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Detection of Pulmonary Tuberculosis by PCR Assay

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ABSTRACT

Background: *Tuberculosis is a major world health problem mainly in the developing countries. Early isolation of infected patients and application of chemotherapy are the main prevention strategies for tuberculosis control. Although acid-fast stained smears and culture of M.tuberculosis are the standard procedures of diagnosis, they are low in sensitivity and time consuming, respectively. Polymerase chain reaction (PCR) technique has simplified and boosted the direct detection of M.tuberculosis in a significantly shorter time than two conventional methods mentioned.*

Materials and Methods: *In this study, which was conducted in 9 months, 211 clinical samples were collected from patients with suspected M.tuberculosis complex who had referred to NRITLD. They were tested by PCR assay, culture technique and Ziehl-Neelsen staining. Two pair oligonucleotides were used in PCR assay, detecting 245bp of IS6110 sequence. Results of PCR assay were compared with those of culture technique.*

Results: *One-hundred and forty five samples (68.7%) were sputum and the other 66 samples (31.3%) were bronchoscopy lavage. Twenty-five samples (11.8%) were positive by PCR assay out of which 21 (84%) were culture positive too.176 from 186 samples with PCR negative results were culture negative too. These 10 samples were examined for PCR inhibitor and identification of M.tuberculosis. Twenty percent (2 samples) of these had PCR inhibitor and 20% (2 samples) were not M.tuberculosis complex. Based on this study, after excluding the samples which had PCR inhibitor and non-tuberculosis complex, sensitivity and specificity of PCR assay in comparison with results of culture and Ziehl-Neelsen staining (as a gold standard) were 93.3% and 98.3%, respectively. Positive and negative predictive values were 84% and 94.6%, respectively.*

Conclusion: *This study showed that there are no significant differences between cultures and PCR methods. For this reason, we can use PCR assay for direct detection of DNA from M.tuberculosis in clinical samples.****(Tanaffos 2005; 4(13): 63-70)***

Key words: *Tuberculosis, Pulmonary TB, PCR, Diagnosis*

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INTRODUCTION

Tuberculosis is a major world health problem mainly in the developing countries. Early isolation of infected patients and application of chemotherapy are the main prevention strategies for tuberculosis control (1). Acid-fast stained smears and cultures of mycobacterium tuberculosis are the standard procedures of diagnosis. Although acid-fast staining is rapid, it is low in sensitivity due to bacterial number limitation, and inefficient species discrimination. Culture protocol is time consuming and usually needs 6-8 weeks before definitive identification (2,3). These technical shortcomings have been known for a long time and now, they need to be replaced with more rapid and reliable laboratory diagnostic methods. This urgent demand has accelerated the efforts on the development of alternative powerful, rapid, sensitive and specific molecular diagnostic procedures namely; nucleic acid amplification and DNA probe hybridization. Polymerase chain reaction (PCR) technique has simplified and boosted the direct detection of Mycobacterium tuberculosis in a significantly shorter time than the two conventional methods mentioned (4). In both in-house and commercial PCR systems, different genetic elements such as IS6110, the 38 (Pab antigen) and 65 KD proteins, and 16S rRNA within the mycobacterial DNA have been used as target sequences in many diagnostic laboratory protocols (2,5). However, these recently developed molecular methods have been criticized because of their variable levels of sensitivity and specificity, or complications arising from the clinical specimens. Also, the need for special laboratory facilities, high skills and prior training, complexity, and high cost of the PCR method, has rendered these assays impractical and only to be performed by few laboratories (and not as a routine diagnostic use). Several trials have been conducted to evaluate the validity and reproducibility of PCR assay in identifying *M.tuberculosis* following participation of different laboratories around the world. In this collaborated and joint study, restricted and unrestricted protocol conditions were considered. The outcoming results of these efforts confirmed that wide deviations in the worked out sensitivity and specificity were due to good laboratory performance, worker experience and the different techniques used for DNA isolation and PCR amplification. It is clear that such conclusion is encouraging if optimal conditions for DNA isolation and PCR method are sought and established (6, 7). Therefore, careful monitoring of clinical samples by superior DNA isolation methods suitable for specimens from different sites of patients may be the best choice for increasing the sensitivity and specificity of PCR technique (7). These and other considerations have prompted us to investigate the validity and clinical usefulness of the PCR assay for mycobacterium tuberculosis detection. This study was performed in two levels. Initially, we set up the PCR assay for detecting *M.tuberculosis* complex by taking advantage of several adapted and modified methods of DNA preparation (showed at a separate report). Secondly, we examined sensitivity and specificity of PCR assay for the detection of *M.tuberculosis* in patients with pulmonary tuberculosis.

MATERIALS AND METHODS

Clinical specimens of sputum and bronchoalveolar lavage were obtained from patients admitted "National Research Institute of Tuberculosis and Lung Diseases" in Tehran suspected of having tuberculosis. For the initial evaluation of the methods of mycobacterium tuberculosis DNA isolation and amplification of specific target sequences, only a subset of weekly received suspected specimens was considered (a total of 211 samples). For some patients, more than one sample was tested during the period of the study. Smear and culture results were not apparent to the person performing PCR technique. For culture, acid-fast smear staining and PCR assay samples were first liquefied and decontaminated following treatment

with 4% NaOH for 15 minutes at room temperature. Aliquots were prepared to be examined with Ziehl-Neelsen staining, inoculating into Lowenstein-Jensen medium culture techniques, and DNA purification.

For DNA isolation from clinical specimens, the decontaminated samples were either directly processed fresh or kept frozen at –20º C for 1 to 5 days before being processed. In the beginning of our study, for mycobacterial genomic DNA extraction, the decontaminated samples were subjected to different DNA isolation methods ranging from short to multi step protocols. Both methods have been reported to have their own advantages and disadvantages. Due to these positive and negative criteria, faster methods were applied which ended up in the multi step procedure as below. Eppendorf cap opener was designed and used to open Eppendorf tubes in order to prevent cross-contamination between specimens by glove fingers.

DNA Extraction: The decontaminated samples were centrifuged at 13500 xg for 10 minutes, and then the pellet was processed with slight modification and adaptation of the previously reported protocols (8, 9). Briefly, the pellet was mixed in 250 ul of lysis buffer containing 100 mM NaOH, 1MNaCl, and 0.5% SDS and incubated at 95º C for 20 minutes. The lysate was neutralized with 1 M Tris-HCl, pH: 7.0 (1:4 of Alkaline lysis buffer). The mixture was first extracted with phenol (1:1 v/v) at 13500 xg for 10 minutes, then a second extraction with one volume of chloroform/isoamyl alcohol (24:1). Ethanol precipitation was continued and the sediment was dissolved in 50 ul or in 100-150 ddH2o when the precipitate was relatively thick. Other further steps were taken in connection with this method. When the DNA precipitate became deeply white, it was diluted 5 to 10 times before using for PCR. Such dilution reduces the DNA amount nearly to the optimum level for the PCR reaction condition. It is clear that the relative large content of DNA most likely belongs to foreign DNA (Human and other saprophytes). Although dilution of this DNA solution lowers the amount of the target mycobacterial genomic DNA, it is suitable for setting up a PCR working reaction. However, when the ethanol sediment was transparent and relatively thick, the precipitate was redissolved in 100 ul of TE (1:10) buffer and proteinase-K was constructed at 100 ul/ml as well as the tube was incubated at 56º C for 30 to 60 minutes, followed by extraction with equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v) and ethanol precipitation. The precipitate was dissolved in 50 to 100 ul of sterile distilled water and used for PCR assay. When the precipitate became yellowish-brown, proteinase-K and 0.5% SDS were included and the same aforementioned extraction and precipitation steps were applied and precipitate dissolved in sterile distilled water, which used or stored at –20º C. (10,11,12,13)

PCR: The PCR was performed with the application of INS1 and INS2 primer pair as follows:
INS1: 5’-TGA CGT TGG CGG AGA CCG-3’
INS2: 5’-ATG GTG CCC TGG TAC ATG-3’

This method was performed according to a study by Sjobring et al. (14). Twenty five ul of DNA isolates were diluted with PCR mixture containing 0.5 nM of each INS1 and INS2 primers, 1.5 mM MgCl2, 2.5 uM of each of the 4 dNTPs (dATP, dGTP, dCTP and dTTP), 5ul of 10X PCR buffer recommended by the manufacturer (GIBCO-BRL, USA) and 2 units of Taq polymerase. The PCR reactions were subjected to 35 amplification cycles in an Eppendorf mastercycler 5330 as follows; 93° C, 2 minutes for DNA denaturation, 65° C, 2 minutes for primer annealing and 72° C, 3 minutes for primer extension.

In each run of PCR, DNA extracted from H37Rv strain and sterile distilled water was used as positive and negative controls, respectively.

PCR product analysis was performed by electrophoresis of 30 ul of amplified product on a 0.5 ug/ml etidium bromide stained 2% ultrapure agarose gel (GIBCO-BRL, Life Technology, USA) at 50 V

for 2 hours, and visualized by UV transilluminator. The band size of the amplified target sequences was determined by comparison with a 100 bp DNA ladder maker (GIBCO-BRL, Life Technology, USA). The presence of a clear band with an accurate size in PCR reaction belongs to the positive mycobacterial DNA in comparison with the DNA marker which is considered as a successful amplification product. The appearance of similar band in PCR reaction of tubes belongs to the tested clinical specimens and was judged as positive tuberculosis.

RESULTS

All samples were tested by PCR assay, Lowenstein-Jensen culture technique and Z.N staining. For some patients, more than one sample was tested during the study period. Two pair oligonucleotides were used in PCR assay, which can detect 245bp of IS6110 sequence. In order to obtain the sensitivity and specificity, results of PCR assay were compared with those of culture technique (the accepted gold standard method). One-hundred and forty five samples (68.7%) were sputum and the other 66 samples (31.3%) were bronchoscopy lavage. Twenty five samples (11.8%) were positive by PCR assay out of which 21 (84%) were culture positive as well. From these 25 samples, 21 (84%) samples were sputum and 4 (16%) were BAL. (Figure 1)

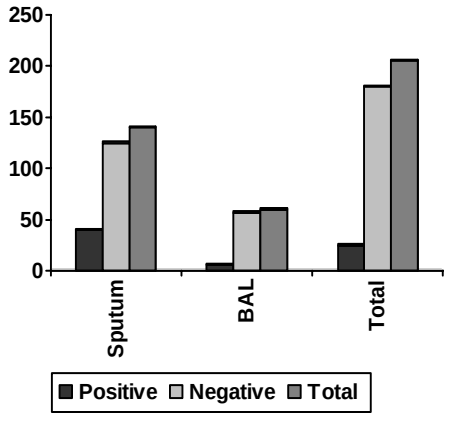

Figure 1. Results of PCR in 211different types of clinical samples

176 from 186 samples with PCR negative result were culture negative too. These 10 samples were examined for PCR inhibitor and identification of *M.tuberculosis*. Twenty percent (2 samples) of these had PCR inhibitor and 20% (2 samples) were not *M.tuberculosis* complex.

This study showed that in order to detect pulmonary tuberculosis, the sensitivity and specificity of PCR were 82.4% and 98.3%, respectively. The positive and negative predictive values were 84% and 94.6%, respectively. (Table 1)

Table 1. Results of PCR in comparison with culture and smear

RESULTS	Culture Positive		Culture Negative		Total
	Smear +	Smear -	Smear +	Smear -	
PCR Positive	14	7	1	3	25
PCR Negative	3	7	7	169	186
Total	17	14	8	172	211

Sensitivity=82.4%

Specifity=98.3%

Positive predictive value=84%

Negative predictive value =94.6%

General efficacy=86.7%

Since 20% of samples had PCR inhibitor and 20% of them were non-tuberculosis complex, we excluded them from the study. After excluding these samples, sensitivity and specificity of PCR assays were 93.3% and 98.3%, respectively. Positive and negative predictive values were 84% and 94.6%, respectively (Table 2).

Table 2. Results of PCR in comparison with culture and smear after deleting PCR inhibitor and non-tuberculosis complex

RESULTS	Culture Positive		Culture Negative		Total
	Smear +	Smear -	Smear +	Smear -	
PCR Positive	14	7	1	3	25
PCR Negative	1	5	7	169	182
Total	15	12	8	172	207

Sensitivity =93.3%
Specifity =98.3%
Positive predictive value =84%
Negative predictive value =96.7%
General efficacy =88.4%

DISCUSSION

Today, polymerase chain reaction (PCR) technique is used to detect *M.tuberculosis* in a significantly shorter time than the two conventional methods, culture and smear (4). However, these recently developed molecular methods have been criticized because of their variable levels of sensitivity and specificity, or complications arising from the clinical specimens. Although several diagnostic health centers have reported successful diagnosis of clinical specimens with the application of PCR amplification kits, several other studies have demonstrated low sensitivity of such commercial PCR systems in comparison with the in-house PCR. The outcoming results of these efforts confirmed that wide deviation in the worked out sensitivity and specifity were due to good laboratory performance, worker experience and the different techniques used for DNA isolation and PCR amplification.

Thus, this study was performed to determine the specificity and sensitivity of in-house PCR assay to detect *M.tuberculosis* after setting up in the laboratory of "National Research Institute of Tuberculosis and Lung Disease". Nevertheless, results of PCR assay were compared with those of culture technique (The accepted gold standard method).

It is clear that if there is an optimal condition for DNA isolation and PCR, the sensitivity and specificity will go up. Although specifity of this test is reported to be about 97%, its sensitivity is variable and reported as 50-75%. This study also showed that the sensitivity of PCR assay to detect pulmonary tuberculosis was 82.4% and its specificity was 98.3%.

Although culture on Lowenstein-Jensen is used as a gold standard, there are some instances that *M.tuberculosis* is unable to grow on them, such as bacilli which were killed during decontamination procedure, paucibacilly cases due to drug ingestion, or introduction of inhibitory substances for proliferation because of sample contamination. According to our results, 2% of samples with positive PCR results were culture negative which is quite reasonable (Table 1).

As you know, IS6110 is an insertion sequence that is repeated many times in the genome of *M.tuberculosis*. It is not found in non-tuberculosis complex such as *M.avium* complex, *M.gastric* etc. Also, numbers of IS6110 copies affect the detection rate of PCR. It means that the lower the number of copies, the lower the sensitivity of PCR for detecting *M.tuberculosis*. Identifying the number of IS6110 was not our objective. Since it needs to be determined by more precise methods such as fingerprinting, we will not elaborate on this issue.

Our study showed that, as a result of biochemical tests, 2 (20%) of these samples with PCR negative and culture positive results, were non-tuberculosis complex.

The variable and unpredictable number of mycobacterium tuberculosis in the clinical sample, makes DNA extraction undependable, since it may end up in a very little or even undetectable amount of

DNA. On the other hand, different types of intrinsically and extrinsically inherited impurities, which may already be present or could be potentially resulted during DNA lysate preparation, impose their inhibitory effects on PCR reaction (15,16,17,18). Also the complexity of the mycobacterial cell wall makes difficult the treatment of clinical specimens for proper DNA release (3). Besides, handling of clinical specimens for DNA preparation is specially laborious and time consuming, which is often the origin of PCR interfering substances or a source of cross-contamination between clinical specimens (19). To overcome these obstacles, short and multistep protocols for mycobacterial genomic DNA have been applied to minimize the loss of DNA, which avoid cross-contamination and eliminate PCR inhibitors. Several trials have been conducted to evaluate the validity and reproducibility of PCR assay in identifying *M.tuberculosis* following participation of different laboratories around the world. In this collaborated joint study, restricted and unrestricted protocol conditions were considered. However, 2 (20%) samples had PCR inhibitor.

By excluding these samples (samples with PCR inhibitor and non-tuberculosis complex), sensitivity and specificity of PCR assay in comparison with results of culture and Ziehl-Neelsen staining were 93.3% and 98.3%, respectively. Positive and negative predictive values were 84% and 94.6%, respectively (Table 2).

It seems that another important factor causing PCR to be negative in spite of positive culture is cross-contamination, a fact that needs more investigation in this regard.

In conclusion, our study shows that using PCR for rapid detection of *M.tuberculosis* is a useful method and can be helpful as a complement to routine methods i.e. smear and culture especially in referral centers.

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