Tuberculosis (TB) is a major health problem in the developing world as well as a disease which is reemerging as a major health threat in the developed world (6). World Health Organization (WHO) statistics indicate that one-third of the world’s population is currently infected (34). TB is rated the second most common infectious disease and has the highest mortality rate of any infectious disease in the world. Currently there are 30 million cases of active TB worldwide, with approximately 8 million new cases and 3 million deaths reported annually. In addition, 50 million people may already be infected with multidrug-resistant (MDR) strains of Mycobacterium tuberculosis. A high prevalence of TB is also associated with human immunodeficiency virus (HIV) infection and AIDS and is now becoming the leading cause of death among HIV-positive individuals, with a fatality rate of 80% (34).

Control of this disease revolves around good patient care and management. In particular, early detection and treatment of tuberculosis can limit transmission of the bacilli. Conventional tests for the diagnosis of tuberculosis include chest X-ray, direct sputum smear for acid-fast bacilli, culture test, and the skin tuberculosis PPD (purified protein derivative) test (29). Among these, the culture method is time-consuming but reliable. PCR and nucleic acid-based methods for detecting M. tuberculosis DNA sequences require complex equipment and are expensive and unsuitable for routine diagnostic testing in developing countries (5). Rapid serological diagnostic tests such as the enzyme-linked immunosorbent assay (ELISA) and membrane chromatography tests, in contrast, are simple and inexpensive, and the latter can be point-of-care devices (3). A major problem encountered in serological techniques is the specificity and reactivity of antigens used. A majority of M. tuberculosis antigens studied to date have homology with analogous proteins of environmental mycobacteria or other bacteria, resulting in unspecific reactivity to antibodies in patients with inactive TB or nontuberculous infections (8, 23). Hence, positive test results produced by these known antigens are generally unreliable, and supplementary tests are required to confirm tuberculosis infection. It was shown that the use of recombinant M. tuberculosis antigens of specific purity or particular epitopes may enhance the specificity and sensitivity of serological testing for TB when used in a panel of recombinant antigens (1). Rapid diagnostic tests that are specific and sensitive would be useful in both seroepidemiological and clinical studies pertaining to tuberculosis control and prevention. In this report, we describe the identification, isolation, and characterization of five recombinant antigens from M. tuberculosis for use as serodiagnostic markers for tuberculosis.

MATERIALS AND METHODS

M. tuberculosis total-protein extraction. M. tuberculosis cells (ATCC 27294) were cultured in MycoFlasks (Gibco, BRL) containing Lowenstein-Jensen medium at 37°C with 10% CO2 in a humidified incubation chamber (Jouan IG/50 model). Confluent cells from six culture flasks were harvested by adding 3 ml of Middlebrook 7H9 medium (Difco Laboratories, Detroit, Mich.) into each flask and gently flushing the surface of the flask. Dislodged cells were placed into sterile plastic tubes (Falcon), and cells were pelleted by centrifugation at 1,100 × g for 5 min. Cells were then washed once in an equal volume of distilled H2O before being resuspended in an equal volume of distilled H2O. The cell suspension was then heated to 90°C for 2 h and frozen at −20°C overnight. Cells were then thawed on ice and pelleted by centrifugation at 20,000 × g for 10 min. Extraction of total protein was performed by adding 500 μl of 8 M urea solution to 0.5 g of cell pellet, vortexing the cell suspension at room temperature for 20 min, and heating it at 90°C for 2 min. Insoluble cellular debris was removed by centrifugation at 20,000 × g for 10 min, and the supernatant containing the extracted total protein was kept at −20°C until further use.

Western blot analysis. The total-protein extract of M. tuberculosis was fractionated on a sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis (SDS–7.5% PAGE) gel (20) and transferred onto a nitrocellulose membrane by Western blotting (33). Strips from the immunoblot were probed against pooled positive (tested positive by skin PPD and culture) and negative sera from nine individuals with active TB and seven healthy individuals, respectively. Incubation with pooled sera (1:100 in 1% skim milk–TBST [10 mM Tris, pH 7.5, 300 mM NaCl, 0.005% Tween 20]) was carried out with rocking for 1 h at room temperature. The blots were then washed four times in TBST before incubation with alkaline phosphatase-conjugated goat anti-human immunoglobulin (Ig) (Harlan Sera Lab, Loughborough, United Kingdom) (1:1000 in 1% skim milk–TBST) for another 1 hr. The strips were again washed four times in TBST followed by

Cloning and Expression of Immunoreactive Antigens from Mycobacterium tuberculosis

RENEE LAY HONG LIM,† LI KIANG TAN,‡ WAI FUN LAU,§ MAXEY CHING MING CHUNG,∥ ROSEANNE DUNN,¶ HENG PHON TOO,‡ AND LILY CHAN†

Bioprocessing Technology Centre† and Department of Biochemistry,‡ The National University of Singapore, Singapore

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Four immunoreactive proteins, B.4, B.6, B.10, and B.M, with molecular weights ranging from 16,000 to 58,000, were observed from immunoblots of Mycobacterium tuberculosis total lysates screened with sera from individuals with active tuberculosis. These proteins were identified from microsequence analyses, and genes of proteins with the highest homology were PCR amplified and cloned into the pQE30 vector for expression studies. In addition, a 37.5-kDa protein, designated C17, was identified from a phage expression library of M. tuberculosis genomic DNA. Preliminary immunoblot assays indicated that these five resultant recombinant proteins could detect antibodies in individuals with active pulmonary and extrapulmonary tuberculosis. The overall ranges of sensitivities, specificities, positive predictive values, and negative predictive values for the recombinant antigens were 20 to 58, 88 to 100, 69 to 100, and 56 to 71%, respectively. The B.6 antigen showed preferential reactivity to antibodies in pulmonary compared to nonpulmonary tuberculosis serum specimens. All of these recombinant antigens demonstrated potential for serodiagnosis of tuberculosis.

Materials and methods

M. tuberculosis total-protein extraction. M. tuberculosis cells (ATCC 27294) were cultured in MycoFlasks (Gibco, BRL) containing Lowenstein-Jensen medium at 37°C with 10% CO2 in a humidified incubation chamber (Jouan IG/50 model). Confluent cells from six culture flasks were harvested by adding 3 ml of Middlebrook 7H9 medium (Difco Laboratories, Detroit, Mich.) into each flask and gently flushing the surface of the flask. Dislodged cells were placed into sterile plastic tubes (Falcon), and cells were pelleted by centrifugation at 1,100 × g for 5 min. Cells were then washed once in an equal volume of distilled H2O before being resuspended in an equal volume of distilled H2O. The cell suspension was then heated to 90°C for 2 h and frozen at −20°C overnight. Cells were then thawed on ice and pelleted by centrifugation at 20,000 × g for 10 min. Extraction of total protein was performed by adding 500 μl of 8 M urea solution to 0.5 g of cell pellet, vortexing the cell suspension at room temperature for 20 min, and heating it at 90°C for 2 min. Insoluble cellular debris was removed by centrifugation at 20,000 × g for 10 min, and the supernatant containing the extracted total protein was kept at −20°C until further use.

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incubation in 1 ml of bromochloroindolyl phosphate-nitroblue tetrazolium substrate (NBT-BCIP; Bio-Rad) for 4 min. The reaction was stopped by washing four times in distilled H2O.

**N-terminal sequencing.** Individual protein bands (which were shown to react positively in the immunoscreening experiment) were excised from several preparative SDS–7.5% polyacrylamide gels and concentrated by redextrophoresis (constant current of 18 mA at 8°C) on a long stacking gel (17 cm of 4% stacking gel, 5 cm of 10% resolving gel). The concentrated protein bands were blotted onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad), stained with Coomassie brilliant blue R-250 (Sigma, St. Louis, Mo.), and excised for N-terminal microsequencing. The protein bands were also blotted onto Hybond-C nitrocellulose membranes (Amersham Life Science, Little Chalfont, United Kingdom) for validation by immunoscreening using the same pooled sera samples as described above.

**Screening of a phage expression library.** An expression library of EcoRI-restricted genomic DNA of *M. tuberculosis* was constructed in lambda ZAP phage expression vector, according to the protocol by Stratagene (ZAP Express cDNA Synthesis manual, Stratagene Cloning Systems, 1998). The resulting library has 98% recombinants (2 × 10⁸ PFU/μg arms) and insert sizes ranging from 0.7 to 2 kb. A lawn of XL1-MRF* host cells infected with about 2 × 10⁹ PFU of the phage stock was prepared on a 150-mm plate and incubated for 6 to 7 h at 42°C. The lawn was then overlaid with a Hybond-C nitrocellulose membrane (Amersham) presoaked in 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for induction of protein expression by further incubation at 37°C for 4 h. The plate and membrane were indexed for matching corresponding plate and membrane position. Approximately 0.4 cm² of each protein antigen was electroblotted onto Hybond-C nitrocellulose membrane (Amersham Life Science, Little Chalfont, United Kingdom) for visualization by staining either with Coomassie brilliant blue R-250 or with silver stain (27).

**SDS-PAGE and Western blotting of *M. tuberculosis* recombinant antigens.** To maintain consistency, Tris-HCl two-dimensional (2D) preparative ready gels (Bio-Rad) were used for Western blotting. A total of 10 μl of purified recombinant antigen was subjected to SDS-PAGE and Western blotted onto Hybond-C nitrocellulose membranes (Amersham) using the Bio-Rad TransBlotter (according to the manufacturer’s protocol). After transfer, the membrane was blocked in 5% skim milk-TBST, and the blots were probed with a positive serum specimen that is reactive to the recombinant protein antigens. A second strip was probed with the commercially available anti-RGS His probe (Qiagen). Screening was carried out by incubating each strip in trays with 1 ml of diluted serum specimen (1:100 in 1% skim milk-TBST) per well for 1 h with rocking at room temperature. The strips were then washed four times in TBST, followed by incubation with alkaline phosphatase-conjugated goat anti-human Ig (Harlan Sera Lab) for 1 h with rocking at room temperature. The strips were again washed four times in TBST and then allowed to develop in 1 ml of NBT-BCIP substrate (Bio-Rad) for 4 min. The reaction was stopped by washing four times in distilled H2O.

**Western blot score.** The reactivities of recombinant proteins to serum specimens were interpreted based on the intensities of bands obtained on an X-Rite 400 densitometer (X-Rite Inc., Grandville, Mich.). The cutoff values (expressed as densities) for each individual recombinant antigen were determined based on the range observed for normal serum specimens.

**Serum specimens.** A total of 139 human sera were used in this study, of which 119 serum specimens were purchased from BioClinical Partners, Inc., Franklin, Mass., whereas 20 were donated by healthy laboratory workers. The control groups consisted of two panels, (i) a panel of 50 serum specimens from healthy individuals, of whom 20 (laboratory workers) had been Mycobacterium bovis BCG vaccinated previously and 30 (BioClinical) had unknown BCG status, and (ii) a panel of 19 serum specimens (BioClinical) from individuals with non-TB respiratory disease (lung cancer). The test group consisted of 48 serum specimens (BioClinical) from bacteriologically confirmed active TB patients and 22 serum specimens (BioClinical) from patients with inactive TB. The active-TB serum specimens comprised 28 from pulmonary-TB and 20 from extrapulmonary-TB patients. Serum specimens from the inactive-TB panel were from patients with positive PPD skin tests but negative acid-fast stains of sputum and bacterial culture. All sera were aliquoted and stored at –70°C before use.
TABLE 1. Results of homology searches against the GenBank protein sequence databases

| Relative molecular size (kDa) | N-terminal sequence | Match (NCBI) |
|------------------------------|---------------------|--------------|
| ~58                          | SKLIEYDELALAEAME     | db: _SKLIEYDETARRAME$_{166}$, 55.7 kDa; GroEL1/ribosomal protein Cpn60 (18, 30); pID = g44601; X60350 (80% match) |
| ~48                          | AEVDAYKFDPDAVD       | db: _AEFDAYRDPMA$_{225}$, probable exported protease, has signal sequence, very similar to three proteases/peptidases from Streptomyces; pID = e235164; MT6427.04c (51% match) |
| ~34                          | MEIDILAVAAP          | db: _IEVDLDDLAD$_{125}$, 33 kDa; mycocerosic acid synthase; pID = g149978; M95808 (56.9% match) |
| ~14                          | ATTLPVQRHDLAR        | db: ATTLPVQRHPRSL, 14/16 kDa (21); pID = g244562; M76712 (69.0% match) |

* Proteins showing the highest homology to the M. tuberculosis proteins excised for N-terminal sequencing are shown.

** Database and software.** Nucleotide and protein sequence analysis was carried out using the basic BLAST 2.0 search program from the National Center for Biotechnology Information (NCBI) and the M. tuberculosis BLAST server at the Sanger Centre (Cambridge, United Kingdom).

** Statistical analysis.** Sensitivities, specificities, and positive and negative predictive values were calculated using the Win Episcope 1.0 (Borland International Inc.).

RESULTS

Identification and isolation of M. tuberculosis antigens. Immunoblot analysis of M. tuberculosis total proteins revealed protein bands that reacted with the pooled active sera but not with the pooled normal sera. When the respective bands were concentrated on a long stacking gel, excised, and Western blotted, these bands were reactive with the pooled active sera but not with pooled normal sera, thus confirming the authenticity of these excised proteins as those initially observed in the primary screening (Fig. 1). These proteins were identified by homology searches against protein sequence databases, which gave a high percentage of homology to Mycobacterium proteins (Table 1). PCR primers were designed to isolate and clone the genes coding for four proteins (B.4, B.6, B.10, and B.M) which gave the highest matches based on a BLAST homology search against the SwissProt database.

Concurrently, primary screening of the phage expression library gave eight reactive phage recombinants, of which six were further confirmed positive by secondary and tertiary screenings (Fig. 2). These clones were subjected to plasmid DNA excision, and restriction enzyme digestions with EcoRI indicated that all the clones contained a 2-kb insert. DNA sequencing revealed that all the clones were identical, having a 1.161-kb open reading frame (in frame with the vector’s ATG initiation codon) which coded for a proline-rich protein. A summary of all five TB antigens, with the respective gene sizes, theoretical molecular masses, and pl values is shown in Table 2.

Expression and purification of recombinant antigens. Expression was detected by probing immunoblots containing these antigens using the commercial anti-RGS His antibody. The levels of expression observed were high for the B.4, B.M, and 38-kDa proteins, moderate for the B.6 and B.10 proteins, and low for the C17 protein (Fig. 3). All of these recombinant proteins, except for C17, were present in the insoluble fraction of an SDS-PAGE analysis (data not shown), indicating that these proteins formed inclusion bodies and were insoluble. As such, these recombinant proteins were purified by Ni-NTA affinity chromatography in 8 M urea, and the SDS-PAGE profile of the purified antigens subsequently used for immunoblots is shown in Fig. 4. The approximate yields of recombinant antigens purified through Ni-NTA were 36 mg/liter for B.4, 0.5 mg/liter for B.6, 0.2 mg/liter for B.10, 15 mg/liter for B.M, <0.1 mg/liter for C17, and 10 mg/liter for the 38-kDa protein.

Reactivities of the recombinant antigens to TB serum specimens. The different reactivities of recombinant TB antigens on immunoblot strips probed with serum specimens and anti-RGS His are shown in Fig. 5. The respective cutoff values for determining reactivity to the different recombinant antigens were obtained based on the mean densities observed in sera from the control group of healthy individuals (n = 50). The cutoff values were densities of >0.04 for the B.4 and B.6 bands, ≥0.04 for the B.10 and B.M bands, ≥0.15 for the C17 band, and >0.15 for the 38-kDa band.

Based on the Western blot assay, the reactivities of these antigens to a panel of active-TB serum specimens are shown in Table 3. Percentages of reactivities and positive and negative predictive values for each antigen were calculated based on sera from infected individuals (n = 48; pulmonary and extrapulmonary TB) and sera from the control group of healthy individuals (n = 50), with a P value of <0.05. The specificities for the B.4, B.6, B.10, B.M, C17, and 38-kDa recombinant antigens are 94, 88, 100, 96, 90, and 98%, respectively. All of the recombinant TB antigens showed substantial reactivity to

(a) Primary screening; (B and C) secondary screening; (D) negative control consisting of plaques of nonrecombinant phages. Arrows indicate plaques containing positive recombinant phage.
active-TB specimens, both pulmonary and extrapulmonary. The B.4 antigen was reactive with 58.3% of the active-TB panel, compared to 37.5% detected by the known 38-kDa antigen. In addition, the B.4 antigen showed reactivity to 27.3% of the inactive-TB specimens compared to other TB antigens, which exhibited lower percentages of reactivity to these specimens (Table 3). The B.6 antigen was found to exhibit specific reactivity to pulmonary-TB specimens (46.4%) compared to extrapulmonary specimens (5%) (Fig. 6). All the other antigens were able to detect antibodies in both pulmonary- and extrapulmonary-TB specimens.

DISCUSSION

A number of M. tuberculosis antigens have been identified and characterized by various methods employing polyclonal antibodies from rabbits or monoclonal antibodies (MAbs) from hybridomas generated from immunized mice. Such antibodies were used widely for identification and purification of protein antigens by affinity chromatography (22), immunoscreening of clones from DNA libraries of M. tuberculosis (24, 37), and analyses of total-cell lysates or secretory proteins from culture medium by both one- and two-dimensional gel electrophoresis (16, 32). Immunogenicity in animals (e.g., mice or rabbits), however, may not reflect relevance to human immune responses. Thus, attempts were made to search for candidate serodiagnostic antigens by directly testing mycobacterial proteins with tuberculous-patient sera on immunoblots of one-

**TABLE 2. TB antigens genes which were cloned and expressed in pQE30**

| Antigen | Size of gene (kb) | Theoreticala: Molecular mass (kDa) | pI |
|---------|------------------|----------------------------------|----|
| B.4     | 1.617            | 55.8                             | 5.12 |
| B.6     | 1.560            | 55.0                             | 5.03 |
| B.10    | 0.903            | 32.9                             | 4.95 |
| B.M     | 0.432            | 16.1                             | 5.00 |
| C17     | 1.161            | 37.5                             | 9.43 |

a The resultant recombinant proteins will be approximately 1.4 to 1.5 kDa larger than the theoretical molecular mass shown, due to the histidine tag at the N terminus.

b Obtained using the software “Compute pI/Mwt” from the ExPASy home page, Swiss Institute of Bioinformatics, Geneva, Switzerland.

FIG. 3. Expression and affinity chromatography purification profiles of the M. tuberculosis antigens expressed in 100 ml of M15/E. coli cultures. Lanes: M and Ms, protein molecular weight markers (M, Kaleidoscope standards; Ms, Sigma Broad range); 1 through 4, aliquots taken at 0, 1, 2, and 3 h, respectively, after induction with 1 mM IPTG; B, total cell lysate before passing through Ni-NTA column; A, total lysate after passing through column; W, wash fractions in 8 M urea buffer (pH 6.5 to 5.9); E1 to E7, eluted fractions in 8 M urea buffer (pH 4.5). The bulk of the recombinant proteins were observed to be eluted in fractions in 8 M urea buffer (pH 4.5). The bulk of the recombinant proteins were observed to be eluted in fractions E2 and E3 (arrows). All the gels were stained with Coomassie brilliant blue, except for C17, which was silver stained.
dimensional and two-dimensional separations of antigenic extracts or culture filtrates of *M. tuberculosis* H37Rv (4, 26).

In this study, we used antibodies present in the sera of infected individuals to screen total-cell lysates and a phage expression library of *M. tuberculosis* DNA. To date, there is no single immunodominant species-specific antigen for detection of tuberculosis. We have chosen to use pooled sera from several infected individuals to allow identification of several immunoreactive antigens reactive to antibodies present in each serum. In addition, the *M. tuberculosis* genome database completed by the Sanger Centre (12) allowed for the rapid identification of these immunoreactive antigens by homology searches against available protein and gene databases, which also facilitated the identification of these gene sequences for cloning.

We have successfully identified and characterized five antigens using a Western blot total-cell-lysate approach; of these, the B.6, B.10, and C17 antigens are novel and showed high degrees of nucleotide identity to unpublished *M. tuberculosis* H37Rv genes. Based on DNA sequencing results, the B.6 antigen gene was found to have 99% nucleotide identity to a gene coding for a protein with homology to exported proteases or peptidases. The B.10 antigen exhibited 99 and 98% nucleotide identities to the *M. bovis* acyl coenzyme A (CoA) synthase (accession no. U75685) and mycocerosic acid synthase (accession no. M95808) genes, respectively. The C17 antigen exhibited 99 to 100% nucleotide identity to a gene coding for PE-PGRS (polymorphic GC-rich repetitive sequence) proteins, a member of PE (proline-glutamic acid) families of clustered genes coding for glycine-rich proteins which may have immunological and pathogenic implications (12).

The B.4 antigen exhibited 99.8% amino acid and 98.9% nucleotide sequence homology to the Cpn-60 protein reported by Kong et al. (18). The diagnostic potential of a 65-kDa protein (also a Cpn-60 family of heat shock proteins) by both serological and PCR methods has been demonstrated (28).

The B.M antigen has 99% nucleotide identity to the reported *M. tuberculosis* 14-kDa antigen gene (accession no. M76712) and the gene for the 19-kDa major membrane protein purified from the virulent Erdman strain of *M. tuberculosis* (21). The serological value of this 19-kDa antigen was shown by 85% reactivity to a panel of 56 sera from individuals with active pulmonary TB (9).

Antigens of diagnostic importance for *M. tuberculosis* identified to date include the 65-, 45-, 30/31-, 19-, and 12-kDa proteins and the 38-kDa lipoprotein (9, 11, 14, 36). Among
these, the 38-kDa protein was shown to be the most specific and sensitive for detecting antibodies against \textit{M. tuberculosis} and is specific for TB complex species (35). As such, we have chosen to clone and express this antigen to be used in immunoscreening against the serum panels for comparison with our recombinant TB antigens. This 38-kDa antigen was expressed as an insoluble protein in the pQE30/\textit{E. coli} system; similarly, Singh et al. reported the expression of this 38-kDa antigen as an insoluble unfused protein in \textit{E. coli} (31).

We have chosen the Qiagen expression system for cloning and expression of the TB antigens. Each expressed recombinant protein contained a nonimmunogenic 6× His tag at the N terminus which could be immunodetected by anti-RGS His. A substantially low expression level was observed for the C17 protein. This may be due to the codon usage of this protein, which is rich in proline (46.6%), as it is reported that the expression level of a gene decreases with an increase in the use of rare codons (17). In addition, the Kyte-Doolittle hydropathy plot revealed that it is very hydrophilic, which explains the soluble nature of this protein (19). As most of the recombinant antigens were insoluble, we have chosen a Western blot assay for preliminary screening against serum specimens.

Immunoblot assays using human sera were described previously for analysis of HPLC-purified 45/47-kDa antigen complex (15). Rovatti et al. reported a semiquantitative Western blot serological test to identify PPD-positive individuals, using a discriminate score for the \textit{M. bovis} BCG antigen complex A60 against MAbs (25). In our immunoblot assay system, we used affinity-purified recombinant proteins to detect antibodies in serum specimens and have included the known 38-kDa lipoprotein as a control for our purification and Western blot assay system. Zhou et al. reported the use of this antigen in a rapid membrane-based assay that gave a specificity of 92%, very close to our in-house immunoblot assay of the 38-kDa protein, which gave 98% specificity (38). The 38-kDa antigen was included in the assay system as a further control for specificity and sensitivity. This was further compared to a commercially available diagnostic test kit which uses two antigens, one of which is the 38-kDa antigen. All 13 of the 18 active-TB serum specimens that tested positive with the 38-kDa protein in our Western blot assay also tested positive with the kit. The percentage of specificity of the 38-kDa antigen in this assay is comparable to that with the kit (98 and 100%, respectively).

Data analysis from our assay system indicated that the B.4, B.6, B.10, B.M, C17, and 38-kDa recombinant antigens reacted with antibodies in serum specimens of TB-infected individuals. The reactivities were sufficiently differentiated from those of serum specimens from healthy individuals and from individuals with inactive TB or lung cancer. Screening of the same serum panels using two commercially available TB diagnostic kits of high specificity indicated the presence of immunologically specific antibodies reacting to these TB antigens (submitted for publication). Reports have also shown that other recombinant antigens did exhibit low levels of cross-reactivities to sera from healthy individuals, which may be due to cross-reactive epitopes or analogues to other bacterial proteins (8). To circumvent this problem, the use of MAbs with recombinant antigens in competition with patient sera as a test assay was reported to give a high degree of specificity (10). Alternatively, we have to further optimize the immunoblot assay to decrease cross-reactivities.

In conclusion, we have demonstrated the identification and cloning of five TB antigen genes for expression in an \textit{E. coli} system and shown the potential of each recombinant antigen as a serodiagnostic marker for detection of TB infections. We are in the process of validating the diagnostic utility of these antigens in various test formats.

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