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Construction of an internal amplification control for *Mycobacterium tuberculosis* polymerase chain reaction (PCR) test

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Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (MTB) which mostly affects the lungs. The disease causes deaths of many people every year. There are different methods to detect MTB such as skin test, staining, culture and molecular techniques. Polymerase chain reaction (PCR) is a simple and rapid method for the detection of MTB; however, positive and negative false results reduce the efficiency of this technique. The aim of this study was to design an internal amplification control (IAC) and apply it in MTB PCR test. PCR technique for MTB was optimized by using specific primers for IS6110 gene. The sensitivity and specificity of the test were determined. IAC was constructed with competitive strategy by PCR-cloning technique and the limitation range was determined. The PCR products of MTB and IAC were 245 and 660 bp, respectively on electrophoresis gel. The IC used in PCR testing of MTB is the competitive form in which the range was between 10 million and 10 bacteria and the most suitable internal control concentration for the mix was 1,000 plasmids. After making IC and using it in MTB amplification, it was observed that IC might guarantee the correctness of PCR reaction.

Key words: *Mycobacterium tuberculosis*, polymerase chain reaction (PCR), internal control.

INTRODUCTION

Tuberculosis (TB) is a lethal infectious disease which after decades of decline, showed an increase simultaneously with human immunodeficiency virus (HIV) incidence (Cole et al., 1998; Noordhoek et al., 1994). World Health Organization (WHO) has estimated that if the disease is not controlled, between 2000 and 2020, nearly one billion people worldwide will be infected with this bacterium, 200 million of this number will get sick and 35 million will die (Pauwels et al., 2001; Sohn et al., 2003). For this reason, TB has been announced a global threat by WHO (Fredricks and Relman, 1999). Nearly 8 million people are infected with tuberculosis every year, and it is estimated that one third of the world's population are infected with latent tuberculosis (Gordin and Masur, 2012). The oldest technique for detection of *Mycobacterium tuberculosis* (MTB) includes microbiological examination and culture. Microbiological examination of sputum is the cheapest and most accessible way in diagnosing pulmonary TB particularly in adults (Eisenach, 1990). Sputum culture is more sensitive than direct tests; however, the results

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need a 4- 8 weeks period. Sputum cultures, moreover, require skilled technicians and equipped laboratories which are not available everywhere (Van Soolhingen et al., 1993). The slow growth and non-sensitive methods particularly in drug-resistant forms in HIV patients have constrained MTB control (Boehme et al., 2010).

Since the old method for detection of drug-resistant MTB takes weeks or even months, rapid molecular techniques have been highly regarded (Barnard et al., 2008). Amplified nucleic acid and hybridization procedures techniques are able to detect microorganisms in patients' samples (Burkardt, 2000). The first and perhaps the best technique is target molecule PCR amplification. Today, this technique has been well positioned in genetic engineering, molecular biology, microbiology, cancer diagnosis and genetical disorders, etc (Nuovo,1995; Persing, 2003).

But the risk of contamination is unavoidable in normal laboratory conditions and can reduce PCR efficiency; this contamination can be from sources such as patient or laboratory work (Millar et al., 2002). One of the disadvantages of PCR is false negative results with a variety of possible reasons including personnel errors, technical problems and low amounts of target DNA in the sample. The most important problem is the lack of internal control (IC) in PCR technique (Burkardt, 2000). IC is a non-target DNA present in the reaction tube and is amplified with target gene. In PCR without IC, a negative result could mean that the target sequence is not in reaction but it can be also because the reaction is inhibited, which may be due to the improper thermal cycler, materials spurious PCR, or existence of inhibitory activity in the sample. But in PCR technique with an internal control, there is always a control signal even when there is no target sequence (Millar et al., 2002).

Recently, various types of IC in PCR technique were highly regarded by researchers. For examples, Rodriguez-Lazaro et al. 2004 used a common strategy to build IC. He used nucleic acid sequence based amplification (NASBA) technique in internal controls. In this year also Wieczorek and Odek (2004) designed an IC for detection of *Escherichia coli* Shiga-toxin producer. The aim of this study was to design an internal amplification control (IAC) for MTB detection to be used in various samples which may be undetected to cause existence of different inhibitors.

MATERIALS AND METHODS

Preparing template DNA

Standard strain, H37Rv DNA was obtained from Mycobacteriology Department of Masih Daneshvari Hospital and DNA was extracted by using DNG-plus kit (CinnaGen, Iran).

Optimization of PCR

Reaction involves 14 µl distilled water, 40 pmol of each INS1 and INS2 primers (17), 0.2 mM dNTP (dATP, dCTP, dGTP, dTTP), 2 U Taq DNA polymerase (BioFlux, Japan), 1.5 mM $Mgcl_2$, 1X PCR buffer and 5 µl template DNA. The mixture was subjected to a denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 45 s, annealing at 72°C for 1 min and final extension at 72°C for 7 min. PCR products were analyzed by electrophoresis and visualized on 1.5% agarose stained with SYBR green (SYBR safe CinnaGen, Iran). PCR target gene was IS6110 gene.

Sensitivity and specificity of PCR testing for MTB

The sensitivity of test was determined by these amounts of MTB DNA: 100,000, 10,000, 1,000, 100 and 50 fg. And also, the specificity of test was determined by these species of *Mycobacterium*: *M. tuberculosis, Mycobacterium chelonae, Mycobacterium fortuitum, Mycobacterium xenopi, Mycobacterium kansasii, Mycobacterium szulgai, Mycobacterium intracellulare, Mycobacterium gordonae* and *Mycobacterium avium*.

Internal control construction

To make IC of *Leishmania* gene, kinetoplast of 620 bp was used. IS6110 primers were added to two ends of this gene that increased the length of the gene to 660 bp (Mahboudi et al., 2001, 2002).

Optimizing PCR test for IC (pICMTB)

The PCR mixture contained 14 µl distilled water, 40 pmol of each primer, 0.2 mM dNTP Mix, 2 U Taq polymerase (BioFlux, Japan), 1.5 mM $Mgcl_2$, 1X PCR buffer and 5 µl template DNA (here IAC). The mixture was subjected to 40 cycles with a denaturation at 93°C for 40 s, annealing at 55°C for 30 s, extension at 72°C for 30 s and final extension at 72°C for 10 min. PCR products were analyzed by electrophoresis and visualized in a SYBR green (SYBR safe CinnaGen, Iran) stained with 1.5% agarose.

For making IAC for MTB, with competitive strategy, *Leishmania kinetoplast* primers were used (*Leishmania major*, P strain) (Mahboudi et al., 2001, 2002). Primer sequences are summarized in Table 1.

Cloning

The PCR product was cloned in pTZ57R vector by using T/A cloning kit (Fermentas, USA) and plasmid was extracted after cloning from recombinant bacteria. Plasmid extraction was performed by alkaline denaturation method and Bioneer kit (K-3112).

The appropriate concentration of the target gene and IC

Mycobacterium containing IC was prepared with different concentrations to optimize PCR testing. 5 fg DNA MTB is equal to one DNA (Pandy et al., 2008). Dilutions were prepared as follows: 100,000, 10,000, 1000, 100 and 50 fg. Also, the following dilutions were prepared for pICMTB: 1,000,000 to 1 pICMTB.

IAC-MTB PCR tests

PCR test for IAC-MTB was carried out in a total volume of 25 µlPCR mixture containing 13 µl distilled water, 1 µl of each ICRMTBF and ICRMTBR primer, 0.2 mM dNTP Mix, 2 U Taq polymerase (BioFlux, Japan), 1.5 mM $Mgcl_2$, 1X PCR buffer, 1 µl IAC and 5 µl template DNA. Temperature profile was the same as optimized temperature for MTB DNA amplification.

Table 1. MTB and IAC primers.

Primers name	Sequence
INS1	5'CGTG AGGG CATC GAGG TGGC3'
INS2	5'GCGT AGGC GTCG GTGA CAAA3'
ICRMTB	5'GCGT AGGC GTCG GTGA CAAA AAAG GGAT TGGT GTAA AATA GGC 3'
ICFMTB	5'CGTG AGGG CATC GAGG TGGC GCTC GCAG AACG CCCC TACC 3'

INS1, MTB forward; INS2, MTB reverse; ICRMTB, IC forward; ICFMTB, IC reverse.

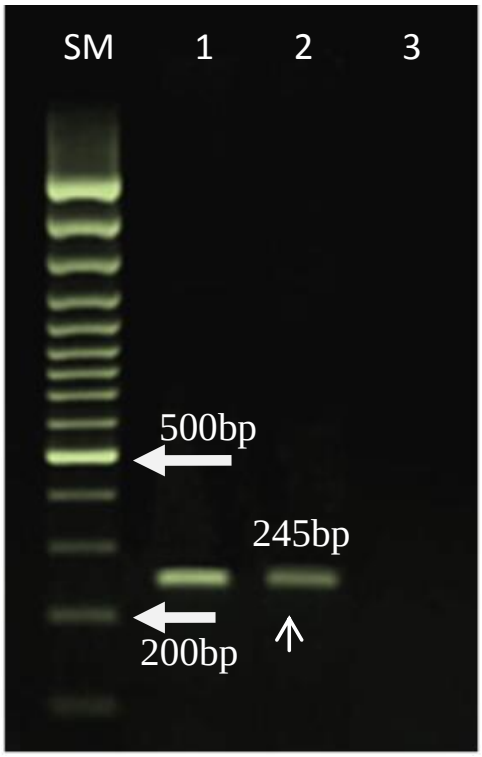

Figure 1. The result of IS6110 PCR on agarose gel 1.5%. SM, 100 bp DNA ladder (Fermentas, USA); 1, 2 , positive controls IS6110 fragment; 4, negative control.

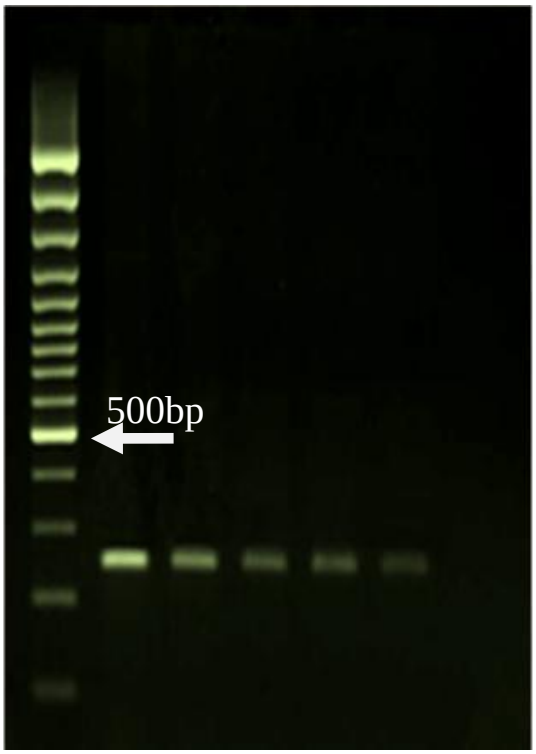

Figure 2. Sensitivity of PCR test for MTB. SM: 100 bp DNA ladder (Fermentas, USA). 1, 100,000 fg; 2, 10,000 fg; 3, 1000 fg; 4, 100 fg; 5, 50 fg; 6, negative control.

RESULTS

MTB PCR test

The target gene in this study was IS6110 (Hasan et al., 2012). The PCR production size of MTB with specific primers by using DNA of MTB (Standard strain, H37Rv by 1000 fg concentration) on agarose gel was 245 bp (Figure 1).

Specificity of the *M. tuberculosis, M. chelonae, M. fortuitum, M. xenopi, M. kansasii, M. szulgai, M. intracellulare, M. gordonae* and *M. avium* test strains were checked with 100% respective results. And sensitivity of the test was determined with different amounts of DNA (100,000, 10,000, 1000, 100 and 50 fg) (Figure 2). *M. tuberculosis* H37Rv DNA was used and the result shows that the sensitivity of test was 50 fg, equal to 10 bacteria.

Internal control construction

After construction of IC and cloning in pTz57R, PCR test was performed for the amplification of IC; the PCR product of IC was 660 bp (Figure 3).

In the cases where the test was positive for the presence of TB, two fragments were seen on the electrophoresis, one of them was 660 bp for IC and the other was 245 bp for IS6110 fragment (MTB). Because of the many differences between these two fragments, PCR products' differentiation was easy.

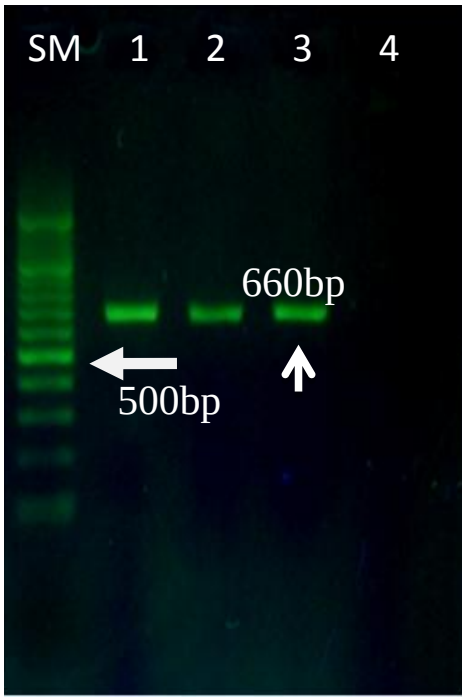

Figure 3. The result of PCR test for three positive clones (pICMTB1-3) on agarose gel 1.5%. SM, 100 bp DNA Ladder (Fermentas, USA); 1 to 3, positive clone (pICMTB1-3); 4, negative control.

The IC amplification

Determining appropriate rate of IC is very important because excessive amounts of IC can cause false negative results. By using different concentrations of IC, the most appropriate IC concentration was gotten. 1,000,000 to 1 plasmids containing IC used in PCR test and 1,000 plasmids containing IC was determined with the appropriate concentration. Then, this concentration of IC and different concentrations of target DNA were used in the PCR tests. In this DNA range, the best competition between target DNA and IC was observed and none of these inhibits DNA proliferation (Figure 4).

DISCUSSION

Many attempts have been made in the detection and treatment of TB, but the disease still remains a major problem for public health (Neonakis et al., 2008). Traditional methods in detecting TB, because of the restrictions created, forced researchers to accurately optimize their research in the field and perform diagnostic tests (Cheng et al., 2005). The most important problem with TB diagnosis is the lack of efficient and simple diagnostic methods with higher sensitivity and specificity than the traditional methods (Neonakis et al., 2008; Poon et al., 2006). Time and detection of TB in patients is very useful in controlling and fighting the disease; therefore, researchers have focused on rapid diagnostic methods for routine diagnosis of TB (Stead et al., 1995; Iwamoto et al., 2003). Nowadays, PCR is widely used for the detection of MTB (Brett-Major and Walsh, 2006; Huggett et al., 2003; Mori and Notomi, 2009). Although, this technique is simple and rapid, it has problems. The most important problem is contamination (foreign bacteria DNA and environmental inhibitors).

In infectious diseases' diagnosis with high incidence such as TB, the inhibitors are responsible for the false negative results (Sachadyn and Kur, 1998). Different primers can be used for a variety of genes that exist in other bacteria which is due to the simultaneous amplification of target DNA with none-target DNA; thus, these problems reduce the efficiency of the PCR test. Additionally, contamination increases false positive results and impairment in molecular tests. The use of IC is essential to overcome such problems (Burkardt, 2000). The aims of this research were to construct and apply internal control in PCR test and provide a solution to eliminate such problems.

Regardless of whether or not there is an IC signal, samples of target signal are considered positive and if there is a signal to the internal control, samples without signal are considered negative. The samples without any signal (no signal for target DNA and no signal for IC) are considered as inhibited samples (Jones et al., 2000).

Various genes can be taken into account for IC; for instance, Jones et al. (2000) used *λ Bacteriofage* to make an IC for validation of detecting congenital *Cytomegalovirus*. This IC was 150 bp and target gene was 100 bp; therefore, it does not seem to be suitable because the size difference between IC and target gene must be relatively high (Jones et al., 2000). Also, Kolk et al. (1994) designed 301 bp IC for MTB and the target gene was 145 bp, lacking sufficient difference in size. In this study, we have used the fragment for IC with suitable difference in the size of the target gene.

Burggraf and Olgmoler used a plasmid as an IC for MTB; they added this IC directly in samples (Burggraf and Olgomoller, 2004), therefore, the rate of IC may be reduced in extraction steps, while in our study, the IC was added in amplification stage, so the sensitivity of the test increased.

Cortez-Herrera (2008) used pAMP linear plasmid and made an internal control that was 660 bp. Our IC was 660 bp too, but we used *Leishmania* kinetoplast gene. The advantage of using this gene is the built IC's ability to amplify the target gene in optimized temperature. Accordingly, this gene was used for making IC of variety of microorganisms such as: *Salmonella*, *Herpes Simplex Virus*, *Mycoplasma* and *Hepatitis B Virus*.

Dreier et al. (2005), used Replicase Bacteriophage gene of MS2 to make a non-competitive IC for real time PCR technique, he also pointed out that the competitive IC can reduce amplification of the target gene and

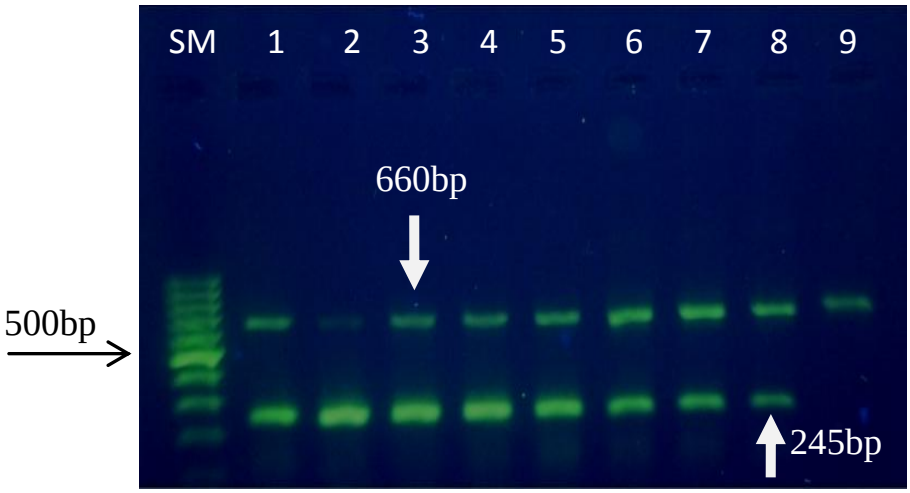

Figure 4. The results of MTB and IC in PCR test. SM: 100 bp DNA ladder (Fermentas, USA). 1, positive control with 1000 MTB DNA and 1,000 plasmids of IC (pIC MTB 1); 2, positive control with 100,000,000 MTB DNA and 1,000 plasmids of IC; 3, positive control with 10,000,000 MTB DNA and 1,000 plasmid of IC; 4, positive control with 1,000,000 MTB DNA and 1,000 plasmids of IC; 5, positive control with 100,000 MTB DNA and 1,000 plasmids of IC; 6, positive control with 10,000 MTB DNA and 1,000 plasmids of IC; 7, positive control with 1,000 MTB DNA and 1,000 plasmids of IC; 8, positive control with 100 MTB DNA and 1000 plasmids containing of IC; 9, positive control with 10 MTB DNA and 1000 plasmids of IC.

detection range (Dreier et al., 2005). In non-competitive strategy explained by Hoorfar (2004), although it is not necessary to design IC, a separate primer is needed for IC (Hoorfar et al., 2004). It seems that using an internal control in each PCR reaction for detection pathogens is evadible. In this study, competitive strategy was used because using IC that has the binding regions to the same primer-binding by target genes can eliminate the need to add a set of specific primers for the IC. Finally, with respect to this result and comparing PCR with IC and without IC, it can be stated that PCR technique with IC in spite of simplicity and not needing other confirmatory tests has high sensitivity over PCR without IC and could be a proper replacement for it.

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