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

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ABSTRACT

Background and Objectives: ESAT-6 (6-kDaearly 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 andCFP-10 proteins of *M. tuberculosis* in soluble form.

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

Results: ESAT-6 andCFP-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 andCFP-10 proteins in rabbit.

Conclusion: In this study, we cloned, expressed and purified sufficient amounts of ESAT-6 andCFP-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-kD a early secretory antigenic target or ESXA) andCFP-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 andCFP-10 proteins.

ESAT-6 andCFP-10 have been described as dominant antigens recognized by T-cells and consider 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). There

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are dilemmas in precise, rapid, and cost-effective diagnostic tools of tuberculosis, therefore evaluation of *M. tuberculosis* specific antigens such as ESAT-6 andCFP-10 to achieve targets for accurate diagnosis is recommended. The aim of this study was to clone, express and purify recombinant ESAT-6 andCFP-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 andCFP-10 were amplified from *M. tuberculosis* H37Rv genomic DNA by PCR using oligonucleotides 5′ CGAGGATCCACAGAGCAGCAGTGGAAT-3′ and 5′GGATCCGCAGAGATGAAGACCGATG-3′ as the forward and reverse primers for ESAT-6; 5′-GCGAATTCATG GCAGAG ATG AAG ACC- 3′ and 5′AAGCTTGAAGCCCATTTGCGAGGAC-3′ as the forward and reverse primers for CFP-10, respectively. These primers were designed to introduce a *Bam*HI site at the 5′-end of forward primers and a *SalI* site at the 5′-end of reverse primer for ESAT-6 as well as *Bam*HI site at the 5′-end of forward primers and a *Hind*III 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 andCFP-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 andCFP-10 genes using specific primers and double digestion. In addition ESAT-6 andCFP-10 were sequenced on both strands.

The restriction digestion was performed using 10U *Bam*HI and 20U *Sal*I for ESAT-6; 10 U *Bam*HI and 20 U *Hind*III forCFP-10, 1 µg DNA and 1X*Bam*HI Buffer (10 mmol ⁄ LTris–HCl (pH 8.0 at 37°C), 5 mmol ⁄ L $MgCl_2$,100 mmol ⁄ L KCl, 0.02% Triton X-100 and 0.1 mg⁄mL bovine serum albumin (BSA). The reaction was incubatedat 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, 0.58 g EDTA, add water to 1 litter and pH adjusted to 8.3). After confirmation, the ESAT-6 andCFP-10 gene were subcloned into pET32a (+) expression vector (Qiagen, USA) using *Sal*I and *Bam*HI for ESAT-6 and *Bam*HI and *Hind*III enzymes forCFP-10.

Protein expression of Recombinant ESAT-6 andCFP-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 andCFP-10. Recombinant ESAT-6 andCFP-10 was purified by nickel-nitrilotri acetic acid (Ni^{2+}-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 andCFP-10 recombinant proteins. Two mature white New Zealand rabbits were immunized with purified ESAT-6 andCFP-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

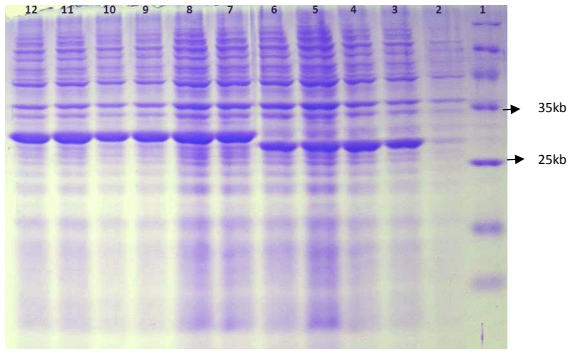

Fig. 1. Purification of CFP10- and ESAT-6 proteins as a soluble recombinant protein: lanes 1, unstained protein molecular weight marker SM0431; lane 2, pET32a(+)-ESAT-6 before induction; lane 3-6, expression of pET32a(+)-ESAT-6 after induction, lane 7-12, expression of pET32a(+)-CFP10 after induction.

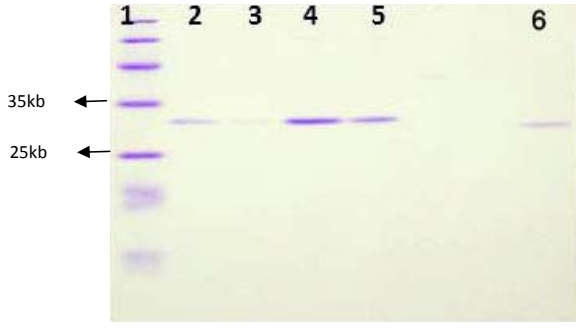

Fig. 2. Purification of CFP 10-and ESAT-6 proteins as a soluble recombinant protein: Lanes 1, unstained protein molecular weight marker SM0431; lane 2,3,4,5 purified CFP-10 proteins, lane 6, purified ESAT-6 protein after Ni-NTA affinity chromatography.

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 andCFP-10 genes. The full-length ESAT-6 andCFP-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 andCFP-10 genes were inserted into the vector.

Protein expression of recombinant ESAT-6 andCFP-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 andCFP-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).

SDS-PAGE analysis of the elution fraction of Ni^{2+}-NTA agarose chromatography showed that recombinant CFP-10 and ESAT-6 proteins were completely purified (Fig. 2).

Analysis of ESAT-6 andCFP-10 by Western blot. ESAT-6 andCFP-10 proteins were purified and detected by anti- ESAT-6 and anti -CFP10 antibodies. Western blot analysis confirmed the presence of recombinant ESAT-6 andCFP-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

Fig. 3. Western blot analysis of recombinant CFP-10 and ESAT-6 proteins; lane 1, purified ESAT-6 proteins; lane 4, 5, purified CFP-10 protein; lanes 2,3,6,7, negative control; lanes 8, prestained protein molecular weight marker #26612 Thermo Scientific.

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 Th_1 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 of 51% to 100% (19). On the other hand, sensitivity ofCFP-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 andCFP-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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