Assessment of a Fusion Polyprotein Based on HspX and Other Eight Antigen Segments for Tuberculosis Serodiagnosis

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Abstract

Background: The lack of suitable diagnostic tools has paved the cornerstone for controlling and treatment of tuberculosis. Serological tests, based on multiple target antigens, represent an attractive option for its rapidity, convenience, and low-cost.

Methods: Measures, including blocking antibodies against common bacteria in serum samples and synthesizing polypeptides covering un-conserved dominant B-cell epitopes of antigens, were attempted to reduce nonspecific reactions and thus improved the specificity. Meanwhile, a fusion polyprotein containing HspX and other eight antigen segments was constructed and expressed to increase the whole sensitivity.

Results: The addition of only E. coli lysate partly increased the specificities. Another, peptides containing un-conserved fragments of TB antigens as well as dominant B-cell epitopes were synthesized and used to effectively reduce non-specific reactions without dramatically decreasing their sensitivities. Meanwhile, a polyprotein fusing HspX and other eight antigen segments was constructed and the sensitivity of the polyprotein was 60.2%, which was higher that of HspX and other individual antigen segments. Importantly, the specificity of the polyprotein was 93.6%, which was not significantly decreased.

Conclusions: Our results demonstrate the roles of fusion polyproteins in the humoral immunity against TB infection and provide a potential novel approach for TB diagnostic development.

Introduction

Despite substantial progress toward TB control over the last decades, TB remains one of the most prevalent infectious diseases and the top 10 causes of death worldwide. In 2016, there were an estimated 10.4 million incident and 1.7 million death TB cases in the world.[1] The increasing emergence of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB), and the spread of HIV/AIDS in TB-endemic regions were impeding efforts to control and eliminate TB and prompted healthy authorities to strengthen and reinforce control strategies to limit their spread.[2, 3]

A rapid, accurate and cost-effective diagnostic tool for TB is urgently required to control this disease. Several methods including the sputum smear and culture tests, chest X-ray and immunodiagnostic detection are currently available. Biomarkers predicting treatment efficacy and cure of active tuberculosis, the reactivation of latent tuberculosis infection, and the induction of protective immune responses by vaccination were also well investigated.[4] However, tuberculosis-specific biomarkers have not yet been discovered and the qualification of biomarkers as a surrogate for a clinical endpoint in tuberculosis is very challenging. Of those, immunodiagnostic detection of antigens or their antibodies in blood of patients has been successfully applied to other pathogens[5] and represents an attractive option for this purpose. However, the sensitivities and specificities of the currently available options based on single or

multiple target antigens are quite variable and not yet to meet the requirements for clinical utility. In 2011, WHO issued a policy recommendation against the use of the various commercial serological tests for TB diagnosis due to the suboptimal sensitivity and specificity.[6] However, further research and development in this field, specifically the identification and screening of novel serodiagnostic antigens, is still highly recommended by WHO.[7]

One major challenge for TB serodiagnosis was false positive reaction with healthy individuals, which reduced the specificity. Apart from cross-reactivity with previous bacilli Calmette-Guérin (BCG) vaccination and other environmental mycobacteria, most TB antigens had homologous sequences with other common bacteria and TB serum antibodies would inevitably cross-react with antigens from these bacteria. One way to reduce nonspecific reactions was to block antibodies against those bacteria. In this study, the serum samples were pre-adsorbed with *Escherichia coli (E. coli)* and other bacteria lysate to block antibodies against *E. coli* antigens and other common bacteria. *Vibrio aquatilis (V. aquatilis), Staphylococcus aureus (S. aureus), Bacillus subtilis (B. subtilis), Proteus vulgaris (P. vulgaris), Staphylococcus albus (S. epidermidis), Enterobacter aerogenes (E. aerogenes),* and *Staphylococcus citreus (S. citreus)* were also included. Another approach to reduce non-specific reactions was selecting un-conserved fragments of TB antigens compared with other bacteria by bioinformatics analysis and simultaneously retained dominant B-cell epitopes as much as possible. However, it was noted that the sensitivity may decrease accordingly. Goyal et al. used B-cell epitopes based peptides of RD1 (ESAT–6, CFP–10) and RD2 (CFP–21, MPT–64) antigens for immunodiagnosis of pulmonary TB (PTB).[8] Afzal et al. constructed fusion proteins tn1FbpC1-tnPstS1 and tn2FbpC1-tnPstS1 with Immunodominant B cell epitope sequences and found that removal of non-epitopic region of FbpC1 from 34–96 amino acids seems to have unmasked at least some of the epitopes, resulting in greater sensitivity.[9]

On the other hand, to data, there was no single TB antigen-based assay has achieved a satisfactory serodiagnostic performance, which impelled us to identify new protein targets and investigate different combinations of currently identified antigens. Thus, strategies including mixture of recombinant antigens, fusion of recombinant antigens (or segments), and peptide-based antibody detection were applied to improve the sensitivity. Zhang et al. mixed and combined three antigens, i.e. Rv3425, 38 kDa, and LAM, and developed a multiple-antigen ELSIA test, which was a potentially useful tool for the serodiagnosis and screening of active TB.[10] Yang et al. constructed a recombinant fusion protein Rv0057-Rv1352 which performed good immunoreactivity with serum from TB patients.[11] A recombinant fusion of three immunodominant antigens 38-kDa–16-kDa-ESAT–6 was also reported by Wu et al.[12] Another approach to TB serodiagnosis was using the fusion of antigen fragments, which contained dominant linear B-cell epitopes, instead of whole protein antigens. Two novel polyproteins, 38kD-ESAT6-CFP10 (38F) and Mtb8.4-MPT64-TB16.3-Mtb8 (64F) was constructed and evaluated by Feng et al. and the novel 38F–64F indirect ELISA assay had effective diagnostic performance.[13] In recent years, several serological tests based on synthetic peptides derived from highly antigenic proteins were also designed and evaluated. Kashyap et al. synthesized four peptides (7–10 amino acids long) corresponding to group-specific epitopes of Ag 85 complex of *M.tb* and identified that the peptide based ELISA was a sensitive, specific,

rapid and cost effective immunoassay for early diagnosis of pulmonary and extrapulmonary TB.[14] In one study, the combination of peptides from B-cell epitopes of ESAT–6, CFP–10, CFP–21 and MPT–64 antigens were evaluated for immunodiagnosis.[8] Our previous study identified a cocktail of serodiagnostic antigens for TB. However, none of any single antigen had enough high sensitivity.[15, 16] In this study, we aimed to combine nine antigens (or segments) with un-conserved dominant B-cell epitopes to be a fusion polyprotein, which may improve the whole sensitivity and specificity compared with individual antigen and other combination forms.

Materials And Methods

Study population

Serum samples from TB patients and healthy individuals were obtained from Shenzhen People's Hospital (Shenzhen, China) from August 2016 to December 2017. All TB Patients were diagnosed as newly treated active TB and has been decided to treat with a full course of TB treatment according to TB diagnostic criteria. Diagnostic criteria included sputum culture or smear positive, typical radiological manifestation and clinical response to anti-TB treatment consistent with active TB. Individuals co-infected with HIV, hepatitis infections or other autoimmune disorders were excluded. Study protocol was approved by the ethics committee of Shenzhen People's Hospital, China. Written informed consent was obtained from all subjects before blood sampling. More details regarding TB patient information were listed in Supplementary Table 1.

Acquisition of the lysates from common bacteria for sera pre-adsorption

Lysates from *V. aquatilis, S. aureus, B. subtilis, P. vulgaris, S. epidermidis, E. aerogenes, S. citreus* and *E. coli* were used to pre-adsorb the serum samples to block antibodies against bacteriaantigens and reduce non-specific reactions.In details,Bacteria were cultured at 100 mL volume overnight at 37°C and then collected after centrifugation at 13,000 rpm at 4°C for 10 min. Bacteria were resuspended with 10 mL PBS for ultrasonic decomposition and then centrifuge at 13,000 rpm to collect the supernatants. 800 ul supernatants, 200 ul sera and 2 mL PBS were mixed at room temperature for 5 h to adsorb antibodies against bacteria proteins.

B-cell epitope selection

ABCpred software was used to screen potential B-cell epitopes.[17] Key algorithms, including probability of surface exposure, local hydrophobicity, beta-turn amino acid sequence, atomic flexibility and experimental high performance liquid chromatography retention times of synthetic peptides, were

considered in this software. For individual antigen, defaults, such as threshold (more than 0.80) and overlapping filter, were set.

Blast for un-conserved sequences

Protein blast from NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to screen each individual antigen's homologous sequences from other bacteria, such as *E. coli.* COBALT software was further employed to compare and select un-conserved sequences. Final ready-to-synthesis sequences were obtained by integration of B-cell epitope prediction and blast results.

Synthesis of polypeptides

Polypeptides of PstS1 and Rv1488 were synthesized by ChinaPeptides ltd (Suzhou, China). The purification of the recombinant proteins was conducted by ion exchange and gel filtration. The purities of each polypeptide were all 95%. The protein concentration was determined by the Bradford method. The concentrations of each polypeptide were all 1 mg/mL.

Three-dimensional (3D) structure prediction of the fusion polyproteins

SWISS-MODEL (https://www.swissmodel.expasy.org/) was used to predict three-dimensional (3D) structure of the fusion polyproteins. SWISS-MODEL is a fully automated protein structure homology-modelling server, accessible via the ExPASy web server, or from the program DeepView (Swiss Pdb-Viewer).[18] The purpose of this server is to make protein modelling accessible to all life science researchers worldwide.

The polyprotein expression

Life technology (Thermo Fisher Scientific, Massachusetts, USA) helped to construct the plasma expression vector to clone and express the polyprotein. Codon was optimized to better express in *E. coli.* In details, the polyprotein expression in *E. coli Rosetta* (DE3; Novagen, Germany) was induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The supernatant or inclusion bodies were stored for further study.

Indirect ELISA

Purified recombinant antigens or polypeptides were diluted into optimized concentrations with the coating buffer (0.05 M Na2CO3-NaHCO3, pH 9.6) and coated on 96-well Immunosorp plates (Nunc, Denmark) overnight at 4°C. After blocking, test serum samples (diluted 1:50 in the blocking buffer) were added and incubated at 37°C for 1 h. HRP-conjugated secondary antibody (1:10,000) was then added for 0.5 h at 37°C. The color reaction was developed by TMB (3, 3', 5, 5'-tetramethylbenzidine) substrate solution and stopped by the addition of 2 N H2SO4. The optical density (OD) at 450 nm was measured using a microplate reader (ELX50, Bio-Tek Instruments, USA).

Statistical analysis

Statistical analyses were performed using SPSS (Version 20.0, SPSS Inc., Chicago, IL, USA), GraphPadPrism 5.0 (GraphPad Software Inc., San Diego, CA), and MedCalc for Windows (Version 17.8, Ostend, Belgium) software. All statistical tests were two-sided and $P < 0.05$ was considered statistically significant. Results are expressed as mean ± standard deviation (SD), unless otherwise specified. A positive antibody test was defined as an OD value greater than the cutoff value, i.e., the mean OD value plus three SD from the negative healthy control serum.

Results

Using lysates from common bacteria for sera pre-adsorption to reduce non-specific reactions

Most TB antigens had homologous sequences with other common bacteria and TB serum antibodies would inevitably cross-react with antigens from these bacteria. One way to reduce nonspecific reactions was to block antibodies against those bacteria. In this study, three serum samples from healthy individuals, including one strong false positive (SFP), one weak false positive (WFP) and one normal, and one serum sample from a positive TB patient were enrolled. The serum samples were pre-adsorbed with *E. coli* and other bacteria lysate to block antibodies against *E. coli* antigens and other bacteria. *V. aquatilis, S. aureus, B. subtilis, P. vulgaris, S. epidermidis, E. aerogenes* and *S. citreus* were included. As shown in Table 1 and Supplementary Table 2, there were no significantly differences after being pre-adsorbed by *V. aquatilis, S. aureus, B. subtilis, S. epidermidis* and *S. citreus.* However, the value of serum samples from SFP and WFP dramatically decreased after pre-adsorbed by *P. vulgaris, E. aerogenes* and *E. coli* compared with groups without adding any bacteria lysate (background groups). Next, we took more attention to these three bacteria. Twelve false positive serum samples from healthy individuals were collected to test the effects. As shown in Table 2, for protein PstS1, the value of 91.7% (11/12) serum samples significantly decreased after adding *E. coli* bacteria lysate, and 88.3% (10/12) decreased after pre-adsorbed by *P. vulgaris* or *E. aerogenes.* However, for Rv1488, as Supplementary Table 3 shown, only 75% (8/12) decreased after being pre-adsorbed by *P. vulgaris.* Together, we selected *E. coli* and *E. aerogene*s for further study. A panel of 96 serum samples, including 72 TB patients and 24 healthy controls were recruited to compare the difference between *E. coli* and *E. coli & E. aerogenes.* Four proteins,

ie. PstS1, Rv1488, PanD and EchA3, were used to validate it. The results showed that there were no significant differences by adding *E. coli & E. aerogenes* lysate compared with only *E. coli,* indicating the redundancy of extra adding *E. aerogenes* lysate (Figure 1 and Table 3). However, in spite of partly increasing the specificities, it was noted that there still a huge of false positive ratio for healthy controls, which impelled us to explore better measures to solve this problem.

Synthesizing polypeptides to reduce non-specific reactions

Another measure to reduce non-specific reactions was synthesizing polypeptides, which contained dominant B-cell epitopes and un-conserved fragments compared with other common bacteria. ABCprid software was used to screen potential B-cell epitopes and COBALT software was further employed to compare and select un-conserved segments of antigens. For example, the full sequence of Rv1488 was submitted into ABCprid software via setting parameters as follow: Threshold (0.80) and overlapping filter. As shown in Figure 2A, 15 B-cell epitopes were predicted. Simultaneously, we used Blast and COBALT from NCBI to screen Rv1488 antigen's homologous sequences compared with other common bacteria, such as *E. coli,* and then selected un-conserved segments. As shown in Figure 2B, the sequence "SPVEDQPKHAADGDDAEVA

GWFSTDTDPSIARAVATAEAIARKPVEGSLGTPPRLTQ" was un-conserved, which contained a dominant B-cell epitope "AEATARKPVEGSLGTP". Thus, final ready-to-synthesis sequence (Pep-Rv1488), i.e. "SPVEDQPKHAADGDDAEVAGWFSTDTDPSIARAVATAE

AIARKPVEGSLGTPPRLTQ" was obtained by the integration of B-cell epitope prediction and un-conserved segments blast results. Similar result was obtained for PstS1 (Supplementary Figure 1).

In order to assess the potential diagnostic value of these polypeptides, a panel of 96 serum samples, including 63 TB patients and 33 healthy individuals, was collected to compare the differences of sensitivities and specificities between these polypeptides and their mirror antigens. As shown in Figure 3, it was noted that both the levels of antibodies against Pep-PstS1 and Pep-Rv1488 in TB patients and healthy individuals groups were significantly decreased when compared with their mirror antigens, which may be due to the loss of some other B-cell epitopes. However, the sensitivities of each polypeptide just slightly decreased (P = 0.38 and P = 0.69) (Table 4). Conversely, some false positive serum samples showed seroconversion when tested by polypeptides, which improved their specificities (Figure 3). The above results indicated that proper polypeptides identified would help to effectively reduce non-specific reactions without dramatically decreasing their sensitivities.

Assembly of the fusion polyprotein

In the search for appropriate diagnostic antigens for TB, it was already known that no single antigen-based assay has so far achieved an optimal serodiagnostic performance due to the complexity of the

human immune response to TB antigens.[19] Thus, strategies using multiple antigens either individually or as fusion polyproteins (or segments) have been recommended. However, when the antigens were mixed together and tested as one for each patient, sensitivity decreased (Supplement Figure 2). Thus, a novel *M. tuberculosis* fusion polyprotein containing multiple polypeptides was preferentially constructed and expressed as an antigen with multi-epitopes in a way described in the Materials and Methods.

As shown in Supplementary Table 4, these nine antigens were previously identified as TB serodiagnostic potential and now would be fused as a polyprotein. Since HSPX was a serodiagnostic antigen with 33.33% sensitivity and 100% specificity[15], and expression of the fusion molecule HSPX with other antigens was increased by about 50% as compared to those of the individual antigen, resulting in cheaper production of the fusion antigens[20], we retained its whole sequence to the fusion protein to enhance immunogenicity. For other antigens, polypeptides were screened as described in the Materials and Methods and fused as an entirety by a sequence linker (GPGPGPGPGP). Finally, a fusion polyprotein containing nine antigens or segments was constructed (Figure 4). The 3-D theoretical structure of the fused polyprotein was predicted and obtained by SWISS-MODEL (Supplement Figure 3). The results showed that the three fragment antigens of the fused polyprotein retained the spatial structure of the original epitope, which suggested that the polyprotein still had good antigenicity. Moreover, the codon usage of the polyprotein was adapted to the codon bias of *E. coli* genes (Supplement Figure 4). Regions of very high (> 80 %) or very low (< 30 %) GC content had been avoided where possible. During the optimization process the following cis-acting sequence motifs were avoided where applicable: internal TATA-boxes, chi-sites and ribosomal entry sites; AT-rich or GC-rich sequence stretches; RNA instability motifs; repeat sequences and RNA secondary structures; (cryptic) splice donor and acceptor sites in higher eucaryotes. The optimization was finally successful: Negative cis-acting sites (such as splice sites, TATA-boxes, etc.) which may negatively influence expression were eliminated wherever possible. GC content was adjusted to prolong mRNA half life. Codon usage was adapted to the bias of *E. coli* resulting in a CAI* value of 0.95. The optimized gene should therefore allow high and stable expression rates in *E. coli.*

Assessment of the sensitivity and specificity of the fusion polyprotein

The optimized gene was amplified by PCR using the following primers: F-*E.coli* 5’CGGATCCGGCTCTAAACCGCCGTCCG3’; R-*E.coli* 5’CCTCGAGGGTTTCGATACGCT

GCTGCAG3’. The constructed plasma expression vector was cloned and expressed in *E. coli Rosetta* and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The purity of the fusion polyprotein was 90.8%, which was identified by Quantity One software (Supplement Figure 5). The indirect ELISA assay was conducted and the sensitivity and specificity of the polyprotein was evaluated. A panel of 192 serum samples, including 128 from TB patients and 64 from healthy individuals, were enrolled. The result was shown in Table 5 and Figure 5. The sensitivity of the

polyprotein was 60.2%, which was higher that of HspX and other individual antigen segments. The specificity of the polyprotein was 93.6%, which was no significantly decreased.

Discussion

Serology-based tests for TB diagnosis, though rapid, efficient and easily implemented, have so far shown unsatisfactory and suboptimal levels of sensitivity and specificity. One possible reason was the heterogeneity of antibody response in TB patients. The number and types of seropositive antigens vary from person to person and this variation may be linked to genetic polymorphisms of the human leukocyte antigen (HLA) class II alleles.[19] Though WHO recommended against the use of the current commercial serological tests for TB diagnosis, further research and development in this field were still encouraged.

Due to having homologous sequences with BCG and other common bacteria, TB serum antibodies would inevitably cross-react with antigens from these bacteria, thus inducing high false positive rate and reducing the specificity. Blocking antibodies against those bacteria was a feasible way to reduce nonspecific reactions. In this study, In addition to *E. coli,* which had been used previously, lysates from other bacteria, including *V. aquatilis, S. aureus, B. subtilis, P. vulgaris, S. epidermidis, E. aerogenes* and *S. citreus,* were used to pre-adsorb the serum samples to block antibodies against bacteriaantigens. Our results showed that there were no significant differences by adding *V. aquatilis, S. aureus, B. subtilis, S. epidermidis,* and *S. citreus,* andthe value of serum samples from strong false positive and weak false positive significantly decreased after pre-adsorbed by *P. vulgaris, E. aerogenes* and *E. coli* compared with groups without adding any bacteria lysate. Further study indicated that *E. coli* and *E. aerogen*es performed better. There were no significant differences by adding *E. coli & E. aerogenes* lysate compared with only *E. coli,* indicating the redundancy of extra adding *E. aerogenes* lysate. However, in spite of partly increasing the specificities, it was noted that there still a huge of false positive ratio for healthy controls, which impelled us to explore better measures to solve this problem. Synthesizing peptides according to immunodominant antigens antigens of *M. tb* would be an alternative and effective approach. These peptides should contain un-conserved fragments of TB antigens compared with other bacteria by bioinformatics analysis and simultaneously retain dominant B-cell epitopes as well as possible. We collected a panel of 96 serum samples, including 63 TB patients and 33 healthy individuals to compare the potential diagnostic value of these polypeptides with their mirror antigens, and found that though the levels of antibodies against these polypeptides in TB patients and healthy individuals groups were significantly decreased, the sensitivities of each polypeptide just slightly decreased ($P = 0.38$ and $P = 0.69$) and their specificities improved. The above results indicated that proper polypeptides identified would help to effectively reduce non-specific reactions without dramatically decreasing their sensitivities.

Since it was impossible to achieve an optimal serodiagnostic performance by using a single antigen-based assay, strategies using multiple antigens either individually or as fusion polyproteins (or segments) have been recommended. Our preliminary experiment showed that crude mixture of several antigens could not significantly increase the positive rates, and even counterproductive (Figure S2). A proper fusion polyprotein containing multiple polypeptides was thus preferentially constructed and expressed as

antigens with a multiepitope. Our previous study identified a cocktail of serodiagnostic antigens for active TB[15] and we currently tried to select the segments from nine antigens with un-conserved dominant B-cell epitopes to be a fusion polyprotein, which may improve the whole sensitivity and specificity compared with individual antigen and other combination forms. Since HspX was a serodiagnostic antigen with 33.33% sensitivity and 100% specificity in our study[15], and expression of the fusion molecule HspX with other antigens was increased by about 50% as compared to those of the individual antigen, resulting in cheaper production of the fusion antigens,[18] we retained its whole sequence to the fusion protein to enhance immunogenicity. pstS1 and mpt64 were the most frequently studied antigens while Rv1488, panD, echA3, cydA, Rv1825 and hns were no vel antigens identified. Finally, a fusion polyprotein containing nine antigens or segments was constructed. The 3-D theoretical structure of the fused polyprotein was successfully predicted and the codon bias of *E. coli* genes was also adapted. The fusion polyprotein was successfully expressed and the indirect ELISA assay was conducted and the sensitivity and specificity of the polyprotein was evaluated. The result showed that the sensitivity of the polyprotein was 56.3%, which was higher that of HspX and other individual antigen segments. The specificity of the polyprotein was 93.6%, which was not significantly decreased.

In summary, Serological tests represented an attractive option for TB diagnosis. However, the unsatisfactory sensitivities and specificities of the currently available options based on single or multiple target antigens not yet meet the requirements for clinical utility, which impelled us to explore better measures to solve this problem. In this study, we tried to use lysates from common bacteria to block antibodies against these bacteria in serum samples and synthesize un-conserved dominant B-cell epitopes of antigens to reduce nonspecific reactions, thus improving the specificity. Meanwhile, we constructed a fusion polyprotein containing HspX and other eight antigen segments and found that the sensitivity of the polyprotein was 56.3%, which was higher that of HspX and other individual antigen segments. The specificity of the polyprotein was 93.6%, which was not significantly decreased. This study will demonstrate the roles of fusion polyproteins in the humoral immunity against TB infection and provide a potential novel approach for TB diagnostic development.

Declarations

Ethics approval and consent to participate

All study procedures were conducted with Internal Review Board approval (Shenzhen People Hospital, China). All participants received information on the aim and procedures of the study, and written informed consent was obtained from all subjects.

Consent for publication

Not applicable.

Competing interests.

The authors have no conflicts of interest to declare.

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Authors' Contributions

1. Z.and W. P. conceived and designed the experiments. F. Z., X. X. and X. C. performed the experiments. F. Z. analyzed the data. F. Z. collected the samples. F. Z. and W. P. drafted the manuscript. All authors reviewed the manuscript.

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Tables

Table 1 Using lysates from various bacteria for sera pre-adsorption to reduce non-specific reactions (for pstS1)

pstS1	*V. aquatilis*	*S. aureus*	*B. subtilis*	*S. epidermidis*	*P. vulgaris*	*E. aerogenes*	*γ-streptococcus*	*S. citreus*	*E. coli*	Mixed	Back-ground	NC
SFP	0.563	0.531	0.592	0.543	0.333	0.399	0.512	0.515	0.318	0.405	0.582	0.016
WFP	0.325	0.283	0.289	0.274	0.133	0.109	0.247	0.223	0.190	0.156	0.271	0.009
SP	0.776	0.784	0.783	0.772	0.618	0.602	0.712	0.678	0.611	0.681	0.698	0.021
HC	0.134	0.139	0.122	0.117	0.088	0.085	0.118	0.115	0.064	0.055	0.114	0.017

Abbreviation: Strong false positive, SFP; Weak false positive, WFP; strong positive, SP; Healthy control, HC; Negative control; *Vibrio aquatilis, V. aquatilis; Staphylococcus aureus, S. aureus; Bacillus subtilis, B. subtilis; Proteus vulgaris, P. vulgaris; Staphylococcus albus, S. epidermidis; Enterobacter aerogenes, E. aerogenes; Staphylococcus citreus, S. citreus; Escherichia coli, E.coli.*

Table 2 Comparison of the effect of pre-adsorption by *P. vulgaris, E. aerogenes* and *E. coli* (for *pstS1)*

pstS1	Background	*E. coli*	*E. aerogenes*	*P. vulgaris*	Mixed	Blank
SFP1	0.625	0.246	**0.565**	**0.565**	0.267	0.024
SFP2	0.541	0.288	0.331	0.294	0.236	0.011
SFP3	0.403	0.268	0.297	0.291	0.269	0.01
SFP4	0.23	0.201	0.187	0.176	0.186	0.01
SFP5	0.373	**0.34**	**0.336**	**0.347**	0.347	0.011
SFP6	0.262	0.174	0.195	0.18	0.177	0.01
SFP7	0.946	0.415	0.468	0.493	0.52	0.017
SFP8	0.283	0.13	0.181	0.166	0.144	0.011
SFP9	1.007	0.246	0.326	0.336	0.51	0.011
SFP10	0.542	0.17	0.247	0.188	0.218	0.009
SFP11	0.513	0.167	0.225	0.218	0.212	0.01
SFP12	0.532	0.181	0.205	0.246	0.259	0.011
SP1	0.504	0.316	0.271	0.263	0.253	0.009
SP2	0.455	0.204	0.259	0.329	0.252	0.009
HC1	0.121	0.086	0.135	0.125	0.087	0.016
HC2	0.109	0.054	0.062	0.063	0.087	0.01

Abbreviation: Strong false positive, SFP; Weak false positive, WFP; strong positive, SP; Healthy control, HC; Negative control

Table 3 Comparison of the effect of pre-adsorption by *E. coli* and *E. aerogenes* & *E. coli*

Rv.	Vs	True positive	False negative	True negative	False positive	Sensitivity (%, 95% CI)	Specificity (%, 95% CI)	P Value
pstS1	*E. coli*	29	43	23	1	40.3(28.9–52.5)	95.8(78.9–99.9)	P1 = 0.69 P2 = 1.00
	E. coli+E. aerogenes	27	45	23	1	37.5(26.4–49.7)	95.8(78.9–99.9)	
Rv1488	*E. coli*	27	45	23	1	37.5(26.4–49.7)	95.8(78.9–99.9)	P1 = 1.00 P2 = 1.00
	E. coli+E. aerogenes	26	46	23	1	36.1(25.1–48.3)	95.8(78.9–99.9)	
PanD	*E. coli*	17	55	22	2	23.6(14.4–35.1)	91.7(73.0–99.0)	P1 = 1.00 P2 = 1.00
	E. coli+E. aerogenes	17	55	22	2	23.6(14.4–35.1)	91.7(73.0–99.0)	
EchA3	*E. coli*	19	53	23	1	26.4(16.7–38.1)	95.8(78.9–99.9)	P1 = 1.00 P2 = 1.00
	E. coli+E. aerogenes	20	52	23	1	27.8(17.9–39.6)	95.8(78.9–99.9)	

*p□0.0001; **p=1.0000 when compared by McNemar’s test. P1 = P value of the sensitivity and P2 = P value of the specificity when compared by McNemar’s test.

Table 4 comparison of the differences of sensitivities and specificities between polypeptides and their mirror antigens

Rv.	True positive	False negative	True negative	False positive	Sensitivity (%, 95% CI)	Specificity (%, 95% CI)	P Value
pstS1	25	38	32	1	39.7(27.6–52.8)	97.0(84.2–99.9)	P1 = 0.38;
Pep- pstS1	22	41	33	0	34.9(23.3–48.0)	100.0(89.4–100.0)	P2 = 1.00
Rv1488	20	43	32	1	31.8(20.6–44.7)	97.0(84.2–99.9)	P1 = 0.69;
Pep-Rv1488	18	45	33	0	28.6(17.9–41.4)	100.0(89.4–100.0)	P2 = 1.00

P value was compared by McNemar’s test by MedCalc. P1 = P value of the sensitivity; P2 = P value of the specificity when compared by McNemar’s test.

Table 5 Sensitivities and specifcities of nine individual antigens (or segments) and the fusion polyprotein with sera from TB patients and healthy controls.

Rv.	Sensitivity (%, 95% CI)	Specificity (%, 95% CI)
HspX	31.3 (23.4–40.0)	100 (94.4–100.0)
pstS1	25.0 (17.8–33.4)	98.4 (91.6–100.0)
Rv1488	23.4 (16.4–31.7)	98.4 (91.6–100.0)
mpt64	18.0 (11.7–25.7)	96.9 (89.2–99.6)
panD	18.0 (11.7–25.7)	96.9 (89.2–99.6)
echA3	12.5 (7.3–19.5)	98.4 (91.6–100.0)
cydA	20.3 (13.7–28.3)	98.4 (91.6–100.0)
Rv1825	15.6 (9.8–23.1)	95.3 (86.9–99.0)
hns	18.0 (11.7–25.7)	98.4 (91.6–100.0)
Polyprotein	60.2 (51.1–68.7)	93.6 (84.3–98.2)

Abbreviation: CI, confdence intervals.

Figures

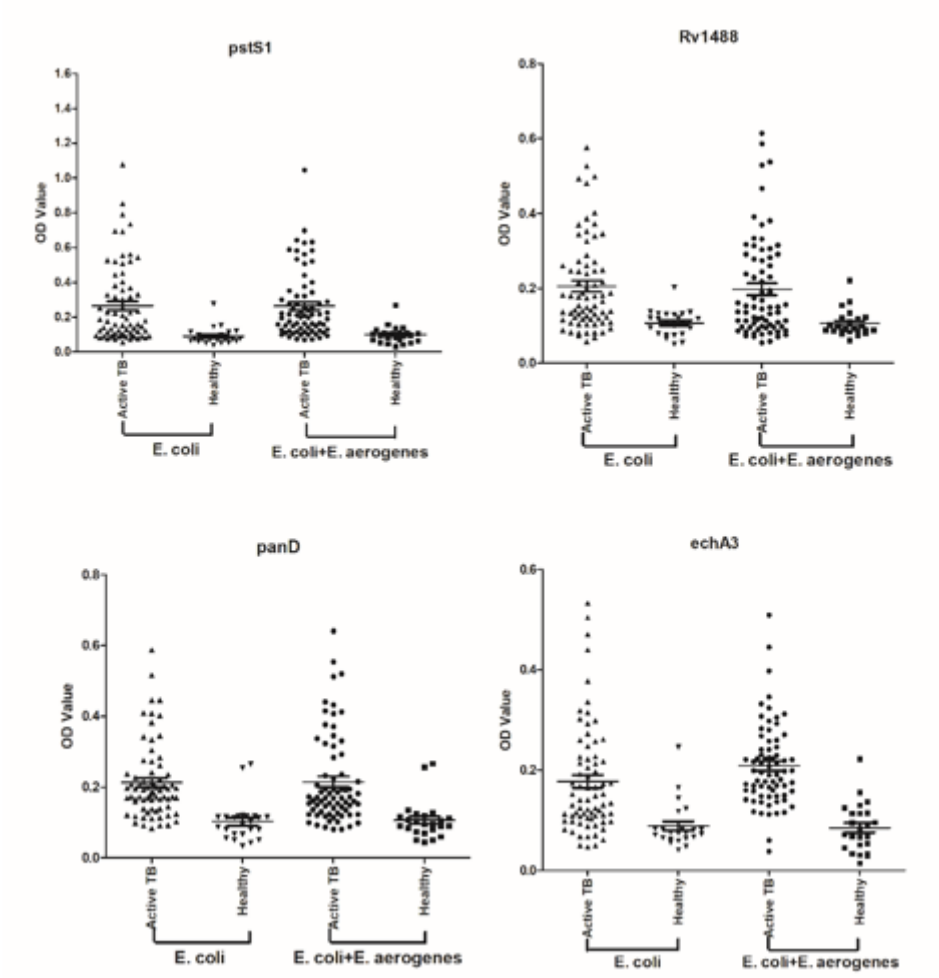

Figure 1

Comparison of the effect of pre-adsorption by E. coli and E. aerogenes & E. coli. A panel of 96 serum samples, including 72 TB patients and 24 healthy controls were recruited. PstS1, Rv1488, PanD and EchA3 four antigens were used to validate it.

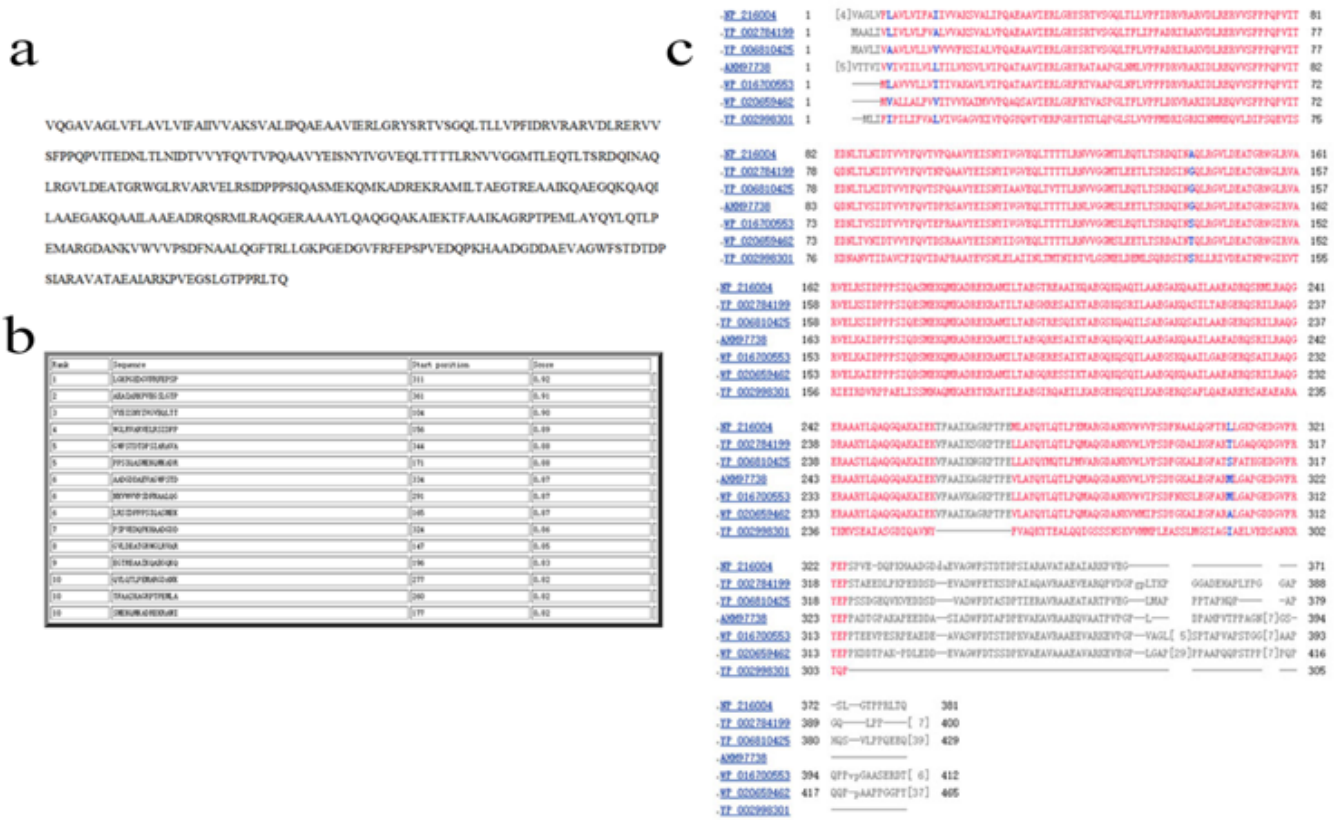

Figure 2

Synthesizing the polypeptide of Rv1488 antigen with dominant B-cell epitopes and un-conserved fragments. a. the full sequence of Rv1488 antigen; b. ABCprid software was used to screen potential B-cell epitopes; c. COBALT software was further employed to compare and select un-conserved segments of antigens.

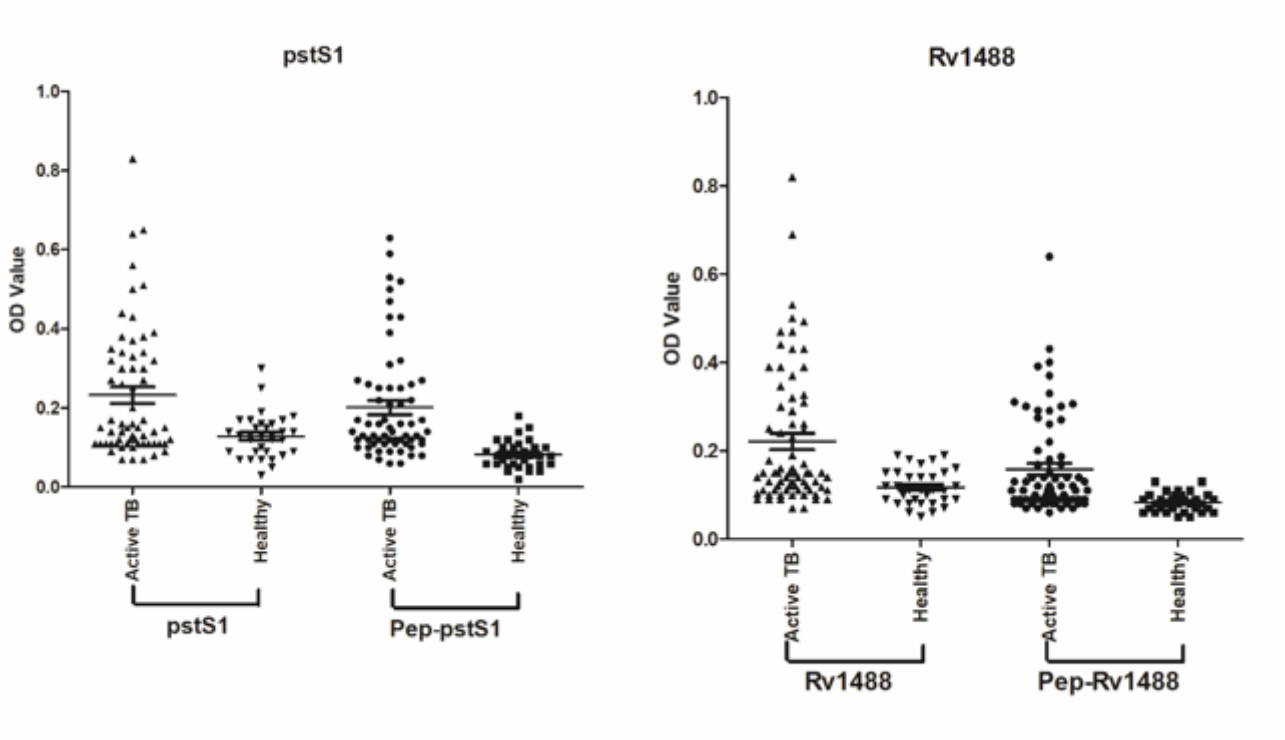

Figure 3

Comparison of the differences of sensitivities and specificities between polypeptides and their mirror antigens. a panel of 96 serum samples, including 63 TB patients and 33 healthy individuals, was collected to compare the differences between Pep-PstS1 and Pep-Rv1488 and their mirror antigens.

Figure 4

Assembly of the fusion polyprotein. a. HspX and other eight antigen segments were fused as an entirety by a sequence linker (GPGPGPGPGP). b. The protein sequence of the fusion polyprotein. c. the gene sequence of the fusion polyprotein.

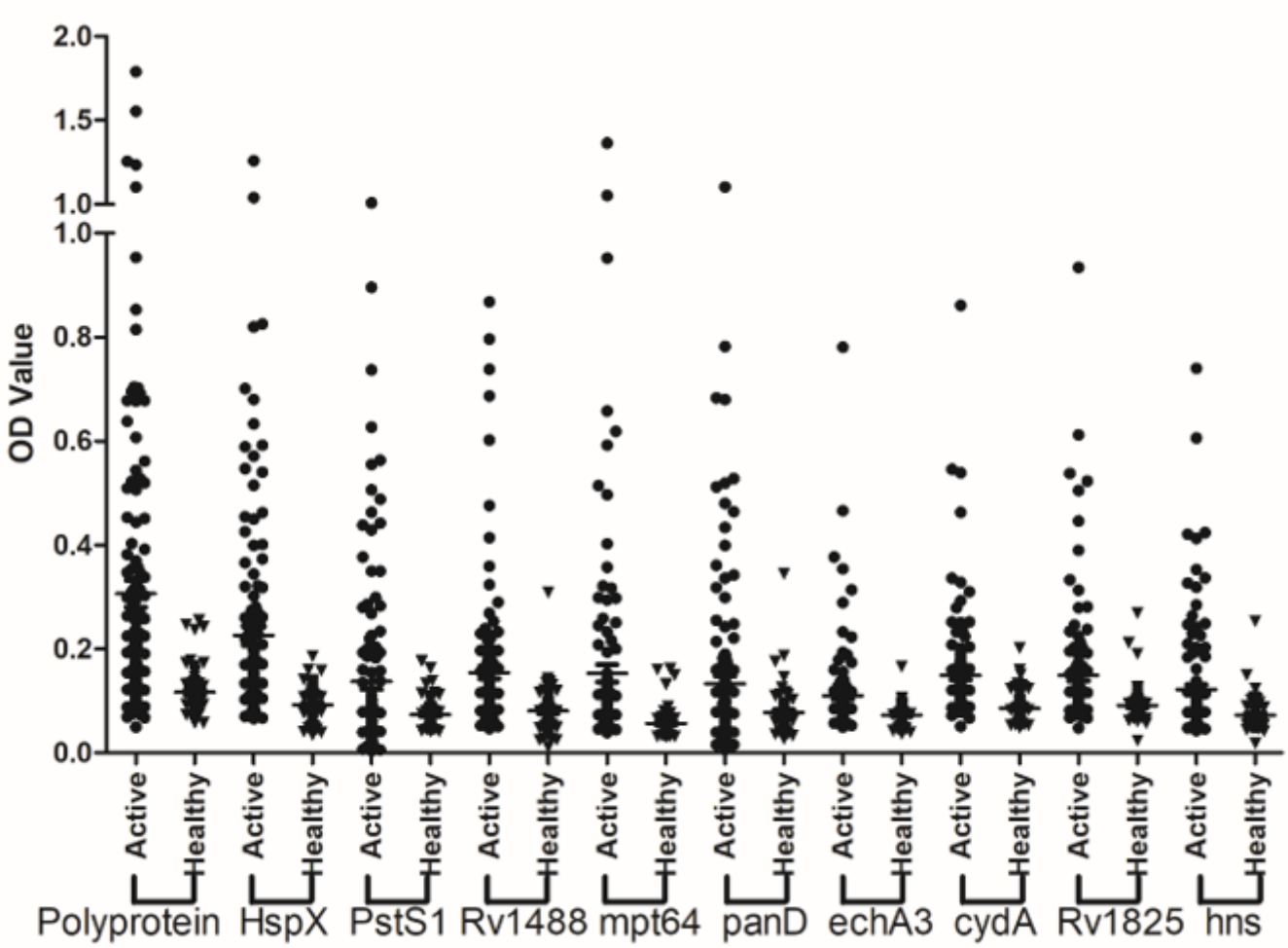

Figure 5

Assessment of the sensitivity and specificity of the fusion polyprotein. The indirect ELISA assay was conducted and the sensitivity and specificity of the polyprotein and each individual antigen or segments was evaluated.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementarymaterials.docx
- Supplementarylegends.docx