Use of enzyme linked immunospot assay (ELISpot) for monitoring treatment response of pulmonary tuberculosis patients

Ramadan M. Nafae 1, Mohammad A. Mohammad 2, Mohammad S. El-Gammal *, Mohammad A.M. Abdullah

Chest Department, Faculty of Medicine, Zagazig University, Egypt

Received 6 February 2013; accepted 9 June 2013
Available online 6 July 2013

Abstract  Background: Tuberculosis (TB) remains one of the major causes of death from a single infectious agent worldwide. The rapid emergence of drug resistant mycobacteria has strengthened the demand for rapid methods for detection of mycobacteria in clinical samples. As prevention of tuberculosis relies on the early detection and cure of the infectious cases, current efforts are focused upon improving the rapidity of identification of Mycobacterium tuberculosis, allowing prompt initiation of appropriate therapy. The rapid enzyme linked immunospot assay (ELISpot) method was developed in the late 1990s based on the numbers of spots made by interferon gamma producing T cells stimulated by culture filtrate protein-10 (CFP-10) or early secretory antigenic target-6 (ESAT-6). Therefore, a T-cell response to these antigens could in theory serve as a specific marker of M. tuberculosis infection.

Aim of the work: Is to assess the potential utility of ELISpot assay for monitoring treatment response of pulmonary tuberculosis patients.

Patients and methods: The study was done on 30 patients diagnosed as pulmonary tuberculosis on clinical, radiological and bacteriological bases. They were collected from Zagazig Chest Hospital and Zagazig University Hospitals from January 2010 to January 2011. A total of 15 healthy volunteers were enrolled in this study as control subjects. The following were performed for all patients before

* Corresponding author. Tel.: +20 1001747101.
E-mail addresses: rnafe@hotmail.com (R.M. Nafae), mohamed-awad266@yahoo.com (M.A. Mohammad), mohamedsobhy1970@yahoo.com (M.S. El-Gammal).
1 Tel.: +20 01223912505.
2 Tel.: +20 01141897861.
Peer review under responsibility of The Egyptian Society of Chest Diseases and Tuberculosis.
Introduction

The World Health Organization (WHO) estimates that more than one-third of the World’s population is infected with Mycobacterium tuberculosis. Even in the developed world, tuberculosis (TB) rates are stable at best, and actually are increasing in many areas. This is due to factors such as immigration, the emergence of drug resistant TB strains, the human immunodeficiency virus (HIV), and other immunosuppressive conditions. Among them, nine million people get new cases of active tuberculosis (TB) annually and 2–3 million people die of TB each year [1].

Tuberculosis is caused primarily by direct inhalation of infective droplet nuclei. Transdermal and gastrointestinal (GI) transmission have also been reported [2]. Classic features associated with active TB are cough, weight loss, anorexia, fever, night sweats, hemoptysis and chest pain. Classic symptoms are often absent particularly in patients who are immunocompromised or elderly. Up to 20% of patients with active TB may be asymptomatic [3].

The only confirmation of TB diagnosis is the bacteriological examination, but a suspicion of TB is frequently based on clinical and radiological grounds [4]. The acid-fast bacilli (AFB) smear of respiratory specimen, including sputum, induced sputum and bronchial washing specimen, is the essential modality for the prompt diagnosis of pulmonary TB. However, the sensitivity of the AFB smear result is known to be poor, varying between 30% and 70% depending on a number of factors relating to how the test is implemented. Even though the culture of tuberculosis bacilli is more sensitive (80–85%), being able to detect as few as 10 bacteria per milliliter of sputum, it usually takes 3–8 weeks to receive culture results [5].

Until recently, the tuberculin skin test (TST) has been used to identify persons infected by Mycobacterium tuberculosis who are at a high risk of the progression to active disease. This method has several limitations especially in immunosuppressed individuals such as HIV infected people who may reveal false negative TST response. Furthermore, false positive TST responses are also possible in cases of Bacille Calmette–Guérin (BCG) vaccination and/or non-tuberculosis mycobacterium (NTM) [7].

Advances in mycobacterium genomics in the late 1990s identified the Region of Difference 1 (RD1), that encodes two highly antigenic proteins, early secretory antigenic target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10) and...
was found to be present in all pathogenic strains of mycobacterium TB[8].

Recently, a new generation of tests has been developed such as QuantiFERON-TB, QuantiFERON-TB Gold (QFN-GOLD) and ELISpot assay (also known as T-Spot. TB). The basis of these tests is the detection in serum of either the release of INF-γ on stimulation of sensitized T-cell by mycobacterium tuberculosis antigen in vitro (QuantiFERON) using a whole blood enzyme linked immunosorbant assay (ELISA) or detection of the T-cells themselves (T-Spot. TB) [9].

The rapid ELISpot assay was developed in the late 1990s by Lalvani et al. [10] who counted individual antigen specific T-cells. T-cells from individuals with mycobacterium tuberculosis infection become sensitized to ESAT-6 or CFP-10 in vivo; when the T-cells re-encounter these antigens in vitro in the overnight ELISpot assay, they release a cytokine, INF-γ. By the next morning each such T-cell gives rise to a dark spot, which is the “footprint” of an individual mycobacterium tuberculosis specific T-cell [10]. T-cell responses to the 1:1 complex of ESAT-6 and CFP-10 were inferior to ESAT-6 alone [11].

However the role of ELISpot assay in monitoring response of TB treatment is less well-established [12].

Aim of the work

The aim of the study is to assess the potential utility of ELISpot assay for monitoring treatment response of pulmonary tuberculosis patients.

Patients and methods

Patients

This study was carried out on 30 patients attending Zagazig Chest Hospital and Zagazig University Hospitals during the period from January 2010 to January 2011. All patients were suspected to have pulmonary tuberculosis on clinical and radiological basis and confirmed by sputum smear Ziehl–Neelsen (Z–N) staining and sputum culture on Lowenstein–Jensen media. Twenty-two of patients were males and the rest were females with a mean age of 41 ± 17 years. Their ages ranged from fifteen to seventy-five years. A total of 15 healthy volunteers were enrolled as control subjects in this study. Eleven of them were males and the rest were females with a mean age of 32 ± 7 years. In principle, we administered four antituberculous drugs to each patient for the initial 2 months (isoniazid, rifampicin, pyrazinamide and ethambutol). The following were performed for all patients before treatment initiation:

- Full history taking.
- Complete clinical examination.
- Chest X-ray, postero-anterior and lateral views:

  - Chest X-rays were used to detect cavitary lesions, associated complications and also grade radiological severity of disease as minimal, moderately advanced and far advanced [13].
  - Tuberculin skin test (TST) by Mantoux technique.
  - 3 successive sputum samples for sputum smear Ziehl–Neelsen (Z–N) staining.

* Sputum collection for Mycobacterium culture on Lowenstein–Jensen media.
* Routine laboratory investigations including complete blood count (CBC), fasting and postprandial blood sugar, serum creatinine, blood urea nitrogen, SGOT, SGPT and albumin.
* Collection of 2 ml heparinized blood for enzyme linked immunospot assay (ELISpot).

The following were performed for all patients after 2 months of therapy:

- 3 successive sputum samples for sputum smear Ziehl–Neelsen (Z–N) staining.
- Sputum collection for Mycobacterium culture on Lowenstein–Jensen media.
- Collection of 2 ml heparinized blood for enzyme linked immunospot assay (ELISpot).

Specimen collection

3 successive sputum samples were obtained from each patient. Patients were instructed to take deep breath, hold it momentarily then cough vigorously into a sterile container. Specimens were either processed at once or after being refrigerated at 2 °C for a maximum period of 2 days.

Specimen processing

1. Decontamination and concentration of specimens
   All specimens, which were likely to contain normal or transient bacterial flora, were decontaminated by standard N-acetyl-L-cysteine-NaOH method [14]. Decontaminated and concentrated sediment was resuspended in 2 ml of sterile 0.67 Magnesium phosphate buffer (pH 6.8) and used for sputum smear Ziehl–Neelsen (Z–N) staining and culture on Lowenstein–Jensen (L.J.) methods.

2. Microscopy
   Smears were made for all the clinical specimens studied and stained with Ziehl–Neelsen (ZN) staining method [14] for AFB as follows:
   - Ziehl–Neelsen stain was prepared by using:
     - Carbol fuchsin which consists of basic fuchsin, ethanol 95%, phenol (melted crystals) and distilled water.
     - Decolorizer which consists of hydrochloric acid, ethanol 95%, methylene blue and distilled water.
     - The stained films were examined microscopically. The number of acid-fast bacilli was reported according to criteria recommended by [15].

3. Culture on Lowenstein–Jensen media (L.J.)
   Principle
   L.J. medium base is a relatively simple formulation that requires supplementation in order to support the growth of mycobacteria. Glycerol and egg mixture are added prior to the inspissation process. The substances provide fatty acids and protein required for the metabolism of mycobacteria. The coagulation of egg albumin during sterilization provides a solid medium for inoculation purposes. L–J medium also

- Sputum collection for Mycobacterium culture on Lowenstein–Jensen media.
- Routine laboratory investigations including complete blood count (CBC), fasting and postprandial blood sugar, serum creatinine, blood urea nitrogen, SGOT, SGPT and albumin.
- Collection of 2 ml heparinized blood for enzyme linked immunospot assay (ELISpot).

Specimen collection

3 successive sputum samples were obtained from each patient. Patients were instructed to take deep breath, hold it momentarily then cough vigorously into a sterile container. Specimens were either processed at once or after being refrigerated at 2 °C for a maximum period of 2 days.

Specimen processing

1. Decontamination and concentration of specimens
   All specimens, which were likely to contain normal or transient bacterial flora, were decontaminated by standard N-acetyl-L-cysteine-NaOH method [14]. Decontaminated and concentrated sediment was resuspended in 2 ml of sterile 0.67 Magnesium phosphate buffer (pH 6.8) and used for sputum smear Ziehl–Neelsen (Z–N) staining and culture on Lowenstein–Jensen (L.J.) methods.

2. Microscopy
   Smears were made for all the clinical specimens studied and stained with Ziehl–Neelsen (ZN) staining method [14] for AFB as follows:
   - Ziehl–Neelsen stain was prepared by using:
     - Carbol fuchsin which consists of basic fuchsin, ethanol 95%, phenol (melted crystals) and distilled water.
     - Decolorizer which consists of hydrochloric acid, ethanol 95%, methylene blue and distilled water.
     - The stained films were examined microscopically. The number of acid-fast bacilli was reported according to criteria recommended by [15].

3. Culture on Lowenstein–Jensen media (L.J.)
   Principle
   L.J. medium base is a relatively simple formulation that requires supplementation in order to support the growth of mycobacteria. Glycerol and egg mixture are added prior to the inspissation process. The substances provide fatty acids and protein required for the metabolism of mycobacteria. The coagulation of egg albumin during sterilization provides a solid medium for inoculation purposes. L–J medium also

- Sputum collection for Mycobacterium culture on Lowenstein–Jensen media.
- Routine laboratory investigations including complete blood count (CBC), fasting and postprandial blood sugar, serum creatinine, blood urea nitrogen, SGOT, SGPT and albumin.
- Collection of 2 ml heparinized blood for enzyme linked immunospot assay (ELISpot).

Specimen collection

3 successive sputum samples were obtained from each patient. Patients were instructed to take deep breath, hold it momentarily then cough vigorously into a sterile container. Specimens were either processed at once or after being refrigerated at 2 °C for a maximum period of 2 days.

Specimen processing

1. Decontamination and concentration of specimens
   All specimens, which were likely to contain normal or transient bacterial flora, were decontaminated by standard N-acetyl-L-cysteine-NaOH method [14]. Decontaminated and concentrated sediment was resuspended in 2 ml of sterile 0.67 Magnesium phosphate buffer (pH 6.8) and used for sputum smear Ziehl–Neelsen (Z–N) staining and culture on Lowenstein–Jensen (L.J.) methods.

2. Microscopy
   Smears were made for all the clinical specimens studied and stained with Ziehl–Neelsen (ZN) staining method [14] for AFB as follows:
   - Ziehl–Neelsen stain was prepared by using:
     - Carbol fuchsin which consists of basic fuchsin, ethanol 95%, phenol (melted crystals) and distilled water.
     - Decolorizer which consists of hydrochloric acid, ethanol 95%, methylene blue and distilled water.
     - The stained films were examined microscopically. The number of acid-fast bacilli was reported according to criteria recommended by [15].

3. Culture on Lowenstein–Jensen media (L.J.)
   Principle
   L.J. medium base is a relatively simple formulation that requires supplementation in order to support the growth of mycobacteria. Glycerol and egg mixture are added prior to the inspissation process. The substances provide fatty acids and protein required for the metabolism of mycobacteria. The coagulation of egg albumin during sterilization provides a solid medium for inoculation purposes. L–J medium also
contains malachite green as an inhibitor to microorganisms other than acid-fast bacilli [16].

0.5 ml of processed specimen was inoculated on two slants of L.J. egg medium and incubated at 37 °C. The slants were inspected every day for first week and then weekly for 10 weeks [17].

(4) Tuberculin skin test (TST)
For TST, 0.1 ml of tuberculin PPD, equivalent to three or five tuberculin units (TU) of purified protein derivative solution (PPD-S) was injected intradermally into the volar aspect of the forearm, and the transverse induration diameter was measured 48–72 h later. The results of the test were interpreted by hospital staff based on the patient degree of risk, according to current guidelines [18].

(5) ELISPOT assay
MTB-specific assay was performed using test – plates from Gen-Probe Diaclone (S100813-Human IFN-gamma ELISPOT Kit). Peripheral blood mononuclear cells (PBMC) were prepared by Ficoll-Hypaque gradient centrifugations from 2 ml of heparinized blood. Briefly, 250,000 PBMC was plated on one plate pre-coated with anti-human IFN-γ antibody after stimulation with ESAT-6 peptide (5 μg/ml final concentration). The plate was incubated for 24 h at 37 °C in a humid atmosphere of 5% CO2. Spot-counting and analysis were performed according to manual’s instruction. For each sample, negative control sample (unstimulated) and a positive control (stimulated with phorbol myristate acetate (P-1585, Sigma) at a final concentration of 20 ng/ml and Ionomycin (I-0634, Sigma) at 1 μM) were included. The test was scored positive if the average number of spot forming cells (SFCs) was higher than the average number of SFCs in controls using ROC curve test. The cut-off value for a positive response was 65 SFCs. Background number of spots in negative control was always less than 10 SFCs and positive control more than 100 SFCs (Fig. 1)[19].

Statistical analysis
Data analyzed using SPSS version 10. Qualitative data were presented as numbers and relative percentage. Comparison between patients and control was done using Chi-square test.

Comparison between the median of ELISPOT of patients and control was done using Mann–Whitney U test. Comparison of ELISPOT in relative to other variables was done either using Mann–Whitney U test or Kruskal–Wallis test. Comparison between ELISPOT pre- and after treatment was done using Wilcoxon W test.

Validity of ELISPOT test in the diagnosis of pulmonary TB was done using receiver operating characteristic curve (Rock curve). The validity of ELISPOT test including sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) was calculated by comparing with the Lowenstein-Jensen media (L.J.).

Results
It is apparent from this table that males constituted the majority of patients (73.3%) while females were only (26.7%) with a mean of 41 ± 17 years. History of household close contact to known tuberculosis patients was found in six patients (20%). Regarding smoking history eight patients (26.7%) were smokers. Ten patients (33.3%) had diabetes mellitus, four patients (13.4%) had hypertension and two patients (6.7%) had rheumatic heart disease. As illustrated in Table 1 it was found that the most prevalent complaints among the thirty patients were cough and expectoration as they were found in all patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years</td>
<td>35 (15–75)</td>
</tr>
<tr>
<td>Sex</td>
<td>41 ± 17</td>
</tr>
<tr>
<td></td>
<td>73.3</td>
</tr>
<tr>
<td></td>
<td>41 ± 17</td>
</tr>
<tr>
<td></td>
<td>26.7</td>
</tr>
<tr>
<td></td>
<td>26.7</td>
</tr>
<tr>
<td>History of household close contact</td>
<td>6</td>
</tr>
<tr>
<td>Co-morbid disease</td>
<td>10</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>33.3</td>
</tr>
<tr>
<td>Hypertension</td>
<td>4</td>
</tr>
<tr>
<td>Rheumatic heart disease</td>
<td>6.7</td>
</tr>
<tr>
<td>Symptoms</td>
<td></td>
</tr>
<tr>
<td>Cough and expectoration</td>
<td>30</td>
</tr>
<tr>
<td>Hemoptysis</td>
<td>8</td>
</tr>
<tr>
<td>Dyspnea</td>
<td>6</td>
</tr>
<tr>
<td>Night fever and night sweats</td>
<td>20</td>
</tr>
<tr>
<td>Tuberculin skin test</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>18</td>
</tr>
<tr>
<td>Negative</td>
<td>12</td>
</tr>
<tr>
<td>Sputum smear AFB</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>18</td>
</tr>
<tr>
<td>+ +</td>
<td>8</td>
</tr>
<tr>
<td>+ + +</td>
<td>4</td>
</tr>
<tr>
<td>Culture on Lowenstein-Jensen media</td>
<td>60</td>
</tr>
<tr>
<td>Positive</td>
<td>30</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 1 ELISpot well (one spot = one T cell).
followed by night fever and night sweats as they were found in 20 patients (66.7%), while hemoptysis was found in eight patients (26.7%), and dyspnea which was found in six patients (20%). Regarding the tuberculin skin test, it was positive in eighteen patients (60%). All thirty patients included in this study were sputum positive smear for AFB and positive culture on L.J. media.

As illustrated in Table 2 it was found that the lesion was more common in the upper lung zone (60%). The lesion also was more common in the right lung (46.7%). Also, it was found that 18 patients (60%) had cavitary lesion on radiology. As regards radiological extent, it was found that eight patients (26.7%) had minimal disease, fourteen patients (46.6%) had moderately advanced disease, and eight patients (26.7%) had far advanced disease.

As illustrated in Table 4 it was found that the number of pre-treatment ESAT-6 ELISpot count in patients with positive tuberculin skin test was significantly higher than those with negative tuberculin skin test. But after 2 months of therapy, there was no significant difference in the number of ESAT-6 ELISpot count between patients with positive tuberculin skin test and patients with negative tuberculin skin test.

As illustrated in Table 5 it was found that the number of pre-treatment ESAT-6 ELISpot count in patients with cavitary lesion was higher than those without cavitary lesion and the difference was highly significant. However, after 2 months of therapy there was no significant difference in decline in the number of ESAT-6 ELISpot count between patients with cavitary lesion and those without cavitary lesion.

It was found also that there was no significant difference in the number of pre-treatment ESAT-6 ELISpot count between patients with infiltration and those without infiltration. Also, after 2 months of therapy, there was no significant difference in decline in the number of ESAT-6 ELISpot count between patients with infiltration and those without infiltration.

As illustrated in Table 7 it was found that the number of pre-treatment ESAT-6 ELISpot count in patients with far advanced disease was higher than patients with minimal or moderately advanced disease. Also, after 2 months of therapy the number of ESAT-6 ELISpot count in patients with far advanced disease showed more decline than patients with minimal or moderately advanced disease. There were high statistical significant differences in patients with moderately advanced and far advanced disease pre and post treatment ESAT-6 ELISpot count.

From Table 8, it was found that ELISpot assay showed sensitivity of (93.3%) while its specificity was (100%) as well as its positive predictive value was (100%) while its negative predictive value was (88.2%).

### Table 2 Radiological findings among the studied patients.

<table>
<thead>
<tr>
<th>Radiological findings</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
</tr>
<tr>
<td><strong>Lung zone (s)</strong></td>
<td></td>
</tr>
<tr>
<td>Upper</td>
<td>18</td>
</tr>
<tr>
<td>Lower</td>
<td>4</td>
</tr>
<tr>
<td>Upper and middle</td>
<td>4</td>
</tr>
<tr>
<td><strong>Site of lesion</strong></td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>14</td>
</tr>
<tr>
<td>Left</td>
<td>8</td>
</tr>
<tr>
<td>Cavitary lesion without infiltration</td>
<td>18</td>
</tr>
<tr>
<td>Cavitary lesion with infiltration</td>
<td>8</td>
</tr>
<tr>
<td>Infiltration without cavity</td>
<td>12</td>
</tr>
<tr>
<td><strong>Radiological extent</strong></td>
<td></td>
</tr>
<tr>
<td>Minimal</td>
<td>8</td>
</tr>
<tr>
<td>Moderately advanced</td>
<td>14</td>
</tr>
</tbody>
</table>

#### Table 3 ESAT-6 ELISpot count before therapy and after 2 months of therapy.

<table>
<thead>
<tr>
<th>ESAT-6 ELISpot count (INF-γ SFCs)</th>
<th>Time of the test</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before therapy</td>
<td>2 months</td>
</tr>
<tr>
<td>Median (range)</td>
<td>500 (35–850)</td>
<td>120 (30–540)</td>
</tr>
</tbody>
</table>

This table illustrated that the median value of ESAT-6 ELISpot count was significantly decreased after 2 months of therapy.

### Table 4 ELISpot assay in relation to tuberculin skin test (TST).

<table>
<thead>
<tr>
<th>Tuberculin skin test</th>
<th>ESAT-6 ELISpot count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-treatment</td>
<td>Post-treatment</td>
</tr>
<tr>
<td>ESAT-6 ELISpot (INF-γ SFCs)</td>
<td>ESAT-6 ELISpot (INF-γ SFCs)</td>
</tr>
<tr>
<td>median (range)</td>
<td>median (range)</td>
</tr>
<tr>
<td>Positive</td>
<td>680 (120–850)</td>
</tr>
<tr>
<td>P-value</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Discussion

Tuberculosis (TB) remains one of the major causes of death from a single infectious agent worldwide. Of great concern for TB control is the emergence of drug resistance. There is concern that they may spread around the world, stressing the need for additional control measures, such as new diagnostics, better drugs for treatment, and a more effective vaccine [20].

Tuberculosis also remains a major health threat, and the rapid emergence of drug resistant mycobacteria has strengthened the demand for rapid methods for the detection of mycobacteria in clinical samples. As prevention of tuberculosis relies on the early detection and cure of the infectious cases current efforts are focused upon improving the rapidity of identification...
Follow-up studies of smear-positive pulmonary tuberculosis (TB) patients receiving short-course antituberculosis therapy have shown relapse rates of 0–6% within 6–30 months indicating that viable Mycobacterium tuberculosis complex (MTB) may remain after treatment in some patients [25]. Methods to identify patients at risk of relapse and methods to monitor response to treatment would be desirable. Clinical evaluation of treatment response can be difficult due to concurrent illness. Since radiographic resolution lags behind clinical improvement microscopy and culture are currently the methods used to monitor the efficacy of treatment and outcome [26].

Monitoring of treatment by smear examination is a crude method since smear microscopy does not distinguish between dead and live MTB, is not specific for MTB, and is insensitive, with an analytical sensitivity estimated to be 5000–10,000 acid-fast bacilli/ml. Sputum culture is superior to smear examination since it is more sensitive and specific. However, culturing is time-consuming and, therefore, supplementary rapid and sensitive monitoring methods would be advantageous [27].

The aim of this study was to assess the potential utility of ELISpot assay as a simple, rapid and easy method for monitoring treatment response of pulmonary tuberculosis patients. In the present study 26.7% of the patients were smokers (Table 1). Alcaide et al. [28] reported cigarette smoking as another risk factor for development of tuberculosis. TB can apparently lie dormant and inactive in the lungs, but smoking reduces the lung’s natural defenses against tuberculosis.

According to the World Health Organization (WHO), the risk of prevalence of TB infection is more among current or ex-smokers than non smokers. The risk of TB is more with the duration of smoking than the number of cigarettes smoked daily [29].

The biological basis by which smoking increases the TB risk may be through a decreased immune response, mechanical disruption of cilia function, defects in macrophage immune responses, and/or CD4+ lymphopenia, thereby increasing the susceptibility to pulmonary TB [30].

Smoking reduces the defences on the surface of the respiratory apparatus, alters the mucociliary apparatus through cell destruction and dysfunction and reduces lysozyme A activity.
As a result, germs and toxic substances reach the alveolar tissue in greater numbers. Smoking also produces alterations in both natural and acquired cell immunity, affecting macrophages and leukocytes. It induces apoptosis in both activated and non-activated macrophages, leading to the multiplication of the bacilli [31].

Smoking influences the clinical progress of TB lesions. Smokers tend to have more cavity disease, and greater severity despite diagnostic delays similar to those among non-smokers [32].

The most prevalent complaints among the thirty patients included in the study were cough and expectoration as it was found in all patients and the least prevalent complaint among patients was dyspnea in 20% of them (Table 1). Seaton et al. [33] stated that there is little specificity about symptoms of tuberculosis but one of the points in history which may make tuberculosis a possibility is gradual onset of symptoms over weeks and months.

In the present study 60% of patients were tuberculin skin test positive (Table 1). This was in agreement with the study performed by Palomino et al. [34] who reported that the sensitivity of the tuberculin skin test is estimated to be around just 60% in known active TB cases; so the test misses up to 40% of activity of the tuberculin skin test is estimated to be around just performed by Palomino et al. [34] who reported that the sensitivity of the tuberculin skin test is estimated to be around just 60% in known active TB cases; so the test misses up to 40% of activity of the tuberculin skin test is estimated to be around just performed by Palomino et al. [34] who reported that the sensitivity of the tuberculin skin test is estimated to be around just 60% in known active TB cases; so the test misses up to 40% of activity of the tuberculin skin test is estimated to be around just performed by Palomino et al. [34] who reported that the sensitivity of the tuberculin skin test is estimated to be around just 60% in known active TB cases; so the test misses up to 40% of activity of the tuberculin skin test is estimated to be around just performed by Palomino et al. [34] who reported that the sensitivity of the tuberculin skin test is estimated to be around just 60% in known active TB cases; so the test misses up to 40% of activity of the tuberculin skin test is estimated to be around just performed by Palomino et al. [34] who reported that the sensitivity of the tuberculin skin test is estimated to be around just 60% in known active TB cases; so the test misses up to 40% of activity of the tuberculin skin test is estimated to be around just performed by Palomino et al. [34] who reported that the sensitivity of the tuberculin skin test is estimated to be around just 60% in known active TB cases; so the test misses up to 40% of activity of the tuberculin skin test is estimated to be around just performed by Palomino et al. [34] who reported that the sensitivity of the tuberculin skin test is estimated to be around just 60% in known active TB cases; so the test misses up to 40% of activity of the tuberculin skin test is estimated to be around just performed by Palomino et al. [34] who reported that the sensitivity of the tuberculin skin test is estimated to be around just 60% in known active TB cases; so the test misses up to 40%

All patients included in this study were positive for acid fast bacilli (AFB) by sputum Ziehl–Neelsen (ZN) stain and confirmed by sputum culture on Lowenstein–Jensen media (Table 1). Gebre et al. [36] noted that the diagnosis of pulmonary tuberculosis relies on bacteriological examination of sputum.

Microscopic examination of sputum smears for the demonstration of acid fast bacilli is believed to be a simple, cheap, quick and the most practicable and effective method for developing countries [37]. Stains used are either Ziehl–Neelsen (ZN) or the fluochrome method [38]. Smear sensitivity varies among different investigators from 22% to 49.7% [39]. Marroro et al. [40] supported continuous use of the acid-fast smear as an aid in the diagnosis of mycobacterial infection.

Culture of sputum is more sensitive than smear examination, but it takes longer time before the result is known [41]. Cultivation of M. tuberculosis from clinical samples is the gold standard for the diagnosis of active TB. It can detect 100 bacilli/ml of sputum in comparison with 5000–10,000 bacilli/ml needed for microscopy [42].

The radiological finding among the thirty patients included in the study were as follows; (60%) showed cavitary lesion without infiltration, (26.7%) showed cavitary lesion with infiltration, (26.7%) had minimal disease, (46.6%) had moderately advanced disease and (26.7%) had far advanced disease (Table 2). Cegielski et al. [43] stated that cavitary lesion is a distinguishing feature of pulmonary TB and it is seen on chest radiographs in about half the cases.

In contrast, Schauf et al. [44] noted that 10–15% of patients with confirmed tuberculosis were thought to have a normal chest radiograph. The Egyptian ministry of health (1994) reported that the diagnosis of pulmonary tuberculosis must not depend upon radiological finding. This is due to the fact that many chest diseases can mimic tuberculosis on X-ray and pulmonary tuberculosis may show many forms of radiological abnormalities. However, the combined positive predictive value of the typical symptoms and the finding of cavitary upper-lobe infiltrates on chest radiographs are high [43].

In the present study, we found that the median IFN-γ ELISpot response to ESAT-6 was significantly decreased after 2 months of antituberculosis therapy (Table 3). So, ELISpot assay can be used as a useful tool for monitoring treatment response of pulmonary tuberculosis patients. In agreement with the findings in this study, Aiken et al. [12] documented a reduction in the magnitude of the IFN-γ ELISpot response to ESAT-6 in response to antituberculosis treatment in almost 80% of patients with culture proven tuberculosis.

In another study, Carrara et al. [24] documented a decrease in the IFN-γ ELISpot response to ESAT-6 following 2 months of antituberculosis treatment in 13 of 18 patients with culture confirmed TB that had a favorable response to treatment. Although various epidemiological factors may have influenced the results of these longitudinal studies, the most consistent finding is a reduction in the magnitude of the IFN-γ response to MTB-antigens during antituberculosis treatment. However in many cases, this reduction does not result in a reversion of the IGRA result.

In contrast, Ferrand et al. [45] investigated the IFN-γ ELISpot response to ESAT-6 at presentation, during and at the completion of antituberculosis treatment in patients with pulmonary TB. In this study IFN-γ ELISpot responses to ESAT-6 were higher at the completion of antituberculosis treatment than at presentation. Similarly, Ulrichs et al. [46] investigated the IFN-γ ELISpot response to ESAT-6 in 10 adult patients with pulmonary TB prior to and 60 days after starting antituberculosis treatment. In these 10 patients, the median IFN-γ ELISpot response to ESAT-6 was significantly higher at 60 days compared to baseline IFN-γ responses. This may be explained by that low numbers of responder T-cells may favor disease or, more likely, that these cells were sequestered at the sites of active disease. Also, it is possible that infection with M. tuberculosis induces energy of T-cells leading to failure of INF-γ production and that treatment of TB would lead to improved immune function and an increase in antigen-specific IFN-γ production by T-cells [47]. But our study support an alternate hypothesis that secretion of M. tuberculosis-specific proteins, such as ESAT-6, requires metabolically active and viable bacilli and thus, INF-γ-producing T-cells specific to ESAT-6 selected epitopes are present at high frequency during the active phase of bacterial replication [48]. However, after re-

Table 8 ELISpot assay sensitivity and specificity in relation to Lowenstein–Jensen media.

<table>
<thead>
<tr>
<th>P-value</th>
<th>Negative predictive value (NPV)</th>
<th>Positive predictive value (PPV)</th>
<th>Specificity</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000</td>
<td>88.2%</td>
<td>100%</td>
<td>100%</td>
<td>93.3%</td>
</tr>
</tbody>
</table>

Use of enzyme linked immunospot assay (ELISpot) for monitoring treatment

415
cept of efficacious therapy, *M. tuberculosis* stops replicating actively and the frequency of the T-cells specific for these epitopes dramatically decreases [49]. Another possible explanation is that ELISpot assay measures the frequencies of effector T-cells sensitized to ESAT-6 and CFP-10 antigens. These cells are thought to closely correlate with antigen load, driven by the burden of *M. tuberculosis* [50]. Therefore it is not surprising that we found significant qualitative and quantitative test reversion with effective therapy. It has been shown that, in the patients with successful treatment for active TB, the release of IFN-γ is absent even after a prolonged 6-day incubation with both ESAT-6 and CFP-10 antigens. It has been suggested that IGRAs based on a prolonged period of incubation with mycobacterial antigens could detect response of central memory T-cells [45] and are more sensitive to identify past latent TB infection; on the other hand, short incubation IGRAs, such as ELISpot assay, mainly detect circulating effector memory T-cells whose numbers correlate with recent or ongoing active *M. tuberculosis* infection [51].

It was found that there was an association between the number of pre-treatment ESAT-6 ELISpot count and presence of cavitary lesion, the extent of the disease in the radiology and the number of AFB (Tables 5–7). Lalvani et al. [52] reported that INF-γ-producing specific T-cells, as measured by the ELISpot assay, may be directly related to bacillary load.

When comparing sensitivity of ELISpot assay with L.J. media it was found to be (93.3%), and when comparing specificity of ELISpot assay with L.J. media it was found to be (100%) (Table 8) which was in agreement with the study performed by Richeldi et al. [2] who reported that the sensitivity of ELISpot assay ranges from 83% to 97%.

So as regards ELISpot assay sensitivity and specificity in relation to L.J. media, it showed that positive predictive value was 100% and negative predictive value was 88.2%. This was in agreement with the study performed by Wang et al. [53] who reported that PPV and NPV of ELISpot assay in detecting active TB approached 100% and 90%, respectively, even in an area with a high incidence of NTM disease.

Conclusions

1. ELISpot assay may be used as a useful tool in the diagnosis of pulmonary tuberculosis (Cut-off value for a positive response was 65 SFCs).
2. The decrease in the *M. tuberculosis*-specific T cell responses following 2 months of successful antituberculosis therapy may have a clinical value as a supplemental tool for the monitoring treatment response of pulmonary tuberculosis patients.
3. ELISpot assay results are directly affected by the antigen burden and subsequently the extent of tuberculosis infection.

Recommendations

1. ELISpot assay may be used to follow up treatment among sputum smear negative pulmonary tuberculosis patients.
2. A longer follow-up period after initiation of treatment might be needed to better appreciate the effect of treatment on T-cell responses, and subsequently to predict complete cure or relapse.
3. A study on ELISpot assay and Quantiferon TB test at the same time should be looked for in the future.

References

Use of enzyme-linked immunospot assay (ELISpot) for monitoring treatment


