Full Length Research paper

Screening novel diagnostic marker of *Mycobacterium tuberculosis*

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The aim of present study was to screen and evaluate the serodiagnostic value of the purified fusion antigens of *Mycobacterium tuberculosis* (Mtb) by enzyme-linked immunosorbent assay (ELISA). A group of vector system which was constructed by cloning Rv1908c, Rv0733, Rv0899, Rv1411c and Rv3914 gene of Mtb into the prokaryotic expression plasmid pET-32b was transformed into *E. coli* for induction and expression fusion antigens to determine their potentiality in diagnostic application. Following purification with the His-select nickel magnetic agarose beads, the expressed fusion proteins showed purity via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot technique. The ELISA plates were coated with these fusion antigens. Through ELISA, the antigen binding with specific IgG levels between active TB patients and healthy controls was compared. The purity and the size of the expressed fusion antigens were confirmed by the Western blot. The ELISA results indicated that IgG levels against Rv1411c-6His, Rv3914-6His and Rv2031c-6His were significantly higher in serum of active TB patients than in that of healthy controls. More interesting, the AUC value of Rv3914-6His (0.7867) was higher than that of Rv2031c-6His (0.754) which was widely used in clinic. The results implied that Rv3914-6His might be a useful candidate antigen in the diagnosis of Mtb infection, especially for active pulmonary TB diagnosis. Its role in serodiagnosis of extra-pulmonary TB is still needed to be validated.

Key words: *Mycobacterium tuberculosis*, expression and purification, antigen, serodiagnosis, tuberculosis.

INTRODUCTION

Tuberculosis (TB) is a chronic infectious disease mainly caused by *Mycobacterium tuberculosis* (Mtb). The bacteria typically attack the lung parenchyma which named pulmonary TB (PTB) by the clinical manifestations (Walzl et al., 2011). Mtb also can attack other part of the body such as lymph nodes, the central nervous system, liver, spleen, kidney, spine, bone and skin which named extrapulmonary TB (EPTB) (Ireton et al., 2010). TB is currently the second-largest killer infectious agent after human immunodeficiency virus (HIV). According to the World Health Organization (WHO) statistics, China has the world’s second largest tuberculosis epidemic. 

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(Wang et al., 2007). Therefore, it is very important to develop new convenient, fast, high sensitivity and specificity diagnosis methods to control the prevalence and spread of tuberculosis.

Although currently bacteriological culture is still considered as the gold standard in clinical laboratory confirmation of TB disease, the laboratory may need more time (4-6 weeks), and the rates of false-positive cultures are too high, which are not suitable for clinical application (Kashyap et al., 2010). The newly developed GeneXpert MTB/RIF assay is a novel nucleic acid amplification diagnostic technique for the diagnosis of tuberculosis and rapid detection of rifampicin (RIF) resistance in clinical specimens (Steingart et al., 2013). But this assay determined the presence or absence of Mtb or rifampicin resistance, the activity of TB bacillus cannot be determined. The QuantiFERON®-TB gold in-tube or T-Spot TB test is an indirect test for measuring the release of interferon-gamma (INF-γ) from the patient's viable T lymphocytes stimulated by a few Mtb-specific antigens (early secreted antigenic target-6 and culture filtrate protein-10) (Rose et al., 2012). INF-γ release assays have excellent sensitivity and specificity with minimizing subjective interpretation and operator bias. However, the assays require expensive instrument, reagents and well trained operators. These requirements restrict their use in China. Serodagnosis has a long history and is characterized by convenient specimens, low costs, and commonly used in clinical laboratories to test numerous TB serum samples in a short time (Steingart et al., 2011). The antibody response to the 88 kDa, 65 kDa Hsp, 45 kDa, 38 kDa lipoprotein (He et al., 2011), 16 kDa HSP (Senol et al., 2009), ESAT-6, CFP-10, and lipoarabinomannan (LAM) (Ben Selma et al., 2010) antigens of Mtb was extensively studied by diagnostic companies and used in the commercial serological tests of TB infection (Flores et al., 2011). However, currently available commercial serodiagnostic tests provided inconsistent and imprecise findings, the WHO issued a policy statement against the use of commercial serological tests for the diagnosis of active PTB (WHO, 2011). Therefore, accurate, rapid and inexpensive antibody-based tests for TB diagnosis are needed urgently (Flores et al., 2011).

The Mtb immunoproteome analyses revealed sera from TB mainly recognized membrane associated and extracellular proteins of the bacterial (Kunnath-Velayudhan et al., 2010). In the study, the successful cloned genes of Mycobacterium tuberculosis envelope proteins RV1908c, RV0733, RV0899, RV1411c and RV3914 were selected for expression and purification and screening serodiagnosis antigens. RV1908c gene name is KatG, antigen name is catalase-peroxidase- peroxynitritase T, molecular mass is about 80.57 kDa, it may play a role in the intracellular survival of mycobacteria within macrophages. RV0733 gene name is adk, antigen name is adenylate kinase, molecular mass is about 20.09 kDa, it is essential for the bacteria in intracellular nucleotide metabolism. RV0899 gene name is ompA, antigen name is outer membrane protein A, molecular mass is about 33.54 kDa, it may protect the integrity of the bacterium. RV1411c gene name is lprG, antigen name is conserved lipoprotein, molecular mass is about 24.55 kDa, the function is still unknown. RV3914 gene name is trxC, antigen name is thioredoxin, molecular mass is about 12.54 kDa, Thioredoxin participates in various redox reactions. Enzyme-linked immunosorbent assay (ELISA) plates were coated with prokaryotic expression and purification of the five antigens, respectively. An indirect ELISA was established for rapid comparison serum IgG responses to the five antigens respectively between patients with active tuberculosis (TB group) and healthy physical examinees with non-tuberculosis (control group). Anti-Rv3914 IgG may become a potential important new serum marker for active tuberculosis diagnosis.

MATERIALS AND METHODS

Plasmids and antibodies

The recombinant prokaryotic expression plasmids pET-32b-Rv0733, pET-32b-Rv1411c, pET-32b-Rv1908c, pET-32b-Rv0899, pET-32b-Rv3914 and RV2031c-6His fusion antigen were kept in our laboratory.

Main reagents and instruments

Mouse monoclonal to 6×His tag antibody was purchased from Abcam, USA. Prestained protein molecular weight marker was purchased from Fermentas, Canada. IRDye 800CW goat antimouse IgG (H+L) was purchased from Licor Biosciences, USA. Plasmid preparation kit and DNA gel extraction kit were purchased from Sigma, USA. Alkaline phosphatase (AP) and IgG (H+L) was purchased from Jackson ImmunoResearch Laboratory, USA. BL21 (DE3) PLYSs competent cells was purchased from Takara, Japan. Amicon ultra-2 centrifugal filter unit with ultracel-3 membrane was purchased from Millipore, USA. Odyssey infrared imaging system was purchased from Licor Biosciences, USA. ELISA plate reader was purchased from Corning Incorporated, USA.

Prokaryotic expression and purification of His-Mtb fusion antigens

Escherichia coli (E.coli) BL21 (DE3) PLYSs was transformed with the recombinant plasmid pET-32b-Rv0733, pET-32b-Rv1411c, pET-32b-Rv1908c, pET-32b-Rv0899, pET-32b-Rv3914 and pET-32b-Rv2031c, respectively. Positive colonies were confirmed by DNA sequencing and performed to verify antigen expression.

Two hundred (200) mL medium LB containing 50 μg/mL ampicillin was inoculated and incubated at 37°C with 200 rpm shaking to the optical density (OD) value approximately 0.4 – 0.6 at 600 nm. IPTG was added to a final concentration of 0.1 mmol/L for inducing expression. Shaking incubation was continued at 30°C for 6 h. Bacterial cells were precipitated by centrifugation (7000 g for 3 min at 4°C) and resuspended in 20 mL lysis buffer containing 50 mmol/L...
NaH₂PO₄, 300 mmol/L NaCl and 10 mmol/L imidazole pH 8.0. The mixture was sonicated and centrifuged (8000 g for 30 min at 4°C). The supernatant containing recombinant Mtb antigens named crude extracts were filtered through a 0.45 μm prefiltre. His-select nickel magnetic agarose beads were uniformly suspended and added 1 mL to the filtrate protein. The mixture gently shaken overnight at 4°C and the affinity gel washed with plenty of wash buffer containing 50 mmol/L NaH₂PO₄, 300 mmol/L NaCl and 20 mmol/L imidazole pH 8.0. The sample was centrifuged and the supernatant containing unbinding contaminating proteins was removed. The His-Tag-C-terminal protein was eluted from the beads with elution buffer (50 mmol/L NaH₂PO₄, 300 mmol/L NaCl, 300 mmol/L imidazole, pH 8.0). Eluted fractions were transferred to Amicon Ultra-2 centrifugal filter unit with ultracel-3 membrane and centrifuged to remove the imidazole and other small molecules. The ultrafiltered samples were sterilized through 0.22 μm filter. The purified proteins were stored in 40% glycerin at -80°C.

SDS-PAGE and Western blotting

The protein concentration of the crude extracts and the purified protein was measured respectively using the Bradford method. An equal amount of total protein content was loaded on each lane and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by staining at 37°C using Coomassie brilliant blue R-250. Rv3914 was used as a given example for assessing the purity effect before and after purification.

For Western blot analysis, five purified antigens were separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes by semi-dry apparatus and nonspecific binding was blocked with PBS containing 3% bovine serum albumin (BSA). The blots were probed with anti-6-His antibodies diluted 1:1000, and the immune complexes were visualized using goat anti-mouse IgG (H+L) IRDye 800 conjugate secondary antibody, according to the manufacturer’s instructions. Blots were digitally photographed using the Odyssey infrared imaging system.

Human serum collection

The research proposal was approved by the ethics committee of Shanghai Junwei Fine Medical Club, China and all participants at Shanghai pulmonary hospital, China provided written informed consents. Thirty-four serum samples were collected from patients with confirmed diagnosis active pulmonary tuberculosis and thirty-five serum samples were obtained from healthy physical examinees. The patients had clinical symptoms, radiological signs of TB, acid-fast stain test of sputum smear, as well as positive culture for Mycobacterium tuberculosis. Serum samples as healthy controls were obtained from healthy physical examinees with no apparent signs of any disease and tuberculosis diagnoses ruled out.

Detection the relative antibody in serum by indirect enzyme-linked immunosorbent assay

Flat-bottom 96-well Costar plates were coated with 100 μL/well of each purified antigen at a concentration of 1 μg/mL in carbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6). The positive control was Rv2031c (16 kDa HSP)-6His antigen, the negative control was serum of healthy physical examinees and blank control was phosphate-buffered saline. After overnight incubation at 4°C, these antigen-coated wells were washed 3 times with PBS, containing 0.05% Tween 20 (PBST), blocked with blocking buffer of 1% gelatin in PBST for an hour at 37°C and followed by three washings. Subsequently, 100 μL of diluted serum samples (1:100 in blocking buffer) was added. Following an incubation period of 1 h at 37°C, the wells were again washed and filled with 100 μL of a 1:2000 dilution of alkaline phosphatase-conjugated goat anti-human immunoglobulin G. After incubation for 1 h at 37°C, the plates were again washed with PBST. The wells were filled with 100 μL of substrate solution (1 mg/mL p-nitrophenylphosphate in 10% diethanolamine buffer containing 0.5 mM MgCl₂, pH 9.8). After 30 min incubation at room temperature in darkness, the reaction was stopped by adding 50 μL/well of 2 M Na₂CO₃. The optical density (OD) of each well was measured at a wavelength of 405 nm in ELISA plate reader. Immunogenic properties of the purified antigens identification were done in ten-fold serial dilutions of serum samples.

Statistical analysis

Data were presented as means and standard errors (mean ± SD). The cut-off value for the optical density (OD) of an ELISA was determined by the mean value of healthy controls plus 3SD. Comparisons between two groups were performed with GraphPad Prism 5 statistical analysis software using the Mann Whitney test, and the level P < 0.05 was considered statistically significant.

RESULTS

Prokaryotic expression, purification and identification of five recombinant antigens of Mycobacterium tuberculosis

Single colonies of BL21 (DE3) PlySS bacteria transformed respectively by pET-32b-Rv0733, pET-32b-Rv1411c, pET-32b-Rv1908c, pET-32b-Rv0899 and pET-32b-Rv3914 were picked up from cultured plates and confirmed by DNA sequencing analysis. Recombinant antigens expressed as COOH-terminally polyhistidine-tagged fusion proteins were induced with IPTG and purified using His-select nickel magnetic agarose beads. SDS-PAGE gels for Rv3914-6His gave representative sample visualization the level of purity required was stained with Coomassie brilliant blue R-250. Taking the molecular weight of the 6×His tag (0.84 kDa) and that of Rv3914 antigen (12.54 kDa) into account, the size of the purified protein showed an expected size of protein band of 13.38 kDa (Figure 1).

Based on the Western blot assay, all of the five purified native fusion proteins could be recognized by His monoclonal antibodies and the expected bands were present respectively (Figure 2). These fusion proteins were Rv0899-6His (34.38 kDa), Rv0733-6His (20.93 kDa), Rv1908c-6His (81.41 kDa), Rv1411c-6His (25.39 kDa) and Rv3914-6His (13.38 kDa), respectively.

Reactivities evaluation of recombinant antigens to human serum specimens

A total of 34 serum samples were collected from 34 patients with a confirmed diagnosis of pulmonary tuberculosis at Shanghai pulmonary hospital between 2009 and 2010.
The clinical and laboratory characteristics of the TB patients and healthy controls were summarized in Table 1. Serum samples as control group were obtained from healthy individuals (n=35) with tuberculosis diagnoses ruled out.

The reactivities of recombinant antigens to human serum specimens were interpreted based on the ELISA assays. For ELISA the positive control antigen Rv2031c-6His (Figure 3) and the representative antigens Rv3914-6His (Figure 4) were used. Rv2031c, the 16 kDa heat shock protein (HSP), one of the three well known diagnostic antigen (Senol et al., 2009), was shown to be specific and sensitive for detecting antibodies against M. tuberculosis. The antigens were coated onto 96-well microplates. A total of 7 human sera were used in this assay, of which 6 serum specimens were from active TB patients, whereas 1 was from healthy individual. Ten-fold serial dilutions of the sera were examined, all reactions were run three times. The results revealed that the purified native Rv3914-6His antigen showed substantial reactivity to active-TB specimens. The OD value of active-TB specimens was significantly increased (0.88-fold for Rv2031c-6His, \( t =-15.433, P=0.0001 \); 0.72-fold for Rv3914-6His, \( t =-8.513, P=0.0004 \)) in microwell plate coated with the antigens at the concentration of 1μg/mL as compared with the concentration of 0.1 μg/mL. The recombinant antigens concentration of 1 μg/mL for ELISA studies was selected as working coated concentration in the following studies.

**ELISA screening of purified recombinant antigens for TB diagnosis**

After confirming the reactivity of purified fusion antigen, a total of 69 human serum samples were assayed by ELISA using the five recombinant antigens, respectively. The Rv2031c-6His (16 kDa antigen) fusion protein was included in the assay system as a positive control for specificity and sensitivity. Each assay was repeated three times. These sera consisted of 34 samples from active TB patients, and 35 samples from healthy physical examinees. The profiles of IgG antibodies against of the purified fusion Mtb antigens were estimated by indirect ELISA in these human serum samples collected (Figure 5). The ELISA with Rv3914-6His, Rv1411c-6His and Rv2031c-6His fusion antigens were useful to discriminate positive and negative TB, a statistically significant difference in IgG level were found between healthy controls and TB patients (\( P_{\text{Rv3914c-6His}}=0.0001, P_{\text{Rv1411c-6His}}=0.0043 \) and \( P_{\text{Rv2031c-6His}}=0.0004 \), respectively). There was no significant difference in the levels of anti-Mycobacterium tuberculosis antibody IgG from those of serum specimens from healthy controls and from active TB individuals by ELISA with Rv0899-6His, Rv0733-6His and Rv1908c-6His fusion antigens (\( P_{\text{Rv0899-6His}}=0.1398, P_{\text{Rv0733-6His}}=0.3060 \) and \( P_{\text{Rv1908c-6His}}=0.0591 \), respectively).

Receiver operating characteristic (ROC) curves is a graph of sensitivity on the y-axis against 100-specificity on the x-axis. It is an excellent way to evaluate the clinical diagnostic value of given biomarker. ROC curves were plotted using GraphPad Prism 5. The areas under the summary ROC curve (AUC) of Rv3914-6His,
Table 1. Clinical and experimental parameters of research cohort.

<table>
<thead>
<tr>
<th>Clinical and laboratory characteristic</th>
<th>Active tuberculosis patients (n=34)</th>
<th>Healthy controls (n=35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years ±SD)</td>
<td>45.4 ± 18.6</td>
<td>42.8 ± 16.2</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>12/22</td>
<td>24/11</td>
</tr>
<tr>
<td>Duration of symptoms (months ±SD)</td>
<td>19.18 ± 2.61</td>
<td>—</td>
</tr>
<tr>
<td>Treatment (months ±SD)</td>
<td>2.45 ± 0.12</td>
<td>—</td>
</tr>
<tr>
<td>Smear positive</td>
<td>20</td>
<td>—</td>
</tr>
<tr>
<td>Sputum culture positive</td>
<td>25</td>
<td>—</td>
</tr>
<tr>
<td>Purified protein derivative (PPD) positive</td>
<td>16</td>
<td>—</td>
</tr>
<tr>
<td>Cavity positive</td>
<td>13</td>
<td>—</td>
</tr>
</tbody>
</table>

Figure 3. Detection of serum IgG against with Rv2031c-6His fusion protein by indirect ELISA. TB: tuberculosis patients; HC: healthy physical examinees.

Figure 4. Detection of serum IgG against with Rv3914-6His fusion protein by indirect ELISA. TB: tuberculosis patients; HC: healthy physical examinees.

Figure 5. Detection of serum IgG against with Mtb antigen-6His fusion protein by indirect ELISA. TB: tuberculosis patients (n=34); HC: healthy physical examinees (n=35).

Rv1411c-6His and Rv2031c-6His ROC were 0.7867, 0.5867 and 0.754, respectively (Figure 6). An AUC of 0.75 or greater is generally considered a good biomarker. Antibody response to the 16 kDa heat shock protein in active TB has been used in assist diagnosis of infectious Mtb (Senol et al., 2009). It was suggested that Rv3914 recombinant protein could be potentially used for the diagnosis of tuberculosis and as a candidate antigen.

DISCUSSION

In recent years, due to movement of population, problems of multi-drug resistant TB and TB-HIV co-infection, tuberculosis continues to be one of major public health burden in China. Rapid and effective diagnosis of infectious cases is crucial to facilitate earlier treatment initiation.
and reducing disease transmission and preventing emergence of resistant strains (Kaushik et al., 2012).

The aim of this study was to screen a specific diagnostic marker from five envelope protein of Mtb which identified by proteomic studies (de Souza et al., 2011; Forrellad et al., 2013). The envelop antigens, such as Rv1908c (Escalante et al., 2013), Rv0899c (Marassi 2011) and Rv3914c (Akif et al., 2008), may play essential roles in the intracellular growth and survival of Mtb within the host. The expression and purification system of five fusion proteins was successfully established and recombinant fusion proteins with high purity and high yield was obtained. The recombinant antigen’s native conformation with antibody binding activity may be lost due to prokaryotic expression system, so the ELISA plates were coated with gradient dilution antigens, and the collected sera (dilution, 1:100) were used as primary antibodies. The result showed the representative Rv3914-6His antigen retained antibody-binding activity. The purified fusion antigens were potentially used as coated antigens in ELISA assays.

Serum specimens (including 34 cases of active TB patients and 35 healthy physical examinees) were tested for the presence of IgG antibodies against these five antigens of Mtb using a quantitative ELISA. The results of ELISA test showed good sensitivity for purified Rv3914-6His and Rv1441c-6His antigen. The levels of antibodies against the two antigens were significantly higher in TB patients than in healthy individuals \( P_{Rv3914c-6His} = 0.0001, \ P_{Rv1411c-6His} = 0.0043 \), respectively. Rv3914c could be selected as an adjunct for TB serodiagnostic assay according to the ROC analysis, the AUC value for Rv3914-6His is 0.7867 and similar to the value for Rv2031c \( \text{AUC}_{Rv2031c-6His} = 0.754 \). It would be of interest to further study how the Rv3914 and Rv2031c antigens perform in combination.

There were no statistically significant differences between the optical density values obtained with sera from TB patients and healthy controls with Rv1908c-6His, Rv0899-6His or Rv0733-6His antigens-coated ELISA plates, respectively. It has been reported previously that mutations in the three antigens’ gene lead to reduce virulence and may cause reduced survival of Mtb in host tissues (Pym et al., 2002; Raynaud et al., 2002; Bellinzoni et al., 2006). These antigens might really begin their work when Mtb were engulfed by macrophages. Therefore, these antigens might be helpful for screening intracellular antibodies of Mtb.

Authors’ contributions

XP designed the research and wrote the paper; XP, SLZ, JLC, BZ, BJP, ZLP, YW, YZ, WF, MC, WQL, XZY, MT, JZ, WS performed research.

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