Original Article

# Designing and construction a DNA vaccine encoding the fusion fragment of *cfp10* and *Ag85A* immunodominant genes of *Mycobacterium tuberculosis*

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## Abstract

**Background:** Pathogenic mycobacteria are one of major causes of human morbidity and mortality. *Mycobacterium tuberculosis* (*M. tuberculosis*) is an etiological agent of human tuberculosis. Designing new vaccines including DNA vaccines may be considered as new approaches for preventing of TB.

**Materials and Methods***:* *M. tuberculosis* H37Rv was grown on Lowenstein Jensen medium for 4 weeks at 37ºC and then DNA was extracted. The *cfp10* gene was amplified by PCR. After digesting the PCR product and the plasmid, *cfp10* fragment was ligated into the vector using *T4 DNA ligase*. Then, *Ag85A* was subcloned into pcDNA*/cfp10*. *Escherichia coli* strain *JM109* bacteria were transformed by the desired construct. Clone confirmations were performed by colony PCR, restriction enzyme digestion and DNA sequencing. Recombinant vector was transfected into HeLa cells and total RNA was extracted, then cDNA was synthesized using *oligo-dT*. Finally PCR was performed by *cfp10* primers.

**Results:** The *cfp10* was amplified by PCR method and the PCR products were visualized by agarose gel electrophoresis. The *cfp10* fragments showed 303 bp in length. The *cfp10* cloned into pcDNA. Then, *Ag85A*was ligated into pcDNA/*cfp10* after digestion correctly. Colony-PCR and restriction enzyme digestion and sequencing confirmed the cloning the fusion *Ag85A/cfp10* fragment. Finally, after cDNA synthesis, expression of vector was confirmed in eukaryotic system.

**Conclusion:** Cloning of *Ag85A/cfp10* genes of *M. tuberculosis* were performed correctly. It can use as a DNA vaccine for investigation the immune responses in animal models in future studies.

**Keywords:** DNA vaccine, *cfp10*, A*g85A*, *Mycobacterium tuberculosis*

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## Introduction

Pathological cause of tuberculosis is *Mycobacterium tuberculosis* (*M. tuberculosis*) which is an optional intracellular bacterium. Tuberculosis (TB) is a worldwide public health problem that is often associated with AIDS and Malaria. *M. tuberculosis* are observed in 8 million new cases and cause 2 million deaths each year (1-4).

Several strategies are being employed for the development of TB vaccines such as subunit vaccines, recombinant BCG vaccines, auxotrophic vaccines, atypical mycobacterial strains, DNA vaccines and heterologous prime boost approach. Scientists around the world are looking for a suitable vaccine against *M. tuberculosis* due to the variable efficacy of BCG vaccine from zero to 80 percent and do not use it in patients who have a weakened immune system. Because of the low ability of BCG vaccine to stimulate CD8+ cells, some researchers have attributed this to the inability of BCG in infection control and they believe the vaccine design that could be create a memory CD8+ cells are helpful (5-8).

BCG is a live attenuated strain of *M. bovis* and is the only available TB vaccine obtained almost one century ago. BCG offers significant protection against disseminated childhood tuberculosis, but is not true for the more prevalent adult pulmonary disease (9, 10).

Currently there are a number of DNA vaccines in the clinical stages which have effects of prevention and treatment. Including the causes that impact on AIDS, herpes virus, influenza virus, malaria and cancer (11).

Epitopes of DNA vaccine encode the protein sequences that bind to MHC molecules of human cells so that T-helper cells identify MHCπ-antigen complex and cytotoxic cells MHC I -antigen complex. Thus, DNA vaccines induce strong CD4+ (Th1) and (CTL) CD8 + against tuberculosis (12). In mice, several antigens *M. tuberculosis* provide immunity against infection including; Mtb 8.4, Ag85, MPT39, MPT63, MPT83, Hsp65, PstS3, ESAT6, MPT64 and lipoprotein 38-kDa (13).

The culture filtrate protein 10 (CFP10) and early secretory antigenic target protein 6 (ESAT6) are encoded by the RD1 genes Rv3874 and Rv3875 of *M. tuberculosis* (14). Researchers demonstrated that CFP10 and ESAT6 might be biological active molecules in vivo when they form a 1:1 heterodimer complex. CFP10 and ESAT6 play an important role in *M. tuberculosis* virulence (15). Studies showed that the ESAT6 and CFP10 are highly immunogenic RD1 region, but not the overall protection (7).

More testing of prepared DNA vaccine contain genes encoding the Ag85 family. All three members of this family have been studied as a DNA vaccine. Ag85A and Ag85B are active while Ag85C is not. Most studies are done on the Ag85A. In the late decade, DNA vaccine encoding Ag85A was produced. This antigen in contrast Ag85C is highly immunogenic and made safety protection in mice that were infected by aerosol with *M. tuberculosis* (7) Vaccination with DNA plasmid encoding Ag85A subunit leads to Th1 and CTL cells strong responses (16).

Ag85 complex is fibronectin-binding and it is effective to induce protective immunity in animal models. Three components of Ag85 complex are 32-30 kDa proteins (Ag85A, Ag85B and Ag85C) (16), that belong to the vast majority of secretory proteins of *M. tuberculosis* and the *BCG* culture products Ag85A (Rv3804c) molecule is a major secretory protein of *M. tuberculosis* with a length of 295 amino acids in addition it has maykolyl transferase activity in the final stage of processing the cell wall of mycobacteria (16, 17). 121 to 145 amino acids (peptides 25 and 27) and 196 to 215 (peptides 40 and 41) make the largest immunogenicity (18).

Several experimental TB vaccines were constructed such as DNA vaccines expressing antigens from the RD1 and RD2 loci such as CFP10 and CFP21 in combination with the secreted protein antigen 85B (19).

Accordingly, vaccination with multiple antigens may improve in high protective effects. In the present study we focus on the *Ag85A* and *cfp10* immunodominant genes in order to produce a vaccine against *M. tuberculosis* H37Rv strain.

# Methods

***2.1 Cells and Plasmids.*** *M. tuberculosis* strain H37Rv obtained from the Pasteur Institute (Tehran, Iran) was used for isolation of *cfp10* gene. Also, pCDNA3.1 (+) vector (Qiagen company, Germany) was used for cloning; pCDNA3.1/Ag85a (GENERAY company, China) was synthesized for our work and was used for subcloning. HeLa cells was used for transfection.

***2.2 Subcloning Ag85A in pcDNA cfp10.*** The pcDNA *cfp10* was used for our work. This vector had been cloned in *E. coli* strain *JM109* and transfected to HeLa cell (20). We subcloned *Ag85a* to pcDNA *cfp10*.

For primer designing of *Ag85A* was done somethings. The upstream *Ag85A* inserted a unique restriction site *Bam*HI and replaced the start codon of the antigen gene with a *Kozak* consensus sequence (ACCATGG) to enhance mammalian expression. In the downstream *Ag85A* was removed the stop codon, and was inserted a second unique restriction site *Eco*RI.

The *Ag85A* gene (1036 bp fragment) from recombinant plasmid pcDNA *Ag85A* and also pCDNA *cfp10* were digested with *Bam*HI and *Eco*RI. The purification procedure was performed using protocol (Invisorb spin DNA extraction kit, Germany) and the products were detected by 1.5% agarose gel electrophoresis.

The digested products were ligated to generate pCDNA *Ag85A/cfp10* by T4 DNA ligase.

*E. coli* strain *JM109* in LB medium was transformed as noted above. Then, extraction of chimeric plasmid was done using the QIAprep Miniprep Kit (Qiagen company, USA).

***2.3Confirmation chimeric plasmid pcDNA Ag85A/cfp10***

***2.3.1 Enzyme digestion.*** The chimeric plasmid was subsequently characterized by restriction enzyme digestion. To make ensuring of the correct orientation of the fragments, chimeric pcDNA *Ag85A/cfp10* plasmid was digested by *Bam*HI and *Eco*RI enzymes and then was electrophoresed on 1% agarose gel. In addition, the pcDNA *Ag85A/cfp10* was digested with *Bam*HI and *Xba*I and in the next step with *Bam*HI and *Eco*RI enzymes to confirm fusion two fragments of *Ag85A/cfp10.*

***2.3.2 Colony- PCR for cfp10.*** The second method for confirmation of cloning *Ag85A* into pcDNA *cfp10* colony-PCR by *cfp10* primers 5’ATTGATAGAATTCGCAGAGATGAAGACCG ATGCCGCT3’ (forward primer) and 5'TCATTAATCTAGATTATCAGAAGCCCATTTG CGAGGACAG 3' (reverse primer). PCR mixture consisted of 10 pmol of each primer, $MgCl_2$ 1.5 mM, 0.2 mM each dNTP, 5U *Taq* DNA polythermase (CinnaGen company, Iran) in a total reaction volume of 25 µl, then a tip of colony was dissolved. The following conditions were applied: initial denaturation at 95°C for 5 min, followed by 35 cycles; denaturation at 95°C for 1 min, annealing at 65°C for 1 min, elongation at 72° for 1 min.

***2.3.3 Sequencing.*** The fusion fragment containing *Ag85A* and *cfp10* was sent for sequencing (Macrogen company, Korea) using the *T7* forward (5' TAATACGACTCACTATAGGG 3') and BGH reverse (5' TAGAAGGCACAGTCGAGGC 3') primers. PCR reaction and it's program was the same of we did for *cfp10* but the difference was at 52° C annealing temperature.

***2.4 Cell transfection.*** HeLa cells were cultured in DMEM medium supplemented with 10% fetal calf serum and 100 units/ml penicillin. The cell culture flask was incubated overnight at 37 ° C, 5% $CO_2$. We checked the cell growth and when the cells reached a confluence level of approximately 70–80%, the transfection was performed.

Actually the cells were transfected with 30 ng/µl the pCDNA *Ag85A/cfp10* expression vector in 125 µl distilled deionized water (DDW) and 25 µl $CaCl_2$ 25mM reagent in a sterile microtube. Then 250 µl 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was added and was maintained in room temperature for 20 min. All of the contents of microtube was poured at cell plates and was incubated for 5h at 37 ° C.

In the next step, the cells were plated in 6-well plates for two overnights at 37 ° C, 5% $CO_2$ according to the protocol in Sambrook book.

***2.5 Confirmation of gene expression in eukaryotic system.*** The confirmation of *Ag85A/cfp10* mRNA expression was carried out by RT-PCR.

RNA extraction was performed by using 10000 transfected cells and 450 µl RNX$^{TM}$-PLUS according to the protocol provided (CinnaGen Company, Iran). For cDNA synthesis, easy cDNA synthesis Kit was used (ParsToos Company, Iran).

In the final step 1 µl DNase1, 1 µl Buffer B, 3 µl Diethylpyrocarbonate (DEPC) water were added to 5 µl of extracted RNA. Due to confirm our test, the similar sample above was selected the control case with exception that RNA plasmid was replaced by DNA plasmid (addition of RNase). We also used of untransfected HeLa cells as another negative control.

Finally, the PCR reaction by *cfp10* primers was performed to prove the pCDNA *Ag85A/cfp10* recombinant vector in HeLa cells had been expressed.

## Results

Before subcloning, digestion of pcDNA *cfp10* with *Bam*HI and *Eco*RI was showed 5700 bp fragment

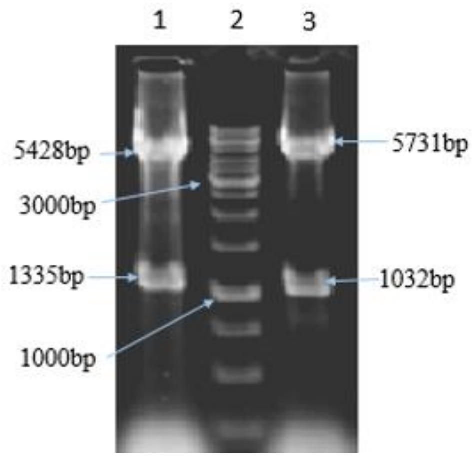


**Figure 1.** Analysis of digested fragments from pcDNA *Ag85A/cfp10*. 1) Double digestion with *Bam*HI and *Xba*I 2) DNA size marker 1kbp (Fermentase company, Germany) 3) Double digestion with *Bam*HI and *Eco*RI

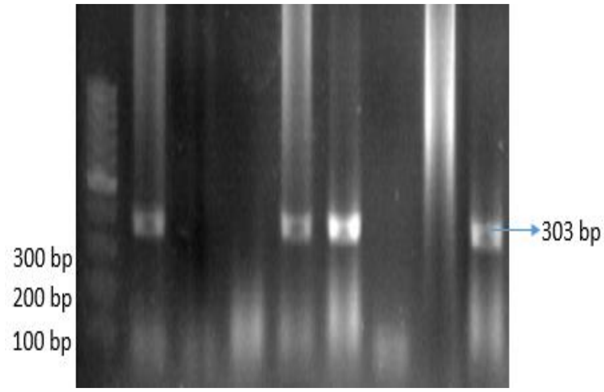


**Figure 2.** Colony-PCR for *cfp10* gene. 303 bp band is related to *cfp10* fragment and DNA size marker is 1oo bp (Fermentase company, Germany)

were related to digested linear pcDNA *cfp10*.

Also the *Ag85A* was separated from pcDNA *Ag85A* so that *Ag85A* by 1032 bp band was observed after digestion by *Bam*HI and *Eco*RI and electrophoresis.

To ensure of subcloning *Ag85A* into pcDNA *cfp10* after ligation, pcDNA *Ag85A/cfp10* vector was digested by the *Bam*HI and *Eco*RI enzymes. The result was the 5731 bp fragment of digested pcDNA/*cfp10* and the 1032 bp was attribute of digested *Ag85A* (Figure 1).

The digestion of extracted pcDNA/*Ag85A/cfp10* vector was also carried out with *Bam*HI and *Xba*I.

The ligation of them and then transformation into *E. coli* were performed so the results showed that the some bacterial cells included pCDNA *Ag85A/cfp10* vector because the colony-PCR for *cfp10* fragment was correctly done (Figure 2).

The sequencing was also done by *T7* and BGH primers. For sequencing assurement the software was DNAMAN. The sequence of fusion fragment was correct according NCBI database.

The total RNA isolated from HeLa cell lines and cDNA was synthesized with reverse transcriptase and then used for PCR analysis.

We determined the consensus sequence of *cfp10* in pCDNA *Ag85A/cfp10* in two different ways. In one approach, we directly sequenced PCR products amplified after RT-PCR. Analysis of the results showed the sequence of *cfp10* was correct. In the second approach, PCR products amplified after RT-PCR, were run on agarose gel and were observed 303bp strand. It is noted that the negative control samples did not have any strand attribute of *cfp10*.

## Discussion

This ability of DNA vaccines to elicit both the arms of adaptive immunity humoral as well as cell mediated immune response, makes them one of the most promising vehicles for antigen delivery and prolonged expression of antigen leads to the production of immunological memory (21, 22).

The vector pcDNA3.1 (+) as a vector for our designing contains the cytomegalovirus immediate early promoter upstream and a bovine growth hormone polyadenylation sequence downstream of the gene of interest. The foreign gene can be inserted in frame between *T7* and BGH promoter.

We fused on two fragments *cfp10* and *Ag85A* in this vector. CFP10 is a protein that is highly susceptible to proteolysis by trypsin digestion. CFP10 is completely destroyed at 4 ° C temperature for 20 min. This protein is highly sensitive due to its unstructured form (23). Three members of Ag85 family are distinguished by different isoelectric points. Monoclonal antibodies against Ag85 in BCG could have cross-reactivity with Ag85 epitopes of *M. tuberculosis* and *M. leprae* (24).

The incidence of immune against Ag85 complex was examined. The plasmids encoding the Ag85A and Ag85B induced the production of TNFα, IL-2, IFNγ but the plasmid encoding Ag85C was non effective (25) therefore, we selected Ag85A that induce high immunity.

In our previous study, we designed *cfp10* in pcDNA3.1 and cloned in *E. coli* strain *JM109* (20) therefore we expanded the work by synthesis a fusion two immunogenic gene containing *cfp10* and *Ag85A* for better inducing of immune system.

Antigenic epitopes containing Ag85A/B, ESAT6 and CFP10 for ECANS DNA vaccine were assigned. These genes were inserted into the *hsp60* of the *M. tuberculosis* genome. Their results showed that T cell responses against the multiepitope DNA vaccine is a stronger than one gene (26), however we did not measure T cell responses in vivo, but we detected production of RNA that related to our targets.

In 2013 the genes encoding Ag85A/MPT64 were amplified by PCR from the genome of *M. tuberculosis* strain H37Rv and it was cloned in the eukaryotic expression vector and then digested with restriction enzymes and this was confirmed by DNA sequencing. The recombinant vector immunogenicity in BALB/c mice were tested and antibody titers were measured by ELISPOT method and their results were compared with the control group showing an increase in immune (23), perhaps fusion *cfp10* and *Ag85A* to be better. We did not inject the DNA vaccine to mouse.

The research was conducted by Macedo *et al*., in 2010 in Brazil. They examined the stimulation of cellular and humoral immunity against antigens Ag85A, Ag85B and ESAT6 of *M. tuberculosis* that it was in patients with pulmonary tuberculosis, extra-pulmonary TB patients receiving chemotherapy and in healthy uninfected individuals. Their results showed that the IgG1 response against Ag85B and ESAT6 were elevated in patients compared to healthy controls. TNFα levels are increased in patients with pulmonary tuberculosis while in patients with pulmonary tuberculosis receiving chemotherapy high levels of IFNγ was observed. This difference was significant in distinguishing patients. According to this study for patient recognition , we can examine the production of IFNγ, TNFα and IgG1 in future against Ag85A and CFP10 antigens (27).

In general, we cloned the two genes in an eukaryotic vector and confirmed RNA expression by RT-PCR in HeLa cells. HeLa cell as an eukaryotic cell was important in our study. The mRNA presence of the fusion protein was detected in HeLa, so we can investigate the fusion protein and interferons in future studies.

## Conclusion

In current study, the *cfp10* and *Ag85A* genes of *M. tuberculosis* were cloned into a eukaryotic plasmid for construction a fusion DNA vaccine.

The fusion fragment was sequenced, so the result was correct after analysis with software. The mRNA related to fusion fragment by RT-PCR was detected.

## Conflicts of Interest

The authors declare that there is no conflict of interest in this study.

## Acknowledgment

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