Original article

Cloning, expression and purification of early secretory antigenic target 6kDa protein (ESAT-6) of *Mycobacterium tuberculosis*

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Abstract

Introduction and objective: The early secretory antigenic target 6kDa protein (ESAT-6) antigen from *Mycobacterium tuberculosis* is a dominant target for cell-mediated immunity in the early phase of tuberculosis (TB) in TB patients. ESAT-6 was found to distinguish TB patients from BCG-vaccinated donors. The aim of this study was cloning and expression of ESAT-6 of *M. tuberculosis*.

Materials and methods: DNA was extracted from *M. tuberculosis* H37Rv. PCR was performed using gene-specific oligonucleotide primers and the PCR products were inserted into the pET102/D vector and transferred into *Escherichia coli* strain TOPO10. The recombinant plasmids transferred into *E. coli* strain BL21.

Results: The transformed plasmid into *E. coli* strain BL21 was effectively expressed. The expressed fusion protein (23kDa on SDS-PAGE) was found almost entirely in the soluble form and the recombinant protein was purified by Ni-NTA column.

Conclusion: We successfully cloned and expressed ESAT-6 protein of *M. tuberculosis* in *E. coli*. As a specific antigen, it can be useful for diagnosis of both active and latent tuberculosis with ELISA in future.

Keywords: ESAT6 antigen, Cloning, *Mycobacterium tuberculosis*

Introduction

Tuberculosis remains a major infectious disease with over eight million new cases and two million deaths annually [1]. Diagnosis of tuberculosis depends upon the culture of *Mycobacterium tuberculosis* and demonstration of acid-fast bacilli in smears. Clinical and radiological findings not

specific for the disease and purified protein derivative (PPD) has some value for diagnosis in developing countries, new serological test for the diagnosis of tuberculosis have been widely explored [2]. In most of the countries where the disease is endemic, facilities for the diagnosis of latent tuberculosis are limited. Tuberculin skin test (PPD), routine serological test, has high rate of false-positive and results is seen in developing countries and some patients with active tuberculosis have tested negative [2].

The RD1 region, highly specific for *M. tuberculosis* complex and absent from *M. bovis BCG* [4], is 9455 bp long, and encompasses nine ORFs (Rv3871–Rv3879c). Rv3874 or EsxB and Rv3875 or EsxA encode the proteins 10-kDa culture filtrate protein (CFP-10) and 6-kDa early secretory antigenic target (ESAT-6), respectively, which play a key role in virulence. Both ESAT-6 and CFP-10 generate a specific Th-1 host immune response and have a strong diagnostic potential for both the virulent form and latent form of *M. tuberculosis* [3]. ESAT-6 is an important T-cell antigen recognized by protective T cells in animal models infected with *M. tuberculosis.* ESAT-6 can elicit strong antibody responses and delayed type hypersensitivity skin reactions in tuberculosis guinea pigs [4].

In this study, we cloned and expressed ESAT-6 protein and refolded the protein and isolated the monomer from a recombinant protein in denatured state to detect the latent tuberculosis with more sensitivity and specificity than other alternative diagnostic procedures.

Materials and methods

Bacterial strains and DNA extraction

M. tuberculosis standard strain H37Rv (ATCC 27294) was purchased from Pasteur Institute of Iran and was cultured on Lewenstien-Jensen medium at 37ºC for a period of four weeks. After this time, cauliflower like colonies stained by Ziel-Neelson and after confirming the acid-fast bacilli, its DNA was extracted by DNA extraction Kit (Cinnagen, Iran) according to the manufacture's instruction. In brief, four to five colonies from bacteria was suspended and homogenized in 500µl sterile PBS buffer and 500µl lysate buffer. Suspended bacteria were boiled at 80ºC for 15min and were centrifuged at 4ºC with 12000rpm for 12min. In the last stage 100µl of lysate buffer and 50µl of mineral oil were added on pellet and suspension was incubated at 95ºC for 30min. The suspension was then centrifuged and soluble phase between pellet and oil that contained DNA was collected.

Primer design and PCR

DNA encoding ESAT-6 sequence is located in RD1 region and called EsxA or Rv3875 (GeneBank accession number BX842584). The DNA fragment encoding ESAT6 Gene consisting of 288 nucleotides retried from NCBI gene bank. Based on this sequence forward 5' "CAC CAT GAC AGA GCA GCA GTG GAA" and reverse 5' "CGC GAA CAT CCC AGT GAC GT" primers were designed by Gene Runner software and applied to amplified specifically ESAT-6 gene, giving a fragment 288 nucleotides in length.

The PCR mixture contained 10 X pfu buffers, 0.1mM dNTP, Mg_2So_4 (1.5 mM), 3µl DNA, 20 pmol of each primer and 1.5 unit of pfu DNA polymerase (Cinnagen, Iran). The final volume of reaction mixture was adjusted to 20µl with sterile distilled water. The PCR cycling program was done with thermocycler (Techne, USA) at 35 cycles comprises 95°C for 30 seconds, 57°C for 45 seconds, 72°C for 30 seconds followed by a final extension at 72°C for 180 seconds [4].

Extraction of PCR product from agarose gel

For removing additional materials such as dNTP, pfu enzyme and nonspecific bands from amplified specific product, PCR total product electrophoresis was performed on low melting 2% agarose gel (Fermentas, UK) and the desired band removed and purified by PCR product DNA extraction kit (Fermentas, UK) and the quality of PCR product assigned. The purified PCR product was then electrophoresed through a 2% agarose gel in TAE buffer (the gel was stained by ethidium bromide) and analyzed by the Kodak 1D 3.5 imaging software (Eastman Kodak company, USA).

Ligation and transformation

Cloning was performed by Champion pET directional TOPO Expression Kit according to kit protocol (Invitrogen TM, USA) to insert and ligate the PCR product into pET102/D expression vector according to kit protocol. Subsequently, recombinant plasmid was transformed into competent *E. coli* TOP10 cells. This organism provides a host for stable propagation and maintenance of recombinant plasmids (Invitrogen TM, USA). Transformed bacteria were then cultured on Luria Bertani agar, LB agar, (Oxoid, UK) media containing ampicillin (0.1mg/ml) [5].

Confirmation of ESAT-6 gene cloning into pET102/D

Plasmid DNA extracted from TOP10 *Escherichia coli* colonies on LB agar media by Qiagen Plasmid Mini Kit (Qiagen, Germany). Electrophoresis of plasmid was performed on 1% agarose gel and PCR analysis of recombinant plasmid was performed by T7 primers provided by Invitrogen kit. Purified recombinant plasmids were sequenced by "Seq Lab" Laboratory (Germany) and the fidelity of the recombinant plasmid was confirmed [5].

Expression and purification of recombinant ESAT-6

Extracted recombinant plasmids were transformed into *E. coli* BL21 by heat shock and were plated on an LB agar containing ampicillin at 37°C overnight. Overnight incubated colonies were inoculated into an LB broth and was grown to OD600 0.55, then isopropyl β-D thiogalactoside (IPTG) added and growth was continued on shaker incubator at 37°C. Then, the total broth media was centrifuged and the bacteria cell pellet was dissolved in a binding buffer (10 mM imidazole, 0.3 M NaCl, 0.1 M KCl, 10% glycerol, 0.5% Triton X-100, 50 mM Tris-HCl, pH 7.6) [5].

Recombinant ESAT-6 (rESAT-6) was purified from the cell-free supernatant by chromatography on an Ni2+-NTA agarose column. After washing the column with 10mM imidazole in a lysis buffer (50mM Tris-HCl, PH=7.8, containing 300mM NaCl, 100mM KCl, 10% glycerol and 0.5% Triton X-100 1% v/v), ESAT-6 was eluted with 200mM imidazole in the lysis buffer. Fraction containing rESAT-6 was dialyzed by a PBS buffer pH=7.5 and was analyzed by SDSPAGE electrophoresis [5].

Results

Amplification of gene encoding ESAT-6

Total DNA was extracted from H37Rv strain of *M. tuberculosis* with Cinnagen protocol. DNA amplification by using specific primers for gene encoding ESAT-6 region was resulted in a single 288bp fragment.

Plasmid electrophoresis result

PCR product was cloned to pET102/D-TOPO vector (Fig.1) and was transformed to TOP10 strain of *E. coli* for propagation and maintained plasmid. Recombinant plasmid (pET102/D-TOPO::ESAT-6) were isolated and electrophoresis was done on 1% agarose gel by using 1Kb DNA ladder.

The molecular weight of bacterial vector was 6315bp (control) and the weight of inserted DNA was 288bp so recombinant plasmid (pET102/D::ESAT-6) was 6603bp on 1% agarose gel (Fig. 1). This implied the presence of inserted fragment (ESAT-6 gene) in mentioned vector. Further analysis of construct by PCR showed a 523bp band on 2% agarose gel electrophoresis, since the interval fragment between forward and reverse T7 primers was 235bp in length and the length of the inserted fragment was 304bp. Therefore, the appearance of 538bp could be due to the presence of the desired insert in the mentioned vector (Fig. 2).

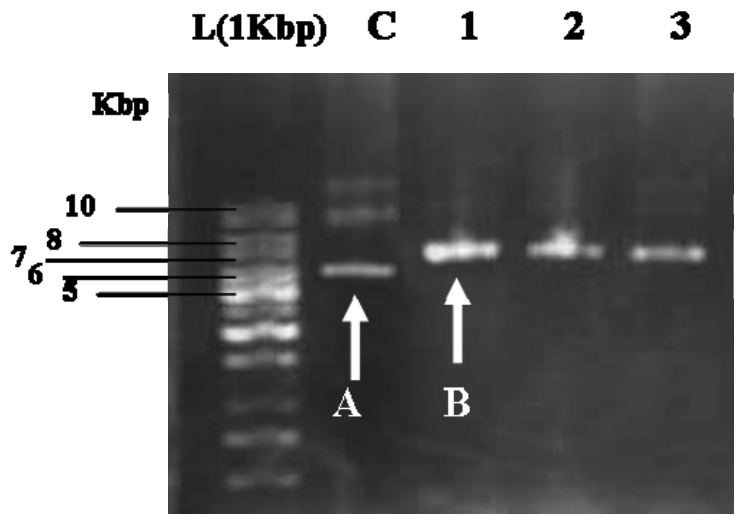

Fig. 1: Confirmation of ESAT-6 gene cloning into pET102/D. (A); confirmation of ESAT-6 gene cloning into pET102/D; recombinant plasmids (arrow B) and empty plasmid (arrow A) indicated a clear difference in electrophoretic mobility on a 1% agarose gel

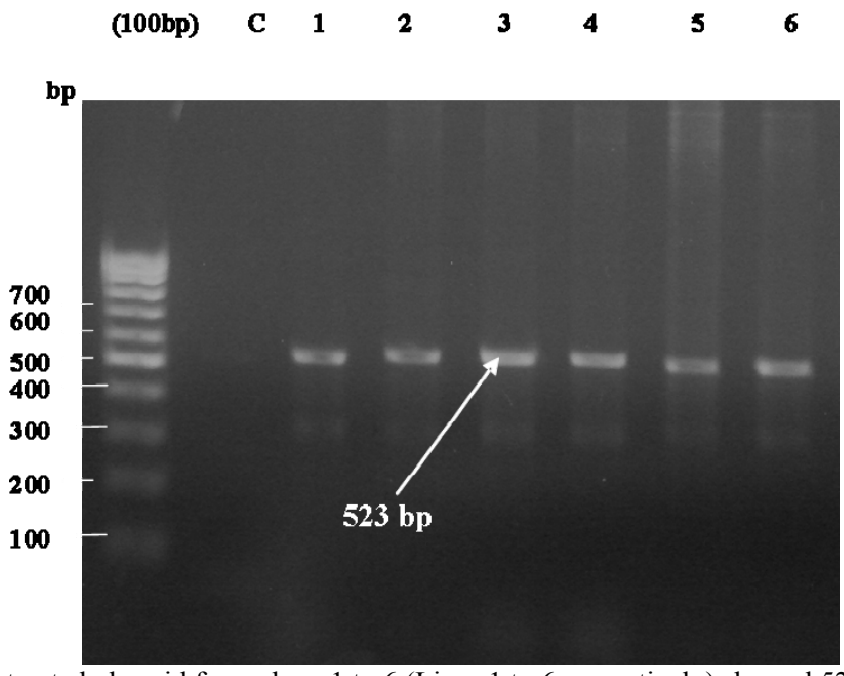

Fig. 2: Extracted plasmid from clone 1 to 6 (Lines 1 to 6 respectively) showed 523bp bands in PCR amplification by T7 primers, corresponding to the ESAT-6 gene and a 203bp fragment from plasmid

Plasmid sequencing was done by SEQ lab laboratory and the result of them was checked in gene bank of NCBI BLAST. Sequencing analysis revealed that the insert corresponds to a 288bp open reading frame which encodes ESAT-6, a 98-amino-acid polypeptide with an average molecular mass of 6kDa. The obtained sequences were searched for homology identity with NCBI BLAST software. The results showed that the sequences were completely matched with the ESAT-6 sequence (Gene Bank Accession Number BX842584). Subsequently we confirmed our gene was in correct frame with the appropriate N and C terminal fusion tag.

Expression and purification result

SDS-PAGE result

Expression of recombinant ESAT-6 protein in BL21 (DE3) strain of *E. coli* was performed with the pET102/D-TOPO expression vector. This protein expression was induced with IPTG. ESAT-6 was expressed at about 22kDa on polyacrylamide SDS-PAGE because the molecular weight of His patch thioredoxin in N terminal was 13kDa and 6xHis tag and v5 epitope in C-terminal was 3kDa. Since ESAT-6 was 6kDa so a fusion protein was obtained with an estimated molecular weight of 22kDa consist of His tag thioredoxin in N-terminal and 6XHis tag and V5 epitopes in C-terminal. We expected a 22kDa band on polyacrylamide gel (Fig. 3).

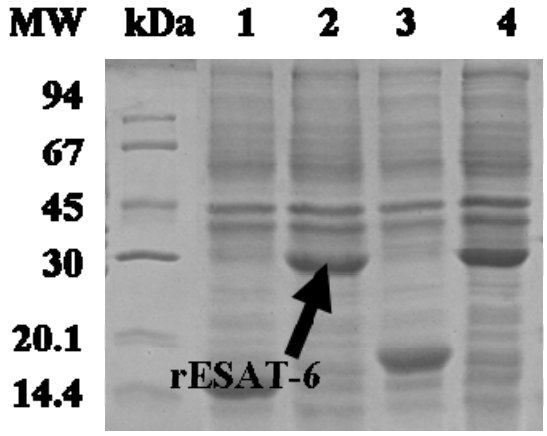

Fig. 3: Expression of rESAT-6 fusion protein in *E. coli* BL21. SDS-PAGE analysis of IPTG-induced BL-21 (DE3) containing recombinant plasmids (Lines 1 and 4) showed a 24kDa band corresponding to the ESAT-6 fusion protein. Uninduced bacteria showed no recombinant protein expression (Line 1 and 3)

SDS-PAGE result for confirmation of ESAT-6 protein in soluble phase

Total broth media was centrifuged and SDS-PAGE electrophoresis performed for soluble fraction of crude lysate of bacteria. This analysis indicated that recombinant protein (rESAT-6) was entirely expressed in soluble fraction (Fig. 4A). This protein was purified from soluble phase with metal affinity chromatography NI-NTA. This protein has 6xHis tag in C terminal so it can band to the resin and may be eluted by competition with imidazole (Fig. 4B).

Discussion

The studies from countries with low prevalence of tuberculosis show that RD1 gene-based diagnosis is more accurate than the TST [6]. The RD1 region in *M. tuberculosis* is 9455bp long, and encompasses nine ORFs (Rv3871-Rv3879c). Rv3874 or EsxB and Rv3875 or EsxA encode the proteins CFP-10 (10-kDa culture filtrate protein) and ESAT-6 (6-kDa early secretory antigenic target), respectively, which play a key role in virulence [3,7]. DNA encoding ESAT-6 called EsxA or Rv3875 that is placed in

RD1 region [8,9]. The ESAT-6 gene, encoding the early-secreted antigenic target 6kDa protein (ESAT-6), is found within RD1 where it was provisionally referred to as orf1C. The ESAT-6 protein is a major T-cell antigen, which has been purified from *M. tuberculosis* short-term culture filtrates (ST-CFs). Purified ESAT-6 stimulates the production of gamma interferon from mice memory immune T lymphocytes and may contribute to the development of anti tuberculosis immunity [9].

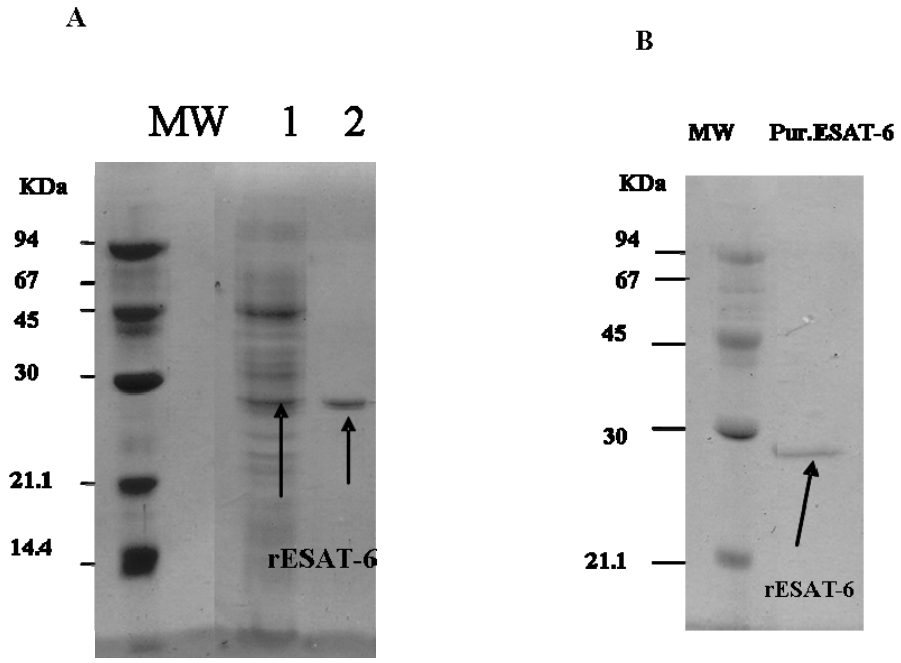

Fig. 4: Purification of ESAT-6 fusion protein as a soluble recombinant protein. The left figure (A) indicates SDS-PAGE analysis of insoluble phase (Lines 1) and soluble phase (Lines 2) of IPTG-induced BL-21 (DE3) containing recombinant plasmids. The right figure (B) shows SDS-PAGE analysis of ESAT-6 fusion protein, purifying by Ni-NTA chromatography

The ESAT-6 protein consists of 95 amino acids and was previously shown to be present in the *M. tuberculosis* ST-CF [7,9]. This protein has already been cloned, expressed and purified with different bacterial and viral vectors. In the year 2004 Bao ling-wan *et al.* [4] amplified the gene ESAT-6 of genome DNA by polymerase chain reaction (PCR) from *M. tuberculosis*. The PCR products were inserted into the pQE30 vector and transferred into *E. coli* strain TG1 and for expression ESAT-6 *E. coli* M15 transformed with pQE30::ESAT-6 were plated on LB solid medium containing ampicillin and Ni-NTA column used to purify ESAT-6 but the expressed fusion protein found almost entirely in the insoluble form (inclusion bodies) in cell lysate.

In the 2006 year in India Meher *et al.* [3] produced fusion protein ESAT-6 with 6xHis tag. They cloned EsxA into pET22b. This cloning strategy added a sequence of eight additional residues at the C-terminus of ESAT-6, the vectors containing EsxA were then transformed into BL21 (λDE3) *E. coli*. ESAT-6 was purified on a Ni-NTA super flow column, under denaturing conditions, as per the manufacturer's instructions except that NaCl and guanidine hydrochloride were excluded from the buffer. Purity of the protein in the eluted

fractions was determined by SDS/PAGE (15% gel). The cloning of ESAT-6 was performed in different expression vectors such as DH5a-pQE30 [10], pUC19 [11], pGEMt-Easy vector, pET23b$^+$ [12], pMCT3 [13], pET23b [14], pRSETB [1], and viral expression vector such as influenza type A virus [15].

In the present study, we used pET directional TOPO expression Kit to directionally clone blunt end PCR product in to pET102/D TOPO vector. This method does not require post PCR procedure, restriction enzyme or ligase. To enable directional cloning successfully we designed forward PCR primer with CACC sequence at the 5' end. The four nucleotides, CACC, base pair with the overhang sequence, GTGG, in pET102/D TOPO vector. In addition, topoisomerase I makes a GTGG overhang on expression vector that annealed to added four bases to forward primer (CACC) and PCR product directly inserted in expression vector without enzymatic digestion [16].

In cloning with other procedures, protein was found almost entirely in the insoluble form (inclusion bodies) in cell lysate and the inclusion bodies were solubilized with 8M urea or 6M guanidine-hydrochloride at pH=7.4, but in our study the interest protein was expressed in soluble phase due to Hispatch thioredoxine. We purified rESAT-6 from soluble phase with metal affinity chromatography or NI-NTA, because Ni-NTA is a nickel-charged agarose resin that has affinity for purification of fusion protein containing the 6xHis tag negative charge. Proteins bound to the resin may be eluted with competition imidazole.

Conclusion

Finally, ESAT-6 was cloned and expressed in a soluble form to be applicable for establishing a sensitive and specific test as an alternative to traditional diagnostic procedures.

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References

1) Fang CM, Zainuddin ZF, Musa M, Thong KL. Cloning, expression, and purification of recombinant protein from a single synthetic multivalent construct of *Mycobacterium tuberculosis*. *Pro Express Purif.* 2006; 47: 341-7.
2) Sada E, Brennan PJ, Herrera T, Torres M. Evaluation of lipoarabinomannan for the serological diagnosis of tuberculosis. *J Clin Microbiol.* 1990; 28: 2587-90.
3) Meher AK, Bal NC, Chary KV, Arora A. *Mycobacterium tuberculosis* H37Rv ESAT-6-CFP-10 complex formation confers thermodynamic and biochemical stability. *FEBS J*. 2006; 273(7): 1445-62.
4) Wang BL, Xu Y, Wu CQ, Xu YM, Wang HH. Cloning, expression, and refolding of a secretory protein ESAT-6 of *Mycobacterium tuberculosis*. *Pro Express Purif.* 2005; 39: 184-8.
5) Sankian M, Yousefi M, Pazouki N, Varasteh A. One-step purification of histidine-tagged profilin with high purity and yield by using metal precipitation. *Biotech Appl Biochem.* 2007; 47: 185-9.
6) Arend SM, Engelhard ACF, Groot G, *et al*. Tuberculin skin testing compared with T-Cell responses to *Mycobacterium tuberculosis*-specific and nonspecific antigens for detection of latent infection in persons with recent tuberculosis contact. *Clin Diag Lab Immunol.* 2001; 8: 1089-96.
7) Renshaw PS, Lightbody KL, Veverka V, *et al*. Structure and function of the complex formed by the tuberculosis virulence factors

CFP-10 and ESAT-6. *EMBO J*. 2005; 24: 2491-8.
8) Brodin P, Majlessi L, Marsollier L, *et al*. Dissection of ESAT-6 system 1 of *Mycobacterium tuberculosis* and impact on immunogenicity and virulence. *Infect Immun.* 2006; 74: 88-98.
9) Berthet FX, Rasmussen PB, Rosenkrands I, Andersen P, Gicquel B. A *Mycobacterium tuberculosis* operon encoding ESAT-6 and a novel low-molecularmass culture filtrate protein (CFP-10). *Microbiology*. 1998; 144: 3195-203.
10) Calderon MN, Parra-López C, Alfonso R, *et al*. *Mycobacterium tuberculosis* ESAT-6 antigen immunogenicity in Owl Monkeys. *NOVA Publicat Cientific*. 2006; 4: 14-26.
11) Brodin P, de Jonge MI, Majlessi L, *et al*. Unctional analysis of early secreted antigenic target-6, the dominant T-cell antigen of *Mycobacterium tuberculosis*, reveals key residues involved in secretion, complex formation, virulence, and immunogenicity. *J Biolog Chem.* 2005; 280: 33953-9.
12) Sinha S, Kosalai K, Arora S, *et al*. Immunogenic membrane-associated proteins of *Mycobacterium tuberculosis* revealed by proteomics. *Microbiol*ogy. 2005; 151: 2411-9.
13) Weldingh K, Rosenkrands I, Okkels LM, Doherty TM, Andersen P. Assessing the serodiagnostic potential of 35 *Mycobacterium tuberculosis* proteins and identification of four novel serological antigens. *J Clin Microbiol*. 2005; 43: 57-65.
14) Mukherjee P, Dutta M, Datta P, *et al*. The RD1-encoded antigen Rv3872 of *Mycobacterium tuberculosis* as a potential candidate for serodiagnosis of tuberculosis. *Clin Microbiol Infect.* 2007; 13: 146-52.
15) Stukova MA, Sereinig S, Zabolotnyh NV, *et al*. Vaccine potential of influenza vectors expressing *Mycobacterium tuberculosis* ESAT-6 protein. *Tuberculosis*. 2006; 86: 236-46.
16) InvitrogenTM. Champion pET directional TOPO expression kits Five-minute, directional TOPO® Cloning of blunt-end PCR products into vectors for high-level, inducible expression in *E. coli* Catalog nos. K100-01, K101-01, K102-01, K151-01, K200-01. USA: Version H; 2006.

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