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Evaluation of *Mycobacterium tuberculosis*-specific antibody responses for the discrimination of active and latent tuberculosis infection

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ABSTRACT

Objectives: The serological antibody detection tests offer several advantages for the rapid diagnosis of tuberculosis (TB). The *Mycobacterium tuberculosis*-specific antibody responses associated with different stages of TB infection remain to be investigated.
Methods: The Pathozyme-Myco IgG (Myco G), Pathozyme TB Complex Plus (TB Complex), IBL *M. tuberculosis* IgG ELISA (IBL), Anda Biologicals TB IgG (Anda-TB), and T-SPOT.*TB* (T-SPOT) tests were performed for 133 active TB patients (ATB group), 131 controls (CON group), and 95 subjects with latent TB infection (LTBI group).
Results: The four serological tests all showed relatively low sensitivity in the ATB group but high specificity in the LTBI and CON groups. The antibody levels of the four serological tests were significantly higher in the ATB group than in the LTBI group. The same trend was observed between the LTBI and CON groups. The four serological tests demonstrated potential diagnostic value in discriminating ATB from LTBI. A combination of the Anda-TB and TB Complex tests exhibited the best diagnostic potential in discriminating ATB from LTBI, with a sensitivity of 89.4% and a specificity of 94.7%. Further, the diagnostic value of Anda-TB and TB Complex were validated in a prospective cohort including 106 patients with suspected ATB. Combined with the T-SPOT test, the tests showed a sensitivity of 87.2% and a specificity of 92.5% for discriminating ATB patients from all ATB suspected cases in the validation group.
Conclusions: The antibody responses of the serological tests all showed significant differences between the ATB and LTBI groups. A combination of Anda-TB and the TB Complex test demonstrated high diagnostic potential in discriminating ATB from LTBI and may be an additional diagnostic tool in the diagnosis of *M. tuberculosis* infection.

Introduction

The global tuberculosis (TB) epidemic has resulted in nearly two million deaths and nine million new cases of the disease per year. It is estimated that two billion people live with latent *Mycobacterium tuberculosis* infection (LTBI) and represent a potential source of future active TB cases (Corbett et al., 2003). Thus, the development of rapid and accurate new diagnostic methods is vital for the global control of TB. However, the diagnostic accuracy of existing tests is inadequate (Wallis et al., 2013). The microscopy method has high specificity in TB-endemic countries, but modest sensitivity and variable results in many settings (Steingart et al., 2006; Urbanczik, 1985). The current immunodiagnostic tests for TB also have considerable limitations. The tuberculin skin test (TST) has limited specificity, and false-positive results can occur as a result of prior bacillus Calmette–Guérin (BCG) vaccination or infection with non-tuberculous mycobacteria (NTM) (Latorre et al., 2010). The T-cell-based interferon-gamma (IFN-γ) release assays (IGRAs) are just as sensitive as and more specific than the TST (Pai et al., 2008).

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However, the current IGRAs cannot discriminate active TB (ATB) from LTBI, which makes them unsuitable for diagnosing active disease, particularly in TB-endemic areas (Mazurek et al., 2007; Mazurek et al., 2010).

In addition to these methods, the development of immune-based tests for the detection of the humoral (serological) antibody immune response to *M. tuberculosis*-specific antigens has been ongoing for decades. The serological antibody detection tests (hereafter referred to as serological tests) offer several advantages (Gennaro, 2000). Detection of the presence of specific antibodies is faster and simpler to perform than most sputum-based and T-cell-based methods (Silva et al., 2003; Rasolofo and Chanteau, 1999; Andersen et al., 2000). In patients suspected of extrapulmonary TB, serological tests may be able to detect antibodies from different sample sources such as urine, cerebrospinal fluid, and pleural fluid, which may reduce or eliminate the need for more invasive tests.

Over the past decade, serological tests for TB have been investigated extensively and several promising candidate antigens have been identified and evaluated, such as 12 kDa, 38 kDa, LAM, 16 kDa, CFP-10, Rv3425, and antigen 60 (Gennaro, 2000; Chan et al., 2000). A number of commercial antibody detection tests have also been developed and evaluated in regions with different TB endemicity rates. However, according to systematic reviews of these studies, the diagnostic accuracy of the commercial serological tests varies widely in performance, with highly inconsistent estimates of sensitivity and specificity, and none of the commercial tests has performed well enough to replace sputum smear microscopy (Steingart et al., 2007a; Steingart et al., 2007c; Steingart et al., 2011). In 2011, the World Health Organization (WHO) policy recommended against the use of these tests for the diagnosis of pulmonary and extrapulmonary TB (World Health Organization, 2011). In spite of this, future use of the serological tests has not been discounted (Ivanyi, 2012; Jacqueline and Anke, 2012). The WHO still strongly encourages improved research on the serological diagnosis of TB, because it may provide many advantages in high-burden, resource-limited regions in the form of point-of-care rapid tests, which are currently missing in the TB diagnostic pipeline (World Health Organization, 2011; Morris, 2011).

The serological tests are also attractive because they may have the diagnostic potential to distinguish between active disease and latent infection, which offers a significant improvement over current routine test methods. The specific antibody responses are rarely detected in non-symptomatic individuals (Daniel et al., 1991). For the most frequently evaluated A60 antigen, several studies have shown that the percentage of positivity and the A60 IgG titers are much lower in inactive post-primary TB patients than in ATB patients (Luh et al., 1996; Gevaudan et al., 1992; Zou et al., 1994). In one study, the antibody levels of 38 kDa and 16 kDa antigens were found to be significantly lower in patients with inactive TB compared to those with ATB, but no significant difference was found between patients with inactive TB and control cases (Senol et al., 2007). A study by Anderson et al. also indicated that the positive rate of the serological test was lower in subjects with latent infection identified by IGRA, and that the serological test may have the ability to aid in the differentiation between ATB and LTBI (Anderson et al., 2008).

These differences in antibody response between active and latent TB are striking, but the diagnostic potential of serological tests to discriminate between active and latent TB infection still requires thorough investigation. Moreover, whether a combination of serological tests and T-cell-based IGRAs could achieve the highest diagnostic efficacy has also not been evaluated to date.

This study recruited subjects with different stages of TB infection, including ATB patients, subjects with LTBI, and controls, to evaluate the diagnostic accuracy of four serological tests. Focus was placed on the evaluation of the serological tests as potential biomarkers in discriminating active TB from latent infection. The serological tests were also used in combination with the T-SPOT.*TB* test (Oxford Immunotech, Oxford, UK) to determine whether the diagnostic performance could be improved. In the next stage of the study, an independent clinical-based validation cohort was recruited to confirm the high performance of the serological tests in discriminating ATB from LTBI.

Materials and methods

Study design and participant selection

In order to investigate the diagnostic performance of the serological tests, two independent study groups were recruited. The subjects in group I were recruited from a cross-sectional study that consisted of three subgroups: active TB patients (ATB group), subjects with LTBI (LTBI group), and non-ATB controls (CON group). Study group II subjects were recruited prospectively from a clinical setting-oriented study to further evaluate the diagnostic performance of the serological tests. The demographic characteristics of the study populations are described in Table 1 and in

Table 1
Characteristics of the study participants.

Characteristic	Study group I			Study group II	
	ATB	LTBI	CON	ATB	NTB
Total number	133	95	131	39	67
Male, *n* (%)	81 (60.9)	49 (51.6)	72 (55.0)	22 (56.4)	32 (47.8)
Median age (range), years	45 (18–75)	47 (22–67)	41 (19–62)	36 (21–73)	41 (19–77)
BCG status					
Unvaccinated	34 (25.6)	16 (16.8)	21 (16.0)	11 (28.2)	15 (22.4)
Vaccinated	95 (71.4)	77 (81.1)	107 (81.7)	25 (64.1)	45 (67.2)
Unknown	4 (3.0)	2 (2.1)	3 (2.3)	3 (7.7)	7 (10.4)
Preexisting conditions and illnesses, *n* (%)					
Previous tuberculosis	5 (3.8)	3 (3.2)	0 (0)	2 (5.1)	7 (10.4)
Diabetes	12 (9.0)	9 (9.5)	7 (5.3)	4 (10.3)	11 (16.4)
HIV-positive	0 (0)	0 (0)	0 (0)	1 (2.6)	2 (3.0)
History of hepatitis B/C	7 (5.3)	6 (6.3)	6 (4.6)	2 (5.1)	5 (7.5)
Pneumonia	3 (2.3)	1 (1.1)	0 (0)	2 (5.1)	1 (1.5)
Chronic liver disease	2 (1.5)	1 (1.1)	1 (0.8)	2 (5.1)	3 (4.5)
Chronic renal failure	1 (0.8)	0 (0)	0 (0)	0 (0)	1 (1.5)
Coronary heart disease	4 (3.0)	2 (2.1)	1 (0.8)	1 (2.6)	2 (3.0)

ATB, active TB patients; BCG, bacillus Calmette–Guérin; CON, controls; LTBI, latent TB infection; NTB, no active TB disease.

Supplementary Material Table S1 in the online version at DOI:10.1016/j.ijid.2018.01.007. All subjects provided written informed consent and the study received ethical approval from the Institutional Review Board (IRB) of Huashan Hospital, Fudan University.

Group I

All 133 patients in the ATB group were recruited from Shanghai Huashan Hospital, Zhuji People's Hospital, and Chongqing Pulmonary Hospital from January 2013 to September 2015. The clinical diagnostic criteria for ATB included the following: (1) clinical signs and symptoms suggestive of ATB including fever, cough, and productive sputum, and (2) a positive acid-fast bacillus (AFB) smear and/or a positive culture for *M. tuberculosis* (Feng et al., 2012). Of the ATB patients recruited, 49 were determined to be smear-positive and culture-positive, 34 were only culture-positive, 29 were only smear-positive, and 21 were negative for both markers but were diagnosed with ATB based on positive histopathological findings, clinical manifestations, and chest radiography. To minimize the effects of anti-TB treatment on the antibody responses, only patients receiving standard anti-TB therapy for <1 week were included in the study.

The LTBI group consisted of 95 household contacts of the smear-positive ATB patients in the ATB group. They had lived with the ATB patients for months before enrollment and had a positive result by T-SPOT assay. None of the LTBI subjects had clinical symptoms or abnormal chest X-ray findings indicating ATB (Wang et al., 2012).

The CON group comprised 81 healthy individuals and 50 non-TB patients (25 with non-TB pulmonary disease and 25 with non-TB non-pulmonary disease). The healthy individuals were recruited from students and staff at Fudan University, China. All subjects answered a detailed questionnaire concerning risk factors for exposure to *M. tuberculosis* and only those with no clinical or radiographic evidence of ATB and no known history of exposure to TB were enrolled. Patients with non-TB disease were diagnosed according to the standard clinical practice for each disease. Detailed information on the subgroups is given in **Supplementary Material** Table S1 in the online version at DOI:10.1016/j.ijid.2018.01.007.

Group II: prospective cohort

Subjects in group II were recruited from an additional prospective study in a validation cohort ($n = 106$) of patients with suspected ATB registered at Huashan Hospital, Shanghai, China. The ATB suspects were defined as patients who presented clinical symptoms (night sweats, weight loss, or cough) or radiographic characteristics consistent with ATB. All of the blood samples were collected before any treatment. After a follow-up of at least 3 months, ATB ($n = 39$) was eventually diagnosed using the same criteria as described above. Among these patients, 15 only had a positive culture for *M. tuberculosis*, 10 only had a positive AFB smear, 12 patients were positive for both, and two patients were diagnosed by positive histopathological findings, clinical manifestations, and chest radiography. The patients who did not meet the criteria for ATB ($n = 67$) were identified as subjects without ATB disease (NTB group). The ATB patients were used as the reference standard for the sensitivity analysis, and the subjects without ATB were used as the reference standard for the specificity assessment.

M. tuberculosis *antibody detection by commercial ELISA*

The *M. tuberculosis* antigen-specific antibody levels were assessed using four commercially available kits. The four kits evaluated were the Pathozyme-Myco IgG test (Myco G), the Pathozyme TB Complex Plus test (TB Complex), the IBL *M. tuberculosis* IgG ELISA test (IBL), and the Anda Biologicals TB IgG test (Anda-TB). The characteristics of the four serological tests, including antigen composition, source of the antigen, immunoglobulin class, and laboratory technique, are shown in Table 2.

The Myco G and TB Complex kits were provided by Omega Diagnostics Limited (Alloa, Scotland). The Myco G test detects the serum IgG antibody to lipoarabinomannan (LAM) and recombinant 38-kDa antigen (Rv0934). LAM is a lipoglycan and major component of the cell wall of all mycobacteria, which is purified from batch-grown *M. tuberculosis* H37Rv. 38 kDa is recombinant and purified from *Escherichia coli*. The TB Complex test detects the IgG antibody to recombinant 38 kDa and 16 kDa (Rv2031c). The result was considered positive when the level of antibody in a sample was >400 serounits/ml for Myco G and >200 serounits/ml for TB Complex.

For the IBL test (IBL-Hamburg, Hamburg, Germany), the microtiter wells were coated with 18-, 36-, and 40-kDa recombinant antigens.[22,33] The serum samples were diluted 1:100 in the sample diluent. The results were expressed in units per milliliter and a value of 1200 U/ml was representative of a positive result.

The Anda-TB test (Anda Biologicals, Strasbourg, France) detects IgG antibodies against antigen 60 (A60). A60 is an antigen complex that is extracted from the cytoplasm of *Mycobacterium bovis* strain BCG. The serum samples were diluted 1:100 in the sample diluent and a value of 120 U/ml was considered positive.

All plasma specimens were tested in duplicate by investigators who were blinded to the study groups. Samples from control subjects and patients were tested together in an interspersed fashion. The assay reproducibility was examined by determining the coefficient of variation (CV%) and the between-assay CV% were 6.8%, 5.9%, 7.1%, and 6.1% for Myco G, TB Complex, IBL, and Anda-TB, respectively.

*T-SPOT.*TB *test (T-SPOT)*

All recruited candidates were screened by T-SPOT.*TB* kit (Oxford Immunotec Ltd, Oxford, UK). The T-SPOT.*TB* test was performed according to the manufacturer's instructions. Briefly, a precoated IFN-γ ELISPOT plate was seeded with 2.5×10^5 peripheral blood mononuclear cells (PBMCs) per well and incubated with medium

Table 2
Commercial serological tests for the diagnosis of tuberculosis.

Name of test	Antigens	Source of antigen	Immunoglobulin class	Laboratory technique
Anda Biologicals TB	Antigen 60	Native from *Mycobacterium bovis* BCG	IgG	ELISA
Pathozyme-Myco	LAM and 38 kDa (Rv0934)	Native LAM from *Mycobacterium tuberculosis* H37Rv and recombinant 38 kDa from *Escherichia coli*	IgG	ELISA
Pathozyme TB Complex Plus	38 kDa (Rv0934) and 16 kDa (Rv2031c)	Recombinant from *Escherichia coli*	IgG	ELISA
IBL *M. tuberculosis*	18, 36, and 40 kDa proteins	Recombinant from *Escherichia coli*	IgG	ELISA

BCG, bacillus Calmette–Guérin; ELISA, enzyme-linked immunosorbent assay; kDa, kilodalton; LAM, lipoarabinomannan.

(Nil), peptide antigens derived from ESAT-6 (Panel A), CFP-10 (Panel B), or phytohemagglutinin (PHA, as a positive control) in a 5% CO_2 atmosphere at 37 °C for 16–24 h. The plate wells were then washed and incubated with a conjugate against the antibody used and an enzyme substrate. Spot-forming cells (SFCs) in T-SPOT Panel A (T-SPOT A) and T-SPOT Panel B (T-SPOT B), representing antigen-specific T-cells secreting IFN-γ, were counted with an automated ELISPOT reader (AID-GmbH, Germany). The test result was considered positive if Panel A – Nil and/or Panel B – Nil ≥8 spots, and negative if both Panel A – Nil and Panel B – Nil ≤4 spots. Results were considered borderline if the Panel – Nil spot count was 5, 6, or 7 spots and was re-tested by collecting another patient specimen. The results were double-checked by other laboratory workers and, if necessary, corrected by manual counting. The laboratory technicians were blinded to the subject identifiers.

Statistical analysis

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated by standard methods to evaluate the diagnostic performance of the various serological tests, T-SPOT, and TST. Ninety-five percent confidence intervals (95% CI) were estimated according to the binomial distribution. The statistical significance of differences in frequency was measured using the Chi-square test or Fisher's exact test, as appropriate. Diagnostic accuracies of the tests were evaluated using the receiver operating characteristics (ROC) curve. The cut-off values were estimated at various sensitivities and specificities and determined at the maximum Youden index (YI) (i.e., sensitivity + specificity − 1) (Youden, 1950). Combinations of tests were identified by decision tree analysis using R 2.12.1 (R Foundation for Statistical Computing). Using this algorithm, the best tree was chosen, as reported in a previous study (Lu et al., 2011). The statistical analysis was performed using GraphPad Prism v. 5.03 software (GraphPad Inc., San Diego, CA, USA). All reported *p*-values were two-sided and statistically significant differences were determined using a *p*-value of <0.05.

Results

Diagnostic performance of the tests in the ATB, CON, and LTBI groups

The positive rates in the ATB, CON, and LTBI groups were calculated according to the antibody levels detected by the four serological tests (Myco G, TB Complex, IBL, and Anda-TB), using the cut-off values provided by the manufacturers. The results are summarized in Table 3.

For the 133 patients with ATB, the sensitivity of the assays ranged from 32.3% to 67.7%, with the highest sensitivity occurring for Anda-TB and the lowest for IBL. The sensitivities of the TB Complex, IBL, and Anda-TB tests were higher in the pulmonary TB group than in the extrapulmonary TB group, but the differences were not significant (Table 3). The patients in the ATB category were then divided into a smear-positive group and a smear-negative group on the basis of AFB smear results. For the 78 smear-positive patients, the sensitivity ranged from 37.2% to 71.8%, with the highest being for Anda-TB and the lowest being for IBL. For the 55 smear-negative patients, the sensitivity ranged from 29.1% to 61.8%, with the highest being for Anda-TB and the lowest being for TB Complex and IBL. None of the four serological tests yielded a significantly higher sensitivity when applied to smear-positive versus smear-negative ATB patients ($p > 0.05$).

The positive rates of the assays in the CON group and LTBI group are also shown in Table 3. All four serological tests showed relatively low positive rates in the CON and LTBI groups. In the CON group, the overall positive rate of the assays ranged from 3.1% to 23.7%, with the highest being for Anda-TB and the lowest being for IBL. In the LTBI group, the overall positive rate of the assays ranged from 3.2% to 15.8%, with the highest being obtained for Anda-TB and the lowest being for Myco G. There were no differences in the positive rate across all assays for the three subpopulations of control cases: those with non-TB pulmonary diseases, those with non-TB non-pulmonary diseases, and healthy subjects ($p > 0.05$). For each serological test, no significant differences in positivity rate were found between the LTBI and CON groups ($p > 0.05$).

To further evaluate the discriminatory ability of the serological tests, ROC curves were constructed by plotting the true-positive rate and false-positive rate with each unique value of the indicator variable for active TB patients (ATB group) and controls (CON group) (**Supplementary Material** Figure S1 in the online version at DOI:10.1016/j.ijid.2018.01.007). Anda-TB showed the highest area under the curve (AUC) among the serological tests (AUC = 0.8691). The optimal positive cut-off values were then determined by ROC analysis using the maximum Youden index, and the sensitivity, specificity, and diagnostic accuracy were calculated (**Supplementary Material** Figure S1 in the online version at DOI:10.1016/j.ijid.2018.01.007). The Anda-TB test achieved a sensitivity of 77.9% and a specificity of 81.8% using the calculated cut-off value (264.8 U/ml), which were higher than those using the manufacturer's cut-off.

Table 3
Results of the four serological tests and the T-SPOT test across subjects.

	Number of subjects (%)	Number of positive (%)[a]				
		Myco G	TB Complex	IBL	Anda-TB	T-SPOT
ATB group						
Pulmonary TB	104 (78.2)	42 (40.4)	39 (36.5)	34 (32.7)	73 (70.2)	94 (90.4)
Extrapulmonary TB	29 (21.8)	13 (44.8)	9 (31.0)	9 (24.1)	17 (58.6)	26 (89.7)
Smear-positive TB	78 (58.6)	35 (44.9)	31 (39.7)	29 (37.2)	56 (71.8)	71 (91.0)
Smear-negative TB	55 (41.4)	20 (36.4)	16 (29.1)	16 (29.1)	34 (61.8)	49 (89.1)
Total	133 (100)	55 (41.4)	47 (35.3)	43 (32.3)	90 (67.7)	120 (90.2)
CON group						
Healthy individuals	81 (61.8)	0 (0)	2 (2.5)	2 (2.5)	18 (22.2)	14 (17.3)
Non-TB pulmonary diseases	25 (19.1)	3 (12)	0 (0)	2 (8)	10 (40)	8 (32)
Non-TB non-pulmonary diseases	25 (19.1)	1 (4)	2 (8)	0 (0)	3 (12)	3 (12)
Total	131 (100)	4 (3.1)	4 (3.1)	4 (3.1)	31 (23.7)	25 (19.1)
LTBI group	95 (100)	3 (3.2)	5 (5.3)	6 (6.3)	15 (15.8)	95 (100)[b]

ATB, active tuberculosis; CON, control subjects; LTBI, latent tuberculosis infection; TB, tuberculosis.
[a] The positive rates of the serological tests and T-SPOT test were calculated using the cut-off values given by the manufacturers.
[b] Only subjects with positive T-SPOT test were included in LTBI group.

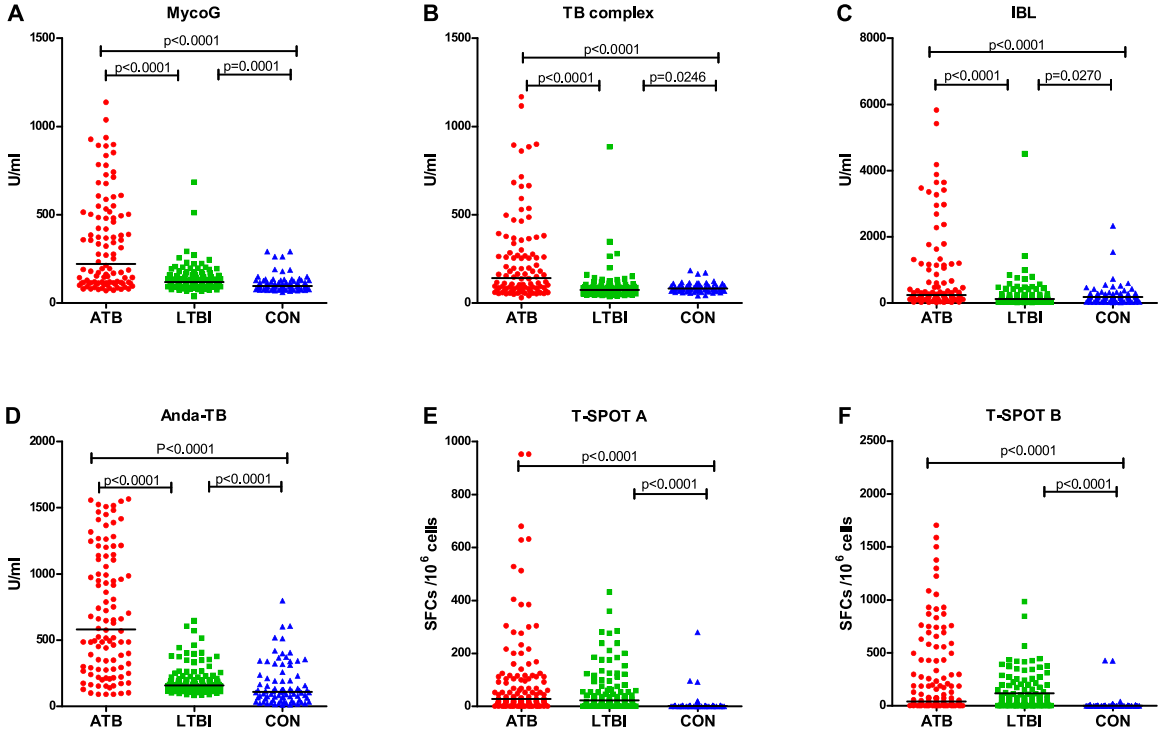


Figure 1. Antibody levels of four serological tests (A–D) and SFCs of T-SPOT (E–F) in patients with active tuberculosis (ATB group), subjects with latent tuberculosis infection (LTBI group) and controls (CON group).
The solid horizontal lines indicate the median amount of antibody levels (A–E) and SFCs (E, F). Kruskal–Wallis tests with Dunn's post tests were used to compare the differences among three groups.

In this study, T-SPOT tests were performed in parallel for all of the subjects. In the ATB group, the positive rate of the T-SPOT was significantly higher than those of all four serological tests ($p < 0.0001$). A significantly higher positive rate for T-SPOT than for any of the four serological tests was also observed in the CON group ($p < 0.0001$). In the ROC analysis, the AUC of T-SPOT A was 0.7279 and of T-SPOT B was 0.7413, which were both significantly lower than the AUC of Anda-TB ($p < 0.01$).

Antibody levels of serological tests in the different groups

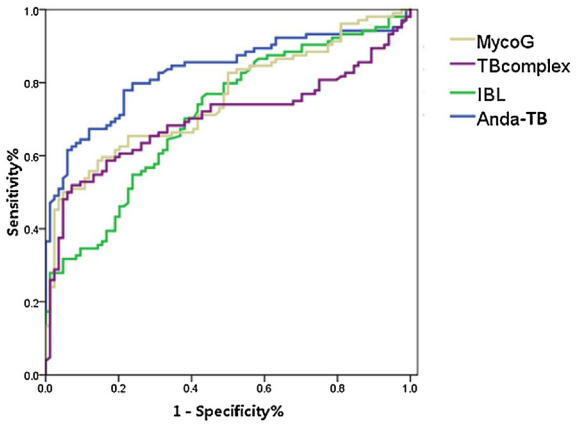
The antibody levels of the serological tests were then compared among the ATB, LTBI, and CON groups, which collectively represent the different stages of TB infection (Figure 1). For the four serological tests including Myco G, TB Complex, IBL, and Anda-TB, the antibody levels in the ATB group were all significantly higher than those in the CON group. Although the positive rates of the four kits showed no significant difference between the LTBI and CON groups, the antibody levels in the LTBI group were all significantly higher than those in the CON group (Figure 1). In order to evaluate the diagnostic potential of the serological tests in discriminating between the ATB and LTBI groups, the antibody levels of the serological tests in the ATB and LTBI groups were then analyzed. Highly significant differences between the ATB and LTBI groups were observed for all of the serological tests ($p < 0.0001$).

For the T-SPOT test, the SFCs of T-SPOT A and T-SPOT B in the ATB and LTBI groups were significantly higher than those in the CON group ($p < 0.0001$). However, no significant difference in SFCs was observed between the ATB and LTBI groups ($p > 0.05$).

Diagnostic performance of serological tests to discriminate between ATB and LTBI

This study sought to determine whether the serological tests could help to discriminate the different stages of TB infection (ATB, LTBI, and CON groups in this study). As expected, the T-SPOT test showed high diagnostic performance for identifying the individuals who were not infected by *M. tuberculosis* (CON group). Therefore, this group was removed from the subsequent analysis and focus was placed on evaluating the diagnostic value of the serological tests in discriminating between the ATB and LTBI groups. For the ROC analysis of each individual serological test, subjects in the ATB group were defined as patients and subjects in the LTBI group were defined as controls. The optimal cut-off values were then determined by ROC analysis. The AUC values, optimal cut-offs, sensitivity, and specificity of the four tests are shown in Figure 2. Anda-TB showed the best diagnostic value with an AUC of 0.8309, achieving a sensitivity of 77.9% and specificity of 78.6% (cut-off 264.8 U/ml). All four serological tests showed an AUC >0.7.

Next, different combinations of the serological tests were analyzed in order to increase the diagnostic accuracy. Data from these tests were subjected to decision tree analysis to identify the ideal combinations and to optimize the discrimination between ATB and LTBI using R program with 15-fold cross-validation. This analysis demonstrated that a combination of the Anda-TB and TB Complex tests provided the best predictive capacity, with as many as 91.7% of individuals being correctly classified. The diagnostic process and cut-off values for each of the tests are shown in Figure 3. The sensitivity and specificity of these two combined serological



Tests	AUC	P	Cut-off	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
Myco G	0.7336	<0.0001	230.8	50.0	40.0-60.0	93.9	85.2-98.3	8.25
TB complex	0.7008	<0.0001	135.2	51.9	41.9-61.8	92.9	85.1-97.3	7.27
IBL	0.7110	<0.0001	133.1	76.0	66.6-83.8	57.1	45.9-67.9	1.77
Anda-TB	0.8309	<0.0001	264.8	77.9	68.7-85.4	78.6	68.3-86.8	3.63

Figure 2. ROC curves for the four serological tests in discriminating between active TB patients and subjects with latent TB infection.
The ROC curves were constructed using data from subjects with active TB (ATB group) as patients and subjects with latent tuberculosis infection (LTBI group) as controls. The cut-off values were estimated by ROC analysis at various sensitivities and specificities and determined at the maximum Youden's index (YI) (i.e. sensitivity + specificity – 1). The sensitivity and specificity here indicated the diagnostic performance of the serological tests in discriminating ATB from LTBI, which were calculated when subjects in the ATB group were defined as patients, and subjects in the LTBI group were defined as negative controls. The tables show the detailed information obtained for each ROC curve including area under the curve (AUC), p values of AUC from ROC analysis, cut-off values, the sensitivity and specificity with a confidence interval (CI) of 95% and the likelihood ratio.

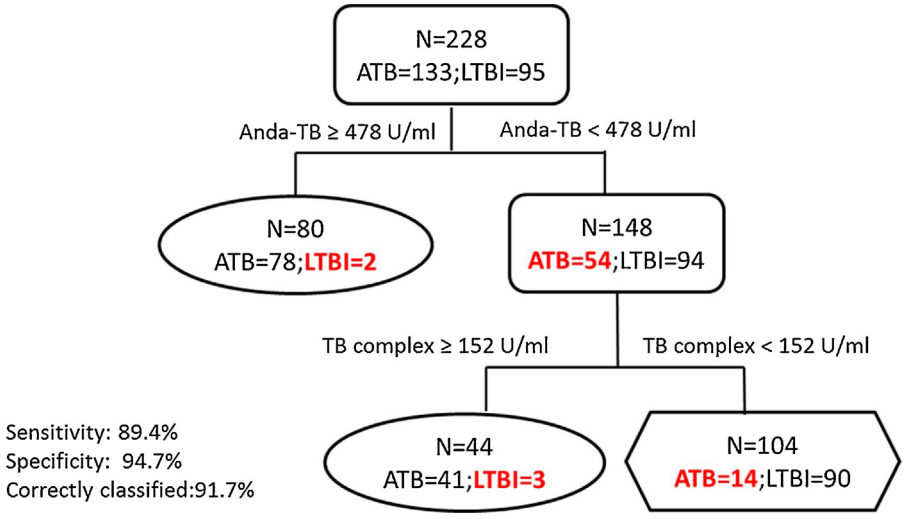


Figure 3. The combination of the Anda-TB and TB complex provides the best discrimination between ATB and LTBI groups.
The ATB group was defined as the patient population and the LTBI group was defined as the control population for the analysis of the diagnostic performance of the biomarkers. The diagnostic strategy and optimum cut-offs for the Anda-TB and TB complex were determined by decision tree analysis. The sensitivity and specificity of the combined tests was 89.4% and 94.7% respectively. 91.7% of individuals were correctly classified according to this method. The misclassified subjects were shown in red. Rectangle: internal nodes; Oval and hexagon: terminal nodes showing the number finally determined as LTBI and ATB, respectively.

tests for the ATB group vs. LTBI group were 89.4% and 94.7%, respectively.

Validation of the serological tests in an independent cohort

In order to further validate the diagnostic performance of the selected serological tests (Anda TB and TB Complex), a clinical setting-oriented study group (group II) was recruited prospectively, including 106 subjects with a suspected diagnosis of active TB (ATB suspects). Among the 106 subjects, 39 were eventually diagnosed as ATB cases based on the criteria described in the Materials and methods section. The serological tests (Anda-TB and TB Complex combined) were performed using the cut-off values established by the decision tree analysis in study group I. Sensitivity and specificity were calculated when ATB patients were defined as patients and subjects without ATB were defined as controls.

At enrollment, all of the 106 patients were tested with the serological tests and T-SPOT. The T-SPOT alone achieved a sensitivity of 89.7%, but a relatively low specificity of 67.2%. The

serological tests achieved a sensitivity of 87.2% and specificity of 82.1%. The diagnostic accuracy of the T-SPOT test was 75.5% and of the serological tests was 84.0%.

The serological tests and T-SPOT test were then combined in a two-step diagnostic procedure, as shown in Figure 4. The result was considered positive when both test results were positive and considered negative when either test was negative. It was found that the T-SPOT combined with the two serological tests could correctly classify 90.6% (96/106) of individuals, with a sensitivity of 87.2% and a specificity of 92.5%. The specificity of the two-step test was significantly higher than that of the T-SPOT test alone ($p < 0.0001$).

Discussion

Serological tests based on the detection of circulating antibodies against *M. tuberculosis*-specific antigens have been investigated widely and have been in use for the last few years. However, a recent systematic review and meta-analysis of previously published studies on various serological tests reported that none of the antigen sensitivities is high enough to replace the current standard sputum smear microscopy examination in both pulmonary and extrapulmonary TB cases (Steingart et al., 2011). Another study conducted by the WHO and TDR (Research and Training in Tropical Diseases) drew similar conclusions (UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, 2008). The results from the study described herein are in accordance with these findings: it was observed that the four serological tests all showed relatively low sensitivity in detecting ATB patients. It was also observed that the diagnostic performance of the four serological tests was not completely consistent with previous studies (Al-Hajjaj et al., 1999; Alifano et al., 1994). According to the literature, the results of these commercial serological tests show highly variable sensitivities and specificities (Steingart et al., 2011). The highly variable sensitivities and specificities of these serological tests are likely due to the heterogeneity of the antigen recognition response exhibited by subjects infected by *M. tuberculosis*, due to the severity of the disease, strain of the bacillus, health of the patient, and bacillary load (Samanich et al., 2001; Abebe et al., 2007). The endemicity rate of TB in the test region could also affect the results of the tests and contribute to their variability. Therefore, it is not likely that any of the serological tests could be used alone for the effective diagnosis of ATB.

Other contributors to the diagnostic variability may be the positivity thresholds provided by the assay manufacturers. Determination of the cut-off value is considered to be the critical issue when defining the diagnostic performance of a serological test. In the present study, the diagnostic accuracy of the Anda TB test was higher using the cut-off value based on the ROC analysis. The reasons for the differences in cut-off point-specific performance remain unclear, but may be related to different levels of *M. tuberculosis* exposure in different regions. For example, it has been demonstrated that the antibody response is higher in healthy individuals from regions where TB is highly endemic, due to a higher chance of exposure to and infection with *M. tuberculosis* (Hoff et al., 2007). Individuals in TB endemic areas may have higher background levels of *M. tuberculosis*-specific antibodies than subjects from low-endemic areas, where the majority of the serological tests used in the present study were developed. Thus, different criteria for positive results should be independently evaluated and used in areas with different TB endemicity rates. Further prospective standardized studies are urgently required to clarify these findings and to determine the optimal rule-in cut-off point in different settings.

This study evaluated the diagnostic performance of four serological tests across different stages of *M. tuberculosis* infection, particularly in subjects with LTBI. In the LTBI group, although all four serological tests showed a low positive rate (which was not significantly different from the CON group), higher antibody levels were observed than in non-TB controls. These results are consistent with those of another report, which demonstrated that significant antibody responses are not restricted to ATB patients but can also reflect subjects with LTBI, particularly in areas with high levels of exposure to *M. tuberculosis* (Hoff et al., 2007). Therefore it was not surprising to find higher antibody levels in household contacts with LTBI.

Another important finding of this study was the diagnostic potential of serological tests in discriminating between ATB and LTBI. It is known that T-cell-based immunological tests such as the T-SPOT assay show no difference in IFN-γ release between active and latent TB infection and fail to discriminate between them (Lalvani and Pareek, 2010). Unlike IGRAs, the *M. tuberculosis*-specific antibody response showed a strong correlation with

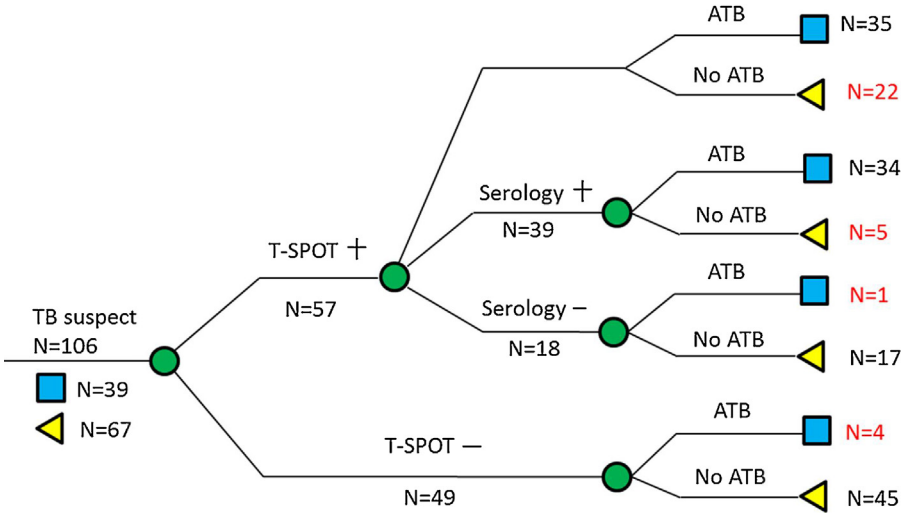


Figure 4. Study decision tree to validate the diagnostic performance of the serological tests using two step strategies.
The 106 individuals suspected of ATB were tested with T-SPOT test at first, and then the subjects with positive T-SPOT TB results were tested with the serological tests (Anda-TB and TB complex tests combined). Finally the positive result were defined when both test was positive and a negative result when either negative. The numbers in red represent false positive or false negative results. Circles: diagnosis nodes; squares: ATB patients; triangles: subjects without ATB.

bacillary burden and the risk of TB disease, and the antibody levels in the ATB group were significantly higher than those in the LTBI group in this study. This finding is consistent with several previous reports, which have shown different antibody responses during different stages of infection (Silva et al., 2003; Davidow et al., 2005; Singh et al., 2005). It is not surprising that subjects with latent infection may harbor a much smaller bacterial population than ATB patients and correspondingly demonstrate antibody levels lower than those of ATB cases. The implications of this are that the current serological diagnostic tests may serve as potential biomarkers to differentiate ATB from LTBI, possibly aiding in early intervention for future TB cases.

In this study, no single serological test achieved satisfactory diagnostic performance in discriminating ATB from LTBI. Therefore a combination of the tests by decision tree was used to improve the accuracy, and the combination of Anda TB and TB Complex was determined to have the best diagnostic performance. A60 used in the Anda-TB test is one of the most studied *M. tuberculosis* antigens used in serological tests for TB (Abebe et al., 2007; Flores et al., 2011). The important diagnostic value of the A60-based Anda-TB IgG test has already been confirmed in many relevant studies, with a sensitivity ranging from 63% to 85% and specificity ranging from 73% to 100% for pulmonary TB (Steingart et al., 2011). The results of the Anda-TB test in the present study are consistent with those of the previous studies and also appear to be superior to those of the other three tests by higher diagnostic accuracy and AUC of ROC analysis. For all of the reasons above, A60 appears to be the most important antigen component in the combined serological tests. 38 kDa and 16 kDa used by TB Complex are also widely used antigens. Although the diagnostic performance of these two antigens varied widely and showed limited sensitivity, they could be used in combination with other *M. tuberculosis* antigens (Abebe et al., 2007). 16 kDa may also have clinical significance for detecting LTBI due to overproduction during the stationary phase of *M. tuberculosis* (Yuan et al., 1996). In the validation group (group II), the candidate serological antigens (A60, 38 kDa, and 16 kDa) were evaluated using two different commercial detection kits. These tests were used separately and the results were combined in a serial way. Further studies are needed to develop and evaluate a single kit with all of these antigens, which could simplify the test procedures.

This study has some inherent limitations. First, since there is currently no gold standard for the diagnosis of LTBI, the T-SPOT test was used in this study as a diagnostic surrogate. This may have introduced some bias into the evaluation of the diagnostic performance of the serological tests in detecting LTBI. In addition, the cross-sectional design may also have led to the risk of over-optimistic cut-off values and overestimated test accuracy. Longitudinal cohort studies will be required to validate the accuracies and cut-off values of the antibody tests, as well as to more carefully determine the predictive value for progression to ATB in a large cohort of subjects at high risk of TB infection. The cross-reactions of the selected antigens (A60, 38 kD, and 16 kD) with other infectious diseases also need to be further evaluated. Future studies should include larger sample sizes and recruit more subjects, especially subjects with lung diseases other than TB, with cured TB, and those with suspected TB relapse. The serological tests should also be evaluated with an emphasis on patients who are particularly at risk of TB infection, such as immunocompromised or immunosuppressed patients (i.e., patients receiving immunosuppressive therapy for rheumatoid arthritis), including those with HIV infection or children (Steingart et al., 2007b). In addition, the serological tests used in this study are all based on the detection of *M. tuberculosis* antigen-specific IgG antibodies. The diagnostic performance of IgM and IgA antibody detection tests for discriminating between ATB and LTBI is also worthy of further investigation.

In conclusion, the diagnostic performance of four serological antibody detection tests was evaluated in subjects with ATB disease, subjects with latent infection, and healthy controls. The serological tests all showed low sensitivity but high specificity with the given cut-off values. Importantly, the antibody levels of the four tests all differed significantly between the ATB and LTBI groups. By ROC and decision tree analysis, a combination of Anda-TB and TB Complex provided the best predictive capacity in discriminating ATB from LTBI. This combination was then validated in a clinical cohort including patients with suspected ATB. The combination of these serological tests with T-SPOT could lead to a substantial improvement in the diagnostic performance, which could consequently improve the detection and treatment of at-risk cases.

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Conflict of interest

No conflict of interest to declare.

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