COMPARISON OF MICROPLATE HYBRIDIZATION WITH GEL ELECTROPHORESIS AND DOT BLOT HYBRIDIZATION FOR THE RAPID DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* PCR PRODUCTS

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Abstract. A microplate ELISA hybridization assay has been developed for the detection of the IS*6110* PCR products of *M. tuberculosis* from sputum specimens. In this study, its efficacy was evaluated by comparison with agarose gel electrophoresis (AGE) and dot blot hybridization (DBH), with culture results as the 'gold standard'. The assay was used with 190 sputum samples: the PCR results detected by ELISA and AGE showed close agreement, with sensitivity, specificity and accuracy of 90%, 100% and 96% respectively. The same values for DBH were 92%, 98% and 96% respectively. The validities of these methods were not statistically significantly different ($p>0.05$). The agreement rates of PCR product detection by AGE comparing with DBH and ELISA were 0.964 and 0.964 respectively, while that of DBH and ELISA was 1.0 by Kappa analysis. The overall agreement was not statistically significantly different ($p>0.05$). Use of DBH or ELISA hybridization increased the sensitivity of detection by AGE 10-fold from 10 pg to 1 pg of purified DNA per reaction; *ie* from about 30 to about 3 organisms. The amount of PCR product detected by ELISA was only one half of that detected by the other methods; the total assay time of ELISA following the PCR was 4 hours. In conclusion, the microplate hybridization assay may replace AGE and DBH for the detection of the PCR products of *M. tuberculosis* because of its sensitivity, specificity and accuracy. Additional advantages of the microplate assay over AGE and DBH include rapidity, ease of use, greater safety, cost effectiveness and greater objectivity in the reading of results; the technique is suitable for use in epidemiological studies for the analysis of a large number of samples.

INTRODUCTION

The resurgence of tuberculosis (TB) has stimulated the development of a large number of molecular diagnostic procedures for the rapid diagnosis of TB, including numerous methods based on the PCR or other amplification methods (Forbes and Hicks, 1993; Nolte *et al*, 1993; Kent *et al*, 1995; Ichiyama *et al*, 1997). Commonly, detection of the PCR product is achieved by ethidium bromide-stained agarose gel electrophoresis (AGE). However, this method has certain disadvantages, including handling time, safety hazards, the risk of nonspecific banding patterns, and subjectivity in the reading of results. The results must be confirmed by hybridization procedures on membranes, *ie* Southern blotting, which are laborious and not suitable for the analysis of large numbers of samples.

We previously developed and optimized a microplate ELISA hybridization assay for the detection of the IS*6110* PCR products of *M. tuberculosis* from sputum specimens. The assay is based on the specific detection with a fluorescein-labeled detection probe of biotinylated PCR products which are captured on avidin-coated microplate. Hybridized products with

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fluorescein were identified by using anti-fluorescein antibody horseradish peroxidase conjugate and colorimetric peroxidase substrate. The assay discriminated perfectly between the positive and negative groups, when an OD at 490 nm of 0.18 was used as the cut-off point. This microtiter plate-based system avoids electrophoresis in a gel and checks the specificity of the PCR products.

In the present study, the assay's efficacy was further evaluated by comparison with agarose gel electrophoresis (AGE) and dot blot hybridization (DBH), using acid-fast staining and culture as the 'gold standard' for the diagnosis of TB. The study hoped to yield a possible alternative assay that would be reliable for the rapid detection of *M. tuberculosis* in sputum specimens.

MATERIALS AND METHODS

Specimen collection, processing and culture

A total of 190 sputum specimens were obtained from newly-diagnosed TB patients who attended the TB Division, Ministry of Public Health, during February and April 1998. The samples (at least 2-3 ml) were collected prior to the administration of medication. Sputum samples were treated with the standard protocol of *N*-acetyl-L-cysteine-4% NaOH and concentrated by centrifugation (3,000*g*). From the sediments smears were made, stained by fluorochrome staining, examined for AFB, and inoculated on 2% Ogawa medium. The remaining sediments were stored at -20ºC until used for PCR amplification. All isolates were examined for growth rate, colony morphology, and pigmentation and any suspected colonies were subjected to conventional biochemical tests (Kent and Kubica, 1985).

DNA extraction

The DNA of two reference strains: *M. tuberculosis* H37Rv and *M. flavescens* ATCC 23035 - for use as the positive and negative control DNA in PCR amplification - were extracted by the proteinase K-phenol-chloroform method described by Tansuphasiri *et al* (1999a). Extraction of DNA from sputum samples was performed according to the method described by Buck *et al* (1992) with the slight modification suggested by Tansuphasiri (2000).

Primers and probe

The primers used for the amplification were originally designed by Tansuphasiri *et al* (1999b) from the IS*6110* reference sequence (accession number X17348) (Thierry *et al*, 1990). The sequences of the primers (synthesized by the National Center of Genetic Engineering and Biotechnology, Ministry of Science, Technology and Environment), which amplify a 377-bp fragment of the IS*6110* sequence were TB1 (5′-CCAACAAGAAGG CGTACTCG-3′, position 961 to 980) and TB2 (5′-GGAGACTCTCTGATCTGAGACC-3′, position 1316 to 1337). The probe, TB3 (5′-TAGGGGATCTCAGTACA-3′, position 1009 to 1025) was internal to the amplified target of the IS*6110* sequence. Primer TB2 was 5′ biotinylated and TB3 was 5′-fluorescein-labeled using the 5′-end labeling kit (Promega).

PCR amplification

Amplification for detection by AGE and DBH followed the optimized PCR condition as previously described (Tansuphasiri *et al*, 1999a). The reaction mixture for PCR (50 μl) consisted of 0.5 μM each of the primer, 1xPCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin], 0.2 mM (each) deoxynucleotide triphosphates, 1 U of *Taq* polymerase (Promega) and 10 μl of sample. Initial denaturation at 94ºC for 5 minutes was followed by 40 cycles of denaturation at 94º C for 1 minute, annealing at 60ºC for 1 minute, and extension at 72ºC for 1 minute. Finally, incomplete PCR products were extended for 10 minutes at 72ºC. Amplification for detection by microplate ELISA hybridization was similar to that described above, except for the primer concentrations: 0.3 μM each of primers TB1 (unlabeled) and TB2 (5′ biotin labeled) were used in the PCR reaction.

Detection and analysis of the PCR products

Agarose gel electrophoresis (AGE): After amplification, PCR products (10 μl) were electrophoresed through a 1.8% agarose gel in TBE buffer (89mM Tris borate, 2mM EDTA, pH 8.2) and run at 100V for 1 hour. The gel was stained with ethidium bromide (0.5 μg/ml) and visualized by UV transilluminator. Positive control, negative control, and reagent control were loaded to all gels. The presence of a band of the expected size (377-bp) was identified by comparison with a molecular mass marker, 100-bp DNA Ladder (Gibco, BRL).

Dot blot hybridization (DBH): To prepare dot blots, 10 μl of amplified product was denatured by boiling in 240 μl of 6xSSC for 10 minutes and the denatured product was spotted onto a nylon membrane by using a manifold system. Positive and negative controls were also performed on each nylon membrane in the same manner. The membrane was air dried and fixed by UV cross-linker. Dots were then prehybridized, hybridized with TB3 probe, washed, and enhanced chemiluminescent (ECL) detection as previously described (Tansuphasiri, 2000). Results were read visually. The intensity of each dot was compared with those of the negative and positive controls.

Microplate hybridization (ELISA): The protocol followed the optimized condition previously described (Tansuphasiri *et al*, 2001). Briefly, the microtiter plate wells (MaxiSorb™, Nunc) were coated with 100 μl of avidin (1.25 μg/ml in 50 mM carbonate-bicarbonate buffer, pH 9.6) at 4ºC overnight or at 37ºC for 2 hours. The wells were rinsed three times in phosphate buffer saline (pH 7.5) and 0.5% (v/v) Tween 20 (PBST), blocked with 100 μl of PBST containing 0.05% bovine serum albumin (BSA) for 1 hour at 37ºC and washed three times. The PCR products were mixed with PBST to 100 μl and dropped into the well. Capture was allowed to proceed for 30 minutes at 37ºC, then the wells were washed three times. The captured biotinylated PCR products were denatured by adding 100 μl of 0.25 M NaOH to each well. Nonbiotinylated DNA strands were removed by washing three times. To each well was added 100 μl of hybridization buffer (0.1 M sodium phosphate, pH 7.0; 0.5 M NaCl; 0.65%(w/v) Tween 20; 0.14 mg Salmon sperm DNA/ml; 2% PEG 4000) containing TB3 detection probe (0.5 μg/ml). After hybridization for 30 minutes at 37ºC, the wells were washed three times with PBST. To each well, 100 μl of anti-fluorescein HRP conjugate (Amersham) diluted 1:500 in PBST buffer containing 0.05% BSA was added. Plates were incubated for 30 minutes at 37ºC, and the wells were washed three times with PBST and 100 μl of O-phenylenediamine substrate (Sigma Fast OPD™, Sigma) were added. The plates were incubated at 37ºC for 1 hour, and the reaction was stopped by adding 100 μl of 3 M H_2SO_4. The A_{490} was read with a microplate reader. Positive and negative hybridization controls were also included in each microplate hybridization assay. They consisted of standard solutions of 10-fold dilution of PCR products derived from 100 pg of H37Rv DNA and *M. flavescens* DNA respectively. Water was used as a negative control.

Stringent contamination control procedures followed those recommended by Kwok and Higuchi (1989). Inhibition of the PCR was checked by spiking duplicates of the tested DNA extracts with approximately 50 fg of H37Rv DNA. Samples showing inhibition were retested at 1:10 dilution.

Sensitivity of detection

To determine the sensitivity of detection, 10-fold serial dilutions of *M. tuberculosis* H37Rv prepared from both bacterial suspension (~10^8 to ~10^2 cells per ml, prepared by just boiling) and purified DNA (concentrations ranging from 100 ng to 0.1 fg per 10 μl) were used as the templates for 40 amplification cycles and the products analyzed by AGE, DBH, and ELISA hydridization. The number of CFU of the original suspension per ml was estimated by routine growing on duplicate Lowenstein-Jensen (LJ) medium and colonies were counted after 6 weeks of growth. The detection limit for each assay was determined by the number of colo-

nies grown on the LJ medium of the corresponding dilution. The sensitivity of detection was also determined by using H37Rv DNA at 10 ng per µl (or 100 ng per reaction) as the template for amplification, and the PCR products were diluted by serial two-fold dilution from undiluted to 1:512 and each dilution was analyzed by AGE, DBH and ELISA hybridization.

Statistical analysis

The agreement rate of all three detection methods for identification of PCR products of MTB from all clinical specimens was determined by Kappa analysis. The validities of the PCR-ELISA, PCR-DBH and PCR-AGE for the direct detection of MTB from all sputum specimens were determined by using the results of AF staining and culture as the 'gold standard'. Statistical comparison was performed by using chi-square analysis; a p of < 0.05 was considered significant.

RESULTS

Culture and smear results

Of the 190 sputum samples from 190 patients, 78 samples (41.1%) were smear positive and culture positive, 1 sample (0.5%) was smear negative and culture positive, 1 sample (0.5%) was smear positive and culture negative, and 110 samples (57.9%) were smear negative and culture negative. Their identities were confirmed by the positive niacin test, the positive nitrate reduction test, and the negative catalase test after being heated at 68ºC. If culture with biochemical confirmation is regarded as the most reliable diagnostic method, then 79 of 190 patients were diagnosed as tuberculous and 111 patients as non-tuberculous.

PCR analysis

Of the 79 specimens that were culture positive for MTB, 71 were positive by PCR with amplicon detection by AGE and 73 by PCR with amplicon by DBH (sensitivities 89.9% and 92.4% respectively). Of the 111 culture-negative samples for MTB, none was positive by PCR in AGE (specificity 100%), while 2 were positive by PCR after hybridization in a dot blot format (specificity 98.2%). The two false-positive samples by the PCR-DBH come from patients suspected as having TB on chest X-ray but found to be negative by AF microscopy and culture. All 8 false-negative samples detected by PCR-AGE as well as the 6 false-negative samples detected by PCR-DBH were from samples that were AF+/Cul+, indicating some PCR inhibitors in the sputum samples.

For PCR with amplicon detection by microplate ELISA hybridization, the mean optical densities (OD) determined at 490 nm, of the negative control, used +3SD as the cut-off point (cut-off OD 490 nm value = 0.18). Then 71 of 190 were in the positive group while 119 were in the negative group. The mean OD at 490 nm of positive group was 1.405 (95% CI = 1.217, 1.592) while that of the negative group was 0.056 (95% CI = 0.047, 0.065). The mean optical densities of the positive and negative groups were significantly different (p=0.000). The result of the test is shown in Table 1.

Of the 79 specimens that were culture positive for MTB, 71 samples were positive by PCR-ELISA hybridization (sensitivity 89.9%). Of the 111 culture-negative samples for MTB, none was positive by PCR-ELISA hybridization (specificity 100%). No false-positive samples were examined, while all eight false-negative samples were from samples that were AF positive and culture positive, indicating some PCR inhibitors in the sputum samples.

Agreement rate of three PCR product detection assays

The correlations between the results from all three PCR product detection methods for 190 sputum specimens are shown in Tables 2-4. The results between AGE and ELISA highly agree. The agreement rate of both methods was 1.0 by Kappa analysis. Comparing between DBH and AGE, 71 were DBH positive and

Table 1
The means of optical density, standard deviations, and 95% confident interval of biotinylated PCR products detection by microplate ELISA hybridization of 190 sputum specimens.

	PCR products detection by microplate ELISA hybridization	
	Positive	Negative
Number	71	119
Means of OD 490 nm	1.405	0.056
Standard deviations of OD 490 nm	0.791	0.051
Minimum OD 490 nm	0.301	-0.043
Maximum OD 490 nm	2.693	0.151
95% CI of OD 490 nm	1.217, 1.592	0.047, 0.065

Table 2
Correlation of PCR product detection between microplate ELISA hybridization and agarose gel electrophoresis for detection of *M. tuberculosis* from 190 sputum specimens.

ELISA hybridization	Agarose gel electrophoresis		
	Positive	Negative	Total
Positive	71	0	71
Negative	0	119	119
Total	71	119	190

Mc Nemar's $\chi^2 = 0.00$, d.f. = 1, p = 1.00, K = 1.0, Z = 13.78, $p < 0.05$

Table 3
Correlation of PCR product detection between agarose gel electrophoresis and dot blot hybridization for detection of *M. tuberculosis* from 190 sputum specimens.

Dot blot hybridization	Agarose gel electrophoresis		
	Positive	Negative	Total
Positive	71	4	75
Negative	0	115	115
Total	71	119	190

Mc Nemar's $\chi^2 = 2.35$, d.f. = 1, p = 0.125, K = 0.956, Z = 13.18, $p < 0.05$

Table 4
Correlation of PCR product detection between microplate ELISA hybridization and dot blot hybridization for detection of *M. tuberculosis* from 190 sputum specimens.

ELISA hybridization	Dot blot hybridization		
	Positive	Negative	Total
Positive	71	0	71
Negative	4	115	119
Total	75	115	190

Mc Nemar's $\chi^2 = 2.35$, d.f. = 1, p = 0.125, K = 0.956, Z = 13.18, $p < 0.05$

AGE positive and 115 were negative by both methods. Of the four samples that were AGE negative and DBH positive, 2 were culture-negative and 2 culture-positive. Comparing DBH and ELISA for 190 samples, 71 were DBH positive and ELISA positive and 115 were negative by both methods. Of the four samples that were ELISA-negative and DBH positive, 2 were culture-negative and 2 culture-positive. The agreement rates of PCR product detection by DBH compared with AGE and ELISA were 0.956 and 0.956 respectively, by Kappa analysis.

Validities of PCR analyzed products by three methods

The validities of PCR analyzed products yielded by the three methods were compared with the results of culture and AF staining as the 'gold standard' for the detection of MTB from 190 sputum specimens is summarized in Table 5. Compared with culture, PCR showed sensitivities, specificities, and accuracies of 89.9%, 100% and 95.8% respectively, for both AGE and ELISA hybridization and 92.4%, 98.2% and 95.8% respectively, for DBH.

For AF smear-positive specimens, the sensitivity, specificity, and accuracy of PCR with detection by both AGE and ELISA were 89.7%, 100% and 89.8% respectively; for DBH, 92.3%, 100% and 92.4% respectively. For AF smear-negative specimens, the sensitivity, specificity, and accuracy of PCR with detection by AGE or by ELISA were all 100% whereas by DBH they were 100%, 98.2% and 98.1% respectively.

Statistical comparison of all validities of PCR by three amplicon detection methods was performed by using the Z test of proportion. All of the validities were not statistically significantly different ($p > 0.05$). For example, in the comparison between AGE and DBH, the sensitivity, specificity, and accuracy were 0.36, 0.24 and 1.0 respectively; between AGE and ELISA they were 1.0, 1.0 and 1.0 respectively; and between DBH and ELISA they were 0.36, 0.24 and 1.0 respectively.

Sensitivity of detection

For heat treatment of bacterial suspension, 10-fold dilutions were made from H37Rv colonies suspended in TE buffer and determined the number of organisms from each dilution growing on LJ medium. A clear PCR signal with all three amplicon detections was observed up to the dilution showing between 3 - 30 CFU/ tube. Under this condition, the limit of detection by AGE, DBH and ELISA hybridization was 30, 3, and 3 bacteria per reaction respectively (data not shown).

The sensitivity of detection as determined by using 10-fold dilutions of H37Rv DNA at a concentration of 10 ng per μl (or 100 ng per reaction) with amplicon detection by AGE was 10 pg per reaction, by DBH was 1 pg per reaction, and by ELISA was 1 pg per reaction (data not shown).

The sensitivity of detection MTB as determined by using genomic DNA purified from H37Rv isolate, and the DNA concentration at 10 ng per μl (or 100 ng per reaction) was used as the template for amplification. The amplification products were diluted by serial two fold dilution from undiluted to 1:512 and each dilution was analyzed by AGE, DBH and microplate ELISA hybridization. The detection limits of AGE, DBH and microplate ELISA hybridization were 1:64, 1:64 and 1:128 respectively (Fig 1).

DISCUSSION

Definitive diagnosis is still based on microscopy and culture, the 'gold standard' for the laboratory diagnosis of MTB infection. However, the time required for conventional laboratory examination remains an obstacle for rapid diagnosis. In practice, conventional culture in solid media is more specific and sensitive, but results require several weeks (2 - 8 weeks) of incubation and an additional 2 - 3 weeks is required for the biochemical identification of isolates. PCR methods generally shorten the clinical detection of MTB from weeks to less than a day. PCR technology has the potential to become an important method for the diagnosis of tuberculosis.

Table 5
Comparison of PCR results by the three amplicon detection methods with confirmed culture results for detection of *M. tuberculosis* in 190 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 (%)		Accuracy (%)
	Positive	Negative			Positive	Negative	
PCR-AGE[b]							
All specimens			89.9	100.0	100.0	93.3	95.8
Positive	71	0					
Negative	8	111					
Smear-positive specimens			89.7	100.0	100.0	11.1	89.8
Positive	70	0					
Negative	8	1					
Smear-negative specimens			100.0	100.0	100.0	100.0	100.0
Positive	1	0					
Negative	0	110					
PCR-DBH[c]							
All specimens			92.4	98.2	97.3	94.8	95.8
Positive	73	2					
Negative	6	109					
Smear-positive specimens			92.3	100.0	100.0	14.2	92.4
Positive	72	0					
Negative	6	1					
Smear-negative specimens			100.0	98.2	33.3	100.0	98.1
Positive	1	2					
Negative	0	108					
PCR-ELISA[d]							
All specimens			89.9	100.0	100.0	93.3	95.8
Positive	71	0					
Negative	8	111					
Smear-positive specimens			89.7	100.0	100.0	11.1	89.8
Positive	70	0					
Negative	8	1					
Smear-negative specimens			100.0	100.0	100.0	100.0	100.0
Positive	1	0					
Negative	0	110					

[a]Using culture as the 'gold standard'.
[b]PCR with amplicon detection by agarose gel electrophoresis (AGE).
[c]PCR with amplicon detection by dot blot hybridization (DBH).
[d]PCR with amplicon detection by microplate ELISA hybridization (ELISA).

Post-amplification, PCR products are commonly detected by agarose gel electrophoresis and ethidium bromide staining of the DNA, which employs hazardous chemicals, may be prone to nonspecific banding products, and requires subjectivity in the reading of results (Nelson *et al*, 1997). This method is considered simple and not expensive, but due to the small number of samples analyzed per gel, the handing time and specific equipment

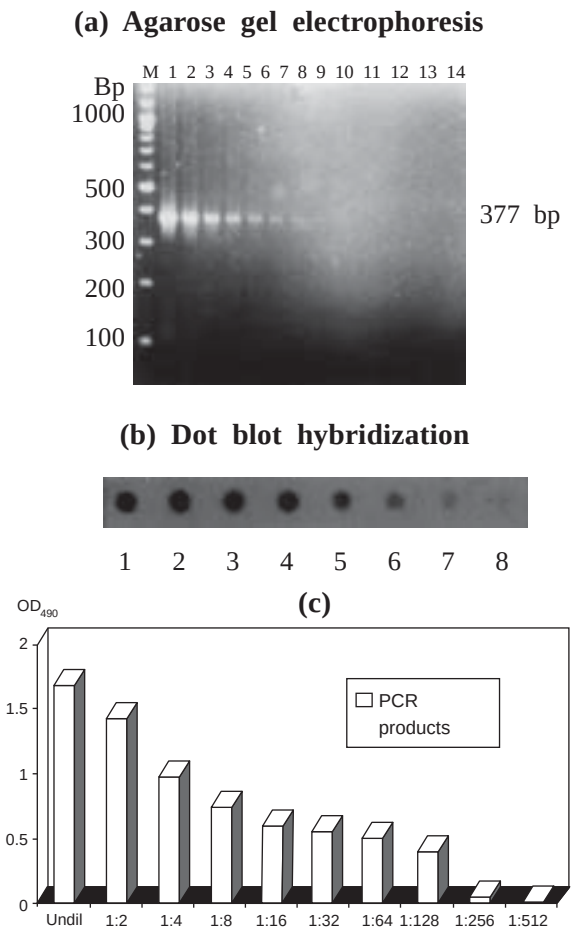

Fig 1–The sensitivity of detection *M. tuberculosis* as determined by using genomic DNA purified from H37Rv isolate, and the DNA at 100 ng per reaction was used as the template for amplification. The PCR products were diluted by serial two fold dilution from undiluted to 1:512 and each dilutions were analyzed by three amplicon detection methods: (a) ethidium bromide-stained agarose gel electrophoresis showing 377-bp PCR products; (b) dot blot hybridization with fluorescein labeled probe and enhanced chemiluminescence detection; and (c) microplate ELISA hybridization with fluorescein labeled probe, and colorimetric detection, and reading the optical density at 490 nm.

required and the need to photograph the gel to document the results, the method is rather expensive and laborious, so rendering PCR difficult to implement in routine diagnostic laboratories.

In the case of doubtful results of gel electrophoresis occuring (more than one band where only one was expected, a diffuse band, or no band) the results are generally confirmed by Southern blot hybridization on membranes with radioactive or nonradioactive-labeled oligomer probes, which increases the sensitivity 10- to 100-fold. However, this is not a desirable approach in the clinical laboratory, due to radioactive chemicals being employed and the somewhat labor intensive and lengthy procedures which increase turnaround-time as well as cost; more over, readings are subjective and are not suitable for the analysis of large numbers of samples or for automation and therefore not always practicable in all laboratories.

To facilitate the detection process for large-scale screening, dot blot hybridization (DBH) with enhanced chemiluminescent (ECL) detection was applied. The agreement rate of PCR product detection by DBH compared with AGE was 0.956. As expected, the DBH was more sensitive than AGE. Use of hybridization with detection by ECL increased the sensitivity of detection by PCR-AGE assay 10-fold from 10 pg to 1 pg of purified DNA per reaction; *ie* from about 30 to about 3 organisms.

However, for the detection of MTB from clinical samples, DBH demonstrated a lack of specificity (98%) compared with AGE (100%), which may be attributed to the use of unpurified amplicons to prepare dot blots or the use of a short probe (17 bp) in hybridization. Analysis with purified or diluted PCR products and hybridization with a specific longer probe may increase the specificity of DBH. However, in the dot method, washing of the solid phase required more time since the reagents must penetrate the membrane during the reaction; therefore, during washing, it is necessary to wait for the unreacted reagents to diffuse out from the inside of the membrane. The required longer washing-time, the high volume of washing buffer and the laborious detection with chemiluminescent made the DBH method more expensive and not suitable for the analysis of large numbers of samples and not always

practicable in all laboratories.

To improve the detection process for the large-scale screening of tuberculosis, a microplate-based DNA hybridization assay with colorimetric detection was applied. For the detection of MTB from clinical samples, the results for AGE and microplate ELISA hybridization are in close agreement. Of these three amplicon detection methods, DBH had higher sensitivity than AGE and ELISA; however ELISA and AGE had higher specificity than DBH. The sensitivity, specificity and accuracy of three amplicon detection methods were rather high and not statistically significantly different ($p>0.05$).

False-negative results were detected by AGE and ELISA hybridization for 8 samples and by DBH for 6 samples. Most samples were from smear-positive specimens and the false negative results were considered to have been due to the presence of PCR inhibitors rather than to the low numbers of organisms in the sputum samples. The presence of inhibitors of PCR in clinical specimens is a well-known diagnostic problem that frequently leads to false-negative results. Although we tried several extraction methods prior to amplification, *ie* phenol-chloroform extraction, QIAamp DNA kit (Qiagen), or incorporation of bovine serum albumin (BSA) according to Forbes and Hicks (1996), in order to remove interfering substances, we could not solve the inhibitor problem.

There are several methods to remove inhibitors by DNA purification, *eg* the 50% sucrose centrifugation step by Victor *et al* (1992). In the report of Forbes and Hicks (1996), BSA was able to override the inhibitor for 21 of 22 inhibitory specimens. Amicosante *et al* (1995) used capture resin [GeneReleaser(GR)] which improved PCR sensitivity from 77% with phenol-chloroform extraction (P-C) alone to 91% (GR without P-C) and 100% (P-C and GR). 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 our study we found that most extraction reagents were able to remove inhibitory substances in some sputum samples that contained a high number of AFB. However, if the specimens had low numbers of AFB, the extraction and purification process may cause bacterial loss and a subsequent decrease in the sensitivity of detection by PCR. Recently, Al-Soud and Radstrom (1998) showed that the PCR-inhibiting effect of various components in biological samples could be eliminated by the use of the appropriate thermostable DNA polymerase.

Another reason for these false-negative results were considered to be the degradation of DNA, since the specimens were processed and DNA was partially purified by extraction with a simple procedure and kept frozen at -20ºC for several months before the PCR was performed. This DNA was degraded, especially in specimens with low numbers of AFB. If the delay in amplification is reduced, the number of false-negative results may be fewer.

For the ELISA hybridization assay described here, the method was rapid with a total assay time following PCR of 4 hours. The assay offers the advantage that it can be applied in the 96-well format of a microtiter plate by using the technology of the ELISA. An additional advantage is that it yields numerical data and therefore does not depend on subjective interpretation; the assay does not require radioisotopes, making it feasible for use in the microbiology laboratory or for large-scale epidemiological studies.

The sensitivity of the ELISA hybridization assay was shown to correspond to that of agarose gel analysis, but the amount of PCR product that could be detected by ELISA assay was one half of that which could be detected by AGE. However, ELISA was less sensitive when compared with DBH. This problem may be explained by the design of the indicator system which is only 1 molecule of fluorescein labeled at the 5′ end of oligonucleotide probe. To overcome this problem, more fluorescein molecules should be labeled on detection probe, *ie* using 3′ end labeling which may increase

the sensitivity of colorimetric detection system. However, the higher sensitivity of DBH than of ELISA was also due to the method of detection by enhanced chemiluminescence: higher sensitivity and more rapidity of detection by ELISA may therefore be obtained by using chemiluminescence detection instead of colorimetric detection.

In conclusion, PCR assay provides early detection of MTB in clinical samples from patients who are at high risk for MTB infection and will greatly benefit patient management; it should however be validated by culture. For the detection of PCR products, the microplate ELISA hybridization confers several advantages over conventional ethidium bromide-stained gel analysis and DBH: it is less cumbersome, cost-effective, quicker, has fewer safety hazards, promotes objectivity in the reading of results and is suitable for use in epidemiological studies for the analysis of a large number of samples; the assay has been shown to be comparable in sensitivity and specificity to the other methods. Because of its high degree of specificity, PCR-microplate hybridization assay could be a confirmatory tool for establishing the presence of MTB in AFB-positive specimens. However, to become a general purpose screening test, it would require improved sensitivity for the reliable detection of specimens containing low numbers of MTB.

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