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Research Article

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Comparison of specific recombinant proteins to *Mycobacterium tuberculosis* skin test in animal model

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ABSTRACT

Detection of tuberculosis infection by delayed-type hypersensitivity skin test with purified protein derivative (PPD) antigen is still used in many countries including Thailand. The problems of this technique are non-specific reactions in individuals who received BCG vaccination and false positive result in previously exposed to non-tuberculous Mycobacteria (NTM) subjects. Several Mycobacterial specific antigens that can distinguish Mycobacterium tuberculosis from BCG and NTM such as ESAT-6, CFP-10, Rv2031c have been reported. This study found that combined-recombinant antigen consists of ESAT-6, CFP-10 and Rv2031c, gave good specific more than individual antigen in distinguishing MTB infection from BCG vaccination by skin testing in guinea pig.

Keywords: *ESAT-6, CFP-10, Rv2031c, PPD, M. tuberculosis.*

INTRODUCTION

Mycobacterium tuberculosis (MTB) is the world's leading cause of the infectious disease tuberculosis (TB) and has an enormous global impact[1]. Bacillus Calmette-Gue´rin (BCG), the world's most widely used vaccine against TB, is derived from *Mycobacterium bovis* and was attenuated after 230 passages over a period from 1908 to 1921[2]. TB is at crisis levels among the poor communities of many countries. Where human immunodeficiency virus (HIV) infection is common, TB spreads and kills rapidly and is responsible for increasing fatalities each year. BCG vaccine has been used since the 1920s and while this vaccine protects very young children from more invasive forms of TB, adolescents are variably protected and remain susceptible to pulmonary diseases caused by *M. tuberculosis*[3]. Most latently infected individuals as well as many patients with active TB are smear-negative and/or culture negative, complicating the diagnosis of TB. The purified protein derivation (PPD) skin test is based on the delayed-type hypersensitivity (DTH) reaction elicited by a mixture of a large number of *Mycobacteria* antigens, and is used widely as an aid in the detection of MTB infection[4]. However, PPD contains antigens that are also present in Non-Tuberculous Mycobacteria (NTM) and in *M. bovis* (BCG), which is used worldwide as a TB vaccine[5]. The skin test can be falsely positive in those who received the BCG vaccines and can be falsely positive in those who have been exposed previously to NTM. As the result, the PPD skin test cannot be used to definitively MTB infection[6]. A more specific skin test, using MTB-specific antigens, has the potential to greatly improve the current methods of TB prevention, diagnosis and disease control[7]. The key proteins in mycobacterial pathogenesis are the virulence determinant protein ESAT-6 and CFP-10. These proteins are encoded by region of difference 1 (RD1), which is the region absent from Bacillus Calmette–Guérin (BCG) and many attenuated strains of MTB[8].

Because of their absence in BCG and NTM strains, ESAT-6 and CFP-10 have been extensively investigated as MTB specific antigens, and have shown the great potential antigens for diagnosis of MTB infection in humans[9]. The ~9.5 kb RD1, encodes for ESAT-6, is a potent human T cell antigen and a putative vaccine candidate that also contributes to virulence, mediates cellular cytolysis, and inhibits immune response by host cells[10,11]. CFP-10, 10-kDa has been shown to stimulate T cells to produce IFN-γ and exhibit CTL (cytotoxic T lymphocytes) activity in animal models and in humans infected with MTB. It is an excellent candidate for inclusion in an antituberculosis subunit vaccine[12]. Rv2031c antigen (16-kDa, α-crystallin homolog heat-shock protein designated HspX) is the dominant protein which produced by MTB during the latent stage of infection. It is essential for bacterial replication inside macrophages[13]. The expression of the gene coding for the 16 kDa antigen, is tightly regulated by the DosR transcriptional regulator[14]. In this study, we evaluated the utility of ESAT-6, CFP-10, Rv2031c and combined antigen (ESAT-6 + CFP-10 + Rv2031c) as stimulating antigens in a skin test for diagnosis of MTB infection.

MATERIALS AND METHODS

Cloning

The cloning of ESAT-6, CFP-10 and Rv2031c genes were performed according to standard procedures[15]. Briefly, the gene encoding the ESAT-6, CFP-10 and Rv2031c were amplified by PCR using specific primers. The forward primer contained a *N*deI restriction enzyme recognition site. The reverse primer contained a *N*otI restriction enzyme recognition site.

Primer	Forward primer	Reverse primer
ESAT-6	5´-TAT ACA TAT GAC AGA GCA GCA GTG-3’	5’-TTT GCG GCC GCC TAT GCG AAC ATC CCA-3’
CFP-10	5’-TAT ACA TAT G GC AGA GAT GAA G-3’	5’-TTT GCG GCC GCG AAG CCC ATT TGC GAG -3’
Rv2031c	5’-TAT ACA TAT G AC GGA AAA CTT GAC -3’	5’-TTT GCG GCC GCG GTA AAC AAC CCG TCG-3’

PCR amplification was proceeded using 35 cycles of denaturation at 94^{o}C for 2 minutes, annealing at 50 ^{o}C for 30 minutes, extension at 72^{o}C for 30 minutes and final extension at 72 ^{o}C for 7 minutes. The 50 ul PCR reaction mixture included DW 22 ul, 2X Mater Mix (Vivantis) 22 ul, primers 1 ul and genomic *M. tuberculosis* H37RV 1 ul. the final products were checked by 1.5% agarose gel electrophoresis under the current of 100 volts for 55 minute. PCR products were purified from the gel using HiYieldTM GEL/PCR DNA Fragment Extraction Kit (RBC bioscience). PCR products and pET 24b(+) were digested with *N*deI and *N*otI (Vivantis). The reaction of 30 ul was carried out overnight at 37 ^{o}C. The specific DNA fragment and pET-24b(+) vector were ligated using Vivantis reagent. The recombinant plasmid was transformed into TOP10 competent cell (Invitrogen) by heat shock method on kanamycin LB agar plate. A single colony was selected to examine the inserted specific DNA positive clones by PCR and sequencing. Plasmid positive clones were extracted with High-Speed Plasmid Mini Kit (Geneaid). The inserted specific gene in pET-24b(+) was verified by sequencing using T7 promoter and T7 terminator primer. DNA sequences from plasmid positive clones were compared with the data from GenBank and transformed into BL21 competent cell (DE3).

Expression protein

The positive ESAT-6, CFP-10 and Rv2031c clones were picked into 200 ml starter media of LB broth containing kanamycin and incubated 37^{o}C with shaking at 200 rpm for 16-18 hours. Each 50 ml starter (10%) was aliquoted into 2 litters of LB broth containing kanamycin which separated into 4 flasks of 500 ml medium. The cultures were shaked with the speed of 200 rpm at 37^{o}C, until the OD values at 600 nm about 0.6 to 0.7. The expression of gene was induced with 1mM IPTG and incubated 37^{o}C with shaking at 200 rpm for 16-18 hours. Pellet cell was harvested by centrifugation and stored at -80 ^{o}C until protein purification.

Protein Extraction and purification

All steps were performed on ice. All buffers were filtered though 0.45 micron membrane. Pellet of cell was resuspended in 20mM sodium phosphate, 0.5 M NaCl pH 7.4 and sonicated 5 times for 20 seconds at a time on the ice for 2 minutes (with 80% amplitude). The suspension was centrifuged at 4^{o}C, 12,000 rpm for 1 hour and supernatant was discarded.

The pellet was dissolved with 20 mM sodium phosphate containing 0.5 M NaCl and 8M urea pH 7.4. The mixture was stirred overnight. After that, the suspension was centrifuged at 4^{o}C, 12,000 rpm for 1 hour. The supernate was collected and filtrated on 0.45 micron membrane. Finally, 1X Protease inhibitor cocktail (Amresco) was added to the suspension and kept cool.

Proteins were purified on 5 ml of Ni-nitrilotriacetic acid resin using a HisTrap HP (GE). The column was equilibrated using 30 ml of 20 mM sodium phosphate containing 0.5 M NaCl and 8M urea pH 7.4. Crude specific protein was loaded into the column and washed with 30 ml of the same bufferand then washed with 30 ml of the same buffer containing 20 mM immidazole. For refolding step, the column was washed with linear gradient of 8-0 M urea by 30 ml of 20 mM sodium phosphate containing 0.5 M NaCl, 8M urea, 20 mM immidazole pH 7.4 and 30 ml 20 mM sodium phosphate containing 0.5 M NaCl, 20 mM immidazole pH 7.4. Elution linear 0-250 mM immidazole was made using 30 ml of 20 mM sodium phosphate containing 0.5 M NaCl pH 7.4 and 20 mM sodium phosphate containing 0.5 M NaCl and 250 mM immidazole pH 7.4. The scan fraction tube OD values at 280 nm was collected and and protein was checked with the SDS-PAGE. The protein was dialysed with PBS buffer pH 7.3 under cold temperatures. The protein concentration could be increased using Viva spin (GE) by 4,000 rpm centrifugation at 4^{o}C. The purified protein was stored at -20 ^{o}C .

Determination of molecular mass

Differentially expressed protein spots were excised from the 2-DE gels and subjected to in-gel tryptic digestion as previously described. Briefly, the excised protein spots were washed three times with 200 ul of 25 mM NH_4HCO_3/50% acetonitrile (ACN). The gel pieces were dehydrated with 200 ul of 100% ACN, rehydrated with 12.5 ug/ml of sequencing grade trypsin (Promega; adison,WI), and incubated at 37°C for approximately 16 hours. The supernatants were then transferred to a new tube and 100 ul of 50% ACN/0.5% formic acid was added. The mixtures were finally dried with speedvac and the resulting peptides were suspended in 10 ul of 50% ACN/0.1% formic acid. The peptides were then analyzed by MS/MS using micrOTOF-Q II™ ESI-Qq-TOF mass spectrometer (Bruker; Berman, Germany) equipped with an online nanoESI source. MASCOT is a software search engine that uses mass spectrometry data to identity proteins form peptide sequence databases. Mass tolerance of parent and fragmented ions were 1.0 Da and 0.6 Da, respectively. MS/MS ions scores ≥38 were considered significant hits. The molecular mass of purified ESAT-6, CFP-10, Rv2031c proteins were determined by mass spectrometry at the Instrument Test Center, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

***M. tuberculosis* strains**

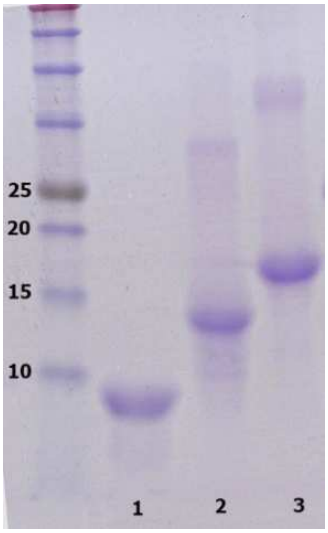
M. tuberculosis H37RV (supported by Faculty of Medical Microbiology, Chulalongkorn University, Thailand) were grown on Lowenstein-Jensen slants at 37^{o}C for 4 weeks and then transferred to Sauton liquid medium at 37^{o}C for 4 weeks without shaking. Powder of *M. tuberculosis* H37RV was killed with heat by autoclave. Then it desiccated and pulverizes into fine powder using mortar with liquid nitrogen. The fine powder was mixed into sterile liquid paraffin by homogenizer[16].

Guinea pig sensitization and skin tests

Various doses (12.5, 25, 50, 100, 200 ug/ml) of ESAT -6, CFP-10, Rv2031c proteins and combined antigens (ESAT-6+CFP-10+Rv2031c) were tested for their ability to produce DTH responses in guinea pigs sensitized with killed *M. tuberculosis* and lived BCG vaccine. Female guinea pigs, weighing 400-450 g, were divided into 3 groups. Group 1 : 16 guinea pigs were sensitized with 0.5 ml of 2 mg/ml killed *M. tuberculosis* H37RV by intramuscular injection into each of two lower limbs[16]. Group 2 : 16 guinea pigs were sensitized with lived BCG vaccine. Sixteen guinea pigs in Group 3 were control group without sensitization. Thirty days after sensitization, guinea pigs were shaved on the abdomen and intradermal injection with 0.1 ml of 12.5 to 200ug the purified ESAT-6, CFP-10, Rv2031c and combined antigens (ESAT-6+CFP-10+Rv2031c) in 0.1 ml, 0.1 ml PBS, 0.1ml of 10 IU PPD (positive control). PPD was produced from Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok, Thailand. The diameters of both axes of skin reaction lesion were measured and recorded within 24-48 hours after inoculation. The skin reaction with more than 5 mm diameter was considered positive.

RESULTS

Fig. 1 : SDS-PAGE analysis of purified ESAT-6, CFP-10 and Rv2031c proteins



The expression protein was purified by His Trap Ni^{2+} column. Lane 1: ESAT-6, Lane 2: CFP-10 and Lane 3: Rv2031c (Kaleidoscope, Bio-Rad is protein molecular weight maker).

Skin reactivity to ESAT-6, CFP-10, Rv2031c and combined antigens (ESAT-6+CFP-10+Rv2031c)

Table 1. Skin reactivity to various doses of ESAT-6, CFP-10, Rv2031c and combined antigens (ESAT-6+CFP-10+Rv2031c) in the guinea pigs (group 1) after sensitization with killed *M. tuberculosis*

injection	Doses	Skin reaction (mean±SD mm. in diameter)	
		24h	48h
PPD	10 IU	9.9±1.6	6.9±1.4
PBS	-	-	-
ESAT-6	12.5 ug	<5	<5
	25 ug	<5	<5
	50 ug	7.4±1.2	5.4±0.5
	100 ug	9.3±1.2	5.4±0.6
	200 ug	9.6±1.5	5.6±0.6
CFP-10	12.5 ug	<5	<5
	25 ug	<5	<5
	50 ug	6.6±0.9	4.9±0.7
	100 ug	7.6±1.4	5.5±0.8
	200 ug	9.6±1.5	5.9±0.7

injection	Doses	Skin reaction (mean±SD mm. in diameter)	
		24h	48h
Rv2031c	12.5 ug	<5	<5
	25 ug	<5	<5
	50 ug	<5	<5
	100 ug	7.1±1.1	<5
	200 ug	7.25±1.1	5.4±0.6
Combined Antigens	12.5 ug	8.8±1.3	<5
	25 ug	10.2±1.0	<5
	50 ug	>10	<5
	100 ug	>10	5.1±0.9
	200 ug	>10	5.5±0.8

PBS, phosphate buffer saline; PPD, purified protein derivative; SD, standard deviation

Table 2. Skin reactivity to various doses of ESAT-6, CFP-10, Rv2031c and combined antigens (ESAT-6+CFP-10+Rv2031c) in the guinea pigs (group 2) after sensitization with live BCG vaccine

injection	Doses	Skin reaction (mean±SD mm. in diameter)	
		24h	48h
PPD	10 IU	9.4±1.5	7.2±1.3
PBS	-	-	-
ESAT-6	12.5 ug	-	-
	25 ug	-	-
	50 ug	-	-
	100 ug	-	-
	200 ug	-	-
CFP-10	12.5 ug	-	-
	25 ug	-	-
	50 ug	-	-
	100 ug	-	-
	200 ug	-	-

injection	Doses	Skin reaction (mean±SD mm. in diameter)	
		24h	48h
Rv2031c	12.5 ug	-	-
	25 ug	-	-
	50 ug	-	-
	100 ug	-	-
	200 ug	-	-
Combined Antigens	12.5 ug	-	-
	25 ug	-	-
	50 ug	-	-
	100 ug	-	-
	200 ug	-	-

PBS, phosphate buffer saline; PPD, purified protein derivative; SD, standard deviation

Table 3. Skin reactivity to various doses of ESAT-6, CFP-10, Rv2031c and combined antigens (ESAT-6+CFP-10+Rv2031c) in the guinea pigs (group 3) without sensitization

injection	Doses	Skin reaction (mean±SD mm. in diameter)	
		24h	48h
PPD	10 IU	-	-
PBS	-	-	-
ESAT-6	12.5	-	-
	25	-	-
	50	-	-
	100	-	-
	200	-	-
CFP-10	12.5	-	-
	25	-	-
	50	-	-
	100	-	-
	200	-	-

injection	Doses	Skin reaction (mean±SD mm. in diameter)	
		24h	48h
Rv2031c	12.5 ug	-	-
	25 ug	-	-
	50 ug	-	-
	100 ug	-	-
	200 ug	-	-
Combined Antigens	12.5 ug	-	-
	25 ug	-	-
	50 ug	-	-
	100 ug	-	-
	200 ug	-	-

PBS, phosphate buffer saline; PPD, purified protein derivative; SD, standard deviation

DISSCUSSION AND CONCLUSION

The optimal condition for ESAT-6, CFP-10 and Rv2031c protein expression were 1 M IPTG induction at 37^{o}C for 16-18 hours and expressed in the inclusion body form of the pellet cell. Length of ESAT-6 , CFP-10 and Rv2031c genes were 288, 303 and 435 bp, respectively. SDS-PAGE pattern of ESAT-6, CFP-10 and Rv2031c proteins were approximately 9, 12 and 16kDa. The purified proteins were confirmed by Mass Spectrometry as ESAT-6, CFP-10 and Rv2031c from protein databases (MASCOT). Sixteen guinea pigs were sensitized with killed *M. tuberculosis* H37RV. Thirty days after the challenge, guinea pigs in were injected intradermally to7 sites on the abdomen of the animals with 0.1 ml of ESAT-6, CFP-10, Rv2031c and combined (ESAT-6+CFP-10+Rv2031c) proteins at 12.5, 25, 50, 100 and 200 ug, respectively. PBS and PPD (10IU) were included as negative and positive control.

The diameters of reactivity in guinea pigs at 24 and 48 hours are shown in Table 1, 2 and 3. A positive result was defined as an average induration greater than 5 mm. Killed *M. tuberculosis* sensitized animals in group 1 had a positive result for PPD, ESAT-6 at over 50 ug, CFP-10 at over 50 ug, Rv2031c at over 100 ug and combined antigen at 12.5 ug (Table 1).

However, the live BCG sensitized 16 guinea pigs, reacted positively only with PPD (Table 2). The animals without sensitization had negative reaction to PPD, PBS, ESAT-6, CFP-10, Rv2031c and combined antigen (Table 3). The size of skin reaction had decreased from 24 hours to 48 hours.The results of skin test showed that combined antigen with the lowest concentration, 12.5 ug, gave the better results than individual protein antigen. At least 50 ug of ESAT-6 and CFP-10 could elicit positive skin test responses, whereas at least 100 ug of Rv2031c was administered for positive skin reaction in guinea pigs (Table1). It showed that ESAT-6, CFP-10 , Rv2031c and combined antigen were capable of eliciting skin test reaction in guinea pigs which immunized with *M. tuberculosis* but could not elicit any reaction in the guinea pigs immunized with BCG vaccine. A positive skin reaction with PPD could not distinguish between *M. tuberculosis* and BCG (Table 1, 2). Our results supported earlier study (Stavri *et al*, 2012) that multi-antigen cocktail (ESAT-6, CFP-10 and MPT64) induced a stronger response in skin test than each of the single antigen. Moreover, recombinant protein mixture could elicit a cellular immune response of the host to TB that will differentiate between TB infection and a successful BCG vaccination[17]. It is expected that the new PPD recombinant protein will probably have a lower price and more affordable diagnostic test for high TB incidence in low income countries in the future, revealed the possibility for developing a new protein which is better than PPD in diagnosis of *M. tuberculosis.*

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REFERENCES

1. Hoft, D. F. Tuberculosis vaccine development: goals, immunological design, and evaluation, *The Lancet*, **372**: 164-175 (2008)
2. Liu, J.Tran, V. Leung, A. S. Alexander, D., and Zhu, B. BCG vaccines: their mechanisms of attenuation and impact on safety and protective efficacy (Review). *Hum Vaccin*., **5(2)**: 70-8 (2009)
3. WHO Informal Consultation on Standardization and Evaluation of BCG Vaccines (Meeting Report), Geneva, Switzerland 22-23 September 2009.
4. Nancy, E. D. John, B. Paula, F. Philip, H. Robert, H. Jr. Max, S., and Patricia, M. S.American Thoracic Society and Centers for Disease control. Diagnostic standards and classification of tuberculosis. *Am Rev Respir Dis*., **142**: 725-35 (1991)
5. Huebner, R. E. Schein, M. F., and Bass, J. B. jr. The tuberculin skin test. *Clin Infect Dis*., **17**: 968-7 (1993)
6. Harboe, M. Antigens of PPD, old tuberculin, and autoclaved *Mycobacterium bovis* BCG studied by crossed immunoelectrophoresis. *Am Rev Respir Dis*., **124**: 80-7 (1981)
7. Wu, X. Zhang, L. Zhang, J. Zhang, C. Zhu, L., and Shi, Y. Recombinant early secreted antigen target 6 protein as a skin test antigen for the specific detection of *Mycobacterium tuberculosis* infection. *Clin ExpImmunol*., **152(1)**: 81-7 (2008)
8. Brodin, P. Eiglmeier, K. Marmiesse, M. Billault, A.Garnier, T.Niemann, S. Cole, S. T.,and Brosch, R. Bacterial artificial chromosome-based comparative genomic analysis identifies *Mycobacterium microti* as a natural ESAT-6 deletion mutant. Infect Immun., **70**: 5568–5578 (2002)
9. Arend, S.M. Andersen, P. van Meijgaarden, K.E. Skjot, R. L.Subronto, Y.W. van Dissel, J.T., and Ottenhoff, T. H. Detection of active tuberculosis infection by T cell responses to early-secreted antigenic target 6-kDa protein and culture filtrate protein 10. *J Infect Dis*., **181(5)**: 1850-4 (2000)
10. Cole, S.T. Brosch, R., and Parkhill, J. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature, **393**: 537–544 (1998)
11. Wang, X. Barnes, P.F. Dobos-Elder, K. M. Townsend, J. C. Chung, Y. T. Shams, H. Weis, S. E., and Samten, B. ESAT-6 inhibits production of IFN-gamma by *Mycobacterium tuberculosis*-responsive human T cells. *J. Immunol*., **182**: 3668–3677 (2009)

12. Arend, S. M. Geluk, A. van Meijgaarden, K. E. van Dissel, J. T. Theisen, M. Andersen, P., and Ottenhoff, T. H. Antigenic equivalence of human T-cell responses to *Mycobacterium tuberculosis*-specific RD1-encoded protein antigens ESAT-6 and culture filtrate protein 10 and to mixtures of synthetic peptides. *Infect Immun*., **68(6)**: 3314-21 (2000)
13. Hu, Y. Movahedzadeh, F.Stoker, N., *and* Coates, A. R. M. Deletion of the *Mycobacterium tuberculosis* α-crystallin-like *hspX* Gene causes increased bacterial growth *in vivo*. *Infect Immun*.,**74***:* 861–8 (2006)
14. Ottenhoff, T. H. M., *and* Kaufmann, S. H. E. Vaccines against tuberculosis: where are we and where do we need to go?. *PLoSPathog.,* **8**: (2012)
15. Michael, R. Green. Joseph Sambrook; *Molecular cloning, a laboratory manual*, Second Edition, Cold Spring Harbor, NY (1989)
16. WHO Technical Report Series, No. **745**: 45 (1987)
17. Henriette, S. Nadia, B. Irina, U. Adriana, C. Loredana, P., and Mircea, I. P. Use of recombinant purified protein derivative (PPD) antigens as specific skin test for Tuberculosis. *Indian J Med Res*., **136**: 799-807 (2012)