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Application of peripheral blood *Mycobacterium tuberculosis* PCR for diagnosis of tuberculosis patients

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Abstract. – BACKGROUND: *Mycobacterium tuberculosis* (MTB) infection is a global health problem. Failure to accurately identify cases of active MTB has serious effects on both patients and the community. Acid-fast bacilli (AFB) smear has poor sensitivity and culture methods have a delay ranging from 1 to 8 weeks for diagnosis. Nucleic acid amplification assays may be suitable candidates for this purpose.

PATIENTS AND METHODS: In a prospective study, we evaluated *Mycobacterium tuberculosis* DNA in peripheral blood samples with PCR technique in 190 patients with pulmonary and extra pulmonary tuberculosis whom were admitted to Tehran Imam Khomeini hospitals during 2006-2010. Three ml citrated blood samples were obtained from cases. DNA extraction was performed by QIAGEN commercial kit and PCR performed with IS1081 Primer.

RESULTS: Fifty six cases had extra-pulmonary tuberculosis and 134 were pulmonary. Overall sensitivity and specificity of the PCR assay was 41.1% and 95.5%, respectively.

CONCLUSIONS: MTB-PCR assay on PBMC using IS1081 primer has a low sensitivity and now can not use as a single or alternative diagnostic test for tuberculosis. However, with regard to its high specificity can use for help diagnosing of TB in cases have no enough sputum (or other specimens) to examination for acid-fast bacilli (AFB) smear and culture.

Key Words:
Mycobacterium tuberculosis, PCR (polymerase chain reaction), PBMC (peripheral blood mononuclear cells).

Introduction

Mycobacterium tuberculosis (MTB) infects one third of the world's population[1]. There were an estimated 8.8 million incident cases of TB (range, 8.5 million-9.2 million) globally in 2010, 1.1 million deaths (range, 0.9 million-1.2 million) among HIV-negative cases of TB and an additional 0.35 million deaths (range, 0.32 million-0.39 million) among people who were HIV-positive[2]. Prevalence of tuberculosis in our country, Iran was 23 per 100 000 population with a mortality rate of 1.8 per 100 000 population[3]. Tuberculosis is second only to HIV as a cause of death worldwide resulting from a single infectious agent. Drug resistant tuberculosis is emerging globally, with approximately 0.5 million new MDR (multidrug resistant) cases annually[1]. In 1993, the WHO declared tuberculosis a global public health emergency[4]. The resulting failure to accurately identify cases of active MTB has profound implications for both the individual and the community. The conventional approach to the laboratory diagnosis of active TB relies on acid-fast bacilli (AFB) smear and culture of a samples. AFB smear – though rapid and inexpensive, has poor sensitivity (requires up to 10,000 bacilli per milliliter of specimen). Culture methods are generally quite sensitive and specific; yet even where they are affordable, a delay ranging from 1 to 8 weeks for diagnosis significantly diminishes the ability to control the spread of TB[5]. Therefore, clinicians need an accurate and rapid and available diagnostic test for timely diagnosis and treatment of patients with tuberculosis. Nucleic acid amplification assays which now widely are available may be suitable candidates for this purpose.

Patients and Methods

To determine the sensitivity of polymerase chain reaction (PCR) in peripheral blood mononuclear cells (PBMC), we have evaluated *Mycobacterium tuberculosis* DNA in peripheral

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blood samples with PCR technique in adult patients with pulmonary and extra-pulmonary tuberculosis. A total of 190 patients with pulmonary and extra pulmonary tuberculosis whom were admitted to Tehran Imam Khomeini hospitals during 2006-2010 enrolled in our study. Case definition for smear positive pulmonary TB was a patients with clinical symptoms suspect to TB plus 2 positive sputum smear or one positive sputum smear and one positive sputum culture or one positive sputum smear and a chest X ray consistent with TB. Case definition for smear negative pulmonary was a patient with clinical symptoms suspect to TB whom had negative sputum smears plus a positive sputum culture or a positive BAL (broncho alveolar lavage) or a chest x ray consistent with TB. Also case definition for extra pulmonary was histopathologic evidences ± positive culture. These case definitions were based on Iranian national protocol for TB control. The patients who had been taking anti tuberculosis drugs excluded from this study. We also enrolled 90 healthy persons (50 male and 40 female with the mean age of 43.2±20.3 years) without any symptoms of tuberculosis as control group. Three ml citrated blood samples were obtained from these cases. The samples were transported to Microbiologic Laboratory of Tehran University of Medical Sciences using tubes with EDTA. Specimens were kept under refrigeration at –20°C for up to 24 hours. DNA extraction was performed by QIAGEN commercial kit (Gaithersburg, MD, USA). The sample was initially lysed and other proteins were denatured in the appropriate buffer. Then the QIAGEN protease was added and incubated at 56°C for 2 hours. The lysate was loaded into the QIAGEN Genomic-tips and the DNA bound to the column, while other cellular components were removed. Following washing to remove other components remaining, the high molecular weight DNA was released and with adding the same volume cold isopropanol and centrifuging at 14,000 rpm for 5 minutes, it was precipitated. Afterward additional materials were taken and DNA pellets were washed with cold 70% ethanol and again the DNA was centrifuged and ethanol was removed. Then we allowed that the DNA pellet dry in room air. At last DNA pellets were solved in 50 micro liter buffer (Tris - EDTA). The time involved in testing for complete process was 150 minutes. Each patient was tested twice to reduce false positive or negative. The PCR testing performed with IS1081 Primer. This specific primer is containing a 344 bp fragment that is located at position 85 to 428 of IS1081 gene. For prevention of cross contamination and reduction of false positive, all steps were performed under laminar hood. We take a written informed consent from all patients, agreeing to participate to the study.

Statistical Analysis

The collected data were analyzed using the SPSS version 13 (SPSS Inc., Chicago, IL, USA). Values are means ± SD. $p < 0.05$ was considered statistically significant.

Results

A total of 190 patients were enrolled in this study that 68 (35.8%) were female and 112 (64.2%) were male. The mean age for female patients was 48.7±24.2 years and for males was 41.8 ± 17.1 years. Pulmonary TB was seen in134 cases (70.47%). Fifty six patients (29.53%) had extra-pulmonary tuberculosis including lumbar spondylodiscitis (14 cases), disseminated (12 cases), peritonitis (11 cases), lymphadenitis (10 cases), pleural effusion (9 cases), pericarditis (4 cases), meningitis (4 cases), arthritis (2 cases). Among patients with pulmonary TB, 82 (61.2%) cases were male and 52 (38.2%) female and among patients with extra pulmonary TB, 40 (71.4%) were male and 16 (28.6%) female. A total of 100 (74.6%) cases with pulmonary TB were sputum smear positive. HIV Elisa test was performed in 111 high risk patients that 45 (44.5%) were HIV positive. PCR of peripheral blood was positive in 78 of 190 (41%) patients and was negative in 112 (59%). Among 134 patients with pulmonary tuberculosis 64 cases (47.8%) had positive PCR while, this test was only positive in 14 (25%) patients with extra-pulmonary (Table I). PCR test was positive in 52 (52%) of sputum smear positive pulmonary TB cases and 12 (35.3%) of sputum smear negative pulmonary TB. The overall sensitivity, specificity, PPV (positive predictive value) and NPV (negative predictive value) of the PCR assay was 41.1%, 95.5%, 95.1% and 43.4%, respectively. Sensitivity, specificity, PPV and NPV for pulmonary TB was calculated 47.8%, 95.5%, 95.1% and 43.1%, respectively and these measurements for extra pulmonary TB were 25%, 95.5%, 77.8% and 67.1%, respectively. Also the PCR test was positive in 26 of 45 (57.8%) HIV infected patients.

Table I. Results of PCR of peripheral blood of patients with tuberculosis in our study.

MTB PCR	Type of tubercolosis			
	Pulmonary	Extra pulmonary	Total	Control group
Positive	64 (47.8%)	14 (25%)	78 (41.1%)	4 (4.5%)
Negative	70 (52.2%)	42 (75%)	112 (58.9%)	86 (95.5%)
Total	134 (100%)	56 (100%)	190 (100%)	90 (100%)

Discussion

Nucleic acid amplification assays including polymerase chain reaction (PCR) tests are suggestive technique for detection of M. tuberculosis in clinical specimens. NAATs (Nucleic acid amplification tests) amplify MTB-specific nucleic acid sequences using a nucleic acid probe[1]. The most commonly used target sequences are IS6110 and a 16s r RNA, which are specific to the MTBC[5]. For smear (sputum) positive specimens, the sensitivity and specificity of nucleic acid amplification exceed 95%. For smear-negative cases, sensitivity has ranged from 40% to 77% and the specificity remains over 95%[1,6]. When there is a high clinical index of suspicion for pulmonary tuberculosis but with a negative acid-fast smear, a positive NAAT is highly predictive of tuberculosis and allows early initiation of therapy[1]. The most investigations in this subject have been performed on the sputum of patients. However, there are some studies which suggest that nucleic acid amplification of non respiratory specimens may help to diagnosis of tuberculosis specially in patients who have no sputum or other suitable specimen including HIV positive and military TB cases[7-11]. In a prospective study, we have evaluated PCR assay of peripheral blood for the diagnosis of tuberculosis including pulmonary or extra pulmonary involvement. The sensitivity and the specificity of PCR test in our cases were 47.8% and 95.5%, respectively. These results have been compared with other similar studies in Table II. The sensitivity of PCR of peripheral blood cells in our patients was partly low. This

Table II. Results of PCR of peripheral blood in this study and their comparison with some other available study.

	Date of publication	Country	Patients number	Targeting genome sequence	Type of tuberculosis	Sensitivity	Specificity
Our study		Iran	190	IS1081	Pulmonary Extra pulmonary	47.8% 25%	95.5% 95.5%
Rolef et al[12]	1995	Germany	160	240bp/244bp/ 158bp	Pulmonary	30-48%	–
Khan et al[11]	2006	India	96	IS6110	Pulmonary	20%	94.4%
Ahmed et al[13]	1998	India	16	IS1081	Pulmonary	43.75%	–
Mirza et al[14]	2003	Pakistan	48	IS6110	Extra pulmonary	62.5%	–
Del Prete et al[15]	1997	Italy	30	IS6110	Pulmonary	87%	–
Mishra et al[16]	2010	India	78	IS6110	Extra pulmonary	32-50%	100%
Scherer et al[17]	2011	Brazil	203	IS6110	Pulmonary	42-67%	76-87%
Amin et al[18]	2011	Pakistan	88	IS6110	Pulmonary/ Extra pulmonary	36.3%	–
Robolloa et al[19]	2006	Spain	57	IS6110	Pulmonary Extra pulmonary	41% 36%	– –
Honore et al[20]	2001	France	20	IS6110	Pulmonary/ Extra pulmonary	30.4% 83.6%	
Taci et al[21]	2003	Turkey	40	IS6110	Pulmonary	40%	100%
Kumar et al[22]	2010	India	102	IS6110	Pulmonary	58.3%	–
Schijman et al[23]	2004	Argentina	103	IS6110	Pulmonary	42.9%	100%
Ritis et al[24]	2000	Greece	11	IS6110	Extra pulmonary	24%	–
Sankar et al[25]	2011	India	84	TRC4/IS6110	Pulmonary	7.14% (TRC4) 41.67% (IS6110)	–

finding is consistent with most studies available in this area[11-25], which can be several reasons such as lake of proper standardization for this test or presence of unknown inhibitors in the serum of TB patients. The sensitivity of test in HIV infected patients was slightly higher (57.8%) that was similar to results of study of Kumar et al[22]. This finding can be due to a more mycobacteremia in HIV positive cases.

Conclusions

MTB-PCR assay on PBMC using IS1081 primer has a low sensitivity and now can not use as a single or alternative diagnostic test for tuberculosis including pulmonary or extra pulmonary. However, with regard to its very high specificity, this test can use for help diagnosing of TB in cases have no enough sputum (or other specimens) to examination for acid-fast bacilli (AFB) smear and culture.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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