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.

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.
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. 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.
TB [PTB(+)], including 26 patients with smear- and culture-positive pulmonary TB [PTB(+)], (ii) 101 patients with sputum 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.

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-B 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 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 4-phenylenediamine dissolved in citrate buffer solution containing 3% H2O2, 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

FIG. 2. Scatter plots of OD492 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.
RESULTS

ROC curves. The areas under curves (AUCs) of 38kDa, 38E6, AH, Rv3612c, 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_{580}, were 0.55, 0.49, 0.67, 0.48, and 0.37, respectively (Fig. 1A). For IgM antibodies, the AUCs of 38kDa, 38E6, AH, Rv3612c, 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 TST- controls (P < 0.01). The specificity provided by IgG against single antigens was considered statistically significant.

IgG antibodies against a combination of two antigens. The reactivity to a combination of two antigens was stronger in PTB(+) patients than in PTB(-) and EPTB patients (Table 1) (P < 0.05). In PTB(+) patients, Rv3612c exhibited a lower sensitivity than 38kDa, 38E6, and Rv3618 (Table 1) (P < 0.05), 38E6 and Rv3618 yielded higher specificities than AH and Rv3612c in PTB(-) patients (Table 1) (P < 0.05). Rv3618 had a higher specificity than the rest of the antigens in EPTB patients (Table 1) (P < 0.01).

The specificities of IgG against AH, Rv3618, and Rv3612c were lower in TST+ controls than in PD patients and TST- controls (P < 0.01), and the specificities of AH, Rv3618, and Rv3612c 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 Rv3612c 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 Rv3612c 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 Rv3612c was lower than that by combinations of Rv3612c and other antigens in PTB(+) patients (Table 1) (P < 0.05). 38kDa, 38E6, AH, and Rv3612c exhibited lower sensitivities than their combinations with Rv3618 in PTB(-) and EPTB patients (Table 1) (P < 0.05).

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

<table>
<thead>
<tr>
<th>Antigen(s)</th>
<th>PTB(+) (n = 50)</th>
<th>PTB(-) (n = 101)</th>
<th>EPTB (n = 88)</th>
<th>Total TB (n = 239)</th>
<th>PD (n = 49)</th>
<th>TST+ controls (n = 50)</th>
<th>TST- controls (n = 151)</th>
</tr>
</thead>
<tbody>
<tr>
<td>38kDa</td>
<td>76.0 (38/50)</td>
<td>37.6 (38/101)</td>
<td>20.5 (18/88)</td>
<td>39.3 (94/239)</td>
<td>89.8 (44/49)</td>
<td>82.0 (41/50)</td>
<td>90.1 (136/151)</td>
</tr>
<tr>
<td>38E6</td>
<td>80.0 (40/50)</td>
<td>44.6 (45/101)</td>
<td>28.4 (25/88)</td>
<td>46.0 (110/239)</td>
<td>93.9 (46/49)</td>
<td>84.0 (42/50)</td>
<td>89.4 (155/151)</td>
</tr>
<tr>
<td>AH</td>
<td>62.0 (31/50)</td>
<td>30.7 (31/101)</td>
<td>26.1 (23/88)</td>
<td>35.6 (85/239)</td>
<td>91.8 (45/49)</td>
<td>20.0 (10/50)</td>
<td>90.7 (137/151)</td>
</tr>
<tr>
<td>Rv3618</td>
<td>68.0 (34/50)</td>
<td>47.5 (48/101)</td>
<td>48.9 (43/88)</td>
<td>52.3 (125/239)</td>
<td>75.5 (36/49)</td>
<td>34.0 (17/50)</td>
<td>90.1 (136/151)</td>
</tr>
<tr>
<td>Rv3621c</td>
<td>48.0 (24/50)</td>
<td>29.7 (30/101)</td>
<td>22.7 (20/88)</td>
<td>31.0 (74/239)</td>
<td>85.7 (42/49)</td>
<td>60.0 (30/50)</td>
<td>88.1 (133/151)</td>
</tr>
<tr>
<td>38kDa + 38E6</td>
<td>84.0 (42/50)</td>
<td>48.5 (49/101)</td>
<td>30.7 (27/88)</td>
<td>49.4 (118/239)</td>
<td>87.8 (43/49)</td>
<td>80.0 (40/50)</td>
<td>88.1 (133/151)</td>
</tr>
<tr>
<td>38kDa + AH</td>
<td>90.0 (45/50) F</td>
<td>52.5 (53/101) BF</td>
<td>37.5 (33/88)</td>
<td>54.8 (131/239)</td>
<td>83.7 (41/49)</td>
<td>16.0 (8/50)</td>
<td>81.5 (123/151) BC</td>
</tr>
<tr>
<td>38kDa + Rv3612c</td>
<td>86.0 (43/50) F</td>
<td>51.5 (52/101) BF</td>
<td>33.0 (29/88)</td>
<td>51.9 (124/239)</td>
<td>75.6 (39/49)</td>
<td>54.0 (27/88)</td>
<td>80.8 (122/151) BC</td>
</tr>
<tr>
<td>38kDa + Rv3618</td>
<td>92.0 (46/50) BF</td>
<td>64.4 (65/101) CE</td>
<td>52.3 (46/88)</td>
<td>65.7 (157/239)</td>
<td>67.3 (33/49)</td>
<td>26.0 (13/50)</td>
<td>82.1 (124/151) BC</td>
</tr>
<tr>
<td>38E6 + AH</td>
<td>90.0 (45/50) F</td>
<td>55.4 (56/101) F</td>
<td>43.2 (38/88)</td>
<td>58.2 (139/239)</td>
<td>85.7 (42/49)</td>
<td>20.0 (10/50)</td>
<td>80.8 (122/151) BC</td>
</tr>
<tr>
<td>38E6 + Rv3612c</td>
<td>96.0 (48/50) BF</td>
<td>69.3 (70/101) EF</td>
<td>58.0 (51/88)</td>
<td>70.7 (169/239)</td>
<td>73.5 (36/49)</td>
<td>30.0 (15/50)</td>
<td>81.5 (123/151) C</td>
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<tr>
<td>AH + Rv3612c</td>
<td>72.0 (36/50) C</td>
<td>46.5 (47/101) C</td>
<td>35.3 (31/88)</td>
<td>47.7 (114/239)</td>
<td>83.7 (41/49)</td>
<td>16.0 (8/50)</td>
<td>80.1 (121/151) B</td>
</tr>
<tr>
<td>AH + Rv3618</td>
<td>72.0 (36/50) C</td>
<td>53.5 (54/101) E</td>
<td>55.7 (49/88)</td>
<td>58.2 (139/239)</td>
<td>71.4 (35/49)</td>
<td>18.9 (9/50)</td>
<td>83.4 (126/151)</td>
</tr>
<tr>
<td>Rv3612c + Rv3618</td>
<td>72.0 (36/50) B</td>
<td>59.4 (60/101) E</td>
<td>52.3 (46/88)</td>
<td>59.4 (142/239)</td>
<td>69.4 (34/49)</td>
<td>28.0 (14/50)</td>
<td>81.5 (123/151) C</td>
</tr>
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</table>

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.

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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 polypeptides 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 close to 1.0, but IgM antibodies may have a very poor discrimination. Five antigens used to detect IgG antibodies are over 0.7, even in clinical practice (26). Our findings suggest that the AUCs of values of AUCs are too low to suggest meaningful usefulness in expected performance. Thus, strategies using multiple antigens either individually or as fusion polypeptides may never achieve satisfactory diagnostic performance.

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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 (74.0%). 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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**REFERENCES**