Cloning, expression and purification of *Mycobacterium tuberculosis* ESAT-6 and CFP-10 antigens

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**ABSTRACT**

Background and Objectives: ESAT-6 (6-kDa early secretory antigenic target) and CFP-10 (10-kDa culture filtrate protein) have been described as dominant antigens recognized by T-cells and considered as virulence factors in *Mycobacterium tuberculosis*. The aim of this study was to clone, express and purify recombinant ESAT-6 and CFP-10 proteins of *M. tuberculosis* in soluble form.

Materials and Methods: ESAT-6 and CFP-10 genes were amplified by PCR, cloned into pET32a (+) vector, and overexpressed using isopropyl-beta-D-thiogalactopyranoside in *E. coli* BL21 (DE3). ESAT-6 and CFP-10 proteins were purified by Ni-NTA affinity chromatography and were detected by anti-ESAT-6 and anti-CFP10 antibodies.

Results: ESAT-6 and CFP-10 genes were successfully expressed and purified. Anti-ESAT-6 and anti-CFP-10 antibodies were produced after induction of immunization against purified ESAT-6 and CFP-10 proteins in rabbit.

Conclusion: In this study, we cloned, expressed and purified sufficient amounts of ESAT-6 and CFP-10 and it would be tested for the development of diagnostic kit for *M. tuberculosis* in future.

**Keywords:** *M. tuberculosis*, ESAT-6, CFP-10

**INTRODUCTION**

Tuberculosis is considered as one of the major health problems throughout the world (1). Nearly 80% of the tuberculosis cases occur in developing countries. Therefore, prompt diagnosis of patients with tuberculosis is vital for reducing the incidence of this disease (2).

Genomic analysis of *Mycobacterium tuberculosis* identified 14 regions of difference named RD1–14. These regions are present in the *M. tuberculosis* H37Rv and are not found in the vaccine strain of *Mycobacterium bovis* var BCG (1). ESAT-6 (6-kDa early secretory antigenic target or ESXA) and CFP-10 (10-kDa culture filtrate protein or ESXB) genes are located in Region of Difference 1 (RD1). In RD1 locus, the *esx* genes are part of a conserved segment encoding members of five additional protein families (3). The *esxA* and *esxB* genes are part of the *esxA*-V gene family, with 23 members in *M. tuberculosis* and code ESAT-6 and CFP-10 proteins.

ESAT-6 and CFP-10 have been described as dominant antigens recognized by T-cells and considered as virulence factors in *M. tuberculosis* (1). These two major proteins facilitate translocation of *M. tuberculosis* from the phagosome into the host cell cytoplasm at later stages of infection (4).
are dilemmas in precise, rapid, and cost-effective diagnostic tools of tuberculosis, therefore evaluation of M. tuberculosis specific antigens such as ESAT-6 and CFP-10 to achieve targets for accurate diagnosis is recommended. The aim of this study was to clone, express and purify recombinant ESAT-6 and CFP-10 proteins of M. tuberculosis in soluble form for future diagnostic purposes by Interferon-γ Release Assays (IGRAs) using the ESAT-6/CFP10 antigens.

MATERIAL AND METHODS

Primer designing and PCR. M. tuberculosis standard strain H37RV DNA was obtained from Pasteur Institute of Iran. Coding sequences of each dominant antigen fragment of ESAT-6 and CFP-10 were amplified from M. tuberculosis H37Rv genomic DNA by PCR using oligonucleotides 5’-CGAGGATCCACAGCAGCAGGATGGAAT-3’ and 5’-GGATCCAGAGATGAAAGCCTGTG-3’ as the forward and reverse primers for ESAT-6; 5’-CGGAATTCGGACATGAAAGCCTGTG-3’ and 5’-AAGCTTGGACAGCAGGAGGAGGAC-3’ as the forward and reverse primers for CFP-10, respectively. These primers were designed to introduce a BamHI site at the 5’-end of forward primers and a SalI site at the 5’-end of reverse primers for ESAT-6 and SalI site at the 5’-end of forward primers and a HindIII site at the 5’-end of reverse primer for CFP-10.

The PCR mixture contained 10 ng M. tuberculosis H37Rv genomic DNA as template in a total volume of 25 μl with 2.0 U of Pfu enzyme. The amplification was carried out with initial heating at 95°C for 4 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 67°C for 30 s, and extension at 72°C for 1 min.

Cloning of ESAT-6 and CFP-10. Each PCR product was ligated into pTZ57R/T cloning vector after purification using QIA quick PCR purification kit (MBI Fermentas, Lithuania). Consequently, ligation mixture was transformed into competent E. coli TOP10 cells. Single colonies were selected on LB plates containing ampicillin (100 μg/mL). Plasmid mini-preps were prepared and purified using commercially available kits (Bioneer, Korea). Plasmid DNA was isolated and analyzed by electrophoresis on agarose gel. The recombinant plasmids were confirmed by PCR for ESAT-6 and CFP-10 genes using specific primers and double digestion. In addition ESAT-6 and CFP-10 were sequenced on both strands.

The restriction digestion was performed using 10U BamHI and 20U SalI for ESAT-6; 10U BamHI and 20U HindIII for CFP-10, 1 μg DNA and 1X BamHI Buffer (10 mmol/L Tris–HCl (pH 8.0 at 37°C), 5 mmol/L MgCl₂, 100 mmol/L KCl, 0.02% Triton X-100 and 0.1 mg/mL bovine serum albumin (BSA). The reaction was incubated at 37°C for 1 hour. The digestion mixture was run on a 0.8% agarose gel in TBE buffer (10.8 g Tris base, 5.5 g Boric acid, 0.1 mg⁄mL bovine serum albumin (BSA). After confirmation, the ESAT-6 and CFP-10 gene were subcloned into pET32a (+) expression vector (Qiagen, USA) using SalI and BamHI for ESAT-6 and BamHI and HindIII enzymes for CFP-10.

Protein expression of Recombinant ESAT-6 and CFP-10. The pET32a(+)—ESAT-6 and pET32a(+)—CFP10 plasmids were transformed into E. coli BL21 DE3 (Novagen, Germany) expression host with antibiotics (100 μg⁄mL ampicillin) and incubated at 37°C until an optical density of 0.6 at 600 nm was reached. Afterwards, 1mM IPTG was added and the culture was allowed to grow for 4 h for induction of the recombinant protein. The protein analysis was carried out by sodium dodecyl sulfate– polyacrylamide gel electrophoresis (SDS–PAGE). Finally, the cells were harvested at 15,000×g for 10 min and stored at -20°C for further steps.

Purification of recombinant ESAT-6 and CFP-10. Recombinant ESAT-6 and CFP-10 was purified by nickel-nitritoltri acetic acid (Ni²⁺–NTA) metal affinity chromatography according to the manufacturer’s recommendations for purification of proteins under soluble conditions (Qiagene, USA). After washing the column, recombinant proteins were eluted with 300 mM imidazole. Purified protein was examined by SDS-PAGE gel electrophoresis followed by Coomassie Brilliant Blue staining of gel.

Production of rabbit antiserum against purified ESAT-6 and CFP-10 recombinant proteins. Two mature white New Zealand rabbits were immunized with purified ESAT-6 and CFP-10 recombinant proteins according to the standard protocols (5). Primary immunization was carried out by 200 μg of each purified protein emulsified in the same volume of incomplete Freund’s adjuvant. Rabbits were boosted for the second and the third recall immunizations.
4 and 8 weeks later. Before immunization, 1.5 mL blood was collected from the marginal vein of the ear of rabbits and was used as a negative control. At the end, serum was separated from these blood samples and analyzed for antibody response by immunoblot techniques.

**Immunoblotting of recombinant proteins.** Western blot analysis with rabbit antisera was performed for evaluation of the antigenic reactivity of both recombinant proteins. The recombinant proteins were electro-transferred onto nitrocellulose membranes in a semi-dry blotting system (Bio-Rad, USA). Protein transfer was checked by Ponceau staining of the membrane. The membrane was blocked with blocking reagent at room temperature for 2 hours using 2% dried skimmed milk in PBS with gentle agitation. The membranes were incubated in diluted (1:200) sera at room temperature for 2 hours and underwent repeated washes prior to incubation with the diluted (1:2000) secondary antibody (horseradish peroxidase (HRP)-conjugated rabbit antihuman IgG, Dako, Denmark) for 1 hour at room temperature. Immunoreactive bands were visualized using 3,3′-Diaminobenzidine (DAB; Sigma, USA) as the color substrate.

**RESULTS**

**Cloning of ESAT-6 and CFP-10 genes.** The full-length ESAT-6 and CFP-10 genes were initially cloned into the pTZ57R/T cloning vector, and then transferred into the bacterial expression vector pET32a(+) . Electrophoresis of digested recombinant plasmids confirmed that plasmids contained the objective gene. The sequencing result showed that the correct sequences of ESAT-6 and CFP-10 genes were inserted into the vector.

**Protein expression of recombinant ESAT-6 and CFP-10.** The recombinant proteins were induced in bacteria using 1 mM IPTG at 37°C for 4 h. After the expression of the recombinant ESAT-6 and CFP-10 proteins by *E. coli* BL21 DE3, recombinant protein bands were detected by SDS-PAGE analysis (Fig. 1). The recombinant proteins found to be present mostly in soluble fraction of expression host. The protein band implied a fusion protein that consists of a CFP-10 protein fused to a 6 His-tag and 109 aa trxA (Trx tag) and ESAT-6 protein fused to a 6 His-tag and 109 aa trxA (Trx tag).

**Analysis of ESAT-6 and CFP-10 by Western blot.** ESAT-6 and CFP-10 proteins were purified and detected by anti-ESAT-6 and anti-CFP10 antibodies. Western blot analysis confirmed the presence of recombinant ESAT-6 and CFP-10 protein (Fig. 3).

**DISCUSSION**

More than 90% of the worldwide burden of tuberculosis occurs in low and middle income countries where the diagnosis of this disease still relies on sputum smear microscopy and clinical finding including chest radiography. There are a great
need for rapid diagnostic tests at all levels of the health system especially in developing counties like Iran (6, 7).

Secreted proteins of *M. tuberculosis* have been reported as rich source of immunogens (8). In the last decades, these proteins have gained specific attention as virulence factors, vaccine and diagnostic candidates (3, 9-13). Although there are highly immunogenic proteins belonged to the 23-membered Esx family in mycobacterial culture filtrate, ESAT-6 and CFP-10 are absent from all *Mycobacterium bovis* BCG vaccine strains (14) and induce potent Th1 responses (8, 15, 16).

ESAT-6 and CFP-10 proteins are recognized by over 70% of tuberculosis patients (9), therefore these two proteins can be used as diagnostic reagents for tuberculosis in both humans and animals (17, 18).

It has been reported that the sensitivity of ESAT-6 antigen for serodiagnosis of tuberculosis varies from 5% to 76% with a specificity ranging from 51% to 100% (19). On the other hand, sensitivity of CFP-10 as diagnostic marker varied from 9% to 78% with specificity range of 55 to 100% (2). Hence, further research is needed to evaluate these two protein as well as other specific recombinant proteins as a diagnostic marker.

In our study, two immunodominant secreted antigens ESAT-6 and CFP-10 were expressed successfully in a soluble form. Until now, ESAT-6 and CFP-10 has been cloned into several vectors (15, 20-22). Some of these studies reported expression of CFP-10 and ESAT-6 as an insoluble protein which requires denaturation (15, 20, 23, 24) whereas there are some reports that expressed this protein as soluble protein (22, 25).

In conclusion, the main focus of the present study and some of our future studies is to develop highly reproducible, robust, cost-effective methods for producing of a large number of *M. tuberculosis* antigens and establish their usefulness for diagnosis of tuberculosis. This will be followed by further evaluation of these specific antigens to develop highly sensitive and specific diagnostic test such as IGRAs for tuberculosis.

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