

# SIMPLE MICROPLATE HYBRIDIZATION ASSAY FOR DETECTION OF AMPLIFIED PRODUCTS OF *MYCOBACTERIUM TUBERCULOSIS*

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**Abstract.** We describe a simple microplate hybridization assay for the rapid detection of the IS6110 PCR products of *Mycobacterium tuberculosis* from clinical cultures and 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 fluorescein were identified by using anti-fluorescein antibody, horseradish peroxidase conjugate and colorimetric peroxidase substrate. The specificity of the assay was assessed by analysis of 56 bacterial strains: the assay discriminated perfectly between the positive and negative groups when an OD<sub>490</sub> of 0.18 was used as the cut-off point. The assay was sensitive enough to detect as little as 1 pg of *M. tuberculosis* H37Rv DNA, which is equivalent to approximately three bacilli.

To evaluate the assay performance clinically, 190 sputum samples from newly diagnosed TB patients were tested; 79 were classified as TB positive, and 111 were classified as TB negative by culture and acid-fast staining as the 'gold standard'. The sensitivity, specificity and accuracy of the PCR-microplate hybridization assay were 90, 100 and 96%, respectively. The total assay time of hybridization following the PCR was 4 hours. The PCR-microplate hybridization assay is fast, simple and accurate and is suitable for use in the microbiology laboratory or for the analysis of large numbers of samples.

## INTRODUCTION

Tuberculosis (TB) remains a significant global health problem and is the leading infectious cause of death worldwide, being responsible for 3 million deaths annually. The World Health Organization has calculated that, unless urgent action is taken, the annual number of deaths could rise from 3 million to 4 million by the year 2004 (WHO, 1994). So serious is the global threat of TB that, in 1993, the WHO took the unprecedented step of declaring this disease a global emergency. The problem is fuelled by the pandemic of HIV infection and AIDS and the emergence of multidrug resistance (Fatkenhever *et al*, 1999). HIV infection renders a person infected by

*Mycobacterium tuberculosis* (MTB) much more likely to develop overt TB, and the evolution of the disease is considerably accelerated.

Attempts to improve laboratory methods for the diagnosis of TB have been made. PCR, a well-developed technic is used extensively for the diagnosis of TB (Forbes and Hicks, 1993; Nolte *et al*, 1993; Kent *et al*, 1995; Ichiyama *et al*, 1997). In general, the analysis of PCR product was confined to agarose gel electrophoresis which has certain disadvantages, including handling time, safety hazards and the risk of nonspecific banding patterns; the results must be confirmed by hybridization procedures on membranes which are laborious and yield subjective readings, not suitable for the analysis of large numbers of samples or for automation, and therefore not practicable in all laboratories. The post-amplification product procedure has been progressively developed as a simplified analytic method using colorimetric detection by ELISA which is a rapid and

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practical procedure that might find routine use in the mycobacteriology laboratory (Landgraf *et al*, 1991; Cho *et al*, 1995; Kox *et al*, 1996; Patel *et al*, 1997).

In this paper we describe another simple microplate hybridization method for the rapid detection of the IS6110 PCR products of MTB, using the 5' end biotin labeled primers designed in the target sequence of IS6110, and a specific oligonucleotide probe labeled with nonradioactive substance. We evaluated the performance of this developed assay in the detection of MTB in clinical sputum specimens by using culture and acid-fast staining as the 'gold standard' for the diagnosis of TB infection.

## MATERIALS AND METHODS

### Bacterial strains

A total of 56 strains of bacteria comprising mycobacteria in *M. tuberculosis* complex (30 strains), non-tuberculous mycobacteria (16 strains), and non-mycobacteria (10 strains) were used in this study to test specificity of the PCR. The reference standard strains and clinical isolates of mycobacteria were provided by the Central Chest Hospital and the TB Division, Ministry of Public Health. Other non-mycobacteria were stock cultures isolated from patients. All MTB isolates were re-identified by microscopic colony morphology, pigmentation, and conventional biochemical tests (Kent and Kubica, 1985).

### Clinical specimens

A total of 190 sputum specimens were obtained from newly-diagnosed TB patients over 15 years of age attending the TB Division, Ministry of Public Health, during February and April 1998. The samples, at least 2-3 ml of either spot or collection sputum, were collected prior to the administration of medication.

### Processing of specimens

The specimens were processed for diges-

tion and decontamination by the N-acetyl-L-cysteine-4% NaOH method (Kubica *et al*, 1963). Briefly, the sputum sample was treated with an equal volume of N-acetyl-L-cysteine-4% NaOH for 15 minutes at room temperature with shaking. The tubes were filled with 10 vol of 0.067 M phosphate buffer, pH 6.8, and centrifuged at 3,000g for 30 minutes. The resulting pellet was resuspended in 2 ml of TE buffer, pH 8.0 (10 mM Tris-HCl; 1 mM EDTA), and 1 ml was used for AF microscopy and routine culture, the remainder was boiled at 80°C for 30 minutes to kill bacteria and then stored frozen at -20°C until detection by PCR procedure.

### Microscopy and culture

The resuspended sediments were screened for the presence of acid-fast bacilli (AFB) by fluorescence microscopy (Bennedsen and Larsen, 1966) and scored as negative, 1+, 2+, and 3+ (NTA, 1969). Culture was performed according to standard technique by inoculating 0.1-0.2 ml of the resuspended sediment onto two Ogawa slants and incubated at 37°C for 8 weeks and examined weekly for growth. All isolates were examined for growth rate, gross and microscopic colony morphology, and pigmentation and any suspected colonies were subjected to conventional biochemical tests (Kent and Kubica, 1985).

### DNA extraction from bacterial cultures and from sputum specimens

The DNAs of two reference strains; *ie M. tuberculosis* H37Rv and *M. flavescens* ATCC 23035 were extracted by a proteinase K-phenol-chloroform method as previously described (Tansuphasiri *et al*, 1999a). DNA extraction from bacterial cultures were performed by the boiling method. Briefly, a few colonies of bacteria which grew on medium were suspended in TE buffer to make a suspension of Mc Farland No.1 (estimated 10<sup>7</sup> cfu/ml) and diluted 10-fold with TE buffer (estimated 10<sup>5</sup> cfu/ml) and the suspension was boiled in a water bath at 95°C for 10 minutes and then placed on wet ice for 5 minutes. The pellet was removed by centrifugation at 12,000g for

5 minutes. Ten  $\mu$ l of the supernatant was used as the template DNA in the PCR. Extraction of DNA from sputum samples was performed according to the method described by Buck *et al* (1992) as slightly modified by Tansuphasiri (2000).

### Primers and probe

Primer TB1 (5'-CCAACAAGAAGGCG TACTCG-3', position 961 to 980) and primer TB2 (5'-GGAGACTCTCTGATCTGAGACC-3', position 1316 to 1337), derived from the IS6110 reference sequence (accession number X17348) which amplify a 377-bp DNA fragment (Thierry *et al*, 1990) were used. The probe, TB3 (5'-TAGGGGATCTCAGTACA-3', position 1009 to 1025) was internal to the amplified target of the IS6110 sequence. All of these primers and the probe were designed by Tansuphasiri *et al* (1999b), and were synthesized by Bioservice Unit (BSU), National Center of Genetic Engineering and Biotechnology. Primer TB2 was 5' biotinylated and TB3 was 5'-fluorescein-labeled using the 5'-end labeling kit (Promega) as described by the manufacturer.

### Optimization of the PCR-microplate hybridization assay

Purified DNA of H37Rv at the concentration of 1 ng/ $\mu$ l was used as the DNA template for optimization of the assay. In general, the condition for amplification followed the optimized condition of previous studies (Tansuphasiri *et al*, 1999a,b), except for the primer concentrations, for which optimization was performed by variation primer concentrations, *ie* 0.1, 0.2, 0.3, 0.4, 0.5 and 1.0  $\mu$ M. The hybridization was optimized in microtiter plate (MaxiSorb™, Nunc) by varying the volume of PCR products (2.5, 5.0 and 10  $\mu$ l), the concentrations of substances using in the reaction, *ie* avidin (1.25, 2.5 and 5.0  $\mu$ g/ml), TB3 probe (0.125, 0.25, 0.5 and 1.0  $\mu$ g/ml), anti-fluorescein-HRP conjugate (1:500, 1:750) and optimization was performed by the check-board titration.

After various trials, the following condi-

tions were found to be optimal for PCR-microplate hybridization assay. Amplification was performed in 1X PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.01% gelatin], 0.2 mM (each) deoxynucleotide triphosphates, 0.3  $\mu$ M each of primers TB1 and TB2, 1 U of *Taq* polymerase (Promega) and a DNA template solution (10 ng/reaction). Initial denaturation at 94°C for 5 minutes was followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, extension at 72°C for 1 minute. Finally, incomplete PCR products were extended for 10 minutes at 72°C.

The purified avidin, 1.25  $\mu$ g/ml (Sigma) was diluted with a coating buffer (50 mM carbonate-bicarbonate buffer, pH 9.6). One hundred  $\mu$ l of the solution were dropped into each well of microtiter plates and incubated for 2 hours at 37°C or overnight at 4°C in moist atmosphere, then the plates were washed three times with PBST buffer (prepared from 10X PBS-0.05% Tween 20; 1X PBS is composed of 1.37 M NaCl; 27 mM HCl; 43 mM Na<sub>2</sub>HPO<sub>4</sub>; 14 mM KH<sub>2</sub>PO<sub>4</sub>), blocked with 100  $\mu$ l of PBST buffer containing 0.05% bovine serum albumin (BSA) for 1 hour at 37°C or overnight at 4°C in moist atmosphere and washed three times. The amplification products were then mixed with PBST buffer to 100  $\mu$ l and dropped into the well. After incubation for 30 minutes at 37°C, the plates were washed three times and 100  $\mu$ l of 0.25 M NaOH were dropped into the well to denature the sens-strand DNA. After 5 minutes at room temperature the microtiter plates were washed three times again and 100  $\mu$ l of the detection probes (0.5  $\mu$ g/ml) diluted in 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) were dropped into the wells and incubated for one half an hour at 37°C then the plates were washed three times. The anti-fluorescein HRP conjugate (Amersham) was diluted with PBST buffer (1:500) containing 0.05% BSA and dropped into the wells, incubated for 30 minutes at 37°C in the dark and washed three times with PBST buffer and the reaction was detected by O-phenylenedi-

amine substrate (0.4 mg/ml OPD, 0.4 mg/ml urea hydrogen peroxide, 0.05 M phosphate citrate buffer, Sigma Fast). After incubation at 37°C for 1 hour, the reaction was stopped by adding 100  $\mu$ l of 3 M H<sub>2</sub>SO<sub>4</sub>. The A<sub>490</sub> was read with a microplate reader (Bio-Kinetics Reader EL321e, Biotex Instrument, USA).

### Determination of sensitivity

To determine the sensitivity of detection of MTB, 10-fold serial dilutions of H37Rv prepared from both bacterial suspension ( $\sim 10^8$  to  $\sim 10^2$  organisms per ml, prepared by just boiling) and purified DNA (the DNA concentrations ranging from 100 ng to 0.1 fg per 10  $\mu$ l) were used as the templates for PCR and hybridization with the optimized condition as mentioned above.

### Control procedures

All reactions were performed under stringent conditions as 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. Positive and negative hybridization controls were also included in each microplate hybridization assay: these 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.

### Statistical analysis

Validation of the PCR-microplate hybridization assay for the direct detection of MTB from all 56 bacterial strains and from 190 sputum specimens was determined by using the results of culture and AF staining as the 'gold standard'. Statistical comparison was performed by using chi-square analysis; a  $p < 0.05$  was considered significant.

## RESULTS

### Optimization of the assay

Since the primer concentrations may influence the amplification efficiency, *ie* a low

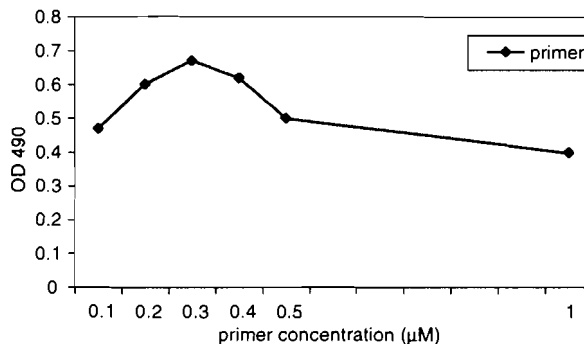


Fig 1—Influence of primer concentrations on the performance of PCR-microplate hybridization assay. Ten nanograms of H37Rv DNA were amplified with various concentrations of primers (TB1-TB2) from 0.1 to 1.0  $\mu$ M, and PCR product was detected with 0.5  $\mu$ g of fluorescein-labeled oligonucleotide probe (TB3) per ml. Data are the means of triplicate values.

primer concentration could result in decreased efficiency, while a high concentration of unincorporated biotinylated primer could compete with the biotinylated segment amplified with TB1-TB2 for avidin on the solid phase. The interaction could reduce the intensity of the specific signal in hybridization. Thus the influence of primer concentration on the efficiency of PCR-microplate hybridization was determined firstly with positive and negative control DNA templates under the standard conditions described in materials and methods. According to the results of these experiments as shown in Fig 1, an optimal concentration of 0.3  $\mu$ M of each primer was selected for testing 56 clinical isolates and 190 clinical specimens.

For microplate hybridization, the influence of avidin concentration as a coating agent was first evaluated. The result of this optimization is shown in Fig 2. Of the various concentrations of avidin-coated plates, the optimal concentration was 1.25  $\mu$ g/ml of carbonate buffer and 100  $\mu$ l was used for coating plates. The varying amounts of PCR products of biotinylated MTB were also evaluated. All dilutions gave the signals in the assay, however undiluted PCR product (10  $\mu$ l) gave the best signal (Fig 3).

MICROPLATE HYBRIDIZATION OF AMPLIFIED PRODUCTS OF MTB

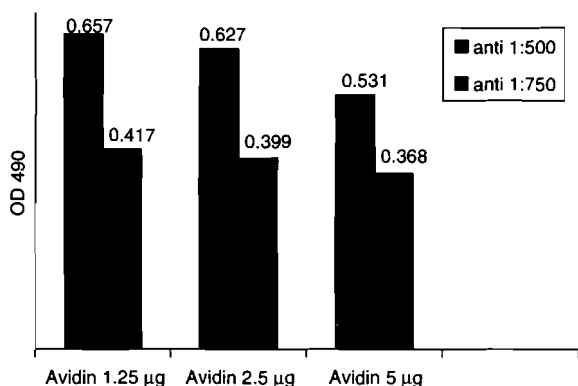


Fig 2—Graph showing the optical density of microplate hybridization assay measured of biotinylated PCR products from MTB DNA following the optimization of avidin and anti-fluorescein-HRP at various concentrations (avidin; 1.25, 2.5 and 5.0 µg/ml, and anti-fluorescein-HRP at dilution 1:500 and 1:750).

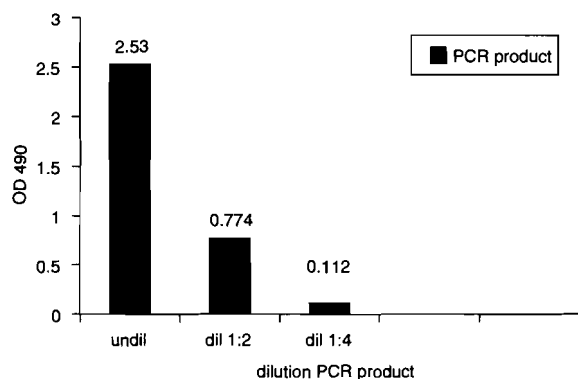


Fig 3—Graph showing the optical density of microplate hybridization assay measured from 2-fold dilutions (undilute, 1:2, and 1:4) of the biotinylated *M. tuberculosis* DNA fragment, which was generated by amplification of H37Rv DNA with primers, TB1 and TB2 biotin labeled and reacted with 0.5 µg/ml of the fluorescein-labeled probe, TB3.

The influence of the detection probe (fluorescein-labeled TB3) was assessed with a various concentrations. The results of these experiments are illustrated in Fig 4. The assay was less sensitive with a probe concentration of less than 0.125 µg/ml with the PCR product diluted 1:100, but not with undiluted or 1:10.

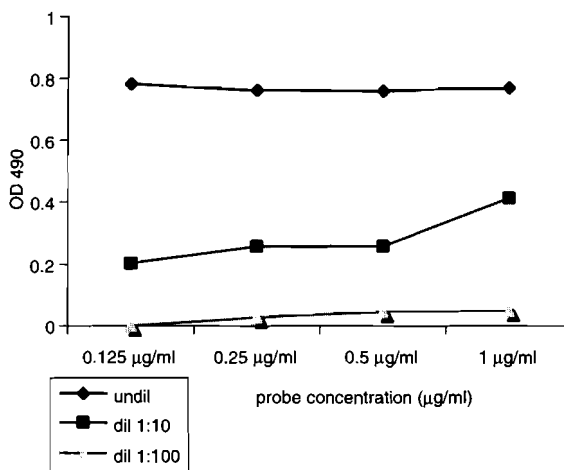


Fig 4—Influence of probe concentrations on detection of biotinylated PCR products. Various concentrations of fluorescein-labeled oligoprobe (TB3) from 0.125, 0.25, 0.5 and 1.0 µg/ml reacted for 1.5 hours at 37°C in 10-fold dilutions (undilute, 1:10 and 1:100 dilution) of the biotinylated *M. tuberculosis* H37Rv DNA fragment.

However for undiluted PCR products, the probe concentration of greater than 0.125 µg/ml did not improve the sensitivity level of the assay, in contrast to 1:10 dilution. The optimal probe concentration for both undiluted and 1:10 dilution was in the range 0.25-0.5 µg/ml. The concentration of probe (0.5 µg/ml) that still gave the best signal was selected for the subsequent studies. For optimal hybridization a temperature of 37°C was selected for all experiments based on the T<sub>m</sub> of the designed oligonucleotide probe (TB3); this temperature suited the shaking incubators, set to 37°C in general laboratories, and did not dry the solution in the wells. Of the two concentrations of anti-fluorescein-HRP that were evaluated, the dilution 1:500 in PBST buffer – 0.05% BSA gave the best signal (Fig 2) and it was selected for all subsequent experiments when 100 µl of this dilution was dropped into each microtiter well.

**Interpretation of OD values**

To define the cut-off OD value, 56 strains proved by culture: MTB complex (30 strains); non-tuberculous mycobacteria (16 strains); and

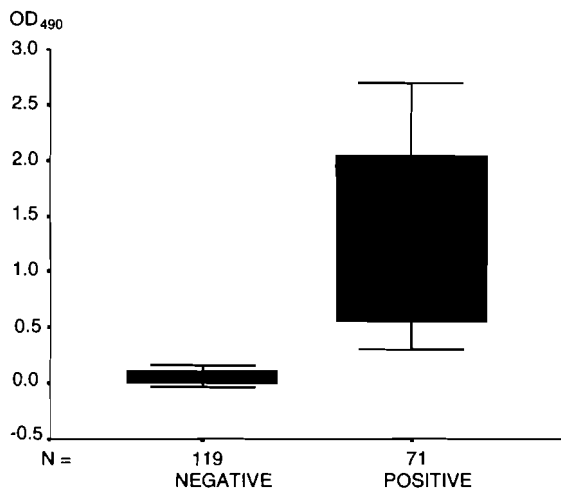


Fig 5—Box plot of the optical densities between *M. tuberculosis* positive and negative groups which the PCR products generated by amplification of crude DNA extraction from 190 clinical specimens, and the amplicon detection by microplate hybridization assay.

non-mycobacteria (10 strains) were analyzed by the PCR-microplate hybridization assay. 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 (mean OD<sub>490</sub> of the negative control + 3 SD). Then 30 of 56 culture isolates were in the positive group while 26 were in the negative group. The mean OD<sub>490</sub> of the positive group (MTB complex) was 1.87 (95% CI = 1.660, 2.086) and that of the negative group was 0.118 (95% CI = 0.111, 0.126).

When the assay was used with 190 clinical sputum samples, it could be identified by microplate hybridization as positive and negative for 71 and 119 samples, respectively (Fig 5). The mean optical density at 490 nm of the positive group was 1.405 (95% CI = 1.217, 1.592) and that of the negative group was 0.056 (95% CI = 0.047, 0.065). The means optical densities of the positive group and the negative group were significantly different ( $p = 0.000$ ).

#### Sensitivity, specificity and limit of detection

Table 1 shows the result of PCR-microplate

hybridization assay compared with culture results for the efficacy of detection of MTB in smear-positive and smear-negative sputum specimens. Of the 79 specimens that were culture positive for MTB, 71 samples were positive by the PCR-microplate hybridization (sensitivity, 89.9%). Of the 111 culture-negative samples for MTB, none was positive by PCR-microplate hybridization assay (specificity, 100%). No false-positive samples by the assay were examined, while all eight false-negative samples detection were from samples with AF smear positive and culture positive for MTB (AF+/Cul+), indicating some PCR inhibitors in the sputum samples. The sensitivity of detection of MTB as determined by using H37Rv DNA was 1 pg per reaction which is equivalent to approximately three bacilli.

#### DISCUSSION

With the increased incidence of TB and the advent of MDR-TB strains, rapid diagnosis and treatment are crucial to reducing mortality and morbidity from TB disease. The use of PCR for the rapid detection of MTB has been demonstrated by several groups, as well as by our studies (Tansuphasiri *et al*, 1999a; Tansuphasiri, 2000). For post-amplification detection, there are several different procedures for the detection of PCR products. The simplest version of product detection relies on agarose gel electrophoresis and ethidium bromide staining of DNA. Despite the fact that this method is not considered expensive, it renders PCR difficult to implement in routine diagnostic laboratories, due to (i) the handling time and specific equipment required, (ii) the use of ethidium bromide with its related handling procedure, (iii) the need to photograph the gel to document the results, (iv) the risk of a non-specific banding pattern.

Subsequent to gel electrophoresis, most researchers propose Southern blot analysis with radioactive or non-radioactive-labeled oligomer probes, which increases the sensitivity 10- to 100-fold. We have previously used a non-radioactive probe method to avoid the disad-

Table 1  
Comparison of the PCR-microplate hybridization assay with confirmed culture results for detection of *M. tuberculosis* from 190 sputum specimens.

Specimen type and PCR result	No. of specimens with the following culture result <sup>a</sup>		Total
	Positive	Negative	
<b>All specimens</b>			
Positive	71	0	71
Negative	8	111	119
Total	79	111	190
<b>Smear-positive specimens</b>			
Positive	70	0	70
Negative	8	1	9
Total	78	1	79
<b>Smear-negative specimens</b>			
Positive	1	0	1
Negative	0	110	110
Total	1	110	111

<sup>a</sup>Culture result was used as the 'gold standard'.

vantage associated with the use of radioactive probes. By employing a fluorescein-labeled probe for Southern blot hybridization with enhanced chemiluminescent (ECL) detection, we increased sensitivity 100-fold from 1 pg by gel electrophoresis to 10 fg after hybridization, and the PCR assay showed overall sensitivity, specificity, and efficiency of 96, 99, and 98%, respectively (Tansuphasiri *et al*, 1999b). However, Southern blot hybridization on membranes is laborious and the readings are subjective, rendering the technic unsuitable for the analysis of large numbers of samples or for automation, and therefore not practicable in all laboratories. To facilitate the detection process for large-scale screening, dot blot hybridization with ECL detection was applied. However, for detection of MTB from clinical sputum samples, dot blot hybridization demonstrated a lack of specificity (85%) compared with gel electrophoresis (100%) (Tansuphasiri, 2000).

In this study, we developed a microplate hybridization assay to simplify the detection of the IS6110 PCR products of MTB by using our previously designed primer pair (TB1-TB2), and compared our developed method with culture and AF staining as the 'gold standard' for diagnosis of TB. To accomplish the re-

quirements of the assay, the 5'-end of downstream primer (TB2) of this primer pair is biotinylated, resulting in biotinylated PCR products. The biotinylated product was quantitatively captured on microtiter plates coated with avidin and then denatured with 0.25 M NaOH. A homogeneous hybridization reaction between PCR products and an internal fluorescein-labeled oligonucleotide probe (TB3) was accomplished under stringent conditions. Following removal of unbound nucleic acids by washing, specific hybrids were detected by reaction with anti-fluorescein antibody conjugated to horseradish peroxidase (HRP) and by the addition of a peroxidase substrate.

When the PCR products of 56 different strains, including reference strains and clinical isolates in the *M. tuberculosis* complex, non-tuberculous mycobacteria and non-mycobacteria, were used for detection by the developed assay, two *M. bovis* strains also proved positive in the microplate hybridization assay. The positive PCR results detection for *M. bovis* was due to the DNA target of amplification, IS6110 which is specific for the *M. tuberculosis* complex. All strains belonging to this group remained positive in the PCR-microplate hybridization as well. However, the incidence

of human TB caused by *M. bovis* has decreased following the pasteurization of milk for human consumption (Liebana *et al*, 1996). In addition, *M. bovis* isolates usually have low copy number of IS6110 element, in contrast to *M. tuberculosis* strains that typically carry multiple copies of this element (McAdam *et al*, 1990).

When 190 sputum samples from newly-diagnosed TB patients were tested in a routine laboratory, in most cases, the culture results and the PCR-microplate hybridization assay results agreed (agreement rate 96%). Of the 79 culture-positive samples, 71 were positive by the PCR-microplate hybridization assay (sensitivity 89.9%) and none of the 111 culture negative samples was positive by this PCR assay (specificity 100%). False-negative results were found in 8 samples: these samples were smear-positive; thus the false-negative results by this PCR detection might indicate the presence of PCR inhibitors rather than the low numbers of organisms in these sputum samples. In this study, we also incorporated 0.2% bovine serum albumin during the extraction process to remove interfering substances from sputum specimens, and those false negative samples by the PCR-microplate hybridization were also retested at dilution 1:10 and checked again by spiking of the previously tested DNA extracts with approximately 100 fg of H37Rv DNA. However the results were negative after dilution because not only the inhibitor was diluted but also the DNA target.

The PCR-microplate hybridization assay was also sensitive enough to detect as little as 1 pg of DNA, which is equivalent to approximately three bacilli. Higher sensitivity and more rapidity of detection may also be obtained by using ECL chemiluminescence detection instead of colorimetric detection. In this study, horseradish peroxidase (HRP)-labeled anti-fluorescein was chosen, therefore the immunoassay could be developed by using either colorimetrically or ECL detection. For the assay described here, the method was quick with a total assay time following PCR of 4 hours. The assay was also simple, less laborious than Southern blot hybridization and more conve-

nient than dot blot hybridization due to easy handling of the microwell plate (multichannel pipets, readers, and other instruments for ELISA techniques can be used). An additional advantage is that the assay yields numerical data and does not depend on subjective interpretation; the assay does not require radioisotopes, making it feasible for use in the microbiology laboratory or for screening a relatively large number of samples in an epidemiologic study (Holmstrom *et al*, 1993). It could become a valuable alternative approach to the diagnosis of TB infections.

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