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A Simple, Single Triplex PCR of IS6110, IS1081, and 23S Ribosomal DNA Targets, Developed for Rapid Detection and Discrimination of *Mycobacterium* from Clinical Samples

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Tuberculosis (TB) is the most common mycobacterial infection in developing countries, requiring a rapid, accurate, and well-differentiated detection/diagnosis. For the rapid detection and discrimination of *Mycobacterium tuberculosis* complex (MTC) from non-tuberculous mycobacteria (NTM), a novel, simple, and primer-combined single-step multiplex PCR using three primer pairs (6110F-6110R, 1081F-1081R, and 23SF-23SR; annealing on each of *IS6110*, *IS1081*, and *23S rDNA* targets), hereafter referred to as a triplex PCR, has been developed and evaluated. The expected product for *IS6110* is 416 bp, for *IS1081* is 300 bp, and for *23S rDNA* is 206 bp by single PCR, which was used to verify the specificity of primers and the identity of MTC using DNA extracted from the *M. tuberculosis* H37Rv reference strain (ATCC, USA) and other mycobacteria other than tuberculosis (MOTT) templates. The triplex PCR assay showed 100% specificity and 96% sensitivity; the limit of detection for mycobacteria was ~100 fg; and it failed to amplify any target from DNA of MOTT (50 samples tested). Of 307 blinded clinical samples, overall 205 positive *M. tuberculosis* samples were detected by single PCR, 142 by conventional culture, and 90 by AFB smear methods. Remarkably, the triplex PCR could subsequently detect 55 positive *M. tuberculosis* from 165 culture-negative and 115 from 217 AFB smear-negative samples. The triplex PCR, targeting three regions in the *M. tuberculosis* genome, has proved to be an efficient tool for increasing positive detection/discrimination of this bacterium from clinical samples.

Keywords: Clinical samples, *Mycobacterium*, single triplex PCR, *IS6110*, *IS1081*, *23S rDNA* genes

Introduction

Mycobacterium tuberculosis is one of the most harmful human pathogens worldwide, causing about 9.4 million incident cases of tuberculosis (TB), coupled with 1.1 million TB deaths among HIV-negative and an additional 0.35 million deaths among HIV-positive people [35]. The diagnosis of tuberculosis is largely based on conventional approaches relying on clinical features and results of microscopy and bacterial culture. Clinical features are not generally diagnostic; and microscopy, while rapid, lacks specificity and sensitivity (detection limit = 7.5×10^3 organisms/ml). The culture is a simple, inexpensive, and reliable method for discriminating *Mycobacterium tuberculosis* complex (MTC) from non-tuberculous mycobacteria (NTM) that can be applied immediately to cultures flagged as positive in liquid media (such as a mycobacterial growth indicator tuber [MGIT] 960 system). The culture methods are specific and sensitive (detection limit = 10 organisms/ml), but they are time consuming (from 2 to 4 weeks) and easily to be contaminated. Among all alternative approaches for rapid and specific diagnosis of *M. tuberculosis*, the most applicable one is DNA sequencing of mycobacterial gene targets such as *16S rRNA* [12], *rpoB* [16], *hap6565* [17], *secA* [37], and the *16S-*

23S internal transcribed spacer (ITS) region [25]. Although a potential for the species-specific identification of nearly all *Mycobacterium* spp., DNA sequencing is not practical to all culture isolates in a clinical diagnostic laboratory, due to its prohibitive cost, particularly in resource-poor developing countries of most common mycobacterial infections [6].

Infections caused by NTM are increasing in immunocompromised individuals, in particular AIDS patients [8]. The treatment protocol depends heavily on the species of *Mycobacterium* isolated and site and severity of infection, and the report of drug sensitivity testing is not always reliable and the duration of the treatment is protracted. Thus, strain identification from the clinical specimens is critically required, particularly in patients not responding to conventional therapy, relapsed, or immunocompromised [34]. PCR has been effectively and early applied for the detection of *Mycobacterium tuberculosis* in clinical specimens, requiring only 3–5 h, with higher specificity and sensitivity comparable to the culture methods [21]. For rapid detection/identification and differentiation of MTC and NTM, PCR/PCR-linked methods have been used simultaneously in routine diagnostic laboratories [11, 13, 20]. One of these molecular approaches is multiplex PCR, targeting a number of different genes, including insertion sequences (*e.g.*, *IS6110*, *IS6100,* and *IS1081*). However, some of these methods yield false-negative results, as the target sequences (such as *IS6110* targets) are not uniformly present in all clinical isolates.

Insertion sequences are repeated sequences for the genetic evolution of microorganisms [2]. In the *M. tuberculosis* complex, repetitive DNA sequences were used as probes and shown to be useful for fingerprinting strains in epidemiological studies [7]. Shortly after the characterization of the insertion sequence *IS6110* [30], an international consensus method, IS6110-RFLP, was adopted almost concomitantly to the World Health Organization declaration of TB as a public health emergency [31]. During recent decades, *IS6100* has also been used to detect *M. tuberculosis* by multiplex PCR, with highly variable sensitivity (11%–81%) in different studies [3, 14, 22]. Because of the rapidly emerging issue that *IS6110* was ineffective in a large part of the world, including South-East Asia (and Vietnam) [9], another insertion sequence, *IS1081* (five to seven repeats), was examined in all strains belonging to the *M. tuberculosis* complex and with low discriminatory power [5]. It was a useful subsidiary marker in detective studies of TB [4, 36] and has been used to help differentiate wild-type *M. bovis* isolates [28].

Although the development of DNA probes has greatly improved mycobacterial identification, particularly MTC, the commercially available AccuProbe DNA probe system (Gen-probe, San Diego, CA, USA), besides being expensive, offers only a limited number of species-specific probes and the clinical isolates are mostly identified as MTC or NTM [1]. Therefore, it is necessary to establish and evaluate PCR assays that can differentiate between these two groups of mycobacteria. This can be achieved by simultaneous amplification of two or more DNA targets. There have also been some reports on the multiplex PCR detection of *M. tuberculosis* strains isolated from the South-East Asian region (including Vietnam). However, single or double IS targets might not be sufficient for accurate amplification for detection of MTC and differentiation between MTC and NTM. Comparison between the *23S rDNA* sequences of *M. tuberculosis* and the corresponding sequences of other mycobacteria and of related non-mycobacterial species revealed a considerable variability, suggesting that the 5′ region of the *23S rDNA* could provide a target for the rapid detection and identification of mycobacteria both at the genus and species level [15].

The aim of this study was to combine *23S rDNA* with the previously selected DNA targets present in multiple copies in the *M. tuberculosis* genome, namely insertion sequences *IS6110* [32] and *IS1081* [33], for development of a sensitive triplex PCR for the simultaneous detection and increased differentiation between the MTC and NTM isolates in clinical fields.

Materials and Methods

Standard Bacterial Strains and Clinical Specimens

The standard strain H37Rv was obtained from the American Type Culture Collection (ATCC), Rockville, MD, USA (http://www.atcc.org/products/all/27294.aspx).

A total of 307 clinical specimens were collected from suspected pulmonary tuberculosis patients in different regions of the country (*e.g.*, National Lung Disease Hospital in North, Hue Central Hospital in Central, and Pham Ngoc Thach Hospital in South), belonging to males and females of different ages and sent for routine mycobacteriology to Hospital 103, Vietnam Military Medical University, were tested in this study. In addition, 50 mycobacteria other than tuberculosis (MOTTs) strains were also examined for this study. All of the samples were isolated from TB patients in Vietnam during 2007–2009 and informed consent was taken in the study.

Processing of Clinical Specimens

After processing for routine mycobacteriology, each specimen was divided into two parts: one part was used for AFB (Acid- Fast Bacilli) smear and culture; and the other was processed for PCR.

The AFB smears were examined for acid-fast bacilli after staining with Ziehl-Neelsen stain and graded according to standard procedures [29]. The cultures were performed in solid Ogawa medium (Korean Institute of Tuberculosis, Korea) and liquid MGIT 960 (Becton Dickinson, Sparks, MD, USA). To differentiate between *M. tuberculosis* and NTM, inoculations were made in media bottles with or without *p*-nitro-α-acetylamine-β-hydroxypropiophenone (NAP) according to the manufacturer's instructions.

Primers for Amplification of Target DNA

Three sets of oligonucleotide primers were used to amplify target DNA from the multiple copy genes *IS6110*, *IS1081*, and *23S rDNA*. The designation and sequence of these primers are given in Table 1. Using *M. tuberculosis* DNA as a template, the primer pairs 6110F-6110R, 1081F-1081R, and 23SF-23SR and were expected to amplify 416, 300, and 206 bp DNA products, respectively.

DNA Extraction from Cultures and Clinical Specimens

From *M. tuberculosis* grown on Ogawa medium, a rapid DNA extraction was performed according to the procedure previously described [18]. The clinical specimens, after processing for routine mycobacteriology, were centrifuged at 10,000 rpm for 10 min; then the pellets were washed twice and DNA was isolated using standard procedures of phenol/chloroform extraction and ethanol precipitation [32]. DNA pellets were dried, suspended in 50 µl of TE, and 5 µl was used for PCR.

Single PCR Assay for *IS6110*, *IS1081*, and *23S rDNA* Genes

For testing primers, the mixture of deionized distilled water, DreamTaq buffer, 2 mM $MgCl_2$, 0.25 mM each deoxynucleoside triphosphate (dNTP), 10 pmol of each primer, 1.25 U DreamTaq DNA polymerase (Fermentas), and DNA template of 50 ng for each *IS6110*, *IS1081*, and *23S rDNA*, respectively, was used in a PCR of 25 µl. The PCR assay was carried out with an initial denaturation (95°C for 5 min), followed by 35 cycles of denaturation (60 sec at 94°C); annealing of 45 sec at 55°C for *IS6110*, 45 sec at 56°C for *IS1081*, and 30 sec at 57°C for *23S rDNA*); and extension (60 sec at 72°C for all); and a final extension of 72°C for 10 min.

Set-Up of Triplex PCR and Condition Optimization

A triplex PCR was set up to simultaneously amplify the three targets, *IS6110*, *IS1081*, and *23S rDNA* genes, for *M. tuberculosis*, using six primers, listed in Table 1. Final conditions were initiated with denaturation at 95°C for 5 min and 35 cycles of denaturation at 94°C for 60 sec, annealing at 56°C for 45 sec, and extension at 72°C for 60 sec. A final extension was carried out at 72°C for 10 min. In each 25 µl of reaction, the mixture consisted of autoclaved deionized distilled water; DreamTaq buffer; 2 mM $MgCl_2$; 0.25 mM each dNTP; primer mix of 10 pmol for *IS6110*, 10 pmol for *23S rDNA*, 15 pmol for *IS1081*; 1.25 U Taq DNA polymerase; and 1 µl (50 ng) of DNA template.

A positive control, using DNA template from the *M. tuberculosis* H37Rv reference strain [18], and a negative control (distilled water), was included in each DNA amplification experiment. The triplex PCR products of expected 416, 300, and 206 bp (as of that from the control amplification), respectively, were visualized on a 2% agarose gel stained with ethidium bromide and viewed under UV light.

Triplex PCR Assay of Detection Limit for Template Sensitivity

The sensitivity of the triplex PCR was assayed for the detection limit from a serially diluted DNA template from the *M. tuberculosis* H37Rv reference strain. A 10-fold serial dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}) was started with 1 µl of 100 ng, providing 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, and 10 fg, from DNA template in each reaction. The assay was performed with 1 µl of the diluted template and a negative (no DNA) control was included. PCR products of 10 µl each were examined on a 1% agarose gel, stained with ethidium bromide and visualized under UV light (BioRad, USA).

Evaluation of Triplex PCR by Comparison with Single PCR, Culture, and AFB Smear Assays for Blinded Clinical Samples

To determine the applicability of triplex PCR in the diagnosis of tuberculosis, the triplex PCR and the other methods were evaluated and compared for their diagnostic potential, using clinical specimens from patients suspected of having tuberculosis. Both single PCR assay for each target (*IS6110*, *IS1081*, and *23S rDNA*) and triplex PCR assay for all of these three regions were carried using DNA template extracted from 307 blinded clinical samples. The efficiency of the triplex PCR from all 307 blinded samples was evaluated using statistical analysis for determination of the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV).

Standard culture technique (see above) was used to culture

Table 1. Primers used for triplex PCR amplification and detection of *M. tuberculosis*.

Target gene	Primer name	Sequence (5′-3′)	Product size (bp)
IS6110	6110F	GGTCGCCCGTCTACTTGGTG	416
	6110R	TGGACGCGGCTGATGTGCTC	
IS1081	1081F	TCGCGTGATCCTTCGAAACG	300
	1081R	CGCAGCTTGGGGATCGCGAC	
23S rDNA	23SF	ACCTGAAACCGTGTGCCTAC	206
	23SR	GGTCCAGAACACGCCACTAT	

M. tuberculosis in 307 clinical samples, and then positive and negative culture results were evaluated. Likewise, the AFB smear method was applied for these clinical samples. Subsequently, triplex PCR was tested to detect *M. tuberculosis* from both the positive and negative cultured and AFB products.

The results obtained from the triplex PCR and other methods (single PCR, AFB smear, and culture) were compared with the calculated 95% confidential intervals using the Stata/SE program for Windows (ver. 9.2; StataCorp LP, College Station, TX, USA).

Results

Testing Primers/PCR Conditions by Amplification of Single and Triple Targets

Template of DNA extracted from the *M. tuberculosis* H37Rv reference strain (ATCC) was used to confirm the primer pairs of each target and the mix of primers for a triplex PCR. A single PCR was performed with template and single target primer pairs; and in a triplex reaction with mixed template and three primer pairs for DNA of *M. tuberculosis* H37Rv strain. A clear DNA product of the expected size (416 bp of *IS6110*, 300 bp of *IS1081*, and 206 bp of *23S rDNA*, respectively) was yielded in each single PCR or triplex PCR (Fig. 1). This completely confirmed the primer specificity for each target in the template, in single PCR or triplex PCR conditions.

Sensitivity Assay of the Triplex PCR

Ten-fold serial dilutions (in a range of 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, and 10 fg) of DNA template of the *M. tuberculosis* H37Rv reference strain were used to assay the analytical sensitivity of the triplex PCR. All experiments were performed in a binary test. The lowest limit of detection was 0.01 ng (*i.e.*, 10 pg) DNA, with three bands of PCR products clearly visualized (Fig. 2). At the dilution of 10 fg DNA template, no bands were present. No DNA product was visualized in the negative control (data not shown). The amplified triplex PCR product could be detected from the 100 fg DNA template concentration, equivalent to about 20 *M. tuberculosis* bacilli (Fig. 2, lane 6).

Identification of *M. tuberculosis* in Clinical Samples

DNAs extracted from 307 clinical samples of suspected pulmonary tuberculosis patients were used as templates by single PCR of each target for identification of *M. tuberculosis* and MOTT strains. Table 2 shows that 106 were detected in the *IS6110*, 182 in the *IS1081*, and 136 in the *23S rDNA* target amplification, giving the overall 205 positive detections for *M. tuberculosis* using the single PCR assay. MOTT samples yielded no DNA band for any *M. tuberculosis* target (Table 2).

Triplex PCR Test for *M. tuberculosis* from Clinical Samples

DNAs of 205 *M. tuberculosis* strains and 50 MOTT strains of (identified by a single PCR, shown in Table 2 above) were used as templates, subsequently tested in the triplex PCR. As a result, all *M. tuberculosis* clinical strains could yield one to three bands of the expected products from any of the three target genes. Of 205 *M. tuberculosis* DNA

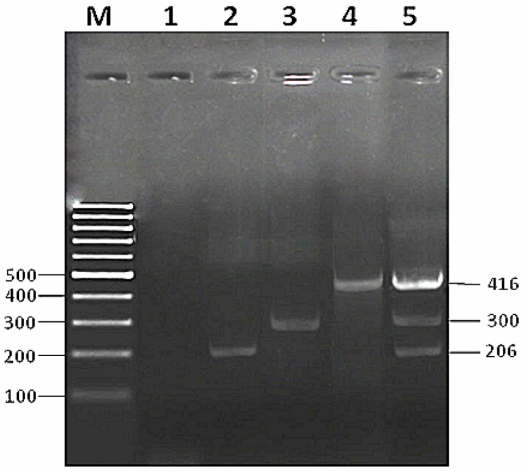

Fig. 1. Triplex PCR optimization using DNA template from the *M. tuberculosis* H37Rv reference strain.
Lane M, 100 bp DNA ladder; lane 1, negative control (water as a template); lane 2, positive *23S rDNA*; lane 3, positive *IS1081*; lane 4, positive *IS6110*; lane 5, triplex PCR with the three targets.

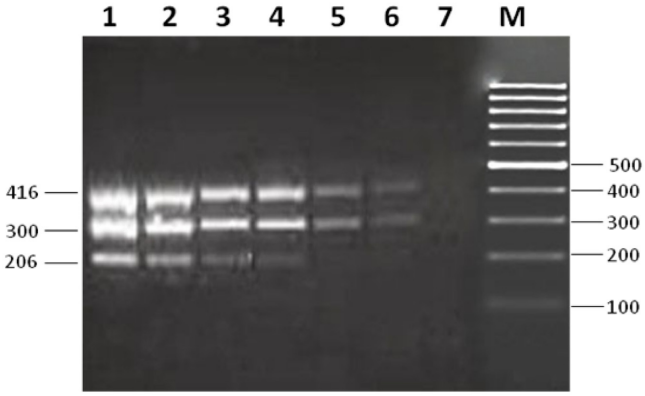

Fig. 2. Sensitivity assay for assessment of triplex PCR using DNA template of the *M. tuberculosis* H37Rv reference strain, visualized on 2% agarose stained with ethidium bromide.
Lanes M, 100 bp DNA ladder; lane 1, 10 ng DNA; lane 2, 1 ng DNA; lane 3, 0.1 ng (100 pg) DNA; lane 4, 0.01 ng (10 pg) DNA; lane 5, 0.001 ng (1 pg) DNA; lane 6, 0.0001 ng (0.1 pg or 100 fg) DNA; lane 7, 10 fg DNA.

Table 2. Positive results by a single PCR for each target and total number for three targets amplified from clinical samples and mycobacteria other than tuberculosis (MOTT) strains.

Samples	Number of samples	By single PCR, the positive PCR bands calculated for			
		IS6110	*IS1081*	*23S rDNA*	Total
Clinical	307	106	182	136	205
MOTT	50	0	0	0	0

Table 3. Results of triplex PCR of 205 samples of *Mycobacterium tuberculosis* complex strains (MTC) identified by a single PCR and mycobacteria other than tuberculosis (MOTT) strains.

Strains	Number of samples	Target DNA		
		IS6110	*IS1081*	*23S rDNA*
MTC	205	201	203	205
MOTT	50	0	0	0

samples tested, triplex PCR could detect 201 of *IS6110*, 203 of *IS1081*, and 205 (all) of *23S rDNA*, respectively. None of the amplification was obtained from non-mycobacterial organisms (Table 3; Fig. 3). These results suggested that the triplex PCR was useful in increasing the positivity of specific identification and differentiation of *M. tuberculosis* complex from other mycobacteria.

Analytical Assays of Triplex PCR, Bacterial Culture, and AFB Smear for Blinded Clinical Samples

Assays for the efficiency assessment of single and triplex PCRs, bacterial culture, and AFB smear of *M. tuberculosis* in blinded clinical samples were performed. DNA templates from positive and negative samples of the single PCR, the culture, and the AFB smear performance were subjected to the triplex PCR amplification. As a result, of 307 clinical specimens, 142 were culture-positive and 165 were culture-negative; 90 were AFB smear-positive and 217 were negative; and by the single PCR assay, 205 were positive and 102 were negative (Table 4).

Triplex PCR could yield product for 195 among 205 positive by a single PCR and none of the amplification from 102 single PCR-negative specimens (Table 4). From culture-positive specimens (n = 142), 140 specimens were positive with *M. tuberculosis* and 2 were negative; and among 165 culture-negative specimens, 55 were positive by triplex PCR. Detection of *M. tuberculosis* by the triplex PCR and AFB smear methods indicated that triplex PCR could yield products from 80 of 90 smear-positive, and 115 from 217 smear-negative specimens (Table 4). These results indicated that triplex PCR had higher sensitivity in detecting *M. tuberculosis* in clinical samples than culture and/or AFB smear methods, even from those of either culture-negative or AFB smear-negative specimens; but it had less efficiency of *M. tuberculosis* detection compared with a single PCR of each target.

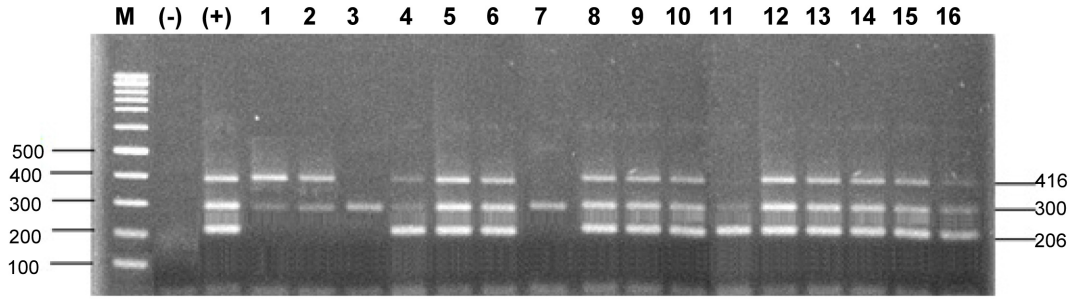

Fig. 3. Testing of applicability of triplex PCR using DNA materials from clinical specimens from patients suspected of having tuberculosis (part of the samples tested).

Lane M, 100 bp DNA ladder; lane 1, negative control (no DNA); lane 2, positive control (*M. tuberculosis* DNA); lanes 3 to 16, samples from each individual patient (16 samples).

Table 4. Detection by triplex PCR from the positive and negative results of clinical tuberculosis patient samples identified by a single PCR or culture or AFB smear method.

	No. of samples	By single PCR		By culture		By AFB smear	
		Positive	Negative	Positive	Negative	Positive	Negative
Total number	307	205	102	142	165	90	217
Triplex PCR (+)	195	195	0	140	55	80	115
Triplex PCR (-)	112	10	102	2	110	10	102

Discussion

The incidence of TB varies considerably around the world, and most mycobacterial infections in the developing countries are still caused by MTC members. Vietnam is one of the high-burden countries of *M. tuberculosis* infection globally, with a prevalence of smear-positive tuberculosis of 89 per 100,000 population. In addition, Vietnam is one of 22 countries in which 80% of the world's new TB cases occur. The rate of case detection in 2010 was estimated to be about 54% (range 43%–71%) [35]. Traditionally, the culture method followed by a panel of biochemical tests has been used for speciation of mycobacteria. This has inherent disadvantages, as most mycobacteria of clinical importance are slow growers and hence are difficult to isolate and to cultivate. The time required for primary isolation ranges from 4 to 6 weeks in the case of solid culture media and 10 to 15 days by radiometric and other automated systems. Moreover, the paucibacillary nature of clinical samples also accounts for the low efficiency of isolation of pathogenic mycobacteria [10, 24, 27]. In addition to difficulties in primary isolation of mycobacterial pathogens, particularly *M. tuberculosis*, there have been reports of difficulty in differentiating closely related mycobacterial species. Therefore, there is a need to develop molecular biological tools like PCR-based assays for reliable, early detection and speciation of mycobacteria in clinical samples and, as a consequence, for determination of the disease burden caused by diverse pathogenic mycobacteria.

Although PCR techniques have been widely evaluated in the diagnosis of tuberculosis, the reports have mostly focused on the detection of *M. tuberculosis* [21]. However, the isolation of NTM from a large proportion of immunocompromised subjects [8, 23] suggests that PCR methods that can differentiate between *M. tuberculosis* complex and NTM are needed. This requires the development of triplex PCR using oligonucleotide primers capable of simultaneously amplifying three specific target genes for *M. tuberculosis* complex. A number of previous studies have demonstrated that the use of *IS6110* as a target for PCR amplification gave the best sensitivity and specificity in the diagnosis of tuberculosis. However, a rapidly emerging issue was that *IS6110* was ineffective in a large part of the world, including South-East Asia (in our case, Vietnam) and India. Therefore, there is a great need to examine another insertion sequence, *IS1081*, that has five to seven repeats in all strains belonging to the *M. tuberculosis* complex and low discriminatory power, and it was used as a useful subsidiary marker in our detective study of *M. tuberculosis*. Moreover, *23S rDNA* also provided a target for the rapid detection and identification of mycobacteria both at the genus and species levels.

In this report, we describe the successful development of a triplex PCR using three target genes (*IS6100*, *IS1081*, and *23S rDNA*) for detection of *M. tuberculosis*. When tested with standard strains obtained from ATCC and *M. tuberculosis* strains and MOTT strains isolated from TB patients in different regions in Vietnam, the assay successfully amplified the positive controls and samples for which nonspecific bands were not observed. In other words, the triplex PCR was able to distinguish *M. tuberculosis* complex from other mycobacteria.

A total of 307 clinical specimens were tested with the newly developed triplex PCR assay. Each sample was also processed by a single PCR. However, the triplex PCR revealed a number of additional TB-positive cases compared with those by the single PCR of *IS6110* or *IS1081* or *23S rDNA*, respectively. This confirmed the improvement in sensitivity of the triplex PCR over that of the PCR with single amplification. Most reports of studies using *IS6110*-based detection have claimed sensitivities from 75% to 92% and specificities approaching 100%. However, the enhanced sensitivity of this triplex PCR detection was obtained using multiple copy genes *IS6110* and *IS1081* simultaneously. The sensitivity (95.12%) and specificity (100%) of the triplex PCR assay developed in the present study for detecting *M. tuberculosis* have proved that the assay is reliable enough for diagnostic application. The detection

limit by triplex PCR amplification was as little as 100 fg DNA. Kidane *et al*. [10] and Shah *et al*. [26] have previously reported a multiplex PCR for the detection and differentiation of *M. tuberculosis* complex and non-tuberculous mycobacteria from clinical and mycobacterial isolates, and the sensitivity assays were limited to detect 10 to 20 pg DNA of the tubercle bacilli [19]. The detection limit of the triplex PCR in our study was 10 times higher compared with previous studies, certainly due to the use of the combination of three targets such as *IS6110* or *IS1081* or *23S rDNA*. This has raised the fact that the *23S rDNA* contributed much to increasing the positivity since not all IS target sequences (such as *IS6110* targets) are present in all clinical isolates.

When compared with the smear or culture results, a number of additional TB-positive cases were detected by triplex PCR, from the negative culture and AFB smear cases, giving a question of reasonability about extremely sensitive multiplexing amplification. The sensitivity of the triplex PCR technique may lead to false positivity based on clinical criteria and in comparison with the smear or culture techniques. The reasons for this could be due to (i) the detection of *M. tuberculosis* in clinical samples by the triplex PCR prior to the onset of clinical signs and symptoms of tuberculosis and/or (ii) the presence of dead, non-cultivatable bacilli in samples. However, the probability of false-positive results being generated by the triplex PCR technique is low, because the likelihood of an incorrect region or nonspecific DNA being erroneously amplified twice is remote [34].

In conclusion, the results presented in this study indicate that the triplex PCR method has reliably improved tuberculosis diagnosis, with the advantage of that this method is more rapid, cost-effective, and sensitive than classical bacteriological methods as commonly applied. This method could also be used directly for differential diagnosis, using a large number of clinical samples in medical and veterinary laboratories.

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