Characterization of a novel antigen of *Mycobacterium tuberculosis* K strain and its use in immunodiagnosis of tuberculosis

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Directed by Professor Sang-Nae Cho

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This certifies that the Master's Thesis
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Paul Jaewook Park

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ABBREVIATIONS

TB, Tuberculosis

***M. tuberculosis*,** *Mycobacterium tuberculosis*

BCG, Bacillus Calmette-Guérin

LTBI, Latent *M. tuberculosis* infection

TST, Tuberculin skin test

IGRA, Interferon gamma release assay

PBMC, Patient peripheral blood mononuclear cells

ESAT-6, 6 kDa early secreted antigenic target

CFP-10, 10 kDa culture filtrate protein

ROC, Receiver operating characteristic curve

AUC, Area under the curve

ABSTRACT

Characterization of a novel antigen of *Mycobacterium tuberculosis* K strain and its use in immunodiagnosis of tuberculosis

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Developing rapid and accurate methods for the diagnosis of *Mycobacterium tuberculosis* infection is a major priority in controlling the disease. The tuberculin skin test, in use for over a century, has remained virtually unchanged and is often complicated by those previously vaccinated against tuberculosis. Recently, assays evaluating a patient's T-cell response to *M. tuberculosis*-specific antigens by measuring interferon-γ (IFN-γ) levels have had promising results. Current methods use the highly immunogenic early-secreted protein ESAT-6 in combination with several other *M. tuberculosis*-specific antigens to elicit an immune response from patient leukocytes.

The Beijing family of *M. tuberculosis* accounts for nearly 50% of all infections in East Asia. In South Korea, a particular strain within this family, the K strain, has been shown to be predominant. Upon comparing the genome of the K strain with the common laboratory strain H37Rv, a K-specific insertion approximately 5.7 kb in size was identified. Within this insertion,

the sequence for an ESAT-6 like protein, *insB*, was found. The purpose of this study was to characterize the immunogenicity of the InsB protein.

The sequence for *insB* was cloned and expressed in *E. coli* BL21(DE3), and the recombinant protein was purified. Mice were immunized with either the ESAT-6 or InsB protein and ELISA was performed using serum samples from the immunized mice to evaluate the humoral response to the antigen. The T cell response was measured using flow cytometry to quantify the cytokine response of isolated splenocytes from the mice. ESAT-6 and InsB were used to measure the antibody in tuberculosis (TB) patients as well.

A significant difference was seen in B cell response in mice prior to and following immunization with InsB. Splenocytes stimulated with InsB showed strong IFN-γ and IL-17 responses and a weak IL-2 response, all of which have been implicated in the diagnosis of tuberculosis. TB patients' serum samples also showed a significant response to InsB when compared to healthy control samples. These results indicate a K strain-specific immunogenicity of InsB that could further improve upon current immunodiagnostic methods, especially for the South Korean population.

Key words: *M. tuberculosis* K strain, antigen, immunodiagnosis, ESAT-6, InsB

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I. INTRODUCTION

Tuberculosis is a major infectious disease and its causative agent, *Mycobacterium tuberculosis,* currently infects about one third of the world's population. According to the World Health Organization, there were approximately 8.8 million incident cases of tuberculosis with nearly 1.1 million deaths due to the disease in 2010[1]. Though a majority of those infected do not proceed to active disease, the organisms remain in a latent phase and can re-emerge if the immune system becomes weakened[2]. In order to control such a disease, rapid and accurate diagnosis of latent *M. tuberculosis* infection (LTBI) is needed for preventive therapy before reactivation.

1. Identification of the K strain in South Korea

Over the years, several distinct strains of tuberculosis have developed. Restriction fragment length polymorphism (RFLP) analyses have been used to find unique polymorphism patterns that differ between strains.

The repetitive DNA element, IS*6110*, is a widely used standard for characterizing tuberculosis families[3]. The Beijing strain, an especially virulent strain, accounts for nearly 50% of infections in East Asia[4]. The Beijing strain has been linked with resistance to antibiotic treatments as well as increased virulence[5]. It has also been suggested that resistance to the Bacillus Calmette-Guérin vaccine (BCG), the only approved vaccine for *M. tuberculosis* in the past century, has led to its selection; the Beijing strain has experienced an approximately 500-fold population increase over nearly two centuries[6, 7].

In Korea, epidemiological studies have shown that one particular strain of the Beijing family, the K strain, is prevalent among TB patients within the country[8,9]. The K strain shares the characteristic IS*6110* RFLP pattern unique to Beijing strains[9]. The K family strains, found more frequently during school outbreaks of TB in South Korea than other strains, are likely more efficient in transmission and were found to be more virulent in a mouse model of TB[10].

Considering the predominance of the K strain, in order to accurately detect tuberculosis infection in Korea, it is important to explore K strain-specific biomarkers in order to increase the sensitivity of current diagnostic tools.

2. Immunology of *M. tuberculosis* infection

Upon entering the body, *M. tuberculosis* is contained by the host immune system in most cases. Uptake by macrophages leads to the formation of granulomas; complex structures involving macrophages, neutrophils, dendritic cells, B cells, T cells, and various other immunologic cells[11]. Within these granulomas, *M. tuberculosis* bacteria are able to persist indefinitely in a hypoxic state, while cytokines such as TNF-α and IFN-γ work to contain the infection from re-activating[12,13]. In this state, disturbances in the immune system such as HIV infection, corticosteroid treatment, or aging, could lead to the release of bacteria and further spread to others[14].

Considering that the bacteria persist mainly within macrophages, it is largely thought that $CD4^+$ T cells are the principal component of the immune

response to infection. Various studies in animal models, as well as studies conducted in HIV-infected patients, reflect higher rates of infection in those with reduced T cell responses[15,16,17] . The T_H2 response and subsequent B cell response, though less studied, have also been suggested to play an important role in managing *M. tuberculosis* infection[18]. A major priority in finding a more effective method of diagnosis is exploring antigen markers that can distinguish these immune responses.

3. Current methods of diagnosis

The tuberculin skin test (TST), which has been used to detect latent *M. tuberculosis* infection (LTBI) for nearly the past century, has much room for improvement in terms of sensitivity and specificity. The test, which evaluates delayed-type hypersensitivity of the patient's cell-mediated immune response to tuberculosis antigens, is often complicated by those previously immunized with BCG[19]. Over the last several decades, new developments have changed the direction of tuberculosis diagnostics. First, assays that measure the antibody titer against *M. tuberculosis* specific antigens are in use today but are still being refined in order to increase diagnostic sensitivity[20]. Secondly, assays that measure the immune response of patient peripheral blood mononuclear cells (PBMC) to *M. tuberculosis* specific antigens *ex vivo* have already been widely adopted in detecting LTBI. Measuring the IFN-γ levels in response to stimulation by *M. tuberculosis* specific antigens has proven to be a more accurate, sensitive method to detect TB infections than TST[21].

The current, widely used IFN-γ release assay (IGRA) diagnostic is known as the QuantiFERON-TB Gold assay. This kit requires incubation of whole blood with *M. tuberculosis*-specific antigens and measures the IFN-γ concentration by ELISA. The antigens used to stimulate the patient PBMC are two proteins secreted by *M. tuberculosis* known as ESAT-6 and CFP-10[22,23]. The ESAT-6 and CFP-10 proteins are part of the ESX-1 secretion pathway, coded in the RD1 region of the *M. tuberculosis* genome. It has been

shown that strains that lack these genes have a significant reduction in virulence[24,25]. In particular the *M. tuberculosis* K strain, when compared to other non-Beijing strains of *M. tuberculosis* such as H37Rv, shows a significant up-regulation of these two proteins[26]. These findings indicate that these two well-characterized antigens could play an important role in identifying K strain infections as well.

4. Purpose

This study aims to characterize a K strain-specific ESAT-6 like protein, InsB, and to explore its potential in improving current immunodiagnostic methods. The completion of the genomic sequence of one of the most widely used laboratory strains of *M. tuberculosis*, H37Rv, has uncovered many new antigen candidates to explore[27]. Upon comparing the H37Rv and K strain genomic sequences, antigen candidates particular to the K strain were identified, among which *insB* was found. PCR was performed on the *insB* sequence and the recombinant protein was expressed in *E. coli*, purified and used to immunize BALB/c mice. ESAT-6 was also included in the study to compare the immunogenicity of the InsB protein with an established biomarker for tuberculosis. Splenocytes from immunized mice were stimulated using the antigens and their cytokine release levels were analyzed. The antigen candidates were also used for antibody detection in serum samples collected from immunized mice as well as TB patient serum samples acquired from Masan National Tuberculosis Hospital in Changwon, South Korea. The immunogenicity of the antigen candidates was analyzed through antibody titer levels using ELISA. By characterizing the immunogenic properties of InsB, we hope to improve both IFN-γ based and antibody-based diagnostics in South Korea.

II. METHODS AND MATERIALS

1. Selection of antigen candidates

Comparison of the partial genome sequences of the K and H37Rv strains revealed a 5.7 kb insertion found only in the K strain. The approximately 5.7 kb sequence, called the insertion region, coded for several proteins of interest found in the PPE protein region. The PE/PPE proteins are known to induce a strong immune response in the host and play an important role in the virulence and pathogenesis of *M. tuberculosis*[28,29]. These protein sequences were then compared to those of established immunogenic antigen markers such as ESAT-6 and CFP-10.

2. Cloning of antigen candidates

M. tuberculosis K strain was grown in Sauton media at 37℃. Its genomic DNA was prepared using *N*-acetyl-*N,N,N*-trimethyl ammonium bromide (CTAB) buffer as described by Somerville, *et al*[30]. Polymerase chain reaction (PCR) was performed to amplify the *insB* sequence. 50 ng K genomic DNA, 10x *Pfu* buffer (Solgent, Daejeon, Korea), 5x Band Doctor (Solgent, Daejeon, Korea), 2.5 mM dNTP (Solgent, Daejeon, Korea), 20 μM each of forward and reverse primer (insB-F : 5'-TTGCATATGACGATCAATTATCAGTTCGG-3' including *Nde*I site; insB-R : 5'-GCGGATCCAGCCCAGCTGGAACCCACT-3' including *Bam*HI site), and 1 ㎕ *Pfu* DNA polymerase (Solgent, Daejeon, Korea) were combined with distilled water for a final volume of 50 ㎕. Denaturation was performed at 94℃ for 5 minutes, annealing was performed at 50℃ for 30 seconds, and amplification was performed at 72℃ for 30 seconds. The PCR product was confirmed on a 1.5% agarose gel and an Expin PCR SV purification kit was used (GeneAll, Seoul, Korea) according to manufacturer's instructions. Sequences containing a 6× histidine tag were inserted into the pET11a_KB vector by incubating with *Nde*I and *Bam*HI

enzymes (NEB, Ipswich, MA, USA) for 1 hour and 30 minutes at 37℃. After the addition of calf intestinal alkaline phosphatase (CIP), the DNA was incubated for an additional 30 minutes at 37℃. The DNA was run on a 1.5% agarose gel and the band was excised and extracted with an Expin Gel SV extraction kit (GeneAll, Seoul, Korea). The vector and DNA inserts were combined in a 1:5 ratio, and then incubated at 60℃ for 5 minutes. Next, 1 ㎕ 10x T4 DNA ligase buffer (NEB, Ipswich, MA, USA), 1 ㎕ T4 DNA ligase (NEB, Ipswich, MA, USA) and distilled water were added for a final volume of 10 ㎕ and incubated for an hour at 25℃ to complete ligation.

A volume of 10 ㎕ DNA was transferred to 100 ㎕ of *E. coli* DH5α and incubated on ice for 20 minutes. The cells were then heat shocked at 42℃ for 90 seconds, placed back on ice, 900 ㎕ Luria-Bertani (LB) broth was added, then the cells were placed in a 37℃ shaking incubator for one hour. The cells were spread onto LB agar plates with ampicillin (100 ㎍/㎖) and incubated at 37℃ overnight. Plasmids were then extracted using an Expin Plasmid purification kit (GeneAll, Seoul, Korea).

3. Expression and purification of antigen candidates

The recombinant protein was expressed in *E. coli* BL21(DE3). *E. coli* BL21(DE3) were transformed with 1 ㎕ DNA following the same protocol as for *E. coli* DH5α Cells were grown in LB broth containing 100 ㎍/㎖ ampicillin at 37℃ for three hours until an OD_{600} of 0.6 ~ 0.7.

Cells were then induced with 1 mM isopropyl-β−D-1-thiogalactoside (IPTG) and incubated again for 4 hours, after which cells were harvested and the pellet was sonicated in a 20 mM Tris buffer (pH 8.0). After centrifugation at 12,000 rpm for 30 minutes, the remaining inclusion bodies were prepared in a 20 mM Tris-HCl (pH 7.5) buffer containing 300 mM NaCl, 5 mM imidazole and 6 M urea. After centrifugation, Ni-NTA resin (Qiagen, Venlo, Netherlands) for histidine affinity purification was added to the supernatant and allowed to bind overnight at 4℃. The resin was then packed in a column

(Biorad, Hercules, CA, USA) for purification and washed with an identical buffer containing 30 mM imidazole. Elutions were performed using a similar Tris buffer containing 200 mM imidazole.

After affinity purification with Ni-NTA resin, fast protein liquid chromatography (FPLC) was used to further purify the recombinant proteins and to eliminate any other contaminants, including endotoxins. FPLC was performed using an ÄKTA-FPLC (GE, Pittsburgh, PA, USA) and a MonoQ anion exchange column (GE, Pittsburgh, PA, USA), in which the NaCl concentration in the Tris buffer was slowly increased to separate individual proteins. Following separation by FPLC, proteins were confirmed with SDS-PAGE for purity. For refolding, samples underwent dialysis overnight at 4℃ in a buffer containing 20 mM Tris (pH 8.0), 20 mM β-mercaptoethanol and 5% glycerol. Samples were then transferred into a similar buffer without β-mercaptoethanol and again placed in 4 ℃ overnight while stirring. A bicinchoninic acid assay (BCA) was performed to quantify the recombinant protein. The assay was performed according to manufacturer's instructions (Pierce, Rockford, IL, USA). Proteins then underwent gamma irradiation for sterilization prior to splenocyte stimulation.

4. Immunization of mice

All mouse experiments were in accordance with IACUC guidelines. BALB/c mice, between 5~6 weeks old, were immunized with a dose of 20 ㎍ of target antigen emulsified with 250 ㎍ dimethyl dioctadecylammonium bromide (Sigma, St. Louis, MO, USA) and adjuvanted with 25 ㎍ monophospholipid A (Sigma, St. Louis, MO, USA)[31]. Five mice were in each immunization group. Control groups, containing four mice each, were injected with either saline or the adjuvant mix only.

The injections (0.2 ㎖/mice) were given subcutaneously on the back three times, in two-week intervals. Serum samples were collected retro-orbitally 3~4 days prior to the initial injection and two weeks following the last injection. Serum was placed at room temperature for 1 hour to allow

clotting, and then placed in 4℃ for two hours for further clotting. The sample was then centrifuged at 4,000 rpm for 20 minutes and the supernatant was transferred to a clean tube for storage at -20℃ until use.

5. Detection of antibodies in mice

Plates were coated overnight at 4℃ with InsB protein at a concentration of 5 ㎍/㎖ (ESAT-6 were coated at 2 ㎍/㎖) in coating buffer (0.5 M carbonate-bicarbonate buffer). After washing with PBST, plates were blocked with PBST with 5% NGS (PBST-NGS) and incubated at 37℃ for 1 hour. The plates were then emptied and incubated with mouse serum samples diluted (1:2,000) in PBST-NGS for 2 hours at 37℃. The plates were washed with PBST, and peroxidase-conjugated anti-mouse IgG enzyme-labeled antibody (Merck, Darmstadt, Germany) diluted (1:10,000) in PBST-NGS was incubated for one hour at room temperature. The remaining procedure was identical to that of the human serum ELISA.

6. Splenocyte stimulation and cytokine analysis

Spleens were removed from the mice two weeks after the last injection and passed through a 40 ㎛ cell strainer (BD Biosciences, San Jose, CA, USA). Cells were suspended in RPMI, which contained 5% fetal bovine serum and 1 unit/㎖ Antibiotic-Antimycotic (Invitrogen, Grand Island, NY, USA). The cells were spun down at 1,200 rpm for 5 minutes and the supernatant was discarded. 2 ㎖ ACK lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA) was then added. After 5 minutes, 8 ㎖ RPMI was added and again spun down at 1,200 rpm for 5 minutes. The supernatant was discarded, 5 ㎖ RPMI was added, and again spun down. The supernatant was discarded and the cells were resuspended in a final volume of 1 ㎖ RPMI. A volume of 100 ㎕ containing 1×10^6 cells were plated in a 96-well

plate and stimulated with either RPMI with 10% FBS or the antigen candidates at a concentration of 5 ㎍/㎕. A positive control group of naïve mouse splenocytes were stimulated with concanavalin A. Cells were then incubated for 48 hours and the supernatant was collected and stored at -75℃. Flow cytometry was performed to quantify cytokine levels using a kit from eBioscience (San Diego, CA, USA) and a BD LSRII flow cytometer (San Jose, CA, USA). The concentration of each cytokine was calculated using FlowCytomix Pro software (eBioscience).

7. Detection of antibodies in human serum samples

All TB patient samples were used according to an IRB-approved protocol. Serum samples were obtained from 86 TB patients and 40 healthy controls. Plates were coated with InsB protein at a concentration of 5 ㎍/㎖ (ESAT-6 coated at 2 ㎍/㎖), diluted in coating buffer (0.5 M carbonate-bicarbonate buffer) and allowed to incubate overnight at 4℃. Plates were then washed with PBS with 0.05% Tween20 (PBST). Patient serum samples were diluted (1:300) in PBST containing 5% normal goat serum (PBST-NGS) and allowed to attach for 2 hours at 37℃.

The plates were washed and incubated again at 37℃ with a peroxidase-conjugated anti-human IgG enzyme-labeled antibody (Merck, Darmstadt, Germany) diluted (1:10,000) in PBST with 5% NGS. Plates were incubated at 37℃ for another hour. After several more washes the plates were visualized using tetramethylbenzidine (KPL, Gaithersburg, MD, USA) and the reaction was stopped with 2.5 N H_2SO4. The absorbance was read at OD_{450}.

8. Statistical Analyses

Statistical calculations were performed using Graphpad Prism 5 (Graphpad Software, La Jolla, CA, USA). Differences in B cell response for

non-immunized and immunized mice were analyzed using the paired Student's T-test. Differences between human active tuberculosis patients and healthy control patients were calculated with the Mann-Whitney test. Differences were considered significant if $P < 0.05$. Diagnostic accuracy of each candidate was evaluated using receiver operating characteristic (ROC) curve analysis, which correlates true and false positive rates [sensitivity and (1-specificity)]. Differences in the area under the curve (AUC) values were calculated as well.

III. RESULTS

1. DNA sequencing

Comparison of the K and H37Rv strain revealed several insertions found only in the K strain. The 5.7 kb insertion found in the PPE region contained a protein with only several amino acid differences compared to the ESAT-6 (Rv3875) protein of the H37Rv strain (Fig. 1, 2). This *insB* gene was selected as the focus of this study due to its similarity in sequence with the well-characterized ESAT-6 antigen, as well as its exclusivity to the K strain. The *insB* gene was chosen for further study because of its similarity in sequence with the well-characterized ESAT-6 antigen and of its presence only in the K strain.

2. Protein expression and purification

Following amplification and insertion of the *insB* gene sequence into the pET11a_KB vector (Fig. 3), the plasmid was successfully expressed in *E. coli* BL21(DE3) with a 6 × histidine tag. The protein was then affinity purified using a Ni-NTA column. The proteins were further purified with FPLC and SDS-PAGE confirmed that the recombinant protein was approximately 6 kDa in size, similar to ESAT-6 (Fig. 4).

3. Characterization of antibody response in mice to the recombinant InsB protein

Following three antigen immunizations over a two-week period, serum samples were obtained from mice prior to the initial immunization and approximately two weeks following the last immunization (Fig. 5). A significant level of antibody production was seen in both mice immunized with ESAT-6 and with the K-specific InsB protein ($P < 0.01$). Analysis with a paired T-test showed a significance of $P < 0.001$ for ESAT-6 and $P < 0.0001$

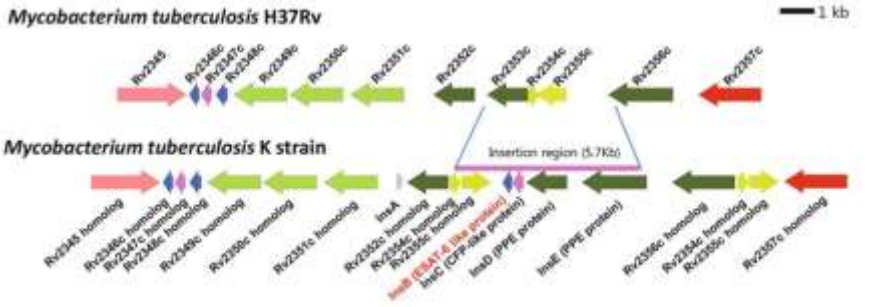

Figure 1. Genetic map of the region containing the ESAT-6 and CFP-10 like proteins in the *M. tuberculosis* H37Rv and K strains. The 5.7 kb insertion region found only in the K strain is indicated by the purple bar. InsB is found in this insertion, while the K strain ESAT-6 protein is found outside the inserted sequence.

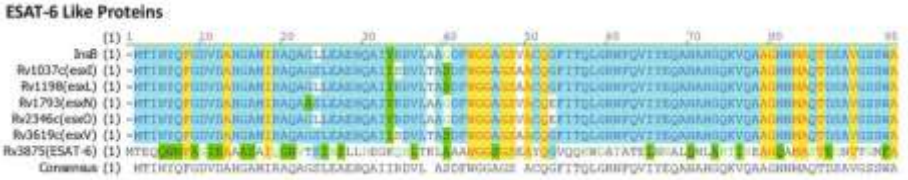

Figure 2. Alignment of InsB compared with related proteins in the family and ESAT-6 of *M. tuberculosis* H37Rv. Sequence analysis of the K-specific InsB protein shows differences only in several amino acids compared to other related ESAT-6 like proteins, except the ESAT-6 sequence in H37Rv (Rv3875). The alignment of amino acid sequences between ESAT-6 and InsB showed only consensus amino acid sequence conservation in each antigen.

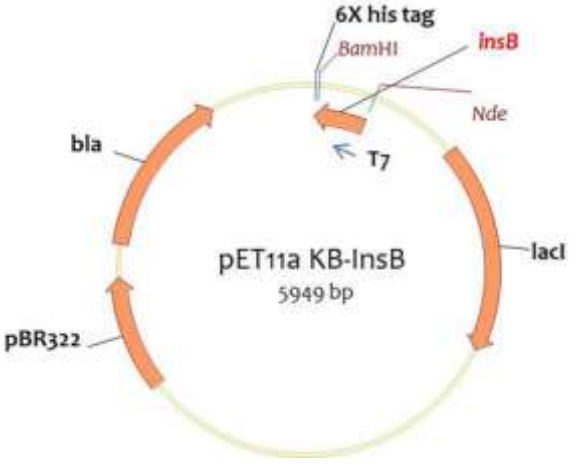

Figure 3. pET11a_KB vector with *insB* insertion. A pET11a_KB vector containing a T7 promoter was used to deliver antigen sequences. ESAT-6 like sequence *insB* was inserted into the pET11a_KB vector with *Nde*I and *Bam*HI restriction enzymes.

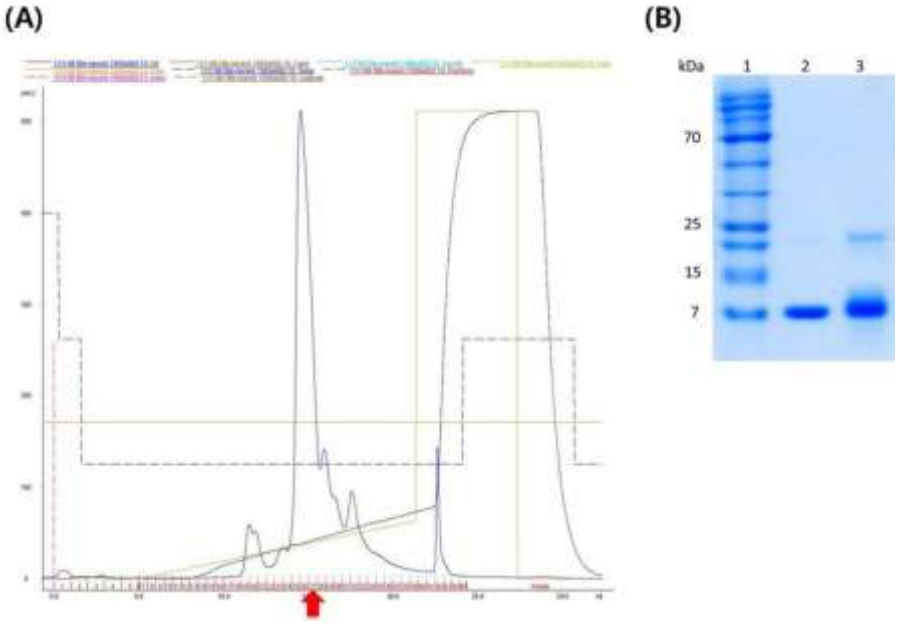

Figure 4. Purification of the recombinant InsB protein expressed in *E. coli* BL21(DE3). (A) Purification of InsB using ÄKTA-FPLC with a MonoQ anion exchange column. (B) SDS-PAGE of purified recombinant InsB after ÄKTA-FPLC. Lanes: 1, Dokdo-MARK Prestained Broad-range Protein Marker (ELPIS Biotech, Daejeon, South Korea); 2, rESAT-6 5 μg; 3, rInsB 5 μg (red arrow).

for InsB (Fig. 6). ELISA with ESAT-6 protein and sera from InsB mice, and vice versa, showed no reactivity. Both ESAT-6 and InsB are immunogenic in elicitng humoral antibody responses, however show no cross-reactivity.

4. Cell-mediated immune response in mice

Splenocytes from mice were isolated two weeks following the third and final immunization. Cells were plated and stimulated with antigen candidates for 48 hours and the supernatant was stored at -70℃. Supernatants were analyzed using a multiplex cytokine analysis kit from eBioscience (San Diego, CA, USA) and a BD LSRII flow cytometer (BD Biosciences, San Jose, CA, USA). A large IFN-γ and IL-17 response was seen in mice immunized with InsB protein and stimulated with the protein. However, cells stimulated with ESAT-6 showed no reactivity. Likewise, for mice immunized with ESAT-6, a moderate response in IL-17 and IFN-γ was seen for those stimulated with ESAT-6, while no response was seen in cells stimulated with InsB. Both groups of mice exhibited a low level response of IL-2 to the respective proteins as well, while there was little or no TNF-α or IL-6 response in either group (Fig. 7).

5. Antibody responses in TB patients

Plates were coated with either ESAT-6 or ESAT-6 like InsB and incubated with human serum samples of 86 active tuberculosis patients from Masan National Tuberculosis Hospital in Changwon, South Korea as well as 40 TB-negative healthy controls. Results were analyzed using the Mann-Whitney test, and revealed a significant response for both ESAT-6 ($P < 0.001$) and the InsB protein ($P < 0.0001$) (Fig. 8). Little correlation is seen between antibody response to ESAT-6 and InsB proteins, indicating a marked difference in B-cell epitopes despite their similarity in amino acid sequence. The ROC curves for each antigen showed a greater sensitivity and specificity

with InsB compared to ESAT-6 in the serum of patients with active TB. The AUC value of ESAT-6 and InsB were 0.62 and 0.75, respectively (Fig. 9).

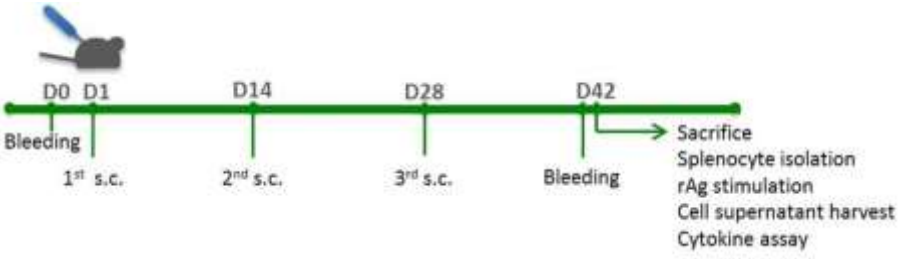

Figure 5. Timeline of mouse immunization and splenocyte harvest. Immunizations were given subcutaneously three times, two weeks apart. Retro-orbital bleedings were performed prior to the first immunization and two weeks following the last immunization. Splenocytes were isolated and cytokine analyses were conducted two weeks following the final immunization.

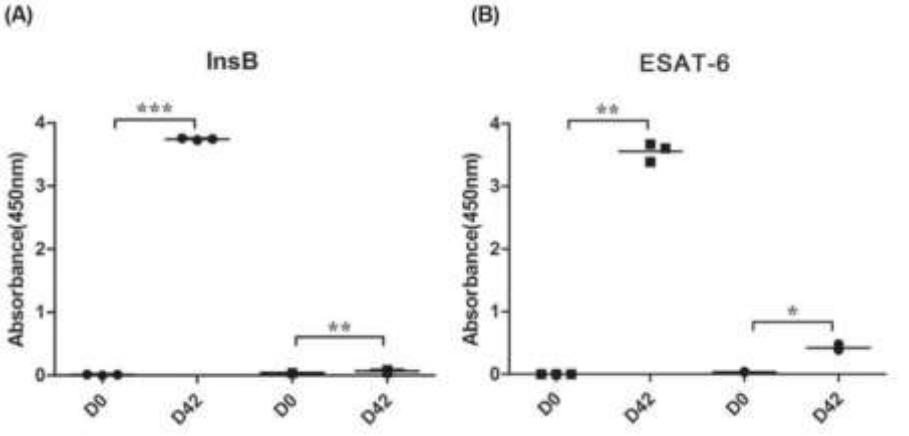

Figure 6. IgG antibody responses determined by ELISA in BALB/c mice immunized with ESAT-6 or InsB. Blood drawn from mice retro-orbitally was collected prior to initial immunization and 2 weeks following final immunization. An enzyme-labeled anti-mouse IgG antibody was used to detect antibody levels (circles : serum from InsB immunized mice, rectangles : serum from ESAT-6 immunized mice) (** = $P < 0.001$, *** = $P < 0.0001$).

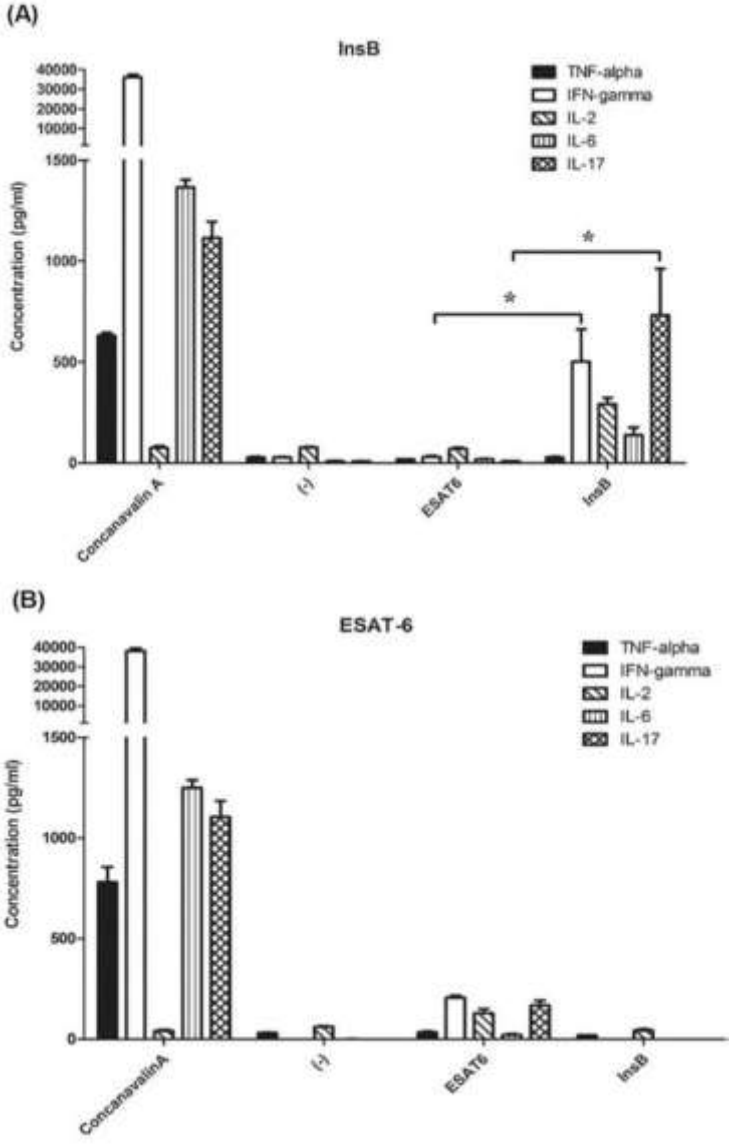

Figure 7. Cytokine analysis of mice immunized with ESAT-6 or InsB protein. Splenocytes from mice immunized with (A) ESAT-6 or (B) InsB were stimulated with either medium (RPMI with 10% FBS), ESAT-6, or InsB, and analyzed by flow cytometry.

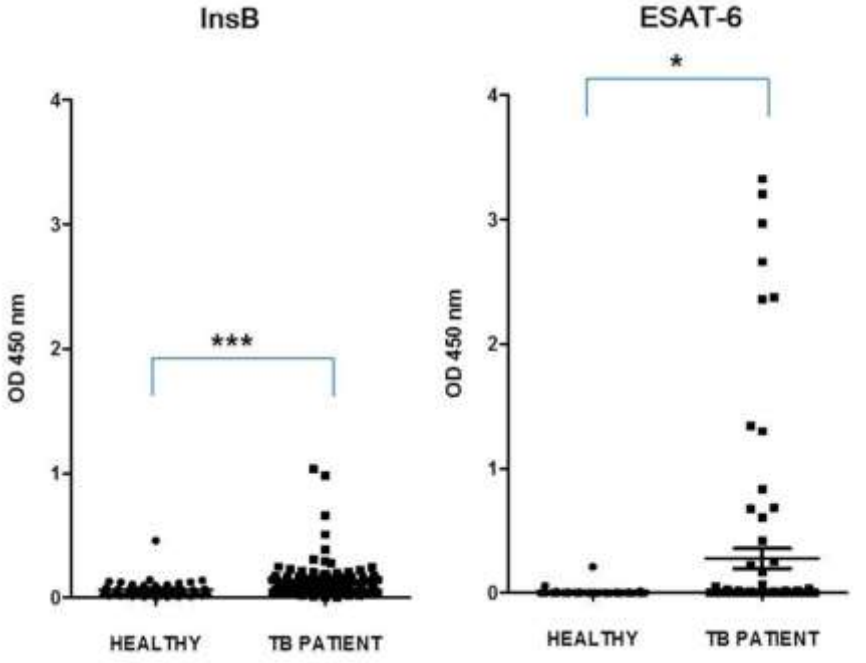

Figure 8. Antibody responses to ESAT6 and InsB in sera from TB patients and healthy controls determined by ELISA. ESAT-6 and InsB show a significant IgG response in serum samples from active tuberculosis patients (n = 86) when compared to healthy controls (n = 40) (* = $P < 0.05$, *** = $P < 0.0001$).

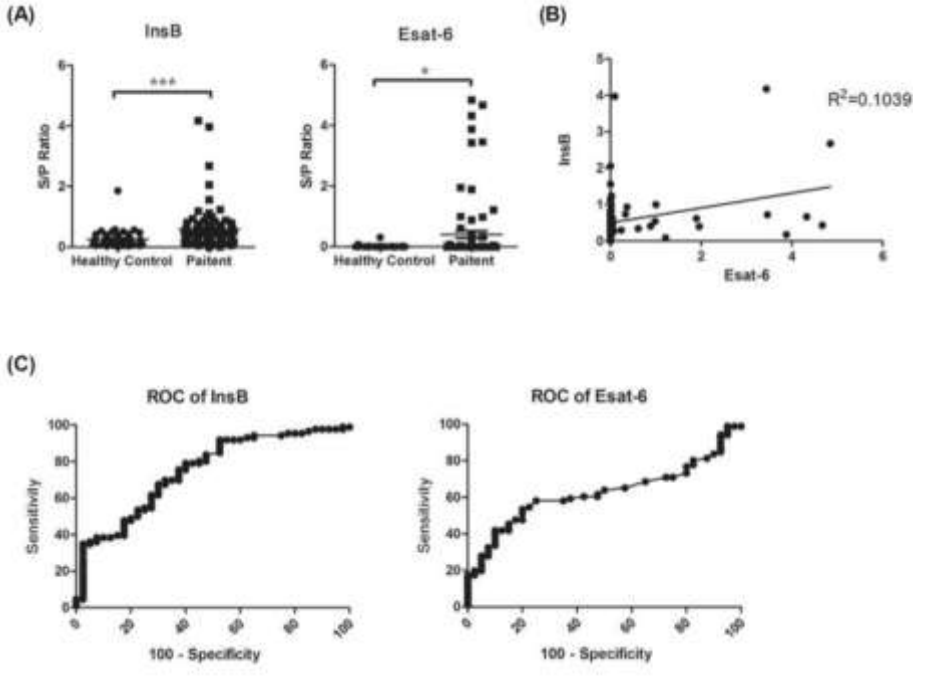

Figure 9. Correlation and receiver operating characteristic (ROC) curves of the antibody responses to ESAT-6 and InsB. (A) S/P ratios of IgG antibodies to ESAT-6 and InsB proteins in TB patients and healthy controls (* $P < 0.05$; *** $P < 0.0001$). (B) Dispersion diagram of correlation between S/P ratios of IgG antibodies to InsB and to ESAT-6 antigens. (C) Receiver operating characteristic (ROC) curves against TB patient serum for the diagnosis of TB. ROC curves were derived by plotting the relationship between the specificity and sensitivity at various cut-off levels. The diagnostic accuracy of the test is expressed by the area under the curve (AUC). InsB had a larger AUC (0.75) than ESAT-6 AUC (0.62).

IV. DISCUSSION

Identifying new antigen candidates that can detect tuberculosis infection is crucial to improving diagnostic methods. The characterization of ESAT-6 and CFP-10 was a major step forward in the immunodiagnosis of latent *M. tuberculosis* infection (LTBI). ESAT-6 is able to elicit a strong T cell response in patients with active tuberculosis, indicated by elevated levels of IFN-γ. For both its similarity to ESAT-6, as well as its specificity to the K strain, InsB was selected for further studies in diagnostic potential. The immunogenicity of InsB was approached from both a humoral and cellular perspective.

Previous studies on the use of the humoral response in evaluating tuberculosis infection differ significantly in their conclusions. Some studies on ESAT-6 claim that the humoral response is not a particularly accurate method of diagnosis, while others believe there is a possible use for antibody measurements as an alternative diagnostic method[32, 33, 34]. Our results showed a significant humoral response ($P < 0.001$) with ESAT-6 in active tuberculosis patient serum samples compared to healthy controls. Interestingly, a more significant difference between active patients and healthy controls was seen with InsB ($P < 0.0001$). The ROC curve analysis demonstrated that the AUC of InsB is approximately 1.2 fold higher than that of ESAT-6. Considering the variability in previous experiments, it is difficult to draw any conclusion on the efficacy of InsB antibody titers as a diagnostic measurement. However, the use of InsB with other antigen candidates to establish an antibody profile may prove to be a more accurate method of diagnosis and warrants further study[34]. In our mouse immunization experiments, we also saw a significant difference in humoral response between serum samples pre- and post- immunization for both ESAT-6 ($P < 0.01$) and InsB ($P < 0.0001$). Though the sample size was too small to accurately illustrate the humoral responses to ESAT-6 and InsB, the results suggest that the mice used for this cytokine analysis experiment were successfully immunized with the antigen candidates.

The cellular immune response to InsB provides more support for its case as a diagnostic biomarker. IFN-γ has been established as a key component in controlling tuberculosis, and its up-regulation during *M. tuberculosis* infection has been established in previous studies. IFN-γ has been the foundation for a new generation of diagnostic tests of LTBI such as the interferon gamma release assay (IGRA) and is an important indicator for any new antigen candidate considered for immunodiagnosis[19,35]. In our experiments, mice immunized with the K-specific InsB antigen showed a strong IFN-γ response, even when compared to those mice immunized with ESAT-6, further supporting its potential use as a biomarker for TB. With the purification and elimination of endotoxins through FPLC, we assume the cytokine release is solely due to stimulation with the target antigens.

Interestingly, InsB also showed a strong response upon stimulation in another cytokine, IL-17. In response to *M. tuberculosis* infection, a population of distinct IL-17 producing T-cells has been shown to increase. The characteristic expression of the chemokine receptor CCR7 in the absence of CD45RA by these IL-17 secreting cells suggests a possible role as long-lived memory cells[36, 37]. *In M. tuberculosis* infection, the release of IL-17 by $CD4^+$ T cells leads to inflammation, as well as the recruitment of neutrophils and granuloma formation. Down-regulation of IL-17 has been shown to decrease proper granuloma formation in mice infected with tuberculosis[38].

IFN-γ has been shown to have a complicated relationship with IL-17. Studies show that IFN-γ can down-regulate IL-17, thus modulating the inflammation response. It has even been suggested that decreases in IFN-γ levels lead to increased tissue damage through the overproduction of IL-17[39]. However, IL-17 has been suggested to have a priming effect for IFN-γ secreting T cells as well. The T_H1 memory response was shown to be accelerated by IL-17. In an experiment involving mice vaccinated with ESAT-6, the recruitment of IL-17 secreting cells preceded the recruitment of IFN-γ cells. Mice with down-regulated IL-17 had difficulty mounting an IFN-γ response, while treatment with exogenous IL-17 restored the recall response of IFN-γ cells in these mice[40].

One study suggests that in combination with other cytokine markers, levels of IL-17 might be used to distinguish between active and latent TB diagnosis, while another indicates varying levels of IL-17 secretion between drug-resistant and drug-susceptible strains[41,42]. IL-17 is an important cytokine in *M. tuberculosis* infection, and a protein that can elicit an IL-17 response could contribute to a more detailed diagnosis of *M. tuberculosis* infection.

An IL-2 response was also shown for both ESAT-6 and InsB proteins in this study. IL-2 is a strong inducer of T cell proliferation, but moderates the immune response by up-regulation of T_{reg} cells as well[43]. Several papers suggest that IL-2 decreases during active disease, while levels increase as treatment progresses or in patients with latent TB[44, 45].

Based on the release of IL-2 and IFN-γ, it would seem that the cytokine response is strongly T_H1-based. However, we see little to no response in TNF-α, a T_H1 cytokine thought to regulate type-1 activation[46]. TNF-α deficient mice infected with *M. bovis* BCG were shown to have increased tissue damage due to the overproduction of IFN-γ and IL-12, while in human patients anti-TNF-α therapy has led to increased cases of reactivation[46,47]. TNF-α release was higher in PBMC and macrophages from active tuberculosis patients compared to healthy controls upon stimulation[48,49]. However, the study by Takashima, *et al.* does indicate higher levels of TNF-α release in newly diagnosed tuberculosis patients versus chronic patients, which might explain why we see decreased levels of TNF-α in our mouse model, whose cytokine release profile was analyzed over a month after initial injection[48]. Also, considering the K strain is associated with multi-drug resistant cases, a study looking at whole blood samples from patients with either multi-drug resistant (MDR) or drug-susceptible tuberculosis found a significant decrease in TNF-α production in drug resistant cases, while comparable levels of IFN-γ were released in both groups[50]. These factors, along with the fact that our studies were conducted in a mouse model with single antigen immunization should be taken into account when comparing our results to experiments with human patients.

V. CONCLUSION

In order to find novel antigen candidates that could improve upon current immunodiagnostic methods, proteins unique to a specific strain of *M. tuberculosis*, the K strain were searched. An ESAT-6 like K strain-specific protein, InsB, was selected for its location in the K strain genome as well as its similarity to the ESAT-6 antigen. The immunogenicity of InsB was tested in a mouse immunization model as well as human serum samples. Immunization with the antigen candidates, followed by ELISA evaluating the B cell antibody response as well as flow cytometry analyzing the T cell cytokine response were performed. B cell antibody responses were tested for in human patient samples as well.

The initial characterization of InsB shows potential as a biomarker for *M. tuberculosis*. The cytokine profile elicited by the protein is comprised of three major cytokines involved in the anti-mycobacterial response, IL-17, IFN-γ, and IL-2. However, these studies were conducted in a mouse model and additional studies in human PBMC would be needed to confirm the protein's cytokine profile in human patients. The ability to elicit a humoral response in human serum samples does illustrate the immunogenicity of InsB, but again further studies would be needed to solidify its role in TB diagnostics. In conclusion, the initial characterization of InsB merits further study of the protein candidate as a diagnostic biomarker for *M. tuberculosis* infection and may lead to a more accurate method of diagnosis, particularly for TB patients in South Korea.

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ABSTRACT (IN KOREAN)

결핵균 K 균주 InsB 항원의 발굴과 면역진단 유용성 분석

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박 재 욱

결핵은 결핵균(*Mycobacterium tuberculosis*)에 의해 유발되는 만성 감염 질환으로 세계보건기구(WHO) 보고에 의하면 세계 인구의 3 분의 1 이 결핵균에 감염되어 있을 정도로 주요한 질병 중 하나이다. 결핵의 효과적인 치료 및 예방을 위해서는 정확하고 신속한 진단법의 개발이 필요하다.

본 연구에서는 최근 국내에서 발생한 결핵 집단감염사례와 결핵 재발환자에게서 높은 빈도로 분리되는, 결핵균 K 균주의 특이적인 항원을 발굴하고 그 특성을 규명하여 면역진단항원으로서의 유용성을 평가하고자 하였다.

이를 위해 결핵균 H37Rv 표준 균주와 K 균주의 유전체 비교 분석을 하여 K 균주에서 5.7 kb 의 유전자 삽입부분을 확인하였고, 이 부위에 삽입되어 있는 여러 개의 ORF 들 중에 현재 IFN-γ release assay (IGRA) 결핵 검사법에 이용되고 있는 ESAT-6 항원과 유사한 아미노산 염기서열을 가진 *insB* 유전자가 삽입되어 있음을 확인하여 이를 연구대상 유전자로 하였다.

InsB 단백질의 면역원성을 확인하기 위하여 *insB* 유전자를 대장균 내에서 발현시키고 Ni-NTA affinity chromatography 와 FPLC 방법으로 정제하였다. ESAT-6 단백질 또한 같은 방법으로 재조합단백질로 생산하여 실험 대조군으로 하여 마우스 면역 모델과 사람의 혈청을 이용한 실험을 진행하였다.

InsB 와 ESAT-6 재조합단백질을 마우스에 각각 2 주 간격으로 3 회 면역시킨 후 얻은 혈청에서 ELISA 를 이용하여 각 항원에 대한 IgG 항체를 검출한 결과 교차 반응 없이 높은 항체가를 확인하였고, InsB 면역 마우스에서의 반응(P<0.0001)이 ESAT-6 면역 마우스에서의 반응(P<0.01)보다 통계적으로 유의함을 확인하였다. 또한 각 항원을 면역시킨 마우스에서 비장세포를 분리한 후 각 항원을 자극시켜 분비되는 cytokine 들을 ELISA 를 이용하여 분석한 결과, 두 그룹간에 교차 반응 없이 각 항원에 대해 특이적으로 cytokine 이 분비되었고, 특히 InsB 면역 마우스에서 ESAT-6 면역 마우스에서보다 많은 양의 IFN-γ, IL-17 그리고 IL-2 가 분비됨을 확인하여 InsB 단백질이 결핵 진단면역항원으로서 이용될 수 있는 가능성을 확인하였다.

실제로 국내의 결핵환자로부터 얻은 86 개의 혈청과 40 개의 건강인의 혈청에서 InsB 와 ESAT-6 항원에 대한 IgG 항체를 검출한 결과, 두 항원 모두 높은 민감도와 특이도를 보여 혈청진단항원으로서 사용될 수 있는 가능성을 확인하였다. 특히 ROC 곡선으로 분석한 결과로 미루어 보아 InsB 항원(AUC=0.75)이 ESAT-6 항원(AUC=0.62)보다 혈청진단에 더 적합함을 알 수 있었다.

본 연구 결과는 국내 임상 분리 균주인 K 균주의 특이적인 InsB 항원을 발굴하여 면역원성을 확인하였으며, 국내 결핵환자를 진단하기 위한 면역진단항원으로서 사용할 수 있는 가능성을 제시하였다. 추후에 보다 많은 환자의 검체를 대상으로 InsB 항원의 유용성을 평가하여 결핵 면역진단방법에 적용될 수 있을 것으로 기대된다.

핵심되는 말 : 결핵균 K 균주, 면역진단항원, ESAT-6, InsB

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