

Full Length Research Paper

# Use of recombinant CFP-10 protein for a skin test specific for *Mycobacterium tuberculosis* infection

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Although the delayed-type hypersensitivity (DTH) skin reaction to tuberculin is used worldwide for tuberculosis (TB) detection, it has poor diagnostic specificity due to the presence of common antigens in tuberculin shared by many mycobacterial species. The problem is noticed, especially in countries where the Bacillus Calmette-Gue´rin (BCG) vaccination is widely practiced. Thus, a new skin test antigen specific for the diagnosis of *Mycobacterium tuberculosis* (MTB) infection is urgently needed. CFP-10, a mycobacterial secretory protein that is absent in *Mycobacterium bovis* BCG and most other mycobacterial species including *Mycobacterium avium*, *Mycobacterium intracellulare*, has been shown to elicit cellular immune responses in MTB infected individuals and can be a good candidate for MTB specific diagnosis. We prepared recombinant MTB CFP-10, rCFP-10, and its utility as specific antigen for TB diagnosis was evaluated by skin testing in guinea pigs sensitized with *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG. Our results show that the purified MTB rCFP-10 antigen elicits a positive skin response only in the guinea pigs sensitized with *M. tuberculosis* and *M. bovis*, and not in the animals sensitized with *M. bovis* BCG vaccine. The data presented in this study supports further testing of the use rCFP-10 as the specific antigen in the skin test for the diagnosis of MTB infection in humans.

**Key words:** Recombinant CFP-10 protein, skin test, delayed-type hypersensitivity, tuberculosis infection, *Mycobacterium tuberculosis*, *Mycobacterium bovis*, Bacillus Calmette-Gue´rin.

## INTRODUCTION

According to the World Health Organization, *Mycobacterium tuberculosis* (MTB) has infected approximately one-third of the world population, and more than 8 million new cases of tuberculosis (TB) worldwide was reported in 2002 (World Health Organization, 2004). It is estimated

that 45% of Chinese population are latently infected with MTB (The Ministry of Health of the People's Republic of China, 2001), among which 10% latently infected individuals may become activated later in their lives. Proper identification of latently infected individuals for prophylactic chemo- therapy is needed to prevent future reactivation of MTB infection and to control TB epidemics in China. At present, the purified protein derivative (PPD, also known as the tuberculin) skin test based on the delayed-type hypersen- sitivity (DTH) reaction is widely used to detect MTB infection (American Thoracic Society and Centers for Disease Control, 1991; Huebner et al., 1993). However, the specificity of PPD skin test is low, because the antigens in PPD are common to many mycobacterial species, including *Mycobacterium bovis* Bacillus Calmette- Gue´rin (BCG), the vaccine strain being used widely for immunization against MTB infection (Huebner et al., 1993; Harboe, 1981). As a result, the PPD skin test cannot be used to definitively identify MTB infection (Harboe, 1981).

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**Abbreviations:** DTH, Delayed-type hypersensitivity; TB, tuberculosis; BCG, Bacillus Calmette-Gue´rin; MTB, *Mycobacterium tuberculosis*; PPD, purified protein derivative; RD1, region of difference 1; NTM, non-tuberculous mycobacteria; HLA, human leucocyte antigen; PCR, polymerase chain reaction; LB, Luria-Bertani; X-Gal, 5-bromo-4-chloro-3-indolyl  $\beta$ -D- galactopyranoside; BLAST, basic local alignment search tool; IPTG, isopropyl-b-D-thiogalactopyranoside; PBS, phosphate-buffered saline; GMP, good manufacturing practices.

Mycobacterial antigens that can distinguish the MTB specific DTH reaction from that induced by the BCG vaccination are highly desirable and their utility in skin test specific for TB diagnosis needs be demonstrated.

The genetic region of difference 1 (RD1) is present in the genomes of MTB and *M. bovis*, but is absent in all strains of *M. bovis* BCG, as well as most non-tuberculous mycobacteria (NTM) including *Mycobacterium avium* and *Mycobacterium intracellulare* (Behr et al., 1999; Behr, 2001; Harboe et al., 1996). Both CFP-10 and ESAT-6 antigens are encoded by Rv3874 and Rv3875 genes, respectively, in one operon within the RD1 region, and are expressed simultaneously at a similar ratio (Berthet et al., 1998). Because of their absence in BCG and NTM strains, ESAT-6 and CFP-10 have been extensively investigated as MTB specific antigens, and shown to have great potential as the specific antigens for the diagnosis of MTB infection in humans (Arend et al., 2000; Goletti et al., 2005; Pai et al., 2004). The immune responses found in experimental animals and humans to the recombinant CFP-10 or ESAT-6 proteins are similar to those found for the CFP-10 or ESAT-6 peptides (Arend et al., 2000; Hill et al., 2005; Skjot et al., 2000; Dillon et al., 2000). However, there were differences in immune responses of human or cattle to individual CFP-10 and ESAT-6 antigens (Hil et al., 2005; Vordermeier et al., 2001). Hill et al., (2005) reported that, as determined by ELISPOT assay, 88 (18%) people tested were positive for CFP-10 peptides, 148 (30%) were positive for ESAT-6 peptides, 161 (33%) were positive for both peptides and 168 (34%) were positive for the ESAT-6/CFP-10 fusion protein; 188 (39%) subjects had either a positive result for a peptide or a positive result for the fusion protein. There was reasonable agreement between individual reactivity against either the peptide or the fusion protein as the antigen. The difference may be explained by polymorphism in the human leucocyte antigen (HLA) type in the population and differences of MTB strains infected in different countries. Previously, we reported the results of using rESAT-6 as the specific antigen in TB skin tests in animals and human volunteers and demonstrated the specificity of rESAT-6 for TB diagnosis (Wu et al., 2008). However, the use of a single antigen in the skin test may exclude those who do not have memory T-cells specific to rESAT-6 even after exposure to MTB due to their unique HLA type. In this study, we evaluated the utility of rCFP-10 protein as a stimulating antigen to improve the sensitivity of rESAT-6 skin test or to find a better antigen than ESAT6 for differential diagnosis of MTB infection.

## MATERIALS AND METHODS

### Bacterial strains and products

*Escherichia coli* strain BL21 (DE3) (Invitrogen, Carlsbad, CA, USA) was grown in Luria-Bertani (LB) liquid and solid media *Mycobacterium* reference strains including MTB (H37Rv), *M. bovis*, and *M. bovis* BCG Danish (chosen because it is widely used

in China), were obtained from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. Stock cultures of MTB, *M. bovis*, and *M. bovis* BCG Danish, were grown on Lowenstein-Jensen slants at 37°C for 4 weeks, and then transferred to Sauton liquid medium at 37°C without shaking for 4 weeks (Chinese Antituberculosis Association, 1995). PPD produced from MTB (50 IU/ml) was purchased from Beijing Gaoke Life and Technology Inc, China.

### Gene cloning, expression and protein purification

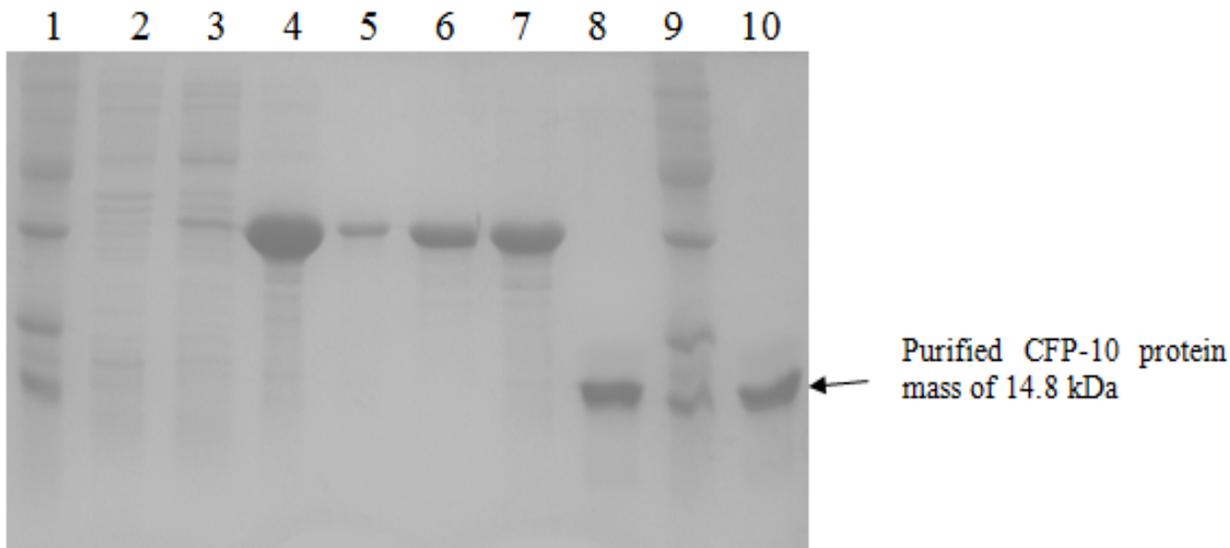
The MTB CFP-10 cloning, expression and purification were performed with standard procedures described by Sambrook et al. (1989). Briefly, the gene encoding the MTB CFP-10 protein was amplified by polymerase chain reaction (PCR) using two primers. The forward primer contained a *Kpn* I restriction enzyme recognition site (underlined) and an enterokinase recognition site (bold letters). 5'-CGAGATCTGGGTACCGACGACGACGACAAGATGGCAGAGATGAAGACCGA-3'. The reverse primer contained a *Eco*R I restriction enzyme recognition site (underlined) 5'-CGGAATTCT CAGAAGCCCATTGCGAG G-3'. PCR amplification was performed by 30 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, extension at 68°C for 1 min, and a final extension at 72°C for 2 min. PCR products were digested by the restrictive enzymes *Kpn* I and *Eco*R I. The resulting fragments were ligated with T4 DNA ligase into a *Kpn* I- and *Eco*R I-digested pET-32a (Novagen, San Diego, CA) plasmid vector containing the kanamycin resistant gene. The recombinant plasmids were transferred into *E. coli* BL21 (DE3), and kanamycin resistant transformants were selected on LB agar plate containing kanamycin (50 µg/ml) and 5-bromo-4-chloro-3-indolyl β-D- galactopyranoside (X-Gal) (20 µg/ml). The plasmid from one kanamycin resistant transformant was sequenced by Canada Sangon Ltd. Beijing, China, using the same primers as for PCR amplification. Sequences were compared with that registered in the GenBank database by Basic Local Alignment Search Tool (BLAST) analysis.

The expression of rCFP-10 as an N-terminal 6×histidine-tagged protein was induced by 1.0 mM isopropyl-b-D-thiogalactopyranoside (IPTG), in soluble form after incubation at 30°C for 4 h. Soluble rCFP-10 was purified by metal chelate affinity chromatography under the native condition, the fusion tag was then cleaved with enterokinase, and the resulting tag-free rCFP-10 was further purified by affinity chromatography according to the purification procedure provided by the expression vector manufacturer (Novagen).

The freshly prepared rCFP-10 protein was suspended in 0.1 mM phosphate-buffered saline (PBS, Ph 7.0), and the solution was filtered by passing it through the Acrodisc Synringe Filter (0.45 µm) with low protein binding (Pall, Life Sciences). The protein concentration was determined spectrophotometrically. The final protein solution was aliquoted into small glass ampoules, each contained 1 mg of purified rCFP-10 protein. The samples were then lyophilized, sealed and stored at -20°C. The protein was reconstituted in 1 ml saline before use.

### Determination of molecular mass and purity

The molecular mass of purified rCFP-10 protein was determined by 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (Sambrook, 1989). The molecular masses of the protein standards ranged from 6 to 97.4 kDa (Sigma, St Louis, MO, USA). Gels were stained with Coomassie blue. The molecular mass and purity of rCFP-10 protein were analyzed and calculated with Biologic software TotalLab v1.11. The molecular mass of purified rCFP-10 protein was further confirmed by mass spectrometry in the Instrument Test Center,



**Figure 1.** Purification of rCFP-10 protein as determined by SDS-PAGE electrophoresis. Lane 1 and 9, molecular weight protein standards (97.4, 66.2, 43, 31, 20.1 and 14.4kDa, Sigma); Lane 2, *E. coli* containing vector pET-32a lysates; Lane 3, *E. coli* lysates; Lane 4, rCFP-10 soluble protein from *E. coli* containing recombinant CFP-10/pET-32a extract; Lane 5 to 7, the purified rCFP-10 fusion protein; Lane 8 and 10, the purified rCFP-10 protein after histidine tag was removed. The gel was subjected to electrophoresis followed by Coomassie blue staining.

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### Guinea pig sensitization and skin tests

Thirty-eight NIH white guinea pigs weighing 250 to 300 g were obtained from the Institute of Epidemic and Microbiology Research, Academy of Chinese Preventive Medicine, Beijing, China, and maintained under specific-pathogen-free conditions. There was a female-to-male ratio of 1:1. In the first experiment, 8 guinea pigs (4 males and 4 females) were sensitized by hypodermic injection of 5 mg autoclaved MTB in 0.1 ml of saline in the groin once weekly for four weeks. In the second experiment, 6 guinea pigs (3 males and 3 females) were sensitized by peritoneal injection of 100 live MTB in 0.5 ml saline and the skin test on these animals was performed 5 weeks after sensitization. In the third experiment, 24 guinea pigs were divided into 3 groups (8 per group, Male : Female = 1:1) and sensitized by hypodermic injection of 0.1 ml live BCG-Danish vaccine (group 1), 5 mg autoclaved MTB (group 2), or 5 mg autoclaved *M. bovis* (group 3) in animal's groin area once weekly for four weeks.

For skin testing, guinea pigs were shaved on the back and injected intradermally with 0.8 to 1.2 µg of the purified rCFP-10 protein in 0.1 ml of PBS or 0.1 ml (5 IU) of PPD or 1.0 µg of the purified rESAT-6 protein in 0.1 ml of PBS as a control for four to eight weeks following sensitization. The diameters of both axes of skin erythema were independently measured and recorded at 24, 48, and 72 h after antigen injection. Results were expressed as means of diameters (in millimeters) of erythema ± standard deviations.

## RESULTS

### Cloning, expression and purification of CFP-10

The nucleotide sequence encoding the rCFP-10 had

100% homologous identity with CFP-10 sequence reported in GenBank database. The molecular mass obtained from rCFP-10 fusion protein as determined by SDS-PAGE was 30.8 kDa. In order to determine an ideal condition for rCFP-10 protein expression, we compared the levels of expression under various IPTG concentrations and different induction time and temperatures. The results showed that IPTG concentrations had no effect on the level of the expression, whereas the induction time and temperature affected the expression level significantly. The amount of rCFP-10 protein that existed in soluble form was at 35 - 50% of the total soluble proteins when the induction took place under 1.0 mM IPTG at 30°C for 4 h. The purified rCFP-10 protein showed only one band when analyzed by 15% SDS-PAGE, with a purity of >90% (Figure 1). The molecular mass of the purified rCFP-10 protein was 14.8 kDa as determined by SDS-PAGE and 13.8 kDa by mass spectrometry.

### DTH reactivity to rCFP-10 in guinea pigs sensitized with killed MTB

Different doses of rCFP-10 protein were tested for their ability to produce DTH responses in guinea pigs sensitized with killed MTB. In the first study, each of the seven guinea pigs (one female guinea pig was dead during the immunization.) was injected intradermally at five sites with PBS as the negative control, PPD (5 IU) as the positive control and 3 doses of rCFP-10 (0.8, 1.0 and 1.2 µg). All three doses of rCFP-10 antigen were able to

**Table 1.** Skin reactivity to different doses of rCFP-10 in guinea pigs sensitized with killed MTB.

Group	Dose	No. of guinea pig	Skin reaction (mean ± SD mm in diameter)		
			24 h	48 h	72 h
PBS	-	7	0	0	0
PPD	5 IU	7	9.5 ± 2.3	7.3 ± 2.5	5.1 ± 1.4
rCFP-10	0.8 µg	7	8.8 ± 1.8	6.6 ± 1.5	4.2 ± 0.6
	1.0 µg	7	8.6 ± 1.6	6.4 ± 1.6	4.1 ± 0.7
	1.2 µg	7	9.1 ± 2.0	7.4 ± 1.8	4.7 ± 1.0

**Table 2.** Skin reactivity to rCFP-10 and rESAT6 in the guinea pig sensitized with live MTB.

Group	Dose	No. of animal	Skin reaction ( mean ± SD mm in diameter)		
			24 h	48 h	72 h
PBS	-	6	0	0	0
PPD	5 IU	6	10.7 ± 2.0	8.2 ± 1.8	7.1 ± 2.6
rESAT6	1.0 µg	6	8.0 ± 4.3	7.0 ± 2.8	6.7 ± 2.9
rCFP-10	1.0 µg	6	10.2 ± 4.8	8.5 ± 3.0	7.5 ± 2.6

defined by an erythema response greater than 5 mm in diameter (Table 1). The difference in skin reaction was not statistically significant ( $P > 0.05$ ). The positive responses measured at the 24 h time point were generally stronger than that at 48 h for all groups, though there was no statistically significant difference. By 72 h, the reaction to PPD had decreased markedly as compared to the reactions at the previous time points. All reactions to rCFP-10 reduced to less than 5 mm in diameter and were therefore considered negative.

#### Skin reactivity to rCFP-10 in guinea pigs sensitized with the live MTB

Next, we determine how the animals sensitized with the live MTB would react to rCFP-10 antigen in the skin test. Six guinea pigs were sensitized by a peritoneal injection of 100 live MTB in 0.5 ml of saline. Five weeks after the challenge, each of the six animals was injected intradermally at four sites with PBS, PPD (5 IU), rESAT-6 (1.0 µg) and rCFP-10 (1.0 µg). DTH responses with similar intensity were elicited by the PPD positive control, rCFP-10 and ESAT6 at all three time points (Table 2). The reactions in the live MTB sensitized animal were stronger than those observed in the animals sensitized with the dead MTB.

#### Specificity of the skin reaction elicited by rCFP-10

Twenty-four guinea pigs were divided into 3 groups of

eight animals per group with half male and half female. One group was sensitized with the live BCG vaccine and the remaining 2 groups were sensitized with killed MTB and *M. bovis*, as listed in Table 3. Each guinea pig was injected intradermally in four separate sites on the back of the animals with 0.1 ml of PBS as the negative control, 0.1 ml of PPD (5 IU) and rESAT-6 (1 µg) as the positive controls, 0.1 ml of rCFP-10 antigen at 10 µg/ml (1 µg total), respectively. The diameters of reactivity in guinea pigs at 24 and 48 h are shown in Table 3. Twenty-four hours following injection, all guinea pigs in three groups had a positive DTH reaction to PPD, while only the guinea pigs sensitized by MTB and *M. bovis*, both of which express ESAT-6 and CFP-10, reacted positively to ESAT-6 and CFP-10. Forty-eight hours following the injection, the size of the skin responses had decreased, some of them were less than 5 mm in size. All animals sensitized with the live BCG vaccine had positive reactions only to PPD, and were negative to ESAT-6 and CFP-10 as well as the negative control.

#### DISCUSSION

It has been shown that ESAT-6 could be used as an antigen in skin test for the detection of MTB infection (Wu et al., 2008; Elhay et al., 1998; Pollock et al., 2003). However, the population with different HLA type may recognize different epitopes. CFP-10 may induce DTH responses in people who do not respond to ESAT-6, overcoming the problems related to genetic restriction in antigen recognition and improving the sensitivity of skin

**Table 3.** Skin reactivity to rCFP-10 in guinea pigs sensitized with three strains of *M. tuberculosis* complex.

Group	Skin reaction (mean $\pm$ SD mm in diameter)					
	24 h			48 h		
	PPD	rESAT-6	rCFP-10	PPD	rESAT-6	rCFP-10
Dead <i>M. tuberculosis</i>	8.4 $\pm$ 1.2	7.0 $\pm$ 1.7	5.6 $\pm$ 0.6	4.8 $\pm$ 1.2	5.1 $\pm$ 1.1	3.7 $\pm$ 0.6
Dead <i>M. bovis</i>	7.0 $\pm$ 1.3	6.2 $\pm$ 0.5	5.4 $\pm$ 0.4	4.4 $\pm$ 0.9	4.4 $\pm$ 0.6	3.5 $\pm$ 0.5
Live BCG vaccine	6.5 $\pm$ 0.9	0	0	5.5 $\pm$ 1.4	0	0

In this study, rCFP-10 protein from *M. tuberculosis* was successfully cloned, expressed and purified. Induction at 30°C produced high level of rCFP-10 in its soluble form. After purification, the rCFP-10 antigen used in this study was greater than 90% pure. The molecular mass of rCFP-10 was 14.8 kDa determined by SDS-PAGE and 13.8 kDa by mass spectrometry.

We found that the recombinant CFP-10 antigen has great potential as a skin test reagent, as demonstrated by our results in guinea pig models. Specifically, 1.2  $\mu$ g of purified rCFP-10 antigen of *M. tuberculosis* produces DTH responses with levels of intensity similar to those induced by PPD and rESAT-6 antigen in guinea pig skin tests. Although the sizes of the reaction to both recombinant antigens, rCFP-10 and rESAT6, were slightly smaller than the size induced by PPD, we found that the difference in size has no statistical significance. The peak DTH response in guinea pigs was observed at 24 h. The responses to all antigens used in our experiments diminished at the 48 h time point and most reactions became negative by 72 h. Our observation on the timing of the DTH response in guinea pigs is consistent with other studies of DTH in animals, which is different from the DTH response observed in humans where the peak reaction occurs at 48 to 72 h (Lyashchenko et al., 1998; Romain et al., 1993).

The rCFP-10 protein as a skin test antigen appeared to be very sensitive to TB infection. The rCFP-10 protein was strongly recognized in all MTB-infected (both live and dead) guinea pigs. One of the main advantages of rCFP-10 is its potential for greater specificity than the widely used mixed protein preparation, PPD. PPD contains many antigens commonly produced by different species of *Mycobacteria*, and therefore can induce cross-reactivity among the animals and people who could either have tuberculous infection, non-tuberculous mycobacterial infection or BCG vaccination. However, the gene coding the CFP-10 protein is located in RD1, which has been identified to be present only in the genome of *M. tuberculosis*, *M. bovis*, *Mycobacterium africanum*, *Mycobacterium kansasii*, *Mycobacterium marinum*, *Mycobacterium szulgai* and *Mycobacterium flavescens*, and is specifically not present

in the BCG vaccine strains as well as not in 90% of the environmental *Mycobacteria* (Behr et al., 1999; Behr, 2001; Harboe et al., 1996). Previous study has also investigated the specificity of CFP-10 as a diagnostic antigen in animal models, and the results from these studies are consistent with what we have found (Colangeli et al., 2000). Colangeli et al. (2000) demonstrated that rCFP-10 is capable of eliciting a skin test reaction in guinea pigs immunized with *M. tuberculosis*, but does not elicit any reaction in the guinea pigs immunized with BCG vaccine and *M. avium*.

In addition to having a greater specificity to MTB infection than PPD, there are many other advantages to developing a skin test that utilizes a single recombinant antigen. Firstly, manufacturing of PPD under good manufacturing practices (GMP) is practically impossible, because of the inconsistent nature of the manufacturing process and the large number of different proteins in the mixture; batch-to-batch variation in the production of PPD at various facilities has been well documented. Secondly, as a single recombinant protein produced in *E. coli*, rCFP-10 being similar with rESAT-6 could be produced in an effective and consistent manner that conforms to GMP standards of the World Health Organization or local regulatory agencies, and is likely to produce consistent and interpretable responses when used. Therefore, the use of purified recombinant antigens should facilitate manufacturing and quality control of skin test reagents.

CFP-10 is currently used as a stimulating antigen in the T-SPOT-TB assay (Oxford Immunotec, Oxford, United Kingdom) and *in vitro* Quantiferon-Gold test (Cellestis); much work has been done with the product that confirms the sensitivity and specificity of this protein as a diagnostic agent (Skjot et al., 2000; Colangeli, 2000). Its use in a skin test could have advantages over Quantiferon in some settings, as a skin test method may be more inexpensive, and does not require a laboratory setting and trained laboratory technicians.

In summary, the present study strongly suggests that recombinant CFP-10 protein could be used in a skin test for the detection of MTB infection. Future evaluation of CFP-10 for its sensitivity, specificity and safety as a diagnostic reagent for skin test in humans will be forth-

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