium* and *M. intracellulare* identified by the multiplex real-time PCR assay.

contained 0.5 μl of 10 μM multiplexed primer mix (Table 1), 5 μl of 2× HRM PCR master mix (Qiagen Inc., Germantown, MD) including EvaGreen fluorescent dye, and 1.5 μl of template DNA in a total volume of 10 μl. The PCR protocol started at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 15 s; then we measured green fluorescence. Following the last cycle, the melting curve was generated by heating from 68°C to 93°C in increments of 0.2°C/s.

Based largely on the frequency and distribution of NTM isolates in South Korea (11, 14, 29, 33), we developed a multiplex real-time PCR assay with melting curve analysis that could identify 23 mycobacterial species. For the correct identification of mycobacteria, the assay was designed to distinguish between any two species that had at least a 2°C difference in the T_m values. The criterion used to make the call of species was a T_m of ±0.6°C. The specificities of six primer mixtures were initially evaluated by performing melting curve analyses on amplicons obtained from 77 bacterial reference strains. Only the expected PCR products were amplified from each reference strain (data not shown; see Fig. S1 in the supplemental material). However, DNA templates from *M. alvei* produced a peak that corresponded to *M. peregrinum* in reaction VI.

The multiplex real-time PCR assay was conducted stepwise to enhance its cost-effectiveness. Step 1 was designed to differentiate between MTC and NTM; because tuberculosis is the most frequent mycobacterial infection in South Korea (11, 14, 15, 17, 27, 29), this reduced the number of species identifications necessary in steps 2 and 3. The step 1 reaction was conducted with primer mixture I. All 189 MTC isolates were correctly identified. A total of 177 of the NTM isolates were detected as NTM, and 3 (1.7%) were identified at the species level as *M. fortuitum*. The step 2 reaction, which included primer mixtures II and III, was conducted to identify the remaining 177 NTM species. This reaction identified 168 (93.3%) NTM species. The step 3 reaction, which included the remaining primer mixtures, identified another 8 (4.4%) NTM species. Of the total 180, only one (0.6%) *M. celatum* remained unidentified.

The results for 11 isolates were discordant with results from 16S rRNA sequencing (five species [Table 2]) and the GenoType assay (six species [Table 3]). The discrepant isolates except one *M. celatum* were further analyzed with ITS sequencing. One *M. avium* isolate identified with 16S rRNA sequencing was identified as *M. intracellulare*; similarly, three *M. fortuitum*/*M. farcinogenes*/*M. senegalense* isolates were identified as *M. conceptionense*. Furthermore, one *M. fortuitum* isolate identified with the GenoType assay was identified as *M. septicum*; the rest of the species were unidentified due to their having mixed sequences.

The developed multiplex real-time PCR assay has distinct ad-

TABLE 3 Comparison of multiplex real-time PCR results with GenoType Mycobacterium assay results for identification of 96 nontuberculous mycobacteria (NTM) isolates

Species identification by real-time PCR	No. of isolates identified by GenoType Mycobacterium assay as:							
	M. abscessus	*M. avium*	*M. intracellulare*	*M. fortuitum*	*M. gordonae*	*M. kansasii*	*M. smegmatis*	NTM species
M. abscessus	12							
M. avium		30	1					
M. intracellulare		1	41	1			1[a]	
M. fortuitum				1				
M. gordonae					3			
M. kansasii						2		
M. septicum				1				
M. peregrinum				1				
M. terrae								1

[a] This is a mixed culture of *M. avium* and *M. intracellulare* identified by the multiplex real-time PCR assay.

vantages over the 16S rRNA sequence analysis and the GenoType Mycobacterium assay. Due to the sequence similarities, 16S rRNA sequencing analysis cannot always distinguish between closely related species, including *M. abscessus* and *M. chelonae*, *M. kansasii* and *M. gastri*, *M. marinum* and *M. ulcerans*, and *M. alvei*, *M. septicum*, *M. peregrinum*, and *M. senegalense* (1, 8, 31). These species typically require additional biochemical or other tests for identification. In contrast, most of our primers were designed based on targeting the ITS gene, which had greater sequence variation than the 16S rRNA gene (22). Thus, our multiplex assay could differentiate between most of those species without additional tests. However, we could not distinguish between *M. alvei* and *M. peregrinum*. To our knowledge, *M. alvei* has never been isolated from clinical specimens in South Korea. Accordingly, we did not design primers to specifically identify this species.

Another advantage of our assay over sequencing analysis was that it could directly identify mycobacteria from primary liquid detection media (in MGIT culture tubes). For accurate identification, sequencing analysis typically requires pure isolates, which are grown in subcultures secondary to the primary liquid detection media. In our evaluation study, we conducted 16S rRNA sequencing on isolates directly derived from primary liquid detection media. A total of 161 isolates were determined to be positive for acid-fast bacilli (AFB), but only 84 species could be identified due to mixed sequences. In contrast, our multiplex PCR assay determined that all 161 of the isolates were NTM, and we could identify each of the species (data not shown). The GenoType assay could also identify isolates directly from primary liquid detection media, but it lacked specificity compared to our multiplex analysis. The GenoType assay did not distinguish *M. septicum* and *M. peregrinum* from *M. fortuitum*. In this study, three isolates that were identified as *M. fortuitum*/*M. farcinogenes*/*M. senegalense* by 16S rRNA sequencing were identified as *M. fortuitum* with the GenoType assay (data not shown). Thus, the GenoType assay could not distinguish *M. farcinogenes* and *M. senegalense* from *M. fortuitum* or *M. septicum* and *M. alvei* from *M. peregrinum* (2, 26). Furthermore, the GenoType assay could not identify *M. terrae*, which has been isolated in South Korea (11, 29).

Several real-time PCR assays have previously been described for mycobacterial identification (3, 13, 18, 19, 23, 30). However, most of those assays were limited in the range of species they could identify. Lim et al. (19) described a real-time PCR assay that could identify 18 mycobacterial species, but there were drawbacks in its applicability to routine identification of cultured isolates. They used 21 pairs of hybridization probes, which made the assay expensive. Furthermore, they did not validate the method with clinical isolates, and they could not differentiate between the following species pairs: *M. kansasii* and *M. gastri*, *M. fortuitum* and *M. peregrinum*, *M. ulcerans* and *M. marinum*. On the other hand, the multiplex real-time PCR assay developed here was a simple, cost-effective, appropriate test for routine identification of most clinically important mycobacterial species in culture.

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