

Design and Construction of a Eukaryotic Cloning Vector Encoding the *mpt51* Gene of *Mycobacterium tuberculosis*

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Abstract

Background: Tuberculosis (TB) is the leading cause of death by infectious diseases worldwide, and especially prevalent in developing countries. Several vaccines against TB have been developed, recently. The aim of the present study was to design and construct a cloning vector encoding *Mycobacterium tuberculosis* (MTB) *mpt51* gene.

Methods: DNA was extracted from MTB H37Rv strain. Gene-specific primers were designed using Gene Runner software and the *mpt51* gene was amplified by PCR. The amplified fragment and pcDNA3.1(+) cloning vector were both digested with restriction enzymes, the *mpt51* fragment was ligated into the vector, and the *Escherichia coli* (*E. coli*) TOP10 strain were transformed by the recombinant plasmid. Positive clones were identified by colony PCR, restriction enzyme digestion, and DNA sequencing.

Results: The *mpt51* gene was successfully cloned into pcDNA3.1(+). A 6400 bp band for the pcDNA3.1(+)/*mpt51* recombinant plasmid and a 926 bp band for *mpt51* were observed by colony PCR, and restriction enzyme digestion on agarose gels. The DNA sequence was 100% homologous with the *mpt51* fragment of H37Rv in GenBank.

Conclusions: In the current study, the *mpt51* gene of MTB was correctly cloned into pcDNA3.1(+). The expression of this recombinant vector can be studied in eukaryotic cells. Moreover, it is possible to determine the efficacy of this vector as a DNA vaccine candidate, and to test its protective function compared to BCG in animal models in future.

Keywords: Molecular Cloning, MPT51 antigen, *Mpt51* gene, *Mycobacterium tuberculosis*.

Introduction

Tuberculosis (TB) is an old contagious disease caused by *Mycobacterium tuberculosis* (MTB) (1, 2), with a high mortality rate. According to the World Health Organization estimations, approximately one-third of the world's population are infected with MTB, and more than 2 million people suffer from active TB (3-5). Besides, TB causes near 3 million deaths annually (6). In general, TB is a chronic disease with slow progression mostly remain undiagnosed for decades. The common form of TB in adults is chronic pulmonary disease, while extra-pulmonary TB is especially

common in children and HIV-co-infected patients (7). A successful tuberculosis control program requires the identification of appropriate cases and the ability to categorize the exact disease situation, due to the lack of full understanding of known immune responses to the disease (8, 9). The only available vaccine for human TB is Bacillus Calmette-Guérin (BCG) (10, 11). According to the vaccination program, BCG is injected immediately after birth or after the first day of exposure to MTB (10). Although BCG administration to infants has been found to be effective against severe childhood

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Received: 12 Jan, 2017; Accepted: Mar 17, 2018

TB in endemic regions, its protection declines gradually with time and may last less than 15 years. This results in highly variable efficiency that seems insufficient to control pulmonary TB in adults (10, 12, 13). In 1992, Tang and colleagues showed that injection of DNA induced immune responses (7, 9). In many studies, it has been observed that DNA vaccines containing MTB antigens induce protection against primary TB infections. Also these vaccines have been used as boosters after BCG vaccination to enhance immune responses (14). Antigens such as Hsp60, Hsp70, Ag85, ESAT-6, and CFP10 are new TB vaccine candidates as well as new diagnostic factors (8). MPT51 is involved in cell wall and possesses a mycolyltransferase activity, which is required for the biogenesis of trehalosedimycolate, also known as cord factor, a dominant structure necessary for the maintenance of cell wall integrity. It is a secretory component and stimulates humoral and cellular immunity (15). The aim of this study was to clone the gene encoding MPT51 (*mpt51*) in pcDNA3.1(+).

Materials and methods

DNA extraction

The standard strain of MTB (H37Rv) was obtained from the Shariati Hospital, Mashhad, Iran. The strain was cultured in Lowenstein Jensen medium (Merck) and incubated at 37 °C for 45 days. Three to four colonies were suspended and homogenized in 400 µl of 100 mM (Tris buffer, 0.05% Tween 20, pH 7.5). Then 20 µl of 18.5 mg/ml proteinase K (Fermentas) was added and incubated at 55 °C for 3 hr. To inactivate the enzyme and DNA extraction, samples were boiled for 10 min and then centrifuged at 21952 g. The supernatant containing the DNA was collected and stored to be used for PCR amplification.

Polymerase chain reaction for *mpt51* amplification

Mpt51-specific primers were designed using Gene Runner software. The PCR mixture contained the following ingredients: 10 pmol/µl of each forward (*mpt51*-F: 5'-AGATAAGGATCCACCATGGGAAGGGTCGGTGGCGCT-3') and reverse (*mpt51*-R: 5'-TCGGCAGAATTCTTAGCGGATCGCACCGACGATATCGC-3') primers (Cinnagen), 1 µl (100 ng) of DNA sample, 25 mM MgCl₂

(Cinnagen), 10 mM of each dNTP (Fermentas), and 5 U/µl of *Taq* DNA polymerase (Cinnagen). The final reaction volume was 25 µl. The first cycle of the PCR was 5 min at 95 °C followed by 40 cycles of one min at 95 °C, 1 min at 58 °C, and 1 min at 72 °C followed by a final cycle of 10 min at 72 °C. Then, total PCR product was electrophoresed on a 1% agarose gel. The amplified *mpt51* fragment was purified with a gel purification kit according to instructions provided by the manufacturer (Bioneer Gel purification kit).

Cloning of *mpt51* into pcDNA3.1(+) vector

Both the purified PCR product and pcDNA3.1(+) were digested with *Eco*RI and *Bam*HI restriction enzymes (Fermentas), electrophoresed, purified from the gel using a gel extraction kit (Bioneer Gel purification kit), and ligated with T4 DNA ligase (Fermentas Company). Competent *E. coli* strain TOP10 cells were prepared using the cold solution of CaCl₂/MgCl₂ (0.1 M) as previously described (16, 17). The cells were transformed using heat shock, and cultured on LB agar containing 100 µg/ml ampicillin (18) for 16 h at 37 °C.

Confirmation of *mpt51* cloning into pcDNA3.1(+) vector

Cloning of *mpt51* into pcDNA3.1(+) was confirmed by colony PCR using *mpt51*-specific primers, enzymatic digestion with *Eco*RI, and DNA sequencing.

Results

Figure 1 shows the result of the electrophoresis of the PCR product. It demonstrates a single band of 926 bp related to *mpt51* gene of interest.

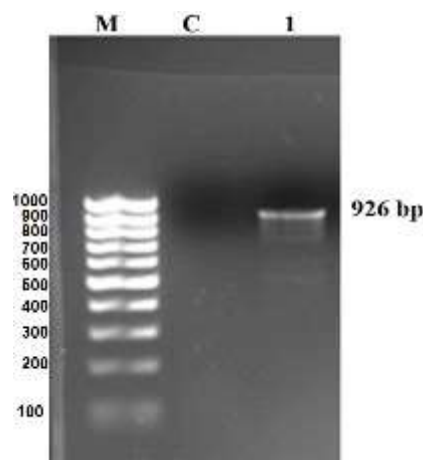


Fig. 1. The figure shows an agarose gel of the *mpt51*PCR product; M: 100 bp DNA size marker (Pars Tous Cat No: DM01-01); C: negative control; 1: 926 bp *mpt51* PCR product.

Transformed colonies were identified by colony PCR, which amplified a 926 bp product and digestion with *EcoRI* (not shown).

The recombinant vector was sequenced (Macrogen) and the *mpt51* sequence was found to be 100% homologous with the *mpt51* fragment of H37Rv in GenBank.

Discussion

BCG vaccine is the only vaccine against tuberculosis that is available. In many countries, this vaccine has made children well-immune, but has not been effective in adults especially against pulmonary TB (21). Efforts have been made to find drugs and vaccines against TB based on identifying new MTB antigens. Complete understanding of the cell wall component of MTB was a great help in introducing these antigens (22, 23). A cell wall protein of MTB named MPT51 along with the antigen 85 complex contributes to a high affinity to fibronectin. This antigen also plays a role in the synthesis of trehalose. It is also essential to maintain the integrity of the cell wall (15).

The secreted protein MPT51 is a major protein in culture filtrates of *Mycobacterium bovis* BCG and immunologically cross reacts with the fibronectin-binding 85 complex secreted by this bacterium. Ag85 complex molecules encode proteins to bind fibronectin and to activate mycoyl transferase which are involved in the final stage of assembly of

mycobacterium cell walls. Ag85A and Ag85B have been shown to stimulate cellular and hemorrhagic immunity. Immunity against these antigens can protect animal models against pulmonary tuberculosis. MPT51 which is dependent on the complex of antigen 85 can also provide protective responses against pulmonary tuberculosis (22).

As it was shown that MPT51 is an immunodominant antigen; the use of the *mpt51*-based serodiagnostic assay could serve as an addendum to sputum microscopy for even confirming the conventional diagnosis of pulmonary TB (23).

In the present study, *mpt51* of MTB was cloned into pcDNA3.1(+), which contains the human cytomegalovirus (CMV) promoter. This promoter allows high expression of recombinant proteins. In this way, this feature can also be used to provide subunits for protein vaccines.

The accuracy of cloning was confirmed by colony PCR using *mpt51*-specific primers, enzymatic digestion with *EcoRI*, and DNA sequencing. The ability of the vector to express protein will be determined in future studies, and it will also be examined as a DNA vaccine candidate to induce effective immune responses.

Acknowledgment

This work was supported financially by the Mashhad University of Medical Sciences, Mashhad, Iran.

References

1. Sato K, Tomioka H, Shimizu T, Gonda T, Ota F, Sano C. Type II alveolar cells play roles in macrophage-mediated host innate resistance to pulmonary mycobacterial infections by producing proinflammatory cytokines. *J Infect Dis* 2002;185(8):1139-47.
2. Akhavan R, Meshkat Z, Khajekaramadini M, Meshkat M. Eight-year study of *Mycobacterium tuberculosis* in mashhad, northeast of iran. *Iranian Journal of Pathology*. 2013;8(2):73-80.
3. Rezaei A, Abdi A. B.C.G usage rate at health centers of ilam medical university. 2002.
4. Asnaashari A, Sadrizadeh A, Ahmadi H, Meshkat M, Gholoobi A, Talab F, et al. The study of *Mycobacterium tuberculosis* in Iranian patients with lung cancer. *Jundishapur J Microbiol*. 2013; 6:237-41.
5. Gholoobi A, Masoudi-Kazemabad A, Meshkat M, Meshkat Z. Comparison of Culture and PCR Methods for Diagnosis of *Mycobacterium tuberculosis* in Different Clinical Specimens. *Jundishapur J Microbiol* 2014;7(2).
6. D'Souza S, Denis O, Scorza T, Nzabintwali F, Verschuere H, Huygen K. CD4+ T cells contain *Mycobacterium tuberculosis* infection in the absence of CD8+ T cells in mice vaccinated with DNA encoding Ag85A. *Eur J Immunol* 2000;30(9):2455-9.
7. Jiang Q, Zhang J, Chen X, Xia M, Lu Y, Qiu W, et al. A novel recombinant DNA vaccine encoding

- Mycobacterium tuberculosis ESAT-6 and FL protects against Mycobacterium tuberculosis challenge in mice. *J Biomed Res* 2013;27(5):406.
8. Tucci P, González-Sapienza G, Marin M. Pathogen-derived biomarkers for active tuberculosis diagnosis. *Front Microbiol* 2014;5.
 9. Walzl G, Haks M, Joosten S, Kleynhans L, Ronacher K, Ottenhoff T. Clinical immunology and multiplex biomarkers of human tuberculosis. *Cold Spring Harb Perspect Med* 2015;5(4):a018515.
 10. Suzuki M, Aoshi T, Nagata T, Koide Y. Identification of murine H2-Dd-and H2-Ab-restricted T-cell epitopes on a novel protective antigen, MPT51, of Mycobacterium tuberculosis. *Infect Immun* 2004;72(7):3829-37.
 11. Baldwin S, D'souza C, Orme I, Liu M, Huygen K, Denis O, et al. Immunogenicity and protective efficacy of DNA vaccines encoding secreted and non-secreted forms of Mycobacterium tuberculosis Ag85A. *Tubercle and Lung Disease*. 1999;79(4):251-9.
 12. Lu J, Wang C, Zhou Z, Zhang Y, Cao T, Shi C, et al. Immunogenicity and protective efficacy against murine tuberculosis of a prime-boost regimen with BCG and a DNA vaccine expressing ESAT-6 and Ag85A fusion protein. *Clin Dev Immunol* 2011;2011.
 13. Montagnani C, Chiappini E, Galli L, de Martino M. Vaccine against tuberculosis: what's new? *BMC Infect Dis* 2014;14(Suppl 1):S2.
 14. Lowrie DB, Tascon RE, Bonato VL, Lima VM, Faccioli LH, Stavropoulos E, et al. Therapy of tuberculosis in mice by DNA vaccination. *Nature*. 1999;400(6741):269-71.
 15. Lew JM, Kapopoulou A, Jones LM, Cole ST. TubercuList-10 years after. *Tuberculosis*. 2011;91(1):1-7.
 16. Baghani A, Youssefi M, Safdari H, Teimourpour R, Meshkat Z. Designing and construction pcDNA3.1 vector encoding cfp10 gene of Mycobacterium tuberculosis Jundishapur *J Microbiol*. 2015;8(10).
 17. Rashidian S, Teimourpour R, Meshkat Z. Designing and construction of a DNA vaccine encoding tb10.4 gene of Mycobacterium tuberculosis. *Iranian Journal of Pathology*. 2016;11(2):112-9.
 18. Teimourpour R, Sadeghian A, Meshkat Z, Esmaelizad M, Sankian M, Jabbari A. Construction of a DNA vaccine encoding Mtb32C and HBHA genes of Mycobacterium tuberculosis. *Jundishapur Journal of Microbiology*. 2015;8(8).
 19. Nabavinia M, Naderi Nasab Mi, Meshkat Z, Derakhshan M, Khaje-Karamadini M. Construction of an Expression Vector Containing Mtb72F of Mycobacterium tuberculosis. *Cell Journal (Yakhteh)*. 2012;14(1):61.
 20. Meshkat Z, Mirshahabi H, Soleimanjahi H, Mohamad Hassan Z. Construction a DNA Vaccine Containing Human Papillomavirus Type 16 Early Genes as a Potential Vaccine for Cervical Cancer Prevention and Therapy. *Iranian Journal of Pathology*. 2009;4(2):65-70.
 21. Deitch EA, Berg R, Specian R. Endotoxin promotes the translocation of bacteria from the gut. *Arch Surg* 1987;122(2):185.
 22. Miki K, Nagata T, Tanaka T, Kim YH, Uchijima M, Ohara N, et al. Induction of protective cellular immunity against Mycobacterium tuberculosis by recombinant attenuated self-destructing Listeria monocytogenes strains harboring eukaryotic expression plasmids for antigen 85 complex and MPB/MPT51. *Infect Immun* 2004;72(4):2014-21.
 23. Achkar M, Dong Y, Holzman R.S, Belisle J, Kourbeti I, Sherpa T, Condos R, Rom W, Laal S. Mycobacterium tuberculosis Malate Synthase- and MPT51-Based Serodiagnostic Assay as an Adjunct to Rapid Identification of Pulmonary Tuberculosis. *Clin Vaccine Immunology*. 2006; 13(11): 1291-93
 24. Nabavinia MS, Nasab MN, Meshkat Z, Derakhshan M, Khaje-Karamadini M. Construction and evaluation of an expression vector containing Mtb32C (Rv0125) of Mycobacterium tuberculosis. *Avicenna Journal of Medical Biotechnology*. 2011;3(4):207.
 25. Silva B, da Silva E, do Nascimento I, dos Reis M, Kipnis A, Junqueira-Kipnis A. MPT-51/CpG DNA vaccine protects mice against Mycobacterium tuberculosis. *Vaccine*. 2009;27(33):4402-7.