**ABSTRACT**

A mycobacterial excretory-secretory protein fraction ESAS-7 purified by 50% ammonium sulphate precipitation followed by SDS-PAGE fractionation was evaluated by penicillinase enzyme linked immuno-sorbent assay (ELISA) for its sensitivity and specificity in the diagnosis of pulmonary tuberculosis. At a "cut off" serum dilution of 600, 38 (90%) of 42 sera from bacteriologically confirmed tuberculosis cases, 15 (100%) of 15 sera from bacteriologically negative but anti tubercular therapy (ATT) responded cases, 3 (7%) of 43 sera from normal healthy subjects and 4 (8%) of 48 sera from non tuberculous disease control cases gave positive reaction for tubercular antibody to ESAS-7 antigen fraction containing predominantly 33-kDa protein with a sensitivity of 90% in bacteriologically confirmed cases and specificity of 92%. Further, this diagnostic assay using the ESAS-7 antigen is more sensitive requiring as little as one nanogram antigen per test compared to use of 100 nanogram EST-6 antigen reported earlier. Thus use of ESAS-7 antigen for antibody detection has good diagnostic potential with improved specificity in pulmonary tuberculosis.

**KEY WORDS:** ELISA, ESAS-7 antigen, Penicillinase.

**INTRODUCTION**

Tuberculosis is responsible for over one-quarter of avoidable deaths from infectious diseases especially in developing countries (1). The increase of multi drug resistant tuberculosis (MDR-TB) especially amongst HIV infected persons has unmasked the deficiencies of current control methods and their application (2). Such developments and statistics serve to underline the need for a quick and reliable method of diagnosis - an "utmost concern".

Inadequacies and limitations of conventional current methods like microscopy, culture techniques and lack of sensitivity and specificity of tuberculin test and X-ray findings are well known. Thus a need exists for a rapid, low cost, sensitive and specific method to detect active tuberculosis.

Over the past several years, a lot of emphasis has been put on serodiagnostic tests. Amongst the serological methods, immunoassays particularly one based on an ELISA system for the measurement of antibodies to mycobacterial antigens seemed to be an appropriate approach (3,4,5). The group of antigens actively secreted by mycobacteria in synthetic medium are more or less similar to the in vivo released mycobacterial antigens in tubercular patients. Recently Nagai et al (6) have described a serial chromatography procedure for the isolation of major protein antigens in culture fluid of *Mycobacterium tuberculosis*.

Excretory - secretory (ES) antigen and its fractions were shown to be useful in the serodiagnosis of tuberculosis in earlier studies from our laboratory (7-13). The present study was undertaken to evaluate the utility of ESAS-7 antigen fraction containing a 33 kDa protein,
isolated from *M. tuberculosis* H$_{37}$Ra culture filtrate in serodiagnosis of tuberculosis by indirect ELISA.

**MATERIALS AND METHODS**

**Human sera**

The study population comprised of patients attending the Kasturba Hospital, Sevagram. Blood samples were collected in sterile vials, serum was separated and stored at -20°C after adding 0.01% sodium azide as preservative.

A total of 148 serum samples were included in this study, of which 57 were obtained from pulmonary tuberculosis cases, 43 from normal healthy volunteers (staff and students of Mahatma Gandhi Institute of Medical Sciences, Sevagram), 48 from cases with non-tuberculous disease controls including leprosy (14), chronic obstructive airway disease (13), bronchial asthma (2), uronchiectasis (1), chronic bronchitis (6), pneumonia (4), pyrexia of unknown origin (1) and pleural effusion (4) and lung abscess (3). The sera from pulmonary tuberculosis cases could be divided into (i) bacteriologically confirmed (S+C+) cases (N=42), and (ii) bacteriologically negative (S-C-) but clinically diagnosed radiologically positive cases with improvement on subsequent antitubercular therapy (ATT) (N=15).

*Mycobacterium tuberculosis* excretory-secretory antigen (M.tb ES Ag)

*Mycobacterium tuberculosis* H$_{37}$Ra strain, procured from Tuberculosis Research Centre (TRC) Chennai was cultured in thyroxine supplemented (8 μg/ml) Sautons medium as described previously (12). The protein content was measured by Lowry's method (14) and the antigen was stored with addition of protease inhibitors (Ethylene diamine tetracetic acid, ethylene glycol tetracetic acid and phenyl methyl sulphonyl fluoride at 1mM concentration and tosyl-L-lysine chloromethyl ketone, tosyl-L-phenylalanine chloromethyl ketone and leupeptin at 0.2 mM, 0.1 mM and 0.1 μM concentration respectively) at -20°C until use.

**M.tb ESAS antigen**

Ammonium sulphate precipitation at 50% saturation of M.tb ES antigen was carried out in water ice bath followed by centrifugation (2000 g) for 30 minutes at 4°C. The supernatant was concentrated by ultrafiltration (Spectropor, USA) and dialyzed against 0.01 M PBS (pH 7.2). The protein content was estimated and labelled as M.tb ESAS antigen.

**SDS - PAGE fractionation**

The M.tb ESAS antigen was fractionated by SDS-Polyacrylamide Gel Electrophoresis (PAGE) on 10% gel and after slicing the gel horizontally at 1 cm intervals, the proteins were recovered from each slice by electroelution (10). The protein each content of the 12 fractions was estimated and labelled as M.tb ESAS 1-12 antigen fractions.

**Anti M.tb ESAS antibodies**

Antibodies to sonicated M.tb H$_{37}$Ra were raised in goat and specific antibodies against M.tb ESAS antigen were isolated from immune sera using antigen coupled CNBr - activated sepharose - 4B (Pharmacia, Sweden) column and glycine-HCl buffer (0.2 M, pH 2.5) for elution of bound antibodies (13).

**Anti human IgG penicillinase conjugate**

Antibodies against human IgG (Sigma, USA) were raised in rabbits. The IgG fraction was isolated from the sera by 33% ammonium sulphate precipitation followed by DEAE ion exchange column chromatography (using Whatmann DE-52) and conjugated with enzyme penicillinase (Hindustan Antibiotics Ltd., Pimpri, India) by single step glutaraldehyde method (15).

**Indirect Penicillinase ELISA**

Stick indirect penicillinase ELISA was carried out for the detection of tubercular IgG antibody as described by Parkhe et al (16). The cellulose acetate membrane squares on plastic sticks sensitized each with five ul of optimally diluted antigen [M.tb ES (200 μg/ml)]/M.tb ESAS (2 μg/ml)/SDS-PAGE fractions of M.tb ESAS (0.2 μg/ml)] in 0.05 M SPB (pH 7.2) and the optimally