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Specific Antigens to Distinguish *M. tuberculosis* from *M. avium*

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ABSTRACT

To distinguish *Mycobacterium tuberculosis* from *Mycobacterium avium*, specific *M. tuberculosis* antigens had been studied for improving the early differential diagnosis effect of tuberculosis caused by different *Mycobacterium*. The rabbit anti-*M. avium* sera and anti-*M. tuberculosis* sera were analyzed for antibody-based reactivity by matrix-assisted laser desorption-ionization mass spectrometry (MALDI-TOF Mass) against *M. tuberculosis* proteins. The immunoreactive spots, which were attributed to the proteins HspX, GroES and CFP-10, were mostly located at 10 - 60 kDa and PI 4 - 6, subsequently Western blotting result proved that HspX and CFP-10 were specific to *M. tuberculosis* and ELISA testing result of 30 *M. avium* positive sera showed that GroES were cross-reactive to *M. avium*. Lastly, positive and negative tuberculosis reference sera and based on the mechanism of indirect ELISA, the specificity and the sensitivity of the methods targeting the antibodies HspX, GroES or CFP-10 were evaluated at 37% and 26%, 12% and 97%, 81% and 98%, respectively. The combination of these three antibody detection methods allowed to reached a specificity of 42%, and of 39% without taken into account of the method targeting the GroES antibody. Using proteomics approach, we found three *M. tuberculosis* specific antigens showed good potential in tuberculosis diagnosis, providing basic study for serodiagnosis of tuberculosis.

Keywords: *Mycobacterium tuberculosis*; *Mycobacterium avium*; Mass Spectrometry; Immunodetection

1. Introduction

Tuberculosis (TB) remains a major cause of death in developing countries. It is also rising throughout the industrialized countries, partly as the cause of human immunodeficiency virus (HIV) infection. *Mycobacterium tuberculosis* complex (MTC) members such as *M. tuberculosis* are the pathogens of TB infection and *Nontuberculous mycobacteria* (NTM) such as *Mycobacterium avium* are responsible of mycobacteria [1,2]. Unfortunately, NTM is resistant to many general anti-TB drugs, leading the patients to suffer a years prolonged course and finally become chronic or refractory cases of mycobacteria [3,4]. However, clinically it is difficult to distinguish between them, because they are similar in clinical manifestations, imaging study, smear and culture, tuberculin tests and pathological examination [5-7]. Therefore, the rapid identification of *M. tuberculosis* and *M. avium* is of extreme importance to diagnosis, effective chemotherapy and control transmission of TB.

Represented by two-dimensional gel electrophoresis (2-D) and mass spectrometry (MS) technology, proteomics has provided some encouraging results in TB research [8-10]. So far, important antigens of *M. tuberculosis* have been identified such as the 38-kDa antigen, early secreted antigenic target (ESAT-6), antigen 85B, the proteins encoded by Rv3872 [11,12]. However, the cross-reaction between *M. tuberculosis* and *M. avium* makes these antigens unable to distinguish these myco-

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bacterial species and consequently differentiate those respective infections [13]. Only a few secreted proteins such as 14-kDa protein and CFP-10 have been further characterized which was demonstrated by Western blotting in culture filtrates of *M. avium* but was not detected in *M. tuberculosis* [14]. CFP-10, molecular weight of 10 kDa, mainly existed in *M. tuberculosis* culture filtrate. Previous data have shown that it is a specific potential antigen to *M. tuberculosis* [15,16]. Renshaw *et al.* [17] reported that BCG and NTM were lack of CFP-10 coding. HspX-14 was predicted to be an important membrane antigen, which increased expression along with *M. tuberculosis* growing into stable phase from logarithmic phase [18] and in anaerobic condition [19]. Some data also showed HspX-14 could induce humoral immune response [20,21]. GroES, a molecular chaperone protein, joints participation in protein folding, assembly, transport and degradation [22]. The high abundance of GroES possibly led antigen presenting cells to secrete many small peptides which could induce the host produced a strong immunological response [23]. These reports suggested that these proteins may be the *M. tuberculosis*-specific antigenicity.

Comparison of immunoblot profiles of rabbit anti-*M. tuberculosis* sera and rabbit anti-*M. avium* sera reacting with extracts of *M. tuberculosis* could detect specific antigens allowing to distinguish easily these two mycobacteria species and helped the serodiagnostic test for tuberculosis.

2. Materials

Mycobacterium tuberculosis H37Rv, rabbit anti-*M. tuberculosis* sera and rabbit anti-*M. avium* sera were obtained from Tuberculosis Research Institute of the People's Liberation Army Hospital 309 (Beijing, China).

300 sera from healthy blood donators were collected from Beijing Red-cross blood Central which were tested negatively to TB by Elisa kits (Chengdu Yongan Pharmaceutical Co. Ltd).

100 sera from *M. tuberculosis* patients were collected in 309 Hospital (Beijing, China). Basis of diagnosis: Two sputum samples examined by smear microscopy-stained for acid-fast bacilli or AFB are positive and chest X-ray visualizes the chest shadow caused by tubercular lesion. Then used COBAS AMPLICOR™ *M. tuberculosis* Kit (Roche) to confirm.

30 sera from *M. avium* patients were collected in 309 Hospital (Beijing, China). Basis of diagnosis: Used COBAS AMPLICOR™ *M. avium* Kit (Roche) to detect *M. avium* and used COBAS AMPLICOR™ *M. tuberculosis* Kit (Roche) to confirm they were not infected by *M. tuberculosis.*

200 sera from purified protein derivative (PPD)-positive healthy body-examining people were collected in Affiliated Hospital of the Academy of Millitary Medical Sciences.

3. Methods

3.1. Protein Preparation

M. tuberculosis H37Rv was cultured in modified Sauton medium for 4 weeks and heat-inactivated at 80˚C for 1 hr. The bacteria were harvested by centrifugation and were washed three times with 10 mM Tris buffer (pH 7.4), then suspended in 500 μl lysate (0.3% SDS, 200 mM DTT, 50 mM Tris, pH 7) and sonicated for 30 min (250 W, 2 sec pulse-on, 4 sec pulse-off intervals), subsequently added the Benzonase nuclease (1:20; Novagen, Merck KgaA, Darmstadt, Germany) and kept at 4˚C for 1 hr, and then centrifuged at 22,300 g for 30 min. Used 2-D Clean-up Kit (GE Healthcare, Fairfield, USA) to purified protein.

3.2. 2-D PAGE

Samples of 200 μg protein suspended in rehydration buffer (6 M Carbamide, 0.71 M SDS, 0.375 M Tris pH 8.8, 20% glycerol) were applied on immobilized pH 4 - 7 linear gradient strips (13 cm; GE Healthcare). Focusing at 20˚C using the following four-step program: a) 50 V, 6 hr; b) 500 V, 1 hr; c) 1000 V, 1 hr; d) 8000 V constant until 66,000 Vh. The current limit was set at 50 μA/strip. After isoelectric focusing (IEF), each strip was equilibrated for 15 min in equilibration buffer I (rehydration buffer, 0.13 M DTT) followed by equilibration buffer II (rehydration buffer, 0.14 M iodoacetamide) for 15 min. The second dimension separation was performed in uniform 12.5% SDS-PAGE gels. Then silver stained the gels.

3.3. Western Blotting

Preparing for Western blotting, proteins was electrophoretically transferred by using TE70 Semi-Dry Transfer (Amersham Pharmacia Biotech, Uppsala, Sweden) to 0.2-μm-pore-size polyvinylidene difluoride membrane (Immun-Blot PDVF membrane; Bio-Rad, Hercules, CA), which was then washed and blocked in Tris-buffered saline-Tween 20 (TBS-T) containing 1% Tween 20 and 5% non-fat dry milk for 120 min, next incubated in the rabbit anti-*M. tuberculosis* and anti-*M. avium* sera (both 1:250 TBS-T diluted) for 2 hr, then reacted with horseradish peroxidase labeled goat anti-rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, USA) (1:2500 TBS-T diluted) for 1 hr, subsequently washed extensively in TBS-T and then add freshly prepared DAB solution (Beijing Saichi Shengwu Keji, Beijing, China) for color. Signal was detected by using UMAX scanner and

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analyzed with ImageMaster 2D software for variance.

3.4. MALDI-TOF MASS

The excised protein spots were destained in 50 mM ammonium bicarbonate/acetonitrile (1:1) until colorless, dehydrated with acetonitrile, reduced in 25 mM ammonium bicarbonate/acetonitrile (1:1). The gel pieces were dried white by acetonitrile and speedvac. Then incubated at 37˚C overnight with 25 mM ammonium bicarbonate diluted trypsin. The reaction was stopped by adding 1% triflouracetic acid (final concentrations 0.1%), Peptide mixtures were applied to AnchorChip (Bruker Daltonics Inc. Billerica, USA) and analyzed by MALDI-TOF (Bruker Daltonik) using a-4-hydroxycinnamic acid as matrix with positive ion detection mode.

For peptide mass fingerprinting (PMF) analysis, MASCOT service provided by the Matrixscience Company (www.matrixscience.com) was used.

3.5. Cloning, Expression, Purification and Immunization Verification of the Recombinant Protein

Obtained from *M. tuberculosis* H37Rv genomic DNA, HspX antigen gene was prepared by amplification using appropriate primers (forward: 5'-CGCAATTCATATG-GCCACCACCCTTCCCGTTC-3' and reverse: 5'-GCCCTAAGCTTTCAGTTGGTGGACCGGATCTG-3') carried Nde I and Hind Ⅲ sites (underlined sequences). GroES antigen gene used forward: 5'-GGGAATCCATATGGTGGCGAAGGTGAACATC-3' and reverse: 5'-CGGAAGCTTCTACTTGGAAACGACGGC-3' carried the same sites (underlined sequences). While CPF-10 antigen gene used forward: 5'-CGTAGCTAGC-GGTGGCGGCATGGCAGAGATGAAGACCGA-3' and reverse: 5'-CCGGAATTCATCATTAACTAGTGCCA-CCGAAGCCCATTTGCGAGGACA-3' carried Nhe I and EcoR I sites.

The gel-purified PCR products were digested by appropriate restriction enzyme, and ligated to pET-28a(+) vector (HspX and GroES PCR products) or pET-30a(+) vector (CFP-10 PCR product) (Novagen).

Escherichia coli BL21 (DE3) harboring recombinant plasmids were grown in LB medium containing 50 mol/l of Kanamycin overnight at 37˚C, then induced with isopropyl thiogalactoside (1 mmol/l) at 37˚C for 4 hr. The harvested cells were resuspended in phosphate-buffered saline (PBS) containing DNAase, and then lysed by sonication (the same model setted above, 20 min). The proteins with His-6 label were further purified by anion exchange chromatography.

Western blots of three proteins were all probed with rabbit anti-*M. tuberculosis* sera (1:1000) and rabbit anti-*M. avium* sera (1:1000) at 37˚C for 2 hr, followed reacted with Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:3500, BGI-GBI Biotech, Beijing, China) at 37˚C for 1 hr. The band density was calculated by image software.

3.6. Immunological Assessment

The reactivity of each protein was tested with 100 patients infected by *M. tuberculosis*, 30 patients infected by *M. avium* and 200 PPD-positive healthy subjects. First, 100 μl of each protein (The optimum concentration after contrast were HspX-14: 0.2 μg/ml, GroES: 5 μg/ml, CFP-10: 2.5 μg/ml) diluted in blocking buffer (0.05% Tween-20, 1% BSA, 0.01 M PBS, pH 7.4) was added to wells of streptavidin coated ELISA plates and incubated at 37˚C for 1 hr. Subsequently, 100 μl of diluted sera (1:50 in PBST, containing 0.1% BSA) from healthy subjects and patients was added and incubated at 37˚C for 1 hr (triplicates). After five times washes with PBST, 100 μl of mixture of HRP-conjugated goat anti-Human IgG (1:40,000, BGI) was added to each well and incubated at 37˚C for 1 hr. After six times washes with PBST, the optical density (OD) value was measured at 450 nm/630 nm. Concerning the sera collected by the Beijing Redcross Blood Central from 300 blood donators, the cutoff value determining the positive responses was the mean optical density plus two standard deviations.

4. Results

4.1. MS Identification of *M. tuberculosis* Antigens

The proteins separated by 2-D PAGE gel (**Figure 1**) were transferred to PVDF membrane and reacted with

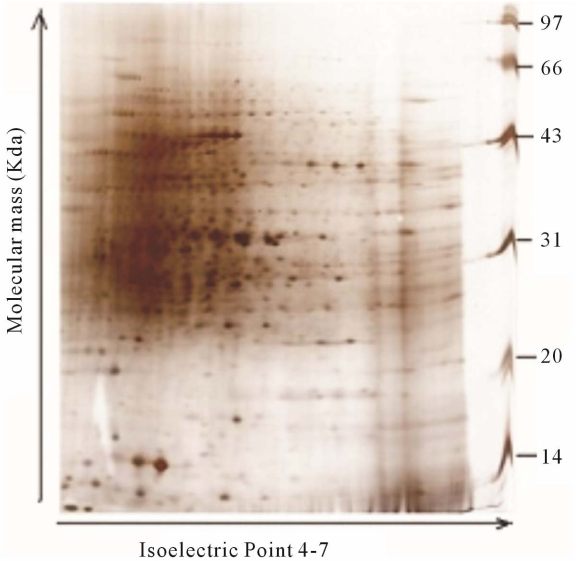

Figure 1. 2-D PAGE gel of *Mycobacterium tuberculosis* H37Rv proteins.

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anti-*M. tuberculosis* and anti-*M. avium* sera respectively. Most immunoreactive spots located at PI 4 - 6 and 10 - 60 kDa in molecular mass. Ten spots were recognized exclusively by anti-*M. tuberculosis* sera and identified as GroES, HspX and CFP-10 by PMF using MALDI-TOF MASS (**Table 1**), the protein corresponding to spots 6-10 were not detected in 2-D PAGE gel, while spots in areas (E-I) were reacted with both anti-*M. tuberculosis* and anti-*M. avium* sera (**Figure 2**, **Table 1**). Spots in areas A - D only reacted with anti-*M. avium* sera.

4.2. Expression, Purification and Immunogenicity of HspX, GroES and CFP-10

The purified HspX protein, appeared as a 17 kDa band on SDS-polyacrylamide gel (**Figure 3**). GroES and CPF-10 were approximately 14 kDa, which were similar to the theoretical molecular weight.

Using Western blotting analysis, rabbit anti-*M. tuberculosis* sera showed strong reactivity with HspX, GroES and CFP-10, whereas rabbit anti-*M. avium* sera did not react with HspX and CFP-10, and showed tenuous reactivity with GroES (**Figure 4**), checking that HspX, CFP-10 and GroES were specific to *M. tuberculosis* compared to avium species.

4.3. Immunological Assessment

The cutoff values for IgG were 0.14 to HspX, 0.12 to GroES and 0.1 to CFP-10. HspX, GroES and CFP-10 proteins were detected in 37% (37 of 100), 26% (26 of

Table 1. List of antigens that reacted only with anti-*M. tuberculosis* sera (spots 1 - 5) and antigens that reacted both with anti-*M. tuberculosis* and anti-*M. avium* sera (areas E - H).

Location	Protein Name	NCBInr Accession no.	Mr	pI	Sequence Coverage	Score[a]
Spot 1	GroES	gi|16796865	10499	4.51	59%	68
Spot 2	CFP-10	gi|15611010	10787	4.59	64%	71
Spot 3/4/5	HspX-14	gi|15609168	162176679	5.00	29%	100
Area E	protein dnaK	gi|15607491	0	4.85	39%	166
Area F	60 KDa chaperonin	gi|1449370	56692	4.85	46%	211
Area G	L Elongation factor Tu	gi|15607825	43566	5.28	87%	267
Area H	35 KDa protein	gi|57117019	29240	5.71	52%	151

[a] The scores greater than 63 are significant ($P < 0.05$).

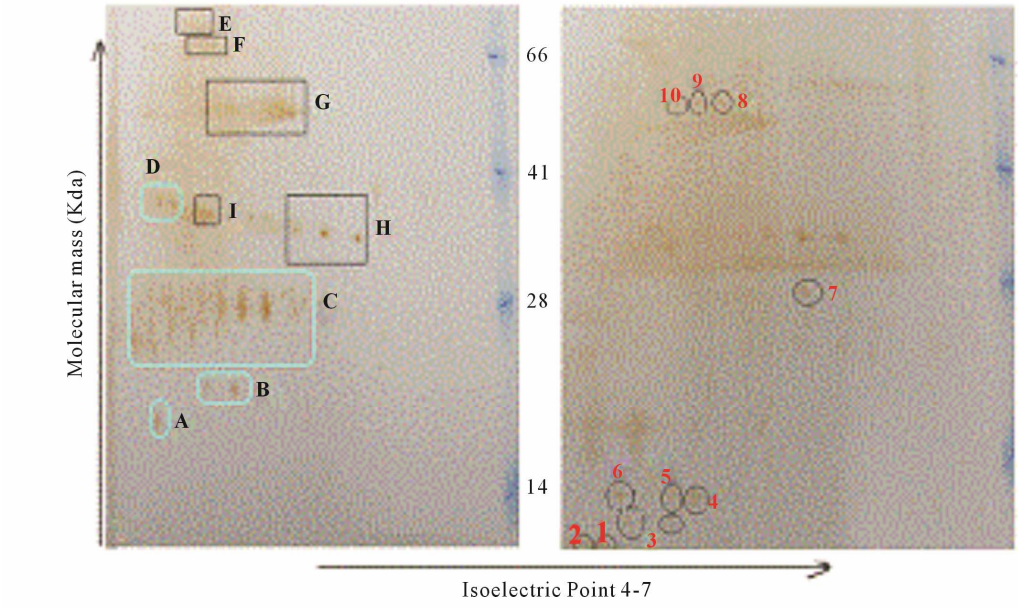

Figure 2. Screening of antigens in *M. tuberculosis* with anti-*M. avium* sera (A) and anti-*M. tuberculosis* sera (B) by 2-D PAGE. Areas E-I reacted with both sera. Spots 1 - 10 showed reactivity with anti-*M. tuberculosis* sera exclusively, while areas A-D only reacted with anti-*M. avium* sera.

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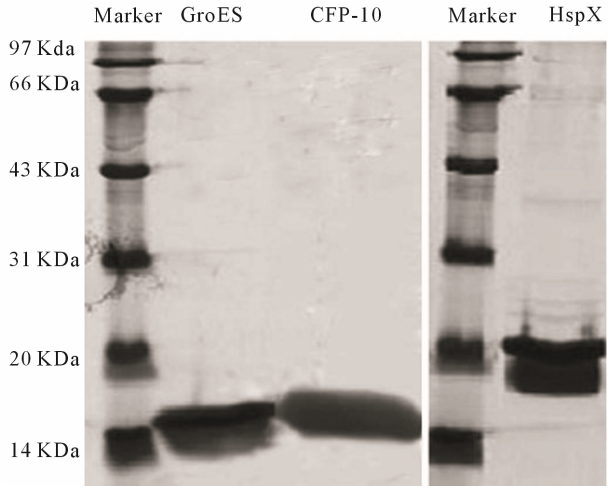

Figure 3. Expression of HspX, GroES and CFP-10 antigens (12.5% SDS-PAGE).

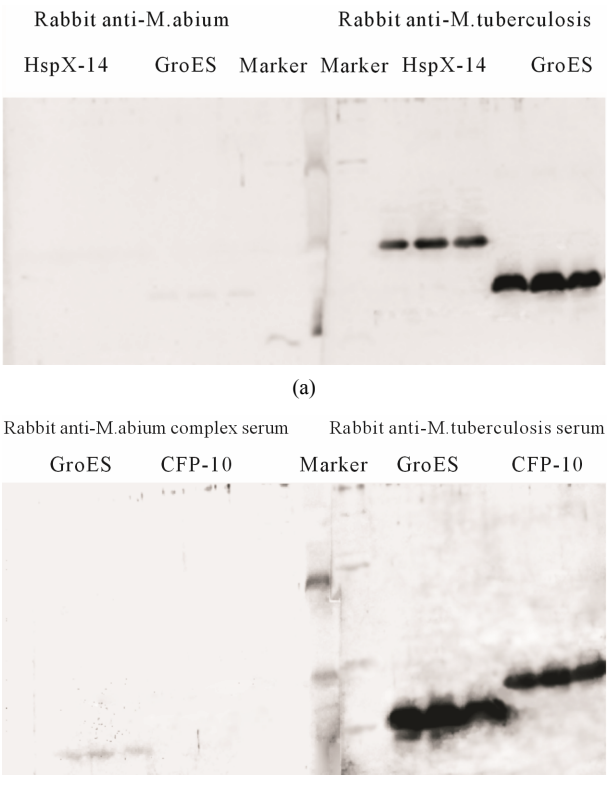

Figure 4. Validation of the antigens specific to *M. tuberculosis*. Western blotting analysis of the equal amount expressed HspX, GroES and CFP-10 proteins separated by SDS-PAGE, were performed with either anti-*M. tuberculosis* or anti-*M. avium* sera for 3 times.

100) and 12% (12 of 100) of *M. tuberculosis* patients sera respectively (**Table 2**). Moreover, two sera of the samples were tested positive with CFP-10 but negative with the other two proteins and another three sera were positive with GroES protein solely. While only GroES protein (7 of 30) was detected in sera of *M. avium* patients.

Table 2. Serological reactivity of CFP-10, GroES and HspX-14 in *M. tuberculosis* patients, *M. avium* patients and healthy PPD+ individuals.

Status	No. of persons	No. of samples positive by ELISA for:						
		C[a]	G[b]	H[c]	C+G	C+H	G+H	C+G+H
***M. tuberculosis*+**	100	12	26	37	28	39	40	42
***M. avium*+**	30	0	7	0	7	0	7	7
Healthy, PPD+	200	3	35	5	36	6	37	38

[a]C: CFP-10; [b]G: GroES; [c]H: HspX-14.

To evaluate the specificity, two hundred of sera from PPD-positive health body-examining people were tested. The specificity of HspX, GroES and CFP-10 were 97.5% (195 of 200), 82.5% (165 of 200) and 98.5% (197 of 200), respectively (**Table 2**). Similarly, 1 sera was tested positive only with CFP-10, while 2 sera only with HspX-14.

5. Discussion

At present, TB remains a serious threat to human health. A rapid diagnostic method of early stage of TB infection will undoubtedly play a decisive role in TB control [24], yet various similar pathological manifestations make them difficult to distinguish [5-7]. Recently, more and more *M. avium* infection has been reported [25-28]. However, the research about the distinction between *M. tuberculosis* and *M. avium* is rare [14].

Using a serological proteomic approach to detect specifically by identifying candidate antigen has been reported by previous research [8-10]. Due to high lipid content of *M. tuberculosis*, we chose 2-D Clean-up kit to purify the crude protein and optimized some other proteomics experimental condition of *M. tuberculosis*, including the IEF voltage settings, staining method, transfer membrane current and time settings. Three proteins (CFP-10, GroES, HspX-14) were identified as potentially specific antigen of anti-*M. tuberculosis* by 2-D PAGE, Western blotting and MALDI-TOF methods in our study. CFP10, one of the antigens selected by our method was consistent with what was reported [14], which suggested the approach could screen out specific antigens.

The reuslt of Immunoblotting assays showed that CFP-10 and HspX-14 only reacted with the anti-*M. tuberculosis* sera while the GroES protein cross-reacted with the anti-*M. avium* sera slightly (**Figure 4**).

The result of testing for specificity of these recombinant proteins showed that the specific response to protein GroES was relatively lower. *M. tuberculosis* GroES,

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having a part homology sequence with human homologous protein, often causes the emergence of spontaneous autoimmune diseases such as rheumatoid arthritis, systemic sclerosis, psoriasis, and ankylosing spondylitis [28, 29]. It may be the causes of lower specificity.

The result of evaluating the sensitivity of theses recombinant proteins showed a low sensitivity (37%, 26%, 12%). This was consitent with the previous *M. tuberculosis* antibody detection studies [16,30,31]. Nonetheless, CFP-10, GroES and HspX-14 proteins as test antigens were complementary in detecting *M. tuberculosis* specific antibodies and could effectively improve the detection of *M. tuberculosis* antibodies (42%). Ignoring the contribution of GroES, the detection ratio of CFP-10 and HspX-14 proteins as a test antigen could reach 39%.

In summary, we detected three *M. tuberculosis* specific antigens, which could be used in order to distinguish *M. tuberculosis* from *M. avium*. By system research of HspX-14, GroES and CFP-10, we found that HspX-14 and CFP-10 showed better potentiality in TB diagnosis.

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7. Disclosure

I certify that all my affiliations with or financial involvement in, within the past 5 years and foreseeable future, any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript are completely disclosed.

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