Assessment of T Cell Response to Novel *Mycobacterium tuberculosis* Synthetic Overlapping Peptides Mixtures (Rv2659 and Rv2660) and ESAT-6 in Egyptian Patients

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Tuberculosis (TB) remains one of the major challenges to the global public health. The most powerful tools in any TB control program are prompt diagnosis and successful treatment of patients with active contagious disease. For almost 100 years the tuberculin skin test (TST) has been used to support the diagnosis of active and latent TB infection. The TST has a number of limitations, most notable low specificity in BCG vaccinated individuals due to cross-reactive components in PPD and the *Mycobacterium bovis* BCG vaccine strain and an intensive search for new and more specific diagnostic antigens has therefore been ongoing. The current diagnostic techniques utilize production of Interferon-gamma (IFN-γ) in response to novel *M. tuberculosis* (*MTB*) synthetic overlapping peptides mixtures to detect *MTB* infection. The aim of this study was to evaluate human immune responses to two novel *Mycobacterium tuberculosis* latency associated antigens Rv2659 Pepmix and Rv2660 Pepmix in comparison with ESAT-6 Pepmix. We compared the production of IFN-γ by ELISA following overnight stimulation with the antigens among the different groups of our study, TST negative healthy subjects (n = 16), TST positive healthy subjects (n = 16) and active pulmonary TB patients (n = 30). Our results showed that in TB patients, a positive IFN-γ response was observed to ESAT-6 by 73% of the donors, 47% responded to Rv2659 and 57% responded to Rv2660 when compared to TST negative controls. In conclusion, the ESAT-6 pepmix is recognized in a greater proportion of TB patients compared to Rv2659 and Rv2660, and levels of IFN-γ in response to ESAT-6 are higher than the levels observed in response to Rv2659 and Rv2660.

Despite the availability of highly efficacious treatment for decades, TB remains a major global health problem. In 1993, the World Health Organization (WHO) declared TB a global public health emergency, at a time when an estimated 7–8 million cases and 1.3–1.6 million deaths occurred each year. In 2010, there were an estimated 8.5–9.2 million cases and 1.2–1.5 million deaths (including deaths from TB among HIV-positive people). TB is the second leading cause of death from an infectious disease worldwide (after HIV, which caused an estimated 1.8 million deaths in 2008) (WHO, 2011). 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 is newly infected with TB bacilli every second. Generally, 5% to 10% of people who are infected with TB become actively sick and contagious (WHO, 2008). However, untreated active infections have more than 50% mortality rate (Crowley, 2007). In Egypt, there is an incidence rate of 20 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 disease. One-third of the World’s
population or two billion people carry the \textit{MTB} bacteria (Report, 2010). Healthy latently infected individuals have approximately 10\% lifetime risk of developing active TB disease (Flynn and Chan, 2001). 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.

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). Although the isolation of tubercle bacilli from clinical specimens is the gold standard for diagnosis, this may not be achievable in every single patient. 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 TB cases (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 existed 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 \textit{Mycobacterium bovis} bacillus Calmette-Guérin (BCG) vaccine strains (Dominguez et al., 2008). Because protein-purified derivative (PPD) originate from culture filtrate of tubercle bacilli and contain numerous antigens shared with the bacille Calmette-Guérin (BCG) vaccine and most nontuberculous mycobacteria, individuals vaccinated with BCG but not infected with \textit{MTB} can test falsely positive using the tuberculin skin test (Richeldi, 2006).

Identification and characterization of some \textit{M. tuberculosis} specific antigens like ESAT-6 and CFP-10 has led to the development of new specific diagnostic tests for infection with \textit{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). Novel interferon gamma (IFN-\gamma) release assays (IGRAs) provide distinct advantages; they are highly \textit{MTB}-specific and thus not confounded in populations containing a high proportion of BCG-vaccinated individuals, avoid boosting of immune responses by \textit{ex-vivo} testing and possess logistical conveniences (Ringshausen et al., 2010). 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 \textit{M. tuberculosis} infection (Demissie et al., 2006). The genome region containing ESAT-6 was lost as one of the first deletion mutations during the attenuation of BCG, (Mahairas et al., 1996).

In latent granulomatous lesions, \textit{MTB} is successfully contained and has to adapt to a hypoxic and nutritionally compromised environment. It has recently been hypothesized that this immune pressure drives \textit{MTB} into a different metabolic state, compared with actively replicating organisms in lesions that characterize early \textit{MTB} infection or active disease. This is supported
by findings from \textit{in vitro} studies which mimic the granuloma environment, where \textit{MTB} was shown to upregulate sets of genes that are distinct from those upregulated in actively replicating organisms; e.g., in nutrient deficient medium, a particular set of genes that were termed the “starvation stimulon” was upregulated. We focused on 2 proteins encoded by the starvation stimulon, Rv2659 and Rv2660, which belong to the RD11 region encoded in \textit{MTB}; however the function of these proteins is unknown (Govender \textit{et al.}, 2010).

The purpose of the present study was to evaluate human immune responses to two novel \textit{Mycobacterium tuberculosis} latency associated antigens Rv2659 Pepmix and Rv2660 Pepmix in comparison with ESAT-6 Pepmix. Our study has allowed comparisons among ESAT-6 Pepmix, Rv2659 Pepmix and Rv2660 Pepmix recognition in TST negative healthy subjects, TST positive healthy subjects and active pulmonary TB patients. Responses were identified by measuring IFN-\(\gamma\) using ELISA technique on whole blood samples after stimulation.

**Subjects and Methods**

**Ethics Statement**

Ethical approval was obtained from the Egyptian Ministry of health and Ain Shams University Ethics Committee. 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 62 BCG vaccinated subjects divided into three groups as follows: Group (1): 16 healthy subjects with negative TST results (9 males and 7 females); whose age 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 age ranged from 13 to 62 years, and without a history of TB infection. Group (3): 30 patients from Cairo Chest Hospital with active pulmonary TB disease (24 males and 6 females); whose age 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.

**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 after collection.

**Mycobacterial Antigens**

- **ESAT-6 Pepmix** (synthetic overlapping peptides mixture)

The amino acid sequences of ESAT-6 Pepmix that were used in the present study are listed in Table 1.

<table>
<thead>
<tr>
<th>Peptide (position)</th>
<th>Amino acid sequence(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>MTEQQWNFAGIEAAASAIQG</td>
</tr>
<tr>
<td>P2</td>
<td>GIEAAASAIQGNVTSI</td>
</tr>
<tr>
<td>P3</td>
<td>SAIQGNVTSIHLDEGKQSLTKLA</td>
</tr>
<tr>
<td>P4</td>
<td>EGKQSLTKLAAAWGGSGSEAYQGVQ</td>
</tr>
<tr>
<td>P5</td>
<td>SGSEAYQGVQOKWDATELNNALQ</td>
</tr>
<tr>
<td>P6</td>
<td>TATELNALONLARTISEAQQAMAS</td>
</tr>
<tr>
<td>P7</td>
<td>NLARTISEAQQAMASTEGNVTGMFA</td>
</tr>
</tbody>
</table>

\(^a\)From the N terminus to the C terminus.

- **Rv2659 Pepmix** (synthetic overlapping peptides mixture)

The amino acid sequences of Rv2659 Pepmix that were used in the present study are listed in Table 2.
Assessment of T cell response to Novel MTB Synthetic Overlapping Peptides Mixtures (Rv2659 and Rv2660) and ESAT-6 in Egyptian Patients

Table 2. Amino acid sequences of Rv2659 Pepmix (P26-P37).

<table>
<thead>
<tr>
<th>Peptide (position)</th>
<th>Amino acid sequence*</th>
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<tbody>
<tr>
<td>P26</td>
<td>GVRDISIPIHLPAIEDIHLH</td>
</tr>
<tr>
<td>P27</td>
<td>LIPAIEDHLHKVNPGRESL</td>
</tr>
<tr>
<td>P28</td>
<td>KHVNPGRESLLFPSVNDPNR</td>
</tr>
<tr>
<td>P29</td>
<td>LFPSVNDPNRLHAPSALYRM</td>
</tr>
<tr>
<td>P30</td>
<td>HLAPSALYRMFYKARKAAGR</td>
</tr>
<tr>
<td>P31</td>
<td>FYKARKAAGRDPDLRVHDLRH</td>
</tr>
<tr>
<td>P32</td>
<td>PDLRVHDLRHSGAVLAASTG</td>
</tr>
<tr>
<td>P33</td>
<td>SGAVLAASTGATLAELMORL</td>
</tr>
<tr>
<td>P34</td>
<td>ATLAELMORLGHSTAGAALR</td>
</tr>
<tr>
<td>P35</td>
<td>GHSTAGAALRYQHAAKGRDDR</td>
</tr>
<tr>
<td>P36</td>
<td>YQHAAKGRDREIAALLSKLA</td>
</tr>
<tr>
<td>P37</td>
<td>EIALLSKLAENQEM</td>
</tr>
</tbody>
</table>

*Rv2660 Pepmix (synthetic overlapping peptides mixture)
The amino acid sequences of Rv2660 Pepmix that were used in the present study are listed in Table 3.

Table 3. Amino acid sequences of Rv2660 Pepmix (P1-P7).

<table>
<thead>
<tr>
<th>Peptide (position)</th>
<th>Amino acid sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>VIAGVDQALAAATGQASQRAA</td>
</tr>
<tr>
<td>P2</td>
<td>ATGQASQRAAGASGGVTVGV</td>
</tr>
<tr>
<td>P3</td>
<td>GASGGVTGVGVGTEQRNLS</td>
</tr>
<tr>
<td>P4</td>
<td>GYGTQQRNLSVQAPSQTF</td>
</tr>
<tr>
<td>P5</td>
<td>VVPQSTFSSRSVPDFVDET</td>
</tr>
<tr>
<td>P6</td>
<td>SRSPDFVDETAGQOWCAILG</td>
</tr>
<tr>
<td>P7</td>
<td>AGQSWCAILGLNQFH</td>
</tr>
</tbody>
</table>

*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, ESAT-6 Pepmix Activated, Rv2659 Pepmix Activated and Rv2660 Pepmix Activated. To each tube 1 ml of heparinized whole blood was added. All the studied antigens (ESAT-6, Rv2659 and Rv2660 Pepmix) were used 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. The limit of detection of the ELISA was determined to be 5 pg/ml. A 1:2 dilution of supernatants was used, which was found optimal in preliminary experiments. 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',5,5'-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.

Statistical Methods
Data analysis was performed using the computer program GraphPad Prism, version 5 (GraphPad Software Inc., San Diego, CA, USA). The Mann–Whitney test was used to test the statistical significance among the different groups under the study. A value of p < 0.05 was considered significant.

Results
Interferon Gamma (IFN-γ) Secretion by T Cells in Response to MTB 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 ESAT-6 Pepmix, Rv2659 Pepmix, or Rv2660 Pepmix. IFN-γ production measured by ELISA in the culture supernatant as described in Subjects and Methods. A values (IFN-γ released in stimulated well minus unstimulated well) for each individual are shown in Figure (1).
Subjects response to ESAT-6 Pepmix

Donors' Groups

Subjects response to Rv2659 Pepmix

Donors' Groups

Subjects response to Rv2660 Pepmix

Donors' Groups

Figure 1. IFN-γ response to M. tuberculosis antigens: IFN-γ production by whole-blood assay after overnight stimulation with M. tuberculosis antigens; ESAT-6, Rv2659, and Rv2660 measured by ELISA. Δ values (IFN-γ released in stimulated well minus unstimulated well) and median for each subject are shown. The Mann–Whitney test was used to test the statistical significance. Dotted lines represent the positive results detection cut-off value calculated from TST -ve group results (mean + 2SD). Solid lines represent the positive results detection cut-off value calculated from TST +ve group results (mean + 2SD).

When Rv2659 Pepmix was tested, no statistical differences were observed between TST -ve controls and TST +ve healthy controls groups ($P = 1.000$), while there was a significant difference between the TST +ve subjects and the TB patients compared to the TST –ve subjects with a $P$ value of 0.024 and 0.011 respectively.

Also, when Rv2660 Pepmix was tested, no statistical differences were observed between TST -ve controls and TST +ve healthy controls groups ($P = 0.331$) or between the TST +ve subjects and the TB patients ($P = 0.067$), while there was a significant difference between the TST -ve subjects and the TB patients with a $P$ value of 0.003.

When ESAT-6 Pepmix was tested, no statistical differences were observed between TST -ve healthy controls and TST +ve healthy controls groups ($P = 0.7864$), but there were significant difference between TST -ve healthy controls and TB patients groups ($P = 0.0001$) and between the TST +ve healthy controls and the TB patients groups ($P = 0.0001$). Overall, the IFN-γ levels induced in active TB patients by the ESAT-6 Pepmix
were higher than the levels induced by Rv2659 and Rv2660 Pepmix (Figure 1).

Diagnostic Performance of ESAT-6, Rv2659, and Rv2660 Pepmix

The Cut-off value used for determine the specificity and sensitivity were calculated by adding 2SD to the mean value of the TST -ve or TST +ve groups. For the Rv2659 the cut-off value for the TST -ve and TST +ve subjects were 22.45 pg/ml and 31.81 pg/ml respectively while for the Rv2660 the cut-off value for the TST -ve and TST +ve subjects were 17.89 pg/ml and 62.68 pg/ml respectively. For the ESAT-6 the cut-off value for the TST -ve and TST +ve subjects were 54.92 pg/ml and 146.14 pg/ml respectively. Based on these cut-off values the sensitivity and specificity was calculated for each Pepmix (Table 4). ESAT-6 was the most frequently recognized antigen in TB patients, but a significant proportion of the patients also recognized the Rv2659 and Rv2660 antigens (47 and 57%, respectively, compared to the TST -ve group).

Table 4. Specificity and sensitivity analysis of IFN-γ released in response to M. tuberculosis antigens in the Egyptian subjects under the study.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>TST -ve group cut-off value</th>
<th>TST +ve group cut-off value</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>% Sensitivity</td>
<td>% Specificity</td>
</tr>
<tr>
<td>ESAT-6</td>
<td>73.3 %</td>
<td>93.8 %</td>
</tr>
<tr>
<td>Rv2659</td>
<td>46.6 %</td>
<td>88.9 %</td>
</tr>
<tr>
<td>Rv2660</td>
<td>56.7 %</td>
<td>87.5 %</td>
</tr>
</tbody>
</table>

Recognition profile of ESAT-6, Rv2659 and Rv2660

Further analysis of the responding TB patients compared to the TST -ve showed that five patients (17%) who did not respond to ESAT-6 responded to either Rv2659 and/or Rv2660, Figure 2A.

The same analysis for the responding TB patients compared to the TST +ve subjects showed that four patients (13%) who did not respond to ESAT-6 responded to either Rv2659 and/or Rv2660, Figure 2B. The unique recognition of Rv2659 and/or Rv2660 in ~15% of donors emphasizes that these antigens has the potential to supplement the recognition observed to ESAT-6.

Figure 2. Recognition of ESAT-6, Rv2659 and Rv2660. Dark grey boxes represent positive responses (above the cut-off level), whereas light gray boxes represent negative responses. Each row shows the response to a single antigen in the 30 patients with active, pulmonary TB. The number of patients responding to each antigen is shown to the right. Each column presents data for one patient. In A, the cut-off level is defined based on TST -ve donors; in B, the cut-off level is defined based on TST +ve donors.
Discussion
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 ESAT-6 Pepmix, Rv2659 Pepmix, and Rv2660 Pepmix, ESAT-6 was included in the study as an immunodiagnostic control antigen. We compared the sensitivity and specificity of those assays for detection of infection with M. tuberculosis in the Egyptian patients.

We were able to detect a clear T cell response by measuring the released IFN-γ in response to ESAT-6 Pepmix stimulation in the subjects under the study. As expected, a large proportion of the TB patients responded to ESAT-6 (73% compared to TST –ve donors). Regarding Rv2659 Pepmix, and Rv2660 Pepmix stimulation; no statistical differences were observed between TST -ve controls and TST +ve healthy controls groups, while there was a significant difference between the TST +ve subjects and the TB patients compared to the TST –ve subjects. Compared to TST –ve donors, 47% of the TB patients recognized Rv2659. Regarding the Rv2660, there was no statistical differences observed between TST -ve controls and TST +ve healthy controls groups or between the TST +ve subjects and the TB patients, while there was a significant difference between the TST -ve subjects and the TB patients. A positive response to Rv2660 was observed in 57% of the TB patients when compared to TST –ve donors.

The ESAT-6 pepmix is recognized in a greater proportion of TB patients compared to Rv2659 and Rv2660, and levels of IFN-γ in TB patients response to ESAT-6 were higher than the levels observed in response to Rv2659 and Rv2660, reflecting the nature of ESAT-6 as an immunodominant T-cell antigen. However, 63% of the active TB patients recognized either Rv2659 or Rv2660 compared to TST-ve controls, and 5 patients (17%) recognized either Rv2659 or Rv2660 but not ESAT-6, emphasizing the potential of the two novel antigens to supplement ESAT-6 recognition. The lower IFN-γ levels and recognition frequency may also reflect that Rv2659 and Rv2660 are latency associated antigens more frequently recognized in persons with latent MTB infection (LTBI), compared with persons with active TB disease.

A limitation of our study was the absence of the LTBI group. Participation of LTBI group should support the hypothesis which suggested that latency antigens will be preferentially recognized by persons with LTBI, compared with persons with active TB disease.

This is the first clinical report of human immune recognition of the Rv2659 and Rv2660 antigens in Egypt. Much more research is needed to determine whether candidate latency-, starvation-, or resuscitation-associated antigens other than Rv2659 or Rv2660 would be needed for better understanding of the role of those latency associated antigens which could enhance the discriminative power of the immunodiagnostic assay to differentiate between active TB disease and latent infection.

In a recent publication, the induction of the Rv2660 locus in Mtb during starvation conditions in PBS was studied and the authors concluded that the upregulated transcript was encoded on the opposite strand to Rv2660. It was therefore questioned if the Rv2660 protein actually exists in Mtb (Houghton 2013). However, when the Rv2660 protein is added to the Ag85B-ESAT6 fusion protein and delivered as a subunit vaccine in mice and Cynomolgus Macaques after Mtb exposure, preventive effects on reactivation were
reported (Aagaard et al., 2011; Lin et al., 2012). More efforts are required to understand this apparent inconsistency, but the human recognition of Rv2660 observed in TB patients from Egypt in this study and in persons with LTBI from South Africa (Govender, 2010) should be addressed in the further elucidation of the role of Rv2660 in latency.

Acknowledgments

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