DOUBLE-STEP MULTIPLEX REAL TIME PCR WITH MELTING CURVE ANALYSIS FOR DETECTION AND DIFFERENTIATION OF MYCOBACTERIA IN SPUTUM

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Abstract. *Mycobacterium tuberculosis (M. tb)* is a causative agent of tuberculosis, a worldwide public health problem. In recent years, the incidence of human mycobacterial infection due to species other than *M. tb* has increased. However, the lack of specific, rapid, and inexpensive methods for identification of mycobacterial species remains a pressing problem. A diagnostic test was developed for mycobacterial strain differentiation utilizing a double-step multiplex real time PCR together with melting curve analysis for identifying and distinguishing among *M. tb*, *M. bovis* BCG, other members of *M. tb*. complex, *M. avium,* and non-tuberculosis mycobacteria. The assay was tested using 167 clinical sputum samples in comparison with acid-fast staining and culturing. Using only the first step (step A) the assay achieved sensitivity and specificity of 81% and 95%, respectively. The detection limit was equivalent to 50 genome copies.

Keywords: *Mycobacterium tuberculosis* complex, DNA melting curve, real time PCR, non-tuberculous mycobacteria

INTRODUCTION

Tuberculosis (TB) remains a major public health problem worldwide. One-third of the world's population is infected with *Mycobacterium tuberculosis* (*M. tb*), and TB causes more adult mortality than does any other single infectious agent (Raviglione *et al*, 1995). It is estimated that nearly one billion people will be newly infected with TB between 2000 and 2020. Furthermore, two hundred million of those people will develop active disease during their lifetime and 35 million will die from TB within this period (Amdekar, 2005).

Recently, the incidence of human mycobacterial infections due to species other than *M. tb* has increased worldwide. These infectious agents include other species of the *M. tb* complex (MTC), as well as non-tuberculous mycobacteria (NTM). The most common pathogens in these two groups are *M. bovis* and the *M. avium* complex, respectively (Nightingale *et al*, 1992; Park *et al*, 2010).

Because disease control depends on appropriate antimicrobial therapy, the precise identification of these clinically important species has become a major

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Table 1
Mycobacterial strains used in the study.

Mycobacterial species (number)	Strains
M. tuberculosis (5)	H37Rv, H37Ra, H37Rvj, V.A.b., ATCC27294
M. bovis BCG (3)	BCG Tokyo KK 1201, BCG ATCC 35740, BCG Pasteur
M. microti (1)	KK 1401
M. kansasii (3)	302, Vincent, ATCC12478
M. szulgai (2)	JATA 3201, 352
M. xenopi (1)	ATCC 19250
M. scrofulaceum (1)	ATCC 19981
M. duvalii (1)	MNC 442
M. avium (3)	ATCC 700898, 212, MAC IWGMT 49
M. intracellulare (2)	ATCC 13950, 71
M. terrae (2)	ATCC 16766, ATCC 15755
M. gordonae (1)	ATCC 11470
M. nonchromo-geium (1)	ATCC 19530
M. austroafricanum (1)	3005
M. neolactis (1)	S152
M. chelonae (1)	ATCC 23016

public health concern, but has been hindered by a lack of specific, rapid, and inexpensive methods (Perez-Martinez *et al*, 2008). Real time PCR has been used as a diagnostic method as it has high sensitivity and specificity, and can even be used for the differentiation of closely related species (O'Mahony and Hill, 2002). Multiplex real time PCR is an extension of the technique in which multiple targets are quantified simultaneously in the same reaction. In this study, we developed a diagnostic test for mycobacterial strain differentiation using double-step multiplex real time PCR coupled with melting curve analysis to identify 1) *M. tb*, 2) *M. bovis* BCG, 3) other species in the MTC, 4) *M. avium,* and 5) non-tuberculosis mycobacteria.

MATERIALS AND METHODS

Sputum specimens and mycobacterial strains

One hundred and sixty-seven clinical sputum samples were tested for mycobacteria using routine TB laboratory techniques, including acid-fast staining, culturing and the niacin accumulation test (Sutantangjai *et al*, 2014). The specimens that were used in this study were from patients at the Disease Prevention and Control 6, Khon Kaen, Thailand and had been previously used for other clinical diagnosis purposes. All samples were stained for acid-fast bacilli (AFB) using the Kinyoun method (Somoskovi *et al*, 2001) and cultured in a BBL MGIT tube (Becton Dickinson, San Jose, CA) containing 7 ml modified Middlebrook 7H9 broth for 42 days or until a positive signal developed. Mycobacterial reference strains used in this study are listed in Table 1. This study was approved by the Khon Kaen University Ethics Committee for Human Research (approval no. HE551091).

Real time PCR procedures

DNA from sputum was performed using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to manufac-

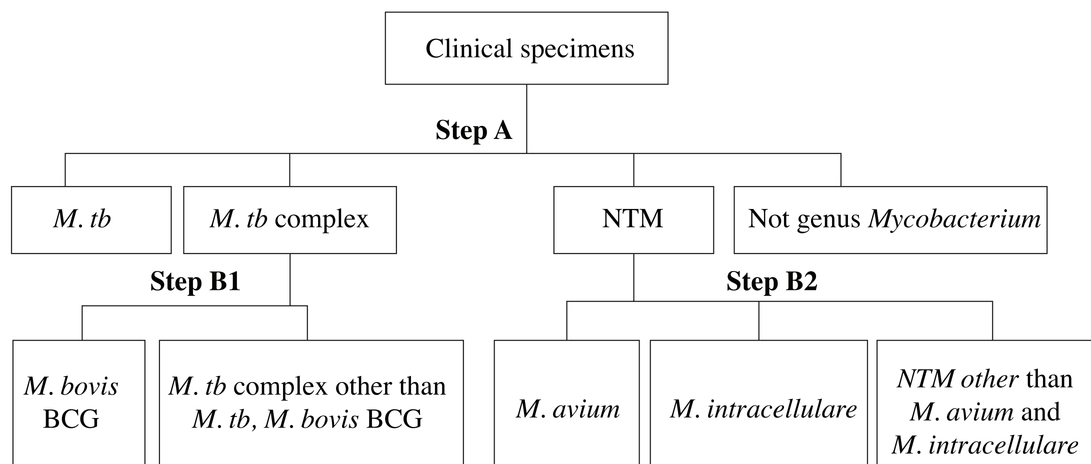

Fig 1–Scheme of multistep multiplex real time PCR coupled with melting curve analysis for detection and differentiation of mycobacteria.

turer's instructions, whereas DNA from stock bacterial strains was performed using proteinase K and lysozyme in order to lyse cells from colonies. Buffering conditions were adjusted to provide optimal DNA-binding conditions and the lysate was loaded onto a DNeasy Mini spin column of the kit. Further procedures were performed according to the manufacturer's instructions. The DNA was stored at -20°C until used.

Primers were selected from published sources (Table 2) and slightly modified to obtain similar melting temperatures (Tms) within each multiplex group. Primers were tested for absence of hairpin loops, self- and hetero-dimers using the Oligoanalyzer website (http://www.idtdna.com/analyzer/applications/oligoanalyzer/). The amplicons generated by these multiplex primers were designed to produce different Tms due to their different lengths.

Three separate multiplex real time PCR assays were designed to include two steps: step A, followed by step B1 or B2 (Fig 1A). For step A, multiplex real time PCR together with melting curve analysis was performed before a decision was made to proceed to step B1 or B2. Three pairs of primers including RD9, MTB, and HSP (specific for *M. tb*, MTC and the genus *Mycobacterium,* respectively) were designed for use in step A (Table 2). The second step was duplex real time PCR, two primer pairs were used in step B1 to distinguish *M. bovis* BCG from members of MTC (RD4 primer pair being species-specific for *M. bovis* and ET primer pair only *M. bovis* BCG). In step B2, four primers that bind to different specific sites of 16S rDNA were used to generate three possible PCR products, specific to *M. avium*, *M. intracellulare* or NTM, which could be differentiated from one another due to their different Tms (Table 2).

PCR conditions were adjusted to equalize the intensity of each amplicon band. In step A, the 20 µl reaction mixture contained 1 µl of 5 µM forward and reverse HSP primers, 2 µl of 5 µM forward and reverse MTB primers, 2 µl of 12.5 µM forward and reverse RD9 primers, 5 µl of 20 ng of DNA template and SsoFast™

Table 2
Multiplex real time PCR primers used in the study.

Step	Primer sequences(5′- 3′)	Amplified region	Amplicon size (bp)	*Mycobacterium* sp	Reference
A RD9-F RD9-R	TTCGAGCCGTAAATTACTGTG GAGCATTCTCGCTCCGAAT	RD9	50	*M. tb*	(Pinsky and Banaei, 2008)
MTB-F MTB-R	GGTATGCTGTTAGGCGACG TCCACCACAAGACATGCATC	16S rDNA	488	*M. tb* complex	(Perez-Martinez *et al*, 2008)
HSP65-F HSP65-R	TCGCCAAGGAGATCGAGCT AGGTGCCGCGGATCTTGTT	HSP65	604	Genus *Mycobacterium*	(Kim *et al*, 2005)
B1 RD4-F RD4-R	AGAAGCGCAACACTCTTGGA AATTGCTGAAAAATGGCTATTGA	16s rDNA	94	*M. bovis* & *M. bovis* BCG	(Pinsky and Banaei, 2008)
ET1 ET3	AAGCGGTTGCCGCCGACCGACC GAGGCGATCTGGCGGTTTGGGG	RD1 delete	150	*M. bovis* BCG	(Talbot *et al*, 1997)
B2 RAC-F RAC-R	TGATCACCGAGAACGTGTTC ACTGGTGCCTCCCGTAGG	16S rDNA	934	Genus *Mycobacterium*	(Perez-Martinez *et al*, 2008)
RAC-F Mycav-R	TGATCACCGAGAACGTGTTC ACCAGAAGACATGCGTCTTG	16S rDNA	900	*M. avium*	
Mycint-F RAC-R	CCTTTAGGCGCATGTCTTTA ACTGGTGCCTCCCGTAGG	16S rDNA	176	*M. intracellulare*	

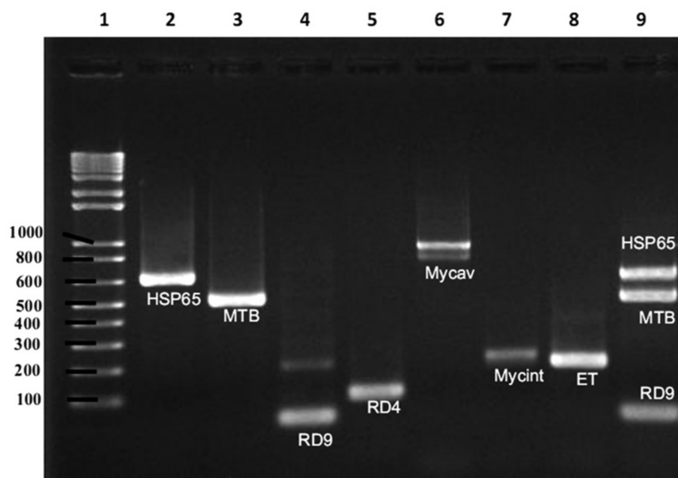

Fig 2–Agarose gel-electrophoresis of amplicons from conventional PCR of mycobacteria. Specific primer pair (labeled in figure) was used to amplify its corresponding mycobacterial DNA as described in Materials and Methods. Lane 1, DNA size markers (bp); lane 2, *M. tb.* H37RV; lane 3, *M. bovis* BCG; lane 4, *M. tb* H37RV; lane 5, *M. bovis* BCG; lane 6, *M. avium*; lane 7, *M. intracellulare*; lane 8, *M. bovis* BCG; lane 9, *M. tb* H37RV (multiplex step A PCR).

EvaGreen® Supermix reagent (Bio-Rad Laboratory, Hercules, CA). In steps B1 and B2, the 20 μl reaction mixture contained 1 μl of water, 2 μl of 5 μM forward and reverse RD4 primers and 2 μl of 5 μM forward and reverse ET primers (for B1) or 2 μl of 5 μM forward and reverse RAC primers, 2 μl of 5 μM forward Mycint primer and 2 μl of 5 μM reverse Mycav primer (for B2), 5 μl of 20 ng DNA template and SsoFast™ EvaGreen® Supermix reagent (Bio-Rad Laboratory, Hercules, CA).

Thermocycling conditions (conducted in a Light-Cycler 480 PCR system; Roche Diagnostic) were as follows: for 2 minutes; 40 cycles of 98ºC for 5 seconds, 53ºC (decreasing from 63ºC to 53ºC by 2ºC/cycle for the first six cycles) for 5 seconds and 72ºC for 24 seconds. Data for the melting curve analysis were collected between 65ºC and 99ºC with a ramp rate of 0.11ºC/sec. Amplicon sizes were confirmed by 1.5% agarose gel-electrophoresis.

RESULTS

The multi-step multiplex real time PCR with melting curve analysis was evaluated using 16 mycobacterial reference strains and 167 isolates from sputum samples, among 126 (75%) were positive for mycobacteria by AFB staining and culturing.

The designed primers were tested by conventional PCR and generated amplicons if the expected sizes as visualized by ethidium bromide staining of 1.5% agarose gel after electrophoresis (Fig 2).

Melting curve analysis after step A PCR using DNA only from *M. tb* generated three peaks with Tms of 80ºC, 86ºC and 91ºC for each of the three 16S rDNA regions amplified, respectively (Fig 3A). Using DNA from other members of MTC Tms of 86ºC and 91ºC were obtained (Fig 3B). Mycobacteria other than MTC generated a single Tm of 91ºC peak using the genus-specific primers (Fig 3C). No specific Tm was found for *Escherichia coli* (Fig 3D).

In step B1, to identify *M. bovis* BCG, Tm of the amplicon generated by the primer pair RD4 (86ºC) and ET (90ºC) was specific to the species and strain, respectively (Fig 3E). In step B2, to differentiate between *M. avium* and *M. intracellulare*, three sets of primers (RAC-FR, RAC-F-Mycav and Mycint-RAC-R) generated amplicons with Tms of 86ºC (*M. avium*), 90ºC (genus-specific) and 95ºC (*M. intra-*

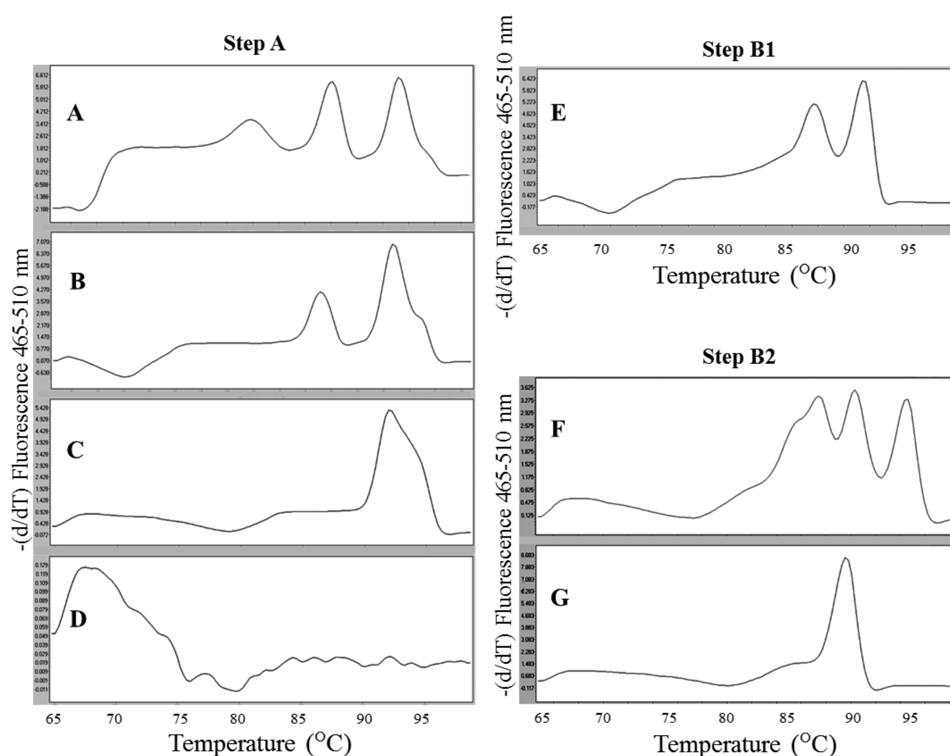

Fig 3–Melting curves of multiplex real time PCR amplicons of mycobacteria. Step A real time PCR was used to detect *M. tb* (A), MTC (*M. bovis* BCG) (B), genus *Mycobacterium* (*M. kansasii)* (C) and non mycobacteria (*E. coli*) (D); step B1 real time PCR to detect *M. bovis* BCG (E); and step B2 real time PCR to detect *M. avium* (F) and *M. intracellulare* (G).

cellulare) (Fig 3F and G).

The specificity of this real time PCR coupled with melting curve analysis was evaluated against a battery of 16 *Mycobacterium* reference strains (Table 1) and other microorganisms (*viz*, *Streptococcus pneumoniae, Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli, Neisseria meningitides, Haemophilus influenzae,* and *Nocardia* spp). All mycobacterial reference strains were correctly identified and none of the other bacterial or fungal strains typically found in sputum samples gave false positive results (data not shown).

Sensitivity of the technique (step A) was determined using chromosomal DNA from *M. tuberculosis* H37RV reference strain at various concentrations and performed detection by using Step A real time PCR. The limit of detection of *M. tuberculosis* H37RV was 0.25 ng, equivalent to about 50 genome copies (based on a genome size of 4.4 million bp (Brosch *et al*, 2000) (data not shown).

Compared with AFB staining of sputum samples, sensitivity of the multiplex real time PCR method was 81% (102/126), specificity 95% (39/41), positive predictive value (PPV) 98% (102/104) and negative predictive value (NPV) 62% (39/63); and when compared with culturing technique, sensitivity, specificity, PPV and NPV was 87% (79/91), 67% (51/76), 76% (79/104) and 81% (51/63), respectively (Table 3).

Table 3
Comparison of multiplex real time PCR method with AFB staining combined with culturing for detection and differentiation of mycobacteria from 167 sputum samples.

		AFB + culture		
		Positive	Negative	Total
Multiplex real time PCR	Positive	102	2	104
	Negative	24	39	63

DISCUSSION

Conventional multiplex and multi-step PCR-based assays have been used for identification of the genus *Mycobacterium*, MTC and seven individual mycobacterial species (Perez-Martinez *et al*, 2008). A stepwise, two-step multiplex real time PCR assay with melting curve analysis has previously been employed for rapid identification from clinical isolates of MTC members at the species level. The primers were designed based on region of differences sequences (Pinsky and Banaei, 2008). In this study, we further developed the assay based on multi-step multiplex real time PCR coupled with a melting curve analysis in order to identified both MTC or NTM from clinical specimen.

The current routine procedure for TB diagnosis differentiates only MTC or NTM (Toihir *et al*, 2011). Our multiplex real time PCR approach provides a more precise diagnosis, allowing identification of *M. tb*, *M. bovis* BCG, members of MTC, *M. avium*, *M. intracellulare* and other NTM members. In order to minimize costs, only the step A of the two-step multiplex real time PCR assay needs to be conducted as the majority of clinical tubercular specimens contain *M. tb*.

Compared with AFB staining and culturing, the sensitivity of our method was only 81%. Generally, sensitivity of singleplex PCR is greater than 95%, but relatively more copies of the template are necessary for a successful multiplex PCR reaction because it uses several pairs of primers and the annealing step is performed at a relatively high temperature in order to avoid non-specific priming. This situation was reflected by the relatively low sensitivity of the multiplex real time PCR technique (50 copies of *M. tb* genome). Compare to the previous study using single tube nested PCR reported better detection limit (Choi *et al*, 2014). However, half of the samples tested in this study had been stored for 3-4 days at 4ºC before DNA extraction, and some of the target DNA could have been degraded prior to this study.

Specificity of our method was better (95%). Two samples were positive by the multiplex real time PCR assay but negative in AFB staining and culturing. The latter approach requires a large number of live cells, whereas PCR detects a relatively low number of both living and dead cells.

A previous study showed that real time PCR cannot distinguish mycobacteria at the species level but can differentiate between MTC and NTM (Pinsky and Banaei, 2008) but our method has the ability of identifying not only *M. tb* but also MTC and NTM, which also is a greater advantage over AFB staining and culturing. The method has a relatively short

turnaround time (4 hours) and a simple protocol. Although the technique is less expensive than probe hybridization, the protocol is less sensitive and is appropriate for use as a primary detection and identification method for mycobacteria. In addition, the assay can be used to identify post-cultured mycobacteria.

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REFERENCES

Amdekar YK. Tuberculosis - persistent threat to human health. *Indian J Pediatr* 2005; 72: 333-8.

Brosch R, Gordon SV, Pym A, Eiglmeier K, Garnier T, Cole ST. Comparative genomics of the mycobacteria. *Int J Med Microbiol* 2000; 290: 143-52.

Choi Y, Jeon BY, Shim TS, Jin H, Cho SN, Lee H. Development of a highly sensitive one-tube nested real-time PCR for detecting *Mycobacterium tuberculosis*. *Diagn Microbiol Infect Dis* 2014; 80: 299-303.

Kim H, Kim SH, Shim TS, *et al*. Differentiation of *Mycobacterium* species by analysis of the heat-shock protein 65 gene (hsp65). *Int J Syst Evol Microbiol* 2005; 55: 1649-56.

Nightingale SD, Byrd LT, Southern PM, Jockusch JD, Cal SX, Wynne BA. Incidence of *Mycobacterium avium-intracellulare* complex bacteremia in human immunodeficiency virus-positive patients. *J Infect Dis* 1992; 165: 1082-5.

O'Mahony J, Hill C. A real time PCR assay for the detection and quantitation of *Mycobacterium avium* subsp. *paratuberculosis* using SYBR Green and the Light Cycler. *J Microbiol Methods* 2002; 51: 283-93.

Park D, Qin H, Jain S, *et al*. Tuberculosis due to *Mycobacterium bovis* in patients coinfected with human immunodeficiency virus. *Clin Infect Dis* 2010; 51: 1343-6.

Perez-Martinez I, Ponce-De-Leon A, Bobadilla M, *et al*. A novel identification scheme for genus *Mycobacterium*, *M. tuberculosis* complex, and seven mycobacteria species of human clinical impact. *Eur J Clin Microbiol Infect Dis* 2008; 27: 451-9.

Pinsky BA, Banaei N. Multiplex real-time PCR assay for rapid identification of *Mycobacterium tuberculosis* complex members to the species level. *J Clin Microbiol* 2008; 46: 2241-6.

Raviglione MC, Snider DE, Jr, Kochi A. Global epidemiology of tuberculosis. Morbidity and mortality of a worldwide epidemic. *JAMA* 1995; 273: 220-6.

Somoskovi A, Hotaling JE, Fitzgerald M, O'Donnell D, Parsons LM, Salfinger M. Lessons from a proficiency testing event for acid-fast microscopy. *Chest* 2001; 120: 250-7.

Sutantangjai M, Faksri K, Chaicumpar K, Chaimanee P, Lulitanond V, Namwat W. Evaluation of an immunochromatographic test kit for detecting *Mycobacterium tuberculosis* complex in sputum samples and on solid and in liquid cultures. *Southeast Asian J Trop Med Public Health* 2014; 45: 357-64.

Talbot EA, Williams DL, Frothingham R. PCR identification of *Mycobacterium bovis* BCG. *J Clin Microbiol* 1997; 35: 566-9.

Toihir AH, Rasolofo V, Andrianarisoa SH, Ranjalahy GM, Ramarokoto H. Validation of an immunochromatographic assay kit for the identification of the *Mycobacterium tuberculosis* complex. *Mem Inst Oswaldo Cruz* 2011; 106: 777-80.