นิพนธ์ต้นฉบับ

การสร้างและผลิตโปรตีน 16 kDa จากเชื้อ *Mycobacterium tuberculosis* เพื่อใช้ในการพัฒนาการตรวจวินิจฉัยวัณโรค

ปนัดดา เทพอัคศร* อณิชา เลื่องชัยเชวง* สกุลรัตน์ พัดพรม* ภัทรพร ทองไทย* เครือวัลย์ พลจันทร* ปฐม สวรรค์ปัญญาเลิศ*

บทคัดย่อ

วัตถุประสงค์ เพื่อสร้างและผลิตโปรตีน 16 kDa ซึ่งเป็นแอนติเจนที่จำเพาะต่อเชื้อ *Mycobacterium tuberculosis* ในหลอดทดลองและตรวจสอบคุณสมบัติเพื่อพัฒนาชุดตรวจวินิจฉัยวัณโรค โดยวิธี serodiagnosis

วิธีการ สร้างรีคอมบิแนนท์พลาสมิด pET21a-hspX และนำเข้าสู่เซลล์แบคทีเรีย *Escherichia coli* BL21(DE3) เพื่อผลิตโปรตีน 16 kDa แอนติเจน จากนั้นนำแอนติเจน ที่ได้ไปทดสอบปฏิกิริยาทางภูมิคุ้มกันกับตัวอย่างซีรัมกลุ่มต่างๆ โดยวิธี ELISA และ Western blot analysis

ผลการทดลอง สามารถสร้างและผลิตโปรตีน 16 kDa ได้ในปริมาณและความบริสุทธิ์สูง โดยอยู่ในรูปของ inclusion bodies และเมื่อทำการแยกสกัดเพื่อใช้ทดสอบกับซีรัมกลุ่มต่างๆ พบว่า สามารถทำปฏิกิริยาทางภูมิคุ้มกันได้อย่างจำเพาะกับซีรัมในกลุ่มผู้ป่วยวัณโรค ได้แก่ ผู้ป่วยวัณโรคปอด (Pulmonary TB) ทั้งที่ตรวจพบและไม่พบเชื้อ โดยการย้อมสี รวมทั้งกลุ่มผู้ป่วยวัณโรคนอกปอด (Extrapulmonary TB)

สรุป จากผลการทดลองพบว่า โปรตีน 16 kDa ของเชื้อ *M. tuberculosis* ที่ผลิตได้มีศักยภาพที่สามารถนำไปใช้พัฒนาการตรวจวินิจฉัยวัณโรค โดยวิธี serodiagnosis ต่อไปได้ **วารสารเทคนิคการแพทย์เชียงใหม่ 2541; 41: 204-213.**

คำสำคัญ 16 kDa antigen, serodiagnosis, *Mycobacterium tuberculosis*, tuberculosis

* ศูนย์เทคโนโลยีชีวภาพด้านการแพทย์และสาธารณสุข กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข

Abstract: Construction and production of 16 kDa antigen from *Mycobacterium tuberculosis* for the development of TB diagnotic test

Panadda Dhepakson* Anicha Luengchaichaweng* Sakulrat Pudprom* Pataraporn Thongthai*
Kruavon Balachandra* Pathom Sawanpunyalert*

Objective To construct and produce protein of 16 kDa antigen from *Mycobacterium tuberculosis* by genetic engineering method and evaluate the potential of the antigen to be used in serodiagnosis of tuberculosis (TB)
Materials and Methods The plasmid pET21a-hspX was constructed and transformed into *Escherichia coli* BL21 (DE3) to express protein of 16 kDa antigen. The isolated antigen was then evaluated for its potential to be used in antibody detection by ELISA and Western blot analysis
Results The protein 16 kDa antigen was expressed at high level in *E. coli* in insoluble form (inclusion bodies). The protein could be obtained at high yield and purity after isolation, solubilization and refolding. By Western blot analysis and ELISA, the 16 kDa antigen showed strong reactivity with serum of TB patients including smear positive pulmonary TB, smear negative and extrapulmonary TB.
Conclusion The 16 kDa TB antigen was successfully cloned and expressed in this study. This antigen showed high potential to be used for serodiagnosis of TB in further study. **Bull Chiang Mai Assoc Med Sci 2008; 41: 204-213.**

Keywords: 16 kDa antigen, serodiagnosis, *Mycobacterium tuberculosis*, tuberculosis

*Medical Biotechnology Center, Department of Medical Sciences, Ministry of Public Health

Introduction

Tuberculosis (TB) remains a major global health problem. Approximately, one-third of the world's population is infected with *Mycobacterium tuberculosis* and it is estimated that 8.3 million new cases and three million deaths occur each year.[1]

More than one half of all infects occur in China, Southeast Asia and the Indian sub continent. Being major of opportunistic infection in human immunodeficiency virus (HIV) infected individuals and increasing multidrug-resistant strains of *M. tuberculosis* have made more alarming of TB in human.[2] To prevent the spread of both diseases, it is needed to develop diagnostic tool that is rapid, inexpensive, but clinically sensitive and specific that can improve upon current methods including culture and acid fast stain.[3] PCR methods are also sensitive but expensive and difficult to apply for field use.[4] The low cost and relatively simple of immunoassays have encouraged investigators to attempt to develop serologic

test for TB diagnosis.[5] However, for the successful assay, immunodominant mycobacterial antigens need to be identified. Among various antigens that have shown promising in serodiagnosis of tuberculosis, the 38-kDa antigen has been found to be very useful. The 38-kDa antigen is a phosphate transport protein (PstS-1).[6-7] It has been used in the development of several commercial assays for the detection of TB.[8-9] While highly specificity for TB, it lacks sensitivity, particularly in the detection of smear negative TB infected individuals.[10] The sensitivity and specificity of 38-kDa antigen also varies with population.[11] Therefore, it can to be used in combination with other *M. tuberculosis* antigens. The 16 kDa antigen is an immunodominant antigen, frequently called 14 kDa protein and belonged to the alpha –crystallin family of low molecular weight heat shock proteins.[12-13] This antigen contains *M. tuberculosis* complex specific B cell epitopes and several studies showed that the combination of this antigen with 38 kDa antigen could increase sensitivity of TB detection.[14-16]

To evaluate the potential of various mycobacterial antigens in serodiagnosis of TB in Thai population, we undertook a study to clone, overexpress, and purify several important mycobacterial antigens in *E. coli* (unpublished data). This paper described cloning, high level expression, and isolation of the His6 tagged 16-kDa antigen of *M. tuberculosis* and determined if the protein could complement 38 kDa antigen for serodiagnosis. The obtained protein showed strong reactivity with antibodies in serum of TB patients that were negative for antibodies to 38 kDa antigen including positive smear, negative smear, and extra-pulmonary tuberculosis patients. The proteins were also evaluated for their specificity of *M. tuberculosis* detection using normal pooled serum.

Materials and Methods

Sample

Serum samples from TB patients and non TB patients were kindly provided from Research Institute of Tuberculosis, Chiang Rai. Data of AFB stains were also recorded, and provided as coded samples without disclosing any information. The normal control serum samples were also obtained from healthy donors and showed negative for antibodies to 38 kDa antigen.

Cloning of 16 kDa antigen. The *hspX* gene encoded for *M. tuberculosis* 16 kDa antigen was amplified from genomic DNA by PCR using primers hspx-BamHI.F : 5' GCC GGA TCC AGG AGG CAT CAA ATG G 3' with BamH I site (underlined) and hspx-Xho I.R : 5' GGC CTC GAG TTG GTG GAC CGG ATC T 3' with Xho I site (underlined) as upsteam and downstream primers, respectively. The amplification reaction was performed in reaction mixture (50 μl) for 5 mins at 95 °C, 30 cycles of 1 min at 95 °C, 1 min at 59 °C, 1 min at 72 °C and a final extension for 10 mins at 72 °C. The PCR product after gel purification was digested with *Xho* I and *Bam*H I and ligated with digested *Xho* I and *Bam*H I expression vector pET 21a (Novagen). The obtained recombinant containing insert size of 435 bps was named pET 21a-hspX.

Expression and Characterization of 16 kDa antigen

For the expression of 16 kDa antigen, *E. coli* BL21 (DE3) cells were transformed with plasmid pET 21a-hspX and the plates were incubated at 37 °C for 16 hrs. Transformed cells from the plates were inoculated in 1L LB medium containing 100 μg/ml

ampicillin and grown at 37°C with vigorous shaking. At an OD 600 nm of 0.8–1.0, the expression of protein was induced by adding isopropyl thiogalactoside (IPTG) (Sigma) to a final concentration of 1 mM and cultured for additional 4 hrs. The pellet was collected and lysed in SDS-PAGE sample buffer. Lysate and lysate pellet were collected and applied to 10% SDS-polyacrylamide gel elctrophoresis Finally, gel was stained with Coomassie Brilliant Blue.

Isolation of inclusion bodies. The induced culture was harvested by centrifugation at 6,500xg for 15 mins at 4 °C. The cell pellet was thoroughly resuspended with 0.1 culture volume of wash buffer (20mM Tris-HCl pH 7.5, 10 mM EDTA, 10% TritonX-100). The cells were lysed by adding lysozyme to a final concentration of 100 µl/ml, mixed by swirling and sonicated on ice until solution was no longer viscous. Crude extract was centrifuged at 10,000xg for 10 mins, the supernatant was discarded and the pellet containing inclusion bodies were kept for solubilization and refolding.

Solubilization and refolding. The insoluble protein (inclusion bodies) was isolated and solubilized under denaturing conditions by adding solubilization buffer (50 mM CAPS, pH 11.0 and 0.3% N-lauroylsarcosine) with gently mixed and incubated at room temperature for 15 mins. After centrifugation at 10,000xg for 10 mins at room temperature, the supernatant containing solubilized protein was transferred to a clean tube and refolded by dialysis with buffer containing 20 M Tris-HCl, pH 8.5, 0.1 mM DTT. At least two buffer changes of mone than 50 volume of the sample were performed.

Turbidity assay for N-lauroylsarcosine. The concentration of residual N- lauroylsarcosine in protein solution after dialysis can be assayed. Briefly, the stock solution of 0.3% N-lauroylsarcosine was prepared and 10 fold serially diluted in deionized water to prepare a series of standards at concentration of 0.3%, 0.03%, 0.003% and 0.0003%, respectively. The solution of protein sample was prepared in the same manber with final volume of 1 ml. HCl (1N) was added 100 µl each. The mixtures were incubated at room temperature for 5 mins and measured at wavelength of 405 nm using deionized water as blank. Standard curve was created and used to determine the N-lauroylsarcosine concentration in the samples.

Determination of specific reactivity of 16 kDa antigen by western blot analysis. Samples of isolated 16 kDa antigen were run onto 10% SDS-PAGE along with a molecular weight marker (Bio-Rad) and transfered electroelectically onto nitrocellulose membrane (Bio-Rad). Membranes were blocked and then reacted with pooled serum (1:1000) and anti-His antibody (1:500) (QIAGEN) using anti 16 kDa antigen monoclonal antibody (AbD Serotec) as positive control. After several washing steps, membranes were incubated with Horseradish peroxidase conjugated rabbit anti-mouse IgG (DAKO, 1:1000) for 30 mins. After washing, membranes were developed using 3,3-Diaminobenzidine (Sigma).

ELISA

Ninety six well microtiter plates (Nunc) were coated overnight at 4 °C with 100 µl of 16 kDa antigen (0.2 ug/well) in 0.1 M carbonate-bicarbonate buffer, pH 9.6. Plates were then aspirated, blocked with phosphate buffer saline containing 5% (W/V) skimmed milk for 1.5 hrs at 37 °C and then washed in PBS containing 0.1% Tween 20 (PBST) pooled. The diluted pooled serum (1:2000 in PBST containing 0.1% BSA) was added to antigen-coated

wells in duplicate and incubated for 45 mins at RT. The plates were washed six times with PBST and incubated with 100 µl of 1:10,000 HRP conjugated goat anti-Human IgG for 30 mins at RT. The plates were washed six times with PBST and three times with PBS. One hundred microliter of Tetramethylbenzidine (TMB) substrate was added, incubated for 30 min at RT. The reaction was stopped by adding 100 µl of 1 N HCl to each well and the optical density at 450 nm was measured with microplate reader (Biotek). The cutoff for the assay was the mean of the negative population plus 3 standard deviations (SD) of the mean.

Results

Cloning and expression of 16 kDa antigen

The recombinant plasmid pET 21a-hspX containing *hspX* gene encoded for 16 kDa antigen was transformed to *Escherichia coli* BL21 (DE3) (Fig.1A). The transformant was selected and used to produce a protein of 16 kDa antigen. The expression protein of 16 kDa antigen was induced by 1 mM IPTG for 4 hrs. at 37 °C. The bacterial cells were lyzed by sonication in lysis buffer and separated into soluble and insoluble fractions by centrifugation. The fractions were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining (Fig. 1B). The result showed that almost of the protein molecular weight 16 kDa was found in the insoluble fraction of the crude extract.

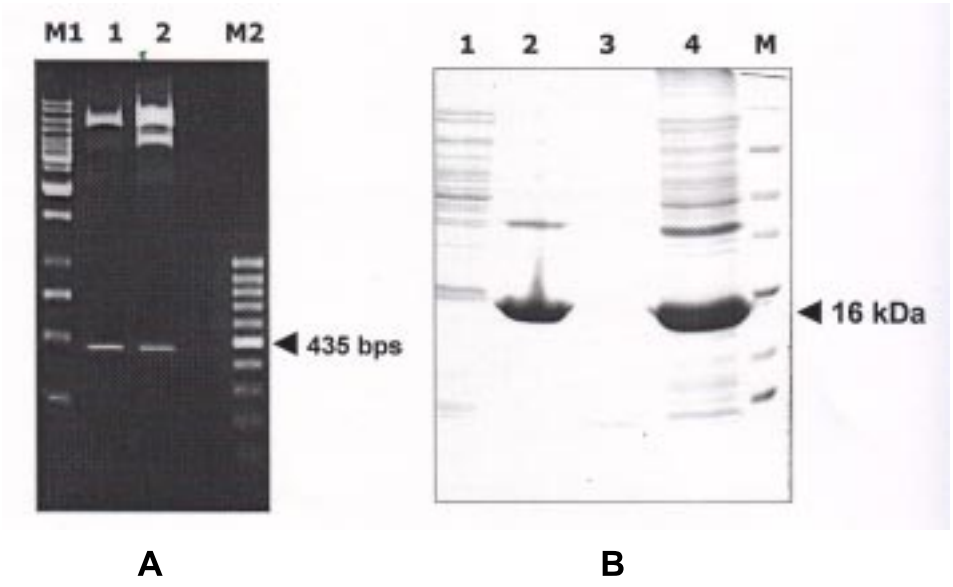

Figure 1. Cloning and Expression of 16 kDa antigen. The transformants were confirmed by double digestion with restriction enzymes *Bam*HI and *Xho*I and analysed by agarose gel electrophoresis (A), Lane M1: 100 bp ladder DNA marker, M2: DNA ladder (mix) marker, Lane 1, 2: plasmid pET 21a-hspX after double digestion with *Bam*HI and *Xho*I showed the insert size of 435 bps. The 16 kDa antigen was expressed in insoluble form (B), Lane1 and 3: cell lysate, Lane 2: cell pellet using lysis buffer containing 1% NP40, Lane 4: cell pellet using lysis buffer containing 1% Triton-X 100, M: Molecular weight marker.

Isolation of inclusion bodies, protein solubilization and refolding

The insoluble protein (inclusion bodies) was successfully isolated in lysis buffer containing lysozyme. The recombinant protein was then solubilized from inclusion bodies with buffer containing 0.3% N-Laurylsarcosine and refolded by dialysis. The obtained protein was confirmed by immunoblotting and probed with anti-His monoclonal antibody. As shown in figure 2A, the molecular weight of the protein on specific band was about 16 kDa as determined by SDS-PAGE in accordance to the predicted molecular weight.

In order to estimate purity and total yield, the refolded protein was run on SDS-PAGE following with Coomassie Brilliant Blue stained using various concentrations of BSA as standard protein. The obtained 16 kDa antigen constituted more than 90% of total protein and exhibited the total yield of 50 mg/L of culture medium (Fig 2B).

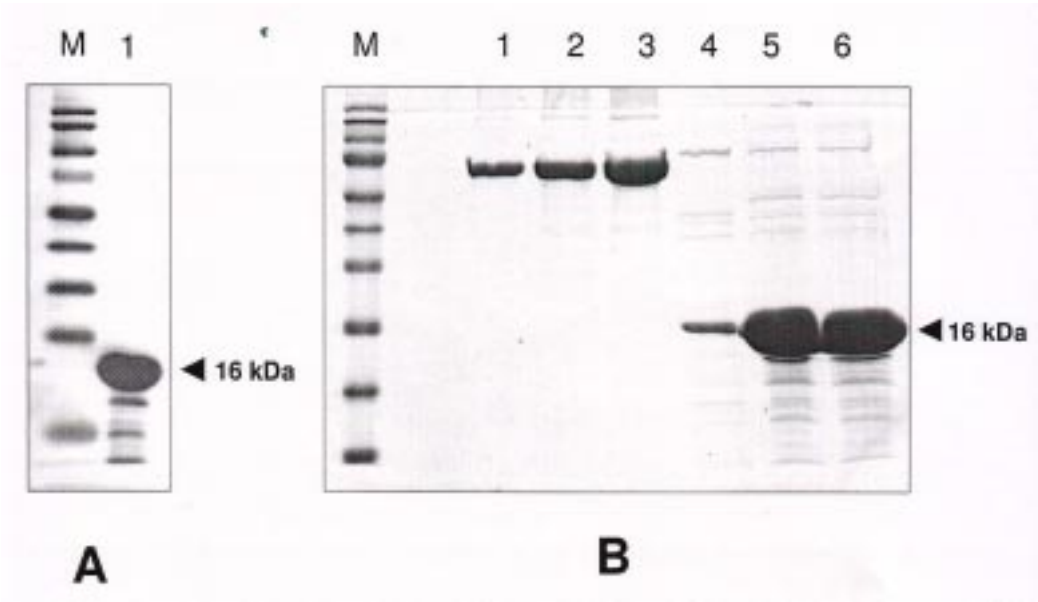

Figure 2. Solubillzation and refolding of 16 kDa antigen. The specificity of recombinant protein of 16 kDa antigen was confirmed by immunoblotting and probed with anti His monoclonal antibody (A), M: Molecular weight marker, Lane 1: the 16 kDa antigen after refolding. The soluble protein could be obtained after solubilization and refolding (B) at yield of 50 mg/L of culture medium, Lane1-3: BSA standard (10, 25 and 50 mg/ml) Lane 4-6: solubilized protein before concentration (Lane 4) and after concentration (Lane 5-6).

Turbidity assay for N-lauroylsarcosine

Turbidity assay was performed in order to determine the residual of N-lauroylsarcosine that may affect biological activity of the protein after refolding. The diagram in Figure 3 showed that the concentration of N-lauroylsarcosine after dialysis was markly reduced to lower than 100 fold of starting concentration in solubilization step.

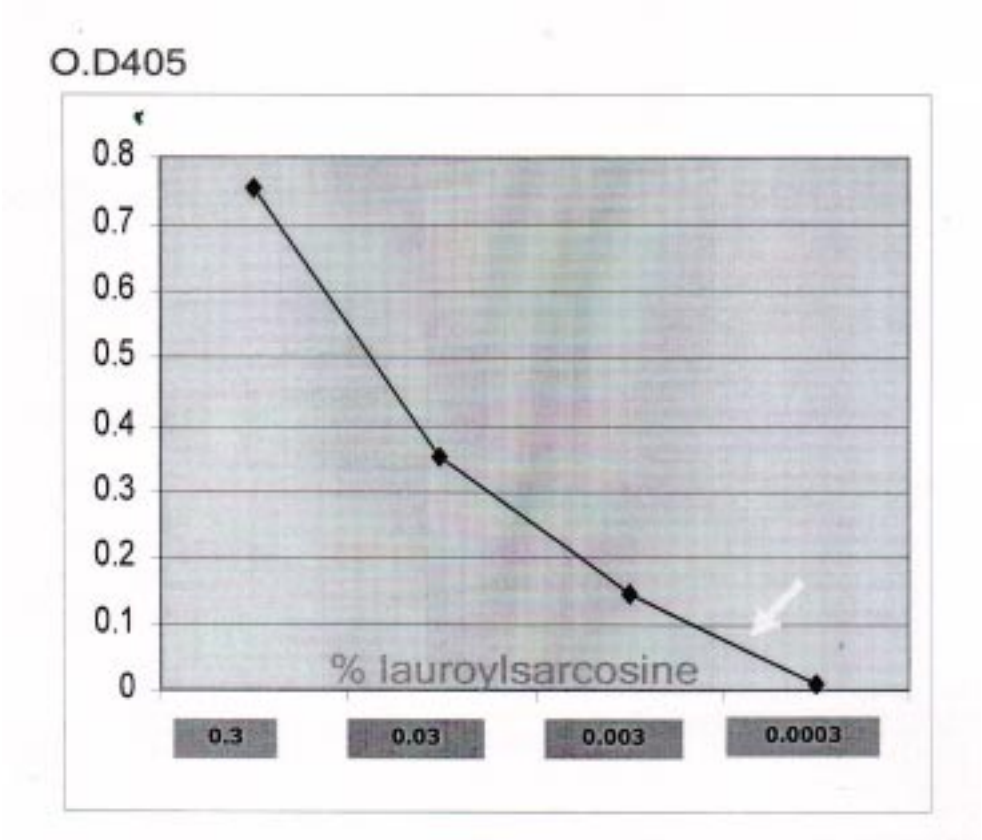

Figure 3. Determination of N-lauroylsarcosine concentration by turbidity assay. The concentration of residual N-lauroylsarcosine in protein solution that contained the 16 kDa antigen after dialysis was lower than 100–fold of starting concentration in solubilization step.(indicated by the arrow)

Determination of specific reactivity of 16 kDa antigen by western blot analysis and ELISA

In order to determine the specific reactivity of the 16 kDa antigen with antibodies in serum, an immunoblotting was performed and reacted with various of serum samples. As shown in Figure 4, by western blot analysis, the 16 kDa antigen reacted with antibodies in serum of TB patients (Fig. 4C-E) using anti-16 kDa monoclonal antibody as positive control (Fig. 4A). In addition, no specific band was observed in normal serum sample. (Fig. 4B).

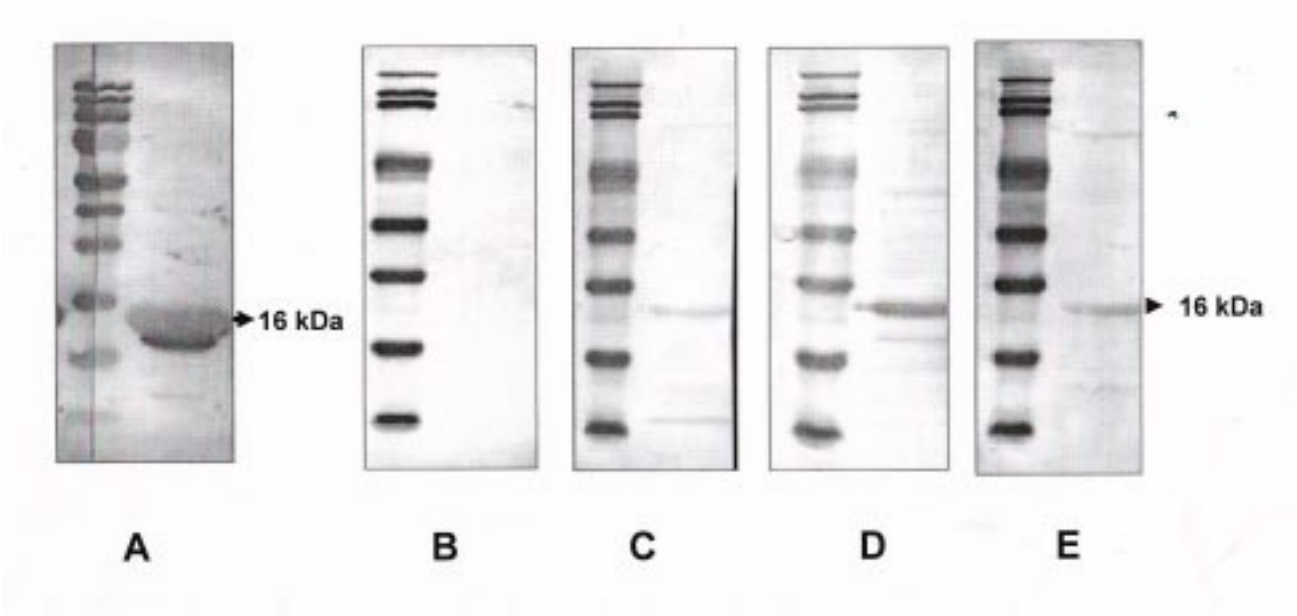

Figure 4. Determination of specific reactivity of 16 kDa antigen by western blot analysis. The specific band of 16 kDa was observed when the membranes were probed with antibodies from: serum of TB patients with positive smear (C), negative smear (D), and extrapulmonary TB (E). No band was observed in normal serum (B), using anti 16 kDa monoclonal antibody as positive control (A).

The 16 kDa antigen was used in ELISA to detect antibodies in sera of patients with tuberculosis. First, serum samples from healthy donors were analysed to calculate mean and SD. The cut off for positive samples was set at mean plus 3 SD. Table 1 shows the reactivity of 16 kDa with sera from tuberculosis patients. The 16 kDa antigen could react with antibodies in samples from pulmonary tuberculosis as well as smear positive smear with reactivity of more than mean plus 3 SD. The similar results were obsorved in sera from patients with extra pulmonary TB. In addition, normal sera was non-reactive with 16 kDa antigen.

Table 1. Determination of specific reactivity by ELISA. The 16 kDa antigen could react with antibodies from sera of TB patients with negative for 38 kDa antigen with no cross reactivity in normal sera.

Serum samples from		ELISA (OD 450)
Pulmonary TB smear positive		2.215
Pulmonary TB smear negative		1.143
Extrapulmonry TB		0.991
Donor		0.109
	cut-off	0.322

Discussion

In this study, 16 kDa antigen, a mycobacterial protein with immunodiagnostic potential have been cloned and overexpressed in *E. coli*. The protein was expressed at high level but accumulated as inclusion bodies. Use of simple protocols including isolation of inclusion bodies by centrifugation, solubilization in denaturing condition using 0.3% N–lauroysarcosine and refolding by dialysis provided almost pure product at the yield of 50 mg/L of culture medium. Previous reports have also shown that 38 kDa antigen was expressed in insoluble form and yielded pure proteins with properties similar to native 38 kDa antigen from *M. tuberculsis* after solubilization and refolding.[17]

To best achievement in development of serological assay for TB, several criteria for selecting of antigens were widely used.[18] The selected gene encoded for protein antigen should be specific to *M. tuberculosis*. The potential antigens should be complementary to the 38-kDa antigen in detecting active TB and could improve detection of disease in smear-negative, extrapulmonary TB, and HIV-TB. Finally, those antigens should not react with sera from BCG vaccinated patients.

To evaluate the potential of 16 kDa antigen in serodiagnosis of TB in Thai population, testing for reactivity against the 16 kDa antigen by Western blot analysis and ELISA were performed using sera that had been tested negative for TB in the 38-kDa antigen immunodiagnostic assay. The results revealed that the protein was a specific antigen among TB patients and provided high potential to be used as antigen for antibody detection in TB diagnosis. Further investigation of individual serum samples and the big sample size will be conducted to determine the variation between samples and the capacity of detection of developed test. In addition, these preliminary results demonstrated that the obtained 16 kDa antigen could complement 38-kDa antigen reactivity in serological testing for detection of active pulmonary and extrapulmonary TB patients and might be used effectively with this antigen in a cocktails to form the unique serological assay.

References

1. Dye C, Scheele S, Dolin P, Pathania V, Raviglione MC. Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. JAMA 1999; 282: 677-86.
2. Harries AD, Hargreaves NJ, Kemp J, Jindani A, *et al*. Deaths from tuberculosis in sub-Saharan African countries with a high prevalence of HIV-1. Lancet. 2001: 357; 1519-23.
3. Foulds J, O' Brien R. New tools for the diagnosis of tuberculosis: the perspective of developing countries. Int J Tuberc Lung Dis. 1998: 2; 778-83.
4. Miller N, Hernandez SG, Cleary TJ. Evaluation of Gen-Probe Amplified *Mycobacterium tuberculosis* Direct Test and PCR for direct detection of *Mycobacterium tuberculosis* in clinical specimens. J. Clin. Microbiol. 1994; 32: 393–7.
5. Silva VMC, Kanaujia G, Gennaro ML, Menzies D. Factor associated with humoral response to ESAT-6, 38 kDa and 14 kDa in patients with a spectrum of tuberculosis. Int J Tuberc Lung Dis. 2003; 7: 478-84.
6. Bothamley GH, Rudd RM. Clinical evaluation of a serological assay using a monoclonal antibody (TB72) to the 38kDa antigen of *Mycobacterium tuberculosis*. Eur Respir J. 1994; 7: 240–6.

7. Chang Z, Choudhary A, Lithigra R, Quiocho FA. The immunodominant 38-kDa lipoprotein antigen of *Mycobacterium tuberculosis* is a phosphate-binding protein. J Biol Chem. 1994; 269: 1956–8.
8. Reddy JR, Kwang J, Lechtenberg KF, Khan NC, R.B. Prasad, Chengappa MM. An immuno-chromatographic serological assay for the diagnosis of *Mycobacterium tuberculosis*. Comp Immunol Microbiol Infect Dis. 2000; 25: 21–7.
9. 9Perkins MD, Conde MB, M, Martins M, Kritski AL. Serologic diagnosis of tuberculosis using a simple commercial multiantigen assay. Chest. 2003; 123: 107–12.
10. Wilkinson RJ, Haslov K, Rappuoli R, *et al*. Evaluation of the recombinant 38-kDa antigen of *Mycobacterium tuberculosis* as a potential immunodiagnostic reagent. J Clin Microbiol. 1997; 35: 553–7.
11. Pottumarthy S, Wells VC, Morris AJ. A comparison of seven tests for serological diagnosis of tuberculosis. J Clin Microbiol. 2000; 38: 2227-31.
12. Raja A, Uma Devi KR, Ramalingam B, Brennan PJ. Immunoglobulin G, A, and M responses in serum and circulating immune complexes elicited by the 16-kilodalton antigen of *Mycobacterium tuberculosis*. Clin Diagn Lab Immunol. 2002; 9(2): 308-12.
13. Verbon A, Hartskeerl A, Schuitema A, Kolk AH, Young DB, Lathigra R. The 14,000-molecular-weight antigen of *Mycobacterium tuberculosis* is related to the alpha crystallin family of low-molecular-weigh heat shock proteins. J Bacteriol. 1992; 174:1352–9.
14. Verbon AR, Hartskeerl A, Moreno C, Kolk HJ. Characterization of B cell epitopes on the 16 K antigen of *Mycobacterium tuberculosis*. Clin Exp Immunol. 1992; 89: 395-401.
15. Senol G, Erer OF, Yalcin YA, *et al*. Humoral immune response against 38kDa and 16 kDa mycobacterial antigens in tuberculosis. Eur Respir J.2007; 29(1): 143-8.
16. Demkow U, Zielonka TM, Nowak-Misiak M, *et al*. Humoral immune response against 38 kDa and 16 kDa mycobacterial antigens in bone and joint tuberculosis. Int J Tuberc Lung Dis. 2002; 6: 1023-8.
17. Singh M, Andersen AB, McCarthy JE, *et al.* The Mycobacterium tuberculosis 38-kDa antigen: overproduction in *Escherichia coli*, purification and characterization, Gene. 1992; 117: 53–60.
18. Houghton RL, Lodes MJ, Dillon DC, *et al.* Use of multiepitope polyproteins in serodiagnosis of active tuberculosis. Clin Diag Lab Immunol. 2002; 9: 883-91.