

Evaluation of Immunodiagnostic Potential of ESAT-6 Synthetic Peptides Mixture in Egyptian Pulmonary Tuberculosis Patients

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Identification of *M. tuberculosis* ESAT-6 proteins has enhanced research for developing new specific and reliable *in vitro* diagnostic tests for pulmonary tuberculosis. The aim of this study was to assess the diagnostic potential of ESAT-6 synthetic overlapping peptides mixture (Pepmix) for diagnosis of pulmonary *M. tuberculosis* infection in Egypt. IFN- γ and IL-17 production was assessed in whole-blood culture assay after overnight stimulation with *M. tuberculosis* crude culture filtrate (CF), purified 38 kDa antigen, and ESAT-6 Pepmix. Concentration of IFN- γ and IL-17 was evaluated in the culture supernatant by ELISA. Patients with active pulmonary TB patients (n = 37), Tuberculin Skin Test (TST) positive healthy subjects (n = 16) and TST negative healthy subjects (n = 16) were included. Results indicated that IFN- γ production following stimulation with crude CF, and 38 kDa purified antigen was not significantly different between patients and TST⁺ healthy controls. In contrast, Pepmix clearly distinguished between patients and TST⁺ healthy subjects with sensitivity of 70.3 % and specificity 96.9 %. The concentration of secreted IL-17 was significantly lower than IFN- γ , and no statistically significant difference was found between groups. It is concluded that IFN- γ assay using ESAT-6 Pepmix, as antigenic stimulus, has the potential to rapidly diagnose pulmonary TB infection.

Infectious diseases remain the highest cause of death in the world. TB remains one of the world's major causes of illness and death. In 1993, the World Health Organization (WHO) declared TB to be a global health emergency. One-third of the world's population or two billion people carry the TB bacteria, more than 9 million of whom become sick each year with "active" TB which can be spread to others, (Vashishtha, 2009), and three million deaths are reported every year around the globe, (Kumar *et al.*, 2010). It is estimated that between 2002 and 2020, approximately 1000 million people will be newly infected, over 150 million people will get sick, and 36 million will die of TB - if control is not further strengthened, (WHO, 2009).

Worldwide, someone newly infected with TB bacilli every second. Generally, 5% to

10% of people who are infected with TB become actively sick, (WHO, 2008). In Egypt, there is an incidence rate of 21 new cases for every 100,000 individual per year, (WHO, 2007). In most cases, *M. tuberculosis* infection is asymptomatic, latent infection that in some instances progress into TB infection. However, untreated active infections have more than 50% mortality rate, (Crowley, 2007). Co-infection with human immunodeficiency virus (HIV) and *M. tuberculosis* increases the risk of developing TB and enhances progression of HIV infection to AIDS, (Kumar *et al.*, 2010). HIV-positive individuals with the latent tuberculosis infection (LTBI) have higher risks of progressing to active TB, about 10% per year, (Zhang *et al.*, 2010). In addition, multidrug-resistant TB is rapidly spreading, (Ravn *et al.*, 1999).

Early and correct diagnosis is essential for infection control and treatment. Although the isolation of tubercle bacilli from clinical specimens is the gold standard for diagnosis, this may not be achievable in every single patient. Chest X-ray, identification of bacilli, and the histopathological detection of granulomatous lesions in addition to clinical findings generally lead to true diagnosis, (Ravn *et al.*, 2004).

The most powerful tools in any TB control program are prompt diagnosis and successful treatment of patients with active contagious disease, (Ravn *et al.*, 2005). Early mycobacterial identification to the species level is important because it would help in the initiation of early and appropriate treatment of patients. However, identification of mycobacteria by conventional methods like bacterial culture, AFB staining, and radiography is time-consuming and not always conclusive, (Varma-Basil *et al.*, 2010). The sensitivity of routine smear-microscopy is approximately 50%, culture techniques take several weeks to yield results, and suitable representative biological samples are frequently unobtainable either due to lack of sputum production or poor sample quality. The HIV pandemic compounds this problem by increasing the incidence of smear-negative and sputum-scare TB, (Cashmore *et al.*, 2010).

Although tuberculin skin test (TST) has long been used for detection of both active and latent tuberculosis, it has a low specificity, (Mori *et al.*, 2004). The tuberculin skin test has been in existence for more than 100 years and has remained more or less unchanged for the last 60 years, (Curley, 2003). The biggest drawback of TST is the cross-reaction with nontuberculous mycobacteria (NTM) or *with Mycobacterium bovis* bacillus Calmette-Gue´rin (BCG) vaccine strains, (Dominguez *et al.*, 2008). Because protein-purified derivative (PPD) is a culture filtrate of

tubercle bacilli containing over 200 antigens shared with the bacille Calmette-Gue´rin (BCG) vaccine and most nontuberculous mycobacteria, individuals vaccinated with BCG but not infected with *MTB* can test falsely positive using the tuberculin skin test, (Richeldi, 2006).

Identification and characterization of some *M. tuberculosis* specific antigens like ESAT-6 and CFP-10 has led to the development of new specific diagnostic tests for infection with *M. tuberculosis*, (Andersen *et al.*, 2000). A number of studies have demonstrated a high sensitivity and specificity when detecting interferon gamma responses to these specific antigens in patients with active TB or unexposed healthy people, (Abdel-Ghaffar *et al.*, 2007).

Cruz *et al.*, 2006, show that IFN- γ -deficient mice have increased numbers of IL-17-producing T cells following infection with *M. bovis* bacille Calmette Gue´rin. Furthermore, exogenous IFN- γ increases IL-12 and decreases IL-23 production by bacille Calmette Gue´rin-infected bone marrow-derived dendritic cells and reduces the frequency of IL-17-producing T cells induced by these bone marrow-derived dendritic cells. These data support the hypothesis that, during mycobacterial infection, both IFN- γ - and IL-17-producing T cells are induced, but that IFN- γ serves to limit the IL-17-producing T cell population. This counter-regulation pathway may be an important factor in limiting mycobacterially associated immune-mediated pathology, (Cruz *et al.*, 2006). Novel interferon gamma (IFN- γ) release assays (IGRAs) provide distinct advantages; they are highly *MTB*-specific and thus not confounded in populations containing a high proportion of BCG-vaccinated individuals, avoid boosting of immune responses by *ex-vivo* testing and possess logistical conveniences, (Ringshausen *et al.*, 2010).

The aim of this study was to evaluate the diagnostic potential of ESAT-6 Pcpmix (synthetic overlapping peptides mixture) in comparison to other CF *MTB* antigens.

Subjects and Methods

Ethics Statement

Ethical approval was obtained from the Egyptian Government / Medical Research Council Joint Ethics Committee, and the General Directorate of Health Affairs, Tuberculosis Control Department in the Egyptian Ministry of Health. Blood samples collected from active pulmonary TB patients in Chest Diseases Hospital in Abbassia, Cairo, Egypt. All subjects provided informed consent for the collection of samples and subsequent analysis.

Study Subjects

This study included 69 subjects divided into three groups as follow: Group (1), 16 healthy subjects with negative TST results (9 males and 7 females); whose ages ranged from 20 to 58 years, and without a history of TB infection, Group (2), 16 healthy subjects with positive TST results (7 males and 9 females) whose ages ranged from 13 to 62 years, and without a history of TB infection and Group (3), 37 patients with active pulmonary TB disease (29 males and 8 females); whose ages ranged from 15 to 73 years, composed of individuals who had an epidemiological history with bacillary TB contact, clinical evidence, chest radiography compatible with TB and a TST > 10 mm. Pulmonary TB was confirmed by culture and microscopic examinations. TB patients with other co-infection that may affect our study like patients with Schistosomiasis, HCV, or HIV were not included in this study.

Bacterial Strain

The bacterial strain used in this study for antigen preparation is the *Mycobacterium tuberculosis* reference (ATCC#HRV37). This reference strain was donated for us through Prof. Robert Cooksey from the Central of Disease Control (CDC), Atlanta, USA. The reference strain was continuously cultivated over Lowenstein solid media. Species confirmation was carried out by microbiological culture and PCR amplification of *IS6110* gene.

Specimen Collection and Preparation

Peripheral blood was collected from study participants in heparinized tubes (Lithium Heparin Vacutainers, 4 ml) as other anticoagulants severely compromise the functional capacity of lymphocytes. Samples were

shipped immediately to the Molecular Immunology Unit (MIU) at Al-Azhar University at room temperature to avoid platelet activation and used within 8 hours of collection.

Mycobacterial Antigens

- Preparation of Culture Filtrate (CF)

Middlebrook broth media was prepared by dissolving 4.7 gm Middlebrook 7H9 (Difco) in 700 ml dH₂O then autoclaved. The media left to cool then 100 ml of Middlebrook ADC Enrichment (Difco) was added and the volume was completed to 1L using dH₂O. Using sterile spatula, five to ten *M. tuberculosis* strain colonies was added to 500 ml Middlebrook broth media. The conical was kept at 37 °C shaking incubator for 3 weeks. The culture filtrate (CF) was centrifuged at 3500 rpm for 20 minutes then filtered using 0.45 µm filters. The bacteria remained after centrifugation was stained to check for the presence of contamination with other bacteria. The CF was cultured over Lowenstein Jensen Solid Media (BioRad) to check for contamination with *M. tuberculosis* and over Blood Agar Media, Nutrient Agar Media, MacConkey Agar Media, and Chocolate Agar Media to check for the presence of other bacteria. The CF was finally aliquoted and stored in -70 °C for later use. The contents of the CF were analyzed using SDS-Polyacrylamide Gel Electrophoresis.

- Purification of 38 kDa Antigen from the Culture Filtrate

Fifty ml of culture filtrate was precipitated by 40% Ammonium Sulphate by adding 11.3 g to the CF step by step with continuous steering in an ice bath and kept on ice for 30 minutes. The mixture was centrifuged at 3500 rpm for 30 minutes and the supernatant was separated and labeled F1 while the precipitate was labeled R1. The F1 was precipitated by 70 % Ammonium Sulphate by adding the Ammonium Sulphate on the F1 step by step with continuous steering in an ice bath and kept on ice for 30 minutes. The mixture was then centrifuged at 20,000 g by an ultra-speed centrifuge; the supernatant was then separated and labeled F2 while the precipitate was labeled R2. The R2 which contained the 38 kDa antigen was taken and dissolved in 10 mM Ammonium Bicarbonate then dialyzed against 10 mM Ammonium Bicarbonate for 3 days. After dialysis, the proteins in the final solution were concentrated by using the speed vacuum concentrator and separated using preparatory SDS-PAGE and the 38 kDa antigen was purified using the chemical elution protocol as follow: The 38 kDa band was excised by a sharp cutter blade (placing the gel on a light box can be helpful in locating the bands). The excised gel was

then sliced as finely as possible then placed in the 15 ml tubes. Gel elution buffer was added to cover the gel completely (almost 2-3 ml was used) and incubated at 37 °C overnight. The solution which contains the eluted protein was removed and concentrated by using the speed vacuum concentrator and stored in -70 °C for later use.

- ESAT-6 Pepmix (synthetic overlapping peptides mixture)

ESAT-6 Pepmix (Batch No.: 080906GK) y was kindly provided by Prof. Peter Andersen, Department of Infectious Diseases Immunology, Statens Serum Institute, Copenhagen, Denmark. The amino acid sequences of the ESAT-6 Pepmix peptides are listed in Table 1.

Table 1. Amino acid sequences of ESAT-6 Pepmix (P1-P7).

| Peptides (Position) | Amino Acid Sequence ^a |
|---------------------|----------------------------------|
| P1 (1–20) | MTEQQWNFAGIEAAASAIQG |
| P2 (10–25) | GIEAAASAIQGNVTSI |
| P3 (16–40) | SAIQGNVTSIHSLLDEGKQSLTKLA |
| P4 (31–55) | EGKQSLTKLAAAWGGSGSEAYQGQVQ |
| P5 (46–70) | SGSEAYQGQVQKWDATATELNNALQ |
| P6 (61–85) | TATELNNALQNLARTISEAGQAMAS |
| P7 (71–95) | NLARTISEAGQAMASTEAGNVTGMFA |

^a From the N terminus to the C terminus.

Whole Blood Assay

In details, four 15 ml polypropylene tubes were labeled as: Unstimulated Negative (Nil) Control, CF Activated, 38 kDa Antigen Activated and ESAT-6 Pepmix Activated. To each tube 1 ml of heparinized whole blood was added. CF was then added to the tube labeled CF Activated in a final concentration of 25 µg/ml, while 38 kDa Antigen was added to the tube labeled 38 kDa Antigen Activated in a final concentration of 5 µg/ml, and ESAT-6 Pepmix was added to the tube labeled ESAT-6 Pepmix Activated in a final concentration of 2 µg/ml.

Each tube was mixed gently using a vortex to ensure proper mixing of the blood with contents and incubated at 37 °C in a humidified atmosphere for 20-24 hours. The tubes were centrifuged at 3,000 g for 10 minutes and the plasma was harvested and immediately frozen at -20°C until further analysis.

IFN-γ ELISA Assay

Microtiter plates (96 well; Maxisorp; Nunc, Denmark) were coated with 1 µg/ml monoclonal rat anti-human IFN-γ Mab M700A (Clone 2G1; Thermo Sci./TriChem). Free binding sites were blocked with 2% (w/v) skimmed milk powder in PBS. Culture supernatants were tested in triplicates. IFN-γ was detected with a 0.15 µg/ml biotin conjugated anti-human IFN-γ M701B antibody (Mab; Clone B133.5; Thermo Sci./Trichem) and horseradish peroxidase conjugated streptavidin (diluted 1:5000 in PBS + 1% BSA) (BD Pharmingen). The enzyme reaction was developed with 3,3',5'-Tetramethylbenzidine, hydrogen peroxide (TMB plus; Kementec, Denmark) and stopped with 0.2 M H₂SO₄. rIFN-γ (BD Pharmingen, USA) was used as a standard. Plates were read at 450 nm with an ELISA-reader and analyzed with Magellan 6 software.

IL-17 ELISA Assay

Commercial enzyme linked immune-sorbent assay (ELISA) kits (Human IL-17A (homodimer) ELISA Ready-SET-Go! purchased from eBioscience®) were used to measure the concentrations of secreted IL-17 in the culture supernatants as specified by the manufacturer. With the above kit, the minimum detectable concentration of IL-17 was 4 pg/ml. Optical densities at 450 nm were determined and converted to IL-17 concentrations from standard curves using Magellan 6 software.

Statistical Methods

Data analysis was performed using the computer program GraphPad Prism, version 5 (GraphPad Software Inc., San Diego, CA, USA). The unpaired student's t-test was used to test the statistical significance among the different groups under the study. A value of p < 0.05 was considered significant.

Sensitivity relates to the test's ability to identify positive results, and it is calculated by the following equation:

$$\text{Sensitivity} = \frac{\text{TP}}{\text{TP} + \text{FN}}$$

Where TP = True Positive and FN = False Negative

Specificity relates to the ability of the test to identify negative results and it is calculated by the following equation:

$$\text{Specificity} = \frac{\text{TN}}{\text{TN} + \text{FP}}$$

Where TN = True Negative and FP = False Positive

Results

Interferon Gamma (IFN- γ) Secretion by PBMC in Response to *M. tuberculosis* Antigens

To determine Interferon gamma (IFN- γ) secretion in response to *M. tuberculosis* antigens, the whole blood from the studied groups were cultured in the presence of crude culture filtrate (CF), purified 38 kDa antigen, or ESAT-6 Peomix. IFN- γ production measured by ELISA in the culture supernatant as described in Subjects and Methods. Δ values (IFN- γ released in stimulated well minus unstimulated well) for each subject are shown in Figure (1). When the CF was tested using the cut-off value (mean \pm 2SD) calculated from the healthy subjects with -TST group results (904.5 pg/ml), or using the cut-off value calculated from the healthy subjects from +TST group results (2929.9 pg/ml), statistical differences were observed between -TST healthy controls and +TST healthy controls groups ($P= 0.002$) and between -TST healthy controls and TB patients groups ($P < 0.0001$), but there was no significant difference between the +TST healthy controls and the TB patients groups ($P= 0.112$). Also, when the purified 38 kDa

antigen was tested using the cut-off value (mean \pm 2SD) calculated from the healthy subjects with -TST group results (357.72 pg/ml), or using the cut-off value calculated from the healthy subjects from +TST group results (1361.34 pg/ml), statistical differences were observed between -TST controls and +TST healthy controls groups ($P= 0.033$) and between -TST healthy controls and TB patients groups ($P = 0.0117$), but there was no significant difference between the +TST healthy controls and the TB patients groups ($P= 0.668$). In contrast, when ESAT-6 Pepmix was tested using the cut-off value that calculated from cut-off value (mean \pm 2SD) calculated from the healthy subjects with -TST group results (72.26 pg/ml), or using the cut-off value calculated from the healthy subjects from +TST group results (121.69 pg/ml), no statistical differences were observed between -TST healthy controls and +TST healthy controls groups ($P= 0.754$), but there were significant difference between -TST healthy controls and TB patients groups ($P= 0.001$) and between the +TST healthy controls and the TB patients groups ($P= 0.001$). The unpaired student's t-test was used to test the statistical significance among the different groups under the study.

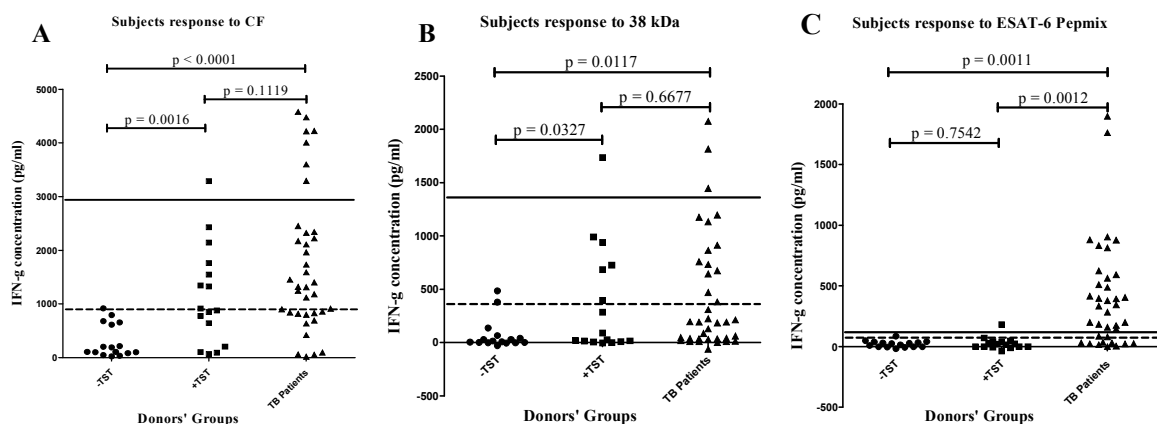


Figure 1. IFN- γ response to *M. tuberculosis* antigens. IFN- γ production by whole-blood assay after overnight stimulation with *M. tuberculosis* antigens; (A) *M. tuberculosis* crude CF, (B) Purified 38 kDa antigen, and (C) ESAT-6 Pepmix. Δ values for each subject are shown. Dotted lines represent the positive results detection cut-off value calculated from -TST group results (mean \pm 2SD). Solid lines represent the positive results detection cut-off value calculated from +TST group results (mean \pm 2SD).

Interleukin-17 (IL-17) Secretion by PBMC in Response to *M. tuberculosis* Antigens

In this study, beside interferon gamma (IFN- γ) we measured interleukin-17 (IL-17) using ELISA technique to determine whether it could increase the sensitivity and specificity of this assay in diagnosing *M. tuberculosis*

infection. Five healthy -TST subjects, five healthy +TST subjects, and fifteen patients with active pulmonary TB were selected to measure IL-17 by ELISA and to compare the released IFN- γ and IL-17 concentrations in response to the different studied antigens (Figure 2).

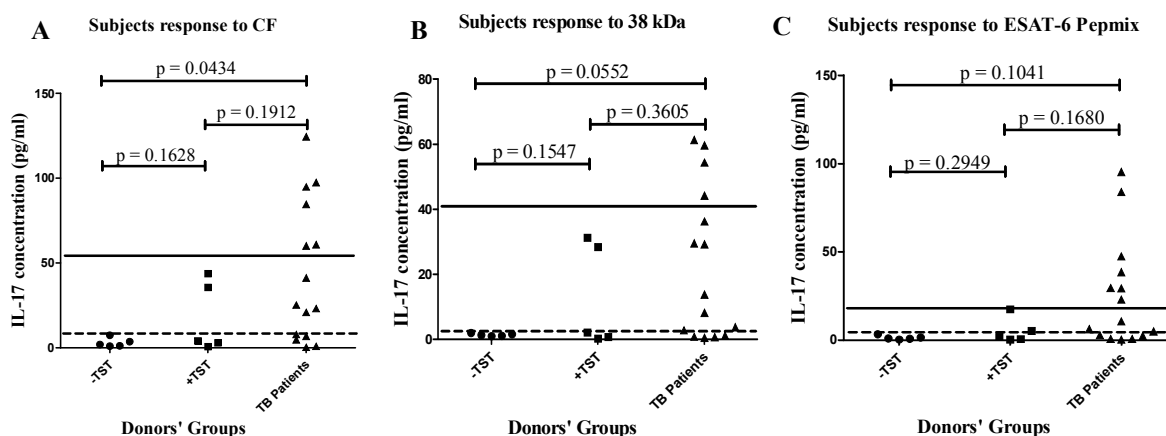


Figure 2. IL-17 response to *M. tuberculosis* antigens. IL-17 production by whole-blood assay after overnight stimulation with *M. tuberculosis* antigens; (A) *M. tuberculosis* crude CF, (B) Purified 38 kDa antigen, and (C) ESAT-6 Pcpmix. Δ values for each subject are shown. Dotted lines represent the positive results detection cut-off value calculated from -TST group results (mean \pm 2SD). Solid lines represent the positive results detection cut-off value calculated from +TST group results (mean \pm 2SD).

To determine interleukin-17 (IL-17) secretion in response to *M. tuberculosis* antigens, the whole blood from the different subjects group were cultured in the presence of crude culture filtrate (CF) purified 38 kDa antigen, or ESAT-6 Pcpmix. IL-17 production measured by ELISA in the culture supernatant as described in Subjects and Methods. Δ values (IL-17 released in stimulated well minus unstimulated well) for each subject are shown in Figure (2). When the CF was tested using the cut-off value (mean \pm 2SD) calculated from the healthy subjects with -TST group results (8.046 pg/ml), or using the cut-off value calculated from the healthy subjects from +TST group results (54.31 pg/ml), statistical differences were observed between -TST healthy controls and the TB patients groups ($P=$

0.0434), but there were no significant difference between -TST healthy controls and +TST healthy controls groups ($P=$ 0.1628), nor between the +TST healthy controls and the TB patients groups ($P=$ 0.1912). But, when the purified 38 kDa antigen was tested using the cut-off value (mean \pm 2SD) calculated from the healthy subjects with -TST group results (2.1 pg/ml), or using the cut-off value calculated from the healthy subjects from +TST group results (40.89 pg/ml), no statistical differences were observed between -TST controls and +TST healthy controls groups ($P=$ 0.155), nor between -TST healthy controls and TB patients groups ($P=$ 0.055), nor between the +TST healthy controls and the TB patients groups ($P=$ 0.361), (Figure 3).

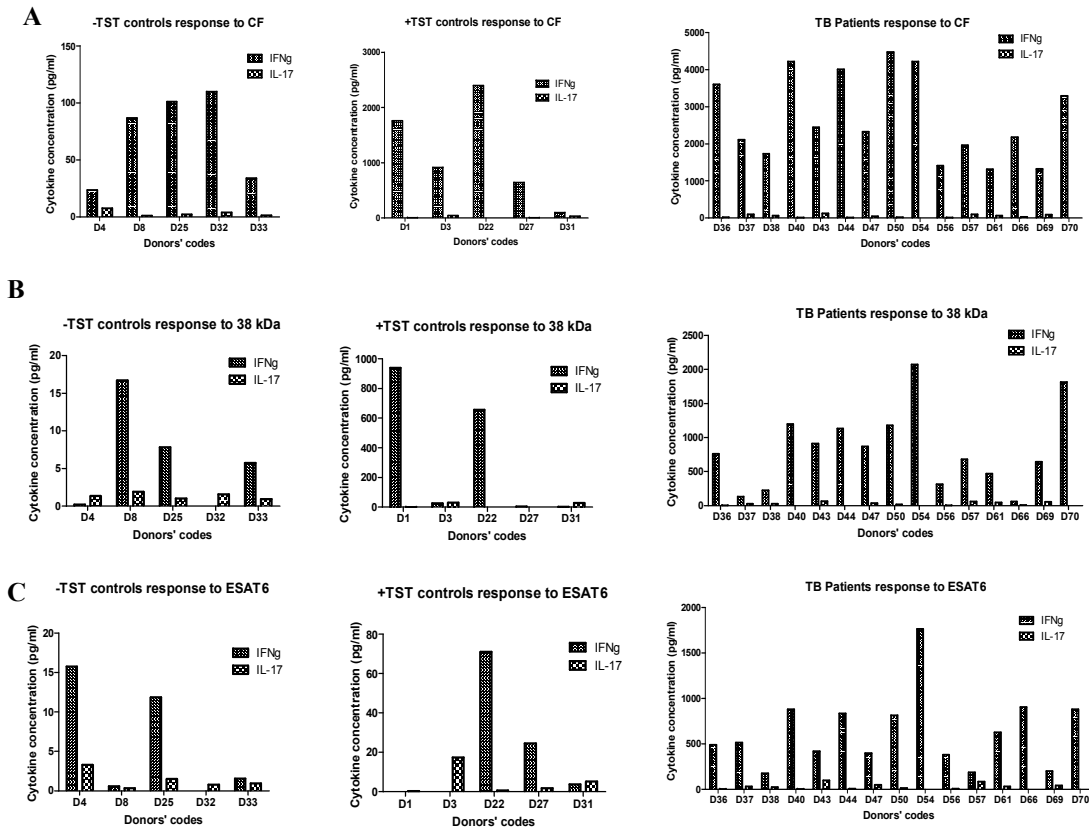


Figure 3. Comparison between IFN- γ and IL-17 concentrations (pg/ml). After overnight stimulation of the different subjects groups with the different antigens used in the study (CF, 38 kDa, and ESAT-6 Pcpmix) the concentration of IFN- γ and IL-17 were evaluated in the supernatants by ELISA as described in Materials and Methods. (A) Whole blood from the studied subjects was stimulated with CF. (B) Whole blood from the studied subjects was stimulated with 38 kDa antigen. (C) Whole blood from the studied subjects was stimulated with ESAT-6 Pcpmix.

Also, when ESAT-6 Pcpmix was tested using the cut-off value (mean \pm 2SD) calculated from the healthy subjects with -TST group results (3.44 pg/ml), or using the cut-off value calculated from the healthy subjects from +TST group results (17.835 pg/ml), no statistical differences were observed between -TST controls and +TST healthy controls groups ($P= 0.295$), nor between -TST healthy controls and TB patients groups ($P= 0.101$), nor between the +TST healthy controls and the TB patients groups ($P= 0.160$). The unpaired student's t-test was used to test the statistical significance among the different groups under the study.

When the purified 38 kDa antigen and ESAT-6 Pcpmix were tested, somewhat lower

levels of IFN- γ responses were found compared to crude CF. But, the quantitation of IFN- γ in the culture supernatants showed that when IFN- γ concentrations were compared for significant differences among the donors groups, only ESAT-6 Pcpmix induced significantly higher concentrations of IFN- γ in TB patients, as compared with healthy subjects.

Diagnostic Performance of Whole Blood Test Based on ESAT-6 Pcpmix

The specificity and sensitivity for diagnosing *M. tuberculosis* infection were analysed using the whole blood interferon gamma release assay with *M. tuberculosis* crude CF, purified 38 kDa antigen or ESAT-6 Pcpmix. Maximal

specificity and sensitivity values with *MTB* ESAT-6 Pcpmix stimulation were obtained in the studied subjects. Levels of specificity were significantly lower to *MTB* crude CF and purified 38 kDa antigens than to ESAT-6 Pcpmix when it compared to -TST group. The sensitivity of the assay was at the highest levels using ESAT-6 Pcpmix compared to either the *MTB* crude CF or the purified 38 kDa antigen (Figure 4). The results obtained in this study indicate that: (1) High ratio of the +TST healthy donors responded to *MTB* CF and purified 38 kDa antigen thereby reducing the specificity and the sensitivity of the assay significantly (especially when it compared to +TST healthy group); (2) Using the ESAT-6 Pcpmix in the whole blood assay increased the specificity and the sensitivity of the test, (Table 2).

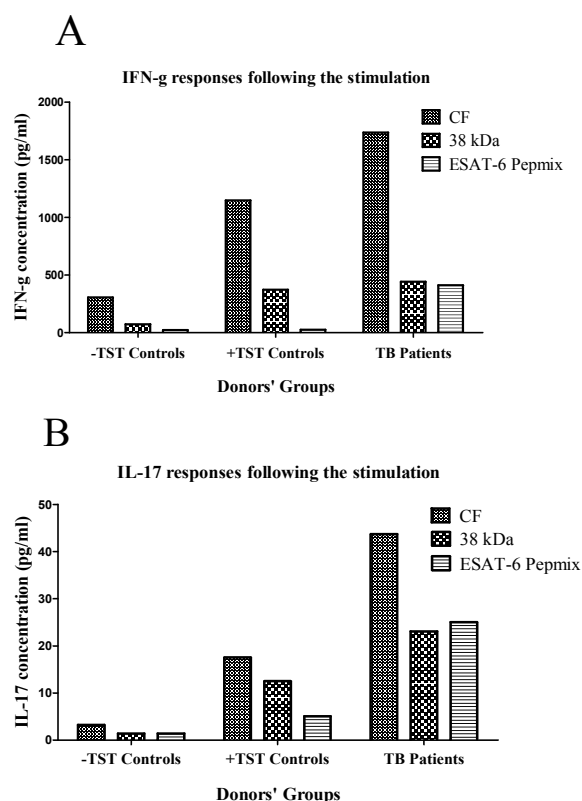


Figure 4. IFN- γ and IL-17 production following stimulation with *M. tuberculosis* antigens. Cytokines production by whole-blood assay after overnight stimulation of subjects groups in response to *M. tuberculosis* antigens. (A) IFN- γ response following the stimulation. (B) IL-17 response following the stimulation. Mean value for each group are shown.

Table 2. Multivariate statistical analysis of IFN- γ released in response to *M. tuberculosis* antigens in the Egyptian subjects under the study.

| Antigen | -TST group cut-off value | | +TST group cut-off value | |
|---------|--------------------------|---------------|--------------------------|---------------|
| | % Sensitivity | % Specificity | % Sensitivity | % Specificity |
| CF | 64.9 % | 71.9 % | 18.9 % | 96.9 % |
| 38 kDa | 37.8 % | 75.0 % | 8.60 % | 96.9 % |
| ESAT-6 | 75.7 % | 93.8 % | 70.3 % | 96.9 % |

Discussion

In most cases, *M. tuberculosis* infection is asymptomatic, latent infection that in some instances progress into TB infection. However, untreated active infections have more than 50% mortality rate, (Crowley, 2007). TB is a leading cause of death among people who are HIV-positive, (WHO, 2009). It is of great importance to diagnose active TB

at an early stage of disease, both for the community, to prevent the spread of infection, and for the individual patient because it would help in the initiation of early and appropriate treatment of patients, (Brock *et al.*, 2001). However, identification of mycobacteria by conventional methods is time-consuming and not always conclusive, (Varma-Basil *et al.*, 2010). The use of the TST has some

limitations for TB diagnosis, mainly due to its low specificity in BCG vaccinated donors or individuals infected or sensitized with nontuberculous mycobacteria, and the subjective nature of placing and reading the test, (Brock *et al.*, 2001). A number of studies have demonstrated a high sensitivity and specificity in *MTB* diagnosis when detecting interferon gamma responses to some *MTB* specific antigens. Novel interferon gamma (IFN- γ) release assays (IGRAs) with using highly *MTB* specific antigens is not confounded in populations containing a high proportion of BCG vaccinated individuals. Immune recognition of ESAT-6 is known to be highly specific for exposure to members of the TB complex, so it serves as a marker for prior *M. tuberculosis* infection, (Demissie *et al.*, 2006).

In this study, we measured the difference in interferon gamma (IFN- γ) production in the culture supernatants after the stimulation of the whole blood samples from Egyptian subjects with *MTB* crude CF, purified 38 kDa antigen, and of ESAT-6 Pcpmix. We compared the sensitivity and specificity of those assays for detection of infection with *M. tuberculosis* in the Egyptian patients. Responses were identified by measuring IFN- γ using ELISA technique. Besides interferon gamma (IFN- γ) interleukin-17 (IL-17) was measured using ELISA technique to determine whether it could increase the sensitivity and specificity of this assay in diagnosing *MTB* infection.

IFN- γ is extremely important for the induction of protective immunity against *MTB*, (Tundup *et al.*, 2008). Our data showed that IFN- γ expression appeared to be the best marker for active TB infection. IFN- γ is very important to be studied because, first, there is general agreement that activation of antimicrobial activities in macrophages by T cell cytokines is involved in *M. tuberculosis*. Accordingly, IFN- γ which is a major

macrophage activating cytokine and other Th1 cytokines are critical. Second, direct killing of mycobacteria by T cells has been demonstrated. Third, mycobacteria reactive T cells lyse infected macrophages. Macrophage lysis appears to be a prerequisite for killing by T cells of microbes residing inside macrophages. Moreover, lysis of infected macrophages could promote release of mycobacteria from incapacitated macrophages to more proficient monocytes, (Kaufmann and Hess, 2000)

A clear T cells response was detected by measuring the released IFN- γ in response to crude CF, 38 kDa antigen and ESAT-6 Pcpmix stimulation in the subjects under study. Regarding CF and 38 kDa antigen stimulation; there were no significant difference between TB patients and +TST healthy controls in the overall T cells response and the cytokines measured. This positive response in the +TST healthy controls is most probably due to the prior exposure to BCG, and reflects similar results to that found with the TST, (Harboe & Wiker, 1992). Unlike CF and 38 kDa, we found that ESAT-6 Pcpmix stimulation was able to distinguish between the TB patients and the +TST healthy controls populations. Egyptian patients with active pulmonary TB infection highly recognized ESAT-6 Pcpmix with sensitivity (75.7 %) and specificity (93.8 %) according to the cut-off value calculated from the -TST group results, and even at the cut-off value calculated from the +TST group results; high sensitivity (70.3 %) and specificity (96.9 %) were found.

Measurement of IFN- γ levels after ESAT-6 Pcpmix stimulation raised the possibility of early diagnosis in the TB patients. Evaluation of the whole blood IFN- γ test for TB diagnosis, based on the specific antigens like ESAT-6 Pcpmix could increase the specificity and the sensitivity of the whole blood test and enhance the discriminative power of the test between TB infection, atypical mycobacterial

reactivity, and reactivity due to BCG vaccination, (van Pinxteren *et al.*, 2000).

The 38 kDa protein is one of the most important antigens of *M. tuberculosis*. It is actively secreted but partly attached to the surface of the mycobacterial cell by a lipid tail that may also be responsible for binding of carbohydrate to the protein. It is a major constituent of *M. tuberculosis* culture fluid after growth and occurs in bacille Calmette-Guérin in far lower concentrations, (Harboe & Wiker, 1992).

In this study, supernatants obtained after overnight stimulation of whole blood with the studied *MTB* antigens were assessed for the secreted IL-17 using ELISA technique. Following stimulation, active TB cases produced significantly lower levels of IL-17 compared with IFN- γ . Measurement of IL-17 yielded no statistically significant differences among the groups, and IL-17 has no greater sensitivity than IFN- γ in active TB patients group.

Recently IL-17, which is produced by Th17 cells, has also been associated with protection against TB but in the latency stage, (Sutherland *et al.*, 2009). IL-17 is an active recruiter of neutrophils to inflammatory sites and IFN- γ regulates the induction of Th17 cells. These facts may explain the damaging inflammatory response seen during mycobacterial infection of IFN- γ -deficient mice in that IFN- γ and IL-17 may counter-regulate each other during chronic mycobacterial infection (Cruz *et al.*, 2006). The more recently described Th17 cells have also been associated with *MTB* infection. IL-17 is produced early during immune response against *MTB* and it has been proposed to be associated with reactivation in latent TB infected individuals, (Marin *et al.*, 2010).

Our data obtained from this study is in agree with many other studies that confirmed the ability of IFN- γ to inhibit IL-17 production in the early stage of *MTB* infection (active

pulmonary TB patients) that protect from inappropriate inflammatory tissue-damaging effect of IL-17. Better understanding of the relationship between IFN- γ and IL-17 could enhance the discriminative power of the immunodiagnostic assay to differentiate between active TB disease and latent infection.

This data is in agreement with Sutherland *et al.*, (2009), who showed that cytokine profiles from culture supernatants were significantly biased toward a Th1-type cytokine response (IFN- γ and IL-12) together with a complete abrogation of IL-17 secretion in the active TB patients at the early stage of the disease, (Sutherland *et al.*, 2009).

Arend *et al.*, 2001, reported that, in humans, T-cell responses to ESAT-6 alone or those to ESAT-6 and CFP-10 were sensitive and specific for detection of active pulmonary or extrapulmonary TB. T-cell responses to a mixture of overlapping synthetic peptides of these antigens yielded results similar to those of the intact recombinant antigen, indicating that, (Arend *et al.*, 2001), peptides have the advantage of faster production at lower cost, (Arend *et al.*, 2000). Thereby suggesting that overlapping synthetic peptides could replace complete antigens in T-cell responses, (Mustafa *et al.*, 2008).

To our knowledge, this study is the first in Egypt that targets assessments of immunodiagnostic potential of synthetic overlapping peptides mixture from *MTB*. Our data raises the possibility of using ESAT-6 Pepmix as a candidate immunodiagnostic reagent to detect the infection with *M. tuberculosis* among Egyptian personals.

The investigation of additional markers or cytokines also could allow us to increase the sensitivity and the specificity of the assay. Using the results from this study, there is considerable scope to streamline this method of diagnosing *MTB* infection. Additional cytokines such as IFN- γ inducible protein (IP-

10) or tumor necrosis factor (TNF- α) could also be measured to possibly increase the assay's usefulness. The addition of other *MTB*-specific proteins such as CFP10 has already demonstrated their usefulness in increasing sensitivity and specificity in the IFN- γ release assay. It offers the potential for improved accuracy in diagnosis of *MTB* infection in individuals, especially those with positive TSTs due to past BCG vaccination, and may enable characterization of the immune response occurring during infection.

Finally, our findings provide proof of concept for an immunodiagnostic test that could potentially allow clinicians to identify an *MTB* infection by ESAT-6 Pcpmix responsiveness and estimate the risk that it is progressive. Such an immunodiagnostic test would make it more feasible to identify and treat active TB at a very early stage, which could finally reduce the transmission of *M. tuberculosis*, (Demissie *et al.*, 2006).

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