Original Article

Efficient ELISA for Diagnosis of Active Tuberculosis Employing a Cocktail of Secretory Proteins of *Mycobacterium tuberculosis*

(tuberculosis / culture filtrate proteins / ELISA / MDR / secretory antigens / serodiagnosis)

D. TIWARI[1,5,6], R. P. TIWARI[2], R. CHANDRA[1], P. S. BISEN[3], S. HAQUE[4]

[1]Dr. B.R. Ambedkar Centre for Biomedical Research, University of Delhi, Delhi, India
[2]Department of Biotechnology, Immunodiagnostic Division, Aspen Laboratories Pvt. Ltd., Delhi, India
[3]School of Studies in Biotechnology, Jiwaji University, Gwalior, MP, India
[4]Department of Biosciences, Jamia Millia Islamia (A Central University), New Delhi, India
[5]School of Chemistry & Physics, [6]Catalysis and Peptide Research Unit, School of Health Sciences, University of KwaZulu-Natal, Durban, South Africa

Abstract. Rapid and accurate diagnosis is important for preventing transmission of *Mycobacterium tuberculosis*. Currently available tuberculosis (TB) diagnostic methods lack desired sensitivity and specificity, and require sophisticated equipment and skilled workforce including weeks' long duration to yield results. In this study, extracellular proteins or secretory protein antigens of *M. tuberculosis* H37Rv have been isolated using ion exchange chromatography, immunocharacterized and exploited for the development of efficient enzyme-linked immunosorbent assay (ELISA) for diagnosis of active TB with enhanced specificity and sensitivity. Apparent molecular masses for purified proteins were found to be 6, 27, 30, 38 and 64 kDa. Out of five purified proteins, one (64 kDa) was found to be novel. Of the five proteins, four (6, 27, 30 and 38 kDa) were found significant to be used in the development of ELISA for pulmonary and extra-pulmonary TB. The immune responses of serum samples of TB patients and other healthy subjects against the above-mentioned antigens' cocktail were evaluated. Critical parameters of newly developed ELISA were optimized and it was observed that the cocktail antigens have a greater specificity (98.06 %) and sensitivity (98.67 %) as compared to other commercially available diagnostic tests. The present findings suggest that the developed ELISA is an effective tool for routine screening and early-stage diagnosis of TB.

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Corresponding author: Shafiul Haque, Department of Biosciences, Jamia Millia Islamia (A Central University), New Delhi-110025, India. Phone & Fax: +91-11-26988335; e-mail: shafiul.haque@hotmail.com

Abbreviations: AFB – acid fast bacilli, BCG – Calmette-Guerin bacillus, BSA – bovine serum albumin, CFPs – culture filtrate proteins, DAB – diaminobenzidine hydrochloride, ELISA – enzyme-linked immunosorbent assay, HRP – horseradish peroxidase, HIV – human immunodeficiency virus, IFA – incomplete Freund's adjuvant, IG – immunoglobulin G, LAM – lipoarrabinomannan, MDR-TB – multidrug-resistant TB, NTM – non-tuberculous *Mycobacteria,* OD – optical density, PBS – phosphate-buffered saline, PBST – phosphate-buffered saline-Tween, SD – standard deviations, SDS-PAGE – sodium dodecyl sulphate-polyacrylamide gel electrophoresis, TB – tuberculosis, TMB – 3,3',5,5'-tetramethylbenzidine, XDR-TB – extensively drug-resistant TB.

Introduction

One-third of the world's population is estimated to be infected with *Mycobacterium tuberculosis* and about eight million new cases of tuberculosis (TB) arise each year (WHO, 2012). India alone has nearly one third of the total global burden of tuberculosis, and the disease is one of the India's most important public health problems (WHO, 2012). In the last few years, there has been an increase in the occurrence of the disease mainly due to the emergence of multidrug-resistant strains of *M. tuberculosis* (MDR-TB) and its association with the human immunodeficiency virus (HIV) infection. Poor sensitivity, lengthy procedure and slow speed of existing diagnostic tools prolongs the diagnosis and treatment of active TB, and diagnosis of extra-pulmonary and pulmonary forms are often especially challenging and emphasize the need for effective prevention and treatment of the disease (Abebe et al., 2007; Tiwari et al., 2007b; Al-Zarouni et al., 2010). Given that *M. tuberculosis* in-

fection is a prerequisite for active TB, reliable determination of the infection status could accelerate diagnostic assessment by enabling rapid exclusion of TB. Early diagnosis of the disease is the prime concern; hence, several studies addressing the issue have been conducted in the past and many are still in progress. Several novel and improved diagnostic approaches have been discovered and they have made a dramatic effect on diagnosing the disease accurately and expeditiously (Garg et al., 2003; Tiwari et al., 2007a, b; Garberi et al., 2011; Suhail, 2011).

Various serodiagnostic tests have been discovered in the last few years (Grange and Laszlo, 1990; Rebeski et al., 1999; Garg et al., 2003; Young et al., 2004; Tiwari et al., 2005; Abebe et al. 2007; Shin et al., 2008) and several researchers have attempted to isolate the species-specific antigen for diagnostic tests (Grange and Laszlo, 1990; Griffin et al., 1991; Daleine, 1995; Jones et al., 1998). However, the task has proved to be tedious because specific antigenic determinants often occur on the same protein molecule as the shared antigen, therefore making it difficult to purify by sensitive techniques, viz., affinity chromatography, etc. Many times an antigenic determinant may be shared among different species of genera *Mycobacterium*, causing incorrect diagnosis of *M. tuberculosis*. Additionally, the given antigenic determinant may be present on a range of molecules of differing physiochemical properties. Thus, preparative techniques such as gel filtration, ion exchange chromatography, etc., have not proved useful (Grange, 1998a, b). Hence, a rapid diagnostic test for the detection of antigen(s) in patients with TB may be the best choice for diagnosis of the disease, as false-positive results have been observed in antibody-based diagnostic tests due to the exposure to environmental non-tuberculous *Mycobacteria* (NTM) or prior Calmette-Guerin bacillus (BCG) vaccination (Fine, 1995). Therefore, the ultimate goal is to minimize the public health burden of TB by developing a sensitive, improved, specific and cost-effective diagnostic tool to combat the menace of the disease.

Secretory protein antigens or extracellular proteins of *M. tuberculosis* appear as culture filtrate proteins (CFPs) in the culture medium in which *M. tuberculosis* is grown, and the mechanism of CFP secretion is still unclear. According to the reports, there are approximately 200 proteins found in the culture filtrate of *M. tuberculosis* (Andersen et al., 1991; Anderson, 1994; Weldingh et al., 1998; Kamath et al., 1999; Kanaujia et al., 2004; Spencer et al., 2004; Young et al., 2004; Sani et al., 2010; Volkman et al., 2010) and some of these proteins are associated with bacterial cells; therefore, the definition of CFP is an operational one. Extensive efforts to devise a sensitive and specific serodiagnostic TB test exploiting CFPs have been made by researchers at several laboratories (Gupta et al., 2005; Harinath et al., 2006; Shin et al., 2008; Beyene et al., 2010; Kumar et al., 2010; Wu et al., 2010). The most promising results for serodiagnosis of TB were obtained with the use of the 38 kDa PhoS protein of *M. tuberculosis*, which provides very high (~98 %) specificity (Wu et al., 2010). However, the sensitivity with this antigen varied from 45 to 80 % for different cohorts, and studies have shown that anti-38 kDa protein antibodies are present primarily in patients with advanced, recurrent, and chronic disease (Daniel and Debanne, 1987; Espitia et al., 1989). Hence, the present study was undertaken with the aim to identify the major immunodiagnostic antigens of *M. tuberculosis* H37Rv for the development of an effective diagnostic tool using a cocktail of secretory protein antigens (CSPs-Ag's) of *M. tuberculosis* with enhanced sensitivity and specificity.

Material and Methods

Bacterial strain, chemicals and kits

M. tuberculosis H37Rv (ATCC 27294) strain was obtained in frozen form (2×10^9 cfu/ml) from National JALMA Institute of Leprosy & Other Mycobacterial Diseases, Agra, India, on Lowenstein-Jensen (L-J) slant. The bacterial strain was grown in 1 liter of Sautons' media at 37 °C for 50 days until late log phase. The bacterial growth pellet was collected by centrifugation (10,000 *g*, Beckman Coulter Inc., Brea, CA) for 20 min at 4 °C and the pellet was further washed twice with 100 ml of phosphate-buffered saline (PBS, pH 7.2). Harvested bacterial cells were resuspended in physiological saline and opacity was adjusted to 1.0 McFarland standard. Strains were stored in 1.0 ml microfuge tubes at -70 °C. All the chemical reagents, biochemicals and media components were purchased from HiMedia laboratories (Mumbai, India), RFCL (New Delhi, India), Bio-Rad Laboratories Inc. (Hercules, CA), Bangalore Genei (India) Private Ltd., (Bangalore, India) and Sigma-Aldrich Corp. (St. Louis, MO).

For the comparative evaluation studies of the newly developed CSP-Ag's ELISA, two commercial kits, i.e., KP-90 ELISA and MycoDot LAM test kits, were purchased from Kreatech (Amsterdam, The Netherlands) and Span Diagnostics (Surat, Gujarat, India), respectively.

Details of the subjects included in this study

A total of 1177 sera samples from Indian patients were collected from Ganghi Medical College, Bhopal, India; TB Hospital, Bhopal, India; DOT Center Hoshungabad, MP, India; S.S. Medical College, Rewa, MP, India; and GTB Hospital, Delhi, India. These 1177 serum samples were divided into four groups (Table 1). A total of 440 serum samples were included from individuals confirmed for active pulmonary TB (group I). All (N = 440) samples were divided into the following subgroups for clinical and laboratory diagnosis., i.e., smear-positive [acid fast bacilli (AFB)-positive] and culture not done (n = 150), smear-positive and culture-positive (these samples were collected from patients admitted to hospital prior to commencement of the treatment)

Table 1. Detailed analysis of the cocktail of secretory protein antigens (CSP-Ag's) with serum samples of pulmonary tuberculosis (Internal studies), extra-pulmonary TB group, normal healthy control subjects (Non-TB group), and other respiratory and common disease group (Internal studies).

Sr. No.	Subjects included in this study	No. of serum samples tested	Category	CSP-Ag's Results		Evaluation parameters of CSP-Ag's
				+Ve	-Ve	
	Pulmonary TB (group I)					**Sensitivity %**
1		150	Smear +ve and culture not done	148	2	98.67%
	Adult TB (N = 250);	50	Smear +ve/Culture +ve	49	1	98.00%
	Children TB (N = 50);	50	Smear -ve /Culture +ve	48	2	96.00%
	HIV co-infected (N = 40);	130	Smear -ve /BACTEC-460 +ve	129	1	99.23%
	MDR-TB (N = 100)	25	PCR +ve /Culture & smear –ve	24	1	96.00%
		35	BACTEC–460 +ve /Smear +ve	34	1	97.14%
Total	**--**	**440**	**--**	**432**	**8**	**98.18%**
	Extra-pulmonary TB (group II)		**--**			
2	Tuberculosis lymphadenitis	46	Clinically +ve/Culture & smear +ve	44	2	95.66%
3	Bone and joint tuberculosis	59	No clinical symptoms/Culture & smear +ve	57	2	96.62%
4	Abdominal tuberculosis	15	Clinically +ve/Culture & smear +ve	14	1	93.34%
5	Tuberculosis pericarditis	35	Culture & smear +ve	34	1	97.15%
6	Genitourinary tuberculosis	28	Clinically +ve/Culture & smear +ve	27	1	96.43%
7	Pleurisy	16	Clinically +ve/Culture & smear +ve	15	1	93.75%
Total	**--**	**199**		**191**	**8**	**95.98%**
	Non TB (group III) (Negative control group)		**Clinical status of the subjects**			**Specificity %**
8	Normal healthy subject	50	Clinically healthy	--	50	100%
9	BCG vaccinated	150	Clinically –ve	2	148	98.67%
10	BCG unvaccinated	50	Clinically –ve	2	48	96.00%
11	Drug-treated clinically –ve	25	Cured TB patients (clinically healthy)	2	23	92.00%
Total	**--**	**275**	**--**	**6**	**269**	**97.82%**
	Other respiratory & common diseases (group IV)		**Sample history**			
12	Lung cancer	32	Symptoms	1	31	96.88%
13	Pulmonary fibrosis	12	Lab analysis	--	12	100.00%
14	Bacterial pneumonia	15	Gram staining	1	14	93.34%
15	Bronchitis	10	Swab culture	2	8	80.00%
16	Common cold/Bronchial asthma	15	Symptoms	--	15	100.00%
17	Other mycobacterial diseases (Leprosy)	50	Clinically & PCR	3	47	94.34%
18	Malaria Pv/Pf	30	Slide/Rapid Test	--	30	100.00%
19	HIV	60	ELISA/Western blot	1	59	98.34%
20	Hepatitis B/C	25	ELISA/Western blot	--	25	100.00%
21	Rheumatoid arthritis	14	Symptoms	--	14	100.00%
Total	**--**	**263**	**--**	**8**	**255**	**96.96%**

(N = 50), smear-negative and culture-positive (samples of patients with chronic TB having history of past chemotherapy) (N = 50), PCR-confirmed positive, smear-negative and culture-negative (samples from these patients were negative by smear and culture but having symptoms of TB with mycobacteria in their sputum samples and confirmed positive with PCR) (N = 25), smear-negative and BACTEC-460-positive (samples of these patients were negative by smear but confirmed positive with BACTEC-460 instrument) (N = 130), and BACTEC-460- and culture-positive (samples of these patients were reported clinically positive; some of them were having latent *M. tuberculosis* infection) (N = 35). Extra-pulmonary TB group (group II) contained 199 patients' samples [tuberculosis lymphadenitis (N = 46), bone and joint tuberculosis (N = 59), abdominal tuberculosis (N = 15), tuberculosis pericarditis (N = 35), genitourinary tuberculosis (N = 28), and pleurisy (N = 16)] and their clinical status was confirmed through smear and tissue biopsy analysis (Table 1).

The negative control group or non-TB group (group III) incorporated 275 samples from subjects with other infections, such as patients with drug-treated and clinically negative TB (N = 25), healthy household contacts of TB patients (N = 50), BCG-vaccinated (N = 150) and BCG-non-vaccinated individuals (N = 50). Two hundred sixty-three serum samples (group IV) were included for specificity study from individuals with respiratory

and non-respiratory diseases [lung cancer (N = 32), pulmonary fibrosis (N = 12), bacterial pneumonia (N = 15), bronchitis (N = 10), common cold/bronchial asthma (N = 15), other mycobacterial diseases (leprosy) (N = 50), malaria Pv/Pf (N = 30), HIV (N = 60), hepatitis B/C (N = 25), and rheumatoid arthritis (N = 14)] and none of the subjects were found to have clinical features of TB.

Bacterial cultivation, isolation and profiling of CFPs

M. tuberculosis H37Rv (~10 mg) was grown in 250 ml of Sauton's medium at 37 °C in stationary conditions for 5–6 weeks (until mid-log phase). The bacterial growth was monitored by measuring absorbance at optical density $(OD)_{580}$ nm against 10 mM phosphate buffer (pH 7.2). Six-week grown *M. tuberculosis* H37Rv was used for isolation of proteins excreted in the culture filtrate. Mycobacterial cell mass was collected after every 48 h by centrifugation and stored at -20 °C. Different batches of CFPs were subjected to ammonium sulphate precipitation. Different concentrations of saturated ammonium sulphate (50, 75 and 80%) were tested for obtaining maximum yield of proteins from the culture filtrate. Finally, the culture supernatant was precipitated with 80% saturated ammonium sulphate at 4 °C overnight and then centrifuged at 12,000 *g*, 18,000 *g*, 28,000 *g*, and 90,000 *g* for 30 min each at 4 °C. Afterwards, the pellet was collected, dissolved in 10 mM phosphate buffer (pH 7.2) and subjected to dialysis (3 kDa cut-off filter) against 10 mM PBS (pH 7.2) at 4 °C overnight. Buffer was changed after every 4 h and it was changed 6–7 times to ensure complete dialysis. Total CFP content was estimated by the Bradford method (Bradford, 1976; Daniel and Debanne, 1987) and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (MiniProtean3, Bio-Rad Laboratories Inc.) analysis following the protocol of Laemmli (1970).

Protein purification and selection of immunoreactives

Individual proteins from the total CFPs were purified following the method of Nagai et al. (1991) using DEAE-Sepharose CL-6B (anion-exchange) equilibrated with 10 mM Tris-HCl buffer containing 3% methylcellulose. Concentrated CFPs were dialysed against Tris buffer and loaded (100 mg) on the equilibrated column. The column-bound proteins were eluted by 50–300 mM NaCl linear gradient prepared in equilibration buffer and absorbance was measured at OD_{280} nm. The purified proteins were concentrated using Amicon unit (3 kDa cut-off filter) and dialysed against PBS at 4 °C overnight. Protein content in each pooled fraction was estimated as stated earlier (Bradford, 1976) and profiling was done by preparative SDS-PAGE. Proteins present in different peaks were isolated in pure form through elution by cutting the individual protein bands from the preparative SDS-PAGE gel and keeping at 4 °C overnight in Tris buffer followed by silver staining. Protein-containing buffer was aspired and subjected to dialysis. Individual purified protein samples were then subjected to SDS-PAGE.

Production of polyclonal antibodies against CFPs

Polyclonal antibodies were raised in rabbits against total CFPs of *M. tuberculosis* strain H37Rv. Antigen mixture was prepared by adding 1 mg protein in 2 ml PBS (pH 7.5), and emulsified thoroughly with incomplete Freund's adjuvant (IFA, Difco Laboratories Inc., Detroit, MI). Immunogen preparation was performed by double hubbed needle method, and it was obtained by mixing equal volumes of reactive immunogens with IFA. The emulsion was tested before its final use for its intact nature by putting one drop of emulsion over water. Five rabbits of 6 months of age were immunized subcutaneously (500 µl/ rabbit) and boosted on 14th and 28th day with IFA to enhance the antibody titre. The animals were bled for sera collection after 14 days after the last dose. Rabbit anti-serum (5 ml) was diluted to 50 ml in 0.1M Tris-HCl (pH 8.2) and loaded on pre-packed protein-A sepharose column XK16/20 (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with Tris-HCl buffer (pH 8.2). Unbound proteins were washed with Tris-HCl and antibodies bound to the affinity column were eluted with 0.1M glycine-HCl buffer (pH 2.5) with flow rate of 1 ml/min into 4 ml fractions. Eluted fractions were adjusted to pH 7.2 using saturated Tris-HCl buffer (pH 9.6). The protein content and purity of each fraction was monitored at OD_{280} nm. Fractions containing purified immunoglobulin G (IgG) antibody were pooled and dialysed against PBS buffer (pH 7.5) for 24 h (with two buffer changes at equal time intervals). Protein content was estimated (Bradford, 1976) and antibody solution was lyophilized and stored at 4 °C.

Western blotting

The purified proteins were run on 12 and 15% SDS-PAGE and then the electrophoresed proteins were transferred to nitrocellulose membrane by trans-blotting. Blotting was conducted under constant electric current of 100 mA for 2 h. Finally, the blots were washed with PBS and soaked for 5–10 min in 0.2% of Ponceau S solution in PBS. A nitrocellulose sheet was transferred to PBS and rinsed for 2 min with several changes of PBS. The membrane was blocked in blocking buffer (PBS with 0.1% Tween-20 and 5% casein digest) overnight at 4 °C over an orbital shaker and then incubated in 10 ml 1 : 50 dilution of primary antibody/serum in dilution buffer (PBS with 0.1% Tween-20) containing 5% casein digest for 2 h at room temperature. At the end of incubation, the membrane was washed five times for 5 min with 50 ml of PBS with 0.1% Tween-20. HRP-anti-human IgG conjugate (Dako Corp., Carpinteria, CA) was diluted 1 : 2500 in dilution buffer (PBS with 0.1% Tween-20) containing 5% casein digest and the mem-

Table 2. Critical parameters considered for ELISA development

Sr. No.	ELISA optimization parameters		Tested conditions
1	Coating buffer	Type	Phosphate & sodium carbonate/ bicarbonate
		pH	7.2 & 9.5
		Ionic strength	10 mM, 50 mM, 100 mM & 200 mM
2	Blocking agent	Type	Normal rabbit serum, bovine serum albumin (BSA) & casein
		Strength	1.0 % of any one of them
		Temperature	37 °C
		Time period	3 h
3	Coating of antigens	Concentration	2–5 µg/well
		Volume	200 ml
		Incubation period	3, 5, 12 and 24 h & 0.1, 2, 3 and 4 h
		Temperature	37 °C
4	Conjugate	Type	Goat anti-human IgG-HRP (Horseradish peroxidase)
		Stoppage time for activity	Ratio of OD_{450} nm at 02, 03, 05, 10, 15, 20, 25 & 30 min

brane was incubated for 2 h at room temperature. At the end of incubation, the membrane was washed five times for 5 min each with 50 ml of washing buffer and finally washed in 10 ml of 10 mM Tris-Cl (pH 7.5) for 5 min. The blots were then developed with 0.8 mM diaminobenzidine hydrochloride (DAB, Sigma-Aldrich Corp.) in the presence of 0.045% H_2O_2 and 0.4 mM nickel chloride for 10–20 min until clear bands were visible. The membrane was kept in PBS to stop the reaction.

Standardization of critical parameters of ELISA

Critical parameters were optimized for the developed ELISA and their details are mentioned in Table 2.

Cocktail antigen ELISA system

The optimal concentration of each antigen, dilution of sera and dilution of labelled goat anti-human IgG conjugate were determined according to the Phalanx titration principle. Combinations which gave the highest signal/noise (S/N) ratio were determined as 'optimum' and the S/N ratios were determined by the ratio of the OD values of the positive control sera to those of the negative control sera at each combination. Polystyrene flat-bottomed microtitre plates (Costar, Cambridge, MA) were coated with either of the five antigens with a concentration of 2 mg/ml and 10 mg/ml lipoarrabinomannan (LAM) (Jiangyin Bio-science Inc., Jiangyin, PR China) diluted in 0.05M carbonate-bicarbonate buffer (pH 7.2), and incubated at 4 °C overnight and washed thrice with washing buffer (phosphate-buffered saline-Tween, PBST) containing 0.05% Tween-20. Further, the plates were kept at 37 °C for 2 h in PBST (pH 7.4) with 3% bovine serum albumin (BSA) also called as 'fraction V' for blocking, washed thrice, and 100 µl of serum (diluted 1 : 100 in PBST buffer, pH 7.4) was added to each well. All samples were tested in triplicate. The plates were incubated at 37 °C for 1 h, washed thrice with washing buffer, and filled with 200 µl of a 1 : 5000 dilution of anti-human-IgG HRP (Sigma-Aldrich Corp.). Afterwards, the plates were kept at 37 °C for 1 h in a water-bath and washed five times, and 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (0.04% TMB, 0.04% urea-peroxide in 0.1M sodium acetate-citric acid buffer, pH 4.0) was added. Subsequently, the plates were kept in the dark for 10 min at room temperature, the reaction was stopped by adding 50 µl of 2M H_2SO_4/well, and absorbance was measured at OD_{450}.

Statistical analysis

The sensitivity and specificity of the assay was calculated as described by Toman (1981) by comparing the results, including patient's history, clinical symptoms, pathological analysis of *M. tuberculosis* infection by PCR and BACTEC-460, and drug administration status.

$$\text{Sensitivity} = \frac{\text{True Positive (TP)} \times 100}{\text{True Positive (TP)} + \text{False Negative (FN)}}$$

$$\text{Specificity} = \frac{\text{True Negative (TN)} \times 100}{\text{False Positive (FP)} + \text{True Negative (TN)}}$$

Mean OD values and standard deviations (SD) were calculated using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, version 16.0.2). Sensitivity is defined as the percentage of individuals in the true-positive group who showed OD values higher than the cut-off value, whereas specificity is defined as the percentage of individuals in the true-negative group who showed lower ELISA values than the cut-off value. The evaluation of positive sera for cocktail-antigen ELISA was based on a positive score derived from the OD values above the cut-off point. The cut-off value was calculated from the mean OD value + 0.2, which was derived from SD taken from the samples of the healthy control group.

Results

Profiling of mycobacterial extracellular proteins

Proteins released into the culture medium from actively growing and lysed mycobacterial cells during different growth phases were investigated over 50 days by determining the total protein yield in the culture filtrate by SDS-PAGE of the samples collected at different time points. The apparent molecular weights of the purified CFPs were 6, 27, 30, 38 and 64 kDa (Fig. 1a,b).

Selection of immunoreactive antigens using TB patients' sera

Western blot analysis of five purified proteins with sera of confirmed TB patients showed that out of the five purified antigens, significant amounts of antibodies were present against 27 and 64 kDa proteins in the sera of the tested patients (Fig. 2). The antibodies were also seen against other antigens in the sera of some patients but not in all tested patients, and in some cases antibodies were present in low count. To develop an efficient ELISA assay for diagnosing the TB patients' sera, a cocktail of purified antigens was also tested along with individual antigen (Fig. 3). The ELISA conditions were optimized for effective detection of antibodies in the sera of TB patients. Further, the serodiagnostic potential of the above-mentioned five purified antigens was also tested for the developed ELISA as a cocktail for specificity and sensitivity using the sera from TB patients. Out of the five antigens, a combination of four antigens, i.e., 6, 27, 30 and 38 kDa, showed enhanced sensitivity and specificity. The 64 kDa antigen was eliminated from the cocktail because of a low signal to noise ratio.

Optimization of ELISA parameters

(i) Blocking agents

In order to enhance the specificity of the developed ELISA, various blocking agents were tested, such as BSA, casein digest and normal rabbit serum. Among the tested agents, 1% casein digest was found to be the best suited blocking agent that gave around zero noise level and showed 100% specificity with process feasibility (data not shown).

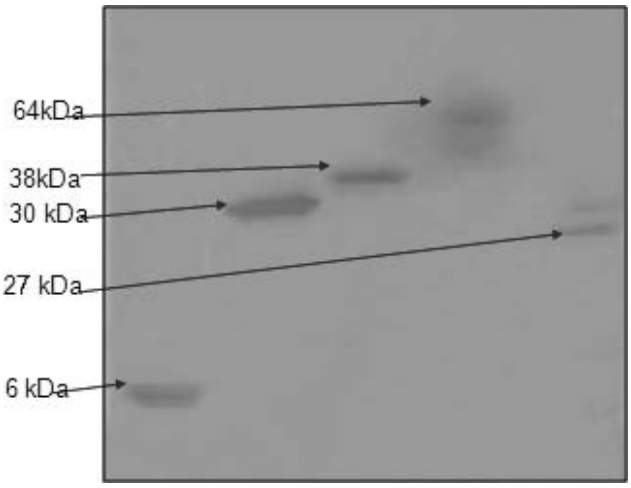


Fig 2. Western blot analysis showing immunoreactivity of five purified mycobacterial secretory proteins with TB patients' sera

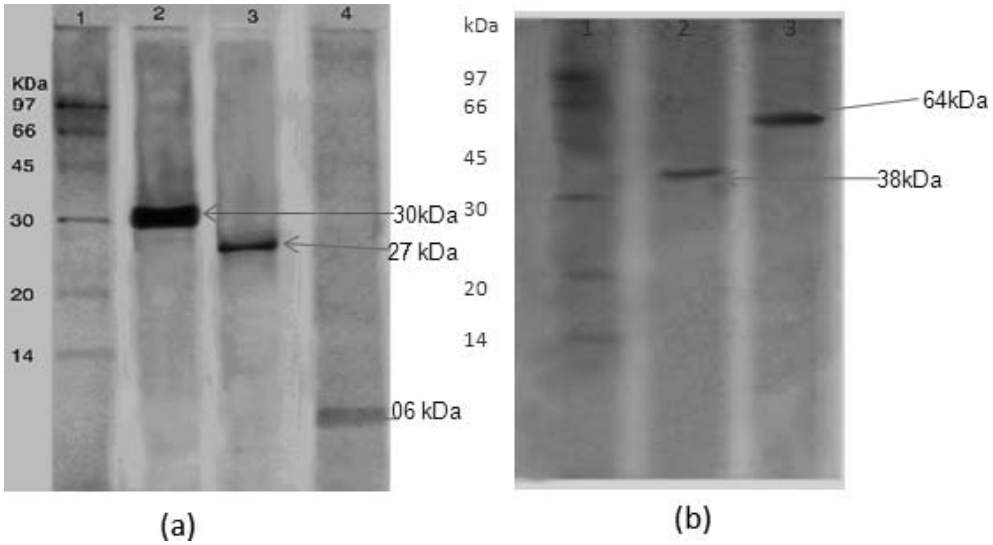
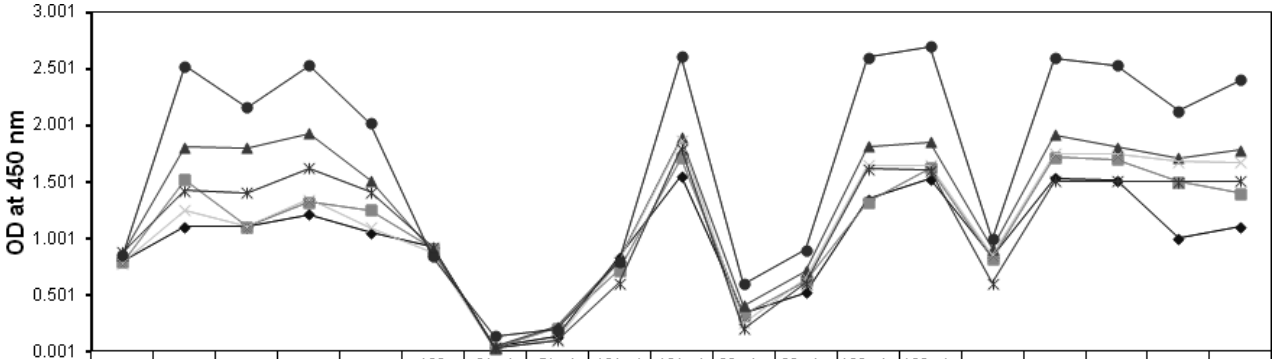


Fig 1. SDS-PAGE analysis of different purified proteins

1(a): Coomassie blue R250-stained 12% SDS PAGE gel showing 30, 27 and 6 kDa purified proteins. Lane M: 100 kDa molecular weight marker (Bio-Rad Laboratories Inc.); lane 1: sample containing 30 kDa purified protein; lane 2: sample containing 27 kDa purified protein; lane 3: sample containing 6 kDa purified protein.

1(b): Coomassie blue R250-stained 12% SDS PAGE gel showing 64 kDa and 38 kDa purified proteins. Lane M: 100 kDa molecular weight marker (Bio-Rad Laboratories Inc.); lane 1: sample containing 38 kDa purified protein; lane 2: sample containing 64 kDa purified protein.



	pH 6.5	pH 7.2	pH 9.5	10 mM	50 mM	100 mM	3 h at 4°C	5 h at 4°C	12 h at 4°C	18 h at 4°C	30 min at 37°C	60 min at 37°C	120 min at 37°C	180 min at 37°C	2.5 µg	5 µg	7.5 µg	10 µg	12.5 µg
64 KDa	0.802	1.103	1.102	1.212	1.051	0.923	0.041	0.133	0.835	1.55	0.335	0.525	1.345	1.525	0.835	1.535	1.515	1.005	1.105
38 KDa	0.791	1.521	1.103	1.321	1.251	0.91	0.031	0.212	0.721	1.712	0.321	0.622	1.322	1.622	0.821	1.721	1.701	1.501	1.402
30 KDa	0.864	1.81	1.805	1.933	1.513	0.893	0.042	0.222	0.81	1.893	0.41	0.713	1.813	1.853	0.91	1.915	1.812	1.712	1.782
27 KDa	0.782	1.251	1.103	1.351	1.1	0.873	0.037	0.116	0.803	1.876	0.253	0.653	1.65	1.652	0.853	1.753	1.743	1.682	1.672
06 KDa	0.879	1.424	1.405	1.624	1.412	0.926	0.035	0.102	0.605	1.795	0.205	0.605	1.615	1.605	0.605	1.505	1.51	1.5	1.507
Antigen	0.853	2.523	2.164	2.531	2.021	0.844	0.14	0.197	0.799	2.608	0.599	0.901	2.601	2.701	0.999	2.599	2.535	2.13	2.403

Various parameters (pH, blocking agent, time duration, temperature & concentration)

Fig 3. Effect of various ELISA parameters (pH and ionic strength of coating buffer, incubation period and temperature, and antigen concentration) for 6 kDa, 27 kDa, 30 kDa, 38 kDa, 64 kDa and CSP-Ag's (designated as Antigen).

(ii) pH of coating buffer

All purified secretory protein(s) showed maximum adsorption on solid phase at pH 7.2. However, the adsorption was quite weak at low pH of 6.5 as it is evident from low absorbance values of all proteins. Hence, pH 7.2 was selected for the immobilization of purified secretory protein(s) and cocktail of secretory antigens (Fig. 3). The secretory proteins of 6, 30 and 64 kDa MW showed maximum adsorption on solid phase at pH 7.2 and pH 9.5. Similarly, the 27 and 38 kDa proteins and cocktail of secretory proteins also showed maximum adsorption on solid phase at pH 7.2.

(iii) Ionic strength of coating buffer

The ionic strength of coating buffer indicated that all purified proteins and cocktail antigens were bound to the surface and showed good absorbance at all three concentrations (10, 50, & 100 mM). However, the best absorbance was noticed at the 10 mM concentration and gradual decrease in absorbance was noticed with increasing concentration (Fig. 3).

(iv) Incubation period

The effect of incubation period for antigen binding on the solid surface was determined by incubating all purified proteins and cocktail of antigens for different time intervals. As shown in Fig. 3, the absorbance values were increased with increasing incubation time and found maximum for the combination when antigens were kept for immobilization for 18 h at 4 °C. Further, there was a rise in the adsorption of the protein antigens on the solid surface up to 120 min at 37 °C; thereafter no further increase in adsorption was observed.

(v) Antigen concentration and conjugate

With an increase in concentration of protein antigens from 2.5 to 7.5 µg/well, a significant increase in the absorbance values was noticed (Fig. 3). No further increase in adsorption/absorbance was noticed after increasing the concentration up to 10 µg/well of antigens used for coating. The extent of the reaction was measured as increase in absorbance from 1.0 within 10 min to 1.9 within 30 min and showed that the selected conjugate, i.e., goat anti-human IgG-HRP, had suitable activity for the developed ELISA (Fig. 4).

Immunodiagnostic efficacy of CSP-Ag's ELISA

A total number of 639 samples from various groups of TB patients [above-mentioned TB patients' groups I (N = 440) and II (N = 199)] were included in internal study and used for 'sensitivity' study (Table 1). Out of 440 samples collected from patients with pulmonary TB evaluated by the developed CSP-Ag's ELISA, 432 were found positive and 8 were found negative (Table 1). In the extra-pulmonary TB group, out of total 199 samples, 191 samples were found positive, whereas 8 were found negative with the CSP-Ag's ELISA (Table 1). However, a total of 538 samples, as mentioned in Table 1 [groups III (N = 275) and IV (N = 263)], were considered for in-house study for determining the 'specificity' of the developed CSP-Ag's ELISA. Among 275 specimens from the non-TB group (Table 1, group III), 269 samples were found negative and 6 samples were found false-positive. Of the 263 samples from respiratory and non-respiratory infections tested, 255 patient samples were found negative and 8 samples were detected as false-positive (Table 1, group IV). An overall sensitivity of approximately 98.00 % was obtained among 639 TB serum samples (Table 1, groups I and II). Of the 639 serum samples, 16 samples showed false-negative results. An overall specificity of ~97.39% was obtained with 538 serum samples collected from non-TB patients, and patients with other respiratory and non-respiratory infections (Tables 1, groups III and IV). Among

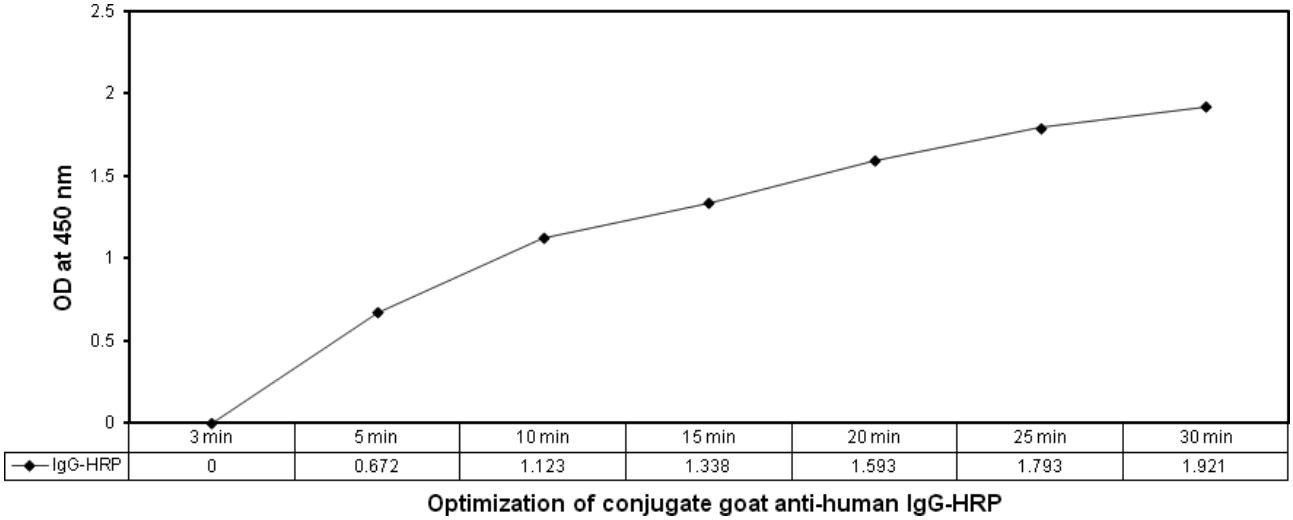


Fig 4. Optimization of conjugate goat anti-human IgG-HRP

538 samples, only 14 were found to be false-positive. Sera from 148 (above 15 years of age) patients immunized with BCG were tested for cross-reactivity and it was noticed that only two samples gave false-positive results. Similarly, no cross-reactivity was found with any of the serum samples collected from the patients of hepatitis B/C virus-positive disease, pulmonary fibrosis, common cold/asthma, malaria, and rheumatoid arthritis.

Comparative evaluation of CSP-Ag's ELISA

Furthermore, a comparative evaluation of CSP-Ag's ELISA (with cocktail of four antigens, viz., 6, 27, 30 and 38 kDa) was performed by using a commercially available ELISA (KP-90) kit and the MycoDot [lipoarrabinomannan (LAM)] test with clinically confirmed TB samples, as stated earlier in the Material and Methods section. A total of 150 samples from patients with confirmed cases of active TB (pulmonary, extra-pulmonary and HIV-*M. tuberculosis* co-infection) were tested by the developed CSP-Ag's ELISA, KP-90 kit and MycoDot sensitivity test. Comparative data showed that out of 150 tested samples, 148 samples were found positive by developed CSP-Ag's ELISA, whereas 107 and 116 samples were found positive with the KP-90 kit and MycoDot test kit, respectively. A total of 413 serum samples including sera from healthy humans (N = 287), BCG-vaccinated individuals (N = 35), patients with leprosy (N = 60) and patients with other common infections (N = 31) were also incorporated in the comparative studies in order to check their efficacies. It was evident from the obtained results that the newly developed CSP-Ag's ELISA was more sensitive (~98.67 %) and more specific (~98.06 %) in comparison to already available commercial tests, i.e., KP-90 ELISA (sensitivity 71.33 %, specificity 95.64 %) and MycoDot LAM (sensitivity 77.33%, specificity 94.43%) test kits (Table 3).

Table 3. Comparative evaluation of the cocktail of secretory protein antigens (CSP-Ag's) ELISA with commercially available KP-90 and LAM test

Serum sample source	**Total No. of samples**	**KP-90 ELISA kit**		**MycoDot LAM test**		**CSP-Ag's ELISA**	
		+ve	**-ve**	**+ve**	**-ve**	**+ve**	**-ve**
Sensitivity study							
Patients with confirmed cases of TB	150	107	43	116	34	148	2
Overall sensitivity (%)		71.33%		77.33%		98.67%	
Specificity study							
Healthy humans (age 15–50 years)	287	00	287	00	287	00	287
Specificity (%)		100%		100%		100%	
BCG vaccinated	35	4	31	6	29	2	33
Specificity (%)		88.57%		82.86%		94.28%	
Patients with leprosy	60	11	49	13	47	5	55
Specificity (%)		81.66%		78.33%		91.67%	
Patients with common infections	31	3	28	4	27	1	30
Specificity (%)		90.32%		87.09%		96.77%	
Total	413	18	395	23	390	8	405
Overall specificity (%)		95.64%		94.43%		98.06%	

Discussion

In this prospective study, efforts were made to evaluate the immunodiagnostic potential of the secretory proteins of *M. tuberculosis* H37Rv by developing a novel ELISA-based serodiagnostic test employing a cocktail of four (6, 27, 30, and 38 kDa) secretory proteins to enhance the sensitivity of the immunoassay, and attempts were also made to check the specificity of the cocktail antigens. During the study, the secretory antigens of *M. tuberculosis* were successfully isolated and well characterized. Five major protein antigens (6, 27, 30, 38 and 64 kDa) were virtually obtained (Fig. 1a,b), and out of all the five proteins, the 64 kDa protein was found novel (Fig. 1b). The reactivity potential of secretory protein antigens was inveterately confirmed through Western blot with sera of Indian tuberculosis-positive population and ELISA. Analysis employing Western blot has shown that purified secretory antigens of *M. tuberculosis* frequently reacted with the TB-positive serum. Since the antibodies to the five purified antigens were seen in the sera of patients, it was worthwhile to test the diagnostic potential of the secretory antigens.

ELISA test is a powerful technique for ng/ml to pg/ml detection of antibodies present in a serum sample; hence, standardization was also done for CSP-Ag's ELISA employed in different conditions. In order to standardize the ELISA for enhanced sensitivity and specificity, the binding capacity of adsorbent plastic surfaces for bio-macro-molecules was also considered; and it is mandatory for activity of immunoassay that it can easily distinguish between the total amount of molecules bound to the plastic surfaces and the amount of molecules that can be bound and still remain biologically active. The said qualities are dependent on the type of the molecules and the nature of the surface. During CSP-Ag's ELISA optimization, two types of adsorbent polystyrene surfaces were tested, i.e., PolySorp surface and MaxiSorp surface (Nunc, Rochester, NY). The PolySorp surface predominantly presents hydrophobic groups, whereas MaxiSorp has some additional hydrophilic groups, which results in a fine patchwork of hydrophobic and hydrophilic binding sites. In our ELISA optimization study, MaxiSorp surface was found more active and used for facilitation of the adsorption of hydrophilic synthetic epitopes, because this surface can easily compete with the water molecules for binding the macromolecules by hydrogen bonds and molecules can also be captured from a much longer distance by the long-range hydrogen bond forces for establishment of both hydrogen bonds and eventually hydrophobic bonds. From the above findings it was concluded that chances of occurrence of peptide epitope binding events were higher with MaxiSorp and adequate incubation conditions could be established more easily.

Antigen coating efficiency is mostly dependent on immobilization pH (coating buffer), ionic strength, immobilization time (incubation period), concentration of antigens, and immobilization temperature. During ELISA optimization, the antigens showed maximum adsorption on solid phase at pH 7.2 and 9.5, whereas the cocktail of antigens also showed maximum adsorption at pH 7.2. There was no significant difference observed in the adsorption between pH 7.2 and 9.5. Hence, the common pH was selected for the immobilization of cocktail of epitopes (Fig. 3). All the antigens showed maximum adsorption on solid phase between 10 to 50 mM concentration of coating buffer and reactivity fell down after 100 mM. The saturation of the adsorption of all the epitopes on solid phase took place within 18 h (Fig. 3). Investigation of immobilization of antigens with variation in the concentration revealed that there was a remarkable rise in the immobilization of all the protein antigens from 2.5 to 7.5 μg/well, and afterwards, there was no effect of higher antigen concentration on immobilization as evident by the experimental data (Fig. 3). Immobilization time at 37 °C temperature showed that there was a significant rise in the adsorption of the antigens on the surface up to 120 min, followed by saturation. However, optimum adsorption took place within 120 min, but it was observed that prolonged incubation period for another 120 min had given better results (Fig. 3).

BSA is the most commonly used blocking agent. However, sometimes more heterogeneous casein and normal rabbit serum are used and may be more effective than BSA (Vogt et al., 1987; Pratt and Roser, 1989). In our study, casein digest, hydrolysate, was found better than BSA (data not shown), which was further found better than normal rabbit serum. Casein digest, hydrolysate, was found to be the best blocking agent amongst all the three tested blocking agents, probably due to its heterogeneous nature (different masses of peptides); perhaps it blocks the inter-epitope space and un-immobilized space on the solid surfaces more firmly than BSA and normal rabbit serum. Probably, casein exerts a lower effect on the steric hindrance in antigen-antibody reaction and has a lower effect on the shielding epitopes. In this regard, 0.1% casein digest, hydrolysate, in 100 mM phosphate buffer (pH 7.2) was found sufficient to serve the purpose of blocking. The developed CSP-Ag's ELISA assay differentiated the reactive from non-reactive samples. Among various reagents used in ELISA, anti IgG-HRP conjugate, solid phase immobilized antigens and the enzyme substrate-chromogen (TMB-H_2O_2) were critical; hence different attempts were made to stabilize them. In the conjugate, HRP was very prone to peroxidation reaction; hence it was protected by adding reducing agent TMB. The protein part of the conjugate was protected from deterioration using protein stabilizers (casein digest, hydrolysate), preservatives (thiol-containing compounds), surfactant (Tween-20) and antibacterial compounds.

The developed serodiagnostic test has a potential to detect the mycobacterial antibodies present in the active phase of TB infection and a capability to clearly differentiate most of the patients with active TB infection from normal subjects or subjects with quiescent lesions,

previous vaccination or infected with other diseases. The comparative performance data of the developed CSP-Ag's ELISA demonstrated an enhanced sensitivity (~98.67 %) and specificity (~98.06 %) among all the enrolled subjects, in comparison to the already available commercial ELISA test (KP-90) and MycoDot. Although in the last few years some studies related to CSP-Ag's of *M. tuberculosis* have been done in the context of sero-reactivity with active TB-affected Indian population (Gupta et al., 2005; Harinath et al., 2006; Kumar et al., 2010), this is the very first time we tested the CSP-Ag's (employing 6, 27, 30 and 38 kDa proteins) with a large sample size of Indian population for serodiagnostic purposes and observed an improvement in the sensitivity and specificity of the developed CSP-Ag's ELISA. Thus, the data obtained from this study clearly indicates that the CSP-Ag's can be utilized for the routine diagnosis of pulmonary and extra-pulmonary TB and it has full potential to easily differentiate healthy subjects and BCG-vaccinated individuals from those with active TB.

Our findings clearly demonstrate that the ELISA test based on the cocktail of all four purified secretory antigens is an effective and receptive tool for screening and diagnosis of samples coming from MDR-TB or XDR-TB (extensively drug-resistant TB) infected patients, and therefore the test has a remarkable potential for detection of suspected TB population especially in developing countries, where TB is still endemic and creates havoc together with HIV infection. Further research studies are under progress to evaluate the compatibility of the developed test for differentiating the TB and atypical TB subjects with special emphasis on sensitivity and specificity, and for selectively diagnosing *M. tuberculosis*-infected subjects. In addition, chances exist that the current findings might be helpful in establishment of a potential diagnostic marker of *M. tuberculosis.*

Conclusion

In this study, a novel CSP-Ag's ELISA was developed and the related parameters were optimized and evaluated for enhanced reactivity, specificity and sensitivity in a pool of confirmed TB patients. Secretory antigens from *M. tuberculosis* H37Rv were characterized and four of them were exploited for improved serodiagnostic test against active TB cases with greater sensitivity and higher specificity as compared to other commercially available tests. Thus, the study suggests possible application of the CSP-Ag's ELISA to routine screening and early-stage diagnosis of active TB.

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