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Effectivity of In-House Developed Sandwich ELISA for Antigen Detection in Resource Constraint Settings

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Abstract---Introduction: Around 26% of World's Tuberculosis (TB) cases are in India. In resource constrained settings, it is difficult to use diagnostic tests like 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. On the basis of that we worked to detect tubercular antigen developing Sandwich ELISA from the serum of tuberculosis patients. Materials & Methods: For the study 50 cases, 50 disease controls, 30 healthy control subjects were taken. Commercially available reagents were used for the development of Sandwich ELISA. The data was statistically analyzed with the help of software SPSS version 16.0. Results: Our effectively developed test had 82% area under the ROC curve. 81% (n=35) patients of PTB cases with non-HIV infection detected by our Sandwich ELISA and 57% cases with the co-infection 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. To

increase the sensitivity and specificity of the Sandwich ELISA, as per the WHO guidelines, further work is needed.

Keywords---antigen, extra-pulmonary, HIV, sandwich ELISA, tuberculosis.

Introduction

Mycobacterium tuberculosis is the causative bacillus for tuberculosis (TB) infection/disease. It is pulmonary TB involving lungs and also extrapulmonary TB involving spine, abdomen, etc. As per 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. Most of the time clinicians treat 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. 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% of worldwide TB cases are present in India. In India, the incidence of TB is 2-2.5 million cases with the prevalence of 2 - 4.6 million cases along with the HIV-TB co-infection having 0.1 million cases. [3] There is 1.0 smear diagnosis laboratory per 1 million population & 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 like extra-pulmonary TB, 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 technique for the detection of antigen. [4] In July 2010 a meeting was held by 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 extra-pulmonary TB by developing Antigen-capture ELISA using tubercular IgG from patient's serum.

Materials and Methods

Institutional Ethical Committee (DMIMS [DU]/IEC/2010-11/15 dated 30.9.2010) gave the clearance for the project to proceed and we completed the project in two years. Selections of the study population were done from rural based tertiary care hospital. 50 patients having Pulmonary tuberculosis (PTB) alongwith HIV infections (n=7), 30 healthy control subjects, 50 disease control patients and also 6 TB patients having focus outside the lungs were included. Subjects who were free from any diseases for the last one 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, in a plain bulb 2 ml blood (venous) from each study participants was taken aseptically. The collected sample undergoes centrifugation to separate the sera at 3000 rpm for 10 minutes. 10% sodium azide (NaN_3) (S D fine chem. Pvt Ltd, Mumbai) 10 μl /ml and 10 μl /ml protease inhibitor cocktail (Sigma) were added in the separated sera samples. Unique numerical coding was given to each sample. Commercially available cocktail TB Ag (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 utilised for this purpose.

We pooled sera samples (pulmonary TB patients) which were well preserved by previous workers of our department and utilized it to isolate anti-TB IgG. Ammonium sulphate 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 elute 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 (ER-2005; -B4B Diagnostic Division); OPD A_{492} primary & A_{620} secondary. SPSS version 16.0 was used for the statistical analysis.

Results

The calculation of mean and standard deviation were done of the ELISA titres for the cases, disease control and healthy controls. The mean and the SD of the ELISA titre for HC group was 0.86 and 0.10 respectively. The cut-off was set to 1.06 which was derived from the data and equals to 'mean + 2SD'. Thus positive result was considered above this cut-off value and negative when the value was below cutoff. PTB cases had 2.25 and 0.93 as the mean and SD of ELISA titre respectively. The ELISA titre of PTB cases was ranged from 0.75 to 4.06. DChad

1.24 and 0.62 as the mean and SD of the titre respectively. The ELISA titre 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%) EP-TB cases were done correctly. The 78% sensitivity was noted to detect TB Ag in PTB cases while in cases of EP-TB it was only 67%. But 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 was 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%. (Fig:3) Sandwich ELISA detected the Ag in HIV non-infected cases- 81% (35) while only 57% (n=4) of the HIV-TB co-infection cases. (Figure 4).

Figures

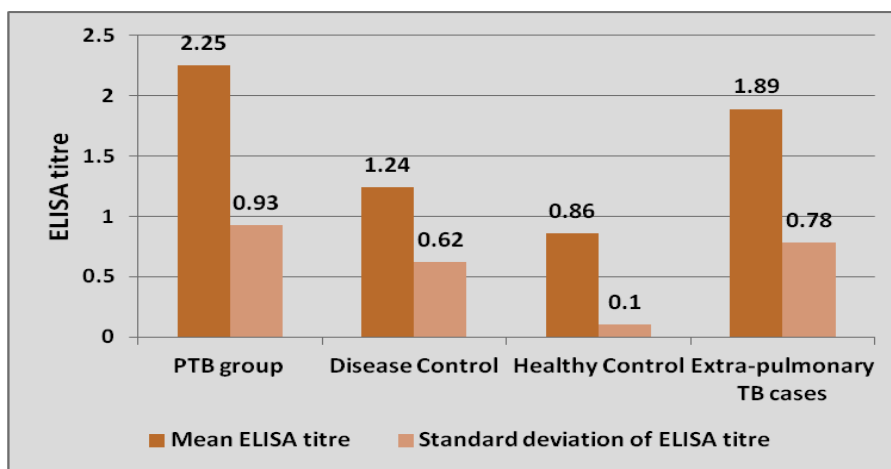


Figure 1. Result of the ELISA titre of mean of ELISA and Standard deviation were compared

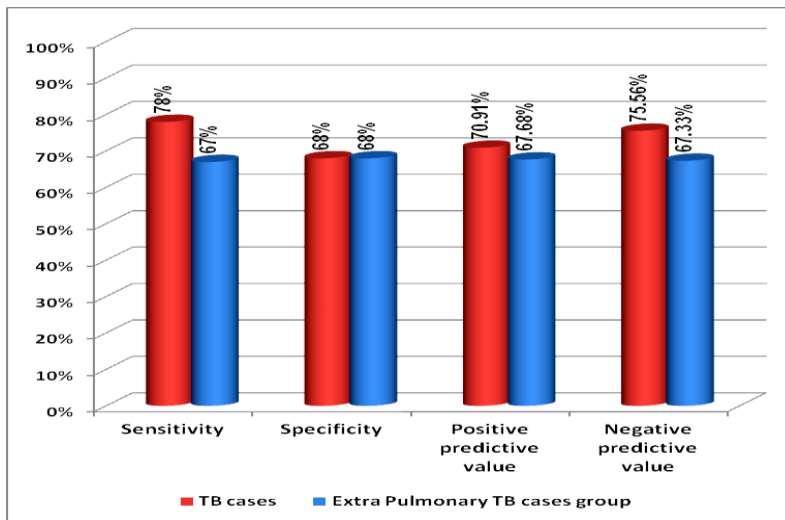


Figure 2. Cases -both Pulmonary TB and Extra-Pulmonary TB: Comparison of Sensitivity and Specificity of the ELISA

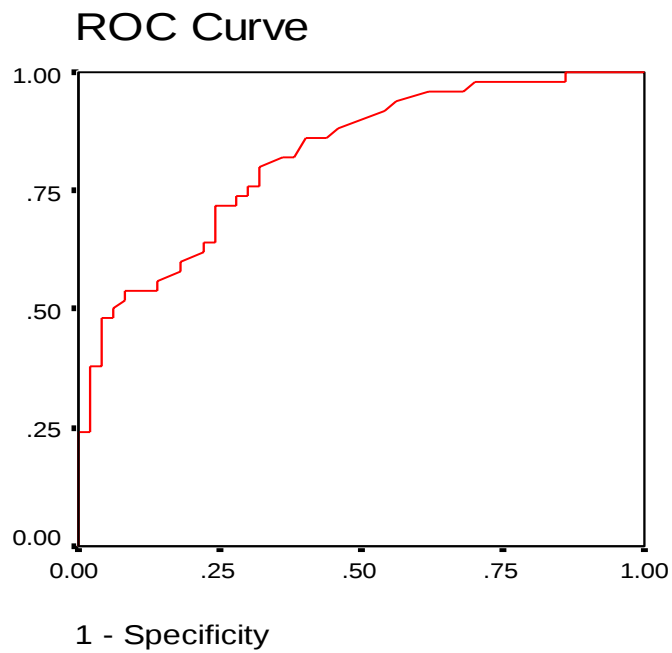


Figure 3. Test sensitivity and specificity represented by receiver operating characteristic curve (Area=0.82)

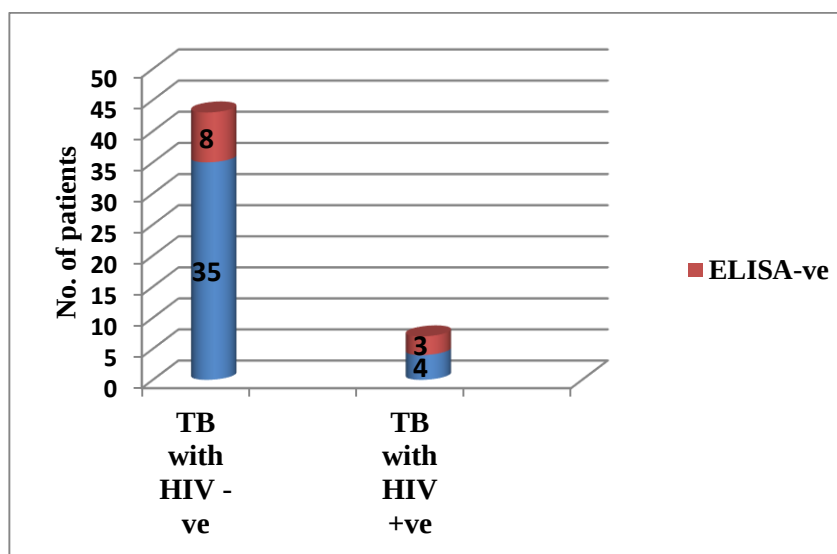


Figure 4. Sandwich ELISA results in PTB cases without HIV and PTB cases with HIV infection

Discussion

In Indian population tuberculosis is predominant. Hemagglutination was used by Arloing in 1898 as a technique to diagnose tuberculosis as a serologic method.[10] The practical utility of antibody detection has not 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, 13, 14,15,16]

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. Also, without any sophisticated technology the antibodies were prepared for use in our Sandwich ELISA. Various immunodiagnostic techniques have not widely clinically utilized for detection of mycobacterial antigens or specific antibodies. Generally 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 i.e., from 37%-95% and the specificity is quite low than the other's work specificities i.e., from 73%-100% respectively.[12-19] But when the specificity was calculated against control groups, 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 co-infection with tuberculosis 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 has achieved by our Sandwich ELISA. [20]HIV-TB co-infected 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 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 tuberculosis cases. [21]. Some of the critical studies on diagnostic aspects of Tuberculosis were reviewed [22-16].

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] 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 WHO guidelines for Sandwich ELISA. However, the described method for sample processing was too laborious for daily use in laboratory which may be a reason of low sensitivity of our Sandwich ELISA. [19] At the last, the enrollment of few cases of HIV-TB co-infection cases and cases with other co-morbidities also decreases its resemblance to most field-use conditions.[27-31]

Conclusion

We have concluded that 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 tuberculosis infection without HIV. Also the test is quite effective in detecting the TB Ag from the HIV-TB co-infected 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 tuberculosis are to be done before the utility of serodiagnosis for tuberculosis to be considered.

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Conflict of interest: Nil.

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