Immunodiagnosis of pulmonary tuberculosis by concomitant detection of antigen and antibodies of excretory-secretory protein of *Mycobacterium tuberculosis* $H_{37}Ra$

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With a view to diagnosing tuberculosis in populations in endemic areas, excretory-secretory antigen fraction (*Mtb* EST-6) of purified *Mycobacterium tuberculosis* $H_{37}Ra$ and affinity purified polyclonal antibodies against *Mtb* EST were used to detect both antibodies and circulating antigen in the sera of patients and disease-free individuals. Indirect stick penicillinase ELISA system using *Mtb* EST-6 detected antigen-specific IgG antibody in 84% of sputum positive, 77% of sputum negative pulmonary tuberculosis patients and 7% of healthy and 11% of subjects with nontuberculosis diseases. Similarly, a sandwich penicillinase ELISA system using affinity purified anti *Mtb* EST antibodies detected circulating antigen in 83% and 61% of sputum positive and negative pulmonary tuberculosis subjects. In contrast only 24% of healthy and 18% of disease controls showed seropositivity. Antibody assay showed higher sensitivity and specificity (83% and 91% respectively) compared to antigen detection (sensitivity of 79% and specificity of 79%). However, by concomitant use of both assays it was possible to enhance the specificity of detection to 98%, though sensitivity was reduced marginally to 70%. The present study confirms the presence of both antigen and specific antibodies in the circulation during clinical disease and draws attention to the utility of *Mtb* EST-6 as a diagnostic marker of pulmonary tuberculosis.

1. Introduction

Tuberculosis remains a major public health problem in many parts of the world. One third of the world's population is exposed to tuberculosis infection and 3 million deaths occur annually. The incidence of tuberculosis is increasing rapidly due to a growing number of people being infected with the human immunodeficiency virus (HIV) (Sudre *et al* 1992). Early diagnosis of active cases of pulmonary tuberculosis is essential for initiating prompt treatment and containment of the disease. A definite diagnosis of pulmonary tuberculosis can be made by demonstrating acid fast bacilli (AFB) in sputum smear. However, problems arise when the bacillary load is low and the sputum smear is negative for AFB. Chest skiagram provides only a probable diagnosis and culture for tubercle bacilli is time consuming. In the last three decades various immunological methods have been explored for the diagnosis of tuberculosis based on the detection of circulating mycobacterial antigens and antibodies (Affronti *et al* 1973; Chaparas 1985). Most of the reported serological assays have exhibited low potential in endemic areas and lacked the required specificity due to exposure to environmental mycobacterial, leading to cross-reactive antibodies (Bardana *et al* 1973; Grange 1984). In the earlier studies from our laboratory *Mtb* EST antigen fractionated by TCA precipitation was analysed on SDS-PAGE. Of the four fractions obtained (EST3, 4, 6 and 10), EST6 was identified to be diagnostically useful in pulmonary tu-

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berculosis (Lodam *et al* 1996). In the present study we have analysed the comparative efficacy of purified *Mtb* EST-6 antigen in indirect ELISA and affinity purified anti *Mtb* EST antigen antibodies in sandwich ELISA system for detection of tuberculous IgG antibody and circulating tubercular antigen respectively in the detection of proven pulmonary tuberculosis.

2. Materials and methods

2.1 *Sera samples*

Blood samples were collected from 71 pulmonary tuberculosis patients. While 58 of them were positive for AFB in sputum smear, the remaining 13 cases were AFB negative both by sputum smear and culture methods. They were diagnosed based on clinical and radiological findings and positive response to antitubercular therapy. Blood samples collected from 29 healthy volunteers of this region and 28 cases of other related diseases viz., leprosy (6), chronic obstructive airway diseases such as chronic bronchitis, bronchial asthma (15), pyrexia of unknown origin (3), lung abscess (2), bronchiectasis (2) served as negative controls. Sera were separated and stored at −20°C with 0·01% sodium azide.

2.2 *Mycobacterium tuberculosis $H_{37}Ra$ excretory-secretory (Mtb ES) antigen*

Excretory-secretory antigen was isolated from 7–9 days old culture medium of *Mtb* $H_{37}Ra$ strain procured from the Tuberculosis Research Centre, Chennai. Bacteria were maintained in logarithmic growth phase in synthetic Sauton medium with minimum lysis by subculturing weekly at 37°C with intermittent shaking (Magnuson and Bentzen 1958). Bacteria were harvested by centrifugation followed by initial filtration through Whatman No. 3 filter paper and then through sterile cellulose acetate membrane filters (Maxflow, Mumbai 0·45 μ). The filtrate was further concentrated by ultra membrane filtration (Millipore Co., Bredford, MA, USA) with a molecular weight cut off of 10 kDa. After extensive dialysis against 0·01 M phosphate buffer saline (PBS), pH 7·2, the protein content was estimated by the method of Lowry *et al* (1951), and stored at −20°C until further use.

2.3 *Mtb EST-6 antigen*

The trichloroacetic acid (TCA) soluble fraction of *Mtb* ES antigen was prepared by adding TCA to a final concentration of 6%, followed by centrifugation at 4000 *g* for 20 min at 4°C. The supernatent fraction (*Mtb* EST antigen) was dialysed against 0·01 M PBS, pH 7·2, and concentrated by ultra filtration with 12–14 kDa membrane. The *Mtb* EST antigen was further fractionated by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) using 10% gel as described by Kharat *et al* (1989). The gel was sliced horizontally into 12 slices at 1 cm intervals and proteins from the 6th gel slice (which had been earlier shown by us to have the antigen) were recovered by electroelution into Tris-glycine buffer (pH 8·3). The eluant was dialysed extensively for complete removal of Tris base against 0·01 M PBS, pH 7·2, and designated as *Mtb* EST-6 antigen.

2.4 *Anti Mtb EST antigen antibodies*

Polyclonal antibodies to *Mtb* $H_{37}Ra$ sonicate antigen were raised in goats by immunizing with sonicate antigen in incomplete Freund's adjuvant as reported earlier (Lodam *et al* 1996).

Antibodies specific to *Mtb* EST antigen were purified by affinity chromatography. In brief, the partially purified *Mtb* EST antigen was coupled with CNBr activated A–H Sepharose (Amino hexane sepharose) beads (1·5 mg of protein/ml of packed volume of sepharose beads). Goat anti serum raised against *Mtb* $H_{37}Ra$ sonicate antigen was passed through the column. The bound antibody was eluted by 0·1 M glacial acetic acid and eluant was collected in Tris-HCl buffer (0·1 M, pH 8·6). The eluant was further concentrated, dialysed against 0·01 M PBS, pH 7·2. After protein estimation it was stored with 0·01% sodium azide at −20°C until further use.

2.5 *Conjugation of proteins to penicillinase*

Conjugation of anti human immunoglobulin G (Lupin Labs Ltd., Bhopal) or anti *Mtb* EST antigen antibodies to enzyme penicillinase (Sigma Chemical Co., St. Louis, Mo, USA) was carried out by a single step glutaraldehyde method of Avrameas (1969).

The starch-iodine-penicillin-V substrate for penicillinase consisted of 150 mg soluble starch in 27·5 ml of 0·25 M sodium phosphate buffer (SPB), pH 7·2, containing 10·65 mg of penicillin-V (Sigma) and 100 μl of 0·08 M iodine in 3·2 M potassium iodide solution.

2.6 *Indirect stick ELISA*

Indirect ELISA was carried out for the detection of tuberculous IgG antibodies as described by Lodam *et al* (1996) using cellulose acetate membrane (CAM) square (5 × 5 mm) fixed onto plastic strips (5 × 70 mm) as the solid support. CAM sticks were coated with optimal concentration of *Mtb* EST-6 antigen (100 ng/5 μl/stick) in 0·05 M SPB, pH 7·2. The unbound sites on CAM squares were blocked by incubating the sticks with 3% gelatin in the same buffer at 37°C for 2 h. After washing the sticks in PBS (0·01 M, pH 7·2) containing 0·05%

(v/v) Tween-20 (PBS-T) 5 times, the sticks were further incubated with 0·5 ml of optimally diluted (1 : 600) and serial two-fold diluted sera in PBS-T at 37°C for 1 h. After incubation the sticks were washed 5 times with PBS-T and incubated further for 30 min at 37°C with 0·5 ml of anti human IgG penicillinase conjugate. After final washing the sticks were incubated with freshly prepared starch-iodine-penicillin-V substrate at 37°C. Complete decolorization of the blue colour or decolorization with only a discernible tinge of the substrate was considered a positive reaction.

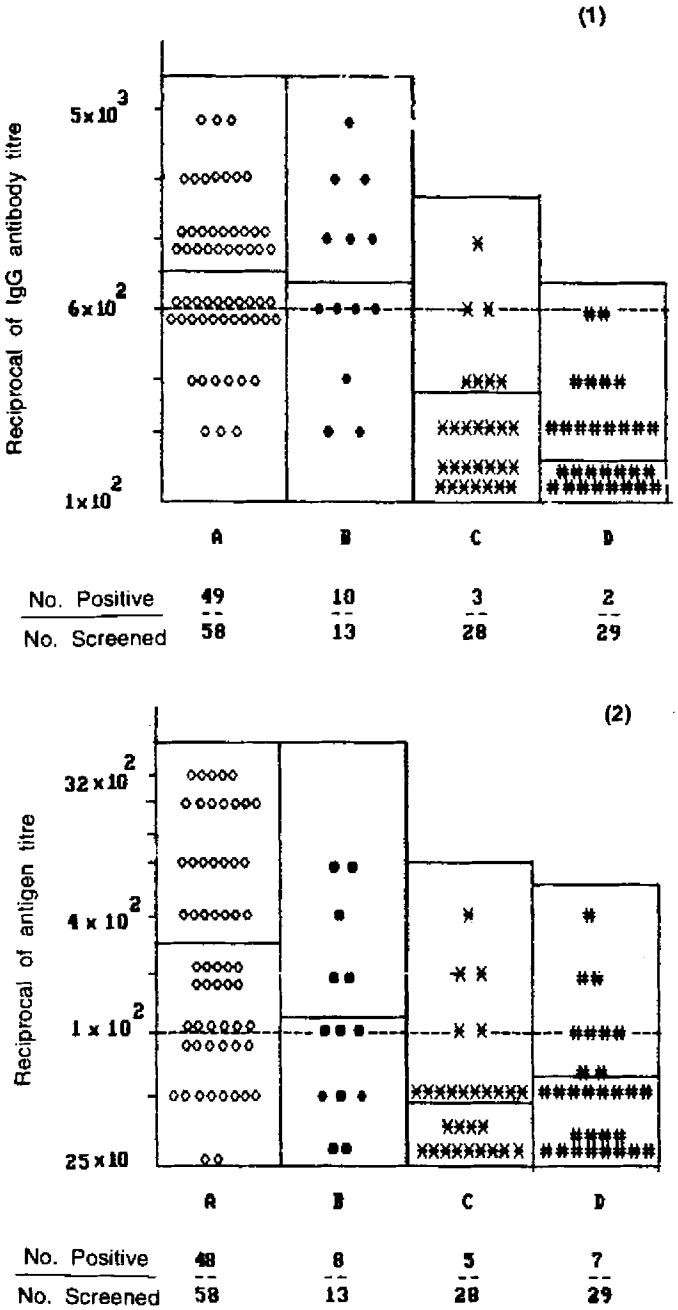
2.7 *Sandwich ELISA*

Sandwich ELISA was carried out for the detection of circulating tubercular antigen as described by Lodam *et al* (1996). The wells in the PVC microtitre plates (Dynatech, Virginia, USA) were sensitized with 50 µl of optimally diluted anti *Mtb* EST antigen antibodies (200 µg/ml in 0·06 M carbonate buffer, pH 9·6), followed by blocking of unbound sites by incubating with 3% gelatin (100 µl/well) in the same buffer. After washing with PBS-T the wells were incubated with 50 µl of optimally diluted (1 : 100) and serial two-fold diluted sera in PBS-T, at 37°C for 2 h. The wells were washed again and further incubated with optimally diluted anti *Mtb* EST antigen antibody penicillinase conjugate (1 : 1000) in PBS-T. Followed by final washing, the immune reaction was observed by incubating the wells with starch-iodine-penicillin-V substrate (50 µl/well). The disappearance of the blue colour at least 5 min before the negative controls denoted a positive reaction.

3. Results

The status of IgG antibodies in the sera of various clinical groups was detected at the threshold dilution (1 : 600) by indirect ELISA using *Mtb* EST-6 antigen (figure 1). Sera from 49 of 58 sputum positive and 10 of 13 sputum negative pulmonary tuberculosis subjects, show detectable anti *Mtb* EST-6 antibodies. In contrast only 3 of 28 disease control sera and 2 of 29 healthy control sera showed a positive reaction in ELISA. Sera from pulmonary tuberculosis patients also showed higher titres of IgG antibodies [geometric mean titres (GMT) 848 and 783 in sera of sputum positive and sputum negative respectively] compared to the antibody titres detected in sera of disease (174) and healthy controls (41).

All the sera samples at the threshold dilution (1 : 100) were also screened for circulating antigen using anti *Mtb* EST antigen antibodies in sandwich ELISA (figure 2). Tubercular antigen was detectable in 48 of 58 sputum positive and 8 of 13 sputum negative pulmonary tuberculosis sera. As before, only 5 out 28 disease and 7 of 29 healthy control sera showed detectable levels of *Mtb* EST-6 antigen. The titres of antigen in sera from sputum positive and sputum negative pulmonary tuberculosis subjects was much higher (387, 117 respectively) as compared to disease (46) controls and healthy volunteers (52).



Figures 1 and 2. (1) Scattergram of tuberculous IgG antibody titres by indirect ELISA using Mtb EST-6 antigen and (2) scattergram of circulating tubercular antigen titres by sandwich ELISA using affinity purified anti Mtb EST antigen antibodies in sera samples belonging to (A) smear positive pulmonary tuberculosis, (B) smear negative pulmonary tuberculosis, (C) disease control and (D) healthy control groups. The geometric mean titre of each group (——) and the threshold antibody titre of 1 : 600 for positivity (– – –) are also shown.

The results of comparative analysis of 128 sera samples for tuberculous IgG antibodies and circulating tubercular antigen are summarized in table 1. Forty four of 58 smear positive pulmonary tuberculosis sera and 6 of 13 smear negative pulmonary tuberculosis sera showed the presence of both anti *Mtb* EST-6 IgG antibodies and the circulating antigen. On the other hand, none of the 28 disease control sera and a negligible 1 of 29 healthy control sera showed concomitant presence of both the antibodies and the antigen.

4. Discussion

A variety of diagnostic tests for tuberculosis have been reported in the literature based on the detection of mycobacterial antigens and/or antibodies. Depending on the type of mycobacterial antigens used, the antibody assays showed a wide range of sensitivity and specificity. Using crude mycobacterial antigens for the detection of antibodies Daniel and Debanne (1987), Hernandez *et al* (1984) and Kumar *et al* (1994) showed a sensitivity of 56% to 94% and specificity of 86% to 100%. Use of purified antigens (Ma *et al* 1986; Sada *et al* 1990; Radhakrishnan and Mathai 1991; Park *et al* 1993) did not significantly improve the sensitivity (50 to 89%) or specificity (78 to 100%).

Mtb ES antigen has been explored in our laboratory (Bhaskar *et al* 1994; Kumar *et al* 1994; Lodam *et al* 1996) in the diagnosis of pulmonary and extrapulmonary tuberculosis.

In the present study a comparative analysis has been carried out to detect both IgG antibody and circulating antigen using purified *Mtb* EST-6 antigen and affinity purified anti *Mtb* EST antigen antibody in indirect and sandwich ELISA respectively.

Using *Mtb* EST-6 antigen in indirect ELISA, tuberculous IgG antibody could be detected in $\geq 77\%$ of proven cases of pulmonary tuberculosis. Only 9% of subjects from control groups showed seropositivity. Of significance was the higher titre of IgG antibodies seen in pulmonary tuberculosis as compared to control subjects [Student's t test ($P < 0.001$)] (figure 1), indicating the importance of antibody response to *Mtb* EST-6 during clinical disease.

Of interest was the concomitant presence of *Mtb* EST antigen in many patients. The 83% of sputum positive and 61% of sputum negative pulmonary tuberculosis and 21% of the control subjects showed the presence of circulating antigen. The difference in the titres of antigens was highly significant between sputum positive and sputum negative pulmonary tuberculosis as compared to the control groups ($P \leq 0.001$) (figure 2).

As many as 33% of leprosy sera showed positive reaction for *Mtb* EST-6 antigens which may be due to the presence of cross reactive epitopes on both pathogens. Such cross reactivity with leprosy sera in immunodiagnostic assays of tuberculosis has been reported by Sood *et al* (1991), Zeiss *et al* (1984) and Agrawal and Moudgil (1988). False positivity in bronchial asthma and healthy control cases was also seen and may be due to subclinical mycobacterial infection. While the antibody assay showed a sensitivity of 83% and a specificity of 91%, the antigen assay gave a lower level of both sensitivity and specificity (79%).

Enzyme immuno assays with variable results have been reported for the detection of mycobacterial antigens in body fluids. Banchuin *et al* (1990) reported a sensitivity of 87% and specificity of 93%. Sood *et al* (1991) reported a sensitivity of 73% and positivity of 16% and 44% in healthy controls and Hansen's disease respectively in detection of tuberculosis antigen. That the improvement of serological diagnosis may be obtained by simultaneous detection of antigen and antibody was indicated by the increased sensitivity observed by Radhakrishnan and

Table 1. Comparative analysis of antibody and antigen positivity in pulmonary tuberculois.

Group	Total number screened	Number (%) positivity for: Tuberculous IgG antibody[a]	Tubercular antigen[b]	Both antibody and antigen
Pulmonary TB (sputum positive)	58	49 (84%)	48 (3%)	44 (76%)
Pulmonary TB (clinically suspected and anti tuberculosis therapy (ATT) responsive)	13	10 (77%)	08 (61%)	06 (46%)
Disease control	28	03 (11%)	05 (18%)	– (–)
Healthy control	29	02 (7%)	07 (24%)	01 (3%)

The serum dilution at [a]1 : 600 and [b]1 : 100 was taken as the threshold level for positivity.

Mathai (1991) and Krombovitis *et al* (1986) using both an antigen capture immuno assay and antibody assay.

The present study on an endemic population indicates that the combination of antigen and antibody detection systems improves specificity of diagnosis of tubercular infection and would be an useful adjunct to the currently available clinical tests. Moreover, it has the potential for monitoring the effectiveness of chemotherapy and the emergence of drug resistance as the alterations in antigen/antibody levels may precede radiological and other clinical changes.

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