Original Article

Serodiagnosis of tuberculous lymphadenitis using a combination of antigens

Demissew Beyene[1,2], Kees Lumc Franken[8], Lawrence Yamuah[1], Abraham Aseffa[1], Harald G.Wiker[3,4], Arend Kolk[7], Howard Engers[1], Paul Klatser[7] and Lisbet Sviland[5,6]

[1]Armauer Hansen Research Institute (AHRI), Addis Ababa, Ethiopia
[2]Centre for International Health, University of Bergen, Bergen, Norway
[3]Section for Microbiology and Immunology, The Gade Institute, University of Bergen, Norway
[4]Department of Microbiology and Immunology, Haukeland University Hospital, Bergen, Norway
[5]Department of Pathology, Haukeland University Hospital, Bergen, Norway
[6]Section for Pathology, The Gade Institute, University of Bergen, Norway
[7]The Royal Tropical Institute (KIT), Biomedical Research, Amsterdam, The Netherlands
[8]Department of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands

Abstract
Background: The diagnosis of extra-pulmonary tuberculosis (EPTB) by conventional methods such as culture and microscopy has low sensitivity and requires an invasive procedure. A simple rapid serological test would be of great value.
Methodology: Six antigens (ESAT-6, Ag85A, TB10.4, Rv3881c, lipoarabinomannan (LAM) and Ara_6-BSA) were tested in an ELISA to detect antigen-specific IgG and IgM antibodies in sera from 54 culture- and histology-confirmed tuberculous lymphadenitis (TBLN) patients as follows: four were HIV seropositive; sera from 25 was smear positive for pulmonary tuberculosis (PTB); 15 were culture- and histology-negative lymphadenitis (non-TBLN) patients; and 22 werehealthy controls (HCs).
Results: The sensitivities of the antigens for the detection of IgG in sera of TBLN patients ranged from 4% to 30%. Specificities ranged from 73% to 100% with sera from non-TBLN patients and 91% to 100% with sera from HCs. Sensitivities of the antigens for detection of IgM ranged from 0% to 15% and specificities ranged from 80% to 100% with sera from non-TBLN patients and 91% to 100% with sera from HCs. LAM was the most potent antigen for detection of IgG. When LAM and ESAT-6 were combined, sensitivity increased up to 43% and specificity with non-TBLN was 80% with HC 96%.
Conclusions: The study suggests that the combined use of LAM and ESAT-6 for IgG antibody detection in sera from TBLN patients could be a supplement to microscopy of fine-needle aspirate (FNA) to diagnose TBLN among patients suspected of TBLN.

Key words: tuberculous lymphadenitis, serological test, antigens

Introduction

Tuberculosis (TB) is a major public health problem in Ethiopia. According to the World Health Organization (WHO) report of 2008 [1], Ethiopia is number 7 among the 22 high TB-burden countries. Primarily considered a pulmonary disease, TB can affect almost any organ. According to the Ethiopian National TB and Leprosy Control program (NTLC) [2], pulmonary TB (PTB) accounts for 63% of all TB cases and extra-pulmonary TB (EPTB) accounts for 37% of cases, with 80% of EPTB being lymph node TB [2]. In spite of these high numbers, diagnosis of tuberculous lymphadenitis (TBLN) remains a challenge.

The clinical signs and symptoms of EPTB are non-specific [3] and both culture and microscopy using Ziehl-Neelsen (ZN) staining to detect acid-fast bacteria (AFB) have low sensitivity as most cases are paucibacillary [4]. The polymerase chain reaction (PCR) is sensitive and specific but expensive and not available in the majority of health care centers in resource-poor countries. Moreover, these tests involve an invasive procedure to obtain clinical specimens [5]. Thus there is a great need to develop simple, rapid, affordable and user-friendly methods to diagnose EPTB. Serological tests would be of great value in resource-poor settings and there are commercial serological tests available that may be used in the diagnosis of EPTB_but they are highly

variable with respect to sensitivity (0 to 100%) and specificity (59 to 100%) [6].

This study evaluated the diagnostic potential of six antigens, early secretory antigenic target-6 (ESAT-6), antigen 85A (Ag85A), TB10.4, Rv3881c, LAM and Ara$_6$-BSA, by using sera from TBLN patients, healthy controls (HCs), non-tuberculous lymphadenitis (non-TBLN) cases and PTB patients. Previous studies have reported that Ag85A, Rv3881c, LAM, Ara$_6$-BSA and ESAT-6 may be useful for the serodiagnosis of pulmonary and bovine TB respectively [7-11]. Ag85A, ESAT-6 and TB10.4 are strongly recognized by T-cells isolated from TB patients and have been seen to induce a robust cell mediated immune response [7,12,13]. However, little is known about the diagnostic potential of these antigens in EPTB. The purpose of the study was to assess the potential of the six antigens and to identify the most potent single and/or combined antigens to differentiate between TBLN and non-TBLN patients using ELISA.

Materials and Methods

Study Participants

Ethical approval was obtained from AHRI/ALERT Ethical Committee (Reg.No.001/05) and the National Ethical Review Committee (NERC), (RDHE/190-64/2005), and the study was conducted from October 2005 until July 2006. Patients 18 years and above attending Butajira hospital, located l35 km south of Addis Ababa, 2) who had enlarged lymph nodes that did not respond to a course of broad spectrum antibiotics and were screened with the current diagnostic algorithm for TBLN in Ethiopia [14]; were considered for the study. All participants signed a written informed consent form. Patients who were critically ill, taking anti-TB drugs, had other forms of TB, were anemic, or had medical contra-indications were excluded from the study. Among 115 TBLN suspects considered for the study, 100 were *M. tuberculosis* culture positive, 54 (proven TBLN) were culture and histology positive, 15 (non TBLN patients) were culture and histology negative, and 46 were excluded from the study by the exclusion criteria. The 54 proven TBLN and all the 15 non-TBLN patients were included in the study. The number of non-TBLN cases was small in number. We therefore included, upon consent, sera from 25 PTB and 22 HCs who were 18 years and above and residing in the same locality. General physical examination confirmed that the HCs did not have signs and symptoms of TB. All of them were BCG

vaccinated at birth as confirmed with BCG scar and/or BCG history. FNA and biopsy specimens were obtained from TBLN suspects and analyzed by culture, ZN staining and histology. Homogenized biopsies were processed and cultured according to the standard method on Löwenstein-Jensen (LJ) media containing glycerol and pyruvate. ZN staining on FNA**s** and sputum smears used the standard hot ZN technique to identify AFB positive cases. Histology was performed on formalin fixed biopsies embedded in paraffin blocks. Sections were cut and stained with haematoxylin and eosin (H&E). The presence or absence of granulomas was noted by a pathologist without the knowledge of culture and ZN staining results. All ZN positive TBLN patients were also positive by culture and/or histology.

Blood samples were collected from TBLN suspects, PTB patients and HCs at the same time when the biopsy and FNA samples were taken for culture and ZN staining respectively. The sera were obtained by centrifugation and stored frozen at -20ºC until used for ELISA.

Collection of blood specimens

HIV counseling was given to the TBLN suspects and all of those included in our study consented to HIV testing prior to the drawing of 10 ml venous blood.

HIV testing

HIV testing was done for all TBLN suspects by using the diagnostic algorithm assay kit according to the manufacturer's directions and following the national guidelines for HIV screening, Ministry of Health, Ethiopia. HIV testing was not done for PTB patients and healthy controls.

ELISA

Six different antigens were used for the ELISA: 4 recombinant proteins, Rv3881c ESAT-6, Ag85A, TB10.4, lipoarabinomannan (LAM) and Ara$_6$-BSA (with BSA as control). To optimize the appropriate antigen concentration, six different antigen concentrations (3 µg/ml, 2 µg/ml, 1 µg/ml, 0.5 µg/ml, 0.25 µg/ml, 0.1µg/ml,) were tested using one TBLN serum, one non-TBLN serum, and one PTB serum. Sera were obtained from patients with or without TB as confirmed by culture, histology and microscopy, and tested in 5 dilutions from 1:100 to 1:500.

The four recombinant proteins were produced as described previously [15,16]. LAM was prepared as described by Hamasur *et al.* [17]. Ara$_6$-BSA, a

synthetic homologue of LAM, was prepared as described by Tong *et al.* [9]. The optimized concentrations, 0.5 µg/ml, 0.25 µg/ml, 0.25 µg/ml, 2 µg/ml 0.5 µg/ml, 0.1 µg/ml,and 0.25 µg/ml of Rv3881c, ESAT-6, Ag85A, TB10.4, LAM, Ara$_6$-BSA and BSA respectively were coated overnight onto flat-bottom ELISA plates (Greiner, Nürtingen, Germany) at 37ºC in a water bath. Plates were then washed four times with phosphate buffered saline (PBS) containing 1% Tween 20 v/v (PBST), 2 x short/2 x soak for 2 to 5 minutes. One hundred µl per well of blocking solution (PBS containing 1% BSA (PBS 0.1% BSA) was added and incubated for one hour at 37ºC. Plates were then washed four times as before and 50 µl of serum diluted 1:200 in PBST with 10% normal goat serum (NGS) was added and incubated at 37ºC for one hour. Accrual of serum samples was random. Plates were then washed four times and 50 µl of peroxidase conjugated rabbit antihuman IgG or IgM peroxidase conjugates (BioRad, France) diluted in PBST with 10% NGS was added to each well. The anti-human IgG conjugate was diluted 1:3000 and the anti-human IgM was diluted 1:1000. After washing four times, 50 µl of tetra methyl benzidine (TMB) substrate solution was added to each well. Optical density (OD) was read at 450 nm wavelength after stopping the reaction with 50 µl of 0.5M H$_2$SO$_4$, when the OD reading of BSA with the standard serum was 0.1 at 630 nm. The person who performed the ELISA was blind to the serum samples tested.

Statistical analysis

Sensitivity was calculated by dividing the number of positive sera by one or by the two combinations of antigens divided by the total number of sera from TBLN patients confirmed with histology and culture or the total number of sera from smear-positive PTB patients x 100%. Specificity was calculated by dividing the number of negative sera by one or by the two combinations of antigens divided by the total number of sera from non-TBLN patients confirmed with culture and histology or the total number of sera from HCs x 100%.

Results

Figure 1 shows the scatter plots for IgG antibody detection against the six antigens using sera from HCs, TBLN patients, non-TBLN cases and PTB patients. Figure 2 shows the scatter plots for IgM antibody detection against the same antigens. Table1 shows the sensitivities with sera from TBLN and PTB cases and specificities with sera from non-TBLN cases and HCs using IgG antibody test results with the six antigens and two combinations of antigens. Sensitivities with sera from TBLN cases for individual antigens ranged from 6 to 30%. Specificities ranged from 73% to 100% with sera from non-TBLN patients and from 91% to 100% with sera from HCs. Of the six antigens tested, LAM was found to be the single most potent antigen with a sensitivity of 30% and specificity of 100%. ESAT-6 followed with a sensitivity of 13% and with a specificity of 96% when HC sera were considered as controls. The specificity was 80% for LAM and 96% for ESAT-6 when sera from non-TBLN patients were considered as a control. Furthermore, sensitivities were improved when different combinations of antigens were used. When LAM and ESAT were combined, sensitivity increased up to 43% and specificity with non-TBLN was 80% with HC 96%.

Table 2 shows the sensitivities with sera from TBLN and PTB cases and specificities with sera from non-TBLN cases and HCs using IgM antibody test results with the six antigens and two combinations of antigens. When LAM was combined with ESAT-6, sensitivity was 9% and specificity 87% with non-TBLN sera and 91% with sera from HCs. When LAM, ESAT-6 and Rv3881c were combined, sensitivity was 15% and specificity 87% with non-TBLN sera and 91% with sera from HC. For all the six single antigens and the two combinations of antigens, IgM antibody detection was more sensitive in PTB patients than in TBLN.

We did not find an association of HIV status with IgG or IgM antibody detection. Of the 54 proven TBLN patients included in our study, only 4 (7%) were AFB positive by ZN staining.

Discussion

This study was conducted to evaluate the diagnostic potential of four recombinant antigens (ESAT-6, Ag85A, TB10-4, and Rv3881c) as well as LAM and its synthetic homologue Ara$_6$-BSA to differentiate between TBLN and non-TBLN patients. Lipoarabinomannan (LAM) was found to be the single most potent antigen among the six antigens tested for IgG antibody detection. ESAT-6 and Rv3881c gave the next highest sensitivity and specificity, respectively, following LAM. Ara$_6$-BSA is among those which showed a relatively lower sensitivity and specificity. This observation could be due to the conformational differences between LAM

Figure 1. Scatter plots for IgG antibody detection using sera from HC, TBLN, non-TBLN and PTB against the six antigens (Rv3881c, LAM, Ar$_{a6-BSA}$, Ag85A, ESAT-6, TB10.4) tested.
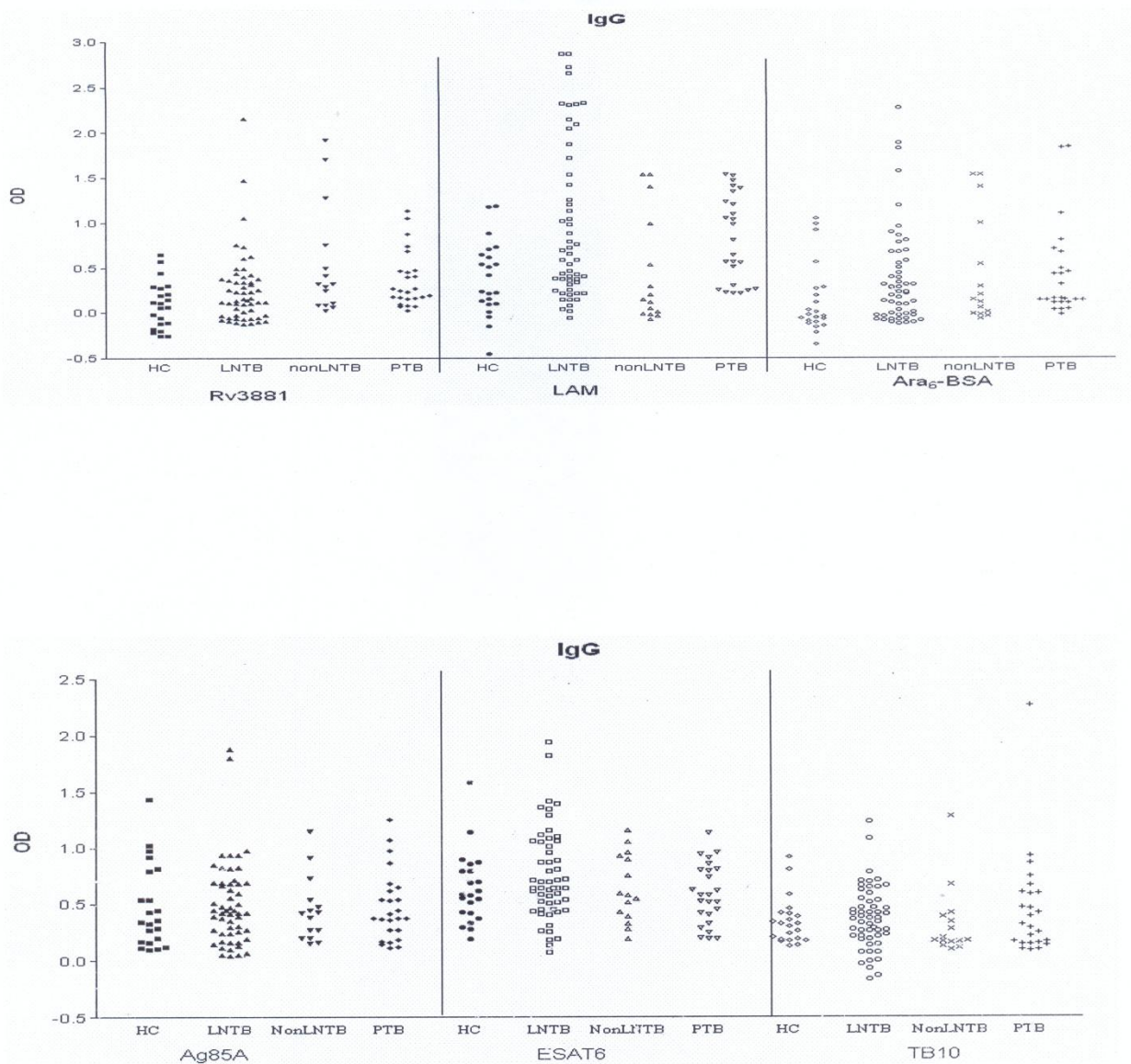

and Ara$_6$-BSA. Antigenic conformation is one of the main attributes that determine formation of stable antigen-antibody complexes [18]. Of the six single antigens and the two combinations of antigens presented in Table 1, IgG antibody detection using the combination of LAM and ESAT-6 was the best to differentiate between TBLN and non-TBLN cases.

A recent systematic review reported by Steingart *et al.* [6] reported a variable estimate of sensitivity and specificity of 21 studies of commercial serological tests. Sensitivity ranged from 0% to 100% and specificity ranged from 59% to 100%, a range within which our findings fall as well.

Toshinobu *et al.* [19] showed that detection of anti-LAM antibody using MycoDot in pleural fluid is a specific diagnostic tool for tuberculous pleurisy and recommended it as simple, rapid, and cost-effective. Our finding that IgG anti-LAM antibody gave better sensitivity and specificity among the six antigens tested suggests its potential role as a target for the serodiagnosis of TBLN.

IgG anti-LAM and anti-ESAT-6 appear more discriminating compared to IgG antibodies of the other four antigens (Rv3881c, TB10.4, Ag85A and Ara$_6$-BSA). Nevertheless, the sensitivities and

Figure 2. Scatter plots for IgM antibody detection using sera from HC, TBLN, non-TBLN and PTB against the six antigens (Rv3881c, LAM, Ara$_{6-BSA}$, Ag85A, ESAT-6, TB10.4) tested.
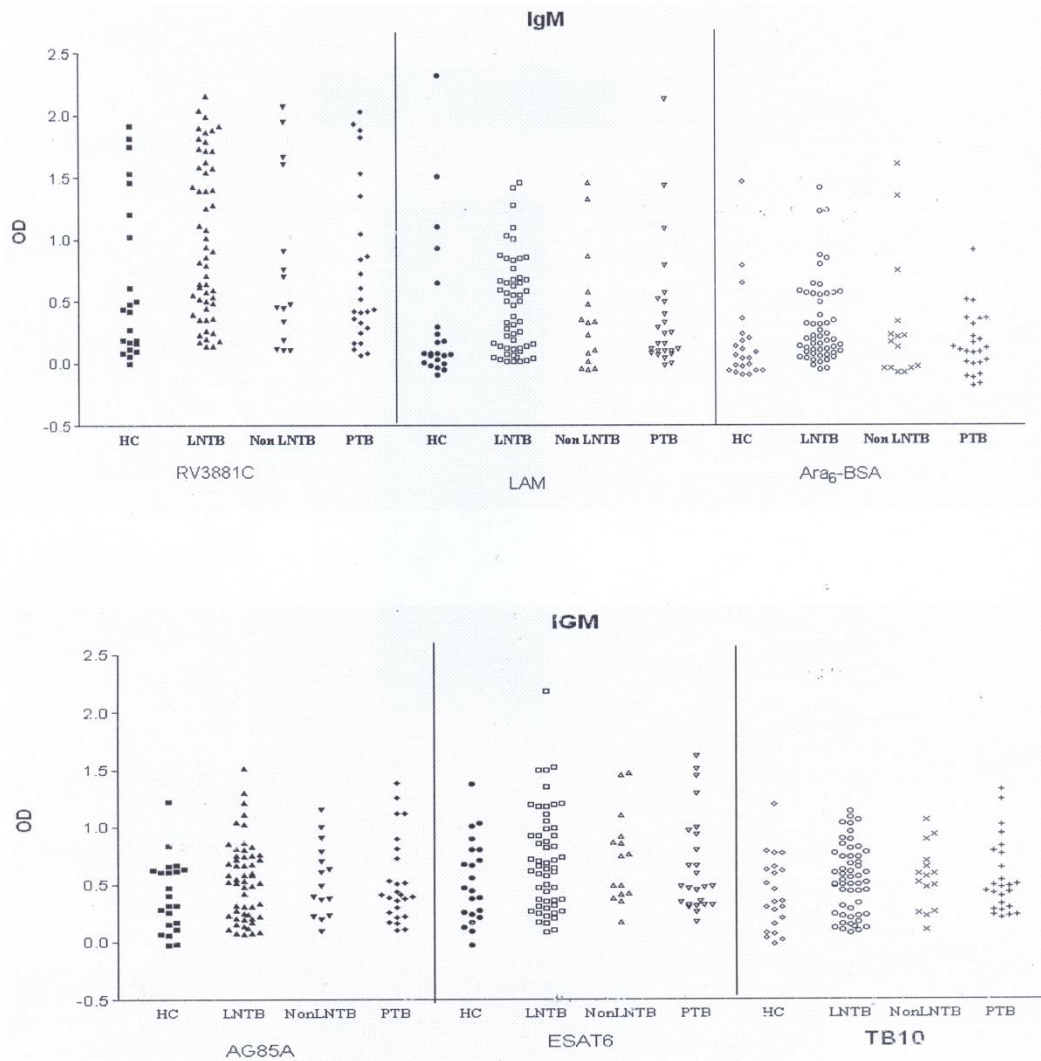
specificities obtained with IgG or IgM antibodies to each of the single antigens suggest that their diagnostic capacity is not optimal. However, the combination of LAM and ESAT-6 showed an increased sensitivity for the detection of IgG antibody when sera from non-TBLN cases were used as a control. Sensitivity increased to 43% and specificity was 80%. Diagnosis of TBLN using the standard ZN microscopy for AFB requires 1×10^4 bacilli per specimen [20]. However, since most TBLN cases are paucibacillary, detection of TBLN by ZN staining is difficult because of its extremely low sensitivity [3,4]. Therefore, since the sensitivity for IgG antibody detection improved when LAM and ESAT-6 were combined, an antibody test on the

combination of these antigens could be a useful supplement to ZN staining of FNA samples if further developed for routine use. However, the number of serum samples tested from both patients and controls was low.

The low sensitivities obtained in our study may be due to the variation in antibody response against different antigens or it could also 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. This profile is modulated when the *in vivo* environment changes [21, 22]. Thus

Table 1. Sensitivities and specificities of IgG antibody assays using sera from TBLN, non-TBLN, HC and PTB.

Antigens	Sensitivity (%) Positive sera TBLN[1]	Specificity (%) Negative sera non-TBLN[1]	Specificity (%) Negative sera HC[1]	Sensitivity (%) Positive sera PTB[1]
ESAT-6	7/54 (13 %)	15/15 (100 %)	21/22 (96 %)	0/25 (0 %)
Ag 85A	2/54 (4 %)	15/15 (100 %)	21/22 (96 %)	1/25 (4%)
TB10.4	3/54 (6 %)	14/15 (93 %)	20/22 (91%)	3/25 (12%)
Rv 3881c	6/54 (11.%)	11/15 (73 %)	21/22 (96 %)	5/25 (20 %)
LAM	16/54 (30 %)	12/15 (80 %)	22/22 (100 %)	7/25 (28 %)
Ara$_6$-BSA	6/54 (11 %)	11/15 (73 %)	20/22 (91 %)	3/25 (12 %)
LAM & ESAT6	23/54 (43 %)2	12/15 (80 %)[2]	21/22 (96 %)2	7/25 (28 %)[2]
LAM, ESAT-6 &Rv3881c	29/54 (54 %) 2	8/15 (53 %)[2]	20/22 (91 %)[2]	12/25(48 %)[2]

[1]TBLN: tuberculous lymphadenitis, Non-TBLN: non-tuberculous lymphadenitis, HC: Healthy control, PTB: Pulmonary tuberculosis
[2]Sensitivity of the combined antigens represented the percentage of patients who were positive by one or by all the antigens combined among histology and culture confirmed TBLN cases. Specificity represented the percentage of individuals who were negative by all the combined antigens among HCs or among culture and histology confirmed non-TBLN cases.

Table 2. Sensitivities and specificities of IgM antibody assays using sera from TBLN, non-TBLN, HC and PTB

Antigens	Sensitivity (%) Positive sera TBLN[1]	Specificity (%) Negative sera non-TBLN[1]	Specificity (%) Negative sera HC[1]	Sensitivity (%) Positive- sera PTB[1]
ESAT-6	5/54 (9 %)	13/15 (87 %)	21/22 (96 %)	4/25 (16 %)
Ag85A	5/54 (10 %)	12/15 (80 %)	21/22 (96 %)	4/25 (16%)
TB10.4	3/54 (6 %)	14/15 (93%)	21/22 (96 %)	2/25 (8 %)
Rv3881c	3/54 (6%)	14/15 (93 %)	22/22(100 %)	1/25 (16 %)
LAM	0/54 (0 %)	15/15 (100 %)	21/22 (96 %)	1 /25 (16 %)
Ara$_6$-BSA	3/54 (6 %)	13/15 (87 %)	21//22 (96 %)	8/25 (32 %)
LAM & ESAT.6	5/54 (9 %) [2]	13/15 (87 %)[2]	20/22 (91 %) [2]	5/25 (20 %)[2]
LAM, ESAT-6 & Rv3881c	8/54 (15 %) [2]	13/15(87 %)[2]	20/22 (91 %) [2]	6/25 (24 %) [2]

[1]TBLN: tuberculous lymphadenitis, Non-TBLN: non-tuberculous lymphadeniti HC: Healthy control, PTB: Pulmonary tuberculosis.
[2]Sensitivity of the combined antigens represented the percentage of patients who were positive by one or by all the antigens combined among histology and culture confirmed TBLN cases. Specificity represented the percentage of individuals who were negative by all the combined antigens among HCs or among culture and histology confirmed non-TBLN cases.

selection of antigens of *M. tuberculosis* based on the antibody response of PTB patients alone might not provide the appropriate materials that can be used for the sero-diagnosis of EPTB.

Although HIV could complicate the diagnosis of TB [23], there was no evidence of association of HIV with IgG or IgM antibody detection with the low number of HIV seropositives in our study, 4/54 (7%). A larger study with more HIV-positive subjects is recommended to assess the influence of HIV co-infection on antibody detection in TBLN patients.

A limitation of this study may be that the ELISA was performed on a limited number of sera, especially those in the non-TBLN patient group. Although we have managed to identify the best single and/or combined antigens, the identification may still be

suboptimal. Our study has investigated the antibody response to six antigens using four groups of sera retrospectively and needs further testing prospectively to confirm the accuracy. Once specific and sensitive antigens have been identified in ELISA, a simple diagnostic test can be developed for routine use.

Rv3881c were a gift of Dr. A. Geluk, University of Leiden, Leiden, The Netherlands.

References

1. World Health Organization (2008) Global Tuberculosis Control: surveillance planning and financing. Geneva, World HealthOrganization. http://www.who.int/tb/publications/global report eng/en/.
2. Ministry of Health (2005/2006) National Tuberculosis and Leprosy Control (NTLC) Ethiopia, Ministry of Health Ethiopia.
3. Beyene D, Ashenafi S, Yamuah L, Aseffa A, Wiker H, Engers H, Klatser P, Sviland L (2008) Diagnosis of tuberculous lymphadenitis in Ethiopia: correlation with culture, histology and HIV status. Int J Tuberc Lung Dis 12: 1030-6.
4. Steingart RK, Ng V, Henry M, Hopewell PC, Ramsay A, Cunningham J, Urbanczik R, Perkins MD, Aziz MA, Pai M (2006) Sputum processing methods to improve the sensitivity of smear microscopy for tuberculosis: A systemic review. Lancet Infect Dis 6: 664-674.
5. Bekedam HJ, Boree M, Kamenya A, Liomba G, Ngwira B, Subramanyam VR, Harries AD (1997) Tuberculous lymphadenitis, a diagnostic problem in areas of high prevalence of HIV. Trans R Soc Trop Med Hyg 91: 294-297.
6. Steingart RK, Henry M, Laal S, Hopewell CP, Ramsay A, Menzles R, Conningham J, Weldingh K, Pai M (2007) A systemic review of commercial serological antibody detection tests for the diagnosis of extra pulmonary tuberculosis. Thorax 62: 911-918.
7. Montgomery DL, Huygen K, Yawman AM, Deck RR, Dewitt CM, Content J, Liu MA, Ulmer JB (1997) Induction of humoral and cellular immune responses by vaccination with *M. tuberculosis* antigen 85 DNA. Cell Mol Biol 43:285-292.
8. Malen H, Søfteland T, Wiker HG (2008) Antigen Analysis of *Mycobacterium tuberculosis* H37Rv Culture Filtrate Proteins. Scandanavan J Immun 67: 245–252.
9. Eleftheriadis T, Tsiaga P, Antoniadi G, Liakopoulos V, Kortszris A, Giannatos E, Barbutis K, Stefanidis I, Vargemezis V (2005) The value of serum antilipoarabidomannan antibody detection in the diagnosis of latent tuberculosis in hemodialysis patients. Am J Kidney Dis 46: 706-712.
10. Tong M , Jacobi CE, van de Rijke FM, Kuijper S, van de Werken S, Lowary TL, Hokke CH, Appelmelk BJ, Nagelkerke NJD, Tanke HJ, van Gijlswijk RPM, Veuskens J, Kolk Arend HJ, Raap A (2005) A multiplexed and miniaturized serological tuberculosis assay identifies antigens that discriminate maximally between TB and non-TB sera. J Immun Method 301: 154-163.
11. Skjøt Rikke LV, Brock I, Arend SM, Munk ME, Theisen M, Ottenhoff Tom HM, Andersen P (2002) Epitope mapping of the immunodominant Antigen TB10.4 and the two homologue proteins TB10.3 and TB12.9 which constitute a subfamily of the ESAT-6 gene family. Infect Immun 70: 5446-5453.
12. Skjøt RL, Oettinger T, Rosenkrands I, Ravn P, Brock I, Jacobsen S, Andersen P (2000) Comparative evaluation of Low molecular mass proteins from *Mycobacterium tuberculosis* identifies members of ESAT-6 family as immunodominant T-cell antigen. Infect Immun 68: 214- 220.
13. Aagaard C, Govaerts M, Meikle V, Vallecillo JA, Gutierrez-Pabello AJ, Suarez-Güemes F, McNair J, Cataldi A, Espitia C, Andersen P, Pollock JM (2006) Optimizing Antigen Cocktails for Detection of *Mycobacterium bovis* in Herds with Different Prevalences of Bovine Tuberculosis: ESAT6-CFP10 Mixture Shows Optimal Sensitivity and Specificity. J Clin Microbiol 44: 4326-4335.
14. Appendices, Manual of the NTLC of Ethiopia (2002) Ministry of Health of Ethiopia.
15. Leyten E M S, Lin M Y, Franken K LM C, Friggen A H Prins C, van Meijgaarden K E, Voskuil M I, Weldingh K, Andersen P, Schoolnik G K, Arend SM, Ottenhoff THM, Klein.M R (2006) Human T-cell responses to 25 novel antigens encoded by genes of the dormancy regulon of *Mycobacterium tuberculosis*. Microbes Infect 8:2052-2060.
16. Franken K L, Hiemstra H S, van Meijgaarden K E, Subronto Y., den Hartigh J, Ottenhoff T H, Drijfhout J W (2000) Purification of his-tagged proteins by immobilized chelate affinity chromatography: the benefits from the use of organic solvent. Protein Expr Purif 18: 95-99.
17. Hamasur B, Källenius G, Svenson Stefan B (2006) A new rapid and simple method for large-scale purification of mycobacterial lipoarabinomannan. FEMS Immun & Med Microbiol 24: 11-17.
18. Bhat TN, Bentley GA, Boulot G, Greene MI, Tello D, Acqua Dall W, Souchon H Schwarz FP, Mariuzza RA, Poljak RJ (1994) Bound water molecules and conformational stabilization help mediate an antigen –antibody association. Proc Natl Acad Sci USA 93: 1089-1093.
19. Toshinobu O, Toru R, Takashi K, Tomoko K, Yuichi O, Hisamichi A (2005) Clinical utility of lipoarabinomannan antibody in pleural fluid for the diagnosis of tuberculous pleurisy. J Infect Chemother 11: 81-83.
20. Yeager H JR, Lacy J, Smith LR, LeMaister CA (1967) Quantitative studies of Mycobacterial population in sputum and saliva. Am Rev Respir Dis 95: 998-1004.
21. Samanich K, Belise JT, Laal S (2001) Homogeniety of antibody responses in tuberculosis patients. Infect Immun 69: 4600- 4609.
22. Singh KK, Zhang X, Patibandla AS, Chien P Jr, Laal S (2001) Antigens of *Mycobacterium tuberculosis* expressed during preclinical tuberculosis. Serological immunodominance of proteins with repetitive amino acid sequences. Infect Immun 69:4185-4191.
23. World Health Organization (2008) Global Tuberculosis Control: Surveillance, Planning, financing. Geneva, World Health Organization. (WHO/HTM/TB/2008.393).

Corresponding author

Demissew Beyene
AHRI, ALERT Campus, Jimma Road
P. O. Box: 1005
Addis Ababa, Ethiopia
Tel no: 251-11-3-211334; Fax no: 251-1-3-211563
Email address: beyene88@gmail.com

Conflict of interests: No conflict of interest is declared.