Int J Pharm Bio Sci 2013 Apr; 4(2): (B) 525 - 533

Research Article **Biochemistry**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

SANDWICH ELISA FOR TUBERCULAR ANTIGEN DETECTION IN AFB SPUTUM POSITIVE TUBERCULAR PATIENTS IN RURAL BASED TERTIARY CARE HOSPITAL.

AJAY MESHRAM[1]* AND UDIT AGRAWAL[2]

1. Professor, Department of Biochemistry, J N Medical College, Sawangi (M), Wardha.
2. Postgraduate student, Department of Biochemistry, J N Medical College, Sawangi (M), Wardha.

ABSTRACT

Introduction: It is estimated that one-third of the world's population is infected with M. tuberculosis. Also we came to know that the numbers of TB cases are on the rise in our rural based tertiary care hospital. We searched for Mycobacterium tuberculosis antigen in the serum of patients with pulmonary TB by developing Sandwich ELISA.
Materials & Methods: We took 50 pulmonary TB patients, 50 disease control patients, 30 healthy control subjects and 6 extra-pulmonary tubercular patients for our study. We procured the commercial cocktail TB Ag of the mark-Hotgen Biotech and isolated the anti-TB IgG from the sera of TB infected patients. We pooled the well preserved sera samples of TB infected patients for the isolation of anti-TB IgG. We have used anti-TB IgG for the development of Sandwich ELISA for tubercular Ag detection.
Results: In 39 out of 50 PTB cases, TB Ag can be detected accurately by our Sandwich ELISA but 16 out of 50 DC cases gave false positive result. Likewise, our test successfully detected TB Ag in 78.12% of PTB cases having 1+ sputum positivity while 83.33% of 2+ and 66.66% of 3+ sputum positive PTB cases.
Conclusion: In association with AFB sputum microscopy, the Sandwich ELISA was used as an effective screening test for tuberculosis diagnosis mainly in pulmonary tuberculosis patients.

KEYWORDS:Tuberculosis, antigen, Sandwich ELISA, Sputum positive.

AJAY MESHRAM
Professor, department of Biochemistry, J N Medical College, Sawangi (M), Wardha.

**Corresponding author*

INTRODUCTION

It is estimated that one-third of the world's population is infected with M. Tuberculosis (1) and that every year 9.2 million new cases of TB are diagnosed, and up to 2.5 million deaths are attributed to the disease (2). Tuberculosis is the leading cause of death in 15 to 45 year age group (3).BCG, the only commercially available vaccine, has been in use since the early 1920s. However, while this vaccine protects children from disseminated TB, it does not prevent adult or pulmonary disease, the latter being the most common and contagious form of TB. In the Indian population, the Mantoux test is used to support the diagnosis of active TB: however, the majority of individuals who are bacilli Calmette-Guérin (BCG) vaccinated or exposed to Mycobacterium tuberculosis may give false positive results to this test. (1)Diagnosis of active TB still relies primarily on the direct finding of the tubercle bacilli either in sputum smears or in culture, procedures that are operator dependent and not sensitive enough to detect more than 65 to 70% of the disease burden (4). Detection by acid-fast bacilli (AFB) staining and culture lacks sensitivity while chest lesions identified by radiographs cannot identify causal agents. The clinical diagnosis of TB is often problematic, and co-infection with other diseases is not uncommon, which leads to difficulty in diagnosis of these cases. Another limitation to control of TB is the lack of a sensitive and reliable diagnostic procedure. Developing countries have experienced a major increase in the burden of TB disease, a problem that is compounded by the emergence of multidrug-resistant TB. Detection of TB using traditional diagnostic tools is a major challenge. Numerous novel diagnostic candidates are currently being pursued. The primary approaches to their discoveries have used the immune response of patients with TB as the readout of the antigen discovery strategies to select the candidate molecules. More efforts are therefore directed towards developing reliable and less costly immunoassays based on the detection of mycobacterial antigens/antibodies in body fluids.However, an interesting alternative approach to this strategy is the direct identification of M. Tuberculosis antigens in the bodily fluids of humans with active disease. A common design for antigen detection test is the Sandwich enzyme linked immunosorbent assay (ELISA) technique, also called the antigen-capture ELISA (5).The WHO Expert Group meeting report July, 2010 strongly encouraged further research to identify new/alternative serological tests with improved accuracy. Further research on accurate point-of-care tests for TB diagnosis was strongly recommended (6). Using this premise, we searched for M. Tuberculosis antigen in the serum of patients with pulmonary TB by developing Sandwich ELISA (4).

MATERIALS AND METHODS

We started our work in October 2010 after getting the clearance of the project from Institutional Ethical Committee and finished it in September 2012. We took 50 Pulmonary tuberculosis (PTB) patients including HIV-TB co-infection cases (n=7), 50 disease control patients, 30 healthy control subjects and 6 extra-pulmonary tubercular patients for our study from the Department of Tuberculosis and Chest Medicine, Acharya Vinobha Bhave Rural Hospital, Sawangi (Meghe), Wardha. Healthy control subjects were apparently healthy at the time of sample collection and not suffering from any diseases from the last one year of duration.The use of microscopy to identify symptomatic patients with acid-fast bacilli (AFB) in sputum smears is an essential component of the World Health Organization's 'directly observed treatment, short course' strategy (DOTS) to control TB (7). We were categorized the pulmonary tuberculosis cases on the basis of DOTS sputum grading system (8).

Collection of blood samples, processing and its storage:2 ml of venous blood was collected from each individual after taking informed consent in a plain bulb with all the aseptic precautions and kept for settling to coagulate for at least 15-20 minutes. The sera was separated from this by centrifuging the coagulated blood at 3000 rpm for 10 minutes and stored.

Mycobacterial strain used: We procured commercially available cocktail TB Ag which is a mixture of recombinant protein having purity > 95% of the mark- Hotgen Biotech Co., from local supplier for our present study to isolate tubercular antigen specific antibody from the patients sera who were diagnosed cases of tuberculosis.. Pooled Sera of the Pulmonary tubercular patients for anti-tubercular (anti-TB) IgG isolation: The sera samples of tubercular patients used by previous workers of the department were well preserved. We pooled those sera samples and then used for anti-TB IgG isolation. Use of well preserved specimens had several advantages including convenience, speed and low cost.The anti-TB IgG was successfully isolated from the total IgG fraction using affinity chromatography with column having cyanogen bromide activated Sepahrose 4B coated with commercial cocktail TB Ag (9). We used BIO-RAD BioLogic LP chromatography instrument along with fraction collector (model 2110) for both the chromatography technique. That anti-Tb IgG was used for two purposes: one for the coating of the well of ELISA plate and second for the conjugate preparation with horse radish peroxidase (9). O-phenylenediamine was used as a substrate for our Sandwich ELISA (10).

Our Sandwich ELISA Protocol for TB Ag detection

1. Tuberculosis Ab (anti-TB IgG) dilution

Prepare 0.2mg/ml anti-tubercular IgG in 0.01M PBS, pH-7.2.

2. Anti-tubercular IgG coating to wells

Dispense 10uL (2ug/well) in each well of the ELISA plate (Himedia Co.) and incubate overnight at 4^{o}C in a humid chamber. Cover the plate properly by paraffin film and then kept for incubation for 24 hrs. Wash the wells with 0.01M PBS-T in ELISA washer for 4 mins x 3 cycles.

3. Blocking free sites of the wells

Dispense 100ul of 0.5% BSA (Himedia) in 0.01M PBS, pH-7.2. Incubate for 2 hrs at 37^{o}C in a humid chamber. Wash with PBS-T for 4 mins x 4 cycles.

4. Capturing of Tuberculosis Ag from serum

Dilute serum 200 times with PBS-T. Dispense 100 uL in each well and incubate in a humid chamber for 1½ hr at 37^{o}C. Each sample was tested in duplicate. Wash with PBS-T for 4 mins x 4 cycles.

5. Visualisation of bound Tuberculosis Ag

a. Binding of conjugate to captured Ag: Dispense 50 uL of 1000 times diluted conjugate in PBS-T. Incubate for 1½ hr at 37ºC in a humid chamber. Wash with PBS-T for 4 mins x 4 cycles. b**.**Substrate addition: Dispense 100 uL of freshly prepared substrate solution of o-Phenylenediamine.2HCl (OPD) and keep in dark till there is good colour differentiation of healthy & tubercular sera (about 15 mins).

6. Stopping the reaction

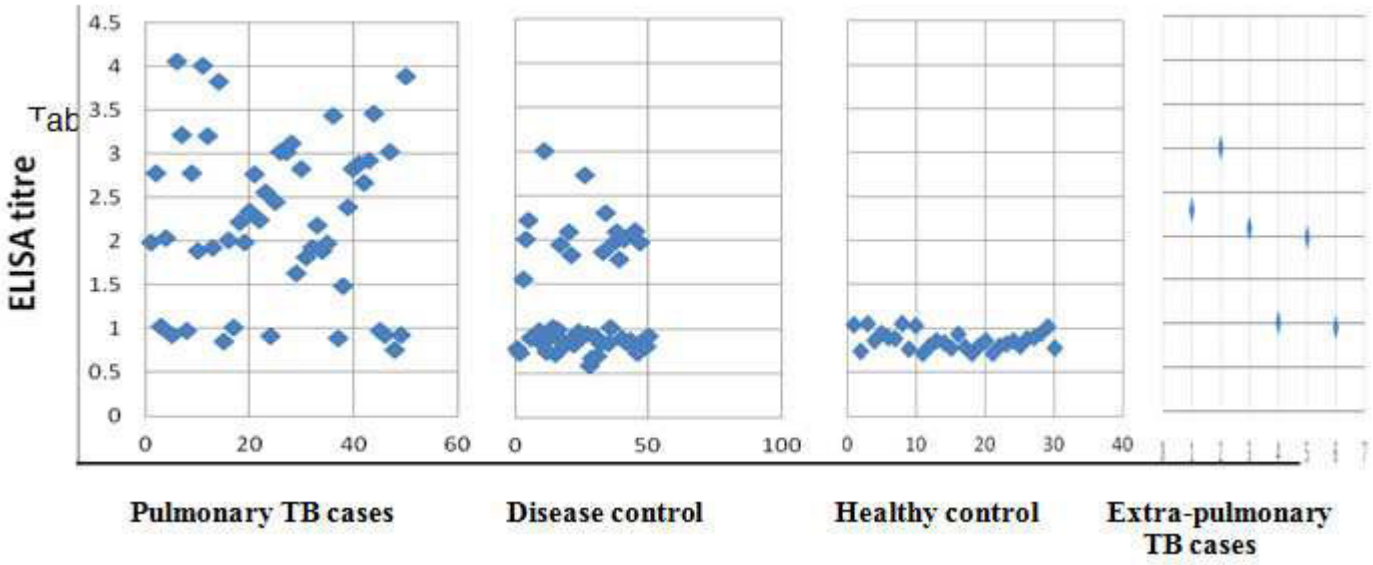
Dispense 100 uL of 2N H_2SO_4 and mix well. Colour changes from yellow to reddish yellow and colour is stable for 25 mins (Fig-9). Take reading by ELISA Reader: For OPD A_{492} primary & A_{620} secondary. The cut-off for each test was determined as mean OD/absorbance 492nm plus 2 standard deviations (SD) of the group of healthy control sera (7). Values above the cutoff value, 1.06, were considered to be positive. The cutoff value was mean (0.86) plus two times the standard deviation (0.10) of the ELISA titre of the serum samples from the 30 Healthy Control subjects. We used ELISA Reader (ER-2005) and Washer (EW-2005) of B4B Diagnostic Division for our Sandwich

ELISA. The data obtained was analysed using - Statistical software SPSS 16.0

RESULTS

In 39 out of 50 PTB cases, TB Ag can be detected accurately by our Sandwich ELISA but 16 out of 50 DC cases gave false positive result. 04 (66.67%) EP-TB cases diagnosed accurately by our test thus proved the effectiveness of the test in serodiagnosis of extra-pulmonary TB cases. None of the HC group subjects had their ELISA titre above cut-off (>1.06) which shows the high specificity of the test in healthy individuals. (Fig-1) It is clear from the table (Table-1) that maximum no of PTB patients (32) was categorized in 1+ DOTS sputum grading while 12 in 2+ and only 6 had 3+ sputum positivity. The mean ELISA titre was also increased with the increment in bacterial load as 2.21 in 1+ while 2.45 in 2+ and 2.32 in 3+ sputum positive patients. Our test successfully detected TB Ag in 78.12% of PTB cases having 1+ sputum positivity while 83.33% of 2+ and 66.66% of 3+ sputum positive PTB cases.The PTB group had newly (41) diagnosed cases more in number & some of relapse (02) and defaulter (07) cases. The mean ELISA titre in newly diagnosed cases was 2.25 which was 2.08 times than the cut-off value while in defaulter cases the mean ELISA titre was 2.53 which was 2.34 times than the cut-off value. (Table-2)PTB cases had a mean age of 42.54 years, whereas healthy control subjects tended to be younger (33.1 years) and patients with non-TB pulmonary disease older (55.4 years). The age wise correlation was found statistically significant ($p<0.0001$). (Fig-2)Our Sandwich ELISA successfully detected 80% (32) of the male patients of PTB cases and 70% (7) of the female patients of PTB cases. 03out of 05 males suffering from EP-TB had detectable TB Ag as shown by our Sandwich ELISA result. The female patient included in our EP-TB study group was only one in number and also our test was quite effective to detect TB Ag in that single tubercular female patients. (Table-3 and fig-3)

Figure 1
Scatter graphic representation of the ELISA titre of all the study groups



(ELISA titre: <1.06=negative result & > 1.06=positive result)

Table-1

Distribution of the Pulmonary TB cases according to DOTS sputum grading system.

DOTS Sputum grading	No.of patients	Mean ELISA titre	Range	Comparison to cut off value (1.06)	Sandwich ELISA result		Sensitivity of the Sandwich ELISA
					Positive	Negative	
1+	32 (64%)	2.21	0.75 - 4.01	2.08 fold	25	07	78.12%
2+	12 (24%)	2.45	0.98 - 4.06	2.31 fold	10	02	83.33%
3+	6 (12%)	2.32	0.89 -3.89	2.18 fold	04	02	66.66%
Total	50 (100%)	2.28	0.75 - 4.06	2.15 fold	39	11	78%

Table 2

Evaluation of the Sandwich ELISA in defaulter, relapse and new pulmonary TB cases.

	Diagnosis			
ELISA Test Result	**Pulmonary TB Relapse cases**	**Pulmonary TB Defaulter cases**	**Pulmonary TB New cases**	**Total**
Mean ELISA titre	1.42	2.53	2.25	-
Comparison to cut off value (titre >1.06)	1.31 fold	2.34 fold	2.08 fold	
Positive	01 (50%)	05 (71%)	33 (80.48%)	39
Negative	01 (50%)	02 (29%)	08 (19.51%)	11
Total	02	07	41	50
Sensitivity of the Sandwich ELISA	50%	71%	78%	78%

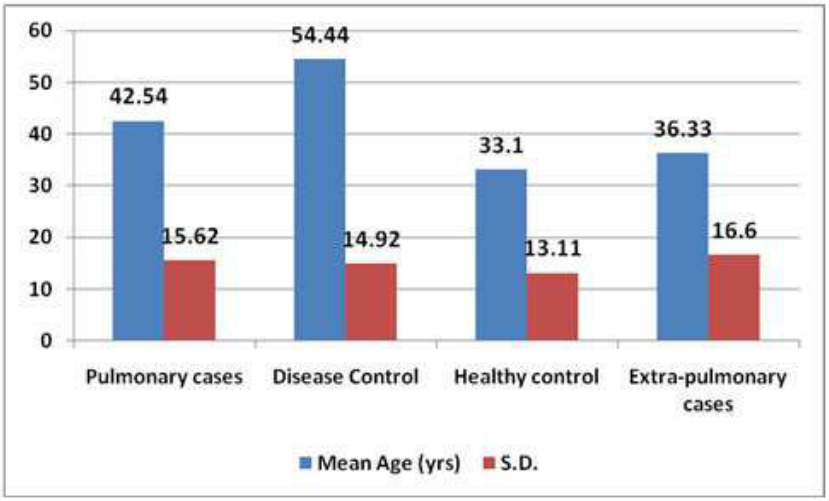


Figure 2

Mean & standard deviation of age of the study group subjects

Table 3
Sex wise distribution of the study group subjects.

	Diagnosis			
Sandwich ELISA Test Result	**PTB male**	**PTB female**	**EP- TB male**	**EP- TB female**
Positive	32	7	3	1
Negative	8	3	2	0
Total	40 (80%)	10 (20%)	5 (83.33%)	1 (16.67%)
Sensitivity of the Sandwich ELISA	80%	70%	60%	100%

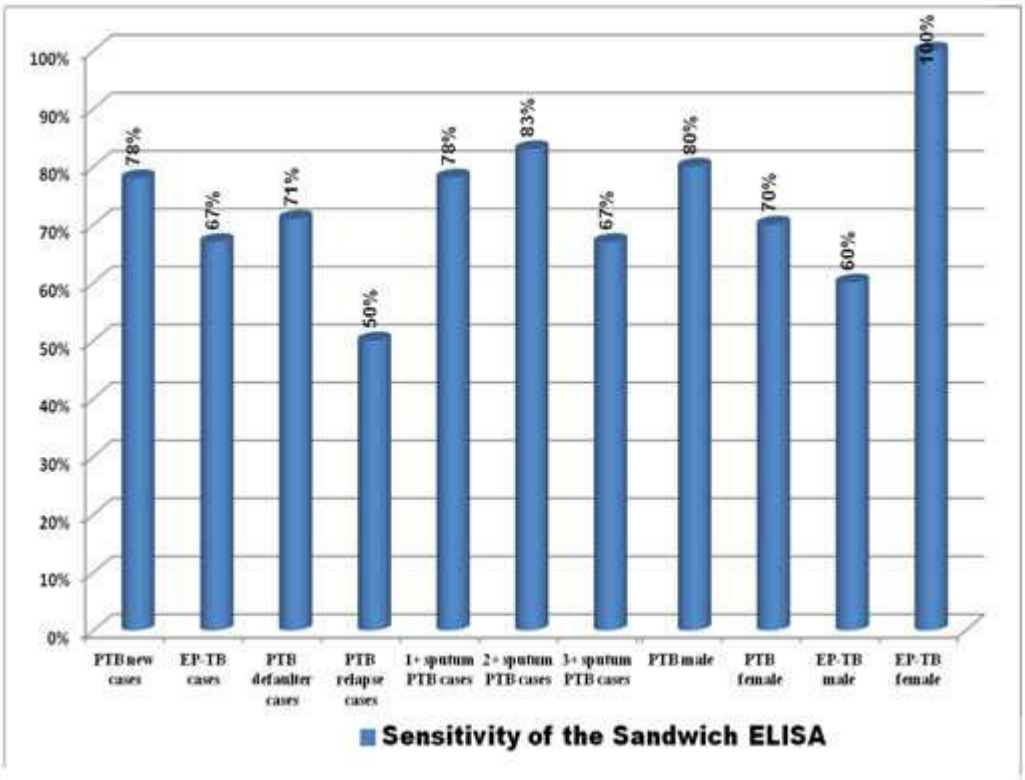


Figure 3
Sensitivities of the Sandwich ELISA in different study groups.

DISCUSSION

India bears one-quarter of world's TB cases which is an alarming situation. Also we came to know that the numbers of TB cases are on rise in our rural based tertiary care hospital. We thought to work on the serodiagnosis of TB and to develop a Sandwich ELISA which will be easy to handle, cost-effective and having better sensitivity and specificity than the test available in the market.Sputum microscopy, currently the sole diagnostic test in most areas where TB is endemic, has several limitations; in particular, the sensitivity compared with that of culture is

variable, multiple patient visits are required, considerable technical training is necessary, and the procedure is labor-intensive (11). Diagnosis using culture method requires laboratory infrastructure that is not widely available in countries with a high burden of TB (12), and results take weeks. Conventional methods used to diagnose MDRTB also rely on culturing of specimens followed by drug susceptibility testing (DST). Chest radiography is normal in up to 10–20% of patients. WHO endorsed Xpert MTB/RIF assay for the rapid diagnosis of TB and rifampicin-resistant TB. These are beginning to be implemented in countries (12), and Xpert MTB/RIF in particular (a fully automated, cartridge-based, nucleic acid amplification test) has the potential to transform the diagnosis of TB and drug-resistant TB. The advent of the nucleic acid amplification test seems to help in the diagnosis of TB, however faced with problems of contamination and sensitivity (13). Further, this technique is expensive, time consuming and requires special expertise and equipment.An ELISA test is relatively simple, inexpensive and does not require sophisticated equipment, and above all, the method is suitable for use in laboratories in low-income countries. Owing to the basic nature of the disease and the diversity of the host immune response, antibody detection has not proved of practical use. Demonstration of antigen in the body fluids would be a better tool for assessing its role in disease activity and also it may correlate with bacterial burden. (14, 15) The antibody present in human serum for MTB was developed naturally in vivo after the infection of tuberculosis to a healthy individual. This naturally developed antibody was more specific for tubercular Ag than the antibody raised in animal bodies of mice, rats, rabbits or goat. So, we isolated the tubercular specific antibody from the sera of tubercular infected patients for the capture of tubercular antigen for our Sandwich ELISA. The result of our Sandwich ELISA have shown significant findings in comparison to age of the different study groups ($p<0.0001$). According to WHO, TB is also more common among men than women, and affects mostly adults in the economically productive age groups; around two-thirds of cases are estimated to occur among people aged 15–59 years (2). The sensitivity our Sandwich ELISA to detect tubercular Ag of PTB in male patients was 80% while of PTB in female patients was 70%, quite supportive of WHO findings. The result of the Sandwich ELISA with Extra-pulmonary TB patients was quite reversed than PTB patients in relation to sex of the patients. As the sample size was very less in extra-pulmonary TB group, so no conclusion can be drawn for the same.All the tubercular cases were categorized on the basis of DOTS sputum grading system. The mean ELISA titre was increased along with the increase in bacterial load when compared with 1+ and 2+ sputum positive PTB cases which was 2.21 and 2.45 respectively. The detection limit of the Sandwich ELISA was also increased with the increase in the bacterial load as it detected only 78% of 1+ while 83% of 2+ sputum positive PTB cases.The overall sensitivity of the Sandwich ELISA used was 78%. Thus along with AFB sputum microscopy, it proved to be an effective screening test for tuberculosis diagnosis particularly in pulmonary tuberculosis patients. Although various immunodiagnostic techniques, based on the detection of mycobacterial antigens or specific antibodies have been reported, they have not come into wide spread clinical use. The reagents used in some of them are not generally available and were difficult to prepare. With the use of generally available reagents, the test would be suitable as a routine diagnostic test of tuberculosis (16). In our study, we had developed the Sandwich ELISA with the use of commercially available routine reagents. The low sensitivity obtained in our study may be due to variations in antigen expression under different conditions. Previous studies have reported that M. Tuberculosis adapts to its environment by altering the profile of the genes that it expresses (17). A second limitation to the study is the lack of inclusion of patients with known non-tuberculous mycobacterial infections, as cross-reactive antibodies to antigens from other

mycobacterial species could result in loss of specificity. The Sandwich ELISA format has the advantages that many serum samples can be tested in parallel and the process can be completely automated, making this technique attractive in the laboratories having minimal facilities. Specificity was very high in healthy individuals and was not compromised by tuberculin sensitivity or childhood BCG vaccination.

CONCLUSION

We have shown that anti-TB IgG is able to capture TB Ag from the serum of tubercular patients and is a sensitive marker of serodiagnosis of active TB infection. In association with AFB sputum microscopy, the Sandwich ELISA was used as an effective screening test for tuberculosis diagnosis mainly in pulmonary tuberculosis patients. 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 feel that larger trials with more representative populations are needed. The most powerful tools in any TB control programme are accurate diagnosis and successful, timely treatment.

ACKNOWLEDGMENT

Authors are grateful to Datta Meghe Institute of Medical Sciences (Deemed University) for funding this work.Conflict of interest: The author(s) declare no conflict of interests.

REFERENCES

1. Kashino SS, Pollock N, Napolitano DR, Rodrigues V and Campos-Neto A. Identification and characterization of Mycobacterium tuberculosis antigens in urine of patients with active pulmonary tuberculosis: an innovative and alternative approach of antigen discovery of useful microbial molecules. British Society for Immunology. Clin and Exper Immun 2008; 153: 56–62.
2. WHO Report 2011. Global Tuberculosis Control: 1-25.
3. WHO Report 2009. Global Tuberculosis Control; A Short Update To the 2009 Report: 4-6.
4. Napolitano DR, Pollock N, Kashino SS, Rodrigues V and Campos-Neto A. Identification of Mycobacterium tuberculosis Ornithine Carboamyltransferase in Urine as a Possible Molecular Marker of Active Pulmonary Tuberculosis. Clin and Vacc Immun 2008; 15 (4):638–643.
5. McNerney R and Daley P. Towards a point-of-care test for active tuberculosis: obstacles and opportunities. Nat. Rev. Microbiol 2011; 9:204–213.
6. WHO-TDR for research on disease of poverty. Commercial Serodiagnostic Tests for Diagnosis of Tuberculosis. Expert Group Meeting Report 2010: 1-72.
7. Chanteau S, Rasolofo V, Rasolonavalona T, Ramarokoto H, Horn C, Auregan G and Marchal G. 45/47 kilodalton (APA) antigen capture and antibody detection assays for the diagnosis of tuberculosis. Int J Tuberc Lung Dis 2000; 4(4):377–383.
8. American Thoracic Society. Diagnostic Standards and Classification of Tuberculosis in Adults and Children. Am J Respir Crit Care Med 2000; 161:1376–1395.
9. Page M and Thorpe R. The Protein Protocols Handbook. 2nd ed. New Jersey: Humana Press Inc, 2002.
10. Bovaird JH, Ngo TT and Lenhoff HM. Optimizing the o-Phenylenediamine Assay

for Horseradish Peroxidase: Effects of Phosphate and pH, Substrate and Enzyme Concentrations, and Stopping Reagents. Clin Chem 1982; 28(12): 2423-2426.
11. Steingart KR, Dendukuri N, Henry M, Schiller I, Nahid P, Hopewell PC, Ramsay A, Pai M and Laal S. Performance of Purified Antigens for Serodiagnosis of Pulmonary Tuberculosis: a Meta-Analysis. Clin and Vacc Immun 2009; 16 (2): 260–276.
12. WHO Report 2011 Global Tuberculosis Control. Chapter 5: New diagnostics and laboratory strengthening for TB: 54-60.
13. Minion J, Leung E, Talbot E, Dheda K, Pai M and Menzies D. Diagnosing tuberculosis with urine lipoarabinomannan: systematic review and meta-analysis. Eur Respir J 2011; 38: 1398–1405
14. Laboratory-based evaluation of 19 commercially available rapid diagnostic tests for tuberculosis. Special Programme for Research & Training in Tropical Diseases (TDR) sponsored by U N I C E F / U N D P / W o r l d B a n k / W H O 2008:1-80.
15. Study of M. tuberculosis ES-31 and ES-20 antigen levels in different pathogenic grades of lymph node tuberculosis. Int J Tuberc Lung Dis 2007; 11(2):222–226.
16. Banchuln N, Wongwajana S, Pumprueg U and Jearanalsilavong J. Value of an ELISA for Mycobacterial Antigen Detection as a Routine Diagnostic Test of Pulmonary Tuberculosis .Asia Pac Jou of Aller and Immu 1990; 8: 5-11.
17. Beyene D, Franken KL, Yamuah L, Aseffa A, Wiker HG, Kolk A, Engers H, Klatser P and Sviland L. Serodiagnosis of tuberculous lymphadenitis using a combination of antigens. J Infect Dev Ctries 2010; 4(2):096-102.