Int J Pharm Bio Sci 2015 July; 6(3): (P) 141 - 147

Research Article **Medical Biochemistry**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

IMMUNODIAGNOSIS OF PULMONARY AND EXTRAPULMONARY TUBERCULOSIS USING *MYCOBACTERIUM TUBERCULOSIS* ES-31/41 ANTIGEN

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ABSTRACT

Tuberculosis is still a major health problem in India and most developing countries and its incidence is rising in many developed countries. This resurgence has been attributed to the HIV epidemic and WHO has declared TB as a global health emergency in 1993. Immunodiagnosis has an important role in screening, diagnosis and management of Tuberculosis. In the present study we have analysed the diagnostic potential for detection of IgG antibody against ES-31/41 antigen by indirect penicillinase ELISA in the clinically and bacteriologically confirmed pulmonary and extrapulmonary tuberculosis cases to determine the usefulness of immunoglobulin in the diagnosis of patients attending the hospital. 47 (90.38%) out of 52 pulmonary tuberculosis cases (clinically diagnosed and/or AFB-positive), 33 (80.48%) out of 41 extrapulmonary tuberculosis cases, only one out of 10 (10%) of healthy group and only four out of 25 (16.0%) disease control cases were positive for IgG antibody to *Mycobacterium tuberculosis* ES antigen. In the present study, a sensitivity of 90.38% and 80.48% for IgG antibody detection was achieved in the pulmonary and extrapulmonary tuberculosis respectively. Hence, the study demonstrated the potential of this assay in the diagnosis of pulmonary and extrapulmonary tuberculosis.

KEY WORDS: **ELISA, ES-31/41, Extrapulmonary tuberculosis, IgG, Pulmonary Tuberculosis.**

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INTRODUCTION

Tuberculosis remains a major health problem in India and developing countries. TB is a leading cause of morbidity in developing countries and ranks eighth most frequent cause of all deaths worldwide. According to WHO estimates, India has the world's largest tuberculosis epidemic[1] and accounts for nearly one third of the global burden of tuberculosis and three million deaths occur annually. In India tuberculosis kills 14 times more people than all tropical diseases combined, 21 times more than malaria and 400 times more than leprosy. It has been estimated that about 30% of the world's tuberculosis patients are residing in India, and 1.5% of India's population is infected with pulmonary tuberculosis. The success of TB control programmes depends not only on successful completion of treatment, but also on the sound support from sensitive diagnostic tests for early diagnosis and constant monitoring. Tuberculosis is a disease of great antiquity and has probably caused more suffering and death than any other bacterial infection[2]. Early diagnosis of this infection is of 'utmost concern' for successful control[3]. The diagnostic problem in extrapulmonary tuberculosis results from the frequent failure of bacteriological methods of smear-microscopy and culture in establishing the diagnosis. Molecular methods like PCR are technically too demanding and expensive. Serological methods have been tried, but no single antigen has been found to be uniformly specific. However, the advantages of simplicity, cost effectiveness and scope of speedy automation make serology an attractive adjunct in diagnosis. Immunodiagnosis has important role in screening, diagnosis and management of tuberculosis. In the earlier studies, the purified ES-31 antigen has been extensively evaluated and found to have diagnostic potential in tuberculosis[4-7]. Similarly, other antigens like ES-41 and ES-43 have been isolated[8,9]. SEVA TB ES-31 antigen has shown potential in detecting tuberculous IgG antibody. The present study was undertaken to analyse, the diagnostic utility of detecting IgG antibody against ES-31/41 antigen by indirect SEVA TB ELISA for diagnosis in patients suffering from pulmonary and extra pulmonary tuberculosis (abdominal tuberculosis, tuberculosis lymphadenitis). Blood samples were obtained from patients with confirmed PTB, EPTB, healthy and disease control patients attending Osmania General Hospital, Hyderabad, Telangana, India.

MATERIALS AND METHODS

The present study was carried out in the department of Biochemistry, Osmania Medical College, Hyderabad, Telangana, India. This study comprised of patients attending TB clinic, Osmania General Hospital and district TB control Office. Normal controls included relatives and friends. The diseased controls included cases of other related diseases viz., bronchial asthma, chronic bronchitis, cirrhotic ascites, theumatoid arthritis, pyogenic arthritis, nonspecific lymphadenitis, pyogenic meningitis, viral encephalitis. All the TB patients included in the present study (Table 1) were selected with strong clinical feature on the basis of positivity of Radiological impression, Bacteriological status of sputum AFB smear and AFB culture, histopathological impression, Fine needle aspiration cytological impression, Mantoux test and ESR. The study population of Tuberculosis was divided into two groups.

Group I – Patients with pulmonary Tuberculosis (number = 52)

Pulmonary tuberculosis patients (n = 52) were further subdivided into two sub groups- fresh (n = 40) and relapse cases who underwent anti tuberculosis treatment (n = 12).

Group II – Patients with extrapulmonary Tuberculosis (n = 41)

Tuberculous lymphadenitis (n = 26) group was further sub divided into two- fresh (n = 18) and relapse (n = 8). This group comprised of Tubercular Meningitis patients (n = 6), abdominal tuberculosis (n = 4), TB spine (n = 2), TB larynx (n = 1), Koch's Gingivitis (n = 1) and Skin TB (n = 1).

Group III – Control Group (n = 10)

This group consisted of asymptomatic healthy individuals (n = 10) who declared that they had no contact with patients with pulmonary tuberculosis, whether relatives or friends, and did not have any clinical symptoms of tuberculosis at the time they were included in our study. Tuberculin tests (PPD) were not carried out in these individuals.

Group IV - Patients with other pneumopathies (n = 39)

This group included patients with non-tuberculous lung diseases (n = 14), chronic non-specific lymphadenitis (n = 6), pyogenic meningitis (n = 3), viral encephalitis (n = 2), Rheumatoid arthritis (n = 3), pyogenic arthritis (n = 4), cirrhosis (n = 7).

Collection of serum samples

Blood samples of 128 subjects were collected in sterile vials, serum was separated and stored at -20°C after adding 20µl of 0.01% sodium azide for each 1ml of serum as preservative. Working sera were stored at 4°C.

ELISA Stick Indirect Penicillinase ELISA for tuberculous IgG antibody detection

The test ELISA for IgG anti ES 31/41 detection was performed in accordance with the guidelines provided by JBTD Research Centre[5, 6, 8]. Optimum individual antigen concentration of 1 ng per stick was used in this assay. In brief, 5 µL of optimally diluted antigen (0.6µg protein/mL) was applied to cellulose acetate membrane coated with antigen ES - 31 / 41 200 sticks (Shri Hari fine Chem., Mumbai) squares fixed to a plastic strip and used along with optimally diluted human serum in the ratio of 1:600. One antigen-coated stick was placed in each vial and incubated at 37°C for 1 h. The sticks were washed 5 times with PBST (40ml of 0.25M sodium phosphate buffer saline + 960ml single distilled water + 9gm NaCl + 0.5ml Tween - 20). The enzyme-conjugate (anti-human IgG penicillinase conjugate) was diluted 1:1000 in PBST. Each stick was incubated with 0.5 ml of diluted conjugate at 37°C for 30 min. The sticks were then washed 9 times in PBST and transferred to labelled glass tubes. Finally, 0.5 ml of starch-iodine penicillin V substrate was added to each tube and incubated at 37°C. The decolorisation time was noted for each tube and the sera samples that showed complete decolorization of blue color of starch iodine penicillin 'V' substrate at least 5 min earlier than the negative control were considered as positive for anti ES- 31/41 IgG antibody (tuberculosis) and that the samples that showed decolorization at least 2 to 3 min earlier than the negative control were considered as border line positive. The test was repeated thrice for reproducible results.

Statistical Index of Diagnostic Accuracy

Sensitivity, Specificity, Positive predictive value and Negative Predictive value were calculated as per Parks text book of Preventive and Social Medicine[10].

Sensitivity

Sensitivity is defined as the ability of a test to identify correctly all those who have the disease, is "TRUE POSITIVE". 90 percent sensitivity means that 90 percent of the diseased people screened by the test will give a "TRUE POSITIVE" result and the remaining 10 percent a "false negative" result.

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive} + \text{False negative}} \text{ X } 100$$

Specificity

is defined as the ability of a test to identify correctly those who do not have the disease, that is, "TRUE NEGATIVES". 90 percent specificity means that 90 percent of the non-diseased persons will give "TRUE NEGATIVE" result, 10 percent of non diseased people screened by the test will be wrongly classified as "Diseased" when they are not.

$$\text{Specificity} = \frac{\text{True negative}}{\text{True negative} + \text{False positive}} \text{ X } 100$$

RESULTS AND DISCUSSION

The analytical results of total 93 sera of different groups of tuberculosis screened in indirect pencillinase ELISA using cellulose acetate membrane coated with antigen ES -31/41 along with 49 control subjects to detect anti tubercular IgG antibodies are summarized in table 1. The sensitivity and specificity of antibody detection by ELISA in tuberculosis patients is presented in Table 2.

Table 1

Seroactivity of M tb Es - 3 1/41 antigen in detection of tubercular IgG antibody by indirect pencillinase ELISA in pulmonary and extrapulmonary Tuberculosis.

Groups	Number screened	Cases-showing positive reaction	Percentage positivity
Total pulmonary Tuberculosis (Fresh & Relapse)	52	47	90.3
Pulmonary Tuberculosis (fresh)	40	37	92.5
Pulmonary tuberculosis (relapse after taking treatment)	12	10	83.3
Extrapulmonary TB (both fresh & relapse cases)	26	21	80.76
TB lymph adenitis (Fresh cases)	18	15	83.33
TB lymph adenitis (Relapse cases after taking anti TB treatment)	8	6	75
TB meningitis	6	5	83.33
TB abdomen	4	3	75
TB spine	2	2	100
Skin TB	1	1	100
TB larynx	1	1	100
TB gingivitis	1	0	0
Total	41	33	80.48
Controls			
Healthy	10	1	10
Disease other than TB			
Non specific lymph adenitis	6	1	16.66
Viral encephalitis	2	0	0
Pyogenic meningitis	3	0	0
Cirrhosis	7	1	14.28
Rheumatoid arthritis	3	1	33.33
Pyogenic arthritis	4	1	25
Total healthy and diseased controls	35	5	14.28
Total	128		

The serum dilution at 1:600 is taken as the threshold, level for positivity for tuberculosis IgG antibody

Immunodiagnosis of pulmonary tuberculosis

In the present study using M.tb antigen ES-31/41 positive reaction for TB IgG antibody was observed in 47 cases out of 52 (fresh + relapse) (Table 1). All the 52 cases of pulmonary TB were bacteriologically confirmed by AFB smear. Out of these, 90.3% were positive for IgG antibody detection. Hence, the sensitivity of the assay was 90.3%. In this hospital based study where samples of different forms of tuberculosis were screened randomly, results of open cases of pulmonary TB are comparable (90.3%) to the results (83.3% positive for IgG) of [11] and 92% positive for IgG antibody to ES-31 antigen[7]. Thirty seven out of 40 fresh pulmonary tuberculosis cases showed positive for indirect ELISA (sensitivity 92.5%). 83.33% (10/12) cases of pulmonary tuberculosis, having relapse after taking treatment showed positive for indirect ELISA. 28.5% (4/14) cases of non tuberculosis disease controls and 10% (1/10) healthy controls showed false positive reaction. 20.83% (5/24) cases of controls (Diseased 14 + Healthy 10) showed false positive reaction. Hence, the specificity of ELISA was 79% (Table 2).

Immunodiagnosis of tuberculosis lymphadenitis

Antibody detection was lower 75% (6/8) in patients with past history of antituberculous treatment (ATT) as compared to 83.33% (15/18) in fresh patients but this difference was not statistically significant. 80.76% (21/26) of tuberculous lymphadenitis (Relapse + Fresh) subjects were positive, similar to 88% in tubercular lymphadenopathy reported by[12.] Out of 6 diseased (non specific lymphadenitis) and 10 healthy controls only one case each showed false positive reaction (16.66% and 10% respectively). 12.5% (2/16) cases of controls (Diseased + healthy) showed false positive reaction. Hence, the specificity of the assay in tuberculous lymphadenitis was 87%. Non specific lymphadenitis had given false positivity

of 16.66% might be due to cross reaction with other pathogenic bacteria, due to exposure to environmental mycobacteria or due to the presence of non- tubercular mycobacteria.

Immunodiagnosis of TB meningitis

83.33% (5/6) of Tuberculosis Meningitis cases were detected to be positive. Our results are in agreement with earlier results of 90% in tubercular meningitis[8] assayed by specific IgG antibody detection to antigen SEVA TB ES - 31/41 by indirect penicillinase ELISA. ELISA to mycobacterial antigen and antibody in the cerebrospinal fluid, the specificity of the test was 96%[13]. Zero out of 5 diseased controls (2 viral encephalitis + 3 pyogenic meningitis) and one out of 10 healthy controls, showed false positive reaction. 6.66% (1/15) cases of controls (5 diseased + 10 healthy) showed false positive reaction. Hence, the specificity of ELISA in T.B. Meningitis cases was 93%. Earlier studies demonstrated that the detectable antiTB antibody was found in cerebrospinal fluid (CSF) [14 - 17]. The detection of antibodies correlated with the disease and was positive in 68 to 80% of the cases. [14 & 18] have shown that antigen or antibody detection is useful in the diagnosis of pulmonary, meningeal, pleural and abdominal tuberculosis by radioimmunoassay. [19] in a limited study of 10 CSF samples from patients with tuberculous meningitis showed the potential usefulness of ELISA in the detection of antigen.

Immunodiagnosis of abdominal tuberculosis patients

75% (3/4) of T.B. abdomen cases showed positive reaction similar to earlier results of 82% in abdominal TB[12]. One each out of 7 cirrhosis (diseased controls) and 10 healthy controls showed positive reaction (14.28% and 10% respectively). From a total of 17 (healthy and diseased) controls 2 cases showed positive reaction. Percentage of false positivity was 11.76% and specificity of ELISA in Tuberculosis abdomen was 88%.

Immunodiagnosis of TB spine, skin TB, TB larynx, TB gingivitis

80% (4/5) cases showed positive reaction. Out of 3 Rheumatoid and 4 pyogenic arthritis (7 diseased controls) and 10 healthy controls one case each showed false positive reaction (33.33%, 25% and 10% respectively). From a total of 17 controls (7 diseased + 10 healthy) 3 cases showed false positive reaction. Hence, percentage false positivity was 17.64% and specificity of ELISA in the above cases was 82% similar to the earlier reports of 85% in bone and joint tuberculosis[12].

Table 2
Sensitivity and specificity of antibody detection by ELISA in tuberculosis patients

Groups	Total number of cases screened	Total number of controls screened (healthy + diseased)	Sensitivity (%)	Specificity (%)
TB (Pulmonary + extra pulmonary)	93	49	86.02	81
Pulmonary TB	52	24	90.3	79
Extrapulmonary TB	41	35	80.48	85
Tuberculous lymphadenitis	26	16	80.76	87
TB meningitis	6	15	83.33	93
TB abdomen	4	17	75	88
Other forms of extra pulmonary				
TB spine	2			
Skin TB	1			
TB larynx	1			
TB gingivitis	1			
Total	5	17	80	82

Early diagnosis of tuberculosis is an essential requirement for initiating prompt treatment and containment of the disease. Although tuberculosis is a curable and to some extent preventable disease, it's diagnosis sometimes, especially in extrapulmonary tuberculosis, HIV-TB, childhood TB, smear negative pulmonary TB becomes difficult. The WHO declared tuberculosis as a global emergency in 1993. Rapid diagnostic tests for tuberculous meningitis are urgently needed because delayed treatment increase the already high mortality rate of this disease. Direct acid-fast staining of cerebrospinal fluid is the only quick method generally available, but it lacks sensitivity. Therefore the use of an enzyme-linked immunosorbent assay (ELISA) to mycobacterial antigen and antibody in the cerebrospinal fluid is of highly beneficial[20]. The use of TB - ELISA tests as a diagnostic tool offer a lot of scope in early diagnosis of serious forms of childhood tuberculosis. The characteristics of these tests have improved with the availability of purified and recombinant antigens. The selection of best combination of antigens for serology prospective clinical trials comparing success rate of serology with the standard different diagnostic procedures are required[21]. An immunodiagnostic test with good sensitivity and specificity would be a boon in these situations. Effective serodiagnosis of tuberculosis can be achieved only by combining detection of both circulating antibodies and antigens using highly specific purified reagents and immune complex - dissociated sera[22]. In the present study, a sensitivity of 90.3% with comparable specificity of 79.0% and a sensitivity of 80.48% with comparable specificity of 85.0% for IgG antibody detection was achieved in the cases of pulmonary tuberculosis and extrapulmonary tuberculosis respectively which is better than reported by other workers in pauci bacillary and smear negative pulmonary tuberculosis[23]. The detection of antibodies to mycobacterium tuberculosis by enzyme - linked immunosorbent assay has proved to be a potentially useful technique for the serodiagnosis of tuberculosis. The technique is capable of full automation.

CONCLUSION

ELISA is an economic, simple and rapid diagnostic test for pulmonary as well as extrapulmonary tuberculosis. *Mycobacterium tuberculosis* ES antigen is having potential with an overall sensitivity of 86.02% and specificity of 81% (Both pulmonary and extrapulmonary together). Sensitivity of 90.3% and 80.48% and specificity of 79% and 85% in pulmonary and extra pulmonary TB (Lymphnode TB, CNS TB, Abdominal TB, other forms of extrapulmonary TB) respectively for IgG antibody detection. This can be utilised at PHC level with minimum laboratory facilities.

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