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A combined application of molecular docking technology and indirect ELISA for the serodiagnosis of bovine tuberculosis

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ABSTRACT

Background: There is an urgent need to find reliable and rapid bovine tuberculosis (bTB) diagnostics in response to the rising prevalence of bTB worldwide. Toll-like receptor 2 (TLR2) recognizes components of bTB and initiates antigen-presenting cells to mediate humoral immunity. Evaluating the affinity of antigens with TLR2 can form the basis of a new method for the diagnosis of bTB based on humoral immunity.
Objectives: To develop a reliable and rapid strategy to improve diagnostic tools for bTB.
Methods: In this study, we expressed and purified the sixteen bTB-specific recombinant proteins in *Escherichia coli*. The two antigenic proteins, MPT70 and MPT83, which were most valuable for serological diagnosis of bTB were screened. Molecular docking technology was used to analyze the affinity of MPT70, MPT83, dominant epitope peptide of MPT70 (M1), and dominant epitope peptide MPT83 (M2) with TLR2, combined with the detection results of enzyme-linked immunosorbent assay to evaluate the molecular docking effect.
Results: The results showed that interaction surface Cα-atom root mean square deviation of proteins (M1, M2, MPT70, MPT83)-TLR2 protein are less than 2.5 A, showing a high affinity. It is verified by clinical serum samples that MPT70, MPT83, MPT70-MPT83 showed good diagnostic potential for the detection of anti-bTB IgG and M1, M2 can replace the whole protein as the detection antigen.
Conclusions: Molecular docking to evaluate the affinity of bTB protein and TLR2 combined with ELISA provides new insights for the diagnosis of bTB.

Keywords: Bovine tuberculosis; molecular docking; recombinant proteins; toll-like receptor 2; enzyme-linked immunosorbent assay

INTRODUCTION

Bovine tuberculosis (bTB), mainly caused by *Mycobacterium bovis*, is a zoonotic disease prevalent worldwide [1-3]. It brings serious economic losses to the animal husbandry and

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: Song S, Wang Z, Chen C; Data curation: Zhang Q, Yang H; Formal analysis: Song S, Guo J; Funding acquisition: Wang Z, Chen C; Investigation: Xu M, Yang N; Methodology: Song S, Wang Y; Project administration: Yi J; Resources: Song S, Guo J; Software: Song S, Yang H; Supervision: Wang Z, Chen C; Validation: Song S, Zhang Q; Visualization: Song S; Writing - original draft: Song S; Writing - review & editing: Wang Z, Chen C.

also pose a threat to human safety [4,5]. Diagnostic methods that depend on purified protein derivative (PPD) have insufficient accuracy [6,7]. The current epidemiological situation requires better diagnostics for effective prevention and control of the disease.

Antibody assays based on humoral immune responses can make up for the shortcomings of PPD. After *M. bovis* infects the body, it secretes various antigens during the reproduction process to induce the body to produce corresponding antibodies, and antibodies of the IgG class predominate mainly in the late stages of infection [8]. Antibodies which induce during infections, can block the proliferation of bacteria or viruses and trigger complement reactions, and the antibody levels are positively correlated with the course of the disease [9]. Therefore, detecting the level of IgG antibodies in serum is of great significance for diagnosis.

The signaling pathway of Toll-like receptor 2 (TLR2) is essential for adequate stimulation of the humoral immune response. For example, bacterial muramyl dipeptide and *Neisseria porin* as adjuvants can activate antigen-presenting cells through TLR2 [10], and TLR2-deficient mice produced significantly lower antibody titers to Borrelia burgdorferi OspA than wild-type mice after immunization [11]. TLR2 is involved in mycobacterial recognition, immune and antigen presentation which affects host defense against invading mycobacteria [12-14]. TLR2 also plays an essencial role in recognizing components including lipoprotein ligands of *Mycobacterium tuberculosis* (Rv3763, Rv1270c, LprA, and Rv1411c, LprG, and PhoS1) and other categories of TLR2 ligands (LAM, LM, PIMs and TDM) can activate macrophages by activating nuclear factor-κB through TLR2 [15,16], which plays an important role in humoral immunity and the expression of immunoglobulins. Therefore, evaluating the affinity between bTB protein and TLR2 is expected to become an important basis for the selection of antigens for the diagnosis of bTB based on humoral immunity.

In this study, we used molecular docking to evaluation the affinity of potential antigens screened among 16 bTB-specific diagnostic antigens with TLR2, combined with indirect enzyme-linked immunosorbent assay (ELISA) for validation of molecular docking results. Our aim was to provide new insight into the screening of bTB diagnostic antigens, to allow its use in improved detection of antibodies specific to bTB.

MATERIALS AND METHODS

Strains and serum samples

M. tuberculosis H37RV standard strain was obtained from the Center of Chinese Disease Prevention and Control (Beijing, China) and cultured in Lowenstein-Jensen Medium Base (Shanghai, China) at 37°C. *Escherichia coli* strains DH5α (Vazyme, China) and C43 (DE3, Vazyme, China) were cultured in Luria–Bertani medium (Supelco, China). We identified 920 positive and 714 negative serum samples by PPD skin test, the IDEXX bTB antibody test, and the interferon (IFN)-γ release test and 2422 untested serum samples from the Northern region of Xinjiang Uygur Autonomous Region, China.

Acquisition of gene sequences and bioinformatics analysis

All the gene sequences encoding AG85A (gene ID: RV3804C), AG85B (gene ID: RV1886C), AG85C (gene ID: RV0129C), CFP-10 (gene ID: RV3874), ESAT-6 (gene ID: RV3875), HSPX (gene ID: RV2031C), MPT64 (gene ID: RV1980C), MPT70 (gene ID: RV2875), MPT83 (gene ID: RV2873), NRDF-1 (gene ID: RV1981C), PPE57 (gene ID: RV3425), PSTS1 (gene ID: RV0934),

LIPC (gene ID: RV0220), RV1985C, RV1987, and RV3807C were found in the national center for biotechnology information (http://www.ncbi.nlm.nih.gov). Physico-chemical parameters was determined by the ProtParam program on the expert protein analysis system (ExPASy) web server. Parameters including theoretical pI, estimated half-life, molecular weight (kDa), grand average of hydropathy (GRAVY) and aliphatic index were predicted. The Immune Epitope Database Analysis Resource (IEDB-AR) tool (http://tools.iedb.org) was used to predict the B-cell epitopes of bTB-specific protein, amplify MPT70 with the primer of MPT70 with linker at the 3' end, amplify MPT83 with the primer of MPT83 with linker at the 5' end, and use overlap polymerase chain reaction (PCR) to connect the two genes of MPT70 and MPT83 to complete the fusion. Primer sequence is shown in **Supplementary Table 1**.

Expression and identification of sixteen *M. tuberculosis* proteins

Subcloned all bTB-specific protein genes into the prokaryotic expression vector pET-28a/30a/32a (+) and protein expression was induced in *E. coli* strains C43. Single colonies were obtained and tested by PCR and sequencing Isopropyl-β-D-thiogalactopyranoside (IPTG, Solarbio, China) was used to induce recombinant expression in this strains. All bTB-specific recombinant proteins were purified by immobilized-metal affinity chromatography using a polyhistidine tag. ESAT-6, CFP-10, MPT64 proteins are expressed in soluble form; AG85A, AG85B, AG85C, PPE57, NRDF-1, HSPX, RV3807C, RV1985C, RV1987, PSTS1, Rv0220, MPT70, MPT83 proteins are all included in precipitation form expression. For purification of soluble proteins, bacterial precipitation was suspended and lysed by sonication on ice. The lysate was centrifuged at 16,500 ×*g* for 30 min, and the supernatant was collected and transferred to a Ni-NTA His column pre-equilibrated with binding buffer (450 mM NaCl, 20 mM Tris, 5 mM imidazole). More than five column-volumes of washing buffer (450 mM NaCl, 20 mM Tris, 20 mM imidazole) was added to remove the nonspecific binding proteins and he target protein was eluted with elution buffer. For purification of precipitation proteins, we suspended the pellet in twice the volume of precipitate washing buffer A (500 mM NaCl, 10 mM Tris, 10 mM imidazole, 8 M Urea), mixed well and place on ice for 30 min with constant shaking, centrifuged at 12,000 for 30 min at 4 °C, and collected the pellets. The supernatant was transferred to the pretreated Ni-NTA His column (after ethanol treatment, 5 times the volume of deionized water was added to the column to wash, and then 10 times the volume of A solution was added for column equilibration), and then precipitate washing buffer B (500 mM NaCl, 20 mM Tris, 500 mM imidazole, 8 M Urea) was added to the Ni-NTA His Band Resin column, supernatant was eluted and protein was collected. The protein was put into the treated dialysis bag and placed in the renaturation buffer for gradient dialysis at 4°C, in which the urea concentration decreased sequentially according to 4 mol/L, 2 mol/L, 1 mol/L, 0.5 mol/L and 0 mol/L. This was followed by protein renaturation, the medium exchange interval was 12 h, the protein was collected, and sucrose was used to absorb water. The final protein products were examined by SDS-PAGE before storing at −80°C.

Western blot analysis

Purified AG85A, AG85B, AG85C, CFP-10, ESAT-6, HSPX, MPT64, MPT70, MPT83, NRDF-1, PPE57, PSTS1, RV1985C, RV1987, LIPC, RV3807C, MPT70-MPT83, dominant epitope peptide of MPT70 (M1), and dominant epitope peptide MPT83 (M2) were separated on 12% SDS-PAGE gels. Protein was then transferred to PVDF membrane. The membrane was blocked for 3 h at room temperature with 2% bovine serum albumin in phosphate-buffered saline (PBS), and then washed three times with PBS containing 0.05% Tween 20 (PBST). Next, the membrane was incubated with positive bovine serum (1: 1,000) at room temperature for 3 h. The membrane was then washed again three times in PBST, followed by incubation with

HRP-conjugated rabbit anti-cattle secondary antibody (1: 1,000). Bands were detected using Protein Simple, FluorChemE.

Molecular docking simulation

For docking of recombinant protein with cattle TLR2, secondary and tertiary structure plot was necessary. PSIPRED online server (http://bioinf.cs.ucl.ac.uk/psipred/) was used for designing secondary structure. We used SWISS-MODEL server (http://swissmodel.expasy.org) to perform homology modeling of recombinant proteins MPT83, M1 and M2. MPT70 protein was searched in Protein Data Bank (PDB) database. HADDOCK server combines GRAMM for global macromolecular docking, scoring with a statistical potential, clustering followed by refinement of best scored docked complexes from biggest clusters, and can deal with a large class of modeling problems including protein-protein, protein-nucleic acids and protein-ligand complexes, including multi-body (N>2) assemblies. Using the docking regions of M1, M2, MPT70 and MPT83 proteins as ligands and cattle TLR2 as receptors, the docking were performed in the HADDOCK server by ambiguous interaction restraints method [17]. For optimization, first extract the ligand molecule, and then repair the side chain, process the end of the main chain, add hydrogen, delete water molecules, specify the atom type, add charge and optimize the energy, etc. All are optimized according to the HADDOCK default values; using HADDOCK the software's protein docking defines below to generate docking activity pockets, and other conditions follow the HADDOCK default values. The total score in the docking result was used as the basis for the final judgment of the docking result.

Establishment and evaluation an indirect ELISA method for bTB

Indirect ELISA test serum from 200 healthy cattle was individually assessed in duplicate to obtain a negative-positive cut-off value (**Table 1**). The mean (M) and standard deviation (S) of the signals obtained with the above 200 negative serum were calculated. For each serum sample, $OD_{450\ nm}$>M+3S was considered positive, and $OD_{450\ nm}$<M+2S was considered negative. For M+2S≤$OD_{450\ nm}$≤M+3S, the sample was considered positive if the sample was still suspicious during a retest. Indirect ELISA detection tests were established with the excellent diagnostic proteins among the above sixteen proteins as antigens, and the serum from 920 bTB positive and 714 healthy cattle were probed. The sensitivity and specificity of each protein for the detection of anti-bTB antibodies was was assessed as a basis for diagnostic efficacy.

Statistical analysis

All the tests were repeated three times. Statistical significance and correlation analyses were calculated with SPSS Statistics 23 (IBM Corp., USA). Student's *t* tests, Student-Newman-Keuls test, and one-way analysis of variance were used for comparisons among different groups. Graphical out-puts were prepared with GraphPad Prism.

Table 1. The threshold for positive and negative samples in ELISA

Protein	IgG		Negative cutoff	Positive cutoff
	M	S		
MPT70	0.21	0.06	0.33	0.39
MPT83	0.22	0.06	0.34	0.4
MPT70-83	0.25	0.1	0.45	0.55
M1	0.15	0.06	0.27	0.33
M2	0.19	0.09	0.37	0.46

ELISA, enzyme-linked immunosorbent assay.

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RESULTS

Bioinformatics analysis and sequence selection

Epitope prediction was used as the basis for evaluating protein immunogenicity and the epitopes of AG85A, AG85B, AG85C, CFP-10, ESAT-6, HSPX, MPT64, MPT70, MPT83, NRDF-1, PPE57, PSTS1, RV1985C, RV1987, LIPC, and RV3807C proteins served as the foundation for construction and expression fusion proteins. The B-cell epitopes of the proteins involved in this article are analyzed by IEDB-AR. Among them, the epitopes of CFP-10, ESAT-6, MPT70, and MPT83 are relatively similar in distribution, and the epitopes are relatively concentrated; although the epitopes of the other proteins are relatively abundant and the number is relatively large but more scattered (**Supplementary Fig. 1**). The positions and lengths of the dominant B-cell epitopes predicted by all proteins are shown in **Supplementary Table 2**. The proportions of α helix, β sheet, β turn, and random coils in the proteins involved in this study are shown in **Supplementary Table 3**. Most of them are α helix and random coils. Among them, AG85C, HSPX, and MPT64 have random coils. The curled structure accounts for a higher proportion, which is relatively loose in the spatial structure.

Physical and chemical analysis of bTB-specific protein

Through the analysis of the physical and chemical properties of the protein (**Supplementary Table 4**), it was found that the PI values of AG85A, AG85B, AG85C, CFP-10, ESAT-6, HSPX, MPT64, MPT70, MPT83, NRDF-1, PPE57, and PSTS1 were less than 7, which showed acidity in a neutral solution; the PI values of LIPC, RV1985C, RV1987, and RV3807C are all greater than 7, which are alkaline in neutral solution. Instability index analysis results show that AG85A, AG85B, AG85C, CFP-10, HSPX, PPE57, RV1985C, RV1987, and RV3807C have instability indexes greater than 40, which are unstable proteins; ESAT-6, LIPC, MPT64, MPT70, MPT83, and NRDF-1 the instability index of PSTS1 is < 40, which indicates a stable protein. The average coefficients of hydrophilicity of AG85A, AG85B, AG85C, CFP-10, ESAT-6, HSPX, LIPC, MPT64, NRDF-1, PPE57, and RV1985C are all < 0, making them hydrophilic proteins; for the hydrophilicity of MPT70, MPT83, PSTS1, RV1987, and RV3807C the average coefficient is > 0, meaning hydrophobic protein.

Sixteen bTB-specific proteins acquisition purification

After IPTG induction, the recombinant plasmids of each expression vector were transferred into *E. coli* DE3 competent cells, resulting in the expression of the bTB-specific proteins. ESAT-6, CFP-10, and MPT64 proteins are expressed in soluble form in the supernatant. AG85A, AG85B, AG85C, PPE57, NRDF-1, HSPX, Rv3807C, RV1985c, RV1987, PSTS1, LIPC, MPT70, and MPT83 proteins were all expressed in bacterial inclusion bodies. After sonication treatment, they were purified and characterized as described in Materials and Methods. The reactivity of bTB positive serum against recombinant bTB-specific proteins by using western blot. We show that all bTB-specific proteins react with bTB positive serum, with all the bTB-specific proteins exhibiting good reactivity (**Fig. 1**).

Determination of optimal antigens for IgG detection of bTB-specific and design of polyprotein fusions for indirect ELISA

Given the heterogeneity of antigen recognition by antibodies in cattle infected with *M. bovis*, optimal sensitivity of serodiagnostic tests requires multiple antigens to cover the breadth of the humoral immune responses. We used the standard reagents such as serum diluent, enzyme-labeled secondary antibody, color developing solution in IDEXX bTB antibody detection kit (indirect method), and pass the determination of P/N (positive/negative) value

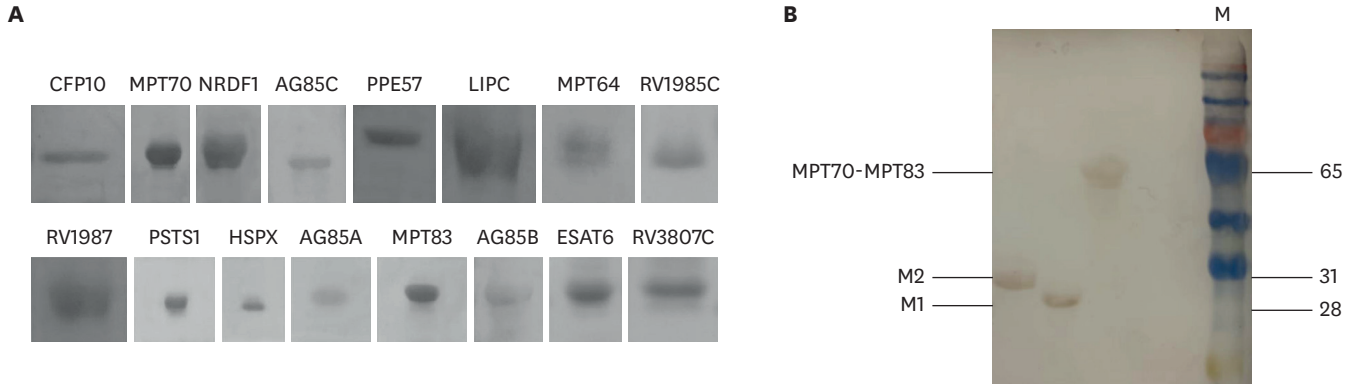

Fig. 1. Western blot analysis of purified proteins. (A) Sixteen tuberculosis-specific proteins were detected with bTB positive serum. (B) the dominant epitope peptide of MPT70 (M1), dominant epitope peptide MPT83 (M2) and the MPT70-MPT83 proteins were detected with bTB positive serum.
bTB, bovine tuberculosis.

of ELISA. The results showed that the P/N values of 16 proteins were AG85A (2.293), CFP10 (0.684), LIPC (0.574), RV3807C (0.904), AG85C (0.886), HSPX (0.513), RV1985C (1.619), MPT70 (7.672), AG85B (1.452), PPE57 (0.989), MPT64 (0.714), RV1987 (1.267), ESAT6 (0.774), PSTS1 (1.17), NRDF1 (1.538), MPT83 (20.179); MPT70 and MPT83 have good diagnostic potential, and their P/N values are several times higher than other proteins. MPT70 and MPT83 proteins known to be major targets recognized by *M. bovis* infected cattle [18]. Therefore, we generated fusion proteins of MPT70 and MPT83 and their respective dominant epitope peptides as candidate diagnostic antigens, and these three proteins could react with bTB-positive serum after purification (**Fig. 1B**).

Docking of recombinant protein with cattle TLR2 receptor and evaluate

Docking of M1, M2, MPT70, MPT83 protein with TLR2 is shown in **Fig. 2**. M1, M2, MPT70, MPT83 have the lowest intermolecular energy as well as the lowest interface root mean square deviation (iRMSD) from the target. This result demonstrates a correlation between the intermolecular energy of our solutions and the iRMSD between these solutions and the target. The best solution of cluster (the lowest in energy) has iRMSD of less than 2.5 A from the reference structure (**Fig. 3**).

Diagnostic value of M1, M2, MPT70, MPT83 and MPT70-MPT83 antigens in indirect ELISA

We used 1,634 sera (920 bTB-positive and 714 bTB-negative sera) to assess the ability of each established indirect ELISA to discriminate between bTB cattle and healthy cattle. Five diagnostic antigens, MPT70, MPT83, MPT70-MPT83, M1 and M2 could effectively distinguish bTB cattle from healthy ($p < 0.0001$) controls. Considering both sensitivity and specificity criteria, the five antigens MPT70, MPT83, MPT70-MPT83, M1 and M2 were effective at detecting IgG antibodies (**Fig. 4A**). Therefore, these could be used for the late diagnosis of bTB infection. We further analyzed the results by drawing scatter plot sand ROC curve for each tested protein and evaluated the potential of each protein candidate for the detection of bTB by calculating the area under the curve (AUC) (**Fig. 4B**). We further analyzed the results by drawing scatter plots and ROC curve for each tested protein and evaluated the potential of each protein candidate for the detection of bTB by calculating the AUC. Five antigen candidates had an AUC > 0.5. Among them, the AUC of MPT70-MPT83 and M2 was > 0.7 (**Table 2**). Considering their sensitivity and specificity, MPT70-MPT83 and M2 have high diagnostic values and can be used for further investigation to develop diagnostic tests based on anti-bTB IgG.

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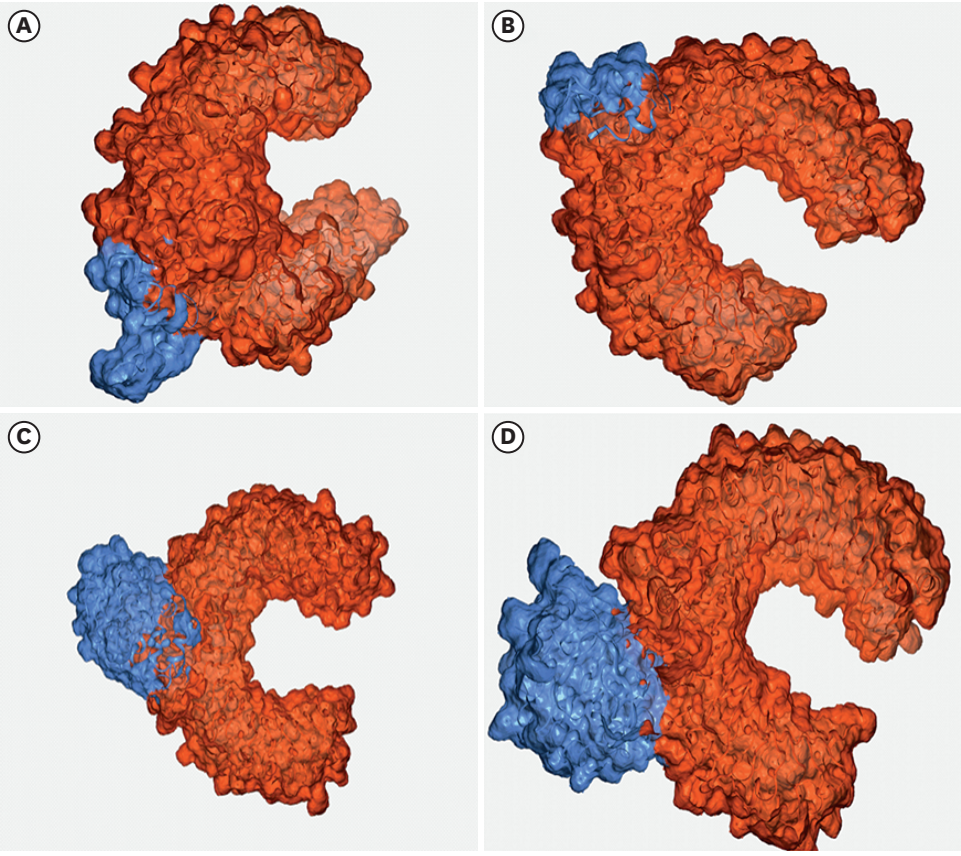

Fig. 2. Modeling diagram of recombinant proteins and TLR2 by molecular docking. (A) Recombinant proteins M1 docking with TLR2. (B) Recombinant proteins M2 docking with TLR2. (C) Recombinant proteins MPT70 docking with TLR2. (D) Recombinant proteins MPT83 docking with TLR2.
TLR2, Toll-like receptor 2.

We then used 2422 sera to compare the different ability of the established indirect ELISA method with IFN-γ assay and IDEXX ELISA kit. IFN-γ assay was 4.00% more positive for bTB, followed by IDEXX ELISA kit at 2.81%; MPT70, MPT83, MPT70-MPT83, M1, and M2 detection rates were 1.24%, 1.07%, 1.82%, 1.03%, and 1.49%, respectively (**Table 3**). The difference between the positive detection rate of IFN-γ assay and ELISA detection rate may be due to serum from different pathological processes of bTB. In general, MPT70-MPT83 and M2 have good application value and development potential in field bTB detection but may have certain limitations in identifying early and late infection of bTB.

DISCUSSION

Conventional methods of cellular immune response are flawed with regard to the detection of animals chronically infected by *M. bovis*. Antibody detection methods based on humoral immune responses just make up for the shortcomings which the detection method based on cellular immunity is not sensitive to open tuberculosis and systemic tuberculosis in the late stage of infection [19]. Therefore, the detection of IgG against *M. tuberculosis* has been widely used in clinical practice to diagnose TB. TLR2 plays a crucial role in recognizing bTB, through their cell wall components, which can activate innate immunity and ultimately mediate humoral immune processes [16]. Therefore, evaluating the affinity of TB protein to

Table 2. The results of IgG antibody test

Protein	Positive serum (N)		Healthy control (N)		AUC	Sensitivity	Specificity
	Positive	Negative	Positive	Negative			
MPT70	448	472	98	616	0.66	48.70%	86.27%
MPT83	452	468	61	653	0.69	49.13%	91.46%
MPT70-MPT83	601	319	18	696	0.82	65.33%	97.48%
M1	397	523	261	453	0.52	43.15%	63.45%
M2	461	459	49	665	0.71	50.11%	93.14%

AUC, area under curve (ROC analysis).

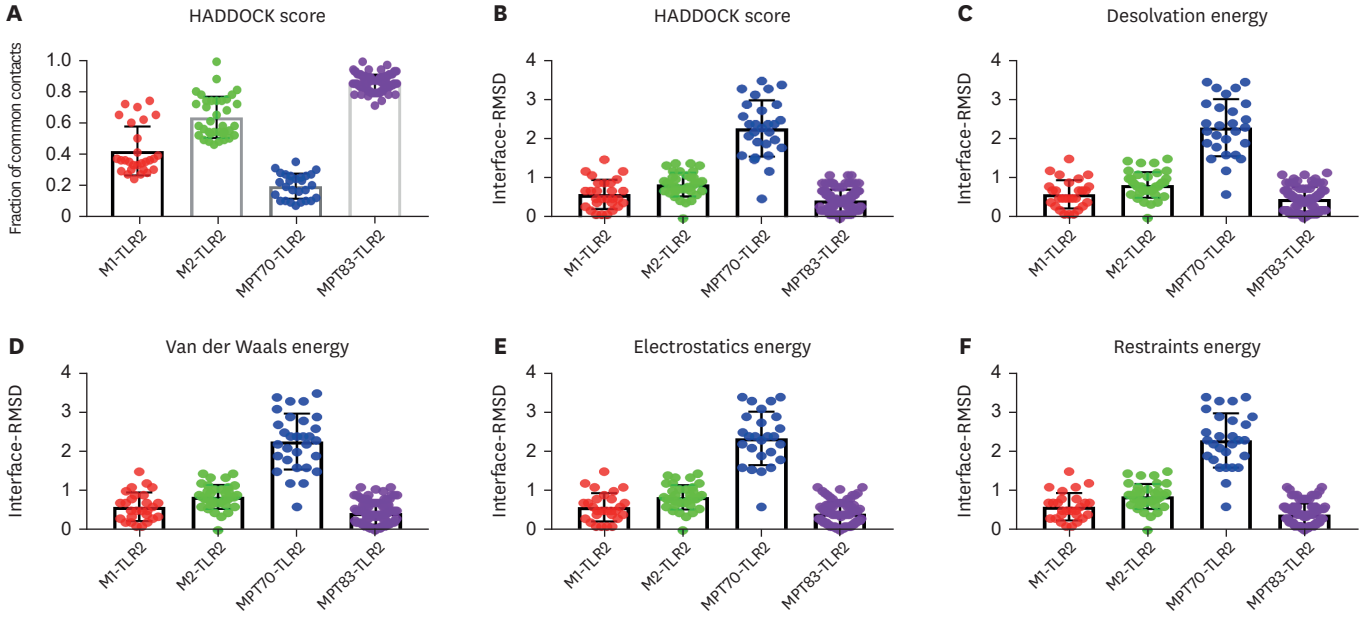

Fig. 3. Intermolecular energy parameters iRMSD for the M1-TLR2 complex of HADDOCK. (A) HADDOCK score versus fraction of common contacts for the M1-TLR2 complex, M2-TLR2 complex, MPT70-TLR2 complex, MPT83-TLR2 complex. (B) HADDOCK score versus iRMSDs for the M1-TLR2 complex, MPT70-TLR2 complex, MPT83-TLR2 complex, MPT83-TLR2 complex. (C) Desolvation energies versus iRMSDs for the M1-TLR2 complex, M2-TLR2 complex, MPT70-TLR2 complex, MPT83-TLR2 complex. (D) Van der Waals energies versus iRMSDs for the M1-TLR2 complex, M2-TLR2 complex, MPT70-TLR2 complex, MPT83-TLR2 complex. (E) Electrostatics energies versus iRMSDs for the M1-TLR2 complex, M2-TLR2 complex, MPT70-TLR2 complex, MPT83-TLR2 complex. (F) Restraints energies versus iRMSDs for the M1-TLR2 complex, M2-TLR2 complex, MPT70-TLR2 complex, MPT83-TLR2 complex.
TLR2, Toll-like receptor 2; iRMSD, interface root mean square deviation.

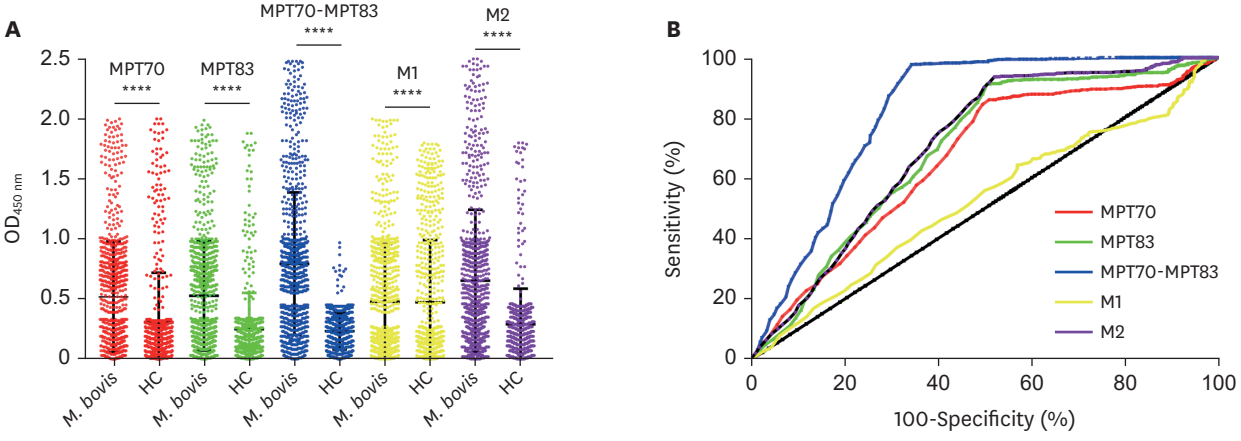

Fig. 4. Diagnostic protein candidates diagnostic efficacy evaluation. (A) Indirect ELISA for detecting the OD value of IgG antibody in candidate diagnostic proteins. (B) ROC curve analysis of diagnostic protein candidates. The AUC shows the effectiveness of 5 diagnostic protein candidates, that is, MPT70, MPT83, MPT70-MPT83, M1, and M2, for the detection of IgG antibodies against *Mycobacterium bovis*. The closer the AUC is to 1, the higher the diagnostic effectiveness; the closer the AUC to identity, the worse the diagnostic effectiveness. The results are from three independent experiments.
ELISA, enzyme-linked immunosorbent assay; ROC, receiver operating characteristic; AUC, area under the ROC curve.

Table 3. Comparative result of established indirect ELISA method with IFN-γ assay and IDEXX ELISA kit

Indirect ELISA	Total number of serum	Number of positive detections	Positive detection rate	Number of negative detections	Negative detection rate
MPT70	2,422	30	1.24%	2,392	98.76%
MPT83	2,422	26	1.07%	2,396	98.93%
MPT70-MPT83	2,422	44	1.82%	2,378	98.18%
M1	2,422	25	1.03%	2,397	98.97%
M2	2,422	36	1.49%	2,386	98.51%
IFN-γ kit detection	2,422	97	4.00%	2,325	96.00%
IDEXX ELISA kit	2,422	68	2.81%	2,354	97.19%

ELISA, enzyme-linked immunosorbent assay; IFN, interferon.

TLR2 receptor may become one of the potential indicators for screening specific diagnostic antigens. Herein, we report the development of an indirect ELISA with MPT70, MPT83, M1, and M2 protein as the coated-antigen by epitope prediction and molecular docking with TLR2. It has been verified by clinical serum samples that M1 and M2 can replace the whole proteins as diagnostic antigens, and the fusion protein MPT70-MPT83 shows good specificity and sensitivity. This finding provides a basis for further development of this method in diagnosing bTB.

Bioinformatics analysis, comparison of protein-associated information, and predictions of epitopes have become important methods in immunological research [20]. A comprehensive analysis of the B-cell epitopes of specific proteins and the physical and chemical properties of secondary structure and hydrophilicity found that 16 bTB-specific proteins were observed to exhibit multiple B-cell epitopes, indicating that they can act as antigens to initiate immune response. These peptides have high hydrophilicity. They are located or rich in random coils in the secondary structure, which is in line with the characteristics of epitope distribution, and the secondary structures such as β-turn and random coils are relatively high. The loose structure can easily be twisted, hovering and protruding to the surface of the protein, which is conducive to chimerization with antibodies and a greater possibility of the protein becoming an epitope. Our results show that the 16 bTB-specific antigen proteins expressed by *E. coli* were used to react with bTB positive serum. We choose MPT70 and MPT83 proteins as diagnostic antigens to evaluate their value in the serological diagnosis of bTB.

Accumulated evidence supports the notion that the activation of TLR2 signaling benefits the host defense against invading pathogens and evaluating the interaction between antigen protein and TLR2 may become an important indicator for screening specific diagnostic antigens based on humoral immunity [15]. The development of bioinformatics and molecular simulation technology provides a new method for simulating intermolecular interactions through computer molecular docking technology [21]. This method effectively makes up for the inadequacy of traditional methods and can obtain detailed results quickly. To date, the interaction between proteins and small molecules by molecular docking has been widely used in food [22], bioengineering [23], medicine and other industries [24], and molecular docking technology has made great progress [25]. The evaluation of the results of docking prediction mainly analyzes the RMSD value of the docking compound. The RMSD value is the most commonly used indicator in structural modeling and prediction, used to measure the degree of difference between two or more structural data [26]. The smaller the RMSD value, the more reliable the modeling or prediction results. Chen et al. [27] believe that after the predicted conformation overlaps with the original crystal conformation, if the RMSD of the C-α atom on the protein-protein interaction surface is less than 2.5 A, the predicted results can be considered correct. MPT70 protein, MPT83 protein, M1, and M2 are used for molecular

docking with TLR2, and the RMSD of the best docking result is less than 2.5 A. Compliance with this criterion shows that these bTB-specific proteins may have good immunogenicity, and the B-cell epitope prediction also shows the same result. Taken together, these results suggest that MPT70, MPT83, M1 and M2 have strong affinity for the TLR2 receptor and may elicit stronger humoral immunity. This is consistent with the current research results. MPT83 and MPT70 are the main specific secretory antigens of *M. bovis*, which can induce humoral immunity *in vivo* and have good immunogenicity and immune protection.

Previous studies have shown that the sensitivity obtained with MPT70 protein in serological diagnosis is between 18% and 73%, and the specificity between 76% and 88% [28]. Variable sensitivities have also been reported by others [29-31]. In our study, when MPT70 protein was used to detect IgG, the sensitivities were 48.70% and the specificity 86.27%. According to other reports, MPT83 protein confers a sensitivity of 37.5% and a specificity of 89% [32]. We found that MPT83 allows IgG detection with a sensitivity of 49.13% and specificity reaching 91.46%. The MPT70-MPT83 fusion protein has a sensitivity of 63% and a specificity of 98% in the detection of naturally infected bTB serum in previous report [33]; here, the MPT70-MPT83 fusion protein has a sensitivity of 65.33% and a specificity of 97.48%. This phenomenon indicates that the same protein can give a broad range of different results, depending on infection stages and individual variability. In addition, the sensitivities were 43.15% and the specificity 63.45% of M1, and the sensitivities were 50.11% and the specificity 93.14% of M2. These results suggest that they can replace MPT70 and MPT83 as potential diagnostic antigens, especially M2. We used 2422 sera to compare the different ability of the established indirect ELISA method with IFN-γ assay and IDEXX ELISA kit. The difference between the positive detection rate of IFN-γ assay and ELISA detection rate may be due to bovine sera from different pathological processes of bTB.

To conclude, there is no infallible test for the diagnosis of bTB. Most of the techniques are used as auxiliary diagnosis. Achieving a definitive conclusion regarding the value of a diagnostic method requires a large amount of clinical diagnostic data. We developed a combined application of molecular docking technology and indirect ELISA for the detection of serodiagnosis of bTB, which could assist in the diagnosis of bTB and help guide future research to achieve the best match between the timing and the method of diagnosis.

SUPPLEMENTARY MATERIALS

Supplementary Table 1

Design of fusion protein specific primers for M1, M2 and MPT70-MPT83

Click here to view

Supplementary Table 2

Epitopes of sixteen tuberculosis-specific proteins B cells

Click here to view

Supplementary Table 3

Secondary structure analysis of sixteen bovine tuberculosis-specific proteins

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Supplementary Table 4

Physical and chemical properties of sixteen bovine tuberculosis-specific proteins

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Supplementary Fig. 1

B-cell epitope predictions of sixteen bovine tuberculosis-specific proteins.

Click here to view

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