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Improved Sensitivity of Diagnosis of Tuberculosis in Patients in Korea via a Cocktail Enzyme-Linked Immunosorbent Assay Containing the Abundantly Expressed Antigens of the K Strain of *Mycobacterium tuberculosis*[▽]

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Tuberculosis (TB) is the leading cause of death from a single infectious agent in Korea. In this study, we compared the proteins present in culture filtrates from *Mycobacterium tuberculosis* strain K, which is the dominant clinical isolate in Korea, with those present in culture filtrates from *M. tuberculosis* H37Rv. Several differences in expression were detected between the two strains for those proteins with a molecular mass of <20 kDa. ESAT-6, HSP-X, and CFP-10 were found to be abundantly expressed in the strain K culture filtrates by liquid chromatography-electrospray ionization-time of flight mass spectrometry. The serodiagnostic potentials of recombinant antigens rESAT-6, rHSP-X, and rCFP-10 and two native antigens (Ag85 and PstS1) were evaluated by Western blot analysis and enzyme-linked immunosorbent assay (ELISA) using sera collected from 46 TB patients with active disease and 46 healthy controls. As for our ELISA results, HSP-X was superior to the other antigens in terms of sensitivity when a single antigen was employed. The results of a receiver operator characteristic analysis revealed that a cocktail ELISA using all five antigens was significantly more sensitive (77.8%) than the use of a single antigen and offered equivalent specificity; moreover, it produced the largest area under the curve (0.91 versus 0.55 to 0.87). Therefore, a cocktail ELISA containing abundantly expressed antigens enhances the sensitivity of a single antigen and can be a useful diagnostic tool for the detection of active TB.

Tuberculosis (TB) is a chronic infectious disease caused by an intracellular pathogen, *Mycobacterium tuberculosis*, and is a major worldwide health problem. According to the World Health Organization (WHO), one-third of the world's population is latently infected with the bacterium and more than 10 million new cases of TB and 2 million deaths occur across the globe annually (11). Controlling the incidence of TB depends on rapid, sensitive diagnostic tests, efficient drug treatment, and the development of an improved vaccine. Currently, attenuated *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is the only available vaccine against TB, but it has proven to be problematic because of inconsistent protection and false-positive results during diagnosis due to cross-reactivity (4, 12). The purified protein derivative of *M. tuberculosis* is the most commonly used antigen for the diagnosis of TB, but its use is also problematic because of poor specificity due to cross-reactivity with antigens from other *Mycobacterium* species (36). Thus, an individual's BCG vaccination status and the prevalence of environmental mycobacteria are important factors to consider in the diagnosis of TB.

Since accurate and reliable diagnostic methods for *M. tuberculosis* infection are urgently required for the global control of this pathogen, the diagnostic potential of *M. tuberculosis*-specific antigens has been explored. In an attempt to identify sensitive and specific serodiagnostic antigens or novel immunogenic antigens of *M. tuberculosis*, culture filtrates (CFs) from *M. tuberculosis* were discovered to contain several highly immunogenic antigens that were recognized by the sera of patients with TB (30, 34). Among those antigens, ESAT-6 and CFP-10 have been noted for their specificity and sensitivity in vitro and in vivo for diagnoses based on interferon stimulation (50, 51). However, the usefulness of these antigens in serodiagnosis is greatly limited in terms of sensitivity (<73%), although both antigens can be used serologically to distinguish between TB and other mycobacterial infections (15, 46). Several studies have suggested that to improve serum-based methods for the detection of TB, a cocktail containing the strongest antigens should be constructed due to the diverse immune responses of individuals (1, 19, 39). The benefits of using combinations of these and other immunogenic antigens should be investigated to overcome problems with sensitivity during serological diagnostic testing.

In the postgenome era, comparative proteomic techniques have been used to identify differentially expressed antigens among endemic, epidemic, and pandemic strains of *M. tuberculosis* in several countries (35, 42). Various studies have shown numerous differences in protein expression between different laboratory strains of *M. tuberculosis*, such as H37Rv and Erdman (23, 31), and these differences may facilitate a

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better understanding of the mechanisms underlying pathogenesis and virulence. Moreover, proteomic analysis of the *M. tuberculosis* strain that is prevalent in a particular country will help to determine which antigens must be considered for the diagnosis of TB. *M. tuberculosis* strain K of the Beijing family is the most-common clinical isolate from TB patients in Korea. Previous studies identified case clustering among patients with pulmonary TB from a screen of Korean high school students (24). The organisms involved in clustered cases of TB are reported to have increased virulence such that they are able to spread across broad areas and produce numerous infections (13).

In this study, we compared the differentially expressed proteins in CFs from *M. tuberculosis* H37Rv and *M. tuberculosis* strain K by using a proteomic approach. We then evaluated the serodiagnostic potential of five of the antigens (recombinant antigens rCFP-10, rESAT-6, and rHSP-X and native antigens Ag85 and PstS1, also known as 30- and 38-kDa antigen) individually or in combination by using an enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Bacteria growth and CF antigen preparation. *M. tuberculosis* H37Rv (ATCC 27294) and *M. tuberculosis* strain K were initially cultivated in 7H9 broth supplemented with 10% (vol/vol) oleic acid-albumin-dextrose-catalase (OADC; Becton Dickinson, Cockeysville, MD) for 1 month at 37°C. Single-cell suspensions of each strain were then prepared by agitation in the presence of glass beads and quantified by plating on 7H10-OADC agar (8). Seed lots of each strain were kept in small aliquots at −80°C until use.

The CF antigens were produced from each strain by inoculating 100 μl of a seed lot culture containing 10^9 CFU/ml into 35 ml of modified Watson-Reid broth (mWR) or Sauton's synthetic medium (44). After incubation at 37°C for 6 weeks, the bacilli were removed by filtration through filter paper (Whatman International Ltd., Kent, United Kingdom). The culture supernatants were sequentially sterilized by using membrane filters (1.2- and 0.2-μm pore size) and concentrated by ultrafiltration (Amicon ultracentrifugal filter unit with a 3-kDa molecular-mass cutoff; Millipore, Bedford, MA). The concentration of each CF antigen was determined by using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

Human sera. To assess the humoral immune response of five proteins (native 30- and 38-kDa antigen plus rHSP-X, rCFP-10, and rESAT-6), serological experiments were conducted using serum samples collected from human immunodeficiency virus-seronegative individuals. Sera were obtained from 46 patients with TB (37 males and 9 females, all Koreans, whose ages ranged from 14 to 80 years) and 46 healthy controls (23 males and 22 females, all Koreans, with a mean [±standard deviation] age of 25 ± 4 years) without a previous history of clinical TB. Patients with active pulmonary TB (n = 46) who had been treated with anti-TB medications for less than 1 month were enrolled at the Department of Internal Medicine of Konyang University Hospital (Daejeon, South Korea). A basic diagnosis of TB was determined by culture and clinical evaluation, such as chest X-ray results. Only six patients had smear positivity and culture negativity. None of the subjects had any previous history of diabetes mellitus or steroid therapy, and all were negative for human immunodeficiency virus. Control sera were obtained from 45 healthy students at Chungnam National University (Daejeon, South Korea) who had no previous history of clinical TB.

Two-dimensional polyacrylamide gel electrophoresis (PAGE). The CF antigens were prepared by using a two-dimensional clean-up kit (Amersham Biosciences, Uppsala, Sweden). Each sample was separated in the first dimension by using 7- or 11-cm immobilized pH gradient (IPG) strips with a pH range of 3.9 to 5.1 or 4 to 7 (Bio-Rad, Hercules, CA). The samples were then focused by using a Protean isoelectric-focusing cell (Bio-Rad) as follows: 250 V for 30 min, from 250 to 4,000 V for 2 h, and 4,000 V for 20,000 Vh in the case of the 7-cm IPG strip and 250 V for 30 min, from 250 to 8,000 V for 2 h, and 8,000 V for 35,000 Vh in the case of the 11-cm IPG strip. The IPG strips were equilibrated prior to running in the second dimension. Electrophoresis in the second dimension was performed as described by Laemmli using 10 to 20% precast gels (Bio-Rad) (25). The gels were stained with 0.25% Coomassie brilliant blue R250 (Bio-Rad).

Protein identification. The identification of the protein spots on the stained gels was performed at the Yonsei Proteomics Research Center (Yonsei University, Seoul, South Korea) by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). Briefly, nano-LC–tandem MS (MS-MS) was performed on an Agilent 1100 series nano-LC and LTQ-MS (Thermo Electron, San Jose, CA). The capillary column used for LC–MS-MS (150 mm by 0.075 mm) was obtained from Proxeon (Odense, Denmark) and slurry packed in house with a 5-μm, 100-Å-pore-size Magic C_{18} stationary phase (Michrom BioResources, Auburn, CA). For LC, mobile phase A was 0.1% formic acid in deionized water, while mobile phase B was 0.1% formic acid in acetonitrile. The chromatographic gradient was set up to produce a linear increase in B from 5 to 35% over 50 min, 40 to 60% over 20 min, and 60 to 80% over 5 min. The flow rate was maintained at 300 nl/min after splitting, and mass spectra were acquired by using data-dependent acquisition with a full mass scan (400 to 1,800 m/z) followed by MS-MS scans. Each MS-MS scan acquired was an average of one microscan on the LTQ. The temperature of the ion transfer tube was maintained at 200°C, and the spray was 1.5 to 2.0 kV. The normalized collision energy was set at 35% for MS-MS. Sequest software was used to identify the peptide sequences. For high-confidence results, the following cutoffs were used for protein identification: deltaCn = 0.1, Rsp = 4, Xcorr = 1.5 with charge state 1+, Xcorr = 2.0 with charge state 2+, Xcorr = 2.5 with charge state 3+, and peptide probability > 0.1. The methionine residues in the peptides were variably oxidized, while the cysteines were variably carboxyamidomethylated or carboxymethylated.

Antigen preparation. Five antigens (two native and three recombinant) were prepared. The two native antigens, 30-kDa antigen and 38-kDa antigen, were purified from *M. tuberculosis* CFs by using the approach described by Lee et al., (27), while the recombinant plasmids encoding *esat-6* and *hsp-x* were provided by Colorado State University (TB Vaccine Testing and Research Materials); *cfp-10* was cloned using pET28. To produce rCFP-10, the corresponding genes were amplified by PCR using *M. tuberculosis* strain K genomic DNA as template and the following primers: forward, 5′-GGC CGG GGA TCC ATG GCA GAG ATG AAG ACC G-3′, and reverse, 5′-GGC CGG GAA TTC GAA GCC CAT TTG CGA GGA C-3′. The products were cut with BamHI and EcoRI and then inserted into pET28a. The recombinant plasmids (encoding *cfp-10*, *esat-6*, and *hsp-x*) were transformed into *Escherichia coli* BL21 cells carrying bacteriophage DE3 for protein overexpression. Cultures were grown at 37°C until the optical density (OD) at 600 nm was 0.4 to 0.5 and then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; ELPIS-Biotech, Daejeon, South Korea). The cells were then harvested by centrifugation; suspended in 20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 20 mM imidazole, and 1 mM phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO); and lysed by sonication. The recombinant proteins were purified by nickel-nitrilotriacetic acid (Ni-NTA) agarose chromatography in accordance with the manufacturer's instructions (Invitrogen, Carlsbad, CA). Each purification step was analyzed by sodium dodecyl sulfate (SDS)-PAGE with Coomassie brilliant blue staining and immunoblotting using anti-His antibodies (Invitrogen).

Antibody production. To obtain antisera for rCFP-10 and rESAT-6, BALB/c mice were immunized intraperitoneally with purified rCFP-10 and rESAT-6 emulsified in incomplete Freund's adjuvant. The mice were injected with the antigen three times at 2-week intervals and were killed 1 week after the final immunization; thereafter, serum samples were collected and stored at −70°C.

Partial purification of native CFP-10 and ESAT-6 complex. The CFP-10 and ESAT-6 antigens were partially purified from CFs of *M. tuberculosis* H37Rv and strain K by a three-step process. In brief, the 0-to-45% ammonium sulfate precipitate of CFs was suspended in 50 mM phosphate buffer (pH 6.8) containing 1 M ammonium sulfate and then loaded onto an Econo-Pac methyl HIC cartridge (Bio-Rad) for hydrophobic interaction chromatography. The cartridge was washed with the same buffer and then eluted with a decreasing ammonium sulfate gradient. The eluates were analyzed by SDS-PAGE. The fractions containing CFP-10 and ESAT-6 complex were pooled and dialyzed against 10 mM phosphate buffer (pH 6.8). Further purification was performed by anion exchange chromatography using gradient elution of 10-to-200 mM phosphate buffer and hydrophobic interaction chromatography using the same conditions as for the first step. The final purified antigens were dialyzed against phosphate-buffered saline (PBS), filter sterilized, and stored at −70°C.

Immunoblotting. Protein transfer from the polyacrylamide gels to nitrocellulose membranes (0.45-μm pore size; Bio-Rad) was performed as described by Davies et al. using a Tris-glycine buffer containing 0.0375% SDS and 20% methanol (9). Prior to the addition of antigen-specific antibodies or human serum, the membranes were incubated for 2 h in blocking buffer (5% skim milk in PBS). The proteins were reacted overnight with the antibodies at 4°C on a rocking platform. For the detection of CFP-10 and ESAT-6, horseradish peroxidase (HRP)-conjugated secondary antibodies against mouse immunoglobulin G

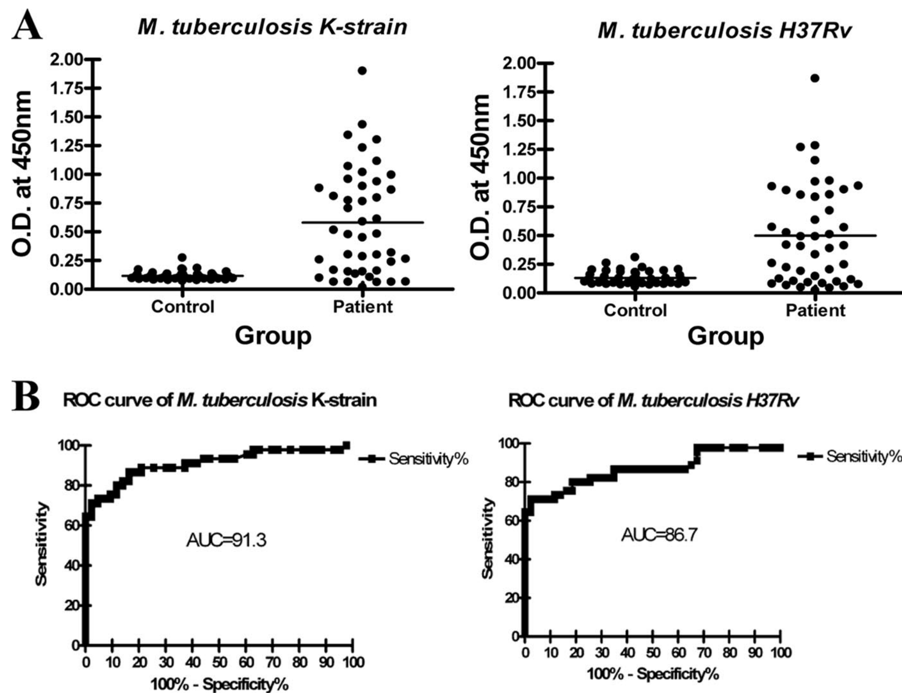

FIG. 1. Antibody responses to CFs of *M. tuberculosis* strain K and *M. tuberculosis* H37Rv using ELISA. The CFs were tested against the sera of 46 patients who were AFB positive with active pulmonary TB and 46 healthy controls. All results are expressed in terms of the S/N ratio (A) and were compared by ROC analysis (B). Significantly higher antibody responses to the CF from *M. tuberculosis* strain K than to the CF from strain H37Rv were detected (AUC = 0.91).

(IgG; Sigma), human serum, and HRP-conjugated secondary antibodies against human IgG (Sigma) were used. All blots were developed by using 3,3′-diaminobenzidine tetrahydrochloride (Sigma) in 20 mM Tris-buffered saline (pH 7.6) with 30% hydrogen peroxide (H_2O_2).

ELISA. ELISA was performed as described by Voller (48). Briefly, polystyrene 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with different antigens overnight at 4°C. ESAT-6, CFP-10, 30-kDa antigen, and 38-kDa antigen, as well as the CFs of *M. tuberculosis* H37Rv and strain K, were each added at 1 μg/ml (0.1 μg/well) in fresh 10 mM PBS (pH 7.4). In each assay, 0.5 μg/ml antigen in the same buffer (0.05 μg/well) was used. The plates were washed three times with PBS plus Tween 20 (PBST) and then blocked with 300 μl/well of 3% (wt/vol) bovine serum albumin in PBST at room temperature for 2 h. After three more washes, 100 μl/well of human serum (1:100 dilution) was added to each plate, and the plates were incubated at room temperature for 1 h. The plates were then washed as described above, and 100 μl of peroxidase-conjugated goat anti-human IgG (1:6,000 dilution; Sigma) was added to each well; the plates were then incubated for an additional 1 h at room temperature. After seven more washes, the reaction was visualized by using tetramethylbenzidine (Sigma) and 0.5% (vol/vol) H_2O_2. The reaction was stopped with 1 N sulfuric acid (H_2SO_4) after 5 min of incubation in the dark. The OD was measured at 450 nm by using an ELISA microplate reader (Molecular Devices, Sunnyvale, CA).

Evaluation of tests and statistical analysis. A receiver operator characteristic (ROC) curve analysis was performed on the ELISA results for each antigen to determine the cutoff point for distinguishing between a positive and negative result. Differences in mean ODs for sera from TB patients and healthy subjects also were evaluated by the Mann-Whitney test. Most statistical analyses were performed by using statistical software (GraphPad Prism version 4.03 for Windows; GraphPad Software, San Diego, CA). The ELISA *a*rea *u*nder the ROC *c*urve (AUC) values were compared by a manual calculation using established methods (14, 17). An ROC curve (a plot of the true positive rate [% sensitivity] against the false-positive rate [100% − % specificity] obtained at each cutoff point) was constructed, and the AUC was determined and compared with the AUC for the acid-fast bacillus (AFB) smear, which was used as an indicator of TB in this study.

RESULTS

Comparison of the CFs from *M. tuberculosis* H37Rv and strain K by serological testing. The CFs from *M. tuberculosis* H37Rv and strain K were tested for their ability to detect antibodies in the sera of 46 patients who were AFB positive with active pulmonary TB and the sera of 46 healthy controls by ELISA. Although similarities were observed in terms of sensitivity and specificity, the results of the ELISA based on the *M. tuberculosis* strain K CF antigens had a higher mean signal-to-noise (S/N) ratio (mean OD of sera from TB patients/mean OD of sera from healthy controls) for the patients with TB than those of the ELISA based on the CF antigens from *M. tuberculosis* H37Rv (mean S/N ratio, 3.34 versus 2.06, respectively; $P < 0.05$). In addition, the CF antigens from *M. tuberculosis* strain K provided a more-accurate ELISA based on the ROC AUC than those from *M. tuberculosis* H37Rv (Fig. 1). These results suggest that some serologically important proteins were comparably included in CFs from *M. tuberculosis* strain K.

Identification of strongly expressed antigens in the CF of *M. tuberculosis* strain K. To investigate the proteins responsible for producing the ELISA results, the CF antigen expression patterns of *M. tuberculosis* H37Rv and strain K were compared. Both strains were cultivated in modified Sauton's (pH 7.2) and mWR (pH 6.0) medium.

The CF antigens from each strain were separated by two-dimensional electrophoresis (2-DE) using IPGs. The results of pilot experiments revealed differential expression of several *M. tuberculosis* proteins in the two strains at a pH of 4 to 7 (Fig.

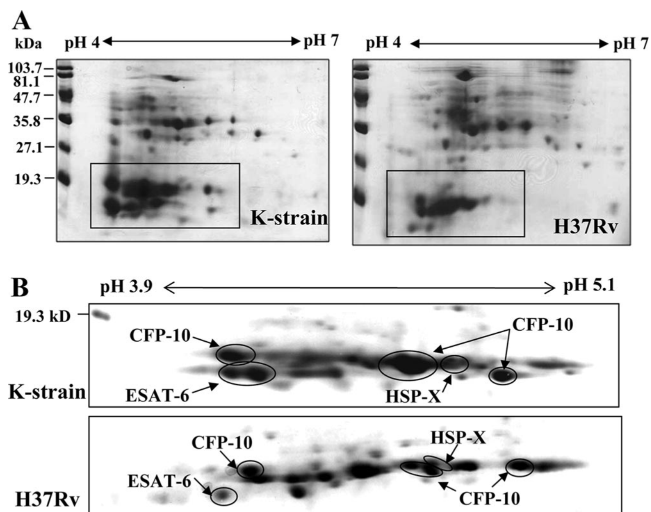

FIG. 2. 2-DE analysis of CFs of *M. tuberculosis* strain K and *M. tuberculosis* H37Rv. (A) CFs were isolated from tubercle bacilli growing in Sauton's synthetic medium and concentrated. The concentrated CFs (300 μg) were then separated by isoelectric focusing using a 7-cm pH gradient strip (pH 4 to 7) in the first dimension and 15% SDS-PAGE in the second dimension. (B) To enlarge the boxed regions in the gels, the proteins were separated again by isoelectric focusing on 11-cm IPG strips (pH 3.9 to 5.1) in the first dimension and 10-to-20% gradient SDS-PAGE in the second dimension. The proteins were stained with 0.25% Coomassie brilliant blue R250, and the spots were identified by LC-ESI-MS.

2A). Those spots with comparable intensities were then expanded by using a pH range of 3.9 to 5.1 and 11-cm IPG strips (Fig. 2B). A similar protein expression pattern was observed regardless of the culture medium; moreover, the differential protein expression between the two strains was most striking at a molecular mass of <20 kDa. The different protein spots in the low-molecular-weight region were further analyzed and identified by LC-ESI-MS. The results of proteomic analysis identified three proteins that were abundantly expressed in *M. tuberculosis* strain K: CFP-10 (Rv3874), Hsp-X (Rv2031c), and ESAT-6 (Rv3875) (Fig. 2A). The relative abundance of these proteins in the strain K CF was confirmed by 2-DE–immunoblot analysis following cultivation in both media. Although the patterns produced by 2-DE of the CF proteins from the mWR medium-cultivated cells were somewhat different from those of the CF proteins from the Sauton's medium-cultivated cells, abundant CFP-10 and ESAT-6 expression in the CF of *M. tuberculosis* strain K was verified by immunoblot analysis (see Fig. 4D and E). Note that the continuous subculture of *M. tuberculosis* strain K in broth medium for more than 6 months resulted in decreased expression of all three proteins (data not shown).

To confirm once again if the CFP-10 and ESAT-6 proteins were more abundantly expressed in strain K than H37Rv, the proteins were partially purified from CFs of *M. tuberculosis* H37Rv and strain K and analyzed by using 2-DE and immunoblotting. The native CFP-10 and ESAT-6 were copurified, as demonstrated previously. On analysis of purified proteins from both strains by 2-DE (Fig. 3A) and immunoblotting using anti-CFP-10 antibody (Fig. 3B) and anti-ESAT-6 antibody (Fig. 3C), we observed again that the CFP-10 and ESAT-6 proteins were more abundantly present in strain K than in strain H37Rv.

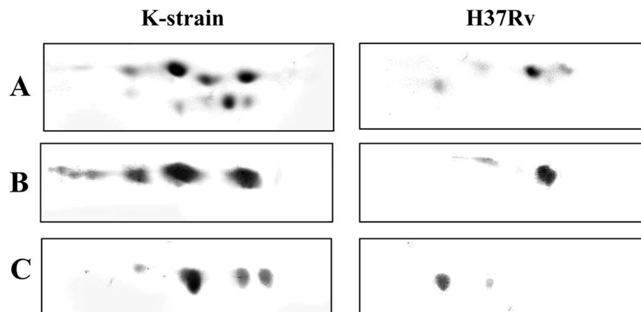

FIG. 3. 2-DE and immunoblot analysis of partially purified native CFP-10 and ESAT-6 complex from *M. tuberculosis* H37Rv and *M. tuberculosis* strain K. The purified native proteins (250 μg) were separated by isoelectric focusing on 7-cm IPG strips (pH gradient of 3.9 to 5.1) in the first dimension and 15% SDS-PAGE in the second dimension. The proteins were analyzed by staining with 0.25% Coomassie brilliant blue R250 (A) and immunoblotting with anti-CFP-10 (B) and anti-ESAT-6 (C) polyclonal antibodies.

Expression, purification, and confirmation of rCFP-10, rHsp-X, and rESAT6. To compare the serological reactivities of ESAT-6, CFP-10, and HSP-X, each protein was expressed as a six-His-tagged fusion protein in *E. coli* BL21 cells and then purified by Ni-NTA affinity chromatography. SDS-PAGE of the recombinant proteins revealed major bands at approximately 6, 10, and 16 kDa, respectively (Fig. 4A to C). rCFP-10 and rESAT-6 were confirmed by comparing them with the native proteins using immunoblot analysis (Fig. 4D and E).

Individual variation in the antibody response to five mycobacterial antigens. We next investigated the serological potential of ESAT-6, CFP-10, and HSP-X; two native proteins frequently used in serological tests were also included (Table 1). ESAT-6, HSP-X, and CFP-10 were abundantly expressed in the CF of *M. tuberculosis* strain K, as were the 30- and 38-kDa antigens, suggesting that they are strong candidates for use in improved serodiagnostic tests for TB. We confirmed that each antigen had the correct band composition; a mixture of the five

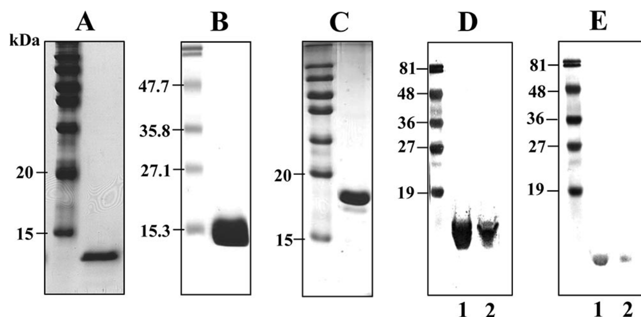

FIG. 4. (A to C) Immunoblot analysis of rCFP-10 and rESAT-6. Six-His-tagged rESAT-6 (A), rCFP-10 (B), and rHSP-X (C) were expressed in *E. coli* cells, purified by Ni-NTA affinity chromatography, and subjected to SDS-PAGE with Coomassie brilliant blue staining. (D and E) Concentrated CFs from *M. tuberculosis* strain K (lane 1) and H37Rv (lane 2) were analyzed by immunoblotting with rabbit anti-CFP-10 (D) and mouse anti-ESAT-6 (E) polyclonal antibodies. Each lane was loaded with 30 μg of proteins. The positions of the molecular standards are shown by bars.

TABLE 1. Antigens used in this study

Antigen	Rv no.	Molecular size (kDa)	Features[a]	Form used
CFP-10	Rv3874	10	Induced strong T-cell response	Recombinant
ESAT-6	Rv3875	6	Heterodimer in tight 1:1 complex	Recombinant
HSP-X	Rv2031c	16	Alpha-crystallin, heat shock protein	Recombinant
Ag85 complex	Ag85A, Rv3804c; Ag85B, Rv1886c; Ag85C, Rv0129c	30–32	Secretory antigen, induced IFN-γ, IL-12p40, and IL-18 production	Native
PstS1	Rv0934	38	Required for phosphate uptake and virulence	Native

[a] IFN-γ, gamma interferon; IL-12 and -18, interleukin-12 and -18.

antigens contained five major bands, while native 30-kDa antigen consisted of three proteins (the Ag85 complex) (Fig. 5A). The individual antibody response to an antigen cocktail consisting of the five mycobacterial proteins was investigated by immunoblot analysis. When the antigens were compared individually, the highest antibody response was obtained for native 30-kDa antigen, which was able to detect 40.0% (18 of 45) of the samples from patients with AFB-positive pulmonary TB. A total of 28 sera reacted to at least one antigen (64.4%), while 6 sera (13.3%) reacted to one of the following: ESAT-6, CFP-10, or HSP-X (Fig. 5B). The multiple additional bands seen in Fig. 5B resulted from minor proteins contaminated in purified native Ag85 complex.

Improved diagnostic sensitivity using all five proteins to create a cocktail ELISA. To evaluate rCFP-10, rESAT-6, and rHSP-X for the serodiagnosis of TB, the human serum IgG level against five antigens (purified rCFP-10, rESAT-6, and rHSP-X and native 30- and 38-kDa antigen) alone or in combination was measured by indirect ELISA using serum samples from 46 patients with active pulmonary TB and 46 healthy controls. All of the antigens tested evoked a significantly higher IgG antibody response in the TB group than in the control group ($P < 0.001$). The greatest AUC value and diagnostic accuracy for a single antigen was obtained using rHSP-X (Table 2). A cocktail ELISA using all five antigens had a higher diagnostic sensitivity than the use of a single antigen (77.8 versus 67.4%) and had equivalent specificity. The results of ROC analysis revealed that the cocktail ELISA also produced the largest AUC (Table 2).

DISCUSSION

Serological tests are attractive for the screening or diagnosis of TB disease or infection because they are quick and relatively

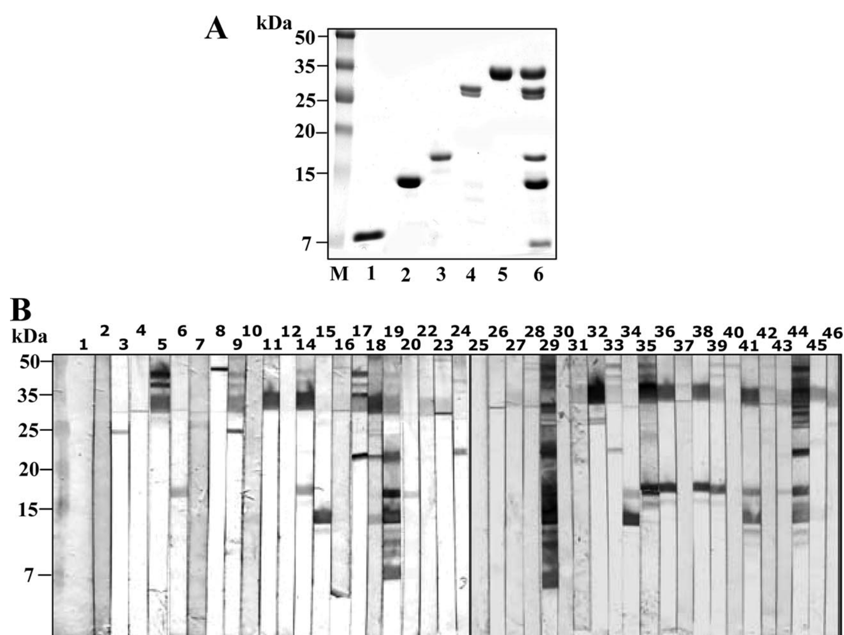

FIG. 5. Variability among the immunoblot profiles of the five antigens. (A) Five antigens, including two native antigens (30- and 38-kDa antigen) and three recombinant antigens (rESAT-6, rCFP-10, and rHSP-X), were subjected to SDS-PAGE and Coomassie blue staining. Lane 1, rESAT-6; lane 2, rCFP-10; lane 3, rHSP-X; lane 4, native 30-kDa antigen; lane 5, native 38-kDa antigen; lane 6, cocktail of the five antigens; lane M, molecular-mass marker. (B) To investigate the reactivity of the five-antigen cocktail against sera from 45 individuals infected with *M. tuberculosis*, each lane was loaded with the same concentration of cocktail (180 μg of each protein). Following 15% SDS-PAGE and protein transfer to nitrocellulose, the sera were applied (1:200 dilution). Subsequently, the membranes were incubated with anti-human IgG HRP conjugate (1:2,000 dilution) and developed using diaminobenzidine.

TABLE 2. Overall comparison using a single antigen alone or combination of all antigens in ELISA for diagnosis of active tuberculosis

Antigen	Cutoff value[a]	Sensitivity	AUC (95% confidence interval)	Avg S/N ratio[b]
CFP-10	0.23	45.87	0.8896 (0.8243–0.9549)	2.45
ESAT-6	0.22	36.96	0.7543 (0.6544–0.8543)	1.86
HSP-X	0.38	67.36	0.9058 (0.8466–0.9650)	3.38
Ag85 complex	0.27	47.83	0.8782 (0.8324–0.9302)	3.13
PstS1	0.18	56.52	0.899 (0.8372–0.9609)	3.15
Combination	0.21	77.78	0.9123 (0.8410–0.9836)	3.619

[a] Cutoff values for each ELISA were determined when the specificity was 100%.

[b] The S/N ratio was considered the standard for overall comparison of antibody response.

simple to perform (6). However, to develop an effective immunological test for TB, one must avoid cross-reactivity with BCG or nontuberculous mycobacteria (4, 12). As a result, researchers are constantly searching for novel immunogenic antigens. A high number of *M. tuberculosis* antigens induce specific humoral and cellular immune responses, and these antigens have been shown to be correlated with proven cases of TB in humans. Previous studies aimed at developing a TB vaccine or immunodiagnostic techniques have focused on the CF antigens released from *M. tuberculosis* because of their immunogenicity (30, 34). In this study, ROC analysis of the CF antigens from *M. tuberculosis* strain K had a higher mean ELISA S/N ratio and more-accurate AUC than ROC analysis of those from *M. tuberculosis* strain H37Rv. Thus, several of the proteins in the CF from *M. tuberculosis* strain K may be useful for immunological testing. *M. tuberculosis* strain K, which belongs to the Beijing family, has been recognized as the most-influential and -prevalent clinical isolate contributing to TB in Korea (24). Consequently, we used a proteomics-based approach to screen for novel strain K antigens that may be useful in the diagnosis of TB. We identified three proteins, HSP-X, CFP-10, and ESAT-6, which were expressed at relatively higher levels in the CF of strain K than in that of strain H37Rv. In particular, CFP-10 and ESAT-6 were abundantly expressed in the CF from strain K, despite the genes encoding CFP-10 and ESAT-6 in both strains of *M. tuberculosis* being identical. Although some proteins were more abundantly expressed in CF antigens from H37Rv than those from strain K (Fig. 2), we were only interested in highly expressed proteins in strain K. Therefore, we did not identify these proteins.

HSP-X is a 16-kDa heat shock protein required for the persistence and growth of mycobacteria within host macrophages in hypoxic microenvironments and is the dominant antigen induced under conditions of reduced O_2 tension (10, 45). CFP-10 (a 10-kDa CF protein) and ESAT-6 (a 6-kDa early secretory antigenic target), both of which are secreted from *M. tuberculosis*, are well-known T-cell-stimulating antigens that lack traditional signal sequences and are exported through an ESX-1 secretion system encoded by *r*egion of *d*ifference 1 (RD1) (16, 32, 40). RD1, which consists of nine genes, is absent from attenuated or avirulent strains, such as *M. bovis* BCG and *Mycobacterium microti*, but is present in all virulent isolates. In previous comparative proteomic analyses of the CF proteins from *M. tuberculosis* K and other virulent strains, CDC1551, ESAT-6, and CFP-10 were included among the CF antigens of the virulent strains more often than among those of strain H37Rv, which is in good agreement with our findings (5). In addition, we found that the abundance of ESAT-6 and CFP-10 in the CF from *M. tuberculosis* strain K decreased following continuous subculture. Taken together, our results suggest that virulent or recently isolated clinical samples of *M. tuberculosis* produce these proteins at higher levels than laboratory-adapted strains, such as H37Rv. Testing whether the virulence of *M. tuberculosis* strain K can be reduced by continuous serial passage may prove interesting.

CFP-10 and ESAT-6 from the two strains were detected at multiple spots with similar or different masses and different pI values by 2-DE. CFP-10 and ESAT-6 are usually bound together as a heterodimer, but the CFP-10:ESAT-6 complex dissociates upon the N-terminal acetylation of ESAT-6 (37, 41). The posttranslational modification of ESAT-6 by acetylation has been shown in several studies (38, 43).

After confirming the strong expression of ESAT-6, CFP-10, and HSP-X in the CF of *M. tuberculosis* strain K, we performed a series of serological tests that included the three proteins plus two native antigens, Ag85 complex and PstS1. Antigen 85 is primarily a candidate for a vaccine against TB, while PstS1 has been commonly pursued for its potential as a serodiagnostic antigen. These antigens induce a strong immune response to *M. tuberculosis* and elicit a protective immune response in animals and humans (3, 18, 21). The Ag85 complex (Ag85A, Ag85B, and Ag85C) includes the 30- and 32-kDa antigens, which are major secretory antigens of *M. tuberculosis* that exhibit mycolyl transferase activity (49). In comparison, PstS1 (a phosphate-specific ABC transporter) is a 38-kDa lipoglycoprotein that may be found as part of a membrane-associated complex or secreted in mycobacterial cultures (7, 20).

Our ELISA results revealed a significantly higher IgG antibody response to all five antigens (CFP-10, ESAT-6, HSP-X, Ag85 complex, and PstS1) in the TB group than in the control group. The greatest AUC and diagnostic accuracy for a single antigen was obtained using HSP-X, whereas the reactivity of 30-kDa antigen (i.e., the Ag85 complex) was the strongest among the five antigens in the immunoblot analysis. During Western blotting, the denatured proteins were probably unable to react normally to the Ig due to the loss of their natural structure or epitope. Thus, the three-dimensional conformation of HSP-X may be important for immunological test development. Serological testing for *M. tuberculosis* using a cocktail of antigens may lead to improved sensitivity (29, 33). Not only the antigen 85 complex and PstS1 (26), but also lipoarabinomannan (22), 34-kDa antigen (2), MTC28, ES-31 and ES-43 from an *M. tuberculosis* H37Ra CF (19), 19-kDa lipoprotein, KatG, and 14-kDa antigen (28, 47) were previously reported to be useful for the detection of TB. In the present study, we confirmed that a cocktail of two well-known potential diagnostic antigens (native Ag85 complex and PstS1) and three additional antigens (rCFP-10, rESAT-6, and rHSP-X) yielded the highest analytical sensitivity. These findings agree with those of previous studies showing that a cocktail of antigens increased the diagnostic sensitivity of serological tests for TB compared to the use of a single antigen.

An important result from this study is the specific CFP-10 or

HSP-X antigen-antibody response. Among the individuals in the TB group, several showed either a CFP-10 or HSP-X antigen-specific antibody response by ELISA and immunoblot analysis. In other words, CFP-10 and HSP-X may further increase the clinical sensitivity of serological tests and help identify patients that do not react to 30- or 38-kDa antigen.

In conclusion, the abundantly expressed immunogenic antigens of geographically prevalent strains of *M. tuberculosis* may be important for the development of serodiagnostic tests for TB; moreover, ESAT-6, CFP-10, and HSP-X may be used to increase assay sensitivity when included alongside well-known serological antigens for the detection of *M. tuberculosis* infection.

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