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The Usefulness of PCR Amplification for Direct Detection of *Mycobacterium tuberculosis* DNA from Clinical Samples

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Abstract: The present study was designed to demonstrate the molecular diagnosis usefulness for direct detection of *M. tuberculosis* from clinical samples. A method of Duplex Polymerase Chain Reaction (DPCR) and Real-time PCR (RTPCR) with primer specific for detection of the IS6110 insertion element of *M. tuberculosis* complex and the *p53* gene of Human Beta Globin (Hbb) were developed. Fifty-four samples consisted of 25 from pulmonary and 29 from non-pulmonary specimens of patients suspected with *M. tuberculosis* infection. In a comparison experiment, 37 and 34 samples were detected as positive by ordinary and duplex PCR (DPCR), respectively, wherein data variation was detected in samples of pleural and other body fluids. For pulmonary specimens, 12/25 (46%) and 5/25 (19%) were positive as detected by DPCR and RTPCR, respectively. On the other hand, the number of positive results, 22/29 (71%) and 8/29 (21%) was higher among non-pulmonary specimens as detected by DPCR and RTPCR, respectively. Of these number, DPCR and RTPCR identified 15/34 (44%) and 8/34 (24%) of specimens, respectively in which Acid Fast Bacilli (AFB) and culture were negative. In addition, 18/19 (95%) and 4/19 (21%) of the AFB positive specimens can be detected by DPCR and RTPCR, respectively. Nevertheless, both PCR amplification methods were able to amplify the IS6110 genes for culture positive specimen. Therefore, duplex PCR method can be a useful additional technique for the diagnosis of *M. tuberculosis* suspected infection. However, RTPCR assay still need to be established.

Key words: Duplex PCR, real-time PCR, *M. tuberculosis*, IS6110, tuberculosis

INTRODUCTION

The occurrences of tuberculosis cause by *Tubercle Bacillus* (TB) is paralleled by high morbidity, with approximately 8 million new cases every year becoming a major concern of public health (Espinal, 2003; Dye, 2006). In Malaysia, there is one death from TB per day (Anonymous, 2006). Without proper treatment and management, most patients would die and any delay in treatment results in significant morbidity (Bhigjee *et al*., 2007). Therefore, the need of rapid and reliable methods, in addition to the standard diagnostic techniques for diagnosis of its causative agent, *Mycobacterium tuberculosis* is vital to save millions of life. In early 1990s, the insertion element IS6110 was used for the first time to obtain the molecular fingerprint of *M. tuberculosis* isolates (Hermans *et al*., 1990; Cave *et al*., 1991). This mobile insertion sequence is found in the members of *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti*). The specificity and repetitive nature of IS6110 make it an ideal target for amplification by PCR. Sense and anti-sense primers for IS6110 yielded a 240 bp-sized product (Rahizan *et al*., 1998a, b). Internal control DNA was constructed based on *p53* region of Human beta globin (Hbb) which yielded a 120 bp product. It will allow the assessment of efficacy of each individual reaction and to ensure that interfering substances does not inhibit the reaction. Therefore, the method for ordinary (without the presence of internal control) and duplex PCR (DPCR) was compared. The conventional methods such as culture and Acid Fast Bacilli (AFB) staining were also been carried out. The real-time PCR (RTPCR) method has been proposed for the rapid detection of many microorganisms including

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Mycobacteria (Cleary *et al*., 2003; Savelkoul *et al*., 2006). The present study was developed the RTPCR method to be used for amount quantification of desired genomic sequence and also eliminates time-consuming post-detection methods. In addition, PCR product was sequence to determine the specificity of the primers used and to validate the PCR method.

MATERIALS AND METHODS

Bacterial isolates and clinical samples: Culture of *M. tuberculosis* strain ATCC 27294 was grown in Ogawa medium and used as positive control. Whereas, *B. subtilis* was grown on blood agar and used as negative control. Samples were sent from various hospitals in Malaysia in 2006. They consisted of pulmonary (n = 25) and non-pulmonary (n = 29) specimens. The samples such as sputum, gastric lavage and urine were decontaminated with 4% NaOH for 15 min before being used. All samples were investigated for the presence of Acid-Fast Bacilli (AFB) by Ziehl-Neelsen and cultured on Loewenstein-Jensen medium.

DNA extraction: Extraction of DNA from bacterial culture and clinical samples were done by using High Pure Viral Nucleic Acid Extraction Kit (Roche Inc.) according to manufacturer's recommendation.

PCR amplifications: The duplex PCR was done using 2 different pairs of primers. The first pair, TB1 (5'-CGTGAGGGCATCGAGGTGGC-3', sense) and TB2 (5'-GCGTAGGCGTCGGTGAC-3', anti-sense) designated to amplify the IS6110 gene in *M. tuberculosis* CDC1551. The second pair, HB1 (5'-CAGAAGAGCCAAGGACAGGTAC-3', sense) and HB2 (5'-AGCAATAGATGGCTCTGCCCTGA-3', anti-sense) were designated to amplify the region of *p53* gene in *Homo sapiens* beta globin. Extracted DNA from all bacterial isolates and clinical samples were subjected to Duplex-PCR using mixture of primers, TB and HB (1:4). The amplification mixture consisted of 5 μL of template DNA, 1X final concentration of MasterMix (Eppendorf) and primers (0.4 pmole μL^{-1}). The amplification was carried out using Master Cycler Gradient Thermocycler from Eppendorf. The cycling parameters were 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 64°C for 45 sec and extension at 72°C for 1 min. Final extension were then carried out at 72°C for 5 min. The PCR product was analysed by a 1.6% agarose gel electrophoresis.

Purification of PCR products: Purification of PCR products was carried out using the Wizard® Genomic DNA purification Kit (Promega) according to the manufacturer's recommendation.

DNA sequencing and analysis: Sequencing was done using the AB3130XL sequencer. Sequence ambiguity was corrected by comparison of both sense and anti-sense products. The sequence data was compared with other *Mycobacterium* gene sequences in GenBank by using the BLAST software (blastn) at the National Center of Biotechnology Information (NCBI) web site (http://www.ncbi.nih.gov/).

Real-time PCR amplification and detection: Probe sequence, TB-P of IS6110 was FAM-GAACGGCTGATGACC AAACT-BHQ-1. 5' end of the probe was labeled with 6-carboxy-fluorescence-dye (FAM) and 3' end was labeled with non-fluorescence dye (BHQ-1). The DNA from bacterial isolates and clinical samples were subjected to Real-time PCR using mixture of TB primers and TB-P probe (1:1). The reaction mixture consisted of 5 μL of template DNA, 10 μL of TaqMan MasterMix (Applied Biosystem), 0.4 pmole μL^{-1} final concentrations of primers and probe. The amplifications were carried out using the ABI7500 Real-Time PCR System from Applied Biosystem. Plus/minus analysis was carried out at 60°C for 1 min. The cycling parameters for Absolute Quantification were done at 50°C for 2 min, 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 sec and annealing at 60°C for 1 min. Data was collected during the annealing steps.

RESULTS AND DISCUSSION

All samples (n = 54) were sent by physicians showing tuberculosis-like symptoms. The pulmonary specimens (n = 25) consisted of sputum (n = 10), pleural fluid (n = 14) and tracheal aspirate (n = 1). The non-pulmonary (n = 29) specimens included pus (n = 3), tissue biopsy (n = 4), bone biopsy (n = 1) and aspirate (n = 1), cerebrospinal fluids (n = 9), urine (n = 2) and others (n = 9).

In a comparison experiment, 37 and 34 samples were detected as positive by ordinary and duplex PCR (DPCR), respectively (Table 1). Data variation was detected in the samples of pleural and other body fluids. This revealed that a false positive arise from the ordinary PCR without the presence of the internal control and was the advantage from the previous study by Rahizan *et al*. (1998a). The PCR products showing both genes will only be considered as positive. The presence of IS6110 gene

without the *p53* gene in the PCR products is considered as inconclusive. Therefore, *p53* genes plays important role as an internal control. Based on the observations from gel, the 240 bp of *IS6110* gene can be easily discriminated from the 120 bp of p53 gene. Figure 1 showed the distance of migration of both genes after amplification of the various sputum samples.

DPCR and conventional method (AFB staining and cultures) were also evaluated. DPCR detected 12/25 (46%) of pulmonary and 22/29 (71%) of non-pulmonary specimens as positive (Table 2). Of these number, DPCR identified 15/34 (44%) of specimens in which Acid Fast Bacilli AFB and culture were negative. It was interesting to note that the PCR methods were able to detect the AFB negative of the non-pulmonary specimens such as CSF, bone biopsy, lumbar puncture, rectal biopsy and lymph nodes. As reported by Davis *et al.* (1993), The CSF of most patients with tuberculosis meningitis contains only 10-10^2 organisms mL^{-1} yet approximately 10^4 organisms mL^{-1} are required for reliable detection with Ziehl-Neelsen

Table 1: PCR amplifications of 54 clinical samples

		Ordinary PCR		Duplex PCR	
Type specimens		+	-	+	-
Pulmonary (n = 25)	Sputum (n = 10)	4	6	4	6
	Pleural fluids and Others (n = 15)	9	6	8	7
Non-pulmonary (n = 29)	Cerebrospinal fluids (n = 9)	8	1	8	1
	Tissue biopsy (n = 4)	3	1	3	1
	Pus (n = 3)	2	1	2	1
	Urine (n = 2)	1	1	1	1
	Other body fluids (n = 11)	10	1	8	3
	Total	37	17	34	20

Table 2: Comparison of DPCR and RTPCR for detection of MTB in clinical samples. The positive and negative samples were indicate as for AFB and culture

	DPCR		RTPCR	
Direct microscopy and culture finding methods	+	-	+	-
Pulmonary Specimen (n = 25)				
AFB+/C- (n = 5)	4	1	1	4
AFB-/C- (n = 20)	8	12	4	16
Total	12	13	5	20
Non-Pulmonary Specimen (n = 29)				
AFB+/C+ (n = 1)	1	0	1	0
AFB+/C- (n = 14)	14	0	3	11
AFB-/C- (n = 14)	7	7	4	10
Total	22	7	8	21

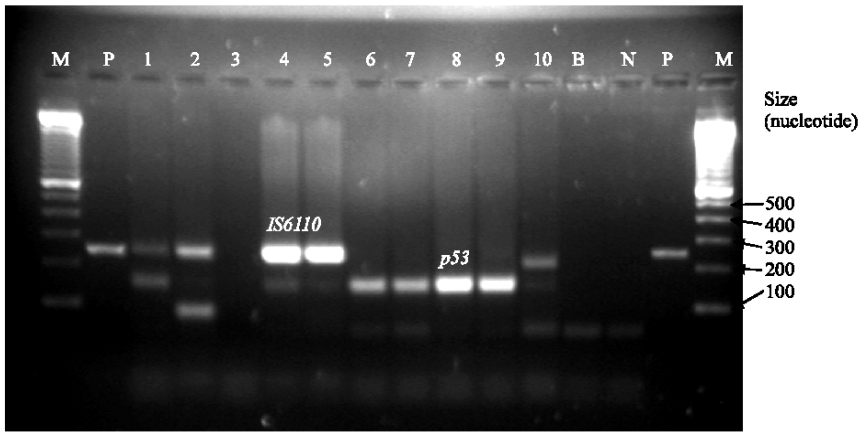

Fig. 1: Agarose gel showing amplification products generated by Duplex PCR. Amplifications were performed with DNA from *M. tuberculosis* ATCC 27297 (P), various sputum samples (1-10), Blank (B), *B. subtilis* (N) and marker 100 bp (M). The *p53* genes have been amplified in all samples except No. 3 and *IS6110* genes have been amplified in samples No. 1, 2, 4, 5 and 10

stains. The CSF PCR assay represents a significant advance in the diagnosis of microbial diseases. The result of PCR studies in CSF has shown 94-100% specificity but sensitivities were from 75% (Caws *et al*., 2000; Nguyen *et al*., 1996). In this study, the sensitivity for non-pulmonary specimen (including CFS) were 89% (Table 1). Nevertheless, culture methods failed to detect the presence of *M. tuberculosis*, except from one urine sample in which it was detected positive by both PCR methods. This was probably due to the fact that the presences of mycobacteria in specimens were below the detection limit of the conventional culture method. It is a known factor that low numbers of bacilli are sufficient to cause tuberculoses damage in peritoneum as well as in non-pulmonary tuberculosis (Hopewell, 1994). Therefore, the DPCR for detection of small amount of bacilli directly from clinical samples is very useful. It can be performed in a small amount of sputum, thus demonstrating the validity of this alternative method for the detection of bacilli in specimens having bacteria undetectable by culture method.

To improve the detection time, we developed RTPCR using the sequence of IS6110 as a primer and probe. The real-time uses fluorescent probes (TaqMan®) which is complimentary of the target sequence have fluorescence promoter and non-fluorescence quencher on 5' and 3' ends, respectively. The result shows that this method was able to detect the culture positive specimens (Table 2), confirming the reliability of the assay but low number of recovery (23%) is statistically insignificant value for this study. It might be due to the effect of decontamination agent to the samples. Long exposure of decontamination agent on the sample has been reported to cause large reduction (80%) of CFU recovered in cultures (Yajko *et al*., 1995). In contrast, low number of inhibitor was detected in both DPCR and RTPCR method because of small amount of DNA used in reaction volume (5 µL of DNA). Nevertheless, the presence of fluorescence probe in RTPCR may increase the specificity of reaction. As a comparison, DPCR analysis was based on the detection of a particular size of amplified fragment. The existence of primer dimer or contaminant DNA that yielding same size of fragment cannot be differentiated with this method. In RTPCR, a probe is used to detect specific sequence in amplified product, confirming the amplification of target sequence (Fig. 2).

In addition, only 8/34 (24%) and 4/19 (21%) were detected positive for AFB negative and AFB positive. As

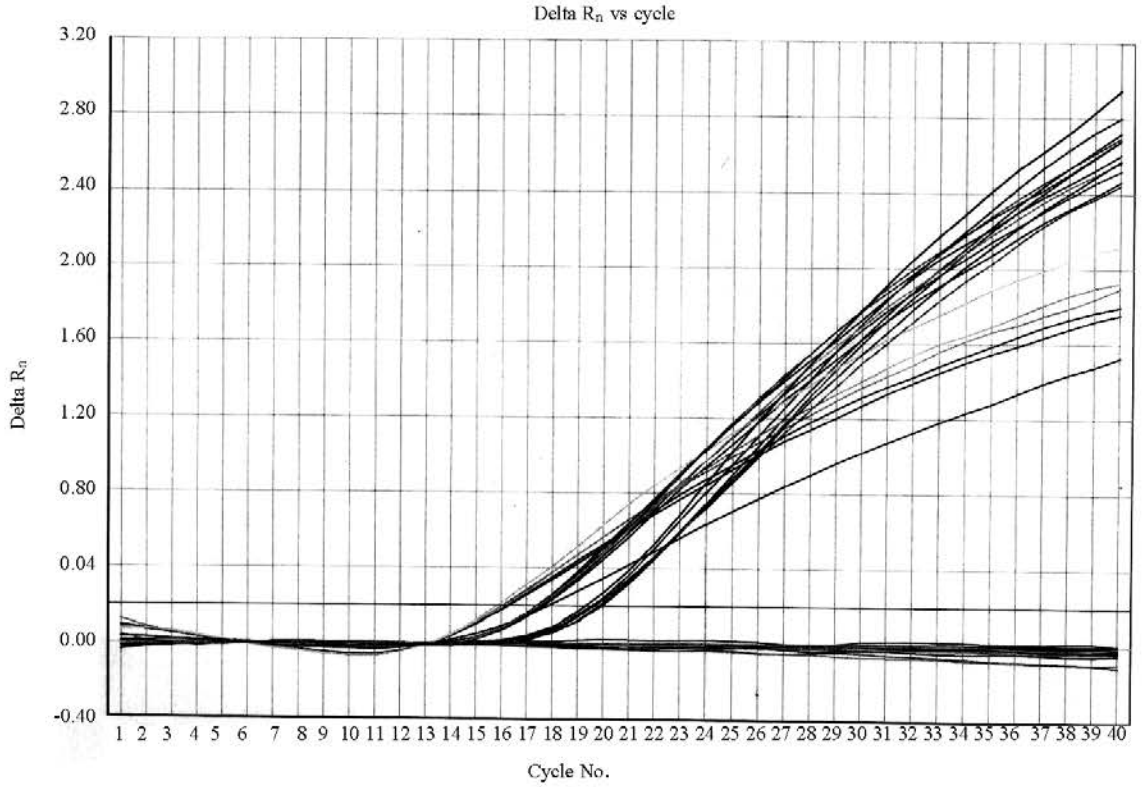

Fig. 2: Real-time PCR response curve. Four dilution series of positive control DNA (ATCC27294), blank, negative control and DNA from six different clinical samples were run together in triplicates

Table 3: Blast results showed high similarities to the target sequence. The specificity of amplified product for both sequence exceed 95% values

Amplicon	Identities (%)	Gaps (%)	Score (bits)	Results
[a]*IS6110*	98	1	353	*M. tuberculosis* CDC1551
	98	1	353	*M. tuberculosis* H37Rv
[b]*p53*	98	1	176	*H. sapiens* isolate HbC-Dgn99 beta globin (Hbb) gene
	98	1	176	*H. sapiens* isolate HbC-Dgn66 beta globin (Hbb) gene
	98	1	176	*H. sapiens* isolate HbC-Ghn154b beta globin (Hbb) gene

a comparison in the case of pulmonary and non-pulmonary specimens, the percentage of positive results were low as shown by 5/25 (19%) and 8/29 (21%), respectively. The low percentage might be due to the low concentration of the template DNA, hence quantification of the DNA was not possible. The quantification for control DNA did work with the FAM standard showing values ranging from 127 to 1145 (data not shown) but if the value was less than 127 or higher than 1145, it will not be detected. It was possible that the same target primer of IS6110 used for DPCR might not suitable for RTPCR. Savelkoul *et al.* (2006) showed that small changes in primer and probe sequences result in differences in the performance of the assay, regardless analytical sensitivity and specificity. Nevertheless, further work need to be carried to find a specific primer for RTPCR.

Blast results from the sequence analysis of both genes showed 98% similarities to *M. tuberculosis* and *H. sapiens* isolates, for *IS6110* and *p53*, respectively (Table 3). The high percentage of similarities confirmed that this assay achieved high specificity in analysis of *M. tuberculosis* in clinical samples (Altschul *et al*., 1997).

CONCLUSION

In conclusion, the duplex PCR method can be useful additional technique for the diagnosis of *M. tuberculosis* suspected infection, in which conventional culture methods failed to detect the presence of *M. tuberculosis*, except from one urine sample. The presence of internal control was important to avoid false positive results. On the other hand, RTPCR with TaqMan® probe can still be improved. Chemical modifications and alterations of oligonucleotide backbone can be done to improve the binding properties to the target template with the specific primer, for the duplex RT PCR.

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