Journal of Microbiology (2015) Vol. 53, No. 1, pp. 38–46
Copyright ⓒ 2015, The Microbiological Society of Korea
DOI 10.1007/s12275-015-4495-8

Performance of a real-time PCR assay for the rapid identification of *Mycobacterium* species

Hye-young Wang[1†], Hyunjung Kim[2], Sunghyun Kim[2,3], Do-kyoon Kim[4], Sang-Nae Cho[5], and Hyeyoung Lee[2*]

[1]*M&D, Inc., Wonju Eco Environmental Technology Center, Wonju 200-722, Republic of Korea*
[2]*Department of Biomedical Laboratory Science, College of Health Sciences, Yonsei University, Wonju 220-710, Republic of Korea*
[3]*Institute for Life Science and Biotechnology, Yonsei University, Seoul 120-749, Republic of Korea*
[4]*Department of Laboratory Medicine, The Catholic University of Korea, St. Vincent's Hospital, Suwon 442-723, Republic of Korea*
[5]*Department of Microbiology, College of Medicine, Yonsei University, Seoul 120-749, Republic of Korea*

(Received Sep 2, 2014 / Revised Oct 27, 2014 / Accepted Oct 28, 2014)

Mycobacteria cause a variety of illnesses that differ in severity and public health implications. The differentiation of *Mycobacterium tuberculosis* (MTB) from nontuberculous mycobacteria (NTM) is of primary importance for infection control and choice of antimicrobial therapy. The diagnosis of diseases caused by NTM is difficult because NTM species are prevalent in the environment and because they have fastidious properties. In the present study, we evaluated 279 clinical isolates grown in liquid culture provided by The Catholic University of Korea, St. Vincent's Hospital using real-time PCR based on mycobacterial *rpoB* gene sequences. The positive rate of real-time PCR assay accurately discriminated 100% (195/195) and 100% (84/84) between MTB and NTM species. Comparison of isolates identified using the MolecuTech REBA Myco-ID® and Real Myco-ID® were completely concordant except for two samples. Two cases that were identified as mixed infection (*M. intracellulare-M. massiliense* and *M. avium-M. massiliense* co-infection) by PCR-REBA assay were only detected using *M. abscessus*-specific probes by Real Myco-ID®. Among a total of 84 cases, the most frequently identified NTM species were *M. intracellulare* (n=38, 45.2%), *M. avium* (n=18, 23.7%), *M. massiliense* (n=10, 13.2%), *M. fortuitum* (n=5, 6%), *M. abscessus* (n=3, 3.9%), *M. gordonae* (n=3, 3.9%), *M. kansasii* (n=2, 2.4%), *M. mucogenicum* (n=2, 2.4%), and *M. chelonae* (n=1, 1.2%). Real Myco-ID® is an efficient tool for the rapid detection of NTM species as well as MTB and sensitive and specific and comparable to conventional methods.

***Keywords*:** *Mycobacterium tuberculosis* (MTB), nontuberculous mycobacteria (NTM), Real Myco-ID®, identification, molecular diagnosis

*For correspondence. E-mail: hyelee@yonsei.ac.kr; Tel.: +82-33-760-2740; Fax: +82-33-760-2561

Introduction

Tuberculosis (TB) remains an important community health problem. The World Health Organization (WHO) estimates that eight million new TB cases are reported annually and are the cause of death in two to three million patients (WHO reports, 2013). Each untreated pulmonary TB patient is responsible for the spread of the disease to 10 to 15 humans over a year. This makes TB one of the most important causes of death from an infectious agent (WHO reports, 2013).

Nontuberculous mycobacterium (NTM) strains causing clinical disease have become increasingly frequent and more varied; therefore, the implementation of strategies for the rapid differentiation between NTM and *Mycobacterium tuberculosis* (MTB) complex for early infection control and choice of antimicrobial therapy is now of primary importance (Alcaide and Santín, 2008; Jarzembowski and Young, 2008; Thanachartwet *et al*., 2014). The incidence of NTM infection has increased in the Korean population, with a simultaneous increase in the elderly and immunocompromised populations (Park *et al*., 2010). The most effective means of protection is early diagnosis and treatment of the disease. Preliminary diagnosis is based on clinical findings, but definite diagnosis is by laboratory methods (Lee *et al*., 2012).

The MTB complex poses diagnostic and therapeutic problems due to low sensitivity of the diagnostic tools available for its identification and discrimination with NTM (Maurya *et al*., 2012). The standard diagnosis for mycobacterial infections relies on clinical presentation, histopathology, acid-fast bacilli (AFB) smears, and the isolation of MTB from culture (Bae *et al*., 2008). These diagnostic methods have limitations. Although the AFB are rapid, sensitivity has not yet been evaluated and does not differentiate between MTB complex and NTM (Maurya *et al*., 2012). The diagnostic delay can affect treatment. Although culture in liquid media allows for the detection of mycobacterial growth at earlier stages, the isolation and phenotypic identification requires several weeks, as does antimicrobial susceptibility testing (AST) (Chakravorty and Tyagi, 2005). Rapid identification of mycobacterial species is important and a simple, sensitive, and specific identification method is required. New techniques are available like chemiluminescent DNA probes (Ichiyama *et al*., 1997), high-performance liquid chromatography (HPLC) (Chen *et al*., 2013), PCR-restriction fragment length polymorphism analysis (PRA) of the *hsp65* (Bannalikar and Verma, 2006), *groES* (Aravindhan *et al*., 2007), and *rpoB* (Lee *et al*., 2000) genes, and sequence analysis of 16S rRNA genes (Pérez-Osorio *et al*., 2012). These techniques are more sophisticated methods but are not cost-effective and require expensive equipment. One of the new techniques, real-time

PCR have eliminated the limitations of techniques and has advantage such as high sensitivity, significantly faster processing time than conventional PCR and other detection methods. However, most of these assays have been used for the detection or quantification of a certain *Mycobacterium* species only, such as MTB, or the detection of drug-resistant MTB (Pérez-Osorio *et al*., 2012; Chen *et al*., 2013). In order to overcome these limitations, a commercial diagnostic kit based on multi-probe real-time PCR, Real Myco-ID® (Optipharm) was developed for the rapid and accurate detection and identification of 17 *Mycobacterium* species including MTB, *M. avium-M. intracellulare*, *M. abscessus-M. massiliense*, *M. chelonae*, *M. fortuitum* complex, *M. ulcerans-M. marinum*, *M. kansasii-M. gastri*, *M. terrae-M. nonchromogenicum*, *M. celatum*, *M. gordonae-M. szulgai*, and *M. mucogenicum* from clinical samples. In the present study, the clinical usefulness of the Real Myco-ID® assay (Optipharm) was evaluated for the rapid differentiation of MTB from NTM and the identification of major NTM species from a total of 279 clinical specimens (94 AFB smear-positives and 185 AFB smear-negatives).

Materials and Methods

Clinical specimens

In order to clinically evaluate the performance of the Real Myco-ID® kit (Optipharm) a total of 279 DNA samples isolated from liquid cultures including 94 AFB smear-positives and 185 AFB smear-negatives were provided from The Catholic University of Korea, St. Vincent's Hospital (Suwon, Korea).

AFB smear and mycobacterial culture

In order to detect AFB, auramine-rhodamine fluorescent staining was performed with respiratory specimens and their results were confirmed by the Ziehl-Neelsen method (Griffith *et al*., 2007). Microscopic results were reported semi-quantitatively, and if more than one AFB per 100 high-power fields were detected, the result was considered as AFB smear positive. A trace of AFB in a specimen is defined by 1 to 2 AFB per ×300 field, 1+ is defined by 1 to 9 AFB per ×100 field, 2+ is defined by 1 to 9 AFB per ×10 field, and 3+ is defined by 1 to 9 AFB per ×1 field. Sputum specimens were decontaminated using the N-acetyl-L-cysteine 2% NaOH method. Mycobacterial culture was performed using the BACTEC™ MGIT™ 960 system (BD Diagnostic System) with decontaminated specimens. The inoculated media were incubated at 37°C and inspected weekly for 8 weeks.

DNA preparation

For the MGIT 960 system, a 1–1.5 ml aliquot of culture broth was centrifuged for 10 min at 13,000 × *g*. The pellet was extracted with DNA extraction solution (Optipharm) and the suspended bacterial solution was boiled for 10 min.

Table 1. Characteristics of study subjects

Characteristics			No.	%
Age (median= 53.0 SD±18.9 yrs)		18–89		
	≥20's		41	14.7
	30's		40	14.3
	40's		38	13.6
	50's		51	18.3
	≥60's		109	39.1
Sex	Male		147	52.7
	Female		132	47.3
Specimens	Sputum		154	55.2
	Bronchial wash specimens		115	41.2
	Pleural		10	3.6
AFB smear	Negative		185	66.3
	Trace		7	2.5
	1+		70	25.1
	2+		15	5.4
	3+		2	0.7
Mycobacterial culture	MGIT960	+	279	100
	Days for positive cultures	≥5	10	3.6
		5-9	73	26.2
		11-19	142	50.9
		20-29	45	16.1
		≥ 30	9	3.2

AdvanSure TB/NTM real-time PCR

DNA samples were extracted from clinical specimens and amplified using the AdvanSure TB/NTM real-time PCR kit (LG Life Science) following the manufacturer's protocol (Kim *et al.*, 2008). The real-time PCR detection system (LG Life Science) was used to measure fluorescence formed during the real-time PCR process. A positive result was indicated when the cycle threshold (C_T) value was less than 35 after observing signal formation of a wavelength from each channel (FAM, HEX, and Cy5).

Multi-probe real-time PCR TaqMan® assay

The multi-probe real-time PCR TaqMan® assay was carried out using the Real Myco-ID® assay (Optipharm), and a CFX-96 real-time PCR system (Bio-Rad) was used for the thermocycling and fluorescence detection. The real-time PCR amplification was performed in a total volume of 20 µl that contained 10 µl of 2× Thunderbird probe qPCR mix (Toyobo), 3.0 µl of primer and TaqMan® probe mixture, 5 µl of template DNA, and ddH_2O added to give a final volume of 20 µl for each sample.

Positive and negative controls were included throughout the procedure. No-template controls with ddH_2O instead of template DNA were incorporated in each run under the following conditions: 95°C for 3 min and 40 cycles of 95°C for 20 sec and 60°C for 40 sec in single real-time PCR. The bacterial load was quantified by determining the cycle threshold (C_T), the number of PCR cycles required for the fluorescence to exceed a value significantly higher than the background fluorescence. A positive result was indicated when the C_T value was less than 35 after observing signal formation of wavelength from each channel.

PCR-reverse blot hybridization assay (PCR-REBA)

To confirm the results of MTB and NTM differentiation and the identification of major NTM species in clinical samples, the MolecuTech REBA Myco-ID® (YD Diagnostics) system was performed according to the manufacturer's instructions (Wang *et al.*, 2014). PCR was performed using 20 µl of reaction mixture (Genetbio) containing 2× master mix, 1× primer mixture, 5 µl of sample DNA, and ddH_2O added to give a final volume of 20 µl. The 40 PCR cycles were comprised of an initial denaturation at 95°C for 30 sec, followed by annealing and extension at 65°C for 30 sec. After the final cycle, samples were maintained at 72°C for 10 min to complete the synthesis of all strands. The amplified target was visualized as a single band corresponding to a length of 250 bp using the Chemi Doc system (Vilber Lourmat). The amplified PCR products were subjected to reverse blot hybridization assays. Hybridization and washing were performed according to the manufacturer's instructions. In brief, the biotinylated PCR products were denatured at 25°C for 5 min in denaturation solution and diluted in 970 µl of hybridization solution along with the REBA membrane strip in the provided blotting tray. Denatured single-stranded PCR products were used to hybridize with the probes on the strip at 55°C for 30 min. The strips were then washed twice with gentle shaking in 1.0 ml of washing solution for 10 min at 55°C, incubated at 25°C with 1:2,000 diluted streptavidin-alkaline phosphatase conjugate (Roche Diagnostics) in conjugate diluents solution (CDS) for 30 min, and then washed twice with 1.0 ml CDS at room temperature for 1 min. The colorimetric hybridization signals were visualized by adding 1:50 diluted alkaline phosphatase-mediated staining solution, NBT/BCIP (Roche Diagnostics) and incubated until

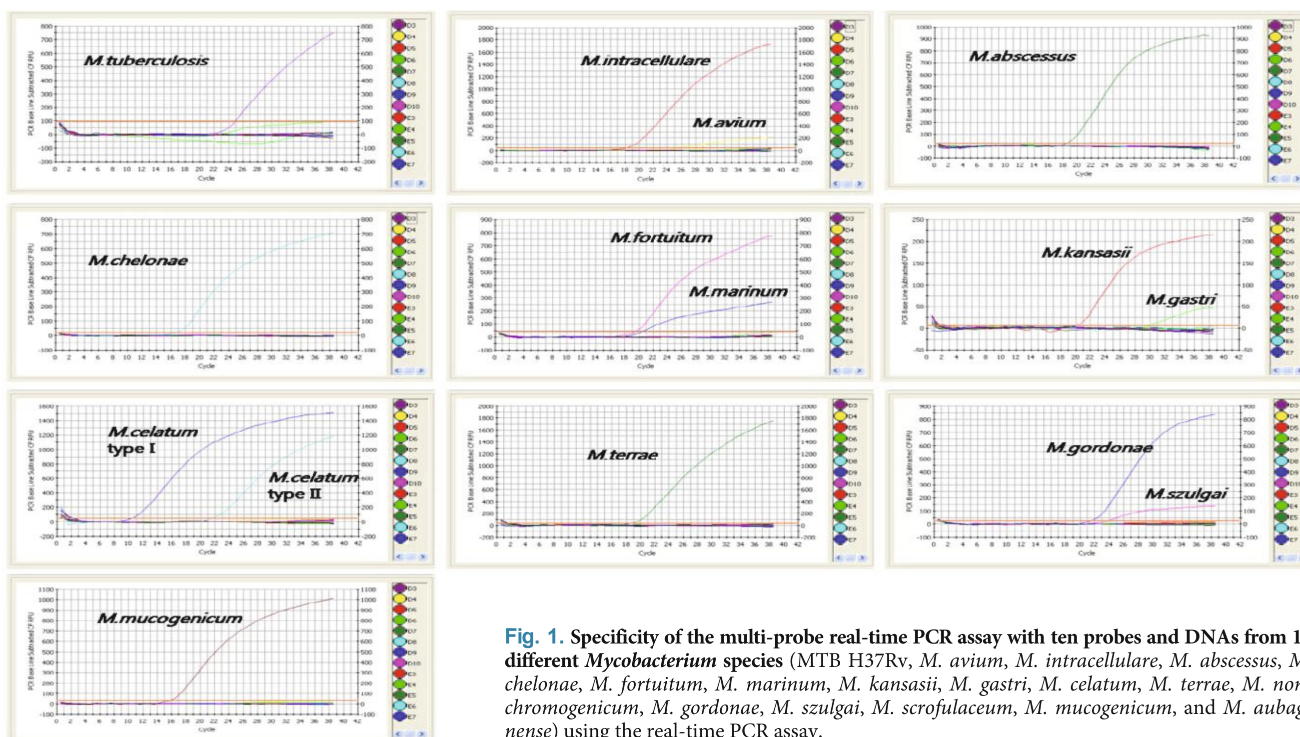

Fig. 1. Specificity of the multi-probe real-time PCR assay with ten probes and DNAs from 17 different *Mycobacterium* species (MTB H37Rv, *M. avium*, *M. intracellulare*, *M. abscessus*, *M. chelonae*, *M. fortuitum*, *M. marinum*, *M. kansasii*, *M. gastri*, *M. celatum*, *M. terrae*, *M. nonchromogenicum*, *M. gordonae*, *M. szulgai*, *M. scrofulaceum*, *M. mucogenicum*, and *M. aubagnense*) using the real-time PCR assay.

Table 2. Specificity of the Real Myco-ID® assay for 63 bacterial reference strains

Genus	Species	Reference strains	Multi-probe real-time PCR TaqMan® assay (C_T value)									
			MTB probe	avi-int probe	abs probe	che probe	fort probe	kan probe	cel probe	ter probe	gor probe	muco probe
Mycobacterial strains												
Mycobacterium	*M. tuberculosis* H37Rv	ATCC 27294	24.9	UD	UD	UD	UD	UD	UD	UD	UD	UD
	M. avium	ATCC 25291	UD	25.9	UD	UD	UD	UD	UD	UD	UD	UD
	M. intracellulare	ATCC 13950	UD	18	UD	UD	UD	UD	UD	UD	UD	UD
	M. abscessus	ATCC 19977	UD	UD	18.6	UD	UD	UD	UD	UD	UD	UD
	M. massiliense	clinical isolates	UD	UD	18.2	UD	UD	UD	UD	UD	UD	UD
	M. bolletii	clinical isolates	UD	UD	21.7	UD	UD	UD	UD	UD	UD	UD
	M. chelonae	ATCC 35749	UD	UD	UD	16.7	UD	UD	UD	UD	UD	UD
	M. fortuitum	ATCC 49403	UD	UD	UD	UD	20.5	UD	UD	UD	UD	UD
	M. marinum	ATCC 927	UD	UD	UD	UD	19.4	UD	UD	UD	UD	UD
	M. mageritense	ATCC 700351	UD	UD	UD	UD	24.9	UD	UD	UD	UD	UD
	M. peregrinum	ATCC 14467	UD	UD	UD	UD	19.3	UD	UD	UD	UD	UD
	M. kansasii	ATCC 12478	UD	UD	UD	UD	UD	18.5	UD	UD	UD	UD
	M. gastri	ATCC 15754	UD	UD	UD	UD	UD	30	UD	UD	UD	UD
	M. celatum	ATCC 51130	UD	UD	UD	UD	UD	UD	21.5	UD	UD	UD
	M. celatum	ATCC 51131	UD	UD	UD	UD	UD	UD	10.4	UD	UD	UD
	M. terrae	ATCC 15755	UD	UD	UD	UD	UD	UD	UD	18.6	UD	UD
	M. nonchromogenicum	ATCC 19530	UD	UD	UD	UD	UD	UD	UD	26.1	UD	UD
	M. gordonae	ATCC 14470	UD	UD	UD	UD	UD	UD	UD	UD	21.5	UD
	M. szulgai	ATCC 35799	UD	UD	UD	UD	UD	UD	UD	UD	23.9	UD
	M. mucogenicum	ATCC 49650	UD	UD	UD	UD	UD	UD	UD	UD	UD	15.8
	M. phlei	ATCC 11758	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	M. smegmatis	ATCC 19420	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	M. triviale	ATCC 23292	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	M. aurum	ATCC 23366	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	M. farcinogen	ATCC 35753	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	M. gilvum	ATCC 43909	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	M. neoaurum	ATCC 25795	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	M. parafortuitum	ATCC 19686	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	M. xenopi	ATCC 19250	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	M. septicum	ATCC 700731	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
Non-mycobacterial strains												
Nocardia	*N. abscessus*	BAA-279	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	N. asiatica	CIP 108374	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	N. asteroides	ATCC 19247	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	N. brevicatena	ATCC 15333	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	N. carnea	ATCC 6847	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	N. cyriacigeorgica	CIP 48295	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	N. elegans	CIP 108553	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	N. farcinica	ATCC 3318	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	N. flavorosea	CIP 104511	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	N. nova	ATCC 33726	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	N. otitidiscaviarum	ATCC 14629	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	N. salmonicida	ATCC 27463	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	N. seriolae	ATCC 43993	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	N. uniformis	CIP 104824	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	N. vaccinii	ATCC 11092	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	N. pseudosporangifera	CIP 104825	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	N. violaceofusca	CIP 104780	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
Escherichia	*E. coli*	ATCC 25922	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	E. coli	ATCC 35218	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
Klebsiella	*K. pneumoniae*	ATCC 13883	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
Pseudomonas	*P. aeruginosa*	ATCC 27853	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
Enterobacter	*E. aerogenes*	ATCC 1304	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD

Table 2. Continued

Genus	Species	Reference strains	Multi-probe real-time PCR TaqMan® assay (C_T value)									
			MTB probe	avi-int probe	abs probe	che probe	fort probe	kan probe	cel probe	ter probe	gor probe	muco probe
Citrobacter	*C. freundii*	ATCC 6750	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
Shigella	*S. boydii*	DML 399*	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	S. dysenteriae	DML 400*	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	S. flexneri	ATCC 9199	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
Salmonella	*S. typhi*	ATCC 19430	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	S. typhimurium	ATCC 13311	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
Staphylococcus	*S. aureus*	ATCC 29213	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	S. aureus	ATCC 25923	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
Enterococcus	*E. faecium*	ATCC 19434	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
Streptococcus	*S. pneumoniae*	ATCC 49619	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
	S. agalactiae	ATCC 13813	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD

ATCC, American type culture collection; CIP, Collection Institut Pasteur; DML, Diagnostic Microbiology Laboratory, Biomedical Laboratory Science, Yonsei University; UD, Undetermined.

the color was detected. Finally, the band pattern was read and interpreted.

rpoB gene sequence analysis

The mycobacterial *rpoB* gene region was sequenced to confirm mismatched samples by the two analysis assays used for NTM identification. The primer sets used to amplify the target *rpoB* gene were 372F (5′-CCTGTTCTTCAAGGAGAAGCGCTACGACCTGG-3′) and 372R (5′-GGACGGATGTTGATCAGGGTCTGCGG-3′), which resulted in a 372-bp PCR product. Amplified DNA (*rpoB* region) was sequenced using an ABI 3730 automated DNA sequencer and the ABI Prism BigDye Terminator kit (Cosmo Genetech). The sequences obtained were compared with sequences in the National Center for Biotechnology Information (NCBI) GenBank database for species assignment.

Results

Population characteristics

A total of 279 participants enrolled with 154 sputum, 115 bronchial wash specimens, and 10 pleural with suspected pulmonary TB. The age range for 279 specimens was 18 to 89 years with a mean age of 53.0 years (SD ± 18.9 yrs). The male-to-female ratio was 1.1:1 (147:132). Number of AFB smear negative, trace, 1+, 2+, and 3+ were 185 (66.3%), 7 (2.5%), 70 (25.1%), 15 (5.4%), and 2 (0.7%), respectively (Table 1).

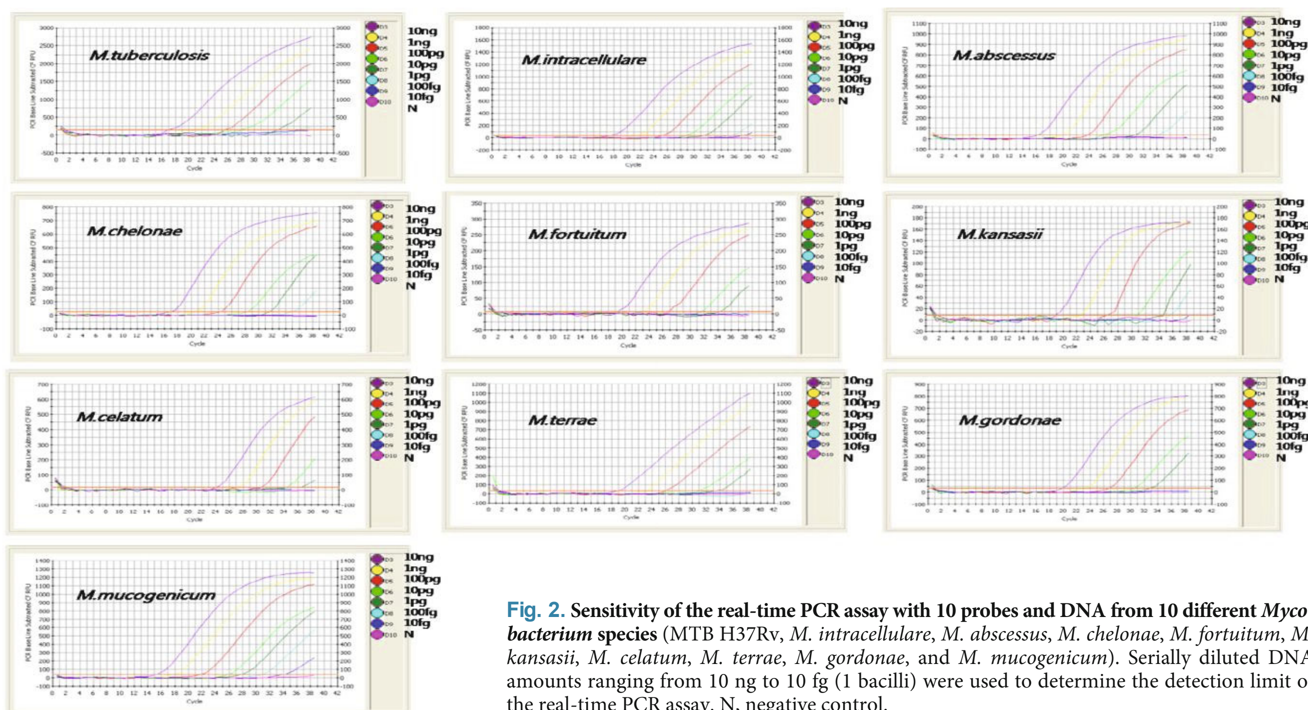

Fig. 2. **Sensitivity of the real-time PCR assay with 10 probes and DNA from 10 different *Mycobacterium* species** (MTB H37Rv, *M. intracellulare*, *M. abscessus*, *M. chelonae*, *M. fortuitum*, *M. kansasii*, *M. celatum*, *M. terrae*, *M. gordonae*, and *M. mucogenicum*). Serially diluted DNA amounts ranging from 10 ng to 10 fg (1 bacilli) were used to determine the detection limit of the real-time PCR assay. N, negative control.

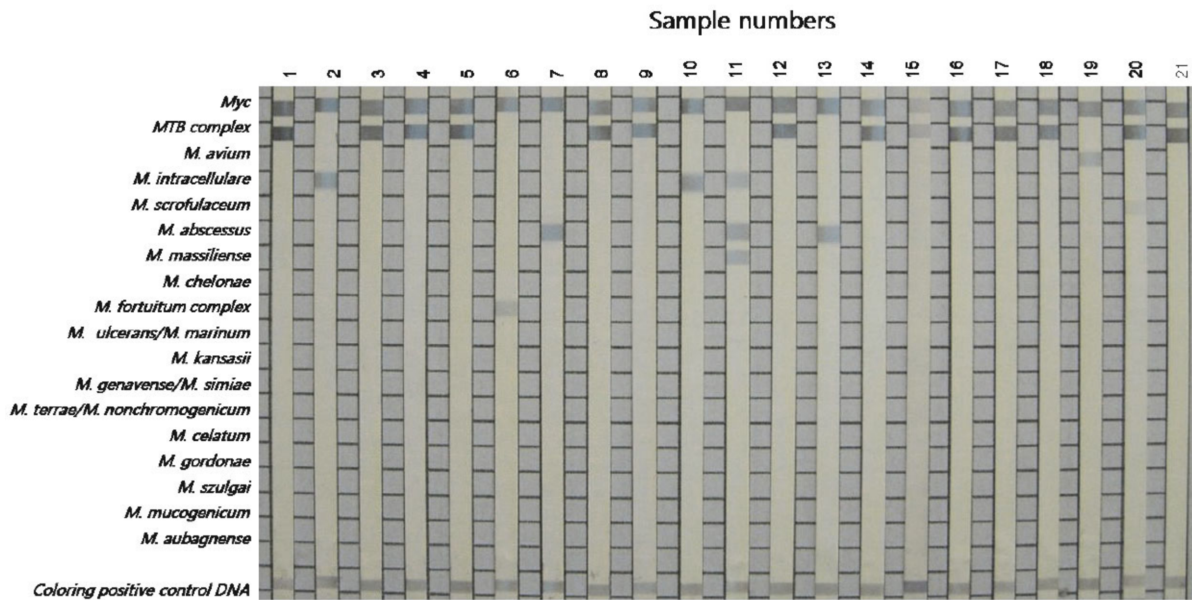

Fig. 3. Examples of the PCR-REBA results with DNA samples isolated from liquid cultures. The species identification of MTB (1, 3-5, 8-9, 12, 14-18, 20-21), *M. avium* (19), *M. intracellulare* (2, 10), *M. fortuitum* complex (6), *M. abscessus* (7, 13), and the *M. intracellulare-M. massiliense* mixed cultures (11) determined by PCR-REBA assay. Data interpretation of *M. abscessus* only show positive band pattern for *M. abscessus* probe and *M. massiliense* shows positive band patterns for *M. abscessus* and *M. massilience* probe.

Specificity of the Real Myco-ID® assay with reference bacterial strains

MTB H37Rv, 29 NTM strains, and 33 non-mycobacterial strains were used to determine the specificity of the Real Myco-ID® assay. The species-specific probes detected MTB H37Rv, *M. abscessus*, *M. chelonae*, *M. terrae-M. nonchromogenicum*, and *M. mucogenicum* accurately without cross-reaction. The C_T values for the species-specific probes of *M. abscessus*, *M. chelonae*, *M. terrae-M. nonchromogenicum*, and *M. mucogenicum* by real-time PCR assays ranged from 17.83 to 30.44, 14.19 to 25.59, and 17.09 to 26.42, respectively. Four probes resulted in positive fluorescence signals for *M. avium-M. intracellulare*, *M. fortuitum-M. marinum*, *M. kansasii-M. gastri*, and *M. gordonae-M. szulgai*, respectively (Fig. 1). The C_T values of the species-specific probes were ranged from 10.4 to 30. Also, these probes did not react with any non-mycobacterial strains (Table 2).

Sensitivity of the Real Myco-ID® assay with reference bacterial strains

The analytical sensitivity of the assay was determined through the use of a 10-fold dilution [10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, and 10 fg (1 bacilli)] standard curve of DNA isolated from MTB H37Rv, *M. intracellulare*, *M. abscessus*, *M. chelonae*, *M. fortuitum*, *M. kansasii*, *M. celatum*, *M. terrae*, *M. gordonae*, and *M. mucogenicum* strains.

The detection limit of the real-time PCR assay was 1 pg (100 bacilli) to 10 fg (1 bacilli). The C_T values for MTB H37Rv, *M. intracellulare*, *M. abscessus*, *M. chelonae*, *M. fortuitum*, *M. kansasii*, *M. celatum*, *M. terrae*, *M. gordonae*, and *M. mucogenicum* with each cell concentrate (10^6–1 bacilli) ranged from 16.9 to 33.4, 17.3 to 35.0, 16.1 to 34.1, 16.9 to 35.0, 19.3 to 34.3, 18.9 to 34.6, 23.9 to 34.3, 18.9 to 33.3, 19.8 to 32.8, and 15.2 to 34.0, respectively (Fig. 2).

Table 3. Results of three molecular assays for detecting MTB and NTM with clinical samples compared to AFB smear scores

AFB smear score (n=279)	Culture (MGIIT 960)		AdvanSure TB/NTM real-time PCR, n (%)		Real Myco-ID® real-time PCR, n (%)		Molecutech REBA Myco-ID® assay, n (%)	
Negative (n=185)	MTB	126 (68.1%)	MTB	126 (68.1%)	MTB	126 (68.1%)	MTB	126 (70%)
	NTM	59 (31.9%)	NTM	59 (31.9%)	NTM	59 (31.9%)	NTM	59 (31.9%)
Trace (n=7)	MTB	4 (57.1%)	MTB	4 (57.1%)	MTB	4 (57.1%)	MTB	4 (57.1%)
	NTM	3 (42.9%)	NTM	3 (42.9%)	NTM	3 (42.9%)	NTM	3 (42.9%)
1+ (n=70)	MTB	55 (78.6%)	MTB	55 (78.6%)	MTB	55 (78.6%)	MTB	55 (78.6%)
	NTM	15 (21.4%)	NTM	15 (21.4%)	NTM	15 (21.4%)	NTM	15 (21.4%)
2+ (n=15)	MTB	10 (66.7%)	MTB	10 (66.7%)	MTB	10 (66.7%)	MTB	10 (66.7%)
	NTM	5 (33.3%)	NTM	5 (33.3%)	NTM	5 (33.3%)	NTM	5 (33.3%)
3+ (n=2)	MTB	0 (0%)	MTB	0 (0%)	MTB	0 (0%)	MTB	0 (0%)
	NTM	2 (100%)	NTM	2 (100%)	NTM	2 (100%)	NTM	2 (100%)

AFB, acid-fast bacilli; MTB, *M. tuberculosis*; NTM, Nontuberculous mycobacteria

Table 4. Comparison of isolates identified by PCR-REBA and multi-probe real-time PCR assays

MTB/NTM differentiation	Identification of mycobacterial species				
TB/NTM PCR assay	PCR-REBA assay with culture samples	n (%)	Multi-probe real-time PCR assay with culture samples		
			n (%)	Ranged C_T value	Mean C_T value
NTM	*M. intracellulare*	38 (45.2)	38 (45.2)	16.9–34.5	21.0
	M. avium	18 (21.4)	18 (21.4)	23.7–33.4	28.6
	M. massiliense	10 (11.9)	10 (11.9)[a]	15.2–21.7	18.2
	M. abscessus	3 (3.6)	3 (3.6)	15.4–21.1	17.9
	M. fortuitum	5 (6)	5 (6)	18.3–23.2	20.8
	M. gordonae	3 (3.6)	3 (3.6)	21.6–31.3	26
	M. kansasii	2 (2.4)	2 (2.4)	21.2–23.5	22.4
	M. mucogenicum	2 (2.4)	2 (2.4)	23.2–26.9	25.1
	M. chelonae	1 (1.2)	1 (1.2)	22.4	22.4
	M. intracellulare and *M. massiliense*	1 (1.2)	1 (1.2)[b]	17	17
	M. avium and *M. massiliense*	1 (1.2)	1 (1.2)[b]	19.7	19.7
Total		84	84		

[a] This culture sample was identified as *M. massiliense* by MolecuTech REBA Myco-ID® but was identified with the *M. abscessus*-specific probe using Real Myco-ID®.
[b] Mixed culture with *M. intracellulare* and *M. massiliense*, *M. avium* and *M. massiliense* by MolecuTech REBA Myco-ID®, but was only detected with the *M. abscessus*-specific probe using Real Myco-ID®.

Results of three molecular assays for detecting MTB and NTM with clinical samples compared to AFB smear scores

In order to confirm the results of the Real Myco-ID® assay, other molecular assays, the AdvanSure TB/NTM real-time PCR assay and MolecuTech REBA Myco-ID® were performed with 279 clinical samples. These results among the AdvanSure TB/NTM real-time PCR, MolecuTech REBA Myco-ID® (Fig. 3), and Real Myco-ID® assay were completely concordant (100%) in all 279 clinical isolates. Of these 279 samples, 195 were MTB and 84 were NTM (Table 2). Among the 185 AFB smear negative cases, 126 (68.1%) were MTB and 59 (31.9%) were NTM. Among the seven AFB smear trace cases, four (57.1%) were MTB and three (42.9%) were NTM. Among the 70 AFB 1+ cases, 55 (51.1%) were MTB and 15 (21.4%) were NTM. Among the 15 AFB 2+ cases, 10 (66.7%) were MTB and five (33.3%) were NTM. Of the two AFB 3+ cases, both (100%) were only NTM (Table 2).

Comparison of results from PCR-REBA and Real Myco-ID® assay with liquid cultures for mycobacterial identification

Among the total 279 samples, 84 samples were detected as NTM by real-time PCR assay and conventional methods. Of these 84 cases, 38 (45.2%) *M. intracellulare*, 18 (21.4%) *M. avium*, 10 (11.9%) *M. massiliense*, five (6%) *M. fortuitum* complex, three (3.6%) of *M. abscessus* and *M. gordonae*, two (2.4%) of *M. kansasii* and *M. mucogenicum*, and one (1.2%) *M. chelonae* was identified by PCR-REBA assay with culture samples (Table 3). Two cases were identified as mixed infections (*M. intracellulare-M. massiliense* and *M. avium-M. massiliense* co-infection). In the Real Myco-ID® assay results, 38 (45.2%) *M. intracellulare* and 18 (21.4%) *M. avium* were all detected using the *M. avium-M. intracellulare* specific probe, 10 (11.9%) *M. massiliense* and three (3.6%) *M. abscessus* were all detected using the *M. abscessus*-specific probe. Also, the results between *M. fortuitum* complex, *M. gordonae*, *M. kansasii*, *M. mucogenicum*, and *M. chelonae* by PCR-REBA assay were completely concordant (100%). Two cases were identified as mixed infection (*M. intracellulare-M. massiliense* and *M. avium-M. massiliense* co-infection) the by PCR-REBA assay were all detected using the *M. abscessus*-specific probe (Table 3).

Discussion

NTM, previously believed to be nonpathogenic, has emerged as a significant cause of infections (Park, 2009; Daley and Griffith, 2010) as the prevalence of NTM infection has been increasing while that of TB has been decreasing from 2002 to 2008 (Park *et al.*, 2010). In the Republic of Korea, the rates of both NTM isolation and NTM-associated lung diseases have increased. The rate of NTM isolation from sputum specimens obtained at a tertiary-care medical center in the Republic of Korea increased from 43% in 2001 to 70% in 2011, and the NTM recovery rate from AFB smear-positive sputum specimens increased from 9% in 2001 to 64% in 2011 (Koh *et al.*, 2013). Since the use of liquid media for mycobacterial culture has increased in the Republic of Korea, the increased rate of NTM recovery may be due, at least in part, to increased culture in liquid media (Jeon *et al.*, 2005). The accurate detection and identification of NTM to the species level is essential because patients with NTM infections show clinical findings that are similar to those of patients with TB, despite the different chemotherapeutic regimens (Brown-Elliott *et al.*, 2012). The rapid identification of mycobacterial species in cultures serves an important role in the clinical decision making process and in public health algorithms. For example, immunocompromised patients with risk factors for TB and a culture positive for mycobacteria would be treated with antibiotics active against MTB and NTM and placed in respiratory isolation until the organism was identified. Thus, rapid identification of the organism not only would shorten unnecessary antibiotic exposure but also could prevent the financially and emotionally costly placement of an individual in respiratory isolation (van Ingen *et al.*, 2012). Specific diagnostic methods for the rapid detection and differentiation of MTB infections from NTM are of

major clinical importance. Identification based on the use of chemical inhibitors of growth is reliable but still has disadvantages because it is time consuming, expensive, and can eventually yield false results (Giampaglia *et al*., 2007). Use of the real-time PCR assay for the diagnosis of many infectious diseases is increasing, as it represents an appealing alternative to conventional PCR assays. It is an improvement over conventional methods because of its increased sensitivity and specificity, low contamination risk, and ease of performance and speed (Espy *et al*., 2006). Recently, new methods reported a real-time PCR method based on melting curve analysis using multiple hybridization probes (HybProbes) and high resolution melting analysis (HRMA) using 16S rRNA-based real-time PCR (Issa *et al*., 2014) for rapid differential identification of mycobacterium species. HybProbe pair based on a T_m comparison among the limited number of different *Mycobacterium* species. However, this method revealed the limitation like a variation of the target sequences potentially leading to a detection failure or misidentification (Lim *et al*., 2008). Also, one limitation of HRMA is that the sensitivity and specificity in an individual clinical diagnostic setting are variable, requires only ng amounts of DNA, and sample size effect on HRMA accuracy (Li *et al*., 2011; Yang *et al*., 2014).

In order to evaluate the clinical usefulness of Real Myco-ID® assay, 10 different *Mycobacterium* species-specific probes were used to evaluate DNA samples isolated from liquid cultures. In the present study, there was 100% concordance in the identification of the mycobacterial species (Table 2) between the results of the AdvanSure TB/NTM real-time PCR and MolecuTech REBA Myco-ID® assays in 279 liquid cultures that were compared with the results of the Real Myco-ID®. Four out of these 10 species-specific probes gave positive fluorescence signals for both *M. avium-M. intracellulare*, *M. fortuitum* complex-*M. marinum*, *M. kansasii-M. gastri*, and *M. gordonae-M. szulgai* mixed cultures. Our study also showed that 38 *M. intracellulare*, 18 *M. avium*, 10 *M. massiliense*, and three *M. abscessus* resulted in positive fluorescence signals with both the *M. avium-M. intracellulare* and *M. abscessus* specific probes, respectively. Although the MolecuTech REBA Myco-ID® is distinguish between *M. avium-M. intracellulare* and *M. abscessus-M. massiliense* co-infections, Real Myco-ID® resulted in both positive signals with the *M. avium-M. intracellulare* complex (MAC) and the *M. abscessus* complex (including *M. massiliense* and *M. bolletii*). In addition, two cases of AFB smear negatives were identified as mixed infections (*M. intracellulare-M. massiliense* and *M. avium-M. massiliense* co-infection) by the MolecuTech REBA Myco-ID® assay; however, only *M. massiliense* was identified using the *M. abscessus*-specific probe by the Real Myco-ID® assay and also *M. abscessus* by *rpoB* gene sequence analysis (Table 3). The reason for the single positive fluorescence signal results was due to the genomic DNA concentration of the *M. massiliense*, which may have contained more DNA than that of the *M. intracellulare* or *M. avium* for target amplification because the rapidly growing mycobacteria (RGM) such as *M. abscessus* and *M. massiliense* can be grown more rapidly than that of the slow growing *Mycobacterium* species such as the *M. avium-M. intracellulare* complex (MAC) in liquid culture.

In many countries, the MAC is the most commonly isolated pathogen in NTM pulmonary disease, followed by *M. abscessus* and *M. kansasii* (Simons *et al*., 2011). Particularly in the Republic of Korea, MTB infections account for more than 93% of mycobacterial infections, and almost 98% of clinically significant NTM infections are known to be caused by *M. avium*, *M. intracellulare*, the *M. abscessus* complex, the *M. fortuitum* complex, and *M. kansasii* (Koh *et al*., 2005). In this study, *M. avium-M. intracellulare* complex (n=56, 66.6%) and the *M. abscessus-M. massiliense* complex (n=13, 15.5%) accounted for the majority of isolated NTM species, while the identification of the *M. fortuitum* complex (n=5, 6%) and *M. kansasii* (n=2, 2.4%) was relatively uncommon.

M. chelonae only identified (1.2%) case and both the *M. terrae-M. nonchromogenicum* complex and *M. celatum* were not detected in this study. In order to consider the importance of accurate and rapid identification of NTM species, further study is therefore necessary to identify these species in direct respiratory specimens. The results show that the multi-probe real-time PCR TaqMan assay is rapid as it usually took no more than 4 h to complete the entire experiment, which included only 1 h of sample preparation and 1.5 h for target DNA amplification because thermal cycling is much faster and amplicon detection is performed in real time. It allowed for the rapid and highly sensitive and specific detection of MTB and NTM and it also could identify mycobacterial species with liquid cultures without the post-PCR process. Therefore, the real-time PCR TaqMan assay using multiple probes may provide essential information for accelerating therapeutic decisions for earlier and adequate prescription of antibiotics in the acute phase of mycobacterial infections.

Acknowledgements

This study was supported by a grant of the Korea Healthcare Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (Grant HI10C1708 [A101750] to H. L.) and supported by a grant of the BK21 Plus Project, Ministry of Education, Republic of Korea (22A20130000035).

References

Alcaide, F. and Santín, M. 2008. Multidrug-resistant tuberculosis. Enferm. *Infect. Microbiol. Clin.* **13**, 54–60.

Aravindhan, V., Sulochana, S., Narayanan, S., Paramasivam, C.N., and Narayanan, P.R. 2007. Identification & differentiation of *Mycobacterium avium* & *M. intracellulare* by PCR-RFLP assay using the *gro*ES gene. *Indian J. Med. Res.* **126**, 575–579.

Bae, E., Im, J.H., Kim, S.W., Yoon, N.S., Sung, H., Kim, M.N., and Shim, T.S. 2008. Evaluation of combination of BACTEC mycobacteria growth indicator tube 960 system and Ogawa media for Mycobacterial culture. *Korean J. Lab. Med.* **28**, 299–306.

Bannalikar, A.S. and Verma, R. 2006. Detection of *Mycobacterium avium* & *M. tuberculosis* from human sputum cultures by PCR-RFLP analysis of *hsp*65 gene & *pnc*A PCR. *Indian J. Med. Res.* **123**, 65–172.

Brown-Elliott, B.A., Nash, K.A., and Wallace, R.J. Jr. 2012. Antimicrobial susceptibility testing, drug resistance mechanisms, and therapy of infections with nontuberculous mycobacteria. *Clin.*

Microbiol. Rev. **25**, 545–582.

Chakravorty, S. and Tyagi, J.S. 2005. Novel multipurpose methodology for detection of Mycobacteria in puilmonary and extrapulmonary specimens by smear microscopy, culture, and PCR. *J. Clin. Microbiol.* **43**, 2697–2702.

Chen, R., Gao, X.B., Liu, Z.H., Shen, X.B., Guo, A.Z., Duan, Y.Y., Liu, Z.L., Wu, X.W., and Zhu, D.Z. 2013. Combination of multiplex PCR with denaturing high-performance liquid chromatography for rapid detection of *Mycobacterium* genus and simultaneous identification of the *Mycobacterium tuberculosis* complex. *Diagn. Microbiol. Infect. Dis.* **77**, 53–57.

Daley, C.L. and Griffith D.E. 2010. Pulmonary non-tuberculous mycobacterial infections. *Int. J. Tuberc. Lung Dis.* **14**, 665–671.

Espy, M.J., Uhl, J.R., Sloan, L.M., Buckwalter, S.P., Jones, M.F., Vetter, E.A., Yao, J.D., Wengenack, N.L., Rosenblatt, J.E., Cockerill, F.R. 3rd, *et al.* 2006. Real-time PCR in clinical microbiology: applications for routine laboratory testing. *Clin. Microbiol. Rev.* **19**, 165–256.

Giampaglia, C.M., Martins, M.C., Chimara, E., Oliveira, R.S., de Oliveira Vieira, G.B., Marsico, A.G., Mello, F.C., de Souza Fonseca, L., Kritski, A., and da Silva Telles, M.A. 2007. Differentiation of *Mycobacterium tuberculosis* from other mycobacteria with p-nitrobenzoic acid using MGIT960. *Int. J. Tuberc. Lung Dis.* **11**, 803–807.

Griffith, D.E., Aksamit, T., Brown-Elliott, B.A., Catanzaro, A., Daley, C., Gordin, F., Holland, S.M., Horsburgh, R., Huitt, G., Iademarco, M.F., *et al.* 2007. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. *Am. J. Respir. Crit. Care. Med.* **175**, 367–416.

Ichiyama, S., Iinuma, Y., Yamori, S., Hasegawa, Y., Shimokata, K., and Nakashima, N. 1997. Mycobacterium growth indicator tube testing in conjunction with the AccuProbe or the AMPLICOR-PCR assay for detecting and identifying mycobacteria from sputum samples. *J. Clin. Microbiol.* **35**, 2022–2025.

Issa, R., Abdul, H., Hashim, S.H., Seradja, V.H., Shaili, N., and Hassan, N.A. 2014. High resolution melting analysis for the differentiation of *Mycobacterium* species. *J. Med. Microbiol.* **63**, 1284–1287.

Jarzembowski, J.A. and Young, M.B. 2008. Nontuberculous mycobacterial infections. *Arch. Pathol. Lab. Med.* **132**, 1333–1341.

Jeon, K., Koh, W.J., Kwon, O.J., Suh, G.Y., Chung, M.P., Kim, H., Lee, N.Y., Park, Y.K., and Bai, G.H. 2005. Recovery rate of NTM from AFB smear-positive sputum specimens at a medical centre in South Korea. *Int. J. Tuberc. Lung Dis.* **9**, 1046–1051.

Kim, Y.J., Park, M.Y., Kim, S.Y., Cho, S.A., Hwang, S.H., Kim, H.H., Lee, E.Y., Jeong, J., Kim, K.H., and Chang, C.L. 2008. Evaluation of the performances of AdvanSure TB/NTM real time PCR kit for detection of mycobacteria in respiratory specimens. *Korean J. Lab. Med.* **28**, 34–38.

Koh, W.J., Chang, B., Jeong, B.H., Jeon, K., Kim, S.Y., Lee, N.Y., Ki, C.S., and Kwon, O.J. 2013. Increasing recovery of nontuberculous Mycobacteria from respiratory specimens over a 10-year period in a tertiary referral hospital in South Korea. *Tuberc. Respir. Dis.* **75**, 199–204.

Koh, W.J., Kwon, O.J., and Lee, K.S. 2005. Diagnosis and treatment of nontuberculous mycobacterial pulmonary diseases: a Korean perspective. *J. Korean Med. Sci.* **20**, 913–925.

Lee, H.Y., Park, H.J., Cho, S.N., Bai, G.H., and Kim, S.J. 2000. Species identification of mycobacteria by PCR-restriction fragment length polymorphism of the *rpoB* gene. *J. Clin. Microbiol.* **38**, 2966–2971.

Lee, S.K., Lee, E.J., Kim, S.K., Chang, J., Jeong, S.H., and Kang, Y.A. 2012. Changing epidemiology of nontuberculous mycobacterial lung disease in South Korea. *Scand. J. Infect. Dis.* **44**, 733–738.

Li, B.S., Wang, X.Y., Ma, F.L., Jiang, B., Song, X.X., and Xu, A.G. 2011. Is high resolution melting analysis (HRMA) accurate for detection of human disease-associated mutations? A meta analysis. *PLoS One* **6**, e28078.

Lim, S.Y., Kim, B.J., Lee, M.K., and Kim, K. 2008. Development of a real-time PCR-based method for rapid differential identification of Mycobacterium species. *Lett. Appl. Microbiol.* **46**, 101–106.

Maurya, A.K., Nag, V.L., Kant, S., Kushwaha, R.A., Kumar, M., Mishra, V., Rahman, W., and Dhole, T.N. 2012. Evaluation of an immunochromatographic test for discrimination between *Mycobacterium tuberculosis* complex & non tuberculous mycobacteria in clinical isolates from extra-pulmonary tuberculosis. *Indian J. Med. Res.* **135**, 901–906.

Park, J.S. 2009. Respiratory review of 2009: nontuberculous Mycobacterium. *Tuberc. Respir. Dis.* **67**, 395–401.

Park, Y.S., Lee, C.H., Lee, S.M., Yang, S.C., Yoo, C.G., Kim, Y.W., Han, S.K., Shim, Y.S., and Yim, J.J. 2010. Rapid increase of nontuberculous mycobacterial lung diseases at a tertiary referral hospital in South Korea. *Int. J. Tuberc. Lung Dis.* **14**, 1069–1071.

Pérez-Osorio, A.C., Boyle, D.S., Ingham, Z.K., Ostash, A., Gautom, R.K., Colombel, C., Houze, Y., and Leader, B.T. 2012. Rapid identification of mycobacteria and drug-resistant *Mycobacterium tuberculosis* by use of a single multiplex PCR and DNA sequencing. *J. Clin. Microbiol.* **50**, 326–336.

Simons, S., van Ingen, J., Hsueh, P.R., Van Hung, N., Dekhuijzen, P.N., Boeree, M.J., and van Soolingen, D. 2011. Nontuberculous mycobacteria in respiratory tract infections, eastern Asia. *Emerg. Infect. Dis.* **17**, 343–349.

Thanachartwet, V., Desakorn, V., Duangrithi, D., Chunpongthong, P., Phojanamongkolkij, K., Jitruckthai, P., Kasetjaroen, Y., and Pitisuttithum, P. 2014. Comparison of clinical and laboratory findings between those with pulmonary tuberculosis and those with nontuberculous mycobacterial lung disease. *Southeast Asian J. Trop. Med. Public Health* **45**, 85–94.

van Ingen, J., Boeree, M.J., van Soolingen, D., and Mouton, J.W. 2012. Resistance mechanisms and drug susceptibility testing of nontuberculous mycobacteria. *Drug Resist. Updat.* **15**, 149–161.

Wang, H.Y., Bang, H., Kim, S., Koh, W.J., and Lee, H. 2014. Identification of *Mycobacterium* species in direct respiratory specimens using reverse blot hybridization assay. *Int. J. Tuberc. Lung Dis.* **18**, 1114–1120.

World Health Organization. 2013. Global tuberculosis report. http://www.who.int/tb/publications/global_report/en/. World Health Organization (ed.) Appia, Geneva, Switzerland: WHO Press, 2013.

Yang, M., Yue, Y.J., Guo, T.T., Han, J.L., Liu J.B., Guo, J., Sun, X.P., Feng, R.L., Wu, Y.Y., Wang, C.F., *et al.* 2014. Limitation of high-resolution melting curve analysis for genotyping simple sequence repeats in sheep. *Genet. Mol. Res.* **13**, 2645–2653.