DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* FROM SPUTUM SPECIMENS USING ONE-TUBE NESTED PCR

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Abstract. One-tube nested PCR was developed for diagnosis of pulmonary tuberculosis using sequences based on the16SrRNA gene. The usage of primers 16SOL, 16SOR, 16SIL and 16SIR with optimized conditions could detect 555 bp DNA band from 21 species, 41 strains of mycobacteria and one isolate of *Nocardia asteroides*. It also revealed a specific 306 bp DNA band from 59 strains of *M. tuberculosis* complex. Cross amplification was observed in *M. marinum*, *M. ulcerans* and a few isolates of *M. fortuitum* complex. The developed method could detect as little as 100 fg of *M. tuberculosis* DNA. The PCR mixtures could be stored at 0ºC for 2 months or at -20ºC for at least 20 months without decrease in sensitivity. Using one-tube nested PCR for detection of *M. tuberculosis* compared with acid fast staining and culture results from 153 sputum specimens revealed 88.6% sensitivity and 89.2% specificity in smear positive specimens and 93.2% sensitivity and 85.0% specificity in culture positive specimens.

INTRODUCTION

Although the incidence of tuberculosis declined dramatically in the industrially developed nations during the 20th century, there has been a distinct increase in incidence in many countries since the mid-1980s. The problem has been exacerbated by the HIV pandemic, increasing urban deprivation and the emergence of multidrug resistance. The worldwide threat to human health is tremendous that, in 1993, the World Health Organization (WHO) declared tuberculosis a global emergency (Collins *et al*, 1997). Tuberculosis is caused by *Mycobacterium tuberculosis*, a pathogenic member among 71 recognized species of the genus *Mycobacterium* (Collins *et al*, 1997). WHO estimated that there are annually about 8 million new active tuberculosis patients. In 1994, about 3 million people, including almost 2 million adults, died of tuberculosis and unless global programs are adequately funded and developed, this mortality rate could rise to 4 million annually by the year 2004 (Grange, 1996). The increase in numbers of tuberculosis patients has stimulated the development of rapid and efficient methods of diagnosis.

An efficient tuberculosis control program relies on case finding and treatment, prophylactic chemotherapy and vaccination (Rodrigues and Smith, 1990). For case finding, microbiological methods are still the gold standard. Microscopy with acid-fast bacilli (AFB) staining is the most rapid laboratory method of diagnosing tuberculosis. Its drawback is low specificity and sensitivity for identification *M. tuberculosis*; at least 10^4 cells/ml of mycobacterium in clinical specimens are essential for positive detection by smear and staining (Hobby *et al*, 1973; Yeager *et al*, 1967). The gold standard for laboratory diagnosis of tuberculosis is the isolation of *M. tuberculosis* from clinical specimens. Culture is more specific and sensitive than staining and can detect 10 organisms/ml of digested, concentrated material (American Thoracic Society, 1990) but it requires about 4-8 weeks and many laborious tests.

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The advent of DNA-based technologies such as PCR, the ligase chain reaction (LCR), strand displacement amplification (SDA), transcription-mediated amplification (TMA), nucleic acid sequence-based amplification (NASBA), Q-Beta replicase amplification, and branched DNA and signal amplification (bDNA) has been a great advantage in the detection of the genetic component of microorganisms (Roth *et al*, 1997; Saiki *et al*, 1988; Bennedsen *et al*, 1996; Jonas *et al*,1993; Lindbråthen *et al*, 1997; Moore and Curry, 1998; Down *et al*, 1996; Smith *et al*, 1997). These methods has been described that they are not only more rapid, but also more sensitive than the culture technique (Chaiprasert *et al*, 1996; Eisenach *et al*, 1991; Manjunath *et al*, 1991; Pierre *et al*, 1991). The sensitivity and specificity of PCR are usually enhanced by performing the second round of amplification with an internal set of primers (nested PCR). These procedures involve extra manipulations and an increased chance of contamination from carry-over of amplicons in the laboratory. Kemp *et al* (1990) developed one-tube nested PCR for detection of HIV to overcome these problems. This technique has been modified for use in the diagnosis of *M. tuberculosis* in sputum samples by amplification of the IS*6110* insertion-like element (Wilson *et al*, 1993a,b). These IS*6110* primers were subsequently shown to produce false positive results with other mycobacteria (Kent *et al*, 1995). Moreover, using the IS*6110* element for the target of PCR should be avoided in some geographical region because there are reported of some strains of *M. tuberculosis* and *M. bovis* carry no copy or single copy of this element (Fomukong *et al*, 1994; van Soolingen *et al*, 1993; Palittapongarnpim *et al*, 1997).

There are also many genes, including those for MPB64, the 65 kDa heat shock protein and the 16S rRNA, to be explored as candidate target genes for PCR (Hance *et al*, 1989; Shankar *et al*, 1990; Böddinghaus *et al*, 1990). Our laboratories has selected the 16S rRNA as gene target for amplification of mycobacterial DNA in sputum samples. Its advantages are that it is an essential gene found in every prokaryotic microorganism and for mycobacteria, the sequences of this gene are available from GenBank database.

In this study, we developed one-tube nested PCR using 16S rRNA-based primers for direct detection of *M. tuberculosis* DNA in sputum. This technique should improve the sensitivity for detection of *M. tuberculosis* DNA in clinical specimens and prevent carry-over of amplicons from conventional nested PCR, thereby increasing the specificity.

MATERIALS AND METHODS

Microorganisms

Mycobacterium tuberculosis H37Rv Japan strain and India strain, non-tuberculous mycobacteria (NTM), some strains of *Mycobacterium*, bacteria and fungi used in this study are shown in Table 1.

DNA isolation

DNA from mycobacteria, gram-positive bacteria and yeasts were isolated by using a modified physical rupture method. Briefly, the suspended samples were transferred to 1.5 ml-microcentrifuge tubes containing 0.5 ml of siliconized glass beads and 400 μl of TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0). The cells were broken by mechanical force on a vortex mixer for 1 minute then placed on ice for 1 minute, alternately a total of 10 times. The cell suspension was boiled in a water-bath for 10 minutes. The mixture was centrifuged at 11,600*g* for 2 minutes and the supernatant was removed to a new 1.5 ml microcentrifuge tube. DNA in the supernatant was precipitated by adding 1/10 volume of 4 M NaCl and 2 volumes of cold absolute ethanol. The DNA pellet was collected by centrifugation at 11,600*g* for 20 minutes and then washed with 70% ethanol, dried and dissolved in 100 μl of TE buffer. The amount of DNA was determined by UV spectrophotometry. One microliter of DNA solution was added to 49 μl of PCR reaction mixture for amplification.

Table 1
Mycobacteria, non-tuberculous mycobacteria (NTM), bacteria and fungi used in this study.

Organisms	No. of strain	Sources
Mycobacteria		
M. bovis, LCDC302	1	BD
M. bovis	4	JICA
M. bovis BCG	1	patient's isolate
M. tuberculosis, H37Rv Japan	1	TB
M. tuberculosis, H37Rv India	1	TB
M. tuberculosis, ATCC27294	1	BD
M. tuberculosis, VA-6	1	BD
M. tuberculosis	42	patient's isolate
M. tuberculosis	4	CCH
Non-tuberculous mycobacteria (NTM)		
M. aurum, MNC974	1	TB
M. austroafricanum, 3005	1	TB
M. avium	1	TB
M. avium, ATCC25291	1	RIT
M. avium, JATA51-01	1	RIT
M. chelonae, ATCC23016	1	TB
M. chelonae	3	patient's isolate
M. duvalii, MNC442	1	TB
M. flavescens, ATCC23035	1	TB
M. flavescens	1	SC
M. fortuitum	6	patient's isolate
M. fortuitum, ATCC6841	1	RIT
M. fortuitum, ATCC23048	1	TB
M. gordonae, ATCC14470	1	RIT
M. intracellulare, ATCC13950	1	RIT
M. intracellulare	1	MCC
M. kansasii, ATCC12478	1	RIT
M. kansasii	1	MCC
M. marinum, ATCC927	1	RIT
M. marinum	2	patient's isolate
M. marinum	1	MCC
M. neolactis	1	MCC
M. nonchromogenicum, ATCC19530	1	RIT
M. phlei, ATCC23042	1	MCC
M. scrofulaceum, ATCC19981	1	RIT
M. simiae, ATCC25275	1	RIT
M. smegmatis, ATCC14468	1	TB
M. szulgai, KK32-01	1	RIT
M. szulgai, 352	1	MCC
M. terrae	1	patient's isolate
M. ulcerans	2	RIT
M. xenopi, ATCC19250	1	RIT

Table 1 (continued)

Organisms	No. of strain	Sources
Bacteria		
Bacteroides melaninogenicus	1	BCC
Branhamella catarrhalis	1	BCC
Corynebacterium diptheriae	1	BCC
Escherichia coli	1	BCC
Klebsiella pneumoniae	1	BCC
Nocardia asteroides	1	MCC
Nocardia caviae	1	MCC
Pseudomonas aeruginosa	1	BCC
Staphylococcus aureus	1	BCC
Streptococcus pneumoniae	1	BCC
Streptococcus viridans	1	BCC
Fungi		
Absidia sp	1	MCC
Aspergillus flavus, CDC-B-15	1	MCC
Aspergillus fumigatus, CDC-B-1172	1	MCC
Aspergillus niger, CDC-107	1	MCC
Basidiobolus ranarum , SIMI-10384	1	MCC
Candida albicans, ATCC10231	1	MCC
Candida tropicalis, SIMI-5	1	MCC
Conidiobolus coronatus, SIMI-10037	1	MCC
Cryptococcus neoformans, SIMI-17	1	MCC
Epidermophyton floccosum	1	MCC
Exophiala jeanselmei	1	MCC
Fonsecaea compactum, B-3318	1	MCC
Fonsecaea pedrosoi, B-2712	1	MCC
Histoplasma capsulatum, SIMI-8838	1	MCC
Mucor sp	1	MCC
Penicillium marneffei, SIMI-10202	1	MCC
Pheoannellomyces werneckii	1	MCC
Phialophora verrucosa, B-2725	1	MCC
Pythium insidiosum, CBS-240-37	1	MCC
Rhizopus sp	1	MCC
Rhodotorula sp	1	MCC
Saccharomyces cerevisiae	1	MCC
Scedosporium apiospermum	1	MCC
Trichosporon sp	1	MCC

BD = Becton Dickinson Diagnostic Instrument System, Maryland, USA (provided by Dr Salman Siddiqi; JICA = Japan International Cooperation Agency, Japan (provided by Dr Masaharu Kanameda); TB = Tuberculosis Division, Department of Communicable Disease Control, Ministry of Public Health, Bangkok, Thailand; CCH = Central Chest Hospital, Department of Communicable Disease Control, Ministry of Public Health, Bangkok, Thailand (provided by Dr Charoen Chuchottaworn); RIT = Research Institute of Tuberculosis, Japan (provided by Dr Chiyoji Abe); BCC = Bacteriology Culture Collection, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University; MCC = Medical Mycology Culture Collection, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, SC = Faculty of Science, Mahidol University.

DNA amplification using one-tube nested PCR

(i) Primer design

This technique aimed to perform nested PCR in the same tube by optimizing the first round of PCR using the outer pair of primers and the second round with the inner pair of primers. For this purpose we synthesized the outer primers with a higher melting temperature or more G-C content than the inner primers by adding a GC clamp (CCGGCGGCCG) to the 5′ terminal of the outer primers. The primers used for amplification were derived from the 16S rRNA gene: the outer primers were 16SOL (5′ TGCACTTCGGGATAAGCCTG 3′, residue 94 to 113), 16SOR (5′ ATTCCAGTCTCCCC TGCAGT 3′, residue 609 to 628) and the inner primers were 16SIL (5′ GGATAGGACCAC GGGAT 3′, residue 133 to 149), 16SIR (5′ TAC CGTCAATCCGAGAG 3′, residue 422 to 438). The outer and inner pairs of primers were expected to be the genus-specific and species-specific primers for 16S rRNA gene amplification, respectively. All primers were synthesized by the Bioservice Unit, National Science and Technology Development Agency Thailand.

(ii) DNA amplification

The PCR reaction was optimized by varying dNTPs concentration, outer primer annealing temperature and primer concentration. The PCR was performed in 50 μl of reaction mixture containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 0.001% gelatin, 25 pmol each of 16SIL, 16SIR primer and optimal concentration of dNTPs (Amersham Pharmacia Biotech, Buckinghamshire, England) and 16 SOL, 16SOR, 1 unit of *Taq* DNA polymerase (Amersham Pharmacia Biotech, Buckinghamshire, England) and 1 μl of purified DNA or 5 μl of DNA extracted from sputum samples. The reaction mixture was initially denatured at 94ºC for 5 minutes, and subjected to 20 cycles of first round PCR comprising denaturation at 94ºC for 1 minute, annealing at 70ºC for 30 seconds and extension at 72ºC for 1 minute, followed by 35 cycles of second round PCR comprising denaturation at 94ºC for 1 minute, annealing at 50ºC for 30 seconds and extension at 72ºC for 1 minute. Finally, the PCR products were detected by electrophoresis of 5 μl reaction mixture in 4% Nusieve agarose gel 3:1 (FMC Bioproducts, Rockland, USA) and examination of the ethidium bromide-stained gel under a UV transilluminator.

DNA preparation from sputum samples

Each sputum sample was decontaminated and digested with an equal volume of 4% NaOH for 15 minutes at room temperature and neutralized with 2 volumes of 0.067 M phosphate buffer, pH 7.0 (Kent and Kubica, 1985). The mixture was centrifuged at 2,020*g* for 15 minutes to collect the cells; then the pellet was resuspended with 1 ml of TE buffer. The treated sputum sample was divided into three parts. The first part of 0.1 ml was processed for direct smear microscopic examination by acid fast staining. The second part of 0.4 ml was cultured for isolation of mycobacteria on Löwenstein-Jensen medium. Mycobacterial species were identified using standard biochemical tests (Kent and Kubica, 1985). The third part (about 0.5 ml) was used for isolation of DNA (Chaiprasert *et al*, 1996). About 5 μl of isolated DNA or diluted DNA from each sputum sample were used for one-tube nested PCR.

Detection of *M. tuberculosis* from sputum specimens

All 153 sputum specimens were collected from newly identified individual patients who received no chemotherapy treatment, HIV negative and symptoms suspected pulmonary tuberculosis. All samples were processed in this study for conventional investigation of acid-fast bacilli by staining and microscopy followed the reporting system of CDC (Kent and Kubica, 1985), isolation of *M. tuberculosis* by cultivation on Löwenstein-Jensen medium, and detection of species specific 306-bp product by one-tube nested PCR. Due to the high risk of contamination of PCR results, we decided to perform pre-PCR, PCR and post-PCR in separate room with each sets of automatic pipette and equipment. The negative reagent

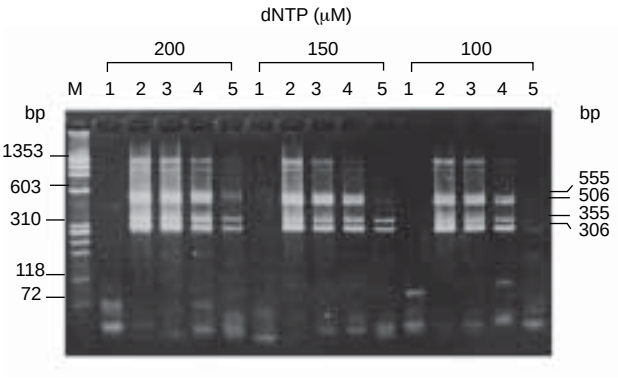

Fig 1–Effect of dNTP concentration on one-tube nested PCR. PCR was performed using 0 (lane 1), 10 pg (lane 2), 1 pg (lane 3), 100 fg (lane 4) and 10 fg (lane 5) *Mycobacterium tuberculosis* H37Rv DNA in the presence of dNTP concentrations of 200 μM, 150 μM or 100 μM and the products were subjected to agarose gel electrophoresis. The amplified product is shown as a group-specific band of 555 bp and a species-specific band of 306 bp. The other product size of 506 bp and 355 bp which generated from cross amplification between outer and inner pair of primers are also shown. The DNA marker of *Hae* III digested øX174 DNA is in lane M.

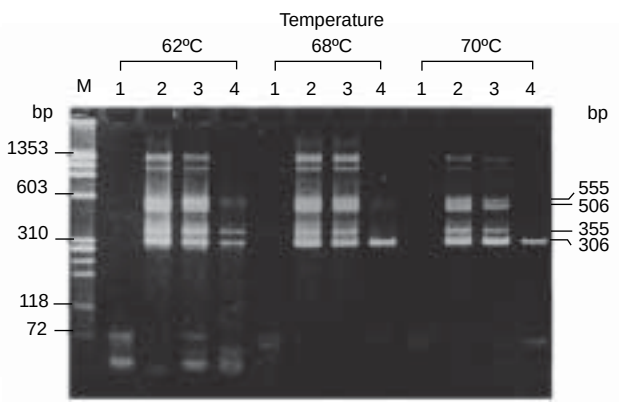

Fig 2–Effect of annealing temperature on one-tube nested PCR. PCR was performed using 0 (lane 1), 1 pg (lane 2), 100 fg (lane 3) and 10 fg (lane 4) *Mycobacterium tuberculosis* H37Rv DNA with an annealing temperature of 62ºC, 68ºC or 70ºC. The amplified product is shown as described earlier in Fig1. The DNA marker of *Hae* III digested øX174 DNA is in lane M.

blank and positive control reaction were processed parallel in each sets of 10-20 specimens. The results which gave the correct negative and positive control in each sets should be used for analysis.

RESULTS

Optimization of amplification conditions

PCR using dNTP concentrations of 150 and 200 μM was shown to give intense signals of specific amplified fragments from *M. tuberculosis* DNA ranging from 10 pg to 10 fg, whereas at 100 μM dNTPs gave lower sensitivity. To ensure that the dNTPs were not limiting for DNA amplification, dNTPs at 200 μM were used in further experiments (Fig 1).

An annealing temperature of 62ºC for first round PCR gave a higher sensitivity than 68ºC or 70ºC (Fig 2). The genus-specific and *M. tuberculosis* complex-specific bands (555 bp and 306 bp, respectively) were observed at the lowest DNA concentration tested when using an annealing temperature for the outer primers of 62ºC. Only the 306-bp band was observed when the annealing temperature was increased. An annealing temperature of 70ºC for 30 seconds was chosen because of its high stringency, which should avoid or decrease the problem of cross amplification and made the test more specific.

For the primer concentration, ratios of outer and inner primer concentrations between 1:12.5 and 1:25 showed both the genus-specific and *M. tuberculosis* complex specific bands, whereas a ratio of 1:50 gave mainly the latter. When the ratio of primer concentration was reduced to 1:100, only the amplified products of 506-bp and 306-bp DNA were seen (Fig 3). The ratio of outer and inner primer concentrations of 1:12.5 was chosen because this allowed the detection of both *Mycobacterium* spp as well as *M. tuberculosis* complex-specific amplified products.

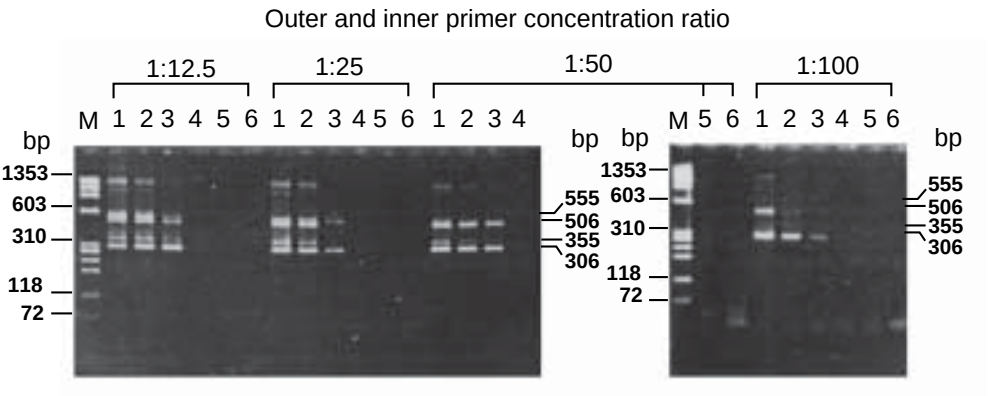

Fig 3–Effect of the ratio of outer and inner primer concentration on one-tube nested PCR. PCR was performed using 10 pg (lane 1), 1 pg (lane 2), 100 fg (lane 3), 10 fg (lane 4), 1 fg (lane5) and 0 (lane 6) *Mycobacterium tuberculosis* H37Rv DNA in the presence of ratios of outer and inner primer concentration of 1:12.5, 1:25, 1:50 and 1:100. The amplified product is shown as described in Fig1. Lane M is *Hae* III digested øX174 DNA marker.

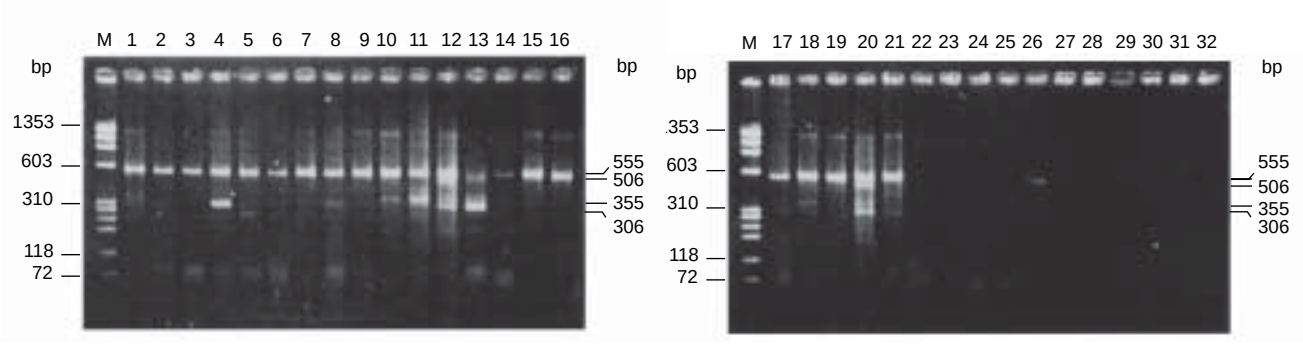

Fig 4–Amplification of DNA template extracted from various isolates of *Mycobacterium* species, bacteria, yeasts and fungi using one-tube nested PCR. PCR was performed with 1 ng DNA of *M. avium* (lane 1), *M. aurum* (lane 2), *M. austroafricanum* (lane 3), *M. chelonae* (lanes 4-5), *M. duvalii* (lane 6), *M. flavescens* (lane 7), *M. fortuitum* (lane 8), *M. gordonae* (lane 9), *M. intracellulare* (lane 10), *M. kansasii* (lane 11), *M. marinum* (lane 12), *M. phlei* (lane 14), *M. scrofulaceum* (lane 15), *M. simiae* (lane 16), *M. smegmatis* (lane 17), *M. szulgai* (lane 18), *M. terrae* (lane 19), *M. ulcerans* (lane 20), *M. xenopi* (lane 21), *Corynebacterium diphteriae* (lane 22), *Streptococcus pneumoniae* (lane 23), *Escherichia coli* (lane 24), *Klebsiella pneumoniae* (lane 25), *Nocardia asteroides* (lane 26), *Pseudomonas aeruginosa* (lane 27), *Candida albicans* (lane 28), *Cryptococcus neoformans* (lane 29), *Aspergillus fumigatus* (lane 30), *Histoplasma capsulatum* (lane 31), *Penicillium marneffei* (lane 32) and 100 fg DNA of *M. tuberculosis* as positive control (lane 13). The amplified product is shown as a group-specific band of 555 bp and a species-specific band of 306 bp. Lane M is *Hae* III digested øX174 DNA marker.

Specificity and sensitivity of one-tube nested PCR

The designed outer primers (16SOL and 16SOR) were genus specific and amplified 555-bp DNA of all mycobacterial species and one isolate of *Nocardia asteroides*. The species-specific primers or inner primers (16SIL and 16SIR) should reveal 306-bp product only from *M. tuberculosis* complex DNAs. In this study, the products of 506 bp and 355 bp were also amplified by crossed annealing of 16SIL-16SOR and 16SOL-16SIR, respectively. Before using developed one-tube nested PCR to detect *M. tuberculosis* DNA in sputum samples,

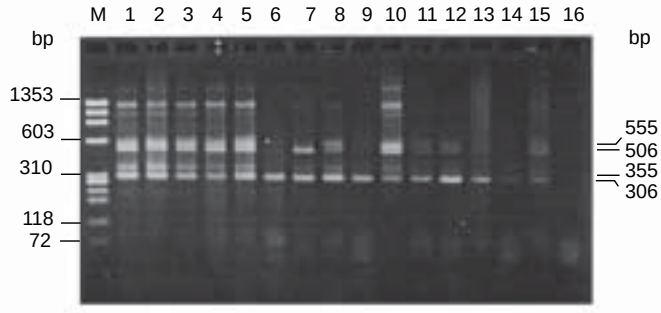

Fig 5–Amplification of DNA template isolated from sputum specimens using one-tube nested PCR. PCR was performed with isolated DNA of sputum specimens (lanes 1-7, 9-15), 100 fg DNA of *M. tuberculosis* as positive control (lane 8) and no DNA as reagent blank control (lane 16). The amplified product is shown as a group-specific band of 555 bp and a species-specific band of 306 bp. Lane M is *Hae* III digested øX174 DNA marker.

the specificity and sensitivity of these primers were evaluated by using reference strains or well identified isolates of mycobacterium and other related microorganisms.

Genomic DNA prepared from 59 strains of *M. tuberculosis* complex, 41 strains (21 species) of non-tuberculous mycobacteria (NTM), 11 other bacteria and 24 fungi were used as DNA templates for PCR (Fig 4). These sets of primers amplified at least single 306-bp DNA from *M. tuberculosis* complex, *M. marinum, M. ulcerans* and some strains of *M. fortuitum* complex. These primers also amplified 555-bp DNA from all mycobacteria except *M. neolactis, M. nonchromogenicum, M. phlei* and one strain of *M. terrae*, whereas no amplified product of the relevant size was observed from other bacteria and fungi. The lower limit of detection of *M. tuberculosis* DNA was 100 fg,

Table 2
Result of PCR after long-term storage of ready-to-use 16S rRNA amplification mixtures.

Storage temperature (°C)	Concentration of DNA template (fg)	PCR result obtained from reaction mixtures after storage (weeks)				
		0	2	4	8	80
-20	1000	+	+	+	+	+
	100	+	+	+	+	+
	10	+	+	+	+	-
	0	-	-	-	-	-
0	1000	+	+	+	+	+
	100	+	+	+	+	-
	10	+	+	-	-	-
	0	-	-	-	-	-

+ = positive for 555 and/or 306 bp amplified product by PCR.
- = negative for 555 and/or 306 bp amplified product by PCR.

Table 3
Comparison of one-tube nested PCR results with AFB smear results for *M. tuberculosis*.

Specimen category (no.)	No. of specimens		Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
	Smear positive	Smear negative				
PCR positive (85)	78	7	88.6	89.2	91.8	85.3
PCR negative (68)	10	58				

Table 4
Comparison of one-tube nested PCR results with culture results for *M. tuberculosis*.

Specimen category (no.)	No. of specimens		Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
	Culture positive	Culture negative				
PCR positive (80)	68	12	93.2	85.0	85.0	93.2
PCR negative (73)	5	68				

which corresponds to the amount of DNA present in approximately twenty mycobacterial cells.

Effect of storage conditions of reaction mixtures for PCR

For the convenience of laboratories that would like to perform PCR for detection of *M. tuberculosis* DNA in clinical samples, the reaction mixtures should be stable for at least 3 months. One hundred and twenty aliquots of reaction mixtures (49 μl per tube) were equally divided and kept at 0ºC and -20ºC. On the preparation day, four tubes from each set were used to amplify *M. tuberculosis* DNA by one-tube nested PCR. The same tests were repeated at up to 80 weeks of storage (Table 2). The criteria for interpretation of positive results was the presence of 555 and/or 306 bp amplified DNA products. Storage of the reaction mixtures at -20ºC gave better results with low concentrations of template DNA.

Detection of *M. tuberculosis* from sputum specimens

One-tube nested PCR was used to detect *M. tuberculosis* DNA from 153 sputum specimens. The amplified products of 555-bp DNA and/or 306-bp DNA observed in sputum specimens were representatively demonstrated in Fig 5. The results were shown to be in good correlation compared with AFB staining of mycobacteria and isolation of *M. tuberculosis* by culture technique. They reveal 88.6% sensitivity and 89.2% specificity compared with smear positive specimens and 93.2% sensitivity and 85.0% specificity compared with culture positive specimens (Tables 3-4).

DISCUSSION

The control of tuberculosis requires rapid identification of infectious cases. Vaccination of such patients with BCG and the implementation of Directly Observed Treatment, Short-course (DOTS) recommended by WHO should virtually reverse the TB epidemic, not only in wealthy countries, but in the world's poorest countries as well (Kochi, 1997). Before application of DOTS, infectious cases are usually identified by microscopic examination of AFB and isolation of *M. tuberculosis* by culture. We have assessed the detection of nucleic acid using PCR amplification for a role in case-finding of tuberculosis patients.

The sensitivity of direct detection of *M. tuberculosis* by PCR depends on the efficiency of DNA isolation from mycobacterial cells, amplification of target DNA and detection of the amplified product. Mycobacterial cell walls are more difficult to break than cell walls of other gram positive and gram negative bacteria. Many procedures have been explored, including boiling in TE-Triton, sonication, enzymatic lysis and physical rupture (Chaiprasert *et al*, 1996; Eisenach *et al*, 1991; Buck *et al*, 1992; Sritharan and Barker, 1991). The physical rupture method provided suitable DNA as revealed by the reproducibility of the amplification of specific product (Chaiprasert *et al*, 1996). This method was also practical, simple and not too expensive for testing large numbers of specimens. Previously simple PCR with or without a hybridization step using repetitive DNA sequence IS*6110* or an other cloned DNA fragment, was shown to detect between 1 fg and

10 fg of purified *M. tuberculosis* DNA (Sritharan and Barker, 1991; De Wit *et al*, 1990; Eisenach *et al*, 1990; Fries *et al*, 1991; Patel *et al*, 1990). Our technique using one-tube nested PCR also provided high sensitivity, between 100 fg to 10 fg, and should avoid false positive results from carryover contamination of conventional nested PCR.

This technique was optimized to provide high specificity and sensitivity and adopted for rapid and direct detection of *M. tuberculosis* DNA from sputum specimens. Although in this study only sputum samples were tested, this technique should be appropriate for detection of *M. tuberculosis* DNA from other types of clinical specimens such as cerebrospinal fluid, granuloma and spine tissues especially if the samples were precultured in liquid media for a week. The best conditions for one-tube nested PCR included a ratio of outer and inner primer concentrations of 1:12.5, which led to amplification of both genus and species specific products. The outer primers 16SOL, 16SOR showed specificity of amplification with 100 mycobacterial strains tested (59 *M. tuberculosis* complex and 41 NTM). Some *M. tuberculosis* strains revealed a faint 555-bp product when using 1 pg DNA templates. This might be due to the degradation of stored DNA, because experiments using freshly prepared *M. tuberculosis* DNA showed consistent detection of the 555-bp DNA band. No amplified product of 555 bp was observed from 10 bacterial species and 24 fungal species studied. Cross amplification was detected in one isolate of *Nocardia asteroides*, which is phylogenetically related to mycobacteria. An exception was also found with one strain of *M. neolactis* which revealed no 555-bp DNA band. This could occur only if mutation of the target DNA at the specific primer-annealing sequence led to no amplification. The inner primers 16SIL, 16SIR could amplify the 306-bp DNA product from 59 isolates of *M. tuberculosis* complex without cross amplification with other bacteria and fungi, except some isolates of *M. fortuitum* complex, *M. marinum* and *M. ulcerans*. The latter two species are closely related to one another and are the two species most closely related to *M. tuberculosis* on the basis of 16S rRNA gene similarity (Tønjum *et al*, 1998). However, *M. marinum* and *M. ulcerans* together with *M. fortuitum* complex, should not interfere with direct detection of *M. tuberculosis* complex in sputum because they usually cause skin diseases and have been rarely reported to cause systemic infection. The optimized one-tube nested PCR using 16SOL, 16SOR, 16SIL and 16SIR primers could detect *M. tuberculosis* DNA at the lowest concentration of 100 fg with consistency. The specific 306-bp product was sometimes observed at a DNA concentration of 10 fg.

The PCR reaction mixture could be prepared and stored at 0ºC for 2 months or at -20ºC for 20 months without decreasing its sensitivity in detection of *M. tuberculosis* DNA. This should be appropriate for use as a diagnostic PCR method in the future. However, some investigator claimed that the PCR mixture in the freeze-dried form could be maintained at 4ºC and at -20ºC for 1 year without decreasing the activity to detect 50 fg of purified *M. bovis* BCG DNA (Klatser *et al*, 1998). The observed results of 10 specimens of PCR negative compared with the AFB staining or 5 specimens compared with the culture technique might be the lower limit of PCR that could detect as low as 20 genomes, whereas the culture could give positive result with the minimum of 10 mycobacterial cells (Chaiprasert *et al*, 1996; Manjunath *et al*, 1991). Moreover, the 5 specimens of smear positive was observed as non-tuberculous mycobacteria (NTM).

This developed PCR showed very good correlation with the gold standard method either culture technique or AFB staining. It revealed high sensitivity and specificity when monitoring the direct detection in sputum and could minimize the time consuming for identification of *M. tuberculosis* in clinical specimens. Many laboratories are currently developing the diagnostic method for direct detection of *M. tuberculosis* in clinical specimens and some commercial diagnostic tests were distributed into the market. The cost of this one-tube nested PCR reaction was estimated as US$ 5. This PCR test was also promising for using

in the laboratory without sophisticated or special instrument or development for high throughout detection in ELISA format. However, the more sensitive and specific PCR technique could be developed further for detection and identification of *M. tuberculosis* in clinical specimens.

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