ONE-TUBE MULTIPLEX PCR METHOD FOR RAPID IDENTIFICATION OF *MYCOBACTERIUM TUBERCULOSIS*

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Abstract. A rapid, inexpensive, simple, and accurate multiplex polymerase chain reaction (PCR) was developed in a single tube for identification of *Mycobacterium tuberculosis*. Assessment of sensitivity and specificity of simple PCR was performed with 116 strains of *M. tuberculosis* complex (MTC) and 144 strains of nontuberculous mycobacteria (NTM) compared with the biochemical method. Specific amplification of KS4, MTC-specific DNA fragment, was found in 98% (114/116) of MTC and not detected in 99% (143/144) of NTM. Amplification of the *mtp40* gene revealed 95% sensitivity (100/105 strains of *M. tuberculosis*) and 77% specificity (not found in 119/155 mycobacterial strains). A multiplex PCR method based on the combination of KS4- and *mtp40*-derived primers was used for identification of *M. tuberculosis*. Crude DNA from slow growing mycobacteria with cream rough colonies that showed both 768-bp amplified product for KS4 and 396-bp for *mtp40* was identified as *M. tuberculosis* whereas that from MTC gave only the 768-bp product.

INTRODUCTION

The *Mycobacterium tuberculosis* complex (MTC), which comprises of *M. tuberculosis, M. africanum, M. bovis, M. bovis* BCG, *M. microti*, and recently recognized *M. canettii* and *M. caprae* (Brosch *et al*, 2002; Mostowy *et al*, 2002), is the causative agent of tuberculosis both in human and animals. It is estimated that there are approximately 8-10 million new cases of tuberculosis, causing 2-3 million deaths annually (Miller and Schieffelbein, 1998). The incidence of tuberculosis and other mycobacterial diseases caused by nontuberculous mycobacteria (NTM) have dramatically increased because of an increase in immunocompromized hosts such as AIDS patients. It is important to differentiate between the disease caused by MTC and that caused by NTM. Rapid identification of the causative agent provides useful information for selection of appropriate and effective treatment regimen. Since most laboratories do not fully identify MTC isolates, the true cause of tuberculosis and its source often remain undiscovered. An important health concern is the zoonotic transmission of some MTC subspecies from animal to human and vice versa. Therefore, the ability to differentiate between members of MTC provides accurate epidemiological data that contribute to appropriate patient treatment and public health measures.

Because of the slow growth rate of MTC, identification by using conventional biochemical tests requires several weeks (Kent and Kubica, 1985). Many molecular techniques have recently been developed as a routine identification procedure. The species-specific AccuProbes (Gen-Probe Inc, San Diego, CA, USA) provide rapid and accurate identification results but can be used for identification of a limited number of

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species and do not differentiate between members of MTC (Lebrun *et al*, 1992). Besides, it is still expensive for routine use in laboratories in developing countries. Restriction digestion of amplified products generated from the 16S-23S spacer region (Lappayawichit *et al*, 1996; Roth *et al*, 2000), 65-kDa heat shock protein-encoding gene (Telenti *et al*, 1993), and *rpoB* gene (Lee *et al*, 2000) are able to identify various species of mycobacteria but cannot distinguish members of MTC. Sequencing of 16S rDNA has been proposed for routine identification of mycobacterial species (Böddinghaus *et al*, 1990), but members of MTC have identical 16S rDNA sequences.

A number of genetic markers, IS*6110*, IS*1081*, *MPB70*, for species differentiation within the MTC have been identified (Radford *et al*, 1990; Thierry *et al*, 1990; Collins and Stephens, 1991; Liébana *et al*, 1996). Most recently, comparative genomics studies have revealed regions of difference (RD) representing the loss of genetic material in *M. bovis* compared to *M. tuberculosis* H37Rv (Brosch *et al*, 1998; Behr *et al*, 1999; Gordon *et al*, 1999). Using the presence or absence of RD loci among the MTC, Huard *et al* (2003) developed a PCR-based system using a panel of seven specific primer pairs and the method could differentiate MTC into subspecies. However, this method may be impractical in some laboratories where a large number of mycobacterial isolates are submitted for identification. A novel DNA strip assay, GenoType MTBC, has been developed and evaluated for differentiation of MTC from acid-fast bacilli positive liquid culture. The test is based on a multiplex amplification of 23S rDNA, *gyrB* gene, and RD1 region, followed by reverse hybridization with specific oligonucleotides immobilized on membrane strips (Richter *et al,* 2004). This novel DNA strip assay is still in the process of evaluation for clinical use. Another specific target used for differentiation between MTC, *mtp40* gene, has been shown to be a species-specific marker for *M. tuberculosis* but not for *M. bovis* (del Portillo *et al*, 1991; Parra *et al*, 1991). Recent study revealed that this gene is found in most, although not all, *M. tuberculosis* strains and is also found in some *M. africanum* and *M. bovis* strains (Liébana *et al*, 1996; Weil *et al*, 1996).

Using *mtp40* gene and KS4, an MTC-specific DNA fragment characterized in our laboratory (Samerpitak, 1992), we describe in this report a simple, rapid and cheap PCR method, which combines in a single tube MTC-specific primers based on the KS4 MTC-specific DNA fragment and *mtp40* for the identification and differentiation of *M. tuberculosis*.

MATERIALS AND METHODS

Mycobacterial strains

Two hundred and eighteen clinical isolates of *M. tuberculosis* complex (MTC) and nontuberculous mycobacteria (NTM) isolated from the Mycobacteriology Laboratory, Department of Microbiology, Faculty of Medicine at Siriraj Hospital, Mahidol University during 1995-2000 and 42 reference mycobacterial strains were used in this study (Tables 1 and 2). All strains were subcultured and reidentified by 9-12 standard biochemical tests (Kent and Kubica, 1985).

Cloning and characterization of *M. tuberculosis* complex-specific DNA fragment

The *M. tuberculosis* complex-specific DNA fragment was generated from the recombinant plasmid pWR6 (Rangsipanuratn, 1990), which was isolated from genomic DNA library of *M. tuberculosis* and shown by hybridization to be specific for *M. tuberculosis* complex (Samerpitak, 1992). After digestion of pWR6 with *Pst*I, the shortest fragment (c.a. 800 bp), designated as KS4, was subsequently subcloned into plasmid pGEM4 and subjected for sequencing by dideoxy chain termination method (Sanger *et al*, 1977). The sequence (Prammananan, 1994; Na Ubol, 2001) was searched for in GenBank database using Nucleotide-nucleotide BLAST (blastn) (Altschul *et al*, 1997).

Preparation of genomic DNA

Mycobacterial DNA was isolated from pure culture grown on LJ slant. In brief, one loop each of mycobacteria was harvested and carefully suspended in 1.5 ml microcentrifuge tube containing 150 µl of TE buffer (10 mM Tris, 1 mM EDTA pH 8.0) in a biological safety cabinet class

II (Gelman BH 2000 series, Gelman Sciences, Australia). The tube was firmly sealed and boiled for 15 minutes for breaking and killing the cells. The cell debris was then pelleted and the supernatant was collected and stored at -20ºC until used.

DNA amplification

Two pairs of primers were used as follows: KS4-based primers TPOL, 5′-CCGGCGCTT GCGGGCGGACCCACCGCC-3′ and TPOR, 5′-CAGGCTGCCCTGCCCC ACGCCCCGGTAG-3′ (Prammananan, 1994); *mtp40*-based primers PT1, 5′-CAACGCGCCGTCGGTGG-3′ and PT2, 5′-CCCCCCACGGCACCGC-3′ (del Portillo, 1991). DNA amplification with each pair of primers was performed in DNA Thermal Cycler 480 (PerkinElmer, Boston, MA, USA) in a 50 µl reaction mixture containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 25 pmol of each primer, 200 µM dNTP (Amersham Biosciences, Piscataway, NJ, USA), 1 U of *Taq* polymerase (Amersham Biosciences, Piscataway, NJ, USA) and 5 µl of DNA template, overlaid with 1 drop of mineral oil. The amplification parameters were as follows: initial denaturation at 94ºC for 5 minutes and 20 cycles of denaturation at 94ºC for 1 minute, annealing at 70ºC for 30 seconds, and extension at 72ºC for 1 minute. After 20 cycles of amplification, the second set of 35 amplification cycles was initiated which consisted of 1 minute at 94ºC for denaturation, 30 seconds at 50ºC for annealing, and 1 minute at 72ºC for primer extension. The PCR products were analyzed in 2.5% agarose gel (Organic Research, Cleveland, Ohio, USA) by electrophoresis (~2.5 V/cm) (Mupid 2, Japan) with ϕX 174-*Hae*III digested DNA ladder (Invitrogen, Carlsbad, CA, USA) for size determination, stained with 0.5 µg/ml of ethidium bromide and photographed under an UV-transilluminator (Spectroline TC-321A, USA). The PCR product was 768 and 396 bp for amplification with TPOL/TPOR and PT1/PT2, respectively.

For multiplex PCR the same reaction mixture was used with some modification. Ten pmol of PT1/PT2 primers were used instead of 25 pmol to obtain an almost equal amount of both amplified products. The PCR was performed in the same manner. This one-tube multiplex PCR was used for identification of slow growing cream-rough colonies isolated from clinical samples in mycobacterial laboratory for more than three years previously.

RESULTS

Cloning and characterization of *M. tuberculosis* complex-specific sequence

The recombinant plasmid pWR6 from *M. tuberculosis* genomic library was digested with *Pst*I to generate 4 different DNA fragments. The shortest DNA fragment, designated as KS4 and shown to be specific for MTC, was subcloned into plasmid pGEM4. The KS4 fragment was subsequently sequenced and the results demonstrated that KS4 was 848 bp in length and consisted of 48 copies of 10-bp consensus tandem repeat, CAACAT/CCGGC, separated by 5-bp variable sequence and flanked with 2 unique sequences (Fig 1) (Prammananan, 1994). Comparison of the nucleotide sequence with DNA sequences deposited in GenBank by BLAST revealed that KS4 was 99.6% (847/849 bp) identical to the MTCY28 BAC clone of *M. tuberculosis* H37Rv that is located in Rv1753c region encoding PPE family protein, the Gly-, Asn-rich proteins (na Ubol, 2001). Comparison of all published complete mycobacterial genome sequences showed homologues of this protein (> 75% identity) only in members of MTC.

Assessment of PCR using primers derived from KS4 *M. tuberculosis* complex-specific sequence

PCR using primers derived from the KS4 fragment, TPOL and TPOR (Fig 1), was performed and evaluated for sensitivity and specificity. Amplification of purified *M. tuberculosis* DNA using TPOL and TPOR primers allowed the detection of the 768-bp amplified product of at least 10 pg (equivalent to 2,000 cells, data not shown). DNA isolated from 116 strains of MTC obtained from patients (101 isolates) and reference strains (15 strains) and 144 isolates of nontuberculous mycobacteria (NTM) (27 reference strains and 117 clinical isolates) that were identified by the conventional biochemical methods were investigated for sensitivity and specificity of the PCR assay. One hundred and fourteen isolates (98%) of MTC showed the 768-bp

Table 1
Mycobacterial reference strains used in the study.

Mycobacterium species	Strain	No. of reference strains
M. africanum	TB division, Thailand	1
M. austroafricanum	3005	1
M. avium	ATCC 25291	1
M. bovis	LCDC 302	1
M. bovis BCG	ATCC 35735, ATCC 35740, ATCC 35743, Pasture, Tokyo KK 12-02	6
M. chelonae	ATCC 23016	1
M. duvalii	MNC 442	1
M. flavescens	ATCC 23035	1
M. fortuitum	ATCC 23048, ATCC 144701	2
M. gordonae	ATCC 144701, 330, Pasture	3
M. intracellulare	ATCC 13950, 71	2
M. kansasii	ATCC 12478, 302, Pasture	3
M. marinum	ATCC 927, 329	2
M. microti	KK 1401, LCDC 203	2
M. neolactis	S 152	1
M. nonchromogenicum	ATCC 19530	1
M. phlei	ATCC 23041	1
M. scrofulaceum	ATCC 19981	1
M. szulgai	352, JATA 3201	2
M. terrae	ATCC 15775	1
M. tuberculosis	H37Rv, H37Ra, ATCC 27294, VA 6	5
M. ulcerans	KK 4301, KK 4302	2
M. xenopi	ATCC 19250	1

ATCC: American Type Culture Collection, Rocksville, Maryland
JATA: Japan Anti-Tuberculosis Association, Tokyo
MNC: Mycobacterium National Culture Collection

Table 2
Mycobacterial clinical isolates used in the study.

Mycobacterium species	No. of clinical isolates	Source of clinical samples
M. avium complex	20	Ascitic fluid (1), blood (17), gastric wash (1), unknown (1)
M. bovis BCG	1	Pus from SCID patient
M. chelonae	44	Bone (1), lymph node (13), pus (1), sputum (11), tissue (11), unknown (7)
M. flavescens	3	Gastric wash (1), sputum (2)
M. fortuitum	17	Lymph node (1), sputum (13), tissue (2), unknown (1)
M. gordonae	7	Sputum (7)
M. kansasii	8	Sputum (8)
M. marinum	10	Tissue (10)
M. scrofulaceum	7	Sputum (7)
M. szulgai	1	Sputum (1)
M. tuberculosis	100	Ascitic fluid (11), blood (5), bronchoalveolar lavage (1), cerebrospinal fluid (28), lymph node (10), pericardial fluid (2), peritoneal fluid (4), pleural fluid (10), pus (12), sputum (10), synovial fluid (1), tissue (3), urine (1), unknown (2)

Table 3
PCR results for KS4 and *mtp40* targets.

Mycobacterium species	No. of isolates with PCR results				Total
	KS4		*mtp40*		
	Positive	Negative	Positive	Negative	
M. tuberculosis complex					
M. africanum	1	0	1	0	1
M. bovis	1	0	0	1	1
M. bovis BCG	7	0	1	6	7
M. microti	2	0	1	1	2
M. tuberculosis	103	2	100	5	105
Total	114	2	103	13	116
NTM					
M. austroafricanum	0	1	0	1	1
M. avium	0	1	0	1	1
M. avium complex	0	20	0	20	20
M. chelonae	0	45	20	25	45
M. duvalii	0	1	0	1	1
M. flavescens	0	4	0	4	4
M. fortuitum	1	18	8	11	19
M. gordonae	0	10	0	10	10
M. intracellulare	0	2	0	2	2
M. kansasii	0	11	3	8	11
M. marinum	0	12	0	12	12
M. neolactis	0	1	0	1	1
M. nonchromogenicum	0	1	0	1	1
M. phlei	0	1	0	1	1
M. scrofulaceum	0	8	0	8	8
M. szulgai	0	3	0	3	3
M. terrae	0	1	0	1	1
M. ulcerans	0	2	2	0	2
M. xenopi	0	1	0	1	1
Total	1	143	33	111	144

amplified product; however, two isolates (1 from LN and 1 from CSF) could not be amplified by this KS4-based PCR assay (Table 2). One hundred and forty-three stains (99%) of NTM showed negative results with this PCR assay. However, the 768-bp amplified product was detected in one strain identified as *M. fortuitum* (Table 3). Thus, the overall sensitivity, specificity, and accuracy of this KS4-based PCR assay was 98%, 99%, and 99%, respectively.

Assessment of PCR using primers derived from *mtp40*

PCR with PT1/PT2 primers was performed with DNA isolated from 116 MTC and 144 NTM isolates. Among 105 *M. tuberculosis* strains, the 396-bp specific product was detected in 100 strains (95%) (Table 3). However, *mtp40* was not detected in five isolates of *M. tuberculosis*. In addition, 1 of 7 strains of *M. bovis* BCG, 1 of 1 strain of *M. africanum*, and 1 of 2 strains of *M. microti* were positive for *mtp40*-based PCR assay. The *mtp40*-based PCR assay was also performed with 19 different mycobacterials species (144 strains). The 396-bp amplified product was detected in 28 of 71 isolates of rapid growing mycobacteria (20 isolates were *M. chelonae* and 8 were *M. fortuitum*) and 5 of 73 isolates of slow growers (three isolates were *M. kansasii* and two were *M. ulcerans*) as summarized in Table 3.

Fig 1–Nucleotide sequence of the *M. tuberculosis* complex-specific KS4 fragment. There are 10-nt tandem repeats (in boxs) of the consensus sequence "CAACAT/CCGGC" separated by 5-nt variable sequences. The forward primer TPOL and reversed primer TPOR are indicated by arrows.

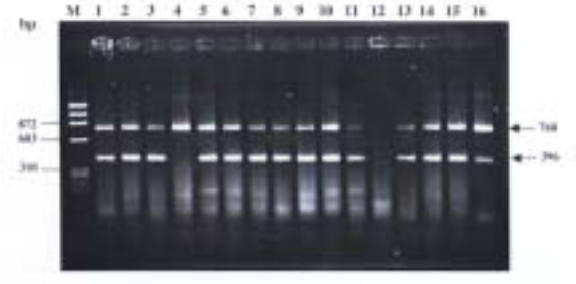

Fig 2–Multiplex PCR of crude DNAs obtained from mycobacterial clinical isolates. The 768-bp amplified product was obtained from *M. tuberculosis* complex-specific primers whereas *M. tuberculosis*-specific primers generated the 396-bp product. Sample that was not amplified by the multiplex PCR was interpreted as nontuberculous mycobacteria (NTM). M: ϕX 174-*Hae*III digested DNA; lanes 1-3: *M. tuberculosis*; lane 4: *M.tuberculosis* complex; lanes 5-11: *M. tuberculosis*; lane 12: NTM; lanes 13-15: *M. tuberculosis*; lane 16: 50 pg *M. tuberculosis* DNA used as the positive control.

Thus, this *mtp40*-based PCR assay had a sensitivity, specificity, and accuracy of 95%, 77%, and 84%, respectively.

Establishment of multiplex PCR

The multiplex PCR based on the combination of KS4- and *mtp40*-derived primers identified all *M. tuberculosis* strains as containing both 768 and 396 bp amplified fragments, whereas other members of MTC gave only the 768 bp amplified fragment (Fig 2). Mycobacteria that were not amplified by this multiplex PCR assay were interpreted as being nontuberculous mycobacteria (Fig 2).

The one-tube multiplex PCR was used for identification of slow grower mycobacteria with rough cream colonies. The tests were performed weekly 234 times with 9,043 isolates. There were 7,248 (80.1%) isolates identified as *M. tuberculosis*, 903 (9.9%) isolates belonging to *Mycobacterium tuberculosis* complex and 517 isolates of NTM. The tests were repeated in 391 (4.3%) iso-

lates due to insufficient amount of DNA or the presence of *Taq* inhibitor in the DNA samples. The identification of such isolates was accomplished by PCR/REA of the *hsp*65 gene and/or *rpoB* gene (data not shown).

DISCUSSION

Identification of mycobacteria by molecular methods has been developed in a past decade in order to replace or complement the biochemical identification. Early methods using hybridization of mycobacterial DNA with species-specific probes had several limitations such as cost, laborious technique used, and the limited number of mycobacterial species identified (Goto *et al*, 1991; Tortoli *et al*, 2001). PCR opened a new era of microbiological laboratory diagnosis, and a number of PCR-based methods have been developed for diagnosis of tuberculosis and identification of mycobacterial pathogens (Kolk *et al*, 1992; Kox *et al*, 1995; Gengvinij *et al*, 2001). PCR approach with species-specific primers or PCR amplification of mycobacterial DNA with genus-specific primers and subsequent hybridization of the amplified products with species-specific probes or digestion of the amplified products with restriction endonucleases have been established for identification of mycobacteria (Plikaytis *et al*, 1992; Lappayawichit *et al*, 1996; Kox *et al*, 1997; Park *et al*, 2000). However, most assays could not differentiate members within the MTC.

The aim of this study was to develop a single-tube PCR method that would be able to detect the presence of KS4-DNA fragment (specific for MTC) and simultaneously identify the species *M. tuberculosis* by means of the presence of a *mtp40* sequence. The KS4 DNA fragment was shown to be specific to MTC (99% specificity and 98% sensitivity). This consensus sequence was found to be identical to the sequence designated as a Major Polymorphic Tandem Repeat (MPTR) by Hermans *et al* (1992), which is in agreement with the results of the hybridization experiment and is a good candidate target for developing a diagnostic tool. Although the *mtp40* gene was originally described as *M. tuberculosis*-specific DNA fragment (del Portillo *et al*, 1991), our study revealed that this gene was found in most, but not all, *M. tuberculosis* strains (identified by all standard biochemical tests) and was also found in *M. africanum, M. bovis* BCG and *M. microti,* which is in agreement with previous studies (Liébana *et al*, 1996; Weil *et al*, 1996). Furthermore, it was shown in this study that the *mtp40* might also be present in some NTM such as *M. kansasii, M. chelonae* and *M. fortuitum* (by revealing the same size of amplified product but not confirmed by DNA sequencing). However, with a 95% sensitivity, *mtp40* is an attractive target for use in diagnostic test, especially when it is used in combination with KS4 fragment for identification of *M. tuberculosis*. The combination of these two sequences in our multiplex PCR method could improve specificity of the approach.

Although the *mtp40* has also been found in some members of MTC, the occurrence in other species causing human tuberculosis is rare, especially in high TB-burden countries. However, for accurate epidemiological data other genetic markers, which are highly specific for *M. tuberculosis* or other species in the complex, should be included in the assay, for instance, the specific spacer region sequences between two direct repeats (DR) or the RD loci of MTC. The spacer regions were reported to be present in *M. bovis* but absent in *M. tuberculosis* and could be used for differentiating among *M. bovis*, *M. bovis* BCG, and *M. tuberculosis* (Liébana *et al*, 1996). The region of difference 1 (RD1) could separate *M. bovis* from *M. bovis* BCG and RD4, RD12, RD13, and RD7-RD10 could differentiate *M. bovis* from *M. tuberculosis* (Talbot *et al*, 1997; Huard *et al*, 2003). However, a single tube PCR assay based on the combination of these primers is, in theory, very difficult to perform and may be useful only in situations where infection caused by *M. bovis* is high. A novel DNA strip assay, GenoType MTBC, was evaluated for differentiation of MTC species and revealed very high sensitivity compared to standard biochemical tests and accuprobe, but its specificity was not assessed (Richter *et al*, 2004).

The single-tube PCR assay developed in this study is rapid, inexpensive and simple, and shown to be an alternative identification method to the biochemical methods for identification of

M. tuberculosis and MTC. Between 40-100 culture isolates could be identified within 1 working day. Basic equipment for DNA amplification and detection by agarose gel-electrophoresis are needed and the cost of reagent and plastic supplies for one test is less than 1 US$. In microbiological laboratories where a large number of mycobacterial isolates are submitted for identification, this assay is able to aid in workload management and provides rapid and accurate identification results.

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