Research Article

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Expression of soluble recombinant *Mycobacterium tuberculosis* early secreted antigen target 6 (ESAT6) using cold-inducible expression system: A prospect for *Mycobacterium tuberculosis* skin test detection

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Tuberculosis (TB) remains a global health issue. One of the major challenge in TB control are over diagnose and underdiagnosed which consequently would lead to increase burden of disease. Therefore, there is urgency for developing a specific and sensitive diagnostic tool. Early secreted antigen target 6 (ESAT6) has been studied extensively as a prospective tuberculosis diagnostic tool. It has the ability to distinguish between TB infection and non TB infection (infection due to non-*Tuberculosis mycobacterium* and previous BCG vaccinated individual). Production of the antigen in a high-level soluble form is required to provide material for skin test as a more accurate diagnostic tool for tuberculosis. In this research, ESAT6 recombinant protein was over expressed primarily as a soluble protein in high-level using combination of *E. coli* BL21-codon Plus RIPL Strain with cold-inducible expression system. Since the BL21 codon plus (DE3)-RIPL cells contain extra copies of argU, ileY, leuW, and proL tRNAs genes, the expression of pLDH which contains 11% of the rare tRNAs was efficiently rescued. Moreover, the use of cold temperature (15^{o}C) has reduced cell growth, slowed down transcription and translation rate, and reduced the strength of hydrophobic interactions that contribute to the protein misfolding. This research provides a convenience method for generation of high quality and quantity of recombinant antigen.

Key words: Tuberculosis, ESAT6, Skin test, *Eschercihia coli* BL21-codon Plus RIPL Strain, cold-inducible expression system.

Tuberculosis (TB) remains a global health issue as a leading cause of morbidity, mortality and has significant economic consequences (WHO, 2012a, Ahlburg, 2000). Globally, TB is the second cause of death due to infectious diseases with an estimation of 9 million new cases and 1.4 million mortality in 2011(WHO, 2012a). According to WHO, South-East Asia region carries 40% of the global burden of TB with 3.5 million new cases in 2010 alone (WHO, 2012b). TB causes an average of 20% to 30% potential loss in household income or approximately 15% of the nation GDP in the developing world (Ahlburg, 2000). Thus holds back economic growth of developing countries (Ahlburg, 2000).

Indonesia is amongst the five countries with the highest TB incidence (0.4-0.5 million cases) and mortality rate due to TB approximately 27 per 100.000 cases per year (WHO, 2011, WHO, 2012b). TB in Indonesia affects the productive age between 15-44 years, consequently suppress human development on a large scale (Soemantri et al. 2011). The estimated national budget for TB has increased from US$ 104 million in 2012 to US$ 117 million in 2013 (WHO, 2011). Based on the health and economic consequences of TB therefore WHO declared

the STOP TB Strategy, focusing on new and effective diagnostic tool as one of the approach for TB control (WHO, 2012a).

Diagnosis of TB has mainly relied on clinical, radiology, sputum and culture examination to determine the present of *Mycobacterium tuberculosis*. However these methods have several limitations including: low sensitivity, less specific and time consuming (Foulds and O'Brien, 1998). Another diagnostic method that has been developed based on intradermal injection of purified protein derivative (PPD). However the tuberculin skin test has many drawbacks, including low specificity, need of patient to return for reading also variability and subjectivity in test application and reading (Howard and Solomon, 1988, Harboe, 1981). Moreover, PPD contains many antigens widely shared among non-tuberculosis, M. bovis bacilli Calmette-Guerin (BCG vaccine) and *M.tuberculosis* (Harboe, 1981). As the PPD skin test may result in false positive reaction in individual prior to BCG vaccination thus cause troublesome in TB diagnosis in adulthood as 80% of infant has been estimated to receive this vaccine (WHO, 2008). Despite these limitations, tuberculin test remains in use, thus reveals the need and urgency for better diagnostic tests for TB.

Early secreted antigen target 6 (ESAT6) has been studied extensively as a prospective tuberculosis diagnostic tool (Arend et al. 2000, Brock et al. 2004). ESAT6 has the potential to deferentiate between TB and non-TB infection. ESAT6 is present in Mycobacterium pathogen such as *M. tuberculosis, M.bovis, M. africanum* and *M. leprae* however is not present in strain *M. bovis* BCG and non-tuberculous *mycobacterium* (Harboe et al. 1996, Behr, 2001). Furthermore, ESAT6 has been recently used as diagnosis of latent tuberculosis infection through interferon gamma release assays (IGRA) (Talati et al. 2011). In previous assay, two antigens were utilized, ESAT6 and CFP10. The drawback of this assay were the need of skilled staff for sample handling, limited time to handle the sample (12 hour time frame while leukocytes were still viable) and costly procedure. ESAT 6 as a potential skin test candidate is suitable to implement in low resource facilities. Therefore this study aims to clone and produce ESAT6 recombinant gene as prospective and specific skin test for tuberculosis diagnosis.

MATERIALS AND METHODS

Isolation of *M. tuberculosis* genomic DNA

Collection and DNA isolation of *M. tuberculosis* sample was performed prior to ESAT-6 gene amplification. *M. tuberculosis* strain H37Rv obtained from BLK Surabaya was isolated and purified using DNeasy tissue kit (QIAGEN, Hilden, Jerman) as per manufacturer's instructions. The integrity of DNA samples isolated was monitored by Agarose gel electrophoresis. The DNA isolated was then further used as template to amplify ESAT 6 fragment.

Amplification of ESAT6

Oligonucleotide primers ESAT6NdeI-F 5'-CATGCCATGGCAGAGCAGCAGTGGA ATTTC-3' and ESAT6EcoRI-R 5'-CCGCTCGAGTGCGAACATCCCAGTGA-3' corresponding to ESAT6 open reading frame (ORF) were constructed based on ESAT6 gene sequence (H37Rv). PCR was performed in 10 µl reaction volume containing 10 pmol of each primer, 1.25 mM $MgCl_2$, 200 µM of dNTPs, 100 ng of *M. tuberculosis* genomic DNA and 1 U of *Pfu* Polymerase. Reaction conditions were as follows: initial denaturation of 3 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 60 °C, 60 s at 72 °C and a 10 min final extension at 72 °C.

Cloning and sequencing of ESAT6 pcoldTF expression vector

The PCR product was digested with *NdeI* and *EcoRI* restriction enzymes, purified, and then ligated with the *Nde*I and *EcoR*I digested pColdTF vector (Takara bio). The plasmid was introduced into competent *E. coli* BL21 (DE3) codon plus-RIPL strain cells (Invitrogen, Carlsbad, CA). Sequencing was performed to confirm the correct sequence.

Expression of recombinant ESAT6

A small scale expression studies were carried out to determine the best incubation temperature, IPTG concentration, and post-induction time. Briefly, expression was performed in 4 ml LB media containing ampicilin to identify the best condition for high level soluble expression of targeted proteins. Analysis for soluble expression was determined by relative percentage distribution

of expressed proteins in soluble and un-soluble fractions in SDS-PAGE. For a large amount of antigens production, stock culture is set up by inoculating 4 ml LB broth supplemented with ampicilin with 4 µl glycerol stock of *E. coli* BL21 (DE3) codon plus-RIPL strain cells containing recombinant plasmids. The culture was grown overnight at 30°C, while shaking at 200 rpm. Approximately 100 ml LB broth containing ampicillin was inoculated with 1ml of overnight culture and allowed to grow at 15°C with shaking at 200 rpm. Incubation temperature, IPTG concentration, post-induction time was set up based on the best results carried out previously (expression studies). Cells were harvested by centrifugation at 6,000 g at 4°C, and the pellet obtained is stored at -20°C until purification.

Purification of recombinant ESAT6

Recombinant ESAT6 was purified by affinity chromatography, using Ni-NTA agarose (QIAGEN) according to the manufacturer's recommendations. The cell was recovered by centrifuging at 3,000 *g* for 10 min at 4°C and suspended in 5 ml ice-cold binding buffer (0.02 M sodium phosphate, 0.5 M NaCl, pH 7.4) and proceed to sonication. Subsequently, the soluble fraction was recovered by centrifugation at 4,000 *g* for 5 min at 4°C. 2 ml of Ni-NTA agarose was added and mixed using a rotator for 1 h at 4°C. The resin was recovered by centrifugation at 900 *g* for 1 min at 4°C, then further packed into a column and washed with 5 ml of washing buffer (binding buffer including 50 mM imidazole). The antigen was eluted and fractionated with 5 ml of elution buffer (binding buffer including 200 mM imidazole). Elution fractions were checked using SDS-PAGE.

Cleavage of recombinant ESAT6

The TF tag of the purified ESAT6 fusion protein can be cleaved and removed by cleaving the protein with factor Xa, thrombin, and HRV 3C protease. In this research we used thrombin to cleavage the fusion protein.

RESULTS AND DISCUSSION

Early secreted antigen target 6 (ESAT6) has been studied extensively as prospective tuberculosis diagnostic tool (Arend et al. 2000, Brock et al. 2004). ESAT6 has the potential to deferentiate between TB and non-TB infection. ESAT6 is present in mycobacterium pathogen such as *M. tuberculosis, M.bovis, M. africanum* and *M. leprae* however is not present in strain *M. bovis* BCG and non-tuberculous *mycobacterium* (Harboe et al. 1996, Behr, 2001). Therefore, this antigen is promising for a more specific skin test for tuberculosis diagnosis.

In this research, we tried to produce ESAT6 antigen as material for TB skin test. To amplify ESAT6 gene, *M. tuberculosis* DNA was obtained from BLK Surabaya using a QIAamp® DNA Blood mini kit as per manufacturer's instructions. The obtained genomic DNA was used as template for PCR amplification of ESAT6.

PCR amplification and cloning of ESAT6

PCR amplification of gene coding for ESAT6 was performed in a total volume of 5 µl with 0.5 unit of *Pfu* DNA polymerase (TaKaRa), 0.2 mM of each dNTPs, 0.2 pM ESAT6NdeI-F and ESAT6EcoRI-R using the following PCR program: 35 cycles of 30 s at 94 °C, 30 s at 60 °C, 60 s at 72 °C and a 10 min final extension at 72 °C. The *M. tuberculosis* contains GC rich fragments thus causes troublesome in PCR amplification due to secondary structure formation and poor denaturation (Poulet and Cole, 1995, Shahdev et al. 2007). Additional buffer such as DMSO 10% was known preferable in disrupting formation of secondary structure therefore improve PCR amplification (Jansen et al. 2010). Using the above optimized condition, we were able to amplify ESAT6 gene from H37Rv strain *M. tuberculosis*. The PCR products obtained the desired molecular weight for ESAT6 as shown in Figure 1.

The pCold TF plasmid was used as expression plasmid. This plasmid is a fusion cold shock expression vector that expresses Trigger Factor (TF) chaperone as a soluble fusion tag. The chaperone is a prokaryotic ribosome-associated chaperone protein (48 kDa) which facilitates co-translational folding of nascent polypeptides. Because of its *E. coli* origin, TF is highly expressed in *E. coli* expression system. The pCold TF vector consists of the cspA promoter plus additional downstream sequences including a 5'-untranslated region (5'-UTR), a translational enhancing element (TEE), a His-Tag sequence, and a multicloning site (MCS).

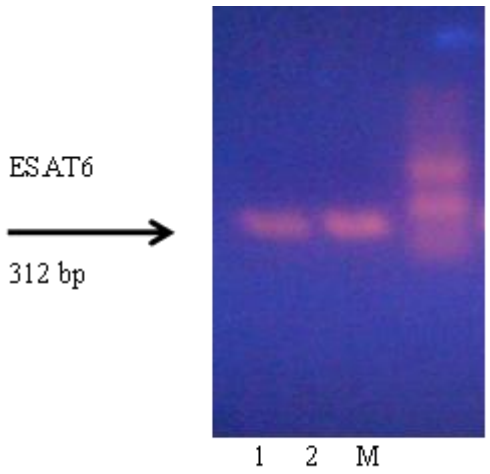

Figure 1: Confirmation of amplified ESAT6 gene fragment product by agarose gel from H37Rv strain M. tuberculosis. Lane 1-2 amplified product of ESAT6 (312 bp), M = Marker.

By using the plasmid, most *E. coli* strains can serve as expression hosts. This vector is depicted in Fig. 2.

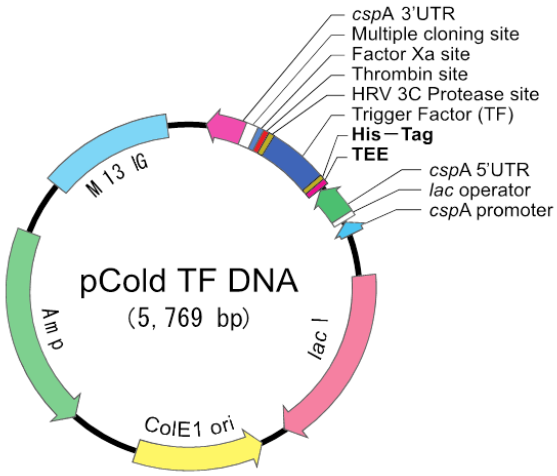

Figure 2: Map of pCold TF vector.

PCR product and pcoldTF plasmid were digested with *NdeI* and *EcoRI* restriction enzyme. pColdTf plasmid was then dephosphorilated with *bacterial alkaline phosphatase* (BAP) to avoid self-ligation. ESAT6 product was subsequently ligated with linearized pColdTF plasmid. Ligation was performed at 12^{o}C for overnight using reaction mixtures as follow: 5x dilution of ESAT6 gene (1.5 µl), 1.5 µl of dephosphorilated pColdTF plasmid, and 2x Mix ligation kit (2.5 µl). By using heat shock transformation technique, the resulted recombinant plasmid (pColdTF-ESAT6) was tranformed into *E. coli* BL21 (DE3) codon plus-RIPL strain competent cells and incubated at 37^{o}C for overnight. Then, a single colony carrying the insert was tooth-picked and used for the next screening using PCR colony.

Once the correct recombinant plasmid was detected, the colony carrying the plasmid was cultured in LB medium containing ampicilin and grown overnight at 37^{o}C in a shaker, and was prepared for sequencing. The obtained DNA was sequenced from both direction using the sequencing primers given in Materials and Methods. The sequencing of pColdTF-ESAT6 revealed that the complete ORF was comprised of 312 base pairs initiated with an ATG start codon and ending with a TGA codon, encoding putative protein of 104 amino acids.

E. coli Bl21 positive clone directly used for the next step in the expression of ESAT6. As described previously, the clone was cultured in LB medium containing ampicilin in cold temperature (15^{o}C). Since the low temperature, the *E. coli* BL21 bearing pCold-ESAT6 was grown slowly. To achieve OD_{600} 0.5, it is needs about 24 h after inoculation.

Expression and purification of ESAT6

Since the ESAT6 molecular weighed 9 kDa and the pCold TF vector bearing the TF chaperone with 48 kDa, the recombinant protein expressed by the pCold-ESAT6 plasmid is expected to encode a fusion recombinant protein with a molecular weight of approximately 57 kDa. To identify positive clones which were capable of expressing the predicted 57 kDa, *E.coli* BL21 codon plus RIPL strain was cultivated in small scale (1.5 ml) LB medium in the presence of inducer (IPTG). The expression conditions were optimized and best result was obtained by induction of 1 mM IPTG concentration at 15^{o}C for 24 h.

Cell lysates containing the soluble fraction of TF-ESAT6 recombinant protein was purified using Ni-NTA agarose (Qiagen, USA). As shown in the SDS-PAGE analysis results in Fig. 3, the Ni-NTA-bound H6TF-fused ESAT6 protein (approximately 57 kD) was purified (Lane 6-8) comparing to flow through fraction (Lane 2) and washing fraction (Lane 3-5)

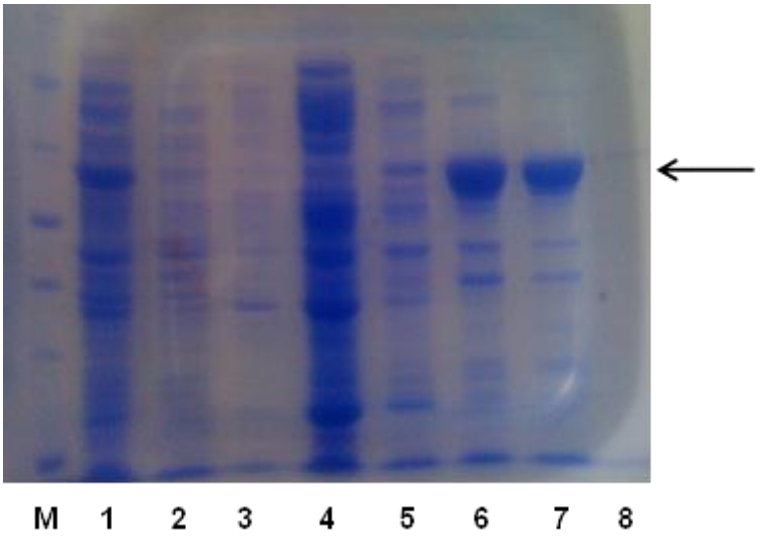

Figure 3: SDS-PAGE analysis of purified recombinant *Esat6*.
Lane 1 = unpurified TF-ESAT6, 2 = flowthrough fraction, 3-5 = washing fraction, 6-7 = elution fraction (purified TF-Esat6).

Interestingly, most of the TF-ESAT6 recombinant protein expressed in this research was expressed primarily as soluble form. These results indicated that the use of combination of pCold TF expression vector, *E.coli* BL21 codon plus RIPL strain, and cold temperature were very suitable to produce high quantity and quality of ESAT6 recombinant.

Cleavage of recombinant protein TF-ESAT6

The TF tag of the purified ESAT6 fusion protein can be cleaved and removed by cleaving the protein with factor Xa, thrombin, and HRV 3C protease. In this research we used thrombin to cleavage the fusion protein. The desired ESAT6 protein could be recovered as a soluble protein even after removal of the TF tag (Fig. 4).

CONCLUSION

In summary, the researchers have achieved to express purified recombinant protein of ESAT6. This study suggests further work including protein expression and purification on a larger scale with previously optimum condition.

ACKNOWLEDGMENT

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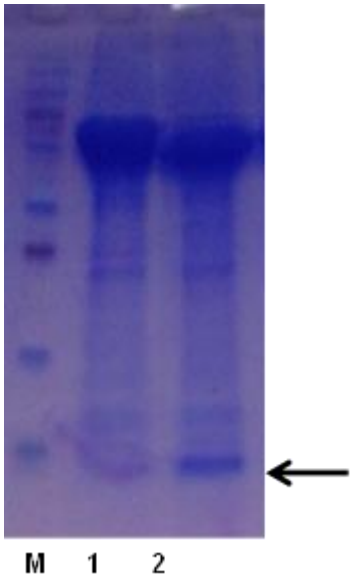

Figure 4: SDS-PAGE analysis of cleavage recombinant Esat6.
Lane 1 = uncleavaged TF-ESAT6
Lane 2 = cleavaged TF-ESAT6

REFERENCES

Ahlburg, D. A. 2000. The Economic Impacts of Tuberculosis. *The Stop TB Initiative Series* [Online].

Arend, S. M., Andersen, P., Meijgaarden, K. E. V., Skojt, R. L. V., Subronoto, Y. W.,

Dissel, J. T. V. & Ottenhoff, T. H. M. 2000. Detection of active tuberculosis infection by T cell responses to early-secreted antigenic target 6-kDa protein and culture filtrate protein. Journal of Infectious Diseases, 181**,** 1850-1854.

Behr, M. 2001. Comparative genomic of BCG vaccine. Tuberculosis, 81**,** 165-168.

Brock, I., Weldingh, K., Lillebaek, T., Follmann, F. & Andersen, P. 2004. Comparison of Tuberculin Skin Test and New Specific Blood Test in Tuberculosis Contacts. American Journal of Respiratory and Critical care Medicine*,* 170**,** 65-69.

Foulds, J. & O'brien, R. 1998. New tools for the diagnosis of tuberculosis:the perspective of developing countries. *The International Journal of Tuberculosis and Lung Disease,* 2**,** 778-783.

Harboe, M. 1981. Antigens of PPD, old tuberculin, and autoclaved Mycobacterium bovis BCG studied by crossed immunoelectrophoresis. The American Review of Respiratory Disease 124**,** 80-87.

Harboe, M., Oettinger, T., Wiker, H., Rosenkrands, I. & Andersen, P. 1996. Evidence for occurrence of the ESAT-6 protein in Mycobacterium tuberculosis and virulent Mycobacterium bovis and for its absence in Mycobacterium bovis BCG. Infection adn Immunity*,* 64**,** 16-22.

Howard, T. P. & Solomon, D. A. 1988. Reading the Tuberkulin Skin Test. JAMA Internal Medicine, 148**,** 2457-2459.

Jansen, M. A., Fukushima, M. & Davis, R. W. 2010. DMSO and Betaine Greatly Improve Amplification of GC Rich Constructs in De Novo Synthesis. PLoS ONE, 5.

Poulet, S. & Cole, S. T. 1995. Characterization of the highly abundant polymorphic GC-rich-repetitive sequence (PGRS) present in Mycobacterium tuberculosis. *Archives of Microbiology,* 163**,** 87-95.

Shahdev, S., Saini, S., Tiwari, P. & Saini, K. S. 2007. Amplification of GC-rich genes by following a combination strategy of primer design, enhancers and modified PCR cycle conditions. Molecular And Cellular Probes, 21**,** 303-7.

Soemantri, S., Senewe, F., Tjandrarini, D., Day, R., Basri, C., Manissero, D., Mehta, F. & Dye, C. 2011. Three-fold reduction in the prevalence of tuberculosis over 25 years in Indonesia. International journal for tuberculosis and lung disease [Online], 11.

Talati, N. J., Gonzalez-Diaz, E., Mutemba, C., Wendt, J., Kilembe, W., Mwananyanda, L., Chomba, E., Allen, S., Rio, C. D. & Blumberg, H. M. 2011. Diagnosis of latent tuberculosis infection among HIV discordant partners using interferon gamma release assays. BMC Infectious Diseases, 11**,** 264.

WHO 2008. Anti-tuberculosis drug resistance in the world. Geneva: WHO.

WHO. 2011. Tuberculosis Profile: Indonesia.

WHO 2012a. Global Tuberculosis Report 2012. Geneva: WHO Press.

WHO 2012b. Tuberculosis Control In South-East Asia Region 2012. Geneva: WHO Press.