Development of PCR-microplate Hybridization Assay for Detection of *Mycobacterium tuberculosis*

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Tuberculosis caused by *Mycobacterium tuberculosis* (MTB) still remains to be the most dreadful infectious disease affecting almost every country. In the present study, we developed a simple and rapid but accurate and sensitive assay method for detecting MTB using microplate hybridization assay. For this, a selective region of the *rpoB* gene was used to design PCR primers, and MTB and *Mycobacterium* genus-specific probe molecules. The specificity of the assay was confirmed using 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, a total of 62 sputum samples with diverse smear scores and culture positive results were used to evaluate the kit performance. In brief, the specificity and the sensitivity of the assay were 100% and 98.4%, respectively.

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 (World Health Organization, 2007; Wenjun et al., 2007). To treat such a number of patients with TB effectively, rapid and accurate detection of *Mycobacterium tuberculosis* 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 (Takeshi et al., 2006).

Worldwide, conventional smear microscopy and culture method are used for detection and differentiation of MTB infection. 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 Ziel-Neilson staining. Furthermore, it is still too insensitive to detect MTB infection at optimum level. 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 (Caws et al., 2000).

As part of an effort to improve such problems, many testing methods based on molecular biological techniques have been developed (Piersimoni et al., 1998; Brown et al., 1999; Oh et al., 2001; Ruiz et al., 2004; Wang et al., 2004; Soo et al., 2006). Of them, polymerase chain reaction (PCR) has been most widely evaluated and implemented in clinical settings, since it can be done in a short time, and

Table 1. Reference strains used in this study

Species	Source	Species	Source
Mycobacterium tuberculosis	ATCC[a] 19420	*E. coli* O157:H7	ATCC 43894
Mycobacterium avium	ATCC 25291	*Shigella flexneri*	ATCC 9199
Mycobacterium intracellulare	ATCC 13950	*Shigella sonnei*	ATCC 25931
Mycobacterium scrofulaceum	ATCC 19981	*Salmonella typhi*	ATCC 19430
Mycobacterium szulgai	ATCC 35799	*Citobacter freundii*	ATCC 6750
Mycobacterium chelonae	ATCC 35749	*Enterobacter aerogenes*	ATCC 13048
Mycobacterium abscessus	ATCC 19977	*Klebsiella pneumonia*	ATCC 35657
Mycobacterium gordonae	ATCC 14470	*Pseudomonas aeruginosa*	ATCC 27853
Mycobacterium kansasii	ATCC 12478	*Yersinia enterocolitica*	ATCC 9610
Mycobacterium celatum	ATCC 51130	*Yersinia pseudotuberculosis*	ATCC 29833
Mycobacterium fortuitum	ATCC 49403	*Enterococcus faecalis*	ATCC 19433
Mycobacterium mucogenicum	ATCC 49650	*Listeria monocytogenes*	ATCC 15313
Mycobacterium senegalense	ATCC 35796		
Mycobacterium genevense	ATCC 51233		
Mycobacterium malmoense	ATCC 29571		

[a]: American type culture collection

give relatively high specificity and sensitivity. 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 better specificity and fluorescent dyes and sensor for better sensitivity has been developed (Kim et al., 2003). 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.

METHERIALS AND METHODS

1. Reference strains

Fifteen different mycobacterial reference strains and twelve different nonmycobacterial reference strains (Table 1) were used to confirm the specificity of the probe molecules. Genomic DNA of reference strains were extracted by chloroform-isoamyl alcohol method as previously described (Parish and Stoker, 2001).

2. Specimens

Sixty-two sputum specimens obtained from Masan National TB Hospital were chosen by being either acid-fast bacterium positive or acid-fast bacterium negative but culture positive. Specimens were decontaminated by the conventional *N*-acetyl-L-cysteine-NaOH method (Deutsches Institut Fur Normung, 1986). After decontamination, the concentrated sediments were suspended in 1.0 to 1.5 ml sterile phosphate buffer (pH 7.0) and smears were processed by the Ziehl-Neelsen staining method (Kent and Kubica, 1985). Specimens were inoculated into liquid medium and onto solid medium and incubated at 37℃ for up to 6 and 8 weeks, respectively. Following inoculation, the leftover sediment of the decontaminated sputum specimens was

used for microplate hybridization assay testing.

3. Primers & Oligonucleotide probes

The primers and probes were prepared based upon the mycobacterial strain sequence obtained from Genebank (http://www.ncbi.nlm.nih.gov). To amplify rpoB gene, the primers were 360LongF (TCAAGGAGAAGCGCTACGA-CCTGGC) and 200R (CGVCGGTTRCCGAAGTGGTCG), and the 5' end of a reverse primer 200R was labelled with biotin. In microplate hybridization assay, the probes were *M. tuberculosis*-specific TB probe (CATGTCGGCGA-GCCC) and mycobacterium genus-specific Myc probe (GACGTCGTCGCCACCATCGA). The 5' end of each probe was labeled with digoxigenin (DIG). These primers and probes were synthesized and labeled by Bioneer (Daejeon, Korea) and Sigma-Proligo (The Woodlands, TX, USA), respectively.

4. PCR amplification

AccPower PCR Premix (Bioneer, Daejeon, Korea) was mixed with 10 pmole of each primer and 5 µl of template DNA so that the total amount reached 50 µl. 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-two cycles were given followed by primer extension at 72℃ for 7 min. 230 base pair sized PCR amplified products were obtained.

5. Microplate hybridization assay

Streptavidin coated microplate wells (Nunc, nunc

immobilizer™ streptavidin, Denmark) were washed three times with phosphate buffer saline (pH 7.5) and 0.5% (v/v) Tween 20 (PBST). 150 µl of PBST containing 0.05% bovine serum albumin (BSA) was dispensed into each well, 5 µl PCR product was added, followed by incubation at 37℃ for 1hr. Then, 100 µl of 0.2 N NaOH was added followed by denaturation at RT for 30 min. The microplate wells were washed with PBST three times, added the diluted probe solution, and incubated at 50℃ for 1 hr.
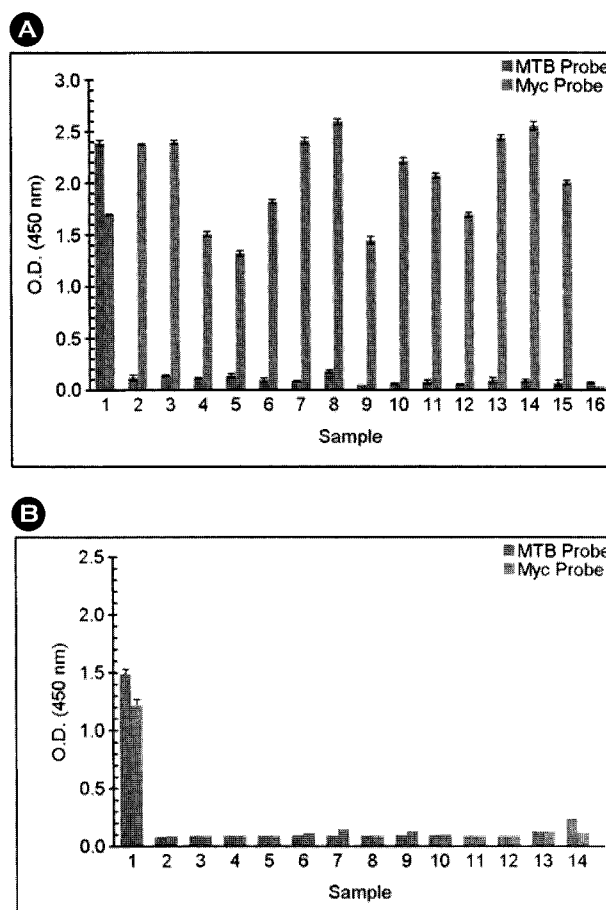
Fig. 1. The specificity of MTB and Myc probe used in this study. MTB probe was positive for only MTB (1-A and B, lane 1) and negative for all the mycobacterial species (1-A, lane 2-15 and 1-B, lane 2-13).

(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) MTB and non-mycobacterial reference strains: 1, *M. tuberculosis*; 2, *E. coli* O157:H7; 3, *S. flexneri*: 4, *S. sonnei*; 5, *S. 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.

Table 2. The performance of the microplate hybridization assay using sputum samples

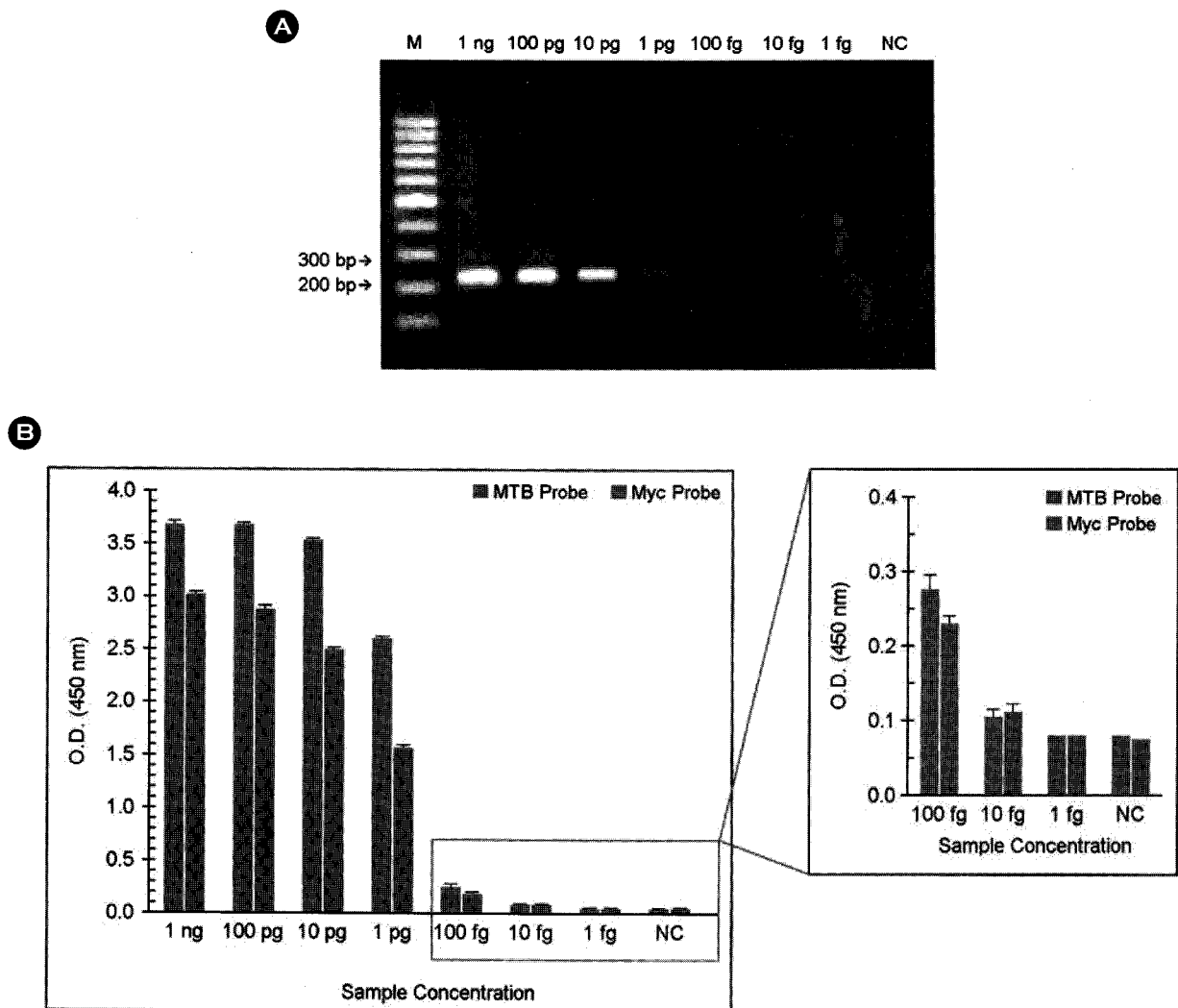| Smear (No. of samples)	Microplate hybridization assay			
	Positive	Negative	Sensitivity (%)	Specificity (%)
− (17)	16	1	94.1	100
± (7)	7	0	100	100
1+ (26)	26	0	100	100
2+ (7)	7	0	100	100
3+ (5)	5	0	100	100
Total (62)	61	1	98.4	100

Fig. 2. The sensitivity of MTB probe used in mycroplate hybridization assay. 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).

Subsequently, the microplate wells were washed with PBST at 50℃ for 10 min three times, added anti-DIG-peroxidase (Roche), and incubated at 37℃ for 30 min, and washed with PBST five times. Finally, the microplate wells were added 100 µl of the mixture (TMB (3,3',5,5'-tetrametyl benzidine, sigma) and 0.1% H_2O_2 in the ratio of 1:1) followed by incubation for 10 min, added 100 µl of 1 N HCl to stop the reaction, and subsequently, measured absorbance values at 450 nm by ELISA reader (Molecular Devices, USA).

RESULTS AND DISSCUSION

The specificity of the MTB and *Mycobacterium*-genus specific probes

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. The specificity of the microplate hybridization using newly designed PCR primers and probe molecules were tested using 15 mycobacterial reference strains

including MTB and 12 non-mycobacterial reference strains (Fig. 1).

While MTB probe only bound to PCR products of reference strain of MTB, it did not bind to any of PCR products of 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 cut-off value used was background signal value + 3 standard deviations (Yoshihiro et al., 2008). The background signal value corresponded to the mean absorbance measured in a well that contained specific probes and PCR negative control products. Accordingly, the cut-off value was set at 0.2. 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.

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 (Fig. 2). 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. The results suggest that the microplate hybridization assay can detect the specimens which could have been false negative by agarose gel electrophoresis assay.

Evaluation of microplate hybridization assay using clinical specimens

Subsequently, the performance of microplate hybridiza-tion assay was evaluated using clinical specimens obtained from Masan National TB Hospital, S. Korea. A total of 62 sputum samples with diverse smear test results and MTB confirmed culture positive results were used in this study. In brief, the results from this study showed that the specificity

and the sensitivity of the microplate hybridization assay with smear positive and culture positive specimens were 100% and 100% (45/45), respectively. In addition, the specificity and the sensitivity of the assay with smear negative and culture positive specimens were 100% and 94.1% (16/17), respectively. Therefore, overall specificity and the sensitivity of the assay were 100% and 98.4% (61/62), respectively. 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.

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