Polymerase Chain Reaction in Pulmonary Tuberculosis

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Objective: **Early diagnosis is one of the most important steps in the management of tuberculosis, so the conventional diagnostic techniques need to be improved. In our study, the diagnostic value of polymerase chain reaction (PCR) in active pulmonary tuberculosis was investigated.**

***Methods*: We have examined 79 cases (61 patients and 18 healthy subjects) between 20-22 ages. In these cases *Mycobacterium tuberculosis complex* DNA specific IS6110 field has been investigated by PCR and the results were compared with the microbiological culture.**

Results: **The sensitivity of PCR results were found to be 96.72% and the specificity was 100%. In 59 out of 61 cases with pulmonary tuberculosis, culture and PCR positivity were shown, PCR was negative for 2 of the culture (+) cases. All control cases were negative in terms of bacteriological culture, PCR, and sputum smear for acid fast bacillus (AFB).**

Conclusion: **PCR is a rapid technique with high sensitivity and specificity. However, there is always a risk of contamination with other specimens and the amplification products. PCR is believed to be useful and time saving if we consider the time needed to wait for the results of microbiological culture, which in turn might cause delay in starting the treatment in time, especially in sputum smear negative cases.**

Key words: *Pulmonary tuberculosis, polymerase chain reaction.*

Effective treatment of contagious diseases is one of the most crucial factors influencing the socio-economic and socio-cultural structure of societies. Tuberculosis has long been considered, almost, out of health problem in developed countries. However, recently it regained its importance at least partly due to mass population movements and AIDS. According to the WHO news in 1990, about 3 million people die of tuberculosis each year. Tuberculosis is 5th among the causes of death in the world (1). In Turkey, it is estimated that around 12 to 15 million people are infected. That means 30-40 thousand new patients each year (2).

It is difficult and time consuming to identify the bacillus microscopically in the sputum, owing to the cellular and structural characteristics of the bacillus. The expected delay, that might be caused by the patient and/or the physician, in determining the sputum Acid Fast Bacillus (AFB) positivity, is approximately 75 days. Patients with sputum AFB positivity infect 2-3 healthy people in developed countries and 3-5 healthy people in the developing countries (3).

Almost everyday new techniques come out in the diagnosis of the diseases. Some of the new techniques related to tuberculosis are serological determination of mycobacterium antigens, gas chromatography - mass spectrometry, and polymerase chain reaction (PCR). It is possible with PCR to detect DNA from different sources and *M. tuberculosis complex (M .tuberculosis, M. bovis, M. bovis BCG, M. microti, and M. africanum*) as well. The specific IS6110 field is one of the most frequently detected common DNA fragments of *M. tuberculosis complex*. That is why we have searched for the fragmentation of IS6110 field of *M. tuberculosis complex* with the PCR technique in the sputum, and compared the results with the bacteriological cultures, and tried to define the sensitivity and the specificity of PCR in active pulmonary tuberculosis.

Material and Method

We enrolled 79 cases (61 with active pulmonary tuberculosis, and 18 healthy controls) to the study. All of the tuberculous cases were male, aged between 20-22, and admitted to hospital with the diagnosis of pulmonary tuberculosis. They had never used anti-tuberculous treatment and they did not have any other coexisting disease other than tuberculosis, such as pneumonia, congestive heart failure, sarcoidosis, immunodeficiency, obstructive lung disease, pneumoconiosis, etc. (Table I).

Control group consisted of cases matching the patient group in terms of sex and age. Control group included those who never had tuberculosis treatment and having no findings of tuberculous and/or any other disease with physical, radiological and biochemical examination.

All of the subjects were taken medical history, and physical examination, PA and lateral chest X-ray, hemogram, erythrocyte sedimentation rate (ESR), routine biochemical tests, and urine analysis were also performed. *M. tuberculosis* was searched with direct microscopy of the morning sputum, culture at Löwenstein-Jensen medium and PCR.

The medical staff dealing with the any step of the PCR process was not informed about the results of AFB smear and sputum culture. We used PCR to amplify the field of IS6110 with the hybridisation technique (4). PCR study was done in three steps:

Table I.General features of cases.

Pulmonary Tuberculosis	61
Control Cases	18
Sex	Male
Age	20-22

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1-DNA isolation

-NaOH as well as the material within the Eppendorf tubes at the rate of 2% was mixed in mixer adding N-Acetyl-L Cystein.

-After 15 minutes, 80 mcl 2 M pH 7.4 tries add per 2% NaOH 100 mcl and mixed.

-The mixture was centrifuged at 12000 G for 15 minutes.

-The top part of it was thrown away and the sediment at the bottom was added 200 mcl distilled apyrogenic water and stirred.

-200 mcl chloroform was added and stirred.

-Kept in the sterilizator at 80°C for 15 minutes.

-This material was centrifuged at 12000 G for 1.5 minute and upper layer was saved for PCR.

2-Reproduction of DNA

Taq DNA polymerase, dATP, dTTP, dGTP, dCTP, reaction buffer, the primers that codify the field of IS6110 of *M. tuberculosis complex* DNA and water were loaded in a tube and 5 mlt of phase which was saved in DNA isolation was added to this. The material prepared for PCR was put into the thermocycler (Minicycler MJ. Research Inc. USA) programmed for 40 cycles. DNA was reproduced through the steps of denaturation in thermocycler, connection and longevity.

3-To show the reproduction

-Sterilizator was set to 68°C, mixture of prehybridization (N-loril-sarkosil, 10% sodium dedoksil sulphate, blocking reagent, 20 X SSC, distilled water) and probe (marked as digoxigein) was taken from deep-freezer.

-Membrane (Boehringer Mannheim Germany) was prepared, loaded (2 mlt) and kept, under UV for 3 minutes.

-Prehibridization mixture was kept at 68°C for 30 minutes. Meanwhile probe was kept at 68°C for 15 minutes and quickly frozen in deep-freezer.

-Probe was kept at 37°C for 2.5 hours.

-Buffer II (5 ml blocking reagent, 45 ml buffer I) was taken out of the freezer and let to melt. Sterilizator was set to 68°C.

-At the room temperature it was shaken twice for twenty minutes in the solution of 2 X SSC (sodium chloride + sodium citrate) + 0.1% SDS.

-It was kept at room temperature in the solution of 0.2 X SSC+0.1 SDS twice for 30 minutes.

-Buffer I (maleic acid, sodium chloride, distilled water, sodium hydroxide, pH 7.4) was shaken at the room temperature for a minute.

-Buffer II was also shaken at the room temperature for 30 minutes.

-Buffer II (15 ml) + Dapcon (3 ml) was also shaken at the room heat for 30 minutes.

-Buffer I was also shaken at the room temperature twice for 15 minutes.

-Buffer III (1M pH 9.5 tries, sodium chloride, 1M magnesium chloride, and distilled water) was also shaken at the room temperature for 2 minutes.

-We waited until seeing the color changed dark in the mixture of buffer III (10 ml) + NBT (45 mcl) + X-phosphate (35 mcl) at 37°C.

-Buffer I was also shaken for 5 minutes.

-Te buffer (2M pH 7.4 tries, 0.5M pH 8.0 EDTA, distilled water) was also kept in the refrigerator.

The results were analysed comparing the positive and the negative controls on the membrane (Figure 1).

While calculating the sensitivity and specificity of PCR we took the culture positivity as the gold standard (5).

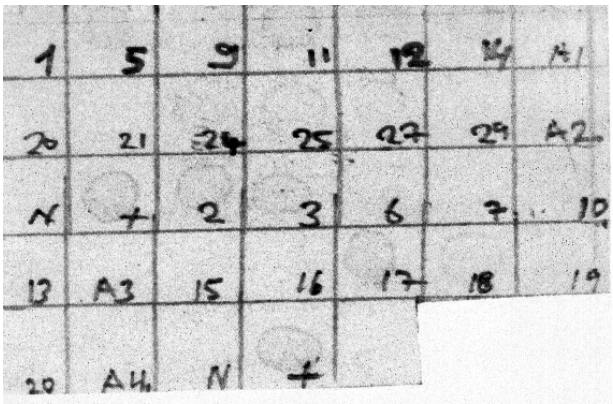

Figure 1. The PCR results on the membrane.

Results

Of 61 cases with active pulmonary tuberculosis 56 were AFB (+) and 5 AFB (-) in sputum smear. In 59 out of 61 cases with pulmonary tuberculosis both culture and PCR examinations were positive but 2 culture positive cases were PCR (-). All control cases were negative in terms of AFB smear, culture, and PCR (Table II).

Considering the culture positivity as gold standard, the sensitivity and specificity of PCR were 96.72% and 100% respectively, for active pulmonary tuberculosis .

Discussion

Although sputum examination is a fast, easy and cheap technique, it has low sensitivity, and there is a need of 5000-10000 bacillus per ml. On the other hand, less than 10 bacillus per ml may not suffice to obtain a culture positivity (6). In the sputum examination, a classical method, it is

Table II. AFB culture and PCR results.

	Patients	Control
AFB Culture (+)	61	0
AFB PCR (+)	59	0
AFB PCR (-)	2	18

possible to show AFB by direct microscopy by the chance of 50-80%, in culture this possibility goes up to 95-98%. Kartaloğlu et al (7) have found the sputum AFB to be positive in 70.8 % of 500 cases with pulmonary tuberculosis.

PCR can be performed with clinical materials such as sputum, pleural fluid, serum, cerebrospinal fluid, urine and biopsy specimens. Savic et al (8) studied sputum by direct microscopy, tuberculostearic acid and PCR. They found the sensitivity and specificity values with direct microscopy 66% and 100% respectively, with PCR 95% and 93% respectively and in tuberculostaric acid 55% and 87% respectively. In a study conducted in Australia with 176 cases, in comparison to the bacteriological culture results, sensitivity of PCR was found 75.5% (9). They claimed that the false positive results might be related to contamination, the cycle number in the process of DNA reproduction and the heat during the connection. Fauville-Dufaux et al (10) found a 94.6% sensitivity and 93.6% specificity for PCR in their study with 200 clinical materials of which 100 were sputum and 11 were pleural fluids. They had given anti-tuberculous treatment to 7 patients with culture (-) but PCR (+) whose clinical findings were in harmony with tuberculosis and clinical and laboratory improvement was observed which implied the diagnosis. In the study of Kocagöz et al (11) conducted in Turkey, 78 sputum PCR was appraised and its sensitivity was found 87% and specificity 96%. In our study, we searched for the existence of IS6110 fragment *in M. tuberculosis complex*. When the bacterial culture was considered as gold standard, sensitivity was found as 96.72% and specificity as 100%.

In the PCR technique there is a need of various procedures for DNA extraction in different materials such as gastric fluid, pleural fluid and cerebrospinal fluid. Some authors who found lower sensitivity and specificity rates in their studies, suggested that this could be related to different materials used in clinical studies (8). Meanwhile, when the culture results were considered to be gold standard, false positivity of PCR increased. When clinical correlation is taken into account in culture (-) but PCR (+) cases, false positivity rate decreases greatly (12).

Even if the basil number is very low (theoretically only one) it can be traced quickly by the PCR technique. The sensitivity of PCR may also increase the risk of contamination. In developing countries the contamination risk is very high due to the fact that in the laboratories they test much more materials, and workload is higher. Therefore it is difficult for PCR to become routine. We think it is useful to use PCR to avoid time waste while waiting for the bacteriological confirmation in cases with suspected tuberculosis but AFB (-), sputum smear and come to a diagnosis so as to start treatment as soon as possible. There are different views and some objections on the cost-effectiveness of PCR. In our opinion this cost can be justified since it reduces the delay by early diagnosis of the disease at the first step.

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