

Research Article

ESAT-6 protein for specific *Mycobacterium tuberculosis* in guinea pigs skin test

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Abstract: Detection of *Mycobacterium tuberculosis* (MTB) infection by delayed-type hypersensitivity skin test with purified derivative (PPD) antigen is still used in many countries including Thailand. However, it is less specificity due to significant cross-reactivity with non-tuberculosis mycobacteria. Antigen such as early secretory antigenic target (ESAT-6) is secreted in the early phase of infection and elicits cell mediated immune responses. Moreover it is found only in *M. tuberculosis*, *M. bovis* and few other mycobacterial species. ESAT-6 protein can be a good candidate for MTB specific diagnosis. Here, we report the cloning, expression, purification of ESAT-6 protein and its utility as specific antigen for MTB diagnosis by skin testing in guinea pig sensitized with killed *M. tuberculosis* and live BCG. Our results show that 200 mg of the purified ESAT-6 protein elicits a positive skin reactivity only in the guinea pigs sensitized with killed *M. tuberculosis* and not in the animals sensitized with live BCG. These data in this study support the use of ESAT-6 protein in specific detection of MTB infection.

Keywords: PPD, ESAT-6, *Mycobacterium tuberculosis*.

INTRODUCTION

Tuberculosis (TB) remains a major global health problem. One-third of the world's population was infected with *M. tuberculosis* and causes tuberculosis. From the Global Tuberculosis Report 2012 (WHO), there were almost 9 million new case in 2011 and 1.4 million TB deaths [1]. Transmission of *M. tuberculosis* occurs via droplets from the throat and lungs of people with the active respiratory disease. There are several tests currently being used for tuberculosis diagnosis such as sputum smear microscopy [2], culture based test, serological test and PCR based test [3]. However, these tests are needed to improved in order to enhance their rapidity, sensitivity and specificity. The results, including inexpensive and less painful, must be concerned [2].

Many tuberculosis subunit antigen types were chosen in the clinical trials of a new TB vaccine. In response to TB infection, all selected antigens, either from active TB or latent TB, could induce Th1-type immune responses. Good antigens, ESAT-6, CFP-10[5], MPT64[6] or Rv3615c, should induce the secretion of IFN- γ and IL-2 for memory T cell responses in both CD4 + and CD8 +[7].

ESAT-6 (early secretory antigenic target), molecular weight of 6 kDa, was generated from the RD1 gene of mycobacterium in *M. tuberculosis*, *M. bovis*, *M. africanum*, *M.kansasii*, *M. marinum*, *M. szulai* and *M. flavescens* strains but absent in BCG vaccine strains. Mycobacteria species commonly found in nature, about 90%[8] of ESAT-6 expressions were continuing to grow both in the process of cell division

and non-cell division [9]. The study by ELISPOT, serum detection method, in patients with tuberculosis showed a response to ESAT-6 up to 96% [10]. Moreover, ESAT-6 could be used not only for human but also for cattle and non-human primates[11]. ESAT-6 was important role in the livelihood of *M. tuberculosis* in the host cells and secretion of ESAT-6 could be recognized by the immune system of the host cell. The expression of ESAT-6 was considered to be a characteristic of the disease phenotype in the perfect *M. tuberculosis* [12]. Some studies of *M. tuberculosis* used ESAT-6 to compare with PPD for delayed-type hypersensitivity (DTH) by using a skin test in guinea pig [6,13]. The results showed that ESAT-6 could isolate *M. tuberculosis* from BCG infected mice and Mycobacteria species natural habitat. It could be concluded that ESAT-6 was highly specific for *M. tuberculosis*. The Tuberculin Skin Test (TST) based on detection of a cutaneous delayed-typed hypersensitivity response to PPD antigen is commonly used in many countries including Thailand. The problems of this test are false positive results by cross-reaction with BCG vaccination and exposition with other non-tuberculosis mycobacteria (NTM). Therefore, a new specific reagent for *M. tuberculosis* diagnosis is needed to overcome the limitations of PPD. The regions of the *M. tuberculosis* genome which are missing from BCG and NTM provides a new opportunity for the development of novel diagnostic reagents. One such region, which is absent in non-tuberculosis mycobacteria as well as in the BCG vaccine but present in the *M. tuberculosis* complex is the RD1 region. ESAT-6 is the gene encoded in RD1 region[8] and is frequently recognized in disease. It has been investigated and shown to have high potential in

the specific *in vitro* diagnosis of tuberculosis infection in humans [14].

Recently, a recombinant ESAT-6 protein was tested as a stimulating antigen in skin test for different diagnosis of TB infection in guinea pig in order to evaluate the sensitivity and specificity. The results showed a positive skin response in animals exposed to TB by using ESAT-6 antigen [13]. Nevertheless, it could distinguish between tuberculosis infection, BCG vaccination, or exposure to environmental mycobacteria. The objective of this study was to determine the diagnostic potential of ESAT-6 antigen and offers a realistic alternative to PPD.

MATERIALS AND METHODS

Cloning

The cloning of ESAT-6 gene was performed according to standard procedures [15]. Briefly, the gene encoding the ESAT-6 protein was amplified by PCR using two primers. The forward primer contain a *NdeI* restriction enzyme recognition site 5'-TAT ACA TAT GAC AGA GCA GCA GTG-3'. The reverse primer contained a *NotI* restriction enzyme recognition site 5'-TTT GCG GCC GCC TAT GCG AAC ATC CCA-3'. PCR amplification was proceeded by using 35 cycles of denaturation at 94 °C for 2 min, annealing at 50 °C for 30 min, extension at 72 °C for 30 min and final extension at 72 °C for 7 min. The PCR reaction mixture (50 ul) included DW 22 ul, 2X Master Mix (Vivantis) 22 ul, primers 1 ul and genomic *M. tuberculosis* H37RV 1 ul. The final products were check by 1.5% agarose gel electrophoresis under the current of 100 volts for 55 min. PCR products were purified from the gel using HiYield™ GEL/PCR DNA Fragment Extraction Kit (RBC bioscience). PCR product and pET 24b (+) were digested with *NdeI* and *NotI* (Vivantis). The reaction of 30 ul was carried out overnight at 37°C. ESAT-6 fragment and pET-24 b (+) vector were ligated using Vivantis reagent. The recombinant plasmid was transformed into TOP10 competent cell (Invitrogen) by heat shock method on kanamycin LB agar plate. A single colony was selected to examine the inserted ESAT-6 positive clones by PCR and sequencing. Plasmid positive clones were extracted with High-Speed Plasmid Mini Kit (Geneaid). The inserted ESAT-6 gene in pET-24b(+) was verified by sequencing using T7 promoter and T7 terminator primer. DNA sequences from plasmid positive clones were compared with the data from GenBank and transformed into BL21 competent cell (DE3) (invitrogen).

Expression ESAT-6 protein

The positive clone was picked into 200 ml starter media of LB broth containing kanamycin and incubated 37°C with shaking at 200 rpm for 16-18 h. Each 50 ml starter (10%) was aliquoted into 2 liters of LB broth containing kanamycin which separated into 4 flasks of 500 ml medium. The cultures were shaken with the speed of 200 rpm at 37°C, until the OD values at 600 nm about 0.6 to 0.7. The expression of ESAT-6 gene was induced with 1mM IPTG and incubated 37°C with shaking at 200 rpm for 16-18 h. Pellet cell was harvested by centrifugation and stored at -80 °C until protein purification.

Protein Extraction and purification

All steps were performed on ice. All buffers were filtered though 0.45 micron membrane. Pellet of cell was resuspended in 20mM sodium phosphate, 0.5 M NaCl pH 7.4 and sonicated 5 times for 20 seconds at a time on the ice for 2 minutes (with 80% amplitude). The suspension was centrifuged at 4°C, 12,000 rpm for 1 hour. The supernatant was discarded. The pellet was dissolved with 20mM sodium phosphate containing 0.5 M NaCl and 8M urea pH 7.4. The mixture was stirred overnight. After that, the suspension was centrifuged at 4°C, 12,000 rpm for 1 hour. The supernate was collected and filtrated on 0.45 micron membrane. Finally, 1X Protease inhibitor cocktail (Amresco) was added to the suspension and kept cool.

Protein were purified on 5 ml of Ni-nitrilotriacetic acid resin using a HisTrap HP (GE). The column was equilibrated using 30 ml of 20mM sodium phosphate containing 0.5 M NaCl and 8M urea pH 7.4. Crude ESAT-6 protein was loaded into the column and washed with 30 ml of the same buffer and then washed with more 30 ml of the same buffer containing 20 mM imidazole. For refolding step, the column was wash with linear gradient of 8-0 M urea by 30ml 20 mM sodium phosphate containing 0.5 M NaCl, 8M urea, 20mM imidazole pH 7.4 and 30ml 20 mM sodium phosphate containing 0.5 M NaCl, 20mM imidazole pH 7.4. Elution linear 0-250 mM imidazole was made using 30 ml 20 mM sodium phosphate containing 0.5 M NaCl pH 7.4 and 20 mM sodium phosphate containing 0.5 M NaCl and 250 mM imidazole pH 7.4. The scan fraction tube OD values at 280 nm was collected and and protein was checked with the SDS PAGE. The protein was dialysed with PBS buffer pH 7.3 under cold temperatures. The protein concentration could be increased using Viva spin (GE) by 4,000 rpm centrifugation at 4°C. The purified protein was stored at -20°C.

Determination of molecular mass

Differentially expressed protein spots were excised from the 2-DE gels and subjected to in-gel tryptic digestion as previously described. Briefly, the excised protein spots were washed three times with 200 µl of 25 mM NH₄HCO₃/50% acetonitrile (ACN). The gel pieces were dehydrated with 200 µl of 100%

ACN, rehydrated with 12.5 µg/ml of sequencing grade trypsin (Promega; Madison,WI), and incubated at 37 °C for approximately 16 h. The supernatants were then transferred to a new tube and 100 µl of 50% ACN/0.5% formic acid was added. The mixtures were finally dried with a speedvac and resulting peptides were suspended in 10 µl of 50% ACN/0.1% formic acid. The peptides were then analyzed by MS/MS using micrOTOF-Q II™ ESI-Qq-TOF mass spectrometer (Bruker; Berman, Germany) equipped with an online nanoESI source. MASCOT is a software search engine that uses mass spectrometry data to identify proteins from peptide sequence databases. Mass tolerance of parent and fragmented ions were 1.0 Da and 0.6 Da, respectively. MS/MS ions scores ≥ 38 were considered significant hits. The molecular mass of purified ESAT-6 protein was determined by mass spectrometry at the Instrument Test Center, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

M. tuberculosis strains

M. tuberculosis H37RV (supported by Faculty of Medical Microbiology, Chulalongkorn University, Thailand) were grown on Lowenstein-Jensen slants at 37°C for 4 weeks and then transferred to Sauton liquid medium at 37°C for 4 weeks without shaking. Powder of *M. tuberculosis* H37RV was killed with heat by autoclave. Then it desiccated and pulverizes into fine powder using mortar with liquid nitrogen. The fine powder was mixed into sterile liquid paraffin by homogenizer [16].

Guinea pig sensitization and skin tests

Various doses (0.25-2.0 mg/ml) of ESAT-6 protein were tested for their ability to produce DTH responses in guinea pigs sensitized with killed *M. tuberculosis* and lived BCG vaccine. Twenty female guinea pigs, weighing 400-450 g were divided into 3 groups. Group 1 : eight guinea pigs were sensitized with 0.5 ml of 2 mg/ml killed *M. tuberculosis* H37RV by intramuscular injection into each of two lower limbs [16]. Group 2 : six guinea pigs were sensitized with lived BCG vaccine. Six guinea pigs in Group 3 were control group without sensitization. Thirty days after sensitization, guinea pigs were shaved on the abdomen and injection intradermally with 0.25 to 2.0 mg of the purified ESAT-6 antigen in 0.1 ml PBS, 10 IU PPD (positive control). PPD produced from Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok, Thailand. The diameters of both axes of skin reaction lesion were measured and recorded within 24-48 h after inoculation. The skin reaction with more than 5 mm in diameter was considered positive.

RESULTS AND DISCUSSION

The optimal condition for ESAT-6 protein expression was IPTG induction at 37°C for 16-18 h. The protein was expressed in the form of inclusion body in the pellet cell (Figure 1).



Figure 1 : SDS-PAGE pattern of crude ESAT-6 protein expressed in soluble form (L1) and in inclusion body form (L2) (Marker: Kaleidoscope, Bio-Rad).

The expression of ESAT-6 protein band (L2) in the form of inclusion body, molecular weight of approximately 10 kDa (including 6 Histidine tag), was better than ESAT-6 protein in a soluble form (L1) on 16% SDS PAGE and Figure 3 is purified by passing HisTrap Ni²⁺ column.

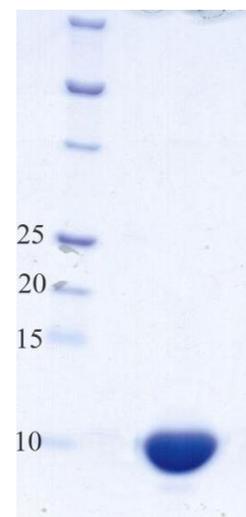


Figure 2: SDS-PAGE analysis of purified ESAT-6 protein. The expressed protein was purified by His Trap Ni²⁺ column. Lane 1 : Protein molecular weight marker (Kaleidoscope, Bio-Rad), Lane 2 : ESAT-6 purified protein.

Skin reactivity to ESAT-6 in guinea pigs sensitized**Table 1 Skin reactivity to various doses of ESAT-6 in the same guinea pig (group 1) sensitized with killed *M. tuberculosis*.**

Injection	Doses	No. of guinea pig	Skin reaction (mean \pm SD mm in diameter)	
			24 h	48h
PPD	10 IU	8	8.87 \pm 1.1	7.25 \pm 0.7
PBS	-	8	0	0
ESAT-6	25 μ g	8	3.25 \pm 0.4	<3.00
	50 μ g	8	5.36 \pm 0.5	3.88 \pm 0.8
	100 μ g	8	6.63 \pm 0.7	5.40 \pm 0.5
	200 μ g	8	7.50 \pm 0.9	5.75 \pm 0.7

PBS, phosphate buffer saline; PPD, purified protein derivative; SD, standard deviation

Table 2 Skin reactivity to ESAT-6 in the guinea pig (group 2) sensitized with live BCG vaccine.

Injection	Doses	No. of guinea pig	Skin reaction (mean \pm SD mm in diameter)	
			24 h	48h
PPD	10 IU	6	9.12 \pm 1.2	7.50 \pm 1.4
PBS	-	6	0	0
ESAT-6	200 μ g	6	0	0

PBS, phosphate buffer saline; PPD, purified protein derivative; SD, standard deviation

Table 3 Skin reactivity to ESAT-6 in the guinea pig (group 3) without killed *M. tuberculosis* and live BCG sensitization.

Injection	Doses	No. of guinea pig	Skin reaction (mean \pm SD mm in diameter)	
			24 h	48h
PPD	10 IU	6	0	0
PBS	-	6	0	0
ESAT-6	200 μ g	6	0	0

PBS, phosphate buffer saline; PPD, purified protein derivative; SD, standard deviation

The ESAT-6 gene was cloned into bacterial expression vector pET-24(+) for protein expression. The recombinant plasmid was transformed into TOP10 competent cell by heat shock method on kanamycin LB agar plate. The inserted ESAT-6 positive clones were examined by PCR and sequencing. The optimal condition for ESAT-6 protein expression was IPTG induction at 37°C for 16-18 h. After expression step, the cells were completely lysed. Then protein in soluble and insoluble fractions were analysed by SDS-PAGE. The SDS-PAGE analysis pattern in figure 1 showed that the ESAT-6 protein was predominantly expressed in the form of inclusion body in the pellet cell. The purified ESAT-6 protein showed a single band of approximate molecular weight of 10kDa (including 6 Histidine tag) on 16% SDS-PAGE (Fig 2). Yield of ESAT-6 protein ranged between 15-20 mg protein/L of culture. The mass spectrometry data (not shown) of purified ESAT-6 protein was identified by using MASCOT search engine. It was found that thirty three amino acid residues at N-terminal were MTEQQWNFAGIEAAASAIQGNVTSHISLLDEGK. The average molecular mass and pI were 9898 Da and 4.48. These results correspond to the sequence GenBank FJ014499 *M. tuberculosis* H37RV.

Twenty female guinea pigs were divided into 3 groups. Eight guinea pigs of group 1 were sensitized with killed *M. tuberculosis* H37RV. Six animals in group 2 were sensitized with live BCG vaccine. Thirty days after the challenge, each guinea pig in group 1 was injected intradermally in six separate sites on the abdomen of the animals with 0.1 ml of PBS as negative control, 0.1 ml (10 IU) of PPD as positive control and 0.1 ml of ESAT-6 protein at 0.25, 0.5, 1.0 and 2.0 mg/ml respectively. The diameters of reactivity in guinea pigs at 24 and 48 h are shown in Table 1, 2 and 3. A positive result was defined as an average induration greater than 5 mm. The killed *M. tuberculosis* sensitized animals in group 1 had a positive result to PPD and ESAT-6 protein at over 1.0 mg/ml (100 μ g) (Table 1). The slightly larger lesion was observed with PPD injection. Group 2, the live BCG sensitized guinea pigs reacted positively only to PPD (Table 2). The animals without sensitization had negative reaction to PPD, PBS and ESAT-6 protein (Table 3). The results at 48 h were similar to that at 24 h, but the size of the skin lesion had decreased. Although the mass of purified ESAT-6 protein in this study was higher than that of native ESAT-6, the pI was almost the same as that of native ESAT-6. However, the mass spectrometry data showed identity of ESAT-6 protein to the sequence of *M. tuberculosis* H37RV. It

has been found that this ESAT-6 protein has great potential as a skin test reagent, as demonstrated by the results in guinea pigs. Two hundred micrograms of purified ESAT-6 antigen produced a slightly lower intensity of reaction than in the skin reactivity to PPD. The concentration of ESAT-6 that produced the positive result is higher than that of ESAT-6 in the previous study of Wu *et al.* [13]. The process of immunization of killed TB including dose of killed *M. tuberculosis* H37RV, single injection of antigen and route of injection, may be effect the ability to response to ESAT-6. In addition, the mass and pI of this ESAT-6 protein are different from the previous ESAT-6 protein.

Because of many antigens common among different species of mycobacteria in PPD, it can therefore induce cross-reactions among animals with tuberculosis infection, non-tuberculous mycobacterial infection and BCG vaccination. A positive skin reaction with PPD (Table 1, 2) cannot distinguish between *M. tuberculosis* and BCG. The gene encoding the ESAT-6 protein is located in RD1 gene of *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. kansasii*, *M. marinum*, *M. sulgai* and *M. flavescens*, and is specifically absent in BCG. Therefore the specificity of ESAT-6 protein can be used to distinguish *M. tuberculosis* from BCG. Our results in animals models are consistent with the previous studies of Wu *et al.* [13]. It showed that ESAT-6 protein is capable of eliciting skin test reaction in guinea pigs immunized with *M. tuberculosis* but does not elicit any reaction in the guinea pigs immunized with BCG vaccine. Moreover having a greater specificity to TB than PPD, there are many other advantages to developing a skin test that utilizes a single recombinant ESAT-6 antigen. Manufacturing of the large number of different proteins in mixture, such as PPD, in compliance with good manufacturing practices (GMP) regulations is practically difficult. The batch-to-batch variation in the production of PPD at various facilities has been well documented. While ESAT-6 protein as a single recombinant protein could be produced in an effective and consistent manner that conforms to GMP standard or local regulatory agencies. Therefore it is likely to produce consistent and more easily interpretable responses when used. In summary, our results here indicate that specificity of ESAT-6 protein can be used for detection of *M. tuberculosis* skin test and it is possible to develop a new testing reagent which will be used instead of PPD for diagnosis of *M. tuberculosis*.

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