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Full Length Research Paper

Serodiagnostic value of 23 *Mycobacterium tuberculosis* proteins and a novel protein combination

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To identify novel serological targets for improving the serodiagnosis of *Mycobacterium tuberculosis* (MTB) infection, a total of 23 MTB proteins were clone into the *Escherichia coli*, and the serodiagnostic values of these proteins and a novel combined protein were evaluated. By a two-step selection process, 21 proteins having cross-reaction with TB antibody were identified. In these proteins, the most frequently recognized antigens were Rv0934 and Rv2185c, which were found in 45.6% of TB patients, while the specificity of Rv0934 was only 93.3% which was lower than that of Rv2185c. Subsequently, Rv2031c, Rv2185c, Rv2537c, Rv2785c and Rv3354 were combined equally and the combined protein had 100% specificity and relatively high sensitivity. The sensitivity of the combined protein was 76% which was close to that of purified protein derivative (PPD) test; the specificity of combined protein was 96~98%, which was higher than that of PPD test (76~92%) in the detection of 150 TB patients and 150 healthy subjects. In addition, the specificity of combined protein was significantly higher than that of PPD in the detection of TB antibody in subjects' negative, positive and strong positive for PPD skin test, but the positive rates of the same protein were not significantly different. Three months after bacillus calmette-guerin (BCG) vaccination, the positive rate of combined protein was lower than that of PPD testing. The combination of proteins with high specificity is an effective method to improve the sensitivity of TB serological diagnosis.

Key words: Mycobacterium tuberculosis, serological diagnosis, protein.

INTRODUCTION

Currently, tuberculosis (TB) affects about one-third of the world's population and the pulmonary TB in 95% patients is MTB. TB has been a leading cause of a single-infection induced death and become a public health concern. According to statistics, the World Health Organization shows there were an estimated 13.7 million prevalent cases of TB in 2007 (206 per 100 000 population), an estimated 1.3 million deaths occurred among human immunodeficiency virus (HIV) negative incident cases of TB (20 per 100 000 population) in 2007. In addition, there were an additional 456 000 deaths among incident TB cases who were HIV positive and these deaths are classified as HIV deaths in the International Statistical Classification of Diseases (World Health Organization, 2009).

Accurate diagnosis of TB infection is critical for the treatment, prevention and control of TB. Currently, TB diagnosis largely depends on clinical and radiographic (X-ray) examinations and the sputum smear microscopy and bacterial culture. However, bacterial culture is not practical as a first-line method because it is time-consuming. At present, the confirmed diagnosis relies on the sputum smear examination, achieving a sensitivity of

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Abbreviations: MTB, Mycobacterium tuberculosis; **PCRs,** polymerase chain reaction; **IPTG,** isopropyl-thio-ß-D-galactopyranoside; **TBST,** tris buffered saline; **OD,** optical density; **SD,** standard deviations; **CMI,** cell-mediated-immune; **TB,** tuberculosis; **BCG,** bacillus calmette-guerin; **HIV,** human immunodeficiency virus; **PPD,** purified protein derivative.

10~20% and low specificity. To more accurately diagnose TB, a rapid and convenient, new diagnostic technique is required. The serological test has been an attractive diagnostic method for its convenience, robustness and easy implementation in developing countries. However, no commercially available serological test has been so far shown sufficient sensitivity and specificity to early detection of MTB infection and differentiation of TB from infection following bacillus calmette-guerin (BCG) vaccination (Hussain et al., 1997). An acceptable improvement in the sensitivity can be achieved by combined application of the best TB related antigens, through using both cocktails of single protein and genetically engineered fusion molecules containing several antigens (Hendrickson et al., 2002; Weldingh et al., 2005).

In order to increase the sensitivity and specificity of serological diagnosis of MTB, three strategies were employed to express the candidate proteins: (1) expression of the proteins that were reported to have high sensitivity or specificity in the TB serological diagnosis, such as ESAT6, CFP10, 38 kDa protein (Brusasca et al., 2001; Vordermeier et al., 2001; Andersen and Hansen, 1989), etc; (2) the CFP10-ESAT6 gene is cloned into pET28b which is then introduced to prokaryotic expression vectors, and then other TB protein genes were introduced to the downstream of CFP10-ESAT6 gene in the vectors which express the protein complex composed of CFP10, ESAT6 and TB protein (data not shown); (3) the genome sequences of H37Rv and BCG is compared through tBLAST, and the genes having identities between H37Rv and BCG genomes of less than 60% are selected. BEPITOPE software is used to predict their antigenic indexes. The proteins with antigenic index of over 3.0 were selected to be expressed in *E. coli*. A total of 21 proteins were purified and their serological diagnostic values were assessed by ELISA.

METHODS

Comparing the identities of proteins encoded by the genomes of H37Rv and BCG-P

The reference sequences of all proteins in MTB H37Rv were compared against those of BCG-P (*Mycobacterium bovis* BCG strain Pasteur 1173P2) in local server with tBLASTn program. All the reference sequences of proteins in H37Rv and the sequences of BCG-P were from NCBI (http://www.ncbi.nlm.nih.gov/genome).

Selecting and predicting the antigenecity of candidate proteins

Firstly, the proteins with low identity (< 60%) between H37Rv and BCG-P were selected and classified into following groups: (1) PE/PPE or MCE protein family; (2) the assumable secretory or transmembrane proteins. Secondly, reported promising antigens or proteins presumably being significantly regulated under different stimuli (such as HSPs) were also selected. Finally, we cloned the candidate proteins with high antigenic index (>3.0) predicted by BEPITOPE software (Odorico and Pellequer, 2003) with the default parameters.

Cloning of mycobacterium tuberculosis (TB) proteins and open reading frames into *Escherichia coli*.

The gene-specific primers for cloning the open reading frames are listed in Table 1. The readers of the index tests and the reference standard were blind to the results. The polymerase chain reaction (PCRs) were conducted in a 50 µl mixture with Taq^+ DNA polymerase (Stratagene, La Jolla, Calif.) and 10 ng of MTB H37Rv DNA. The primer concentrations were 0.4 µM, and the nucleotide concentrations 250 µM. The PCRs were initiated by a denaturation step at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55 to 65°C for 30 s and extension at 72°C for 90 s followed by a final extension at 72°C for 10 min. The products were cloned into N-terminal His-tagged expression vector pET24b and pET28b at the restriction sites (Table 1) and then transformed into *E. coli*.

Expression and purification of recombinant proteins

E. coli strain BL21-Gold (DE3) (Stratagene, USA) was used as the cloning host to over-express all recombinant proteins except for Rv1984c, Rv2537c and Rv2785c proteins, which were expressed in *E. coli* strain Rosetta (DE3) (Novagen). Expression was induced with 1 mM isopropyl-thio-β-D-galactopyranoside (IPTG) for 3 to 5 h at 37°C. Bacterial pellets were harvested by centrifugation at 6000 rpm for 10 min at 4°C, washed in buffer (20 mM Tris-Hcl, 0.5 M NaCl, pH 8.0). Pellets were thawed and re-suspended in Buffer A (20 mM Tris-Hcl, 0.5M NaCl, 5 mM imidazole, pH 8.0) and lysed using a JY92-II Ultrasonic Crasher (Ningbo Scientz Biotechnology Co. Ltd., China) with 60 rounds of 5 s sonication/10 s ice incubation. All the following centrifugations were performed at 12000 rpm for 20 min at 4°C.

Soluble Rv2031c, Rv2185c and Rv2376c proteins were purified as follows: *E. coli* over-expressing recombinant proteins was lysed by sonication (Qiagen, Germany) in Buffer A. The supernatants were loaded onto a Ni-NTA column and the bound proteins were eluted stepwise with Buffer A containing 5~300 mM imidazole.

The rest recombinant proteins were purified as follows: *E. coli* with high expression levels of recombinant proteins were sonicated in wash Buffer. The lysate was centrifuged and the supernatants were removed. The remaining pellets were re-suspended with Buffer B (50 mM Tris-Hcl, 5% Triton X-100, 50 mM NaCl, 5 mM EDTA, pH 8.0) and centrifuged, and these procedures were repeated twice. After washing, the pellets (inclusion body) were lysed in Buffer C (20 mM Tris-Hcl, 0.5 M NaCl, 8 M urea, pH 8.0) and centrifuged. The supernatants were loaded onto a Ni-NTA column, and the bound proteins were eluted stepwise with Buffer C containing 5~200 mM imidazole (Andersen and Hansen, 1988).

Proteins selected by Western blot

Western immunoblotting of recombinant proteins was performed as follows. Electrophoretically separated proteins were transferred onto nitrocellulose membranes (0.45 µm pore size, Bio-Rad Laboratories) using a tank style blotting system at 100 volts for 1 h and block for 1 h at 37°C in 7.4 pH, Tris buffered saline (TBST) (0.02 M Tris, 0.8% [w/v] sodium chloride, 0.02% [w/v] potassium chloride, 0.3 [v/v] Tween 20). The nitrocellulose was incubated with rabbit anti-tuberculosis polyclone antibody (1:200) in TBST overnight at room temperature. Nitrocellulose was washed with TBST, incubated with alkaline phosphatase conjugated goat anti-rabbit IgG (1:5000) in TBST for 2 h at room temperature. Phosphatase substrate (5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium) was added to the nitrocellulose for 10 to 15 min and the reaction was stopped with addition of de-ionized water. The optical density of the bands was then determined (Rosenkrands et al., 2000).

Table 1. Primers for PCR amplification and cloning.

Gene	Forward (5' 3')	Reverse (3' 5')
Rv0934	atgcCATATGGTGAAAATTCGTTTGCATAC	ccgCTCGAGGCTGGAAATCGTCGCGAT
Rv1980c	atgcCATATGGTGCGCATCAAGATCTTCATGC	ccgCTCGAGGGCCAGCATCGAGTCG
Rv3875	gctaGCTAGCATGACAGAGCAGCAGTGG A	ccgCTCGAGTGCGAACATCCCAGTGAC
Rv3874	atgcCATATGATGGCAGAGATGAAGACC GAT G	ccgCTCGAGGAAGCCCATTTGCGACCA
Rv3804c	atgcCATATGCAGCTTGTTGACAGGGTTCGTGGCGCC	ccgCTCGAGGGCGCCCTGGGGCGCGGGCC
Rv1186c	atgcCATATGACAGACGTGAGCCGAAAGATTCG	ccgCTCGAGGCCGGCGCCTAACGAACTC
Rv1174C	atgcCATATGAGGCTGTCGTTGACCGCATTGAGC	ccgCTCGAGATAGTTGTTGCAGGAGCCGGCA
Rv2873	atgcCATATGATCAACGTTCAGGCCAAACCGGC	ccgCTCGAGCTGTGCCGGGGGCATCAGCACCG
Rv1196	atgcCATATGGTGGATTTCGGGGCGTTACCACCG	ccgCTCGAGGCCGGCCGCCGGAGAATGCGGCA
Rv1984c	atgcCATATGACTCCACGCAGCCTTGTTCGCATCGTT	ccgCTCGAGTCCGGCGTGATCGAGCCTGTTCG
Rv1636	atgcCATATGAGCGCCTATAAGACCGTGGTGGTAGGA	ccgCTCGAGGGTGGTGTGCACGATCAGCACG
Rv1926c	atgcCATATGAAGCTCACCACAATGATCAAGACG	ccgCTCGAGCGGCTCCCAAATCAGCAGATCC
Rv2185c	atgcCATATGGCGGACAAGACGACACAGACG	ccgCTCGAGGCCCTCGACTCGTTTCTTCAGA
Rv0652	atgcCATATGGCAAAGCTCTCCACCGACG	ccgCTCGAGCTTGACGGTGACGGTGGCGCCGGCG
Rv1636	atgcCATATGAGCGCCTATAAGACCGTGGTGGTAG	ccgCTCGAGGGTGGTGTGCACGATCAGCACGT
Rv1243c	atgcCATATGGCCTCCCCGATGCAAGCGG	atgcGAATTCGCCGTTCAGGCCGTGCTGACCG
Rv1966	atgcCATATGAGCTTGCGGTCGACTGTGCCGG	atgcGAATTCTGGCTGCTCCCCCGCCGCGGGG
Rv1968	atgcCATATGCAGCGGCTGCCGTTTTTCAACC	atgcGAATTCCGGTTGGTCTCCCGGCTGCTG
Rv1971	atgcCATATGCATTTCGTGAGGCTGCCGGCG	atgcGAATTCGCTGCCCGGCGGCAGCAGCATC
Rv1973	atgcCATATGTTGCTGAAATGGCAGGACGGCG	atgcGAATTCGATCGGTTCGAATTGCGAGATCAGCC
Rv198	atgcCATATGACGGCCAGGGCGACGTTG	atgtGCGGCCGCGGTGCAAGGATATTGCCCGTTGAGC
Rv2031c	atgcCATATGGCCACCACCCTTCCCGTTCAGC	atgtGCGGCCGCGTTGGTGGACCGGATCTGAATGTG
Rv2376c	atgcCATATGGTGACTTCGATCATGGCTGGCGG	atgcGAATTCTCCGGCGTGATCGAGCCTG
Rv2537c	atgcCATATGAGCGAACTGATCGTGAACGTG	atgcGAATTCCGTCCCGACATGCTCAGCTAGG
Rv2785c	atgcCATATGGCGCCTGACAGCCGAGCAAAAAAAGG	atgcGAATTCGCGACGCAGGCCCAGACGCTCGATGAGTGAACG
Rv2875	atgcCATATGGATCTGGTGGGCCCGGGCTG	atgcGAATTCCGCCGGAGGCATTAGCACGC
Rv2878c	atgcCATATGGAACGTCTGCAGTTCACCGCAACC	atgcGAATTCGGACGTCAGCGCAGCCACCC
Rv3590c	atgcCATATGGCGTCGCCGCTGCAAGCCGTGG	atgcGAATTCGCCGGGTAGGCCGTCCGCACC
Rv3354	atgcCATATGAACCTACGGCGCCATCAGACCC	atgcGAATTCCTAGGACCCGGGCAGCCCCGGCATGT

PPD which was purchased from Chinese fortune Biological Products Co., specifications 50 U/ml was intradermal injected into the left volar forearm with 0.1 ml (5 µ). Injection site reactions were checked after 72 h. The diameter of the induration was measured and recorded in mm. Negative PPD skin test refers to no response or induration diameter <5 mm; generally positive refers to induration diameter ≥5 mm and <20 mm; strong positive refers to induration diameter ≥20 mm or with the existence of blisters, necrosis and lymphangitis.

Study population

Sera were collected from four groups: (i) TB patients group (n=150); there were 78 males and 72 females who were recruited from the 309th hospital of People's Liberation Army, China. The diagnosis of TB was based on findings on X-ray and/or clinical examination and/or medical history. Among these patients, 16 had extrapulmonary TB and the remaining pulmonary TB. The mean age was 39 years. Sputum smear microscopy

showed positive in 51 patients and negative in 99.
(ii) Normal group (n=90): the healthy volunteers (51 males and 39 females) were recruited from the medical examination center of our hospital without known exposure to MTB and no PPD skin test. Findings on X-ray and/or clinical examination and/or medical history were employed to rule out TB.
(iii) The third group, 300 serum from recruits from all parts of China (all males), PPD skin test showed negative in 100 subjects, among whom 55 had not been vaccinated with M. bovis BCG, and 45 were vaccinated with BCG more than 16 years earlier; PPD skin test revealed positive in 100 subjects, among whom 18 had not been vaccinated with M. bovis BCG, and 82 were vaccinated with BCG more than 16 years earlier; 100 were PPD skin test showed strong positive in 100 subjects, among whom 30 had not been vaccinated with M. bovis BCG, and 70 were vaccinated with BCG more than 16 years earlier. The mean age was 17 years.
(iv) BCG-vaccinated group (n=100): PPD skin test in all male subjects showed negative and then they were BCG-vaccinated. The sera were collected at 3 month-s after BCG vaccination. The mean age was 17 years. The patients come from all over the country and all the selected patients were diagnosed as TB with a complete clinical data. The negative, positive and strong positive PPD skin test populations were indeed selected from the recruits. Because general healthy people usually do not check the PPD skin test, so this serum is difficult to collect. The study was approved by the ethics committee of our hospital.

Antibody detection by ELISA

Preparation of 1:100 dilution of serum was made and these sera were used for detection in duplicates. The ELISA procedures were as follows: 96-well polystyrene microtiter plates (Nunc, Roskilde, Denmark) were coated with 100 µl of antigen solution (10 mg/ml) in 0.05 M carbonate buffer (pH 9.6) overnight at 4°C and then washed with phosphate-buffered saline (PBS; pH 7.2)-0.05% Tween 20 (PBS-T) three times. The serum samples were diluted 1:100 in PBS and added to the plates followed by incubation for 1 h at room temperature. Then, these plates were incubated for 1h with rabbit anti-human immunoglobulin G antibody conjugated to horseradish peroxidase (D0336; Dako, Hillerød, Denmark) (1:1,000) in PBS-T. Subsequently, the plates were washed with PBS-T followed by incubation with tetramethylbenzidine peroxidase substrate (100 µl/well) for 30 min at room temperature. Then, 50 µl of 8 N sulfuric acid were added to stop the reaction and the optical density (OD) was measured at 405 nm.

Statistical analysis

The ELISA results were analyzed by using cutoff values equal to the mean OD for the healthy control serum samples plus 2.576 standard deviations (SD). If the mean OD of any serum of healthy control is greater than the cutoff, the serum was considered as positive. The differences between TB patients and BCG-vaccinated individuals or healthy controls were compared with the nonparametric Mann-Whitney rank sum test by using the SigmaStat software package (SPSS Inc., Chicago, USA). A value of $P<0.05$ was considered statistically significant.

RESULTS

Expression, purification and preliminary serological screening of candidate proteins

Among the 28 proteins purified, genes of 3 proteins (Rv1243c, Rv1968 and Rv3590c) failed to be cloned and another 2 proteins (Rv1987, Rv2875) failed to be purified. Finally, a total of 23 proteins were successfully cloned and purified. The genes of all proteins were cloned into vector pET24b or pET28b, most of genes were expressed in the inclusion body, the recombinant proteins carried their tags in the C-terminal or N-terminal, and all proteins were purified with Ni-NTA column. During the first round of screening, we attempted to identify the proteins capable to hybridize with TB antibody in the Western blotting using rabbit anti-TB polyclonal antibody and results showed positive in 21 proteins and negative in only 2 proteins (Rv1966 and Rv1971) (Table 2).

Sensitivity and specificity of candidate proteins in the serological diagnosis

The diagnosis values of 21 proteins with cross-reaction with TB antibody and PPD were evaluated with the sera collected from 90 TB patients (including 9-extra pulmonary TB patients) and 90 healthy volunteers. These proteins and PPD were evaluated independently. The results showed the mean OD of sera of TB patients was significantly higher than that of healthy volunteers ($P<0.05$). In these proteins, the most frequently recognized antigens were Rv0934 and Rv2185c, which were identified in 45.6% of the sera of TB patients, while the specificity of Rv0934 was 93.3%, which was lower than that of Rv2185c.

The sensitivity of each protein for TB antibody in TB patients was only up to 45.6% (Table 3), which cannot meet the clinical requirement. Combining proteins with high specificity may improve the detection sensitivity but may decrease the specificity. Thus, the proteins with high specificity were selected and combined to form a protein combination. Shown in Table 3, 8 proteins (Rv1174C, Rv2031c, Rv2185c, Rv2537c, Rv2785c, Rv3354, Rv3874 and Rv3875) had the specificity of 100%, but the sensitivity of Rv1174C, Rv3874 and Rv3875 was less than 20%. Thus, Rv2031c, Rv2185c, Rv2537c, Rv2785c, Rv3354 combined equally, with the specificity of 100% and relatively high sensitivity in the detection of 150 TB patients and 150 healthy subjects. As shown in Table 4, the sensitivity of the combined protein was 76%, which is close to the sensitivity of PPD test. The specificity of the combined protein was 96.7% which is higher than that of PPD test.

Diagnostic performance of serological analysis with the combined protein and PPD in subjects with various responses to purified protein derivative (PPD) skin test

We previously speculated that the poor specificity of PPD test in TB diagnosis was due to wide application of PPD vaccination in China and the result of serological testing with PPD might be parallel to that of PPD skin test. That is to say, the antibody positive rate is relatively high in the

Table 2. Expression, purification and serological screening of candidate proteins.

Gene	Protein	vector	expression	purification	Western blot	Reference(s)
Rv0652	TB13.4	pET24b	Inclusion body	Ni-NTA column	+	9
Rv0934	38-kDa protein	pET24b	Inclusion body	Ni-NTA column	+	7,10
Rv1174C	MTB8.4	pET24b	Inclusion body	Ni-NTA column	+	11
Rv1186c	Ag85B	pET24b	Inclusion body	Ni-NTA column	+	12
Rv1196	PPE18	pET24b	Inclusion body	Ni-NTA column	+	13
Rv1243c	PE-PGRS23	pET28b	failure	Ni-NTA column	N	Computational prediction
Rv1636	TB15.3	pET24b	Inclusion body	Ni-NTA column	+	14
Rv1926c	MPT63	pET24b	Inclusion body	Ni-NTA column	+	15
Rv1966	mce3A	pET28b	Inclusion body	Ni-NTA column	_	16
Rv1968	mce3C	pET28b	failure	Ni-NTA column	N	Computational prediction
Rv1971	mce3F	pET28b	Inclusion body	Ni-NTA column	_	Computational prediction
Rv1973	Possible mce	pET28b	Inclusion body	Ni-NTA column	+	Computational prediction
Rv1980c	MPT64	pET24b	Inclusion body	Ni-NTA column	+	17,18
Rv1984c	CFP21	pET24b	Inclusion body	Ni-NTA column	+	19
Rv1987	Possible chitinase	Pet28b	Inclusion body	Unsuccessful	N	Computational prediction
Rv2031c	hspX	pET28b	supernatants	Ni-NTA column	+	20
Rv2185c	TB16.3	pET28b	supernatants	Ni-NTA column	+	14
Rv2376c	Cfp2	pET28b	supernatants	Ni-NTA column	+	21
Rv2537c	aroD	pET28b	Inclusion body	Ni-NTA column	+	Computational prediction
Rv2785c	rpsO	Pet28b	Inclusion body	Ni-NTA column	+	Computational prediction
Rv2873	MPt83	pET24b	Inclusion body	Ni-NTA column	+	22
Rv2875	MPT70	pET28b	Inclusion body	Unsuccessful	N	23
Rv2878c	MPT53	pET28b	Inclusion body	Ni-NTA column	+	24
Rv3354	TB9.7	pET24b	Inclusion body	Ni-NTA column	+	25
Rv3590c	PE-PGRS58	pET28b	failure	Ni-NTA column	N	Computational prediction
Rv3804c	Ag85A	pET24b	Inclusion body	Ni-NTA column	+	26
Rv3874	CFP10	pET24b	Inclusion body	Ni-NTA column	+	27
Rv3875	ESAT6	pET24b	Inclusion body	Ni-NTA column	+	28

samples showing strong positive in the PPD skin test. Thus, the diagnostic performance of combined protein and PPD (purchased from Chinese medicine and biological products standardization station, 2 mg/ml) was investigated in 100, 100 and 100 subjects showing negative, positive and strong positive, respectively, in the PPD skin test. As shown Figure 1, the specificity of combined protein was statically higher than that of PPD, but the positive rates of the same protein in the detection of TB antibody in subjects negative, positive and strong positive for PPD skin test were not significantly different: the positive rate of PPD test was about 10% and that of combination protein about 3%. Thus, we concluded that the serology immunity of TB was not parallel to the cellular immunity.

Diagnostic performance of serological analysis with the combined protein and PPD in subjected at 3 months after bacillus calmette-guerin (BCG) vaccination

The sera were collected from 100 subjected at 3 months after BCG vaccination, and serological analysis with combined protein and PPD was performed independently. Four subjects were positive for the combined protein detection, and 24 positive for PPD testing. In addition, this positive rate of PPD testing was statistically higher than that in subjects at more than 16 years after BCG vaccination. The sensitivities of detection with the combined protein were therefore compared with samples from 3 months after BCG vaccination and more than 10 years of BCG vaccination.

DISCUSSION

In the recent years, antigens with performance better than that of Rv0934 (38 kDa) protein in the detection of TB have been identified (Weldingh et al., 2005). To identify additional potentially promising serological antigens, comparative genomics, bioinformatics and ELISA were employed to identify new seroantigens. With the computational predication and tracking documents, 28 candidate proteins were identified and introduced to *E.*

Table 3. Sensitivity and specificity of candidate protein for serological diagnosis.

Protein name	90 TB serum		90 control serum		Sensitivity (%)	Specificity (%)
	Positive numbers	Negative numbers	Positive numbers	Negative numbers		
TB13.4	18	72	3	87	20	96.7
38-kDa protein	41	49	6	84	45.6	93.3
MTB8.4	12	78	0	90	13.3	100
Ag85B	38	52	10	80	42.22	88.9
PPE18	23	67	5	85	25.6	94.4
TB15.3	28	62	3	87	31.1	96.7
MPT63	29	61	10	80	32.2	88.9
Possible mce	32	58	10	80	35.6	88.9
MPT64	27	63	7	83	30	92.22
CFP21	23	67	3	87	25.6	96.7
hspX	35	55	0	90	38.9	100
TB16.3	41	49	0	90	45.6	100
Cfp2	22	68	5	85	24.4	94.4
aroD	29	61	0	90	32.2	100
rpsO	23	67	0	90	25.6	100
MPt83	31	59	7	83	34.4	92.2
MPT53	22	68	4	86	24.4	95.6
TB9.7	31	59	0	90	34.4	100
Ag85A	36	54	9	81	40	90
CFP10	17	73	0	90	18.9	100
ESAT6	18	72	0	90	20	100
PPD						

Table 4. Sensitivity and specificity of combined protein for serological diagnosis.

Protein name	150 TB patients		150 healthy controls		Sensitivity (%)	Specificity (%)
	Positive numbers	Negative numbers	Positive numbers	Negative numbers		
Combined protein	114	36	5	145	76	96.7
PPD	120	30	17	133	80	88.7

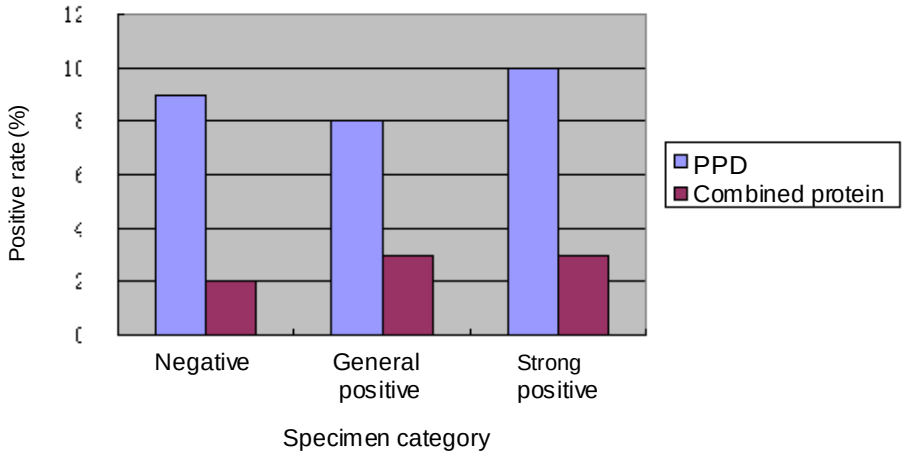

Figure 1. The specificity of the combined protein and PPD. The positive rate of PPD test was about 10% and that of combination protein about 3%.

coli among whom 23 were successfully expressed or purified, and 2 failed to cross-react with rabbit anti-tuberculosis polyclonal antibody in the Western blot assay.

In the present study, ELISA was used to evaluate the serological responses to 21 recombinant antigens of MTB, 18 of which were reported to be promising antigens. After the second round of evaluation, the TB16.3 antigen was identified as the most important antigen, with levels of recognition of 45.6% and maintenance of 100%. The remaining proteins had poor sensitivity or specificity in TB diagnosis.

The 38-kDa antigen is the most frequently studied serological antigen and a critical component in several different commercial TB serological tests (ICT Tuberculosis AMRAD-ICT, RAPID test TB and PATHOZYME-MYCO). By using the native purified antigen, the sensitivity of 68~94% and the specificity of 91% were yielded in the direct ELISA (Balestrino et al., 1984; Benjamin and Daniel, 1982; Espitia et al., 1989). By a competition ELISA with soluble MTB extract-coated plates and monoclonal antibody specific for the 38-kDa antigen in the detection of competing antibodies in human sera, 85% of smear-positive pulmonary TB patients, 74% of extra-pulmonary TB patients and 70% of smear-negative TB patients showed positive (Wilkins and Ivanyi, 1990). However, in this study, the 38-kDa antigen obtained 45.6% sensitivity and 93.3% specificity in the detection of TB, which were lower than the previously reported. Similar results were also found in some other proteins. This may be explained by the following reasons. First, the recombinant proteins were used in this study and the posttranslational modification of native protein was not considered for the recombinant antigens produced in *E. coli* (Young and Garbe, 1991). In this regard, Samanich et al. (2001) reported that, there was difference in the humoral recognition of recombinant reagents and native antigens, most likely as a consequence of the lack of posttranslational modification and structural differences in the recombinant proteins. Furthermore, it is possible that the antigens purified from a complex of MTB mixture may be contaminated by trace amounts of other MTB proteins which thereby lead to false-positive result. It is therefore likely that the different performances of the same antigen may reflect differences in the source of antigen used. Secondly, the difference in the residential area of the TB patients may also contribute to the different sensitivity and specificity of the same antigen.

Single protein in the present study cannot meet the requirement of clinical diagnosis. Combined proteins can improve the detection sensitivity, but may decrease the specificity. Thereby, the proteins (AroD, rpsO, TB16.3, TB9.7 and hspX) with 100% specificity and relatively high sensitivity were selected for the first round of evaluation. In the following experiment, the AroD, rpsO, TB16.3, TB9.7 and hspX were equally combined to detect sera from 150 TB patients and 150 healthy subjects. The sensitivity of combined protein was 76% which was close to that of PPD testing; the specificity of combined protein was 96.7%, which was higher than that of PPD testing. Thus, the combined protein has potential clinical application. The combination of TB antigens with high specificity is an alternative and effective method to improve the diagnostic sensitivity.

TB infection may initiate both humoral and cellular immunity. We previously postulated that TB could induce humoral and cellular immunity although not completely consistent, at least in parallel relationship, that is to say, the antibody positive rate is relatively high in the subjects showing strong positive in PPD skin test. In order to confirm our assumption, individuals showing negative, positive and strong positive in PPD skin test were recruited, and the serum and peripheral blood lymphocytes were collected to detect the TB antibody and γIFN following antigen stimulation by ELISA and ELISPOT, respectively. The results showed that all the positive rates among these individuals were about 10% in PPD test, and the around 3% in the detection with combination protein showing no significant difference. The γIFN level produced by peripheral blood lymphocytes following PPD challenge in these three groups is basically the same to that after PPD skin test, and the γIFN positive rate after stimulation by ESAT6 and CFP10, was also similar to that after PPD skin test results. Furthermore, the subjects negative for PPD skin test usually had negative result in the detection of γIFN of peripheral blood lymphocytes by ELISPOT. This might be attributed to that the TB antibody in humans following MTB infection or BCG vaccination, but the antibody level declined over time. In this regard, Hu et al (2008) dynamically determined the level of TB antibody in 190 TB patients with recovery. Their results showed the positive rates of TB antibody were 72.2 and 23.1% at 6 and 9 months after discharge, respectively, and the positive rate of TB antibody in these patients was not significantly different from that in healthy controls. That is to say, the TB antibody can maintain for 9 to 12 months after recovery. The serological testing can directly detect the antibodies in humans, without stimulating the memory immune cells. Therefore, the serum level of antibody in three groups was basically identical. However, the cell-mediated-immune (CMI), PPD skin test and ELISPOT require TB antigen to stimulate the memory immune cells to initiate immunity, and the pre-existing infection or BCG vaccination may cause false positive results. It is generally recognized that individuals in the clinical latency of infection are positive for CMI-based assays using ESAT-6 and CFP10 as antigens (Lalvani et al., 2001; Ravn et al., 1999).

The advantages of antibody-based assay over CMI-based technique are that a serological assay does not require the stimulation of memory immune cells, reduces the probability of false positive rate, is better than cell-

mediated-immune in the specificity, and can be developed into a simple, convenient and robust kit for use, especially in developing countries.

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