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Rapid immunodiagnostic assays for *Mycobacterium Tuberculosis* infection

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ABSTRACT

Purpose: There is a need for a continued effort to develop rapid immunodiagnostic assays for tuberculosis (TB) infection with greater sensitivity and specificity that can be used in the field and in the laboratory and that can be formatted for use with multiple species. This would help to obtain definitive early diagnosis of TB. The present study was developed to determine the role of using early secreted antigenic target-6 (ESAT-6) in immunodiagnosis of *Mycobacterium tuberculosis*. Methods: Serum samples were obtained from TB infected patients and normal healthy controls. Two rapid immunodiagnostic assays (Enzyme-linked immunosorbent assay (ELISA) and Immunoblotting) were performed. Results: The sensitivity of immunoblotting assay was 100%; however, ESAT-6 antigen was not able to discriminate between patients and normal controls. Application of direct ELISA using ESAT -6 antigen yielded 97.6% sensitivity and 75% specificity for the diagnosis of TB infection. Conclusion: In conclusion, the detection of antibodies against ESAT-6 antigen in the sera of TB patients by direct ELISA could be used as a preliminary assay for diagnosis of human *M. tuberculosis* infection. A combination of the ELISA with either radiological or microscopic examination is required to overcome the low specificity of the assay for negative results.

Keywords: Tuberculosis; Diagnosis; ESAT-6; ELISA; Immunoblotting

1. INTRODUCTION

Tuberculosis (TB) is a global health problem with one-third of the world's population latently infected with *Mycobacterium tuberculosis* (MTB) and about 8 million cases of active disease occurring each year [1,2]. Due to the increasing numbers of persons with MTB in the last years, the worldwide dissemination of HIV infection, the strongest risk factor for MTB development, and the continual migration of people from areas with a high incidence of TB to highly industrialized countries, MTB is considered a global emergency [3-6]. In general, infection by MTB is controlled initially by host defenses, and the infection remains latent. However, latent TB infection has the potential to develop into active TB at any time. Because active TB is infectious and leads to the spread of MTB, rapid diagnosis and effective treatment of individuals with active TB are the most important component of TB control programs. Moreover, identification and treatment of persons with latent MTB infection who are at high risk of progressing to active disease, also may contribute to TB control [7].

Diagnosis of MTB based on clinical symptoms, chest radiography and sputum microscopy is sensitive but not specific [8]. Culture of bacteria is time-consuming, and usually the bacillus is not cultured [9], whereas nucleic acid-based methods such as polymerase chain reaction (PCR) are not consistently accurate enough for the diagnosis of smear-negative pulmonary TB [10]. The tuberculin skin test (TST), using purified protein derivative (PPD), is largely used for both diagnosis and screening. The greatest drawback of PPD is its broad cross-reactivity with antigens derived from several mycobacterial species, e.g., attenuated *M. bovis* bacillus Calmette-Gue´rin (BCG) used for vaccination, greatly decreasing the specificity of the TST [11,12]. Moreover, 10-25% of TB patients have a negative TST result, and this percentage increases up to 50% in patients with advanced disease or with immunodeficiency due to HIV coinfection [13,14]. MTB infection evokes a strong cell-mediated immune response, and detection of specific T cells might be a mean to detect infection [15-17]. Because of their ease of performance and cost effectiveness, serodiagnostic tests constitute a

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promise for early detection of TB. In fact, no serological test is commonly used in the diagnosis of TB [18].

In an effort to develop more accurate diagnostic tools, recent studies have led to the identification of the genomic segment BCG-region 1 (RD1), present in MTB but absent from all strains as well as almost all environmental mycobacteria [19-21]. Therefore, RD1 gene products offer the potential for the development of new diagnostic tests that may differentiate MTB infection from BCG vaccination as well as exposure to environmental mycobacteria. Two secretory, low molecular mass proteins, early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) have been identified as a product of this gene [22,23]. The antigens have already shown great potential for tuberculosis diagnosis [24,25]. Thus, the purpose of this study was to evaluate the diagnostic potential of two rapid immunodiagnostic assays (Immunoblotting and Enzyme-linked immunosorbent assay (ELISA) for the detection of *M. tuberculosis* infections using ESAT-6 antigen.

2. MATERIALS AND METHODS

2.1. Human Sera

Forty-two serum samples from patients with pulmonary tuberculosis were obtained from Mamora Chest Hospital (Alexandria, Egypt). They were diagnosed by a specialist using smear and/or sputum culture (Ziehl-Neelsen stain) [26] and confirmed by chest X ray. Sixteen control sera were collected from laboratory staffs (who never suffer from TB infection and did not get a vaccination). All investigations were done in accordance with the Ministry of Health**,** health and human Ethical Clearance Committee guidelines for clinical researches. Minufiya University local ethics committee approved the study protocol. All patients and controls agreed to be enrolled in this study.

2.2. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

ESAT-6 antigen was kindly prepared and provided by Dr. William C. Davis (College of Veterinary Medicine, Washington State University, Pullman, WA, USA). Protein content was estimated by the Bradford method [27]. Antigen was analyzed by 12% SDS-PAGE using a discontinuous SDS-PAGE system [28] and stained with Coomassie brilliant blue (Sigma Chemical Co., St. Louis, MO, USA). The protein molecular weight standard was from invitrogen (Invitrogen Corporation Carlsbad, CA, USA).

2.3. Immunoblotting

ESAT-6 was subjected to 12% SDS-PAGE, electrotransferred onto 0.22µm nitrocellulose (NC) membrane (Bio-Rad Laboratories, Richmond, CA, USA) [29]. Electrophoretic transfer was accomplished in 1h at 4°C with a constant 200V. After electrotransfer, the sheet was cut into 0.2cm wide strips. The resolved bands on NC membrane were visualized with specific immunological detection as described by [29-30]. Briefly, the antigen-containing strips were incubated with sera of MTB infected patients diluted 1:50 in blocking buffer (phosphate-buffered saline (PBS) containing 5% non-fat milk and 0.3% polyoxyethylene-20 (Tween-20) (Sigma) for 1h. All incubations were carried out at room temperature on an orbital shaker (Bellco, Vineland, NJ. USA). After incubation, the NC strips were washed with hot (65°C) washing buffer (PBS/0.3%Tween-20) and incubated for 1h with peroxidase-labeled goat anti-human IgG (GAH-GPOD) (kindly supplied by Dr. Victor Tsang, Division of Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, GA) diluted 1:1000. The strips were then washed 3 times with washing buffer and once with PBS only. Reactive bands were visualized by incubating the NC strips with freshly prepared substrate solution [50mg of 3, 3'-diaminobenzidine (DAB) and 5µl H_2O_2 (30%) dissolved in 50ml PBS, pH 7.2) (Sigma Chemical Company, St. Louis, MO]. Positive reaction bands appeared within 10 min. The reaction was then stopped using distilled water.

2.4. Enzyme Linked Immunosorbent Assay (ELISA)

The ELISA was performed in 96 well flat bottomed microplates (Griener Labortechnik, Kremsmunster, Austria). The optimum antigen, serum and conjugate concentrations and the incubation times were determined by checkerboard titration. The wells were coated with 100 µl of ESAT-6 diluted in PBS (pH 7.2). The plates were incubated 1h at 37°C then overnight at 4°C. After one wash with PBS containing 0.05% Tween-20 (PBS/Tw), 200 µl of 5% non-fat milk diluted in PBS/Tw were added to each well and incubated for 1.5 h at 37°C. After one wash with PBS/Tw, tested TB-infected and normal healthy control sera (100 µl/well) diluted in dilution buffer (PBS/Tw containing 1% non fat milk) were added in duplicates and incubated at 37°C for 1.5h. At the end of the incubation period, plates were washed 3 times with PBS/Tw and 100 µl GAHG-POD were incorporated to each well at dilution 1:1000 in dilution buffer. After 4 washes with PBS/Tw, 100 µl of TMB (3, 3', 5, 5'-tetramethyl benzidine) and H_2O_2 (0.02% in citric acid buffer) substrate (Kirkegrd and Perry Lab, Caithersuburg, MY, USA) were added to each well. The incubation time for the substrate was 15min. at room temperature. The optical density was measured at 620nm using the UV-max ELISA plate reader (Molecular Devices Corp.).

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2.5. Statistical Analysis

Data are expressed as mean OD ± SD and were analyzed using the statistical software package for social science (SPSS). Suitable cutoff value for the ELISA was determined by receiver operating characteristics (ROC) analysis. Comparisons of patients and normal control groups were performed by the Student's t-test. The differences were considered significant if the probability was associated with $p<0.05$.

3. RESULTS

3.1. Gel Electrophoresis

ESAT-6 was subjected to SDS-PAGE followed by Comassie brilliant blue staining. As the results in **Figure 1** showed, 2 bands were identified in ESAT-6 (7.1 and 6.5 kDa).

3.2. Immunoblotting

On screening of the TB infected sera, the immunoblotting assay sensitivity was 100% as all TB infected patients reacted with ESAT-6. However, the antigen was not able to discriminate between patients and normal controls (**Figure 2**).

4. ELISA

Each serum sample was tested for its reactivities against ESAT-6. **Figure 3** showed the reactivity of TB infected sera and uninfected controls against ESAT-6. The mean absorbance value in the TB patients was 0.566 ± 0.017, significantly higher ($p<0.01$) than that in normal control group (0.360 ± 0.029). After ROC analysis (**Figure 4**), the area under the curve and standard error of the area under the curve were 0.961 (95% confidence interval [CI], 0.918 to 1.005) and 0.022, respectively. Among different cutoff values from the ROC analysis, a cutoff value of 0.351 was selected, as the sensitivity of 97.6 % and specificity of 75% at the chosen cutoff was optimal for our ELISA. Out of 42 TB infected sera, only 1 serum sample showed a negative result at the cutoff value (1/42) (false negative). On the other hand, out of 16 normal

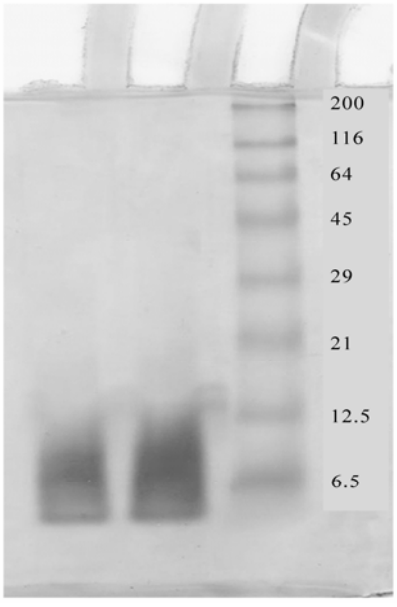

Figure 1. Coomassie blue stained 12% SDS-PAGE gel of ESAT-6 antigen.

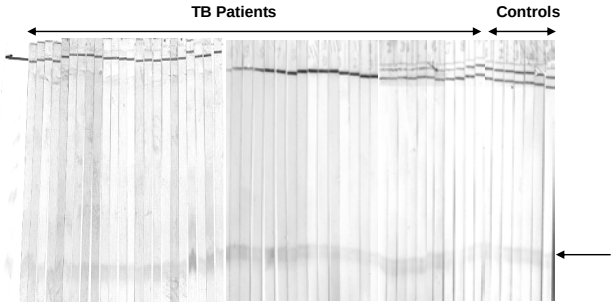

Figure 2. Western blot profile of *M. tuberculosis* patients and normal controls. ESAT (0.1 µg/mm) were separated by 12% SDS-PAGE, electrotransferred onto NC sheets, cut into 0.2 cm identical strips and reacted with patients and normal human serum (last 8 strips) diluted 1:50 in PBS/0.3%Tween-20/5% non-fat milk. Arrow pointed to the reactive band (≅6 kDa).

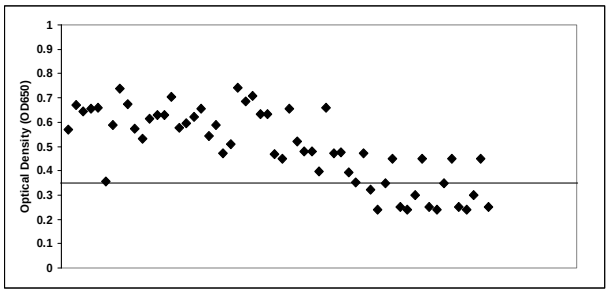

Figure 3. Reactivity of sera from *M. tuberculosis* infected patients against ESAT-6 antigen (as shown by direct ELISA).

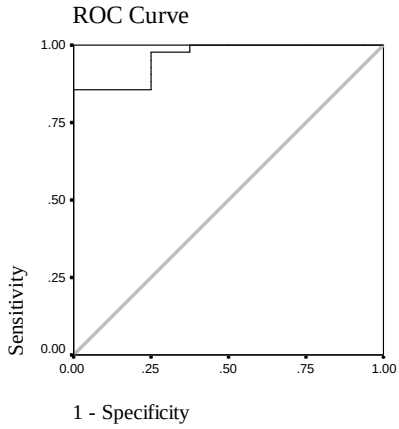

Figure 4. ROC curves for ELISA with ESAT-6.

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controls, 4 serum samples showed a positive result at the cutoff value (4/16) (false positive).

5. DISCUSSION

Along with HIV and malaria, MTB has been declared a global enemy [31]. Delay in diagnosis is significant regarding not only disease prognosis at the individual level but also transmission within the community and the productive rate of TB epidemic [32,33]. An effective TB control program requires early diagnosis and immediate initiation of treatment. To control tuberculosis (TB), it is still necessary to find diagnostic methods that are both more rapid to carry out and more sensitive than traditional methods (smear and culture) but which are simpler and less expensive than the new molecular diagnostic tests that are based on the amplification of nucleic acids. Serological methods, which seem to be the ideal choice, are usually based on the detection of free soluble circulating antibodies against mycobacterial antigens. Thus, there is a need for a continued effort to develop rapid immunodiagnostic assays with greater sensitivity and specificity that can be used in the field and in the laboratory and that can be formatted for use with multiple species. Several secreted antigens have been characterized, and their utility for diagnostic testing as well as their attributes as vaccine components have been exploited [34-36].

An antibody detection test can be developed into several formats depending on the membrane, antigen(s) coating, and incubation technique. Common designs include ELISA and immunoblotting formats (commercial serological). TST has many drawbacks, such as the need for patients to return for test reading, as well as variability and subjectivity in test application and reading. Most importantly, TST has low specificity as PPD, the antigen used for the test, is a mixture of mycobacterial antigens also present in non tuberculous mycobacteria and in the BCG vaccine strains [11]. Although both assays (TST and ELISA) do not offer a good specificity in detection of *M. tuberculosis* infection, ELISA has many other advantages over the TST. Objective quantitative results can be obtained the day after blood sampling, and time spent on return visits to have the TST read is spared. Furthermore, since no antigen is injected, the problem of a booster affect on sequential skin tests is avoided. The test is simple to perform, and can be used even in countries with less-developed infrastructure.

ESAT-6 protein is secreted at an early or active phase of mycobacterial infection. It is virtually specific for *M. tuberculosis* complex representing a potential candidate for use in early detection as substitute or as improved skin test antigen [21-23,37-39]. Here in, we investigated the human antibody response against ESAT-6 antigen for the detection of *M. tuberculosis* infection using different immunoassays (immunoblotting and ELISA). Serial samples of sera collected from *M. tuberculosis* infected patients were subjected to our developed immunoblotting and ELISA to determine the serological response to ESAT6. Western blot analysis showed a reactive band at ≅6 kDa with all serum samples. This may suggest that, using immunoblotting format assay, ESAT-6 antigen is not only recognized with individuals with TB infection but also with normal healthy controls. This result merely reflects a case of cross reaction that may returned to shared epitope identification afforded by this assay. Thus, our work showed that western blotting using ESAT antigen is not the suitable choice for diagnosis of *M. tuberculosis* infection and just used to support its diagnosis. On the other hand, application of ELISA has increased the sensitivity and specificity for diagnosis of *M. tuberculosis* infection to 97.6% and 75%, respectively.

ESAT-6, for comparison, is broadly recognized early during disease in different species infected with *M. tuberculosis* or *M. bovis* [37,40,41] and this antigen is generally reported to trigger the release of high levels of IFN-γ by sensitized peripheral blood mononuclear cells (PBMC) from TB patients [42,43]. This antigen discriminates TB patients from both BCG-vaccinated and M. avium patients and has therefore been suggested as a candidate for in vitro TB diagnosis [42,43]. The diagnostic potential of low molecular weight culture filtrate antigens (ESAT-6 and CFP-10) of *M. tuberculosis* has been reported in earlier studies [24,25,44]. It has been previously reported that subjects with latent TB infection may respond to ESAT-6 peptides and to the whole ESAT-6 protein [45-49]. Vincenti *et al.* [50] demonstrated that, patients with active TB recognized the whole ESAT-6 protein or PPD. The lack of response in some TB patients may be due to their particular state of immune suppression [43-50].

Although serologic testing can detect specific antibodies to mycobacteria in serum and is attractive diagnostic method due to its ease of application, they present low sensitivity and specificity due to the great heterogeneity of the humoral response in patients with TB [51]. It is noteworthy that, in patients suffering from AIDS, in whom the number of T cells is decreased or even null, determining humoral response can be an invaluable tool in making an early diagnosis and gaining epidemiological control over TB [52]. Thus, even with 75% specificity, getting 97.6% sensitivity with ESAT-6 in ELISA format has improved the diagnostic potential of this antigen for TB diagnosis. Imaz *et al.* [18] suggested the use of multi-antigen cocktail to improve the diagnostic utility of the ELISA. The design of antigen combinations would achieve the high diagnostic accuracy of the assay.

In conclusion, this study offers the potential for developing relatively rapid assays (ELISA and western blotting) for detection of *M. tuberculosis* infection. We have tested the application of antibody reactivity against ESAT-6 antigens as a serodiagnostic marker. The results showed that the use of ESAT-6 improves the diagnosis's

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sensitivity and provide an ELISA as a simple method that could be applied in TB diagnosis. To overcome the low specificity of the assay, a combination of the ELISA with either radiological or microscopic examination is required for negative results.

6. ACKNOWLEDGMENTS

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