

Assessment of a Protein Cocktail-Based Skin Test for Bovine Tuberculosis in a Double-Blind Field Test in Cattle

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Bovine tuberculosis (bTB) is a worldwide zoonosis caused mainly by *Mycobacterium bovis*. The traditional diagnostic method used often is the tuberculin skin test, which uses bovine purified protein derivatives (PPD-B). However, it is difficult to maintain uniformity of PPD-B from batch to batch, and it shares common antigens with nonpathogenic environmental mycobacteria. To overcome these problems, *M. bovis*-specific antigens that showed good T cell stimulation, such as CFP-10, ESAT-6, Rv3615c, etc., have been used in the skin test, but there have been no large-scale clinical studies on these antigens. In this study, two combinations (CFP-10/ESAT-6/TB10.4 protein cocktail and CFP-10/ESAT-6/Rv3872/MPT63 protein cocktail) were developed and used as stimuli in the skin test. Cattle were double-blind tested to assess the efficiency of the protein cocktail-based skin tests. The results showed that the CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test can differentiate TB-infected cattle from *Mycobacterium avium*-infected ones and that it shows a high degree of agreement with the traditional tuberculin skin test ($\kappa = 0.8536$) and gamma interferon (IFN- γ) release assay ($\kappa = 0.8154$). Compared to the tuberculin skin test, the relative sensitivity and relative specificity of the CFP-10/ESAT-6/TB10.4-based skin test were 87% and 97%, respectively. The relative sensitivity and relative specificity of the CFP-10/ESAT-6/TB10.4-based skin test were 93% and 92%, respectively, on comparison with the IFN- γ release assay. The correlation between the increases in skin thickness observed after the inoculation of stimuli was high (PPD-B versus CFP-10/ESAT-6/TB10.4, Spearman r of 0.8435). The correlation between the optical density at 450 nm (OD₄₅₀) obtained after blood stimulation with PPD-B and the increase in skin thickness observed after inoculation of the CFP-10/ESAT-6/TB10.4 protein cocktail was high (Spearman $r = 0.7335$). Therefore, the CFP-10/ESAT-6/TB10.4-based skin test responses correlate to traditional measures of bovine TB evaluation, including skin test and gamma interferon release assay.

Mycobacterium bovis is a Gram-positive, acid-fast bacterium that belongs to the *Mycobacterium tuberculosis* complex (MTC) (including *M. bovis*, *M. tuberculosis*, *M. microti*, *M. africanum*, *M. pinnipedii*, *M. caprae*, *M. canetti*, and *M. mungi*) (1,2). *M. bovis* mainly infects cattle, causing bovine tuberculosis (bTB), and occasionally affects other species of mammals (3). *M. bovis* can also infect humans through the inhalation of aerosols or the ingestion of unpasteurized dairy products (4,5). The eradication of bovine tuberculosis is based on reliable diagnostic methods.

The traditional diagnostic method of identifying bTB is the tuberculin skin test, which is based on the detection of delayed-type hypersensitivity (DTH) induced by bovine purified protein derivative (PPD-B). The tuberculin skin test has been used worldwide and is economical and easily applied. However, PPD-B is a poorly defined mixture of proteins, lipids, and carbohydrates obtained from a virulent *M. bovis* culture, the uniformity of which is difficult to maintain from batch to batch (6). Moreover, the use of virulent *M. bovis* during the production of PPD-B may potentially harm workers. Importantly, shared antigenic components in PPD-B and nonpathogenic environmental mycobacteria can reduce the specificity of the tuberculin skin test (7,8). Although the gamma interferon (IFN- γ) release assay (IGRA) showed higher specificity than the single intradermal skin test, the high price of this kit limits its use in developing countries such as China (9,10). To overcome the drawbacks of the traditional diagnostic methods, scientists have focused on screening new *M. bovis*-specific antigens to substitute for PPD-B as stimuli in the skin test (11–13).

Recent studies focused mainly on the development of skin test reagents to discriminate infected from vaccinated cattle (DIVA

test), in which *M. bovis*-specific antigens, such as CFP10 and ESAT-6, were often selected as stimuli for the skin test (6, 11, 14–16). Those antigens were located in RD1, deleted in *M. bovis* BCG strains, and could elicit strong DTH in TB-infected cattle. To increase the sensitivity of CFP-10/ESAT-6 peptides or a protein cocktail-based skin test, some other antigens were added, such as Rv3615c. Some of these antigens may exist in other mycobacterial strains but could not trigger DTH in healthy or *M. avium*-infected animals (6, 14, 17).

Because of the lack of vaccination of cattle in China and the existence of *M. avium* in some local areas, we focused on screening antigens that could be used to differentiate TB-infected cattle from *M. avium*-infected ones. CFP-10 and ESAT-6 are expressed in MTC strains and can induce DTH in *M. bovis*-infected cattle (6, 15). Flores-Villalva et al. found that the cocktail of CFP-10 and ESAT-6 could be used as a stimulus in the skin test with acceptable specificity, but the sensitivity (48/63) was not satisfactory (15). Casal et al. found that the addition of Rv-3615c and Rv3020c

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TABLE 1 Characteristics of selected antigens

Gene	Gene product	Primer sequences ^a	Comments (references) ^b
<i>esxB</i>	CFP-10	Sense, 5'-CGCGGATCCATGGCAGAGATGAAGACCGAT-3'; antisense, 5'-CCC <u>AAGCTT</u> TTCAGAACCCATTTGCGAGGAC-3'	Belongs to ESAT-6 family, RD1 region, equivalent to Rv3874 (6, 14–16, 25)
<i>esxA</i>	ESAT-6	Sense, 5'-CGCGGATCCATGACAGAGCAGCAGTGGAAAT-3'; antisense, 5'-CCC <u>AAGCTT</u> TGCGAACATCCCACTGACGT-3'	Belongs to ESAT-6 family, RD1 region, equivalent to Rv3875 (6, 14, 15, 24, 25)
<i>esxH</i>	TB10.4	Sense, 5'-CGCGGATCCATGTCGCAATCATGTACAAC-3'; antisense, 5'-CCC <u>AAGCTT</u> CTAGCCGCCCATTTGG-3'	Belongs to ESAT-6 family, equivalent to Rv0288
<i>mpt63</i>	MPT63	Sense, 5'-GCGAATTCATGAAGCTCACCACAAT-3'; antisense, 5'-CCGCTCGAGTTAGGGAAGCTGCCTCGCCAG-3'	Equivalent to Rv1926c, exists only in <i>M. tuberculosis</i> complex
<i>Pe35</i>	PE family-like protein	Sense, 5'-CGCGGATCCATGGAAAAATGTCACATGATCCG-3'; antisense, 5'-CCC <u>AAGCTT</u> TTAGGCGAAGACGCCGGC-3'	RD1 region, equivalent to Rv3872

^a Restriction sites used are underlined.

^b Rv, *M. tuberculosis* gene numbering was used; RD1: region of difference 1, which exists only in virulent *M. tuberculosis* complex.

could enhance the DTH reaction and improve the sensitivity (13/14) of the skin test based on the cocktail of CFP-10 and ESAT-6 (6, 14). These data indicated that the *M. bovis*-specific proteins used as stimuli in the skin test are promising. However, thus far there has not been a large-scale clinical assessment in the field to demonstrate feasibility and validation in a double-blind testing (6, 15).

In this study, we chose three *Mycobacterium*-specific proteins, namely, TB10.4, MPT63, and Rv3872 (Table 1), to supplement CFP-10 and ESAT-6 in order to strengthen the sensitivity of the skin test. TB10.4 is encoded by *esxH*, which exists in both *Mycobacterium tuberculosis* complex and *Mycobacterium avium* complex (MAC), and can induce higher levels of IFN- γ in TB patients than ESAT-6 and CFP-10 (18, 19). Thus, TB10.4 has potential to enhance the sensitivity of the skin test but may cause a loss of specificity. MPT63 and Rv3872 can induce a high level of IFN- γ release in TB patients but not in MAC-infected persons or healthy ones (20–22), so the addition of MPT63 and Rv3872 may increase the sensitivity of the skin test and not sacrifice specificity. According to the characteristics of these proteins, a CFP-10/ESAT-6/TB10.4 protein cocktail and a CFP-10/ESAT-6/Rv3872/MPT63 protein cocktail were prepared and used as stimuli in the skin test. Our present study was to assess the efficiency of protein cocktail-based skin tests in a total of 1,097 cattle, with the tuberculin skin test and IFN- γ release assay as references.

MATERIALS AND METHODS

Ethical approval. All animals 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, China.

Bacterial species and plasmids. *Mycobacterium bovis* strain 68002 was obtained from the China Institute of Veterinary Drug Control, Beijing, China. It is a clinical isolate with high virulence from *M. bovis*-infected cattle and is used for preparation of bovine tuberculin in China. *M. avium* strain P18 (with high virulence) was isolated from an *M. avium*-positive chicken and is used for preparation of avian tuberculin in China. Plasmid pET32a(+) (Novagen, Madison, WI) was used as the expression vector, and *Escherichia coli* strain BL21(DE3) was used for protein expression. 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. Five *M. bovis*-specific antigens were selected for expression and purification using a prokaryotic expression system (Table 1). The primers were designed based on gene sequences in *M. bovis* strain AF2122/97 and synthesized by Invitrogen (Beijing, China). The primer sequences are listed in Table 1. Genomic DNA was isolated from *M. bovis* strain 68002 using a Genomic DNA Mini Preparation Kit with

Spin Column (Beyotime Institute of Biotechnology, Beijing, China) according to the manufacturer's instructions and was stored at -80°C . The expression of CFP-10, ESAT-6, TB10.4, Rv3872, and MPT63 was induced in *E. coli* BL21(DE3), and the proteins were purified by metal chelate affinity chromatography (HisTrap FF crude; 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).

Endotoxin was removed from purified recombinant proteins 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) (23). All purified proteins were filtrated by using 0.22- μm sterile filter membrane and analyzed by 12% SDS-PAGE. The concentrations of proteins were determined by bicinchoninic acid (BCA) assay. All purified proteins were diluted to a final concentration of 0.5 mg/ml in sterile PBS (pH 7.4) and stored at -80°C .

Skin test procedure. The tuberculin skin test was performed according to the Chinese standard diagnostic technique for tuberculosis in animals (GB/T 18645-2002). To evaluate the efficiency of recombinant proteins, up to four intradermal injection sites on the same side of each cattle neck were used, and distances between the injection sites were at least 15 cm apart. PPD-B was always included in each test, and all antigens (including PPD-B, 2,500 IU/animal) were injected in a volume of 0.1 ml. The skin thicknesses at injection sites were measured before and at 72 h after the skin test by the same operator, using calipers. Results were expressed as the difference in skin thicknesses (mm) between the readings before and after the skin test. For the tuberculin skin test, if the difference in skin thicknesses is equal to or greater than 4 mm, the animal is considered TB infected, and if the difference is less than 2 mm, the animal is considered free of TB. When the skin thickness difference is greater than or equal to 2 mm and less than 4 mm, the result is considered inconclusive, and the animal should be retested after an interval of 60 days. In the second tuberculin skin test, if the difference is greater or equal to 2 mm, the animal should be considered TB infected. All clinical skin tests were operated as double-blind tests by Dairy Cattle Research Center, Shandong Academy of Agricultural Sciences, China.

IFN- γ release assay. All cattle tested by skin test were also diagnosed by using IGRA (*Mycobacterium bovis* gamma interferon test kit for cattle; Bovigam, Prionics AG, Schlieren, Switzerland) according to the manufacturer's instructions. Heparinized whole blood of each animal was collected within 30 days after the skin test. The whole blood from each animal was dispensed into a 24-well tissue culture tray (1.5 ml/well, 3 wells for each animal) and stimulated with 100 μl PPD-A (avian tuberculin PPD, 300 mg/ml, Australia), PPD-B (bovine tuberculin PPD, 300 mg/ml, Australia), and PBS. The plasma of each well was collected after the culture was incubated for 20 to 24 h at 37°C in 5% CO_2 and tested using the *Mycobacterium bovis* gamma interferon test kit. Cattle with PPD-B-sim-

ulated blood plasma having an optical density (OD) greater than 0.100 above those of PPD-A and PBS were considered TB infected.

PCR analysis. TB infection was confirmed by PCR as described by Flores-Villalva et al. (15). Briefly, nasal swabs or rectal swabs were collected and submerged in 2 ml of sterile phosphate-buffered saline. DNA was extracted from the sediments and suspended in 30 μ l of water. A nested PCR was performed to amplify a region of the *mpb70* gene of MTC. First, a single PCR was run by amplifying a 372-bp segment of the *mpb70* gene using specific primers M70F (5'-GAACAATCCGGAGTTGACAA-3') and M70R (5'-AGCACGCTGTCAATCATGTA-3'). Then, a nested PCR was run using 1 μ l of the previous reaction mixture to amplify a 208-bp fragment within the 372-bp region of the *mpb70* gene with the primers M22F (5'-GCTGACGGCTGCACTGTCGGGC-3') and M22R (5'-CGTTGGCCGGCTGGTTTGGCC-3'). The products were analyzed in 2% agarose gels.

The 16S rRNA genes were amplified and sequenced to identify *Mycobacterium* strains. A single PCR was run by amplifying a 580-bp segment of the 16S rRNA gene using specific primers 16sF (5'-CACATGCAAGTCGAACGAAAGG-3') and 16sR (5'-GCCCCGATCGCCCGCACGCT-3') and the following protocol: a first cycle of 5 min at 95°C; 30 cycles of 1 min at 94°C, 1 min at 63°C, and 1 min at 72°C; and a final cycle of 5 min at 72°C. The PCR products were purified by using the E.Z.N.A. gel extraction kit (Omega) and then sequenced by Sangon (Beijing, China). The sequences were compared with the 16S rRNA gene sequences reported in GenBank by using LaserGene MegAlign.

Bacteriology analysis. Six experimentally infected cattle, four naturally TB-infected cattle, and five *M. avium*-infected cattle were slaughtered after the experiment. Tissue samples were taken for *Mycobacterium* culture as OIE standards. The isolates were identified by PCR (*mpb70* and 16S rRNA gene) as described above.

Design of recombinant protein-based skin test. Twenty TB-infected cattle (from a farm where TB was prevalent) and 20 healthy cattle (from a TB-free farm) were selected by using the tuberculin skin test, IFN- γ release assay, and PCR. The cattle were randomly divided into four groups (five TB-infected and five healthy cattle for each group).

Ten *M. avium*-infected cattle (from a farm where *M. avium* subsp. *paratuberculosis* was prevalent) were screened by using the tuberculin skin test, IFN- γ release assay, PCR analysis, and 16S rRNA gene sequencing. All cattle were retested 2 to 3 months after the initial tuberculin skin test.

To confirm the infection, four TB-infected cattle (one animal in each group) and five *M. avium*-infected cattle were slaughtered after the experiment, and the lesions or lymph nodes were collected for *Mycobacterium* culture. The bacteriology analysis confirmed that isolate strains from TB-infected cattle belonged to the MTC and that isolate strains from *M. avium*-infected cattle belonged to *M. avium*.

To verify whether TB10.4, Rv3872, or MPT63 could strengthen the DTH induced by the cocktail of CFP-10 and ESAT-6, each animal in group 1 was administered the skin test with PPD-B, a mixture of CFP-10 and ESAT-6 (designated CE) (the concentration of total proteins was 0.5 mg/ml, and the volume ratio of the components was 1:1), a mixture of CFP-10, ESAT-6, and TB10.4 (designated CET) (at 0.5 mg/ml with a ratio of 1:1:1), and a mixture of CFP-10, ESAT-6, Rv3872, and MPT63 (designated CERM) (0.5 mg/ml with a ratio of 1:1:0.5:0.5) at different sites on the same side of the neck. The thicknesses of skin indurations at injected sites were measured before and at 24, 48, and 72 h after the injections.

To achieve the best detectable DTH induced by the CFP-10/ESAT-6/TB10.4 protein cocktail, the concentration of total protein and the ratio of each component were optimized. Each animal in group 2 was intradermally (i.d.) administered PPD-B and the CFP-10/ESAT-6/TB10.4 protein cocktail (0.5 mg/ml at 1:1:1 and 0.5 mg/ml at 1:1:2). Cattle in group 3 were i.d. administered PPD-B and CET (0.2 mg/ml at 1:1:1, 0.5 mg/ml at 1:1:1, and 0.7 mg/ml at 1:1:1).

To test the specificity of the CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test, cattle in group 4 were i.d. administered PPD-B and PET [the purified pET32a(+) tag, 0.5 mg/ml]. Ten *M. avium*-infected cattle

were i.d. administered PPD-A, PPD-B, and the CFP-10/ESAT-6/TB10.4 protein cocktail (0.5 mg/ml, 1:1:1) at different sites on the neck. The thicknesses of skin indurations of TB-infected animals in groups 2, 3, and 4 and of *M. avium*-infected cattle were measured before and at 72 h after injection.

To determine the cutoff value for the CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test, 125 healthy cattle (from a TB-free herd and shown to be negative by the tuberculin skin test, IGRA, and PCR analysis) and 117 TB-infected cattle (shown to be positive by the tuberculin skin test, IFN- γ release assay, and PCR analysis) were administered the skin test using the CFP-10/ESAT-6/TB10.4 protein cocktail (0.5 mg/ml, 1:1:1) and PPD-B as stimuli at different sites on the same side of neck. The differences in skin thickness were analyzed by receiver operator curve (ROC) analysis (GraphPad Prism 5 software). The cutoff value for the CFP-10/ESAT-6/Rv3872/MPT63 protein cocktail-based skin test (0.5 mg/ml, 1:1:0.5:0.5) was calculated by analyzing the differences in skin thicknesses of 67 healthy cattle (from a TB-free herd and shown to be negative by the tuberculin skin test, IFN- γ release assay, and PCR analysis) and 148 TB-infected cattle (showed to be positive by the tuberculin skin test, IFN- γ release assay, and PCR analysis) by using the program mentioned before.

Animal infection. To evaluate the efficiency of the protein cocktail-based skin tests with true TB-infected cattle, nine male Friesian cattle aged 1 to 2 months from a tuberculosis-free dairy farm were randomly selected and raised in isolated pens under strict pathogen-free conditions to prevent infection. All animals were identified as being free of tuberculosis, brucellosis, or bovine viral diarrhea. Three cattle were infected with 10^6 CFU of *M. bovis* strain 68002, three cattle were infected with 10^6 CFU of *M. avium* p18, and the other three cattle were used as healthy controls. Animals in each group were monitored clinically for 1 year and tested using the tuberculin skin test, IGRA, and optimized protein cocktail-based skin tests at 0, 2, and 8 months postinfection. *M. bovis*- and *M. avium*-infected cattle were slaughtered after the experiment and analyzed using PCR and *Mycobacterium* culture.

Recombinant protein-based skin test used at clinic testing. To evaluate the effects of the CFP-10/ESAT-6/TB10.4 and CFP-10/ESAT-6/Rv3872/MPT63 protein cocktail-based skin tests, 1,097 cattle (125 males and 972 females) (679 for CFP-10/ESAT-6/TB10.4 and 418 for CFP-10/ESAT-6/Rv3872/MPT63, containing the data used for ROC analysis) were tested by the protein cocktail-based skin test, tuberculin skin test, and IFN- γ release assay.

Evaluation of diagnostic test and statistical analysis. Quantitative outcomes (increase in skin thickness induced by PPD-B or protein cocktails and OD₄₅₀ of IGRA induced by PPD-B, data obtained from the cattle used for ROC analysis and experimentally infected cattle) and qualitative outcomes (data obtained from all naturally TB-infected cattle, including the cattle used for ROC analysis) of the skin tests (with PPD-B and protein cocktails used as stimuli) and IGRA were analyzed using GraphPad Prism 5 software. The correlation of quantitative outcomes was assessed using the Spearman coefficient. Agreement between tests was evaluated using the kappa (κ) coefficient.

RESULTS

Design and verification of protein cocktail-based skin test procedure. To prepare the antigens used in the skin test, a fusion protein strategy was adopted to increase expression, and each recombinant protein was expressed with thioredoxin, S, and His tags (the total molecular mass of the tags was 18 kDa) at the carboxyl terminus. Proteins were run in a 12% polyacrylamide gel (data not shown) and the molecular mass of each protein matched to its predicted molecular mass. The recombinant proteins, with a purity of >90% and final concentrations of 0.5 to 1.5 mg/ml, were used for subsequent experiments. The endotoxin level for each protein was <2 endotoxin units (EU)/mg.

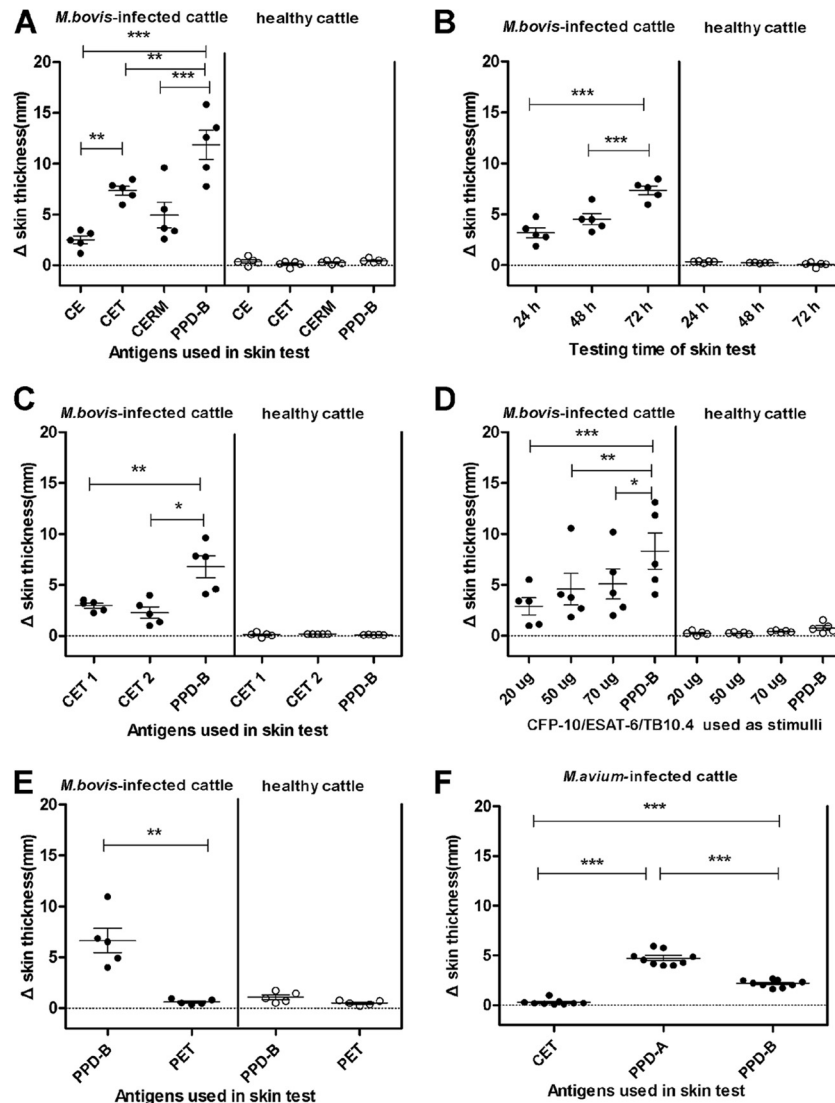


FIG 1 Optimization of protein cocktail-based skin tests. (A) Group 1, showing the recombinant proteins used as stimuli in skin tests. (B) Group 1, showing the optimization of testing time for the CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test. (C) Group 2, showing different volume ratios of the CFP-10/ESAT-6/TB10.4 components. The total protein concentration of each combination was 0.5 mg/ml. CET 1 represents a CFP-10/ESAT-6/TB10.4 volume ratio of 1:1:1; CET 2 represents a CFP-10/ESAT-6/TB10.4 volume ratio of 1:1:2. (D) Group 3, showing the optimization of the concentration of the CFP-10/ESAT-6/TB10.4 protein cocktail, with a CFP-10/ESAT-6/TB10.4 volume ratio of 1:1:1. (E) Group 4, showing the purified pET32a(+) tag protein (PET) used as the stimulus in the skin test. The total protein concentration of PET was 0.5 mg/ml. (F) Group 5, showing *M. avium*-infected cattle administered skin tests using PPD-A, PPD-B, and the CFP-10/ESAT-6/TB10.4 protein cocktail as stimuli. The total protein concentration of the CFP-10/ESAT-6/TB10.4 protein cocktail was 0.5 mg/ml, and the CFP-10/ESAT-6/TB10.4 volume ratio was 1:1:1. The horizontal lines show the means ($\pm$ standard errors of the means [SEM]), with results expressed as the difference in skin thicknesses (mm) between the readings before and after the skin test. The statistical difference between responses induced by various stimuli was determined by using analysis of variance (ANOVA) (Tukey's multiple-comparison test) (*, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$), except for group 4, which was analyzed by using the paired t test.

To select the best antigens and test procedure for the recombinant protein-based skin test, optimizations of the antigen components, the volume ratio between the components, the detection time, and the concentration of antigen were performed. As shown in Fig. 1, the CFP-10/ESAT-6, CFP-10/ESAT-6/TB10.4, and CFP-10/ESAT-6/Rv3872/MPT63 protein cocktails could induce DTH in TB-infected cattle, while the purified PET [pET32a(+) Tag protein], as a control, could not induce DTH in TB-infected or healthy cattle. Among these three combinations of the recombinant proteins, the CFP-10/ESAT-6/TB10.4 protein cocktail showed a better reactivity, but it was still weaker than that of

PPD-B. The indurations induced by the injection of the CFP-10/ESAT-6/TB10.4 protein cocktail in TB-infected cattle became stronger over time and were strongest at 72 h postinjection. The DTHs induced by different concentrations of the CFP-10/ESAT-6/TB10.4 protein cocktail or various ratios of its components were not significantly different, indicating that relatively minor changes in the concentration of each component in the CFP-10/ESAT-6/TB10.4 mixture do not change the effect of the protein cocktail. Considering that a higher CFP-10/ESAT-6 ratio might ensure the specificity of the CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test, the volume ratio of the components was set at 1:1:1.

TABLE 2 Sensitivities and specificities of protein cocktail-based skin tests estimated by ROC analysis^a

Diagnostic antigen	Area under the ROC curve	Sensitivity (%)	95% confidence interval (%)	Specificity (%)	95% confidence interval (%)	Cutoff value (mm)
CFP-10/ESAT-6/TB10.4 protein cocktail	0.9841	94.02	88.06–97.56	95.2	89.84–98.22	>1.0
		92.31	85.90–96.42	97.3	93.15–99.5	>1.10
		88.03	80.74–93.30	99.2	95.62–99.98	>1.4
CFP-10/ESAT-6/Rv3872/MPT63 protein cocktail	0.9711	95.04	90.04–97.98	95.52	87.47–99.07	>0.8125
		92.91	87.34–96.55	97.01	89.63–99.64	>1.0
		83.69	76.54–89.37	99.9	94.64–100	>1.42

^a The cutoff value for the CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test was obtained from 125 healthy cattle (from a TB-free herd and shown to be negative by the tuberculin skin test, IFN-γ release assay, and PCR analysis) and 117 TB-infected cattle (shown to be positive by the tuberculin skin test, IFN-γ release assay, and PCR analysis); the cutoff value for the CFP-10/ESAT-6/Rv3872/MPT63 protein cocktail-based skin test was obtained from 67 healthy cattle (from a TB-free herd and shown to be negative by the tuberculin skin test, IFN-γ release assay, and PCR analysis) and 148 *M. bovis*-infected cattle (shown to be positive by the tuberculin skin test, IFN-γ release assay, and PCR analysis). CFP-10/ESAT-6/TB10.4, a protein cocktail consisting of CFP-10, ESAT-6, and TB10.4; the concentration of total proteins was 0.5 mg/ml, and the volume ratio of the components was 1:1:1; CFP-10/ESAT-6/Rv3872/MPT63, a protein cocktail consisting of CFP-10, ESAT-6, Rv3872s and MPT63; the concentration of total proteins was 0.5 mg/ml, and the volume ratio of the components was 1:1:0.5:0.5. Boldface indicates the cutoff values used in the field trial.

Considering the cost of production and the DTH response intensity, the total protein concentration was set at 0.5 mg/ml. CFP-10/ESAT-6/TB10.4 did not induce DTH in *M. avium*-infected cattle, suggesting its specificity toward TB-infected cattle.

To obtain the target test specificity of 97%, the cutoff value for the CFP-10/ESAT-6/TB10.4-based skin test should be set at 1.1 mm, while the cutoff for the CFP-10/ESAT-6/Rv3872/MPT63-based skin test should be set at 1.0 mm. According to the ROC analysis, the area under the curve (AUC) indicated that the CFP-10/ESAT-6/TB10.4 protein cocktail had a better diagnostic outcome in the detection of bovine tuberculosis than the CFP-10/ESAT-6/Rv3872/MPT63 protein cocktail (Table 2).

Efficiency of protein cocktail-based skin test with experimentally infected cattle. To evaluate the efficiency of the protein cocktail-based skin test with true infected cattle, cattle were experimentally infected with *Mycobacterium* and slaughtered after the experiment. The infection of these cattle was confirmed by comparative tuberculin test, IGRA, PCR, and *Mycobacterium* culture (data were not shown). Three *M. bovis* strain 68002-infected cattle were positive on *M. bovis* culture, but only one of them showed

lung lesions compatible with tuberculosis. Three *M. avium* p18-infected cattle were positive on *M. avium* culture, but none of them showed lesions in the intestine. Cattle were tested using PPB-B, PPD-A, and protein cocktails at 2 months and 8 months postinfection. The results showed that both the CFP-10/ESAT-6/TB10.4 and CFP-10/ESAT-6/Rv3872/MPT63 protein cocktails could induce detectable DTH in *M. bovis*-infected but not in *M. avium*-infected or healthy cattle (Fig. 2) and could be used to differentiate *M. bovis* infection from *M. avium* infection.

Double-blind testing using the protein cocktail-based skin test with a large number of cattle. To assess the application of the protein cocktail-based skin tests, 1,097 cattle in total (679 for CFP-10/ESAT-6/TB10.4 and 418 for CFP-10/ESAT-6/Rv3872/MPT63, containing the data used for ROC analysis) from 8 different herds were tested using the protein cocktail-based skin tests (CFP-10/ESAT-6/TB10.4 or CFP-10/ESAT-6/Rv3872/MPT63), tuberculin skin test, and IFN-γ release assay. The CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test showed a higher correlation with traditional diagnostic tools than the CFP-10/ESAT-6/Rv3872/MPT63-based skin test. The correlation between the increases in

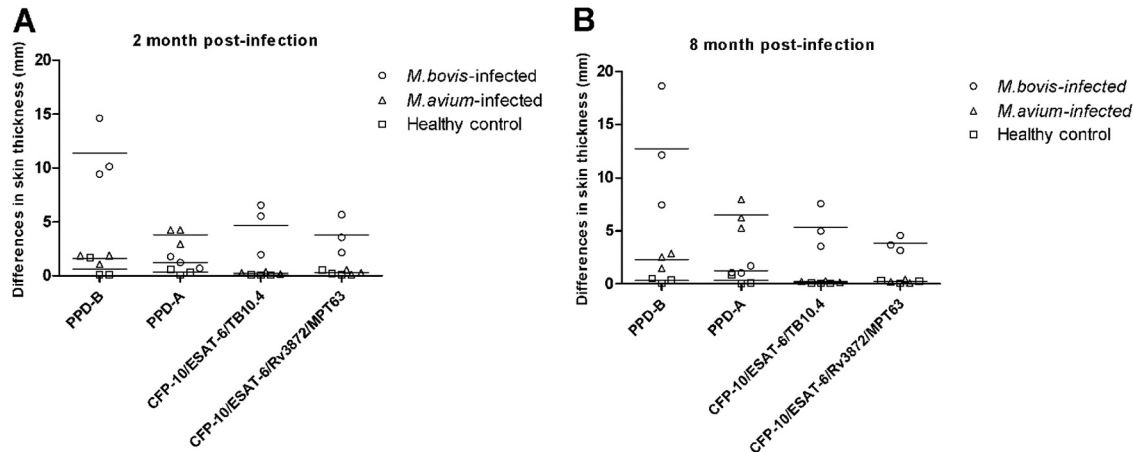


FIG 2 Protein cocktail-based skin tests with experimentally infected cattle. (A) Experimentally infected cattle tested at 2 months postinfection. (B) Experimentally infected cattle tested at 8 months postinfection. Infection was confirmed by using PCR, IGRA, and *Mycobacterium* culture. The CFP-10/ESAT-6/TB10.4 protein cocktail consists of CFP-10, ESAT-6, and TB10.4, in which the concentration of total proteins was 0.5 mg/ml and the volume ratio of the components was 1:1:1. The CFP-10/ESAT-6/Rv3872/MPT63 protein cocktail consists of CFP-10, ESAT-6, Rv3872, and MPT63, in which the concentration of total proteins was 0.5 mg/ml and the volume ratio of the components was 1:1:0.5:0.5.

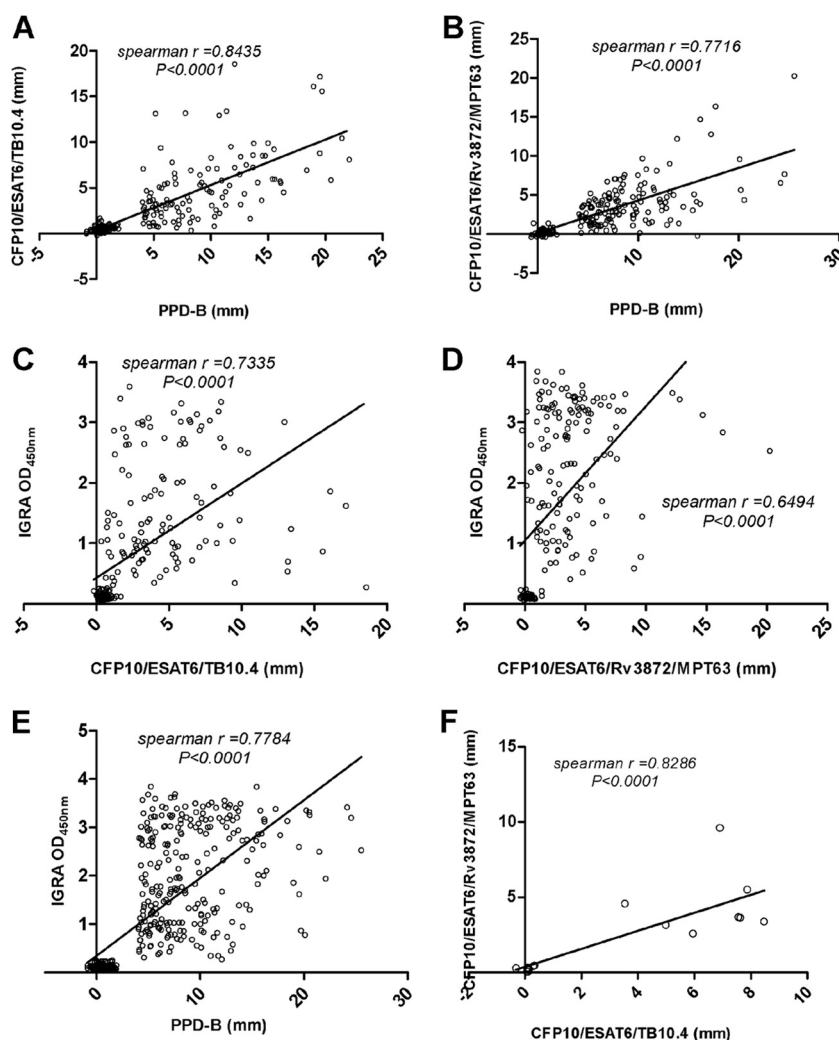


FIG 3 Correlation between protein cocktail-based skin tests and traditional diagnostic methods. (A) Correlation between the CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test and the tuberculin skin test. (B) Correlation between the CFP-10/ESAT-6/Rv3872/MPT63 protein cocktail-based skin test and the tuberculin skin test. (C) Correlation between the CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test and IGRA. (D) Correlation between the CFP-10/ESAT-6/Rv3872/MPT63 protein cocktail-based skin test and IGRA. (E) Correlation between the tuberculin skin test and IGRA. (F) Correlation between the CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test and the CFP-10/ESAT-6/Rv3872/MPT63 protein cocktail-based skin test. For panels A and C, 117 TB-infected cattle and 125 TB-free cattle were included to analyze the correlation between the CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test and the tuberculin skin test or IGRA. For panels B and D, 148 TB-infected cattle and 67 TB-free cattle were included to analyze the correlation between the CFP-10/ESAT-6/Rv3872/MPT63 protein cocktail-based skin test and the tuberculin skin test or IGRA. For panel E, 265 TB-infected cattle and 192 TB-free cattle were included to analyze the correlation between the tuberculin skin test and IGRA. For panel F, 8 TB-infected cattle (three were experimentally infected cattle and confirmed by PCR and *Mycobacterium* culture; five were naturally infected cattle and confirmed by PCR, with only one confirmed by *Mycobacterium* culture) and 8 TB-free cattle (from TB-free cattle confirmed by PCR, IGRA, and tuberculin skin test) were included to analyze the correlation between the CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test and the CFP-10/ESAT-6/Rv3872/MPT63 protein cocktail-based skin test.

skin thickness observed after the inoculation of stimuli was high (PPD-B versus CFP-10/ESAT-6/TB10.4, Spearman $r = 0.844$; PPD-B versus CFP-10/ESAT-6/Rv3872/MPT63, Spearman $r = 0.772$; CFP-10/ESAT-6/TB10.4 versus CFP-10/ESAT-6/Rv3872/MPT63, Spearman $r = 0.8286$) (Fig. 3). The correlation between the OD₄₅₀ values obtained after blood stimulation with PPD-B and the increases in skin thickness observed after inoculation of stimuli was also high (IGRA versus CFP-10/ESAT-6/TB10.4, Spearman $r = 0.7335$; IGRA versus CFP-10/ESAT-6/Rv3872/MPT63, Spearman $r = 0.649$; IGRA versus PPD-B, Spearman $r = 0.76$). The protein cocktail-based skin tests showed good agreement with traditional tests (CFP-10/ESAT-6/TB10.4 versus tuber-

culin skin test, $\kappa = 0.8563$; CFP-10/ESAT-6/TB10.4 versus IGRA, $\kappa = 0.8154$; CFP-10/ESAT-6/Rv3872/MPT63 versus tuberculin skin test, $\kappa = 0.8082$; CFP-10/ESAT-6/Rv3872/MPT63 versus IGRA, $\kappa = 0.8270$). Compared to the traditional diagnostic methods (tuberculin skin test and IFN- γ release assay), the relative sensitivity of the CFP-10/ESAT-6/TB10.4-based skin test was more than 87% and its relative specificity was more than 92%, while the relative sensitivity and relative specificity of the CFP-10/ESAT-6/Rv3872/MPT63-based skin test were higher than 89% and 91%, respectively (Tables 3 and 4). Therefore, based on the field trial, the protein cocktail-based skin tests are promising methods to use for the field test.

TABLE 3 Numbers of tested cattle and agreement between CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test and tuberculin skin test or IFN- γ release assay^a

CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test result	Tuberculin skin test			IGRA		
	No. positive	No. negative	Total	No. positive	No. negative	Total
Positive	205 (108 ^b)	20 (2 ^{b,d})	225	180 (108 ^b)	45 (2 ^{b,d})	225
Negative	28 (9 ^b)	426 (205 ^c)	454	20 (9 ^{b,c})	434 (205 ^c)	454
Total	233	446	679	200	479	679

^a Cattle from six different farms were tested. For the CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test, the protein cocktail consisted of CFP-10, ESAT-6, and TB10.4, with the concentration of total proteins at 0.5 mg/ml and the volume ratio of the components at 1:1:1. The tuberculin skin test used PPD-B as the stimulus. IGRA, IFN- γ release assay using the *Mycobacterium bovis* gamma interferon test kit. The cutoff value for the CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test was set as 1.1 mm. Results for the tuberculin skin test were evaluated as follows: if the difference in skin thicknesses was equal to or greater than 4 mm, the animal was considered infected with TB; if the difference was smaller than 2 mm, the animal was considered free from TB; if the difference was greater than or equal to 2 mm and less than 4 mm, the result was considered inconclusive and the animal should be retested after an interval of 60 days, and then if the difference is greater or equal to 2 mm, the animal should be considered TB infected. κ values were as follows: CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test and tuberculin skin test, κ = 0.8536; CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test and IFN- γ release assay, κ = 0.8154. The relative specificities of the CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test compared to the tuberculin skin test and IFN- γ release assay were 97% and 92%, respectively. The relative sensitivities of the CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test compared to the tuberculin skin test and IFN- γ release assay were 87% and 89%, respectively.

^b Confirmed TB positive by PCR analysis.

^c Confirmed TB free by PCR analysis.

^d Cattle from a farm with a low TB prevalence.

DISCUSSION

CFP-10 and ESAT-6 could be used as stimuli in the skin test and IFN- γ release assay, showing high specificity but lower sensitivity than the PPD-based tuberculin skin test (6, 16, 24). Several antigens have been added to increase the sensitivity of CFP-10- and ESAT-6-based skin tests, such as MPB70, MPB83, Rv3020c, Rv3615c, etc., but only a few of them have undergone field trials (14, 15, 17, 25). In this study, two combinations (the CFP-10/ESAT-6/TB10.4 protein cocktail and the CFP-10/ESAT-6/Rv3872/MPT63 protein cocktail) were developed and used as stimuli in the skin test. The addition of TB10.4 or a mix of Rv3872 and MPT63 could elicit stronger DTH in TB-infected cattle than CFP-10/ESAT-6 used alone (Fig. 1), especially TB10.4. Compared to the tuberculin skin test, the CFP-10/ESAT-6/TB10.4- and CFP-10/ESAT-6/Rv3872/MPT63-based skin tests showed higher relative sensitivities (87% and 89%, respectively) than the CFP-10/ESAT-6 protein cocktail-based skin test (76.2% and 48/63 [15])

based on overall field trials. Further, the ROC analysis based on PCR-confirmed TB-infected cattle showed that the sensitivities of the CFP-10/ESAT-6/TB10.4- and CFP-10/ESAT-6/Rv3872/MPT63-based skin tests were 92.31% and 92.91%, respectively. Although the T cell-stimulated activities of Rv3872 and MPT63 are weaker than that of TB10.4, the CFP-10/ESAT-6/Rv3872/MPT63-based skin test showed sensitivity similar to that of the CFP-10/ESAT-6/TB10.4-based skin test. This may be related to higher antigen numbers in CFP-10/ESAT-6/Rv3872/MPT63, even though the concentration of total proteins is the same (0.5 mg/ml) (17). The CFP-10/ESAT-6/TB10.4- and CFP-10/ESAT-6/Rv3872/MPT63-based skin tests detected more positive cattle than traditional diagnostic methods in a farm with a low TB prevalence. However, it is unfortunate that we were not able to kill positive cattle in the farm with a low TB prevalence to confirm the infection. Flores-Villalva et al. also found that the CFP-10/ESAT6-based skin test can identify more positive cattle that were con-

TABLE 4 Numbers of tested cattle and agreement between CFP-10/ESAT-6/RV3872/MPT63-based skin test and tuberculin skin test or IFN- γ release assay^a

CFP-10/ESAT-6/Rv3872/MPT63 protein cocktail-based skin test result	Tuberculin skin test			IGRA		
	No. positive	No. negative	Total	No. positive	No. negative	Total
Positive	179 (131 ^b)	20 (4 ^{b,c} , 3 ^{b,d})	199	177 (131 ^b)	22 (4 ^{b,c} , 3 ^{b,d})	199
Negative	22 (10 ^b)	197 (63 ^c)	219	15 (10 ^b)	204 (63 ^c)	219
Total	201	217	418	192	226	418

^a Cattle from three different farms were tested. For the CFP-10/ESAT-6/Rv3872/MPT63 protein cocktail-based skin test, the protein cocktail consisted of CFP-10, ESAT-6, Rv3872, and MPT63, with the concentration of total proteins at 0.5 mg/ml and the volume ratio of the components at 1:1:0.5:0.5. The tuberculin skin test used PPD-B as the stimulus. IGRA, IFN- γ release assay using the *Mycobacterium bovis* gamma interferon test kit. The cutoff value for the CFP-10/ESAT-6/Rv3872/MPT63 protein cocktail-based skin test was set as 1.0 mm. Results for the tuberculin skin test were evaluated as follows: if the difference in skin thicknesses was equal to or greater than 4 mm, the animal was considered infected with TB; if the difference was smaller than 2 mm, the animal was considered free from TB; if the difference was greater than or equal to 2 mm and less than 4 mm, the result was considered inconclusive and the animal should be retested after an interval of 60 days, and then if the difference is greater or equal to 2 mm, the animal should be considered TB infected. κ values were as follows: CFP-10/ESAT-6/Rv3872/MPT63 protein cocktail-based skin test and tuberculin skin test, κ = 0.7987; CFP-10/ESAT-6/Rv3872/MPT63 protein cocktail-based skin test and IFN- γ release assay, κ = 0.8223. The relative specificities of the CFP-10/ESAT-6/Rv3872/MPT63 protein cocktail-based skin test compared to the tuberculin skin test and IFN- γ release assay were 91% and 90%, respectively. The relative sensitivities of the CFP-10/ESAT-6/Rv3872/MPT63 protein cocktail-based skin test compared to the tuberculin skin test and IFN- γ release assay were 89% and 92%, respectively.

^b Confirmed TB positive by PCR analysis.

^c Confirmed TB free by PCR analysis.

^d Cattle from a farm with a low TB prevalence.

firmed by PCR than the tuberculin skin test (15). This may be related to the lower cutoff value of protein cocktail-based skin tests than of PPD-B.

The tuberculin skin test is used widely in the world to diagnose TB infection, but its specificity is often reduced by sensitization to environmental *Mycobacterium* or infection with *M. avium* subsp. *paratuberculosis*. Avian tuberculin was usually used as a contrast stimulus in IGRA or comparative skin tests to remove the interference. However, comparative tests could be confused by coinfection with TB and *M. avium*, and the high price of the IGRA kit encourages development of a more effective diagnostic method. *M. bovis*-specific antigens were screened and used as stimuli in skin test to improve the specificity of traditional diagnostic methods. In this study, two kinds of protein cocktails were used as stimuli in skin tests and compared with PPD-B with the aim of assessing the cocktails in herds with different bTB prevalence rates and presence of *M. avium*. The main benefit of the application of protein cocktails was observed in the *M. avium*-infected cattle, because CFP-10/ESAT-6/TB10.4 and CFP-10/ESAT-6/Rv3872/MPT63 did not induce detectable DTH in *M. avium*-infected cattle, unlike when PPD-B was used as the stimulus (Fig. 1F and 2B). Although the *esxH* gene, which codes for TB10.4, exists in *M. bovis* and *M. avium*, the addition of TB10.4 did not induce DTH in *M. avium*-infected cattle (Fig. 1F and 2B). Further, the clinical data showed that the relative specificity of the CFP-10/ESAT-6/TB10.4-based skin test (compared to tuberculin skin test, 97%; compared to IFN- γ release assay, 92%) is similar to that of CFP-10/ESAT-6 (96%, 28/29 [15]), and ROC analysis based on PCR-confirmed TB-free cattle (from TB-free herds) showed that the specificities of the CFP-10/ESAT-6/TB10.4- and CFP-10/ESAT-6/Rv3872/MPT63-based skin tests were 97.3% and 97.01%, respectively. The reasons for the good specificity of TB10.4 are unknown. It may be related to its lower concentration in *M. avium* than *M. bovis* or in the *M. avium* strains prevalent in China.

In this study, 1,097 cattle were double-blind tested to assess the efficiency of protein cocktail-based skin tests, and the results indicated that the CFP-10/ESAT-6/TB10.4 protein cocktail and the CFP-10/ESAT-6/Rv3872/MPT63 protein cocktail can be used as possible alternates for the traditional tuberculin skin test. Although the DTHs induced by the protein cocktails were significantly weaker than that induced by PPD-B, the DTH induced by the protein cocktails showed good correlation to that from PPD-B (Spearman $r > 0.75$). The protein cocktail-based skin tests also showed reasonably good agreement with the tuberculin skin test and IGRA ($\kappa > 0.8$); this was better than that for the ESAT-6/CFP-10/Rv3615c-based skin test (tuberculin skin test, $\kappa = 0.714$; IFN- γ release assay, $\kappa = 0.742$), which was reported as the best protein cocktail-based skin test so far (6). However, the CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test showed relatively higher agreement with traditional diagnostic methods than the CFP-10/ESAT-6/Rv3872/MPT63 protein cocktail-based skin test (Tables 3 and 4). Further, considering the presence of fewer components in the CFP-10/ESAT-6/TB10.4 protein cocktail and its stronger DTH responses (Fig. 1 and 2), the CFP-10/ESAT-6/TB10.4 protein cocktail was considered to be better than the CFP-10/ESAT-6/Rv3872/MPT63 protein cocktail as stimuli in skin tests based on overall field trials.

Unfortunately, it was impossible to slaughter all the cattle used in this study to confirm the TB infection, but the PCR analysis of nasal swabs helped us to confirm some results achieved with the

protein cocktails in the skin test and to calculate the sensitivities of the protein cocktail-based skin tests using ROC analysis. However, 825 cattle in TB or *M. avium* prevalent herds were analyzed by PCR. Only 205 cattle were confirmed TB positive. Two TB-free herds were selected to calculate the specificity of the protein cocktail-based skin tests, and the animals were confirmed free from TB by using PCR, the tuberculin skin test, and IGRA, but a negative result in PCR does not mean that the animal is not infected, because the cattle may be in a nonshedding pattern.

Conclusion. TB10.4, MPT63, and Rv3872 could strengthen the DTH in *M. bovis*-infected cattle, and the CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test could differentiate *M. bovis*-infected cattle from *M. avium*-infected ones and showed better agreement with the traditional tuberculin skin test and IFN- γ release assay than the CFP-10/ESAT-6/Rv3872/MPT63 protein cocktail-based skin test. In conclusion, the CFP-10/ESAT-6/TB10.4-based skin test responses correlate to traditional measures of bovine TB evaluation but still require further validation using gold standard methods.

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We have no competing interests.

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