Microplate Hybridization Assay for Detection and Differentiation of *Mycobacterium tuberculosis* from Non-tuberculous Mycobacteria

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Dedicated to my family and my friends.

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ABBREVIATION

AIDS	:	Acquired immune deficiency syndrome
BSA	:	Bovine serum albumin
DIG	:	Digoxigenin
ELISA	:	Enzyme-linked immunosorbent assay
GAPDH	:	Glyceraldehyde 3-phosphate dehydrogenase
HIV	:	Human immunodeficiency virus
MAC	:	*Mycobacterium avium* complex
MTB	:	*Mycobacterium tuberculosis*
NTM	:	Non-tuberculous mycobacteria
PBST	:	Phosphate bufferd saline tween-20
PCR	:	Polymerase chain reaction
rpoB	:	RNA polymerase beta subunit
SDS	:	Sodium dodecyl sulfate
SSPE	:	Saline-sodium phosphate-EDTA
TMB	:	3,3',5,5'-tetramethyl benzidine

ABSTRACT

Microplate Hybridization Assay for Detection and Differentiation of *Mycobacterium tuberculosis* from Non-tuberculous Mycobacteria

Tuberculosis caused by *Mycobacterium tuberculosis* (MTB) still remains to be the most dreadful infectious disease affecting almost every country. In addition, with the recent increase in the frequency of non-tuberculous mycobacterial infections, it is becoming increasingly necessary to differentiate MTB from non-tuberculous mycobacteria (NTM) infections because the remedies for diseases resulting from NTM should be different from those for MTB infection.

In the present study, a simple, rapid, accurate and sensitive microplate hybridization assay for detecting MTB was developed. For this, a region of the *rpoB* gene was used to design PCR primers, MTB probe and *Mycobacterium* genus-specific probe molecules. The specificity of the assay was confirmed using DNA extracted from fifteen different mycobacterial reference strains and twelve different non-mycobacterial

reference strains, and the sensitivity was determined to be 100 fg using genomic DNA of MTB reference strain, H37Rv. Subsequently, DNAs extracted from 62 sputum samples with diverse smear scores and culture positive results were used to evaluate the assay performance. In brief, the sensitivity of the assay were 98.4% (61/62).

Next, a microplate hybridization assay for identifying NTM species was developed. For this, the species-specific probes for 13 different NTM species which are frequently detected in clinical practice were designed based on a region of the rpoB gene. The specificity of the individually probes was confirmed using DNA extracted from 15 mycobacterial reference strains including MTB, and 12 non-mycobacterial reference strains.

The findings from the experiment above suggest that microplate hybridization assay is capable of isolating and differentiating MTB from NTM, as well as molecular diagnostic method with good sensitivity identification of NTM. From this, it is expected that the microplate hybridization assay developed in this study may be a useful.

Key words: Microplate hybridization assay, *Mycobacterium tuberculosis*, Nontuberculosis mycobacteria, PCR

CHAPTER I

A Microplate Hybridization Assay for Detection and Differentiation of *Mycobacterium tuberculosis* from Non-tuberculous Mycobacteria

I. INTRODUCTION

Tuberculosis is known as one of the most life-threatening infectious diseases, which is prevalent across the world. According to a report released by WHO in 2007, about 8.8 million people develop active TB and 1.6 million people die from the infection in each year (1, 2). The resurgence of tuberculosis (TB) in developed as well as developing countries since 1980 has been associated with the HIV epidemic, the emergence of drug-resistant strains, and increases in emigration from regions with high rates of disease endemicity (3-5). To treat such a number of patients with TB effectively, rapid and accurate detection of *Mycobacterium tuberculosis* (MTB) is absolutely essential. In addition, with the recent increase in the frequency of non-tuberculous mycobacterial infections, it is also becoming increasingly important to differentiate MTB from non-tuberculous mycobacteria (NTM) infections, in order to treat patient effectively, remedies for diseases resulting from NTM should be different from those for MTB infection (6).

Worldwide, conventional smear microscopy and culture method are used for detection and differentiation of MTB infection (7). However, conventional smear microscopy, although simple and economical, can not differentiate MTB from NTM, since both of them are acid-fast

positive bacilli by the standard Ziehl-Neelson staining. Furthermore, it is still too insensitive to detect MTB infection at optimum level (about 5,000-10,000 bacilli per ㎖ of sputum) (8, 9). On the other hand, even though it is more sensitive and accurate than smear test, culture takes at least 3-6 weeks of time, and still needs subsequent biochemical tests for differentiation of MTB from NTM (10).

As part of an effort to improve such problems, many diagnostic methods based on molecular biological techniques have been developed (11-16). Of them, polymerase chain reaction (PCR) has been most widely evaluated and implemented in clinical settings. For many years, the PCR results have been confirmed by gel electrophoresis in most laboratories.

Although the specificity and the sensitivity of the PCR employing gel electrophoresis have been considered to be acceptable, it has some limitations. First of all, since the gel electrophoresis only confirms the size of the PCR amplicons, the specificity of the assay can be often decreased when false priming occurs during the amplification. Secondly, the sensitivity of the PCR assay can also be decreased, since the detection of amplicons by gel electrophoresis depends merely on the laboratory personnel's vision. A cut-off value would be useful if it is introduced to interpret PCR results.

Recently, real-time PCR which employs specific probe molecules to obtain higher specificity and sensitivity using fluorescent dyes and sensor has been developed (17). However, it requires expensive equipments, which makes it difficult to be applied for routine clinical use especially in countries with high burden of TB problem.

As an effort to overcome the problems with PCR employing gel electrophoresis and offer an alternative inexpensive method with advantage of real-time PCR, this study was set to develop specific and sensitive way of analyzing PCR amplicons using microplate hybridization assay.

II. MATERIALS AND METHODS

1. Genomic DNAs from reference strains

DNAs extracted from fifteen different mycobacterial reference strains (Table 1) and twelve different nonmycobacterial reference strains (Table 2) were used to conform the specificity of the probe molecues. The genomic DNAs of reference strains were obtained from M&D (Wonju, Kangwon, Korea).

2. Genomic DNAs from clinical specimens

DNAs extracted from sixty-two sputum specimens chosen by being either acid-fast bacterium positive or acid-fast bacterium negative but culture positive were obtained from Masan National TB Hospital. The DNAs extracted from sputum specimens were used for microplate hybridization assay testing.

Table 1. Mycobacterial reference strains used in experiments

Species	Source
Mycobacterium tuberculosis	ATCC[a] 19420
Mycobacterium avium	ATCC 25291
Mycobacterium intracellulare	ATCC 13950
Mycobacterium scrofulaceum	ATCC 19981
Mycobacterium szulgai	ATCC 35799
Mycobacterium chelonae	ATCC 35749
Mycobacterium abscessus	ATCC 19977
Mycobacterium gordonae	ATCC 14470
Mycobacterium kansasii	ATCC 12478
Mycobacterium celatum	ATCC 51130
Mycobacterium fortuitum	ATCC 49403
Mycobacterium mucogenicum	ATCC 49650
Mycobacterium senegalense	ATCC 35796
Mycobacterium genevense	ATCC 51233
Mycobacterium malmoense	ATCC 29571

[a] : American Type Culture Collection.

Table 2. Nonmycobacterial reference strains used in experiments

Species	Source
E. coli O157:H7	ATCC [a] 43894
Shigella flexneri	ATCC 9199
Shigella sonnei	ATCC 25931
Salmonella serovar Typhi	ATCC 19430
Citobacter freundii	ATCC 6750
Enterobacter aerogenes	ATCC 13048
Klebsiella pneumonia	ATCC 35657
Pseudomonas aeruginosa	ATCC 27853
Yersinia enterocolitica	ATCC 9610
Yersinia pseudotuberculosis	ATCC 29833
Enterococcus faecalis	ATCC 19433
Listeria monocytogenes	ATCC 15313

[a] : American Type Culture Collection.

3. Primers & oligonucleotide probes

The primers and probes were prepared based upon the mycobacterial strain sequence and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene sequence obtained from Genebank (http://www.ncbi.nlm.nih.gov). The amplification of *rpoB* gene and *GAPDH* gene were performed with the primers shown in Table 3. The 5'end of each reverse primer was labelled with biotin. The probes used in microplate hybridization assay shown in Table 4. The 5' end of each probe was labelled with digoxigenin (DIG). These primers and probes were synthesized and labelled by Bioneer (Daejeon, Korea) and Sigma-Proligo (The Woodlands, TX, USA), respectively.

Table 3. Sequences of primers used for amplification of target gene

Target gene	Primer name	Sequence (5' → 3')
rpoB	360Long-F	TCAAGGAGAAGCGCTACGACCTGGC
	200-R*	CGVCGGTTRCCGAAGTGGTCG
GAPDH	IC-F	CCATCTTCCAGGAGCGAGATCC
	IC-R*	ATGGTGGTGAAGACGCCAGTG

* : biotin-labelled primer

Table 4. Sequences of probes used in microplate hybridization assay

Probe name	Sequence (5' → 3')
M. tuberculosis-specific MTB* (MTB)	CATGTCGGCGAGCCC
Mycobacterium genus-specific Myc* (Myc)	GACGTCGTCGCCACCATCGA
GAPDH-specific IC* (IC)	TGCTGGCGCTGAGTACGTCGTGGA

* : digoxigenin-labelled probe

4. PCR amplification

AccPower PCR Premix (Bioneer, Daejeon, Korea) was mixed with 10 pmole of each primer and 5 ul of template DNA so that the total amount reached 50 ul. For PCR cycle, a single cycle was made up of denaturation at 95℃ for 5 min.; denaturation at 95℃ for 30 sec.; primer annealing at 65℃ for 30 sec.; and finally, primer extension at 72℃ for 30 sec. Thirty-five cycles were given followed by primer extension at 72℃ for 7 min. 230 base pair sized PCR amplified products were obstained.

5. Microplate hybridization assay

Pre-incubation : Streptavidin coated microplate wells (Nunc, Nunc immobilizerTM streptavidin, Kamstrupvej, Denmark) were washed three times with phosphate buffer saline (pH 7.5) and 0.5% (v/v) tween 20 (PBST).

PCR product binding : One hundred fifty microliters of PBST containing 0.05% bovine serum albumin (BSA, Roche, Indiana, USA) was dispensed into each well, 5 ul PCR product was added, followed by incubation at 37℃ for 30 min.

Denaturation of PCR product : One hundred microliters of 0.2 N NaOH was added followed by denaturation at RT for 15 min. The microplate wells were washed with PBST three times

Probe hybridization : The microplate wells were added the diluted probe solution, and incubated at 50℃ for 30 min. Subsequently, the microplate wells were washed with PBST at 50℃ for 5 min three times.

Enzyme incubation : The microplate wells were added anti-DIG-peroxidase (Roche, Indiana, USA), and incubated at 37℃ for 30 min. Finally, the microplate wells were washed with PBST five times, added 100 ul of the mixture (TMB (3,3',5,5'-tetrametyl benzidine, Sigma-Aldrich, Saint Louis, USA) and 0.1 % H_2O_2 in the ratio of 1:1) followed by incubation for 10 min, added 100 ul of 1 N HCl to stop the reaction, and subsequently, measured absorbance values at 450 nm by ELISA reader (Molecular Devices, California, USA). Schematic diagram of the expriment is shown at Figure 1.

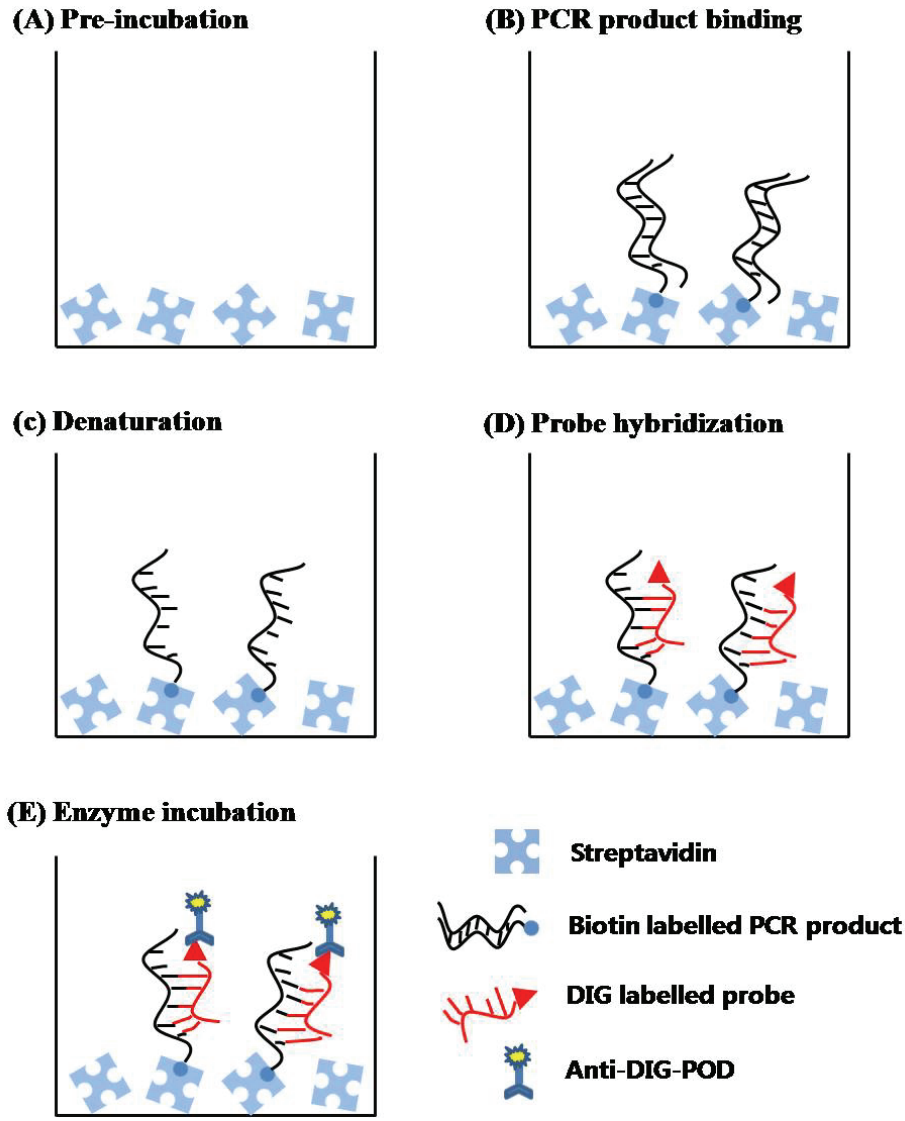


Figure 1. Diagrammatic representation of microplate hybridization assay.

6. Data presentation

All assays were set up in triplicate, and the results were expressed as mean ± SD. Statistical significance for chemical or temporal effect was examined by independent pairs Student's *t*-test at P = 0.05 or 0.01.

III. RESULTS

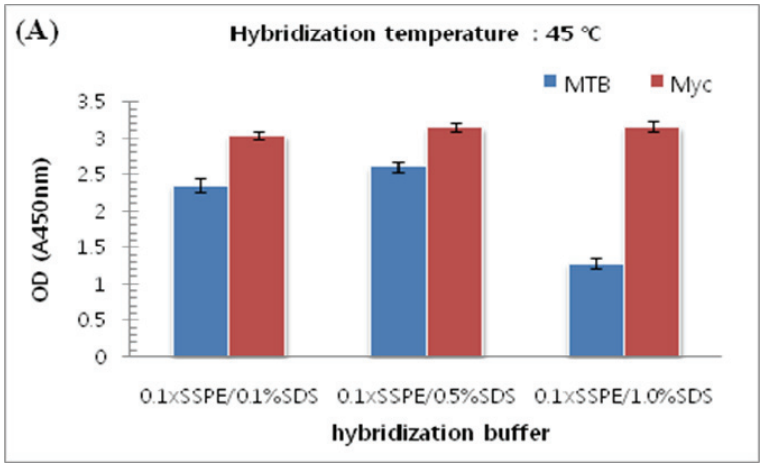
1. Optimal conditions for the microplate hybridization assay

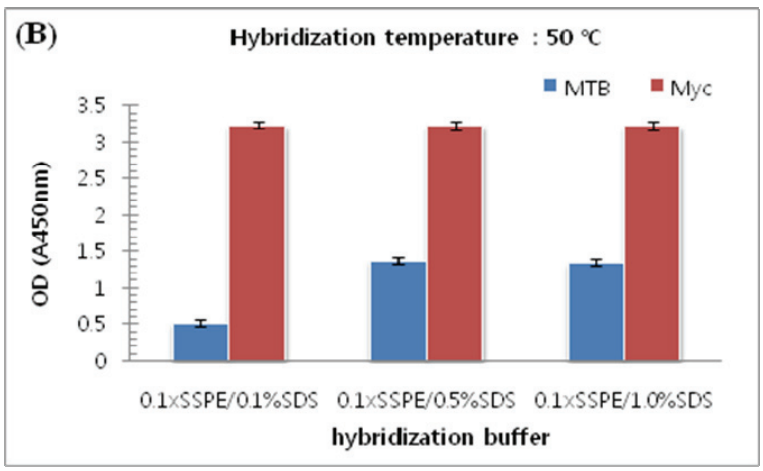
In order to develop microplate hybridization assay which can detect MTB as well as differentiate MTB from NTM simultaneously, a selective region of the *rpoB* gene was used to design PCR primers, and MTB-specific (MTB probe) and *Mycobacterium* genus-specific probe (Myc probe) molecules, respectively.

Several conditions were experimented to determine the optimal conditions for microplate hybridization assay. First, the hybridization conditions of the designed probe were experimented, suggesting that the optimal reaction was induced in 0.1x SSPE/0.1% SDS at 50℃ (Figure 2). Next, washing conditions were experimented, showing that washing three times for five minutes at 50℃ was optimal (Figure 3). In addition, different conditions including the concentrations of probe and anti-DIG-peroxidase were also tested (data not shown). Based upon the combination of the above results, the optimal conditions for microplate hybridization assay were finally determined (Table 5).

And the cut-off value determined in microplate hybridization assay was background signal value + 3 standard deviations (18). The background signal value corresponded to the mean absorbance measured

in a well that contained specific probes and PCR negative conrol products. Accordingly, the cut-off value was set at 0.2 (data not shown).


(A)
Hybridization temperature : 45 ℃
MTB
Myc
OD (A450nm)
3.5
3
2.5
2
1.5
1
0.5
0
0.1xSSPE/0.1%SDS
0.1xSSPE/0.5%SDS
0.1xSSPE/1.0%SDS
hybridization buffer


(B)
Hybridization temperature : 50 ℃
MTB
Myc
OD (A450nm)
3.5
3
2.5
2
1.5
1
0.5
0
0.1xSSPE/0.1%SDS
0.1xSSPE/0.5%SDS
0.1xSSPE/1.0%SDS
hybridization buffer

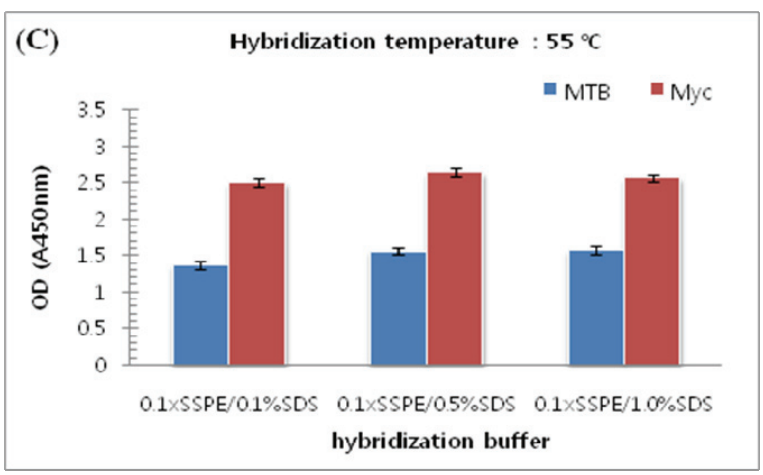
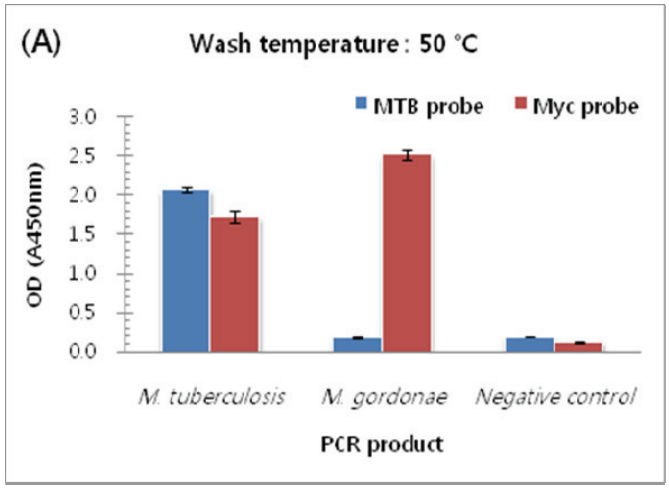
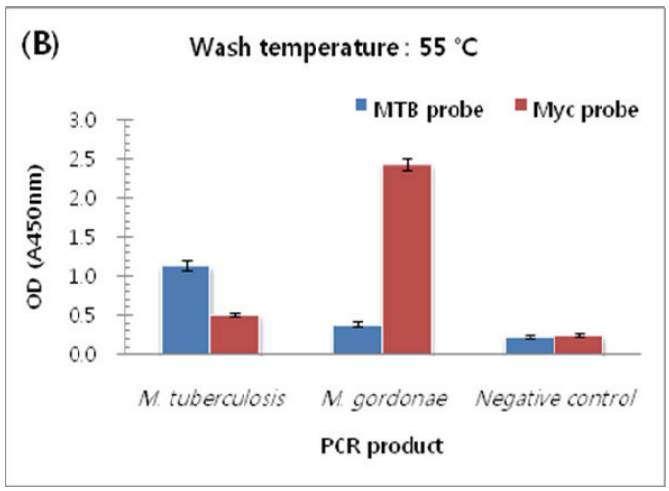

(C)
Hybridization temperature : 55 ℃
MTB
Myc
OD (A450nm)
3.5
3
2.5
2
1.5
1
0.5
0
0.1xSSPE/0.1%SDS
0.1xSSPE/0.5%SDS
0.1xSSPE/1.0%SDS
hybridization buffer

Figure 2. Condition of the microplate hybridization assay (1). The hybridization conditions of the designed probes were tested with DNAs extracted form *M. gordonae*, and it was found that the optimal conditions are given in 0.1x SSPE/0.1% SDS at 50℃. Experimental procedure: (1) pre-incubation: wash three times with PBST.; (2) PCR product binding: at 37℃ for 30 min.; (3) denaturation: place in 0.2 NaOH at RT for 15 min, and then, wash three times with PBST.; (4) probe hybridization: set aside for 30 min under the respective specified conditions, followed by washing three times with PBST.; (5) enzyme incubation : after incubation at 37℃ for 30 min, wash five times with PBST.; and, (6) detection : following substrate treatment, leave as it is at RT for 10 min, stop the reaction with 1N HCl, and measure the O.D. at 450 nm.


(A)
Wash temperature : 50 °C
MTB probe
Myc probe
OD (A450nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
M. tuberculosis
M. gordonae
Negative control
PCR product


(B)
Wash temperature : 55 °C
MTB probe
Myc probe
OD (A450nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
M. tuberculosis
M. gordonae
Negative control
PCR product

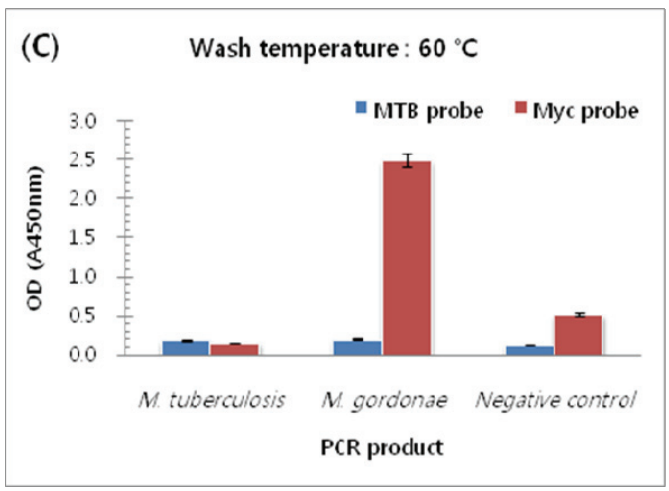

(C)
Wash temperature : 60 °C
MTB probe
Myc probe
OD (A450nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
M. tuberculosis
M. gordonae
Negative control
PCR product

Figure 3. Condition of microplate hybridization assay (2). The washing conditions of the designed probes were tested with DNAs extracted from *M. tuberculosis* and *M. gordonae*, and it was found that washing three times with PBST at 50℃ for 5 min is optimal. Experimental procedure: (1) pre-incubation: wash three times with PBST.; (2) PCR product binding: incubation at 37℃ for 30 min.; (3) denaturation: place in 0.2 NaOH at RT for 15 min, and then wash three times with PBST.; (4) probe hybridization: place in 0.1x SSPE/0.1% SDS at 50℃ for 30 min, and then wash three times with PBST for 5 min at the respective temperatures.; (5) enzyme incubation: after waiting at 37℃ for 30 min, wash five times with PBST.; (6) detection: following substrate treatment, leave as it is at RT for 10 min, stop the reaction with 1N HCl, and measure the O.D. at 450 nm.

Table 5. Procedure of the microplate hybridization assay

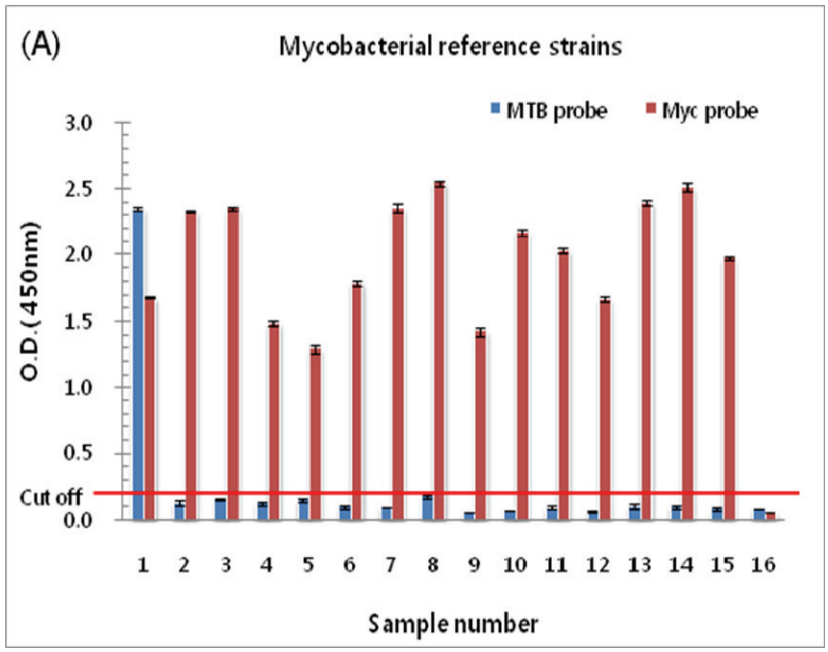
Step	Procedure
1. Pre-Incubation	Wash 3 times with PBST
2. PCR product binding	Incubation at 37 ℃ for 30 min.
3. Denaturation	Place in 0.2 NaOH at RT for 15 min.
	Wash 3 times with PBST
4. Probe hybridization	Place in 0.1x SSPE/0.1% SDS
	at 50 ℃ for 30 min.
	Wash 3 times with PBST at 50 ℃ for 5min
5. Enzyme incubation	Incubation at 37 ℃ for 30 min.
	Wash 5 times with PBST
	Incubation at RT for 10 min.
6. Detection	Stop the reaction with 1N HCl
	Measure the O.D. at 450 nm

2. The specificity of probes used in microplate hybridization assay

The specificity of the microplate hybridization assay with newly designed PCR primers and probe molecules were tested using DNAs extracted from 15 mycobacterial reference strains including MTB and 12 non-mycobacterial reference strains (Figure 4).

While MTB probe only bound to PCR products of reference strain of MTB, it did not bind to any kind of PCR products from other bacterial strains. On the other hand, Myc probe bound to PCR products of all mycobacterial reference strains including MTB, and did not bind to those of other non-mycobacterial reference strains.

The results from this study suggested that two specific probes used in this microplate hybridization assay could specifically detect MTB and differentiate it from NTM as well.


(A)
Mycobacterial reference strains
MTB probe
Myc probe
O.D.(450nm)
3.0
2.5
2.0
1.5
1.0
0.5
Cut off
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Sample number

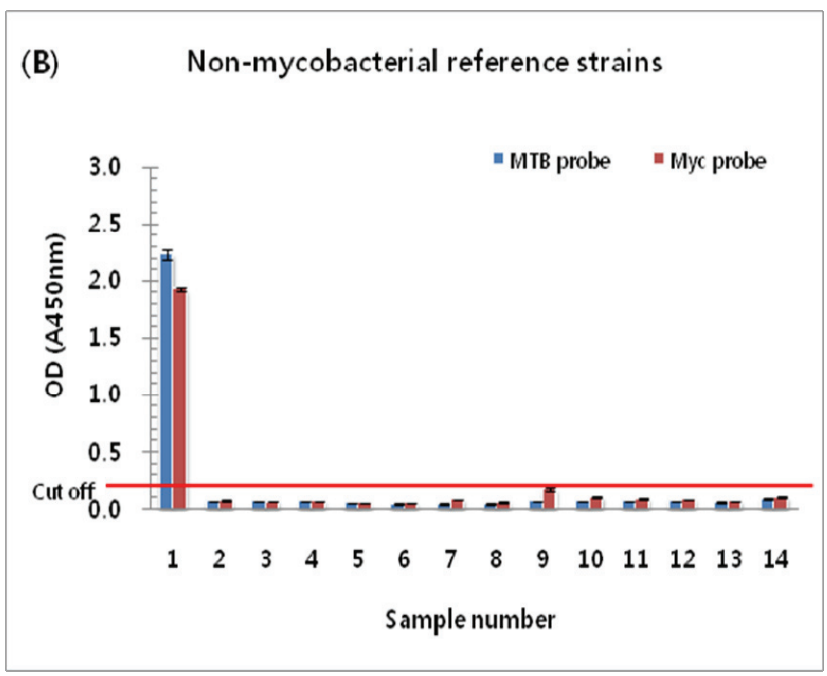

(B)
Non-mycobacterial reference strains
MTB probe
Myc probe
OD (A450nm)
3.0
2.5
2.0
1.5
1.0
0.5
Cut off
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Sample number

Figure 4. The specificity of MTB and Myc probe. MTB probe was positive for DNAs extracted from only MTB (A and B, lane 1) and negative for DNAs extracted from all the mycobacterial species and non-mycobacterial species. (A, lane 2-15 and B, lane 2-13). Myc probe was positive for all the mycobacterial species (A, lane 1-15 and B, lane 1) and negative for all the non-mycobacterial species (B, lane 2-13). (A) Mycobacterial reference strains: 1, *M.tuberculosis*; 2, *M. avium*; 3, *M. intracellulare*; 4, *M. scrofulaceum*; 5, *M. szulgai*; 6, *M. chelonae*; 7, *M. abscessus*; 8, *M. gordonae*; 9, *M. kansasii*; 10, *M. celetium*; 11, *M. fortuitum*; 12, *M. mucogenicum*; 13, *M. senegalense*; 14, *M. genavense*; 15, *M. malmoense*; 16, negative control. (B) MTB and non-mycobacterial reference strains: 1, *M. tuberculosis*; 2, *E. coli* O157:H7; 3, *S. flexneri*: 4, *S. sonnei*; 5, *S.* serovar Typhi; 6, *C. freundii*; 7, *E. aerogenes*; 8, *K. pneumonia*; 9, *P. aeruginosa*; 10, *Y. enterocolitica*; 11, *Y. pseudotuberculosis*; 12, *E. faecalis*; 13, *L. monocytogenes*; 14, Negative control.

3. The sensitivity of MTB-specific probe

To determine the sensitivity of MTB-specific probe, serial 10-fold dilutions of reference strain of MTB genomic DNA were used for PCR amplification. The PCR products were then visualized both using agarose gel electrophoresis and microplate hybridization assay (Figure 5). The results showed that while agarose gel electrophoresis detected PCR products with 1 pg of genomic DNA, the microplate hybridization assay detected PCR products with 100 fg of genomic DNA. In other words, it was revealed that the sensitivity of microplate hybridization assay is 10 times higher than that of agarose gel electrophoresis assay. This results suggests that the microplate hybridization assay can detect the specimens which could have been false negative by agarose gel electrophoresis assay.

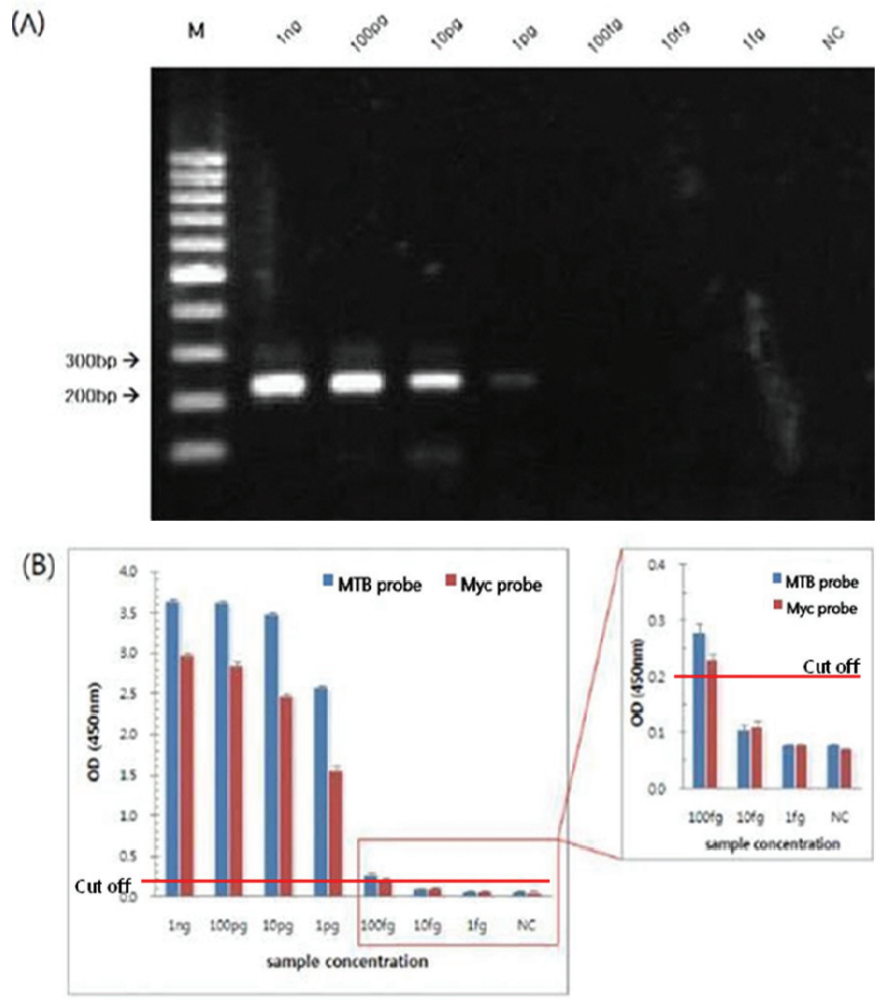
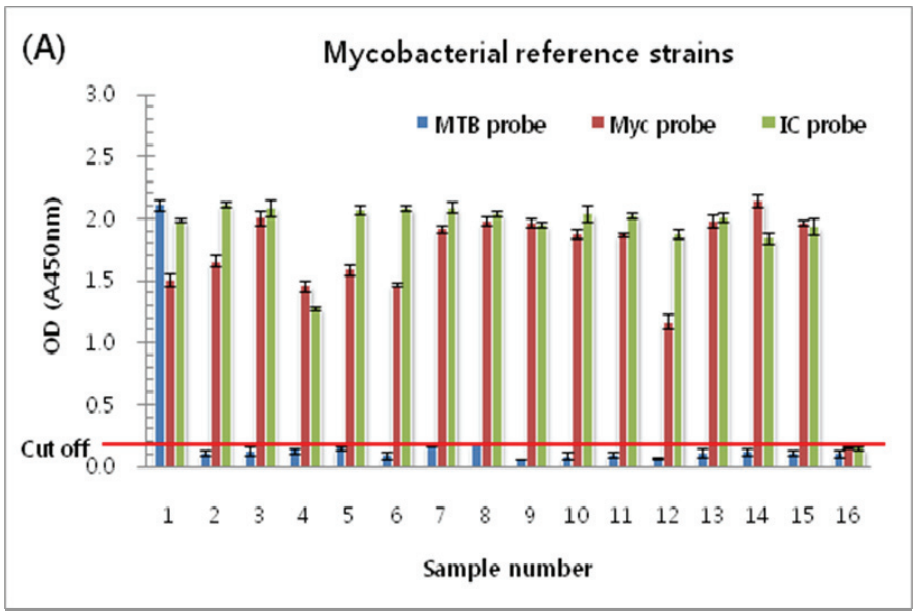


Figure 5. The sensitivity of MTB probe and Myc probe. A series of diluted *M. tuberculosis* DNA (1 ng ~ 1 fg) was used for PCR amplification. After amplification, PCR products were visualized (A) using ethidium bromide stained agarose gel electrophoresis and (B) using microplate hybridization assay. While agarose gel electrophoresis detected PCR products with 1 pg of genomic DNA, microplate

hybridization assay detected PCR products with 100 fg of genomic DNA (cut-off value = 0.2 at 450 nm).

4. Internal control to microplate hybridization assay

A primer which amplifies a specific site in *GAPDH* gene to yield 98 base pairs of products was designed. A specific probe capable of detecting the amplified products was designed, also. These designed primer and probe were tested with DNAs extracted from 15 mycobacterial reference strains including MTB and 12 non-mycobacterial reference strains to ascertain their availability as internal control (Figure 6).


(A)
Mycobacterial reference strains
MTB probe
Myc probe
IC probe
OD (A450nm)
3.0
2.5
2.0
1.5
1.0
0.5
Cut off
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Sample number

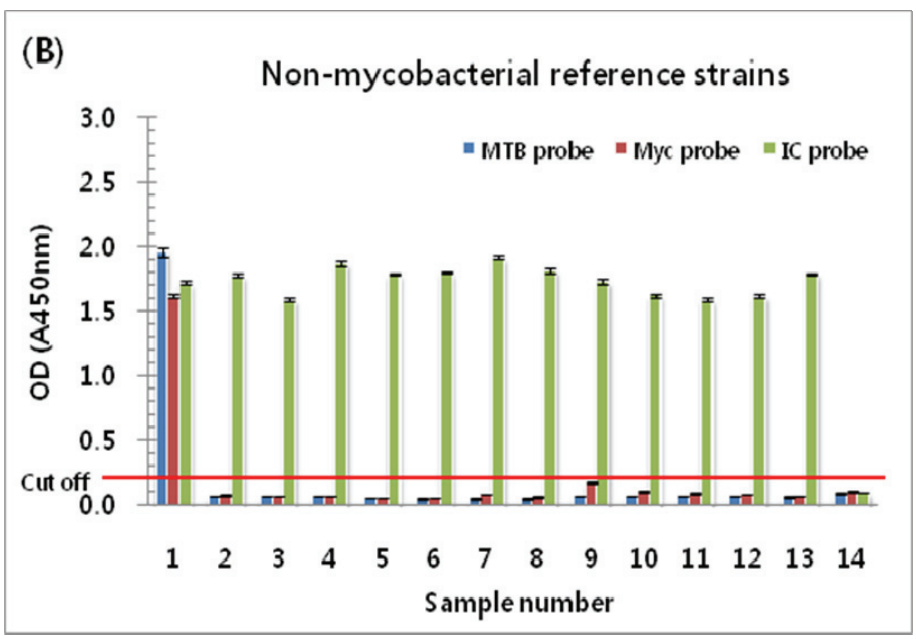

(B)
Non-mycobacterial reference strains
MTB probe
Myc probe
IC probe
OD (A450nm)
3.0
2.5
2.0
1.5
1.0
0.5
Cut off
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Sample number

Figure 6. Internal control to microplate hybridization assay. MTB probe was positive for DNAs extracted from only MTB (A and B, lane 1) and negative for DNAs extracted from all the mycobacterial species and non-mycobacterial species. (A, lane 2-15 and B, lane 2-13). Myc probe was positive for all the mycobacterial species (A, lane 1-15 and B, lane 1) and negative for all the non-mycobacterial species (B, lane 2-13). Internal control probe was positive for all the species (A, lane 1-15 and B, lane 1-13). (A) Mycobacterial reference strains: 1, *M.tuberculosis*; 2, *M. avium*; 3, *M. intracellulare*; 4, *M. scrofulaceum*; 5, *M. szulgai*; 6, *M. chelonae*; 7, *M. abscessus*; 8, *M. gordonae*; 9, *M. kansasii*; 10, *M. celetium*; 11, *M. fortuitum*; 12, *M. mucogenicum*; 13, *M. senegalense*; 14, *M. genavense*; 15, *M. malmoense*; 16, negative control. (B) MTB and non-mycobacterial reference strains: 1, *M. tuberculosis*; 2, *E. coli* O157:H7; 3, *S. flexneri*: 4, *S. sonnei*; 5, *S.* serovar Typhi; 6, *C. freundii*; 7, *E. aerogenes*; 8, *K. pneumonia*; 9, *P. aeruginosa*; 10, *Y. enterocolitica*; 11, *Y. pseudotuberculosis*; 12, *E. faecalis*; 13, *L. monocytogenes*; 14, Negative control.

5. Evaluation of microplate hybridization assay using clinical specimens

Subsequently, the performance of microplate hybridization assay was evaluated using DNAs extracted from clinical specimens obtained from Masan National TB Hospital, S. Korea. DNAs extracted from 62 sputum samples with diverse smear test results and MTB confirmed culture positive results were used in this study (Table 6). In brief, the results from this study showed that the sensitivity of the microplate hybridization assay with smear positive and culture positive specimens was 100% (45/45). In addition, the sensitivity of the assay with smear negative and culture positive specimens was 94.1% (16/17). Therefore, overall sensitivity of the assay was 98.4% (61/62).

Table 6. The performance of the microplate hybridization assay using culture positive sputum samples

Smear (No. of samples)		Microplate hybridization assay		
		Positive	Negative	Sensitivity (%)
-	(17)	16	1	94.1
trace	(7)	7	0	100
1+	(26)	26	0	100
2+	(7)	7	0	100
3+	(5)	5	0	100
Total	(62)	61	1	98.4

IV. DISCUSSION

It is well-known that tuberculosis affects one third of the world population and is one of severe infectious diseases to the extent that the annual number of deaths associated with the disease exceeds a million. In addition, many researchers have suggested that rapid and accurate diagnoses are critically important for effective tuberculosis treatment and have mentioned the several disadvantages of acid-fast stain and culture as a conventional diagnostic technique. Recently the CDC recommends that clinical specimens received be analyzed simultaneously by culture, acid-fast stain, and nucleic acid amplification protocols (19).

As part of an effort to improve such problems, many testing methods based on molecular biological techniques have been developed (11-16). Of them, PCR has been most widely evaluated and implemented in clinical settings.

In this study, a microplate hybridization assay in order to make up for such the disadvantages of conventional TB diagnostic techniques was developed. For this, a region of the *rpoB* gene was used to design PCR primers, MTB probe and *Mycobacterium* genus-specific probe molecules. The selective region of the *rpoB* gene used for PCR

amplification in this assay method has an advantage of facilitating isolation and identification of NTM than 16S rRNA gene or IS*6110* employed in the above-mentioned molecular biological technique (5, 20, 21). Thus, the primers designed in this study were also used when developing the microplate hybridization assay to isolate and identify NTM afterwards.

Besides, the specificity of the probe used in this assay method was confirmed with DNAs extracted from some reference strains, and it was found that it has the sensitivity to detect up to 100 fg in the reference strains. In other words, this assay method showed 10-fold higher sensitivity than that of the widely accepted technique of detecting PCR products by electrophoresis.

Subsequently, DNAs extracted from 62 sputum samples with diverse smear scores and culture positive results were used to evaluate the microplate assay performance. In brief, the results from this study showed that the sensitivity of the microplate hybridization assay with smear positive and culture positive specimens was 100% (45/45). In addition, the sensitivity of the assay with smear negative and culture positive specimens was 94.1% (16/17).

In conclusion, the microplate hybridization assay developed in this study seems to be highly specific and sensitive for detection of MTB.

Since the assay was shown to be more sensitive than conventional PCR-agarose gel electrophoresis assay from this study, it may be an alternative new sensitive method for detecting MTB with direct specimens such as sputum

CHAPTER II

A Microplate hybridization assay for Identification of Non-tuberculous Mycobacteria

I. INTRODUCTION

Although MTB is the main cause of mycobacteriosis in humans, other species of mycobacteria may also cause infections (22). The increasing importance of NTM in the clinical laboratory is now generally recognized. Among the many factors that may contribute to such an increase are the HIV/AIDS pandemics and other immunocompromising diseases, the technical improvements in NTM recovery and identification, the increased interest in NTM identification and the improvement of public health services for tuberculosis (23, 24).

Unlike infection by MTB, person-to-person transmission is thought not to occur, and thus the segregation of infected patients is not required. It is generally assumed that most are infected by environmental NTM. Of the many possible sources of infection, airborne NTM may play an important role in respiratory disease (25, 26). The most frequent pathogens are *M. avium* complex (MAC), *M. kansasii, M. abscessus, M. xenopi, and M. malmoense* in NTM pulmonary disease. NTM pulmonary disease in the United States is most commonly due to MAC, followed by *M. kansasii* (27). In the United Kingdom, *M. kansasii* is the most common pathogen in England and Wales, whereas *M. malmoense* is most common in Scotland, and *M. xenopi* in southeast England (28).

In Japan, MAC is the most common cause of NTM pulmonary disease, followed by *M. kansasii* (29). In Korea, MAC is the most common cause of NTM pulmonary disease, follow by *M. abscessus, M. fortuitum, M. kansasii* (30, 31).

NTM organisms are identified by their patterns of pigmentation, growth characteristics and microscopic appearances, and by biochemical tests such as niacin production, nitrate reduction, tween-80 hydrolysis etc. However, this strategy is time consuming and inconclusive for many isolates with variable characters. The rise in NTM isolation demands faster methods for their identification and for selection of appropriate therapy. In clinical practice, unnecessary or inappropriate antituberculosis treatment has been instituted in many patients with NTM pulmonary diseases (32).

Thus, more rapid discriminatory systems are being developed (26, 27). *16S rRNA* sequencing of a PCR product from NTM has become the gold standard for identification of NTM species (33, 34). However, if a limited segment of the gene is examined identical sequences may be present in 2 different species, thus precluding molecular differentiation. One such example is *M. marinum and M. ulcerans* (35). In the case of *M. absecessus and M. chelonae*, that share the relevant gene sequence, a supplementary sequencing using another well defined segment of *16S*

rRNA gives the specific diagnosis (36).

Recently, GenoType Mycobacterium CM (Hain Lifesciences, Nehren, Germany) and INNOLiPA (Innogenetics, Ghent, Belgium) were developed as molecular diagnostics using reverse DNA hybridization. However, these assay also have the disadvantage of the ability to isolate and identify just a limited range of strains due to the above-mentioned problems for *16S rRNA*. For this reason, the present study used a selective region of the *rpoB* gene for PCR amplification so as to improve such the disadvantage. And, specific probes were designed to identify thirteen different NTM strains which are frequently detected in clinical practice. With the designed primers and probes, microplate hybridization assay was developed which is capable of isolating and identifying NTM.

II. MATERIALS AND METHODS

1. Genomic DNAs from reference strains

Genomic DNAs extracted from reference strains used in chapter I were used to conform the specificity of the probe molecues.

2. Primers & oligonucleotide probes

The primers and probes were prepared based upon the mycobacterial strain sequence obtained from Genebank (http://www.ncbi.nlm.nih.gov). The amplification of *rpoB* gene was performed with the primers used in chapter I. In microplate hybridization assay, the probes shown in Table 7. The 5' end of each probe was labelled with digoxigenin (DIG). These primers and probes were synthesized and labelled by Bioneer (Daejeon, Korea) and Sigma-Proligo (The Woodlands, TX, USA), respectively.

Table 7. Sequences of probes used in microplate hybridization assay

Probe name	Sequence (5' → 3')
M. avium probe*	GCC GGT GAG CCG ATC ACC A
M. intracellulare probe*	CGG CCT GCA CGC GGG CGA G
M. scrofulaceum probe*	AAG CCC GTA CGG ATG GCC AGC
M. szulgai probe*	AAA GAA CGT CGG CGA GCC G
M. chelonae probe*	AAA TGG TGA CTG CCA CCA CG
M. abscessus probe*	AAA AGG TGA CCA CCA CCA CC
M. gordonae probe*	AAA GTC GGC GAT CCG ATC A
M. kansasii probe*	AAA GGC CAC GAT GAC CGT G
M. celatum probe*	CGA GAG CCC AAT CAC CAC C
M. fortuitum probe*	CCT GAA CGC CGG CCA G
M. mucogenicum probe*	CTG CAC GAC GGC AAC CCG
M. genevense probe*	CCA GCC GAC GAT GAC G
M. malmoense probe*	GAG TCG GCC GTA CCC GCC TCG A

* : digoxigenin-labelled probe

3. PCR amplification

PCR amplification carried out under conditions which are identical with chapter I.

4. Microplate hybridization assay

Microplate hybridization assay carried out under conditions which are identical with chapter I.

5. Data presentation

All assays were set up in triplicate, and the results were expressed as mean ± SD. Statistical significance for chemical or temporal effect was examined by independent pairs Student's *t*-test at P = 0.05 or 0.01.

III. RESULTS

1. Designing specific probes for identification of non-tuberculous mycobacteria

For NTM identification, the specific probes were designed for a selective region of the *rpo*B gene in thirteen different NTM strains which are frequently detected in clinical practice. The respective probes were modified so that they can be applied to the assay conditions determined in Chapter I (Table 7).

And the cut-off value used in microplate hybridization assay was background signal value + 3 standard deviations (18). The background signal value corresponded to the mean absorbance measured in a well that contained specific probes and PCR negative conrol products. Accordingly, the cut-off value was set at 0.2 (data not shown).

2. The specificity of probes used in microplate hybridization assay

The specificity of the microplate hybridization using newly designed probe molecules were tested using DNAs extracted form 15 mycobacterial reference strains including MTB and 12 non-mycobacterial reference strains (Figure 7 ~ 19). While each probe only bound to PCR products of target strain, it did not bind to any of PCR products of other bacterial strains. The results suggested that each probe used in this microplate hybridization assay could identification of NTM as well.

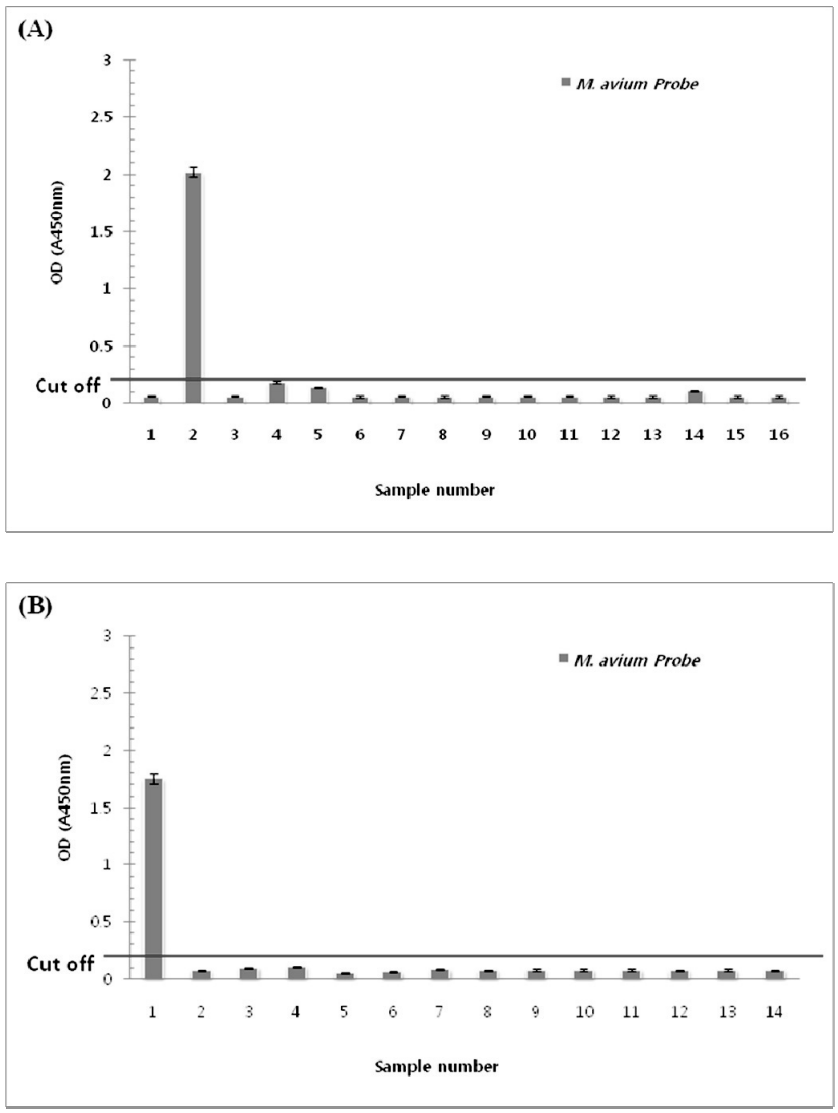
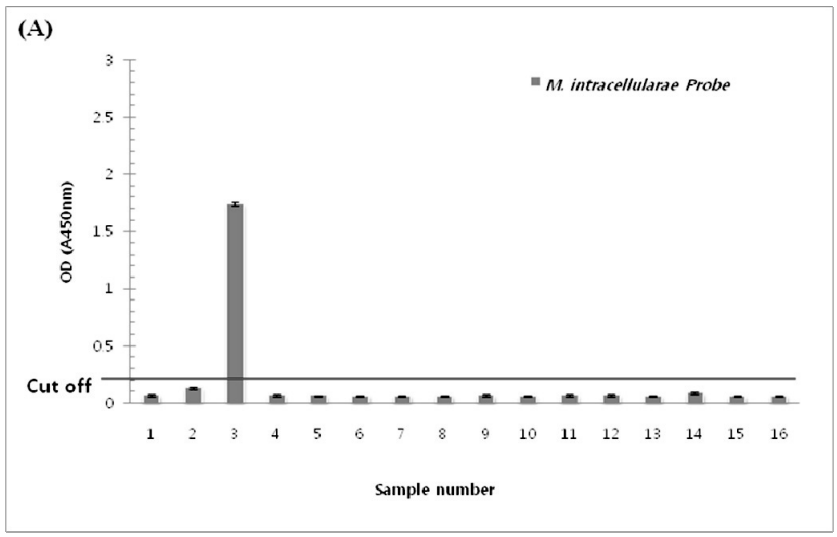


Figure 7. The specificity of *M. avium* probe used in experiments. *M. avium* probe was positive for only *M. avium* (A, lane 2 and B, lane 1) and negative for other bacterial strains (A, lane 1 and lane 3-15, and B, lane 2-13). (A) Mycobacterial reference: 1, *M.tuberculosis*; 2, *M. avium*;

3, *M. intracellulare*; 4, *M. scrofulaceum*; 5, *M. szulgai*; 6, *M. chelonae*; 7, *M. abscessus*; 8, *M. gordonae*; 9, *M. kansasii*; 10, *M. celetium*; 11, *M. fortuitum*; 12, *M. mucogenicum*; 13, *M. senegalense*; 14, *M. genavense*; 15, *M. malmoense*; 16, negative control. (B) *M. avium* and non-mycobacterial reference strains: 1, *M. avium*; 2, *E. coli* O157:H7; 3, *S. flexneri*: 4, *S. sonnei*; 5, *S.* serovar Typhi; 6, *C. freundii*; 7, *E. aerogenes*; 8, *K. pneumonia*; 9, *P. aeruginosa*; 10, *Y. enterocolitica*; 11, *Y. pseudotuberculosis*; 12, *E. faecalis*; 13, *L. monocytogenes*; 14, Negative control.



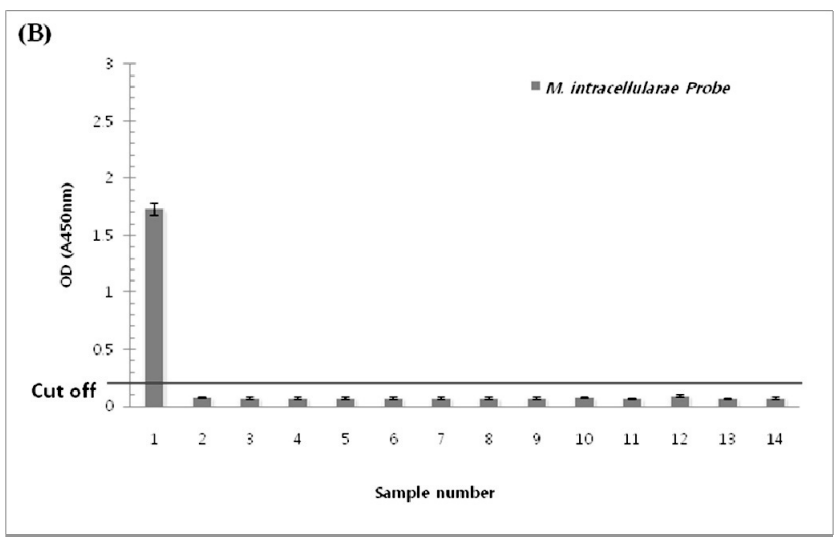
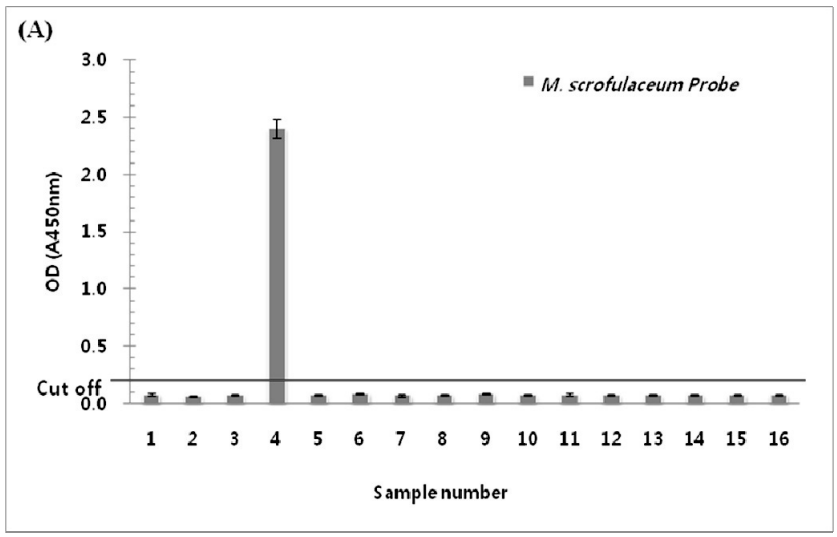


Figure 8. The specificity of *M. intracellulare* probe used in experiments. *M.intracellulare* probe was positive for only *M. intracellulare* (A, lane 3 and B, lane 1) and negative for other bacterial strains (A, lane 1-2 and lane 4-15, and B, lane 2-13). The experiment carried out under conditions which are identical with figure 7.



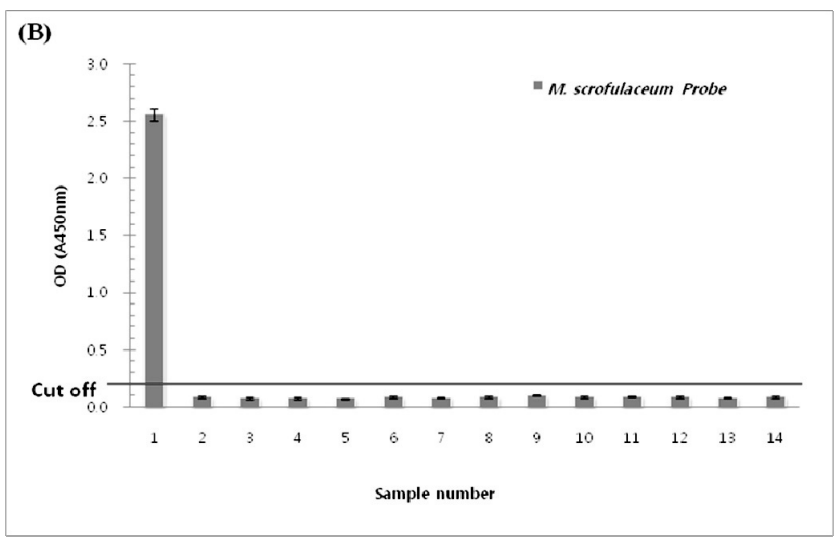
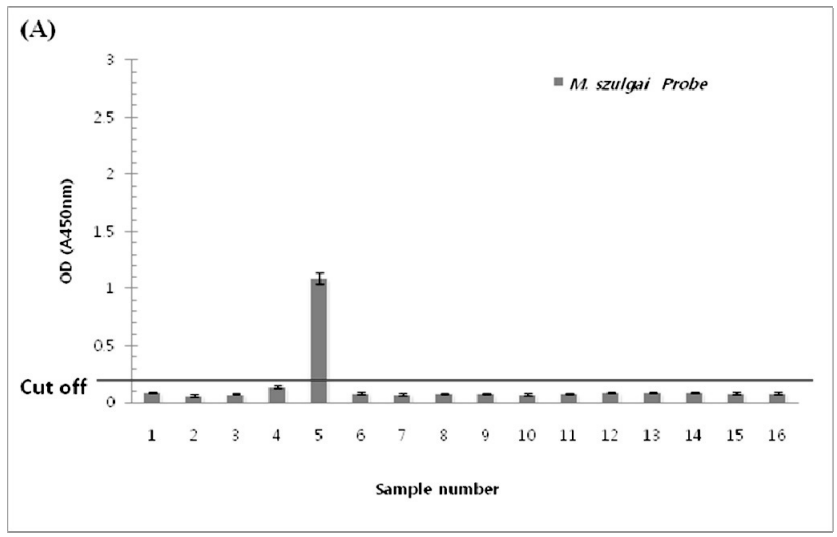


Figure 9. The specificity of *M. scrofulaceum* probe used in experiments. *M. scroflulaceum* probe was positive for only *M. scroflulaceum* (A, lane 4 and B, lane 1) and negative for other bacterial strains (A, lane 1-3 and lane 5-15, and B, lane 2-13). The experiment carried out under conditions which are identical with figure 7.



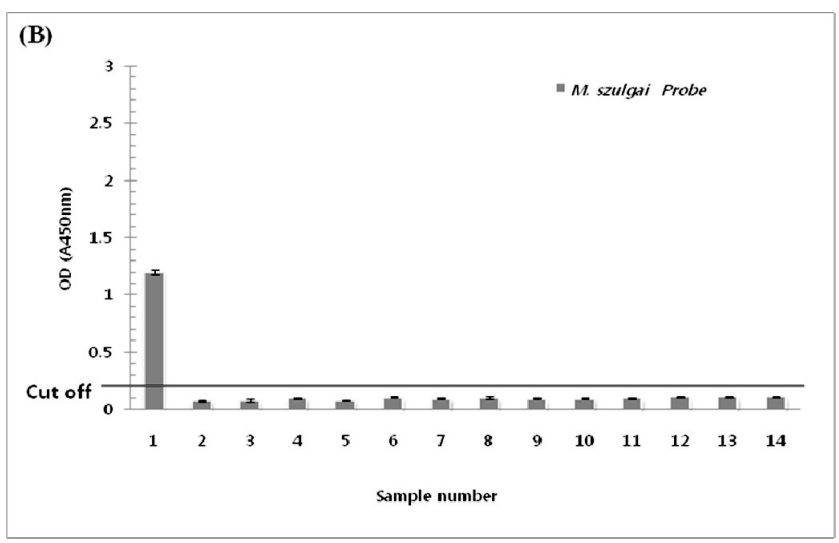


Figure 10. The specificity of *M. szulgai* probe used in experiments. *M. szulgai* probe was positive for only *M. szulgai* (A, lane 5 and B, lane 1) and negative for other bacterial strains (A, lane 1-4 and lane 6-15, and B, lane 2-13). The experiment carried out under conditions which are identical with figure 7.

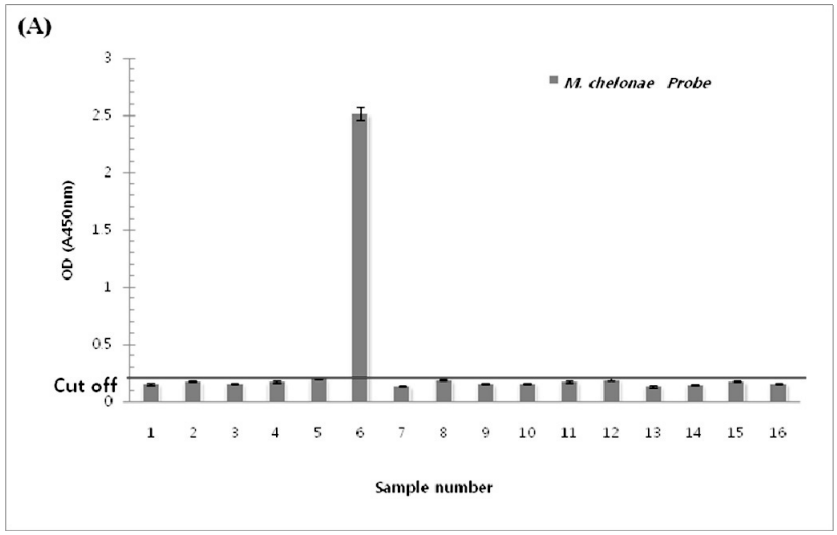


Figure 11. The specificity of *M. chelonae* probe used in experiments. *M. chelonae* probe was positive for only *M. chelonae* (A, lane 6 and B, lane 1) and negative for other bacterial strains (A, lane 1-5 and lane 7-15, and B, lane 2-13). The experiment carried out under conditions which are identical with figure 7.

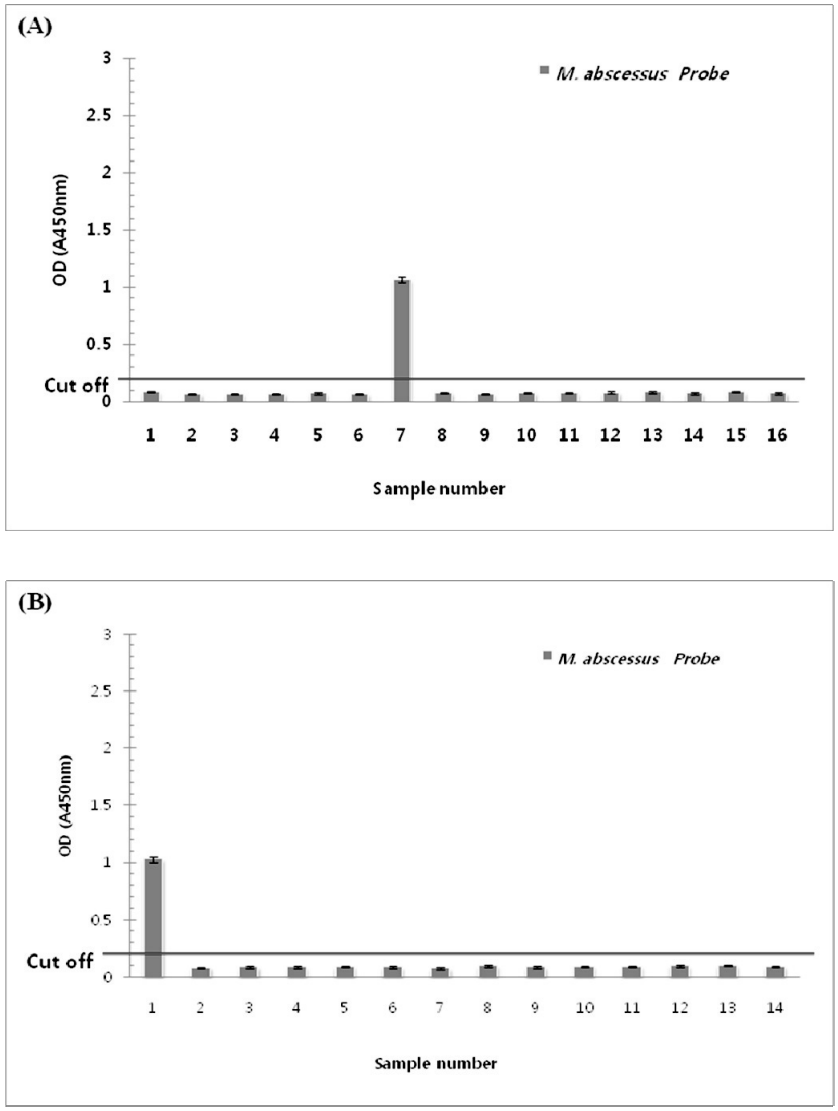


Figure 12. The specificity of *M. abscessus* probe used in experiments. *M. abscessus* probe was positive for only *M. abscessus* (A, lane 7 and B, lane 1) and negative for other bacterial strains (A, lane 1-6 and lane 8-15, and B, lane 2-13). The experiment carried out under conditions which are identical with figure 7.

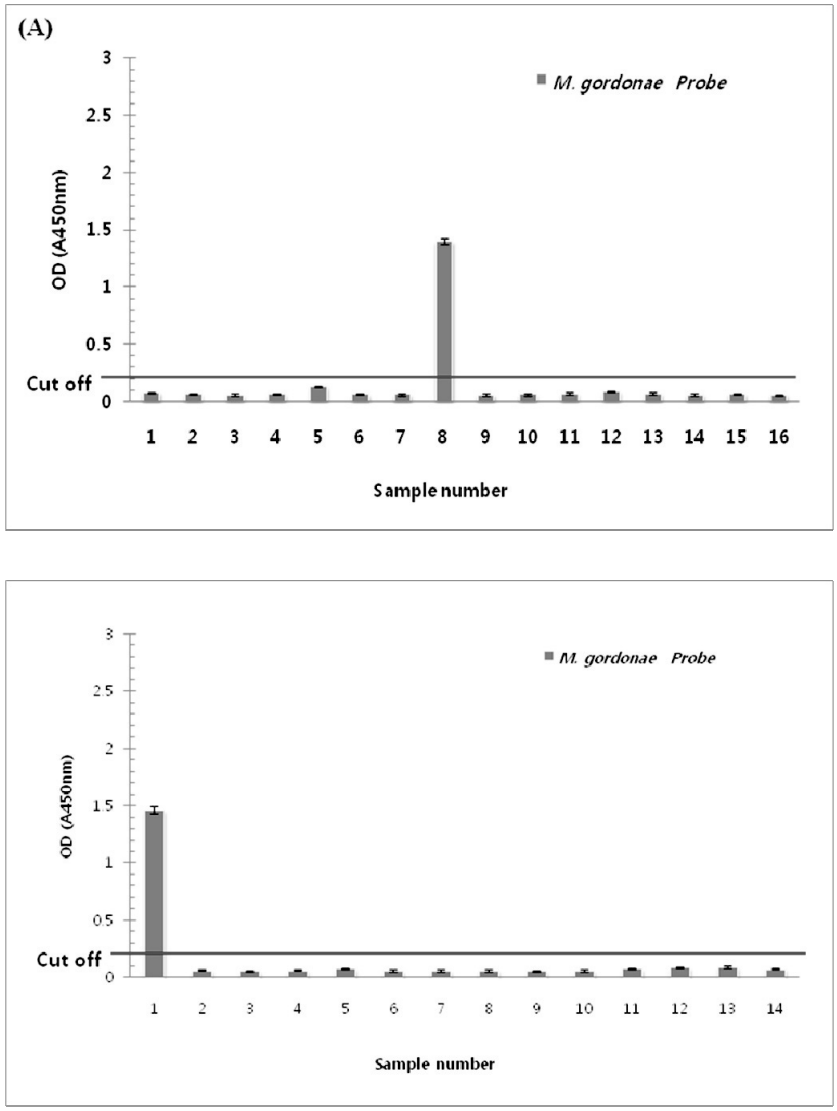
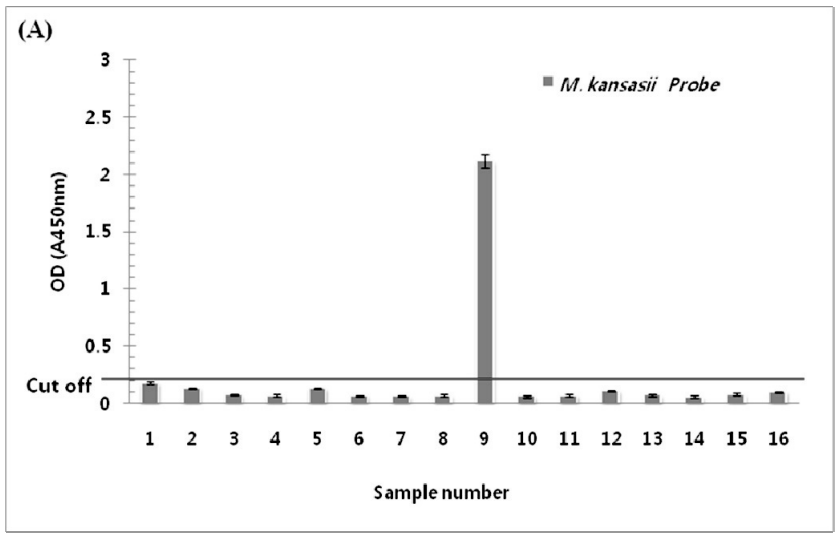


Figure 13. The specificity of *M. gordonae* probe used in experiments.

M. gordonae probe was positive for only *M. gordonae* (A, lane 8 and B, lane 1) and negative for other bacterial strains (A, lane 1-7 and lane 9-15, and B, lane 2-13). The experiment carried out under conditions which are identical with figure 7.



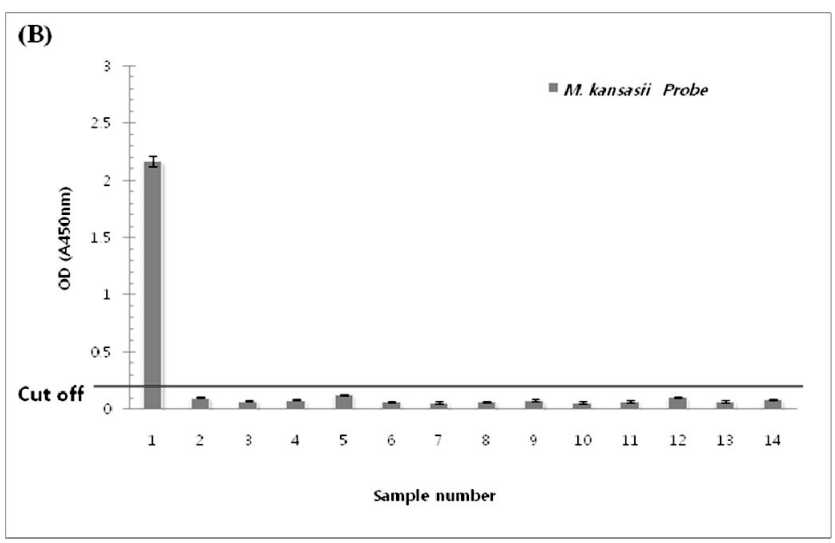


Figure 14. The specificity of *M. kansasii* probe used in experiments. *M. kansasii* probe was positive for only *M. kansasii* (A, lane 9 and B, lane 1) and negative for other bacterial strains (A, lane 1-8 and lane 10-15, and B, lane 2-13). The experiment carried out under conditions which are identical with figure 7.

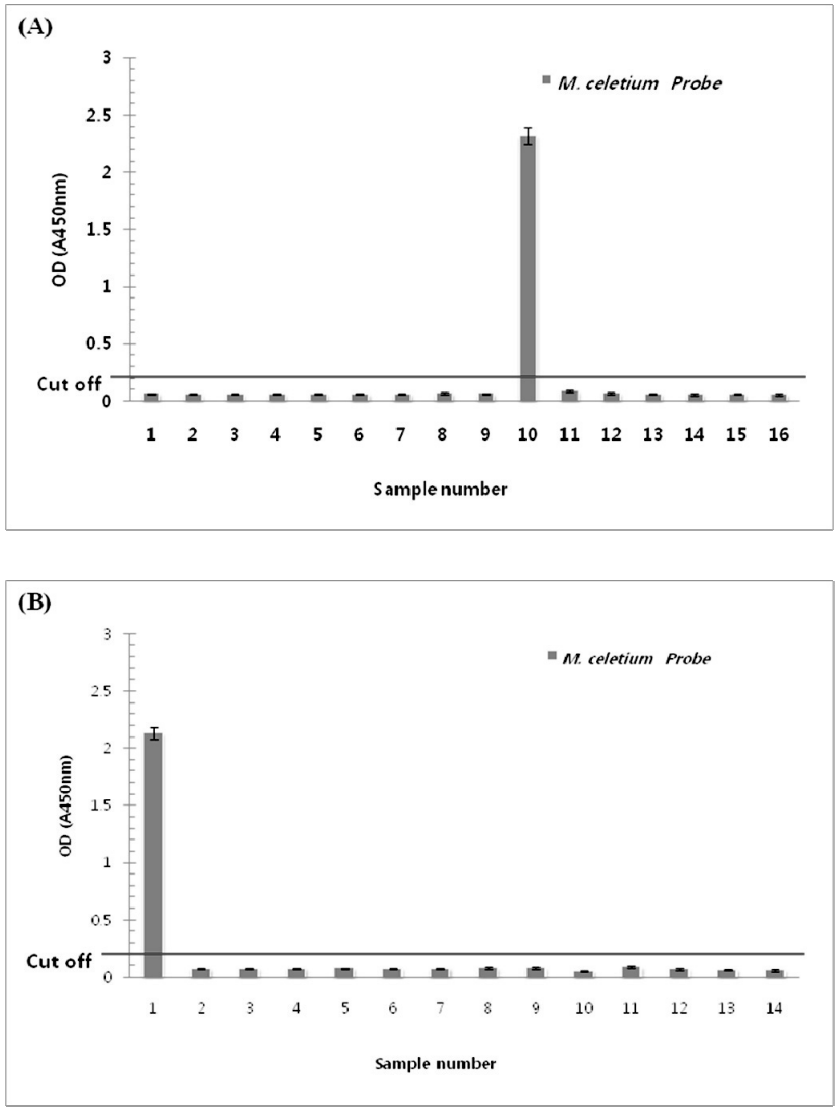


Figure 15. The specificity of *M. celetium* probe used in experiments. *M. celetium* probe was positive for only *M. celetium* (A, lane 10 and B, lane 1) and negative for other bacterial strains (A, lane 1-9 and lane 11-15, and B, lane 2-13). The experiment carried out under conditions which are identical with figure 7.

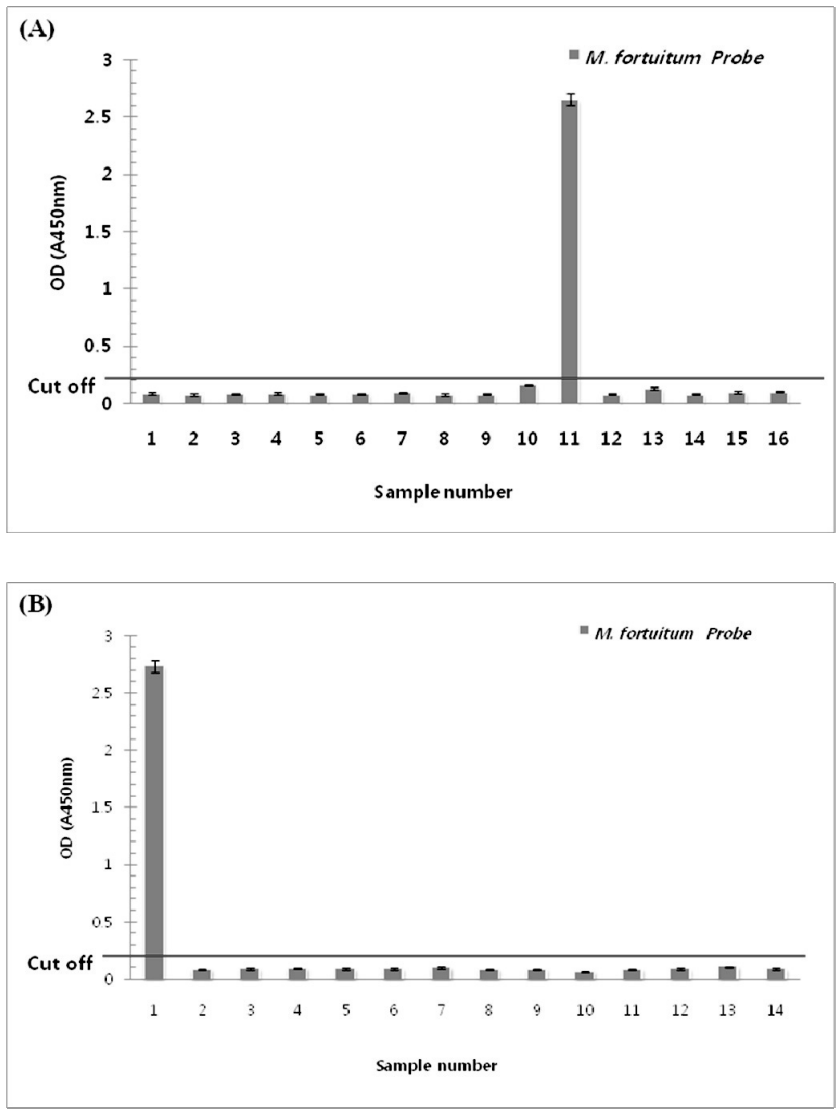


Figure 16. The specificity of *M. fortuitum* probe used in experiments. *M. fortuitum* probe was positive for only *M. fortuitum* (A, lane 11 and B, lane 1) and negative for other bacterial strains (A, lane 1-10 and lane 12-15, and B, lane 2-13). The experiment carried out under conditions which are identical with figure 7.

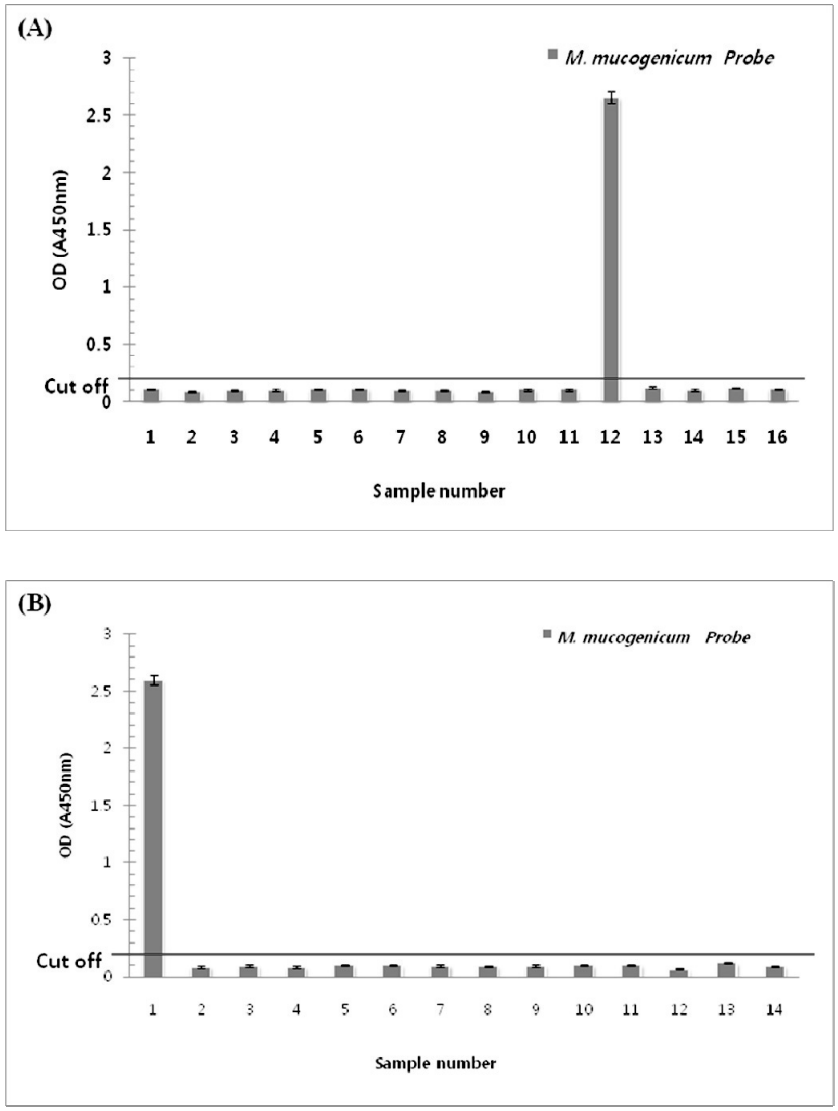


Figure 17. The specificity of *M. mucogenicum* probe used in experiments. *M. mucogenicum* probe was positive for only *M. mucogenicum* (A, lane 12 and B, lane 1) and negative for other bacterial strains (A, lane 1-11 and lane 13-15, and B, lane 2-13). The experiment carried out under conditions which are identical with figure 7.

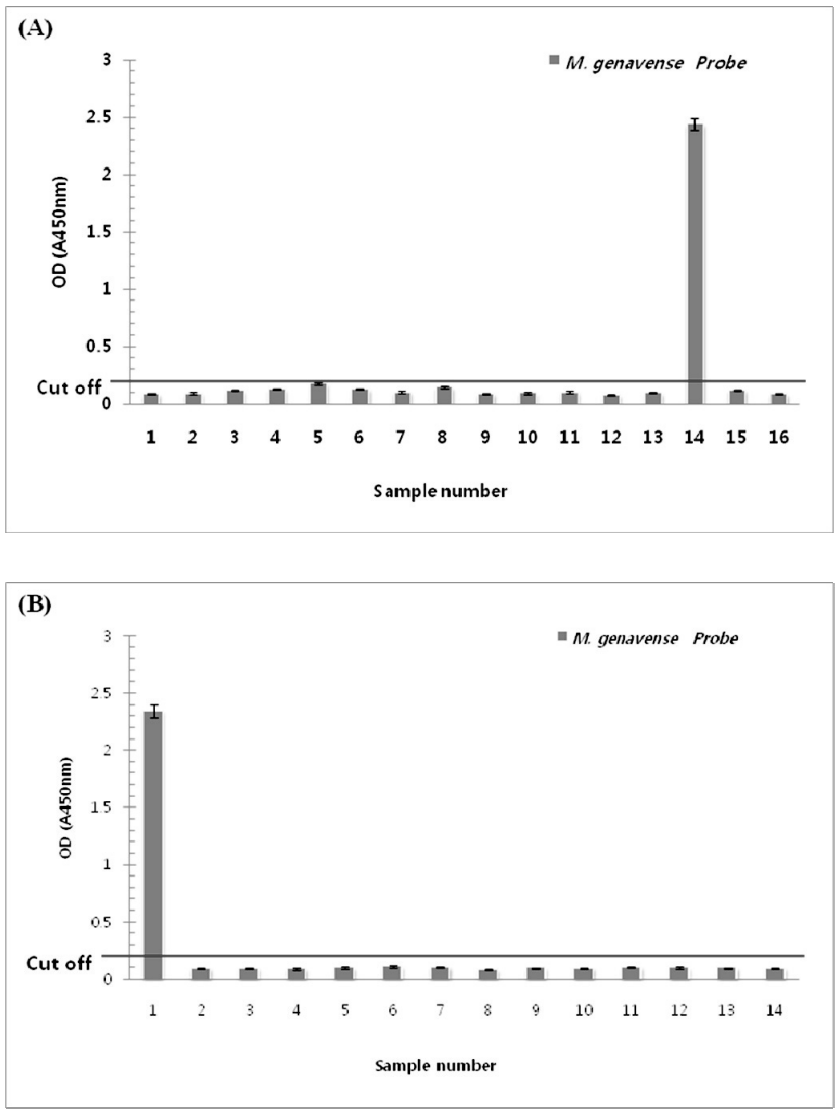


Figure 18. The specificity of *M. genavense* probe used in experiments. *M. genavense* probe was positive for only *M. genavense* (A, lane 14 and B, lane 1) and negative for other bacterial strains (A, lane 1-13 and lane 15, and B, lane 2-13). The experiment carried out under conditions which are identical with figure 7.

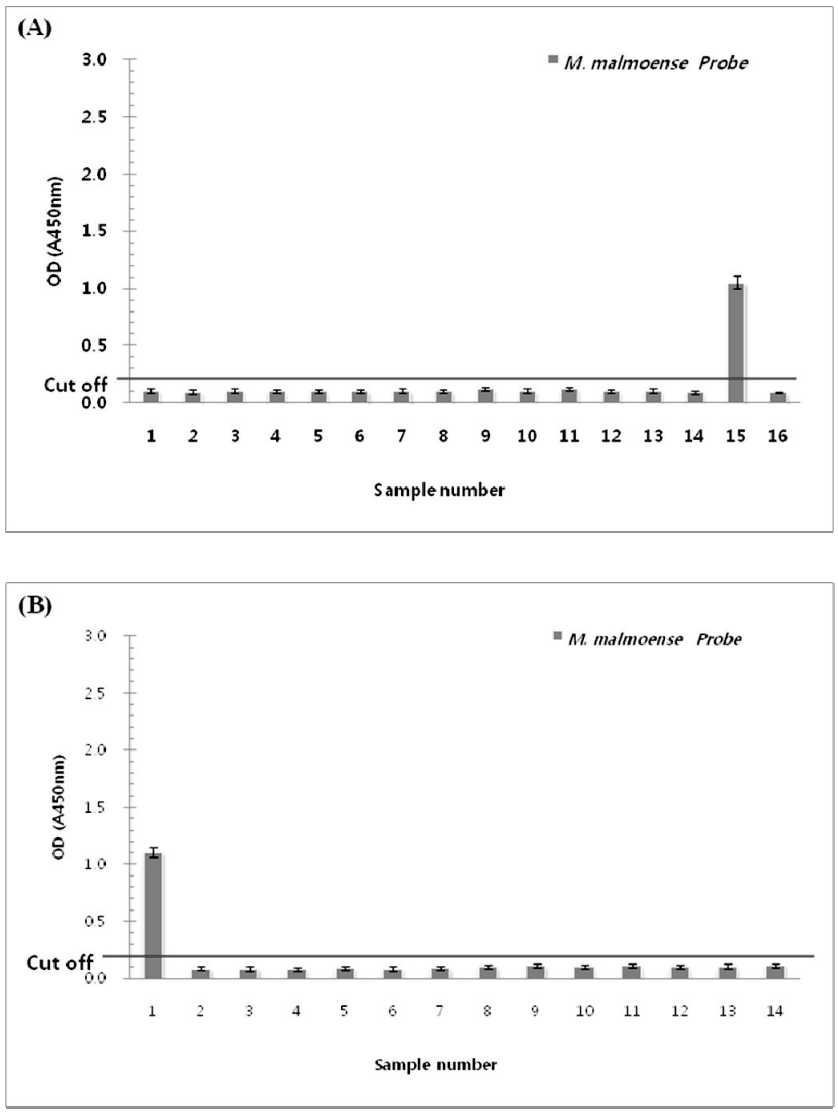


Figure 19. The specificity of *M. malmoense* probe used in experiments. *M. malmoense* probe was positive for only *M. malmoense* (A, lane 15 and B, lane 1) and negative for other bacterial strains (A, lane 1-14 and B, lane 2-13). The experiment carried out under conditions which are identical with figure 7.

IV. DISCUSSION

Tuberculosis is seriously influencing the health of world people, such that above 9 million persons have been infected and above 1 million persons have died every year. MTB is known as the main causative bacteria of tuberculosis. Recently, however, the increasing number of patients with immunocompromising diseases as well as HIV infection led to the increased infections by NTM, which brought about more importance of NTM identification (23, 24).

It is generally assumed that most are infected by environmental NTM (25, 26). The most frequent pathogens are *M. avium* complex (MAC), *M. kansasii, M. abscessus, M. xenopi, and M. malmoense* in NTM pulmonary disease.

Traditionally, NTM organisms are identified by their patterns of pigmentation, growth characteristics and microscopic appearances, and by biochemical tests. However, this method is time consuming and inconclusive for many isolates with variable characters.

Thus, more rapid molecular biological methods have been developed, which could improve the disadvantage of the traditional method that have been used for isolation and identification of NTM. These have targeted the 16S rRNA gene. However, if a limited segment of the gene

is examined identical sequences may be present in 2 different species, thus precluding molecular differentiation (36). For the microplate hybridization assay, developed in this study, the *rpoB* gene was used as subject to supplement such the disadvantage. And, the specific probes were designed to identify thirteen different NTM strains which are frequently detected in clinical practice (Table 7). These specific probes were designed suitably for the experimental conditions of the microplate hybridization assay, which has already been developed. And the cut-off value of each probe was set to 0.2.

Also, for each probe, specificity was confirmed using DNAs extracted from 15 mycobacterial reference strains and 12 non-mycobacterial reference strains. As a result, each probe was shown to be positive only to the target strain and negative to the other species. These results assumed that the microplate hybridization assay using these probes will be available in NTM identification clinical specimens. The availability of this assay is to be further evaluated for clinical specimens.

CONCLUSIONS

As an effort to overcome the problems with PCR employing gel electrophoresis and offer an alternative inexpensive method with advantage of real-time PCR, this study was set to develop specific and sensitive assay of analyzing PCR amplicons using microplate hybridization assay.

1. A specific primer (360LongF and 200R) was designed which is intended to amplify selectively *rpoB* gene of *Mycobacterium* genus.

2. *M. tuberculosis* specific probe (MTB) and mycobacterium genus specific probe (Myc) were designed to distinguish *M. tuberculosis* and non-tuberculous mycobacteria from the amplified products.

3. The optimal conditions for microplate hybridization assay with the designed primer and probe were definitely set up.

4. The specificity of the probe used in microplate hybridization assay was examined in 15 mycobacterial reference strains including MTB

and 12 non-mycobacterial reference strains.

5. The sensitivity of this assay is 10 times higher than that of PCR assay. This suggests the potential to detect correctly them even in the specimens which have false negative results by PCR assay.

6. The sensitivity of the microplate hybridization assay with smear positive and culture positive specimens was 100% (45/45).

7. The sensitivity of the assay with smear negative and culture positive specimens was 94.1% (16/17).

8. For NTM identification, the specific probes were designed for a selective region of the *rpo*B gene in thirteen different NTM strains which are frequently detected in clinical practice.

9. The specificity of the individually designed probes was confirmed using DNAs extracted from 15 mycobacterial reference strains including MTB and 12 non-mycobacterial reference strains.

The findings from the experiment above suggest that microplate hybridization assay is capable of isolating and differentiating MTB from NTM, as well as molecular diagnostic method with good sensitivity identification of NTM. From this, it is expected that the microplate hybridization assay developed in this study may be a useful.

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국문 요약

비결핵 마이코박테리아로부터 결핵균의 분리 동정을 위한 microplate hybridization 검사법

결핵균에 의해 야기되는 결핵은 여전히 거의 모든 나라에서 문제를 일으키는 심각한 감염성 질병이다. 또한, 최근 비결핵 마이코박테이리아 감염의 빈도가 증가하여 결핵에 의한 감염과의 구별의 필요하게 되었다. 그것은 결핵균과 비결핵 마이코박테리아의 치료법이 다르기 때문이다.

결핵균을 검출하기 위해 microplate hybridization 검사법을 이용한 간단하고 빠르며 정확하고 민감한 검사법을 개발하였다. 또한 이를 위해 *rpoB* 유전자 부위를 대상으로 primer와 결핵균 및 마이코박테리아속에 특이적인 probe를 디자인 하였다. 이 검사법의 특이도는 15종의 마이코박테리아 표준 균주와 12종의 비마이코박테리아 표준 균주로 부터 추출한 genomic DNA를 대상으로 확인하였다. 그리고 결핵균 표준 균주인 H37Rv의 genomic DNA를 사용하여 검사한 결과 100 fg를 나타내었다. 이어서 배양양성이며 다양한 smear 결과를 나타내는 62개의 객담검체로부터 추출한 DNA를 대상으로 이 검사법을 평가하였다. 그 결과, 이 검사법의 민감도는 98.4% (61/62)를 나

타내었다.

다음으로 비결핵 마이코박테리아의 동정하기 위한 microplate hybridization 검사법을 개발하였다. 이를 위해 rpoB 유전자부위를 대상으로 임상에서 자주 분리되는 13종의 비결핵 마이코박테리아에 대한 특이 probe를 디자인하였다. 각각의 프루브의 특이도는 결핵균을 포함한 15종의 마이코박테리아 표준 균주와 12종의 비마이코박테리아 표준 균주로부터 추출한 DNA를 대상으로 확인하였다.

위의 실험 결과로 볼 때, microplate hybridization 검사법은 비결핵 마이코박테리아로부터 결핵균의 분리동정이 가능할 뿐만 아니라 비결핵 마이코박테리아의 동정에 좋은 민감도를 가지는 분자진단법이라고 생각되어진다. 따라서, 앞으로 microplate hybridization 검사법이 유용하게 사용될 것으로 생각된다.

핵심어: Microplate hybridization 검사법, 결핵균, 비결핵 마이코박테리아, 중합효소연쇄반응