



Tuberculosis Diagnosis by Multiplex PCR Techniques with Three Target Genes *IS6110*, *IS1081*, *23S rDNA*

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Abstract In this study, 100 specimens from tuberculosis were confirmed by the patients and suspected patients who were treated in Hai Duong Tuberculosis and Lung Disease Hospital to perform 3 simultaneous *M. tuberculosis* detection methods as multiplex PCR assay, direct sputum smear (AFB) and bacterial culture (BK). The results have shown that the multiplex PCR assay was the most effective with three target genes *IS6110*, *IS1081*, *23S rDNA*, identified 55/100 (55%) patients with the positive for TB. The diagnostic time was simultaneously shortened in just a few hours. Meanwhile, culture revealed 35/100 (35%) cases. 22/100 (22%) cases were positive only on direct sputum smear.

Keywords *Mycobacterium tuberculosis*, multiplex PCR assay, target genes, *IS6110*; *IS1081*; *23S rDNA*.

1. Introduction

Tuberculosis (TB) is a leading cause of death from infectious agents in the world. It is the second only to AIDS. Over 95% of cases and deaths are occurred in the developing countries. TB is caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) which divides every 15-20 hours, and is extremely slow and able to persist in dry conditions for weeks due to the lipid-rich cell wall. The *M. tuberculosis* genome consists of 4 million base pairs and contains approximately 4,000 genes. It is estimated by World Health Organization (WHO) that Vietnam ranks 12th among the 22 high-TB burden countries. In the Western Pacific region, Vietnam ranks third only after China and Philippine [6]. According to the summary report of National Tuberculosis Control Program (Ministry of Health), over 105.839 TB cases including new and recurrent infectious cases occurred in Vietnam in 2016 [7].

Currently, the traditional procedures of TB diagnosis are used by most TB hospitals, mainly based on: Ziehl-Neelsen method staining technique is used to stain *Mycobacterium*. However, this method can only detect TB cases with *tuberculosis* bacteria $\geq 10^4$ AFB/1ml per specimen. Hence, lots of TB patients are left due to false

Due to negative diagnosis result. On the other hand, AFB has to be stained much waste time by this method, at least 3 times for 3 mornings consecutively [2, 4]. The culture in Lowenstein - Jensen medium remains the gold standard for TB detection whereas it takes 4 weeks to get results with the traditional culture. The mean times to detection by MGIT Bactec were 1 - 2 weeks in normal cases with less bacillus, so TB supervision and control can hardly be met [1,10]. As the previous reports, the sensitivity of the traditional TB detection methods is not high (AFB test), it requires a long time to report (10 - 15 days for incubation) or easily to mistake with other specimens (lung X-rays, mantoux). Detection of *mycobacterium tuberculosis* complex using real-time polymerase chain reaction (Multiplex PCR) to amplify *IS6110*, *IS1081* and *23S rDNA* for TB rapid diagnosis and avoiding patient omission [8]. Therefore, the objective of the current research was to diagnosis by multiplex PCR techniques with three target genes *IS6110*, *IS1081*, *23S rDNA*.



2. Materials and Methods

2.1. Subjects and methods

* Subjects

M. tuberculosis complex was collected from TB patients at Hai Duong Tuberculosis and Lung Disease Hospital. These samples were tested for all three target genes including: IS6110, IS1081 and 23S rDNA and they were also used as positive controls. Also, we collected 100 specimens from over sixteen-year-old patients examined and treated in Hai Duong Tuberculosis and Lung Disease Hospital. Most of these specimens were mainly sputum treated and DNA extracted for diagnostic samples.

* Study methods

Sample processing and DNA extraction

We underwent pulmonary sputum ingestion for 100 patients, at least 5 ml in volume and in a container with screw caps. Samples (approximately 3 mL) and positive samples were extracted according to the procedures of Amita et al. [11] Nottle et al. [12] to improve their methods to fit our laboratory conditions.

Solution used for DNA extraction: Solution 1 (Trizol): Phenol 38%, guanidium thyocyanate 0.8M, glycerol 5%, pH 8.0. Solution 2: Chloroform. Solution 3: Isopropanol precipitation. Solution 4: Ethanol 70%. Solution 5: TE 1X (Tris 0,1M - EDTA 0,001M).

A volume of 100 μ sample was added into 900 μ L of 38 % Phenol, vortex for 30 seconds, in 10 min. Later, 200 μ L of Chloroform was well - mixed. Centrifugation for 10 min at 13,000 rpm, 600 μ L of floating solution was collected, 600 μ L of Isopropanol was added and mixed, centrifuged for 10 min at 13,000 rpm, for blue residue. Then, 900 μ L of 70 % Ethanol was added, centrifuged for 5 min at 13,000 rpm to collect residue and dry at 60 $^{\circ}$ C for 10 min. 50 μ of TE 1X (Tris 0,1M - EDTA 0,001M) was added and gently mixed.

The PCR product was extracted on 1.2% agarose gel electrophoresis, EtBr stain, observed and photographed under ultraviolet; wavelength of 260/280 nm was used to assess the purity of DNA. The DNA extraction of *M. tuberculosis* from specimens or positive samples was performed in only 1 to 1.5 h.

Primer design

We used primer design software such as Primer3, DNACLUB, Oligo to generate suitable primers for research and remove non-optimal primers based on the sequence genes (*IS6110*, *IS1081*, *23S rDNA*) on gene bank. We designed primer pairs IS6110, IS1081 and 23S to amplify target genes based on genome sequence of strains on gene bank as NC_000952; CP000611.1; AM408590.1; AB244270.1; AB244268.1; AB244265.1.

The IS6110 was designed from the very conservative sequence published on genbank. This primer copies the target segment of the IS6110 with 416 bp. The 23S rDNA primer pair was designed to replicate the 23S rDNA target genome with 206 bp. Mekonnen Kurabachew et al [5] showed that the target gene was in all pathogenic strains of TB [5]. In this study, we designed IS1081-F/IS1081-R primers based on nucleotide sequences of international strains to overcome the lack of target genes IS6110 in some strains in India, Southeast Asia and Vietnam. These primer pairs were used to amplify the target gene with 300 bp [9], as shown in Table 1.

Table 1: Primer Pairs Sequence used in this study

Target Genes	Primers	Sequence 5'-3'	Size (bp)
IS6110	IS6110-F	5' GGT CGC CCG TCT ACT TGG TG 3'	416
	IS6110-R	5' TGG ACG CGG CTG ATG TGC TC 3'	
IS1081	IS1081-F	5' TCG CGT GAT CCT TCG AAA CG 3'	300
	IS1081-R	5' CGC AGC TTG GGG ATC GCG AC 3'	
23S rDNA	23S-F	5' ACC TGA AAC CGT GTG CCT AC 3'	206
	23S-R	5' GGT CCA GAA CAC GCC ACT AT 3'	

Using Multiplex PCR technique with 3 target genes IS6110, IS1081, 23S rDNA for Mycobacterium Tuberculosis detection

Multiplex PCR was performed for all three target genes IS6110, IS1081 and 23S rDNA after optimizing the components and thermal cycles of the PCR reaction. Total of 25 μ L for multiplex PCR consists of 3.25 μ L Buffer1x; 2.5 μ L dNTP (2.5 μ M). Reaction mixtures contained 1.0 μ L of each forward prime and reverse primer (0.4 μ M) for 23S and IS6110; 1.5 μ L each of each forward primer and reverse primer, 0.6 μ M of IS1081



primer; 2.0 μL MgCl_2 25 μM (2.5 μM); 0.25 units of Taq - polymerase (1.25 units); 3.0 μL mold DNA (150 ng); 7.0 μL H_2O and additives. The thermal cycling conditions used were as follows: 95°C for 5 min; 35 cycles x (94°C for 1 min, 56°C for 45 s, 72°C for 1min 10 s); 72°C for 10 min.

As PCR terminated, 10 μL of PCR product was extracted on 1.5 % agarose gel electrophoresis with 100 bp molecular marker. By comparing the bands in a sample to DNA ladder, we were able to determine amplified DNA fragment (Figure 1).

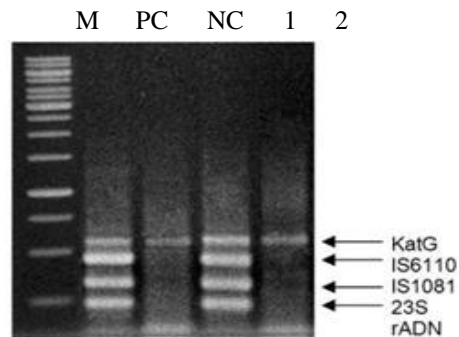


Figure 1: 1.5 % agarose gel electrophoresis of Multiplex PCR

M: DNA ladder 1kb; (*PC*): Positive Controls; (*NC*): Negative Controls; 1,2: samples

The samples (lane 1) appeared in 4 sizes corresponding to 3 target genes: 23S rDNA, IS6110, IS1081 and internal IC-KatG equivalent to 206 bp, 416 bp, 300 bp and 684 bp. The result showed one of three lanes corresponding to 3 lanes 23S rDNA, IS6110, IS1081 (*PC*) was positive TB. Without target genes in lanes of *NC*, the result showed negative tuberculosis (lane 2). We used direct sputum smear (Acid Fast Bacilli – AFB) and culture for the remaining specimens to compare the results of TB detection between 3 methods (AFB, BK culture and multiplex PCR)

2.2. Results and discussion

DNA extraction and purification

After DNA extraction, the samples were examined for purity with spectrophotometer. The spectral absorption of DNA was measured at 260 nm and the protein was at 280 nm. DNA concentration was calculated by spectral absorption at 260 nm, purity as measured by the $\text{OD}_{260\text{nm}}/\text{OD}_{280\text{nm}}$. The $\text{OD}_{260}/\text{OD}_{280}$ ratio of 1.8 - 2.0 was considered to be pure; free of protein and other impurities [4]. These DNA samples can be used for further experiments (Figure 2).

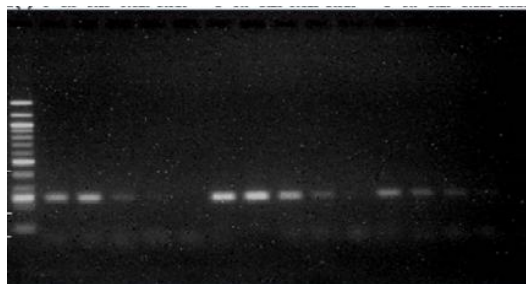


Figure 2: Amplified product of DNA extracted from specimens

M. tuberculosis detection with 3 target genes IS6110, IS1081, 23S rDNA

In this study, we simultaneously used 3 specific primer pairs including 3 target genes IS6110, IS1081, 23S rDNA in multiplex PCR to detect *M. tuberculosis*. The results showed in Figure 3. We used multi-primer PCR assay (using multiple pairs of specific primers simultaneously, multiplying different gene fragments on a DNA molecule) for *M. tuberculosis* detection.

The most important of this method is to design primer pairs with the same pairing temperature on the target DNA, also these primers are not paired together in the same PCR. The 23S rDNA sequenced 23S-F: 5' ACC TGA AAC CGT GTG CCT AC 3'; 23S-R: 5' GGT CCA GAA CAC GCC ACT AT 3' multiplied the fragment gene of 206bp. The IS1081-F: 5' TCG CGT GAT CCT TCG AAA CG 3'; IS1081-R: 5' CGC AGC TTG GGG



ATC GCG AC 3' multiplied the fragment gene of 300 bp. The IS6110-F: 5' GGT CGC CCG TCT ACT TGG TG 3'; IS6110-R: 5' TGG ACG CGG CTG ATG TGC TC 3' multiplied the fragment gene of 416 bp.

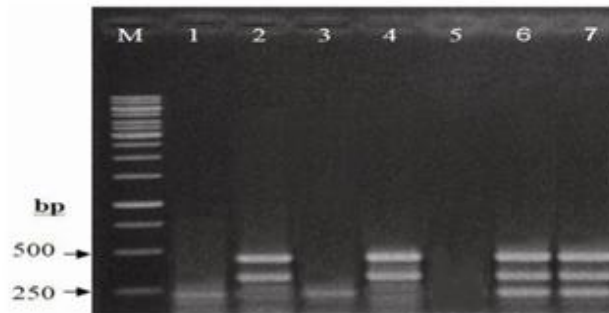


Figure 3: Amplified product of DNA extraction for *M. tuberculosis* detection
M: DNA ladder 1kb; 7: Positive Control; 1,2,3,4,5,6: specimens

As aforementioned, the samples appeared one of three lanes corresponding to one of three target genes 23S rDNA, IS6110, IS1081 (PC) with the size of 206 bp, 416 bp, 300 bp was positive TB. Without target genes in lanes of NC, the result showed negative tuberculosis. As shown on Figure 3, specimens in lanes 1,3 appeared 1 DNA band which coincides with the position of the 23S rDNA and the estimated size was 206 bp compared with molecular Marker.

The results on the electrophoresis (Figure 3) showed that samples from wells 1,3 showed only a DNA band, which coincides with the position of the 23S rDNA target gene and is estimated to be 206 bp compared to molecular marker. Two DNA bands were identified in lanes 2, 4 coinciding with the position of IS1081 and IS6110, which were estimated to be 416 bp and 300bp against the molecular marker. Three DNA bands in lane 6 coinciding with the position of 23S rDNA, IS108 and IS6110 which were estimated to be 206 bp, 300 bp and 416 bp. We did not find any DNA bands in lane 5, so it was negative tuberculosis.

The results showed that 55 samples (55%) were positive, 45 samples (45%) were negative among 100 specimens from tuberculosis confirmed patients and suspected patients. One hundred specimens were not only for multiplex PCR but also for BK culture and direct sputum smear AFB to detect *Mycobacterium tuberculosis* and compared the results of three methods. The results are showed in Table 2.

Table 2: Comparison of BK culture, multiplex PCR and AFB

Methods	BK culture	Multiplex PCR	AFB
Results			
Positive	35	55	22
Negative	65	45	78
Total	100	100	100

The results revealed that, multiplex PCR for *M. tuberculosis* was the highest (55%). BS culture was 35%. AFB only detect 22% as it only detects with the TB bacteria $\geq 10^4$ AFB/mL specimen. The results were similar to the report of Lazraq et al [3] when evaluating the efficiency of *M. tuberculosis* complex compared to direct methods of culture and culture on 102 samples and 41 extra-pulmonary specimens [3].

Currently, many scientists still consider culture method to be "the gold standard". In fact, many cases have clear TB symptoms but the culture was still negative due to few bacteria in the sample, some are suppressed during clinical specimen process, others due to infectious bacteria during culture and mycobacteria, which do not detect TB bacteria, so the sensitivity of culture is not absolute. Negative culture does not rule out the possibility of TB infection. So far, no method has actually been considered "the gold standard" for TB diagnosis. Moreover, PCR is capable of amplifying millions of times a target gene in the sample,

While PCR with the principle of gene amplification is capable of amplifying millions of times a target gene in the sample. It was not depended on whether the bacteria are alive or dead. During clinical treatment,



theoretically, PCR is more sensitive than culture. Hence, there should be negative culture cases except PCR results.

3. Conclusions

In summary, we applied Multiplex PCR technique for TB diagnosis successfully with 3 target genes *IS6110*, *IS1081*, *23S rDNA* in Hai Duong Tuberculosis and Lung Disease Hospital. The results disclosed that positive patients identified by multiplex PCR for *M. tuberculosis* percentage was the highest (55%) compared to AFB (22%) and BK culture (35%). The diagnostic time was simultaneously shortened in just a few hours.

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