

EVALUATION OF CULTURE AND PCR-BASED ASSAY FOR DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* FROM SPUTUM COLLECTED AND STORED ON FILTER PAPER

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Abstract. We evaluated the use of culture and PCR-based assay for the direct detection of *Mycobacterium tuberculosis* (MTB) from sputum collected and stored on filter paper at room temperature for 5 days; the results were compared with those of staining and conventional culture of fresh sputum before storage (the 'gold standard'). Out of 231 sputum specimens examined, MTB was recovered from 124 samples by culture before storage. The culture positivity rate was significantly decreased to 70% after 5 days storage. For PCR assay, a fragment of 377 bp of the IS6110 sequence was amplified and detected using three methods : first PCR product combined with agarose gel electrophoresis (AGE); first PCR product with dot blot hybridization (DBH); nested PCR with AGE. Compared with culture, the sensitivity, specificity, and efficiency for first PCR with AGE were 71.8, 100 and 84.9% respectively; PCR with DBH gave results of 89.5, 96.3 and 92.6% respectively; the same values for nested PCR were 96.0, 97.2, and 96.5% respectively. Of these three methods, nested PCR gave excellent sensitivity and specificity with no significant difference ($p = 0.727$) from conventional culture. The storage of sputum on filter paper and storage at room temperature for 5 days had no apparent effect on the performance of nested PCR. We propose that this collection and storage method be considered for transporting sputum specimens from peripheral health centers or from the field; specimens may be sent by post to a central point for both culture and PCR analysis by trained technicians supervised in accordance with a well-established quality control system.

INTRODUCTION

Tuberculosis (TB) remains a major world-wide health problem. It affects one-third of the world's population, and there are 8 to 10 million new patients every year, 95% of whom live in developing countries (Narain *et al*, 1992). This situation is likely to deteriorate in the future, with the annual disease rates expected to rise from 8.8 million in 1995 to 11.9 million in 2005 (Raviglione *et al*, 1995). In Thailand, TB has re-emerged as a major disease, after considerable decline during past decades and remains the leading cause of death due to

notifiable infectious disease. Although national average figures declined again during 1994 and 1995, areas with a high incidence of HIV infection have experienced a continuing increase in the number of TB cases (Payanandana *et al*, 1999). In addition, there appears to be an increase of multidrug-resistant (MDR) cases. MDR-TB figures in the high HIV-burden areas, eg Chiang Rai have shown an alarming upward trend and are 3 times higher than the national rate (6-7%) (Payanandana *et al*, 2000). To help control the spread of TB, case finding and treatment must be rapid and practicable; prompt and accurate diagnosis is essential.

Laboratories at peripheral health centers are often unable to culture MTB and determine drug susceptibilities. Therefore, in many in-

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stances, sputum specimens collected at these health centers must be sent to central laboratories for TB isolation, identification, and susceptibility testing. However, delays in culturing sputum specimens because of lengthy transportation can result in a significant loss of recovery of MTB (Babakhani *et al*, 1995); moreover, when transportation creates higher than usual temperatures, another problem arises contamination. To overcome these problems, we proposed in a previous study a method of sputum collection on filter paper for transporting sputum from peripheral centers to central laboratories (Tansuphasiri *et al*, 1997). We designed an experiment for storage sputum aliquots on filter papers and compared this method with the collection in a plastic cup before storage and after storage for up to 5 days at room temperature. There was no significant difference between the two storage methods; a significant loss of viability after storage for 5 days was found. However, the filter paper method has some advantages: it provides a simple and inexpensive way to store and transport sputum samples without refrigeration and makes for convenient culture with less contamination, although the transport of sputum specimens over long distances to a central laboratory may affect the recovery of organisms. A PCR-based assay was considered as an alternative method for the detection of MTB in sputum specimens collected by the filter paper method.

In the present study, we evaluated the use of both culture and PCR-based assay for the direct detection of MTB in sputum collected on filter paper at room temperature for 5 days. The PCR assay is based on the IS6110 sequence using our designed primer pair (Tansuphasiri *et al*, 1999a) and the products are detected using three methods : first PCR combined with agarose gel electrophoresis (AGE); first PCR product with dot blot hybridization (DBH); nested PCR with AGE. The results of PCR assay and culture for detection of MTB from sputum collected and stored on filter paper were compared with those of staining and conventional culture before storage (the 'gold standard').

MATERIALS AND METHODS

Sputum collection

A total of 231 sputum samples were collected from newly-diagnosed patients of the TB Division, Ministry of Public Health. The samples were either collection or spot sputum (at least 2-3 ml). One fraction (0.5 ml) was separated and stored on filter paper at room temperature for 5 days. The remaining part of the fresh sputum samples was used to make smears for fluorochrome staining and then processed for culturing on the day of collection.

Storage of sputum on filter paper

The procedure for collection and storage of sputum specimen on a filter paper was as follows : a Whatman No. 2 filter paper (11 cm diameter) was folded in half twice to form a cone; at least 0.5 ml of sputum is put into the bottom of the cone; the cone is closed using staples, air-dried and then put into a plastic bag, the bags is placed in an aluminum box; the box is placed in dark storage at room temperature; after 5 days storage, each specimen is processed for culture and PCR examination.

Processing of fresh sputum samples for culture

Sputum samples were examined firstly as fluorochrome-stained smears (Bennedsen and Larsen, 1966) and scored as negative, 1+, 2+, and 3+ (NTA, 1969); sputum was then processed for culture by a modification of the method described by Petroff (1915): the sputum was treated with an equal volume of N-acetyl-L-cysteine 4% NaOH for 15 minutes at room temperature with rocking and then inoculated onto 2 slants of 2% Ogawa medium, incubated at 37°C for 8 weeks, and examined weekly for growth. Positive cultures were examined for growth rate and colony morphology. Cultures were identified by conventional biochemical tests (Kent and Kubica, 1985).

Processing of sputum collected on filter paper for culture and PCR assay

Individual filter papers were removed from

the plastic bags, 2 cm was then cut away from the cone tip and placed into a centrifuge tube containing 5 ml of N-acetyl-L-cysteine 4% NaOH. The sputum on the filter paper was eluted by shaking at room temperature for 15 minutes and 4 drops were then inoculated onto each of 2 slants of 2% Ogawa medium. The method for culture and identification was similar to that described above. The results for colony growth were recorded and scored according to the CDC (Nassua, 1958). The remaining part of the processed sputum sample was then washed with 25 ml of 0.067M phosphate buffer (pH 6.8) and centrifuged at 2,400g for 15 minutes. The sediment was resuspended in 1 ml of TE buffer (10 mM Tris HCl, 1mM EDTA, pH 8.3) and stored at -20°C for DNA extraction.

DNA extraction from bacterial strains and clinical specimens

Purified DNA was isolated from reference strains; *ie M. tuberculosis* H37Rv and *M. flavescens* ATCC 23035; there were used as a positive and the negative controls in PCR amplification. Extraction of DNA from sputum samples collected on filter paper was performed according to the method described by Buck *et al* (1992), with a slight modification by Tansuphasiri (2000).

Primers and probe

Two sets of primers derived from the IS6110 reference sequence X17348 (Gen Bank) (Thierry *et al*, 1990), designated TB1-TB2 and TB3-TB4 were used in the initial PCR reaction and the nested PCR reaction respectively. In addition, an oligonucleotide primer, TB3 was also used as a probe in the dot blot hybridization (DBH) to test the specificity of the PCR product. All of the primers and the probe (shown in Table 1) were designed by Tansuphasiri *et al* (1999a).

Conventional PCR with product detection by AGE and DBH

The first PCR reaction mixture, in a final volume of 50 µl, followed the optimized PCR

conditions described by Tansuphasiri *et al* (1999a,b). The reaction mixture consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 0.2 mM deoxynucleotide triphosphates, 0.5 µM of each of the primers TB1 and TB2, 1 U of *Taq* polymerase (Promega), and a DNA template solution. PCR was conducted in an automatic thermal cycler (Perkin-Elmer Cetus). The samples were denatured at 94°C for 5 minutes; 40 amplification cycles were performed. Each cycle consisted of denaturation at 94°C (1 minute), annealing at 60°C (1 minute), and extension at 72°C (1 minute). After the final cycle, all reactions were incubated at 72°C for an additional 10 minutes. For detection, 10 µl of PCR product was electrophoresed on a 2% agarose gel containing ethidium bromide and viewed under UV light.

To prepare dot blots, 10 µl of the amplified product of initial PCR was denatured by boiling in 40 µl of 6X SSC for 10 minutes; and the denatured product was the spotted onto a nylon membrane using a manifold system (Hybridot Manifold, Life Technologies; BRL). Positive and negative controls were also provided on each nylon membrane in the same manner. The membrane filter was air-dried and fixed by UV cross-linker. The dot blots were then prehybridized, hybridized with a fluorescein labeled probe (TB 3; 10 ng/ml) for 2 hours at 48°C, washed, and subjected to chemiluminescent detection as described previously (Tansuphasiri, 2000). The intensity of each dot was compared with those of negative controls and with those of various dilutions of positive controls. Results were read visually.

Nested PCR with product detection by AGE

A nested PCR detection protocol comprised the first-round used primers (TB1 and TB2, which generated a product of 377 bp), and the second-round used primers (TB3 and TB4, which were internal to the product amplified by TB1 and TB2, and which generated a product of 137 bp). The reaction mixture of the second round in a final volume of 25 µl contained the same chemicals and

concentrations as the first round and 5 µl volume of the first-round reaction mixture was added to each reaction mixture as target DNA. Reaction conditions for the second round were: 94°C for 5 minutes; 40 cycles of 94°C for 1 minute; 45°C for 1 minute; 72°C for 1 minute; a final extension phase of 72°C for 10 minutes. For detection, PCR product was electrophoresed on 2% agarose gel as described above.

Control procedures

To avoid possible contamination of the PCR mixture, all reactions were performed under the stringent conditions recommended by Kwok and Higuchi (1989). All available specimens that were culture positive for MTB but negative by PCR were retested. Inhibition of the PCR was checked by spiking duplicates of the previously tested DNA extracts with approximately 100 fg (in 10 ml of TE buffer) of *M. tuberculosis* H37Rv DNA (Schirm *et al*, 1995). Samples showing inhibition were retested at two different dilutions: 1:5 and 1:10.

Statistical analysis

Statistical comparisons were made by using the McNemar chi-square test; p of < 0.05 was considered significant.

RESULTS

Analysis of fresh sputum and sputum stored on filter paper for 5 days by culture

Of the 231 fresh sputum specimens tested, 53.7% (124) were culture positive for MTB and 46.3% (107) were culture negative for MTB (Table 2). Smears for AFB were positive for 96% (119 of 124) and negative for 4% (5 of 124) of the culture positive specimens. After these specimens were stored on filter paper for 5 days, MTB were recovered from a total of 87 samples (time to positive culture: 2 to 8 weeks; median time: 21 days), while culture was negative in 127 samples, 17 samples were contaminated.

Table 3 shows the culture positivity rates for the detection of MTB from sputum samples

stored on filter paper, as classified by the AFB smear grading results (*ie*, negative, 1+, 2+ and 3+) of the 124 culture positive specimens. In group smear negative and 1+, the culture positivity rates were 40 and 55.6% respectively. Correspondingly, in groups with smear 2+ and 3+, the culture positivity rates were substantially increased: 70.5 and 77.2% respectively. In general, the results of colony grading of culture (*ie*, actual count, 1+, 2+, 3+ and 4+) from storage sputum was correlated with that of AFB smear results of fresh sputum a high grading smear gave a higher-graded colony of culture. Compared with the culture positive results of fresh sputum before storage, the culture positivity rate after storage was 70.2% (87 of 124), the contamination rate was 13.7% (17 of 124), and culture negative rate was 16.1% (20 of 124). There was a significant difference in the positivity rate between the two periods of culture detection ($p < 0.001$).

Analysis of sputum collected on filter paper for 5 days by PCR assay

For the first PCR with primer TB1-TB2, (products analysed by AGE) bright sharp and clearly visible bands with a molecular size of 377-bp were shown; however specimens with AF 3+ produced more bright sharp bands than those with AF1+ or AF negative (Fig 1). Eighty-nine of the 124 culture positive specimens were positive by first PCR-AGE, while none of the 107 culture negative specimens was PCR positive. Most of FN samples (33 of 35 FN) were from smear positive specimens, indicating that some PCR inhibitors were present in sputum rather than low number of organisms in these samples.

For the first PCR with detection by DBH using a specific fluorescein labeled probe and ECL detection, dark spots without any nonspecific reaction were shown (Fig 1). Of the 124 culture positive specimens, 111 were PCR-DBH positive and 4 of 107 culture negative samples were PCR-DBH positive. All 13 FN samples were from samples which were smear positive and culture positive. In addition, 4

Table 1
PCR primers and probe.

Primer set	Primer and probe name	Primer sequence 5' to 3'	PCR product (bp)
First PCR	TB1	CCAACAAGAAGGCGTACTCG (Sn 961-980; 20 bp)	377
	TB2	GGAGACTCTCTGATCTGAGACC (Asn 1316-1377; 22 bp)	
Nested PCR	TB3*	TAGGGGATCTCAGTACA (Sn 1009-1025; 17 bp)	137
	TB4	GCTCGGTCTTGTATAGGC (Asn 1128-1145; 18 bp)	

*TB3 was also used as a probe in the dot blot hybridization (DBH).

Table 2
Results for acid-fast microscopy and culture of fresh sputum after confirmed as
M. tuberculosis (MTB) from 231 sputum specimens.

Acid-fast (AF) microscopy and culture results	No. of specimens	No. (%) of specimens with confirmed culture result	
		MTB positive	MTB negative
Culture positive (n = 124)			
AF negative	5	5 (2.2)	0 (0.0)
AF positive	119	119 (51.5)	0 (0.0)
Culture negative (n = 107)			
AF negative	105	0 (0.0)	105 (45.4)
AF positive	2	0 (0.0)	2 (0.9)
Total	231	124 (53.7)	107 (46.3)

Table 3

The culture positivity rates for detection of MTB from sputum samples after storage on filter paper for 5 days, classified by AF smear grading results of the 124 culture positive of fresh sputum samples before storage.

Culture results after storage sputum 5 days	No. of samples by AF smear grading results				
	Negative (n = 5)	1+ (n = 18)	2+ (n = 44)	3+ (n = 57)	Total (n = 124)
Contamination	0	4	7	6	17
Negative	3	4	6	7	20
Positive*	2	10	31	44	87
Actual count	2	6	13	7	28
1+	0	3	11	15	29
2+	0	1	4	9	14
3+	0	0	3	6	9
4+	0	0	0	7	7
Culture positivity rate (%)	40	55.6	70.5	77.2	70.2

*Grading numbers of colonies of the positive culture results according to CDC (Nassua, 1958).

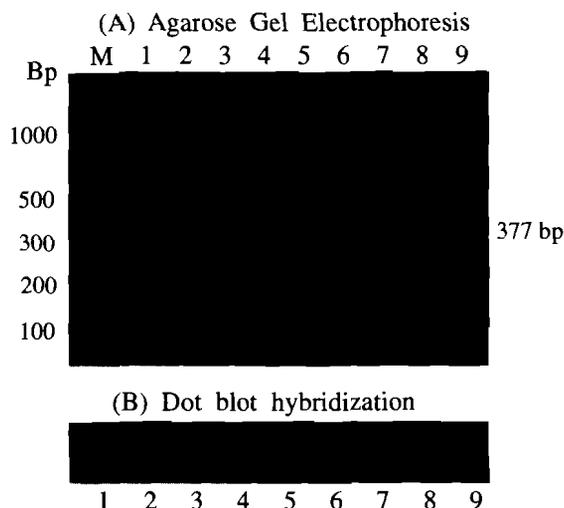


Fig 1—Representative results of PCR amplification for detection of MTB from some clinical sputum specimens storage on filter paper 5 days; the products were analyzed by : agarose gel electrophoresis (A), and dot blot hybridization with fluorescein labeled oligoprobe and enhanced chemiluminescent detection (B). The number of dots corresponds to number of lanes. Lanes 1 and 2: DNA from non TB patient specimens. Lane 3: DNA from *M. flavescens* as negative DNA control. Lanes 4 and 5: DNA from specimens that were AF 2+ and culture positive. Lanes 6 and 7: DNA from specimens that were AF 3+ and 1+, respectively and culture positive. Lanes 8 and 9: DNA from *M. tuberculosis* H37Rv as positive control and nuclease free water as reagent control, respectively. Lane M: molecular size marker (100-bp DNA ladder).

FP samples were from smear negative and culture negative specimens.

The second PCR round used primers TB3-TB4 which were internal to the product amplified by TB1-TB2 and then nested PCR products were analyzed by AGE, a clearly visible band of 137-bp was produced (Fig 2). For 35 FN by first PCR-AGE, 30 samples became positive after nested PCR (Fig 3). In the same way of 13 FN by first PCR-DBH, 8 became positive after nested PCR. Of the 124 culture positive specimens, 119 were positive by nested PCR; whereas 3 of 107 culture-negative samples were positive by nested PCR. All 5 FN samples

were from smear positive culture positive samples. Likewise, 3 FP samples were from smear negative culture negative samples.

The correlation and agreement rates of all PCR detection assays were determined by using the Mc Nemar χ^2 test. Of 231 samples, 111 were positive by both nested PCR and PCR-DBH, and 105 were negative by both methods (data not shown). Eleven samples were nested PCR positive but PCR-DBH negative, while 4 were nested PCR negative but PCR-DBH positive. The agreement rate of nested PCR comparing with first PCR-DBH was 0.870 by Kappa analysis. The correlation of both methods was not statistically significantly different ($p = 0.118$).

In comparison of nested PCR and first PCR-AGE, 89 were positive by both nested PCR and PCR-AGE and 109 were negative by both methods. Thirty-three samples were nested PCR positive but first PCR-AGE negative. The agreement rate of nested PCR comparing with first PCR-AGE was 0.718 by Kappa analysis. In comparison between first PCR detection by either AGE or DBH, 87 samples were positive and 114 were negative by both methods; while 28 samples were AGE positive but DBH negative, and 2 were AGE negative but DBH positive. The agreement rate was 0.740 by Kappa analysis. The correlation between nested PCR with conventional PCR-AGE, or between conventional PCR detection by either AGE or DBH were statistically significantly different ($p < 0.001$).

The validities of these PCR assays performed on 231 sputum specimens stored on filter paper for 5 days versus those of culture results of fresh sputum specimens as the 'gold standard' are summarized in Table 4. The sensitivity, specificity and efficiency of first PCR were 71.8, 100 and 84.9% respectively with detection by AGE and 89.5, 96.3 and 92.6% respectively with detection by DBH. The same values for nested PCR were 96.0, 97.2 and 96.5% respectively.

For AF smear-positive specimens, the sensitivities of conventional PCR with detection

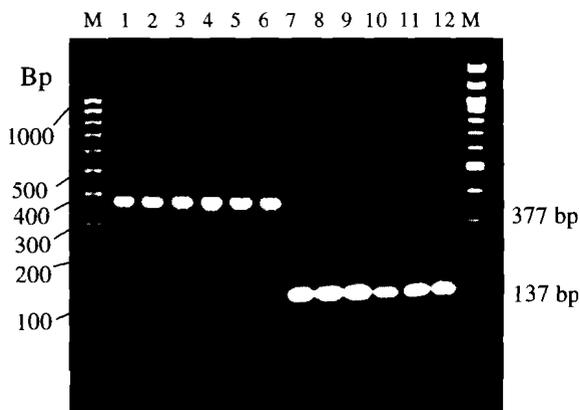


Fig 2—PCR products of MTB from sputum specimens storage on filter paper for 5 days, analyzed by first PCR and nested PCR assay. Lanes 1 to 6: DNA of first PCR products; molecular size 377-bp. Lanes 7 to 12: DNA of nested products, molecular size 137-bp. Lane M: molecular size marker (100-bp DNA ladder).

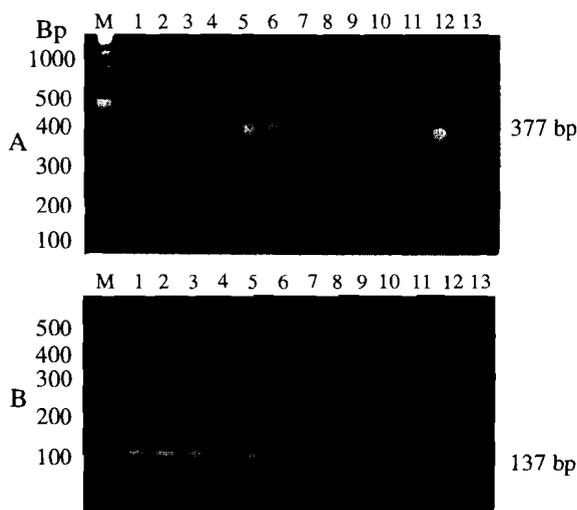


Fig 3—PCR products from sputum specimens by first PCR (A) and nested PCR (B) with detection by agarose gel electrophoresis. Lanes 1 to 3: DNA from TB positive specimens, by which the first PCR were negative and nested PCR became positive. Lane 4: DNA from *M. flavescens* as negative DNA control. Lanes 5 and 6: DNA from sputum specimens, positive results by culture, first PCR and nested PCR. Lanes 7 to 11: DNA from specimens, negative results by culture, first PCR and nested PCR. Lanes 12 and 13, DNA from *M. tuberculosis* H37Rv and water as positive control and reagent control respectively. Lane M: molecular size marker (100-bp DNA ladder).

by AGE and DBH were 72.3 and 89.0% and nested PCR was 95.8%, while specificities were 100% for all PCR methods; efficiencies were 72.7 and 89.3% by first PCR with detection by AGE and DBH and 95.9% by nested PCR. For AF smear-negative specimens, first PCR-AGE was the least sensitive (60%), while PCR-DBH and nested PCR gave equal sensitivity (100%). However, specificities were 100 and 96.2% by first PCR with AGE and DBH detection respectively and 97.1% by nested PCR; efficiencies of these methods were in the range 96 - 98%.

Statistical comparison using the Mc Nemar χ^2 test revealed that there was a statistically significance difference of sensitivities and efficiencies between first the PCR (either detection by AGE or DBH) and the nested PCR ($p < 0.05$). Furthermore, specificities of conventional PCR-AGE and nested PCR were significantly different ($p < 0.05$), except PCR-DBH and nested PCR ($p > 0.05$).

DISCUSSION

The re-emergence of TB and increasing MDR strains poses a public health threat of global concern; the rapid detection of MTB is an important issue that may improve the management of infected patients and facilitate infection control procedures. In practice, sputum specimens collected in plastic container are sent to central laboratories for culture. In a previous study, we proposed the use of filter paper as an alternative means for the collection of sputum specimens, which may provide a simple and inexpensive way to collect and transport sputum sample by post without refrigeration, at peripheral health centers or collected in the field to be sent to a central point for culture.

However, the transport of sputum specimens over a long distance may affect the culture recovery; moreover, the time required for conventional culture remains an obstacle to rapid diagnosis. In this study, a PCR-based assay was considered as an alternative means for the rapid detection of MTB from sputum collected

Table 4

Summary of the validities of PCR assays performed on sputum collected and stored on filter paper for 5 days compared with culture results of fresh sputum as the 'gold standard' for detection of MTB in 231 sputum specimens and in smear-positive and smear-negative specimens.

Specimen type and PCR product detection by	No. of specimens with the following culture result ^a		Sensitivity (%)	Specificity (%)	Predictive value (%)		Efficiency (%)
	Positive ^b	Negative			Positive	Negative	
PCR-AGE^c							
All specimens			71.8	100.0	100.0	75.4	84.9
Positive	89	0					
Negative	35	107					
Smear-positive specimens			72.3	100.0	100.0	5.7	72.7
Positive	86	0					
Negative	33	2					
Smear-negative specimens			60.0	100.0	100.0	98.1	98.2
Positive	3	0					
Negative	2	105					
PCR-DBH^d							
All specimens			89.5	96.3	96.5	88.8	92.6
Positive	111	4					
Negative	13	103					
Smear-positive specimens			89.0	100.0	100.0	13.3	89.3
Positive	106	0					
Negative	13	2					
Smear-negative specimens			100.0	96.2	55.5	100.0	96.4
Positive	5	4					
Negative	0	101					
Nested PCR^e							
All specimens			96.0	97.2	97.5	95.4	96.5
Positive	119	3					
Negative	5	104					
Smear-positive specimens			95.8	100.0	100.0	28.6	95.9
Positive	114	0					
Negative	5	2					
Smear-negative specimens			100.0	97.1	62.5	100.0	97.3
Positive	5	3					
Negative	0	102					

^aUsing culture results of fresh sputum samples as the 'gold standard'.

^bCulture positive for MTB in 124 specimens (119 smear-positive and 5 smear-negative specimens).

^cFirst PCR with amplicon detection by agarose gel electrophoresis (AGE).

^dFirst PCR with amplicon detection by dot blot hybridization (DBH).

^eNested PCR with amplicon detection by agarose gel electrophoresis.

by this filter paper method. The efficacy of detection by PCR and by culture from sputum collected and stored on filter paper at room temperature for 5 days were investigated. The results were compared with those of staining and conventional culture of fresh sputum before storage as the 'gold standard'.

In comparison with culture results of fresh sputum samples, the positivity rate by culture of sputum collected on filter paper for 5 days was 70.2%. The culture positivity rate of sputum storage on filter paper by this study was higher than previously described (70% compared with 50%) (Tansuphasiri *et al*, 1997).

This may be explained by differences in the number of specimens with various smear grading results analyzed. In this study, sputum samples with grading smear 2+ and 3+ were found in higher proportion than smear 1+ and smear negative. In general, the culture positivity rate decreased to 70% when sputum specimens were kept on filter paper at room temperature for 5 days but the advantage of this method was low contamination (7%; 17 of 231 specimens). In the study of Paramasivan *et al* (1983), the contamination rates were 5, 7, 12 and 18% after storage for 0, 3, 5 and 7 days respectively.

For amplification, the IS6110-PCR based method using our designed primer pair permitted the amplification of 377-bp DNA fragment located at the end region of IS6110 sequence and demonstrated good sensitivity and specificity for MTB (Tansuphasiri *et al*, 1999a,b; 2000). Since the sensitivity and specificity of PCR assay also vary according to not only the target region of nucleic acid but also the PCR techniques, including the DNA release techniques and the methods used to detect amplified products. In this study, we compared three PCR detection methods: first PCR combined with AGE, first PCR with DBH, and nested PCR with AGE.

For detection of amplified product of first PCR by conventional gel analysis, the result primarily showed low sensitivity (50%) but high specificity (100%). Inhibitor and sensitivity of detection by gel method were considered. Since the presence of inhibitors of PCR in clinical specimens is a well-known diagnostic problem that frequently leads to FN results. To address this problem, PCR inhibitors must be diluted, inactivated or removed from the samples. In this study, those samples negative on AGE were retested at dilutions of 1:5 and 1:10. 4 of 5 inhibitory specimens in 1:10 dilution produced a correct band and only one specimen remained negative. The PCR assays were checked again by spiking of the previously tested DNA extracts with 100 fg of H37Rv DNA. However the results were negative after dilution because not only the

inhibitor was diluted but also the DNA target.

To solve this problem, bovine serum albumin (BSA) was incorporated with these specimens to override the presence of interfering substances as recommended by Forbes and Hicks (1996). The result showed that 21 of 22 inhibitory specimens with 0.02% BSA incorporation became positive. Thus in processing sputum for all PCR assay, prior to boiling, 100 µl of 0.02% BSA were added in 1:2 dilution. By this treatment, the sensitivity of detection by first PCR-AGE was increased to 71.8% while the specificity remained 100%. However, mostly of FN samples (33 of 35 FN) analyzed by PCR-AGE were from smear positive specimens, indicating that some PCR inhibitors were still present rather than low number of organisms in these samples. These inhibitors may persist even after DNA purification. Several methods to remove the inhibitors by DNA purification had been studied (Victor *et al*, 1992; Amicosante *et al*, 1995). However, some techniques are labor intensive, costly or incorporate multiple steps that may cause bacterial loss and a subsequent decrease in the sensitivity of detection. In addition, many of the reagents used for nucleic acid extraction or to prepare samples for PCR can inhibit PCR when present at contaminating levels (Rossen *et al*, 1992).

We also tried several extraction methods prior to amplification, *ie* phenol-chloroform extraction, single-step DNA extraction using QIAamp DNA kit (Qiagen), or incorporation of BSA to remove interfering substances from some culture-positive sputum specimens. It was found that most extraction reagents were able to remove inhibitory substances in some sputum samples that contained high number of AFB. However if the specimens were contained low number of AFB, the extraction and purification process may cause bacterial loss and a subsequent decrease in the sensitivity of detection by PCR. Recently, Abu Al-Soud and Radstrom (1998) have shown that the PCR-inhibiting effect of various components in biological samples can be eliminated by the use of an appropriate thermostable DNA polymerase.

As previously, stated by many investigators, the hybridization method, either a Southern blot or dot blot format, was more sensitive than AGE. PCR showed sensitivity and specificity of 55 and 98% respectively, for AGE and 74 and 95% for DBH respectively (Shawar *et al*, 1993). In like manner, Tansuphasiri (2000) compared PCR with culture, PCR showed sensitivity and specificity of 88 and 100% respectively, for AGE and 96 and 85% for DBH respectively. Use of hybridization with detection by ECL increased the sensitivity of detection by PCR-AGE assay by 100-fold from 1 pg to 10 fg of purified DNA per reaction.

Thus, in this study all amplified products were also analyzed by DBH with ECL detection. As expected, the DBH was more sensitive than AGE (89.5% by DBH compared with 71.8% by AGE). In addition, DBH could process more specimens than AGE. However, for detection of MTB from sputum samples on filter paper, DBH demonstrated a lack of specificity (96.3%) in comparison with AGE (100%). This was probably due to the technique of using unpurified amplicons to prepare dot blots and/or hybridization with a rather short internal oligonucleotide probe, which is usually associated with a high background since non-specific product or DNA from several organisms normally present in normal throat specimens can be hybridized with this probe. DNA of *Escherichia coli* in IS3411 sequence were homologous with some regions of IS6110 of MTB complex (Kent *et al*, 1995).

Since the FN by conventional PCR with detection by either AGE or DBH for detection of MTB from sputum on filter paper were rather high (28.2 and 10.5% respectively), due to either the remnants of inhibitor or the inadequate sensitivity of the detection method used. Nested PCR was considered as another alternative detection method which gave high sensitivity and specificity (96 and 97%). Our nested PCR result was also in accordance with Miyazaki *et al* (1993), the nested PCR correlated well in both sensitivity and specificity with conventional culture and there was no significant difference between the results ob-

tained with nested PCR from sputum after storage on filter paper and conventional culture of fresh sputum before storage ($p=0.727$).

Three FP samples (2.8%) by nested PCR were from smear-negative and culture-negative samples. This technique increased sensitivity, but there is a greater risk of cross-contamination. As a result, single one-tube nested PCR should be used to avoid the risk of cross-contamination. Five FN (4%) were also detected by nested PCR. These factors may be traced to the number of organisms, the presence of inhibitor, the lack of IS6110 in the genome and the degrading of the specimen. However, all 5 samples were from smear-positive culture-positive samples; and 3 of these specimens were also positive by culture after 5 day storage. Thus, these specimens contained high number of organisms. All isolates obtained from culture of fresh sputum samples also gave positive results by PCR based on IS6110, therefore these strains contained IS6110 sequence. The presence of inhibitors might account for the problem. However, when all specimens were diluted in 10-fold; again none became positive by PCR. Beside these were spiked duplicates of DNA extracts with small amount of H37Rv DNA and no inhibitors were found. Another reason of these FN results may also due to DNA degrading since all samples after DNA extraction were stored at -20°C for month before amplification.

This study has demonstrated that sputum specimens collected on filter paper and stored at room temperature for 5 days can be used for rapid detection of MTB by IS6110-PCR based method. Of these PCR methods, nested PCR with detection by AGE showed outstanding sensitivity (96.0%), specificity (97.2%) and efficiency (96.5%); furthermore no difference was found when compared with the conventional culture of fresh sputum. Thus PCR can be considered to be the method of choice for the diagnosis of TB in combination with culture from mailing specimens, either sputum storage in a plastic cup or on filter paper; sputum storage on filter paper has the advantage of being convenient for mailing and for culture.

A practical use for PCR assay would be for patients who are at high risk for MTB infection and whose sputum smears are negative; whereas for the specimens positive by AF smear, PCR permits distinction between MTB and other mycobacteria rapidly and thus may be helpful for the optimal management of specific drug therapy and the isolation of infectious individuals as soon as possible. Since diagnosis of TB by culture may take up to 8 weeks and the species identification procedures extend the diagnosis time even further, while the average time for detection of MTB by nested PCR is 4-6 hours when analyzed by AGE. In cases of false negative PCR result from clinical specimens, analysis of multiple sputum specimens should maximize the detection of MTB.

In general, PCR is a valuable adjunct to the laboratory diagnosis of TB but it cannot be expected to replace cultures because of the need for isolation of organisms for susceptibility testing. PCR can detect the DNA of nonviable or qualitatively irrelevant MTB pathogens that would not cause disease or require treatment. PCR results were positive whereas culture and AF smear were negative in patients with anti-tuberculosis medication. The contamination with DNA is necessary to strictly control procedure in PCR assay that may be FP. Finally, the cost of PCR is lower since small reaction volumes of 25-50 µl are adequate to test one sample but the cost of PCR is higher than culture. However, if single one-tube nested PCR is developed and large numbers of samples are tested, the costs will be reduced. Further improvements of the assay, especially at the detection step by using ELISA hybridization method, may lead to a method which is less cumbersome, quicker, safer, cost effective, and which allows objectivity in reading results. In addition, the equipment for PCR is more expensive although PCR laboratories are not necessary in every province and TB zone; they may be set in principle cities that have adequate personnel, material and equipment (*ie* Tuberculosis Division) and act as a reference lab to sputum specimens are sent from peripheral laboratories. In study, sputum

specimens that were stored at room temperature for 5 days had no adverse effect on the performance of PCR. This storage method may find a use in epidemiological studies of the analysis of large number of samples collected in the field and to sent reference laboratories for PCR analysis, where PCR technique may be used in the routine laboratory diagnosis of TB.

ACKNOWLEDGEMENTS

This study was supported by a grant from Mahidol University. We are grateful to the Director of the Tuberculosis Division, Ministry of Public Health for providing clinical samples.

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