

Cloning and expression of *Mycobacterium tuberculosis* ESAT-6 in prokaryotic system

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ABSTRACT

The identification of a large number of antigens with potential for development of new tuberculosis vaccine has been accomplished in recent years. This study was designed for cloning and expression of ESAT-6 as a potent antigen of *Mycobacterium tuberculosis*. Selected gene (Rv3875) was amplified by PCR and product was ligated into expressing plasmid vector pQE30 and recombinant pQE30-ES plasmid was constructed. This hybrid vector was transformed in *E. coli* M15 and expressed in optimal condition. The expressed protein was analyzed on SDS-PAGE and confirmed by western blotting using specific antisera to ESAT-6. We successfully cloned and expressed ESAT-6 (His)₆ from *M. tuberculosis* H37Rv genome. As well as usage for serodiagnosis, this recombinant protein offers the potential development of other vaccine formats such as DNA or subunit vaccines against tuberculosis.

Keywords: *Mycobacterium tuberculosis*, ESAT-6, recombinant protein, cloning

INTRODUCTION

Mycobacterium tuberculosis infects more than one-third of the world's population and causes 2–3 million deaths annually (Smith *et al* 2003, Baumann *et al* 2006). The trials have shown that attenuated *Mycobacterium bovis* Bacillus Calmette Guerin (BCG) vaccine does not provide consistent protection against TB (Barreto *et al* 2006, Fine 1995). The studies to identify *M. tuberculosis* antigens and epitopes as candidates for new protective vaccines for TB have led to the identification and characterization of several major antigens of *M. tuberculosis* (Martin *et al* 2005, Doherty *et al* 2004). Some of these antigens (such

as hsp60, Ag85 and ESAT-6, etc) have shown promise as new candidate vaccines (Ulmer *et al* 1997, Nasser Eddin *et al* 2006, Kaufmann *et al* 2006). On the basis of such proteins or their peptide was created a number of diagnostic systems and vaccines, among which are most interesting those of them, which were created on the basis of the secretory proteins ESAT6 or Cfp-10 (Sable *et al* 2007). The 6 KDa early secretory antigenic target (ESAT-6) from *Mycobacterium tuberculosis* can be isolated from a highly stimulatory low molecular mass fraction of short term culture filtrate (ST-CF), and this antigen is strongly recognized in TB patients, in cattle infected with *M. bovis* and in several strains of TB-infected mice. This protein is absent in *M. bovis* and the majority of

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nonpathogenic mycobacteria (Okkels *et al* 2004). The mycobacterial antigen ESAT-6 is target for T cells in the early phases of infection (J.M. Pollock *et al* 2003. Brandt *et al* 1996). Furthermore, ESAT-6 has recently been demonstrated to induce protective immunity when administered as either a subunit or a DNA vaccine (Brandt *et al* 2000. Xu *et al* 2006). Because ESAT-6 is such a broadly and strongly recognized antigen in several species, a role for this molecule in future vaccines against tuberculosis have previously suggested, and this antigen has shown promise when delivered as a DNA vaccine (Olsen *et al* 2004, Xu *et al* 2008. Nosareva *et al* 2008). The purpose of the present work was to construct recombinant genes encoding the ESAT-6 antigen and study their expression in the prokaryotic System. The data would allow us to develop methods for constructing recombinant vaccine and use it for creation of tuberculosis and related infectious recombinant vaccines.

MATERIALS AND METHODS

Materials. All the chemicals were purchased from Sigma (Sigma Aldrich) except when otherwise noted. Restriction endonucleases and T4 DNA ligase used in all routine cloning and transformation experiments were procured from Fermentas GmbH (Germany) Plasmid Maxi kit and QIAquick Gel Extraction Kit were obtained from Qiagen (GmbH, Hilden, Germany). Vector pQE30 was obtained from Iranian Recombinant Gene Bank (Institute Pasteur, Tehran). Oligonucleotides were custom synthesized by cinnagen (Tehran, Iran). Mycobacterium tuberculosis H37Rv genome was obtained from Tuberculosis Research Laboratory (Masih Daneshvari Hospital).

Construction of expression vector containing ESAT-6 gene. The Rv3875 gene from the M. tuberculosis H37Rv genome, which encodes the ESAT-6 protein, was used to design primers for

PCR amplification of the gene. The sequences of the forward and the backward primers are as below:

ESAT6-F:

ACGAGATCTACAGAGCAGTGGAATTTTC(BglII)

ESAT6-R:

ACGGGATCCTGCGAACATCCCAGTGA(BamHI)

PCR was performed using standard conditions (94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min, 35 cycles). The 288 bp PCR product was purified through Qiagen mini columns as per manufacturer's instruction. Plasmid DNA was digested with BglII and BamHI. After running on 1% Agarose, the fragments were purified and cloned in the pQE30 vector pre digested with the BamHI. Prior to ligation, the linearized plasmid DNA and insert DNA were gel purified using the QIAquick Gel extraction kit. The ligation of ESAT-6 into pQE30 was performed using T4 DNA ligase and transformed into E. coli DH5 α cells. The transformants were screened with RE analysis using EcoRI and HindIII and confirmed by DNA sequencing. The plasmid pQE30-ES from the correct transformant was purified from an over night culture of recombinant E. coli DH5 α cell and then transformed into the competent E. coli M15 cells cells and screened on LB plates containing 50 μ g/ml ampicillin and 30 μ g/ml Kanamycin.

Expression of Recombinant Plasmid pQE30-ES.

The transformed E. coli M15 cells harboring the pQE30-ES plasmid was used in the expression. Several transformants were cultured in 50 ml LB medium supplemented with ampicillin (50 μ g/ml) and Kanamycin (30 μ g/ml) at 37 °C. When A600 reached 0.55, isopropyl-D-thiogalactopyranoside (IPTG) was added to final concentration of 1mM and incubation was continued for 3 h. sampling were performed before and after induction and was designed as T₀, T₁, T₂, and T₃. Protein expression was checked by electrophoresis in 15% SDS-polyacrylamide gel and 5% stacking gel. The bacterial cells were centrifuged at 13,000 rpm for 5 min; the precipitate was resuspended in 50 μ l of

buffer as a sample-buffer, and boiled for 5 min. The samples were clarified by centrifugation under the same conditions, and 10- μ l samples were placed onto the gel.

Western Blot Analysis. Samples from the acrylamide gel were transferred on nitrocellulose membrane in semidry transfer condition with transfer buffer. The membrane was blocked with 1% solution of bovine serum albumin for 30 min and incubated for 1 h with operating dilutions of polyclonal mouse sera to the ESAT-6-(His)₆ at 37°C. Antigen-antibody complex was detected using Rabbit antimouse antibodies conjugated with horseradish peroxidase (Sigma Aldrich) in the operating dilution. The complex was incubated with the conjugate for 1 h at 37°C. After washing, the membrane was stained in solution of a horseradish peroxidase substrate, 3, 3'-diaminobenzidine tetrahydrochloride.

RESULTS

Amplification of ESAT-6 gene. The gene encoding the mature protein of ESAT-6 was amplified by PCR. After running a standard PCR protocol with designed primers from *Mycobacterium tuberculosis* genome, a distinct band after 250 bps area was appear which corresponded with ESAT-6 gene with 288 nucleotides (Rv3875) (Figure 1).

Construction of expression vector pQE30-ES. In this study, ESAT-6 gene was ligated into the prokaryotic expression vector pQE30. The clones were screened first with RE analysis using EcoRI and BamHI. EcoRI was used because it is a unique site in the backbone of pQE30 vector which can be used to determine the orientation of the cloned gene. These REs will produce an approximately 350 bps band of the ESAT-6 gene together with a part of the pQE30 backbone and a 4.12 kb band for the remaining backbone of pQE30 vector. RE analysis of the clones gave the expected size of the

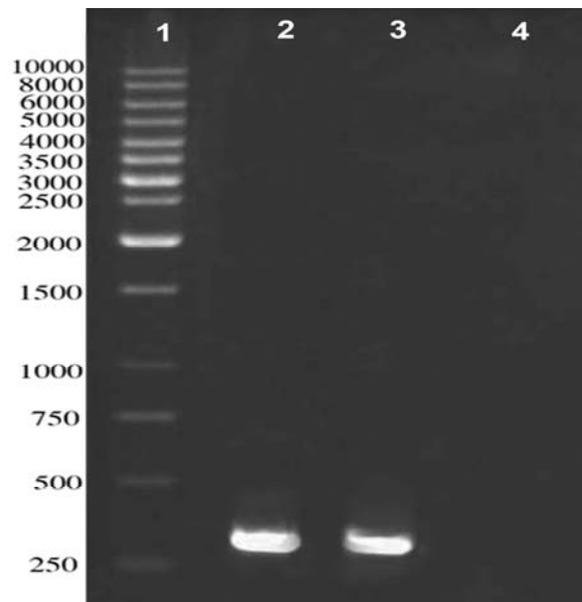


Figure 1. Amplifications of ESAT-6 gene from *M. tuberculosis* genome by PCR against 1kb DNA ladder. Distinct band in approximately 290 bps was seen in lane 2 and 3. Lane 4 is negative control.

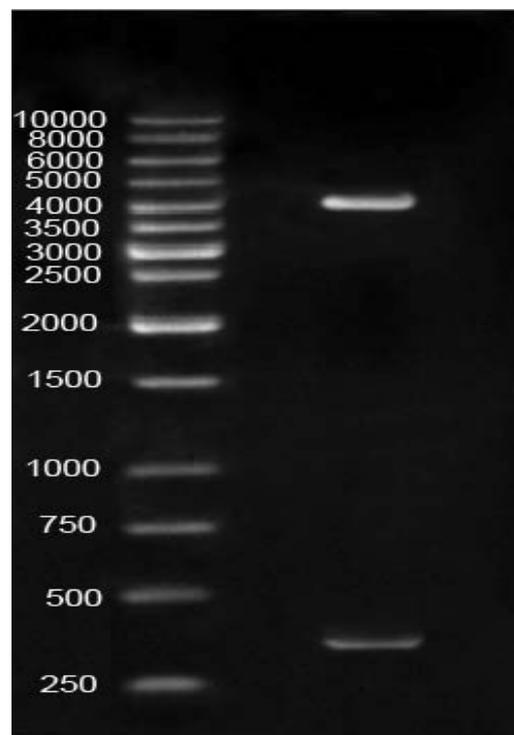


Figure2. Restriction analysis of recombinant plasmid pQE30-ES by EcoRI and BamHI against 1kb DNA ladder. Two separate fragments was seen. Lower is approximately 350 bps band of the ESAT-6 gene together with a part of the pQE30 backbone and upper is 4.12 kb band for the remaining backbone of pQE30 vector.

two bands (Figure 2). DNA sequencing confirmed that the ESAT-6 gene was successfully cloned into pQE30 in the correct orientation (Data not shown).

Expression of the protein ESAT-6. ESAT-6 protein was recombinantly produced in *E. coli* using the His-tag cloning system, pQE30. The expression vector was chosen based on the presence of sequences encoding six histidine residues at N-end of the multiple cloning sites (MCS). Insertion of the PCR product into the plasmid vector pQE30 resulted in a hybrid gene encoding the protein with the calculated molecular weight around 10 kDa, which consisted of the mature protein ESAT-6 and a sequence of six His on the N-end of the molecule. After electrophoresis (15% SDS-PAGE) in the cell plate of the *E. coli* M15 culture upon induction with IPTG, a band was detected which corresponded to the recombinant protein ESAT-6-(His)₆ (Figure 3).

This expected distinct band around 10 kDa was observed in T₁, T₂, and T₃ of the pellet samples. This band was not observed in negative control samples (Figure 3). Therefore, the results obtained from SDS-PAGE suggest that the ESAT-6 recombinant protein has been successfully expressed in *E. coli* M15. Western blotting was performed to confirm the expression of the fusion protein by probing immunoblots containing this antigen using the commercial anti-ESAT-6 antibody and 10kDa band was observed in expressed fractions (Figure 4).

DISCUSSION

Research on ESAT-6 and the ESAT-6 system-1 has become an important topic for studies on the pathogenesis and immunogenicity of *M. tuberculosis* infections because of the outstanding role of ESAT-6 in these processes (Skjot *et al* 2000, Brodin *et al* 2004). This antigen has been demonstrated recently to be one of the major targets for memory effector cells during the recall

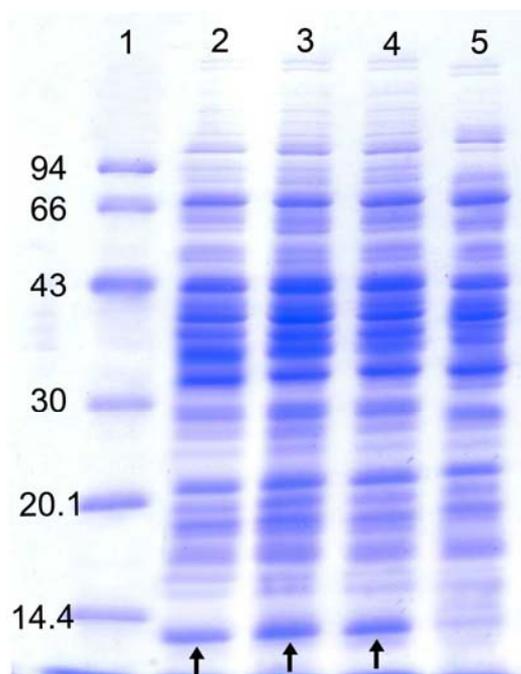


Figure 3: SDS-PAGE analysis of recombinant *M. tuberculosis* ESAT-6 expression from pQE30-ES plasmid in *E. coli* M15. (Shown with arrow) Lane 1, molecular weight standard; lane 2, 3 hour after induction with IPTG; lane 3, 2 hour after induction with IPTG; lane 4, 1 hour after induction with IPTG; lane 5, before induction with IPTG. Protein bands were visualized by Coomassie blue staining.

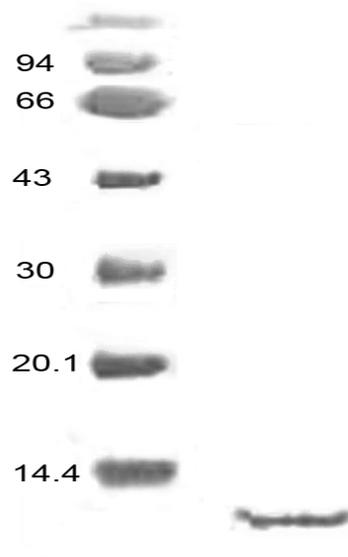


Figure 4. western Blotting analysis of expressed ESAT-6 recombinant protein by anti-ESAT-6 antibody. An expected band in approximately 10kDa was seen.

of memory immunity in a mouse model of tuberculosis (Brodin *et al* 2004). ESAT-6 is a secretory protein of *M. tuberculosis* with a small amount in the culture medium (Okkels *et al* 2004). The availability of sufficient amounts of ESAT-6 from *M. tuberculosis* is an essential step to studying the immunological and biological features of this antigen. As it is impossible to obtain enough ESAT-6 for research due to a slow growth of *M. tuberculosis*, it was greatly recommended for recombinant production of this antigen (Wang *et al.* 2005, Lim *et al* 2000). In this work, we successfully amplified *esat-6* gene from *M. tuberculosis* H37Rv genome DNA by PCR. The gene encoding this protein was isolated, and data obtained from DNA sequencing studies were compared with results from amino acid analyses of the native protein. The structural gene of ESAT-6 was identified as a DNA sequence encoding a polypeptide of 95 amino acids. We observed a high degree of consistency between the cloned gene of ESAT-6 and the gene bank results. Furthermore, this fragment was inserted to pQE30, as an expression vector. Finally recombinant ESAT-6 was highly expressed from engineered hybrid plasmid. Our study demonstrated that recombinant ESAT-6, like many proteins, can be efficiently expressed in prokaryotic system (*E. coli* M15). This was confirmed in western blot analysis by specific monoclonal antibody against ESAT-6. Application of ESAT-6 as a single-component vaccine or as a member of a multiple-component vaccine in conjunction with the appropriate adjuvant is presently being investigated (Nosareva *et al* 2008, Xu *et al* 2006). Another feasible possibility to be considered is that of engineering a DNA vaccine that includes the ESAT-6-encoding gene (Kamath *et al* 1999, Mustafa 2005). Recent studies with the mouse model of tuberculosis have shown that immunization with ESAT-6 protects the animals against challenge with viable *M. tuberculosis* (Reed S. *et al* 2005). ESAT-6

therefore may be considered for inclusion as a subunit vaccine, either by itself or in combination with other relevant antigens of *M. tuberculosis* inducing protective immunity (Mustafa 2002). However, since not all subjects infected with *M. tuberculosis* respond to single antigens, including ESAT-6, a combination of several promising antigens may constitute a better candidate vaccine (Lim *et al* 2000, Baumann *et al* 2006). This view is strengthened by a recent report showing the greater protective efficacy of DNA vaccines expressing multiple antigens (i.e. HSP70, MPT-64, and Ag-85B) in comparison with the protection afforded by immunization of mice with DNA vaccines expressing any single antigen (Luby *et al* 2008, Mustafa 2005, Olsen *et al* 2004). However, as well as diagnostic value of purified protein derivative (Van-Lume *et al* 2008), we could use this cloned gene or recombinant antigen for DNA or subunit vaccine study for tuberculosis.

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