Monitoring the advancement and evaluation of a novel diagnostic method for rapid detection of tuberculosis

Amrita Masanta[1], K.Gopinath Achary[2], Shikha Singh[3*]

[1]*Centre for Biotechnology, Siksha O Anusandhan (Deemed to be University), Bhubaneswar, Odisha, India*

[2]*Imgenex India Pvt.Ltd, E-5 Infocity, Bhubaneswar, Odisha, India*

[3]*Rama Devi Women's University, Bhubaneswar, Odisha, India.*

Abstract

Evaluation of the clinical usefulness for detection of isolated serum from infected tuberculosis (TB) individuals was raised against mycobacterial antigenic protein molecular weight of 14 KDa, 16KDa, 110KDa and 190 KDa for possibility of robust diagnosis & discrimination between tuberculosis and other pulmonary diseases. Under optimum experiment conditions, mycobacterial antigenic proteins was identified by using indirect ELISA with high sensitivity detection limit of 0.1 mg at 1:5000 dilution. Statistical analysis report shows the correlation coefficient among various phases of tuberculosis infected urine samples assembled from Balasore, Odisha, India. Proteins ranges from 14KDa -190KDa were got separated and identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Western blotting results shows positive reaction against the mycobacterial antigenic proteins & also revealed immune-reactive bands with high antibody retort contrast to the proteins with molecular weight of 35KDa, 66KDa and 110KDa, along with 4 additional reactive bands molecular weight of 14KDa, 16KDa, 110KDa, and 190KDa. The outcomes suggest that the TB serum could be fortunately employed for the specific immunological detection against mycobacterial antigenic proteins at an early phase of tuberculosis infection.

Keywords: Tuberculosis; mycobacterial antigenic protein; TB serum; SDS-PAGE; indirect ELISA; Western blotting.

1. Introduction

Tuberculosis (TB) is still continue as a serious public health disaster faced by globally, with estimated more than 10 million person infected worldwide in 2017, together with 5.8 million men, 3.2 million women and 1 million children (age ≤ 15 years) [1]. Since, it is the leading cause of death & mortality rate reached 16% in 2017 [2]. These death rates are due to late or missed diagnosis. By enhancing the performance of diagnostics and their accessibility can be the key for reduction of global morbidity and mortality.

However, *Mycobacterium tuberculosis* as a causative agent of tuberculosis has been characterized by its ability for transmission from infected individual to another host. The detection of tuberculosis for those individual, mainly living in resource limited countries is more challenging that principle setback in diagnosis and treatment of disease, hence increased in mortality. Microbiological confirmation through microscopic examination of sputum along with Ziehl-Neelsen staining is a quite easy, fast & low cost test convenient but it qualifies only 60-70% sensitive for detection of pulmonary tuberculosis [3]. Mycobacterial cell culture is quite sensitive almost takes measurably 15-20 days or longer period if solid media are

employing and culture facilities are not accessible in many countries. The shortfalls of chest radiography appearance specific to tuberculosis cause a prospect instinctive & falsity practice [4]. Another major aspect, with developed HIV co-infection & change the clinical demonstration of tuberculosis and reduce the sensitivity of classical microbiological techniques [5,6,7]. Accordingly, in resource-limited countries, it is exceptionally crucial to have an economical, simple and robust method for tuberculosis detection and diagnosis of tuberculosis so that each individual can be isolated & treated immediately [8].

Apart from these factors, there are many other obstacles that face successful field detection and treatment of tuberculosis like rising costs of the first line anti-TB drugs like isoniazid, rifampicin, pyrazinamide, ethambutol for tuberculosis treatment to suppress resistance [9,10,11]. Other techniques like flowcytometry, radiometric detection, latex agglutination etc. hold their own set of imperfections. Yet, the efficiency and fidelity of these approaches depends on the experienced and skilled person for disease detection. An explanation to this is an immunological assays that have been showed with remarkably specific, sensitive, simple, fast & affordable that can be automated for initial detection.

Study of this polypeptide could be utilized for the development of a robust with potential of early detection of tuberculosis designed by immunological assay. The present study focus on how a blood serum isolated from tuberculosis infected individual used against the mycobacterial antigenic protein at various stages of disease to evaluate the specificity & sensitivity of the isolated serum & to regulate the utility of ELISA method for rapid detection of mycobacterial antigenic proteins by using the isolated serum.

2. Methodology

2.1. Assortment of infected urine & blood

The present research was regulated from September 2017 to June 2018 in the laboratory of Cell Culture, Department of Biotechnology, Siksha O Anusandhan (Deemed to be University), Bhubaneswar, Odisha, India and Monoclonal division, Imgenex India Pvt.Ltd, E-5 Infocity, Bhubaneswar, Odisha, India. Informed written consent from each individual prior to urine & blood sampling, and all samples were collected from UMANG, a social health welfare committee for Tuberculosis (TB), organized by Lion's Club of Balasore, Odisha, India.

A total of 86 samples have been collected: 4 from cases with active tuberculosis (TB), 82 from patients without sign of tuberculosis. All individuals had already been vaccinated by BCG. None of the subjects, along with individual with active TB and controls, receive a history of serious pathologies, along with HIV and cardiovascular disease. Demographic information, including name, sex, age and clinical type of TB, are mentioned in **Table 1.** All blood serum were gather previously any analysis and stored at ─80℃ until immunological trial. All 86 urine samples were aliqouted and kept in ─80℃. Urines collected from healthy patients were considered as a positive control. Total protein content was calculated by following Bradford (1976) [12] utilizing Bovine Serum Albumin (BSA) as the standard.

Table: 1 Population analysis and scientific evidence for tuberculosis victim and healthy controls.

Disease type	Infection site	No. of cases	No. of male	No. of female	Age group, yrs. (range)
Pulmonary TB	Lungs	3	2	1	45-55
Ertra Pulmonary TB	Lymph nodes	1	_	1	42
Non- TB	COPD	24	15	9	19-81
Asthma		8	3	5	32-70
Cancer		16	6	10	21-68
Mycosis		11	7	4	32-45
Urinary infection		14	_	14	25-70
Sarcoidosis		7	4	3	28-42

Note: Non-TB, Pulmonary non tuberculosis disease; COPD, chronic obstructive pulmonary disease

Urines collected by tuberculosis infected individuals were classified into 3 various stages depends on the antigenic loads. The total concentrations of mycobacterial antigenic proteins are depicted in **Table 2.**

Table: 2 Tuberculosis infected urine sample collection from Balasore, Odisha, India. Total protein content (mg/ml) of TB infected urine sample at various stages.

District	**State**	**Sample codes**	**Stages**	**Conc. of *Mycobacterium tuberculosis* (mg/ml^{-1})**
Balasore	Odisha	TBUS Ba1	III	5.026 ± 0.044
		TBUS Ba2	II	3.962 ± 0.061
		TBUS Ba3	III	5.319 ± 0.095
		TBUS Ba4	I	2.092 ± 0.089

Notes: Mean ± SD; n = 3. Stages I, II and III represent stages of TB. All stages are characterized by results of clinical, radiological and pathological examination of an individual.

2.2. *Separation of mycobacterial antigenic proteins by SDS-PAGE*

Separation and studying of mycobacterial antigens was performed by polyacrylamide gel electrophoresis (PAGE) using 12% gels (W/V) along with 5% (W/V) stacking gels, consisting of Sodium Dodecyl Sulfate (SDS) (Laemmli,1970) [13]. The proteins concentration ranges from 0.120 mg/ml to 10.148 mg/ml. Established by antigenic loads, 8 µg (mild) to 100 µg (chronic) proteins were weighted into each wells. For the determination and estimation of molecular weight of the antigens, a standard protein ladder was used. Separation of protein was performed in a Bio-Rad Mini-PROTEAN vertical electrophoresis system below with a sustained volt of 120 V for 1 hr. the electrophoresed gels were stained with Coomassie Brilliant Blue R-250 (Sigma) to envision of the mycobacterial antigen with visible protein ladder.

2.3. Preparation of isolated blood serum for ELISA screening test

Tuberculosis infected serum were isolated from blood by centrifuging at 20000 rpm for 30 mins at 4°C and serum was collected and stored at ─80°C. TB serum has been raised against pooled antigenic proteins of *Mycobacterium tuberculosis.* As hypothetically, TB serum holds the proficiency to identify mycobacterial proteins up to 0.1 mg at 1:5000 dilutions. The collected TB serum was employed for indirect ELISA.

2.4. Enzyme Linked Immunosorbent Assay

Indirect ELISA was performed by coating mycobacterial antigens (8-100 µg/ml) with diluted 1X 50 mM carbonate coating buffer at pH 9.6 and incubated at 4°C for 12 hr. After incubation, was the plates for three times with 1X Phosphate Base Saline Tween 20(PBST) and then incubate the microtitre plate for 45 min at room temperature by adding blocking buffer into the coated wells with 2.5% of skimmed milk in PBS. After repeatedly washing thrice with PBST, 200µl of TB serum that rose against mycobacterial antigenic proteins were diluted in 1% of skimmed milk in PBS & subsequently added to the wells followed by incubation for overnight at room temperature. The plate was washed for three times with PBST; 100µl of diluted horseradish peroxidase- conjugated anti human IgG (Bio-Rad) was added to each well at a dilution point of 1:10000 and incubated for 1 hr at room temperature. After washing the plate three times with PBST vigorously, 200µl of H_2O_2/TMB (Hydrogen peroxide/3,3´,5,5´-Tretramethybenzidine) substrate was added to each well and incubate for 10 min in dark for color development. The reaction was stopped by adding 100 µl of stop solution (2 N Sulfuric acid, H_2SO_4) and optimum absorbance was measured at 450 nm in a ELISA Microplate Reader (Mindray MR-96A).

2.5. Determination of serum specificity by Immunoblotting

Mycobacterial antigenic proteins used in the qualitative assessment was prepared by pooling equal volumes of samples from each phase. The SDS-PAGE separated proteins were transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore) using mini trans blot cell module (Bio-Rad). Immunoblotting was carried for 90 min at a constant voltage of 60 V. The PVDF membranes were stained using Amido-black (Sigma) and destained with 10% methanol and 10% acetic acid and distilled water. The transferred membrane were cut into strips and then blocked by blocking buffer (Tris-buffered Saline Tween 20 (TBST); 0.02 M Tris-HCl, 0.15M NaCl, 0.05% Tween 20, pH 8), including 3% skimmed milk with slight shaking (50rpm). After vigorously washing of strips in TBST buffer, the TB serum with 1:2500 dilutions were added to strips and incubated for 2 hr at room temperature. The strips were then again washed for three times and 1:10000 dilution of horseradish peroxidase-conjugated anti human IgG (Bio-Rad) was added and incubated for 1 hr at room temperature. Visible band were expanded after vigorously washing of strips with TBST followed by submerging in chemiluminescent substrate solution (Pierce ECL Western Blotting Substrate, Thermo Scientific). The delineated band was developed by (Hyper-Film, Kodak using Developer & Fixer) in darkroom.

2.6. Validation of specificity and sensitivity of ELISA

The specificity, sensitivity and accuracy of tuberculosis detection for disease diagnosis were validated using Graph pad Prism 7 software. Diagnostic specificity [true negative/ (false positive + true negative)], sensitivity [true positive/ (false negative (false negative + true positive)] and accuracy [(true positive + true negative) / (true positive + true negative + false positive + false negative)]. All the calculation of results was depicted in percent (%).

3. Results and discussions

3.1. Analysis of SDS-PAGE

The banding pattern of mycobacterial antigenic protein allowed the identification of multiple visible protein bands as depicted in **Figure 1**. The molecular weight of *Mycobacterium tuberculosis* proteins ranges from approximately 10 KDa to 190 KDa. Analysis of mycobacterial antigenic proteins showed several specific visible protein bands which were completely absent in healthy urine sample. The number and intensity of bands in the antigenic protein isolated from chronic stage of TB infection were shows higher in comparison to stage I & stage II of TB infection.

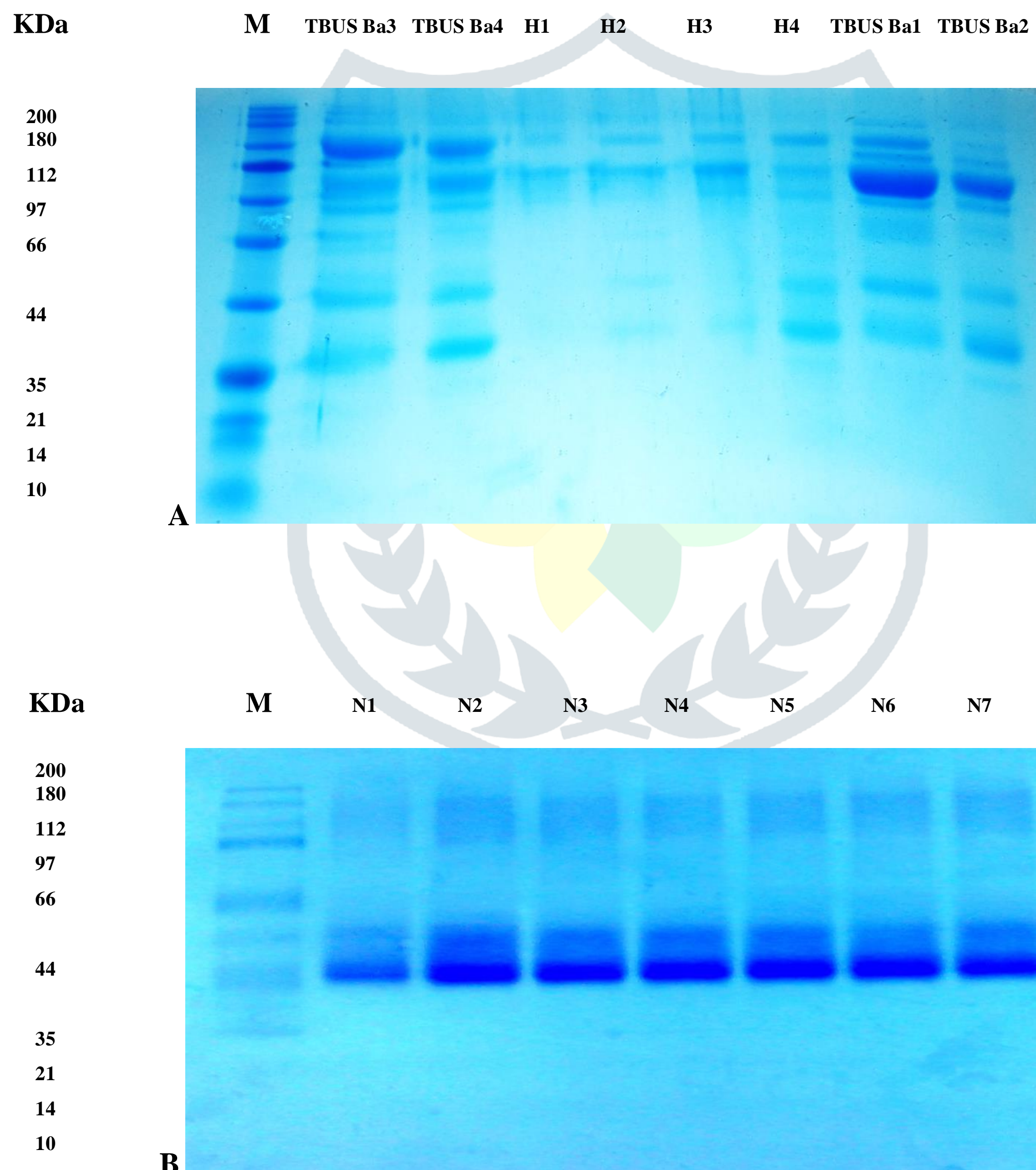

Figure 1. Visible bands of TB infected and non-TB urine separated in a (12 %) SDS-PAGE. (A) Banding pattern of mycobacterial antigenic protein, lanes: (TBUS Ba1 - TBUS Ba4) and healthy controls (H1 - H4); M: marker; and (B) Protein bands of non- TB infected (negative control), lanes: (N1, N2, N3, N4, N5, N6, N7); M: marker; Molecular weight (MW) of standard protein is indicated on left margin.

3.2. Report of Enzyme- linked Immunosorbent assay

From the analysis result of ELISA, the isolated blood serum raised against the mycobacterial antigenic protein recognized from the co-related titration in the dilution of 1:1000, 1:2500, 1:5000 &1:10000. Under favorable investigational condition, the ELISA test designed against mycobacterial antigenic protein showed a good sensitivity with limit of detection (LOD) of 0.05 mg at 1:5000 dilution (R^2 = 0.984), as depicted in **Figure 2.** From the **Figure 3,** the result shows that the TB serum used at a dilution 1:5000 provide optimal reactivity with the antigens when the integration or absorbance value was approximately in between 2-4 units. All reaction was identified after 10 min of the substrate added with no change in readings for the negative control, as observed after 30 min of incubation. The reactivity of the mycobacterial antigenic proteins with the TB serum. The isolated TB serum also showed immunological reaction with antigenic proteins isolated from various infected TB urine samples & the titration outline of TB serum is represented in **Figure 4.**

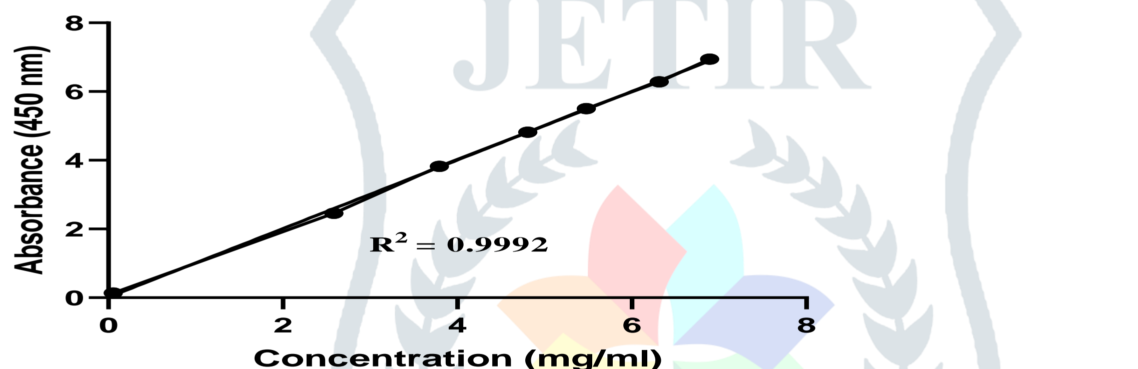

Figure 2. Standard curve of indirect ELISA test perform beneath optimal conditions. All the valuations were (mean ± SD).

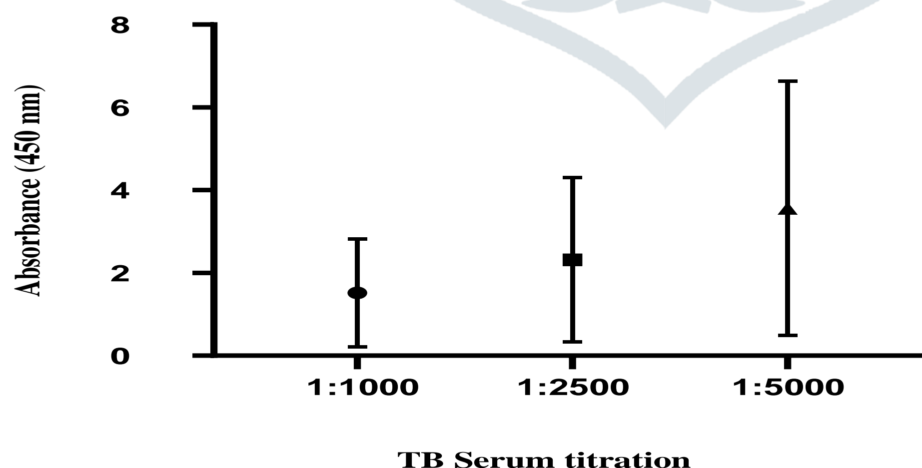

Figure 3. Evaluation of indirect ELISA report of isolated TB serum with mycobacterial antigenic proteins by different dilution ratio.

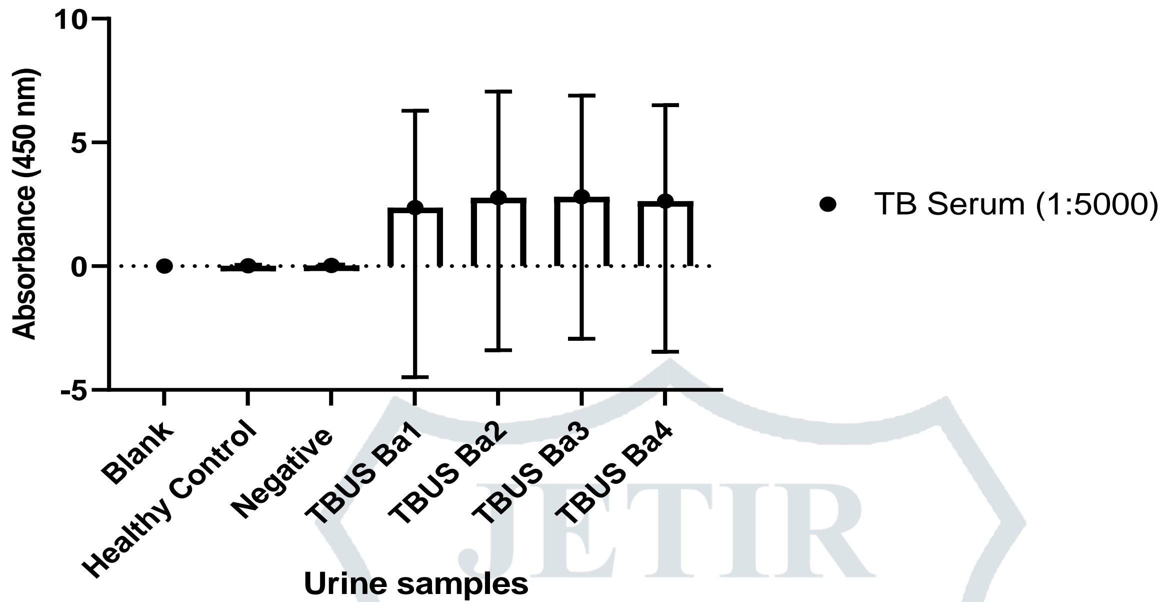

Figure 4. Data of indirect ELISA exhibiting the titration at wavelength of 450 nm between the isolated TB serum and urines samples collected from TB and non-TB individuals

Table: 3 Sensitivity test of isolated serum against mycobacterial antigenic proteins by indirect ELISA.

Antigens	Stages of infection	ELISA reaction at 1:2500 dilution (TB serum)
TBUS Ba1	III	+++
TBUS Ba2	II	++
TBUS Ba3	III	++
TBUS Ba4	I	+
Healthy urine (control)	NA	0
Negative urine (negative control)	NA	0

Notes: Assessment was listed after 10 mins of incubation.

Absorbance at 450 nm: > 5 (+++); 5-3 (++) ; < 3 (+)

NA = not applicable

3.3. Analysis of Western Blotting

The analysis report of western blot of isolated TB serum against the mycobacterial proteins at distinct stages produced extraordinary variations in the identification profile. The isolate TB serum interpreted with multiple bands extent from 10 KDa to 190 KDa in all three stages of TB. The serum elevated alongside *Mycobacterial tuberculosis* showed the maximum counter reaction against the antigens (35 KDa, 66 KDa, 110 KDa) including the additional bands of reactivity (14 KDa, 16KDa, 110 KDa & 190 KDa), shown in **Figure 5.**

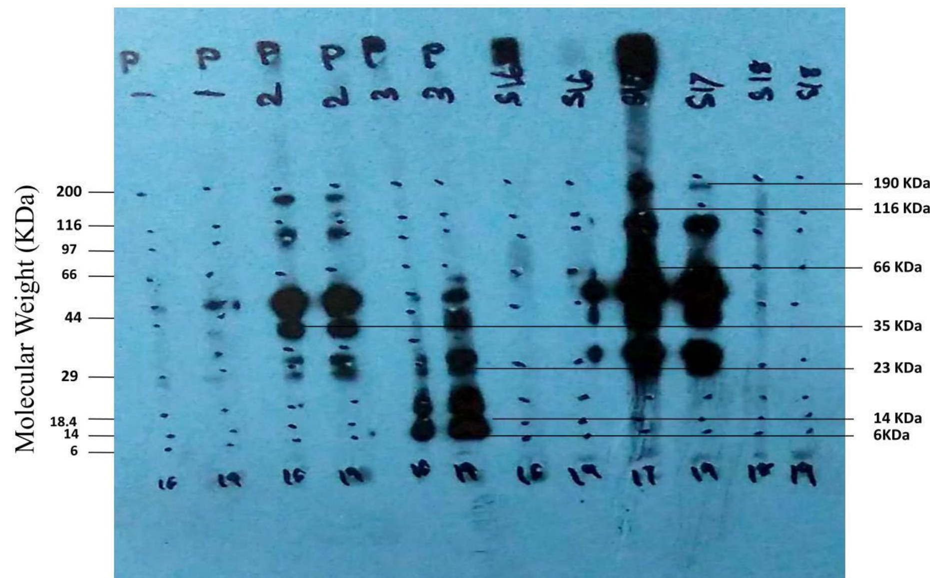

Figure 5 Western blotting analysis of Ab to pooled mycobacterial antigens from various stages of infection. Proteins were blotted onto a nitrocellulose membrane and incubated with TB serum. The Ab rebound to Ag was distinguised and seen from 10-120 kDa. The Ab response was high against the antigen 35 kDa, 66 kDa, 110 kDa and seen additional bands of reactivity, including 14 kDa, 16 kDa, 190 kDa and 110 kDa. Molecular weight of standard protein is indicated on left margin.

3.4. Recognition of Enzyme –linked Immunosorbent assay technique

By assuming the set of outcomes from TB infected & non-infected urine samples, the sensitivity, specificity and accuracy of the ELISA assay was calculated. A sample size was taken as 86, sorting of both infected and healthy control. The outcomes of the figures are depicted in Table 4**.** Consequently, it is accomplished that the accumulated TB serum raised against *Mycobacterium tuberculosis* were carrying immense potential to detect the mycobacterial proteins though its highly sensitive, specific & accurate that could be helpful for early disease diagnosis of tuberculosis.

Table: 4 Interpretation of diagnostic ELISA test for detection of mycobacterial antigenic protein from infected and non-infected sample with isolated TB serum.

Disease type	**No. of positive case**	**ELISA test cut-off value of isolated TB serum**		
		Sensitivity (%) [b]	Specificity (%) [c]	Accuracy (%) [d]
pTB + epTB [a]	4	80 %	98.73 %	97.61 %

Notes: (a) pTB, Pulmonary Tuberculosis; epTB, Extra Pulmonary Tuberculosis; (b) Sensitivity (%)[a] = True positive/ (False negative + True positive) × 100; (c) Specificity (%)[b] = True negative/ (False positive + True negative) × 100; and (c) Accuracy is an ability of a test to provide true positive and true negative results (%)[c] = (True positive + True negative)/ (True positive + True negative + False positive + False Negative) × 100.

4. Conclusion

The current research characterized the advancement of specific, sensitive & reliable immunological assays for early detection of *Mycobacterium tuberculosis* from TB infected urine sample. These immunological approaches demonstrated that the diagnosis is self-supporting of antigenic load or the stages of TB. The obtained result shows the mycobacterial antigenic proteins approximate at the beginning stage of tuberculosis can be employ for primary identification. The discoveries of this challenge proposed that the concentration of mycobacterial antigenic protein with suitable dilutions of antisera is necessary element that affects early detection. Moreover, the method has outstanding capability for direct quantification of the antigens and, hence, it can be used as robostic tool for TB diagnosis and treatment.

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The authors declare no competing financial interest.

Conflict of interest

The authors declare that no competing interests exist.

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