DEVELOPMENT OF SINGLE-TUBE MULTIPLEX PCR FOR CLASSIFICATION OF *MYCOBACTERIUM TUBERCULOSIS* LINEAGES BASED ON LARGE SEQUENCE POLYMORPHISMS

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Abstract. Large sequence polymorphisms (LSPs) or regions of differences (RDs) are molecular epidemiological and evolutionary markers used to classify *Mycobacterium tuberculosis* (MTB) into East Asian (Beijing), Indo-Oceanic (IO), Euro-American (EuA) and East African Indian (EAI) lineages. The most used method is separate PCR and sequencing for each RD. We developed a single-tube multiplex PCR using four primer pairs specific to the four MTB lineages and a primer pair for species-specific RD9 with genomic DNA extracted from isolated colonies. The single-tube multiplex PCR produced lineage-specific amplicon patterns capable of differentiating the four MTB lineages. Sensitivity and specificity of the assay were 100% when differentiating MTB lineages from other species and strains of bacteria. The limit of detection of genomic MTB DNA was 12.5 ng. This single-tube multiplex PCR method offers a simple, rapid and reliable method for classification of MTB lineages based on LSPs.

Keywords: *Mycobacterium tuberculosis*, large sequence polymorphism, multiplex PCR, region of differences

INTRODUCTION

Tuberculosis (TB) remains one of the major causes of global public health problems, with 1.3 million deaths and 8.6 million new cases annually (Millet *et al*, 2012). *Mycobacterium tuberculosis* (MTB) is the major causative agent of human TB. Genetic markers and molecular epidemiology of this pathogen have been extensively studied (Thierry *et al*, 1990; Groenen *et al*, 1993; Small *et al*, 1994; Frothingham and Meeker-O'Connell, 1998; Fleischmann *et al*, 2002; Tsolaki *et al*, 2004). One class of genotypic marker that has been used to differentiate MTB into lineages consists of large sequence polymorphisms (LSPs) or regions of differences (RDs) (Fleischmann *et al*, 2002; Tsolaki *et al*, 2004). LSPs have been used as one of the main tools for identifying genomic variability in MTB (Brosch *et al*, 2002; Tsolaki *et al*, 2004). Indels of large

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DNA sequences have been employed to classify MTB into four global lineages (Gagneux *et al*, 2006; Gagneux and Small, 2007). RD105, RD239, RD750 and 7-bp deleted (del) *pks15/1* are specifically absent, or present in a deleted form, in the East Asian (or Beijing), Indo-Oceanic (IO), East African Indian (EAI) and Euro-American (EuA) lineages, respectively (Gagneux *et al*, 2006; Gagneux and Small, 2007). These genotypic markers have been associated with geographical regions and human ethnic groups and are indicators of host-pathogen co-evolution (Gagneux *et al*, 2006; Gagneux and Small, 2007).

The MTB Beijing lineage is more capable of causing disease with higher severity than other lineages. This lineage is responsible for major worldwide outbreaks (Bifani *et al*, 2002; Glynn *et al*, 2002) and is associated with hypervirulent phenotypes (Reed *et al*, 2004), treatment failure (Lan *et al*, 2003) and multidrug-resistant TB (Drobniewski *et al*, 2005). In contrast, the EuA lineage is suggested to be the most benign MTB lineage based on its association with decreased numbers of cases of disseminated disease (Caws *et al*, 2008; Thwaites *et al*, 2008).

The molecular technique generally used to classify MTB lineages based on LSPs involves a separate PCR for each RD (Gagneux *et al*, 2006; Faksri *et al*, 2011; Lu *et al*, 2012; Rindi *et al*, 2012). PCR coupled with a sequencing technique is commonly used to identify the 7-bp deletion of *pks15/1* (Tsolaki *et al*, 2005; Chaiprasert *et al*, 2006b; Gagneux *et al*, 2006; Caws *et al*, 2008; Zenteno-Cuevas *et al*, 2013), and a multiplex, allele-specific PCR (MAS-PCR) (Caws *et al*, 2008) and an RD-specific hybridization probe technique (Fleischmann *et al*, 2002; Alland *et al*, 2007) have also been applied. However, these techniques are complicated, time-consuming and, in the latter method, require relatively large amounts of DNA.

As the single-tube multiplex PCR, a modification of PCR that has been successfully applied in many areas of mycobacterial testing (Chaiprasert *et al*, 2006a; Aloyce *et al*, 2012), it was selected for this study as the tool for LSP-based MTB lineage classification. Due to its simplicity, rapidity and reliability, this method should facilitate epidemiological and evolutionary studies of MTB.

MATERIALS AND METHODS

Bacterial strains and sample preparation

Eighty-eight bacterial strains comprising 10 species of nontuberculous mycobacteria (NTM); 2 species of MTB complex (MTC), namely, *M. bovis* BCG and *M. microti*; 66 MTB strains; and 10 bacterial species other than mycobacteria, were used in this study (Table 1). In addition, one human buccal DNA sample was employed as a no-target control (NTC). The 66 MTB strains consisting of 28 Beijing, 21 IO, 13 EuA and 4 EAI lineages were classified using standard typing methods [IS*6110* RFLP, spoligotyping, VNTR typing and LSPs (by a separate PCR for each RD and by PCR/sequencing methods)] as reported previously (Faksri *et al*, 2011). Genomic DNA was extracted from bacterial colonies by an enzymatic method (van Embden *et al*, 1993). Briefly, lysozyme, RNase A, sodium dodecyl sulfate (SDS), and proteinase K were sequently added and processed with the bacterial colonies for DNA extraction.

This study was approved by the Khon Kaen University Ethics Committee for Human Research (approval No. HE551091).

Table 1
Bacterial species and strains used to evaluate the multiplex PCR method.

Species	Strains
M. tuberculosis [a,b]	Clinical specimens
M. astroafricanum[b]	Clinical specimen (3005)
M. avium	ATCC 700898
M. bovis BCG	ATCC 35740
M. chelonae	ATCC 23016
M. flavescens	ATCC 23035
M. fortuitum	ATCC 20348
M. intracellulare	ATCC 13950
M. kansasii	ATCC 12478
M. marinum[b]	Clinical specimen (92-03776)
M. microti[b]	Clinical specimen (KK 1401)
M. smegmatis	ATCC 16941
M. xenopi	ATCC 19250
Burkholderia pseudomallei[c]	Clinical specimen
Escherichia coli[c]	Clinical specimen
Haemophilus influenzae[c]	Clinical specimen
Klebsiella pneumoniae[c]	Clinical specimen
Nisseria meningitidis[c]	Clinical specimen
Nocardia spp[c]	Clinical specimen
Pseudomonas aeruginosa[c]	Clinical specimen
Staphylococcus aureus[c]	Clinical specimen
Streptococcus pneumoniae[c]	Clinical specimen
Streptomyces spp[c]	Clinical specimen

[a]Beijing (n = 28), Indo-Oceanic (n = 21), Euro-American (n = 13) and East African Indian (n = 4) lineages (Faksri *et al*, 2011). [b]Identified using PCR and restriction enzyme analysis (PCR-REA) of *hsp*65 and *rpo*B (Cheunoy *et al*, 2005). [c]Identified using the VITEK® 2 system (BioMérieux, Lyon, France) and biochemical tests.

Bioinformatics and primer design

The identification and selection of RDs as targets for MTB lineage classification were based on the results of previous studies (Constant *et al*, 2002; Gagneux *et al*, 2006; Caws *et al*, 2008). Lineage-specific RD sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov), and the primer sets corresponding to each RD were designed using OligoAnalyzer 3.1 (http://www.idtdna.com/analyzer/applications/oligoanalyzer/), based on the selection of primer pairs that provided distinguishable product sizes and were predicted to have similar melting temperatures (Tm) (Table 2). Specificities of the selected primer pairs were analyzed using Nucleotide BLAST and Primer-BLAST analysis.

Singleplex PCR for LSP determination

Singleplex PCR for each RD was performed in a 25- l mixture of 1X PCR buffer containing 0.2 U *Taq* DNA polymerase, 0.2 mM dNTP mixture, 3 mM $MgCl_2$ (Invitrogen, Carlsbad, CA), appropriate concentrations of primers, 5 ng of DNA

Table 2
Primers used in multiplex PCR for lineage classification of *M. tuberculosis* based on LSPs.

LSP markers	Primer sequence	Amplicon (bp)	Primer Tm (ºC)	Interpretation
RD9	F: 5′ATGGTCAACACCACTACGCGG 3′	78	68	(-)=Others
	R: 5′CGTGGTCAGCATGGCCAGATG 3′		69.2	(+)=MTB
RD105	F: 5′TGT GGT GCT TGG GCC TGA GAG3′	435	70.5	(-)=Beijing
	R: 5′AAT CGT GGT GAT CCC GGA GCG3′		70.1	(+)=non Beijing
RD239	F: 5′GTGAGCTGAAGAAGCTCGTCCC 3′	1,053/210	67.6	(210 bp)=IO
	R: 5′ATCACCGTCAAACCGTTCACGAC 3′		67	(1,053 bp)=non IO
RD750	F: 5′CACAGACAATTCCAACCCATTTGG3′	948/158	64	(158 bp)=EAI
	R: 5′CGCATGCCGCACATGCAAGTAC 3′		69.5	(948 bp)=non EAI
7bp *pks15/1*	F: 5′GGCCGCGGCCCGCGGCC3′	332	85.9	(-)=EuA
	R: 5′GGCTCAATCAAGTCGAACATGGG 3′		65.7	(+)=non EuA

(-), absent; (+), present. Others, other organisms; MTB, *M. tuberculosis*; IO, Indo-Oceanic; non IO, not Indo-Oceanic; EuA, Euro-American; non EuA, not Euro-American; EAI, East African Indian; non EAI, not East African Indian.

template and distilled water. The primer concentration used for detection of RD9, RD105 and RD239 was 100 nM, and that for 7 bp del *pks15*/1 was 400 nM. For negative controls, distilled water was used as a no-DNA control (NDC), and human buccal DNA as a NTC. Amplification (using a C1000™ Thermal Cycler; Bio-Rad, Hercules, CA) conditions consisted of 5 minutes at 95ºC; followed by 40 cycles of 1 minute at 95ºC, 1 minute at 60, 60, 63, 64 and 66ºC for the annealing of primers specific to RD9, 7-bp del *pks15/1*, RD750, RD105 and RD239, respectively, and 1 minute at 72ºC; with a final heating for 7 minutes at 72ºC. PCR amplicons were subjected to 2% agarose gel-electrophoresis and visualized using UV light after staining with ethidium bromide.

Single-tube multiplex PCR assay

Single-tube multiplex PCR was performed as described above with some modifications. The multiplex PCR solution contained 100, 100, 100, 200 and 800 nM primers for RD9, RD105, RD239, RD750 and 7-bp del *pks15/1*, respectively, and 50 ng of DNA template. The annealing temperature was set at 60ºC.

Data analysis

Sensitivity was calculated using true positive (TP) divided by TP plus false negative (FN), and specificity was calculated using true negative (TN) divided by TN plus false positive (FP).

RESULTS

Singleplex PCR and verification of amplification targets

Singleplex PCR generated all possible amplicons of the four global (Beijing, IO, EuA and EAI) MTB lineages (Fig 1 and Table 3). Amplification of RD9, RD105 and 7-bp del *pks15/1* resulted in amplicons of 78, 435 and 332 bp, respectively. Amplification of intact and deletion forms of RD239 and RD750 resulted in amplicons of 1,053 and 210 bp, respectively for the

Table 3
Specific PCR patterns and product sizes of *M. tuberculosis* lineages based on LSPs using the multiplex PCR method.

LSP markers	Amplicon pattern and size (bp)							
	Beijing		Indo-Oceanic		Euro-American		East African Indian	
	Expected pattern	Observed pattern	Expected pattern	Observed pattern	Expected pattern	Observed pattern	Expected pattern	Observed pattern
RD9	78	78	78	78	78	78	78	78
RD105	absent[a]	absent	435	435	435	435	435	435
RD239	1,053	absent	210[a]	210	1,053	absent	1,053	absent
RD750	948	948	948	948	948	948	158[a]	158
7-bp del *pks15/1*	332	332	332	332	absent[a]	absent	332	absent

[a]MTB lineage-specific.

Table 4
Sensitivity and specificity of a single-tube multiplex PCR for classifying *M. tuberculosis* lineage from bacterial cultures.

Tested organisms	Sensitivity	Specificity
Beijing (n = 28)	28/28	-
IO (n = 21)	21/21	-
EuA (n = 13)	13/13	-
EAI (n = 4)	4/4	-
Mycobacterium spp (n = 12)	-	12/12
Other bacteria (n = 10)	-	10/10
Human buccal DNA (n = 1)	-	1/1
Total	66/66 (100%)	23/23 (100%)

Mycobacterium spp consisted of *M. astroafricanum, M. avium, M. bovis* BCG, *M. chelonae*, *M. flavescens*, *M. fortuitum*, *M. intracellulare, M. kansasii, M. marinum*, *M. microti*, *M. smegmatis* and *M. xenopi*. Other bacteria consisted of *B. pseudomallei, E. coli, H. influenzae*, *K. pneumoniae*, *Nisseria* spp, *Nocardia* sp, *P. aeruginosa, S. pneumoniae*, *S. aureus* and *Streptomyce*s spp. IO, Indo-Oceanic; EuA, Euro-American; EAI, East African Indian.

former and 948 bp and 158 bp, respectively for the latter. The lower detection limit of singleplex PCR for each RD was 0.5 ng of genomic DNA from all four MTB lineages (data not shown).

Single-tube multiplex PCR assay of MTB lineage

Four well-characterized MTB strains of each lineage (n = 16) were subjected to the single-tube multiplex PCR assay. The amplicon patterns obtained from the multiplex PCR were slightly different from the expected patterns due to the absence of certain amplicons (Fig 2 and Table 3): the 332-bp amplicon of the EAI strain and 1,053-bp of the Beijing, EuA and EAI strains. However, RD9, a species-specific

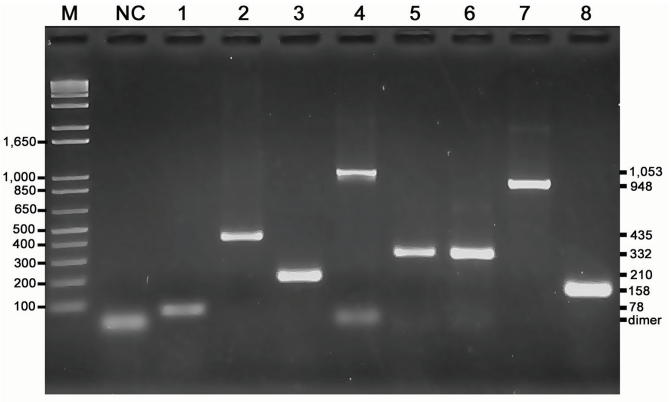

Fig 1–Singleplex PCR for lineage classification of *M. tuberculosis* based on LSPs. Primers are listed in Table 2. Lane M, 1-kb plus DNA ladders; lane NC, negative control; lane 1, 78-bp amplicon of intact RD9 from the Indo-Oceanic lineage; lane 2, 435-bp amplicon of intact RD105 from the Euro-American lineage; lane 3, 210-bp amplicon of deleted RD239 from the Indo-Oceanic lineage; lane 4, 1,053-bp amplicon of intact RD239 from the Beijing lineage; lane 5, 332-bp amplicon of intact 7-bp del *pks15/1* from the Indo-Oceanic lineage; lane 6, 332-bp amplicon of intact 7-bp del *pks15/1* from the East African Indian lineage; lane 7, 948-bp amplicon of intact RD750 from the Indo-Oceanic lineage; lane 8, 158-bp amplicon of deleted RD750 from the East African Indian lineage.

marker, was specifically amplified in all MTB strains but not in other species of mycobacteria and other bacteria tested.

In order to evaluate the multiplex PCR assay, 66 well-characterized MTB strains were tested, and the assay exhibited 100% sensitivity and specificity (Fig 3 and Table 4). The detection limit of the multiplex PCR was 12.5 ng of genomic DNA of all four MTB lineages (a typical result for the Beijing lineage is shown in Fig 4).

DISCUSSION

The global lineages of MTB (Beijing, IO, EuA and EAI) can be classified using RD markers (Fleischmann *et al*, 2002; Tsolaki *et al*, 2004). To solve the various difficulties in the classification of MTB lineages based on LSP using separate PCRs for each RD (Brosch *et al*, 2002; Flores *et al*, 2007; Rindi *et al*, 2012), by PCR/sequencing of the 7-bp del *pks15/1* (Tsolaki *et al*, 2005; Chaiprasert *et al*, 2006b; Gagneux *et al*, 2006; Caws *et al*, 2008; Zenteno-Cuevas *et al*, 2013), or by the hybridization technique (Fleischmann *et al*, 2002; Alland *et al*, 2007), in this study we selected single-tube multiplex PCR as the method for classifying LSP-based MTB lineages. Single-tube multiplex PCR constitutes a simple, rapid and easy-to-perform method. With its ability to simultaneously detect multiple gene targets, this method has been widely applied in various types of molecular studies, such as in the detection and characterization of indels (Pocsai *et al*, 2001), mutations (Shuber *et al*, 1993) and infectious diseases (McAuliffe *et al*, 2013).

Our single-tube multiplex PCR system consisted of five primer pairs, each specific for a particular RD. All MTB lineages produced the RD9 78-bp amplicon. Although singleplex PCR could provide specific amplicons for all MTB lineages, multiplex PCR produced the expected amplicon patterns for only the IO lineage and failed to generate all the expected amplicons for the other three MTB lineages. The absence of the RD239 amplicon (1,053 bp) in our multiplex PCR system might due to the limited processivity of *Taq* DNA polymerase. The absence of the 332-bp

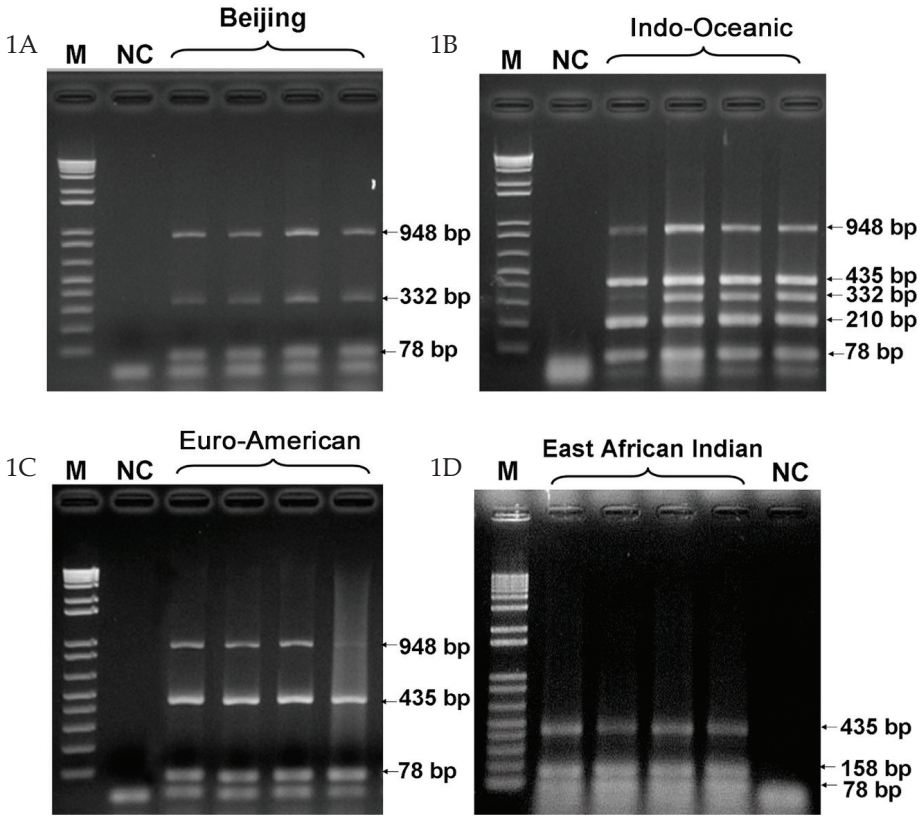

Fig 2–Single-tube multiplex PCR for lineage classification of *M. tuberculosis* based on LSPs. Primers are listed in Table 2. 1A. Beijing lineage. 1B. Indo-Oceanic lineage. 1C. Euro-American lineage. 1D. East African Indian lineage. Four well-characterized strains of each MTB lineage group were tested. Lane M, 1-kb plus DNA ladder; lane NC, negative control.

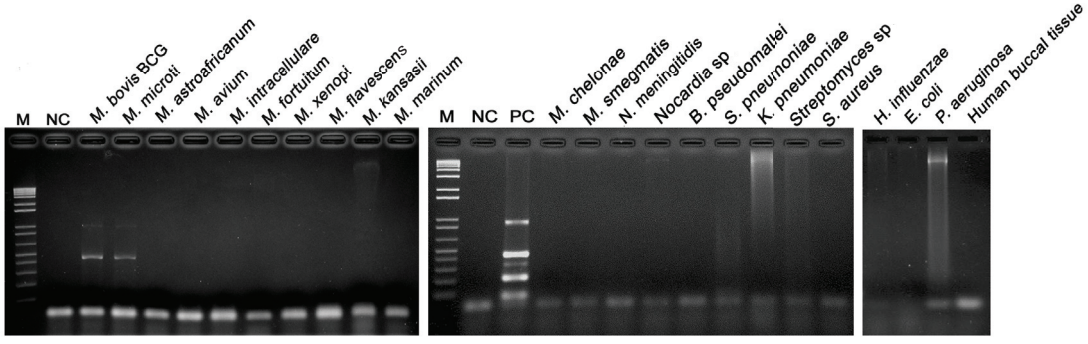

Fig 3–Specificity of single-tube multiplex PCR in classifying MTB lineages based on LSPs**.** Primers are listed in Table 2. Lane M, 1-kb plus DNA ladder; lane NC, negative control; lane PC, positive control of the MTB Indo-Oceanic lineage. The 435-bp (RD105) and 948-bp (RD750) amplicons were amplified in *M. bovis* BCG and *M. microti,* respectively. The lowest band corresponds to primer dimer.

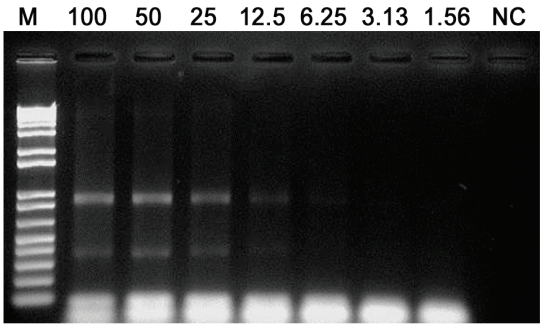

Fig 4–Sensitivity of single-tube multiplex PCR. Primers are listed in Table 2. Number above the lane indicates the concentration (ng) of MTB Beijing lineage DNA. Lane M, 1-kb plus DNA ladders; lane NC, negative control.

amplicon of 7-bp del *pks15/1* in the EAI lineage but not in the Beijing and IO lineages in the multiplex PCR, despite being amplified in singleplex PCR, may reflect suboptimal design of this primer pair. It should be noted that the primer concentration that was required to amplify 7-bp del *pks15/1* in both singleplex and multiplex PCRs was the highest among the five primer pairs. Although optimizations of the multiplex PCR by varying the annealing temperatures and PCR components, including using a 'touch-down' technique, were attempted, the problem of uneven amplification was not completely solved. Nonetheless, the resulting amplicon patterns were consistent and sufficient to differentiate all four MTB lineages, which was the major objective of this study.

Although our single-tube multiplex PCR was successful using genomic DNA extracted directly from MTB colonies, detection/identification of MTB lineages from DNA isolated from AFB-positive sputum was not fruitful (data not shown). This was not due to the presence of inhibitors in the sputum samples, as spiked MTB DNA could be detected. It is still worthwhile, in our opinion, to improve the multiplex PCR technique so as to enable MTB lineage differentiation in clinical samples.

In summary, we have developed a single-tube multiplex PCR as a platform to differentiate each lineage of MTB in DNA samples extracted from bacterial culture. This method is rapid, simple and reliable and can be used for epidemiological and evolutionary studies of MTB based on LSPs.

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