Mycobacterium tuberculosis Secreted Proteins As Potential Biomarkers for the Diagnosis of Active Tuberculosis and Latent Tuberculosis Infection

Caiqin Zhang,[1,†] Xiaoqin Song,[1,†] Yong Zhao,[1] Hai Zhang,[1] Shanmin Zhao,[1] Fengfeng Mao,[1] Bing Bai,[1] Shaoping Wu,[2]* and Changhong Shi[1]*

[1]*Division of Infection and Immunology, Laboratory Animal Center, Fourth Military Medical University, Xi'an, P.R. China*

[2]*Department of Radiology, First Affiliated Hospital, Chengdu Medical College, Chengdu, P.R. China*

Background: The detection of *Mycobacterium tuberculosis* (*Mtb*) specific human antibodies has been an important diagnostic aid in the diagnosis of tuberculosis (TB) cases with smear-negative sputum samples, especially for the screening of high-risk population. This study focused on the analysis and comparison of the four potential *Mtb*-secreted proteins (ESAT6, CFP10, Ag85B, Hsp16.3) and the fusion protein Ag85B-Hsp16.3 as new markers in the serodiagnosis between active TB and latent TB infection (LTBI). *Methods:* These five recombinant proteins were produced and used in optimized ELISA to detect IgG serum antibodies against the four secreted proteins. The capacity of identifying infection was evaluated either in active TB patients or LTBI individuals, which was compared with the control groups consisting of hospitalized non-TB individuals. *Results:* The results showed that Ag85B-Hsp16.3/ESAT6 and Hsp16.3/ESAT6 were the best-associated antigens for serology diagnosis of the active TB and LTBI individuals because of their specificity, sensitivity, YI values, and positive rates, respectively. ELISA test demonstrated that 41.67% (25/60) of blood donors respond to Ag85B-Hsp16.3/ESAT6. The consistency of this positive respond with clinical diagnosis almost reached 84% (21/25). *Conclusion:* Thus, a combined test of multiple *Mtb*-secreted proteins Ag85B, Hsp16.3, and ESAT6 may be the ascendant preliminary screening antigens for active TB or LTBI patients. J. Clin. Lab. Anal. 29:375–382, 2015. © 2014 The Authors. *Journal of Clinical Laboratory Analysis.* Published by Wiley Periodicals, Inc.

Key words: *M. tuberculosis* (*Mtb*); tuberculosis (TB); ESAT6; CFP10; Ag85B; Hsp16.3; diagnosis

INTRODUCTION

Tuberculosis (TB) remains one of the leading causes of death in the world. About one-third of the world's population is latently infected with *Mycobacterium tuberculosis* (*Mtb*). Recently, World Health Organization (WHO) estimated that each year about 9.2 million diseases and 1.7 million deaths were attributed to TB (1). Early diagnosis and treatment of TB patients is crucial for the control of TB spreading (2). In many countries, especially in developing countries, because of resource constraint the diagnosis of TB largely relies on the detection of acid-fast bacilli in sputum in conjunction with assessment of clinical symptoms and X-ray radiographic evidence. However, these evaluations are time-consuming and unable to distinguish between active TB and latent infection. In recent years, the detection of *Mtb*-specific human antibodies

TABLE 1. Clinical Characteristics of the Serum Samples

Tuberculosis type	Total number of sera	Smear positive	Culture positive	ELISPOT positive
Active TB	60	42	60	/
LTBI	30	0	0	30
TB⁻HP	92	0	0	0

LTBI, latent TB infected individuals; TB⁻ HP, TB negative healthy persons;
"/," the 60 active TB patients weren't detected by ELISPOT, because they were positive by smear and culture.

has been an important diagnostic aid in the diagnosis of TB cases with smear-negative sputum samples (3), especially for the screening of high-risk population. Although there are plenty of antibody tests in the market with poor sensitivity and specificity, and WHO has warned their use in the diagnosis of TB, efforts have been made to develop and accelerate the diagnosis tests for TB.

Currently, the antigens including 38kD, 16kD, ESAT-6, MPT63, 19kD, MPT64, MPT32, Rv1009, MTB48, Mtb81, MTC28, Ag85B, and KatG have been evaluated for their serodiagnostic potential (4–12). The use of any single TB antigen as a diagnosis marker generated a false-positive rate of 30~40% (13), but a combined test of multiple antigens improved the positive diagnostic rate. Some research groups (10, 14, 15) reported that a combination of multiple antigens could improve the diagnosis of pulmonary TB. In this study, we tested the recombinant proteins ESAT6, CFP10, Ag85B, Hsp16.3, and the fusion protein Ag85B-Hsp16.3 by ELISA to evaluate the usefulness of different combinations of these antibodies in diagnosing active TB patients and latent TB infected individuals.

MATERIALS AND METHODS

Collection of Blood Serum

A total of 242 serum samples of TB patients and volunteers from west of China were selected to analyze, which included 60 samples from active TB patients who were having treatment for TB; 30 samples from latent TB infected individuals who had been normative TB treated for over 2 years or contacted with active TB patients but had no TB clinical symptom, and 92 from TB-negative healthy persons (Table 1) who were diagnosed by IGRA tests (T-SPOT-TB™, Oxford Immunotec, UK) (16). In addition, 60 samples from blood donors were included. All patients and healthy controls in this study were negative for HIV antibodies.

Bacterial Stains

Mtb strain H37Rv was provided by the Institute of Drug and Biological Products Checking (Beijing, China) (17).

Escherichia coli strain DH5a was provided by the Laboratory Animal Center of the Fourth Military Medical University (Xi'an, China), and was used for the expression of recombinant proteins Ag85B-Hsp16.3, Ag85B, Hsp16.3, ESAT6, and CFP10.

Reagents

Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Sigma-Aldrich (St. Louis, MO). Glutathione Sepharose 4 Fast Flow was purchased from GE Healthcare (Shanghai, China); Protein Refolding Kits were purchased from Novagen (Darmstadt, Germany); BCA Protein Assay Kit was purchased from Beyotime Institute of Biotechnology. HRP-conjugated goat-anti-human IgG was purchased from Dingguo Biotech (Beijing, China).

Expression and Purification of *Mtb* Ag85B-Hsp16.3, Ag85B, Hsp16.3, ESAT6, and CFP10

The recombinant proteins Ag85B-Hsp16.3, Ag85B, Hsp16.3, ESAT6, and CFP10 were expressed and purified as described previously (18–20). The recombinant proteins Ag85B-Hsp16.3, Ag85B, Hsp16.3, and CFP10 were purified by metal–chelate affinity chromatography using Ni^{2+}-NTA resin, according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Finally, for the recombinant proteins Ag85B-Hsp16.3, Ag85B, Hsp16.3, and CFP10, N-terminal His tags were removed by TEV digestion and purified by exclusion from a Ni^{2+}-NTA agarose beads (16). The purification of the recombinant protein ESAT6 was performed according to the Protein Refolding Kits and Glutathione Sepharose 4 Fast Flow manufacturer's protocol. The concentrations of recombinant proteins were determined by BCA Protein Assay Kit and the purities were estimated by SDS-PAGE. Those recombinant proteins had been folded correctly via biophysical analyses and then were stored at –80°C for further use.

Antibody Detection by ELISA in a Total of 242 Serum Samples

To detect the specific IgG antibodies, 100 µl of individual recombinant proteins (2.5 µg/ml for Ag85B-Hsp16.3, 5 µg/ml for Ag85B, 5 µg/ml for Hsp16.3, 0.5 µg/ml for ESAT6, and 1 µg/ml for CFP10) were used to coat the 96-well plates overnight at 4°C. Then the wells were blocked with 300 µl of 20% fetal bovine serum dilution with PBS. Hundred microliters of serum (diluted 1:100) was triplicate added and incubated for 1 h at 37°C. After washing, HRP-conjugated goat-anti-human IgG was used as the secondary antibody incubated for 1 h at 37°C. Hundred microliters of enzymatic substrate solution containing OPD (1 mg/ml) and 30% H_2O_2 were added and
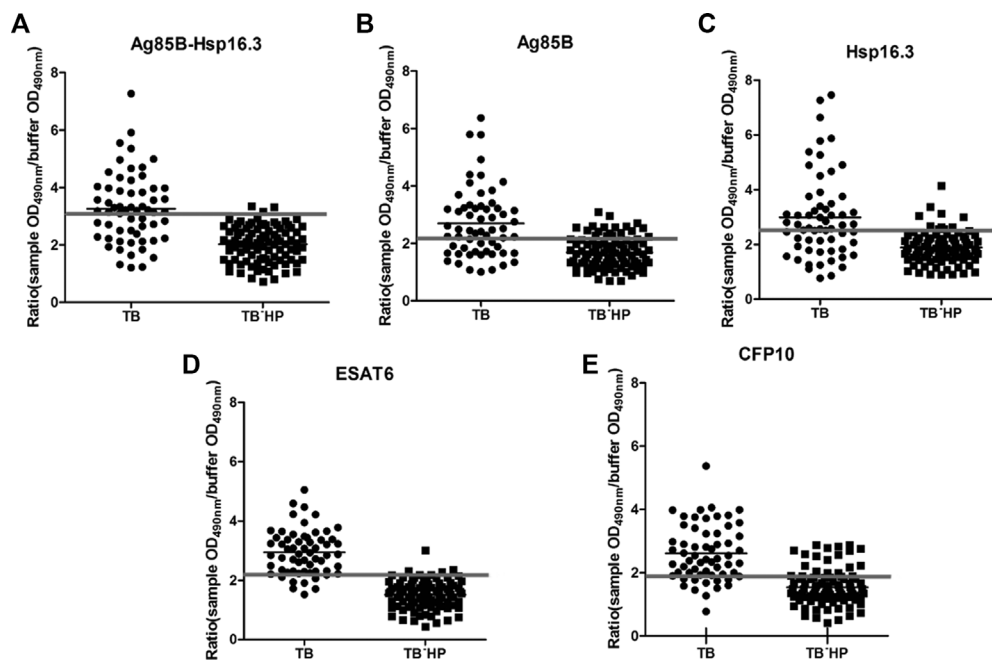
Fig. 1. Comparison of the antibody IgG response to the recombinant five antigens in groups of TB patients and healthy individuals. (A) Ag85B-Hsp16.3, (B) Ag85B, (C) Hsp16.3, (D) ESAT6, (E) CFP10. Black horizontal lines indicate the mean value, whereas gray lines indicate the cutoff values, derived from the ROC curves. TB, active TB patients ($n = 60$), TB⁻ HP, non-TB healthy population ($n = 92$).

incubated for 10 min at 37°C. The response of specific antibodies was measured at a wavelength of A490 nm in Bio-Rad 680.

Statistical Analysis

The ratio value corresponding to the sample OD_{490nm}/buffer OD_{490nm} was determined for each sample. The mean and standard deviation of the ratio of individual groups for antibody/antigen responses to each marker were calculated using GraphPad Prism 5.0 software, and the receiver operating characteristic (ROC) curves of the ratio values for antibody responses to each marker were plotted. The area under the curves (AUCs) and 95% CI for responses to each antigen were calculated. Individuals were scored as positive for the specific antibody response when their ratio value was greater than or equal to the cutoff value. Difference of each group was compared using Student's t-test and χ^2 test.

RESULTS

Expression and Purification of the Five Proteins

The purified proteins Ag85B-Hsp16.3, Ag85B, Hsp16.3, ESAT6, and CFP10 were obtained with the purity of more than 95%, and their concentrations were 1.2 mg/ml, 3.6 mg/ml, 1.0 mg/ml, 2.4 mg/ml, and 2.5 mg/ml, respectively.

IgG Antibody Response Against the Five Antigens in Active TB Patients

The five individual indirect ELISAs were optimized and used for analyzing the samples from distinct patient groups (Fig. 1). Antibody responses to the antigens were analyzed by the ROC curves of the ratio values. The ROC curves described the relationship between the sensitivity and specificity at any cutoff values, which are depicted in Figure 2. The AUC of each antibody response is provided in Table 2. The AUC for Ag85B-Hsp16.3, Ag85B, Hsp16.3, ESAT6, and CFP10 were 0.82 (0.75–0.89), 0.78 (0.70–0.85), 0.75 (0.66–0.83), 0.96 (0.93–0.99), and 0.86 (0.80–0.92), respectively. The optimal combination of specificity (95.65%) and sensitivity (86.67%) (Table 4) were generated from cutoff levels based on the AUC values. The levels of antibodies against each antigen in TB patients were significantly higher than those in healthy controls ($P < 0.0001$, Table 2).

Serological Responses in Latent TB Patients

The specific IgG responses against the five antigens in the samples from latent TB infected individuals are described in Figure 3, and the cutoff values (Table 4) were obtained from the ROC curves (Fig. 4). The AUC values were from 0.65 (0.51–0.79) to 0.79 (0.69–0.89) for the five antigens, which were
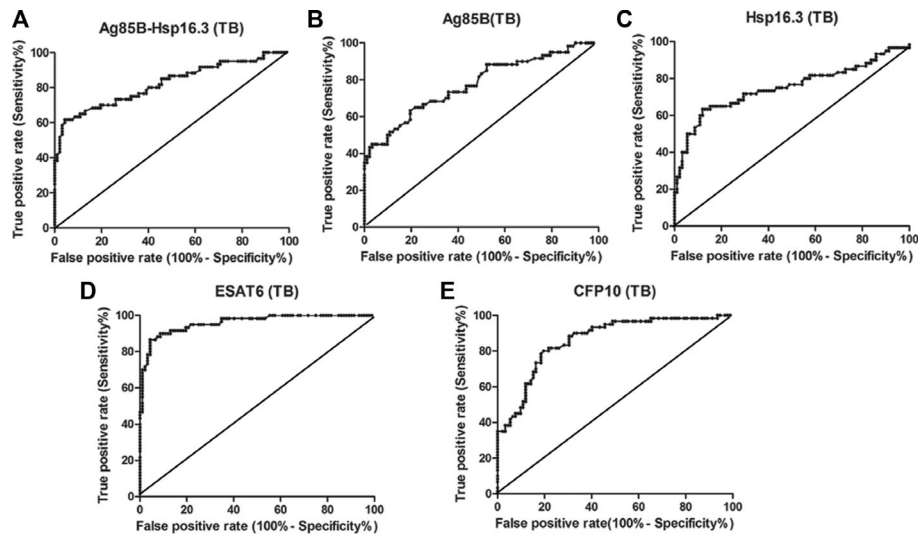
Fig. 2. ROC analysis of the antibody response against five antigens in TB patients and healthy individuals. Comparison of the ROC curves for IgG detection specific to Ag85B-Hsp16.3 (A), Ag85B (B), Hsp16.3 (C), ESAT6 (D), and CFP10 (E).

TABLE 2. ROC analysis of the antibody response against five antigens in TB patients and healthy individuals

Antigens	Groups (number)	SD	AUC	95%CI	P values
Ag85B-Hs p16.3	TB (60)	1.212	0.82	0.75–0.89	<0.0001
	TB$^-$ HP (92)	0.6216			
Ag85B	TB (60)	1.196	0.78	0.70–0.85	<0.0001
	TB$^-$ HP (92)	0.5399			
Hsp16.3	TB (60)	1.540	0.75	0.66–0.83	<0.0001
	TB$^-$ HP (92)	0.5743			
ESAT6	TB (60)	0.7538	0.96	0.93–0.99	<0.0001
	TB$^-$ HP (92)	0.4740			
CFP10	TB (60)	0.8783	0.86	0.80–0.92	<0.0001
	TB$^-$ HP (92)	0.5716			

SD, standard deviation; AUC, area under the ROC curves; CI, confidence interval.

lower than the AUC values from the ROC curves (Fig. 2). The levels of antibodies against Ag85B-Hsp16.3, Ag85B, ESAT6, and CFP10 antigens in LTBI individuals were significantly higher than those in healthy controls ($P < 0.01$, Table 3). However, the level of antibody against Hsp16.3 in LTBI patients was significantly higher than that in healthy control groups ($P < 0.05$, Table 3). The optimal combination of specificity (97.83%) and sensitivity (53.33%) (Table 4) was generated from the ROC curves (Fig. 4).

Evaluation of the Diagnostic Value of the Five Proteins Between Active TB and LTBI

The AUC values and 95% CI of TB and LTBI groups are calculated in Table 2 and Table 3, respectively. The optimal cutoff values were chosen when the Youden index (YI) value was maximum (Table 4). The specificity for detecting TB antibody responses to antigens Ag85B-Hsp16.3, Ag85B, Hsp16.3, ESAT6, and CFP10 was 95.65%, 80.43%, 88.04%, 95.65%, and 80.43%, respectively, and the sensitivity was 61.67%, 63.33%, 63.33%, 86.67%, and 80.00%, respectively. Furthermore, the YI values were 0.573, 0.438, 0.514, 0.823, and 0.604, respectively. The optimal IgG antibody responses were ESAT6 or CFP10 by χ^2 test.

The serological responses against antigens Ag85B-Hsp16.3, Ag85B, Hsp16.3, ESAT6, and CFP10 when detecting LTBI individuals showed that the specificity was 73.91%, 97.83%, 88.04%, 84.78%, and 69.57%, respectively, and the sensitivity was 60.00%, 53.33%, 53.33%, 60.00%, and 73.33%, respectively. Furthermore, the YI values were 0.339, 0.512, 0.414, 0.448, and 0.429, respectively (Table 4). It had no statistical significance between the antibody response results of detecting LTBI patients against the five antigens.

To improve the efficiency of the serodiagnostic test, the associated parallel tests were designed. Any two antigens were combined to detect active TB patients and LTBI individuals (Tables 5 and 6). In Tables 5 and 6, either of the combination antigens was positive by ELISA then the serum sample was diagnosed positive. The specificity and sensitivity of the combination antigens Ag85B-Hsp16.3/ESAT6 were 93.33% and 92.39%, respectively, which was the optimal antigens combination to diagnose active TB in Table 5. YI value of Ag85B-Hsp16.3/ESAT6 was 0.857, which was higher than the YI value (0.823, Table 4) of ESAT6. However, the positive rates have no difference between associated antigen Ag85B-Hsp16.3/ESAT6 and single ESAT6 ($P > 0.1$) in active TB group. In Table 6, the optimal antigens combination to diagnose LTBI was Hsp16.3/ESAT6, the specificity and sensitivity of which were 75.0% and 76.67%,
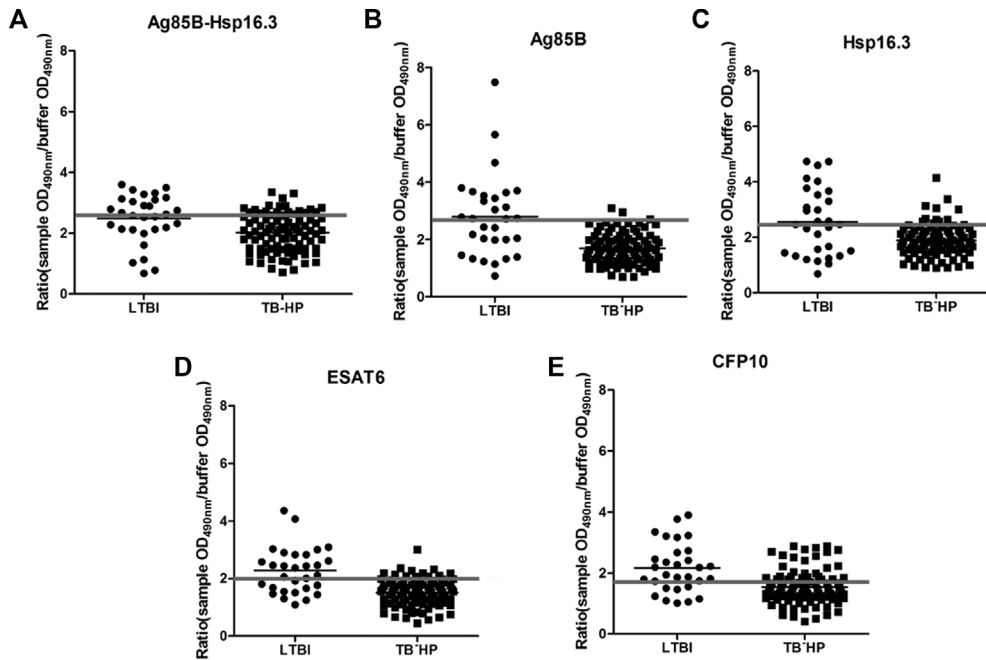
Fig. 3. Comparison of the antibody IgG response to the recombinant five antigens in groups of LTBI and healthy individuals. (A) Ag85B-Hsp16.3, (B) Ag85B, (C) Hsp16.3, (D) ESAT6, (E) CFP10. Black horizontal lines indicate the mean value, whereas gray lines indicate the cutoff values, derived from the ROC curves. LTBI, latent TB infected patients ($n = 30$), TB$^-$ HP, non-TB healthy population ($n = 92$).

respectively. YI value of Hsp16.3/ESAT6 was 0.517, and a little higher than the YI value (Table 4) of single antigen Ag85B. However, the positive rate of Hsp16.3/ESAT6 was found significantly different from that of single Ag85B ($P < 0.05$) in latent TB-infected group.

The IgG Antibodies Against the Five Antigens of the 60 Blood Donors

Sixty samples from blood donors were detected against the Ag85B-Hsp16.3, Ag85B, Hsp16.3, ESAT6, and CFP10 antigens of MTB, and the results are shown in
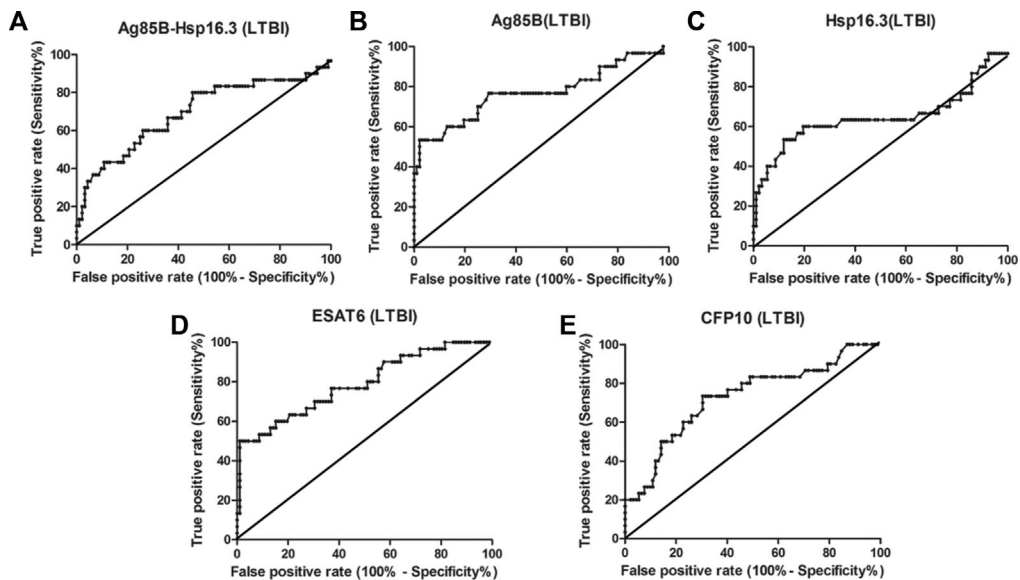

Fig. 4. ROC analysis of the antibody response against five antigens in LTBI and healthy individuals. Comparison of the ROC curves for IgG detection specific to Ag85B-Hsp16.3 (A), Ag85B (B), Hsp16.3 (C), ESAT6 (D), and CFP10 (E). The black lines represent the no-discrimination lines from the left bottom to the top right.

TABLE 3. ROC analysis of the antibody response against five antigens in LTBI and healthy individuals

Antigens	Groups (number)	SD	AUC	95%CI	P values
Ag85B-Hsp16.3	LTBI (30)	0.7946	0.70	0.57–0.82	0.001375
	TB⁻ HP (92)	0.6216			
Ag85B	LTBI (30)	1.429	0.77	0.66–0.89	<0.0001
	TB⁻ HP (92)	0.5399			
Hsp16.3	LTBI (30)	1.203	0.65	0.51–0.79	0.01608 [a]
	TB⁻ HP (92)	0.5743			
ESAT6	LTBI (30)	0.7880	0.79	0.69–0.89	<0.0001
	TB⁻ HP (92)	0.4740			
CFP10	LTBI (30)	0.8050	0.73	0.62–0.84	0.0001930
	TB⁻ HP (92)	0.5716			

SD, standard deviation; AUC, area under the ROC curves; CI, confidence interval.

Table 7. The maximum TB-positive rate was 41.67% (25/60) against associated Ag85B-Hsp16.3/ESAT6 antigens, including 15 samples (25.0%, 15/60) that were positive against associated Hsp16.3/ESAT6 antigens. The positive rates against ESAT6, CFP10, and ESAT6/CFP10 were 13.33% (8/60), 8.33% (5/60), and 15.0% (9/60), respectively. In addition, there were seven samples (11.67%, 7/60) that were positive against Ag85B-Hsp16.3 and Hsp16.3 at equal pace. Further, smear culture results showed that six of those 25 positive donors were active TB patients, and 15 were latent TB infected individuals who were diagnosed by IGRA tests.

DISCUSSION

Although the golden standard for diagnosing active TB should be cultured and should remain so especially in the light of the increasing burden of MDR-TB-XDR-TB. The serological method is an effective diagnostic of TB in vitro, which would be an attractive progress as immunoassays are simple, rapid, inexpensive, and may offer the possibility to detect cases missed by standard spu-

tum smear microscopy (21). Selection-specific antibody against *Mtb* proteins is very important for serological diagnosis of TB. The *Mtb* life cycle can be separated into three main stages: latent, reactivating, and active TB. Each stage represents differences in *Mtb* gene expression and therefore we can determine the immune response to stage-specific antigens (22). Hsp16.3 is secreted during the latency phase of mycobacterial growth and is an important component that facilitates the survival of MTB during latent human infection (23). Immune responses to *Mtb* antigens ESAT6/CFP10 and Ag85B have been shown to be significantly higher in active TB than in LTBI (24). Thus, it was rational to evaluate the value of those *Mtb*-secreted antigens in serodiagnosis of active TB or LTBI.

In this study, five recombinant *Mtb* proteins—Ag85B-Hsp16.3, Ag85B, Hsp16.3, ESAT6, and CFP10—were successfully obtained and evaluated for their diagnostic potential in detecting serum antibodies by ELISA in a population of active TB patients, latent TB infected individuals, and healthy controls. We found that both active TB and LTBI individuals had higher levels of antibodies against these five individual antigens, compared with that in healthy controls ($P < 0.05$, Tables 2 and 3). Although the sensitivity and specificity were lower than Wu X' study (3), the difference may be attributed to differences in the populations, optimization of ELISA, and purity of recombinant proteins (16).

Although the YI value of ESAT6 (0.823) was lower than that of the associated Ag85B-Hsp16.3/ESAT6 (0.857), their positive rates had no difference in active TB patients ($P > 0.1$), indicating that ESAT6 was the optimal antigen for the detection of active TB from the results in Table 2. As shown in Table 5, Ag85B-Hsp16.3/ESAT6 was the best-associated antigen for serology diagnosis of the active TB patients.

The results presented in Table 3 demonstrated that the antibody response against ESAT6 was the optimal to detect LTBI individuals according to the values of SD,

TABLE 4. ROC analysis of the antibody response against five antigens in active TB patients, LTBI, and healthy individuals

Antigens	Groups	Cutoff values	Specificity (%)	Sensitivity (%)	YI values	Likelihood ratio
Ag85B-Hsp16.3	TB	2.905	95.65	61.67	0.573	14.18
	LTBI	2.510	73.91	60.00	0.339	2.30
Ag85B	TB	2.165	80.43	63.33	0.438	3.24
	LTBI	2.710	97.83	53.33	0.512	24.53
Hsp16.3	TB	2.455	88.04	63.33	0.514	5.30
	LTBI	2.455	88.04	53.33	0.414	4.46
ESAT6	TB	2.200	95.65	86.67	0.823	19.93
	LTBI	2.000	84.78	60.00	0.448	3.72
CFP10	TB	1.895	80.43	80.00	0.604	4.09
	LTBI	1.720	69.57	73.33	0.429	2.41

YI, Youden index.

TABLE 5. The results of two antigens parallel tests TB and healthy individuals

Two associated antigens	ESAT6/Ag85B-Hsp16.3	ESAT6/Ag85B	ESAT6/Hsp16.3	ESAT6/CFP10	Ag85B-Hsp16.3/Hsp16.3
Specificity (%)	93.33	77.7	83.70	78.26	86.96
Sensitivity (%)	92.39	93.33	93.33	91.67	80.00
YI values	0.857	0.71	0.770	0.699	0.670

TABLE 6. The results of two antigens parallel tests LTBI and healthy individuals

Two associated antigens	ESAT6/CFP10	Ag85B/Hsp16.3	Hsp16.3/CFP10	Ag85B/CFP10	Hsp16.3/ESAT6
Specificity (%)	59.78	84.78	59.78	69.57	75.00
Sensitivity (%)	80.00	63.33	80.00	80.00	76.67
YI values	0.398	0.480	0.398	0.496	0.517

AUC, and 95%CI. However, the YI value of Ag85B was higher than that of ESAT6 antigen (Table 4), and the positive rate of ESAT6 had no difference from that of Ag85B in latent TB infected patients ($P > 0.5$). The detection of antibodies directed against multiple antigens could provide an improvement in sensitivity compared to single antigen (12, 25). As showed in Table 6, the YI value of the associated Hsp16.3/ESAT6 (0.517) was higher than single Ag85B (0.512), and the positive rate of Hsp16.3/ESAT6 was significantly different from that of single Ag85B antigen in latent TB infected individuals ($P < 0.05$). Hsp16.3/ESAT6 was the best-associated antigen for serology diagnosis of LTBI patients.

The antibody response of the 25 sera from 60 blood donors was positive against Ag85B-Hsp16.3/ESAT6, including 15 sera that were positive against Hsp16.3/ESAT6. It indicated that those positive 25 blood donors were a high-risk population and misfit to be blood donors. The follow-up experiments show that in those 25 blood donors, 6 were active TB patients and 15 were latent TB infected individuals who were diagnosed by IGRA tests. The positive rate of blood donors respond to Ag85B-Hsp16.3/ESAT6 is 41.67%, and the actual clinical diagnosis positive rate is 35%. The consistency (%) of those two tests reached 84% (21/25.). Thus, the combination of Ag85B-Hsp16.3 and ESAT6 shows promising performance with high sensitivity and specificity index for the discrimination between active TB patients and health individuals with a negative ELISPOT.

ELISA is a simple, rapid, and inexpensive test of TB diagnosis, thus this protocol should be the prime screening method for TB, especially for LTBI. In China, the HIV-positive patients, immune-suppressed individuals, infants, and severely ill patients—also solid organ donors—most often are highly considered for *Mtb* screening. People with positive results should be considered for high risk of TB, further differential clinical diagnosis should be performed, which is significantly important for controlling TB (26).

In summary, the results of this study showed *Mtb*-secreted proteins Ag85B, Hsp16.3, and ESAT6, which should be the ascendant preliminary screening antigens of active TB patients and LTBI individuals or as an additional test after smear microscopy. This test may also be used for early diagnosis of the TB infection or screening the high-risk population (2, 27, 28).

ACKNOWLEDGMENTS

CONFLICT OF INTEREST

All authors declare that they have no conflict of interest. All patients and healthy controls in this study had written informed consent. This study was approved by the ethics committee of the Fourth Military Medical University.

TABLE 7. The results of 60 blood donors by ELISA

Antigens	Ag85B-Hsp16.3	Ag85B	Hsp16.3	ESAT6	CFP10	ESAT6/CFP10	Ag85B-Hsp16.3/ESAT6	Hsp16.3/ESAT6
Positive number	23	16	11	8	5	9	25	15
Positive rate (%)	38.33	26.67	18.33	13.33	8.33	15.00	41.67	25.00

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