

## A multiple-antigen detection assay for tuberculosis diagnosis based on broadly reactive polyclonal antibodies

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### ABSTRACT

**Objective(s):** Detection of circulating *Mycobacterium tuberculosis* (*M. tuberculosis*) antigens is promising in Tuberculosis (TB) diagnosis. However, not a single antigen marker has been found to be widely expressed in all TB patients. This study is aimed to prepare broadly reactive polyclonal antibodies targeting multiple antigen markers (multi-target antibodies) and evaluate their efficacies in TB diagnosis.

**Materials and Methods:** A fusion gene consisting of 38kD, ESAT6, and CFP10 was constructed and overexpressed. The fusion polyprotein was used as an immunogen to elicit production of multi-target antibodies. Their reactivities were tested. Then, the multi-target antibodies and three corresponding antibodies elicited by each single antigen (mono-target antibodies) were evaluated with sandwich ELISA for detecting *M. tuberculosis* antigens. Their diagnostic efficacies for TB were also compared.

**Results:** The polyprotein successfully elicited production of multi-target antibodies targeting 38kD, ESAT6, and CFP10 as analyzed by Western blotting. When used as coating antibodies, the multi-target antibodies were more efficient in capturing the three antigens than the corresponding mono-target antibodies. By testing clinical serum, the multi-target antibodies demonstrated significantly higher sensitivity for clinical TB diagnosis than all three mono-target antibodies.

**Conclusion:** The multi-target antibodies allowed detecting multiple antigens simultaneously and significantly enhanced TB detection compared to routine mono-target antibodies. Our study may provide a promising strategy for TB diagnosis.

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### Introduction

Tuberculosis (TB) is still one of the world's most serious infectious diseases (1, 2). The rapid and accurate identification of infected individuals, which allows initiation of treatment when the patient is still accessible, is important for TB control (3, 4). However, point of care (POC) TB-test remains a challenge in the clinical laboratory (5). Presently, the vast majority of TB patients live in low- and middle-income countries where TB diagnosis still mainly depends on direct microscopy of sputum smear, whose sensitivity is far from satisfactory (40 to 60%, at best) (6, 7). As the gold standard of TB diagnosis, confirmation with organism growth in selective media is a costly and complex technique and thus not ubiquitously available. More importantly, it is very time-consuming due to slow growth of mycobacteria and requires up to several weeks before a definite diagnosis is established (8). Other methods, such as nucleic acid amplification test and tuberculin skin

test (TST), are either sophisticated in technique and infrastructure, or limited in diagnostic performance, and not suitable to be widely used for TB detection. Consequently, a simple and reliable diagnostic tool is still urgently needed (9-11).

Serologic techniques based on enzyme-linked immunosorbent assay (ELISA) have brought great progress in TB diagnosis (12-14). However, it was demonstrated that the antibody repertoire of TB is highly diverse and varies greatly in different patients (13). Detection of circulating *Mycobacterium tuberculosis* (*M. tuberculosis*) antigens is another approach in serodiagnosis of TB. Notably, antigens released during active disease is independent of host immune response and therefore, allows specific diagnosis of active TB to be made. Serologic detection of antigens was therefore considered as a specific and promising approach in serodiagnosis of TB (13, 15). However, until now, not a single antigen has been found that is widely expressed and present in all TB patients

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due to the heterogeneity of *M. tuberculosis*. Serodiagnosis with multi-target antibodies or a cocktail of mono-target antibodies targeting multiple TB antigens would cover more patients and is conceptually promising and deserves in-depth studies (13, 16).

In the past years, much effort has been put into serodiagnosis of TB and several notable TB antigens were identified. Among these antigens, ESAT-6, CFP10, and 38kD were most widely reported. ESAT-6 and CFP10 were identified from a low-molecular-mass fraction of *M. tuberculosis* culture filtrate. Interestingly, the two genes could not be demonstrated in any BCG vaccine strains, promising to discriminate TB patients from BCG-vaccinated individuals (17). 38kD is another TB immunodominant antigen; it contained species-specific epitopes which were significant in diagnosis (2). In our previous report, we have prepared a tri-fusion antigen containing 38kD, CFP10, and ESAT-6 (18, 19). When applied to detect serum antibodies for TB diagnosis, the tri-fusion antigen demonstrated significantly enhanced sensitivity compared with each of the single antigens. In this study, the polyprotein was further used to immunize animals, aiming to produce multi-target antibodies with reactivity to 38kD, CFP10, and ESAT-6. Then, the multi-target antibodies and corresponding mono-target antibodies were systematically evaluated and compared in Elisa measurement of antigens for TB diagnosis.

## Materials and Methods

### **Expression and purification of ESAT-6, CFP10, 38kD, and polyprotein consisting of the three antigens**

The sequences of 38kD, ESAT-6, and CFP10 were obtained from Genbank database. B-cell epitopes were predicted from protein sequence of antigens using the BioSun version 3.0 software (developed by the Center for Computational Biology, Beijing Institute of Basic Medical Sciences, Beijing, China) and then, the target antigens were prepared as described in our previous report (19). Each single antigen and the tri-fusion antigen were purified using ion exchange and gel filtration. The purity of the protein was analyzed by Gel-Pro Analyzer version 3.1.00.00 (Media Cybernetics, Silver Spring, MD) and the protein concentration was determined by the Bradford method (Pierce, Rockford, IL).

### **Production, purification, and characterization of antibodies**

Antibodies were raised in rabbits by immunizing intramuscularly with single antigens and tri-fusion antigen, respectively. Briefly, 1 mg antigens were mixed with 1 ml of Freund's complete adjuvant. After emulsification in an agitator, the mixture was subcutaneously injected into rabbits along spine bilaterally in 20 sites. Each animal received 1 ml injection and rabbits were immunized again after 4 weeks in the same way. 8 weeks after the first

immunization, the third immunization was performed by intramuscular injection of 1 mL antigen solution. Immune sera were collected at 2 weeks after the last immunization. Antibodies were purified from serum with Montage antibody purification prosep-G kit according to the manufacture's instruction. The purified proteins were measured with ultraviolet spectrophotometer at 280 nm and antibody titers were measured with indirect ELISA. Antibodies were characterized by SDS-PAGE electrophoresis. The reactivities of antibodies were determined by Western blotting using ESAT-6, CFP10, 38kD, and tri-fusion proteins.

### **Preparation of horseradish peroxidase-labeled antibodies**

Antibodies against 38kD, ESAT6, CFP10, and polyprotein were labeled with horseradish peroxidase (HRP) using modified sodium iodate method. Briefly, dissolving 5 mg HRP into 0.5 ml demineralized water and then 1 ml 0.06 mol/l NaIO<sub>4</sub> solutions was added. The mixture was gently agitated in darkness at room temperature for 30 min. 1 ml 0.16 mol/l ethanediol was added and gently agitated at room temperature for 1 hr to terminate the reaction. The solution was transferred into bag filter (molecular weight cutoff: 8000-14000) and dialyzed in 1000 mL buffer bicarbonate (0.01 mol/l, pH 9.5) at 4 °C for 24 hr. The buffer bicarbonate was changed three times at 6 hr intervals. 3 ml above HRP solution of hydroformylation was added into 1 ml buffer bicarbonate containing 5 mg antibodies followed by 2~3 hr gentle agitation at room temperature in darkness. Then, 5 mg NaHB<sub>4</sub> was added and the solution was kept at 4 °C overnight. The solution was transferred into bag filter for dialysis again at 4 °C for 24 hr with dialysate changed three times at 6 hr intervals. After that, solutions were centrifuged at 3000 rpm for 30 min and the supernatant was collected. Glycerine of equal volume was added, aliquoted and stored at -20 °C until usage.

### **Participants clinical isolates and specimens for validation**

Forty healthy and fifty patients with active pulmonary TB were enrolled from the center for disease control of Chaoyang district, Beijing, China. Diagnosis of TB was based on clinical symptoms, such as cough, fever, fibrocavitary lung infiltrates on chest radiograph, etc. For the suspected TB cases, sputum smear and culture test were performed for confirmation. 3 ml of fasting peripheral venous blood was drawn and collected from each participant. The samples were centrifuged at 1,200 g for 10 min at 4 °C to remove the blood cells within 4 hr and the supernatant was transferred into an RNase/DNase-free tube and stored at -80°C until use.

The study was approved by the Ethics Committee of Chaoyang District Centre for Disease Control and Prevention and Beijing institute of basic medical

sciences, Beijing, China. Written informed consent was obtained from each participant.

### Double antibody sandwich enzyme-linked immunosorbent assay (Elisa)

Antibodies against 38kD, ESAT-6, CFP10, and polyprotein were prepared into 2.5 µg/ml solution in coating buffer (0.1 M carbonate/ bicarbonate, pH 9.6). The four antibody solutions were added into microplates (100 µl/well) for coating. After being kept overnight at 4°C, the microplates were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST). Two hundred microliters PBST containing 1% bovine serum albumin was added to each well. Then, the plates were sealed and incubated at 37°C for 1 hr followed by washing three times. One hundred ml serum sample diluted at 1:10 in PBST containing 1% BSA was added to the antibody-coated well. The plates were sealed and incubated at 37 °C for 30 min followed by three times washing again. 100 µl horseradish peroxidase-conjugated corresponding antibodies (prepared as above described) was added to each well. After sealing and incubation at 37°C for 30 min, the plates were washed three times. For routine Elisa (termed ELISA bellow), freshly-prepared tetramethylbenzidine (TMB) substrate was added, and the plates were incubated at room temperature for 20-30 min. Then, 0.1 N sulfuric acid solution was added to stop the reaction and the optical density was measured at 450 nm using an automatic microplate reader (Bio-Rad, USA). For chemiluminescent Elisa, the chemiluminescent substrate was added according to the manufacturer's guidance. The signals were detected using PerkinElmer 2030 multilabel reader (PE, USA).

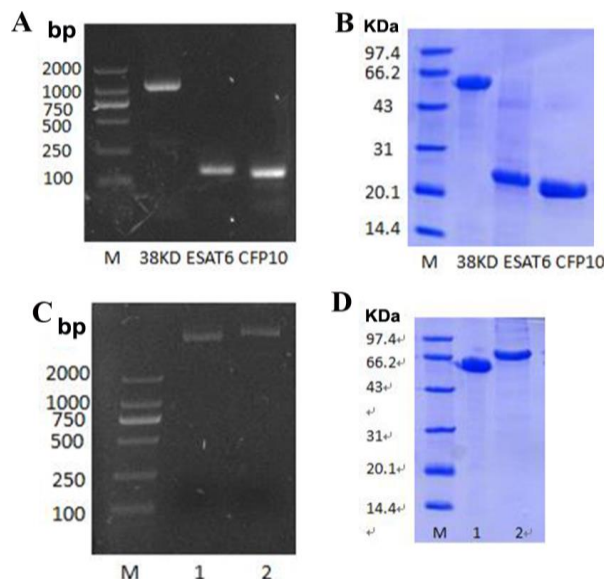
### Statistical analysis

Data was expressed as Mean±SD. Mapping of ROC and statistical analysis were performed using GraphPad Prism 4.0. Comparison of the minimum limit of detection between routine Elisa and Chemiluminescence Elisa was performed using Student's t test.  $P < 0.05$  was considered as statistically significant.

## Results

### Generation and characterization of antibodies

Based on the epitope curve, the fragments containing the dominant B-cell epitopes with higher peak value for 38kDa, ESAT-6, and CFP10 were determined. It was 21-374 aa for 38kDa antigen, 20-95 aa for ESAT-6, and 55-100 aa for CFP10. Bioinformatics analysis showed that fragments of each antigen infused polyprotein retained the spatial structure of the original epitope, suggesting good and multiple antigenicities of fused polyproteins (see our previous report) (19). The detailed information about each single antigen and polyprotein could also be found in our previous report (19). The



**Figure 1. Preparation and characterization of antigens.** A) The genes of 38kD, ESAT6, and CFP10 were amplified from *M. tuberculosis* H37Rv genomic DNA by PCR; B) 38kD, ESAT6, and CFP10 were overexpressed prokaryotic expression plasmid. The obtained proteins were characterized by SDS-PAGE electrophoresis and typically straps of corresponding sizes were verified; C) Two recombinant plasmids carrying the 38kD-ESAT6-CFP10 fusion gene were constructed and stored in our lab (pBVIL1-38kD-ESAT6-CFP10 and pGEX-38kD-ESAT6-CFP10). Lane1: plasmid pBVIL1-38kD-ESAT6-CFP10; Lane2: plasmid pGEX-38kD-ESAT6-CFP10; D) Both of the fusion genes in two plasmids were expressed and purified. SDS-PAGE electrophoresis demonstrated typical straps of polyproteins at corresponding sizes

characterization for 38kD, ESAT-6, CFP10, and polyprotein was shown in Figure 1.

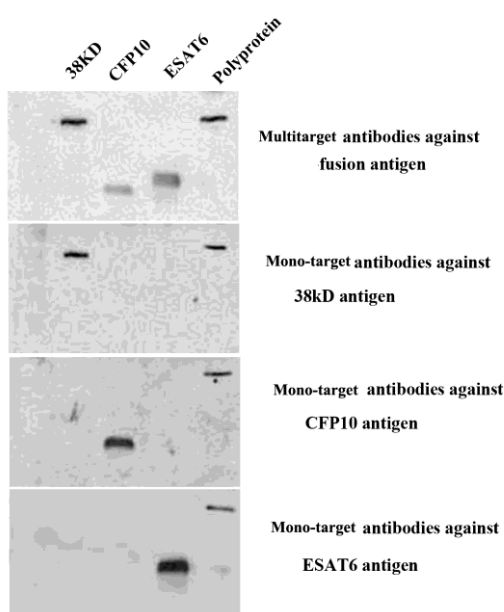
After successful immunization of rabbits, the immune sera were collected. The antibodies against 38kD, CFP10, ESAT6, and polyprotein were produced in different animals. The corresponding antibodies were collected and purified for further characterization. The antibody concentration for 38kD, ESAT6, CFP10, and polyprotein were 1.9 mg/ml, 2 mg/ml, 1.8 mg/ml, and 2.5 mg/ml, respectively. SDS-PAGE electrophoresis of obtained antibodies showed two clean straps, which is the typical characteristic of immunoglobulin: heavy chain (55 kD) and light chain (25 kD), indicating little contamination by foreign proteins in the target proteins. Western blotting showed that the reactivity of antibodies produced by polyprotein immunization was multi-target, targeting all the three antigens, 38kD, ESAT6, and CFP10 (Figure 2), while antibodies produced by single antigens were mono-target, only targeting the corresponding antigen, 38kD, ESAT6, or CFP10 (Figure 2).

### Establishment and optimization of double antibody sandwich ELISA

The above mono-target and multi-target antibodies were used as capture antibodies to coat microplates. The HRP-labeled corresponding antibodies were used to detect target antigens. The concentration of coating

**Table 1.** The detection limit of different antibodies in *Mycobacterium tuberculosis* antigen detection with ELISA

Antigen	Detection antibodies	Coating antibodies	The minimum limit of detection
38kD	HRP-38kD-Ab	38kD- Ab	5 ng/ml
		38kD-ESAT6-CFP10- Ab	1 ng/ml
ESAT6	HRP-38kD-ESAT6-CFP10- Ab	38kD- Ab	100 ng/ml
		38kD-ESAT6-CFP10- Ab	50 ng/ml
	HRP-ESAT6- Ab	ESAT6-antibody	1 ng/ml
		38kD-ESAT6-CFP10- Ab	1 ng/ml
CFP10	HRP-CFP10- Ab	ESAT6- Ab	1 ug/ml
		38kD-ESAT6-CFP10- Ab	1 ug/ml
	HRP-38kD-ESAT6-CFP10- Ab	CFP10-antibody	100 n/ml
		38kD-ESAT6-CFP10- Ab	50 ng/ml
		CFP10- Ab	5 ug/ml
		38kD-ESAT6-CFP10- Ab	1 ug/ml



**Figure 2. The reactivities of mono-target antibodies and multi-target antibodies.** Poly-target antibodies raised by the polyprotein (38kD-ESAT6-CFP10) and mono-target antibodies raised by 38kD, ESAT6, and CFP10 were tested by Western blotting for their reactivity with 38kD, ESAT6, and CFP10. It demonstrated each mono-target antibody only reacts with corresponding antigen and polyproteins, while multi-target antibodies could react with all the three antigens and polyproteins.

antibodies and was optimized as 2.5 ug/ml. During our experiment, coating antibody was combined with its corresponding HRP-labelled antibodies and HRP-labelled multi-target antibodies for antigen detection with the purpose of optimizing the couple antibodies for further application. As shown in Table 1, each antigen was detected in four systems and the detection sensitivities were compared among them.

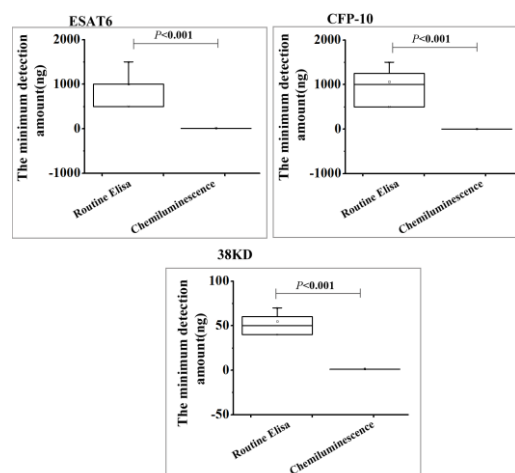
The minimum limit of detection for all three antigens (38kD, CFP10, and ESAT6) was obtained when the corresponding mono-target antibodies were used as detection antibodies and multi-target antibodies were used as coating antibodies, indicating that multi-

target antibodies were more efficient than the mono target ones to capture each antigen as coating antibodies.

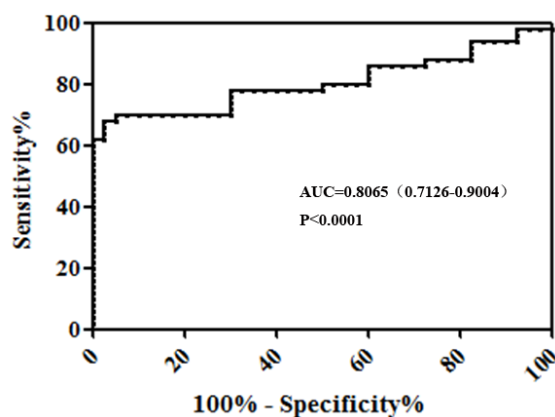
Based on the optimized system described above, we employed chemiluminescence in Elisa (chemiluminescence Elisa) aiming to further improve the detection sensitivity. As shown in Figure 3, for each target antigen, the detection sensitivity was significantly higher by chemiluminescence ELISA than that by routine ELISA. In the following experiments, chemiluminescence Elisa was thus selected as a diagnosis tool for all clinical specimens.

### Clinical serodiagnosis of TB

With multi-target antibodies as capture antibodies, each single mono-target antibody and multi-target antibody was evaluated for its performance



**Figure 3. Comparison of the minimum limit of detection for antigens between routine ELISA and chemiluminescence ELISA.** The multi-target antibodies were respectively used as coating antibodies and detection antibodies in routine ELISA and chemiluminescence ELISA to detect TB antigens ESAT6, CFP10, and 38kD. The minimum limit of detection for EAST6 was  $1000 \pm 408.2$  ng/ml by routine ELISA and  $10 \pm 7.1408.2$  ng/ml ( $P < 0.001$ ); The minimum limit of detection for CFP10 was  $1062 \pm 427$  ng/ml by routine ELISA and  $1.5 \pm 0.6$  ng/ml ( $P < 0.001$ ); The minimum limit of detection for 38kD was  $55 \pm 12.9$  ng/ml by routine ELISA and  $1.25 \pm 0.5$  ng/ml ( $P < 0.001$ )



**Figure 4. ROC analysis of multi-target antibodies for tuberculosis diagnosis with serum samples.** ROC analysis demonstrated a good diagnostic performance of multi-target antibodies (AUC=0.8065,  $P<0.0001$ ).

in TB diagnosis with serum samples. As shown in Figure 4, ROC analysis showed that area under the curve (AUC) was 0.8065(0.7126-0.9004, 95% CI,  $P<0.0001$ ). From the ROC, we calculated Youden's index for each point and determined the optimal operating point (OOP) (where Youden's index was maximum). The first two maximums of Youden's index were 0.655 and 0.65. At the cutoff value when Youden's index equaled 0.655, the sensitivity of the multi-target antibodies for TB were 68% (53.30% to 80.48%, 95%CI) with 97.5% specificity (86.84% to 99.94%, 95% CI); at the cutoff value when Youden's index equaled 0.65, the sensitivity was 70% (55.39% to 82.14%, 95% CI) with 95% specificity (83.08% to 99.39%, 95% CI). The diagnostic performance was clinically significant. However, no more than 40% sensitivity was obtained with each mono-target antibody. The results validated the superiority of multi-target antibodies in clinical practice.

## Discussion

Traditional diagnostic methods for TB, such as TST, sputum smear, and sputum culture, were of low efficacy or time-consuming. This promoted the development of novel diagnostic technology. In recent years, several newer methods have been developed for the diagnosis of TB, such as T cell-based assays and polymerase chain reaction (PCR) (5, 20). The Xpert MTB/RIF was a typical representative of test kits that were recently endorsed by the WHO. Several studies have systemically evaluated the diagnostic performance of the Xpert MTB/RIF kit by Meta-analysis and found the pooled sensitivities were ~50% to ~90% (specificity>90%) (21, 22). Thus, the Xpert MTB/RIF kit was considered the most promising development in TB diagnostics in the USA and Europe (23). However, high specialty and high cost made these techniques not suitable for extended use in resource-limited

countries where the vast majority of TB patients are distributed. Thus, a rapid and cost-effective method for TB diagnosis is still urgently needed for TB control (5). As cost-effective and easy-to-use methods, serologic tests hold great potentials in developing a rapid POC test in TB diagnosis (24, 25). The multiple-antigen detection assay we developed in this study was a serological test. The diagnostic performance for TB was ~70% sensitivity with 95% specificity as evaluated in our study, which was comparable with the Xpert MTB/RIF kit as reported to some extent. Of course, the efficacy of our method needs to be validated in a wider context. Importantly, the method in the present study was based on the ELISA assay which was rapid, inexpensive and simple compared with nucleic acid amplification. At present, though WHO has not yet recommended the use of commercial serological tests for the diagnosis of active TB, it has stressed again the importance of continued research on developing relevant point-of-care tests for TB in 2011 (26).

Compared with TB-related antibodies, *M. tuberculosis* antigens in serum of infected individuals is independent of intact host immune response and thus varies less in patients (27). In addition, the appearance of antigens after *M. tuberculosis* infection and their disappearance after cure were earlier than antibodies. These properties made the serologic test of *M. tuberculosis* antigens more suitable for TB diagnosis. Over the past decades, a number of mycobacterial antigens have been explored for their potentials in TB diagnosis (28, 29). However, none of them demonstrated uniform results in all hands and under all conditions. Therefore, it was widely accepted that single antigen-based TB diagnosis was not sufficient to acquire satisfactory sensitivity and specificity, developing serologic tests targeting multiple antigen markers should be a more rational option (2, 28). 38kD, CFP10, and ESAT6 were all important *M. tuberculosis*-related antigens that possessed potent immunogenicity. The 38kD antigen is a phosphate transfer protein which was located in plasmalemma. It was specific for mycobacteria which could evoke humor and cellular immunity. Thus, the 38kD antigen is one of the most frequently investigated serological antigens for TB identification (19). ESAT6 is an early secreted protein and was found in short-term *M. tuberculosis* culture filtrates. It was confirmed as a potent T-cell protein antigen and could be able to discriminate between infected and BCG-vaccinated humans. CFP10 has been identified in the low-molecular-mass fraction of *M. tuberculosis* culture filtrate. Its encoding gene is located in the same operon as ESAT-6 (30). Both EAST-6 and CFP10 have been identified to be amongst the earliest proteins produced by *M. tuberculosis* during culture in bacteriological media (31). Thus, measurement of these antigens was

valuable for early infection of *M. tuberculosis*. Naturally, 38kD, CFP10, and ESAT6 proteins were encoded by separate genes and thus no 38kD-CFP10-ESAT6 fusion antigen would be produced from *M. tuberculosis*. In this study, the fusion antigen containing 38kD, CFP10, and ESAT6 were prepared by gene engineering and expressed in *Escherichia coli*. We have described the detailed method for preparing the fusion antigen in our previous report (19). Then, we prepared multi-target antibodies targeting three *M. tuberculosis* antigens 38kD, ESAT6, and CFP10 by using 38kD-ESAT6-CFP10 fusion polyprotein as an immunogen. The broad reactivity of the multi-target antibodies allowed detecting multiple antigens simultaneously in a single plate well. The measurement of multiantigens with broadly reactive polyclonal antibodies was mainly intended to utilize the potential complementation of individual antigen. This is significant in clinical practice. On one hand, targeting multiple antigen markers would achieve a higher sensitivity. On the other hand, it would be more convenient and cost-effective in practice than using combinational antibodies (13). To our knowledge, this is the first report about the preparation and diagnostic usage of TB-related multi-target antibodies, which may indicate a feasible strategy to develop sensitive and cost effective methods for serodiagnosis of TB.

In fact, targeting multiple antigen markers by combinational mono-target antibodies, or termed cocktail of antibodies has already been reported in the past years (13, 16). In a study by Harinath, et al (13), a cocktail of affinity-purified antibodies (target ES-31, ES-43, and EST-6) was prepared by mixing each mono-target antibody in equal proportion and further used for TB diagnosis. Though a significantly higher sensitivity was acquired by using the cocktail of antibodies, the procedure was rather complicated from the preparation of antibodies to the diagnostic application, which would inevitably result in the higher cost. In the present study, we have also tried to use the cocktail of three corresponding mono-target antibodies for TB diagnosis and compared its efficacy with that of multi-target antibodies, because we found that the sensitivity of each mono-target antibody was higher than that of multi-target antibodies in detecting the corresponding single antigens (38kD, ESAT6, or CFP10, Table1). However, when used for the clinical sample, we found that it was difficult to determine the suitable concentration and proportion of each antibody in the cocktail, and mutual interference among mixed antibodies may also exist, leading to false results (data not shown). We supposed that although the successful application of antibody cocktail in TB diagnosis has been reported previously, the method may not be suitable for all antibodies. In addition, by testing sputum culture samples, we demonstrated that the

TB detection rate by multi-target antibodies was even higher than the total of those by three mono-target antibodies. One possible explanation was that the level of each single antigen may be undetectable in some clinical samples, but the total levels of multiple antigens were sufficient to be detected and the multi-target antibodies detected the total levels of each single antigen. This should be another advantage of using multi-target antibodies against using multiple mono-target antibodies.

It is noteworthy that though the diagnostic sensitivity of the multi-target antibodies for TB was significantly higher than mono-target antibodies, the sensitivity of the multi-target antibodies to 38kD, ESAT6, and CFP10 was lower (larger detection limit) than that of corresponding mono-target antibodies, as shown in Table 1. The main reason for the low detection sensitivity of multi-target antibodies compared with mono-target antibodies should be ascribed to their efficacies for individual protein. It was supposed multi-target antibodies produced by fusion protein reacted with multiple proteins but were of relatively low efficacy for each protein (recognize fewer epitopes of individual protein), while mono-target antibodies produced by individual protein only reacted with a single protein but were of relatively high efficacy (recognize more epitopes of individual protein). Therefore, multi-target antibodies produced by fusion protein were more suitable to be used as coating antibodies to capture target, because more epitopes could be left for detection antibodies. If mono-target antibodies were used as coating antibodies, fewer epitopes would be left for detection antibodies. In that case, both multi-target antibodies and mono-target antibodies would be of low sensitivity. However, whether multi-target or mono-target antibodies were used as coating antibodies, mono-target antibodies may demonstrate a higher sensitivity than multi-target antibodies when used as detection antibodies, because mono-target antibodies possessed a higher efficacy and should be more effective for the left epitopes of the target protein. That may be why the sensitivity of multi-target antibodies produced by fusion was low. Therefore, it could be supposed that if the reactivity of the multi-target antibodies was enhanced, a further sensitivity may be achieved. Thus, further study is needed to improve the fusion technique or enhance the immunogenicity of polyproteins by employing adjunctive technologies, such as nanotechnology (32). This is clinically significant and deserves in-depth investigation.

## Conclusion

This study demonstrated the superiority of the multi-target antibodies in the clinical diagnosis of TB to multiple mono-target antibodies that were mostly used previously, providing a promising strategy to

develop sensitive and cost effective methods for TB diagnosis.

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### Conflict of interest statement

No competing interests exist.

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