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Construction of a Novel DNA Vaccine Candidate Encoding an HspX-PPE44-EsxV Fusion Antigen of *Mycobacterium tuberculosis*

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Abstract

Background: *Mycobacterium tuberculosis* is the causative agent of tuberculosis (TB). *Bacille Calmette-Guerin* (BCG) vaccine, is not effective in adults, therefore, many efforts have been made to produce an effective adult TB vaccine. The aim of this study was to develop a new tuberculosis DNA vaccine candidate encoding a recombinant HspX-PPE44-EsxV fusion antigen of *M. tuberculosis*.
Methods: A fusion DNA segment consisting of HspX, linker, PPE44, linker, and EsxV, after codon optimization, was designed. The fusion DNA was cloned and its sequence confirmed. Then, expression of a recombinant pcDNA3.1 (+)/HspX-PPE44-EsxV plasmid in Chinese hamster ovary (CHO) cells was verified by RT-PCR and Western-blot analysis.
Results: A 1968 bp band in RT-PCR and a 68 kDa band on Western-blot analysis confirmed transcription and expression of recombinant *hspX-ppe44-esxV* in eukaryotic cells.
Conclusions: A recombinant DNA segment encoding the HspX-PPE44-EsxV fusion antigen of *M. tuberculosis* was constructed and considered to be tested as a new TB DNA vaccine candidate.

Keywords: DNA vaccine, EsX, Hsp, *Mycobacterium tuberculosis*, PPE44

Introduction

Mycobacterium tuberculosis, the cause of tuberculosis (TB) in humans, is a serious public health threat (1). The World Health Organization (WHO) estimates that one-third of the world's population is infected with *M. tuberculosis* (2-6). In addition, infections with multi-drug resistant (MDR) and extensively-drug resistant (XDR) strains of *M. tuberculosis* have widely increased (4). Global control of TB faces serious problems, including the difficulty of rapid diagnosis, lack of efficient vaccines, concurrent TB and HIV infections, prolonged chemotherapy, and MDR and XDR resistance. The design and production of a new vaccine to reduce the 8-10 million new TB infections each year is essential (7, 8).

In 2012, about 8.6 million new infections were reported (9).

The aim of the WHO is to reduce TB infections, and three tools are required to achieve that: 1) development of rapid diagnosis, 2) development of more effective vaccines than those presently available, and 3) provision of an effective treatment regimen (7, 8). To date, various types of vaccines against *M. tuberculosis* have been designed to establish immunity. These vaccine types include subunit, recombinant, attenuated, and DNA (10). Currently, DNA vaccines have shown the most promise for long-lived and stable immune responses. They are also safer than attenuated vaccines, particularly

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in patients with AIDS, and easier to produce and maintain without the need for refrigeration (11, 12). Long lasting immunity due to the ongoing genes expression has been an advantage of DNA vaccines, as seen with the plasmid containing ESAT-6, Ag85A, and hsp genes, which reduced the bacterial load in a murine model, so DNA vaccines can also act as adjutants (13-17). DNA vaccines containing antigens of *M. tuberculosis* conferred protection against primary infection and their use as boosters greatly increased cellular immune responses simultaneously with or after BCG administration (9, 10). *M. tuberculosis* is an intracellular pathogen; therefore, cellular immune responses have important roles in infection control, particularly by secreted IFN-γ from CD4+ and CD8+ T-cells. Mycobacterium antigens such as Hsp60, Hsp70, ESAT-6, PPE44, and HspX, which can induce cellular immune responses, are new candidates for vaccine production and TB diagnosis (14, 18). Studies have shown that stronger immunity is induced with multiple than with single antigens (19). The *M. tuberculosis* PPE44antigen is a protein with Pro-Pro-Glu at the N-terminus. This antigen has specified epitopes that are exclusively detectable by MHC I and MHC II, and when used as a subunit or DNA vaccine, has provided good protection (9, 18). Esat-6 family proteins also are immunodominant antigens that trigger IFN-γ production by memory T cells and effectively protect against TB (20). In a study of TB patients, it was reported that EsxV (Rv19c) and EsxW (Rv3620c) proteins are suitable vaccine candidates because they are specific T cell targets (21). Another potential candidate is HspX, a 16-kDa antigen that induces host immunity against primary and activated secondary TB infection (15). Synthesis of this protein increases during the stationary growth phase (22, 23). A recent study showed that IFN-γ levels related to HspX are higher in TB patients than in BCG-vaccinated individuals (24). Because of the low efficiency of the BCG vaccine, a new TB vaccine is urgently needed. Therefore, construction of a novel DNA vaccine was the aim of this project. In this study a DNA vaccine encoding an *M. tuberculosis* HspX-PPE44-EsxV fusion antigen was constructed. This vaccine candidate contains a new fusion antigen with both antigenic and immunogenic properties.

Materials and Methods

Design of the HspX-PPE44-EsxV fusion construct

The HspX-PPE44-EsxV fusion DNA containing *hspX*, linker, *ppe44*, linker, *esxV* sequences, and a poly-histidine tag was designed. Optimization and rare codon analysis of the fusion segment was performed by software and online tools (http://www.genscript.com/cgibin/tools/rare_codon_analysis). *Bam*HI and *Xba*I restriction enzyme sites and the Kozak sequence were considered for the pcDNA3.1(+) vector. To allow the appropriate function of each component, linkers were inserted between HspX, PPE44, and EsxV sequences and six histidine sequences were added to the segment end (Fig. 1).

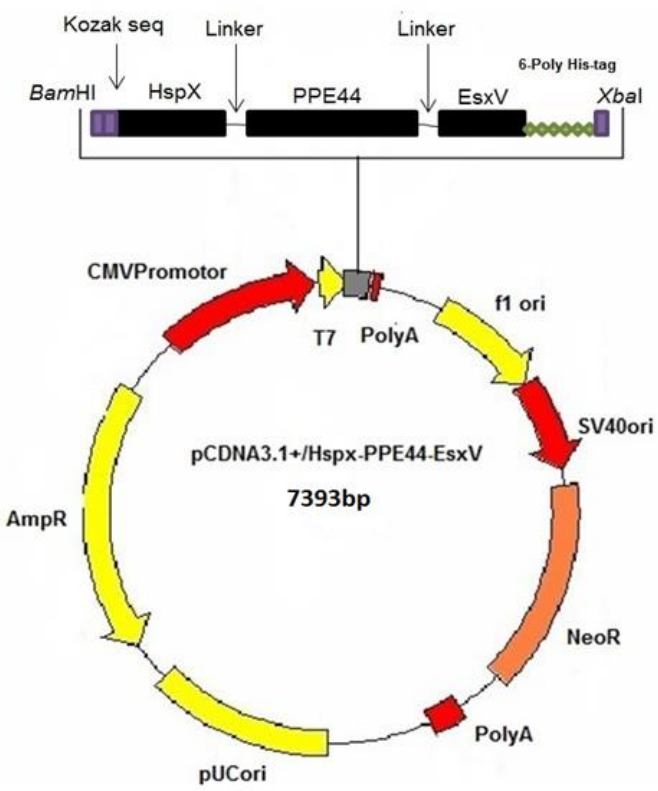

Fig. 1. Schematic map of the pcDNA3.1 (+)/HspX-PPE44-EsxV-His plasmid. The fusion segment consisting of hspX, linker, ppe44, linker, esxV sequences, and a 6-polyhistidine-tag was designed between the *Bam*HI and *Xba*I restriction sites of pcDNA3.1 (+) downstream of the CMV promoter.

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Construction of the recombinant pcDNA3.1 (+) /HspX-PPE44-EsxV vector

Our optimized HspX-PPE44-EsxV fusion segment was synthesized at the Generay Company (China). After synthesis, the fusion segment was cloned into the pGH vector (Generay Biotech Co, China) at *Bam*HI and *Xba*I restriction sites at the 5´and 3´ ends. Sequencing showed that the fusion DNA was inserted correctly into the multi-cloning site of the vector. Then the HspX-PPE44-EsxV fusion segment was sub-cloned into pcDNA3.1 (+) at the *Bam*HI and *Xba*I sites. Sequencing confirmed the correct insertion of the fusion construct in pcDNA3.1 (+). To propagate the construct, competent *E. coli*, Top-10 strain were transformed with the recombinant vector. Then, an AccuPrep Nano-Plus Plasmid Extraction Kit (Bioneer, South Korea) was used to extract pcDNA3.1 (+) carrying the fusion DNA.

Colony-PCR and restriction enzyme analysis

To verify the presence of the fusion segment in pcDNA3.1 (+) in the growing colonies, colony-PCR using the T7 forward primer and BGH reverse primer was performed and the products digested with *Bam*HI and *XbaL* (Fermentas, Germany). For colony-PCR, the T7 forward primer (5'-TAATACGACTCACTATAGG -3') and the BGH reverse primer (5'-TAGAAGGCACAGTCGAGG -3'), each at 10 pmol/µl (Macrogen, South Korea), Master mix (Pars-Toos, Iran), and a single bacterial colony as the source of the template DNA, were used. DNA was amplified for 25 cycles (95 °C for 1 min, 57 °C for 1 min, and 72 °C for 1 min). For restriction enzyme analyses, a mixture of pcDNA3.1(+)/HspX-PPE44-EsxV, 10x buffer, and *Bam*HI and *Xba*I restriction enzymes were incubated at 37 °C for 2 hours.

Detection of recombinant pcDNA3.1 (+)/HspX-PPE44-EsxV plasmid expression

For transfection, 10^6 Chinese hamster ovary **(**CHO**)** cells were seeded into a 6-well micro-plates and incubated overnight in RPMI 1640 (Bioidea, Iran) with 10% fetal bovine serum. When the cells reached 80% confluency, they were transfected with 1 µg of recombinant pcDNA3.1 (+)/PPE44-EsxV-HspX plasmid in 2 µl of Escort-IV transfection reagent (Sigma Aldrich, USA). After 48 hours, the cells were collected**.**

To verify the entrance and transcription of pcDNA3.1 (+)/HspX-PPE44-EsxV-, RT-PCR was performed. RNA from transfected cells was extracted using an RNA extraction kit (Pars-Toos, Iran). Two µl of purified RNA were added to the final mixture for cDNA synthesis. Then the cDNA was used as the template to amplify the gene using hspX forward (5'-TGGGATCCATCATGGCTG -3') and esxV reverse primers (5'-TGTCCATGGGCATTGGCTTG -3'), each at 10 pmol/µl (Macrogen, South Korea).

To verify expression of the HspX-PPE44-EsxV fusion protein, Western blots were performed. First the CHO cell extracts were prepared. To prevent protease activity, phenylmethylsulfonyl fluoride (PMSF) (Invitrogen, USA) was added to the cell extracts. Then, the cellular extracts were heated at 95 °C for 10 min and subjected to 12% SDS-PAGE. The proteins were transferred to polyvinylidine difluoride (PVDF) membranes (BioRad, USA) using a dry electroblot system (BioRad, USA) and transfer buffer containing 25 mM Tris (pH 8.3), 192 mM glycine, and 20% methanol at 0.35 A for 40 min at room temperature. The membrane was blocked for 16-18 hours with 5% (w/v) bovine serum albumin (BSA) in TBS-T buffer (0.5 M NaCl, 0.02 M Tris pH 8.5, 0.05% Tween 20) and then then incubated for 2 hours at room temperature with mouse anti-His Tag antibody (AbD Serotec, UK), diluted 1:5000 in TBS-T. After incubation with initial antibody, the blot was washed four times with TBS-T and then incubated with peroxidase-conjugated rabbit-anti-mouse IgG antibody (AbD Serotec, UK), diluted 1:5000 in in TBS-T. The blot was washed four times with TBS-T and then incubated with substrate for 2 minutes. Bands were detected immunochemically by enhanced chemiluminescence (ECL) (Thermo Fisher Scientific, USA).

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Results

Construction of the recombinant pcDNA3.1 (+)/HspX-PPE44-EsxV vector

The optimized HspX-PPE44-EsxV fusion segment was synthesized in the pGH vector by Generay Company (China) in *Bam*HI and *Xba*I restriction sites at the 5´and 3´ ends. Then the generated HspX-PPE44-EsxV, along with vector, was digested with *Bam*HI and *Xba*I restriction enzymes and ligated into pcDNA3.1 (+) (Invitrogen, USA) in *Bam*HI and *Xba*I restriction sites at the 5´and 3´ ends (Fig. 2). This plasmid was used to transform competent *E. coli*, Top-10 strain. Agarose gel electrophoresis of the *Bam*HI and *Xba*I digested plasmid showed the expected size bands of 1968 and 5428 bp, representing the insert and pcDNA3.1(+), respectively (Fig. 3a). Undigested plasmid electrophoresed as a single band bp (Fig. 3b). Also sequencing showed that the fusion DNA was inserted correctly into the multi-cloning site of pcDNA3.1 (+) .

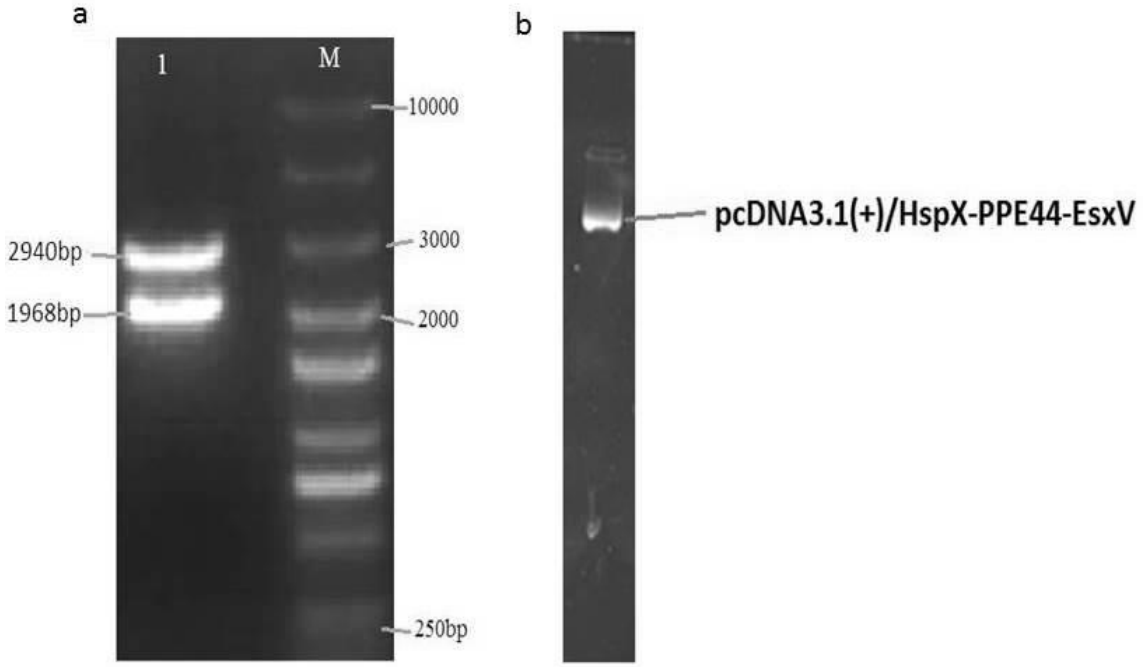

Fig. 2. Agarose gels of recombinant vector. a: digestion of pGH/HspX-PPE44-EsxV by *Bam*HI and *Xba*I. lane 1: pGH (2940 bp) and HspX-PPE4-EsxV (1968 bp); lane; M: 1kb DNA size marker. B: Ligated pcDNA3.1 (+)/hspX-ppe44-esxV. The band is Recombinant pcDNA3.1 (+) /HspX-PPE4-EsxV plasmid after extraction from transformed top-10 strain.

Colony-PCR and restriction enzyme analysis

To verify the presence of the fusion segment in pcDNA3.1(+) in the growing colonies, colony-PCR using the T7 forward and BGH reverse primers, followed by enzymatic digestion with *Bam*HI and *Xba*I were performed. Colony-PCR and double-digestion products were visualized on 1.2% agarose gels. In the Colony-PCR, a 1968 bp band representing the HspX-PPE44-EsxV fusion segment was seen, in the agarose gels, (Fig. 3a, lane 1). In the restriction enzyme analysis the bands of 1968 and 5428 bps representing the fusion construct and pcDNA3.1 (+), respectively, resulted digestion of the purified plasmid with *BamH*I and *XbaL* were seen. (Fig. 3b, lane 1).

Detection of pcDNA3.1 (+)/HspX-PPE44-EsxV expression

10^6 CHO cells/well were seeded into wells of 6-well micro-plates and incubated overnight. When the cells reached 80% confluency, they were transfected with pcDNA3.1 (+)/HspX-PPE44-EsxV in Escort IV transfection reagent (Sigma Aldrich, USA) according to the manufacturer's instructions.

To confirm HspX-PPE44-EsxV transcription, RT-PCR was performed on RNA isolated from the transfected cells. Agarose gel electrophoresis of the RT-PCR product identified a band of the expected size of 1968 bp (Fig. 4, lane 1).

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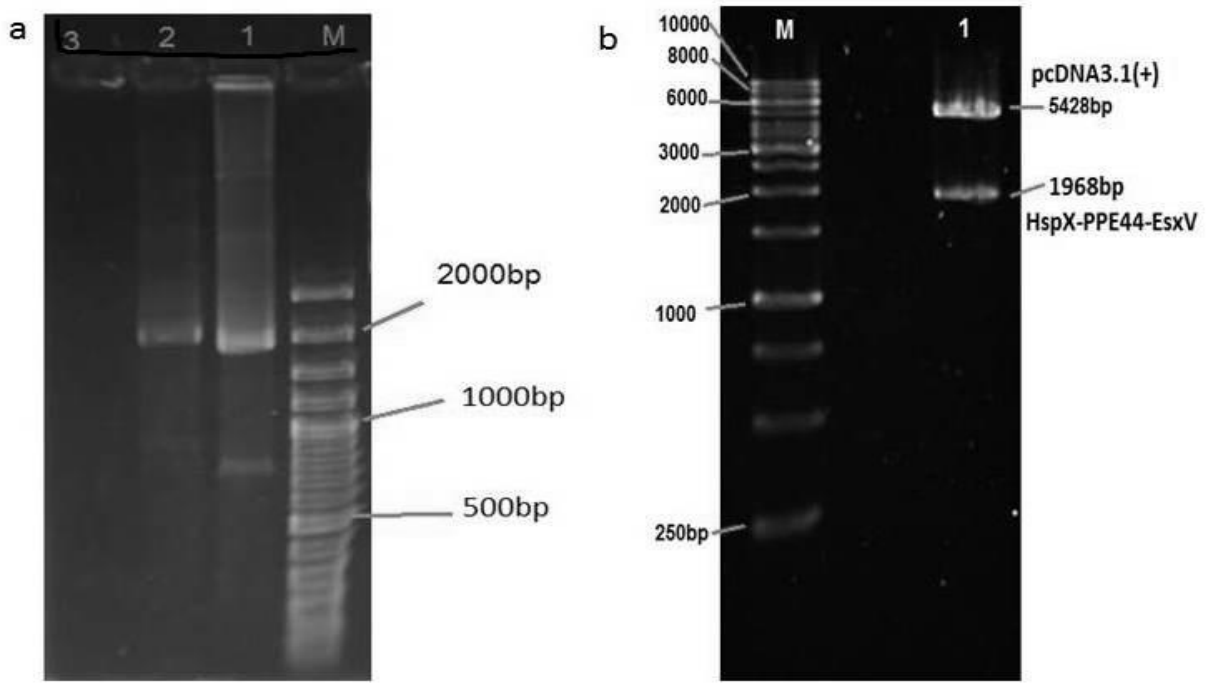

Fig. 3. Colony-PCR and digestion products visualized on 1.2% agarose gels. A: Agarose gel electrophoresis of colony-PCR product. Lane 1: 1968 bp PCR product of fusion fragments, M: 100 bp-plus DNA size marker; B: *Bam*HI and *Xba Xba*I digestion products of recombinant vector. Lane1: vector and hspX-ppe44-esxV fusion gene. Expected bands of 1968 and 5428 bp representing hspX-ppe44-esxV and pcDNA3.1 (+) are seen. Lane M: 1 kb DNA marker.

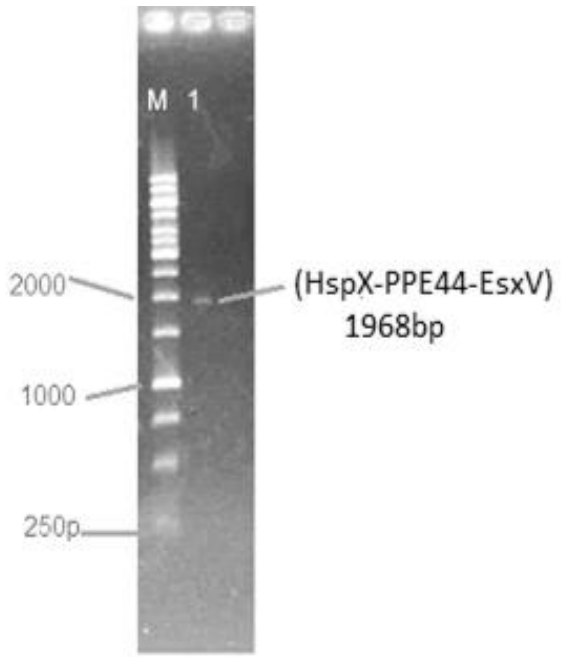

Fig. 4. Agarose gel electrophoresis of RT-PCR product from RNA isolated from pcDNA3.1(+)/*hspX-ppe44-esxV*-transfected CHO cells by RT-PCR analysis using *hspX* forward and *esxV* reverse primers. Lanes 1: a 1968 bp band of RT-PCR product; lane M: 1 Kb DNA size marker.

To verify protein expression of HspX-PPE44-EsxV, Western blots were performed. Western blots rabbit anti-mouse IgG antibody (AbD Serotec, UK), showed an immunoreactive band of the expected size of 68 kDa (Fig. 5).

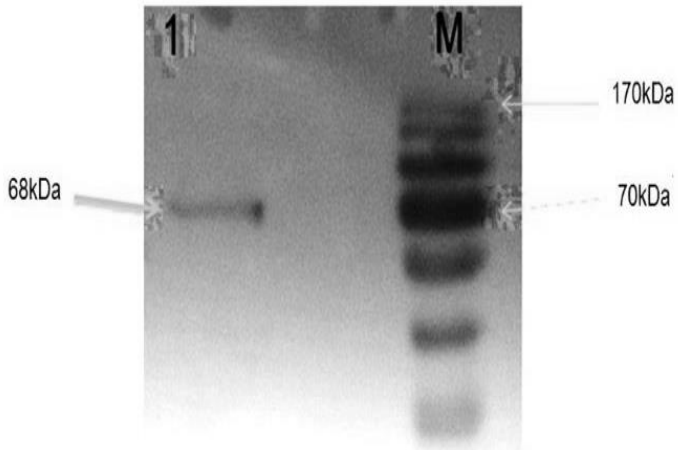

Fig. 5. Western blot of pcDNA3.1(+)/HspX-PPE44-EsxV-transfected CHO cell lysate immunoblotted with a rabbit anti-mouse IgG antibody: Lane 1: 68kDa HspX-PPE44-esxV protein fusion, lane M: Protein size marker.

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Discussion

Presently, BCG is the only commonly used vaccine against TB; however, BCG does not provide completely immunity against TB, and its protection varies among populations, with an average protection rate of 50% (25). Many efforts have been made to develop a safe and effective vaccine against TB. A new strategy is to construct DNA vaccines encoding *M. tuberculosis* genes and use them via the prime boost method. Such DNA vaccines could be used to increase the effectiveness of BCG. In the prime boost method antigens whose immunogenicity has been confirmed in previous studies are used. Such antigens include HspX, Esat-6, PPE44, and EsxV (9, 26-29). In this study, we constructed a new DNA vaccine encoding *hspX*, *ppe44*, and *esxV* of *M. tuberculosis*. These genes are not expressed in BCG and are suitable for prime-boost evaluation. Because *M. tuberculosis* is an intracellular pathogen, TB should be controlled by cellular immune responses, particularly by secreted IFN-γ from (30, 31). It has been revealed in several studies that CD4+ and CD8+ T cells secrete IFN-γ and IL-2, and activate phagocytes (32). Therefore, identification of antigens inducing CD4+ T cells responses is critical to the design and construction of effective TB vaccines. In our study HspX, PPE44, and EsxV, three immunogenic antigens, were selected and optimized. PPE44, a protein in the PPE family, with Pro-Pro-Glu at the N-terminus, is an important *M. tuberculosis* antigen (33, 34). PPE44 expression in the initial phase of TB stimulates dendritic cells to activate macrophages (35). Few studies have examined PPE44 as a DNA vaccine. In studies in which PPE44 DNA has been analyzed, immune responses have been increased. In one study, immunization with a PPE44 DNA vaccine increased expression of IFN-γ, IL-12, and TGF-β. These cytokines play important roles in the increasing bactericidal activity of macrophages against TB and in cellular proliferation and differentiation (9, 18). In another study, co-administration of BCG and a plasmid encoding PPE44 increased T- and B-cell responses in C57BL/6 mice greater than the administration of BCG alone (9). Therefore, co-administration with the prime boost method increased the efficiency of the DNA vaccine in stimulating immune responses. These findings suggest that PPE44 can induce immune responses against TB and may be used as a DNA vaccine against TB (36). ESAT-6 family antigens have also been shown to trigger IFN-γ production by memory T cells (20). The ESAT-6 protein family includes 23 secretory proteins that are produced in the early stage of TB (37). In a study of TB patients, it was demonstrated that EsxV (Rv3619c), an ESAT-6 protein and a specific T cell target, induces CD8+ T cell responses. In addition, researchers recently showed that combining EsX antigens with BCG in the prime-boost method significantly induced immune responses and cytokine production via activation of CD8+ T cells. These results suggested that EsX antigens are suitable vaccine candidates (21, 38). In a study by Hanif and Mustafa, EsxV (Rv3619c) induced cellular immune responses, consistent with our study. These results indicated that EsxV stimulates host cellular immune responses appropriately, and can be considered as a safe and novel vaccine candidate (39, 40). HspX, an antigenic *M. tuberculosis* protein expressed during the latent phase of TB infection, is another potential vaccine candidate. HspX mediates long-term stability of *M. tuberculosis* within macrophages (41, 42) and stimulates immune system responses in the latent phases of infection. These responses activate T and B cells in individuals with active and latent TB (43). Recently a few studies investigated immune responses to HspX vaccination. In the Roupie study, HspX was used to design and produce a new fusion vaccine, as in our study (42). This study showed high IFN-γ and IL-2 production, verifying strong cellular immune responses. These findings indicate that proteins expressed during the latent phase of TB infection have potential as antigens in TB vaccine development (44). In light of these and other studies (44) we expect our construct to induce protective immune responses. Studies have shown that HspX, PPE44, and EsxV can activate strong cellular immune responses. A vaccine containing these three antigenic proteins in the prime-boost method likely can increase the efficiency of BCG.

In this study we report the production of a new potential DNA vaccine candidate. Further studies are

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needed to investigate its efficiency. To increase its efficiency in the proposed studies, it is also proposed that nano-particles such as mannosylated PLGA, nanospheres, chitosan, and liposomes to be used. The efficacy of nano-particles via intranasal and injection routes has been demonstrated. In addition, to evaluate the vaccine in *M. tuberculosis*-infected mice, immune responses and bacterial load in the mouse lungs will be investigated.

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