Archives of Razi Institute, Vol. 78, No. 3 (2023) 815-821

DOI: 10.22092/ARI.2022.360063.2543

Original Article

Comparative Evaluation of Specific Antibody against Mycobacterium tuberculosis ESAT-6 Recombinant Antigen in Healthy Subject with Positive and Negative Skin Test

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Received 18 September 2022; Accepted 3 October 2022
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Abstract

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (*M. tuberculosis*). The laboratory diagnosis of the disease includes various bacteriologic and immunologic methods. Despite the effectiveness of many of these methods in diagnosing active TB, their high cost and time-consuming nature have led researchers to adopt more accurate and rapid screening methods based on specific antigens for *M. tuberculosis*. The present study aimed to measure specific antibody serum levels against the early secretory antigenic target 6 kDa (ESAT-6) recombinant protein in healthy people and compare it to TB patients. The target population included 27 TB patients and 87 healthy individuals with no clinical TB symptoms. The healthy population was divided into two groups, including positive purified protein derivative (PPD) and negative PPD (35 and 52 people, respectively), using the Tuberculin skin test. The specific antibody level against the ESAT-6 recombinant antigen and the PPD protein was measured using an indirect Enzyme-Linked Immunosorbent Assay (ELISA) test. The results of the study showed that the majority of the healthy population with no symptoms of clinical TB and having negative skin test results did not have antibodies against the recombinant ESAT-6 (98%) and PPD (96%) antigens. On the other hand, there was a high level of the specific antibody of the ESAT-6 recombinant and PPD antigens in TB patients (77%). It is notable that in people with positive skin test results, the level of the antibody against the ESAT-6 recombinant antigen and PPD antigen was 94%. The results demonstrated that the ELISA method based on the measurement of antibodies against the ESAT-6 recombinant antigen can be a proper diagnostic method for rapid and accurate screening of healthy from infected people.

Keywords: Antibody, ELISA, ESAT-6, *Mycobacterium tuberculosis*

1. Introduction

Tuberculosis (TB) is a major health problem worldwide, with around 80% of its cases occurring in developing countries (1, 2). According to the WHO (3) guidelines, the control and treatment of active TB strongly depend on rapid and accurate diagnosis. However, current diagnostic methods hold some disadvantages.

Traditionally, microscopic examination of sputum smear has been the most applied method for TB diagnosis. However, the results of the test could be negative if run in the early stages of the disease. The sputum culture, as a standard diagnostic method, is carried out in the Lowenstein Jensen environment. Despite its high sensitivity, this method is very time-consuming given the time required for the development

of the bacterium. Molecular methods require modern equipment and highly experienced staff. Therefore, methods with high sensitivity, which are also quick, can play a crucial role in the treatment and control of TB (4).

Tuberculin skin test is still applied, along with other diagnostic tests. The test measures the delayed hypersensitivity to the injection of purified protein derivative (PPD). However, this method does not enjoy high sensitivity and specificity. Furthermore, it can lead to false positive results in cases with previous encounters with non-pathogenic *Mycobacteria* and a history of Bacillus Calmette-Guerin (BCG) vaccination. It can also yield false negative results in immunodeficient patients (5, 6).

The most recent test for TB diagnosis approved by the Food and Drug Administration is based on the Interferon Gamma Release Assay, which is related to the cellular immune response against *Mycobacterium tuberculosis* (*M. tuberculosis*) specific antigens (7). Although this method offers valid indicators of the disease, it is relatively expensive. This and other immunologically related methods are based on the identification of specific antigens of *M. tuberculosis*, which have great sensitivity and specificity (4, 7).

Genomic studies carried out on the pathogenic strains of *M. tuberculosis* have shown that they contain three distinct genetic areas, called the regions of difference, which are absent in nonpathogenic *Mycobacteria* and vaccinal strains. Among different antigens of this area, the early secretory antigenic target 6 kDa (ESAT-6) and culture filtrate protein 10 kDa (CFP-10) have been considered great immunogenic antigens which are present in all pathogenic mycobacterial strains of the TB family, including *M. tuberculosis*, *M. bovis*, and *M. Africanum*.

About 96% of TB patients respond to the ESAT-6 antigen. Such a response has not been reported for any *Mycobacteria* antigens so far. In addition to cellular immunity, ESAT-6 can induce humoral responses. The levels of specific antibodies against this antigen have been noteworthy for those infected with *M. tuberculosis*. Since ESAT-6 is not present in the BCG strains, it can be used as an indicator for the diagnosis of patients from healthy people with a history of BCG vaccine (8).

The CFP-10 and ESAT-6 have a hydrophobic structure and are composed of alpha-helix structures consisting of 100 amino acids. The analysis of the resonance structure of the complex shows that two hairpins, which form distinct proteins, are placed side by side unilaterally and form a batch of four helixes. A long flexible arm has risen from the four helixes, containing the same seven amino acids forming the carboxyl (C) end of the CFP-10 molecule, which are essential for attaching to the surface of the host's white blood cells, such as macrophages and monocytes. The secretion and attachment of the CFP-10/ESAT-6 complex to the infected host cell highlight the role of this molecule in the pathogenesis of *M. tuberculosis* (9, 10).

With respect to these reasons and for the assessment of ESAT-6 antigen as a potential capture antigen in the Enzyme-Linked Immunosorbent Assay (ELISA) method, the present study aimed to evaluate antibody levels against ESAT-6 antigen in normal subjects, compared to TB-infected patients.

2. Materials and Methods

2.1. Sample Collection

A total of 27 serum samples were collected from the TB-suspected patients referred to Tehran hospitals, and the infection was confirmed through various diagnostic methods. The other test group consisted of 19 individuals from the personnel working in the Tuberculin Department at Razi Vaccine and Serum Research Institute, Karaj, Iran, who had long been exposed to various antigens associated with *M. tuberculosis* and had shown positive skin test results. The control group, on the other hand, included 68 healthy individuals with no history of previous TB or exposure to TB patients and showed negative results in their skin test. To check the subjects' responses to the PPD antigen in the control group, the antigen was

injected into the subjects subcutaneously. In this test, cellular immunity fights with the antigen; therefore, if there is the initial contact with *M. tuberculosis* or other bacteria from the *Mycobacterium* family, hypersensitivity is developed by the antigen injection, leading to redness and inflation in the injected area. The PPD readings were performed in two stages (48 and 72 h), and a stiffness diameter of larger than 10 mm was considered positive. All subjects were informed of the study conditions, and they all completed a consent form before being included in the examination. During the study, 5 ml of blood sample was collected from each subject, and the serum samples were collected and kept at -20°C, according to the safety principles before the test.

2.2. Preparation of the Recombinant ESAT-6 Antigen

The production and purification of the ESAT-6 recombinant antigen were performed as described previously. Briefly, the gene encoding ESAT-6 from *M. tuberculosis* H37Rv was amplified by polymerase chain reaction (PCR) methods, and the PCR product was cloned into pQE30 (Qiagen, USA) as the expression vector. The constructed plasmid was transformed into E. coli M15 [pREP4], and the protein expression was induced by isopropyl-beta-D-thiogalactopyranoside (IPTG) SDS-PAGE analysis. The overexpressed proteins were purified by affinity chromatography using the Ni-NTA resin, and the related band was observed by 15% sodium dodecyl-sulfate (SDS) polyacrylamide gel electrophoresis. The presence of the recombinant protein was confirmed by the Western Blot analysis using the anti-6His monoclonal antibody (Novagen, German). Finally, the protein concentration was determined by the Bradford Protein Assay Reagent Kit (Bio-Rad, USA).

2.3. Evaluation of Antibody against the ESAT-6 and PPD Antigen with ELISA

The specific antibody against the recombinant ESAT-6 protein and PPD antigen was measured using indirect ELISA. The optimum concentration of the capture antigens, anti-human conjugated antibody, and suitable dilutions of the serum sample were determined by checkerboard titrations.

The flat-bottomed 96-well plates were coated with 100 µL of ESAT-6 (500 ng/well) or PPD (1.4 µg/well) in Carbonate-Bicarbonate buffer pH 9.6. The plates were covered and incubated at 4°C overnight. The contents of the wells were then completely removed and washed three times with phosphate-buffered saline containing 0.05% Tween-20 (PBS-T) as the washing buffer. The plates were blocked with 300 µL of 3% bovine serum albumin in PBS-T and incubated for 1 h at 37°C. After washing the wells, 100 µL of serum samples (1:100 diluted) were added to the respective wells and placed in an incubator at 37°C for 1 h. The plates were washed three times with the washing buffer, and 100 µL of Horseradish Peroxidase-conjugated goat anti-human IgG (1:50,000 diluted) was added to each well, followed by 1 h incubation at 37°C. After five times of additional washing steps, 100 µL of tetramethylbenzidine substrate was added to each well and incubated for 20 min at room temperature and in the darkness. The reaction was then stopped by a 10% sulfuric acid solution, and the optical absorbance (OD) of the samples was measured at 450 nm with an ELx800 ELISA reader (BioTek, USA).

2.4. Statistical Tests

All the experiments were repeated three times, the results of which were analyzed by one-way ANOVA and t-test, using the SPSS (version 16.0) and GraphPad Prism (version 6.0) softwares. The significance level was $P<0.05$ for all tests. A cut-off point was also used to determine positive and negative values. The cut-off point was defined as the average OD of the negative samples plus two standard deviation units.

3. Results

The concentrations of the purified recombinant ESAT-6 protein and PPD antigen used in this study were determined as 2800 and 6456.3 µg/mL, respectively. After performing the SDS PAGE on the

15% gel, a distinct single was observed with an expected size of 12 kDa and 93.6% purity. However, the corresponding band in the same molecular weight was detected on the nitrocellulose membrane after the Western Blot analysis (data not shown).

3.1. Subcutaneous Injection of the PPD Antigen

Among 68 healthy subjects, who were examined after the intracutaneous injection of PPD antigen, 16 (23.52%) were diagnosed based on positive skin test results with an induration size of greater than 15 mm. The other 52 (76.47%) subjects had negative skin test results, and the results were non-detectable or lower than 14 mm.

3.2. Evaluation of the Antibodies Produced against the Recombinant ESAT-6 Protein and PPD Antigen

The specific antibody levels against the recombinant ESAT-6 protein and PPD antigen were estimated by the indirect ELISA test in the serums collected from patients and healthy subjects.

The results showed that, except for one sample, all subjects with negative PPD skin test results had no antibodies against the recombinant ESAT-6 antigen. However, when PPD proteins were used as a capture antigen in the ELISA test, two of the 52 normal subjects (3.8%) had greater antibody levels than the cut-off point (Figure 1).

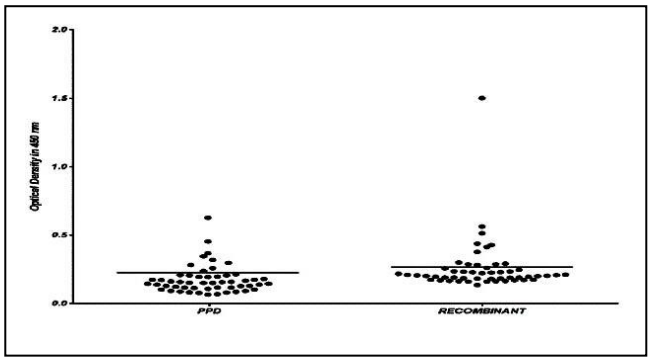

Figure 1. Optical absorbance graph for the samples of the subjects with negative skin test results against the PPD antigen in the ELISA test versus the recombinant ESAT-6 protein and PPD antigen at 450 nm

On the other hand, the specific antibody levels against the PPD proteins and the recombinant ESAT-6 antigens for the 35 subjects with positive skin results were approximately the same as those for subjects with negative skin test results. Most of the studied subjects were below the cut-off line, except for two cases for each antigen; therefore, the test specificity for both tests was 94% (Figure 2).

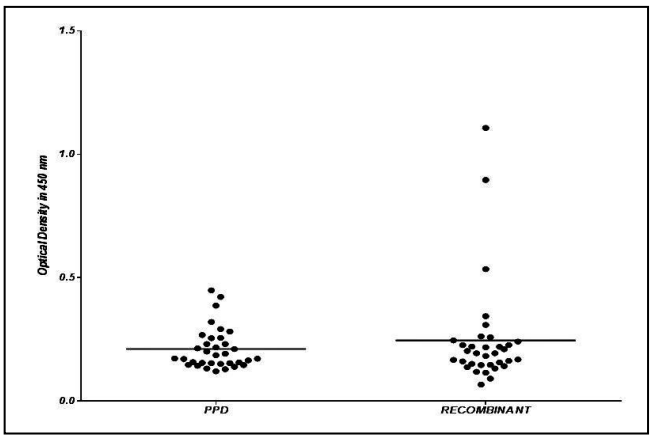

Figure 2. Optical absorbance graph for the samples of the subjects with positive skin test results against the PPD antigen in the ELISA test versus the recombinant ESAT-6 protein and PPD antigen at 450 nm

The results of the 27 TB patients showed that most had high levels of the specific antibody against PPD and ESAT-6 antigens. According to the results, the test sensitivity for both the recombinant and PPD antigens was 77% (Figure 3).

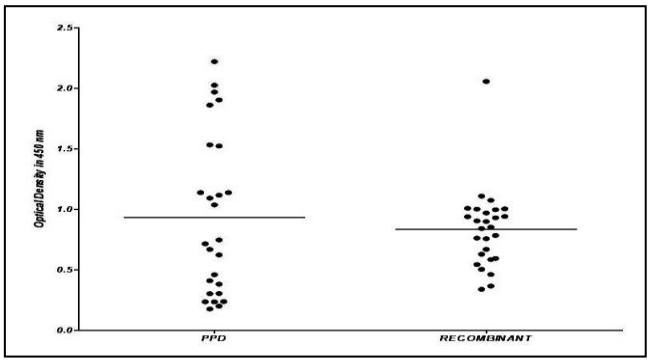

Figure 3. Optical absorbance graph for the patient samples versus the recombinant ESAT-6 protein and PPD antigen at 450 nm

The results indicated a significant difference in the specific anti-ESAT-6 antibody between the healthy

group and the patients (P<0.01). However, there was no difference between the healthy subjects with positive skin test results and those with negative skin test results (P>0.05) (Figures 4 and 5).

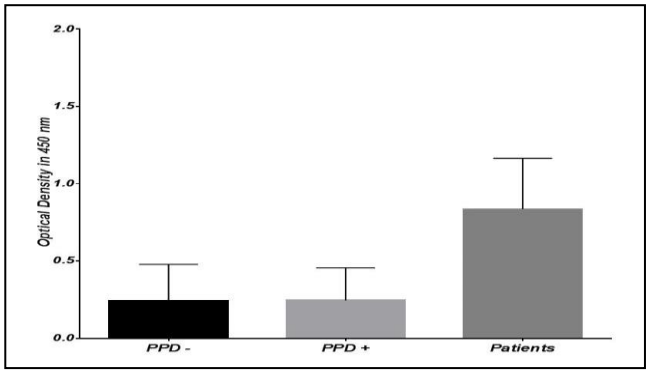

Figure 4. Comparison of the groups (PPD-, PPD+, and patients) for presence of the recombinant antigen

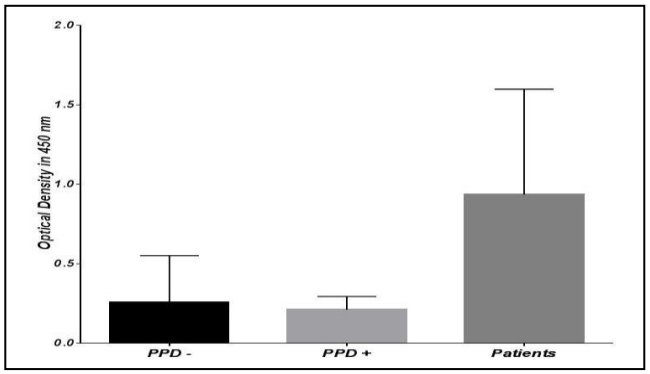

Figure 5. Comparison of the groups (PPD-, PPD+, and patients) for presence of the PPD antigen

4. Discussion

According to the complex structure of *M. tuberculosis*, some diagnostic tests have been developed for its detection. Despite the acceptable indicators provided by some of these tests, there is a need for rapid, accurate, and cheap diagnostic methods. Among different techniques which have been used for TB diagnosis, the ELISA test has been noted by many researchers, especially in developing countries. The ease of use, rapidness, design capability for different kinds of antigens, the ability to simultaneously evaluate a vast number of samples, and cost-effectiveness are some of the advantages of this test.

This study benefited the immunogenic *M. tuberculosis* ESAT-6 antigen. This antigen is present in all *Mycobacterium* pathogenic strains but is absent in vaccine strains and peripheral *Mycobacteria*. According to the studies carried out in different locations with a high and low incidence of this disease, T-cell response to the ESAT-6 antigen is reported in about 65% of the overall positive PPD subjects (10). Therefore, it can be suggested for an early diagnosis of TB for its acceptable sensitivity and specificity.

One of the comprehensive studies conducted on the presence of antibodies against ESAT-6 and CFP-10 antigens in healthy people and patients in Tanzania, Ethiopia, Brazil, and Denmark revealed that antibodies had a desirable level in TB patients and people with latent TB infection. They concluded that the test yielded better results in endemic areas (11).

Wu, Yang (12) investigated the presence of antibodies against the ESAT-6 and CFP-10 antigens in a population of patients infected with *M. tuberculosis* and healthy people in China and showed that it can be a suitable method for the evaluation of suspected people with negative smear and culture. Kassa, Ran (13) applied the ELISA test to measure the level of antibodies produced against the ESAT-6 and CFP-10 antigens and confirmed a 70% specificity for this method. They reported that 84.8% of the samples responded to these antigens positively. In another study examining the immune response against the ESAT-6 protein, a sensitivity rate of 96.6% was reported for the ELISA test. They also reported a similar sensitivity (97.4%) for the immune response against the Ag85A antigen (14).

Another report investigating specific antibody levels against ESAT-6 and CFP-10 antigens in the serum of healthy people and TB-infected patients revealed that this method yields better results in patients recently infected with TB (15, 16). However, similar hopeful

results have been obtained based on our previous results of the serum antibody levels against the ESAT-6 and CFP-10 recombinant antigens to identify TB-infected from healthy people through indirect ELISA test.

The results of the present study indicated that approximately all healthy people with negative PPD skin test results had no observable antibody levels against the recombinant ESAT-6 antigen and PPD protein.

On the other hand, based on our findings, people with positive skin test results showed the same results as those with negative skin test results. This shows that humoral immunity did not conduct a similar activity in cellular immunity in the skin test, and most of the studied subjects stood below the cut-off point, except for two cases in each antigen. However, it is notable that two subjects with elevated antibodies against the ESAT-6 antigen had been long working in the Tuberculin Department and had probable previous contact with *M. tuberculosis*.

The results for the TB patients showed that the majority had high titers of specific antibodies against the PPD and ESAT-6 antigens. Based on the results, the sensitivity of the test was 77% for both the recombinant ESAT-6 and PPD antigens. The negative predictive value of the experiment was 91.5% for both the recombinant ESAT-6 and PPD antigens. The positive predictive value was 90% for the recombinant ESAT-6 antigen and 85.3% for PPD. The relationship between the predictive value and the prevalence of the disease was 91% for the recombinant ESAT-6 antigen and 90% for PPD.

Overall, based on the results of this study and previous reports, it can be concluded that the examination of specific antibodies against ESAT-6 recombinant antigen could be noted as an appropriate candidate for TB diagnosis or examination of the course of the disease in patients being treated

Authors' Contribution

Study concept and design: M. T. and S. A.
Acquisition of data: S. A.
Analysis and interpretation of data: M. T. and S. A.
Drafting of the manuscript: S. A.
Critical revision of the manuscript for important intellectual content: M. T. and N. M.
Statistical analysis: S. A.

Ethics

This study was approved by the Ethics Committee of Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran.

Conflict of Interest

The authors declare that they have no conflict of interest.

Grant Support

The current study was supported by a teaching and research scholarship grant from the Agricultural Research, Education and Extension Organization.

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