A prospective study of inhouse developed SEVA TB ELISA using cocktail of antigens and their immunoglobulins in the diagnosis of tuberculosis suspected patients in a tertiary care hospital located in rural area.

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Abstract

An inhouse developed SEVA TB ELISA using cocktail of Mycobacterium Excretory-Secretory antigens (ES-31, ES-43 & EST-6) for antibody detection and their affinity purified antibodies for antigen detection was prospectively evaluated during January-August 2008 in TB suspected patients in tertiary hospital located in rural area. Antibody or antigen positivity is considered as ELISA positive. 238 suspected cases of pulmonary and extra-pulmonary tuberculosis were screened. ELISA test showed 100% correlation (12 patients) with AFB positivity. ELISA test also showed 100% correlation (42 patients) with anti-tubercular therapy (ATT) treated cases. 110 out of 238 cases showed negative ELISA test and these cases were also not recommended ATT. 86 out of 238 cases were neither AFB positive nor ATT treated, but these cases were ELISA positive. These cases may possibly have dormant infection and need further follow up for showing disease, if any, in due course of time and its usefulness for confirmation of clinical suspicion. This study has shown usefulness of SEVA TB ELISA in diagnosis of suspected patients of Pulmonary and Extrapulmonary tuberculosis

Key Words: Excretory secretory antigen, cocktail antigen, affinity purified antibody ELISA, Pulmonary tuberculosis, Extra-pulmonary tuberculosis

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Introduction

Tuberculosis (TB) is one of the curable infectious diseases, remain the leading disaster in front of the world inspite of availability of treatment. Based on surveillance and survey data, WHO estimated that 9.2 million new cases of TB occurred in 2006, including 4.1 million new smear-positive cases. India, China, Indonesia, South Africa and Nigeria rank first to fifth in terms of incident cases. Among these new cases around 7.7% were HIV-positive [1]. Latent tuberculosis is another burden on TB control programme. Currently, about 2 billion people worldwide are believed to have latent TB. Of those, 8 million to 9 million will develop TB each year and 1.6 million of them will die [2]. The successful control of TB depends on early detection of cases and effective treatment. [3,4].

Diagnosis of TB using acid-fast staining of sputum smear or standard culture is considered as the ‘gold standard’, however the sensitivity of detection has been shown to be only 40-75% and hence clinicians have to treat the suspected patient based on clinical judgment [5-7]. Empirical treatment increases public health expenditure and the risk of drug side-effects that may be fatal [8].

The advent of the nucleic acid amplification test seems to help in the diagnosis of TB, however faced with problems of contamination and sensitivity [9]. Further, this technique is expensive, time consuming and requires special expertise and equipment. Hence, there is an urgent need for improved and easily accessible diagnostic methods that are simple, rapid, inexpensive, reliable and suitable for use in the developing world.

Serological tests are relatively simple to use, inexpensive and easy to interprete. As they do not depend on the site of infection, serological tests may be better for detecting extra-pulmonary TB and for children or uncooperative patients, among whom collection of clinical samples may be difficult. Many groups have explored culture filtrate
antigens of *Mycobacterium tuberculosis* (M.tb.) in ELISA for serodiagnosis of TB [10,11]. In earlier studies from our laboratory, we have shown diagnostic usefulness of M. tb. excretory secretory (ES) antigens ES-31, ES-41 and ES-43 in antibody detection by sandwich penicillinase ELISA (Pen-ELISA) [12-15]. Antigen EST-6 containing 38 and 41kDa proteins was also explored for antibody detection by Pen-ELISA [16]. A cocktail of ES-31, ES-41 and ES-43 antigens had shown improved sensitivity of Pen-ELISA compared to single ES-31 antigen in antibody detection in pulmonary TB (PTB) [17]. Further, a cocktail of affinity purified antibodies against ES-31, ES-43 and EST-6 antigens was explored for circulating free and IC antigen detection in TB by Indirect ELISA [18]. Hence we made a prospective study to find the usefulness of this inhouse developed SEVA TB ELISA using cocktail of antigens (ES-31, ES-43 and EST-6 antigens) for antibody detection and their immunoglobulins for antigen detection in TB suspected cases in tertiary hospital located in a rural area.

Materials and Methods

Patients and clinical case selection

The present prospective study was carried out for the duration of 8 months, between January 2008 and August 2008. Patients attending Kasturba Hospital of Mahatma Gandhi Institute of Medical Sciences (Tertiary hospital), Sevagram, located in rural India, who were suspected of having tuberculosis on clinical examinations and other laboratory investigations participated in the present study. Sputum microscopy and culture test for AFB are routinely done by Microbiology Department of this Institute. Sera samples were received from 238 patients, out of which 154 were suspected for having PTB while 84 for EPTB. Patients and clinical case selection

Clinical history, physical examination, baseline laboratory investigations [hemogram, tuberculin skin test, chest skigram, urinanalysis], microbiological (AFB smear and culture), histological and cytological (FNAC) investigations, ultrasound or computerized tomographic scanning (CT) depending upon the location of the infection in patients, clinicians high degree of suspicion of TB depending on clinical sign and symptoms and response to ATT or ATT treated were considered as the basis for selection of cases.

Isolation of M. tb. ES-31, ES-43 and EST-6 antigens and their antibodies

ES-31 antigen was isolated from M.tb. H_{37}Ra ES antigen by affinity chromatography using anti ES-31 antibody coupled Sepharose-4B column (Pharmacia Biotechnology AB, Uppsala, Sweden) [19]. Briefly, Cyanogen bromide-activated Sepharose 4B beads were coupled with purified anti ES-31 antibody. DSS antigen was passed through column and ES-31 antigen was eluted by glycine HCl buffer (0.01 mol/ L, pH2.5) and collected in Tris-HCl buffer (0.01M, pH8.6). Similarly ES-43 and EST-6 were isolated by affinity chromatography using anti ES-43 or anti-EST-6 antibody coupled Sepharose-4B column. Cocktail antigen (ES-31, ES-43 and EST-6) was prepared by mixing the individual antigen in equal proportion.

M. tb. H_{37}Ra detergent soluble sonicate (DSS) antigen, was prepared from M.tb. H_{37}Ra bacilli. Briefly, bacilli were 5% phenol inactivated in 0.5M phosphate buffer (PBS, pH7.2) and incubated with sodium dodecyl sulphate (SDS) extraction buffer. The supernatant as dialysed against 0.01M PBS, pH 7.2 and used as an antigen source [18]. Anti-DSS IgG antibodies were raised in goat by immunizing intramuscularly with 500 µg protein/mL. DSS antigen with 1 ml Freund’s incomplete adjuvant on days 0, 20, 33 and 45. Immune sera were collected on days 32, 44, 57, 60 and thereafter fortnightly and anti-SDS IgG was isolated by 33% saturation with ammonium sulphate under ice, followed by diethyl aminoethyl-cellulose ion exchange column chromatography as described earlier [18]. Anti-ES-31, anti-ES-43 and anti-EST-6 antibodies were isolated from anti-DSS IgG by affinity chromatography using ES-31, ES-43 or EST-6 antigen coupled Sepharose-4B column [19]. Anti-cocktail antibody (anti-ES-31, anti-ES-43 and anti-EST-6) was prepared by mixing individual antibody in equal proportion.

Penicillinase ELISA

Detection of Antibody

Indirect Penicillinase ELISA was performed for detection of antibody using cocktail antigen. Optimum individual antigen concentration of 1ng per stick was used in this assay. In brief, 5µL of optimally diluted antigen (0.6µg
protein/ml) was applied to Cellulose acetate membrane squares fixed to a plastic strip and used along with optimally diluted human serum (1:600). Serum incubation was done at 37°C for 1 hr. After washings, strips were incubated with anti-human IgG Penicilllinase conjugate (1:1000) at 37°C for 30 minutes. Finally, strips were incubated with blue color starch-iodine-penicillin ‘V’ substrate at least 5 min earlier than the negative control was considered as positive.

Detection of circulating free and immune complexed antigen

Sandwich penicilllinase ELISA was performed for detection of circulating free and immune complexed antigen using cocktail of affinity-purified antibodies to ES-31, ES-43 and EST-6 antigens. Optimum individual antibody concentration of 1µg protein per stick was used in the assay. In brief, 5µL of optimally diluted antibody (0.6 mg protein/mL) was applied to cellulose acetate membrane squares fixed to a plastic strip and used along with optimally diluted human serum (1:300). Serum incubation was done at 37°C for 1 h. After washings, strips were incubated with goat anti-cocktail IgG penicilllinase conjugate (1:1000) at 37°C for 1 hr. Finally, strips were incubated with blue color starch-iodine-penicillin ‘V’ substrate at least 5 min earlier than the negative control was considered as positive.

For detecting IC antigen, serum samples were pretreated with Glycine-HCl buffer (0.1mol/L) followed by heating at 65°C for 15 minutes.

Results

During the 8 month (January to August 2008) follow up study, 238 TB suspected patients were screened for the presence of antibody, circulating free and immune complexed antigens in the sera by penicilllinase ELISA (Table 1). Sera showing presence of antibody or antigen or IC-antigen, are considered as ELISA positive. Out of 238 suspected TB cases screened, only 12 cases were AFB positive. All of these 12 cases were positive for ELISA test, thus ELISA test showed 100% correlation with AFB positivity (Table 2). All these 12 AFB positive cases were ATT treated. Out of 238 screened cases, a total of 42 cases were ATT treated on the basis of sputum/culture positivity or other clinical investigations and all of these 42 cases were ELISA positive (Table 2). Out of 238 screened cases 110 cases showed negative ELISA test and these cases were not recommended ATT. Out of 238 cases, 86 cases which were neither AFB positive nor ATT treated, but these cases were ELISA positive.

<table>
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<th>Group</th>
<th>Total No. Screened</th>
<th>AFB positive*, ELISA positive and ATT treated</th>
<th>AFB negative**, ELISA positive and ATT treated</th>
<th>AFB negative, ELISA negative and no ATT</th>
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<tr>
<td>PTB†</td>
<td>154</td>
<td>11</td>
<td>14</td>
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<td>54</td>
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<td>84</td>
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<td>2</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
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<td>0</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
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<td>2</td>
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<tr>
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<td>4</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>TB lymphadenitis</td>
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<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
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*Pulmonary TB, ‡: extra-pulmonary TB, *Acid fast bacilli positive
**Acid fast bacilli negative.

Table 2: Analysis of ELISA, AFB positivity and ATT treated in suspected PTB and EPTB patients*
### Discussion

In current clinical practice diagnosis of TB depends on clinical information and various laboratory tests including smear, culture and FNAC for AFB, chest radiology, computer tomography, ultrasonography and response to ATT. Although AFB smear microscopy and culture are valuable for confirmative diagnosis of tuberculosis, low bacillary load, extent of TB disease extrapulmonary site of the infection do make the AFB test undependable. Further, It is very difficult to obtain sputum specimen in children. Based on suspected clinical symptoms and time consuming culture methods there is always a delay in diagnosis. Therefore immunodiagnosis seems to be ideally suited as a diagnostic method. Serodiagnostic tests like ELISA can show promise because of their ease of performance in field laboratories and cost-effectiveness.

Over a period of decade, our laboratory reported usefulness of various mycobacterial excretory secretory antigens in the diagnosis of TB by ELISA. Cocktail of different antigens has shown to be more useful than single antigen assay [20]. Assay for detection of free circulating cocktail antigen (ES-31, ES-43 and EST-6) was found useful for PTB cases with 91% sensitivity and 97% specificity for sputum positive AFB positive cases [18]. In EPTB and latent TB cases the diagnosis is difficult due to the insensitive sputum and culture methods. This cocktail antigen assay showed 70% sensitivity and 100% specificity in EPTB cases [20].

In the present study the inhouse developed Pen-ELISA test was evaluated prospectively for the diagnosis of PTB and EPTB cases attending Kasturba Hospital, a tertiary hospital located in rural area. Assay showed 100% correlation with AFB positivity as well as with ATT treatment. A total of 42 cases of PTB and EPTB were administered ATT. Out of the 12 AFB positive cases, 11 cases were of PTB and one case was of EPTB. Based on other clinical investigations and ELISA positivity, 31 out of 84 EPTB patients were treated for TB showing the usefulness of ELISA test in extrapulmonary tuberculosis. 110 out of 238 cases (46.21%) cases were suspected of having tuberculosis were negative for TB by Pen-ELISA and were not treated for TB. 86 out of 238 cases (36.13%) were neither AFB positive nor ATT treated, but these cases showed ELISA positivity. The probable reason of this finding might be the low sensitivity of sputum microscopy and mycobacterial culture [8]. At concentrations below 1000 organisms per ml, the chance of observing bacilli in a smear becomes less than 10% while detection limit of mycobacterial culture is around 100 organisms per ml [21]. Even the smear examinations has proven less sensitive in HIV-TB coinfection due to absence of cavity formation secondary to poor immune status, resulting in lower bacillary count [22]. Expanded case definition and response to treatment (RTT) have been advocated to enhance the diagnosis of PTB and EPTB in HIV infected adults in resource limited settings [23, 24]. The other probable reason might be that these cases may be harboring latent TB infection. These suspected TB cases need further followup for showing clinical disease if any in due course of time.

Thus, this inhouse developed simple and less expensive SEVA TB ELISA has shown to be user friendly and may be used as an adjunct test with smear microscopy or culture techniques for routine screening of patients suspected of pulmonary and extrapulmonary tuberculosis. AFB negative but ELISA positive patients may be followed and can promptly be administered treatment with the development of the disease.

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### References


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