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ORIGINAL ARTICLE

# Evaluation of *Mycobacterium tuberculosis* Early Secreted Antigenic Target 6 Recombinant Protein as a Diagnostic Marker in Skin Test

Jale Moradi, Nader Mosavari, Mahmoud Ebrahimi, Reza Arefpajohi, Majid Tebianian*

*Department of TB, Razi Vaccine and Serum Research Institute, Karaj, Iran.*

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**Abstract**
**Objectives:** Tuberculosis (TB) is the leading infectious disease in the developing world. Delayed-type hypersensitivity skin test diagnoses TB using tuberculin purified protein derivative (PPD), but this test is incapable of distinguishing *Mycobacterium tuberculosis* (MTB) infection from bacillus Calmette–Guérin (BCG) vaccination or an infection caused by nontuberculous mycobacteria (NTM). This study was performed to evaluate the use of recombinant early secretory antigenic target 6 (rESAT-6), a secretory protein found only in MTB, *Mycobacterium bovis*, and few other mycobacterial species, as a skin marker for MTB in guinea pigs.
**Methods:** We prepared recombinant MTB ESAT-6 and evaluated its use as a specific antigen for MTB in guinea pigs.
**Results:** Our results show that the purified MTB rESAT-6 antigen is capable of inducing a positive reaction only in guinea pigs sensitized to MTB. No such reaction was observed in the animals sensitized to *M. bovis*, BCG vaccination, or NTM (*Mycobacterium avium*).
**Conclusion:** Our study results confirm that the ESAT-6 antigen is more specific to MTB infection than PPD and could be used in more specific skin tests for detection of MTB in large animals and in humans.

## 1. Introduction

In 2012, estimates indicated 8.6 million new TB cases and 1.3 million TB-related deaths, of which there were 1 million human immunodeficiency virus (HIV)-negative patients, with the remaining 0.3 million being HIV-positive patients. These data indicate that TB is one of the important health problems [1]. Furthermore, a pandemic of tuberculosis (TB) is influenced by increases in the HIV/acquired immunodeficiency syndrome cases and emergence of multidrug-resistant and extensively drug-resistant strains, all of which aggravate the

*Corresponding author.
E-mail: M.tebianian@rvsri.ac.ir

problem. Therefore, there is a necessity to identify strategies for controlling TB infection [2].

TB-controlling strategies are based on accurate diagnosis, but this is not possible with the classical common technique that is used to screen patients for TB [3]. Tuberculin purified protein derivative (PPD) test is a delayed-type hypersensitivity reaction that is widely used for screening patients with exposure to *Mycobacterium tuberculosis* (MTB) for several decades [4]. PPD is a mixture of large number of MTB antigens that are present in nontuberculous mycobacteria (NTM) and *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) [5]. Therefore, the PPD skin test can produce a false-positive reaction in patients who have received the BCG vaccination or have had exposure to NTM [5]. The *early secretory antigenic target 6* (*ESAT-6*) gene is located in the RD1 region and is present in the pathogenic strains of *Mycobacterium* such as MTB, *M. bovis*, *Mycobacterium africanum*, *Mycobacterium kansasii*, *Mycobacterium marinum*, *Mycobacterium szulgai*, *Mycobacterium flavescens*; however, this gene is absent in all strains of *M. bovis* BCG and in a large number of NTM [6]. The gene activates one of the most important virulence factors in MTB and is responsible for inducing secretion of interleukin-8 (neutrophils) and T-lymphocyte chemotactic cytokine [7,8]. In recent years, this gene was evaluated as a diagnostic tool for detection of MTB infection in enzyme-linked ImmunoSpot assay technique and as a candidate for vaccination [9]. We have successfully cloned and expressed ESAT-6 protein and evaluated its sensitivity and specificity as a skin test antigen and compared recombinant ESAT-6 (rESAT-6) skin test reaction with locally prepared PPD in guinea pigs.

## 2. Materials and methods

### 2.1. Bacterial strains and product

MTB H37Rv genome, *Mycobacterium avium*, and *M. bovis* BCG were obtained from the Tuberculosis Research Laboratory of Razi Vaccine and Serum Research Institute (Karaj, Iran). The vector pQE60 was obtained from the Iranian Recombinant Gene Bank (Institut Pasteur, Tehran, Iran). PPD produced from MTB (50 IU/mL) was obtained from Razi Vaccine and Serum Research Institute. *Escherichia coli* strains M15 and XL1-blue were grown in Luria-Bertani liquid media.

### 2.2. Cloning, expression, and purification

The *ESAT-6* gene from H37Rv strains of MTB was amplified by polymerase chain reaction (PCR). Forward and reverse primers have sites for *Bgl*II and *Bam*HI. After digesting PCR products with appropriate enzymes, the fragments were ran on 1% agarose gel and purified. The *ESAT-6* gene was ligated to the pQE60 vector using

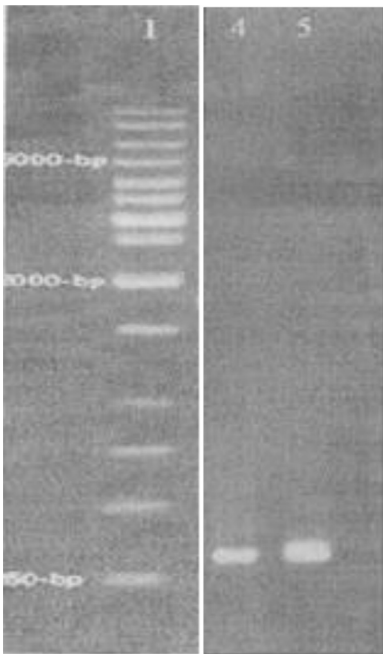


**Figure 1.** Evaluation of recombinant early secretory antigenic target 6 (*rESAT-6*) gene as polymerase chain reaction product. Lanes 4 and 5 = *rESAT-6* gene.

T4 DNA ligase and transformed into *E. coli* XL1-blue cells. Restriction enzyme analysis was used to screen the transformants using *Eco*RI and *Hind*III and these were confirmed by sequencing. The pQE60-E6 was purified from the culture of recombinant *E. coli* XL1-blue and transformed into the competent *E. coli* M15 cells. The transformants were placed on lysogeny broth plates containing 50 μg/mL ampicillin and 30 μg/mL

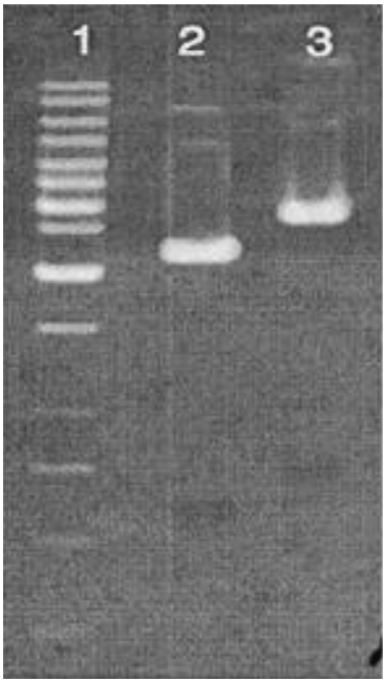


**Figure 2.** Electrophoresis of recombinant plasmid after digestion by restriction endonucleases.

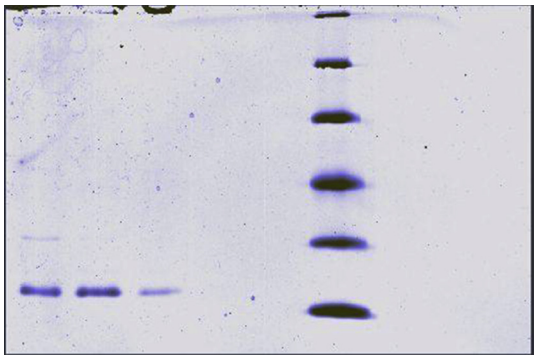

**Figure 3.** Purification of recombinant early secretory antigenic target 6 (rESAT-6) protein as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

kanamycin. Recombinant histidine (His)-tagged ESAT-6 (rESAT-6) protein was expressed in these cells and purified by nickel-nitrilotriacetic acid (Ni-NTA) column. Purified proteins were ran on sodium dodecyl sulfate (SDS) page and confirmed by Western blotting (Figures 1–3).

### 2.3. Sensitization of guinea pigs and skin test

White guinea pigs ($n$ = 37; weight, 250–300 g) were obtained from Razi Vaccine and Serum Research Institute (Karaj, Iran). First, nine guinea pigs were sensitized by intradermal administration of 0.5 mL sensitization solution (100 mg MTB, 50 mL paraffin, and 50 mg pumice stone) and maintained for 45 days under specific pathogen-free condition. After the sensitization period, guinea pigs were shaved on the back and flanks, and then each animal, based on the potency test table (Table 1), received 0.1 μg of protein randomly. The diameter of reactions on the skin was measured 24 hours after the injection of the sensitization solution. During skin testing, 28 guinea pigs were tested; seven groups were formed, with each group consisting of four guinea pigs. The first group was the control group, which was sensitized to 0.1 mL of phosphate-buffered saline. The second group was sensitized to 0.1 mL of live *M. bovis* BCG. The third group was sensitized to 5 mg of *M. avium*. The fourth, fifth, and six groups were sensitized to 5 mg of PN, DT, and C strains of MTB, respectively. Guinea pigs were maintained under specific pathogen-free condition for 45 days. After the sensitization period, each guinea pig received six injections of PPD (5–25–125 tuberculin units) and rESAT-6 (0.1–1–10 μg). Twenty-four to forty-eight hours after the last injection, both axes of the erythema were measured in millimeters by digital palpation.

## 3. Results

### 3.1. Cloning, expression, and analysis of rESAT-6

The *ESAT-6* gene from the MTB H37Rv genome was amplified and cloned in the pQE60 expression vector. The recombinant ESAT-6 protein successfully expressed in these cells and three-level usage of elution buffer in Ni-NTA purification column was carried out. A single band of rESAT-6 protein (approximately 10 kDa) was determined by SDS page and confirmed by anti-ESAT-6 antibody in Western blotting.

### 3.2. Potency test

In this test, nine guinea pigs sensitized to three strains of MTB randomly received six injections. The potency of the injections was measured by statistical analysis. Our results show that the guinea pig model evaluated in this study is accurate for detection of rESAT-6.

**Table 1.** Potency test.

| | (Standard) PPD | | | (Test) ESAT-6 | | |
|---|---|---|---|---|---|---|
| Guinea pigs | 1 | 2 | 3 | 4 | 5 | 6 |
| No | 1/100 | 1/500 | 1/2500 | 1/100 | 1/500 | 1/2500 |
| 830 | 22 | 20.5 | 17 | 24 | 20.8 | 16 |
| 831 | 18.5 | 18 | 15 | 19 | 18.2 | 14.5 |
| 832 | 27 | 24.5 | 16.5 | 27.5 | 23.2 | 14 |
| 838 | 26.5 | 23 | 17 | 27.2 | 23 | 14.5 |
| 839 | 25.5 | 24.5 | 17.5 | 26.2 | 24.2 | 15.5 |
| 840 | 25.5 | 24 | 20 | 26.8 | 24.8 | 19.5 |
| 841 | 25 | 23.5 | 20 | 26.7 | 24 | 9.5 |
| 842 | 23.5 | 21 | 19 | 24.8 | 22.1 | 18 |
| 843 | 24 | 21 | 19.5 | 24.8 | 23 | 18 |
| Total | 217.5 | 200 | 161.5 | 227 | 203.3 | 139.5 |
| Mean | 24.17 | 22.23 | 17.94 | 25.22 | 22.58 | 15.5 |
| | Total for standard = 579 | | | Total for test = 569.8 | | |

ESAT-6 = early secretory antigenic target 6; PPD = tuberculin purified protein derivative.

**Table 2.** Skin test reaction to rESAT-6 and PPD in sensitized guinea pigs (measured in millimeter).

| | | | *Mycobacterium tuberculosis* strains | | | | |
|---|---|---|---|---|---|---|---|
| | Buffer | BCG | D4 | PN | DT | C | C, DT, PN |
| Groups | $N = 4$ | $N = 4$ | $N = 4$ | $N = 4$ | $N = 4$ | $N = 4$ | $N = 4$ |
| 5 TU PPD | — | 12.67 | 13.83 | 16.32 | 15.96 | 16.20 | 16.37 |
| 25 TU PPD | — | 14.67 | 16.33 | 21 | 20.90 | 21.07 | 21.5 |
| 125 TU PPD | — | 16.33 | 18.16 | 22.90 | 23 | 23.2 | 23.5 |
| 0·1 rESAT-6 | — | — | — | 14 | 14.02 | 13 | 14.75 |
| 1 mg rESAT-6 | — | — | — | 18.11 | 18.5 | 18.80 | 18.63 |
| 10 mg rESAT-6 | — | — | — | 20.90 | 20.53 | 20.68 | 21 |

BCG = bacillus Calmette–Guérin; PPD = tuberculin purified protein derivative; rESAT-6 = recombinant early secretory antigenic target 6; TU = tuberculin units.

### 3.3. Delayed-type hypersensitivity reaction

The hypersensitivity reaction was measured in seven groups of guinea pigs and analyzed 48 hours after receiving the injection. The first group (control) that received the buffer did not show any reaction to rESAT-6 and PPD (Table 2). The groups sensitized to MTB strains showed relative sensitivity to PPD and rESAT-6. Groups sensitized to BCG vaccine and *M. avium* D4 strain as an NTM showed reactivity to PPD, but no reactivity to rESAT-6 (Figure 4).

## 4. Discussion

TB is considered a major global health problem. It infects millions of people each year and in terms of mortality rate, TB is the second leading infectious disease, after HIV [1,10]. TB-controlling strategies are based on accurate diagnosis, but this is not possible with the classical common technique that is used to screen patients for TB [3]. Delayed-type hypersensitivity skin reaction test uses tuberculin PPD, and is widely used for

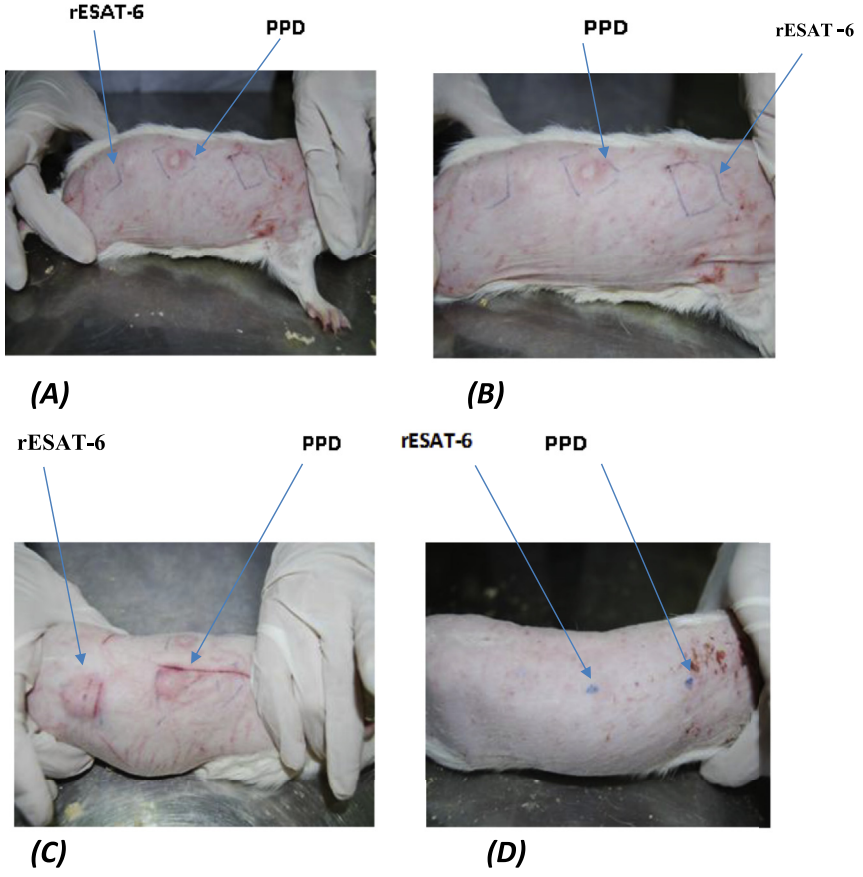


**Figure 4.** Skin test reaction results: (A) Sensitized to *Mycobacterium avium*; (B) sensitized to bacillus Calmette–Guérin; (C) sensitized to *Mycobacterium tuberculosis*; and (D) sensitized to phosphate-buffered saline. PPD = tuberculin purified protein derivative; rESAT-6 = recombinant early secretory antigenic target 6.

screening patients with TB [4]. PPD contains many antigens that are common among different species of *Mycobacterium*, and thus there is a possibility of cross reactivity between TB and NTM or BCG vaccination in animals and humans [6]. This has reduced the specificity of PPD for the diagnosis of TB, and thus many groups have studied various methods for accurate diagnosis of TB with other specific MTB antigens. One of the best candidates for this purpose is the ESAT-6 antigen, whose gene loci are located in the RD1 region of MTB, *M. bovis*, *M. africanum, M. kansasii, M. marinum, M. szulgai*, and *Mycobacterium flavescens*.

However, this antigen is absent on BCG and in 90% of NTM [11]. In this study, the ESAT-6 antigen of MTB was successfully cloned and expressed in a prokaryotic system and purified with a simple one-step purification system based on affinity chromatography. Several studies have been carried out regarding recombinant expression and purification of MTB ESAT-6 in various systems [12]. Previous studies have shown that injection of MTB-specific antigens, such as rESAT-6, have good potential to stimulate immune response [13−15]. Therefore, ESAT-6 can be an appropriate alternative to PPD for TB diagnosis [16]. The present study confirmed the results of previous studies. We found that rESAT-6 could only elicit positive skin reactions in MTB, but not in *M. avium* or BCG-sensitized guinea pigs. In addition, we have shown that the use of rESAT-6 as an antigen in TB skin test was highly sensitive to infection. The guinea pigs in the test were sensitive to rESAT-6 and the size (diameter) of skin reaction was very close to that of PPD. In summary, the results of this study showed that the TB-specific skin test based on ESAT-6 antigen had accurate diagnostic ability. Thus, based on results from present and previous studies, we strongly suggest the use of rESAT-6 antigen in skin test in large animals and human volunteers.

## Conflicts of interest

All contributing authors declare no conflicts of interest.

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