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Original Article

Mycobacterium tuberculosis Detection based on *mpt64* amplification by Nested-PCR in Sputum samples

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Abstract

Introduction: Tuberculosis (TB) is an old problem that is currently considered as a great challenge, mostly in developing countries. It may be a lethal disease. Thus, rapid diagnosis of *Mycobacterium tuberculosis (MTB)* infection plays a critical role in controlling the spread of TB, whereas conventional methods may take up to several weeks or longer to diagnose the infection. Hence, nested polymerase chain reaction (NCR) assay was applied for direct identification of the *MTB* DNA presence in sputum samples. The aim of the study was the development of a direct NCR method using *mpt64* specific primers for rapid diagnosis of *MTB* infection.

Materials and Methods: To development of study, eight positive and negative sputum specimens obtained from Masih Daneshvari hospital pulmonary TB center, were studied. After smear preparation genomic DNA was extracted and *mpt64* was amplified using NCR method. While doing work we paying attention to PCR standardization and precautions to avoid sample contamination.

Results: After evaluation gained appropriate results from purified genomic DNA by AGE and biophotometer, the standardized NCR products were evaluated by Agarose Gel Electrophoresis. Five of 7 positive samples were positive, and one of the negative samples was negative using our NCR assay.

Conclusion: Based on the results of this study, we could be successful in the NCR technique's optimization to our system for disese detection, while it can be apply as a more rapid, accurate, inexpensive, and specific diagnostic assay for direct detection of *MTB* DNA.

Keywords: *Mycobacterium tuberculosis*, Nested PCR, MPT64, Pulmonary tuberculosis

1. Introduction

Tuberculosis (TB), a chronic infectious disease threatening public health, is currently one of the leading causes of worldwide mortality, especially in developing countries [1-5]. Human immunodeficiency virus-AIDS pandemic, the emergence of multidrug-resistant (MDR) *Mycobacterium tuberculosis (MTB)* strains [4, 5], blindness by ocular manifestations [6], pulmonary TB [2, 7-9], nervous system TB [10], and TB-related meningitis [3, 11], have further contributed to the spread of TB.

Delay in *MTB* diagnosis leads to a neurologic sequel in patients who do not receive early treatment [3]. Microbiological *MTB* detection is insufficient for the early

diagnosis because there are too few organisms in cases for consistent demonstration by direct smear and culture identification take a long time [1, 3, 7, 9, 12-14].

In recent years, the advent of molecular techniques, based on DNA amplification, e.g. polymerase chain reaction (PCR), to detect small amounts of genomic DNA from body fluids or tissues have let us to diagnose and confirm infections that were previously difficult to detect[6,8]. The *mpt64* has been extensively used for molecular research of *MTB* strains in pulmonary TB, especially [5, 9, 15, 16].

The aim of the present study was the development of a direct Nested-PCR (NCR, N-PCR) method using *mpt64* specific primers for rapid diagnosis of *MTB* infection.

2. Materials and Methods

2.1. Subjects and clinical samples

This original article was done on Agust 2014 until April 2015 in Tonekabone universiy's labratory on basis of the collected sample in 2011 for the another original article, 3000 patients with suspected tuberculosis referred to health and care centers in Iran, that were found positive for *M. tuberculosis* on direct smear[17]. Seven culture- positive clinical sputum specimens and one culture-negative specimen were selected among of the collected samples in 2011 from TB patients from the Masih Daneshvari pulmonary TB center, Tehran, Iran. [18]

All subjects were informed and were approved by ethics committee of Islamic Azad University of Tonekabon.

2.2 Smear preparation, Extraction and purification of DNA

Sputum samples were homogenized and decontaminated using sodium and N-acetyl-L-cysteine. After Ziehl Nelson staining, the smears were examined for the presence of acid fast bacilli. [2, 3, 8-10, 12-14, 19, 20]

DNA extraction was performed by using a commercial column extraction kit (Roche, Ltd. Germany) for genomic DNA extraction according to the manufacturer instructions.

The quantity of extracted DNA was assessed by spectrophotometery (Eppendorf Biophotometer DNA/RNA UV calculator, Germany) on diluted extract samples. [12]

The quality of the extracted DNA was determined by agarose gel electrophoresis (AGE) on diluted purified samples. [12, 17]

2.3. mpt64-based N-PCR assay on MTB samples

Two sets of specific primers for the gene encoding the MPT64 protein of *MTB* (*mpt64*; GenBank accession no. NC_000962) [5, 11, 13, 15, 16, 20], used in two consecutive rounds of amplification using NCR assay, are shown in table 1, 2. The amplicon has a length of 123 bp.

Table 1. *MTB* First-Step PCR Primers Sequence (Outer Primers)

	Tm	5'-3' Seq
F_1	73.1	5'-ATCCGCTGCCAGTCGTCTTCC-3'
R_1	72.6	5'-CTCGCGAGTCTAGGCCAGCAT-3'

Tm: temperature; Seq: sequence; F: forward; R: reverse

Table 2. *MTB* Second-Step PCR Primers Sequence (Inner Primers)

	Tm	5'-3' Seq
F_2	68.9	5'-CATTGTGCAAGGTGAACTGAGG-3'
R_2	72.6	5'-AGCATCGAGTCGATCGCGGAT-3'

Tm: temperature; Seq: sequence; F: forward; R: reverse

The reactions contained 75 mM Tris-HCl (pH 8.0), 20 mM Ammonium sulfate, 0.01% Tween 20, 2 mM MgCl2, 5 mM dNTP, 10 pM of the primers F_1 and R_1 at the first-step and the primers F_2 and R_2 at the second-step, and 2.5 U of *Taq* DNA polymeras (hot-start). 5µl of the extracted DNA specimen at the first step and 3µl of the single PCR product at the second step were used in the PCR mixture as the template (overall volume was 25µl). These preparations were exposed to two subsequent optimized PCR amplification protocols that shown in Table 3, 4. The N-PCR products were evaluated using electrophoresis on a 2.5% agarose gel and stained with ethidium bromide. [1, 2, 6-9, 14, 16-18, 21]

Table 3. Optimization of PCR First-Step

	Initial-Denaturation		Denaturation	Annealing-Extension		Final-Extension	
1	95°×3'	1	95°×40"	68°×50"	30	72°×5'	1
2	95°×4'	1	95°×30"	72°×25"	30	72°×5'	1
3	95°×4'	1	95°×20"	72°×15"	30	72°×5'	1
4	95°×4'	1	95°×20"	72°×25"	30		
5*	95°×12'	1	95°×25"	72°×30"	30		
6**	95°×12'	1	95°×25"	72°×30"	25		

* Hot-Start *Taq* Enzyme, ** Hot-Start *Taq* Enzyme, Optimized Situation

Table 4. Optimization of PCR Second-Step

	Initial-Denaturation		Denaturation	Annealing-Extension		Final-Extension	
1	95°×3'	1	95°×40"	72°×50"	30	72°×5'	1
2	95°×4'	1	95°×30"	72°×25"	30	72°×5'	1
3	95°×4'	1	95°×20"	72°×15"	35	72°×5'	1
4	95°×3'	1	95°×20"	72°×20"	35	72°×5'	1
5*	95°×12'	1	95°×25"	72°×30"	35		
6**	95°×12'	1	95°×25"	72°×30"	35		

* Hot-Start *Taq* Enzyme; ** Hot-Start *Taq* Enzyme, Optimized Situation

2.4 Standardization of PCR

For N-PCR assay, three important topics may affect the assay conditions; the annealing temperature, the $MgCl_2$ and primer concentration, the number of amplification cycle of two-step PCR, and especially employing the hot-start *Taq* DNA Polymerase enzyme. These were detected by the following series of primary experiments.

In the initial experiments, the routine concentration of $MgCl_2$ (25mM/2mM) and primers (10pmol/1pmol) was used, and to detection the optimal number of amplification cycle and temperature six tests were performed with different number of annealing and extension cycles from 25 to 35 with different annealing and extension time (Table 3,4). [3, 12]

2.5. Precautions to elude sample contamination

To elude contamination, the following precautions were considered; use of a pre-PCR cabin in every step of PCR and separate areas in every stage of experimental procedure, preparation of PCR master mix solution with specific primers without the *Taq* DNA polymerase and DNA template for each PCR step, application of the no-template control (NTC) and positive samples in each PCR assays to eliminate the

risk of carryover and cross-contamination. [16, 3]

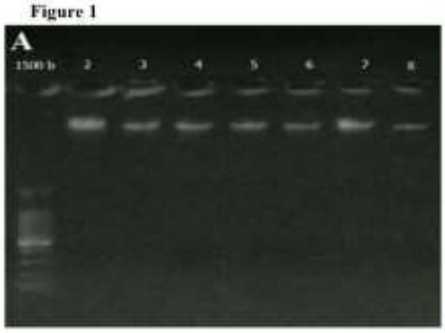
3. Results

Clinical samples and DNA extraction

The total genomic DNA purified was evaluated by AGE (Figure 1A) that electrophoresis of extracted genomic DNA shows genomic DNA with a high molecular weight than the DNA Ladder. and Biophotometer (Figure 1B) that appears spectrophotometric analysis of extracted genomic DNA. The results indicate the appropriate quality and quantity of the extracted DNA.

Figure 1

A



B

DNA Concentration	9 µg/ml
260/280*	1.50
260/230**	0.87

*260/280: DNA/Protein OD ratio
**260/230: DNA/Carbohydrate OD ratio

Figure 1. *MTB* clinical samples and DNA extraction

Optimization and *mpt64*-based N-PCR assay

In the NCR assay, several rounds of PCR were performed with different thermal and time criteria with distinctive concentration of reagents. Results are shown in figures 2A- G. *(A)* Observation of 123bp amplicons of *mpt64* in samples 4, 6 and NTC with undesirable bands and smear in condition one (table 3,4), Indicates the attachment of primers to non-specific sites and amplification of other irrelevant segments in addition to the target segment. The reason of the negative result in sample 5 is DNA deterioration during conservation period, and master mix contamination resulted in the appearance of 123bp band in NTC. *(B)* Based on the results of the first NCR, the Tm of the reactions was increased, and the annealing time was decreased to remove the unspecific bands and smear (condition 2, table 3, 4). After these changes, unspecific band and smear were observed in positive control as well as smear in NTC. Nonetheless, the number of non-specific bands was reduced. *(C)* To further optimize the NCR reaction, we reduced the time of annealing (condition 3 in table 3, 4). The positive control has no unspecific band though with lower intensity and an observable smear, which indicates the annealing time was reduced too much. The NTC has a non-specific band, which shows the contamination of NCR mix. *(D)* Based on the results of the condition 3, the annealing time was increased and final extension step was removed to optimize the NCR reaction (condition 4, table 3, 4). Non-specific band and smear re-appeared, and the NTC has 123bp band that shows the contamination of the NCR mix. *(E)* In the condition 5 (shown in table 3, 4) we used a Hot-start Taq DNA polymerase. In addition, annealing time was increased. Positive

control still shows non-specific band and smear. However the smear and the non-specific bands are less than the previous condition. NTC is still positive. *(F)* In addition to using hot-start polymerase, the number of NCR cycles was also reduced (condition 6, table 3, 4). In Sample 7, the 123 bp band is observed with no non-specific bands. In sample 8, 123bp band and non-specific bands are observed, which can be due to high concentration of the sample. This condition was considered as the optimal condition of NCR for further experiments. *(G)* The optimum condition (condition 6, table 3, 4) was used to perform the NCR assay on the samples. Samples 6 and the NTC has no bands, and samples 2, 3, 7, and 8 has the 123bp band. The absence of 123bp band in sample 6 can be due to the deterioration of samples after a long time of storage. These results conforms that the NCR condition is optimal, and there is no contamination in the NCR mix, which means handling of samples and reagents were acceptable. The application of hot-start *Taq* DNA polymerase enzyme in optimal temperature (Tm) and timing (1st step: 95˚×25" and 72˚×30" at 25 cycles, 2nd step: 95˚×25" and 72˚×25" at 35 cycles) provided the amplification of the specific band at 123bp without undesirable product amplifications and smear (Figure 2G).

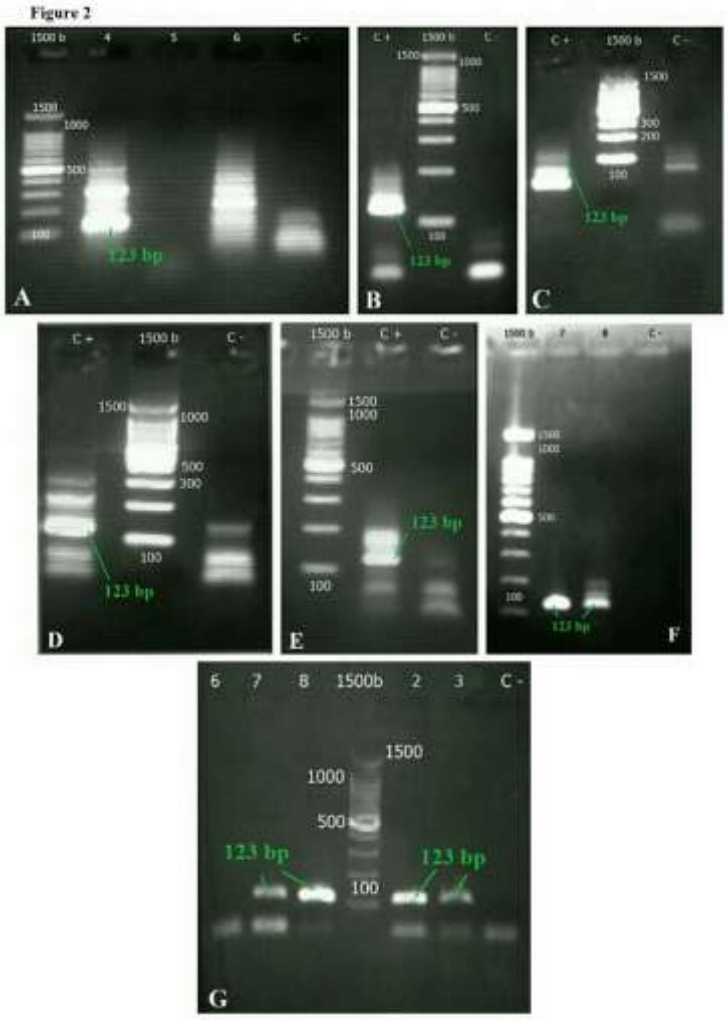


Figure 2. Optimization of mpt64-based N-PCR assay

4. Discussion

The purpose of this study was an attempt to the development of a direct NCR method using *mpt64* specific primers for rapid diagnosis of *MTB* infection to induce a cheap quantitative *mpt64*-based NCR assay.

Recently, in the diagnosis of TB, PCR has been widely used for the detection of *MTB* DNA as a more rapid, sensitive, and specific diagnostic method compared to the traditional microbiology detection likes direct smear and culture[1,2,7,11-13,16,18]. Using NCR assay has increased sensitivity and specificity of DNA amplification than the conventional single-step PCR assay [7, 8, 11, 16]. *MTB* DNA has been detected in different samples such as cerebro-spinal fluid (CSF), sputum, and blood [1, 2, 6]. Although CSF specimens are still used in *MTB* diagnosis, there is a great chance of false-negative results and contamination because of the laborious and invasive sample collection and low amount of the bacteria in it [11, 16]. Previously, many specific regions of *MTB* genome, including *IS6110* insertion elements, 65-kDa heat shock protein, 16SrRNA gene, MPT64 secreted protein, and others were evaluated by molecular assays [5, 9, 13, 15, 16].

The investigators suggest that MPT64 is one of the secreted proteins from *MTB* that has an important role in disease pathogenesis and may function as a specific virulence factor during infection. Therefore, the *mpt64* has been proved a useful target to diagnose TB patients, especially extra-pulmonary, using PCR method [5, 9, 13, 15, 16].

In this study by system optimization and development, due to the low concentration and complexity of the DNA septum, we investigated the ability of a simplified *mpt64*-based NCR assay for direct *MTB* DNA detection in positive pulmonary TB patients previously analyzed by Tavakkoliamol etal [17]. We also confirmed with high specificity of approximately 100% that these samples were *MTB*-positive using the simple and rapid *mpt64*-based NCR methods.

To our knowledge, there have been no reports that an inexpensive and accurate *mpt64*-based NCR method has been applied to directly detect *MTB* in sputum samples. Although there are a number of molecular studies that used a conventional single-step and real-time PCR to quantitatively determine various infectious pathogens in different biological samples, they are inaccurate or expensive and time-consuming [1, 3, 11, 13, 16-20].

5. Conclusion

By employing this optimized NCR assay we considered the ability of NCR to detect *MTB* DNA in sputum samples with small number of bacteria. We analyzed the clinical usefulness of this direct technique with regard to rapidity and accuracy of *MTB* diagnosis compared to other conventional methods, and we found out that our method is rapid, specific, accurate, and inexpensive[1,2,6,7,8,10,12,16,18,21].

Therefore, NCR assay based on *mpt64* has been shown to be a powerful tool for *MTB* detection in clinical laboratories.

The advantages of this molecular assay are rapid detection of *MTB* since the samples are analyzed directly, lowering the cost by using a non-probe-based method, and increasing the rapidity and precision of NCR method instead of routine bacteriological methods. In addition, using sputum samples, as an alternate for CSF, can reduce the damage stress to the patients.

The authors suggest developing the assay by using more clinical samples, preparing serial dilutions to determine the sensitivity and limit of detection of the assay, and using two genomic regions to eliminate false negative and false positive results. In addition, we suggest the preparation of a positive control by cloning a PCR amplified standard.

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Conflict of interest

The authors declare no conflict of interest.

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