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Plasma Antibody Profiles as Diagnostic Biomarkers for Tuberculosis▽

Imran H. Khan,[1,2]*† Resmi Ravindran,[1]† Viswanathan V. Krishnan,[1,2,3] Irum Nawaz Awan,[4] Syed Kumail Rizvi,[4] Muhammad Arif Saqib,[4] Mirza Imran Shahzad,[4] Sabira Tahseen,[5] Greg Ireton,[6] Celia W. Goulding,[7] Phil Felgner,[8] Kathy DeRiemer,[9] Azra Khanum,[4] and Paul A. Luciw[1,2]

Center for Comparative Medicine,[1] Department of Pathology and Laboratory Medicine,[2] and Department of Public Health Sciences,[9] University of California, Davis, Davis, California; Department of Chemistry, California State University, Fresno, California 93740[3]; Department of Biochemistry, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi, Rawalpindi, Pakistan[4]; National TB Control Program, Islamabad, Pakistan[5]; Infectious Disease Research Institute, Seattle, Washington[6]; and Departments of Molecular Biology & Biochemistry and Pharmaceutical Sciences[7] and Department of Medicine,[8] University of California, Irvine, Irvine, California

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Two billion people are infected with *Mycobacterium tuberculosis*, the etiological agent of tuberculosis (TB), worldwide. Ten million to 20 million of the infected individuals develop disease per year. TB is a treatable disease, provided that it is diagnosed in a timely manner. The current TB diagnostic methods are subjective, inefficient, or not cost-effective. Antibody-based blood tests can be used efficiently and cost-effectively for TB diagnosis. A major challenge is that different TB patients generate antibodies against different antigens. Therefore, a multiplex immunoassay approach is needed. We have developed a multiplex panel of 28 *M. tuberculosis* antigen-coated microbeads. Plasma samples were obtained from over 300 pulmonary TB patients and healthy controls in a country where TB is endemic, Pakistan. Multiplex data were analyzed using computational tools by multivariate statistics, classification algorithms, and cluster analysis. The results of antibody profile-based detection, using 16 selected antigens, closely correlated with those of the sputum-based diagnostic methods (smear microscopy and culture) practiced in countries where TB is endemic. Multiplex microbead immunoassay had a sensitivity and specificity of approximately 90% and 80%, respectively. These antibody profiles could potentially be useful for the diagnosis of nonpulmonary TB, which accounts for approximately 20% of cases of disease. Since an automated, high-throughput version of this multiplex microbead immunoassay could analyze thousands of samples per day, it may be useful for the diagnosis of TB in millions of patients worldwide.

More than one-third of the world's population is infected with *Mycobacterium tuberculosis* (7, 26a). Annually, 10 million to 20 million of these individuals develop clinical symptoms, and about 2 million die of tuberculosis (TB) (4, 17a). The infected host typically mounts a vigorous immune response (25). Nevertheless, 10% of all infections result in active disease within 2 years. Another 10% of cases may experience disease after a latent phase spanning many years (8, 17a). Several *Mycobacterium* species (e.g., *M. tuberculosis*, *M. bovis*, and *M. africanum*) can infect and cause disease in humans (2, 24). In about 80% of active TB cases, direct involvement of the lung results in pulmonary disease (4a). However, *M. tuberculosis* can spread to other organs. In approximately 20% of cases, *M. tuberculosis* may cause nonpulmonary disease in various organ systems (urogenital system, nervous system, digestive system, skeletal system, etc.) with or without the lung involvement (7, 18). TB is a treatable disease, provided that a timely and appropriate diagnosis is made (4a). Commonly used sputum-based methods for pulmonary TB diagnosis are subjective, insensitive, and/or inefficient. Furthermore, for the detection of pediatric pulmonary TB, a major limitation is that children often have difficulty producing usable quantities of sputum.

Sputum smear acid-fast bacillus (AFB) microscopy is recommended by the World Health Organization (WHO) as the first-line diagnostic procedure for pulmonary disease. Although relatively specific, this method is subjective, inconsistent, and not very sensitive (globally, 30 to 70% sensitivity) (26a). Bacterial culture is considered a "gold standard" for TB diagnosis, but because *M. tuberculosis* is a slow-growing organism, the standard culture methods can take up to 8 to 12 weeks to obtain results (9).

The complete genome sequences of *M. tuberculosis* (H37Rv, virulent laboratory strain) have been determined (3). More recently, specific and sensitive TB diagnostic tests have been developed by taking advantage of advances in sequencing and annotation of the *M. tuberculosis* genome, which has revealed approximately 4,000 open reading frames (http://genolist.pasteur.fr/TubercuList/). These diagnostic tests include nucleic acid amplification of *M. tuberculosis* but are limited to use with processed sputum samples. Disease diagnostics based on blood tests are advantageous because they are minimally invasive, rapid, and cost-effective and are useful for nonpulmonary and pediatric TB. Detection of anti-*M. tuberculosis* antibodies (plasma or serum) is more suitable for implementation in a variety of clinical laboratory settings. Despite efforts to develop TB diagnostics based on serology, there are challenges facing

* Corresponding author. Mailing address: Center for Comparative Medicine, University of California at Davis, Hutchison Rd. and County Rd. 98, Davis, CA 95616. Phone: (530) 752-7200. Fax: (530) 752-7914. E-mail: ihkhan@ucdavis.edu.
† These authors contributed equally to this work.
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this approach. Not all patients produce antibodies against the same *M. tuberculosis* antigens, and exposure to environmental mycobacteria and *M. bovis* BCG (bacillus Calmette-Guérin) vaccination can potentially lead to confounding results. We reasoned that these challenges can be overcome by a user-friendly and cost-effective multiplex method that employs dozens of *M. tuberculosis* antigens for detecting profiles of anti-*M. tuberculosis* antibodies. Detection of antibodies against multiple *M. tuberculosis* antigens has been fruitful in the detection of *M. tuberculosis* infection (16).

Ideally, a multiplex platform selected for a clinical diagnostic test should be suitable for the entire process from assay development to clinical validation and implementation. It should additionally be amenable to high throughput, robust, and flexible; readily deployable in low-resource settings; require minimal training; and be cost-effective. A multiplex microbead immunoassay based on the xMAP technology platform (Luminex Corp, Austin, TX) satisfies all of the above-described requirements for a useful infectious disease diagnostic. Discovery platforms such as 2-dimensional protein array (21) are useful in the initial selection of target proteins (antigens) but are inflexible, require sophisticated laboratory infrastructure, and are not cost-effective.

In our study, antibody profiles generated by multiplex microbead immunoassay and multivariate and cluster analyses enabled differentiation of TB patients from healthy indigenous individuals. The xMAP platform used in this study has a high capacity for analysis of hundreds to thousands of samples from patients and control groups per day, making it applicable for use as a first-line diagnostic in countries where TB is endemic.

MATERIALS AND METHODS

***M. tuberculosis* antigens.** Recombinant antigens from 28 *M. tuberculosis* genes were expressed in *Escherichia coli*. Plasmid vectors for expression of eight recombinant antigens were obtained from the TB Resource Center at Colorado State University (Fort Collins, CO): Rv3875 (ESAT6), Rv3874 (CFP10), Rv2031c (HspX), Rv3804c (antigen 85a [Ag85a]), Rv1886c (Ag85b), Rv0129c (Ag85c), Rv3841 (Bfrb1), and Rv3418c (GroES). The recombinant antigens were expressed and purified at the University of California, Davis (UCD; Davis, CA), and the University of Arid Agriculture (UAAR), Rawalpindi, Pakistan, as described elsewhere (http://www.cvmbs.colostate.edu/mip/tb/sop.htm). Several other recombinant antigens ($n = 14$) were expressed and purified at the Infectious Disease Research Institute (IDRI; Seattle, WA) as previously described (13): Rv2875 (MPT70), Rv1984c (CFP21), Rv1980c (MPT64), Rv0934 (P38 or PstS1), Rv1860 (MPT32), Rv0054, Rv3874-Rv3875 (CFP10-ESAT) fusion, Rv3873, Rv3619, Rv2220, Rv0831c, Rv1009, Rv1099, and Rv2032. The antigens Rv1926c, Rv2878c, Rv1677, Rv3881c, Rv1566c, and Rv3507 were expressed and purified at the University of California, Irvine (Irvine, CA), as previously described (10, 11).

Sample groups. Blood samples from TB patients and healthy individuals were obtained under the protocols approved through the institutional review boards (IRBs) at UCD and UAAR. The HIV status of healthy controls and TB patients was unknown. Samples were obtained from the four main groups described below.

(i) Healthy control samples. The first control group ($n = 87$) comprised individuals of both sexes (ages, 21 to 25 years) from the same geographical area as the TB patients (Rawalpindi/Islamabad, Pakistan, where TB is endemic). This group was termed the "indigenous healthy (IH) group." BCG coverage in Pakistan since 1978 has been over 80% (19a). The second group of healthy donors of both sexes (ages, 21 to 65 years) comprised individuals from the California Central Valley (Delta Blood Bank, Stockton, CA) and was termed the "nonindigenous healthy (NH) group" ($n = 146$); the BCG status of this group is unknown.

(ii) TB patient samples. Plasma samples were collected from AFB microscopy-positive (AFB^+) TB patients ($n = 243$) at the Federal Government TB Hospital, Rawalpindi, Pakistan. At this hospital, TB diagnosis is primarily based on AFB microscopy (on the basis of WHO-recommended criteria). Three consecutive, daily sputum specimens were used to determine positivity by AFB microscopy. A subgroup of AFB^+ patients consisted of those undergoing directly observed treatment, short course (DOTS), termed the "DOTS group." Blood samples were drawn at four time points: (i) before DOTS (including all the AFB^+ patients, $n = 243$) and (ii) 2 months ($n = 52$), (iii) 4 months ($n = 31$), and (iv) 6 months ($n = 21$) post-DOTS initiation. Patients from whom post-DOTS samples were taken were subsets of the original AFB^+ patients ($n = 243$). Because visits to the Federal Government TB Hospital, Rawalpindi, by the original AFB^+ patients decreased over time due to poor compliance, there was a progressive decrease in the number of samples available at the later time points. Also included in the study are a group of TB patients that were negative by AFB microscopy but positive by culture, designated the "AFB^-/Cul^+ group" ($n = 17$).

Blood sample collection, processing, and storage. A 5-ml blood sample was collected from each individual through venipuncture and placed into a Vacutainer tube (EDTA; catalog no. 367899; BD, NJ). Plasma was separated by centrifugation at $1,000 \times g$, 10 min, room temperature) within 2 h of collection and immediately frozen in aliquots at −80°C until use.

Microbead coating with *M. tuberculosis* antigens. Microbead sets (Luminex Corp, Austin, TX) were coupled through carbodiimide linkages with each of the *M. tuberculosis* recombinant antigens as previously described (14–16).

Multiplex assay conditions and data collection. Multiplex assays were performed and data (median fluorescence intensity [MFI]) were collected as previously described (16). Cutoff values were calculated for each antigen-coated microbead set; MFI values that represented the reactivity of each microbead set to healthy control samples from the IH group were used for calculations, as follows: mean MFI + ($2 \times$ standard deviation).

Statistical analysis. Multivariate analysis was performed to select useful antigens. Fold increases of antibodies in patient samples were calculated across conditions by fitting a linear model to the data (antibodies to 28 *M. tuberculosis* antigens with technical duplicates) for each patient relative to the healthy groups. A significantly positive signal was detected in at least one comparison between control and patient groups (e.g., IH versus AFB^+ groups). Differential expression across the multiple comparisons was detected by the *F* test; separate *F* tests were performed for each antibody; and *P* values were adjusted using the Benjamini-Hochberg procedure to correct for multiple comparisons (1, 5, 8).

Cluster analysis was performed to define natural groupings of antigens and TB patients (AFB^+ group) analyzed with respect to healthy controls (IH group) as previously described (20). Briefly, data sets with which to perform the cluster analysis were generated by scaling the MFI levels of each sample in the AFB^+ group with reference to the baseline value for each microbead set. Hierarchical cluster analysis was performed using a Euclidean distance metric, without standardization. For visual depiction, results of the cluster analysis were presented as dendrograms, and heat maps were generated by a combination of codes using the Matlab (19) and SigmaPlot (23) programs.

To calculate assay predictive values, sensitivity, and specificity, multiplex data were first analyzed by two regression model-based classification algorithms (random forest [RF] and sequential minimal optimization [SMO]). These analyses were performed on data from IH controls and AFB^+ patients with 20-fold cross validation employing Waikato Environment for Knowledge Analysis (WEKA; version 3.6.2) software (6, 12). Analysis by classification algorithms yielded the following measures: true positive (TP), true negative (TN), false positive (FP), and false negative (FN). Calculations were performed using the following formulas: sensitivity = TP/(TP + FN), specificity = TN/(FP + TN), positive predictive value (PPV) = TP/(TP + FP), and negative predictive value (NPV) = TN/(FN + TN). Because the total number of samples in the AFB^-/Cul^+ group was relatively low ($n = 17$) and, therefore, analysis by classification algorithms was unsuitable, sensitivity calculations for this group were performed on TP and FN values determined from the cutoff points for tier 1 antigens (Fig. 1).

RESULTS

Detection of antibodies in TB patients. We examined the serological response to 28 recombinant *M. tuberculosis* antigens in patients with TB ($n = 243$) diagnosed by AFB microscopy (AFB^+ group) and IH controls ($n = 87$). Using a multiplex microbead immunoassay and multivariate analysis, antibodies against 16 *M. tuberculosis* antigens were detected in the AFB^+ group (Table 1). Results for the NH controls ($n = 146$) were similar (Table 1). The other 12 antigens either re-

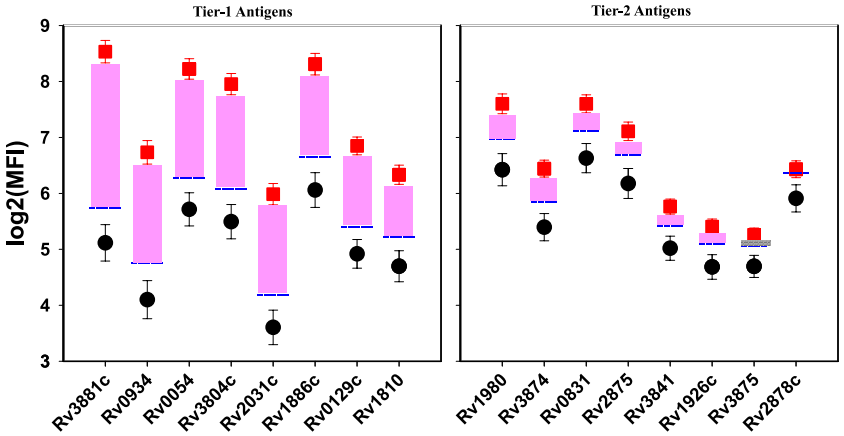

FIG. 1. Capacity of *M. tuberculosis* antigen-coated microbead sets to discriminate between TB patients (AFB^+ group) and healthy controls (IH group). Black filled circles and red filled squares, average reactivity (MFI) of each antigen-coated microbead set to IH group ($n = 87$) and AFB^+ group ($n = 243$), respectively; thin bars, standard deviations; blue dashed lines, baseline for each antigen-coated microbead set [mean MFI of IH group + (2 × standard deviation)]; lavender wide bars, region of discrimination of each microbead set to detect antibody-positive patient samples.

acted nonspecifically (Rv1009, Rv1099, Rv3507, Rv1677) or displayed negligible reactivity (Rv3874-Rv3875 fusion, Rv3619, Rv2220, Rv2032, Rv1984, Rv3873, Rv1566c, Rv3418c) (data not shown).

Important antigens. The 16 useful antigens were further divided, on the basis of the antibody detection signal (MFI fold change [FC]), into two categories, tier 1 and tier 2 (Table 1).

TABLE 1. Fold increases in patient plasma antibodies[a]

Antigen group and antigen	Fold change			
	IH group		NH group	
	AFB^+	AFB^-/Cul^+	AFB^+	AFB^-/Cul^+
Tier 1 antigens				
Rv3881c	10.56	4.92	8.00	3.73
Rv0934	6.06	13.93	6.06	13.93
Rv0054	5.66	1.32[b]	4.92	1.15[b]
Rv3804c	5.66	5.28	4.59	4.59
Rv2031c	5.28	5.66	4.29	4.29
Rv1886c	4.92	4.92	4.00	4.00
Rv0129c	3.73	2.30[b]	1.74	1.00[b]
Rv1860	3.03	3.73	2.83	3.48
Tier 2 antigens				
Rv1980c	2.30	3.25	2.64	3.73
Rv3874	2.00	3.03	1.52	2.14[b]
Rv0831c	2.00	2.00[b]	2.83	2.83
Rv2875	1.87	1.07[b]	1.87	1.15[b]
Rv3841	1.62	1.74[b]	1.52	1.52[b]
Rv1926c	1.62	1.74[b]	1.62	1.62[b]
Rv3875	1.52[b]	1.32[b]	1.41[b]	1.32[b]
Rv2878c	1.41[b]	1.87[b]	1.15[b]	1.41[b]

[a] *M. tuberculosis* antigens important in the detection of anti- *M. tuberculosis* antibodies in TB patients were selected on the basis of multivariate analysis. Assignment of antigens in the two categories (tier 1 and tier 2) was based on the fold change (FC) of the respective antibodies in patient groups (AFB^+ and AFB^-/Cul^+) in comparison to the IH control group. Antibodies against tier 1 and tier 2 antigens were at least 3-fold (P value < 0.001) and 1.5-fold (P value < 0.01) higher, respectively, in patients. Comparisons of patient groups to NH controls are also shown. Though Rv3875 and Rv2878c do not follow the selection criteria of tier 2 antigens, there are included because they are valuable in a subset of patients.

[b] Nonsignificant P values.

The extent of discrimination between antibody-positive and -negative samples was much larger for tier 1 antigens than for those in tier 2 (Fig. 1). Differences in antibody responses in the two groups of TB patients (AFB^+ and AFB^-/Cul^+) were also noted (Fig. 2; Table 1). For example, antigen Rv0054 (tier 1) was of high value for the AFB^+ group of patients (5.66 FC and 42% positive) but was not significant in the AFB^-/Cul^+ group (1.32 FC and 12% positive).

Antibodies in TB patients post-DOTS group. Over a 6-month period post-DOTS, antibody profiles did not change for the post-DOTS group as a whole or in individual patients (data not shown). The impact of DOTS on the outcome of disease in these patients is not known; it is assumed to be an effective treatment.

Antibody profiles. The serology information based on cutoff values for each antigen-coated microbead set (Fig. 1) was incorporated in the cluster analysis first to identify antibody-positive patient samples in the AFB^+ group and then to iden-

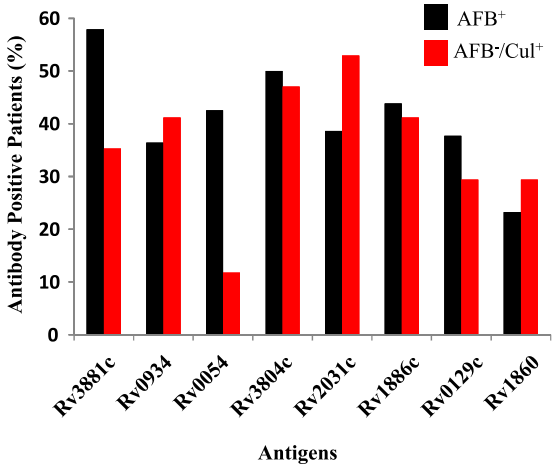

FIG. 2. Percentage of patients positive for antibodies to tier 1 antigens relative to the baseline values in Fig. 1. Black bars and red bars, AFB^+ and AFB^-/Cul^+ patient groups, respectively.

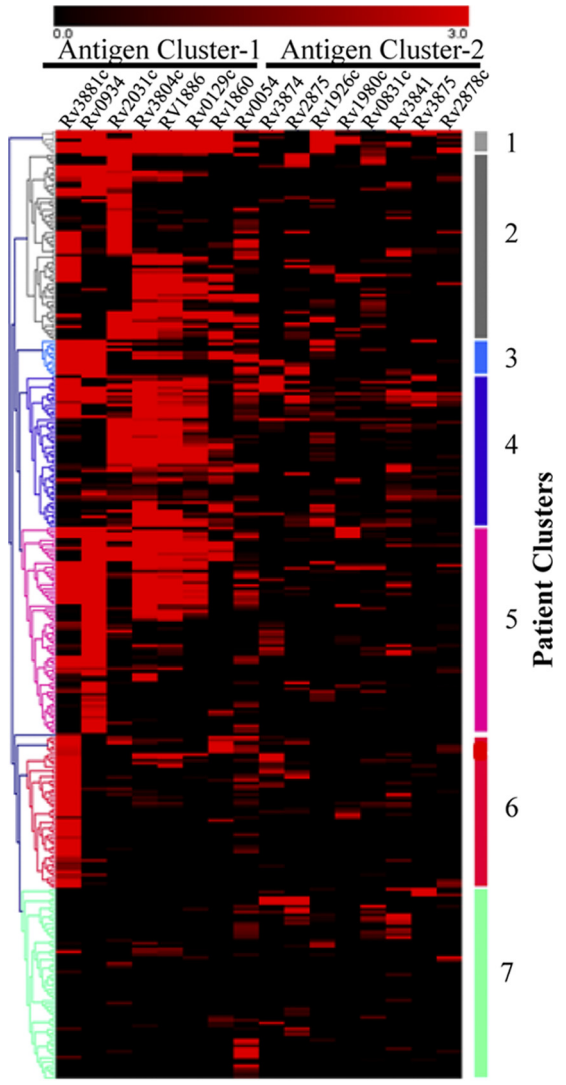

FIG. 3. Natural groupings (clusters) of antigens and patients (AFB^+ group). *M. tuberculosis* antigens are listed at the top of the heat map, and two major clusters of antigens are shown. Patient clusters are indicated by color-coded dendrograms on the left side and numbered on the right side of the heat map. The reactivity of each antigen to each patient sample is shown in red. The intensity scale at the top shows the extent of reactivity in arbitrary units from black (background or cutoff level) to shades of red to a maximum value of 3 (level of antibody signal).

tify reactivity with specific antigens and classify antigens and patients into natural groupings by computer-assisted clustering.

(i) Antigen clusters. The relative strength of reactivity of each antigen in individual patient samples is shown in a heat map by color intensity (red) above the cutoff values (black) (Fig. 3). Profiles of antibodies against the same 16 antigens identified by the multivariate analysis emerged as two major clusters (clusters 1 and 2; Fig. 3); clusters 1 and 2 were the same as tiers 1 and 2 (Table 1), respectively.

(ii) Patient clusters. TB patients with similar antibody profiles classified into seven major clusters (Fig. 3). Antibodies against Rv3881 can be visualized in the largest number of patients, followed by antibodies against the rest of the antigens, as in Fig. 2. In most of the patient clusters (e.g., clusters 1 to 6), each cluster was dominated by antibodies against at least one tier 1 antigen (Fig. 3). Because tier 1 antigens demonstrated strong discrimination between TB patients and healthy controls (Fig. 1), these patient clusters were well-defined. Patient cluster 7 was an exception; antibodies against no single antigen dominated, and most of the patients contained antibodies against cluster 2 antigens.

Predictive modeling. Two classification algorithms (i.e., RF and SMO) were used to classify antibody-positive and -negative samples among the patient and healthy control samples. Data from these classification analyses were used for determining predictive values, as well as assay sensitivity and specificity.

(i) Positive and negative predictive values. The ability of the multiplex microbead immunoassay to predict whether individuals were infected with *M. tuberculosis* (PPV) or healthy (NPV) was assessed. PPVs for tier 1 antigens alone and tier 1 plus tier 2 antigens together were 91 to 95% and 93%, respectively. NPVs were 77 to 78% and 78 to 80%, respectively (Table 2).

(ii) Assay sensitivity and specificity. The sensitivity of the multiplex microbead immunoassay (tier 1 antigens) for the detection of *M. tuberculosis* infection in the AFB^+ group was 91 to 92%. Similar levels of sensitivity were observed with tier 1 and tier 2 antigens together (92 to 93%) (Table 2). Sensitivity for the detection of *M. tuberculosis* infection in the AFB^-/Cul^+ group of patients was 88% (tier 1 antigens). The specificity of the multiplex immunoassay assay ranged from 75 to 85% for tier 1 antigens and was 79% for tier 1 and tier 2 antigens together (Table 2).

DISCUSSION

Sample sets and data analysis. Samples from healthy individuals from the same geographical region (IH group) as the TB patients were used as controls. A second control group (NH group) showed that the differences in background levels in two geographically distinct healthy populations were minimal. Data were analyzed with three statistical approaches, each highlighting a specific aspect of the diagnostic power of the multiplex microbead immunoassay coupled with appropriate

TABLE 2. Positive and negative predictive values, sensitivity, and specificity[a]

Antigen group	PPV (%)		NPV (%)		Sensitivity (%)		Specificity (%)	
	RF	SMO	RF	SMO	RF	SMO	RF	SMO
Tier 1	91.1	94.5	77.4	77.9	92.2	91.4	74.7	85.1
Tiers 1 and 2	92.6	92.5	80.2	77.5	93	91.8	79.3	79.3

[a] Classification of antibody-positive and -negative samples among patients (AFB^+ group) and healthy controls (IH group) was performed on the data from multiplex microbead immunoassay. Two classification algorithms (RF and SMO) were used. Percent predictive values were computed from the resulting classification data. Results for tier 1 and tier 1 and 2 antigens are shown. Percent sensitivity and specificity for detection of *M. tuberculosis* infection by the multiplex microbead immunoassay were computed from the classification data, as described for the predictive values.

computational tools. First, multivariate statistical methods were used to compare the antibody levels between the study groups. Linear modeling with the empirical Bayesian method (8) enabled identification of antibodies with high fold changes and significant *P* values (Table 1). Second, a clustering scheme was used to categorize the profiles of antibodies (and their levels) with natural groupings of antigens and patients (Fig. 3). Third, two distinctly different but high-performing algorithms (RF, SMO) were used for classification of antibody-positive and -negative samples (Table 2). An approach based on the receiver optimization characteristic (ROC) curve is useful for binary distinction of a single antibody. In contrast, the regression-based classification algorithms used in this study provided a powerful summary of prediction and performance in the presence of multiple positive signals. In the development of a computational data-mining system, the above-described algorithms provided the most accurate results with data from several hundred samples (unpublished data) analyzed by multiplex serodetection (10 infectious pathogens) in a validated mouse model of infectious diseases (20).

Anti-*M. tuberculosis* antibody profiles. Two independent statistical approaches used for the selection of antigens useful for serodetection of *M. tuberculosis* infection in AFB^+ patients (multivariate and cluster analysis) identified the same sets of antigens. In fact, antigens in tiers 1 and 2 were identical to those in clusters 1 and 2, respectively. In addition, the ability of antigen-coated microbead sets to discriminate between antibody-positive and -negative samples (Fig. 1) is consistent with the value of each antigen prioritized on the basis of multivariate and cluster analyses (Table 1 and Fig. 3). We did not observe significant changes in the antibody profiles post-DOTS. This observation suggests that once generated, antibodies are sustained over many months.

Antigens used in this study were chosen on the basis of previous studies that have shown the utility of *M. tuberculosis* antigens in serodiagnosis of TB in various immunoassay formats either individually (e.g., enzyme-linked immunosorbent assay) or in combination (e.g., two-dimensional chip array) (13, 26, 27). More recently, a comprehensive multiplex study based on two-dimensional chip array analysis of gene expression products of over 4,000 open reading frames in the *M. tuberculosis* genome was reported (17). Results showed that only a subset of *M. tuberculosis* antigens (about a dozen) may be useful in serodetection of TB. The selected antigens were as follows: Rv3881, Rv3804, Rv1860, Rv2031c, Rv0934, Rv1980c, Rv1411, Rv3616c, Rv3864, Rv0632, Rv2873, Rv3874, and Rv1984c. The first five of these selected antigens (Rv3881, Rv3804, Rv1860, Rv2031c, Rv0934) are common with those in tier 1 reported in this study, and the sixth antigen (Rv1980c) is in tier 2. There were a few differences in the results of the two multiplex studies. For example, we identified three additional antigens (Rv0054, Rv1886c, and Rv0129c) to be highly discriminatory between TB patients and healthy individuals (Fig. 1). Additionally, antibodies against Rv3874 in our study were not highly discriminatory between TB patients and healthy individuals; therefore, it was included in tier 2 (Fig. 1). Antibodies against Rv1984c were not at all detectable in our study. These differences in results may be due to the different technology platforms used in the two studies. Five other selected antigens (Rv1411, Rv3616c, Rv3864, Rv0632, and Rv2873) listed in the previous study (17) were not included in our study.

Sensitivity and specificity. Data analyses based on two classification algorithms show that the assay sensitivity for TB patients in the AFB^+ group was approximately 90%, while the assay specificity was approximately 80%. Due to a smaller number of patients ($n = 17$), assay sensitivity for the AFB^-/Cul^+ group was calculated on the basis of cutoff values for each microbead set, and it was similar to that for the AFB^+ group (88%). Although inclusion of tier 2 antigens (e.g., Rv3874 and Rv3875) somewhat reduced assay specificity and a relatively small number of patients contained antibodies against them (Fig. 3), Rv3874 and Rv3875 are useful antigens since they are specific to *M. tuberculosis*/*M. bovis* and are absent in environmental mycobacteria and BCG. Certain other antigens (e.g., Rv3804c, Rv1886c, Rv0129c, and Rv1860) are also found in environmental mycobacteria. Therefore, detection of antibodies against these antigens may contribute to a decrease in assay specificity.

Overall, the following conclusions can be drawn: (i) the sensitivity and specificity of multiplex microbead immunoassay strongly suggest that this approach will be useful for TB serodiagnostics in regions of the world where TB is endemic, (ii) antigens selected in this study are sensitive for the detection of *M. tuberculosis* antibodies in TB patients, and (iii) the inclusion of multiple antigens is necessary to achieve specificity and sensitivity comparable to those of sputum methods of smear microscopy and culture.

Results presented in this report clearly demonstrate that the selected antigens and the xMAP technology platform are highly suitable for TB serodiagnostics in pulmonary TB patients. Most importantly, although nonpulmonary TB patients were not included in this study, it is reasonable to propose that this multiplex microbead immunoassay will also be useful for the serodiagnosis of this manifestation of *M. tuberculosis* infection. Approximately 20% of TB cases do not have lung involvement (or have minimal lung involvement) (22). These cases may go undetected by conventional diagnostic methods for long periods of time. A research grant has been awarded by the Regional Office for the Eastern Mediterranean (EMRO) of WHO to perform a field validation study of the multiplex serodiagnostic system in Pakistan. We will assess application of this novel assay for the detection of several forms of TB, including pediatric and nonpulmonary cases, by analysis of a total of 1,200 TB patients and matched controls.

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Any opinions, findings, conclusions, or recommendations expressed are those of the authors and do not necessarily reflect the views of the project sponsors.

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