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## Document heading

# Prospective study of SEVA TB peroxidase assay for cocktail antigen and antibody in the diagnosis of Tuberculosis in suspected patients attending a tertiary care hospital located in rural area

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## ABSTRACT

**Objective:** To evaluate inhouse developed SEVA TB peroxidase enzyme immunoassay using cocktail of mycobacterial excretory-secretory antigens (ES-31, ES-43 & EST-6) for antibody detection and their affinity purified antibodies for antigen detection in tuberculosis suspected patients. **Methods:** Inhouse developed SEVA TB peroxidase enzyme immunoassay was evaluated prospectively in 73 suspected pulmonary and 46 extra-pulmonary tuberculosis patients during November 2008–March 2009 in a tertiary hospital located in rural area. **Results:** Assay on prospective analysis showed 100% correlation of pulmonary tuberculosis (PTB) and extra-pulmonary tuberculosis (EPTB) acid fast bacilli positivity and antitubercular treatment in 11 cases. Thirty nine PTB and 12 EPTB cases showed negative for ELISA test and were also not given antitubercular therapy. However 30 PTB and 27 EPTB cases showing ELISA positivity were neither acid fast bacilli positive nor antitubercular therapy treated. These cases may possibly have dormant infection and need further diagnosis. In EPTB cases ELISA was observed to be more useful than AFB smear test. **Conclusions:** This inhouse developed user-friendly peroxidase ELISA can be used as an adjunct test of smear microscopy or culture techniques for routine screening of patients suspected of PTB or EPTB.

## 1. Introduction

Tuberculosis (TB) is an airborne infectious disease that is preventable and curable. But it still remains as a major predicament to intercontinental health systems. As per global tuberculosis control – a short update to the 2009 report[1]; World Health Organization (WHO) estimated 9.4 million incident cases of TB globally in 2008, most of which occurred in Asia (55%) and Africa (30%). But the number of notified cases of TB in 2008 was only 5.7 million, equivalent to 55–67% of all incident cases. India and China alone account for an estimated 35% of TB cases worldwide. Among these new cases around 15% were human immunodeficiency virus (HIV) positive. Despite widespread introduction of the Directly Observed Therapy Short-course (DOTS) program for TB, incidence of the disease continues to rise, exacerbated by high levels of HIV[1]. Latent TB is another burden on TB control programme. The control of TB depends on early detection and effective treatment[2, 3]. Diagnosis of TB using acid-fast staining of sputum smear and standard culture is

considered as the gold standard, but it miss a large number of cases because of handling problem and lack of good sensitivity (sensitivity 40–75%)[4]. Clinicians either have to treat based on clinical judgment or wait for culture results, which may take up to 6 weeks[5, 6]. Empirical treatment increases public health expenditure and the risk of drug side-effects that may be fatal[7]. Nucleic acid amplification test seems to help in the diagnosis of TB[8]. However, this technique is expensive, requires special expertise and equipments. Excessive delays in correct TB diagnosis and treatment remain a weakness of the control strategy. Such delays are critical as infectious individuals provide more opportunities for transmission of the disease. 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 simple to use and inexpensive. As they do not depend on the site of infection, serological tests may be better for detecting EPTB and for children or uncooperative patients, among whom collection of clinical samples may be difficult. In earlier studies from our laboratory, we have shown diagnostic usefulness of *Mycobacterium tuberculosis* (*M. tuberculosis*), excretory secretory (ES) antigens ES-31, ES-41 and ES-43 in antibody detection by penicillinase ELISA (Pen-ELISA)[9–12]. Antigen EST-6 containing 38 kDa and 41 kDa proteins was also explored for antibody detection by Pen-ELISA[13]. A cocktail of ES-31, ES-41 and ES-43 antigens

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had shown improved sensitivity of Pen-ELISA compared to single ES-31 antigen in antibody detection in PTB [14]. Further, a cocktail of affinity purified antibodies against ES-31, ES-43 and EST-6 antigens was explored for circulating free and immune complexed (IC) antigen detection in TB by sandwich ELISA [15]. The usefulness of inhouse developed penicillinase ELISA using cocktail of antigens (ES-31, ES-43 and EST-6 antigens) and their immunoglobulins was also shown in a prospective study which was carried out at a tertiary care hospital located in rural area [16]. All these assays were based on penicillinase ELISA, which is sensitive but semi-quantitative and subjective assay. Microtitre plate peroxidase sandwich ELISA for detection and quantitation of circulating free and IC ES-31 antigen was also shown to be useful in diagnosis of PTB cases [17]. Mass screening suitable, user friendly SEVA TB peroxidase enzyme immunoassay was developed using cocktail of antigens (ES-31, ES-43 and EST-6 antigens) and their immunoglobulins for the detection of TB cases [18]. In the present study, this inhouse developed SEVA TB peroxidase enzyme immunoassay was analyzed prospectively to find its usefulness in suspected TB cases in tertiary hospital located in a rural area.

## 2. Materials and methods

### 2.1. Patients and clinical case selection

The present prospective study was carried out during November 2008 ~ March 2009. Patients attending Kasturba Hospital of Mahatma Gandhi Institute of Medical Sciences (Tertiary hospital), Sevagram, located in rural India, who were suspected of having TB on clinical examinations and other laboratory investigations participated in the present study. Sera samples were received from 119 patients, out of which 73 were suspected for having PTB while 46 for EPTB. EPTB included bone and joint TB (29), tuberculous lymphadenopathy (7), genitourinary TB (4), abdominal TB (3), ocular TB (1) and tuberculous meningitis (1). Data of their acid fast bacilli (AFB) status and antituberculosis therapy (ATT) was collected.

Sera samples were stored at our Center's sera bank in 0.5 mL aliquots at -20 °C with 0.1% sodium azide until use. All cases included in this study had history of BCG vaccination. Each sera sample was assayed for presence of cocktail antibody, circulating or IC-cocktail antigen as described above.

Clinical history, physical examination, baseline laboratory investigations (hemogram, tuberculin skin test, chest skigram, urinalysis), 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 treatment were considered as the basis for selection of cases.

The study was done prospectively in blinded manner in which clinical diagnosis was not available to the laboratory personnel prior to the assay. In the present study, each serum sample had been assayed in duplicate.

### 2.2. Isolation of *M. tuberculosis*, ES-31, ES-43 and EST-6 antigens and their antibodies

ES-31 antigen was isolated from *M. tuberculosis* H<sub>37</sub>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. detergent soluble sonicate (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. tuberculosis* H<sub>37</sub>Ra DSS antigen, was prepared from *M. tuberculosis* H<sub>37</sub>Ra bacilli. Briefly, bacilli were 5% phenol inactivated in 0.5 M phosphate buffer (PBS, pH7.2) and incubated with sodium dodecyl sulphate (SDS) extraction buffer. The supernatant was dialysed against 0.01M PBS, pH 7.2 and used as an antigen source [15]. 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 day 0, 20, 33 and 45. Immune sera were collected on day 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 [20]. 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 [20]. Anti-cocktail antibody (anti-ES-31, anti-ES-43 and anti-EST-6) was prepared by mixing individual antibody in equal proportion [19].

### 2.3. SEVA TB peroxidase enzyme immunoassay for cocktail antibody, circulating free and IC-antigen

Indirect peroxidase ELISA was performed for detection of antibody using cocktail antigen (ES-31, ES-43 and EST-6). The wells of ELISA plates (NUNC) were sensitized with optimally diluted concentration of cocktail antigen 3 µg / 100 µL/well in 0.06 M carbonate buffer pH9.6 overnight at 4 °C, followed by blocking with BSA 1% for 2 hours at 37 °C. Plate was washed twice with PBS containing 0.05% Tween 20 (PBS/T) followed by addition of sera (dilution 1:50) in PBS/T for 1 hour at 37 °C, then washed 3 times. After that the wells were exposed to 1:1000 diluted anti-human IgG peroxidase conjugate for 1 hour at 37 °C. The wells were washed five times with PBS/T with 1 minute interval. The color was developed using TMB substrate (20X concentration) followed by using 50 µL stop solution (2N H<sub>2</sub>SO<sub>4</sub>). Then mean optical density at 450 nm was read with ELISA reader.

The detection of circulating cocktail antigen (ES-31, ES-43 and EST-6) using affinity purified anti-cocktail antibody (anti-ES-31, anti-ES-43 and anti-EST-6) was performed by sandwich plate peroxidase ELISA. The procedure was the same as that of indirect peroxidase ELISA mentioned above. The anti-cocktail antibody filled in the ELISA wells was 50 µg / 100 µL/well, and the wells were finally exposed to 1:1000 diluted goat anti-cocktail antibody IgG peroxidase conjugate.

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

## 3. Results

During the prospective study, 119 suspected TB patients were screened for the presence of antibody, circulating free and immune complexed cocktail antigens in the sera by peroxidase immunoassay (Table 1). Sera showing presence of

**Table 1**

Immunoscreening of suspected cases of tuberculosis attending tertiary care hospital during November 2008 to March 2009.

Group	Total No. screened	ATT Treated		No ATT	
		ELISA positive	ELISA negative	ELISA positive	ELISA negative
PTB	73	4	0	30	39
EPTB	46	7	0	27	12
Bone and Joint TB	29	6	0	17	6
Lymphnode TB	7	0	0	6	1
Genitourinary TB	4	0	0	2	2
Abdominal TB	3	0	0	1	2
Ocular TB	2	0	0	1	1
TB Meningitis	1	1	0	0	0

ELISA test positivity either for antibody or free antigen or immune-complexed antigen is considered ELISA positive.

antibody or antigen or IC-antigen, are considered as ELISA positive.

There were a total of 73 suspected PTB cases, of which 15 were screened for presence of AFB, but none was positive. Of the 46 suspected EPTB cases, 3 were screened for presence of AFB, only one was positive (It was also ELISA positive). Four of the PTB cases and 7 of the EPTB cases were administered with ATT on the basis of sputum/culture or other clinical investigations and all of these cases showed ELISA positivity. Thus, assay showed 100% correlation between acid fast bacilli positivity and antitubercular treatment. 39 PTB cases and 12 EPTB cases showed negative ELISA and these cases were not given ATT. 30 PTB and 27 EPTB cases were neither AFB positive nor ATT treated, but these cases showed ELISA positivity.

Of the 29 suspected cases of bone and joint TB cases screened, 23 cases were ELISA positive. However only 6 were administered with ATT, 3 of the 6 were ELISA positive as well as ATT treated but they were negative for AFB, histopathology and FNAC (Cytology) for tubercular pathology. In these cases, strong clinical suspicion was correlated with ELISA positivity. Out of 7 suspected lymphnode TB cases screened, 6 were ELISA positive, and 1 was also positive on FNAC (Cytology) for tubercular pathology, however none of the seven cases were administered with ATT. Two of the 4 genitourinary cases, 1 of the 3 abdominal, 1 of 2 ocular TB cases were ELISA positive but not ATT treated. One case of TB meningitis showed ELISA positivity and was ATT treated.

#### 4. Discussion

In current clinical practice, the diagnosis of TB depends on clinical findings and various laboratory tests. Although AFB smear microscopy and culture are valuable for confirmative diagnosis of TB; low bacillary load and extent of TB disease at extrapulmonary site of the infection do make the AFB test not reliable. Further, it is very difficult to obtain sputum specimen in children. Based on no proper history of BCG vaccination, evening rise of fever, sweating, loss of appetite etc and ruling out other causes children are suspected of having TB Meningitis. Therefore immunodiagnosis seems to be ideally suited as a diagnostic method. Serodiagnostic test like ELISA can show promise because of its ease of performance in field laboratories and cost-effectiveness. Over a decade, our laboratory reported usefulness of various mycobacterial excretory secretory antigens in the diagnosis of TB by penicillinase enzyme immunoassay. Assay for detection of free circulating cocktail antigen (ES-31, ES-43 and EST-6) by SEVA TB penicillinase ELISA was found useful for PTB cases with 91% sensitivity and 97% specificity for sputum positive AFB positive cases and also useful in

detection of EPTB cases[15, 21]. But, the penicillinase assay is subjective and semiquantitative. User friendly, quantitative microtitre plate peroxidase sandwich ELISA was explored by using affinity purified anti ES-31 antibody for detection of circulating antigen in TB sera[17]. SEVA TB Peroxidase enzyme immunoassay was shown 80% sensitivity and 90% specificity for detection of PTB cases and proven the usefulness of assay of cocktail antigen over assay for single antigen. This assay showed a sensitivity for detection of very low concentration of cocktail antigen (0.25  $\mu$ g/ mL cocktail antigen) [18].

In this tertiary hospital located in rural area, patients usually visit with chronic symptoms or progressive TB disease. Clinicians send samples for testing on clinical suspicion based on some clinical signs. In the present study, the inhouse developed SEVA TB peroxidase enzyme immunoassay was evaluated prospectively. Enzyme immunoassay showed 100% correlation with AFB positivity as well as with ATT treated cases of PTB and EPTB. Seven ATT treated EPTB (Bone and joint and TB Meningitis) cases showed ELISA positivity. This may be due to presence of tubercular foci at extrapulmonary sites. Thus in ATT treated AFB negative cases, ELISA positivity can be correlated with histopathological, FNAC or radiological findings and supports the clinical diagnosis. It is of interest that peroxidase assay could detect antigen in AFB negative but clinically diagnosed and ATT treated cases. In AFB negative patients; though bacillemia is low, antigen level is significant, possibly due to antigen released by death of bacilli or slow clearance of antigen in these patients. This needs further extensive study of AFB negative clinically suspected and ATT responding PTB & EPTB cases.

Thirty PTB and 27 EPTB cases 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[7]. At concentrations below 1 000 organisms per mL of sputum, the chance of observing bacilli in a smear becomes less than 10% while detection limit of mycobacterial culture is around 100 organisms per mL[22]. 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. 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 reason behind positivity of ELISA in these cases might have indicated presence of latent TB. Such cases need further follow-up for showing clinical disease if any in due course of time.

The diagnosis in AFB negative PTB and EPTB cases is difficult. Most of the EPTB cases are AFB negative as proper tissue sample or fluid is not available for AFB testing. Such cases may not show presence of AFB, but on histopathology

or FNAC, tubercular pathology can be seen. Usually the classical symptoms or signs suggestive of TB such as a productive cough for more than 2 weeks, which may be accompanied by other respiratory symptoms (shortness of breath, chest pains, haemoptysis) and/or constitutional symptoms (loss of appetite, weight loss, fever, night sweats and fatigue) are not present in EPTB cases [25]. Based on presentation of chronic fever, pain in joints, lesions etc and ruling out other causes patients are suspected of bone & joint TB. Instead of starting ATT in these AFB negative cases, often clinicians prescribe broad-spectrum antibiotics (excluding anti-TB drugs and fluoroquinolones and aminoglycosides) for 4 to 6 weeks. If patient does not respond then as per WHO guidelines, clinicians prescribe ATT considering the case as AFB negative but clinically suggestive [25]. Such delay frequently made on circumstantial evidence alone and hindrance in diagnosis might have life threatening consequences. In such AFB negative PTB and EPTB cases, serodiagnostic tests may act as supportive substantiation for starting of ATT. However extensive study and follow up on more suspected patients is needed.

Thus, this inhouse developed less expensive, user-friendly peroxidase ELISA may be used as adjunct test of smear microscopy or culture techniques for routine screening of patients suspected of PTB as well as EPTB cases.

### Conflict of interest statement

We declare that we have no conflict of interest.

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### References

[1] World health organization. *Global tuberculosis control – a short update to the 2009 report*. P-4–5. [online] Available from: [http://www.who.int/tb/publications/global\\_report/2009/update/tbu\\_9.pdf](http://www.who.int/tb/publications/global_report/2009/update/tbu_9.pdf) [Accessed on 11th April, 2009].

[2] Frieden TR, Sterling TR, Munsiff SS, Watt CJ, Dye C. Tuberculosis. *Lancet* 2003; **362**: 887–99.

[3] Grzybowski S, Barnett GD, Styblo K. Contacts of cases of active pulmonary tuberculosis. *Bull Int Union Tuberc* 1975; **50**: 90–106.

[4] Kim TC, Blackman RS, Heatwole KM, Kim T, Rochester DF. Acid-fast bacilli in sputum smears of patients with pulmonary tuberculosis. Prevalence and significance of negative smears pretreatment and positive smears post-treatment. *Am Rev Respir Dis* 1984; **129**: 264–8.

[5] Millen SJ, Uys PW, Hargrove J, van Helden PD, Williams BG. The effect of diagnostic delays on the drop-out rate and the total delay to diagnosis of tuberculosis. *PLoS ONE* 2008; **3**(4):e1933.

[6] Storla DG, Yimer S, Bjune GA: A systematic review of delay in the diagnosis and treatment of tuberculosis. *BMC Publ Heal* 2008; **8**:15.

[7] Centre for Disease Control and Prevention. Update: Fatal and severe liver injuries associated with rifampin and pyrazinamide for latent tuberculosis infection, and revisions in American Thoracic Society/CDC recommendations—United States, 2001. *MMWR* 2001; **50**: 733–5.

[8] Yee YC, Gough A, Kumarasinghe G, Lim TK. The pattern of utilization and accuracy of a commercial nucleic acid amplification test for the rapid diagnosis of Mycobacterium tuberculosis in routine

clinical practice. *Singapore Med J* 2002; **43**: 415–20.

[9] Banerjee S, Gupta S, Shende N, Kumar S, Harinath BC. Serodiagnosis of tuberculosis using two ELISA systems. *Ind J Clin Biochem* 2003; **18**: 48–53.

[10] Banerjee S, Gupta S, Kumar S, Shrikhande A V, Reddy MVR, Harinath BC. Seroreactivity of 31 kDa and 41 kDa mycobacterial secretory proteins isolated from culture filtrate in extra pulmonary tuberculosis. *Ind J Pathol Microbiol* 2003; **46**: 261–4.

[11] Gupta S, Shende N, Kumar S, Hadnath BC. Antibody response to lrvLb.HjIRa excretory-secretory ES43 and ES-31 antigens at different stages of pulmonary tuberculosis. *Biomed Res* 2004; **15**:76–9.

[12] Bhatia AS, Gupta S, Shende N, Kumar S, Harinath BC. Serodiagnosis of Childhood Tuberculosis by ELISA. *Ind J Pediatr* 2005; **72**(5): 383–7.

[13] Lodam AN, Reddy MVR, Narang P, Gupta OP, Harinath BC. Fractionation analysis and diagnostic utility of Mycobacterium tuberculosis H37Ra excretory secretory antigen in pulmonary tuberculosis. *Indian J Biochem Biophys* 1996; **33**:67–71.

[14] Gupta S, Shende N, Kumar S, Harinath BC. Detection of antibodies to a cocktail of mycobacterial excretory secretory antigens in tuberculosis by ELISA and Immunoblotting. *Curr Sci* 2005; **88**: 1825–7.

[15] Harinath BC, Kumar S, Roy SS, Hirudkar S, Upadhye V, Shende N. A cocktail of affinity purified antibodies reactive with diagnostically useful mycobacterial antigens ES-31, ES-43 and EST-6 for detecting the presence of Mycobacterium tuberculosis. *Diag Microbio Inf Dis* 2006; **55**:65–8.

[16] Majumdar A, Upadhye V, Harinath BC. A prospective study of inhouse developed SEVA TB ELISA using cocktail of antigens and their immunoglobulin in the diagnosis of tuberculosis suspected patients in a tertiary hospital located in rural area. *Biomedical Research* 2009; **20**(1); 56–63.

[17] Majumdar A, Upadhye V, Harinath BC. Peroxidase enzyme immunoassay for circulating SEVA TB ES-31 antigen in pulmonary tuberculosis sera. *Biomedical research* 2008; **19**(3):201–6.

[18] Majumdar A, Kamble PD, Harinath BC. Detection of circulating free and immunocomplexed antigen in pulmonary tuberculosis using cocktail of antibodies to Mycobacterium tuberculosis excretory secretory antigens by Peroxidase enzyme immunoassay. *Ind J Tuberc* In press.

[19] Nair ER, Banerjee S, Kumar S, Reddy MVR, Harinath BC. Isolation of Mycobacterium tuberculosis 31 kDa antigen protein of diagnostic interest from culture filtrate using anti ES-31 antibody by affinity chromatography. *Indian J Clin Biochem* 2001; **16**: 132–5.

[20] Saha-Roy S, Shende N, Kumar S, Harinath BC. Effectivity of crude versus purified mycobacterial secretory protein as immunogen for optimum antibody production. *Ind J Exp Biol* 2005; **43**: 1196–8.

[21] Upadhye V, Shende N, Kumar S, Harinath BC. Detection of antibody and antigen in extrapulmonary tuberculosis patients' sera using a cocktail of mycobacterial excretory secretory antigens and their antibodies. *Biomed Res* 2007; **18**: 161–6.

[22] Frieden T Ed. What is role of mycobacterial culture in diagnosis and case definition? In: *Toman's tuberculosis case detection, treatment and monitoring – questions and answers*. 2nd ed. Geneva: World Health Organization; 2004, p.35–43.

[23] Wilson D, Nachega J, Morroni C, Chaisson R, Maartens G. Diagnosis smear-negative tuberculosis using case definitions and treatment response in HIV-infected adults. *Int J Tuberc Lung Dis* 2006; **10**: 31–8.

[24] Maris B, Schaaf S, Hesselting A, Gie R. Tuberculosis case definition: time for critical re-assessment? *Int J Tuberc Lung Dis* 2008; **12**: 1217–9.

[25] World health organization. *Treatment of tuberculosis: Guidelines*. [Online] Available from: [http://whqlibdoc.who.int/publications/2010/9789241547833\\_eng.pdf](http://whqlibdoc.who.int/publications/2010/9789241547833_eng.pdf). [Accessed on 11th, April 2009].