

Short Communication:

Cloning, production and responses of the sMTL-13 protein (13 kDa lectin secretion) *Mycobacterium tuberculosis* against Interleukin 6

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Abstract. Agus R, Purnamasari WOS, Parura RD, Silang F, Massi MN. 2022. Short Communication: Cloning, production and responses of the sMTL-13 protein (13 kDa lectin secretion) *Mycobacterium tuberculosis* against Interleukin 6. *Biodiversitas* 23: 6208-6212. The Bacillus Calmette-Guérin (BCG) vaccine protected effect against meningitis and disseminated Tuberculosis TB in children. However, it does not prevent primary infection and reactivation of latent pulmonary infection. Therefore, an effective vaccine is needed to prevent tuberculosis. One of the potentials is the sMTL-13 protein (13 kDa lectin secretion), which causes an increase in interleukin 6 (IL-6) as a proinflammatory cytokine and shows high titers in the serum of TB patients. This study aimed to clone, express and evaluate the sMTL-13 recombinant protein. The research steps were ligation of Rv1419 to pQE-30Xa, transformation to *Escherichia coli* BL-21, and production of recombinant protein with IPTG induction. Furthermore, immunoreactivity tests with the serum of TB patients and healthy serum using ELISA. The results showed that the PCR product Rv1419 showed a band of 474 bp and white colonies as a recombinant clone of pQE-30Xa-Rv1419. The production of sMTL-13 recombinant protein has been successfully carried out and produces 13 kDa protein. The results of the immunoreactivity test showed that the serum level of IL-6 in patients with active TB was 84.22 pg/mL and the serum level of IL-6 in healthy people was 63.74 pg/mL.

Keywords: BCG, clone, expression, lectin, sMTL-13

INTRODUCTION

Tuberculosis is caused by *Mycobacterium tuberculosis* (Mtb). These bacteria usually attack the lungs but can attack other body parts such as the kidneys, spine, and brain (CDC 2020). In 2020, 30 countries accounted for 86% of new TB cases. More than 95% of cases and deaths occur in developing countries (WHO 2021). The Bacillus Calmette-Guérin BCG is the most widely used vaccine in the world but its effectiveness varies, between 0 and 80% (Heemskerk et al. 2015). BCG can prevent TB, but its effectiveness varies in high-burden countries and is not effective for preventing of pulmonary TB in adults (WHO. 2019; Wilkie and McShane. 2015). Recent data show that 154 countries have BCG vaccination policies for the entire population. There are 53 countries reporting BCG vaccination coverage of at least 95% and in 2019 there were 31 countries with a decrease in BCG coverage of 5% or more (WHO 2021). Some of the side effects caused by the BCG vaccine include hardness at the injection site, an ulcer arising from blisters at the injection site and an abscess at the injection site (Vaccine Knowledge Project. 2019). Although more than 20 vaccines are currently in clinical trials, it is very important to choose a TB vaccine candidate that is effective and safe against TB infection, especially in the presence of MDR TB and HIV-TB

coinfection (WHO 2019). Therefore, developing new vaccines for TB prevention and control is very important.

Surface and secreted mycobacterial proteins play a major role in the infection process by mediating macrophage-bacterial interactions and cell death. One of the potential *M. tuberculosis* proteins is the 13-kDa lectin (sMTL-13) encoded by the Rv1419. Bioinformatics analysis showed that sMTL-13 belongs to the ricin-type β -trefoil family of proteins containing a Sec-type signal peptide present in Mtb complex species. The sMTL-13 causes an increase in IFN- γ production by PBMCs from active tuberculosis patients (Nogueira et al. 2010). sMTL-13 has an R-type lectin on the surface of Mtb cells and functions in interaction with macrophages. R-type lectins have been detected in plants, animals, and bacteria (Kolbe et al. 2019). In addition, lectins can induce the production of proinflammatory cytokines by APCs and cause the proliferation of CD4⁺ T lymphocyte cells indicating that sMTL-13 has immunogenic activity (Souza 2012).

Interleukin-6 (IL-6) is a cytokine with high serum levels in active pulmonary tuberculosis (TB). IL-6 screening in patients in contact with TB is useful for monitoring the infectious process and for observing the risk of disease progression (Lopes et al. 2013). Interactions between macrophages infected with *M. tuberculosis* and cell T lymphocytes will cause the release of various

mediators such as IL-1 β , IL-6, IL-10 and TNF- α (Wahyuniati 2017). Interleukin-6 (IL-6) is a proinflammatory cytokine produced by many cell types and expressed during states of cellular stress, such as inflammation, infection, wound sites, and cancer. IL-6 levels can increase several thousand-fold and may help coordinate responses to dysregulation of tissue homeostasis (Choy and Stevan 2017). IL-6 is a cytokine with pleiotropic activity; it induces the synthesis of acute-phase proteins such as CRP, serum amyloid A, fibrinogen, and hepcidin in hepatocytes, and inhibits albumin production. IL-6 also plays a role in the stimulation of antibody production and the development of effector T cells (Tanaka et al. 2022). IL-6 expression was higher in newly diagnosed TB patients. Therefore, IL-6 differentiate active and latent TB and monitor treatment efficiency (Seyedhosseini et al. 2019). The aim of this study was to clone, express and evaluate the response of the sMTL-13 protein to the IL-6.

MATERIALS AND METHODS

Bacterial strains and plasmids

The sample is a clinical isolate of *Mycobacterium tuberculosis* obtained from the Medical Research Center (Hum-RC) at Hasanuddin University, Makassar, Indonesia. *Escherichia coli* BL21 as the host cell and pQE-30 Xa as the expression vector.

Amplification of Rv1419

The Rv1419 was amplified using forward primer 5'-GATCGCTAGCATGGGTGAATTACGGTTG-3' and reverse primer 5'-TATCTCGAGCGGCACGCTATCCCA-3'. The *NheI* site GCTAGC was inserted in the forward primer and the *XhoI* site CTCGAG was inserted in the reverse primer. The PCR reaction was repeated for 30 cycles. One cycle consisted of pre-denaturation at 94°C for 10 minutes, denaturation at 95°C for 45 seconds, annealing at 60°C for 1 minute and extension at 72°C for 1 minute. The target gene is Rv1419 with a length of 474 bp (Patra et al. 2010).

Competent cell

The competent cells used were *E. coli* BL21. A single colony of *E. coli* BL21 was grown in 5 mL of LB (Luria Bertani) medium. Shaker at 37°C for 18 hours at 150 rpm then into 20 mL of LB, 2% culture was added and incubated at 37°C for 2 hours. Cultures were kept on ice for 10 min and centrifuged at 4,000 rpm for 10 minutes. Into the pellet 300 μ L CaCl₂ was added, resuspended and centrifuged at 4000 rpm for 10 minutes. Add 50 μ L cold CaCl₂ into pellets and incubated overnight at 4°C (Sambrook et al. 1989).

Cloning of Rv 1419 in *Escherichia coli* BL 21

Cloning was initiated by cutting the pQE-30Xa expression vector with the appropriate restriction enzymes. The target DNA (Rv 1419) was ligated to the vector with the T4 NA ligase to obtain the pQE-30Xa-Rv1419 recombinant plasmid. Transformation into competent *E.*

coli BL21 host cells by heat shock method (Sambrook et al. 1989). The ligation product was put into competent *E. coli* BL21, incubated on ice for 30 minutes, in a water bath at 42°C for 90 seconds, and incubated on ice for 1 hour. Liquid LB medium was added and incubated at 37°C for 3 hours at 150 rpm. Then it was centrifuged for 1 minute at 10,000 rpm. The pellets as transformation products were spread into solid Luria Bertani medium containing ampicillin, 5-Bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-Gal) and Isopropyl β -D-1-thiogalactopyranoside (IPTG), then incubated at 37°C for 16-18 hours.

Characterization of recombinant clone

The characterization of recombinant clones can be done by colony PCR and restriction analysis (Thermo Scientific 2012). The white colonies grown on Luria Bertani medium and ampicillin were recombinant clones (pQE-30Xa-Rv 1419). First, the white colonies were put into nuclease-free water and then PCR was performed with the primers and the previous conditions. Before restriction analysis, plasmid extraction from white colonies was performed using a plasmid extraction kit. Then, restriction analysis was carried out by cutting the recombinant plasmid with the previous restriction enzyme.

Expression of sMTL-13 protein

Single colonies containing recombinant plasmid were cultured on 15 mL LB broth and ampicillin (100 μ g/mL) and shaker incubated overnight at 37°C. Then 300 μ L of the cultures were inoculated into 2 mL LB broth containing IPTG 1,0 mM. Incubation was continued at 37°C with shaking and lysis cells by sonication technic. The sMTL-13 was characterized by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE).

Responses of sMTL-13 with ELISA (Enzyme-Linked Immunosorbent Assay)

ELISA test begins with coating with sMTL-13 protein on the plate with IL-6 cytokine. A total of 14 healthy serums and 14 active TB serums were inserted into the plate, and the results were read in the Elisa reader.

RESULTS AND DISCUSSION

Amplification Rv1419

The results of PCR amplification of clinical isolates of *M. tuberculosis* can be seen in Figure 1.A. The PCR product of Rv1419 obtained a band measuring 474 bp.

Cloning of Rv1419 in *Escherichia coli* BL21

Cloning was performed by ligation the inserted DNA (Rv1419) to the expression vector (pQE-30Xa), resulting in the recombinant plasmid pQE-30Xa-Rv1419. Furthermore, the recombinant plasmid was transformed into competent *E. coli* BL21 cells. In Figure 2, it can be seen that the petridish is overgrown with white colonies carrying recombinant clones. White colonies indicate that the inserted DNA was successfully inserted into the plasmids, while the blue colonies were plasmids without DNA insert.

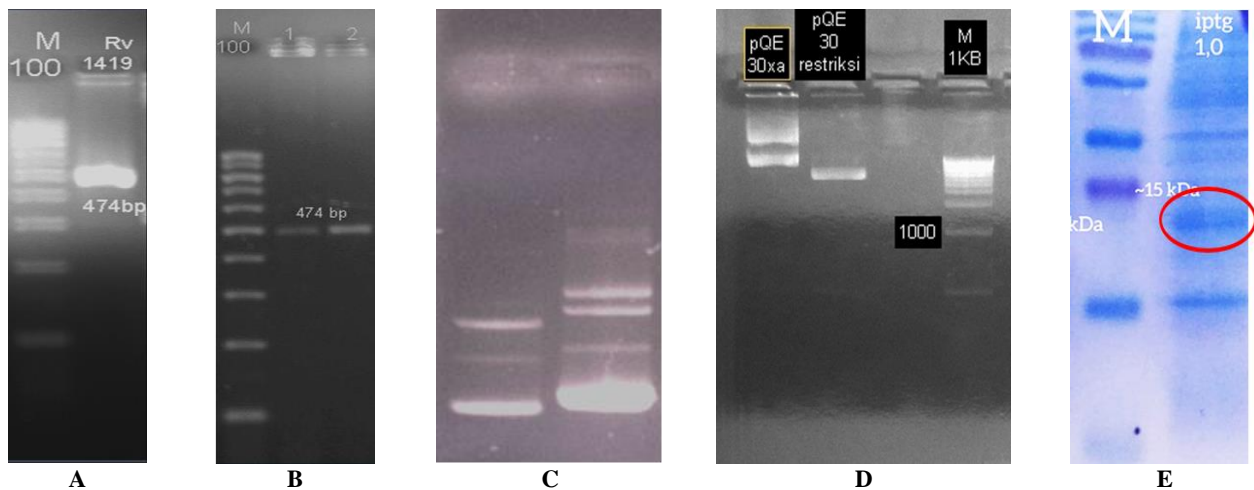


Figure 1. A. PCR product Rv 1419. M = marker 100 bp Rv1419 =clinical isolate. B. Colony PCR. M: Marker = 100 bp 1,2 : Rv 1419. C. Recombinant plasmid pQE-30Xa-Rv1419. 1 = plasmid of the blue colony, 2 = plasmid of the white colony. D. Restriction analysis in the recombinant plasmid. pQE30Xauncut pQE30Xa cut Marker 1 kb. E. SDS PAGE of the sMTL-13 protein

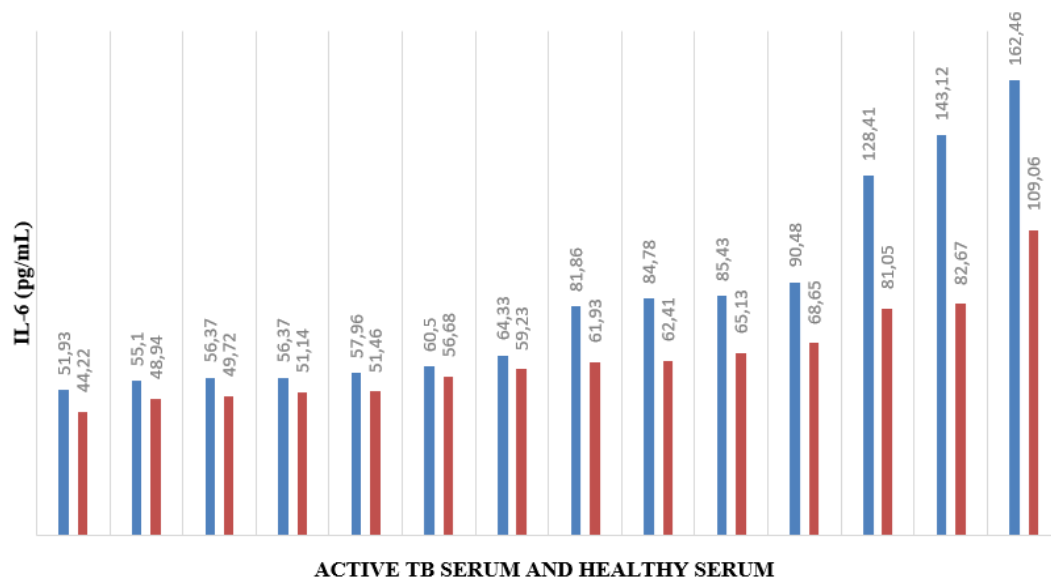


Figure 3. Responses sMTL-13 against IL-6 with healthy and active TB using ELISA. ■ Serum level of IL-6 in active TB; ■ Serum level of IL-6 in healthy

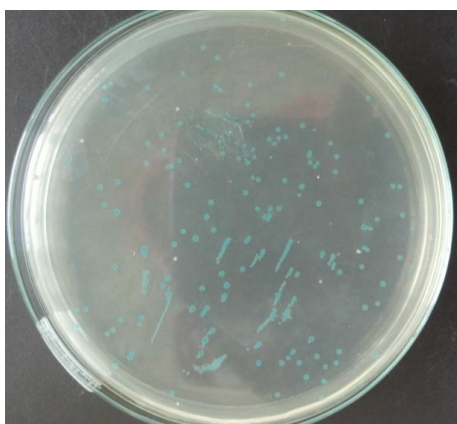


Figure 2. Transformation of the recombinant plasmid into *E. coli* BL21

The purpose of plasmid extraction was to obtain recombinant plasmid and confirm the presence of recombinant plasmid in white colonies. The recombinant plasmid consisted of DNA insertion of Rv1419 and pQE-30Xa expression vector. The results of the plasmid extraction obtained can be seen in Figure 1.C. Restriction analysis was performed on recombinant plasmids using the same restriction enzymes as previously. The results can be seen in Figure 1.D.

Production of sMTL-13 protein

White colonies were grown in LB medium containing ampicillin and induced with IPTG. This study carried out IPTG for protein expression with 0.5 mM, 1 mM and 1.5 mM. Optimal results were obtained with a concentration of 1mM. The growth of *E. coli* BL21 colonies carrying

recombinant plasmids was indicated by turbidity in the medium. Recombinant proteins were obtained by lysing host cell membranes and characterized by SDS-PAGE (Figure 1.E).

Responses of sMTL-13 against IL-6

The results of the responses of healthy and active TB with sMTL-13 protein against IL-6 cytokines by ELISA test can be seen in Figure 3.

Discussion

The results of the PCR of clinical isolates of *M. tuberculosis* can be seen in Figure 1.A. The PCR product of Rv1419 obtained a band measuring 474 bp. These results are the same as those obtained by Patra et al. 2010 using *M. tuberculosis* H37Rv.

The transformation results can be seen in petri dishes with white colonies and blue colonies (Figure 2). The white colonies indicated that the inserted DNA was successfully inserted into the plasmid, while the blue colonies were plasmids without the inserted DNA. The recombinant plasmid pQE-30Xa-Rv1419 has been successfully transformed into *E. coli* BL21 cells were indicated by the presence of white colonies on LB media + ampicillin. Recombinant plasmids can be grown on LB media with ampicillin because the plasmid pQE-30Xa has a marker for ampicillin.

The characterization of recombinant clones was carried out by adding chromogenic substrate (X-Gal) to petridish. When β -galactosidase is produced, X-gal is hydrolyzed to form 5-bromo-4-chloro-indoxyl, which indirectly dimerizes to obtain the insoluble blue pigment 5-bromo-4-chloro-indoxyl so that the colonies formed are colonies of recombinant cells (white colonies) and non-recombinant (blue colonies) (Merck 2022). The white colony shows that the DNA insert has been ligated in the multi - cloning site (MCS) found in the *lacZ* area of pQE-30Xa. The DNA insertion will inhibit the *lacZ* gene from encoding the β -galactosidase subunit so that the enzyme does not degrade the galactosidase substrate. Blue bacterial colonies showing no insertion DNA can degrade galactose substrates.

Characterization of recombinant clones was carried out on white colonies carrying DNA inserts. Colony PCR was performed using the same primers and conditions previously to amplify Rv1419. Colony PCR results from the recombinant plasmid pQE-30Xa-Rv1419 found Rv 1419 as insertion DNA (Figure 1.B).

The results of plasmid extraction showed that the white colony containing Rv1419 as insertion DNA had a larger size than the blue colony which was the pQE-30Xa vector. That is indicated by the migration rate of white colonies being slower than blue colonies (Figure 1.C).

The insertion DNA analysis was performed with restriction enzyme *NheI*. This enzyme was chosen because this enzyme is present in the forward primer and the pQE-30Xa vector has a cleavage site for this enzyme. The insertion DNA was cut with restriction enzymes so that the inserted DNA was separated from the vector, and the recombinant plasmid shape became linear to be compared with the marker. The recombinant plasmid successfully cut

was linear and measured more than 3000 bp and the DNA insert was at the bottom but the band was less clear (Figure 1.D).

Expressing recombinant proteins in *E. coli* is an inexpensive and easy to-use method for large-scale protein production (Panah et al. 2017). The study's purpose is to determine the choice between the original antigen and the recombinant antigen. If the researcher develops an immunodiagnostic test, the native antigen is considered more suitable. However, the original antigen is sourced from biological raw materials, so the product is limited especially after the product reaches commercialization. If researchers need positive controls for applications such as Western blot or ELISA, recombinant antigens are often used (Craggs 2019).

Recombinant clones of *E. coli* BL21 were cultured in LB media and induced with IPTG. The efficiency of recombinant protein expression is influenced by expression vectors, compatible hosts, and cultural conditions (Kaur et al. 2018). In addition, the productivity of recombinant protein expression can be improved by optimizing external factors, such as bacterial growth temperature, expression level induction, and duration of induction (Larentis et al. 2014).

The induced lac promoter is one of the most commonly used promoters for heterologous protein expression in *E. coli*. Currently, Isopropyl- β -D-thiogalactopyranoside (IPTG) is the most efficient molecular inducer to regulate the transcriptional activity of this promoter (Briand et al. 2016). This compound is a molecular mimic of allolactose, which is lactose that can trigger transcription of the *lac* operon, so it is used to induce the expression of *Escherichia coli* protein which is controlled by the lac operator (Biologics International Corp 2018). Transcription of the lac promoter is regulated by the *lac* repressor, which is a product of the *lac I* gene. If the inducer (i.e lactose or IPTG) is absent, the *lac* repressor will inhibit transcription by binding to the operator region. When the repressor binds to the operator, the RNA polymerase will not go to the promoter region so that transcription of the lac gene does not occur. However, because there is a chemical balance between the bound and unbound repressor molecules the operator is not constantly occupied by the lac repressor causing low transcriptional levels of the *lac* gene.

Expression protein was started by lysis cells using the sonication method. The method's principle broke the cell wall mechanically by ultrasonic frequency. The characterization of the sMTL-13 protein was carried out by sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) electrophoresis. The sMTL-13 protein showed 13 kDa as size (Figure 1.E). These results are the same as the expression of the sMTL-13 protein on Mtb H37Rv also measuring 13 kDa. The sMTL-13 protein was also expressed in Culture Filtrate Protein (CFP) from Mtb, but not found in the non-TB *Mycobacteria* (NTM) species *Mycobacterium avium*, *Mycobacterium kansasii* and *Mycobacterium fortuitum* (Nogueira et al. 2010).

Figure 3 shows that the serum level of IL-6 in patients with active TB is 84.22 pg/mL and the serum level of IL-6 in healthy is 63.74 pg/mL. IL-6 is an important cytokine

with high levels in active pulmonary TB (Lopes et al. 2013). Martinez et al. (2013), stated that IL-6 is an important cytokine because it can provide very high resistance to tuberculosis and IL-6 also provides a protective immune response against Th1 cells after vaccination.

Because lectins are present on the surface of mycobacterial cells, they can be easily recognized by immune cells so that lectins can be used as potential vaccine candidates. Several studies have shown binding epitopes of MHC-I and MHC-II to mycobacterial lectins (Sundar et al. 2021). A DNA vaccine from Rv1419 has been successfully developed and can induce a weaker humoral immune response and a stronger cellular immune response in mice. It mainly induces Th1 and has a TB immunotherapy effect in mice. In the future, Rv1419 could be one of the therapeutic vaccines against TB (Liang et al. 2016).

In conclusion, the sMTL-13 protein from *M. tuberculosis* has been successfully cloned and expressed in *E. coli* BL21 with a size of 13 kDa. Furthermore, this protein is recognized in the serum of TB patients more strongly than healthy serum after being reacted with IL-6.

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