

Effectiveness of in-house developed Sandwich ELISA for antigen detection of tubercular antigen in resource constraint setting



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ABSTRACT

Background: Around 26% of World's tuberculosis (TB) cases are in India. In resource constrained settings, it is difficult to use diagnostic tests such as nucleic acid amplification technique and mycobacterial culture due to their complexity and expensiveness. Developing an assay using polyclonal antibody may help to solve this problem. **Aims and Objectives:** On the basis of this, we worked to detect tubercular antigen by developing Sandwich ELISA from the serum of TB patients. **Materials and Methods:** For this study, 50 cases, 50 disease controls, and 30 healthy control subjects were taken. Commercially available reagents were used for the development of Sandwich ELISA. The data were statistically analyzed with the help of software SPSS version 16.0. **Results:** Our effectively developed test had 82% area under the ROC curve. About 81% (n=35) patients of pulmonary TB cases with non-HIV infection detected by our Sandwich ELISA and 57% cases with the coinfection of HIV-TB. Developed test had 70.91% positive predictive value and 75.56% negative predictive value. **Conclusion:** We successfully developed Sandwich ELISA for tubercular antigen detection and to increase the specificity of the Sandwich ELISA, as per the WHO guidelines, further work is needed for the detection of tubercular antigen.

Key words: Antigen; Extra-pulmonary; HIV; Sandwich ELISA; Tuberculosis

INTRODUCTION

Mycobacterium tuberculosis is the causative bacillus for tuberculosis (TB) infection/disease. It is pulmonary TB (PTB) involving lungs and also extra PTB (EPTB) involving spine, abdomen, etc. As per the World Health Organization since 1993, TB is the most emergent disease before COVID-19.

In India, TB is the epidemic disease and for this delay in diagnosis is a crucial factor. Stopping the TB, we need effective and early diagnostic modalities which are to be secured by complete drug therapy. "Gold standard" for the TB diagnosis was sputum smear staining for acid-fast bacilli or standard culture, but only 40–75% sensitivity of these

tests has been there. The most of the time clinicians treats the suspected patients on their clinical acumen.¹

In resource-constrained settings, nucleic acid amplification tests and mycobacterial culture like diagnostic tests are difficult to be available to use as they are too costly and complex for day-to-day TB control programs. An additional barrier is lack of accessibility for the diagnostic services. The WHO endorsed Xpert MTB/RIF (Cepheid, Inc., Sunnyvale, CA) is quick and utmost sensitive to detect TB, but it is highly expensive, again preventing its use in resource-constrained epidemic zones.²

Around 26% – worldwide TB cases are present in India. In India, the incidence of TB is 2–2.5 million cases with the

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prevalence of 2–4.6 million cases along with the HIV-TB coinfection having 0.1 million cases.³

There is 1.0 smear diagnosis laboratory per 1 million population and only 0.1 culture and drug sensitivity testing laboratory per 5 million populations available in India.³ Therefore, there is a need for fast and trustworthy diagnostic tests for TB depending on the examination of sputum, blood, and other clinical specimens.

Serological tests are comparatively user friendly and interpretable. Serological tests are somewhat better for the detection as they are independent in relation to the infection site for the conditions such as EPTB, uncooperative patients, for children, and also from the patients having difficulty in collection of clinical samples. Antigen-capture ELISA or Sandwich ELISA is one of the common techniques for the detection of antigen.⁴

In July 2010, a meeting was held by the WHO Expert Group. They emphasized for the development of sensitive and specific serological test.⁵

Development of assay based on the use of polyclonal antibody may help to solve this problem. On the basis of that we worked to detect tubercular antigen from the serum of pulmonary and EPTB by developing Antigen-capture ELISA using tubercular IgG from patient's serum.

Aims and objectives

The aim of the study was to check the effectiveness of in-house developed Sandwich Elisa for antigen detection of tubercular antigen in resource constraint setting.

MATERIALS AND METHODS

Institutional Ethical Committee (DMIMS [DU]/IEC/2010-11/15 dated September 30, 2010) gave the clearance for the project to proceed and we completed the project in 2 years. Selections of the study population were done from rural based tertiary care hospital. Fifty patients having PTB along with HIV infections ($n=7$), 30 healthy control subjects, 50 disease control patients, and also six TB patients having focus outside the lungs were included in the study. Subjects who were free from any diseases for the past 1 year and also looking healthy at the time of sample collection were selected as healthy control.

Selection of Cases: Clinical and treatment history, sputum sample tests, radiological investigations, and blood investigations (HIV-ELISA) were also done in patients and to be considered for selection of cases.

After taking informed consent and with all aseptic precaution, 2 ml blood (venous) was taken in a plain

bulb from each study participants. The collected sample undergoes centrifugation to separate the sera at 3000 rpm for 10 min. About 10% sodium azide (S D fine chem. Pvt Ltd, Mumbai) 10 μ l/ml and 10 μ l/ml protease inhibitor cocktail (Sigma) were added in the separated sera samples. Unique numerical coding was given to each sample.

Commercially available cocktail TB antigen (purity >95%, mixture of recombinant protein) was procured from local supplier for the isolation of IgG which was TB Ag specific. The sera of already diagnosed cases of TB was utilized for this purpose.

We pooled sera samples (PTB patients) which were well preserved by the previous workers of our department and utilized it to isolate anti-TB IgG.

Ammonium sulfate precipitation method was used to isolate the immunoglobulin rich fraction from those pooled sera.⁶ After that, we used ion-exchange chromatography with DEAE-Sephadex beads column to separate the total IgG from the immunoglobulin rich fraction.⁷ Then, affinity column chromatography (BIO-RAD BioLogic LP) with cyanogen bromide activated Sepahrose 4B coated with commercial cocktail TB Ag was used to successfully eluted the anti-TB IgG from the total IgG fraction.⁷

Anti-Tb IgG utilized for dual purpose: (i) One for the conjugate preparation with horse radish peroxidase and (ii) for the coating of the wells of ELISA plate.⁷ Our Sandwich ELISA used O-phenylenediamine as a substrate.⁸ We used the mentioned referenced Sandwich ELISA protocol to detect Tb Ag.⁹

ELISA Reader and Washer (Model No:-ER-2005;-B4B Diagnostic Division): OPD A_{492} primary and A_{620} secondary.

SPSS version 16.0 was used for the statistical analysis.

RESULTS

The calculation of mean and standard deviation was done of the ELISA titers for the cases, disease control, and healthy controls. The mean and the SD of the ELISA titer for HC group were 0.86 and 0.10, respectively. The cutoff was set to 1.06 which was derived from the data and equals to "mean+2SD." Thus, positive result was considered above this cutoff value and negative when the value was below cutoff. PTB cases had 2.25 and 0.93 as the mean and SD of ELISA titer, respectively. The ELISA titer of PTB cases was ranged from 0.75 to 4.06. DC had 1.24 and 0.62 as the mean and SD of the titer, respectively. The ELISA titer of DC was ranged from 0.59 to 3.01 (Figure 1).

Sandwich ELISA detected accurately TB Ag in 39 out of 50 PTB cases but false positivity had been shown in 16 out of 50 disease control. Effectiveness of the test had been proven as the detection of 04 (66.67%) EPTB cases was done correctly.

The 78% sensitivity was noted to detect TB Ag in PTB cases while in cases of EP-TB, it was only 67%. However, the 68% specificity was noted for both the cases though pulmonary or extra-pulmonary. The 75.56% negative predictive value and 70.91% positive predictive value were recorded (Figure 2).

The ROC curve plotting used to check the performance of the Sandwich ELISA. The area under the curve represents the performance of the test. Effectiveness of our developed Sandwich ELISA had been proven as the area covered was 82% (Figure 3).

Sandwich ELISA detected the Ag in HIV non-infected cases – 81% (35) while only 57% (n=4) of the HIV-TB coinfection cases (Figure 4).

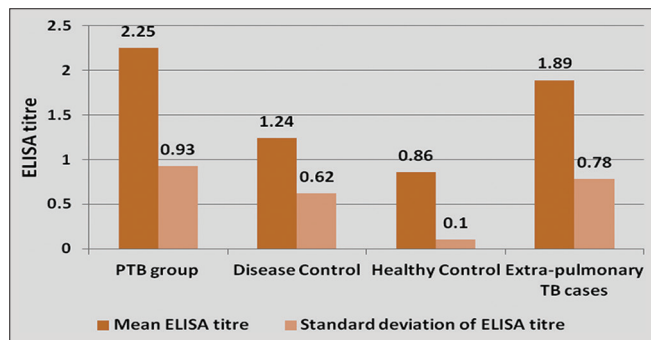


Figure 1: Result of the ELISA titer of mean of ELISA and standard deviation was compared

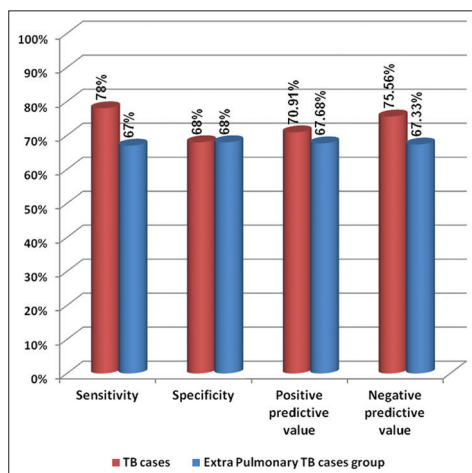


Figure 2: Cases-both PTB and EPTB: Comparison of sensitivity and specificity of the ELISA. PTB: Pulmonary tuberculosis, EPTB: Extra pulmonary tuberculosis

DISCUSSION

In Indian population, TB is predominant. Hemagglutination was used as a technique to diagnose TB as a serologic method.¹⁰ The practical utility of antibody detection has not been proven due to the diversity of the host immune response and the basic nature of the disease. Bacterial burden may be correlated with antigen detection in the body fluids and also for the assessment of disease activity.¹¹ Different antibodies have been evaluated by various researchers in their studies to detect TB antigen.¹²⁻¹⁶

For our Sandwich ELISA to capture the tubercular antigen, isolation of the TB specific IgG was done with the serum of TB infected individuals. Detection of *M. tuberculosis* antigens with the use of such specific antibodies would be quite easy and specific. Furthermore, without any sophisticated technology, the antibodies were prepared for use in our Sandwich ELISA.

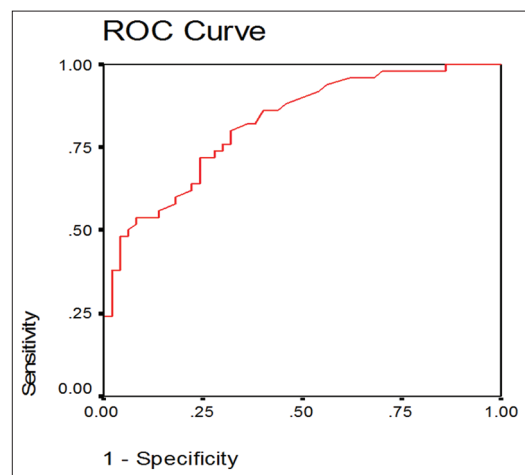


Figure 3: Test sensitivity and specificity represented by Receiver Operating Characteristic curve (Area=0.82)

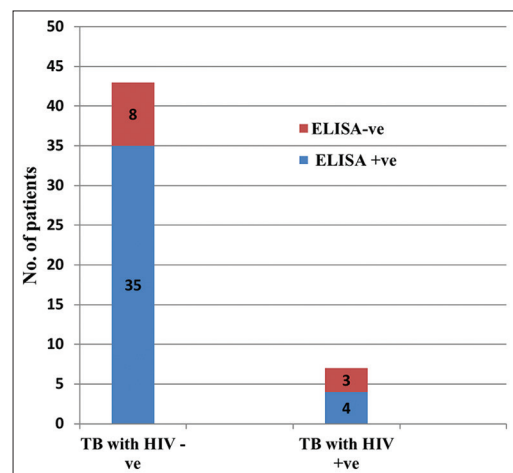


Figure 4: Sandwich ELISA results in PTB cases without HIV and PTB cases with HIV infection. PTB: Pulmonary tuberculosis

Various immunodiagnostic techniques have not widely clinically utilized for detection of mycobacterial antigens or specific antibodies. In general, available reagents are not used in some of them and also in some conditions, preparations were difficult. The test which uses routinely obtainable reagents would be appropriate to be used for TB diagnosis.¹⁷ The TB antigen detection Sandwich ELISA was developed by us using routinely obtainable commercial reagents.

The sensitivity of our Sandwich ELISA is well within the range of the sensitivities of other worker's antigen detection ELISA, that is, from 37% to 95% and the specificity is quite low than the other's work specificities, that is, from 73% to 100%, respectively.¹²⁻¹⁹ However, when the specificity was calculated against control groups and disease control as well as healthy control, it came 80% which was well within the range of the specificities of the other workers.¹²⁻¹⁹ Developed test had 70.91% positive predictive value and 75.56% negative predictive value.

Human immunodeficiency virus coinfection with TB globally increases the morbidity and mortality in many parts of the world.¹⁸ The WHO recommendations for the serological test sensitivity to diagnose PTB cases without HIV have achieved by our Sandwich ELISA.²⁰ HIV-TB coinfecting patients have low serum antigen load than TB cases without HIV infection, though the percentage of mycobacteria is probably more in HIV infected individuals. Suppressed immune response of HIV patients may be unable for mycobacterial degradation and thus not able to secrete antigens in the quantity needed to detect the test of Sandwich ELISA. This was one of the reasons of low sensitivity of developed Sandwich ELISA in respect to HIV infection in TB cases.²¹

The developed ELISA detected 16 positive of the 50 disease control patients, all of whom were diagnosed with non-tubercular pulmonary disorders. In the assay, use of first and second antibodies from the same species could be the reason for false positive reactions.¹⁷

Limitations of the study

We have some limitations in the developed Sandwich ELISA. Freeze-thaw cycle should be avoided to increase the effectivity of the test. Sensitivity of the developed test may be increase with the use of fresh serum. This might be one of the reasons for not achieving required sensitivity limit for serological test of TB diagnosis as per the WHO guidelines for Sandwich ELISA. At the last, the enrollment of few cases of HIV-TB coinfection cases and cases with other comorbidities also decreases its resemblance to most field-use conditions.

CONCLUSION

Anti-TB IgG is able to capture TB Ag from the serum of tubercular patients and is very much useful for serodiagnosis of active TB infection. Our Sandwich ELISA had a high negative predictive value which makes it a potential test to be considered for screening of active TB in a population at risk. Screening of sera for tubercular Ag by our Sandwich ELISA was sensitive and specific to detect TB infection without HIV. Furthermore, the test is quite effective in detecting the TB Ag from the HIV-TB coinfecting cases. In HIV-uninfected patients, combined use of serology and sputum microscopy was as sensitive as culture, thus representing an opportunity to greatly shorten the time for diagnosis in a substantial subset of patients.

Our conclusion for the developed test is that for increasing the sensitivity of the test, we were unable to achieve the WHO recommended specificity for the TB serodiagnostic test. We proposed that the work carried out for TB Ag detection from the serum using anti-TB IgG by Sandwich ELISA is more sensitive, specific, and worthy than TB antibody detection by other serodiagnostic test. We recommend that trials with more number of participants having TB are to be done before the utility of serodiagnosis for TB to be considered.

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Authors Contribution:

UA- Conceptualized the study, curated the data, and prepared the original draft; **ShA** – Reviewed the literature; **VA, SP,** and **SA**- Discussed the methodology and analyzed the data; and **SA** and **SP**- Provided the valuable inputs toward designing the manuscript. All the authors read and approve the final version of the manuscript

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