

Efficiency of PCR Method for Screening Pulmonary Tuberculosis Patients under DOTs Protocol Therapy

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Abstract

Tuberculosis has increased in the recent years. Use of a sensitive screening method, can be one of the influential parameters in reduction of pulmonary tuberculosis. Current screening method in the laboratories is based on observation of bacilli in stained smear by Ziel-Neelsen procedure. The aim of this research was to study the PCR efficiency in all confirmed patients of Hamadan province. Twenty eight patients were registered as having pulmonary tuberculosis by the tuberculosis comity of the Hamadan province. All these patients had positive results for staining and culture. Amontst these registered patients, we could access only to the 12 patients during the study. Sputums were collected from early stage of diagnosis and continued during treatment at the end of each month. All these patients were under the treatment by the DOTs protocols. All samples were tested by PCR and the results were compared with Ziehl-Neelsen staining method that is recommended procedure by WHO. Compared results showed none of the samples were positive by staining method at the end of second month up to the last month of treatment, while PCR changed to negative gradually. It was negative in 5 and 4 patients at the end of the third and fourth month, respectively. Specimens of the three remained patients were continuously positive up to the end of treatment period. Gradually changing PCR results to negative in three fourth of studied patients means it can be applicable screening tools, but one fourth remained positive cases needs more study for the evaluation of target gene role and efficiency of the test.

Keywords: *Tuberculosis disease, Screening method, PCR*

Introduction

Tuberculosis kills about 2 million people each year (1). The number of new smear positive tuberculosis cases is reported 23 per 100,000 populations in Iran, while it is an important public health problem in the Eastern Mediterranean region with increasingly incidence. Low incidence rate in Iran may be due to increasing DOTs (Direct Observation Therapy) population coverage and case detection rate from 28% and 7% in 1997 to 100% and 28% in 2001, respectively (2, 3). One of the important affecting parameters on rising the infection is HIV infection in some countries. It is reported that 95% of

HIV infected tuberculosis cases are attributable to HIV infection (4).

At present, DOTs treatment procedure is based on the result of microscopic examination by Ziehl-Neelsen staining method (5). According to the reports of Iran Health Centers, most sputum smears are negative at the end of the second month of treatment. On the other hand, relapse has been observed in these patients with microscopic and culture negative results (6), because of low efficiency of staining method.

Use of PCR test was investigated in treatment evaluation by different researchers. Some reports underline mycobacterium DNA clears af-

ter commencing treatment whereas others insist on the persisting of DNA for a while. Yaun and his colleague report Mycobacterial DNA remained only four weeks after commencing treatment in 29 patients out of 41 using PCR targeting 38- kDa gene (7). In another study, Kennedy and his colleague reported DNA could remain 1 to 2 months after commencing treatment, using PCR targeting IS6110 gene (8). Levee also reports PCR test, targeting IS6110 would stay positive 2-3 month after treatment in 13% of patients and in one out of 14 patients after six months (9).

The aim of this project was to know what the result of PCR was and how its negativation speed in patients was under treatment in comparison with the current screening Ziehl-Neelsen staining method.

Materials and Methods

Twenty-eight patients were registered with pulmonary tuberculosis based on smear and culture positive results by the tuberculosis Committee of Hamden province. They were under treatment on the basis of DOTs protocol for six months. These patients were entered to the study during one-year, each followed up during treatment course. We could follow up only 12 patients. The rest were either moved out from Hamedan province or insufficiency of specimens (Table 1).

Patient selecting criteria

- 1) Two smear-positive sputum;
- 2) One smear positive sputum with X-ray confirmation;
- 3) One smear positive sputum with isolation of organism from culture;

Specimens Three sputum specimens were collected at diagnosis stage before receiving antibiotics and five specimens from the end of second month to six month. Samples were collected by the health service centers in the Hamadan province. These specimens were examined for isolation and microscopic observa-

tion after staining by Ziehl-Neelsen staining method. All isolated organisms were tested for a range of differential tests including niacin and nitrate reduction (10). Finally they were tested by PCR method.

DNA extraction method of the samples DNA was extracted by extraction kit (provided from Sinnagen Co.). Five hundreds μ l of lysis solution (provided in extraction kit) were added to 500 μ l of sputum sample and heated for 10 min in 80° C. Mixture was centrifuged at 10000 rpm for 15 min. In the next step, 100 μ l of lysis solution was added to the pellet and incubated in 95° C for 30 min. Finally the mixture was centrifuged at 10000 rpm for another 15 min and supernatant was stored at- 20° C after suspending in TE buffer.

PCR Method *Mycobacterium* PCR diagnosis kit (Sinnagen Co.) was used to test the samples. Primers were designed to amplify 163 bp region of transposable element (IS6110). Provided kit contained taq polymerase, PCR mixture buffer, dNTP, and primers. PCR mixture reaction volume was 25 μ l for each test and following amplification program was used: 95° C for 40 s (120 s for the first cycle), 72° C for 40 s and 60° C for 40 s. Ten μ l of the products was mixed by 2 μ l of loading buffer, then was analyzed by agarose gel electrophoresis in TBE buffer (89 mM Boric acid, 89 mM tris pH8.0, and 10mM EDTA) (11), DNA was finally stained with ethidium bromide and observed on transilluminator.

Results

All 12 patients had three positive smears at the diagnosis stage. Culture method revealed that isolated organisms were *Mycobacterium tuberculosis* in all 12 patients.

Staining results during treatment Five collected specimens from second to the end of treatment were stained in all 12 patients. Smear examination results proved they were changed to negative at the end of the second month (Table 2).

PCR results PCR was positive in all patients at the diagnosis stage (Fig. 1, Table 2). The test was positive up to the end of the second month,

but it was changed to negative gradually from the third month (Table 2).

Table 1: Number of studied and final remaining patients in this study from different parts of Hamadan province

	Hamadan	Razan	Malayer	Bahar	Toossercan	Kaboodarahang	Total
Entered to study	13	3	3	2	1	6	28
Remained in study	8	1	-	-	-	3	12

Table 2: Results of samples tested by the PCR and staining

Test	No of Patients with positive results in the first three received specimens			No. of patients with positive results at the end of each month					
	One sample positive	Two samples positive	Three samples positive	2	3	4	5	6	
Staining	-	-	12	-	-	-	-	-	
PCR	-	2	10	12	7	3	3	3	

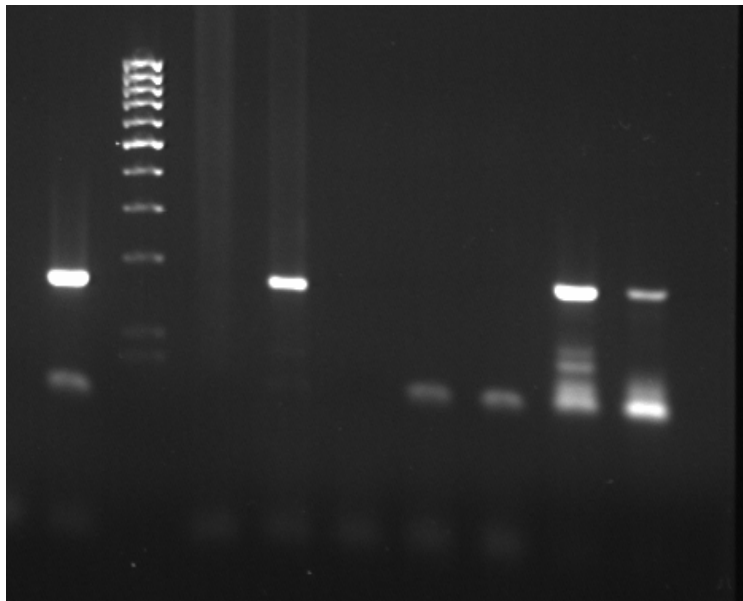


Fig. 1: PCR results of clinical samples. Lanes 1, 4, 8 and 9 clinical samples with positive results, Lanes 3, 5, 6 and 7 those with negative results. Lane number 2 100 kilo base pair ladder (Sinna Gen)

Discussion

Transposable element IS6110 of *M. tuberculosis* has been used as molecular marker in epidemiological studies. Up to 25 virtually identical copies are found in various genomic locations (12, 13). Different PCR protocols have been reported to diagnose *Mycobacterium* using this fragment gene.

Reported sensitivity and specificity are ranging from 30% to about 95% (14, 15). Results of this study shows nagtivation speed of staining method are more rapid than PCR.

At present, WHO recommend Ziehl-Neelsen staining method as a standard screening method during treatment course. Treatment monitoring by smear examination is unspecific and also is a crude method since dead and live *M. tuberculosis* complexes are not distinguishable. This method also is not sensitive enough and estimated sensitivity is 5,000 to 10,000 acid-fast bacilli/ ml. That is why some smear negative samples are grown in culture.

The question is the possibility of replacement PCR by staining method. But result of PCR must be first proved as true positive. On the other hand it has been shown that PCR remains positive in some cases up to the end of treatment in different studies.

Another question is about the relapse rate. Does delayed negativation of PCR results mean facing with live *Mycobacterium*?

It is reported that relapse may be observed in those patients with smear and even culture negative results with 6% rate within 6 to 30 months after finishing anti-tuberculosis therapy (16). It means viable *M. tuberculosis* complex may remain after treatment. Also it is reported that some patients with culture negative results may have PCR positive results (5, 6). In another study, Chierakul et al. compared conversion rate for culture and smear with PCR (17). Their results also reported 4 PCR positive patients at the end of treatment period while only 1 patient had relapse. Hence, those remained PCR posi-

tive cases could be considered as unsuccessfully treatment.

Since the numbers of studied cases were only 12, further investigation is recommended for correlation relapse with positivity of the PCR results in these patients.

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