Original Article

Diagnosis of Tuberculosis by PCR-based Amplification of mpt64 Gene from Peripheral Blood

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Background: **Using blood to diagnose tuberculosis (TB) infection has been advocated for years, but the results were unsatisfactory. In this study, we modified the nucleic acid extraction and amplification methods to see if diagnostic sensitivity could improve.**

Materials and Methods: **We prospectively enrolled 74 patients suspected of having TB infection. The blood was collected from each patient. Modified Triton X-100 differentiated lysis method was used to purify mycobacterial nucleic acid from the cytoplasm of mononuclear blood cells. Real-time polymerase chain reaction (PCR) of *mpt64* gene fragment were carried out on both mononuclear cells and plasma samples of each patient.**

Results: **The sensitivity, specificity, positive and negative predictive values of peripheral blood real-time PCR for diagnosing TB infection was 30.8, 88.8, 95.2 and 15.1%, respectively. The diagnostic sensitivities of pulmonary, extra-pulmonary and disseminated TB were 26.9, 45.4 and 50%, respectively. Of the 20 TB patients whose mycobacterial DNA were detected in blood, 11 (55%) had detectable DNA only in their plasma, 7 (35%) only in mononuclear cells, and 2 (10%) in both plasma and cells. When the PCR results of both plasma and white cell were put together, the sensitivity (30.8%) was more than that of either one alone (13.8% for white cell only, 20% for plasma only).**

Conclusion: **Peripheral blood *mpt64* PCR amplification can diagnose some TB patients, especially those with extra-pulmonary and disseminated TB. Although the method is not sensitive enough to diagnose pulmonary TB, it may be a potentially useful tool in diagnosing extra-pulmonary and disseminated TB. Mycobacterial DNA could be detected in both plasma and white cells. Combining the PCR results of both plasma and white cells could increase the diagnostic sensitivity for TB.**

Key words: blood buffy coat, clinical laboratory techniques, real-time polymerase chain reaction, tuberculosis

Introduction

About 2 billion people, equal to one-third of the world's population, are infected with tuberculosis (TB). There were 9.4 million new TB cases in 2008[1]. It poses a great threat to global public health. Traditional diagnosis of pulmonary TB using sputum acid-fast stain is insensitive. Although sputum *Mycobacterium tuberculosis* (MTB) culture is more sensitive and specific, it takes up to 8 weeks to yield a positive result[2]. It has been known for decades that MTB could be detected in peripheral blood of some TB patients[3-4]. Schluger *et al.* success-

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fully detected the presence of IS*6110* DNA sequence of MTB in the buffy coat layer of TB patients' peripheral blood by polymerase chain reaction (PCR) amplification[5]. With the advantage of more rapid turnaround time, it was once regarded as a promising diagnostic tool. However, subsequent studies revealed it's not sensitive either, except for AIDS patients[6-7]. Most of these studies were performed by isolation of mononuclear cells by Ficoll-Hypaque technique, followed by destruction of all the nuclear and plasma membranes by detergents such as Tween 20, Nonidet P-40 or sodium dodecyl sulfate. Nucleic acids extracted by these methods contained not only mycobacterial, but also host DNA. DNA amount was estimated to be around 150-300 μg/mL whole blood. The amount was far from ideal for a PCR reaction. Such large amount of mainly human DNA made PCR primers hard to efficiently detect their specific mycobacterial targets. In this study, we collected 74 blood samples from clinically suspected TB patients and modified previous method by using Triton X-100 to destroy plasma membrane and left nuclear membrane intact. *Mycobacterium tuberculosis* MPT64 protein is an MTB-specific antigen[8] secreted during bacterial growth[9]. It has been proved useful in identification of MTB[10]. The *mpt64* was targeted for PCR amplification in this study. We also applied real-time PCR methods to both plasma and mononuclear cells to observe their differential abilities in detecting TB infections.

Material and Methods

Patients and specimens for acid-fast stains and culture

From July to Nov 2009, we prospectively enrolled 74 non-AIDS patients suspected of TB infection in China Medical University Hospital. The study was approved by the Institutional Review Board of China Medical University Hospital. Informed consents were obtained from all participants. The diagnosis of TB infection was made when positive bacteriologic culture confirmed it (49 patients met this criteria) or when the attending clinicians suspected it and embarked anti-TB therapies (16 such clinically diagnosed TB patients). Sputa were collected for acid-fast stain and mycobacterial culture from all participants except for one missed acid-fast stain. Most of the participants have received anti-TB therapies for less than one week when enrolled.

DNA extraction from peripheral blood

Ten ml of whole blood from each patient was collected in tubes containing ethylenediaminetetraacetic acid (EDTA). Subsequent processing was performed within 30 minutes after blood collection. All tubes were centrifuged at 3000 *g* for 10 minutes to separate plasma and buffy coat. We transferred 100 μl of plasma to a new microcentrifuge tube containing 5 μl of proteinase K (20 mg/ml, Roche) and 500 μl of digestion buffer (100 mM Tris pH 7.5, 10 mM EDTA, 50mM $NaCl_2$, 0.1 % linear polyacrylamide (LPA), 2% SDS) and incubated it at 55ºC for at least 30 minutes. Two ml of buffy coat was carefully layered over a solution of Ficoll-Hypaque (specific gravity, 1.077, GE) and centrifuged at 400 *g* for 40 minutes at room temperature. The mononuclear cells were washed with phosphate buffered saline (PBS) and then centrifuged with 100 *g* for 10 minutes. After discarding the supernatant, cells were re-suspended in 500 μl of Triton X-100 solution (0.32 M sucrose, 5mM $MgCl_2$, 1% Triton X-100, 10mM Tris HCl, pH 7.8) and incubated at room temperature for 10 minutes. Then, the mixture was centrifuged at 1,200 g for 1 minute. About 500 μl supernatant was transferred to a new microcentrifuge tube containing 5 μl of proteinase K (20 mg/ ml, Roche) and 100 μl of 10% SDS solution. It was incubated at 55ºC for 30 minutes.

Then, the digestion mixture from plasma or mononuclear cells was mixed with 200 μl of 8M NaH_4OAc and 200 μl of chloroform. After vigorously vortex for 20 sec, it was centrifuged at maximum speed (larger than 12,000 g) for 5 minutes. An aliquot of 700 μl of supernatant was transferred to a new microcentrifuge tube. Two μl of 1% (w/v) linear polyacrylamide (LPA) and 550 μl of 100% isopropanol were added to the tube. The tube was inverted for 20 to 30 times. After centrifuging at maximum speed for 10 minutes, the supernatant was discarded. The pellet of DNA and LPA carrier was washed once with 70 % ethanol, followed by air-dry and re-suspension in a final volume of 25-50 μl of TE buffer (10 mM Tris, 0.1 mM EDTA, pH7.6). The extracted DNA was stored at -20ºC until PCR amplification.

PCR procedures

Nucleic acid amplification was performed as previously described[11].The first PCR was to detect a 239 bp region of MTB complex-specific *mpt64* gene sequence.[26]

The first PCR reactions were preformed in a volume of 20 µl, containing 1 U of *Taq* polymerase (Finnzymes, Finland), 200 µM (each) deoxynucleoside triphosphates (Viogene, Taiwan), and 250 nM (each) primer in reaction buffer (1.5 mM $MgCl_2$ and 50 mM KCl in 10 mM Tris-HCl, pH 8.3). Five µl of the extracted DNA was added to a 15-µl aliquot of PCR master mixture solution. The sequence of the outer forward primer (F-1) was 5'-ATCCGCTGCCAGTCGTCTTCC -3' and outer reverse primer (R-1) was 5'-CTCGCGAGTCTAGGCCAGCAT-3'. After initial denaturation at 95ºC for 3 minutes, the reaction was perform with 35 cycles of denaturation at 95ºC for 30 seconds, annealing at 60ºC for 30 seconds, and extension at 72ºC for 1 minute. An additional final extension was performed at 72ºC for 10 minutes. The nested real time PCR was to detect a 77-bp internal region of the first PCR product. The PCR reaction solution used was DyNAmo^{TM} Flash Probe qPCR kit (Finnzymes, Finland) containing 500 nM (each) forward primer (TqMn-F) 5'-GTGAACTGAGCAAGCAGACCG-3', reverse primer (TqMn-R) 5'-GTTCTGATAATTCACCGGGTCC-3' and 250 nM TaqMan probe (TqMn-W-FAM) 5'-FAM-TATCGATAGCGCCGAATGCCGG-BHQ-3. After initial denaturation at 95ºC for 10 minutes, the reaction mixture ran through 40 cycles of denaturation at 95ºC for 10 seconds, annealing and extension at 60ºC for 1 minute. Either mononuclear cell or plasma with detectable mycobacterial DNA was considered as a positive result.

Results

Patient description

Among the 74 enrolled patients, 65 of them were diagnosed to be TB infections and the other 9 were excluded. Of the 65 TB patients, 52 were pulmonary TB, 11 were extra-pulmonary TB and 2 were disseminated (mixed intra- and extra-pulmonary) TB. Of the 65 TB patients, 49 sputum cultures were positive for TB bacilli. 10 sputa yield non-tuberculous mycobacterium growth (Four of them were excluded from TB later, but the other 6 patients were still diagnosed to be clinical TB patients and treated accordingly). The sensitivity and specificity of acid-fast stain was 43.8 and 55.6%, respectively (only 73 samples were examined by acid-fast stain).

Diagnostic Accuracies of Real-Time PCR

The results of *mpt64* PCR on 74 blood specimens of patients with or without TB were shown in the Table 1. The sensitivity, specificity, positive and negative predictive values of peripheral blood real-time PCR for diagnosing TB infection was 30.8, 88.8, 95.2 and 15.1%, respectively. Table 2 demonstrates PCR detection results of different forms of TB infections. The diagnostic sensitivities of extra-pulmonary and disseminated TB were 45.4 and 50%, which were higher than that of the pure pulmonary TB (26.9%). Of the 20 TB patients whose mycobacterial DNA were detected in blood, 11 (55%) had detectable DNA only in their plasma, 7 (35%) only in mononuclear cells, and 2 (10%) in both plasma and cells. The information provided by plasma and cellular DNA were partially complementary to each other. When they were put together, the sensitivity (30.8%) was more than that of either one alone (13.8% for white cell only, 20% for plasma only). The effects of anti-TB medications on diagnostic sensitivities were presented in Table 3. Those who did not previously receive any anti-TB medication while PCR was undertaken had the highest diagnostic sensitivities (75%). After anti-TB medications, the sensitivities dropped to between 17 to 35%. There was no evident treatment duration-dependent difference. If only bacteriological proved TB cases was considered infection, the sensitivity and specificity were 32% and 75%, respectively.

Correlations between plasma and mononuclear cells in PCR results

By Fisher's exact test, the p value was less than 0.001. So both results were closely associated.

Discussion

After the first introduction of blood PCR for TB diagnosis in 1994[12], there have been several reports about its clinical applications. The highest sensitivity was reported to be 95% among human immunodeficiency virus (HIV) infected patient by Condos *et al*[13]. As for non-HIV infected patients, the sensitivities was usually below 50% and ran a gamut from 22% to 87%[7 14-15]. Despite the refurbished methodologies we applied, our sensitivity was only 30.8%, similar to earlier reports. Such a low sensitivity may partly be related to the fact that most of patients have already received therapies when their bloods were drawn. Although previous data suggested the detectable MTB DNA segment will per-

sisted for up to 4 months after initiating anti-TB therapy[13], we found the sensitivity was significantly higher among the 4 patients not given therapy when their blood were drawn (3 of them were positive by blood PCR detection).

In spite of the low overall sensitivities, this method has been reported to be especially sensitive for extra-pulmonary and disseminated TB infections. The reported sensitivities ranged from 33 to 63%[7 15-16]. In our study, the sensitivities of diagnosing extra-pulmonary and disseminated TB were 45.4 and 50%, respectively. Since the surge of AIDS, the portion of extra-pulmonary and disseminated TB increased significantly[17]. Extra-pulmonary TB is hard to diagnose. Its symptoms and signs are non-specific. Bacteriologic or histologic confirmation often requires invasive procedures, which are not always accessible in some part of the world[18]. Because of such diagnostic difficulties, extra-pulmonary TB is among major causes of delayed or mis-diagnosis of tuberculosis[19]. Although serological antibody detection tests are commercially available, their diagnostic accuracies for extra-pulmonary TB are unreliable and cannot be routinely used in clinical practice[20]. The pathogenesis of extra-pulmonary TB involves transient MTB bacteremia[21]. That may explain why blood PCR method is especially useful in this subgroup of TB patients.

The specificity of our peripheral blood PCR method was 88.8%. The result was also similar to most previously reported figures, ranging from 83.6 to 100%[6-7 14 22]. Most control group of previous studies included healthy subjects, whereas ours contained only TB suspects. Khosla *et al.* reported the specificity of peripheral blood PCR for TB pleurisy was only 56.l8% in India, a TB-ravaged country[23]. In that study, the DNA contents were further quantified by comparative PCR method. False positive patients contained MTB DNA contents significantly lower than the true patient counterparts. Maybe many control subjects encountered MTB earlier in their lives and had detectable mycobacterial DNA for a protracted period and contributed the false positive results. The prevalence of TB infection in Taiwan is high[24] and that may be a cause of our false positive result.

Almost all previous studies amplified DNA within white blood or mononuclear cells. One study amplified all the DNA extracted from whole blood showed a sensitivity of 20% and a specificity of 94.4%, not quite different from the results obtained from white blood cell[25]. Our data showed MTB DNA was also present in the plasma of patients. Furthermore, there were more (55%) detectable MTB DNA in plasma than in white cells (35%) (Table 2). The plasma and cellular information were partially complementary to each other. When they were combined together, the diagnostic sensitivities increased from 13.8 or 20% to 30.8%.

The study was carried out in a group of hospital-based patients suspected of having TB infection. So, the results are not applicable to general population. There were only 9 (about 12%) non-TB patients in this study. This uneven disease proportion among participants also limits the inferences of the results.

In conclusion, we found peripheral blood *mpt64* PCR amplification can be used to diagnose some TB patients, especially those with extra-pulmonary and disseminated TB. Although the method is not sensitive enough to diagnose pulmonary TB, it may be a potentially useful tool in diagnosing extra-pulmonary or disseminated TB. Mycobacterial DNA could be detected in both plasma and white cells. Combining the PCR results of both plasma and white cells could increase the diagnostic sensitivity for TB.

Table 1. Diagnostic Accuracies of *mpt64* PCR from blood of patients with or without TB.

mpt64 PCR	TB patients	Non-TB patients	Total
Positive	20	1	21
Negative	45	8	53
Total	65	9	74

Table 2. Numbers of samples detected by *mpt64* nested PCR on mononuclear cell or plasma from 65 patients infected with various forms of TB.

		mpt64 nested PCR			
	Total	Cell(-) Plamsa(-)	Cell(-) Plamsa(+)	Cell(+) Plamsa(-)	Cell(+) Plamsa(+)
Pulmonary	52	38	7	5	2
Extra-pulmonary	11	6	3	2	0
Disseminated	2	1	1	0	0
Total	65	45	11	7	2

Table 3. Effects of anti-TB medications on diagnostic sensitivities.

Duration of therapy (day)	Patients (n)	Patients infected with TB (n)	Patients with PCR (+) (n)	Sensitivity (%)
0	4	4	3	75
1-2	9	9	2	22
3-4	7	6	1	17
5-6	14	12	3	25
7-8	40	34	12	35

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