



Expression and diagnostic value of proteins in *Mycobacterium tuberculosis*

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ABSTRACT. We constructed a prokaryotic expression vector expressing the *Mycobacterium tuberculosis* protein TB16.3, as well as 3 other proteins, including TB15.3, CFP-10, and Rv2626C, which were purified and analyzed for their effectiveness as detection antibodies. The *TB16.3* genes of *M. tuberculosis* H37Rv genomic DNA were amplified by polymerase chain reaction, inserted into the expression vector pET-30a, and expressed in *Escherichia coli*. An enzyme-linked immunosorbent assay was used to detect the 4 *M. tuberculosis* antibodies. Engineered *E. coli* bacteria expressing TB16.3 and the 3 other proteins were constructed and found mainly to be soluble. For recombinant TB16.3 proteins, serum samples of 118 tuberculosis (TB) patients and 96 healthy controls were analyzed. Sensitivity, specificity, and adjusted concordance rate for the TB16.3 antibody were 72.9, 86.5, and 79.6%, respectively. The positive rate of Rv2626C antibody in TB patients (44.1%) was significantly lower than that in normal controls (75.0%, $\chi^2 = 20.8$, $P < 0.01$). TB15.3 and TB16.3 were used for simultaneous detection and showed sensitivity, specificity, and repeatability rates of

69.4, 96.9, and 83.7%. The antibody positive rate and specificity for patients with lung disease was 9.6 and 90.4%, respectively. TB15.3 and TB16.3 were mixed and detected simultaneously. Combined with the results for TB15.3, the sensitivity, specificity, and concordance rates were 82.2, 95.9, and 88.9%, respectively. The concordance rate was the highest value observed. Target genes were cloned into a host strain and expressed successfully. The TB16.3 recombinant protein may be used as a new serological antigen for tuberculosis diagnosis.

Key words: *Mycobacterium tuberculosis*; TB16.3 protein; Prokaryotic expression vector; Serological diagnosis

INTRODUCTION

Tuberculosis (TB), which is caused by *Mycobacterium tuberculosis*, is one of the most infectious diseases and currently has one of the highest mortality rates of any disease. It has been estimated that TB infection affects one-third of the global population, with 8.9-9.9 million new TB cases reported annually. Approximately 1.3 million people die of the disease. Thus, TB is a global public health problem (WHO, 2010). The most critical aspect of TB prevention and control is early detection and correct medication. Methods for early diagnosis of TB are urgently needed. Sputum smear detection of acid-fast bacilli remains the preferred method of TB diagnosis and therapy assessment in many developing countries. However, the limitations of this method include its low sensitivity, multiple testing, and loss of manpower, among others (Steingart et al., 2006). Recently, serological methods for TB diagnosis have been applied, and early laboratory diagnosis of TB has achieved good results; however, several limitations remain (Zhu et al., 2004; Steingart et al., 2009; Ben-Selma et al., 2011). Identification of stable and efficient specific antigens or antigen combinations has been challenging. Thus, the development of satisfactory antigens and antigen combinations with high sensitivity and specificity has become a focus in the serological diagnosis of TB. In this study, TB16.3 was examined for its ability to detect TB and Bacille Calmette-Guérin (BCG) genetic variation. Using genetic engineering to directly express the antigen, the serological diagnostic effectiveness of TB16.3 was analyzed. TB16.3 is not purified from *M. tuberculosis* culture filtrate. We cloned and expressed TB16.3 as well as 3 other proteins (Liu et al., 2011; Zhang et al., 2011) to detect TB antibodies in human serum.

MATERIAL AND METHODS

Strains and vectors

M. tuberculosis H37Rv, *Escherichia coli* BL21 (DE3) cells, and the pET-30 expression vector were stored in the clinical laboratory department of our hospital.

Reagents

Isopropyl β -D-1-thiogalactoside (IPTG) and kanamycin (Kan) were purchased from Shanghai Sangon Biological Engineering Technology & Services Ltd. (Shanghai, China).

DNA polymerase, T4 DNA ligase, restriction endonucleases, and the DNA marker were purchased from Takara Co., Ltd. (Shiga, Japan). The pre-stained protein marker was purchased from Beijing Bai Tai Ke Biotechnology Co., Ltd. (Beijing, China). An agarose gel DNA extraction kit was purchased from Beijing Tian Gen Biotechnology (Beijing, China). Columns for 6x His-tagged Ni-NTA Agarose protein purification were obtained from QIAGEN (Hilden, Germany). Common high-purity biochemical reagents were purchased from Biosharp (Hefei, China), and other reagents were of analytical grade. Horseradish peroxidase-labeled anti-human immunoglobulin (Ig) was purchased from Solarbio (Beijing, China). The chromogenic substrate was purchased from InTec Technology, Ltd. (Xiamen, China).

Serum source

Serum from 118 diagnosed TB patients was collected from the Haikou Tuberculosis Control Department, including serum from 83 males and 35 females. Twenty patients had smear-positive samples and 98 patients had smear-negative samples. Sixty-six patients were ≤ 40 years old, 29 patients were aged 41-59 years, and 23 patients were ≥ 60 years old. Fifty-three patients did not have TB but had other lung diseases; 12 patients had community-acquired pneumonia, 26 patients had chronic obstructive pulmonary disorder, 8 patients had lung infection, and 6 patients had lung cancer. All were hospital inpatients. Serum from 96 normal controls was collected from healthy subjects.

Primers

According to the gene sequence of *TB16.3* in standard strains of *M. tuberculosis* H37Rv (GenBank sequence No. CAD97060; Garnier et al., 2003), the following primers were synthesized: for the 5' end (TB16.3F), 5'-CGGAATTCATGGCGGACAAGACGACACA-3', and for the 3' end (TB16.3R), 5'-CCGCTCGAGTCAGCCCTCGACTCGTTTCT-3'. Underlined bases indicate *EcoRI* and *XhoI* restriction sites. Primers were synthesized by GenScript, Ltd. (Piscataway Township, NJ, USA).

TB16.3 gene amplification and cloning

H37Rv genomic DNA was used as a template, and *TB16.3* gene fragments were amplified by polymerase chain reaction (PCR). The reaction conditions were as follows: 95°C for 5 min, 95°C for 30 s, 53°C for 30 s, and 72°C for 1 min, for a total of 35 cycles. After this, the temperature was held at 72°C for 7 min and then maintained at 4°C. PCR products were purified using an Agarose DNA extraction kit (Tian Gen Biotechnology, Beijing, China). After restriction enzyme digestion, the fragments were ligated into the expression plasmid pET30a using T4 DNA ligase. Ligation products were transformed into *E. coli* BL21 (DE3) competent cells, and positive recombinants were selected for sequencing by Nanjing GenScript Biological Engineering Co., Ltd. (Nanjing, China).

Induced expression of target proteins

Sequence-verified recombinants were inoculated in Luria-Bertani (LB) liquid me-

dium containing 50 mg/L Kan. After shaking the culture at 37°C, the absorbance at 600 nm (A_{600}) was adjusted to 0.6-0.8. IPTG was added to reach a final concentration of 1 mM, the culture was incubated at 37°C for 1 h from the start of induced expression, and the samples were collected at 1-h intervals until 6 h of expression. The bacterial cells were harvested for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Expression and purification of recombinant proteins

The bacterial cells were collected following inducible expression, sonicated, and centrifuged to remove cell debris. The pellet and supernatant were collected. The SDS-PAGE analysis was used for target protein expression. The QIAGEN 6 x His-tagged Ni-NTA Agarose protein purification column was used for the purification of His-tagged recombinant proteins.

Detection of recombinant TB16.3 protein antigens by enzyme-linked immunosorbent assay (ELISA)

Using the matrix method, the optimum antigen coating concentration and the secondary antibody dilution times were determined. The optical density at a wavelength of 450 nm (OD_{450}) was measured using a microplate reader. OD_{450} values ≥ 2.1 times the negative control value were defined as positive based on previously described methods (Liu et al., 2011; Zhang et al., 2011).

Statistical analysis

Sensitivity, specificity, positive and negative predictive values, and diagnostic accuracy were calculated based on methods described in the literature (Lv et al., 1991). Statistical significance was evaluated using the chi-square test, with $P < 0.05$ considered to be statistically significant. The SPSS18.0 statistical package was used for analysis (SPSS, Inc.; Chicago, IL, USA).

RESULTS

Construction and identification of the recombinant plasmid

The *TB16.3* gene was successfully amplified from H37Rv genomic DNA. The length was 435 base pairs (bp), which was consistent with the design. The constructed expression vectors pMD-19T-TB16.3 and pET-30a-TB16.3 were digested using restricted enzymes after purification. The 435-bp fragments are displayed in Figure 1A and B. Sequencing results showed that the sequence of the gene inserted into the vector was 100% consistent with the reported sequence (Garnier et al., 2003).

Optimization of expression conditions

A 37-kDa protein band was observed on the SDS-PAGE gel after IPTG induction (Figure 2A). Maximum expression was observed 5 h after induction (Figure 2B). Inducible expression occurred at 28°, 37°, and 42°C. Although the TB16.3 protein was expressed at the

3 different temperatures, the expression reached a maximum at 37°C (Figure 3A). Analysis of expression at 3 different concentrations of IPTG showed that 1 mM IPTG most effectively induced protein expression (Figure 3B).

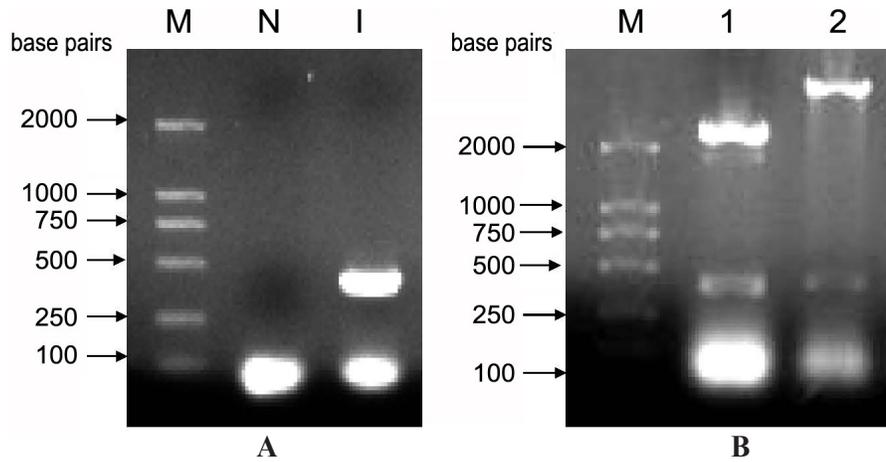


Figure 1. PCR amplification of the target band and restructuring pMD-19T with pET-30a plasmid digestion results. **A.** Lane *M* = DNA marker; lane *N* = negative control; lane *I* = TB16.3 PCR product (435 bp); **B.** Lane *M* = DNA marker; lanes 1 and 2 = pMD-19T-TB16.3 and pET-30a-TB16.3 after double-digestion of the double bar belt.

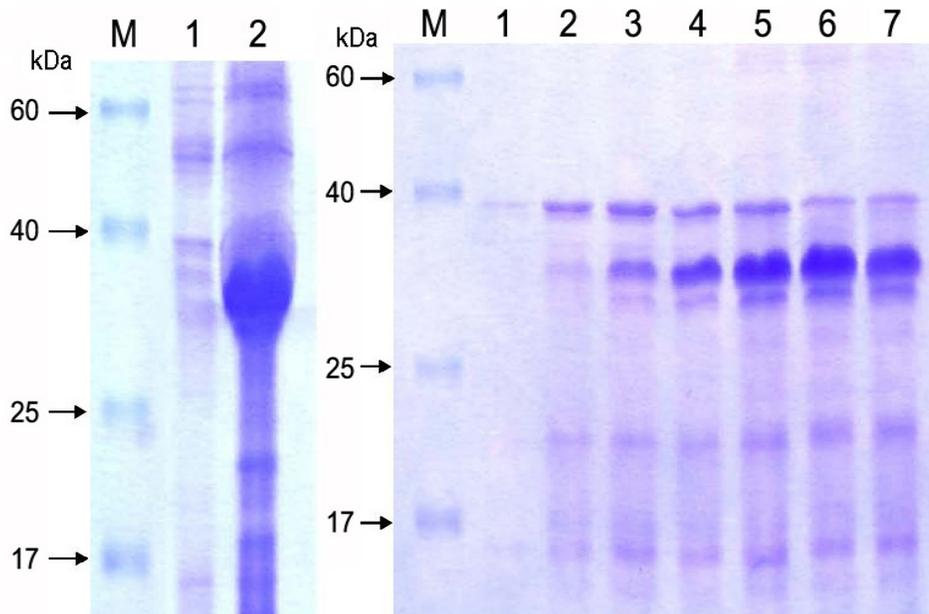


Figure 2. Protein expression of antigen and induction time gradient results. Lane *M* = pre-stained protein marker; **A.** lane 1 = uninduced engineered bacteria; lane 2 = engineered bacteria induced after 4 h; **B.** lane 1 = uninduced engineered bacteria; lanes 2-7 = 1-6 h induced engineered bacterial culture supernatant.

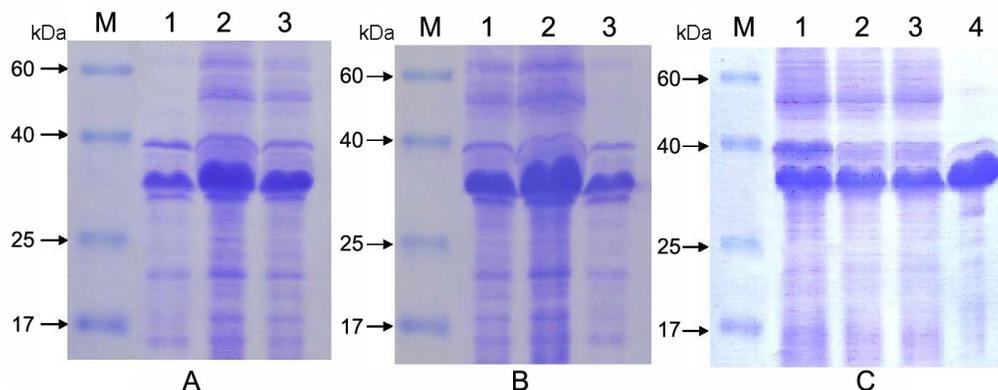


Figure 3. Induced expression optimization results. Lane M = pre-stained molecular weight marker; **A.** lanes 1-3 = cloned bacteria at 28°, 37°, and 42°C induction results. **B.** lanes 1-3 = 0.5 and 1.5 mM IPTG for TB16.3 antigen expression. **C.** Lane 1 = engineered bacterium culture supernatant; lane 2 = engineered bacterium broken precipitation; lane 3 = engineered bacterium broken supernatant; lane 4 = purified rTB16.3 peptides.

Soluble analysis and purification results of TB16.3 peptides

While the highest expression was observed at 37°C (Figure 3B), the recombinant antigens were mainly present in inclusion bodies. When the temperature was reduced to 28°C, the recombinant antigens were mainly in the form of soluble protein. Therefore, we used a temperature of 28°C to expand fermentation. The supernatants were collected after expression, and the TB16.3 peptides were purified using a nickel agarose gel purification column to obtain highly purified TB16.3 (Figure 3C).

Establishment of anti-rTB16.3 detection technology using ELISA

Using matrix methods, the optimum coating concentration of recombinant TB16.3 peptides (rTB16.3) was found to be 0.1 µg/well. The optimum working concentration of mixed antigens was 0.1 µg/well. The content of the 2 recombinant proteins was 0.05 µg/well. The optimum working concentration of horseradish peroxidase secondary antibody was 1:1000.

Detection of TB using TB16.3

The results of TB16.3 protein-coating and serum results for TB patients using ELISA are shown in Table 1 and Figure 4. TB patients with sputum smear-positive results showed the highest TB16.3 sensitivity. The positive rate of Rv2626C antibody in TB patients (44.1%) was significantly lower than that in normal controls (75.0%, $\chi^2 = 20.8$, $P < 0.01$). CFP-10 and TB16.3 showed higher sensitivity. Without the sputum smear test, the CFP-10 antibody positive rate was the highest. The sensitivities of detection using TB15.3, TB16.3, and TB15.3 + TB16.3 are shown in Figure 4; their specificities were 99.0, 96.9, and 86.5%, respectively.

Table 1. Performance index of detection using different antigens.

Capture antigens or antigen combinations	Sensitivity (%)		Specificity (%) (96)	Positive predictive value (%)	Negative predictive value (%)	Adjusted concordance rate (%)
	Total positive (118)					
	Positive (20)	Negative (98)				
TB15.3	75.0 (15)	40.8 (40)	99.0 (1)	98.3	60.1	76.0
TB16.3	95.0 (19)	68.4 (67)	86.5 (13)	86.9	72.2	79.6
CFP-10	70.0 (14)	76.5 (75)	55.2 (43)	67.4	64.6	65.7
Rv2626c	45.0 (9)	43.9 (43)	25 (66)	41.9	26.7	34.4
TB15.3+TB16.3	95.0 (19)	64.2 (63)	96.9 (3)	96.5	72.1	83.7
CFP-10+Rv2626c	80.0 (16)	76.5 (75)	23.2 (72)	55.2	45.2	50.2
TB15.3 and TB16.3	70.0 (14)	28.6 (28)	86.5 (13)	76.4	52.2	62.7
TB15.3+TB16.3 and TB15.3	70.0 (14)	21.4 (21)	95.9 (4)	89.9	52.6	67.0
TB15.3+TB16.3 and TB16.3	90.0 (18)	48.0 (47)	86.5 (13)	83.4	61.1	71.5
TB15.3 and TB16.3 and CFP-10	40.0 (8)	17.3 (17)	80.0 (19)	56.5	45.2	50.7
TB15.3 and TB16.3 and Rv2626c	35.0 (7)	13.3 (13)	25.0 (72)	21.7	19.7	20.8
TB15.3 or TB16.3	100.0 (20)	78.6 (77)	85.4 (14)	87.4	79.6	83.7
TB15.3+TB16.3 or TB15.3	100.0 (20)	78.6 (77)	95.9 (4)	96.0	81.4	88.9
TB15.3+TB16.3 or TB16.3	100.0 (20)	89.8 (88)	83.3 (16)	87.1	88.9	87.7

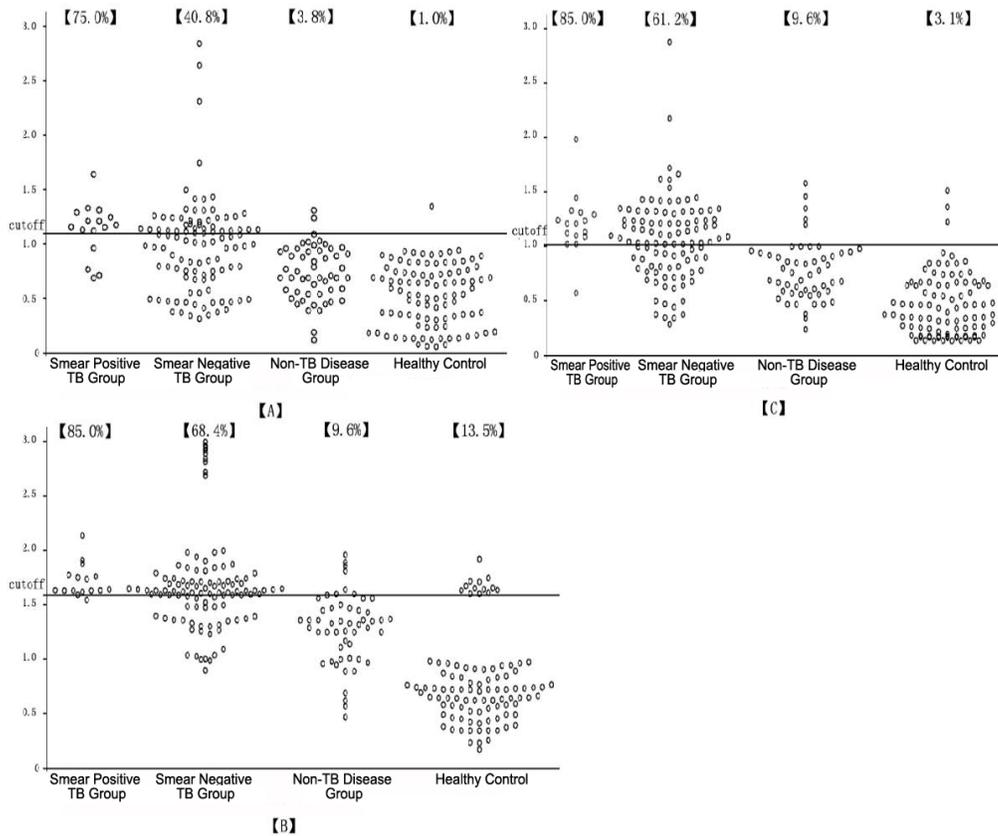


Figure 4. Tuberculosis patients' TB15.3 and TB16.3 antibody results by ELISA. **A.** **B.** and **C.** show antibody sensitivity and specificity using ELISA in smear-positive, smear-negative TB patients, the non-TB acute disease group, and healthy serum for anti-TB15.3, anti-TB16.3, and anti-TB15.3 + TB16.3.

Four recombinant antigen combinations in TB antibody detection

The results of the combined antigen ELISA test of serum in TB patients are shown in Table 1. An equal mixture of Rv2626C and CFP-10 was detected, with seropositive rates very similar between TB patients and normal controls (77.1 and 76.8%, respectively). If TB15.3, TB16.3, and Rv2626C were detected, the positive results of the 3 was defined as positive; the positive rates of TB patients and controls were 16.9% (20/118) and 75.0% (72/96), respectively ($\chi^2 = 72.8$, $P < 0.001$). This difference was very significant, indicating that the Rv2626C antibody can be used as an indicator of improvement in patients.

The data in Table 1 also show that TB15.3 and TB16.3 were detected at the same time, with sensitivity, specificity, and consistency rates of 69.4, 96.9, and 76.0%, respectively. TB15.3 and TB16.3 were both detected individually as well. If either of the 2 antibody results were positive, the test results were judged as positive. The sensitivity, specificity, and consistency rates were 82.2, 85.4, and 83.7%, respectively. If a mixture of TB15.3 and TB16.3

was detected, the test results or TB16.3 individual test results were defined as positive; the sensitivity, specificity, and consistency rates were 91.5, 83.3, and 87.7%, respectively. If the mixture of TB15.3 and TB16.3 was detected, the test results or TB15.3 individual test results were defined as positive; the sensitivity, specificity, and consistency rates were 82.2, 95.9, and 88.9%, respectively. The concordance rate was the highest value among antigen or antigen cocktail tested.

DISCUSSION

Laboratory diagnosis of TB is crucial to controlling the disease. Early detection of row bacteria in patients and early treatment can prevent the spread of TB. Significantly blocking the spread of multi-drug-resistant TB is even more important. The means of diagnosing TB in the laboratory currently include the following. First, direct or enriched smear microscopy can intuitively discover pathogens, with the drawback of a low positive rate (Steingart et al., 2006). Second, TB culture *in vitro* can improve positive rates; however, although susceptibility testing and species identification can be performed, this test is time-consuming, taking as long as 6-8 weeks. Liquid culture techniques can significantly shorten the time to diagnosis, but TB patients typically cannot afford the expensive equipment and reagents. Third, molecular biology techniques are used for TB diagnosis, which can improve sensitivity and show high specificity, and even detect resistance genes. However, the high false-positive results and the high expense of such tests may prevent the widespread use of this method (Perkins and Cunningham, 2007). Fourth, immunological diagnostic methods are used, which can be divided into cellular immunity and humoral immunity diagnostic methods. Cell immunological diagnostic methods, such as ELISPOT and TB-IGRA tests, are only suitable for TB screening in countries with low TB incidence. Because the false positive diagnosis rate is high and the tests are costly, prospects for applying this method in developing countries appear to be limited.

In TB-specific antibody detection, the immunological tests are fast and simple, with no specific equipment or facilities required. These tests can be performed for a large population. Tuberculosis antibody detection technology primarily includes ELISA, colloidal gold filtration assay, and protein chip technology (Zhu et al., 2004). The use of antigens has shifted from purified proteins of mycobacterial culture to genetically engineered antigens. The currently used TB antigens include the 38-kDa protein (Silva et al., 2003; Ben-Selma et al., 2011), the 16-kDa protein (Senol et al., 2007; Ben-Selma et al., 2011), lipoarabinomannan, HspX, MTB48 (Lodes et al., 2001), and Mtb81 (Silva et al., 2003; Perkins et al., 2003; Ben-Selma et al., 2011). Despite significant research efforts, the sensitivity, specificity, sputum acid-fast bacilli-positive concordance rate, and relevance of a serious condition reveal little useful information. Positive antibodies are not useful as a primary method for diagnosing TB. Therefore, screening for TB proteins is used in antibody detection, which is currently an important area of study.

The TB16.3 protein is encoded by the TB gene *Rv2815C*, producing a mature protein of 144 amino acids with a molecular weight of 16.3 kDa; thus, the protein was named TB16.3. Because it cannot be separated from the H37Rv protein filtrate or cell detritus, it has been referred to as a hypothetical protein (Garnier et al., 2003). The amino acid sequence is identical to that in *Mycobacterium bovis* and is similar to the ML0889 protein expressed in *Mycobacterium leprae* (Garnier et al., 2003). Sable et al. (2005) used ion-exchange chromatography

and high-resolution gel separation technology to purify 104 peptide chains. In the detection of peptide chains with a molecular weight of 16.9 kDa, Rv2185C was considered to be the expression product that stimulates the patients' peripheral blood lymphocytes to produce higher levels of interferon (IFN)- γ . The ELISA method can be used to detect TB, suggesting that TB16.3 can stimulate the body to produce TB-specific cellular and humoral immunity. Gasitsiwe et al. (2010) used the peptide chip (peptide microarray) to analyze TB16.3 epitopes. They found that of the 44 12-amino acid peptide segments in the protein, 5, 2, and 1 could bind with DR1, DR2, and DR4 in MHCII antigens, respectively. This accounted for 18% (8/44) of the epitopes. The positive rate was among the highest in the 61 TB proteins tested. Weldingh et al. (2005) cloned and expressed TB16.3, which was used as a capture protein. An antibody test based on an ELISA showed that the sensitivity was 44-55% in Danish TB patients. The sensitivity was 88-98% in TB patients in Africa, while the specificity reached 97%. This indicated that TB16.3 could be used as a diagnostic protein.

In this study, the TB16.3 gene *Rv2185C* was PCR-amplified from H37Rv genomic DNA, and an expression vector was constructed. Sequencing and restriction digestion indicated the proper construction of the expression vector. The molecular weight of TB16.3 was found to be 37 kDa using SDS-PAGE, which differs from the theoretically predicted size. Some of this increase in size might be due to the inserted amino acids and His-tag used in constructing the expressed vector, but the increase might also be associated with the protein band structure or charge abnormalities. The data in Table 1 show the reactivity of TB16.3 and TB serums, and indicate that we expressed the correct protein. Through induction under different conditions, we confirmed that the expression vector could express the TB16.3 protein; we also determined optimum preparation and purification conditions for the protein.

The results showed that using an established ELISA method for detecting TB in serum, TB16.3, which we cloned and expressed, showed an antibody positive rate of 72.9% and specificity of 86.5%. The sensitivity rate was higher than that of the Danish anti-TB16.3 antibody-positive rate as reported by Weldingh et al. (2005). However, the sensitivity and specificity rate was lower than the antibody-positive rate in the African population. This may be due to the strength of the immune response is inconsistent in different regions or ethnicities. We also observed that the Rv2626C antibody-positive rate in normal subjects was significantly higher than that in patients with TB. The TB-positive rate was 16.9%, which was lower than that in normal subjects in which the positive rate was 75.0%. The Rv2626C protein may not be expressed in TB-infected non-TB patients or TB-recovered patients. Further studies are necessary to determine whether the protein with the corresponding antibodies may be used as a recovery indicator in these patients.

Our data reflect that the expressed proteins, particularly TB16.3 and TB15.3, can be used to improve detection sensitivity after mixing coating detection for joint or separate detection. This test can be used to determine whether TB patients are discharging bacteria. Thus, using existing tests, a combination of TB protein antibody detection was found to be valuable for diagnosing TB. This method can also be used for our listed products upgrading (Zhu et al., 2004), making the product more competitive.

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