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# Expression of Recombinant TB10.4 of *Mycobacterium bovis* and the Preliminary Study of its Potential in Diagnosis of Bovine Tuberculosis

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**Abstract:** Bovine tuberculosis caused mainly by *Mycobacterium bovis* is a serious zoonosis in the world that can infect cattle and humans as well. Prevention and eradication of this disease require effective and sensitive diagnosis method. While the traditional tuberculin skin test and IFN-γ release assay stimuli as PPD-B may share common antigens with *Mycobacterium avium* which could cause false positive results. To overcome this problem, studies are focus on screening more specific proteins to replace PPD for stimuli recently. Previous research showed that TB10.4 could activate T cell stimulation therefore, researchers optimized the expression of TB10.4 by comparing three different recombinant proteins and using as supplementary antigen stimuli was detected by in diagnosis of bovine tuberculosis. The pET-32a (+) plasmid and pET-28a (+) plasmid were chosen which are different in the solubility of the recombinant protein. After transformation, expression and purification of the recombinants, the recombinant proteins rTB10.4-1 and rTB10.4-3 were obtained successfully. In order to analysis the importance of the C terminal for the activity of TB10.4, the initiation and termination codon were also introduced into the N and C terminal of *TB10.4* gene and the rTB10.4-2 lack of His tag protein in the C terminal was obtained. The result of SDS-PAGE showed that rTB10.4-1, TB10.4-2 and rTB10.4-3 were 30, 30 and 12 ku, respectively which were just the same size as expected. The biological activities of these recombinant-proteins were compared by IFN-γ release assay, real-time PCR and Western blot and the results showed that all of the proteins had good T cell activity and B cell activity. In comparison, rTB10.4-1 possessed strongest activity while rTB10.4-3 was the lowest one as the inclusion body. The skin test on guinea pigs showed that the DTH in *M. bovis*-infected group could be detected intensively by using mixture of recombinant proteins rTB10.4-1 and rCE in skin test compared to control group. In summary, the recombinant protein of TB10.4 could be obtained successfully and be used as antigen stimuli in the skin test which would lay the foundation for manufacture of the diagnosis kit for bovine tuberculosis with characteristics of high specificity, sensitivity and reproducibility.

**Key words:** Bovine tuberculosis, TB10.4, T cell activity, B cell activity, the gamma interferon (IFN-γ) release assay

## INTRODUCTION

*Bovine tuberculosis* (bTB) is a very important zoonosis caused by infection of the *Mycobacterium bovis* with the features of weight loss, tubercle formation, caseous lesions and calcification in the diseased tissues (Suzuki *et al*., 2010). In developed countries, the incidence of bTB significantly reduced becsuse of the eradication programs however, the infection of wild animal made it difficult to eliminate such disease. The bTB harmed the development of cattle industry seriously caused the economic loss and threatened human life and health. Therefore, the establishment of efficient methods for diagnosis had great implications in bTB prevention and the tuberculosis research.

At present, the primary method for bTB detection was the tuberculin skin test and the gamma Interferon (IFN-γ) Release Assay (IGRA) in China which based on the detection of Delayed Type Hypersensitivity (DTH) triggered by Bovine Purified Protein Derivative (PPD-B). Nowadays, the skin test by the stimuli of PPD-B had low specificity while IFN-γ release assay could not be widely used due to its high cost in developing countries (Lee *et al*., 2011; Jeong *et al*., 2012). Recent studies mainly concentrated in looking for alernative stimuli of PPD-B such as CFP10 and ESAT-6, both of which were expressed in MTC strains and could induce DTH in *M. bovis*-infected cattle (Casal *et al*., 2012; Wilcke *et al*., 1996; Jones *et al*., 2012; Flores-Villalva *et al*., 2012; Bergstedt *et al*., 2010). However, the skin test results

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showed that the effect of cocktail stimulus of CFP-10 and ESAT-6 was moderate specificity and poor sensitivity (48/63) (Flores-Villalva *et al*., 2012). To increase the sensitivity of the skin test using cocktail stimulus of CFP-10 and ESAT-6, other antigens were added which existed in other mycobacterial strains but could not trigger DTH in healthy or *M. avium*-infected animals (Lee *et al*., 2011; Jones *et al*., 2012; Lyashchenko *et al*., 1998).

TB10.4, encoded by the *Rv0288* gene and expressed in both *M. tb* and BCG was identified recently as secretory antigen. The amino acid sequence of TB10.4 was highly conserved in *M. tuberculosis* isolated from clinical samples (Skjot *et al*., 2002; Dietrich *et al*., 2005). Billeskov *et al*. (2007) revealed that the coding sequences of Rv0288 had multiple repeats in virulent strains (H37Rv) and lost in attenuated strain (H37Ra) of *M. tuberculosis* which suggested that the TB10.4 protein might play important role in mycobacterial pathogens. Indeed, TB10.4 contains a number of MHC-I and MHC-II restricted epitopes distributed throughout the protein sequence that could activate T cells (Majlessi *et al*., 2003; Hervas-Stubbs *et al*., 2006; Billeskov *et al*., 2010). The most active epitopes were located in the N-terminus (Skjot *et al*., 2000). Skjot and Dietrich reported that TB10.4 alone or together with Ag85B as the subunit vaccine could generate protective immunity against to *Mycobacterium tuberculosis* infection in mice (Skjot *et al*., 2002; Dietrich *et al*., 2005). TB10.4 could also lead to the release of IFN-γ in TB patients (Uma Devi *et al*., 2002). There were a number of reports about the function of TB10.4 but as the antigen candidate many details of TB10.4 were still not clear particularly on the aspect of lacking any posttranslational modification. All these researches were established on the basis of the biological activity of recombinant TB10.4 proteins.

In this study, three different recombinant TB10.4 proteins were obtained by the prokaryotic expression system and the activity of these proteins were evaluated in T and B cell. Finally, researchers found that the recombinant protein rTB10.4-1 possessed excellent biological activity and could be used as potential stimuli for supplementary antigen in rCE/rTB10.4 protein cocktail-based skin test.

## MATERIALS AND METHODS

**Ethical approval:** All guinea pigs used in this research were treated with care and with the approval of the Animal Care and Use Committee of the Chinese Academy of Agricultural Sciences (CAAS) in China.

**Bacterial species:** *Mycobacterium bovis* strain 68002 was obtained from the China Institute of Veterinary Drug Control in Beijing and this clinical isolate expressed a high virulence from *M. bovis*-infected cattle and was used for preparation of bovine tuberculin in China. Bovine tuberculin (PPD-B; Harbin Pharmaceutical Group, Heilongjiang Province, China) and avian tuberculin (PPD-A; China Institute of Veterinary Drug Control, Beijing, China) were used in the tuberculin skin test.

**Preparation of antigens:** The specific antigens TB10.4 were chosen for expression and purification using a prokaryotic expression system. TB10.4 encoded by the *Rv0288* gene which was amplified using 2 sets of primers basing on gene sequences in *M. bovis* strain AF2122/97. The primer sequences are listed in Table 1. Genomic DNA was isolated from *M. bovis* strain 68002 using a Genomic DNA Mini Preparation kit (Beyotime Institute of Biotechnology, Beijing, China) according to the manufacturer's instructions. Compared to the primers 2, the primers 1 contained initiation and termination codon. The PCR consisted of a denaturation step at 94°C for 30 sec an annealing step at 55°C for 45 sec and extension steps at 72°C for 1 min performed for 35 cycles. The amplified 285 and 291 bp products were inserted into pET-32a (+) plasmid and pET-28a (+) plasmid, respectively. Then, researchers got three recombinant-plasmids as follows: pET-32-TB10.4-1, pET-32-TB10.4-2, pET-28-TB10.4-3, pET-32-TB10.4-4 and the recombinant plasmids were transformed into *E. coli* BL21 and selected on LB agarose plates supplemented with ampicillin (100 μg $mL^{-1}$) and kanamycin (25 μg $mL^{-1}$) for the selection of pET32a and pET28a derivatives, respectively. After transformation, expression and purification of the recombinants, recombinant-proteins were obtained including rTB10.4-1, rTB10.4-2, rTB10.4-3 and rTB10.4-4. These proteins were purified by metal chelate affinity chromatography Ni-NTA (GE Healthcare, Germany). All purified proteins were exchanged into sterile Phosphate-Buffered Saline (PBS) (pH 7.4) using a HiPrep 26/10 desalting column (GE Healthcare, Germany). pET32a tag protein (32a) and a CFP10:ESAT6 recombinant fusion protein (rCE) were provided by Dr. Ting Xi which was used in the tuberculin skin test before (Xin *et al*., 2013).

Then, purified recombinant proteins removed endotoxin using Triton X-114 two-phase separation and tested by the chromogenic endpoint Tachypleus amebocyte lysate method (Chinese Horseshoe Crab Reagent Manufactory Co., Ltd. China). All purified proteins were filtrated by using 0.22 μm sterile filter

Table 1: The primers of TB10.4

| The name of primers | The sequence of primers | Values (bp) |
|---|---|---|
| TB10.4-F1 | CGCggatccATGTCGCAAATCATGTACAAC | 291 |
| TB10.4-R1 | CCCaagcttCTAGCCGCCCCATTTGG | |
| TB10.4-F2 | CGCggatccTCGCAAATCATGTACAACTAC | 285 |
| TB10.4-R2 | CCCaagcttGCCGCCCCATTTGGCGG | |

membrane and analyzed by 15% SDS-PAGE. Researchers determined the concentrations of proteins by Bicinchoninic Acid (BCA) assay.

**IFN-γ release assay with three rTB10.4 proteins:** Three *M. bovis*-infected cattle and three heath cattle according were selected to TST and IFN-γ release assay (BOVIGAM). Then whole blood was collected from each animal and dispensed into a 48 well tissue cultue tray with the total volume of 750 μL in each well. The 100 μL of either rTB10.4-1, TB10.4-2, rTB10.4-3, 32a, rCE, PPD-A or PPD-B were added to appropriate wells containing the blood. After incubating for 24 h at 37°C, plasma was collected and the quatity of IFN-γ release was detected using *Mycobacterium bovis* Gamma Interferon Test kit (IGRA) (*Mycobacterium bovis* gamma interferon test kit for cattle; Bovigam, Prionics AG, Schlieren, Switzerland) according to the manufacturer's instructions. The result was credible according the $OD_{450}$ value of negative control <0.130 and the positive control >0.700. The three protein was diluted into equal molarity by PBS, the final concentrations of rTB10.4-1, rTB10.4-2, rTB10.4-3, rCE and 32a (the purified pET32a (+) tag) were 10, 10, 1.33, 0.67 μg $mL^{-1}$, respectively. Cattle with PPD-B-simulated blood plasma having a value of optical density ($OD_{450}$) >0.100 earlier those of PPD-A and PBS were considered TB infected, meanwhile, blood plasma of cattle by recombinant protein simulation having a P/N value ≥2.1 were considered TB infected (p = the value of $OD_{450}$ by recombinant protein simulation, the value of $OD_{450}$ by pET32a tag protein simulation; N = the value of $OD_{450}$ by PBS simulation).

**Real-time PCR:** The bovine peripheral blood lymphocytes (PBMC) from heparinized blood of each cattle was separated and added 50 μL of either protein rTB10.4-1, TB10.4-2, rTB10.4-3, 32a, rCE, PPD-A or PPD-B into the appropriate wells then incubated for 6 h at 37°C. Finally, the total RNA of stimulated PBMC was extracted using TRIZOL and the quantity of IFN-γ mRNA was detected using real-time PCR. PBS-stimulated PBMC was as control sample. *β-actin* gene was as reference (Table 2).

**Western blotting:** Immunoblot analyses were carried out following the procedures described earlier (Skjot *et al*., 2002). Briefly, after electrophoresis, the gels were soaked in transfer buffer (0.025 M Tris-HCl, pH 8.3 containing 0.192 M glycine and 20% methanol) for 30 min and electro-transfer onto PVDF membrane (Mollopore Immobion-P#IPVH 000 10) using a transblot apparatus (Bio-Rad, USA) at 100 V for 70 min. Non-specific binding sites of the PVDF were blocked by incubating with 5% skimmed milk powder in TBS with 0.05% Tween 20 (TBST). After washing, strips were cut and incubated overnight at 4°C with rabbit anti-mouse His antibody at 1:500 (Signalway Antibody, Maryland, USA) or positive sera from TB-infected cattle at 1:100. Antibodies were detected with HRP-conjugated anti-rabbit IgG secondary Ab or HRP-conjugated anti-bovine IgG secondary Ab at 1:5000 (all from Pierce, Rockford, IL). The peroxidase-positive strips were detected using the Diamino Benzidine (DAB) (Pierce, Rockford, IL).

Table 2: The sequence of *β-actin* and *IFN-γ* gene

| Names | The gene sequences |
|---|---|
| β-actin-Forward primer | 5-GCCCTGAGGCTCTCTTCCA-3 |
| β-actin-Reverse primer | 5-GCGGATGTCGACGTCACA-3 |
| β-actin-Taqman-probe | 5-CATGGAATCCTGCGGCATTCACG-3 (BHQ) |
| IFN-r-Forward primer | 5-GCTGATTCAAATTCCGGTGGA-3 |
| IFN-r-Reverse primer | 5-CAGGCAGGAGGACCATTACG-3 |
| IFN-r-Taqman-probe | 5-TCTGCAGATCCAGCGCAAAGCC-3 (BHQ) |

**Guinea pigs skin test:** A number of 20 healthy Guinea pigs were divided into two groups randomly. Each Guinea pig was injected 0.1 mL inactivated *M. bovis* as the allergens in the deep muscle of thigh. Researchers detected the sensitization by PPD-B stimulation. Then 6 Guinea pigs were selected, all of which were at sensitivity status in PPD-B skin test. Researchers used recombinant rTB10.4-1, rTB10.4-2, rTB10.4-3, rCE, protein Tag 32a and PPD-B as stimulus for skin test, respectively. Then, researchers equimolarly mixed the three recombinants with rCE for skin test also. The diameter of lesions were measured at stimulated of 24, 48 and 72 h. The skin test was performed according to the Chinese standard diagnostic technique for tuberculosis in animals (GB/T 18645-2002). Results were expressed as the difference in skin thicknesses (mm) between the readings before and after the skin test. If the difference in skin thicknesses is ≥4 mm, the animal is considered TB infected, however the animal is considered free of TB when the difference is <2 mm. The result is considered inconclusive if the skin thickness difference is ≥2 mm and <4 mm and the animal should be redetected in the second skin test after an interval of 60 days. If the difference is ≥2 mm, the animal should be considered TB infected. All clinical skin tests were operated as double-blind tests by Dairy Cattle Research Center in Shandong Academy of Agricultural Sciences.

## RESULTS AND DISCUSSION

**Expression and purification of recombinant TB10.4 proteins:** The gene of *TB10.4* was amplified using 2 sets of primers basing on gene sequences in *M. bovis* strain AF2122/97 and the PCR product of TB10.4 was cloned into pET-32a (+) and pET-28a (+) plasmid, respectively. Four recombinant-plasmids were obtained as follows: pET-32-TB10.4-1 (285 bp), pET-32-TB10.4-2 (291 bp),

pET-28-TB10.4-3 (291 bp) and pET-28-TB10.4-4 (291 bp). All of these recombinant plasmids were verified by sequencing.

The evaluation of protein expression was performed. The results of expression trials for pET-32-TB10.4-1, pET-32-TB10.4-2, pET-28-TB10.4-3 and pET-28-TB10.4-4 were analyzed by SDS-PAGE. The rTB10.4-3 was used for the following experiments because both rTB10.4-3 and rTB10.4-4 were identical protein with the same tag protein. The result of proteins expression was correct and the molecular weights of pET-32-TB10.4-1, pET-32-TB10.4-2 and pET-28-TB10.4-3 were accorded with 30, 30 and 12 ku, respectively (Fig. 1).

To evaluate the solubility of the protein, the whole cell lysates, supernatant samples and sediment samples were determined by IPTG induction as the analysis by SDS-PAGE (Fig. 2) the pET-32-TB10.4-1 and pET-32-TB10.4-2 were soluble products but the pET-28-TB10.4-3 was the inclusion body.

Then the recombinant protein rTB10.4-1 and rTB10.4-2 were purified by Gradient Linear Method and Urea Gradient Elution Method was used to purifying the recombinant protein rTB10.4-3. The recombinant proteins, rTB10.4-1, rTB10.4-2 and rTB10.4-3 with a purity of >90% and final concentrations of 0.5-1.5 mg $mL^{-1}$ were used for subsequent experiments. The endotoxin level for each protein was <2 Endotoxin Units (EU)/mg.

**T cell activity identification of rTB10.4:** The negative control and the positive control were detected to ensure the effectiveness of result. The $OD_{450}$ value of negative control <0.130 and the positive control >0.700 indicated the result of experiment was credible. As the Table 3 and 4 according to the manufacturer's instructions of *Mycobacterium bovis* gamma interferon test kit (IGRA), the cows of number 1, 2, 3 were considered TB infected, the cows of number 4, 5, 6 were considered free of TB. rTB10.4-1, rTB10.4-2, rTB10.4-3, rCE, 32a, PPD-A and PPD-B were used as stimulus for skin test, the results showed that the P/N values of rTB10.4-1, rTB10.4-2, rTB10.4-3 as stimulus with TB infected cows were 8.64, 7.01 and 4.37, respectively which in the healthy cattle were 0.08, 0.20 and 0.91, respectively. While the purified 32a (pET32a (+) Tag protein) as a control could not induce a high expression of IFN-γ in TB-infected or healthy cattle. Meanwhile, the P/N value of rCE was up to 31.27 as stimulus with TB-infected cattle and low to 0.38 with the cows free of TB. The experimental data showed that rTB10.4-1, TB10.4-2, TB10.4-3 could induce the expression of IFN-γ from TB-infected cattle but no effect from healthy cows. All of these proteins had T cell activities, rTB10.4-1 showed stronger activity than rTB10.4-2 and rTB10.4-3. Meanwhile, these effects of stimulation were inferior to the PPD-B or rCE which were fairly accorded with experimental results reported in literatures.

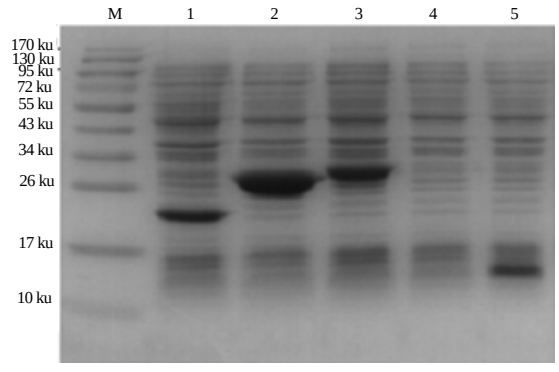


Fig. 1: The SDS-PAGE analysis of expressed recombinant proteins. M: the marker of protein; Line 1: the expression of pET-32a (+) empty vector by IPTG induction; Line 2: the expression of pET32-TB10.4-1by IPTG induction; Line 3: the expression of pET32-TB10.4-2 by IPTG induction; Line 4: the expression of pET-28a (+) empty vector by IPTG induction and Line 5: the expression of pET28-TB10.4-3 by IPTG induction

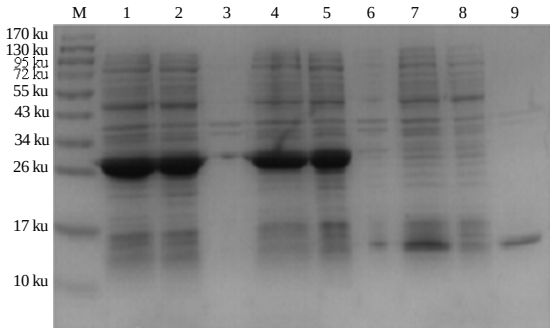


Fig. 2: The solubility analysis of expressed recombinant proteins by SDS-PAGE. M: the marker of protein; Line 1: the total protein expression of rTB10.4-1; Line 2: the protein expression of rTB10.4-1 in the supernatant of cell lysis after centrifugation; Line 3: the protein expression of rTB10.4-1 in the sediment of cell lysis after centrifugation; Line 4: the total protein expression of rTB10.4-2; Line 5: the protein expression of rTB10.4-2 in the supernatant of cell lysis after centrifugation; Line 6: The protein expression of rTB10.4-2 in the sediment of cell lysis after centrifugation; Line 7: the total protein expression of rTB10.4-3; Line 8: the protein expression of rTB10.4-3 in the supernatant of cell lysis after centrifugation and Line 9: the protein expression of rTB10.4-3 in the sediment of cell lysis after centrifugation

Table 3: The Optical Density (OD) value of recombinant proteins by IFN-γ release assay

| P/N | PPD-B | PPD-A | PBS | 32a | rCE | rTB10.4-1 | rTB10.4-2 | rTB10.4-3 |
|---|---|---|---|---|---|---|---|---|
| **Positive cattle** | | | | | | | | |
| 1 | 1.5856 | 0.8755 | 0.0609 | 0.1883 | 2.2082 | 0.6351 | 0.5620 | 0.2869 |
| 2 | 1.9938 | 0.2665 | 0.0624 | 0.1840 | 2.3217 | 0.8297 | 0.7040 | 0.3418 |
| 3 | 1.3159 | 0.8523 | 0.0624 | 0.1772 | 1.8229 | 0.6914 | 0.5858 | 0.3689 |
| Average | 1.6318 | 0.6648 | 0.0619 | 0.1832 | 2.1176 | 0.7187 | 0.6173 | 0.3325 |
| **Negative cattle** | | | | | | | | |
| 4 | 0.1553 | 0.1254 | 0.0714 | 0.1066 | 0.1744 | 0.0940 | 0.1250 | 0.1216 |
| 5 | 0.1235 | 0.1154 | 0.0632 | 0.1524 | 0.1532 | 0.1600 | 0.1568 | 0.1351 |
| 6 | 0.1586 | 0.1204 | 0.0641 | 0.1332 | 0.1455 | 0.1516 | 0.1497 | 0.1221 |
| Average | 0.1458 | 0.1204 | 0.0662 | 0.1307 | 0.1577 | 0.1352 | 0.1438 | 0.1263 |

Table 4: The evaluation of recombinant proteins by IFN-γ release assay

| P/N | CE | rTB10.4-1 | rTB10.4-2 | rTB10.4-3 |
|---|---|---|---|---|
| **Positive cattle** | | | | |
| 1 | 33.17 | 7.34 | 6.14 | 3.71 |
| 2 | 34.26 | 10.35 | 8.33 | 4.48 |
| 3 | 26.37 | 8.24 | 6.55 | 4.91 |
| Average | 31.27 | 8.64 | 7.01 | 4.37 |
| **Negative cattle** | | | | |
| 4 | 0.95 | -0.18 | 0.26 | 0.70 |
| 5 | 0.01 | 0.12 | 0.07 | 1.14 |
| 6 | 0.19 | 0.29 | 0.26 | 0.90 |
| Average | 0.38 | 0.08 | 0.20 | 0.91 |

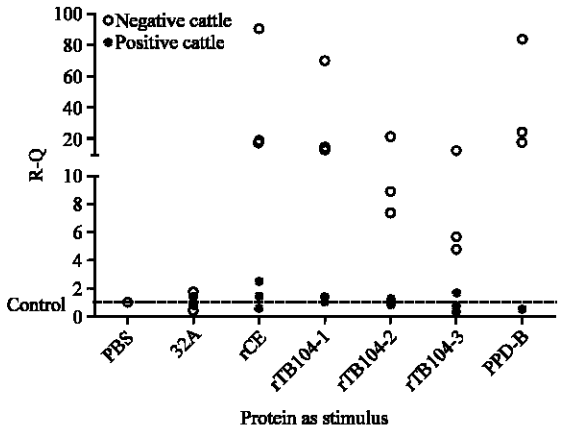


Fig. 3: IFN-γ mRNA expression level by fluorescence quantitative PCR

The T cell activity of these recombinants was detected by real-time PCR (Fig. 3). The result showed that the transcriptional level of IFN-γ mRNA stimulated by rTB10.4-1 was the highest one comparing with the rTB10.4-2 or rTB10.4-3. Due to individual differences the RQ value of IFN-γ mRNA stimulated by rTB10.4-1 was better than the level induced by rCE and B-PPD with TB-infected cow number 3. These results indicated these recombinant-proteins possess high T cells activities and the rTB10.4-1 could be as a supplementary antigen for rCE in IFN-γ release test.

**B cell activity identification of rTB10.4:** The B cell activity of the recombinants was detected by Western blot as the (Fig. 4a) all of the proteins could be recognized by the antibody-His tag and the protein rTB10.4-1 had a strongest reaction with positive serum of *Mycobacterium bovis* (Fig. 4b) but rTB10.4-3 had only a minimal effect. The results indicated these recombinant proteins had B cell activity, rTB10.4-1 possess high B cells activities.

**Guinea pigs skin test with recombinant protein:** In the skin test, 6 Guinea pigs in each group were infected with inactivated *M. bovis* at sensitivity status. The diameter of lesions were measured at stimulated of 24, 48 and 72 h. As Fig. 5 using PPD-B as stimulus for skin test, all of 6 Guinea pigs had reaction as Delayed Type Hypersensitivity (DTH) rCE could caused the DTH reaction with 4/6 Guinea pigs in 24 h but disappeared within 48 h. Researchers found there was no DTH reaction induced by single protein rTB10.4-1, rTB10.4-2, or rTB10.4-3 as stimulus in skin test for various times (Fig. 5). It was worth mentioning that all Guinea pigs had reaction as DTH using the mixture proteins as stimulus for skin test by rCE with rTB10.4-1, TB10.4-2 or rTB10.4-3, respectively (the concentration of total proteins was 0.5 mg $mL^{-1}$ and the volume ratio of the components was 1:1). The strongest DTH reaction was induced by rTB10.4-1:rCE as stimulus for skin test in 24 h (Fig. 6) the average thickness of skin was up to 10 mm, similar to the reaction by PPD-B induction (12 mm). The protein Tag 32a could not lead to the DTH as stimulus in skin test at each time points. All these data indicated that there was not satisfactory with single recombinant protein as the stimulus in skin test which were fairly accorded with experimental results reported in literatures but the rCE/rTB10.4-1 protein cocktail could induce strongest detectable DTH in *M. bovis*-infected in 24 h.

Current methods for diagnostic of *Bovine tuberculosis* mainly depended on the skin test for the whole group monitoring and suspected case of TB-infected cattle were further confirmed by the gamma Interferon (IFN-γ) release assay (IGRA, BOVIGAMTM). Both the Skin test and IGRA were based on PPD-B (Purified Protein Derivative) as the stimulus source. However, PPD-B is a poorly defined mixture of proteins, lipids and carbohydrates obtained from a virulent

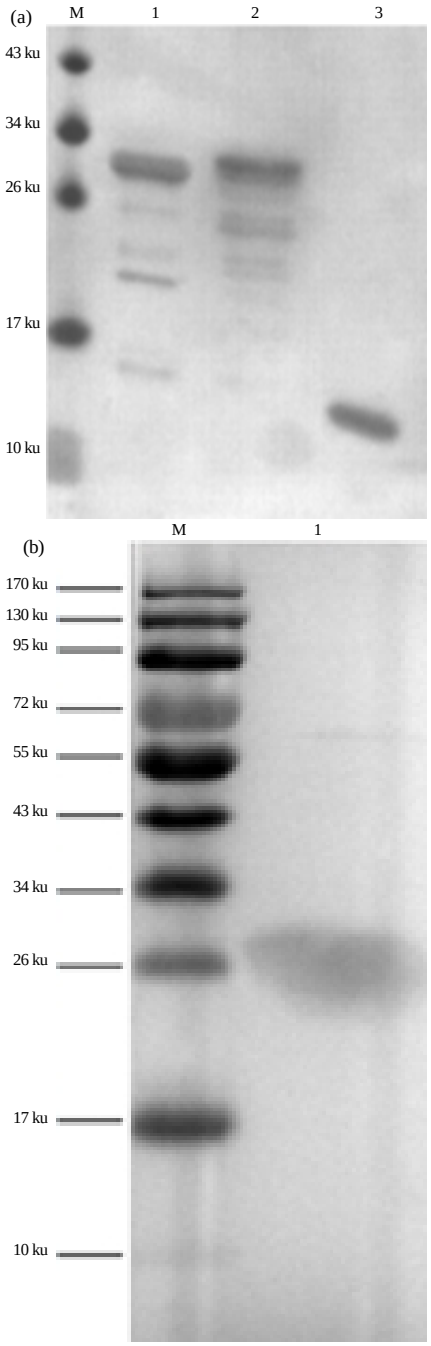


Fig. 4: a) Western blot analysis of rTB10.4-2, rTB10.4-2 or rTB10.4-3 with His tag antibody. M: the marker of protein; Line 1: the protein expression of rTB10.4-1after purification; Line 2: the protein expression of rTB10.4-2 after purification and Line 3: the protein expression of rTB10.4-3 after purification; b) Western blot analysis of rTB10.4-1 with positive serum of *Mycobacterium bovis*. M: the marker of protein; Line 1: Western blot analysis of rTB10.4-1by serum from TB-infected cattle

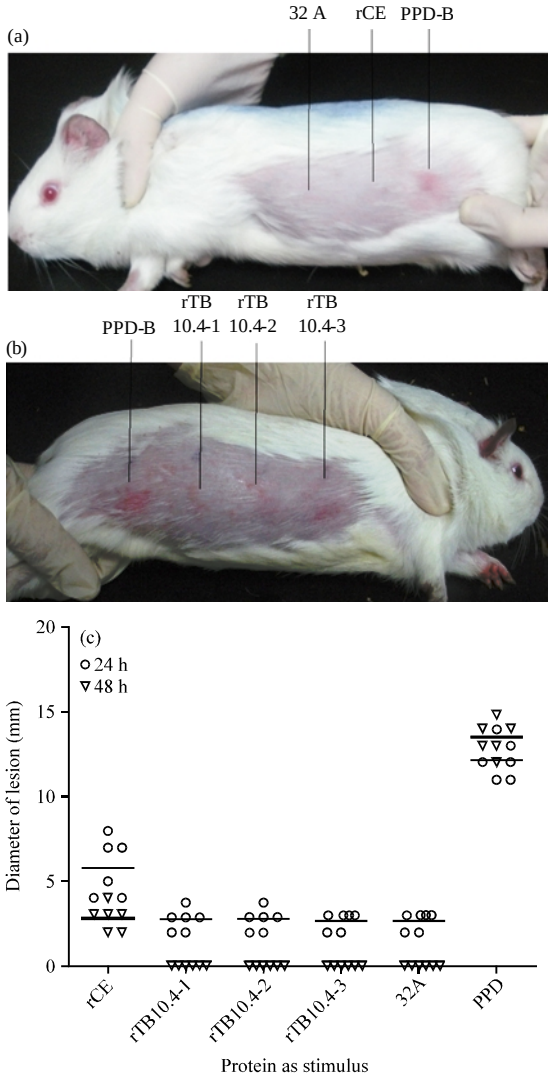


Fig. 5: TST results images of single protein stimulates; a) the images of TST result induce by 32a, rCE and PPD-B, respectively; b) the images of TST result induce by PPD-B, rTB10.4-1, rTB10.4-2 and rTB10.4-3, respectively; c) diametre of lesion induce by proteins stimulation in TST at various times

*M. bovis* culture, the use of virulent *M. bovis* during the production of PPD-B exists latency risk. Importantly, shared antigenic components in PPD-B and nonpathogenic environmental mycobacteria can reduce the specificity of the tuberculin skin test (Xin *et al*., 2013). Recent researches mainly focused on improving the relative specificity, sensitivity and reproducibility of the skin test. *M. bovis*-specific antigens CFP10 and ESAT-6 were often chosen could effectively improved the relative specificity of the skin test as stimulus source substitute PPD (Casal *et al*., 2012; Wilcke *et al*., 1996;

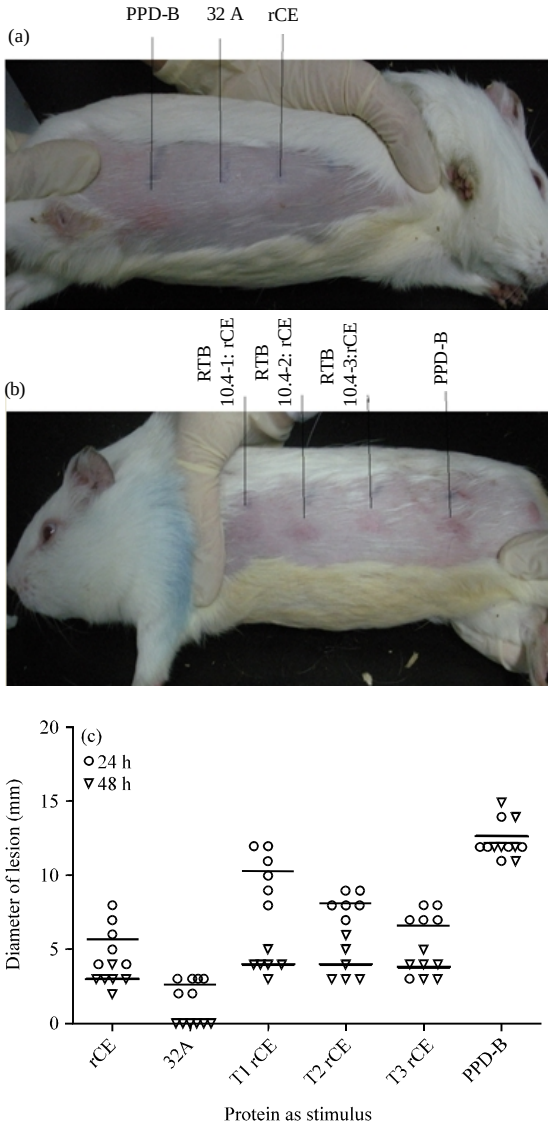


Fig. 6: TST results images of protein combination stimulates; a) the images of TST result induce by PPD-B, 32a and rCE, respectively; b) the images of TST result induce by rTB10.4-1:rCE, rTB10.4-2:rCE, rTB10.4-3:rCE and PPD-B, respectively and c) diametre of lesion induce by proteins stimulation in TST at various times

Flores-Villalva *et al*., 2012; Bergstedt *et al*., 2010). However, it was crucial to supplement antigen for enhancing the relative sensitivity of the skin test.

Taken into account the production and natural conformation, the prokaryotic system was used to express the recombinant protein TB10.4 with pET32a vector which contained TRX tag to promote the expression of solution by formation of the two disulfide bonds. The C terminal sequence of TB10.4 was found potentially related to the activity of the protein (Ilghari *et al*., 2011) 2 sets of primers for TB10.4 were designed. One pair primers contained the initiation codon and termination codon, other primers did not have. Due to reading the initiation and termination codon frame, the expression of the recombinant protein rTB10.4-1 carried Trx, His, S tag at N termini, without any tag at C terminal, a His tag at C terminal was obtained by the expression of rTB10.4-2. Prifying with His FF crude affinity chromatography and Hiprep 26/16 desulting affinity chromatography, soluble expression of the rTB10.4-1 and rTB10.4-2 were received. However, a larger tag protein (20 ku) carried by pET-32a (+) vector may affect the recombinant protein on folding into a native state. Therefore, pET-28a (+) vector was chosen to construct the recombinant pET-28-TB10.4-3 which contains a tag protein only 2 ku at the N terminal (His and T7). Then, rTB10.4-3 was purified as an expression of inclusion body by urea gradient elution. The recombinant protein rTB10.4-1, rTB10.4-2 and rTB10.4-3 were successfully obtained in this study, lay the foundation for the subsequent activity analysis.

Combining with the results of IFN-γ release test, fluorescence quantitative PCR and Western blot detection, recombinant proteins rTB10.4-1, rTB10.4-2 and rTB10.4-3 possessed the activities of T and B cells. rTB10.4-1 showed the highest expression of IFN-γ and strongest reaction with positive serum of TB-infected cattle possibly because of the soluble expression of recombinant. The activity of rTB10.4-1 was better than rTB10.4-2 due to lack of the tag protein at C terminal, rTB10.4-1 was ensured the expression of activity domain without interference by insertion sequence. This result indicated the carboxyl terminus sequences of TB10.4 might be the main domain of the biological activity which was fairly accorded with the analysis by Ilghari *et al*. (2011). rTB10.4-3 obtained the lowest activity because of the expression as inclusion body which could not fold exactly in renaturation process. The protein Tag 32a (the total molecular mass of the tags was 18 ku) could not had a reaction in the skin test of IFN-γ expression or with positive sera of TB-infected cattle. However, lack of the protection polypeptide at the carboxyl terminus, possibly made the rTB10.4-1 easy to degrade and affect the protein storage.

In the skin test, there was no DTH reaction to single recombinant protein-stimulated with Guinea pigs, probably because PPD-B is a complex mixture from a virulent *M. bovis* culture, single protein might not be sufficient to cause the DTH reaction, induced by synergistic effect with multiple proteins. CFP10:ESAT6

recombinant fusion protein (rCE) was found to have a high relative specificity of the tuberculin skin test, the DTH reaction was induced in a short time by the mixture of rCE with rTB10.4-1, rTB10.4-2 or rTB10.4-3, respectively as stimuli. Among of three mixture proteins, rCE with rTB10.4-1 (rCE/rTB10.4-1) protein cocktail could induce strongest detectable DTH in *M. bovis*-infected and the pET32 protein Tag (32a) could not cause DTH reflection at all.

## CONCLUSION

This result indicated that the recombinant protein rTB10.4-1 possessed a good biological activity to strengthen the DTH induced by t rCE used as potential stimuli for supplementary antigen in rCE/rTB10.4 protein cocktail-based skin test.

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