Development of a Quantitative Sandwich Enzyme-Linked Immunosorbent Assay for Detecting the MPT64 Antigen of Mycobacterium tuberculosis

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Purpose: Tuberculosis (TB) is a major infectious disease and is responsible for two million deaths annually. For the identification and quantitation of Mycobacterium tuberculosis (M. tuberculosis), a causative agent of TB, a sandwich enzyme-linked immunosorbent assay (ELISA) against the MPT64 protein of M. tuberculosis, an antigen marker of the M. tuberculosis complex, was developed. Materials and Methods: The MPT64 protein was expressed, and anti-MPT64 monoclonal antibodies were prepared. A sandwich ELISA was established using recombinant MPT64 protein and anti-MPT64 monoclonal antibodies. The sandwich MPT64 ELISA was evaluated using reference and clinical mycobacterial strains. Results: The sandwich MPT64 ELISA detected MPT64 protein from 2.1 ng/mL to 250 ng/mL (equivalent to 1.7×10^4 CFU/mL and 2.0×10^6 CFU/mL). All 389 clinical M. tuberculosis isolates tested positive in the sandwich MPT64 ELISA (sensitivity, 100%), and the assay showed no cross reactivity to any tested nontuberculous mycobacterial strain (specificity, 100%). Conclusion: The sandwich MPT64 ELISA is a highly sensitive and quantitative test for MPT64 protein, which can identify M. tuberculosis.

Key Words: Mycobacterium tuberculosis, MPT64 protein, sandwich ELISA

INTRODUCTION

Tuberculosis (TB) is a major infectious disease that infects one-third of the world’s population. Mycobacterium tuberculosis (M. tuberculosis), a cause of TB, infects 30 million people every year and causes 8 million new TB cases and 3 million deaths annually.1,2 The situation has been worsened by the appearance of multi-drug or extended drug-resistant M. tuberculosis strains, as well as by the combination with human immunodeficiency virus infection.3 Diagnosis of TB relies mainly on microbiological tests, such as smear microscop-
Sandwich ELISA Against MPT64

Bacterial strains and growth conditions
M. tuberculosis H37Rv (American Type Culture Collection) was used as a reference strain, and was also used for cloning of the MPT64 protein. Five reference strains of M. tuberculosis, 46 NTM reference strains, 389 clinical M. tuberculosis isolates, and 27 M. avium isolates, were used for this study (Table 1). Of the clinical isolates, 231 clinical M. tuberculosis isolates grown on 3% Ogawa medium (Asan Pharmaceutical, Seoul, Korea) and 158 clinical M. tuberculosis strains grown in the BacT/ALERT Automated System (BioMérieux, Durham, France) were used in this study. All clinical NTM isolates were grown on 3% Ogawa medium. All clinical isolates were identified by Ziehl-Neelsen staining, the AdvanSure TB/NTM real-time PCR kit (LG life science, Seoul, Korea), and REBA Myco-ID® (M&D, Wonju, Korea).

PCR amplification and cloning of mpt64 of M. tuberculosis
The mpt64 gene was amplified by PCR using oligonucleotide primers designed to include an EcoRI restriction enzyme site at the 5’ end and an XbaI restriction enzyme site at the 3’ end. The sequences of the primers were 5'-GAA TTC GGC CCC AAG ACC TAC TGC GAG-3' (EcoRI/ MPT64-F) and 5'-TCT AGA CTA GGC CAG CAT CGA GTC GAT C-3' (XbaI/MPT64-R). The amplified mpt64 gene was ligated into the pT7 Blue vector (Novagen, Darmstadt, Germany), and their sequences were confirmed.

Expression and purification of recombinant MPT64
The mpt64 gene was ligated into the pMAL-p2x expression vector (New England Biolabs, Beverly, MA, USA), and MPT64 protein was expressed using E. coli TB-1 (Invitrogen, San Diego, CA, USA). The recombinant MPT64 protein was purified using affinity chromatography with an amylose resin column (New England Biolabs) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and a Western blot assay using mouse polyclonal anti-M. tuberculosis antibody, which was kindly provided by Prof. S.N. Cho (Yonsei University, Seoul, Korea).

Production of anti-MPT64 monoclonal antibodies
Ten eight-week-old female BALB/c mice (Orient Bio, Seongnam, Korea) were immunized intraperitoneally (i.p.) three times at two-week intervals with 40 µg of recombinant MPT64 protein emulsified in incomplete Freund’s adjuvant (Sigma-Aldrich Co., St. Louis, MO, USA). Spleen cells were isolated and fused with SP2/0 myeloma cells at a ratio of 5:1 in the presence of polyethylene glycol 1500 (Roche Diagnostics GmbH, Mannheim, Germany). The hybridomas were selected in HAT medium (hypoxanthine-aaminopterin-thymidine medium) and screened by measuring their binding activity to recombinant MPT64 protein by...
introduced antibodies were enriched in ascitic fluid from BALB/c mice pretreated with 1.0 mL of Pristance (Aldrich, Milwaukee, WI, USA), and the immunoglobulins were purified by chromatography on a protein G-Sepharose 4B flow (Amersham Bioscience, Piscataway, NJ, USA).

**Sandwich enzyme-linked immunosorbent assay for MPT64 protein**

Initially, anti-MPT64 monoclonal antibodies were screened for their reactivity to recombinant MPT64 protein, and highly reactive anti-MPT64 monoclonal antibodies were tested for their suitability for the sandwich ELISA. The optimum dilutions of these reagents were selected by checkerboard titration. Next, the sandwich ELISA was performed as follows: briefly, 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with anti-MPT64 monoclonal antibody in a suitable concentration and incubated at 4°C overnight. After blocking with non-fat dry milk, recombinant MPT64 protein in phosphate buffered saline (PBS) was added and incubated for 2 h at 37°C. Subsequently, wells were washed four times and incubated with other horseradish peroxidase (HRP)-conjugated anti-MPT64 monoclonal antibodies for 1 h at 37°C. Finally, after six washes, 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate was added to the wells, the plates were incubated for 20 min in the dark, and absorbance was read at 450 nm after stopping the reaction with 2.5 N H₂SO₄.

For generation of a standard curve, 1.0 µg/mL to 1000 µg/mL of recombinant MPT64 protein was used in the sandwich ELISA. The detection limit of the assay was defined as the mean value of blank plus three times its standard deviation.

**Table 1. List of Mycobacterial Strains**

| Mycobacterial species        | Reference no. | Mycobacterial species        | Reference no. |
|------------------------------|---------------|------------------------------|---------------|
| *M. tuberculosis* H37Rv      | ATCC27294     | *M. intracellulare*          | KCTC9514      |
| *M. tuberculosis* H37Ra      | ATCC25177     | *M. intracellulare* (27)     |               |
| *M. tuberculosis* Erdman     | ATCC35801     | *M. kansasii*                | KCTC9515      |
| *M. tuberculosis* CDC1551    |               | *M. marinum*                 | ATCC11564     |
| *M. tuberculosis* HN878      |               | *M. marinum*                 | ATCC927       |
| *M. tuberculosis* (389)      |               | *M. molmoense*               | ATCC29571     |
| *M. abscessus*               | ATCC19977     | *M. morkaense*               | KCTC9516      |
| *M. abscessus*               | ATCC23003     | *M. mucogenicum*             | KCTC19088     |
| *M. abscessus* (12)          |               | *M. mucogenicum*             | ATCC49650     |
| *M. acapulcensis*            | KCTC9501      | *M. neoaurum*                | ATCC25795     |
| *M. agri*                    | KCTC9502      | *M. nonchromogenicum*        | ATCC19530     |
| *M. asiaticum*               | KCTC9503      | *M. peregrinum*              | ATCC14467     |
| *M. austroafricanum*         | KCTC9504      | *M. phlei*                   | KCTC2192      |
| *M. avium*                   | ATCC25291     | *M. porcinum*                | KCTC9517      |
| *M. avium* (25)              |               | *M. pulveris*                | KCTC9518      |
| *M. branderi*                | ATCC51788     | *M. scrofulaceum*            | ATCC19981     |
| *M. celatum*                 | ATCC51133     | *M. senegalense*             | ATCC35796     |
| *M. chelonae*                | KCTC9505      | *M. shimoidei*               | ATCC27962     |
| *M. dierhotheri*             | KCTC9506      | *M. simiae*                  | ATCC15080     |
| *M. flavescens*              | ATCC14474     | *M. simiae*                  | ATCC25275     |
| *M. flavescens*              | ATCC23008     | *M. smegatis*                | KCTC1057      |
| *M. fortuitum*               | KCTC1122      | *M. sphagni*                 | ATCC33027     |
| *M. fortuitum*               | KCTC9510      | *M. szulgai*                 | KCTC9520      |
| *M. gilvum*                  | KCTC9512      | *M. terrae*                  | ATCC15755     |
| *M. gordonae*                | KCTC2026      | *M. triviale*                | ATCC23292     |
| *M. gordonae*                | KCTC9513      | *M. vaccae*                  | ATCC15843     |
| *M. interjectum*             | ATCC51457     | *M. xenopi*                  | ATCC19250     |
| *M. intermedium*             | ATCC51846     |                             |               |

ATCC, American Type Culture Collection; KCTC, Korean Collection for Type Culture.

( ): Number of strains.
Evaluation of MPT64 sandwich ELISA using clinical Mycobacterium isolates

To determine the detection limit of the MPT64 sandwich ELISA, a series of diluted culture suspensions of *M. tuberculosis* H37Rv were applied to the MPT64 sandwich ELISA, which was counted by inoculation onto Middlebrook 7H10 agar (Difco, Detroit, MI, USA) plates. To determine the sensitivity and specificity of the tests, 389 clinical *M. tuberculosis* isolates, five *M. tuberculosis* reference strains, 46 NTM reference strains, and 64 clinical NTM isolates were tested (Table 1). Three or four colonies from 3% Ogawa medium were collected and suspended in 200 µL of PBS, and then 100 µL of the suspension was used as a sample. The samples were classified as positive when the absorbance value was greater than or equal to twice the absorbance value of the negative control.

Statistical analysis

Statistical analysis was performed using Microsoft® Office Excel (2010) and the GraphPad Prism (version 4.0) program.

**RESULTS**

Cloning and expression of the mpt64 gene

The gene coding for the MPT64 protein was amplified from the *M. tuberculosis* H37Rv genome by PCR, and the sequence was confirmed (data not shown). The expressed recombinant MPT64 protein has a molecular weight of approximately 65 kDa and is reactive with the mouse polyclonal anti-*M. tuberculosis* antibody (Fig. 1).

Characterization of anti-MPT64 protein monoclonal antibodies

Prepared hybridomas were screened for their ability to secrete monoclonal antibodies against the MPT64 protein. Two antibodies (1A4 and 2E9) from screened hybridomas showed the highest reactivity in the immunoassay (data not shown). The immunoglobulin isotype of both antibodies belongs to the IgG1 subclass having the lambda light chain (data not shown).

Establishment of a sandwich ELISA for MPT64 protein

A sandwich ELISA for the MPT64 protein was established using a 1A4 monoclonal antibody as a capture antibody for the MPT64 protein and HRP-conjugated 2E9 monoclonal antibody as a detection antibody for MPT64 protein, which produced minimal background, as well as the highest sensitivity and accuracy for the quantification of the MPT64 protein. The optimal concentrations of the capture antibody 1A4 and detection antibody 2E9 were 2 µg/mL and 4 µg/mL, respectively, and the secondary antibody was diluted to 1:20000. A typical calibration curve for quantification of the MPT64 protein was generated by plotting the concentration of recombinant MPT64 protein versus the absorbance, which yielded a coefficient of determination ($R^2$) of 0.9943 (Fig. 2A). The lower limit of detection of this assay was 2.1 ng/mL, equivalent to an absorbance value of 0.102, which was the mean signal of the blank plus three times its standard deviation, and this assay was linear over the range of 3.8 to 250 ng/mL. Therefore, this assay might be a highly sensitive assay to quantify the MPT64 protein of *M. tuberculosis*.

Detection limit of MPT64 sandwich ELISA and MPT64 ICA

To evaluate the ability of the MPT64 sandwich ELISA to detect secreted MPT64 protein from growing *M. tuberculosis*, the sequential culture supernatants of *M. tuberculosis H37Rv* were tested. The MPT64 sandwich ELISA detected levels from $1.7 \times 10^4$ CFU/mL of *M. tuberculosis* as positive, based on a cut-off value of 0.102, which is equivalent to 2.1 ng/mL (Fig. 2B). The upper limit of detection of the
The sensitivity and specificity of these tests were evaluated using 51 reference strains of *M. tuberculosis* and NTMs, 389 clinical *M. tuberculosis* isolates, and 64 clinical NTM iso-

![Graph A](image-url)

**A** Calibration curve of the developed sandwich ELISA for MPT64 protein (A), and detection limits of the assay (B). The calibration curve was obtained from the mean value of six sandwich ELISAs with the anti-MPT64 antibodies Mab-1A4 and Mab-2E9 under optimized conditions (A). Culture supernatants of *M. tuberculosis H37Rv* in Middlebrook 7H9 broth with enrichment were serially diluted and applied to the test. The cut-off of the MPT64 sandwich ELISA is indicated by a dotted line. ELISA, enzyme-linked immunosorbent assay; CFU, colony-forming unit; OD, optical density.

**B** Absorbance at 450 nm

![Graph B](image-url)

**Table 2.** Comparison of the Results Obtained by the MPT64 Sandwich ELISA and the Immunochromatographic TB Ag MPT64 Rapid Test for *M. tuberculosis* and Other Nontuberculous Mycobacterial Strains

| Species (no. of isolates) | MPT64 sandwich ELISA | Immunochromatographic TB Ag MPT64 Rapid Test |
|--------------------------|----------------------|---------------------------------------------|
| *M. tuberculosis (5)*†    | 5 (100)              | 5 (100)                                     |
| *M. tuberculosis (231)*†  | 231 (100)             | 231 (100)                                   |
| *M. tuberculosis (158)*†  | 158 (100)             | 158 (100)                                   |
| NTMs (46)*                | 0 (0)                | 46 (100)                                    |
| *M. abscessus (12)*†      | 0 (0)                | 12 (100)                                    |
| *M. avium (25)*†          | 0 (0)                | 25 (100)                                    |
| *M. intracellulare (27)*† | 0 (0)                | 27 (100)                                    |

NTM, nontuberculous mycobacteria; ELISA, enzyme-linked immunosorbent assay; TB, tuberculosis.

*Reference mycobacterial strains.

†Clinical isolates grown on 3% Ogawa medium.

‡Clinical isolates grown in the BacT/ALERT Automated System.
In this study, we developed a highly sensitive and quantitative sandwich ELISA for the MPT64 protein of M. tuberculosis using anti-MPT64 monoclonal antibodies. This MPT64 sandwich ELISA could measure the quantity of secreted MPT64 protein from M. tuberculosis, which differentiates M. tuberculosis from NTM. The lower limit of detection of the MPT64 sandwich ELISA was 2.1 ng/mL, and this assay could detect 1.7×10^4 CFU/mL of M. tuberculosis. Recently, Liu, et al. developed an MPT64 ELISA using polyclonal antibodies, and its lower limit of detection was 10 ng/mL, which is approximately five times higher than ours. A possible explanation for this is that the anti-MPT64 monoclonal antibodies used for the sandwich ELISA might have a higher affinity than polyclonal antibodies. The MPT64 ICA could detect 1×10^4 CFU/mL of M. tuberculosis as positive. This suggests that the MPT64 sandwich ELISA might be a more sensitive assay than MPT64 ICA for the detection of MPT64 protein. The sensitivity and specificity of the MPT64 sandwich ELISA were both 100%. These results seem to be comparable to or more sensitive than MPT64 ICA, which has a reported sensitivity of 92-99% and a specificity of 97-100%. This indicates that the MPT64 protein is highly specific for the M. tuberculosis complex and is therefore a comparably more sensitive target analyte.

In addition, the MPT64 sandwich ELISA has an advantage over the MPT64 ICA, in that the MPT64 sandwich ELISA is able to quantify the MPT64 protein of M. tuberculosis, which is significantly correlated with the count of M. tuberculosis (R^2=0.998, p<0.001). This would be very useful for measuring counts of M. tuberculosis, because it takes three to six weeks for enumeration of M. tuberculosis and also could contribute to the monitoring of the therapeutic effects of anti-mycobacterial drugs, as well as to the more rapid screening of anti-mycobacterial drug candidates.

In conclusion, a MPT64 sandwich ELISA, developed using recombinant MPT64 protein and anti-MPT64 monoclonal antibodies, was shown to be a highly sensitive and specific assay for the identification of M. tuberculosis via detection of the MPT64 protein, and may be a quantitative assay for measuring the MPT64 protein of M. tuberculosis.
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