

## Original Article

# Cloning and characterization of Clp protease proteolytic subunit 2 and its implication in clinical diagnosis of tuberculosis

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**Abstract:** Objective: To clone, express, and characterize *Mycobacterium tuberculosis* (Mtb) ClpP2, and evaluated the potential usage of ClpP2 in clinical diagnosis of tuberculosis. Methods: Mtb ClpP2 was cloned into recombinant plasmid pET32a (+) and transformed into *E. coli* BL21 (DE3). SDS-PAGE and Western blot analysis were performed to detect the expression of the recombinant protein. The immunogenicity of Mtb ClpP2 was assessed with epitope prediction and antibody titer assay. Quantitative real-time PCR was performed to detect the influence of stress conditions on ClpP2 expression. ClpP2 antigen and antibody in patients with pulmonary diseases were detected by indirect ELISA. ROC curve was constructed to assess the diagnostic accuracy of Mtb ClpP2 for tuberculosis. Results: We had cloned and expressed recombinant Mtb ClpP2 in *E. coli*. Our results showed that Mtb ClpP2 had potent immunogenicity, and our own prepared polyclonal antibody could be used in detection and diagnostic tests. Results from Western blot showed that ClpP2 was mainly located in *M. bovis* BCG cytoplasm, and real-time PCR indicated that stress conditions could enhance the mRNA expression of ClpP2. Indirect ELISA suggested that, in tuberculosis patients, both the levels of ClpP2 antigen and antibody were increased, and the positive rates of ClpP2 were elevated. ROC curve had demonstrated satisfactory sensitivity and specificity of ClpP2-based diagnosis for tuberculosis. Conclusion: Our results suggest that Mtb ClpP2 antigens would be used as a biomarker in tuberculosis pathogenesis. These findings highlight the feasibility of the application of Mtb ClpP2 in the clinical diagnosis of tuberculosis.

**Keywords:** Clp protease proteolytic subunit 2 (ClpP2), *Mycobacterium tuberculosis* (Mtb), tuberculosis, serological diagnosis

## Introduction

Nowadays, one-third of the world's population is estimated to be infected by *Mycobacterium tuberculosis* (Mtb), 10% of which carry the risk of ending up with tuberculosis [1]. Although tuberculosis is a curable and preventable disease, the rapid rise of multidrug- and extensively drug-resistant Mtb complicates the disease control. Prevention of tuberculosis infection mainly depends on cellular immune responses. On the other hand, humoral immune would help to evaluate tuberculosis diagnosis and vaccines [2, 3]. Therefore, identification of antigenic proteins is very important for assessing the immune responses against tuberculosis, and for understanding the disease pathogenesis.

Caseinolytic proteases (Clp) are ATP-dependent enzymes, which are first discovered in *Escherichia coli*, and then found to be present in various eukaryotic and prokaryotic organisms. Clps are comprised of several proteolytic and regulatory subunits [4]. Mtb Clp system consists of two paralog genes encoding putative peptidases, i.e., Clp protease proteolytic subunit 1 (ClpP1) and ClpP2, which are essential for the optimal growth of the bacteria [5-7]. ClpP1 and ClpP2 are co-transcribed to form active proteolytic complex [8, 9]. A growing body of evidence suggests that ClpP1 and ClpP2 might be actively involved in Mtb pathogenesis [6, 10]. However, the immunological characteristics of particular ClpP2 and its implication in

the clinical diagnosis of tuberculosis have not yet been fully elucidated.

In this study, we obtained and characterized Mtb Rv2460c-encoded recombinant ClpP2, and detected the immunoreactivity of the target protein with serum from patients with pulmonary diseases. The potential usage of ClpP2 in tuberculosis diagnosis was also evaluated. Our results suggest ClpP2 might be used as a detection target in the rapid and accurate diagnosis of tuberculosis.

## Materials and methods

### *Strains and animals*

*M. bovis* BCG strain Pasteur (Danish strain) was purchased from Shanghai Institute of Biological Products, Shanghai, China. *Escherichia coli* competent bacteria, *Escherichia coli* BL21 (DE3) were obtained from Novagen, Madison, WI, USA. Four male New Zealand white rabbits, weighing 1-1.5 kg, were purchased from the Animal Center of Chongqing Medical University. All animal experiments were in compliance with guideline of the Ethics Committee of Chongqing Medical University.

### *Patients*

All human serum samples used herein were from the First Affiliated Hospital of Chongqing Medical University. Tuberculosis diagnosis was determined by culture/smear and clinical evaluation (chest X-ray examination and clinical symptoms, etc.). Normal control subjects had all been vaccinated with BCG, without tuberculosis history. Non-tuberculosis patients with other pulmonary diseases had to be tuberculosis free, as confirmed by sputum acid-fast staining and culture. Tuberculosis suspects had typical tuberculosis symptoms (such as fever, night sweats, and coughing, etc.), without etiological evidence. All subjects should be negative for HIV. Prior written and informed consent were obtained from every patient and the study was approved by the ethics review board of Chongqing Medical University.

### *Cloning, expression, and purification of Mtb ClpP2*

Mtb ClpP2 primers (forward: 5'-GGGGTACCGTGAATCCCCAAATTCT-3', and reverse: 5'-CCCAA-GCTTTCAGGCGGTTTGCG-3') with engineered

enzyme recognition sites, KpnI and HindIII, were used to amplify the open reading frame (ORF) of ClpP2-coding Rv2460c gene from Mtb strains H37Rv. After purification and digestion, PCR product was cloned into the pET32a vector (Novagen). Then the recombinant plasmids ClpP2-pET32a were transformed into *E. coli* BL21 (DE3) for expression. Transformed *E. coli* BL21 cells were grown in LB medium containing kanamycin at 37°C, until OD<sub>600nm</sub> reached 0.6. Protein expression was induced with 1 mmol/L IPTG at 30°C for 5 h. *E. coli* BL21 cells were harvested by centrifugation at 5,000 g for 10 min, and then resuspended in lysing buffer, followed by sonication.

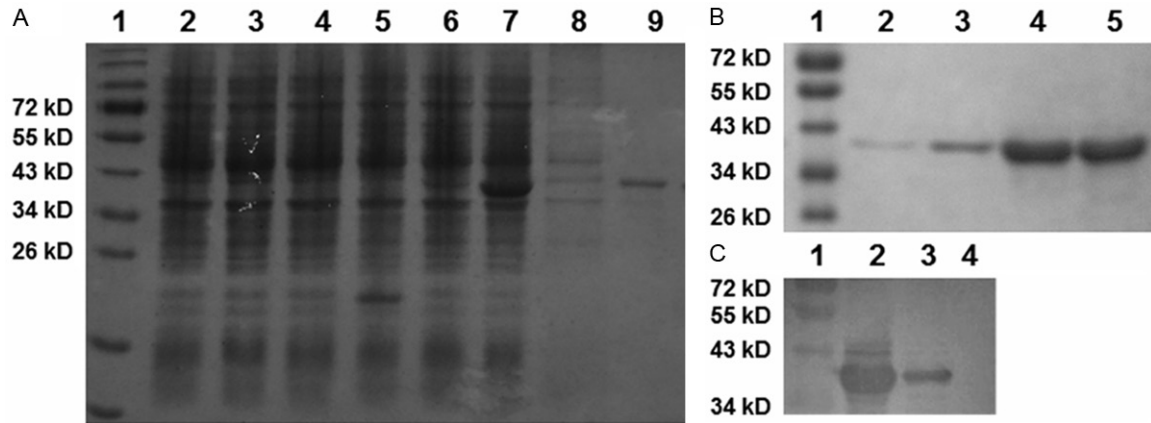
For purification, the cell pellet was lysed in binding buffer at room temperature for 1 h, and then centrifuged at 10,000 g for 20 min. Recombinant proteins were purified using purification system Ni-Agarose His Tag under denaturing conditions with binding buffer containing 8 M urea. Those fractions with a single band of the correct molecular size were collected, and then dialysed with decreasing concentration gradients of urea (6 M, 4 M, 2 M, 0 M) at 4°C. Protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA), according to the manufacturers' instructions.

### *Preparation of rabbit anti-ClpP2 antibodies*

Rabbits were injected with emulsified recombinant protein ClpP2 (0.5 mg each animal) by Freund's adjuvant every two weeks for four times, at animal back, groin, and other sites. Control animals were injected with emulsified saline by Freund's adjuvant instead. One week after the last injection, cardiac blood was collected. Serum was obtained and stored at -80°C. The titer evaluation was performed by ELISA in recombinant protein-coated microtiter plates, with serial dilutions of rabbit serum as the primary antibody, and HRP-conjugated goat anti-rabbit IgG as the secondary antibody. Maximum antibody dilution that fulfilled criteria ( $OD_{positive}/OD_{negative} > 2.1$ ) was considered as the antibody titer, in which serum collected before immunization was used as negative control.

### *Protein epitope prediction*

VaxiPred software was used for Mtb ClpP2 epitope prediction. B-cell epitope prediction web-



**Figure 1.** SDS-PAGE and Western blot analysis of recombinant ClpP2 expression. A. SDS-PAGE analysis of rClpP2. Lanes: 1, protein marker; 2, non-induced *E. coli* BL21 (DE3); 3, induced *E. coli* BL21 (DE3); 4, non-induced *E. coli* BL21 (DE3) with pET32a (+); 5, induced *E. coli* BL21 (DE3) with pET32a (+); 6, non-induced *E. coli* BL21 (DE3) with Mtb ClpP2-pET32a (+); 7, induced *E. coli* BL21 (DE3) with Mtb ClpP2-pET32a (+); 8, supernatant of induced *E. coli* BL21 (DE3) with Mtb ClpP2-pET32a (+); 9, precipitation of induced *E. coli* BL21 (DE3) with Mtb ClpP2-pET32a (+). B. SDS-PAGE analysis of purified rClpP2. Lanes: 1, protein marker; 2-5, purified rClpP2. C. Western blot analysis of purified rClpP2. Lanes: 1, protein marker; 2, induced rClpP2/His fusion protein; 3, purified rClpP2/His fusion protein; 4, negative control (induced *E. coli* BL21).

site was <http://www.imtech.res.in/raghava/abcpred>; T-helper (Th) epitope prediction website was <http://www.imtech.res.in/raghava/propred>; cytotoxic T-lymphocyte (CTL) epitope prediction website was <http://www.imtech.res.in/raghava/ctlpred>.

#### ELISA

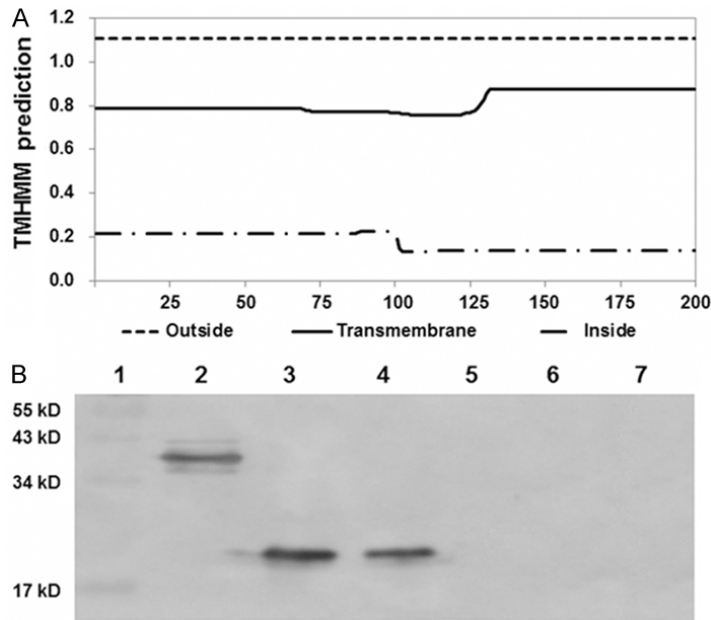
Serum levels of ClpP2 antigen and antibody from patients were analyzed by indirect ELISA. For the assessment of anti-ClpP2 antibody in serum, 100  $\mu$ l purified recombinant ClpP2 (10  $\mu$ g/ml) was used to prepare coated 96-well microtiter plates at 4°C overnight. After washing with PBS (pH 7.4), 300  $\mu$ l PBS with 2% BSA was added for blocking at 37°C for 2 h. After washing with 0.05% Tween-20 PBS (pH 7.4), 100  $\mu$ l serum sample (1:100 dilution) from Mtb-infected patients or healthy control subjects was added. After washing, HRP-conjugated goat anti-human IgG (1:2000 dilution) was added for incubation at 37°C for 1 h. TMB was used for chromogenic detection at 37°C for 15 min. 2 M  $H_2SO_4$  was added to stop the reaction, and  $OD_{450nm}$  was read. For the evaluation of ClpP2 antigen in serum, purified recombinant ClpP2, human serum samples (1:100 dilution), and HRP-conjugated goat anti-human IgG in the above description were replaced by standardized serum, rabbit polyclonal anti-ClpP2 antibody, and HRP-conjugated goat anti-rabbit IgG, respectively.

#### Quantitative real-time PCR

Total RNA was extracted with Trizol reagent. Synthesis of cDNA was performed with PrimeScript™ RT reagent kit (Takara, Shiga, Japan). Quantitative real-time PCR reaction system (20  $\mu$ l) was as follows: 10  $\mu$ l SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA), 2  $\mu$ l cDNA template, 1  $\mu$ l of each primer, and 6  $\mu$ l RNase-free water. Amplification reaction conditions consisted of denaturation at 95°C 30 s; 95°C for 5 s, 60°C for 10 s, for a total of 30 cycles; extension at 65°C for 10 s. ClpP2 primers were forward 5'-GAGTCCAATCCATACAACAAAGC-3' and reverse 5'-CTCCAACACCAGCAACTGTG-3', and internal control SigA primers were forward 5'-GACGAAGACCACGAAGACCTC-3' and reverse 5'-TCATCCCAGACGAAATCACC-3'.

#### SDS-PAGE and Western blot analysis

Subcellular localization of ClpP2 in *M. bovis* BCG strain Pasteur was investigated by SDS-PAGE and Western blot analysis. *M. bovis* BCG were harvested and subjected to centrifugation to separate the precipitate and supernatant. 100  $\mu$ l DB with 20% sucrose (Shanghai Huashun Biological Engineering Company, Shanghai, China) was added into the precipitate, followed by 400  $\mu$ l lysozyme solution (25 mg/ml), and then mixed and placed in 37°C bath for 48 h. After centrifugation, supernatant containing cell wall constituent was collected and stored



**Figure 2.** Localization of ClpP2 in *M. bovis* BCG. A. Transmembrane prediction of ClpP2 with TMHMM. B. Western blot analysis of cellular fractions from *M. bovis* BCG. Purified rClpP2 (~37 kD) was used as positive control. Lanes: 1, protein marker; 2, positive control; 3, whole cells; 4, cytoplasm; 5, membrane; 6, cell wall; 7, culture supernatant.

at -80°C, and 1 ml lysing buffer (0.5 M NaCl, 20 mM Tris-Cl, 5 mM iminazole, pH 7.9) was added into the precipitate before ultrasonication. The lysate was centrifuged, and supernatant containing cytoplasmic components was collected and stored at -80°C. These samples were then subjected to SDS-PAGE and Western blot analysis.

After mixed with SDS loading buffer, the protein samples were subjected to 12% SDS-PAGE, and then Coomassie blue staining was performed. For Western blot analysis, protein samples were transferred onto a PVDF membrane. 5% (w/v) non-fat milk was used to block the membrane at room temperature for 2 h, and the PVDF membrane was then incubated with rabbit anti-rClpP2 antibody (1:200 dilution) at 4°C overnight. After washing with PBST, HRP-conjugated goat anti-rabbit IgG (1:5000 dilution; ZSGB-BIO, Beijing, China) was used to incubate the membrane at room temperature for 2 h. The blots were then developed using DAB reagents.

#### Statistical analysis

Data were expressed as mean  $\pm$  SD. Independent t test and Kruskal Wallis test (NonParam-

etric ANOVA) with the Dunnett post test were used for comparison.  $P < 0.05$  was considered statistically significant.

#### Results

##### *Cloning, expression, and characterization of recombinant Mtb ClpP2*

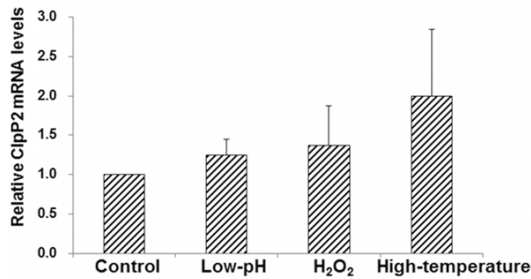
*Mtb* ClpP2 was first cloned into recombinant plasmid pET32a (+) and transformed into *E. coli* BL21 (DE3). SDS-PAGE and Western blot analysis were performed to detect the expression of the recombinant protein. After IPTG induction at 37°C for 2 h, cells were harvested and lysed, and then subjected to SDS-PAGE analysis. The results showed a single band with expected molecular weight (~37 kD), indicating the expression of rClpP2 (Figure 1A). After purification, rClpP2 with purity greater than

90% was obtained (Figure 1B). Moreover, Western blot analysis showed a clear band at ~37 kD, which was in line with the expected molecular weight (Figure 1C). In the following experiments, characterization of the recombinant protein was performed, and its polyclonal antibodies were prepared. The potential usage of rClpP2 in tuberculosis diagnosis was also evaluated.

##### *Epitope prediction and immunogenicity analysis of recombinant Mtb ClpP2*

To evaluate the immunogenicity of recombinant *Mtb* ClpP2, epitope prediction was first performed. ABCpred server was used for B-cell epitope prediction. Results showed that there might be three B-cell epitopes (aa 45-60; aa 176-191; aa 197-212) in the sequence of *Mtb* ClpP2. Prediction of cytotoxic T-lymphocyte (CTL) epitopes using neural networks [11] indicated three peptide sequences with high scores, i.e., aa 16-24 (LPSFIEHSS), aa 105-113 (LGQAASAAA), and aa 22-30 (HSSFGVKES). On the other hand, on-line program suggested three distinct T-helper (Th) epitopes in *Mtb* ClpP2, namely DRB1\_0101, DRB1\_0102 and DRB1\_0103 [12]. The prediction indicates that *Mtb* ClpP2 might have the potential to induce





**Figure 3.** Effects of stress conditions on ClpP2 expression in *M. bovis* BCG. *M. bovis* BCG was subjected to low-pH (pH 5.5), 10 mM H<sub>2</sub>O<sub>2</sub>, and high-temperature (45°C) treatments, respectively, for 24 h. Then real-time PCR was performed to detect the mRNA expression levels of ClpP2 in *M. bovis* BCG. Compared with normal control, \**P* < 0.05.

cellular and humoral immunity. Next, the immunogenicity of recombinant Mtb ClpP2 was evaluated, and the polyclonal antibody was generated, in New Zealand white rabbits. Rabbits were immunized subcutaneously with Mtb rClpP2 and Freund's adjuvant, and the antibody titer was assessed by ELISA. Our results showed that the serum antibody titer was 1:64000 with rClpP2 and Freund's adjuvant, while no antibody response was observed in the serum with Freund's adjuvant alone (antibody titer lower than 1:100). These results confirmed the potent immunogenicity of recombinant Mtb ClpP2, and the polyclonal antibody was used in the following detection and diagnostic tests.

#### Localization and induced expression of ClpP2 in *M. bovis* BCG

The subcellular localization of ClpP2 in *M. bovis* BCG was next investigated. Using hidden Markov models (TMHMM), transmembrane prediction showed that ClpP2 protein lacked transmembrane helices (**Figure 2A**), indicating that ClpP2 might be a cytoplasmic or secreted protein. To further confirm the localization of ClpP2 in *M. bovis* BCG, bacterial components were collected and subjected to Western blot analysis, using our own prepared rabbit anti-ClpP2 polyclonal antibody. Our results showed that there were no detectable bands in the lanes of cell membrane, cell wall, and culture supernatant, while there was a clear band in the cytoplasm lane (**Figure 2B**).

To investigate the influence of stress conditions on ClpP2 expression, the gene mRNA expres-

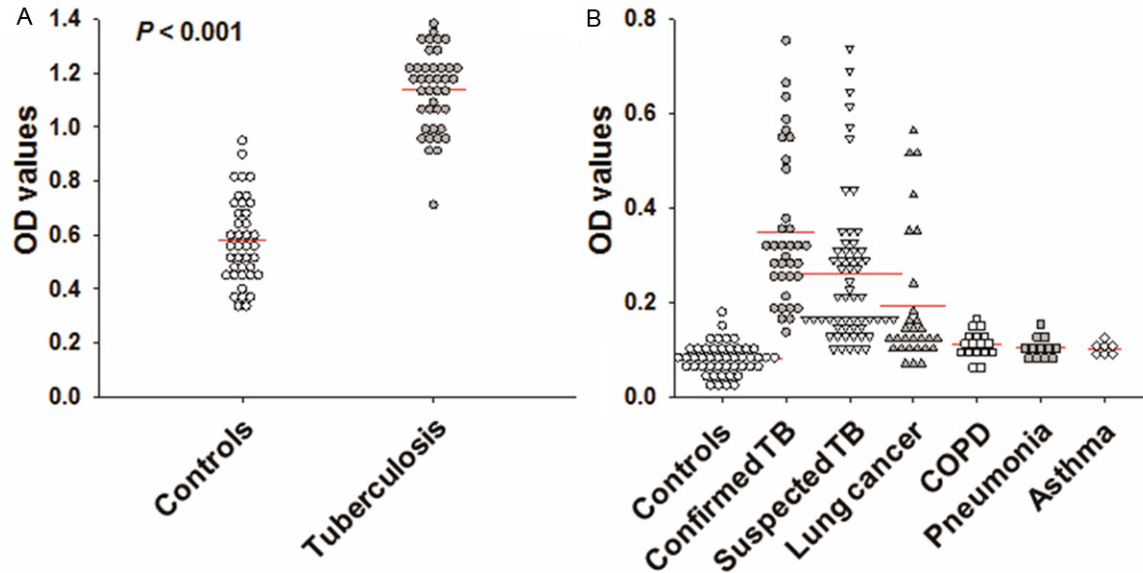
sion in *M. bovis* BCG was assessed with quantitative real-time PCR under limiting growth conditions. The bacteria were subjected to low-pH (pH 5.5), 10 mM H<sub>2</sub>O<sub>2</sub>, and high-temperature (45°C) treatments, respectively, for 24 h. Our results showed that, under stress conditions, the transcriptional levels of ClpP2 were significantly elevated, which might contribute to the protection against adverse environment (**Figure 3**). These results suggest that ClpP2 is mainly located in the cytoplasm in *M. bovis* BCG, and stress conditions could enhance the mRNA expression of ClpP2.

#### Analysis of ClpP2 in serum from patients with clinical tuberculosis

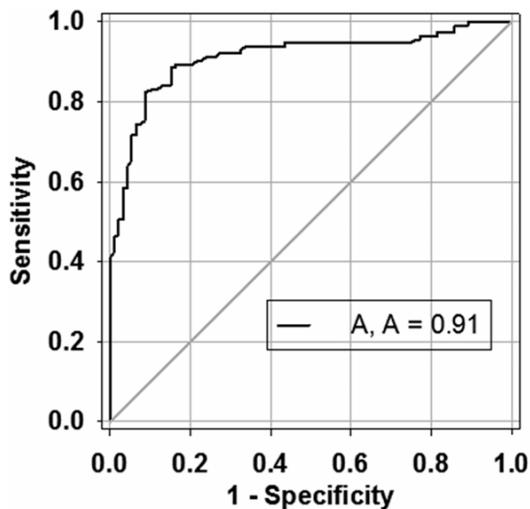
To investigate the immune responses against Mtb ClpP2 in tuberculosis patients, anti-ClpP2 IgG in serum was detected by indirect ELISA. Our results showed that the mean optical density at 450 nm (OD<sub>450nm</sub>), indicating the antibody level, in tuberculosis patients was  $1.136 \pm 0.147$ , which was significantly higher than the normal control subjects ( $0.579 \pm 0.15$ ) (*P* < 0.01; **Figure 4A**).

Considering the enhanced antibody responses against ClpP2 in patients with tuberculosis, we wondered whether Mtb ClpP2 antigen existed in the serum of these patients. Therefore, 96-well microtiter plates were coated with our prepared rabbit anti-ClpP2 polyclonal antibody, and then Mtb ClpP2 antigen levels in the serum samples from patients with various pulmonary diseases were detected by indirect ELISA. Totally, 93 tuberculosis serum samples (35 confirmed and 58 suspected) and 113 non-tuberculosis serum samples (12 pneumonia, 7 asthma, 17 COPD, 29 lung cancer, and 48 healthy individuals) were tested. As shown in **Figure 4B**, the mean OD<sub>450nm</sub> of tuberculosis patient ( $0.293 \pm 0.162$ ) was significantly higher than the non-tuberculosis subjects ( $0.119 \pm 0.094$ ) (*P* < 0.001). Among the non-tuberculosis subjects, OD<sub>450nm</sub> values were decreased in the following order: lung cancer ( $0.193 \pm 0.145$ ), COPD ( $0.111 \pm 0.029$ ), pneumonia ( $0.104 \pm 0.023$ ), asthma ( $0.101 \pm 0.012$ ), and healthy individuals ( $0.081 \pm 0.032$ ).

Furthermore, the positive rates of ClpP2 antigen in serum samples from the confirmed and suspected tuberculosis patients, and non-tuberculosis patients were 100% (35/35), 55.2% (32/58), and 8.0% (9/113), respectively,



**Figure 4.** Enzyme-linked immunosorbent assay detecting *Mtb* ClpP2 antibody and antigen levels in patients with pulmonary diseases. The anti-rClpP2 IgG in serum from tuberculosis patients (A) and ClpP2 antigen levels in serum of patient with various pulmonary diseases (B) were detected by indirect ELISA. Each point denotes an individual's data, and the short red line denotes the mean absorbance value in each group. (A) Tuberculosis patients,  $n = 40$ ; healthy control subjects,  $n = 40$ . (B) Confirmed tuberculosis,  $n = 35$ ; suspected tuberculosis,  $n = 58$ ; pneumonia,  $n = 12$ ; asthma,  $n = 7$ ; COPD,  $n = 17$ ; lung cancer,  $n = 29$ ; healthy individuals,  $n = 48$ . Tuberculosis patients vs. healthy individuals,  $P < 0.001$ .



**Figure 5.** Receiver operating characteristic (ROC) curve for *Mtb* ClpP2 antigen in tuberculosis diagnosis. ROC curve was constructed to investigate the diagnostic accuracy of *Mtb* ClpP2 for tuberculosis, and the area under the curve (AUC) was calculated accordingly.

while there were no ClpP2-positive cases in healthy individuals. The above results suggest that both the levels of ClpP2 antigen and anti-

body are increased, and the positive rates of ClpP2 are elevated, in tuberculosis patients, implying that ClpP2 might be involved in tuberculosis pathogenesis and helpful in the clinical diagnosis.

#### Receiver operating characteristic (ROC) curve construction of *Mtb* ClpP2 antigen in tuberculosis diagnosis

Infection could always be confirmed by the existence of antigens of certain pathogens, while the antibody tests might not precisely reflect the disease progression [13]. Therefore, *Mtb* ClpP2 immunodominant antigens would be used a biomarker in diagnosis of tuberculosis. To investigate the diagnostic accuracy of *Mtb* ClpP2 for tuberculosis, the ROC curve was constructed, and the area under the curve (AUC) was calculated accordingly. Our results showed that the AUC value for *Mtb* ClpP2 was 0.911 (95% CI: 0.869-0.953), and our method yielded 72.2% sensitivity and 91.3% specificity for tuberculosis diagnosis (Figure 5). These results further demonstrate the feasibility of the application of *Mtb* ClpP2 in the clinical diagnosis of tuberculosis.

## Discussion

Tuberculosis is responsible for 1.5 million deaths worldwide every year, which is only second to HIV/AIDS as the leading cause of death due to single infectious agent [1]. Tuberculosis is typically caused by *Mtb* infection. Increasing evidence has demonstrated that antigen-specific cellular and humoral immunity plays an important and indispensable role in protecting against *Mtb* infection [14, 15]. Antigen is the key element in successfully developing diagnostic methods and/or vaccines. Immunological diagnostic tests usually involve recombinant *Mtb* proteins produced under various growth conditions, which can be detected *in vitro* or *in vivo* at different stages of infection. The association of proteins could identify tuberculosis-infected individuals at different disease development stages. However, few antigens are available, and a systematic study on *Mtb* ORFeome-based antigen screening is still needed. At present, tuberculosis diagnosis still mainly depends on the PPD skin test as well as sputum smear and culture, which are time-consuming, with rather low sensitivity and unsatisfactory specificity [16]. Instead, serological tests may be superior to those traditional methods, and more and more recombinant antigens have been shown to be suitable for the test [17-19]. Even though World Health Organization (WHO) has been currently holding reservations on the usage of commercially available serodiagnostic tests for tuberculosis diagnosis [20], antigen detection tests are actually very effective in confirming active pulmonary tuberculosis [13]. Previous proteomic studies have discovered several candidate antigens/antigen combinations that would be used in pulmonary tuberculosis serological tests [21]. Therefore, it is important to identify suitable antigens that can distinguish active tuberculosis patients from Bacille Calmette-Guerin (BCG)-vaccinated individuals.

ClpP2 protease, an important participant in *Mtb* growth, is essential for the protease specificity, which would be up-regulated during reaeration and/or under stress conditions [22, 23]. Reaeration response proteins are related to the relapse of *Mtb*-infected diseases, which has been thought to be helpful in developing new and effective diagnostics [24]. Therefore, reaeration response proteins, including ClpP2, might be used to identify latently infected indi-

viduals, and these patients would benefit from the preventive therapy. In the present study, ClpP2 was used to evaluate the immune response of the body against tuberculosis infection. Under various stress conditions (heat, acid, and oxidative stress), the expression of *Mtb* ClpP2 was enhanced, indicating that they would contribute to the protection against adverse environment. These results suggest that ClpP2 would play an important role in the survival of the bacteria under a wide range of stress conditions.

In recent years, bioinformatics methods, combined with scientific researches and experiments, has become an important approach to study protein structure, as well as immunogenicity. The prediction of *Mtb* ClpP2 plasticity and antigenic determinants indicated that this protein might have multiple potential epitopes, i.e., 3 CTL epitopes, 3 Th epitopes, and several B-cell epitopes. These results suggest that, ClpP2 could be recognized not only by CD4<sup>+</sup> Th cells and CD8<sup>+</sup> cytotoxic T cells, but also by B cells, indicating that the protein can potentially induce cellular and humoral immune responses. Based on these results, the immunogenicity of ClpP2 was further studied. Subcutaneous immunization of ClpP2 could induce high levels of humoral immune responses in rabbits. Moreover, our results indicated that *Mtb* ClpP2 also displayed strong and specific immunoreactivity against serum from clinically active tuberculosis patients, suggesting that *Mtb* ClpP2 may have pathogen-specific epitopes.

ClpP2 had been identified as cytoplasmic proteins in *M. bovis* BCG. Although the protein is not located on the surface of bacteria, significant antibody responses could be induced, which is in line with the fact that in convalescent serum, the antibody against cytoplasmic antigens would be detectable [2, 25, 26]. One of the explanations would be that, after early treatments, a large number of infected macrophages might undergo autolytic degradation together with pathogens, leading to high level of antibody responses in these tuberculosis patients. Moreover, drugs like isoniazid could destroy mycobacterial cell wall structure, resulting in the appearance of tuberculosis antigens in the patients [27]. Genes encoding ClpP2 are present in the chromosomes of *Mtb* and *M. bovis*, with the protein sequences of nearly 100% homology. However, majority of bacteria

do not possess the ClpP2 gene. Considering its limited distribution in prokaryotes and its potential to induce antibody responses, we evaluated the application of Mtb ClpP2 in serological diagnosis. We described the sensitivity and specificity of ClpP2 to distinguish tuberculosis patients from those infected with other pathogens. ROC curve analysis of ClpP2 antigen also suggested that it had a good accuracy in diagnosing tuberculosis. These results highlight the sensitive and specific characteristic of ClpP2, indicating its promising usage in the serodiagnosis of tuberculosis. Mtb ClpP2 antigen detection might be an attractive diagnostic alternative for the early and rapid diagnosis of tuberculosis. For the first time, we demonstrated the potential of ClpP2 in diagnosing active pulmonary tuberculosis. Taking into account the important role they play in proteolysis, these proteases possess potent antigenic properties, making them particularly valuable in distinguishing tuberculosis patients from those healthy individuals vaccinated with BCG, and from patients with other infections. Mtb ClpP2 may also be involved in regulating the body's immune function. However, further studies are still needed to validate ClpP2-induced cellular immune function.

In conclusion, we had cloned, expressed, and characterized recombinant Mtb ClpP2, and prepared its polyclonal antibody. Our results showed that ClpP2 was mainly located in the cytoplasm in *M. bovis* BCG, and stress conditions could enhance its expression. In tuberculosis patients, both the levels of ClpP2 antigen and antibody were increased, and the positive rate of ClpP2 was also elevated. Taken together, these results highlight the feasibility of the application of Mtb ClpP2 in the clinical diagnosis of tuberculosis.

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## Disclosure of conflict of interest

None.

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