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Research Paper

Isolation and Evaluation of Diagnostic Potential of Tubercular ES-6 Antigen and Its Antibody

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Abstract: *An Excretory Secretory protein antigen of M.tbH_{37}Ra 6kDa (ES-6) was isolated from Mycobacterium tuberculosis H_{37}Ra culture filtrate and its purity was checked by SDS-PAGE. The antibody against M.tbH_{37}Ra ES-6 antigen was isolated using affinity chromatography. SEVA TB penicillinase ELISA was used to detect seroreactivity in TB and HIV-TB coinfection patients. The results had shown good sensitivity and specificity in relapse pulmonary TB and HIV-TB coinfection comparatively. It could be envisaged that isolated MtbH_{37}Ra ES-6 kDa antigen and its affinity purified ES-6 antibody could be explored and detect reactivated TB and TB infection in HIV-TB coinfected patients*

I. INTRODUCTION

TB is one of the oldest and widespread infectious disease in the developing countries affecting both the public health and economy. According to WHO global report 2010, in 2009 there were an estimated 9.4 million incident cases of TB , out of which 1.98 million were estimated to have occurred in India. Demonstration of the tubercle bacillus in clinical specimens by microscopy and cultural techniques is an indication of an active TB infection but unfortunately, microscopy is relatively insensitive and culture of M. tuberculosis on egg or agar media is slow and time consuming. Accurate detection and diagnosis of the infection is necessary to monitor the chemotherapy and prevent spread of the disease. Accordingly, there develops a considerable interest in the development of immunodiagnostic techniques based on humoral immune to detect TB infection. Over the years various mycobacterial antigens ranging from crude (culture filtrate, whole sonicates, PPD) to purified antigens (Antigens 5, glycolipid antigens, lipoarabinomannan, etc.) have been attempted and utilized for the serological diagnosis of TB. The group of secreted proteins had attracted special attraction to many workers, being more or less similar to the in *vivo* released mycobacterial antigens. The culture filtrate are the first protein to provide stimulus in vivo for the humoral response[1] and may be valuable in a serological test.[2] In the previous study, Andersen *et al*[3] had shown that proteins of low molecular weight 4-11kDa and 26-35kDa had a significant stimulatory properties of which ESAT-6 had been characterized as M. tuberculosis specific. In our Lab, the M.tbH_{37}Ra 31kDa antigen and its affinity purified anti ES 31 antibody was shown as a useful diagnostic potential in pulmonary TB. Hence, In this study an attempt was made to isolate MtbH_{37}Ra ES-6 antigen, a low molecular culture filtrate protein of 6kDa and its antibody and further evaluate its diagnostic potential by detecting tubercular antibody and antigen in Pulmonary TB, Extra-pulmonary TB and HIV-TB coinfection.

II. MATERIALS AND METHODS

The present prospective study was carried out in the department of Biochemistry during January 2009 to December 2010. TB Patients attending Kasturba Hospital, Sevagram (Tertiary Hospital) and District Tuberculosis Hospital, Wardha participated in the study. Blood samples were collected after taking informed written consent from 50 patients of pulmonary TB which were classified into fresh and relapse cases and 25 patients of extrapulmonary TB and HIV-TB coinfection following the Revised National Tuberculosis Control Programme (RNTCP) criteria. The study was commenced after obtaining clearance from the institutional Human ethical committee.

Fresh case - An individual who is freshly diagnosed with sputum microscopy by the presence of at least 1 AFB bacilli or an individual who had been given ATT for TB for less than four weeks.

Relapse- Relapse is defined as a patient cured under DOTS who had two sputum samples positive for AFB by direct smear, one smear and one culture positive from separate samples, or two cultures positive.

Extrapulmonary TB of 25 sera samples were subdivided into 6 different forms. Viz; Tubercular pleural effusion, Tuberculoma, Tubercular lymphadenopathy, pott's spine Abdominal TB, Genito-urinary TB.

Total 25 sera samples were collected from **HIV+ individual** and who developed TB during the course of HIV-progression.

Blood samples were also collected from 25 patients each of non tubercular diseases simulating pulmonary TB and extrapulmonary TB serving as disease control and from 25 healthy individuals of the locality as healthy control. The sera was stored separately in a plastic vial at -20^{o}c after adding 5% sodium azide as preservative.

2.1 Isolation of MtbH_{37}Ra ES-6Antigen and its antiES-6 antibody

The M.tbES antigen was isolated from the 10 days spent culture (Sauton medium) of exponentially growing tubercle bacilli as described by Lodam *et al*.[4] The bacilli were separated from the medium by filtrating with Whatman 3 filter paper. The culture filtrate was concentrated 100 fold by ultrafiltration (Millipore USA) using a membrane of 10-12 kDa molecular weight cut off followed by freeze drying for 24 hrs at 4^0C. The filtrate containing low molecular weight antigen (<10-12 kDa membrane) obtained was further concentrated in stirred cells (Millipore,USA) using a membrane of 1 kDa (YM1) molecular weight cut-off and was dialyzed extensively against 0.01M PBS (pH 7.2). The filtrate obtained was estimated for the protein concentration by Lowry's method[5] and labelled as M. tb ES-6 antigen. Antigenic activity was checked by Indirect ELISA using pooled positive and negative control sera and confirmation of its molecular weight is checked by SDS PAGE using a 15 % non gradient slab gel as described by Laemmli.[6] M. *tb* ES Ag (1000µg/500µl) was diluted with an equal volume (1:1) of SDS- sample buffer, mixture was run by stacking at a constant current of 20mA and separated at 25mA until the tracking dye (bromophenol blue) reaches the 12 cm length in the resolving gel. After the separation, following electrophoresis,

the gel was cut and stained in a coomasie blue (R-250) for about an hour. M.tuberculosis H_{37}Ra detergent soluble sonicate antigen (DSS) was used as an immunogen and was prepared from M.tbH_{37}Ra bacilli taken from 2 weeks –incubated thyroxine supplemented Lowenstein-Jensen slant.[7] Polyclonal antibodies against sonicate extract of M. tuberculosis H_{37} Ra strain were raised in goats by immunizing intramuscularly with 500µg protein/ml of DSS antigen in 4 equal doses with incomplete Fruend's adjuvant on days 0, 20, 33, and 45. The immune sera were collected on days 32,44,57,60 and thereafter fornightly and immunoglobin fraction was isolated with 33% saturation with ammonium sulphate. The IgG fraction was isolated from goat sonicated Ig by DEAE cellulose column chromatography. The IgG fraction was used for isolating anti ES-6 antibody by affinity chromatography using the sepharose 4-B beads coupled ES-6 antigen as described by Nair *et al*.[8]

2.2 SEVA TB Penicillinase enzyme immunoassay for anti ES-6 antibody and ES-6 antigen

Indirect ELISA was performed as described by Nair et al, to evaluate the reactivity of ES-6 antigen for the detection of tuberculous IgG antibodies in human sera**.** The first coating of isolated antigen showed reactivity at optimal concentration of 1ng/ml concentration by indirect ELISA using pooled positive tuberculosis and negative sera. Optimal diluted human sera (1:600) and antihuman IgG penicillinase conjugate (1:1000) were used. The disappearance of blue color substrate at least 5 minutes before the negative control denoted a positive reaction. Sandwich ELISA was carried out using anti-ES 6 affinity purified antibody to detect tuberculous antigen. Optimal concentration of the first coating anti ES-6 antibody to the CAM stick showed reactivity at 1µg/ml by Sandwich ELISA, using pooled positive tuberculosis and negative sera. Optimal diluted human sera (1:300) and anti-ES-6 penicillinase conjugate (1:1000 dilutions) in PBS/T were used. The disappearance of blue color substrate at least 5 minutes before the negative control denoted a positive reaction.

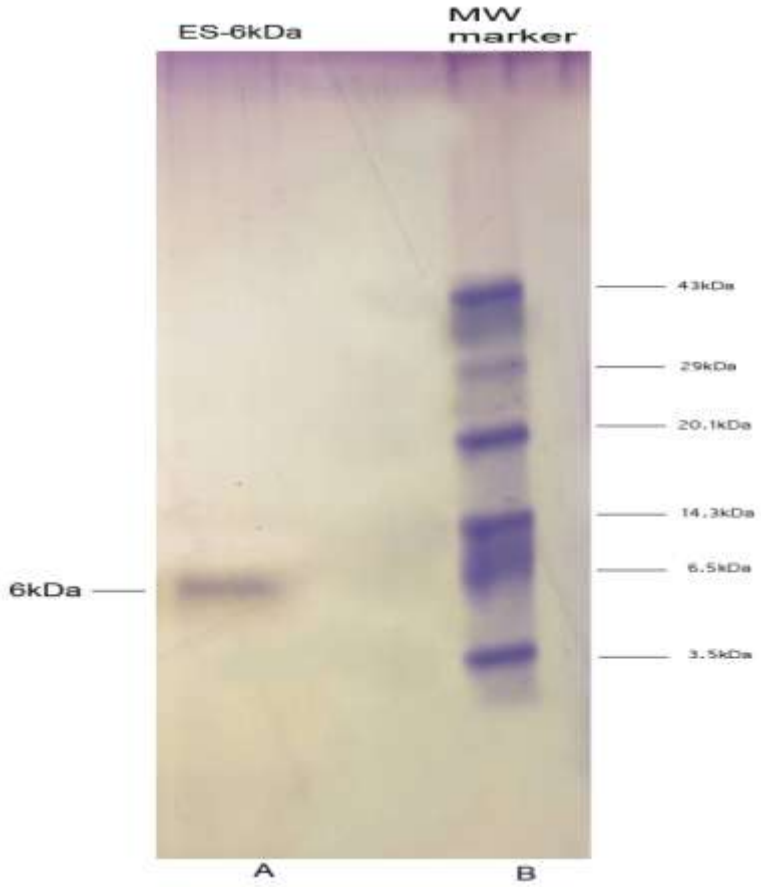
III. Results

In the present study, MtbH_{37}Ra ES-6 antigen and its anti ES-6 antibody were isolated and employed for the detection of tubercular antibody and antigen by Indirect and Sandwich ELISA. Sera dilution for the antibody and antigen assay was 1:600 and 1:300 respectively. The culture filtrate obtained using 1kDa (YM1) membrane concentration was labeled as low molecular weight containing ES-6 antigen and average yield in culture filtrate protein was found to be ~500 µg /2.2. When 30 µg of the antigen was run on SDS-PAGE, the protein was found to be a single band with 6 kDa molecular weight protein. The optimal concentration of the first coating antigen of ES-6kDa to the CAM stick showed reactivity at 1ng/ml concentration by indirect ELISA using pooled positive tuberculosis and negative sera. The percentage recovery of anti-ES 6 antibody protein using using ES-6 antigen coupled CNBr-activated sepharose 4B by affinity chromatography was found to be 32%. Optimal concentration of the first coating anti ES-6 antibody to the CAM stick showed reactivity at 1µg/ml by Sandwich ELISA, using pooled positive tuberculosis and negative sera. A total of 50 pulmonary TB cases including 25 each of fresh and relapse cases along with 25 diseases and 25 age, sex matched healthy controls were screened for antigen and antibody. 15 out of 25(60%) showed positive reaction for tubercular IgG antibody in fresh cases and 18 out of 25(72%) showed positive reaction in relapse cases whereas, 3 cases out of 25(12%) disease control and 2 cases out of 25(8%) showed positive reaction in healthy controls. Sensitivity was 88% while

specificity 70% in fresh case and in relapse case sensitivity was 90% while specificity 77%. The potential of detecting tubercular antigen in the sera samples of pulmonary TB were also explored in the study. In the assay for detection of antigen 15 out of 25(60%) in fresh cases and 19 out of 25(76%) in relapse cases showed positive reaction respectively. Whereas 4 cases out of 25(16%) disease control and 3 out of 25(12%) healthy control showed positive reaction. Sensitivity was 83% while specificity 70% in fresh case whereas, sensitivity was 88% while specificity 79% in relapse case. Out of 25 fresh TB cases, 17 were AFB smear positive, out of which 11(65%) were positive for antibody and 12(71%) were positive for antigen. While remaining 8 cases were found AFB smear negative, out of which 4(50%) were positive for antibody and 3(38%) were positive for antigen.

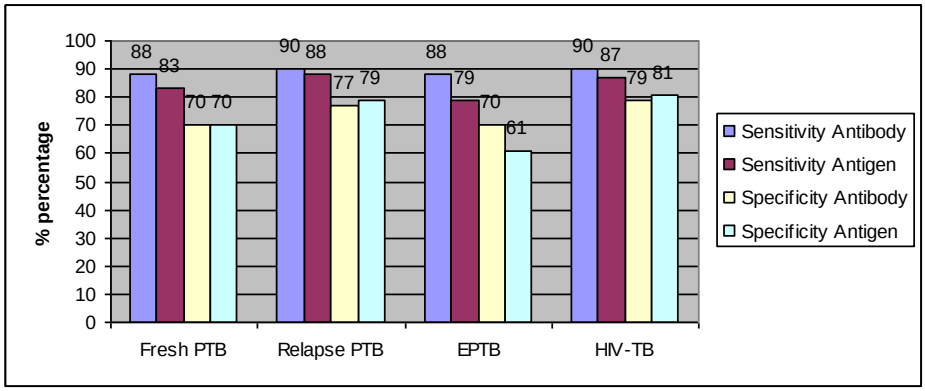
Sera samples from 25 cases of EPTB were taken for screening along with 25 cases suffering from other diseases mimicking EPTB cases and 25 age, sex matched healthy controls. In the detection of tubercular IgG antibody 15 out of 25(60%) showed positive reaction whereas 2 out of 25(8%) showed positivite reaction in both disease and healthy control. Sensitivity was 88% while specificity 70%. Potential of detecting tubercular antigen were also explored in the sera samples. In the assay for the detection of antigen assay 11 out of 25(44%) showed positive reaction whereas 4 out of 25(16%) disease control and 3 out of 25(12%) healthy control showed positive reaction. Sensitivity was 79% while specificity 61%. Total 25 sera samples of HIV-TB coinfection cases were screened for detection of antibody and antigen assay. The tubercular IgG antibody showed positive reaction, 19 out of 25(76%) showed positive reaction for HIV-TB coinfection. Whereas, 3 out of 25(12%) disease controls and 2 out of 25(8%) healthy controls were shown positive reaction. Sensitivity was 90% while specificity 79%. Potential of detecting tubercular antigen was also explored, 20 out of 25(80%) HIV-TB coinfection cases were shown positive reaction. Whereas 4 out of 25(16%) disease controls showed and 3 out of 25(12%) in healthy controls showed positive reaction respectively. Sensitivity was 87% while specificity 81%.

Fig 1. Sds page profile of es-6 kda antigen



Lane A: Purified M.tbH_{37}Ra ES-6kDa antigen (6kDa) **Lane B:** Molecular weight markers; Ova albumin (43kDa); Carbonic anhydrase (29kDa); Soya bean trypsin inhibitor (20.1kDa); Lysozyme (14.3kDa); Aprotonin, 6.5kDa; Insulin (3.5kDa)

Fig- 2 Efficacy of $MtbH_{37}Ra$ ES-6 antigen and anti ES-6 antibody



Eptb- Extra-pulmonary Tuberculosis, PTB-pulmonary tuberculosis

Table- 1 Percentage recovery anti $MtbH_{37}Ra$ ES-6 antibody from 1mg column bound ES-6 antigen by affinity chromatography

MtbES-6 Antigen bound to the column	AntiDSS antibody applied	AntiES-6 tubercular antibody recovered	Percentage of recovery	Total anti ES-6 antibody Recovered from 20 Batches by affinity chromatography
1 mg	1mg	0.320mg	32%	4.642mgs

Table- 2 Percentage Recovery of protein of $MtbH_{37}Ra$ ES-6 antigen from 1mg column bound anti ES-6 antibody by affinity chromatography

Anti-MtbES-6 antibody bound to the column	Concentrated Low molecular Filtrate applied	ES-6 antigen recovered	Percentage of recovery	Total ES-6 antigen Recovered from 20 Batches by affinity chromatography
1mg	1mg	0.16mg	16%	2.315mgs

Table- 3 Optimal Reactivity Concentrations Of Isolated Purified Fraction Of $Mtbh_{37}ra$ Es-6 Antigen By Indirect Elisa

Concentration of ES-6 antigen Coated	Time taken for Pooled TB positive Sera (minutes)	Time taken for Pooled TB negative Healthy normal sera (minutes)	Differences in time Between positive and negative (minutes)
10ng	22	24	2
5ng	18	20	2
3ng	20	23	3
1ng	20	25	5

Table- 4 Optimal reactivity concentrations of isolated $MtbH_{37}Ra$ anti ES-6 antibody by Sandwich ELISA

Concentration of Anti ES-6 antibody Coated	Time taken for Pooled TB positive Sera (minutes)	Time taken for Pooled TB negative Healthy normal sera (minutes)	Differences in time Between positive and negative (minutes)
10 µg	20	19	1
5 µg	19	16	3
4 µg	20	17	3
1µg	21	15	6

Table - 5 Seroanalysis for $MtbH_{37}Ra$ ES-6 antigen and anti ES-6 antibody in the detection of tubercular antibody and antigen in Pulmonary and Extrapulmonary TB cases

Sl no.	Case Group	No. of Sera Samples	No. of sera showing +ve reaction	
			Antibody*	Antigen**

1	Pulmonary TB	50		
	Fresh	25	15(60)	15(60)
	i) Sputum +ve	17	11(65)	12(71)
	ii) Sputum –ve	8	4(50)	3(38)
	Relapse	25	18(72)	19(76)
2	Extra-pulmonary TB	25	15(60)	11(44)
3	HIV-TB coinfection	25	19(76)	20(80)
4	Disease Control			
	Pulmonary TB	25	3(12)	4(16)
		25	2(8)	4(16)
	Extrapulmonary TB	25	3(12)	4(16)
	HIV-TB coinfection			
5	Healthy control	25	2(8)	3(12)

Cases	Sensitivity		Specificity		PPV		NPV		Likelihood Ratio		Fischer Exact Test	
	Ab	Ag	Ab	Ag	Ab	Ag	Ab	Ag	Ab	Ag	Ab	Ag
Fresh PTB	88	83	70	70	60	60	92	88	2.91	2.66	0.0002 p<0.05	0.0009
Relapse PTB	90	88	77	79	72	76	92	88	3.85	4.03	p<0.0001	p<0.0001
EPTB	88	79	70	61	60	44	92	88	2.91	2.02	0.0002	0.02
HIV-TB	90	87	79	81	76	80	92	88	4.37	4.69	p<0.0001	p<0.0001

*Sera showing positive reaction at 1:600 dilution
**Sera showing positive reaction at 1:300 dilution
Figure in parentheses denote percentage positivity

Table – 6 Evaluation efficacy

PTB- Pulmonary Tuberculosis
EPTB- Extra-pulmonary Tuberculosis
PPV- Positive predictive value
NPV- Negative predictive value
Ab- Antibody
Ag- Antigen

IV. Discussion

Tuberculosis (TB) continues to be a major public health problem.. Presently diagnosis of tuberculosis largely depends upon clinical, radiological, cytological and bacteriological examinations. Direct microscopy of sputum for bacilli is not sensitive and culture method is cumbersome and takes lengthy periods of time. Nucleic acid amplification tests is costly and requires specialized laboratory. Hence, Immunodiagnosis appears to be a promising approach for the diagnosis of TB infection. But due to low sensitivity of most of the serological tests isolation of a purified antigen for use in serological test is a major concern. Amongst, various mycobacterial antigens culture filtrate antigens are most extensively studied and several hundreds have been identified which replicate in liquid media.[9,10] Earlier studies in our laboratory had reported the potential of culture filtrate M.tbH_{37}Ra excretory secretory antigens in diagnosing pulmonary and extrapulmonary tuberculosis.[11,12,13] Thus, in the present study, isolation of MtbH_{37}Ra ES-6 antigen and its anti ES-6 antibody using affinity chromatography was performed and explore for the detection of antibody and antigen in pulmonary TB, extrapulmonary TB and HIV-TB coinfection. In the present study the diluent containing the low molecular weight ES-6 antigen was found to be 6kDa antigen when run in 15% non gradient SDS- PAGE. Several studies done before also used SDS-PAGE to separate low molecular proteins with antigenic properties. Sharon *et al* [14] also showed separation of at least 10 bands including 6kDa protein using Tris-Triscine SDS-PAGE ranging from 2kDa to 14kDa in 6-8 weeks culture filtrates proteins of Mycobacterium spp having antigenic properties. The use of affinity chromatography had helped in an increased recovery of proteins and obviates the need for time consuming conventional purification steps. In the present study, affinity chromatography was used to isolate the anti-ES-6 antibody using ES-6 bound sepharose-4B-column. The percentage protein recovery of anti-ES 6 antibody was 32%. In the study analysis of sera from pulmonary tuberculosis patients detection of tubercular IgG antibody in fresh cases showed 60%(15/25) positive reaction. Sensitivity was 88% while specificity 70%. The PPV&NPV for IgG detection were 60% and 92% respectively whereas in detection of tubercular antigen 60%(15/25) showed positive reaction. Sensitivity was 83% and while specificity 70%.

The PPV & NPV were 60% and 88%. The detection of tubercular antibody and antigen assay was also explored in relapse case of pulmonary TB. The detection of tubercular antibody showed 72%(18/25) positive reaction. Sensitivity was 90% while specificity 77%. The PPV & NPV were 72% and 92%. In the detection of tubercular antigen 76%(19/25) showed positivite reaction. Sensitivity was 88% while specificity 79%. The PPV & NPV were 76% and 88% respectively. Silva *et al*[15] have shown that low molecular weight ESAT-6 and 14 kDa were seroreactive in inactive rather than active TB infection and may be associated with reactivation of the disease. Importantly, a recent study in Canada that compared antibody responses to ESAT-6, the 38-kDa antigen and a 14-kDa antigen (*Rv0455c*) in patients with a spectrum of TB-related conditions, showed that ESAT-6 was associated with risk factors for future active disease, suggesting the potential of this antigen as a marker for latent or reactivation of TB.[15] A report by Imaz *et al*[16] had shown that 14kDa a low molecular weight when compare antibody responses before and after treatment suggested that higher levels of antibody responses against this antigen were associated with active TB (newly acquired or relapse). Our study co-relates with the finding that ES-6 antigen and its antibody showed better response in the detection of relapse case of TB in comparison with fresh pulmonary TB and this antigen could used in detecting reactivation of TB. Various studies done before using low molecular antigens have been shown be similar to our study. In the study, the isolated ES-6 antigen and its affinity purified antiES-6 antibody were also explored for detection of tubercular antibody and antigen in extra-pulmonary tuberculosis (EPTB). Out of 25 cases of EPTB, 60% (15/25) showed positive reaction for tubercular IgG antibody. Sensitivity was 88% while specificity 70%. The overall PPV & NPV were 60% and 92%. In the detection of tubercular antigen 44%(11/25) cases showed positive reaction. Sensitivity was 79% while specificity 61%.

The PPV&NPV were 44% and 88% respectively. Our study in EPTB cases showed low positivity in both antibody and antigen detection. Earlier study in our Lab on low molecular weight antigen, ES-20 antigen and its antibody showed sensitivity of 83% and 80% in pulmonary TB and 90% in lymph node TB.[17] Wilkins and Ivanyi[18] had reported sensitivity of 81% using 38kDa In TBLN and in patients with extra-pulmonary TB using 16kDa antigen a low molecular antigen sensitivity of 56%, 65% and 43% were obtained from bone and joint, kidney, lymph node and pleural TB respectively whereas the specificity was 98%.[19] The spread of HIV infection in the population that has the highest TB incidence is making an increasing contribution of TB cases worldwide. It has created a need for the test that that can identify co-infected individual at a risk of progression of TB. In the present study, detection of antibody using the ES-6 antigen and antigen using the affinity purified anti ES-6 antibody was explored in sera of 25 TB patients with HIV co-infection. In the detection of tubercular IgG antibody 76% (19/25) showed positive reaction. Sensitivity was 90% while specificity 79%. The overall PPV & NPV were 76% and 92%. Detection of tubercular antigen showed 80%(20/25) positive reaction. Sensitivity was 87% while specificity 81% and with PPV& NPV of 80% and 88% respectively. Our study showed varied seroreactivity in antibody and antigen detection in HIV-TB coinfected cases. The antibody detection showed good sensitivity but low specificity whereas the antigen detection showed good level of sensitivity and

specificity. Weldingh *et al*[20] analysed the serodiagnostic potentials of TB9.7(Rv3354) and TB16.3(2185c) and showed that the sensitivities of antigens ranges from 31% to 93% and with a specificity of at least 97% on panels of sera from sputum smear-positive and sputum smear-negative TB patients from TB endemic and non-endemic areas coinfected with HIV. Krishna *et al*[21] had shown that the difference in antigens response in sputum positive TB, HIV-negative and HIV-positive TB patients was the lack of cavitary lesions in the latter group and the expression and replication of bacteria is possibly enhanced in the cavitary environment. The main possible reason for good antigen detection in the study could be that this particular antigen could detect TB prior to the cavitary formation in the alveoli and this hypothesis is further enhanced by the observation of ~75% of antiES-6 antibody detection in the sera samples. In support of this, another Cohort studies done in the USA, India, Uganda, South Africa and Brazil indicated that theMTB81(88kDa) antigen is immunodominant in HIV-TB co-infected patient and these study also indicates that MTB81 is present in HIV-TB coinfected patients prior to manisfestations of clinical TB.[22] The outcome of our study showed that ES-6 antigen and its antibody can be preferentially recognized by serum antibodies and antigen in the detection of relapse pulmonary TB case and The study also showed this particular antigen could also be used to detect TB infection prior to clinical manisfestations of TB in HIV-TB coinfected patients. But, the study limits itself and needs expansion by increasing number of cases and controls to claim its promising diagnostic potential.

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