MYCOBACTERIUM TUBERCULOSIS H₃₇Ra ESAS-7 - AN EXCRETORY - SECRETORY ANTIGEN FRACTION OF IMMUNODIAGNOSTIC POTENTIAL IN PULMONARY TUBERCULOSIS

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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,

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isolated from *M. tuberculosis* H₃₇Ra 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), bronchiectasis (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 H₃₇Ra 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 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 H₃₇Ra 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 µl 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

diluted sera (1:600) and antihuman IgG penicillinase (1:1000) were used in this assay. Pooled healthy volunteers sera were used as negative control. The immune reaction was observed by incubating the sticks each with 0.5 ml of starch-iodine-penicillin 'V' substrate at 37°C. The sera showing complete decolourisation of blue colour substrate at least 5 minutes earlier than the pooled negative control denoted a positive reaction. The IgG antibody titre was determined by serial two fold dilutions of the test sera.

Sandwich Penicillinase ELISA

Antigenic activity in different SDS-PAGE fractions of M.tb ESAS antigen was determined in Sandwich ELISA, as described in our earlier study (10) with some modifications. Affinity purified goat anti M.tb ESAS antibody was conjugated to enzyme penicillinase (15). The wells in PVC microtitre plate (Dynatech, USA) coated with 1 µg/100 µl of anti M.tb ESAS antibodies were used to screen serially diluted (10 fold) M.tb ESAS 1-12 fractions. The immune reaction was probed using optimally diluted (1:500) M.tb antibody penicillinase conjugate and starch-iodine-penicillin substrate. The disappearance of blue colour at least 5 minutes before the no antigen control wells denoted a positive reaction.

Statistical analysis was done using student's t test to compare the group means.

RESULTS

The sera belonging to different groups viz; pulmonary tuberculosis (smear positive, culture positive and smear negative, culture negative but ATT responded) and controls (disease and healthy) were tested carrying out indirect stick penicillinase ELISA using M.tb ES and M.tb ESAS antigens. The results are summarized in Table 1. The M.tb ESAS antigen showed a higher geometric mean titre of tuberculous IgG antibody in sera compared to that given by M.tb ES antigen. The M.tb ESAS antigen also showed higher percentage of positivity for tubercular antibodies in S+C+(90%) as well as S-C- (66%) tuberculosis sera compared to the positivity of 80% and 50% respectively in these two groups of sera shown by M.tb ES antigen. Simultaneously ESAS antigen also showed cross

reaction in less number of control sera (9/44) compared to ES antigen (16/44).

SDS-PAGE fractionation of M.tb ESAS showed three prominent protein bands with molecular masses 47 kDa, 33 kDa and 16 kDa (Fig. 1). Eluates of 12 SDS-PAGE fractions were analyzed for tuberculosis antigen activity by Sandwich ELISA using affinity purified goat anti M.tb ESAS antibodies. Four fractions viz; ESAS-2 (95-160 kDa), ESAS-6 (37-45 kDa), ESAS-7 (31-37 kDa) and ESAS-10 (17-20 kDa) showed positivity each with a reciprocal antigen titre of 10³ (Table 2).

Table 1. Detection of tuberculous IgG antibodies in human sera by stick indirect ELISA using Mtb ES and Mtb ESAS antigens.

Sera group	Total No. screened	Sera positive for tuberculous IgG antibodies* using			
		Mtb ES antigen		Mtb ESAS antigen	
		No. +ve	GMT	No. +ve	GMT
Pulmonary TB (S+C+)	20	16 (80%)	909	18 (90%)	1242
Pulmonary TB (S-C-; ATT responded)	06	03 (50%)	600	04 (66%)	600
Disease control	24	11 (45%)	400	06 (25%)	259
Healthy control	20	05 (25%)	261	03 (15%)	235

*The sera dilution at 1:600 was taken as the threshold level for positivity.

(Values in parentheses show percentage of positivity)

Table 2. Analysis of SDS-PAGE fractions of Mtb ESAS antigen by Sandwich ELISA for tuberculous antigenic activity

Fraction Number	Approximate Mol. mass (X-10) ³ kDa	Reactivity with affinity purified goat anti Mtb ESAS antibodies*
1	>160	100
2	95 - 160	1000
3	68 - 95	100
4	56 - 68	0
5	45 - 56	100
6	37 - 45	1000
7	31 - 37	1000
8	25 - 31	0
9	20 - 25	100
10	17 - 20	1000
11	14 - 17	0
12	12 - 14	100

* The reciprocal of the highest dilution of fraction showing positive reaction. The initial protein concentration of each fraction was 10 µg/ml.

When these four fractions of SDS-PAGE were employed in indirect penicillinase ELISA to analyze their immunodiagnostic potential in pulmonary tuberculosis with a limited number of sera, ESAS-7 antigen showed a higher geometric mean titre of tuberculous IgG antibody (GMT - 1378) compared to GMT-1200 for ESAS-6, GMT - 424 for ESAS-2 and GMT-689 for ESAS-10 antigens (Fig. 2). Hence further ESAS-7 antigen was explored for its diagnostic potential with increased number of sera and compared with our earlier reported M.tb EST-6 antigen. In ESAS-7 fraction a prominent band of molecular weight 33 kDa was observed in the corresponding position in the stained part of the gel (Fig. 1).

M.tb ESAS-7 antigen fraction detected antibodies in 90% of AFB positive (S+C+) and 100% of AFB negative (S-C-) but ATT responded tuberculosis sera, 8% of disease controls and 7% of healthy controls. The difference in the GMT of tuberculous IgG antibodies in pulmonary tuberculosis and control groups was significant by student's t test ($p < 0.001$). Between the two groups of tuberculosis sera S+C+ group had a higher geometric titre of tuberculous IgG antibodies (1486) compared to S-C- group (GMT-791) (Fig.3).

DISCUSSION

In the immunodiagnostic studies of tuberculosis the detection of tuberculous antibody against different preparations of mycobacterial antigens has been of considerable interest. Many workers have explored the use of culture filtrate antigens in ELISA in different immunodiagnostic techniques for the diagnosis of tuberculosis (17,18) and reported the sensitivity and specificity varying between 48-94% and 87-100% respectively (19-23). The antigen preparations explored by most of these workers were crude. The use of purified, characterized antigen could be of help to overcome the lack of sensitivity and improve specificity. Many workers have explored purified fractions of mycobacterial antigens in ELISA (21, 5) but still no purified fraction of mycobacterial antigens has been found to be satisfactory for diagnosis.

In the present study, we fractionated and analyzed the diagnostic potential of M.tb ES antigen. Earlier studies from our laboratory showed the usefulness of M.tb ES antigen in the diagnosis of pulmonary tuberculosis in adults (9) and pulmonary and extrapulmonary tuberculosis in children (8). A 33 kDa antigen containing fraction (ESAS-7) was isolated by ammonium sulphate precipitation followed by SDS-PAGE fractionation

Fig. 1. SDS-PAGE of (A), M.tb ESAS antigen and (B), molecular weight markers.

Fig. 2. Geometric mean titres of tuberculous IgG antibody in different groups of sera using M.tb ESAS-2,6,7 and 10 Ag.

Fig. 3. Scattergram of tuberculous IgG antibody levels against M.tb ESAS-7 Ag. in sera samples of; (A) S+C+ pulmonary tuberculosis; (B) S-C- pulmonary tuberculosis; (C) healthy controls; (D) leprosy; (E) COAD; (F) bronchial asthma; (G) bronchiectasis; (H) chronic bronchitis; (I) pneumonia; (J) pyrexia of unknown origin; (K) pleural effusion; (L) Lung abscess. horizontal line represents cut off serum dilution (1:600).

of ES antigen from short term culture fluid of M.tb H₃₇Ra. This antigen showed a high reactivity with sera of tuberculosis patients. Das et.al. (24) have shown that the major proteins found in culture filtrates were those of molecular masses 28-33 kDa secreted by living mycobacterial cells and explored in diagnosis of leprosy.

In our previous study in which EST-6 antigen was used in ELISA to measure anti-EST-6 antibody in tuberculosis sera, we observed that EST-6 antigen cross reacted with 10% of normal sera and 13% of disease control group giving a sensitivity of 94% compared to sputum AFB positivity and specificity of 88% (10). In the present study ESAS-7 antigen reacted with a smaller number (7%) of normal sera and only 8% of disease control group. The sensitivity of the present assay was found to be 90% in bacteriologically confirmed cases (S+C+) pulmonary tuberculosis cases and a specificity of 92%, an improvement over the specificity of the assay system using EST-6 antigen. The specificity of the diagnostic test is quite important as indiscriminate use of antituberculosis drugs causes various adverse reactions. Further ESAS-7 is also more sensitive requiring only one nanogram antigen concentration per test compared to use of 100 nanogram EST-6 antigen.

In another study Sada et al (22) analyzed ammonium sulphate precipitated purified 30 kDa antigen and reported a sensitivity of 70% in sputum positive active pulmonary tuberculosis cases and specificity of 100% in control subjects. Alamelu Raja et al (25) evaluated, the 30 kDa antigen purified by DEAE-cellulose chromatography in ELISA and reported a sensitivity of 89% (S+C+) and 32% (S-C-). The results obtained evaluating our ESAS-7 antigen fraction in ELISA were comparable with 30 kDa antigen in detecting tubercular IgG antibody in S+C+ cases (90%) and further quite promising in detecting S-C- but ATT responded cases (100%). The over all specificity using ESAS-7 was 92% compared to 75% obtained with 30 kDa antigen thus showing a good diagnostic potential for tuberculosis.

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