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Evaluation of In-House Optimized Semi-Nested PCR and EIA for Direct Detection of Mycobacterial DNA in CSF

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SUMMARY **A rapid and correct diagnosis of mycobacterial infections is important for effective patient treatment. Semi-nested-PCR with FI-16 SOL, 16SOR and 16SNSR primers based on the 16S rRNA gene, under optimized conditions, can detect 499 bp amplified products from all tested mycobacteria. The assay could detect as little as 100 fg of mycobacterial DNA except for rapid growing mycobacteria, whose detection limits ranged from 1 ng to 10 pg. The specificities of the capture probes were assessed with 96 mycobacterial strains (22 species) and 33 nonmycobacterial strains (30 species). The specificities of pAll1, pTbc1 and pMar1 were 94%, 93% and 82%, respectively, and that of pAvi1, pInt1, pChe1 and pFor1 were 100%. The pTbc1 and pAvi1 were tested with DNA from 108 CSF samples, and the sensitivity and specificity of the detection method were 56% and 84% compared to culture and patient histories. The assay should be used for rapid detection and concurrent identification of slow growing mycobacteria without parallel conventional culture verification.**

The global burden of tuberculosis remains enormous, mainly because of poor control in Southeast Asia, sub-Saharan Africa and eastern Europe, and because of high rates of *M. tuberculosis* and HIV coinfection. New cases of tuberculosis are estimated to be 7.96 million per year including 3.52 million (44%) cases of infectious pulmonary disease (sputum smear-positive). Dye *et al.*[1] reported 16.2 million existing cases of disease in 1999 with a global case fatality rate of 23%. In the last few years, the incidence of infections due to nontuberculous mycobacteria (NTM) has increased significantly, especially in immunocompromised patients.[2-4] The rising incidence of NTM signifies the need for rapid and accurate diagnosis of mycobacterial infections, because the treatment of tuberculosis differs from that of the disease caused by NTM, which are often resistant to antituberculous drugs.[5-7] The most common *in vitro* diagnostic method for mycobacterial infection is microscopic examination of a sputum smear stained using the acid-fast procedure. This method is rapid and cost-effective, but lacks both sensitivity and specificity. A large number of organisms (> 10^4 CFU/ml) must be present in order to be detected and the species cannot be identified.[8,9] *Mycobacterium* culture is the "gold standard" of laboratory diagnosis. This method has a high sensitivity and specificity but taking long time of incubation. Moreover, the differentiation of *Mycobacterium* species is usually performed by the time-consuming evaluation of phenotypic and biochemical characteristics. Many molecular genetic methods have been introduced, hoping to replace the time-consuming and laborious procedures. PCR is a technique for *in vitro* amplification of DNA. It is a rapid method, which is independent of culture.[10] The sensitivity of PCR is usually enhanced by subjecting the product to a second round of PCR with an internal set of primers (nested-PCR).[11] However, these procedures involve an increased chance of contamination. Wilson *et al.*[12] and Gengvinij *et al.*[13] used a one-tube nested-PCR technique in order to decrease the problem

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of carryover contamination, which might occur with the conventional nested-PCR.

A semi-nested-PCR technique has been described by Patel *et al.*[14] The principle of this technique is based on using three primers in a one-tube reaction mixture. Amplified products formed in the first profile using outer primers would act as a target for the reverse and forward inner primer in the secondary PCR. Many genes and sequences have been used as target DNA for amplification of mycobacterial DNAs in clinical samples.[15-17] The 16S rRNA gene is used and assessed as target DNA for amplification of mycobacterial DNAs in this study. The reasons of using this target are as follows:- it is an essential gene that is found in every prokaryotic organism and rRNA functions have been highly conserved and contains hypervariable regions which exhibit species-specific characteristics.[18,19] These species-specific regions can be detected in several ways, the simplest one is by hybridization to a species-specific probe.[20-23]

In this study, the combination of semi-nested-PCR and an enzyme immunoassay (EIA) capture probe are modified for the direct detection and identification of mycobacterial DNA in cerebrospinal fluid (CSF) samples.

MATERIALS AND METHODS

Microorganisms

The *Mycobacterium* reference strains, *Mycobacterium* clinical isolates, other bacteria and fungi used in this study are shown in Table 1.

Clinical Samples

One hundred and eight CSF samples were obtained for a retrospective blind study from individual patients with suspected mycobacterial meningitis. DNA from clinical samples was isolated by using a modified physical rupture method.[13]

DNA extraction

DNA from mycobacteria, bacteria and fungi were isolated by using a modified physical rupture method.[13] Briefly, three to four loopfulls of microbial cells were transferred to a 15 ml centrifuge tube containing glass beads (450-500 µm in diameter) and 1.5 ml of TE buffer. The cell suspension was heated to 80°C in a waterbath for 20 minutes to kill the living cells. It was then vortexed for 1 minute alternated with placing it on ice for 1 minute (repeated 10 times), in order to break the cells. Consecutively the suspension was centrifuged at 5,000 x *g* for 5 minutes. The supernatant was pipetted into a new 1.5 ml microcentrifuge tube. The protein was digested by adding a mixture of 10% SDS and 10 mg/ml proteinase-K, vortexing briefly and incubating at 65°C for 10 minutes. Later, 100 µl of 5 M NaCl were added, mixed thoroughly and 100 µl of CTAB/NaCl solution, (which was prewarmed to 65°C) were added and mixed again until the liquid became milky white. It was then incubated at 65°C for 10 minutes. Protein extraction was accomplished by adding chloroform-isoamyl alcohol (24:1), mixed for at least 10 seconds and centrifuging at room temperature at 10,000 x *g* for 5 minutes. The supernatant was put into a new microcentrifuge tube. Fifteen microliters of RNaseA were added and the liquid was mixed and then incubated at 37°C for 1 hour. Two volumes of absolute ethanol were added and mixed gently, centrifuged at 12,074 x *g* for 10 minutes at 4°C after which most of the supernatant was discarded. The DNA pellet was washed using 1 ml of cold 70% ethanol, centrifuged for 5 minutes at 4°C and the supernatant was discarded. The DNA pellet was dried at 56°C and redissolved in TE buffer. The DNA concentration was determined using a UV-spectrophotometer, by assuming that 1 OD at a wavelength of 260 nm corresponded to 50 µg/ml of DNA. The DNA solution was stored at 4°C or -20°C for further use.

DNA amplification using semi-nested-PCR

Primer design

The primers used for amplification were derived from the 16S rRNA gene. The 16SOL (5'-TGCACTTCGGGATAAGCCTG-3', residues 94 to 113) and 16SOR (5'-ATTCCAGTCTCCCCTGCAG- T-3', residues 609 to 628) primers were genus specific for mycobacterial species and were used as the outer primers for the first amplification.[13] The 5' end of these primers were added to a GC clamp (CCGGCGGCCG) to increase the melting temperature for drop-in amplification. The 16SOL primer was labelled with fluorescein at the 5' end and the 16SOR was the unlabelled reverse primer. The inner reverse primer 16SNSR (5'-CGCTCACAGTTAAGCCGTGAG-3', residues 562 to 582) was genus specific and could amplify the primary PCR product by using the Fl-16SOL primer as a forward primer.

DNA amplification

The semi-nested-PCR was optimized by varying some parameters, such as the Fl-16SOL primer concentrations (12.5, 25, 37.5 pmole) and the 16SOR primer concentrations (2.5, 1.25, 0.83, 0.625 and 0.5 pmole). The PCR was performed in 50 µl of reaction mixture containing 10 mM Tris-HCl (pH 8.4),

Table 1 *Mycobacterium* reference strains, *Mycobacterium* clinical isolates, other bacteria and fungi used in this study

Organisms	No. of Strains	Sources
Mycobacterium		
M. austroafricanum, 3005	1	TBD
M. avium, ATCC 25291	1	RIT
M. avium, 212	1	RIT
M. bovis, KK 12-05	1	RIT
M. bovis, LCDC 302	1	BD
M. bovis BCG, Pasteur	1	SC
M. bovis BCG, Tokyo KK 12-02	1	RIT
M. bovis BCG, ATCC 35733	1	RIT
M. chelonae, ATCC 23016	1	TBD
M. chelonae	9	Patient's isolate
M. duvalii, MNC 442	1	TBD
M. flavescens, ATCC 23035	1	TBD
M. fortuitum, ATCC 23048	1	TBD
M. fortuitum, ATCC 6841	1	RIT
M. fortuitum	8	Patient's isolate
M. gordonae, ATCC 14470	1	RIT
M. gordonae, 330	1	MCC
M. intracellulare, ATCC 13950	1	RIT
M. intracellulare, 71	1	MCC
M. kansasii, ATCC 12478	1	RIT
M. kansasii, 302	1	MCC
M. marinum, ATCC 927	1	RIT
M. marinum, 329	1	MCC
M. marinum	6	Patient's isolate
M. microti, LCDC 203	1	BD
M. microti, KK 14-01	1	RIT
M. neolactis, S 152	1	MCC
M. nonchromogenicum, ATCC 19530	1	RIT
M. phlei, ATCC 23042	1	TBD
M. scrofulaceum, ATCC 19981	1	RIT
M. smegmatis, ATCC 16941	1	TBD
M. szulgai, JATA 32-01	1	RIT
M. szulgai, 352	1	MCC
M. terrae, ATCC 15775	1	TBD
M. tuberculosis, H37Rv Japan	1	TBD
M. tuberculosis, H37Rv India	1	TBD
M. tuberculosis, VA-6	1	BD
M. tuberculosis, H37Ra ATCC 21566	1	SC
M. tuberculosis	10	Patient's isolate
M. xenopi, ATCC 19250	1	RIT
MAC	27	CCH (Patient's isolate)

50 mM KCl, 2.5 mM $MgCl_2$, 0.001% gelatin, 200 µM of dNTPs 1 unit of *Taq* polymerase, 25 pmole of 16S-NSR primer and appropriate concentrations of Fl-16SOL and 16SOR primers and 1 µl of purified DNA or 5 µl of DNA extracted from clinical samples. The reaction mixture was overlaid with mineral oil and performed in a DNA thermal cycler (Perkin Elmer 480). The reaction mixture was initially denatured at 94°C for 5 minutes followed by 20 cycles of amplification by outer primers (Fl-16SOL and 16SOR). Each cycle consisted of denaturation of DNA for 1 minute at 94°C, annealing at 70°C for 30 seconds and primer extension at 72°C for 1 minute, then followed by 35 cycles for the other set of primers (Fl-16SOL and 16SNSR), denaturation of DNA for 1 minute at 94°C, annealing at 60°C for 30

Table 1 *Mycobacterium* reference strains, *Mycobacterium* clinical isolates, other bacteria and fungi used in this study (continued)

Organisms	No. of Strains	Sources
Bacteria		
Corynebacterium diphtheriae	1	BCC
Escherichia coli	1	BCC
Haemophilus influenzae	1	BCC
Klebsiella pneumoniae	1	BCC
Neisseria meningitidis	1	BCC
Nocardia asteroides	2	MCC
Pasteurella multocida	1	BCC
Pseudomonas aeruginosa	1	BCC
Rhodococcus equi	2	CCH
Salmonella Typhimurium	1	BCC
Staphylococcus aureus	1	BCC
Fungi		
Absidia species	1	MCC
Aspergillus flavus,CDC-B-15	1	MCC
Aspergillus fumigatus, CDC-B-1172	1	MCC
Basidiobolus ranarum, SIMI 10384	1	MCC
Candida albicans, ATCC 10231	1	MCC
Cladosporium carrionii	1	MCC
Conidiobolus incongruus,SIMI 10037	1	MCC
Cryptococcus neoformans	2	MCC
Epidermophyton floccosum	1	MCC
Fonsecaea pedrosoi, CDC-B-2712	1	MCC
Histoplasma capsulatum, SIMI 8838	1	MCC
Penicillium marneffei, SIMI 10202	1	MCC
Phialophora verrucosa, B-2725	1	MCC
Prototheca wickerhamii	1	MCC
Pythium insidiosum, CBS-240-37	1	MCC
Rhizopus species	1	MCC
Scedosporium apiospermum	1	MCC
Trichophyton mentagrophytes	1	MCC
Trichosporon beigelii	1	MCC

ATCC = American Type Culture Collection; BD = Becton Dickinson Diagnostic Instrument System, Maryland, USA (provided by Dr. Salman Siddiqi); TBD = Tuberculosis Division, Department of Diseases Control, Ministry of Public Health, Bangkok, Thailand (provided by Mr. Somsak Rienthong); CCH = Chest Diseases Institute, Department of Diseases 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 (provided by Dr. Prasit Palittapongarnpim).

seconds and a primer extension at 72°C for 1 minute. Finally the mixture was incubated at 4°C before checking the PCR products.

Product detection

Gel electrophoresis

The amplified products were detected by electrophoresis of 5 µl of the reaction mixture on 3% ultra pure agarose or other equivalent type of agarose (i.e. Noble agar, Difco). After this, the gel was stained with ethidium bromide and viewed on an UV-transilluminator. The sizes of the amplified products were estimated by comparing the distance they had moved to those of the molecular weight marker (100 bp DNA ladder).

Capture probe hybridization

The capture probes used in this study were based on the 16S rDNA sequence[14] and each was produced with an incorporated biotin at the 5′ end (Table 2).

The hybridization was optimized by varying some parame-

Table 2 The nucleotide sequences of capture probes[23] used for hybridization in this study

Oligonucleotide probe	Sequence 5′ ⟶ 3′	Tm (°C)	Residues
pAll1[a]	GGTATTAGACCCAGTTTCCCA	62	155-175
pTbc1[b]	ACAAGACATGCATCCCGTGGT	64	183-203
pAvi1[c]	AGAAGACATGCGTCTTGAGGT	62	183-203
pInt1[d]	CTAAAGACATGCGCCTAAAGG	62	184-204

[a]pAll1 = probe for *Mycobacterium* species, [b]pTbc1= probe for *M. tuberculosis* complex
[c]pAvi1= probe for *M. avium*, [d]pInt1 = probe for *M. intracellulare*

ters, including the amounts of PCR products (1, 2, 3, 4 and 5 µl) and the probe concentrations (20, 15, 10, 5 and 1 pmole). The 5′-biotinylated probes were stuck on to the wells of a streptavidin-coated microtiter plate as follows: Fifty microliters of phosphate-buffered saline containing 0.1% Tween-20 (PBST) combined with an optimal concentration of species-specific probe were added to each well, and the wells were incubated at 37°C for 30 minutes. To prevent secondary structure formation of probes, 50 µl of 0.1 N NaOH were added. Then, the plate was washed twice with 1 x SSC and twice with PBST and cooled on ice. Appropriate volumes of the PCR products were denatured in a boiling waterbath for 5 minutes and then chilled on ice. Three hundred microliters of ice-cold hybridization buffer (5 x SSC, 2% skim milk, 0.1% N-lauroylsarcosine, 0.02% SDS) were added to the denatured PCR product. Two hundred microliters of this mixture were added to each well, and the plate was incubated at 55°C for 30 minutes. The plate was washed three times with 2 x SSC-0.5% SDS and three times with 1 x SSC. Hybridization of the labelled PCR product to the probe was detected colorimetrically with an anti-fluorescein conjugated with horseradish peroxidase (POD). The anti-fluorescein conjugate was diluted 1:2,000 in conjugate buffer (100 mM Tris-HCl, 150 mM NaCl, pH 7.5: 1% skim milk in a ratio of 1,800 µl: 200 µl) and 100 µl of diluted conjugate was added to each well. The plate was incubated at 37°C for 30 minutes and washed twice with 1 x SSC and three times with PBST. One hundred microliters of 3, 3′, 5, 5′ tetramethylbenzidine (TMB) solution were added to each well, and color was allowed to develop for 30 minutes at room temperature. The reaction was stopped by adding 100 µl of 2 M H_2SO_4, after which the optical density was measured with a plate reader (ELx800 UV, BIO-TEK Instruments INC) at 450 nm.

Term storage of coated microtiter plate

To determine the length of time that a probe-coated microtiter plate could be stored for further use, 5′-biotinylated probes immobilized to the wells of a streptavidin-coated microtiter plate were prepared. After washing, the plates were stored at 4°C and -20°C. The performance of the plates was tested at 3 months and 6 months after storage.

RESULTS

Optimization of amplification conditions

The oligonucleotide Fl-16SOL and 16SOR could be used to amplify the DNA template in the first amplification and to produce a PCR product of 555 bp. Whereas, the oligonucleotide Fl-16SOL and 16SNSR were used to amplify the PCR product in the secondary amplification and to produce a PCR product of 499 bp.

In this experiment, the concentrations of the 16SOR primer at 2.5, 1.25 and 0.83 pmole revealed two genus specific bands of amplified products of 555 bp and 499 bp from 1 pg and 100 fg of *M. tuberculosis* DNA. Furthermore, concentrations of the 16SOR primer of 0.625 and 0.5 pmole produced an intense band of 499 bp. The 16SOR primer at a concentration of 0.5 pmole was chosen because this concentration still enabled the amplified product of the 499 bp to be detected (Fig. 1). In order to prevent interference from fluorescein labelling, the concentration of the Fl-16SOL primer was optimized. The Fl-16SOL primer at a concentration of 12.5 and 25 pmole showed an intensely amplified DNA band of 499 bp, whereas at 37.5 pmole it revealed a paler band (Fig. 2). Therefore, the Fl-16SOL primer at a concentration of 12.5 pmole was used in further experiments.

Sensitivity and specificity of semi-nested-PCR

Before using this semi-nested-PCR to detect DNA of *Mycobacterium* species in clinical specimens, its sensitivity and specificity were evaluated. The sensitivity and specificity of these primers were evaluated by amplification of purified DNA samples of 1 ng to 100 fg of genomic DNAs prepared from 60 strains of mycobacterial clinical isolates, 36 mycobacterial reference strains, 1 ng of genomic DNA from 13 strains of bacteria and 20 strains of fungi. The

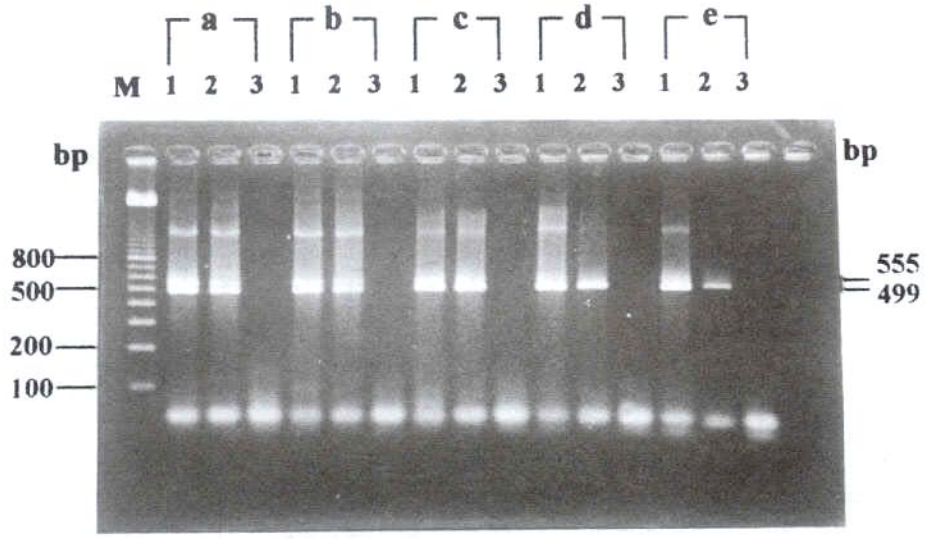

Fig. 1 *In vitro* amplification of *M. tuberculosis* H37Ra DNA by semi-nested-PCR with different concentrations of 16SOR primer. (a) 16SOR primer at a concentration of 2.5 pmole, (b) 16SOR primer at a concentration of 1.25 pmole, (c) 16SOR primer at a concentration of 0.83 pmole, (d) 16SOR primer at a concentration of 0.625 pmole, (e) 16SOR primer at a concentration of 0.5 pmole. Lane M = 100 bp ladder DNA markers, Lane 1 = *M. tuberculosis* DNA 1 pg, Lane 2 = *M. tuberculosis* DNA 100 fg, Lane 3 = blank control.

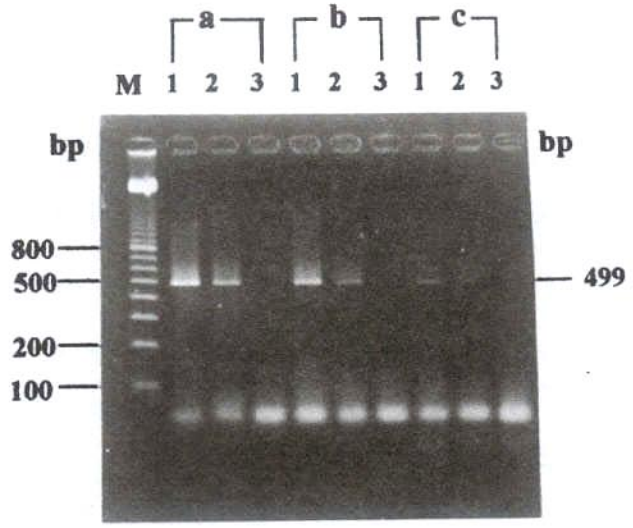

Fig. 2 *In vitro* amplification of *M. tuberculosis* H37Ra DNA by semi-nested-PCR with different concentrations of FI-16SOL concentrations. (a) FI-16SOL primer at a concentration of 12.5 pmole, (b) FI-16SOL primer at a concentration of 25 pmole, (c) FI-16SOL primer at a concentration of 37.5 pmole, Lane M = 100 bp ladder DNA markers, Lane 1 = *M. tuberculosis* DNA 1 pg, Lane 2 = *M. tuberculosis* DNA 100 fg, Lane 3 = blank control.

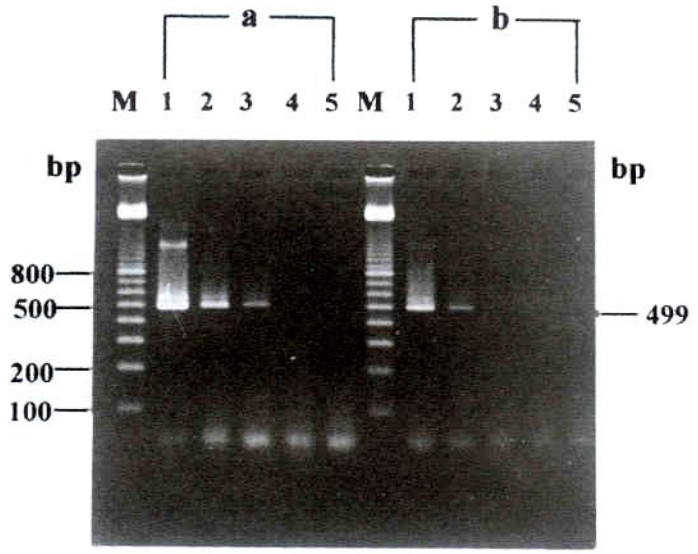

Fig. 3 *In vitro* amplification of *M. tuberculosis* DNA by semi-nested-PCR. (a) *M. tuberculosis* H37Rv Japan DNA, (b) *M. tuberculosis* H37Ra ATCC 21566 DNA, Lane M = 100 bp ladder DNA marker, Lane 1 = *M. tuberculosis* DNA 1 pg, Lane 2 = *M. tuberculosis* DNA 100 fg, Lane 3 = *M. tuberculosis* DNA 10 fg, Lane 4 = *M. tuberculosis* DNA 1 fg, Lane 5 = blank control.

lower limit of DNA detected from slow growing mycobacteria was as little as 100 fg (or equal to 20 mycobacterial genomes), which showed a consistent 499 bp band (Fig. 3). Whereas, the detection limit for DNA of rapid growing mycobacteria varied from 1 ng to 10 pg, which showed 555 bp and 499 bp or a pale 499 bp bands. In addition, the primers could amplify a 555 bp band from *Rhodococcus equi* and *Nocardia asteroides*.

Optimization of hybridization conditions

The cut-off value used for PCR-EIA in this study was 0.306 OD at 450 nm. This value was derived from the hybridization of each specific and non specific probe to mycobacterial species, and calculated by the following formula, cut-off value = X + 3 SD.

The amount of amplified product obtained was investigated by hybridizing it with 25 pmole of pTbc1 (capture probe). The PCR product at a volume of 1 µl and 2 µl produced a slightly weaker signal, whereas volumes of 3, 4 and 5 µl showed a more intense signal, but they were not significantly different from one another. Thus, a volume of 3 µl of PCR product was used in further experiments. In chosing an appropriate concentration of biotinylated capture probe for hybridization it was noted that concentrations of probe of 20, 15, 10 and 5 pmole showed no difference in the signal generated, whereas a concentration of 1 pmole gave a slightly weaker signal (Fig. 4). All tested concentrations of biotinylated capture probe revealed a consistently intense signal which was more than 0.306 OD when hybridized with amplified products from both the 100 fg and 10 fg DNA template. A probe concentration of 4 pmole was therefore chosen for reasons of cost and avoiding human error in dilution.

The sensitivity and specificity of the optimized microtiter plate hybridization method

The sensitivity and specificity of the semi-nested-PCR-EIA was tested with DNA from each species as described in detail before. The amplified products were detected by microtiter plate hybridization with a specific panel of probes. The detection limit was as little as 10 fg of DNA which is equivalent to 2-3 mycobacterial cells (except for the rapid growing mycobacteria). The results suggest that microtiter plate hybridization is 10 times more sensitive than agarose gel electrophoresis. The sensitivities of pAll1, pTbc1, pAvi1, pInt1 were 100%, whereas the specificities were 94%, 93%, 100%, 100%, respectively. The probe Tbc1 cross-hybridized with *M. terrae, M. austroafricanum* and *M. marinum* amplified DNA and probe All1 (genus specific) cross-hybridized to *Rhodococcus equi*.

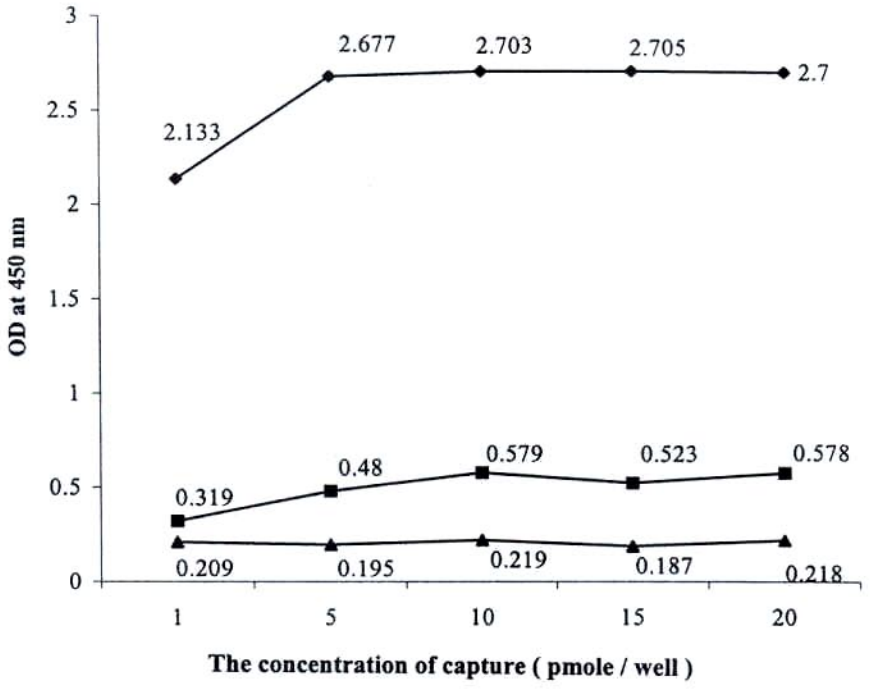

Fig. 4 The evaluation of different concentrations of Tbc1 capture probe. ▲—▲ = 3 μl of PCR product from 100 fg of *M. tuberculosis* H37Ra ATCC 21566 DNA, ■—■ = 3 μl of PCR product from 10 fg of *M. tuberculosis* H37Ra ATCC 21566 DNA, ◆—◆ = blank control.

Effect of storage on coated microtiter plate

In order to make the laboratory testing with PCR and microtiter plate hybridization for identification of *Mycobacterium* species in clinical samples more convenient, the Tbc1 oligonucleotide probe was adhered to the wells of a microtiter plate and stored for testing. Coated microtiter plates stored at 4°C and -20°C could be used to detect amplified products from as little as 10 fg of DNA template after 3 months of storage. Whereas, after 6 months of storage both the 4°C and -20°C coated plate showed a positive signal only at a concentration of ≥ 100 fg of DNA template (Table 3).

Comparison of semi-nested-PCR-EIA results for the detection of *Mycobacterium* in CSF samples with the results of culture

The semi-nested-PCR-EIA developed in this study was used to detect mycobacterial DNA, namely, *M. tuberculosis* complex and *M. avium* in CSF samples in a completely blinded study without previous knowledge of culture results. In this study, a total of 108 DNA specimens from CSF samples were tested. The sensitivity and specificity of the PCR-EIA for pTbc1 were 40% (8/20) and 77% (20/88), respectively (Table 4). An additional 6% (6/108) of PCR-EIA were positive for *M. avium*, whereas culture revealed only *Cryptococcus neoformans*. The specificity for pAvi1 was 94% (Table 5). Only 24 samples from 24 patients that were PCR-EIA positive were culture negative. Reviewing the case notes, there were only 15 patients available for analysis. Of these, 7 patients had a past history of tuberculosis and received antituberculous drugs. Therefore, the infecting organisms might have been dead and hence showed no growth of *M. tuberculosis* by culture. Six patients were not treated with antituberculous drugs, and 2 patients had a diagnosis of cryptococcal meningitis. One patient showed a positive PCR-EIA for *M. avium* and another was positive for both *M. tuberculosis* complex and *M. avium*. Two patients did not improve after administration of antifungal drug therapy and finally died. Of the remaining 9 CSF samples which showed positive results for PCR-EIA; 5 with *M. tuberculosis* complex, 3 with *M. avium* and one with both organisms, the case record forms were lost and could not be analyzed.

DISCUSSION

Current microbiological methods are insufficient for the rapid diagnosis of meningitis caused by *Mycobacterium*, because for consistent demonstration by direct smear there are too few organisms in the cerebrospinal fluid and

Table 3 Results of PCR-EIA for long-term storage of the coated microtiter plate for hybridization

Storage temperature (°C)	Concentration of DNA template[a]	PCR-EIA results[b] for times of storage	
		3 months	6 months
4	1 pg	+	+
4	100 fg	+	+
4	10 fg	+	-
4	1 fg	-	-
4	Blank	-	-
-20	1 pg	+	+
-20	100 fg	+	+
-20	10 fg	+	-
-20	1 fg	-	-
-20	Blank	-	-

[a] DNA of *M. tuberculosis* H37Ra ATCC 21566
[b] + = OD ≥ 0.306, - = OD < 0.306

because identification by culture takes at least 6-8 weeks. Several new techniques for the rapid diagnosis of mycobacterial meningitis have been developed with different sensitivities and specificities.[24-26] In this experiment, the semi-nested-PCR using Fl-16SOL, 16SOR and 16S-NSR primers was optimized. The optimal combined concentrations were 12.5 pmole of Fl-16SOL, 0.5 pmole of 16SOR and 25 pmole of 16SNSR, which allowed detection of amplification products about 499 bp in length. A ratio of 1:50 of 16S-OR and 16SNSR primer concentrations was chosen to enhance the annealing of the 16SNSR primer in the second amplification. The fluorescein label might hamper the annealing of the primer, so when the concentration of primer was decreased to 12.5 pmole, the amplified products of 499 bp showed an intense band. The optimized semi-nested-PCR could detect slow growing mycobacterial DNA at a minimum concentration of 100 fg consistently. In contrast, these primers could only detect the rapid growing group of mycobacteria at higher concentrations of DNA and exhibited both 555 bp and 499 bp bands. These results indicate that the 16SNSR primer was inappropriate for use with rapid growing mycobacteria. Therefore, the 16S-NSR primer should be changed to another region of the 16S rRNA gene or attempts should be made to increase the sensitivity of detection such as decreasing the annealing temperature or increasing the annealing times. These primers showed specificity of amplification with 60 strains of clinical mycobacterial isolates and 36 mycobacterial reference strains. There were no amplified products from the other bacterial and fungal strains studied, except for *Nocardia asteroides* and *Rhodococcus equi*, which have been classified as phylogenetically related to *Mycobacterium*.[27]

Table 4 Comparison of the detection of *M. tuberculosis* complex in CSF samples by PCR-EIA and culture methods

Culture for *M. tuberculosis* complex	PCR-EIA with pTbc1		Total
	Positive	Negative	
Positive	8	12	20
Negative	20	68	88

Table 5 Comparison of the detection of *M. avium* in CSF samples by PCR-EIA and culture methods.

Culture for *M. avium*	PCR-EIA with pAvi1		Total
	Positive	Negative	
Positive	0	0	0
Negative	6	102	108

To improve the sensitivity

of detection and identification of mycobacterial species in clinical specimens, the semi-nested-PCR combined with microtiter plate hybridization (EIA) has been developed. This method used amplified products, which were labelled with fluorescein and hybridized to a species-specific biotinylated probe. The hybridized complex was detected by a colorimetric endpoint. The semi-nested-PCR-EIA was able to amplify small quantities of mycobacterial DNA, which allowed the identification of species from as little as 10 fg of DNA (equivalent to the DNA from 2 or 3 mycobacterial cells). As expected, the hybridization method examined in this study was 10 times more sensitive than agarose gel electrophoresis, which is in accordance with other studies.[21-23] All these species were specifically identified by their corresponding species-specific probe under the hybridization conditions selected, except for pTbc1 which showed cross-hybridization with *M. marinum*, *M. austroafricanum*, and *M. terrae*. In addition to this, the pAll1 probe was found to cross-hybridize with *Rhodococcus equi*. This was due to the strong similarity of the 16S rRNA gene among closely related species. However, these species were rarely reported as pathogens in the same group of specimens containing *M. tuberculosis*, which made this cross-hybridization less important in laboratory diagnosis. The coated microtiter plate can be prepared and stored at 4°C or -20°C for 3 months without decreasing sensitivity for detection of *M. tuberculosis* DNA at a level of 10 fg. After 6 months of storage the sensitivity decreased by 10-fold. Storage for more than 3 months should be investigated further.

The developed semi-nested-PCR-EIA showed a sensitivity and specificity of 40% and 77% respectively, compared to the culture for the *M. tuberculosis* complex. The results after analysis of the data, together with available patients' histories showed a 56% sensitivity and 84% specificity. Kunakorn *et al.*[28] used a PCR-Southern blot hybridization assay for the diagnosis of extrapulmonary tuberculosis, and their results showed 85.7% sensitivity and 99.5% specificity in tuberculosis patients. This outstanding difference between their study and this study was due to the chosen target gene or DNA extraction method for amplification. In this study, the sensitivity was quite low for direct detection in CSF samples, which contained very few organisms. To increase the sensitivity, the samples could be precultured in liquid media for a week before taking 1 ml of the culture media for PCR-EIA. Still, this method can be used as another option for the rapid diagnosis of severe mycobacterial infection in the routine laboratory. Whether this method is more useful for rapid detection and identification of mycobacterial species than the conventional culture method still needs further investigation.

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