



Excretory Secretory Proteins Released during Growth of *Mycobacterium tuberculosis* (H37Ra), With Diagnostic Potential in Pulmonary and Extra Pulmonary Tuberculosis

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Abstract

TB immunodiagnostics based on antibody and antigen detection for early detection and monitoring tubercular infections at low cost and flexible to adapt to field laboratories are a boon to developing countries. In this context we have explored in-house developed penicillinase based ELISA assays for the detection of antigen, antibody as well as immune-complexed antigen using various excretory secretory antigenic proteins and specific antibodies. Excretory secretory protein antigens such as ES-31, ES-41, ES-43, ES-6, ES-20, ES-100 and EST-6 were studied extensively and found to be useful in various pulmonary and extra pulmonary cases of tuberculosis infection. ES-31 has shown its diagnostic potential in chronic PTB cases, ES-43 in relapse cases and ES-41 in bone and joint TB. Elevated level of ES-20 antigen was observed in patients with weak immune response in TB lymphadenitis. Detection of ES-100 antigen and antibody by penicillinase ELISA was observed to be useful in detection of TB meningitis. ES-6 antigen was shown to be useful in detection of latent infection. These proteins with antigenic properties are utilized for production of specific antibodies against them and observed to be useful in immune diagnostics for detection of specific circulating and immune complexed antigens. Further user friendly peroxidase immunoassay has been standardised and is being routinely used for suspected TB cases attending 1000 bedded Kasturba Hospital attached to medical institute on physician's request.

Keywords Immunodiagnostics; Excretory Secretory Protein antigens; Antibodies; ELISA; Pulmonary and Extra pulmonary tuberculosis.

Introduction

Despite of declaration of tuberculosis (TB) as global emergency by World Health Organization in 1993, the problem continues to exist with 9.6 million new patients and 1.5 million deaths in the year 2014, including 0.4 million deaths among human immunodeficiency virus (HIV) positive individuals [1]. Nearly, 33% of the global population is considered to be infected with *Mycobacterium tuberculosis* (MTB). A shocking fact is that, TB is the disease that is treatable and completely curable but still continued to be a major health problem worldwide. Many factors are associated with this condition, starting from its accurate diagnosis to start and completion of the anti-tuberculosis treatment. The problem is getting worsened due to newly emerging drug resistant tuberculosis and extremely drug resistant tuberculosis and HIV TB co-infections. HIV with immunocompromised state is getting attention for increasing prevalence of extra-pulmonary tuberculosis. According to the World Health Organization in 2012, 8.7 million new TB cases have TB including 13% with HIV TB co-infection [2]. EPTB constitutes almost 15-20% of all cases of tuberculosis while it is more than 50% in HIV positive individuals [3]. Increasing trend of EPTB is an area of concern especially in developing countries like India. Thus, intricated tuberculosis scenario has definitely complicated the way for its control.

The existing gold standard test for confirmation of tuberculosis is AFB screening in specimen samples for both pulmonary and EPTB cases. Detection of acid fast bacilli limits itself requiring bacillary density >10,000 bacilli/ml of sputum sample [4]. However procuring specimen samples is difficult in EPTB as these cases have atypical presentations, and may yield poor positive result [3]. New technologies like the Xpert MTB/RIF assay, which enables simultaneous detection of *Mycobacterium tuberculosis* (MTB) bacilli and rifampicin (RIF) resistance, was endorsed by world Health Organization. This assay was specifically recommended for use as the initial diagnostic test for suspected drug-resistant or HIV-associated pulmonary tuberculosis. Although the development of the Xpert MTB/RIF assay is undoubtedly a landmark event, clinical and control programmatic effects and cost-effectiveness remain problems in resource limited developing countries [5]. Three commercially available serologic assays were evaluated for detection of antibodies in active TB infection namely, InBios Active TB detect IgG ELISA, IBL M. tb IgG ELISA and Anda Biologics TB ELISA [6]. InBios Active TB detect IgG ELISA, which is quite superior to other kits utilizes several antigens like Mtb81, Mtb8, Mtb48 and MPT 32, 38 KDa antigen and two additional proprietary antigens. Based on excretion of LAM antigen in urine, a commercial kit marketed as Clearview® TB ELISA was evaluated in culture positive TB cases and observed low sensitivity and not suitable in the current format [7].

A rapid, accurate point-of-care diagnostic test that is affordable and can be readily implemented is urgently needed. Simple serological test can be an answer for getting prompt diagnostic help, as all proposed definitive methods like culture taking 2-4 weeks and thus these tests

may help clinicians as adjunct test with other set of investigations to take right decision within a short time. More over serological tests based on antigen or antibody assays have a potential for providing simple and inexpensive tests for diagnosing tuberculosis both PTB and EPTB. As serological tests are simple in use and are also easy to interpret and most important is that they are not dependent on the site of infection and hence more suitable for EPTB cases as obtaining a sample for screening is the biggest challenge in EPTB diagnosis. The most notable feature to be considered for such diagnostic tests is that identification of key biomolecule in context to its immune responses from human body and also should give an idea about the degree of infection. This review discusses results and future prospects for our work.

Proteins released by *M. tb* bacilli during growth

Bacilli release a number of proteins during growth in surrounding media [8]. These excreted and secreted proteins are responsible for rapid recognition of bacilli by host lymphocytes as an antigen and thus play an important role in developing immune responses. Purification of such mycobacterial antigens is essentially required for defining structural probe and dissecting basic mechanism of the immune response to mycobacteria. It has also caught the attention of researchers as it has potential application in serodiagnosis, and also as new drug targets. The isolation and characterization of antigens released by *M. tuberculosis* in short term cultures have been an active area of research in our laboratory for more than 20 years. These antigenic proteins have their own significance as they are secreted by intact viable mycobacteria and are available for immune recognition in early stage of infection [9], hence can be targeted in early diagnosis and response to treatment. It is well accepted that antigen detection certainly presents more direct evidence for the presence of mycobacteria in body and helps in diagnosis of active infection. As these circulating antigens released during active infection are independent of host's immune response [10] and hence considered as an important approach to serodiagnosis of tuberculosis. Moreover it is very important to consider infected host's response for *M. tb* infection in different tuberculosis cases like pulmonary TB, abdominal tuberculosis, lymphnode tuberculosis, bone and joint tuberculosis. Different sites of infection may alter the body immune responses against the *M. tb* bacilli depending on their embryological tissue origin. Attention has been focused on recognition of protein molecule which will be useful as diagnostic marker in specific tissue infected cases (e.g. bone) of tuberculosis, diagnosis of early stage of infection, immune response to monitor chemo therapeutic treatment in tuberculosis [11].

Very few studies are available in context of different levels of antigen expression in different stages of *M. tb* infection. A study using ES-38 kDa antigen and anti-ES-38 antibodies have been reported primarily in patients with recurrent advanced chronic TB infection [12]. Laal et al. [13] have reported the presence of 88 kDa secreted antigen as a surrogate marker for identifying HIV infected persons with active, subclinical disease who are at the higher risk of developing clinical tuberculosis. A study by Kaplan et al. [14] showed difference in antibody levels in patients of new and relapse TB cases using crude culture filtrate antigen by immunodiffusion test. A sensitive serodiagnosis has been reported by Mukharjee et al. [15] in 2007 using recombinant RD1-encoded antigens of *Mycobacterium tuberculosis*. Simultaneously in the year 2007, monoclonal antibodies against antigen 85 complex have been used for serodiagnosis by indirect

ELISA method [16]. LED based fluorescence microscopy has been reported to be 10% more sensitive than ZN microscopy [16]. The transformation in TB diagnosis was brought about with the introduction of Genexpert test, which diagnoses TB by detecting the presence of TB bacteria, as well as testing for resistance to the drug Rifampicin [17].

Methodology

Excretory secretory (ES) proteins isolated from 10 day culture fluid of *M. tuberculosis* H37Ra bacilli grown in Soutan's media supplemented with thyroxin at concentration of 4 µg/ml [18] by precipitation with 50% ammonium sulphate and the soluble proteins were labelled as ESAS antigens [4,19]. ESAS antigen was then further fractionated by SDS PAGE (10%). The 2nd, 6th, 7th and 10th SDS-PAGE fractions from ESAS proteins were isolated and screened for antigenic activity. ESAS-6 antigen on fractionation on resource Q (1 ml) anion exchange column by FPLC, gave ESAS-6A containing 43 kDa protein [20]. ESAS-7 antigen on fractionation on resource S (1 ml) cation exchange column by FPLC, gave ESAS-7F (31 kDa) protein fraction having ES-31 antigen [21,22]. Isolation of ES-20 antigen was done by fractionation on FPLC using Superdex 200 HR gel filtration column followed by resource Q 1 ml anion exchange column [23]. For isolation of ES-6, the concentrated filtrate was fractionated by FPLC using Superdex HR 10/30 gel filtration column [24]. The 2nd SDS-PAGE fraction from ESAS antigen was isolated and labelled as ES-100 antigen [19]. Culture fluid ES proteins were precipitated with 6% trichloro acetic acid and the soluble antigens were further fractionated on SDS-PAGE (10%). EST 6 comprising ES-38 and ES-41 were recovered by electroelution [19].

Antibodies specific to the above mycobacterial antigens were raised in goats by immunizing them with respective antigen in incomplete Freund's adjuvant on day 0, 20, 33 and 45 intramuscularly. Immune sera were collected on day 32, 44, 57, 60 and thereafter fortnightly. Anti DSS IgG was isolated from immune sera by 33% saturation with ammonium sulfate followed by DEAE ion exchange column chromatography [25]. Further, antibodies specific to the antigens were isolated by sepharose column bound to the different antigens. The bound antibodies were eluted using 1 M acetic acid and neutralised with tris buffer (0.1 M, pH 8.6). The eluents were concentrated by ultra-filtration [20] and stored at -200°C till its use.

Penicillinase ELISA method was standardised for detection of specific antigens and antibody. In short, indirect ELISA for detection of antibodies in serum samples of pulmonary as well as extra pulmonary TB cases was performed using cellulose acetate membrane (CAM) sticks [20]. In brief 5 µl of optimally diluted specific antigens, anti-human IgG penicillinase conjugate and freshly prepared starch-iodine-penicillin-V substrate was used. Complete decolorizations or decolorization with slight tinge of substrate colour denoted a positive reaction. Similarly Sandwich ELISA was performed for antigens [20]. For convenience and bulk sample analysis, PVC microtitre plates were used. Briefly wells were sensitised with specific antibodies followed by incubation with optimally diluted human test serum, anti-MTB-antigen-antibody penicillinase conjugate and starch-iodine-penicillinase-V substrate. The disappearance of blue color at least 2 mins before the wells without antigen denoted positive reaction [20].

All these assays based on penicillinase ELISA, which is sensitive but semi-quantitative and subjective assay. Hence peroxidase ELISA, more user friendly and objective assay was further standardised for antigen

and antibody assays. However it requires 1 µg antigen/well as well as 1:50 serum dilution and with less sensitivity compared to penicillinase assay [25,26].

Diagnostically important excretory secretory (ES) proteins

Immune response to purified ES proteins and their utility in screening of suspected cases of tuberculosis has been extensively studied at JBTDR, Sevagram. From our laboratory different antigens have shown variable immune response namely ES-31 in chronic PTB cases, ES-43 in relapse cases and ES-41 in bone and joint TB which should be utilized for improved diagnosis [8,9,14]. Elevated ES-20 antigen level was observed in weak immune patients of TB lymphadenitis [27]. As antigen is better marker, detection of immune complexed antigen as well improved the sensitivity in detection of TB. Detection of antigen and immune complexed antigen using anti ES-31 antibody increased the sensitivity of the immunoassay [28]. Antigen assay was found to be more sensitive than antibody assay in immune compromised HIV infected sera [29].

A cocktail of antigens ES-31, ES-43 and ES-41 detecting antibody showed improved sensitivity (96%) in PTB cases [27]. A cocktail of affinity purified antibodies to antigens ES-31, ES-43 and EST-6 (ES-41+ES-38) showed a sensitivity of 91% for detection of antigen and 97% for IC-Ag with specificity of 95% and 99% respectively by sensitive penicillinase immune assay. Similar observations were made for detection of antibody and antigen in patients of extra pulmonary tuberculosis [30,31]. In a prospective study, the in house developed SEVA TB ELISA using cocktail of ES-31 and EST-6 antigens showed 100% correlation (42 cases) with AFB positivity and ATT treatment [32]. The study further showed importance of ELISA with 80% positivity in AFB negative TB but clinically diagnosed and ATT treated cases [32,33]. This can make an important contribution in confirming TB and correlating with clinical decision in smear negative TB cases in PTB and EPTB.

Detection of ES-100 Ag and antibody by penicillinase ELISA was found to be useful in detection of TB meningitis [33]. On the other hand, ES-31 and ES-43 antigens showed good immune response in chronic and relapse cases respectively while ES-6 antigen showed increased seroreactivity in household contacts of pulmonary tuberculosis patients. ES-6 antigen was shown to be useful in detection of latent infection for predicting active disease in course of time [27]. A newly developed peroxidase enzyme immunoassay was explored using cocktail of ES-31, ES-43 and EST-6 antigens for antibody detection and affinity purified antibody for antigen detection in tuberculosis patients at Kasturba Hospital [33]. This assay on prospective analysis showed 100% correlation of PTB and EPTB acid fast bacilli positivity and anti TB treatment in 11 cases [32]. The same cocktails of antigens and antibodies were explored and found significant correlation (91/95) with ELISA negativity and no ATT advised to clinically suspected bone and joint and abdominal etc. EPTB cases [26].

At 1000 bedded Kasturba Hospital, a tertiary healthcare hospital located in rural area, no commercial TB test kit is used. Detection of antigen, antibody and circulating free and immune complexed antigen is done for TB suspected patient on the request of the clinicians [34]. After unsuccessful response from the treatment with broad spectrum antibiotics in suspected cases of tuberculosis, clinicians prefer to send serum samples for the SEVA TB ELISA, and treat the patient accordingly. Thus SEVA TB ELISA has made an important

contribution in confirming TB and correlating with clinical decision in smear negative TB cases in PTB and EPTB [35]. Reason for failure of serology, is expectation that it should be similar to sputum smear or culture, the gold standard, in spite of limitations of its insensitivity in paucibacillary PTB and EPTB and decreased use in hospital setting for time constraint and delay in diagnosis. Antigen clearance by infected individual may also affect detection of antigenic markers. Therefore immune complexed antigen detection assays are also needed to be explored. Lastly the reasons for failure are lack of thorough evaluation and follow ups in hospital setting.

Our approach to immunodiagnostics cannot be mathematical in terms of percentage sensitivity and specificity but should also take a feedback from clinicians which were actually dealing with such cases. Here the clinical data suggests that cocktails of these antigens, assays of antibody, free and immune complexed antigens with ES proteins and specific antibodies in a hospital setting will definitely help in developing a successful immunodiagnostic test profile for tuberculosis.

Conclusion

Analysis of immune response in pulmonary and extra pulmonary TB cases is important in early diagnosis, prevention of spread of disease and its recurrence. Use of mycobacterial ES antigens and antibodies in immune screening of tuberculosis patients for over two decades at this centre, have shown that assay of immune response to specific antigen may be a potential marker for diagnosis as observed with ES-31 in pulmonary and some forms of extra-pulmonary TB, ES-41 in abdominal and bone and joint tuberculosis, ES-43 in relapse cases and ES-6 in contact cases. ES-31 was also found to be useful in immuno monitoring, assessing effectiveness of chemo therapy and patient's compliance. Cocktail of ES-31 and EST-6 antigens is in routine use on request of clinicians for diagnosis of TB in TB suspected patients attending Kasturba Hospital. However challenge there is a need for a large scale sample evaluation in multicentric study for confirming the usefulness of mycobacterial ES protein antigens in diagnosis, prognosis and prediction of disease development in tuberculous infection.

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