Detection of *Mycobacterium Tuberculosis* DNA from peripheral blood in patients with HIV-seronegative and new cases of smear-positive pulmonary tuberculosis by polymerase chain reaction

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Abstract Tuberculosis is still one of the most important cause of mortality and morbidity in many countries and there is a need for new methods for accurate and rapid diagnosis of tuberculosis. To determine the sensitivity and specificity of polymerase chain reaction (PCR) method, we have evaluated *Mycobacterium tuberculosis* DNA in peripheral blood samples with PCR technique in adult patients with human immunodeficiency virus (HIV)-negative and new cases of smear-positive pulmonary tuberculosis. We investigated the relationship between characteristic of the patients, radiological extension of the disease, sputum smear grade, presence of cavity, body-mass index (BMI), serum albumin level, total delay time and PCR positivity. Forty patients (33 male and 7 female; mean age 37.8 ± 14.1) and 20 healthy control subjects (13 male and 7 female; mean age 35.6 ± 7.3) were enrolled in this study. PCR was positive in 16 of 40 (40%) patients with pulmonary tuberculosis and negative in 24 of 40 (60%). None of the healthy controls had positive PCR results. The overall sensitivity, specificity and accuracy of the PCR assay was 40, 100 and 60 %, respectively. We found the positive correlation between PCR positivity and sputum smear grade (r = 0.46, P=0.003), radiological extension of the disease (r = 0.69, P=0.001), presence of cavity (r = 0.90, P=0.001). We conclude that the detection of *M. tuberculosis* DNA from peripheral blood by PCR technique is useful for the rapid diagnosis of tuberculosis patients with HIV-negative. Hematogenous dissemination was important in tuberculosis patients and peripheral blood samples were suitable and easy materials. However, standardization of the PCR method must be ensured for the diagnosis of tuberculosis.

doi:10.1053/rmed.2003.1500, available online at http://www.sciencedirect.com

Keywords tuberculosis; blood; polymerase chain reaction.

INTRODUCTION

Tuberculosis continues to be an important cause of mortality and morbidity in developing countries. In the developed countries, especially with the spread of HIV in the world, tuberculosis problem has reached a dramatic dimension (1).

The most effective means of preventing tuberculosis are early diagnosis and giving appropriate treatment. For this reason, a rapid and reliable diagnosis of the disease is required (2). For the detection of tuberculosis bacilli, rapid and highly sensitive and specific methods were investigated and PCR method was developed (3,4). Since the bacteriologic examination of sputum, which is the most frequently used material in the diagnosis of pulmonary tuberculosis, carries a transmission risk for both the collecting and examining staff and as it cannot be obtained from some patients, the need for investigation of different materials arose. It has been shown that, especially in HIV-positive pulmonary tuberculosis patients, the detection of *M. tuberculosis* DNA in peripheral blood mononuclear cells may be helpful in the diagnosis of tuberculosis (5). However, studies concerning the diagnostic effectiveness of this method in immunocompetent patients are limited.

The purpose of this study was to search *M. tuberculosis* bacilli in the peripheral blood with PCR technique, to determine sensitivity and specificity of PCR and to evaluate the clinical importance of PCR positivity by examining the association with radiologic extension of the disease, the presence of cavity, body-mass index, total delay time and albumin level in patients with

Received 12 June 2002, accepted in revised form 4 November 2002
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HIV-negative and new cases of smear-positive pulmonary tuberculosis.

MATERIALS AND METHODS

Patients

Forty new cases of smear-positive patients not receiving prior antituberculosis therapy before applying to Atatürk Chest Diseases and Chest Surgery Education and Research Hospital between February and September 2001, and 20 healthy subjects as control group were enrolled in our study.

Patients who had at least two sputum smear acid-fast bacilli (AFB) positive and never had prior treatment with antituberculosis drugs were accepted as new case of smear-positive pulmonary tuberculosis. Patients and healthy subjects are HIV-negative and patients have no extra-pulmonary involvement and disseminated disease. Patients younger than 18 years of age, or patients with pregnancy, extra-pulmonary or disseminated tuberculosis, any systemic disease or drug use leading to immunosupression were not included in the study. All of the patients and healthy control subjects gave their informed consent, having been informed about the details of the study.

Age, sex, total delay time, albumin level, radiologic extension of the disease and sputum AFB smear results were recorded. Weight and height of patients were measured and their body-mass index were calculated by using the equation; weight (kg)/ heigth $(m)^2$ (6). Total delay time is accepted as the time (day) from the beginning of symptoms of tuberculosis to the initiation of treatment. The disease was considered extensive if cavities totalled $\geq$ 15 cm in diameter and/or moderately dense infiltrates involved more than 75% of lung fields on chest X-ray; without these two criteria it was considered as limited disease (7). Sputum smear grade was evaluated: 1–9 bacilli in 100 oil immersion fields=+1, 1–9 bacilli in 10 oil immersion fields=+2, 1–9 bacilli in all oil immersion fields=+3, more than 9 bacilli in all oil immersion fields=+4 (8).

PREPARATION OF SAMPLES FOR PCR

Five milliliters of peripheral blood were taken from all cases into EDTA-containing tubes and *M. tuberculosis* DNA was searched by PCR method in Refik Saydam Hifzisihha Center Tuberculosis Research and Reference Laboratory.

DNA isolation method

Thirty five milliliters of distilled water was added into 5 ml EDTA-containing blood. They were mixed for 10 s by handling up and down. Then 2 ml of 3.08 M NaCl (it is prepared by dissolving 17.99 g of NaCl in 82.01 ml of distilled water and by autoclaving it 15 min at 121°C) was added and mixed. It was centrifuged at 1200 rpm for 10 min and the supernatant was discarded. Fifty milliliters of phosphate buffered saline (PBS) was added to the pellet and it was centrifuged at 2000 rpm for 12 min. After the supernatant was discarded, again 50 ml of PBS was added and centrifuged at 2000 rpm for 12 min and the supernatant was discarded again. 0.5 ml of 1xTris EDTA was mixed with sediment. Then Triton tris buffer was added until the total volume reached 1.5 ml and transferred to eppendorf tube. It was centrifuged at 13 000 rpm for 5 min. The supernatant was discarded and the pellet was again washed with triton tris buffer. Then the pellet was dissolved in distilled water so that it became 20–50 μl and boiled at 100°C for 15 min (9).

PCR procedure

Amplification was performed as previously described (10). The assay detects a 245-bp region from the *M. tuberculosis* specific insertion sequence IS986. The reaction components; 10μl of template DNA, 2 μl of 5 mM INS1 primer (5' CGTGAGGGCATCGAGGTGGC 3') (11), 2 μl of 5 mM INS2 primer (5' GCGTAGGCGTCGGTGACAAA 3')(11), 4 μl of 25 mM $MgCl_2$, 15 μl of 10 x Master Amp. Enhancer® (Epicentre Tecnologies Corp., Wisconsin, U.S.A.), 4 μl of dNTP mixture (dATP, dCTP, dGTP, dUTP each 20 mM), 0.5 μl of Tfl DNA polymerase®[final concentration 0.5 U] (Epicentre Tecnologies Corp., Wisconsin, U.S.A.) and 12.5 μl of sterile water were put into PCR tube and placed in the termocycler apparatus (Mastercycler gradient®, eppendorf AG 22331, Hamburg) (10). Since the apparatus is heated from above and it has a cover, vaporization is prevented. For this reason, mineral oil preventing vaporization is not added to tubes. So contamination risk with mineral oil has been decreased. After the tubes are placed in the termocycler apparatus, DNA amplification procedure was started. During DNA amplification was carried out in 30 cycles, each consisting of at 94°C for 1.5 min for denaturation, at 65°C for 2 min for hybridization and at 72°C for 3 min for synthesis (10). After the tubes are brought to room temperature, amplification products are subjected to agarose gel electrophoresis.

Detection of PCR products in agarose gel electrophoresis

Four grams of agarose was mixed with 200 ml of 1xTBE in 500 ml flask. It was placed in a water bath and boiled at 100°C for 20–30 min; then it was placed in water bath at 60°C and cooled. Ethidium bromide was added so that the final concentration would become 0.5 μg/ml and it was poured into casettes and coombs are placed. Thirty

to sixty minutes later coombs are taken out. It was placed with 0.5xTBE in electrophoresis apparatus. Thirty microliters of PCR end product was mixed with 6 μl of blue/orange 6xloading dye® (Promega, U.S.A.) and placed in holes over agarose gel. This was subjected to electrophoresis for 45–60 min under 110 V constant current. Then it was demonstrated by photographing with special polaroid camera under UV transluminator (10).

In PCR study, sterile distilled water was used as negative control, and *M. tuberculosis* H37 Ra strain was used as positive control.

Used buffers

Tris borate buffer (5x TBE): 54 g of Tris base, 27.5 g of boric acid, 20 ml of 0.5 M EDTA (pH:8.0),

tris EDTA: 10 mM tris hydrochloride (pH:8.0), 1 mM EDTA (pH:8.0),

triton tris buffer: 99 ml of 20 mM tris–HCL (pH:8.3), 1 ml of triton x 100,

phosphate buffer saline (PBS): 8 g of NaCl + 0.2 g of KCl + 1.44 g of Na_2HPO_4 + 0.24 g

of KH_2PO_4 mixture is solved in 800 ml of distilled water and is treated with HCl until the pH becomes 7.4. H_2O is added until 1 lt. It is autoclaved at 121 °C for 20 min.

The sensitivity, specificity and accuracy of PCR method was calculated. The association between PCR results and patients' age and sex, radiological extension of the disease, the presence of cavity, sputum smear grade, BMI, total delay time and albumin level was evaluated. This study was conducted in accordance with the Declaration of Helsinki amended the 52nd WMA General Assembly (Edinburgh, 2000) and approved by local ethics committees.

Statistical method

Statistical evaluations are made using Statistical Package for Social Sciences (SPSS Inc.) software. Statistical significance was taken as $P < 0.05$ (two sided). The comparison between patient and control groups was made using chi-square and Mann–Whitney *U*-tests, and the correlation between PCR results and various factors was investigated by Spearman correlation test.

RESULTS

A total of 40 patients and 20 healthy subjects as control group were enrolled in this study. The demographic features are similar in the two groups (Table 1). The PCR results studied to identify mycobacterial DNA in blood of patients and control group are given in Table 2.

Based on the above results, in blood samples, the sensitivity of PCR assay in patients with smear-positive pulmonary tuberculosis is 16/40=40%, specificity is 20/20=100% and accuracy is 16+20/60= 60%. The appearance of PCR-amplified *M. tuberculosis* DNA in agarose gel electrophoresis is shown in Fig. 1.

Table 1. Characteristics of patients and control group

	n	Age (yr) (range)	Sex (M/F) *n*
Patients group	40	37.8 ± 14.1 (18–64)	33/7
Control group	20	35.6 ± 7.3 (24–55)	13/7

Data are presented as mean ± SD, M: male; F: female.

Table 2. The PCR results of patients and control group

	n	PCR positive *n* (%)	PCR negative *n* (%)
Patients group	40	16/40 (%40)	24/40 (%60)
Control group	20	0/20 (%0)	20/20 (%100)
Total	60	16/60 (%26.7)	44/60 (%73.3)

In patients with pulmonary tuberculosis, the correlation between sputum smear grade and PCR positivity was found to be statistically significant (r=0.46, P=0.003) (Table 3). In patients with extensive radiologic lesions, PCR positivity was found to be significantly higher than the limited disease (r=0.69, P=0.001) (Table 4).

The correlation between PCR positivity and the presence of cavity in chest X-ray is statistically significant (r=0.90, P=0.001) (Table 5). In patients with pulmonary tuberculosis; total delay time was 91.8 ± 75.5 days, BMI was 20.1 ± 2.7 kg/m^2, serum albumin level was 3.6 ± 0.5 g/dl. In patients with smear-positive pulmonary tuberculosis, no significant correlation was found between PCR results and variables such as sex, age, BMI, serum albumin level and total delay time.

DISCUSSION

The definitive diagnosis of pulmonary tuberculosis is the bacteriologic identification of the bacilli (2). For this purpose, the most frequently used material is sputum. However, it is not possible to obtain sputum from all patients suspected pulmonary tuberculosis or extra-pulmonary tuberculosis patients. Also because of the low sensitivity and specificity of microscopic examination of sputum and since the isolation of the bacilli by the Löwenstein–Jensen culture takes a long time up to 8 weeks, the investigation of different materials and techniques are required in the diagnosis of pulmonary tuberculosis. Until the HIV epidemic, blood cultures were not considered to be important in the diagnosis of mycobacterial infections. The studies showed that the blood culture results were in correlation with the degree of immunosupression and the dissemination of tuberculosis (11,12).

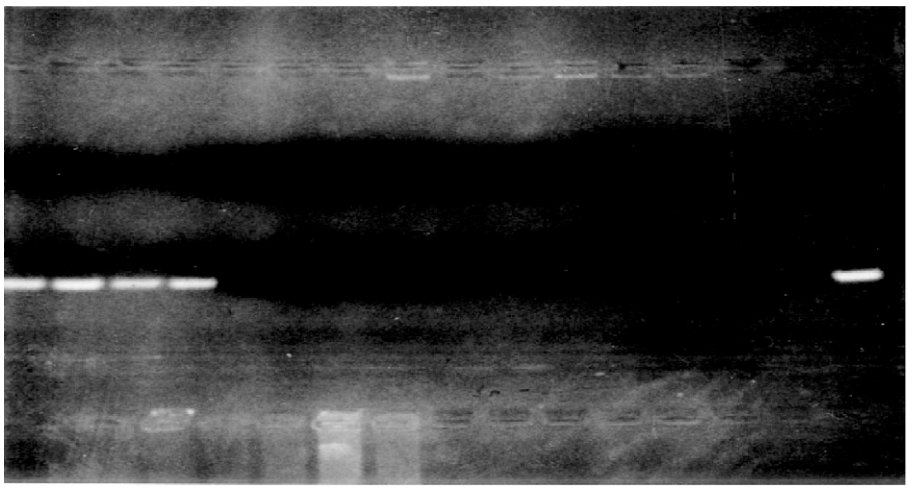

Fig. 1. Analysis of PCR products by agarose gel electrophoresis. PCR-amplified *M. tuberculosis* DNA are seen in lanes 1–4. Lane 15: Negative control and Lane 16: positive control. In the other lanes, PCR is negative.

Table 3. Correlation between PCR results and sputum smear grade

Sputum smear grade	PCR positive *n* (%)	PCR negative *n* (%)	Total *n*
+4	7/8 (%87.5)	1/8 (%12.5)	8
+3	4/12 (%33.3)	8/12 (%66.7)	12
+2	4/10 (%40.0)	6/10 (%60.0)	10
+1	1/10 (%10.0)	9/10 (%90.0)	10
Total	16/40 (%40.0)	24/40 (%60.0)	40

Sputum smear grade: 1–9 bacilli in 100 oil immersion fields =+1, 1–9 bacilli in 10 oil immersion fields=+2, 1–9 bacilli in all oil immersion fields =+3, more than 9 bacilli in all oil immersion fields =+4.

Table 4. Correlation between PCR results and radiological extension of the disease[a]

	n	PCR positive *n* (%)	PCR negative *n* (%)
Extensive disease	12	11/12 (%91.7)	1/12 (%8.3)
Limited disease	28	5/28 (%17.9)	23/28 (%82.1)
Total	40	16/40 (%40.0)	24/40 (%60.0)

[a]The disease was considered extensive if cavities totalled = 15 cm in diameter and/or moderately dense infiltrates involved more than 75% of lung fields on chest X-ray; without these two criteria it was considered as limited disease.

Table 5. Correlation between PCR results and cavity

	n	PCR positive *n* (%)	PCR negative *n* (%)
Presence of cavity	18	16/18 (%88.9)	2/18 (%11.1)
Absence of cavity	22	0/22 (%0)	22/22 (%100)
Total	40	16/40 (%40.0)	24/40 (%60.0)

Jones *et al*. (13) reported that the blood cultures were only sensitive enough in HIV-positive patients with CD_4 T-lymphocyte count $<100/\mu l$, suggesting that CD_4 lymphocyte play a critical role in hematogenous dissemination.

In HIV-negative patients, however, information is limited about the identification of tuberculous bacilli in blood cultures. In some studies, very low positive rate could be obtained, whereas in some studies high positive rate was reported (4,5,12,14,15). In our study, *M. tuberculosis* DNA was found to be 40% positive with PCR method in HIV-negative patients. Niyaz Ahmed *et al*. (14) isolated *M. tuberculosis* DNA in 7 of 16 (43.75%) HIV-negative, immunocompetent pulmonary tuberculosis patients. The patients had antituberculosis treatment for 2 weeks in their study. They reported that the investigation of bacilli DNA by taking blood before antituberculosis treatment could increase the sensitivity. Our study consists of a larger patient population and blood samples were taken before the initiation of treatment from all patients to investigate bacilli DNA by PCR technique. Despite this, the sensitivity was found similar in our and Ahmed's studies. In the study reported by Condos *et al*. (4), among 15 patients with positive PCR results, all had still positive results after one month of treatment; whereas at the 4th month, the blood PCR results became negative in 13 of 15 patients. These studies suggest that treatment in the early period may not effect

the sensitivity of PCR. In the study of Folgueira *et al*.(5), PCR was found positive in 82% of HIV-positive patients and in 33% of HIV-negative patients. PCR was positive in all patients with disseminated tuberculosis, whereas among patients with extrapulmonary tuberculosis, PCR was positive in 100% of HIV-positive patients and in 27% of HIV-negative patients. They reported that, especially in HIV-infected patients, *M. tuberculosis* amplification from blood samples is important in rapid diagnosis and that there is more hematogenous spread than previosly thought in HIV-negative patients. They concluded that *M. tuberculosis* DNA in peripheral blood mononuclear cells may be a helpful method in the rapid diagnosis especially in HIV-positive patients with disseminated and extrapulmonary tuberculosis forms. Although we did not include patients with extrapulmonary, miliary or disseminated tuberculosis, the detection of *M. tuberculosis* DNA in 40% of cases in blood samples by PCR assay is important to show the passing of bacilli into blood in HIV-negative pulmonary tuberculosis patients. Schluger *et al*.(16), in their study with eight pulmonary tuberculosis patients, found *M. Tuberculosis* DNA positive in blood samples by PCR in all patients. However, the population of patients is small and six patients were HIV-positive. In the study of Condos *et al*.(4), 39 of 41 (95%) patients with active pulmonary tuberculosis had positive PCR results. 43% of patients were HIV-positive, 40% were negative and in 17% of patients the result was not known. They reported that positive PCR results were not affected by HIV status. In our study, all patients were HIV-negative. However, we could not find PCR positivity as high as the studies of Condos *et al*. and Schluger *et al*. This supports that, as stated by Folgueira *et al*., hematogenous spread is more frequent in HIV-infected tuberculosis patients and suggests that CD_4 lymphocyte count may be important in hematogenous dissemination. How the tuberculosis bacilli are passed into blood in immunocompetent patients or why bacil DNA cannot be shown in blood in all patients with pulmonary tuberculosis is still discussed. In contrast to the previous thoughts, it is stated that the escape of tubercle bacilli from alveolar spaces to the bloodstream may be more common (3). Another possibility is that monocyte-macrophages or polymorphonuclear phagocytes that migrated into inflammatory area, return into blood circulation after phagocyting mycobacteria (17). In our study, we found that PCR results were more likely positive in patients with higher sputum smear grade. This suggests that how much there is bacilli load in the patients, the escape of tubercle bacilli from alveolar spaces to bloodstream will be more frequent. When we evaluated the pulmonary lesions radiologically, we found that PCR positivity was higher in patients with extensive lesions. None of our patients had extrapulmonary tuberculosis. However, the escape of bacilli into bloodstream may be facilitated in the extensive disease. The reason may be that the bacilli load may be higher in extensive disease and that the spread of infection may not be controlled by cellular immune mechanisms in the alveoli and bacilli may show hematogenous dissemination. We found higher positive PCR result in patients with cavity. Because cavity formation is an effort of the organism to limit the infection, we expect that tubercle bacilli escapes less into bloodstream in patients with cavity. However, in our study the patients with cavity also had diffuse parenchymal infiltration in chest X-ray. When we assessed the BMI of our patients, we found that 65% had a lower than normal value. Low BMI and the presence of hypoalbuminemia representing the bad nutritional status, can lead to a decrease in CD_4 T lymphocytes in HIV-seronegative patients and cause development of tuberculosis and hematogenous dissemination (18). However, we could not find a statistically significant relationship between PCR results and BMI, serum albumin level.

We found that total delay time was 91.8 ± 75.5 days in our study. A longer total delay time, may cause an increased rate in escape of bacilli into bloodstream by a deterioration in the both of immunity and in nutritional status with a widespread disease. However, we could not find a statistically significant association between total delay time and PCR results. Also there was no significant relationship between age, sex and PCR positivity.

When the used primers are not always homologous with target DNA, this can cause false negativity. When four primers (nested PCR) are used instead of two primers, the sensitivity of the test increases (19). Schluger *et al*. (16) used four primers in their study and amplified bacilli DNA in blood in all eight patients Miyazaki *et al*. (19) amplified *M. tuberculosis* DNA in different clinical specimens with 97% sensitivity and 92% specificity by nested PCR method. We used two primers in our study and found the sensitivity 40% and the specificity 100%. These results show that the use of nested PCR increases sensitivity.

The blood-based PCR assay has some advantages. Since blood contains less PCR inhibitory substances than sputum, the isolation of *M. tuberculosis* DNA from peripheral blood mononuclear cells requires more simple procedures than sputum (3,4). Also it is easier to obtain blood sample from patients.

One of the most important problems encountered in PCR studies, is the contamination of reaction products obtained one-step earlier (20). In our study, in order to prevent contamination, we carried out the study in four separate rooms for the admission and preparation of samples, the preparation of solutions, the amplification, and the observation of results, respectively. We used positive and negative controls for each study series. Pipette tips, distilled water and buffers were used after sterilization in autoclave and we used separate pipettes, gloves, etc. for each procedure. Our negative controls gave negative results. Also in our healthy control group, all results

were found negative. This supports that there was no cross-contamination during the preparation of samples.

In conclusion, the study demonstrated that the detection of *M. tuberculosis* DNA from peripheral blood by PCR technique is useful for the rapid diagnosis of tuberculosis patients with HIV-negative. Hematogenous dissemination was important in tuberculosis patients and peripheral blood samples were suitable and easy materials. However, the lack of achievement of universal standardization in PCR studies yet, creates problem. With continuing studies in this field, the position of this test in routine use will be more clearly defined.

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