Evaluation of whole blood IFNγ test using PPD and recombinant antigen challenge for diagnosis of pulmonary and extra-pulmonary tuberculosis

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Quantiferon TB gold (QFT-G) with recombinant antigen cocktail is well evaluated for diagnosis of pulmonary tuberculosis (PTB). However, diagnosis of extra-pulmonary tuberculosis (EPTB) is more difficult due to limitations of conventional techniques. This study compares recombinant antigens based QFT-G and low cost PPD based interferon test for the diagnosis of PTB and EPTB. IFNγ release, with recombinant antigens and PPD, was assayed by ELISA from 140 cases of EPTB, 100 cases of PTB along with acid fast bacillus (AFB) detection, AFB culture on LJ and MGIT BACTEC. Sensitivity and specificity for QFT-G recombinant antigens was 84.29% and 96%, while for PPD based interferon was 70% and 84% for EPTB group. The sensitivity was far superior to AFB smear and culture for both the antigens. Nine samples were identified as non-tubercular mycobacteria (NTM) in the EPTB group and all were negative for QFT-G, but six of them were positive for PPD based test. Results of the study show that QFT-G using recombinant antigen is sensitive and specific for both PTB and EPTB diagnosis. The PPD based test is economic and offers comparable performance for PTB and EPTB diagnosis and also useful for diagnosis of NTM.

Keywords: CFP-10, ESAT-6, Extra-pulmonary tuberculosis, IFNγ, Non-tubercular mycobacteria, PPD, TB

One-third of the world’s population is infected with Mycobacterium tuberculosis1, and tuberculosis (TB) remains a major worldwide cause of morbidity and mortality. It is estimated that extra-pulmonary tuberculosis (EPTB) constitutes 20% of all cases of tuberculosis in immunocompetent patients and accounts for more than 50% of cases in HIV positive individuals2-10.

Clinical presentation together with radiological and laboratory findings make the diagnosis of pulmonary tuberculosis (PTB) easier. However, the diagnosis of EPTB is usually more challenging as radiographic analysis is often not conclusive, the site of infection relatively inaccessible and the site may be fewer bacilli can cause considerably greater harm because of the types of organs infected. Invasive procedures are therefore often needed for confirmation of tentative diagnosis11.

Detection of cell-mediated immunity by tuberculin skin test (TST) has for nearly a century been used as a surrogate marker for infection with Mycobacterium tuberculosis, both as a diagnostic aid and in epidemiological studies. The main drawback with the clinical use of TST is lack of specificity due to cross-reactivity with other mycobacteria such as Mycobacterium bovis12,13. High endemicity and mass BCG vaccination are the other limitations.

A simple diagnostic test based on whole blood was used in an attempt to overcome some of the limitations of TST14-18. The Quantiferon TB gold in tube test (Cellestis, Australia) measures interferon gamma (IFNγ) production after in vitro stimulation of whole blood cells with Mycobacterium tuberculosis specific antigen cocktail mixture containing early secreted antigenic target 6 (ESAT-6), culture filtrate protein 10 (CFP-10) and TB7.7 (p4). ESAT-6 and CFP-10 antigens are encoded by genes located within the region of difference 1 (RD1) segment of the M. tuberculosis genome and are more specific because they are not shared with any of the BCG vaccine strains and most of the non-tuberculous mycobacteria (NTM). TB7.7 (Rv2654) is termed as peptide-4 which is specific for M. tuberculosis complex. Several studies have documented high sensitivity and specificity for detection of active PTB infection14-18, but the data is scanty for the diagnosis of EPTB19, 20. The aim of the present study was to evaluate the response of lymphocytes from EPTB and PTB cases to M. tuberculosis specific antigens cocktail mixture (ESAT-6, CFP-10, TB7.7) the tube using the
Quantiferon TB test and its comparison to PPD antigen challenge. The data was analyzed in the background of AFB smear and culture findings for the cases.

**Materials and Methods**

**Specimens for study**

Cases of EPTB (140) referred to Choithram Hospital and Research Center, (CHRC), Indore, India were included in the study. Males (98) and females (42) between the age group of 18-50 years. Four groups were involved in the study—healthy volunteers, EPTB cases, bacteriologically confirmed PTB cases and non tubercular lung infection cases (Table 1). The study, conducted from January 2007 to July 2008, was approved by the Research and Ethical Committee of CHRC, Indore, India. The Ethical Committee has guidelines based on Helsinki declaration.

**Healthy volunteers group**—Healthy voluntary blood donors from Indore city and the adjoining area who came for blood donation at the blood bank of CHRC were included as normal population. Volunteers (50) were in the age group of 22-40 years and neither had no history of any notable infection in the past 2 years, nor had symptomatic tuberculosis in the lifetime.

**PTB group**—100 cases of PTB were included in the study. Criteria for inclusion were—active PTB infection, based on clinical, radiological and sputum AFB smear positivity. All the cases had cough (>3 week duration) with expectoration, weight loss, fever, tiredness, loss of appetite, breathlessness, and chest pain. Chest X-rays showed a classical pattern of upper lobe infiltrate/bilateral infiltrate/cavitation with or without pulmonary fibrosis and shrinkage.

**Non-tubercular lung group**—Another control group of 50 cases had lung infection wherein tuberculosis was ruled out on the basis of AFB smear negativity in three consecutive samples and subsequently AFB culture negativity. Their diagnosis is mentioned in Table 1.

**EPTB group**—149 cases of EPTB were included in the study. Diagnosis of EPTB was made on the basis of clinical manifestations consistent with TB and positivity for one or more of the following parameters—AFB microscopy, AFB culture, histological or biopsy findings and raised ADA, lymphocytes and proteins and response to anti-TB drugs.

**Specimen collection and processing**

Specimens were decontaminated and concentrated using NaOH-NALC method. Centrifuged deposit with about 2 mL supernatant mixed by vortexing formed the concentrate. An aliquot of 0.5 mL concentrate was inoculated over Lowenstein Jensen (LJ) slant and MGIT BACTEC tube. The LJ slant were incubated at 37°C for 4-8 weeks and observed weekly. The MGIT tube was observed on alternate days using micro MGIT fluorescent reader (BD BBL, USA). Another aliquot of 0.5 mL concentrate was recentrifuged in microtubes at 6000 rpm for 5 min and the deposit was used for AFB smear. The smear stained with Auramin “O” (Hi-Media, India) was seen under fluorescent microscope. Identification of non-tubercular mycobacteria (NTM) was based on biochemical tests [niacin, catalase, para-nitro benzoic acid (PNB)]. The blood samples were collected for T cell Interferon gamma assay.

**T cell interferon-γ assay**

**Activation of T cells in tuberculosis cases**—Aliquots (1mL) of heparanized whole blood were incubated with (i) recombinant antigen [ESAT-6, CFP-10, TB7.7(p4)], (ii) PPD (10 TU-PPD, 2μg/mL) (Span Diagnostics, India) and (iii) nil control. Following 16 to 24 hr incubation plasma was removed and the amount of IFN-gamma was quantified by ELISA.
Measurement of IFN-gamma levels—Interferon gamma levels were measured by enzyme linked immunosorbent assay (ELISA) method using Quantiferon TB Gold kit as per the protocol suggested by the manufacturer. The conjugate (anti-human IFNγ HRP) dilutions were prepared with diluent (bovine casein, normal mouse serum) and 50 μL were added to the wells coated with monoclonal mouse anti-human IFNγ. Test plasma (50 μL) and standards were added to the wells and incubated for 120 min at room temperature. Wells were washed for six times. Enzyme substrate solution (tetra-methly-benzidine) (100 μL) was added to the wells and incubated for 30 min. at room temperature. Stop solution (H2SO4) (50 μL) was added and OD measured at 450 nm with 620 nm reference filter. Standard curve was constructed as per the manufacturers instructions to quantify the IFNγ concentration (IU/mL) for each of the test plasma samples. Cutoff value for a positive response was set as 0.35 IU/mL for recombinant antigens (ESAT-6, CFP-10 and TB7.7) as per the manufacturer instructions. The cut of 0.5 IU/mL for PPD was obtained by adding mean ± 2 SD values from healthy volunteers group.

Statistical analysis—Data was analysed using diagnostic test 2 × 2 contingency tables to calculate sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and likelihood ratios (LR) using StatsDirect statistical software version 2.7.2, UK. Differences between various diagnostic methods were measured using McNemar X² test (P values <0.05 were considered significant).

Results
Out of the 140 cases of EPTB, AFB staining was positive in only 42 cases whereas 54 samples were found culture positive among the EPTB Group. In the EPTB group 9 cases were found to be NTM based on biochemical identification of the isolates. All 9 NTM isolates were rapid growers—*M. fortuitum* (8) and *M. chelonae* (1). Positivity for various tests and NTM isolation among EPTB cases are shown in Table 2.

In PTB group among the 4 parameters the highest sensitivity was recorded for T-cell IFNγ (96%) using recombinant antigens followed by, T-cell IFNγ (82%) using PPD, culture by LJ and MGIT-BACTEC (84%) and AFB by fluorescent microscopy (74%). Sensitivity, specificity, PPV, NPV and likelihood ratio values for different tests for samples from PTB, healthy volunteers and non-TB lung infection samples are shown in Table 3.

Among the 4 parameters in EPTB group the highest sensitivity was recorded for T-cell IFNγ (84.29%) using recombinant antigens (ESAT-6, CFP-10 and TB7.7). In the EPTB group T-cell IFNγ using recombinant antigens (ESAT-6, CFP-10 and TB7.7) had high sensitivity than AFB smear, culture by LJ media and MGIT-BACTEC (95% CI, \( P<0.0001 \), McNemar Test). Similarly T-cell IFNγ using PPD antigen had high sensitivity than AFB smear, culture by LJ media and MGIT-BACTEC (95% CI, \( P<0.0001 \), McNemar Test). Sensitivity, specificities and likelihood ratio values were high for T-cell IFNγ using recombinant antigens than for PPD but the differences by two-sided McNemar test were not significant (95% CI, \( P=0.31 \)). Data for both EPTB group, healthy volunteers and non-TB lung infection group with their sensitivity, specificity, NPV, PPV and likelihood ratio for different parameters are given in Table 4.

Among the 9 cases infected with NTM in the EPTB group, 6 were found positive by PPD–T-cell IFNγ assay, whereas all 9 NTM were found negative by recombinant T-cell IFNγ assay.

### Table 2—Positivity for various parameters and NTM isolation among EPTB cases

<table>
<thead>
<tr>
<th>EPTB Category</th>
<th>AFB smear positive case</th>
<th>Culture by LJ and MGIT-BACTEC positive cases</th>
<th>No. of NTM isolated</th>
<th>T-Cell IFNγ using combined antigens (ESAT-6, CFP-10 and TB7.7) positive cases</th>
<th>T-Cell IFNγ using PPD antigen positive cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB-lymphadenitis (55)</td>
<td>22</td>
<td>24</td>
<td>4</td>
<td>50</td>
<td>44</td>
</tr>
<tr>
<td>TB pleuritis (34)</td>
<td>9</td>
<td>11</td>
<td>1</td>
<td>30</td>
<td>25</td>
</tr>
<tr>
<td>Bone TB (20)</td>
<td>6</td>
<td>8</td>
<td>1</td>
<td>17</td>
<td>14</td>
</tr>
<tr>
<td>Genitourinary TB (12)</td>
<td>3</td>
<td>5</td>
<td>2</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Abdominal TB (10)</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>TB meningitis (5)</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Milliary TB (3)</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Ocular TB (1)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total No. of EPTB cases (140)</td>
<td>42</td>
<td>54</td>
<td>9</td>
<td>118</td>
<td>98</td>
</tr>
</tbody>
</table>

Number of volunteers in each category is given in parenthesis.
Discussion

Rapid diagnosis and treatment of tuberculosis is crucial to reduce morbidity and mortality from untreated TB. EPTB accounts for approximately one third of all cases of tuberculosis. Conventional methods have limitations for the diagnosis of EPTB. The smear for AFB was negative for 98 of the 140 samples from EPTB cases at our end and pointed out the low diagnostic yield for the parameter. The culture test requires 50 to 1,000 bacilli/mL in the sample. Yield of AFB cultures by LJ and MGIT-BACTEC was 38.57%. Prescription of ofloxacin or ciprofloxacin as empirical antimicrobial therapy for respiratory tract and other infections in our area appeared to be one of the limiting factors for culture.

Tuberculin skin test (TST) has been used for almost a century to support the diagnosis of TB infection. However, TST has limitations such as low specificity among BCG vaccinated individuals or sensitized with NTM. A more specific diagnostic assay should preferably be based on antigens that are present exclusively in MTB but not in BCG. Because of advances in molecular biology and genomics, a major breakthrough in recent years has been the development of in vitro assays that measure T-cell release of interferon γ in response to stimulation with antigens such as ESAT-6 and CFP-10. ESAT-6 and CFP-10 antigens are encoded by genes located within the region of difference 1 (RD1) segment of the M. tuberculosis genome and are more specific because they are not shared with any of the BCG vaccine strains and from most of the NTM species. TB 7.7 (peptide 4) is added to kit by manufacturer as it is specific to M. tuberculosis complex and will detect some sites which are missed by ESAT-6 and CFP-10. These antigens are considered to be potential candidates for immunodiagnosis of tuberculosis.

There have been several reports to support the utility of T-cell release of IFNγ for the diagnosis of acute PTB. However, the reports over the evaluation of TB Quantiferon test for the diagnosis of EPTB have been fever. In the present series the PTB group was included for comparing the diagnostic utility of the test for EPTB cases. Sensitivity for T-cell interferon test using specific antigen combination (ESAT-6, CFP-10 and TB 7.7) was 84.29% for EPTB population while 96% for PTB population. Specificity for both groups

Table 3—Sensitivity, specificity, PPV, NPV and LR values for different parameters for samples from PTB, healthy volunteer group and non-tuberculous lung infection samples

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Healthy volunteer group (50)</th>
<th>Positive cases in non-TB group (50)</th>
<th>Positive cases in PTB group (100)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>LR</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB smear</td>
<td>Nil</td>
<td>Nil</td>
<td>74</td>
<td>74</td>
<td>100</td>
<td>100</td>
<td>65.78</td>
<td>10.35-inf</td>
</tr>
<tr>
<td>Culture by LJ and MGIT-BACTEC</td>
<td>Nil</td>
<td>Nil</td>
<td>84</td>
<td>84</td>
<td>100</td>
<td>100</td>
<td>75.75</td>
<td>11.7-inf</td>
</tr>
<tr>
<td>T-cell- IFNγ (ESAT-6, CFP-10 and TB 7.7)</td>
<td>2</td>
<td>2</td>
<td>96</td>
<td>96</td>
<td>96.15</td>
<td>97.95</td>
<td>95.59</td>
<td>24.93</td>
</tr>
<tr>
<td>T-cell- IFNγ (PPD)</td>
<td>8</td>
<td>8</td>
<td>82</td>
<td>82</td>
<td>84</td>
<td>91.11</td>
<td>73.52</td>
<td>5.12</td>
</tr>
</tbody>
</table>

PPV–positive predictive value; NPV–negative predictive value; LR–likelihood ratio
Number of volunteers in each category is given in parenthesis

Table 4—Sensitivity, specificity, PPV, NPV and LR values for different parameters in EPTB, healthy volunteer group and non-tuberculous lung infection samples

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Healthy volunteer group (50)</th>
<th>Positive cases in non-TB group (50)</th>
<th>Positive cases in EPTB group (140)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>LR</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB smear</td>
<td>Nil</td>
<td>Nil</td>
<td>42</td>
<td>30</td>
<td>100</td>
<td>100</td>
<td>33.78</td>
<td>4.18-inf</td>
</tr>
<tr>
<td>Culture by LJ and MGIT-BACTEC</td>
<td>Nil</td>
<td>Nil</td>
<td>54</td>
<td>38.57</td>
<td>100</td>
<td>100</td>
<td>36.6</td>
<td>5.38-inf</td>
</tr>
<tr>
<td>T-cell- IFNγ (ESAT-6, CFP-10 and TB 7.7)</td>
<td>2</td>
<td>2</td>
<td>118</td>
<td>84.29</td>
<td>96</td>
<td>98.33</td>
<td>68.57</td>
<td>21</td>
</tr>
<tr>
<td>T-cell- IFNγ (PPD)</td>
<td>8</td>
<td>8</td>
<td>98</td>
<td>70</td>
<td>84</td>
<td>92.45</td>
<td>50</td>
<td>4.37</td>
</tr>
</tbody>
</table>

PPV–positive predictive value; NPV–negative predictive value; LR–likelihood ratio
Number of volunteers in each category is given in parenthesis
was 96% and 96.15%, respectively. Thus, the diagnostic efficiency of the test appears equal for both PTB and EPTB cases. Similar observation was reported by Nishimura et al.\textsuperscript{19} from Japan. Ravan et al.\textsuperscript{17} also reported greater sensitivity of the test for EPTB cases as compared to PTB cases. However, the population size included in the study was much smaller.

The notable observations in the present study was that sensitivity and specificity of the T-cell interferon release following PPD challenge appears lower than with specific combination antigen (ESAT-6, CFP-10, TB 7.7) challenge in Quantiferon test. However, the differences between both the challenges were not found to be statistically significant ($P=0.31$). The observation has immense financial bearing since the cost of locally available PPD antigen drastically reduced the reagent cost in comparison to imported Quantiferon kit (Rs 250 vis-a-vis Rs 1000). Brock et al.\textsuperscript{16} reported similar sensitivity for PPD antigen and ESAT-6 or CFP-10 antigen. However, PPD antigen had shown positivity of 47\% among BCG vaccinated group with no active TB infection in Danish population. In present study, only 8 of the 50 (16\%) cases in healthy volunteers had shown positivity for PPD antigen based interferon rise. Eight of the 50 (16\%) non-TB lung infection cases had shown positive for PPD antigen based interferon rise. It was not possible to ascertain whether these non-TB cases had latent TB infection.

The most striking observation was the elevation of T-cell interferon following PPD challenge among 6 of 9 cases infected with NTM, while none of the NTM cases showed rise in interferon following ESAT-6, CFP-10 and TB 7.7 antigen challenge. Thus, the Quantifieron test confirmed the specificity of the antigen combination for detecting exclusively M. tuberculosis, but will be missing interferon caused by NTM. Our earlier study has pointed out prevalence of NTM among EPTB cases to be 38\%.\textsuperscript{25}

Based on present observation, it is expressed that the PPD based challenge to mononuclear blood cells and measurement of interferon can be a reasonable test for the diagnosis of EPTB caused by both M. tuberculosis and NTM and can prove much more economical compared to Quantiferon TB Gold test. Multicentric trials for the PPD based interferon test are suggested in developing countries before its final recommendations. Diagnostic utility of the test also needs to be evaluated among HIV or immunocompromised groups.

**Acknowledgement**

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**References**

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