

Humoral Immune Responses against the *Mycobacterium tuberculosis* 38-Kilodalton, MTB48, and CFP-10/ESAT-6 Antigens in Tuberculosis[∇]

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Received 13 July 2009/Returned for modification 3 August 2009/Accepted 28 December 2009

The diagnosis of smear-negative and culture-negative patients with active tuberculosis (TB) is challenging. The detection of *Mycobacterium tuberculosis*-specific antibodies in human sera has been an important diagnostic aid. However, detection of antibody responses to a single antigen usually has a low sensitivity for diagnosis of TB. In this study, humoral immune responses against recombinant *M. tuberculosis* 38-kDa, MTB48, and CFP-10/ESAT-6 (culture filtrate protein 10/6-kDa early secreted antigen target of *M. tuberculosis*) antigens in 250 Chinese TB patients and 260 healthy subjects were evaluated by an enzyme-linked immunosorbent assay (ELISA). The levels of antibodies against those antigens in TB patients, even in bacterium-negative ones, were significantly higher than those in healthy subjects ($P < 0.001$). The serodiagnostic sensitivities to detect antibodies against individual antigens, i.e., recombinant *M. tuberculosis* 38-kDa, MTB48, and CFP-10/ESAT-6 antigens, in TB patients were 73.6%, 73.2%, and 60.4%, respectively, with specificities of 85.4%, 77.7%, and 73.8%, respectively. Importantly, the sensitivity to positively detect humoral responses to one of the antigens increased further. Our data suggest that the humoral immune responses to *M. tuberculosis* antigens in TB patients are heterogeneous. The 38-kDa, MTB48, and CFP-10/ESAT-6 antigens can be used as the cocktail antigens in the serodiagnosis of active TB, especially for smear- or culture-negative TB cases.

The control of tuberculosis (TB) remains challenging in China (18). Currently, the diagnosis of active TB mainly relies on clinical symptoms, radiologic findings, and the detection of *Mycobacterium tuberculosis* in clinical samples using smear staining and mycobacterial culture. However, the diagnosis of TB in smear- and culture-negative TB patients is difficult. The detection of *M. tuberculosis*-specific antibodies in human sera has been an important aid in diagnosis of TB. Notably, several antigens have been demonstrated to have merit in TB diagnosis, including the 38-kDa protein, which is commonly used in serodiagnostic tests (4, 5, 8, 13, 19, 22, 23). Previous studies suggest that the antibody responses to *M. tuberculosis* antigens are heterogeneous among individuals (17) so that the detection of antibodies against a single antigen usually has a low sensitivity for diagnosis of TB, especially for bacterium-negative cases. Therefore, it may be valuable to evaluate antibodies against the 38-kDa antigen and other major antigens for the diagnosis of active TB (14, 15).

Notably, the MTB48, CFP-10 (culture filtrate protein 10), and ESAT-6 (6-kDa early secreted antigen target of *M. tuberculosis*) genes are conserved in *M. tuberculosis* and *Mycobacterium bovis* isolates but partially deleted or absent in *M. bovis* BCG as well as in most nontuberculous mycobacteria (NTM) (1–3, 10, 16). Importantly, the proteins encoded by these genes are immunogenic (7, 9, 12, 16). In this study, we cloned the 38-kDa, MTB48, CFP-10, and ESAT-6 genes and generated

recombinant 38-kDa, MTB48, and CFP-10/ESAT-6 fusion proteins in *Escherichia coli*. Subsequently, we developed an enzyme-linked immunosorbent assay (ELISA) for the characterization of serum antibodies against 38-kDa, MTB48, and CFP-10/ESAT-6 antigens in a population of 250 active TB patients and 260 healthy subjects. We found that characterization of antibodies against multiple *M. tuberculosis* antigens were valuable for the diagnosis of active TB.

MATERIALS AND METHODS

Bacterial strains. *Escherichia coli* strain BL21(DE3) (Invitrogen, Carlsbad, CA) was cultured in Luria-Bertani (LB) medium. *Mycobacterium tuberculosis* reference strain (H37Rv) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China, and cultured on Lowenstein-Jensen slants at 37°C for 4 weeks (6).

Generation of recombinant *M. tuberculosis* antigens. The procedures for the cloning, expression, and purification of *M. tuberculosis* 38-kDa, MTB48, and CFP-10/ESAT-6 antigens were described previously (15, 21, 24). Briefly, the genes encoding the *M. tuberculosis* 38-kDa, MTB48, CFP-10, and ESAT-6 proteins were amplified by PCR using specific primers. The sequences of these primers were as follows. For the 38-kDa antigen, the forward primer was 5'-GGTATTCATATGTTGGCTCGAAACCACCGAGC-3', and the reverse primer was 5'-GCAGTGACGAATTCCTGGAAATCGTCGCGATCAAC-3'. The *NheI* and *EcoRI* restriction enzyme sites, respectively, are underlined. For MTB48, the forward primer was 5'-GCTAGCCAGTCGACAGACCGTGACG-3', and the reverse primer was 5'-CCCAGCTTCTTCGACTCCTTACTGTCT-3'. The *NheI* and *HindIII* restriction enzyme sites, respectively, are underlined. For CFP-10, the forward primer was 5'-CCGGATCCATGGCAGAGATGAAGAC-3', where the *BamHI* restriction enzyme site is underlined, and the reverse primer was 5'-GCTGCCGCCACCGCCGCTTCCGCCACCGCCGCTTCCACCGCCACCGAA GCCATTTCGAGGACAGCGCCT-3'. For ESAT-6, the forward primer was 5'-GGTGGCGGTGGAAGCGGCGGTGGCGGAAGCGGCGGTGGCGGCA GCATGACAGAGCAGCAGTGGAAATTCGCGG-3', and the reverse primer was 5'-CCAAGCTTTCGCAACATCCCAGTGA-3', where the *HindIII* restriction enzyme site is underlined. The PCR products of the *M. tuberculosis* 38-kDa and MTB48 DNA fragments were digested with *NdeI/EcoRI* or *NheI/HindIII* enzymes and cloned into pET24b (Novagen, San Diego, CA), followed by trans-

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∇ Published ahead of print on 6 January 2010.

formation into *E. coli* BL21(DE3), respectively. The CFP-10 and ESAT-6 DNA fragments were used as the templates, together with a DNA strand linker encoding glycine-glycine-glycine-glycine-serine-glycine-glycine-glycine-serine-glycine-glycine-glycine-glycine-serine, for generating the CFP-10-ESAT-6 fused gene by PCR using the following primers: forward (5'-CCGGATCCATG GCAGAGATGAAGAC-3') and reverse (5'-CCAAGCTTTGCGAACATCCC AGTGA-3'). The PCR products were digested with BamHI and HindIII, gel purified, and cloned into pET-28a (Novagen, San Diego, CA), followed by transformation into *E. coli* BL21(DE3). After DNA sequencing, individual transformants were cultured in LB medium containing kanamycin (50 µg/ml) overnight and treated with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h to induce the expression of recombinant proteins. The recombinant proteins were purified by metal chelate column chromatography using Ni-nitrilotriacetic acid (Ni-NTA) resin, according to the manufacturer's protocol (Qiagen). The concentrations of the recombinant proteins were determined by spectrometry, and their molecular weights and purities were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue, as described previously (21). Individual preparations with a purity of > 90% were further aliquoted at 1 mg/tube, lyophilized, and then stored at -80°C.

Collection of blood samples. Blood samples were collected from individual TB patients in the hospital and control subjects at the clinical laboratory of the 309th Hospital of Chinese PLA from January to April 2007, and their sera were prepared by centrifugation and then stored at -80°C. The clinical data were surveyed retrospectively. A total of 250 serum samples were selected from the patients with pulmonary TB, including 119 sera from smear- or culture-positive patients and 131 from smear- or culture-negative patients confirmed retrospectively. The acid-fast staining of bacillus smears and mycobacterial culture of sputa were performed in our laboratory according to the *Chinese Laboratory Science Procedure of Diagnostic Bacteriology in Tuberculosis* (6). An additional 260 serum samples from healthy volunteers were selected and used as controls. All patients and healthy controls in this study were negative for HIV antibodies. This study was performed in accordance with the guidelines of the Research Ethics Committee of the 309th Hospital of Chinese PLA.

PPD skin tests. The purified protein derivative (PPD) from *M. tuberculosis* (50 IU/ml) was purchased from Beijing Gaoke Life and Technology, China. Healthy individuals or subjects were injected intradermally with 0.1 ml of 5 IU PPD in the left forearm (Mantoux technique). The diameters of both axes of skin induration were measured and recorded by a certified doctor 72 h postinjection. A positive response to PPD was defined as an induration greater than 5 mm in diameter.

ELISA. The humoral responses of individual subjects to *M. tuberculosis* antigens were determined by an ELISA as described previously (10). In brief, individual recombinant proteins (10 µg/well) were used to coat the wells in 96-well microtiter plates (Nunclon; Nunc, Roskilde, Denmark) overnight at 4°C. After the wells were washed, they were blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h at room temperature. Subsequently, individual serum samples (1:100) were added in triplicate to the wells and incubated for 1 h at 37°C. After the wells were washed, the bound antibodies were detected by horseradish peroxidase (HRP)-conjugated goat-anti-human IgG (1:10,000) for 1 h at 37°C and developed with *o*-phenylenediamine and hydrogen peroxide, followed by the addition of 2 mol/liter sulfuric acid and measurement at an optical density of 492 nm (OD₄₉₂) in an ELISA plate reader.

Data management and statistical analysis. All data were entered into a Microsoft Office Excel file. The mean and standard deviation of the optical density of individual groups for antibody responses to each of the *M. tuberculosis* antigens were calculated. The differences between the values for the groups were analyzed by Student *t* test. Furthermore, the receiver operating characteristic (ROC) curves of the optical density values for antibody responses to each *M. tuberculosis* antigen were plotted using SPSS 9.06 software and the area under the curves and 95% confidence intervals (95% CIs) for responses to each *M. tuberculosis* antigen were calculated. In addition, the optimal cutoff values were chosen when Youden's index (sensitivity + specificity - 1) was maximum. Subsequently, individuals were scored as positive for the specific antibody response when his/her optical density value was greater than or equal to the cutoff value, and the positive rates and 95% CIs of individual groups for antibody responses to each *M. tuberculosis* antigen were calculated and differences for the values of the groups were analyzed by χ^2 test.

RESULTS

***M. tuberculosis* antigens.** After the DNA fragments were cloned by PCR, the individual positive colonies, which expressed each recombinant protein with six consecutive histi-

TABLE 1. Serum antibody responses to recombinant *M. tuberculosis* 38-kDa, rMTB48, and rCFP-10/ESAT-6 antigens

Group and status	No. of subjects	Serum antibody response to antigen ^a (OD ₄₉₂ [mean ±SD])		
		r38kD ^b	rMTB48	rCFP-10/ESAT-6
Healthy controls				
PPD ⁻	129	0.064 ± 0.04	0.052 ± 0.03	0.040 ± 0.03
PPD ⁺	131	0.082 ± 0.07 ^c	0.077 ± 0.06 ^c	0.049 ± 0.05
Total	260	0.073 ± 0.06	0.064 ± 0.05	0.044 ± 0.04
TB patients				
Bacterium ⁻	131	0.177 ± 0.13 ^d	0.150 ± 0.16 ^d	0.119 ± 0.16 ^d
Bacterium ⁺	119	0.254 ± 0.26 ^e	0.241 ± 0.29 ^e	0.226 ± 0.39 ^e
Total	250	0.213 ± 0.20 ^f	0.193 ± 0.23 ^f	0.170 ± 0.30 ^f

^a Serum antibody responses to recombinant *M. tuberculosis* 38-kDa, rMTB48, and rCFP-10/ESAT-6 antigens were measured by determining the optical density at 492 nm (OD₄₉₂).

^b r38kD, recombinant 38-kDa antigen.

^c *P* < 0.05 versus the PPD⁻ healthy subjects.

^d *P* < 0.001 versus healthy controls.

^e *P* < 0.05 versus bacterium-negative TB patients.

^f *P* < 0.001 versus healthy controls.

dine residues at the carboxyl terminus, were confirmed by DNA sequencing. Expression of the recombinant 38-kDa, recombinant MTB48 (rMTB48), and recombinant CFP-10/ESAT-6 (rCFP-10/ESAT-6) antigens were induced in *E. coli* BL21(DE3), and the recombinant proteins were purified using metal-chelate affinity chromatography. Individual preparations of recombinant protein antigens with a purity of >90% were used for subsequent experiments. The successful generation of recombinant 38-kDa, rMTB48, and rCFP-10/ESAT-6 proteins provided a basis for the characterization of antibody responses in different groups of subjects by an ELISA.

Serum antibody reactivities to *M. tuberculosis* antigens. The levels of antibodies against the recombinant 38-kDa, rMTB48, and rCFP-10/ESAT-6 antigens in individual sera were measured by an ELISA. The levels of antibodies against the recombinant 38-kDa, rMTB48, and rCFP-10/ESAT-6 antigens in TB patients were significantly higher than those in healthy controls (*P* < 0.001) (Table 1). Furthermore, antibody responses to those antigens in the bacterium-positive TB patients were stronger than those in the bacterium-negative TB patients (*P* < 0.05). Importantly, the levels of antibodies against these antigens in bacterium-negative TB patients were significantly higher than those in healthy controls (*P* < 0.001). In addition, the levels of antibodies against the recombinant 38-kDa antigen and rMTB48, but not rCFP-10/ESAT-6, in the PPD-positive subjects were significantly higher than those in the PPD-negative ones (*P* < 0.05). Apparently, characterization of antibodies against those *M. tuberculosis* antigens can effectively distinguish between TB patients and healthy controls.

Furthermore, individual values of antibodies against recombinant 38-kDa, rMTB48, and rCFP-10/ESAT-6 antigens were used for the generation of ROC curves (data not shown). The areas under the curves and 95% CIs were calculated in Table 2. Accordingly, the optimal cutoff values of the optical densities of antibody responses to the 38-kDa, MTB48, and CFP-10/ESAT-6 antigens for diagnosing TB patients were 0.0852, 0.0718, and 0.0690, respectively. As a result, the sensitivities of detecting antibody responses to the 38-kDa, MTB48, and CFP-

TABLE 2. Areas under the curve of antibody responses to *M. tuberculosis* antigens^a

Antigen(s)	AUC ^b	SEM	95% CI
r38kD ^c	0.788	0.021	0.747–0.829
rMTB48	0.773	0.021	0.732–0.814
rCFP-10/ESAT-6	0.762	0.022	0.720–0.805

^a The *P* values for TB patients versus healthy controls were <0.001.

^b AUC, area under the curve.

^c r38kD, recombinant 38-kDa antigen.

10/ESAT-6 antigens were 61.2%, 74.4%, and 73.2%, respectively, and the specificities were 85.4%, 77.7%, and 73.8%, respectively. The overall positive rates of the detection of antibodies against recombinant 38-kDa, rMTB48, and rCFP-10/ESAT-6 antigens by an ELISA were calculated in Table 3. Clearly, the positive rates in active TB patients were significantly higher than those in healthy controls (*P* < 0.001), and the values in the PPD-positive subjects were significantly higher than those in the PPD-negative subjects (*P* < 0.05). Importantly, the positive rates for antibody responses to two or more antigens were significantly higher than those to single antigen (*P* < 0.05), respectively. Therefore, characterization of serum antibody responses to multiple antigens may be valuable for the diagnosis of *M. tuberculosis* infection and TB in patients.

DISCUSSION

Although the role of humoral immunity in host defense against *M. tuberculosis* is poorly understood, the *M. tuberculosis*-specific antibodies were present in TB patients, suggesting that serological characterization of *M. tuberculosis*-specific antibodies may be valuable in the diagnosis of TB in humans. In this study, we successfully generated the recombinant 38-kDa, rMTB48, and rCFP-10/ESAT-6 proteins and evaluated their diagnostic potential by ELISA in detecting antibodies against *M. tuberculosis* antigens in sera from confirmed TB cases and healthy controls. We found higher levels of antibodies against individual recombinant 38-kDa, rMTB48, and rCFP-10/ESAT-6 antigens in patients with active TB than in healthy controls, consistent with a previous report (17). The levels of

antibodies against these antigens in bacterium-positive TB patients were significantly higher than those in bacterium-negative patients. Importantly, the levels of antibodies against these antigens in bacterium-negative TB patients were significantly higher than those in healthy controls, indicating their values in the diagnosis of smear/culture-negative patients (11, 19, 20, 22). Therefore, characterization of serum antibodies against recombinant 38-kDa, rMTB48, and rCFP-10/ESAT-6 antigens are effective in the diagnosis of TB in *M. tuberculosis* smear- and culture-negative and -positive patients in the clinic.

Further analysis revealed that the sensitivities of the ELISA for detecting humoral immune responses to the recombinant 38-kDa, rMTB48, and rCFP-10/ESAT-6 antigens were 61.2%, 74.4%, and 73.2%, respectively, with specificities of 85.4%, 77.7% and 73.8%, respectively. Importantly, the positive rates of the ELISA in active TB patients were significantly higher than those in healthy controls (*P* < 0.001), and the positive rates for antibody responses to two or more antigens were significantly higher than those to single antigen (*P* < 0.05). Therefore, characterization of antibody responses to two or more *M. tuberculosis* antigens can sensitively differentiate patients with active TB from healthy controls. Notably, the sensitivities for detection of antibodies against these antigens in our assays were higher than those reported previously (5, 9, 10, 12, 13, 16). The high sensitivity may stem from different genetic backgrounds of the individuals studied or different antigens used in the assays. We are interested in further directly comparing the commercial antigens if available and the antigens we generated in characterizing humoral immune responses.

We found that the levels of antibodies against recombinant 38-kDa or rMTB48 antigen and the positive rate in PPD-positive healthy controls were significantly higher than those in PPD-negative ones. These results indicated that some subjects might be at a latent stage of *M. tuberculosis* infection or recovering from previously unrecognized TB. Alternatively, the humoral immune responses to those antigens in the PPD-positive subjects may result from previous BCG vaccination, as infection with mycobacteria can induce long-term memory responses in humans.

A serological test with appropriate sensitivity and specificity can have significant advantages over currently available tests.

TABLE 3. Positive rates of antibody responses to *M. tuberculosis* antigens

Group and status	No. of subjects	Positive rate of antibody response to <i>M. tuberculosis</i> antigen (95% CI)					
		Single antigen			Combinations of antigens		
		r38kD ^a	rMTB48	rCFP-10/ESAT-6	r38kD and rMTB48	r38kD and rCFP-10/ESAT-6	r38kD, rMTB48, and rCFP-10/ESAT-6
Healthy controls							
PPD ⁻	129	16.3 (10.4–23.8)	16.3 (10.4–23.8)	7.8 (3.8–13.8)	28.7 (21.1–37.3)	22.5 (15.6–30.7)	34.1 (26.0–43.0)
PPD ⁺	131	26.7 ^b (19.4–35.2)	36.6 ^b (27.7–44.7)	20.6 ^b (14.0–28.6)	44.3 ^b (35.6–53.2)	34.4 ^b (26.3–43.2)	49.6 ^b (40.8–58.5)
Total	260	21.5 (16.6–27.0)	26.2 (20.9–31.9)	14.2 (10.2–19.1)	36.5 (30.7–42.7)	28.5 (23.1–34.4)	41.9 (35.9–48.2)
TB patients							
Bacterium ⁻	131	73.3 (64.9–80.6)	72.5 (64.0–80.0)	55.0 (46.0–63.4)	83.2 (75.7–89.2)	77.1 (69.0–84.0)	84.7 (77.4–90.4)
Bacterium ⁺	119	73.9 (65.1–81.6)	73.9 (65.1–81.6)	66.4 (57.2–74.8)	84.9 (77.2–90.8)	86.6 (79.1–92.1)	90.8 (84.1–95.3)
Total	250	73.6 ^c (67.7–79.0)	73.2 ^c (67.3–78.6)	60.4 ^c (54.0–66.5)	84.0 ^c (78.9–88.3)	81.6 ^c (76.2–86.2)	87.6 ^c (82.9–91.42)

^a r38kD, recombinant 38-kDa antigen.

^b *P* < 0.05 versus PPD⁻ controls determined by χ^2 test.

^c *P* < 0.001 versus healthy controls determined by χ^2 test.

Unlike the tuberculin skin test (TST) and Quantiferon test, characterization of antibody responses to *M. tuberculosis* antigens is less invasive and dangerous (adverse effects) and saves time. Furthermore, it does not require the patient to return to the clinic for evaluation. In addition, the serological assay can be developed into a user-friendly and field-ready format. Notably, T-cell and macrophage-related inflammation is critical for the pathogenesis and control of TB in humans. Accordingly, the results from TST and the Quantiferon test can reflect T-cell immunity and the status of inflammation in *M. tuberculosis*-infected individuals. The Quantiferon test measures systemic T-cell immunity to the purified protein derivative of *M. tuberculosis*, which can help in diagnosis of latent TB infection and evaluation of TB progression. Our findings indicated that simultaneous characterization of antibody responses to multiple *M. tuberculosis* antigens effectively distinguished patients with active TB from healthy controls. However, besides the value of TB diagnosis, the implication of positive antibody responses to *M. tuberculosis* antigens in the pathogenesis and progression of TB in patients with active TB is unclear. Given that antibody responses to *M. tuberculosis* antigens usually depend on T cells, the results from our experimental system may partially reflect systemic immune responses to *M. tuberculosis*. Therefore, characterization of antibody responses to multiple *M. tuberculosis* antigens should aid in the diagnosis of active TB in clinic. We are interested in further simultaneous evaluation of the serological assay, TST, and the Quantiferon test in a larger population.

In summary, our data suggest that characterization of antibodies against 38-kDa, MTB48, and CFP-10/ESAT-6 antigens can increase the sensitivity and specificity for the diagnosis of active TB. Therefore, these *M. tuberculosis* antigens can be used as cocktail antigens in the serodiagnosis of active TB, especially for smear- or culture-negative TB cases. We are interested in further screening and evaluating the serological responses to other antigens to identify the optimal combinations of antigens and/or polyproteins for the sensitive and specific diagnosis of active TB.

ACKNOWLEDGMENTS

This study was supported by the Serious Infectious Diseases Foundation of China (2008ZX-10003-001) and the Beijing Science and Technology Foundation of China (D08050700640802).

REFERENCES

- Behr, M. A., M. A. Wilson, W. P. Gill, H. Salamon, et al. 1999. Comparative genomics of BCG vaccines by whole genome DNA microarray. *Science* **284**:1520–1523.
- Behr, M. A. 2001. Comparative genomics of BCG vaccines. *Tuberculosis* **81**:165–168.
- Berthet, F. X., P. B. Rasmussen, I. Rosenkrands, et al. 1998. A *Mycobacterium tuberculosis* operon encoding ESAT-6 and a novel low-molecular-mass culture filtrate protein (CFP-10). *Microbiology* **144**:3195–3203.
- Chan, E. D., L. Heifets, and M. D. Iseman. 2000. Immunologic diagnosis of tuberculosis: a review. *Tuber. Lung Dis.* **80**:131–140.
- Chan, S. L., Z. Reggiardo, T. M. Daniel, et al. 1990. Serodiagnosis of tuberculosis using an ELISA with antigen 5 and a hemagglutination assay with glycolipid antigens. Results in patients with newly diagnosed pulmonary tuberculosis ranging in extent of disease from minimal to extensive. *Am. Rev. Respir. Dis.* **142**:385–389.
- Chinese Antituberculosis Association. 1995. Chinese laboratory science procedure of diagnostic bacteriology in tuberculosis, p. 9–21. Chinese Antituberculosis Association, Beijing, China.
- Cooper, A. M., and J. L. Flynn. 1995. The protective immune response to *M. tuberculosis*. *Cure Opin. Immunol.* **7**:512–516.
- Demkow, U., T. M. Zielonka, J. Strzalkowski, D. Michalowska-Mitczuk, et al. 1998. Diagnostic value of IgG antibody levels against 38 kDa mycobacterial antigen. *Pneumonol. Alergol. Pol.* **66**:509–516.
- Dillon, D. C., M. R. Alderson, C. H. Day, et al. 2000. Molecular and immunological characterization of *Mycobacterium tuberculosis* CFP-10, an immunodiagnostic antigen missing in *Mycobacterium bovis* BCG. *J. Clin. Microbiol.* **38**:3285–3290.
- Harboe, M., T. Oettinger, H. G. Wiker, I. Rosenkrands, and P. Andersen. 1996. Evidence for occurrence of the CFP-10 protein in *Mycobacterium tuberculosis* and virulent *Mycobacterium bovis* and for its absence in *Mycobacterium bovis* BCG. *Infect. Immunol.* **64**:16–22.
- Harboe, M., and H. G. Wiker. 1992. The 38-kDa protein of *Mycobacterium tuberculosis*: a review. *J. Infect. Dis.* **166**:874–884.
- Houghton, R. L., M. J. Lodes, D. C. Dillon, et al. 2002. Use of multi-epitope polyproteins in serodiagnosis of active tuberculosis. *Clin. Diagn. Lab. Immunol.* **9**:883–891.
- Jackett, P. S., G. H. Bothamley, H. V. Batra, et al. 1988. Specificity of antibodies to immunodominant mycobacterial antigens in pulmonary tuberculosis. *J. Clin. Microbiol.* **26**:2313–2318.
- Julián, E., L. Matas, J. Alcaide, and M. Luquin. 2004. Comparison of antibody responses to a potential combination of specific glycolipids and proteins for test sensitivity improvement in tuberculosis serodiagnosis. *Clin. Diagn. Lab. Immunol.* **11**:70–76.
- Li, B. Y., D. W. Li, Z. Wang, et al. 2007. Research on the high expression of Rv3881c in *E. coli* and the antigenicity. *Practical Med. J.* **23**:1605–1607. (In Chinese.)
- Lodes, M. J., D. C. Dillon, R. Mohamath, et al. 2001. Serological expression cloning and immunological evaluation of MTB48, a novel *Mycobacterium tuberculosis* antigen. *J. Clin. Microbiol.* **39**:2485–2493.
- Lyashchenko, K., R. Colangeli, M. Houde, et al. 1998. Heterogeneous antibody responses in tuberculosis. *Infect. Immunol.* **66**:3936–3940.
- National Technical Steering Group of the Epidemiological Sampling Survey for Tuberculosis. 2002. Report on the fourth national epidemiological sampling survey of tuberculosis. *Chin. J. Tuberc. Respir. Dis.* **25**:3–7. (In Chinese.)
- Pottumarthy, S., V. C. Wells, and A. J. Morris. 2000. A comparison of seven tests for serological diagnosis of tuberculosis. *J. Clin. Microbiol.* **38**:2227–2231.
- Samanich, K. M., M. A. Keen, V. D. Vissa, J. D. Harder, et al. 2000. Serodiagnostic potential of culture filtrate antigens of *Mycobacterium tuberculosis*. *Clin. Diagn. Lab. Immunol.* **7**:662–668.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Senol, G., O. F. Erer, Y. A. Yalcin, M. Coskun, et al. 2007. Humoral immune response against 38-kDa and 16-kDa mycobacterial antigens in tuberculosis. *Eur. Respir. J.* **29**:143–148.
- Wilkinson, R. J., K. Haslov, R. Rappuoli, F. Giovannoni, et al. 1997. Evaluation of the recombinant 38-kilodalton antigen of *Mycobacterium tuberculosis* as a potential immunodiagnostic reagent. *J. Clin. Microbiol.* **35**:553–557.
- Wu, X., Q. Li, Y. Yang, et al. 2009. Latent tuberculosis infection among new recruits to the Chinese army: comparison of ELISPOT assay and tuberculin skin test. *Clin. Chim. Acta* **405**:110–113.