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Assessment of Five Antigens from *Mycobacterium tuberculosis* for Serodiagnosis of Tuberculosis▽

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Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is a major public health issue, particularly in developing countries, and thus effective diagnostic methods for TB remain a central theme in basic and clinical research. To evaluate five antigens (38-kDa protein [38kDa], Rv3621c, Rv3618, 38kDa-ESAT-6 [38E6], and Ag85B-HBHA [AH]) in serological tests for TB patients, we recruited 288 patients and 201 healthy controls. The median IgG reactivity to 38kDa, 38E6, and AH was higher than that to Rv3618 and Rv3621c in pulmonary TB. 38kDa and 38E6 provided high sensitivities in pulmonary TB but low sensitivities in extrapulmonary TB (EPTB). The specificities achieved by 38kDa and 38E6 ranged from 82.0% to 93.9% in patients with non-TB respiratory disease (PD) and in controls. 38kDa and 38E6 exhibited lower sensitivities and higher specificities than their combinations with Rv3618. These findings provide useful information on the relative importance of the above five antigens and suggest that combinations of Rv3618 with 38kDa and 38E6 can increase their sensitivities, but their specificities need to be further increased.

Tuberculosis (TB) is a chronic respiratory disease caused by *Mycobacterium tuberculosis* and also a serious public health issue (11, 23); on the other hand, there exists only a 10% lifetime chance of a person with latent TB infection (LTBI) developing an active TB disease (17). Thus, it appears to be important to distinguish active TB from LTBI. There are diverse diagnostic methods for TB, like chest radiography, sputum smear microscopy, mycobacterial culture, nucleic acid amplification, serological detection, phage-based TB detection, Mantoux test (tuberculin skin test [TST]), and cytokine detection (27). However, these methods have their own limitations and cannot meet target specification.

Mycobacterial culture is usually considered the diagnostic gold standard for TB, but its sensitivity is low in TB patients with low mycobacterial burdens. It is also difficult to recruit samples from patients with extrapulmonary TB (EPTB) for mycobacterial culture. Cytokine detection with RD1 antigens and TST cannot distinguish active TB from LTBI (7, 27). The humoral responses correlate with the progression of the infection to active TB disease (6). Serodiagnosis is characterized by convenient sampling, low costs, easy operation, and rapid determination. Several antigens from *M. tuberculosis* have been developed into commercial kits, like MycoDot kit (which uses lipoarabinomannan [LAM]), InBios Active Tb*Detect* IgG enzyme-linked immunosorbent assay (ELISA), IBL *M. tuberculosis* IgG ELISA, Anda Biologicals TB ELISA, and Linonex TB kits (2, 16). However, commercially available serological tests for pulmonary TB and EPTB have variable accuracies and a limited clinical role (20, 21, 22). Consequently, it is required to screen effective antigens for serodiagnosis of TB.

Previous studies have usually focused on those antigens that can distinguish active TB from LTBI and improve the sensitivity and specificity of the TB diagnosis (8, 10). In this study, two fusion proteins and three proteins were chosen as potential candidates for serodiagnosis based on the following reasons. The 38-kDa protein (38 kDa; Rv0934), developed into a commercial kit for TB identification (2), is the most widely studied antigen. The sensitivity of anti-38kDa IgG antibody is 61.0% in patients with culture-positive pulmonary TB, with a specificity of 100.0% (25). Rv3621 belongs to the *M. tuberculosis* PPE family and is an Ala- and Gly-rich protein (http://www.sanger.ac.uk). Rv3618 is a monooxygenase of *M. tuberculosis*. The gold nanoparticle probes for Rv3618 can detect *M. tuberculosis* complex and *M. tuberculosis*, with 94.7% sensitivity and 99.6% specificity for *M. tuberculosis* detection (19). ESAT-6 (Rv3875) and Ag85B (Rv1886c), secreted antigens of *M. tuberculosis*, have been evaluated as vaccine candidates (4). IgG positivity in ELISA for ESAT-6 antigen is 75.4% in pleural tuberculosis patients (24). The sensitivities of Ag85B and ESAT-6 antigens are 34.0% and 64.9%, and their specificities are 74.1% and 88.9%, respectively (9). Heparin-binding hemagglutinin (HBHA; Rv0475) of *M. tuberculosis* can agglutinate red blood cells and aggregate mycobacteria, and TB patients develop strong humoral responses to methylate HBHA (30). The purpose of this study was to evaluate the serodiagnostic potential of three single antigens (38kDa, Rv3621c, and Rv3618) and two fusion antigens (38kDa-ESAT-6 [38E6] and Ag85B-HBHA [AH]).

MATERIALS AND METHODS

Antigen preparation. Recombinant *Escherichia coli* BL21(DE3) expressing 38kDa, Rv3621c, Rv3618, 38E6, and AH antigens was prepared. 38kDa and 38E6 were purified with ion-exchange and hydrophobic chromatography,

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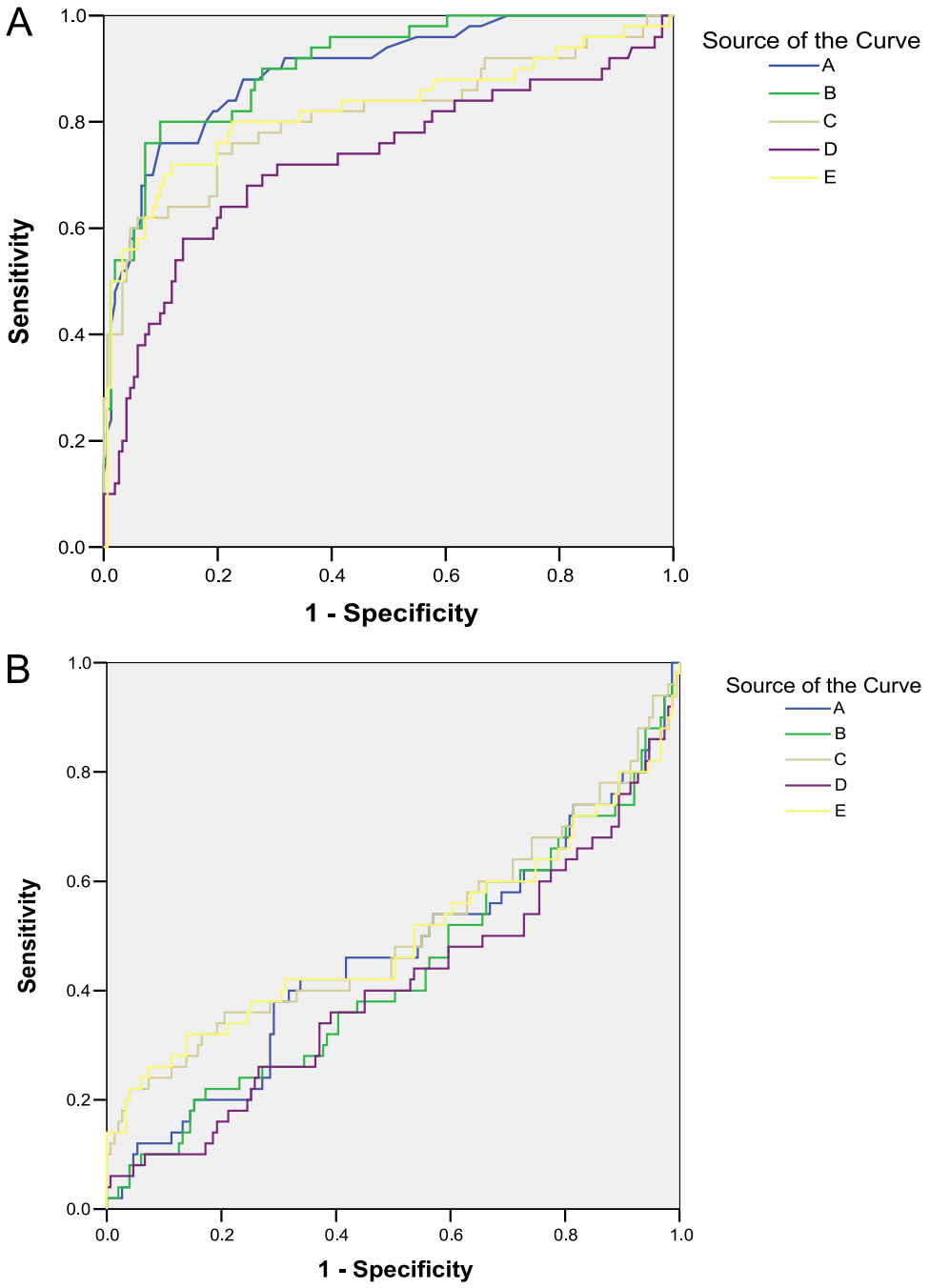

FIG. 1. ROC curves of 38kDa, 38E6, AH, Rv3621c, and Rv3618 performance for serodiagnosis of PTB(+) patients and TST$^-$ controls. A, B, C, D, and E under "source of the curve" in the panels stand for 38kDa antigen, 38E6 fusion protein, AH fusion protein, Rv3621c antigen, and Rv3618 antigen, respectively. (A) ROC curves for IgG detection. The AUCs of 38kDa, 38E6, AH, Rv3621c, and Rv3618 antigens were 0.91, 0.92, 0.81, 0.73, and 0.84, respectively. (B) ROC curves for IgM detection. The AUCs of 38kDa, 38E6, AH, Rv3621c, and Rv3618 antigens were 0.46, 0.43, 0.50, 0.41, and 0.49, respectively.

Rv3621c and Rv3618 were purified with Ni affinity and ion-exchange chromatography, and AH was purified with heparin affinity and ion-exchange chromatography. The purities of these five recombinant proteins ranged from 85.2% to 92.5%.

Subjects. Two hundred thirty-nine TB patients, 49 patients with nontuberculous pulmonary disease (PD), and 201 healthy subjects were recruited from the 309th Hospital of Chinese People's Liberation Army from June 2009 to July 2010. Specifically, all the participants were HIV negative by routine AIDS tests that were required to be conducted at government-sponsored hospitals. Pulmonary TB patients were identified according to the guidelines for pulmonary TB diagnosis and therapy authored by the Tuberculosis Branch Association of the Chinese Medical Association, including clinical presentation, chest X-ray examination, Ziehl-Neelsen-stained sputum smear, and mycobacterial culture. Extrapulmonary tuberculosis (EPTB) was identified according to clinical presentation, smear or culture positive in samples from corresponding organs. Nontuberculosis pulmonary disease was diagnosed via clinical presentation, chest X ray, and pathological examination. Here, we classified all the participants into six groups: (i) 50 patients with sputum culture- or smear-positive pulmonary

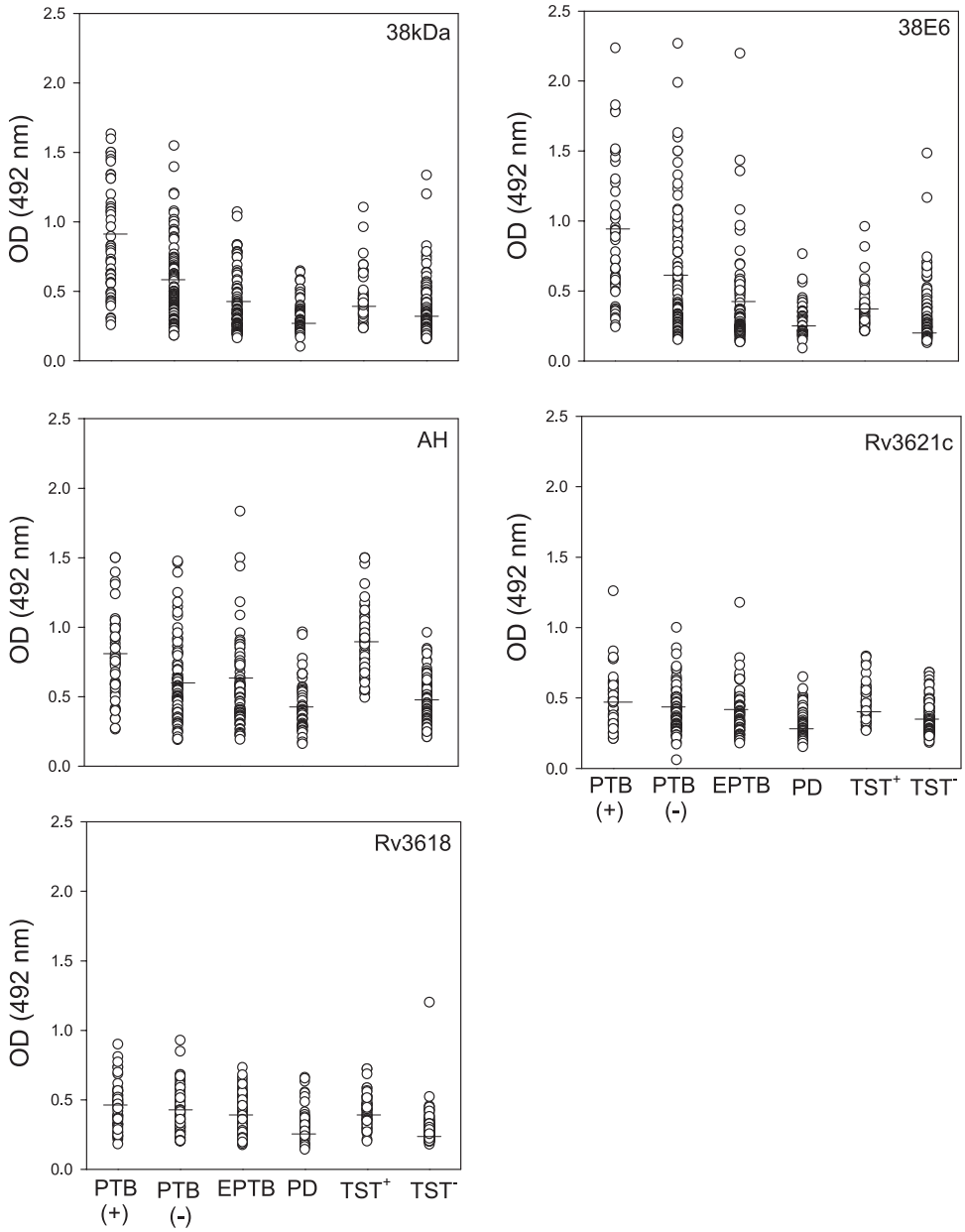

FIG. 2. Scatter plots of OD_{492} values of IgG against 38kDa, 38E6, AH, Rv3621c, and Rv3618 in patients with culture- or smear-positive pulmonary TB [PTB(+)], culture- and smear-negative pulmonary TB [PTB(−)], extrapulmonary tuberculosis (EPTB), nontuberculosis pulmonary disease (PD), and healthy subjects with positive tuberculin skin tests (TST^+ controls) and negative tuberculin skin tests (TST^- controls). The median value of each group is indicated by a solid horizontal line.

TB [PTB(+)], including 26 patients with smear- and culture-positive pulmonary TB, (ii) 101 patients with sputum culture- and smear-negative pulmonary TB [PTB(−)], (iii) 88 EPTB patients, (iv) 49 PD patients, including lung cancer and pneumonia, (v) 151 TST-negative (induration area < 5 mm) healthy subjects (TST^- controls), and (vi) 50 TST-positive (induration area > 10 mm) healthy subjects (TST^+ controls). Serum samples of TST^- and TST^+ controls were obtained at 72 h after TST. Approval was granted by the 309th Hospital of Chinese People's Liberation Army. All participants provided informed consent.

Serum samples. Two milliliters of venous blood was harvested and coagulated for 2 to 3 h. The sera were obtained by centrifugation at 3,000 × *g* for 15 min, and 50-μl aliquots of serum were stored at −80°C until ELISA.

ELISA. Ninety-six flat-bottom enzyme immunoassay (EIA)/radioimmunoassay (RIA) plates (Costar product number 3590) were coated with individual antigens at 5 μg/ml (100 μl/well) and stored at 4°C overnight. The plates coated with antigens were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST) for 5 min each time. Two hundred microliters of PBST containing 1% bovine serum albumin (PBST-B) was added to each well, and the plates were sealed and incubated at 37°C for 1 h in a moisture bath. The plates were washed three times. One hundred microliters of serum diluted at 1:200 in PBST-B was added to the antigen-coated well. The plates were sealed and incubated at 37°C for 2 h in a moisture bath and then washed three times. One hundred microliters of horseradish peroxidase-conjugated anti-human IgG antibody or IgM antibody (Jackson ImmunoResearch) diluted at 1:30,000 in PBST-B was added to each well, and the plates were sealed and incubated at 37°C for 1 h in a moisture bath. The plates were washed three times. Substrate solution was prepared with 40 mg *O*-phenylenediamine dissolved in citrate buffer solution containing 3% H_2O_2, 100 μl substrate solution was added, and the plates were protected from light for 20 to 30 min until the color was seen with the

TABLE 1. Sensitivity and specificity of IgG against single antigens or combinations of two antigens[a]

Antigen(s)	% sensitivity				% specificity		
	PTB(+) (n = 50)	PTB(−) (n = 101)	EPTB (n = 88)	Total TB (n = 239)	PD (n = 49)	TST$^+$ controls (n = 50)	TST$^-$ controls (n = 151)
38kDa	76.0 (38/50)	37.6 (38/101)	20.5 (18/88)	39.3 (94/239)	89.8 (44/49)	82.0 (41/50)	90.1 (136/151)
38E6	80.0 (40/50)	44.6 (45/101)	28.4 (25/88)	46.0 (110/239)	93.9 (46/49)	84.0 (42/50)	89.4 (135/151)
AH	62.0 (31/50)	30.7 (31/101)	26.1 (23/88)	35.6 (85/239)	91.8 (45/49)	20.0 (10/50) D	90.7 (137/151)
Rv3618	68.0 (34/50)	47.5 (48/101)	48.9 (43/88) D	52.3 (125/239) D	75.5 (36/49) A	34.0 (17/50) D	90.1 (136/151)
Rv3621c	48.0 (24/50) D	29.7 (30/101)	22.7 (20/88)	31.0 (74/239)	85.7 (42/49)	60.0 (30/50) A	88.1 (133/151)
38kDa+38E6	84.0 (42/50)	48.5 (49/101)	30.7 (27/88)	49.4 (118/239) B	87.8 (43/49)	80.0 (40/50)	88.1 (133/151)
38kDa+AH	90.0 (45/50) F	52.5 (53/101) BF	37.5 (33/88) B	54.8 (131/239) EF	83.7 (41/49)	16.0 (8/50) E	81.5 (123/151) BC
38kDa+Rv3621c	86.0 (43/50) F	51.5 (52/101) BF	33.0 (29/88)	51.9 (124/239) EF	75.6 (39/49)	54.0 (27/50) E	80.8 (122/151) B
38kDa+Rv3618	92.0 (46/50) BF	64.4 (65/101) CE	52.3 (46/88) E	65.7 (157/239) EF	67.3 (33/49) E	26.0 (13/50) E	82.1 (124/151) BC
38E6+AH	90.0 (45/50) F	55.4 (56/101) F	43.2 (38/88) BC	58.2 (139/239) EF	85.7 (42/49)	20.0 (10/50) E	80.8 (122/151) BC
38E6+Rv3621c	90.0 (45/50) F	55.4 (56/101) F	37.5 (33/88) C	56.1 (134/239) BF	75.6 (39/49) B	50.0 (25/50) E	81.5 (123/151)
38E6+Rv3618	96.0 (48/50) BF	69.3 (70/101) EF	58.0 (51/88) E	70.7 (169/239) EF	73.5 (36/49) E	30.0 (15/50) E	81.5 (123/151) C
AH+Rv3621c	72.0 (36/50) C	46.5 (47/101) C	35.3 (31/88)	47.7 (114/239) EF	83.7 (41/49)	16.0 (8/50) F	80.1 (121/151) B
AH+Rv3618	72.0 (36/50)	53.5 (54/101) E	55.7 (49/88) E	58.2 (139/239) E	71.4 (35/49) E	18.0 (9/50)	83.4 (126/151)
Rv3621c+Rv3618	72.0 (36/50) B	59.4 (60/101) E	52.3 (46/88) E	59.4 (142/239) E	69.4 (34/49)	28.0 (14/50) B	81.5 (123/151) C

[a] A, compared with 38kDa; B, compared with the first component of a combination; C, compared with the second component of a combination; D, compared with 38kDa; E, compared with the first component of a combination; F, compared with the second component of a combination. A, B, C, $P < 0.05$; D, E, F, $P < 0.01$.

naked eye. The optical densities were measured at 492 nm (OD_{492}) after stopping the reaction by adding 50 μl of 2 M H_2SO_4. This test was a single-blinded study, where clinicians were responsible for harvesting clinical data and sera and marking serial numbers of serum samples and laboratory technicians were responsible for ELISA according to serial numbers of serum samples and did not know the participants who provided sera.

Data analyses. Receiver operator characteristic (ROC) curves were constructed by plotting the true-positive rate (sensitivity) and false-positive rate (1-specificity) with each unique OD_{492} of IgG and IgM antibodies against five antigens for PTB(+) patients and TST$^-$ controls, respectively. ROC curves were used to determine cutoff values. The positive predictive value (PPV) was determined as the ratio of the number of the true-positive samples to the sum of true- and false-positive samples, and the negative predictive value (NPV) was determined as the ratio of the number of true-negative samples to the sum of true- and false-negative samples. True positive referred to the number of PTB(+) patients who had positive IgG responses (conversely, false negative); true negative referred to the number of TST$^-$ controls who had negative IgG responses (conversely, false positive). One-way analysis of variance with least-significant-difference (LSD) comparisons was used to test differences among OD_{492} values. Statistical analyses of sensitivity and specificity were performed with a chi-square test. All analyses were performed with SPSS 13.0. P values of <0.05 were considered statistically significant.

RESULTS

ROC curves. The areas under curves (AUCs) of 38kDa, 38E6, AH, Rv3621c, and Rv3618 antigens used to detect IgG were 0.91, 0.92, 0.81, 0.73, and 0.84, respectively, and the thresholds of these five antigens, indicated by OD_{492}, were 0.55, 0.49, 0.67, 0.48, and 0.37, respectively (Fig. 1A). For IgM antibodies, the AUCs of 38kDa, 38E6, AH, Rv3621c, and Rv3618 were 0.46, 0.43, 0.50, 0.41, and 0.49, and their thresholds were 0.49, 0.49, 0.62, 0.63, and 0.58, respectively (Fig. 1B).

IgG antibodies against single antigens. The median IgG reactivity to a single antigen was stronger in PTB(+) and PTB(−) patients than in PD patients and TST$^-$ controls (Fig. 2) ($P < 0.05$). IgG antibody response to AH was greater in TST$^+$ controls than in PTB(−) and EPTB patients (Fig. 2) ($P < 0.01$).

The sensitivity provided by a single antigen was higher in PTB(+) patients than in PTB(−) and EPTB patients (Table 1) ($P < 0.05$). In PTB(+) patients, Rv3621c exhibited a lower sensitivity than 38kDa, 38E6, and Rv3618 (Table 1) ($P < 0.05$). 38E6 and Rv3618 yielded higher sensitivities than AH and Rv3621c in PTB(−) patients (Table 1) ($P < 0.05$). Rv3618 had a higher sensitivity than the rest of the antigens in EPTB patients (Table 1) ($P < 0.01$).

The specificities of IgG against AH, Rv3618, and Rv3621c were lower in TST$^+$ controls than in PD patients and TST$^-$ controls ($P < 0.01$), and the specificities of AH, Rv3618, and Rv3621c also were lower than those of 38kDa and 38E6 in TST$^+$ controls ($P < 0.05$) (Table 1). Rv3618 yielded a lower specificity in PD patients than in TST$^-$ controls ($P < 0.01$), and the specificity of Rv3618 also was lower than that of 38kDa, 38E6, and AH in PD patients ($P < 0.05$) (Table 1).

The PPV of 38kDa, 38E6, AH, Rv3618, and Rv3621c were 71.7% (38/53), 71.4% (40/56), 68.9% (31/45), 69.4% (34/49), and 57.1% (24/42), respectively. The NPV of 38kDa, 38E6, AH, Rv3618, and Rv3621c were 91.9% (136/148), 93.1% (135/145), 87.8% (137/156), 89.5% (136/152), and 83.6% (133/159), respectively.

IgG antibodies against a combination of two antigens. The sensitivity provided by Rv3621c was lower than that by combinations of Rv3621c and other antigens in PTB(+) patients (Table 1) ($P < 0.05$). 38kDa, 38E6, AH, and Rv3621c exhibited lower sensitivities than their combinations with Rv3618 in PTB(−) and EPTB patients (Table 1) ($P < 0.05$).

38kDa, 38E6, and AH provided higher specificities than their combinations with Rv3618 in PD patients (Table 1) ($P < 0.05$). 38kDa and 38E6 had higher specificities than their combinations with AH, Rv3618, and Rv3621c in TST$^+$ controls (Table 1) ($P < 0.05$). 38kDa had a higher specificity than its combinations with AH, Rv3618, and Rv3621c in TST$^-$ controls (Table 1) ($P < 0.05$).

Combination of IgG and IgM antibodies against single antigens. Some sera negative for IgG were found to be positive for IgM. By combining results of IgG and IgM (IgG+IgM), the sensitivities provided by 38kDa, AH, and Rv3621c increased in EPTB patients ($P < 0.05$), in PTB(−) patients ($P < 0.01$), and in PTB(+) patients ($P < 0.01$), respectively (Tables 1 and 2). The specificity for IgG+IgM antibodies was lower than that for

TABLE 2. Sensitivity and specificity of combinations of IgG and IgM antibodies against single antigens

Antigen	% sensitivity			% specificity		
	PTB(+) (n = 50)	PTB(−) (n = 101)	EPTB (n = 88)	PD (n = 49)	TST^+ controls (n = 50)	TST^- controls (n = 151)
38kDa	82.0 (41/50)	45.5 (46/101)	35.2 (31/88)	67.3 (33/49)	76.0 (38/50)	80.1 (121/151)
38E6	82.0 (41/50)	48.5 (49/101)	40.9 (36/88)	71.4 (35/49)	70.0 (35/50)	82.1 (124/151)
AH	72.0 (36/50)	59.4 (60/101)	55.7 (49/88)	44.9 (22/49)	18.0 (9/50)	82.1 (124/151)
Rv3618	76.0 (38/50)	57.4 (58/101)	56.8 (50/88)	46.9 (23/49)	24.0 (12/50)	82.8 (125/151)
Rv3621c	52.0 (26/50)	31.7 (32/101)	29.5 (26/88)	55.1 (27/49)	56.0 (28/50)	80.8 (122/151)

IgG with 38kDa, 38E6, AH, and Rv3621c in PD patients ($P < 0.01$) and with 38kDa, 38E6, and AH in TST^- controls ($P < 0.05$) (Tables 1 and 2).

DISCUSSION

It is already known that single dominant species-specific antigens can never achieve satisfactory diagnostic performance. Thus, strategies using multiple antigens either individually or as fusion polyproteins have been recommended (12). Additionally, it is important to screen new antigens, including multiple antigens. In previous studies, much attention has been given to 38kDa antigens, while relatively limited attention has been paid to Rv3621c, Rv3618, 38kDa-ESAT-6 (38E6), and Ag85B-HBHA (AH). Our findings add to the understanding of effectiveness of these five antigens in serodiagnosis for TB.

The value of AUC usually indicates diagnostic accuracy, because AUC can convert a two-dimensional depiction of classifier performance into a single scalar value representing expected performance (5). No realistic classifier should have an AUC less than 0.5. As Van Der Schouw et al. pointed out, the values of AUCs are too low to suggest meaningful usefulness in clinical practice (26). Our findings suggest that the AUCs of five antigens used to detect IgG antibodies are over 0.7, even near 1.0, but IgM antibodies may have a very poor discriminatory ability, with all AUCs close to 0.5.

The real value of serodiagnosis for TB should be tested through cross-sectioned studies. As Rutjes et al. pointed out, the accuracy of the serodiagnosis is overestimated in the comparison between the TB case and healthy controls (case-control studies) (15). Since *Mycobacterium bovis* BCG vaccination, environmental mycobacteria, HIV infection, and other respiratory diseases may influence the results of the serodiagnosis of TB, case-control studies may provide high sensitivities and overestimate the diagnostic value. The real values of any antigens need to be ascertained via cross-sectioned studies in clinical settings where the TB suspects include patients with a variety of respiratory diseases (1). However, previous knowledge stems mainly from case-control studies (14, 22, 28, 31). Rv3618 was a good antigen for ELISA detection of IgG antibodies based on case-control studies; however, it gave a lower specificity in sera from PD patients (75.5%) and TST^+ controls (34.0%). AH produced a higher specificity for PD patients (91.8%) and TST^- controls (90.7%) but a lower specificity for TST^+ controls (20%). Ag85B is able to discriminate TST^+ animals from TST^- animals in an indirect ELISA to diagnose bovine TB (18). Anti-AH IgG antibody responses may not distinguish active TB from LTBI, but they can distinguish LTBI from PD patients and TST^- controls.

There appears to be trade-offs between sensitivity and specificity. For example, recombinant 38kDa yielded a sensitivity and specificity of 37% to 74% and 98% to 78%, respectively (29, 32). In our study, 38kDa gave a total sensitivity of 39.3% and a specificity of 90.1% in TST^- controls. These results suggest that the combination of IgG and IgM antibodies or multiple antigens may increase sensitivity but decrease specificity. In comparison with anti-30kDa antibodies, the combination of IgG, IgA, and IgM antibodies improved the sensitivity from 67.0% to 74.0% and decreased the specificity from 99.0% to 92.0% (13). In comparison with single lipoarabinomannan (LAM) (30%) and ESAT-6 (13%), the LAM combination with ESAT-6 increased the sensitivity (43%) (3). Rv3425, LAM, and 38kDa antigens provided sensitivities of 32%, 35%, and 21% in active TB patients, respectively, and the multiple antigens (Rv3425+38kDa+LAM) provided a sensitivity of 44% in PTB(−) patients (32).

In summary, 38kDa and 38E6 may have the potential value to detect IgG antibody in active TB. Specifically, they exhibited high specificities in PD patients and healthy controls and high positive rates in PTB(+) patients. Rv3618 may be suitable to detect PTB(−) and EPTB patients but give lower specificities in PD patients and TST^+ controls. AH seems to be able to screen LTBI. We note that most TB patients enrolled are not confirmed by culture, the gold standard method for TB diagnosis in this study. The potential serodiagnosis of the five antigens awaits further efforts.

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