DETECTION OF TUBERCULAR ANTIBODY AND ANTIGEN IN SERA OF BONE AND JOINT TUBERCULOSIS

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ABSTRACT

Trichloroacetic acid (TCA) solubilized and DEAE fractionated *Mycobacterium tuberculosis* H₃₇Ra excretory-secretory (ES) antigen viz., Mtb EST DE1 and affinity purified goat antibodies to the TCA solubilized ES antigen (Mtb EST) were explored in detecting tubercular antibody and antigen respectively in sera of bone and joint tuberculosis by indirect and sandwich ELISA. Out of total 36 bone & joint tuberculosis cases, tubercular antibody was detected by indirect ELISA in 30 patients (sensitivity 83%), while circulating tubercular antigen was detected by sandwich ELISA in 27 patients (sensitivity 75%). Out of 34 non tubercular disease control cases, 10 patients showed positive reaction for antibody while only 4 patients showed positive reaction for antigen. In another group of 34 healthy subjects who were screened, 4 individuals showed positive reaction for tubercular antibody and 2 cases for antigen. This study shows that antigen detection assay using affinity purified anti Mtb EST antigen antibody is superior with overall specificity of 91% as compared to antibody detection assay with 75% specificity in bone & joint tuberculosis.

KEY WORDS : *Mycobacterium tuberculosis*, bone & joint tuberculosis, excretory-secretory antigen, anti Mtb EST antigen, antibody, indirect ELISA, sandwich ELISA.

INTRODUCTION

Tuberculosis is reemerging as a major public health problem in developing and developed countries alike (1,2). With the spread of HIV infection worldwide, various forms of extra pulmonary tuberculosis have been reported to be increasing at an alarming rate in recent years (3-5). Extra pulmonary tuberculosis is a paucibacillary condition and commonly presents with diagnostic difficulties (6). Routine microbiological methods are inadequate for early diagnosis of extra pulmonary tuberculosis particularly in bone & joint tuberculosis (7). Histopathological confirmation of

clinical diagnosis of bone & joint tuberculosis is often problematic due to poor accessibility to the site of infection and lack of patient compliance towards invasive procedures (8). CT, MRI, radio-nucleide bone scan are obviously helpful in critical cases (9) but expensive and not available widely. Although there is no substitute for culture demonstration of tubercle bacilli in definitive diagnosis of tuberculosis, highly sensitive immunodiagnostic techniques such as ELISA for detection of tubercular antibody, antigen or both have been reported to be useful in pulmonary and extra pulmonary tuberculosis (10-16).

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However few studies have been reported in bone & joint tuberculosis (8,17,18). In the earlier studies from this laboratory, *Mycobacterium tuberculosis* H₃₇Ra excretory-secretory antigen (Mtb ES antigen) used in indirect ELISA for detection of tubercular IgG

antibody was found to be useful in diagnosis of pulmonary tuberculosis (19-23).

In the present study a partially purified *M. tuberculosis* H₃₇Ra ES antigen (Mtb EST - DE1) and goat affinity purified antibodies against trichloroacetic acid solubilized ES antigen (EST) have been explored for the immunodiagnosis of bone and joint tuberculosis.

MATERIALS AND METHODS

Patient's sera

Sera were collected from different groups of patients attending orthopaedics department of this Institute with their prior consent. Thirty six patients of bone and joint tuberculosis included were biopsy proven with histopathological evidence of tuberculosis and disease control group consisted of 34 cases with 11 of rheumatoid arthritis, 11 of pyogenic arthritis, 5 of multiple myeloma and 7 of filarial arthritis. Healthy control group consisted of 34 cases who were found healthy on medical examination and volunteered for it. Sera were separated and stored at -20°C after adding 0.01% sodium azide until use.

M. tuberculosis excretory-secretory (M.tb ES) antigen

Mtb ES antigen was prepared essentially as described by Pramanik et. al. (24). Briefly, *M. tuberculosis* H₃₇Ra strain was maintained at mid logarithmic growth phase in thyroxine supplemented (8 µg/ml) Sauton medium with intermittent shaking. Short term (7-9 days) spent culture fluid was separated from bacilli by centrifugation followed by filtration through Whatman No. 3 filter paper, Seitz filter and 0.45 µ membrane (Maxflow India), and concentrated by ultrafiltration (Millipore, USA). The concentrated culture fluid was dialysed extensively against 0.01M PBS (pH 7.2), [protein concentration

was estimated by Lowry's method (25)] and stored at -20°C after adding 0.01% sodium azide until use.

Purification of Mtb ES antigen

Mtb ES antigen was partially purified by 6% trichloroacetic acid (TCA) precipitation as described by Lodam et. al. (21). The TCA soluble fraction was separated by centrifugation, dialysed, concentrated and labelled as Mtb EST antigen. The Mtb EST antigen was further fractionated on DEAE (Diethyl amino ethyl) cellulose (Whatman DE 52 R) column as described by Lodam et. al. (22). Briefly, 2 mg of Mtb EST antigen was applied on to a DEAE cellulose column (1.2 x 20 cm) and unbound fraction was collected after elution at a flow rate of 10 ml/hr with 0.05M sodium phosphate buffer (pH 7.2) and was further dialysed, concentrated and labelled as Mtb EST-DE₁ antigen.

Anti Mtb EST antigen antibodies

Polyclonal antibodies to *M. tuberculosis* H₃₇Ra sonicate antigen were raised in goats by immunizing with sonicate antigen in incomplete Freund's adjuvant (on contract from Lupin Lab., India). Antibodies specific to Mtb EST antigen were isolated by affinity column chromatography. Briefly, Mtb EST antigen was coupled with CNBr activated A-H sepharose (Sephacrose CL 6B) beads (1.5 mg of protein/ml of packed volume of sepharose beads). Goat anti Mtb H₃₇Ra sonicate immunoglobulins were passed through the column. The antibody bound to the column was eluted out by 0.1M glacial acetic acid and collected in Tris-HCl buffer (0.1M, pH 8.6) to neutralize the eluant. The eluant was dialysed, concentrated and stored at -20°C after adding sodium azide (0.01%) until use.

Conjugation of proteins to penicillinase

Conjugation of anti human immunoglobulin G

(Lupin Lab., India) and anti Mtb EST antigen antibodies to penicillinase enzyme (Sigma Chemical Co., USA) was carried out by single step glutaraldehyde method of Avrameas (26).

Indirect Stick ELISA

Indirect ELISA was carried out for detection of tubercular IgG antibodies as described elsewhere (22) using cellulose acetate membrane (CAM) squares (5 x 5 mm) fixed on to plastic strips (5 x 70 mm) as the solid support and coated with five μ l of optimally diluted antigen (Mtb EST-DE₁ antigen of 20 μ g/ml in 0.05M SPB, pH 7.2). Complete decolorization of blue colour of the substrate denoted a positive reaction.

Sandwich ELISA

Sandwich ELISA was carried out for detection of circulating tubercular antigen as described by Lodam et. al. (23) using the PVC microtitre plates (Dynatech, USA) sensitized with 50 μ l of optimally diluted anti Mtb EST antigen antibodies (200 μ g/ml in 0.06M carbonate buffer, pH 9.6).

RESULTS

In the present study, Mtb EST DE₁ antigen was used in indirect ELISA for detection of tubercular IgG

antibody and affinity purified anti Mtb EST antigen antibody was used in sandwich ELISA for detection of circulating tubercular antigen and the results are summarised in Fig. I A & B. Out of 36 cases of bone & joint tuberculosis screened, 30 cases showed positive reaction for tubercular antibody (sensitivity 83%), while 29% (10/34) of disease control cases and 12% (4/34) of healthy control subjects also showed positive reaction (Table). Thus in bone & joint tuberculosis cases overall 79% of specificity was observed when antibody detection assay was used. Rheumatic arthritis (4/11) and pyogenic arthritis (3/11) cases within the disease control group showed relatively more false positive reaction.

Out of 36 tubercular cases screened by sandwich ELISA, 27 cases showed positive reaction for tubercular antigen (sensitivity 75%). However, only 12% (4/34) disease control cases and 6% (2/34) of healthy control subjects showed positive reactions (Table). The fact that not more than 6% of healthy normals are positive for tubercular antigen in this study of population who have been vaccinated with BCG, suggests that tuberculin positivity may not be interfering with results of our tests. Therefore, overall 91% of specificity was observed using antigen detection assay. Among the disease control cases pyogenic arthritis (2/11) showed relatively more false positive reaction for tubercular antigen compared to others.

Table 1. Detection of tuberculosis antigen and antibody in osteoarticular tuberculosis using affinity purified anti M.tb EST antibodies and M.tb EST-DE₁ antigen respectively.

Groups	Total No. screened	No. of cases showing positive * reaction for Antigen by Sandwich ELISA	Antibody by Indirect ELISA
A. Diagnosed TB cases (Spine=7, hip=10, knee=13, others=6)	36	27(75%)	30(83%)
B. Non TB disease control cases (Spine=12, hip=6, knee=12, others=4)	34	4(12%)	10(29%)
C. Healthy control	34	2(6%)	4(12%)

* The serum dilutions at $\geq 1:100$ and $\geq 1:600$ were taken as the threshold levels for positivity for antigen and antibody respectively.

Figure : Scattergrams of (A) circulating tubercular antigen titres and (B) tubercular IgG antibody titres in different groups viz., OTB = Osteoarticular tuberculosis; HC = Healthy control; RA = Rheumatoid arthritis; PA = Pyogenic arthritis; MM = Multiple myeloma and FA = Filarial arthritis.

* The sera showing antigen titre of > 100 and antibody titre of > 600 are considered as positive.

DISCUSSION

Bone and joint tuberculosis cases are being commonly observed in the hospital setting. Routine microbiological techniques are inadequate in diagnosis of this form of extra pulmonary tuberculosis. Moreover, all those tuberculous patients suffering from concomitant HIV infection further distract clinicians from the proper diagnosis due to uncommon clinical presentations (7). Hence, there is a pressing need for evaluation of various newer diagnostic tools for this disease.

Tubercular antibody and or antigen detection assays have been extensively evaluated in pulmonary tuberculosis and some of the forms of extra pulmonary tuberculosis (e.g. tubercular meningitis) and encouraging results have been reported (12, 13, 15, 27). Similar immunodiagnostic studies in bone & joint

tuberculosis are surprisingly few (8, 18). Among those rare studies, Stroebel et. al., (8) have shown promising results using antigen-6 in detection of tubercular IgG antibody in this form of extra pulmonary tuberculosis (sensitivity 94%, specificity 100%). In a different study the same group of investigators (17) have shown that the level of antibody to antigen-6 falls gradually during antitubercular therapy in bone & joint tuberculosis cases with few exceptions and thus further substantiated the diagnostic as well as prognostic usefulness of tubercular antibody detection assays in this disease. Few inherent disadvantages in antibody detection assays have been reported in recent years (13), and in patients with impaired humoral immunity, these assay systems have shown little diagnostic value. Detection of circulating mycobacterial antigen in tuberculosis has been regarded as the direct evidence of the pathogen in the host and reported to be useful even in HIV infected

individuals (13). Simultaneous use of antibody and antigen detection assays have been argued to be more useful in both pulmonary and extra pulmonary tuberculosis (14, 16, 28).

A purified fraction of *M. tuberculosis* H₃₇ Ra excretory- secretory antigen (Mtb EST- DE₁ antigen) when used in the present study for detection of tubercular IgG antibody in bone and joint tuberculosis, showed sensitivity of 83% and specificity of 79%. In an earlier study Patond and Prakash (18) used antigen 60 for detection of circulating tubercular antibody and showed relatively lower sensitivity (76%) but higher specificity (98%) in this disease. This may be due to the fact that no disease control cases were included in that study group.

Affinity purified specific antibody to Mtb EST antigen when used for detection of tubercular antigen in this study, showed relatively low sensitivity (75%) and high specificity (91%). In another study from this laboratory, this assay showed 79% sensitivity and specificity in pulmonary tuberculosis (23). This reflects the need for consideration of pulmonary and extra pulmonary tuberculosis as two different entities while evaluating immunoassays for their diagnosis .

Earlier, in the two different studies Radhakrishnan et. al. (27, 29) used polyclonal antibody to cell free culture filtrate antigen and affinity purified specific

antibody to antigen-5 for detection of tubercular antigen in cerebrospinal fluid of tubercular meningitis cases and reported low sensitivity (68% and 49% respectively) and high specificity (100% in both).

To our knowledge, there is hardly any published report available on immunodiagnostic studies in bone & joint tuberculosis using tubercular antigen detection assay. However, in the present study it was interesting to note that antigen detection assay showed only few (12%) of the disease control cases as positive, while the overall result of antibody detection assay was less discriminatory (Figure 1A & B).

From the present study it can be inferred that the tubercular antigen assay with specificity better than the antibody assay, is useful in diagnosis of bone and joint tuberculosis in the absence of facilities for invasive procedures.

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