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Cloning, expression and purification of a gene encoding CFP-10 and ESAT-6 protein of *Mycobacterium tuberculosis* in pET SUMO plasmids as Vaccine Candidates

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Abstract: The Bacillus Calmette-Guerin vaccine did not show consistent results in preventing tuberculosis, with an effectiveness ranging from 0% to 80%. More effective proteins are needed as vaccine candidates to eliminate *tuberculosis*. Culture filtrate proteins 10-kDa(CFP-10) and Excretory Antigen Target 6-kDa(ESAT-6) demonstrated very strong antigenicity against T and B cells. These proteins are secreted during the early phase of infection and could potentially be used for vaccine candidates. This study aims to clone and express the CFP-10 and ESAT-6 proteins on the pET SUMO Plasmid. PCR with specific primers was used to amplify the genes encoding CFP-10 and ESAT-6 proteins. The PCR products were cloned into pET SUMO plasmids for transformation into competent cells *E.coli BL21*(DE3). Recombinant protein expression was induced in LB medium using 1 mM IPTG. The yield of recombinant proteins was visualized by SDS-PAGE and western blotting using an Anti-HisTaq Antibody. PCR results showed the target gene sizes to be 304 bp (CFP-10) and 291 bp (ESAT-6). SDS-PAGE and Western blotting revealed proteins at 22kDa (CFP-10) and 18kDa (ESAT-6). The genes encoding the CFP-10 and ESAT-6 proteins of *M. tuberculosis* have been successfully cloned into the pET SUMO plasmid and expressed in *E. coli* BL21(DE3) with molecular weights corresponding to the target genes.

Keywords: CFP-10; ESAT-6; *M. tuberculosis*; Vaccine Candidates

Introduction

Tuberculosis (TB) remains a very serious health problem and is a risk factor for long-term respiratory impairment that needs treatment and attention (Gyimah and Dako-Gyeke, 2019), since it is responsible for millions of deaths per year worldwide (Ravimohan et al., 2018), particularly in developing countries. The TB disease in humans is a directly contagious disease caused by *Mycobacterium tuberculosis* (*M. tuberculosis*), which mainly infects the lungs, but can also attack other organs. The World Health Organization (WHO) reported there were 10.4 million people infected by *M. tuberculosis*, consisting of 5.9 million (56%) men, 3.5 million (34%) women, and 1 million (10%) children (Jones-Lopez et al., 2013; WHO 2018). It is predicted that the number of individuals developing tuberculosis may increase by over one million each year during the period of 2020–2025 (Kim et al., 2022). Indonesia has the second largest number of TB cases in the world after India, with 130 new cases per 100,000 population and 41 deaths per 100,000 population in 2018. (WHO 2018; Ministry of Indonesia Health 2018). Due to its characteristics, such as slow bacterial growth, and highly varied strains, which require patience in the treatment, *M. tuberculosis* is the main problem in TB treatment. Additional challenges include the presence of a resistant strain of tuberculosis (MDR-TB), infection of Human

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Immunodeficiency Virus/Auto Immune Deficiency Syndrome (HIV/AIDS), presence of latent infection reaching to 40-50% of infection cases, as well as decreased effectiveness of vaccines and diagnostic methods which have been generally ineffective in developing successful treatments (Yuen et al., 2014; WHO 2018). Several strategies have been investigated to control and eliminate TB. Among these strategies, searching for more effective vaccine candidates is important to execute, including vaccine sub-units (Jiang et al., 2013). To date, Bacillus Calmette-Guerin (BCG) is the only TB vaccine used in humans (Fihiruddin et al., 2020) and has been used worldwide since 1921 (Chatterjee et al., 2011).

The BCG vaccine is effective in preventing infection in newborns (Jiang et al., 2013), but provides poor protection and highly variable efficacy against TB infection in adults (Purwanti et al., 2023). Elimination of the large number of TB cases globally depends on early detection, diagnosis, and handling of cases (Xu et al., 2012). ESAT-6 and CFP-10 are proteins secreted by *M. tuberculosis* in the early phase of infection that have the potential to be developed as promising antigens for tools in serodiagnostic tests (Ruhwald et al., 2017) and recombinant DNA vaccines (Jiang et al., 2013). ESAT-6 and CFP-10 have a very dominant antigen that is recognized by T cells and plays a role as a virulence factor of *M. tuberculosis*. These proteins transfer bacteria from the phagosome to the cell cytoplasm at the time of initial infection (Mahmoudi et al., 2013). ESAT-6 and CFP-10 strongly induce T cells and Interferon-gamma (INF-γ) (Bates et al., 2023) because they contain epitopes recognized by T cells and B cells in the patients. These proteins are encoded by a gene that is located in the RD1 genome and found only in the virulence factors of *M. tuberculosis* (Xu et al., 2012). The production of recombinant proteins from the CFP-10 and ESAT-6 encoding genes could create a better diagnostic test or develop a combination of vaccines (Kuo et al., 2013; Bates et al., 2023). The use of a plasmid as a carrier medium for genes to be inserted will determine the success of the formulation of these recombinant proteins (Piubelli et al., 2013). Each plasmid is equipped with different peptide tags. The vector pET SUMO is used as a recombinant plasmid to produce recombinant proteins efficiently. One of the advantages of using the pET SUMO plasmid as a cloning vector is its ability to obtain recombinant proteins without the presence of additional amino acids (Rosano and Ceccarelli 2014; Ceylan and Erdogan 2017). Also, it does not require the digestion of the restriction enzyme for the polymerase chain reaction (PCR) product that would be inserted (Gopal and Kumar 2013; Ceylan and Erdogan 2017). This study aimed to study the cloning, expression, and purification of CFP-10 and ESAT-6 recombinant proteins in pET SUMO plasmids from MDR-TB of *M. tuberculosis* samples from clinical isolates as candidates for serodiagnostic tests and recombinant vaccines.

Method

Extraction and measurement concentration of DNA

Isolates of *M. tuberculosis* were obtained from Balai Besar Laboratorium Kesehatan (BBLK) Surabaya. The bacterial control used was *M. tuberculosis* strain H37Rv. The identification of *M. tuberculosis* used The BACTEC MGIT 960 System (BD) methods. Isolates were sub-culture on Lowenstein-Jensen (LJ) medium and BACTEC MGIT 960 System (BD), and confirmed by GeneXpert® System (Cepheid USA) test. The isolates of *M. tuberculosis* were subcultured (refreshed) in the Microbiology Laboratory of BBLK Surabaya to obtain isolates in 2-3 weeks of log phase growth, and the molecular test was conducted on 3-week average growth of isolates. The process of extracting *M. tuberculosis* from the medium was conducted using the Dnesay Blood and Tissue Kit from Qiagen. The concentration of bacterial DNA from the extraction was measured with the NanoDropTM2000/ 2000c Spectrophotometer from Thermo Fisher Scientific.

Design of primers and Polymerase Chain Reaction (PCR)

The area to be amplified was determined by the genome sequence of *M. tuberculosis* H37Rv (ATCC 27294) that was obtained from GenBank. Primary forward used was: 5'-CAG CAT GGC AGA GAT GAA GAC CGA T-3 ', reverse primer: 5'- GAA GCC CAT TTG CGA GGA C-3', (Primer for CFP-10) and forward 5'-ACA GAG CAG CAG TGG AAT-3 ' and the reverse primer was: 5'- GTT GCC CTA TGC GAA CAT CC -3' (Primer ESAT-6) (Zarif et al., 2013). Amplification of CFP-10 and ESAT-6 encoding genes was performed using a PCR Amplitron (Thermolyne) machine in 25 µL reaction volume consisting of 12.5 µL PCR mix, 2 µl primary forward, 2 µl primary reverse, 6.5 µl ddH2O and 2 µl DNA template. Optimization was performed in 35 cycles which consisted of pre denaturation for 5 minutes at 95^{o}C; each cycle consisting of denaturation for 30 seconds at 95^{o}C, annealing for 30 seconds at 56^{o}C, and extension for 1 minute at 72^{o}C. Extension stage was conducted in 10 minutes at 72^{o}C, then the tube cooled for 10 minutes at 12^{o}C. The PCR products were analyzed by electrophoresis on 1% agarose gel and observations were conducted in ultraviolet (UV) light which showed specific bands for CFP-10 was 304 bp and for ESAT-6 was 291 bp.

Cloning and Transformation

The process of ligation was performed by mixing plasmid DNA with fresh PCR product inside a tube with

composition: 2 µl fresh PCR product, 1 µl 10x ligation buffer, 2 µl pET Sumo Vector, 4 µl sterile water, and 1 µl T4 DNA ligase. Plasmid DNA was incubated at 15°C for at least 4 hours or 15°C/overnight in a water bath. The ligation results were stored at -20°C, ready for transformation (Champion™ pET SUMO Protein Expression System, Invitrogen). Transformation was done by taking the blue tube (One Short®Mach1TM-T1R) and placing it in an ice container, allowing it to melt and adding 10 µl plasmid from the ligation result. This was then mixed until homogeneous without using a vortex and incubated in ice for 30 min, then heat shocked cell for 30 seconds at 42°C temperature and immediately stored in an ice tube. Next, 250 µl SOC medium was added, the tube was closed tightly and incubated at 37°C for 1 hour at shaker at 200 rpm.

The results of transformation were then spread on LB agar media each as much as 50 µl, 100 µl and 150 µl in LB plate medium on a petri dish with cover glass, then incubated at 37°C for 24 to 48 hours. The growth of colonies in the LB medium was observed and isolation DNA of plasmid was performed with PureLinkTM HQ Mini Plasmid Purification Kit. Transformation for expression proteins was done by taking a chocolate cap tube (competent cell *E. coli* BL21 (DE3) One Short® cells), adding 5-10 ng plasmid DNA. This was mixed with tips, incubated on ice for 30 minutes, heat shocked cell for 30 seconds at 42°C then immediately stored on ice in a tube. Next, 250 µl SOC medium was added, the tube was closed tightly and incubated at 37°C for 1 hour in shaker at 200 rpm. Finally, the transformation reaction was ready for removal to a Luria Bertani (LB) agar plate.

Recombinant proteins tested by SDS-PAGE

CFP-10 and ESAT-6 recombinant proteins were purified using nickel column chromatography (Ni-NTA Purification System, NOVEX by Lab Technologies Cat No. K950-01). The preparation of a 15% gel gradient and 3% gel stacking was performed for protein electrophoresis. The polymerization of the gel gradient occurred over the next 4-5 hours.

The gradient gel was cleaned with Aquadest on the surface of the gel to remove the remains of butanol. The protein samples were taken and then added with loading dye at a ratio of 1:4 (10 µl loading dye: 40 µl sample) to the tube, homogenized, and then heated to the boiling point of water at 80°C for 5 min. The sample was allowed to cool, and then the sample was loaded into the electrophoresis well. Electrophoresis was performed at 120 volts for 2 hours. After completion of electrophoresis, the gel was taken and stained with Coomassie blue for 1 hour using a shaker. The gel was washed with aquabidest, then washed with a destaining solution (30% methanol and 10% acetic acid solution) for 30 min until the band was visible, then cleaned with water to remove any remaining color.

Western blotting of recombinant proteins of CFP-10 and ESAT-6

The result of the SDS-PAGE that contained protein bands was transferred to polyvinylidene fluoride (PVDF) paper using Bio-Rad's semi-dry blotter tool with an electric current of 500 mA for 120 min. In the next step, the PVDF paper was blocked by Bovine serum albumin 1%, then added with anti-histidine Tag monoclonal antibody and secondary antibody (Goat IgG anti-mouse). After the washing process, this reaction was ended by adding NBT/BCIP substrate for 2-5 minutes.

Result and Discussion

The recombinant plasmid containing the inserted gene encoding the CFP-10 and ESAT-6 proteins was substantially transformed into the competent cell *E. coli* BL21 (Figure 1). Amplification of genes encoding CFP-10 and ESAT-6 proteins produced a single fragment with a length of 304 bp and 291 bp, respectively (Figure 2). The result of amplification of the encoding genes of CFP-10 and ESAT-6 protein by PCR of fresh product was used as DNA insertion in the ligation process. The ligation process used the pET SUMO vector as a cloning vector. This vector was designed to facilitate the cloning of PCR products directly. The pET SUMO vector had a nucleotide size of 5643 bp. The encoding genes CFP-10 and ESAT-6 of *M. tuberculosis* were successfully inserted into the SUMO pET vector and transformed into competent cells of *E. coli* BL21. The transformation growth on LB plate medium with the addition of kanamycin 50 µg/ml resulted in large amounts of bacterial colonies. *E. coli* BL21 colony growth on selection medium with kanamycin was promoted by *E. coli* BL21 bacteria carrying the pET SUMO vector. This vector has a kanamycin-resistant marker gene. Screening test results from several colonies of competent cell *E. coli* BL21(DE3) growth on LB medium showed that amplification with specific primers CFP-10 and ESAT-6 had specific bands sized at 304 bp and 291 bp, respectively. A positive deoxyribonucleic acid (DNA) plasmid that was carrying the CFP-10 and ESAT-6 genes was confirmed using SUMO forward and T7 reverse primers to find the inserted gene in the right frame. Several amplified colonies showed that the encoding genes CFP-10 and ESAT-6 proteins inserted in the pET SUMO vector were transformed well in competent cells.

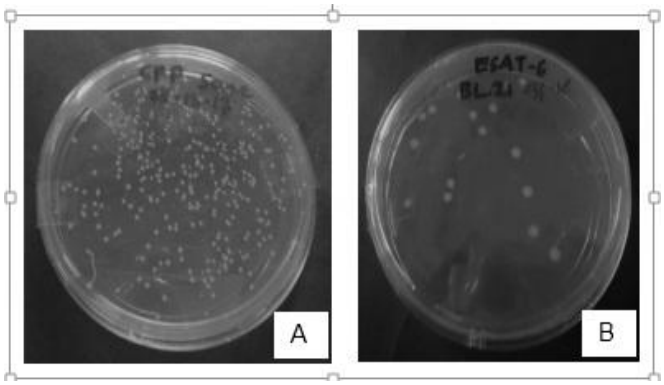

Figure 1. The growing of competent cell of *E.coli BL21 (DE3) One Short® cells* on LB agar. The colonies of *E.coli* competent cells containing a pET SUMO plasmid inserted with *esxB gene* (CFP-10) (A) and *esxA gene* (ESAT-6) (B) of *M. tuberculosis*.

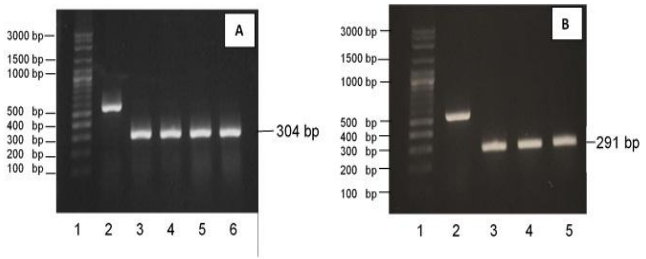

Figure 2. Results of PCR from bacterial colonies grown on LB agar medium. Line 1: DNA ladder, line 2: plasmid control primers, line 3,4,5 and 6: *esxB gene* (304 bp) (A). Line 1: DNA ladder, line 2: Plasmid control primers, and line: 3, 4, and 5: *esxA gene* (291 bp) (B) of *M. tuberculosis*.

The amplified gene that encodes the CFP-10 and ESAT-6 proteins from the competent cells *E. coli* BL21 (DE3) (Figure 2) showed it was successfully inserted into the pET SUMO plasmid. Amplification of the gene insertion area by using primers of plasmids (sumo protease forward and T7 reverse) showed the size of the gene that was inserted according to the length of the insertion gene size. The compatibility of the insertion gene size from the PCR examination results of bacterial colonies grown on LB medium was proven by sequencing. The result of gene sequencing inserted on the pET SUMO plasmid is shown in Figures 3 and 4. The complete genome of *M. tuberculosis* comprises 4,000 genes, which include 200 of the genes located in 16 local regions of difference (RD) locus.

The RD has been studied due to its role related to pathogenesis and the determinants of virulence in disease pathogenesis (Anes et al., 2023). The RD1 locus was found in *M. tuberculosis* of the H37Rv strain but not found in the *Mycobacterium bovis* vaccine strain (Abdallah et al., 2015). The RD1 region has a length of 9.5 kb, containing 9 open reading frames, which among others are the complex Early Secretory Antigen Targets-6 and Culture Filtrate Proteins. An investigation was conducted against ESAT-6 and CFP-10 and used to obtain ideal sensitivity and specificity for detecting *M. tuberculosis* either in single or recombinant antigens (Shen et al., 2011; Lu et al., 2025). In this study, the encoding genes CPF-10 and ESAT-6 of *M. tuberculosis* proteins were successfully amplified by specific primers designed from the *M. tuberculosis* genome strain H37Rv. The results of the amplification of encoding genes CFP-10 and ESAT-6 from *M. tuberculosis* clinical isolates obtained specific bands with nucleotide sizes of 304 bp and 291 bp, respectively.

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CLUSTAL O(1.2.4) multiple sequence alignment

Original :  ---ATGGCAGAGATGAAGACCGATGCCGCTACCCTCGCGCAGGAGGCAGGTAATTTCGAG  57
Forward  :  AGCATGGCAGAGATGAAGACCGATGCCGCTACCCTCGCGCAGGAGGCAGGTAATTTCGAG  60
Reverse  :  AGCATGGCAGAGATGAAGACCGATGCCGCTACCCTCGCGCAGGAGGCAGGTAATTTCGAG  60
            ************************************************************

Original :  CGGATCTCCGGCGACCTGAAAACCCAGATCGACCAGGTGGAGTCGACGGCAGGTTCGTTG  117
Forward  :  CGGATCTCCGGCGACCTGAAAACCCAGATCGACCAGGTGGAGTCGACGGCAGGTTCGTTG  120
Reverse  :  CGGATCTCCGGCGACCTGAAAACCCAGATCGACCAGGTGGAGTCGACGGCAGGTTCGTTG  120
            ************************************************************

Original :  CAGGGCCAGTGGCGCGGCGCGGCGGGGACGGCCGCCCAGGCCGCGGTGGTGCGCTTCCAA  177
Forward  :  CAGGGCCAGTGGCGCGGCGCGGCGGGGACGGCCGCCCAGGCCGCGGTGGTGCGCTTCCAA  180
Reverse  :  CAGGGCCAGTGGCGCGGCGCGGCGGGGACGGCCGCCCAGGCCGCGGTGGTGCGCTTCCAA  180
            ************************************************************

Original :  GAAGCAGCCAATAAGCAGAAGCAGGAACTCGACGAGATCTCGACGAATATTCGTCAGGCC  237
Forward  :  GAAGCAGCCAATAAGCAGAAGCAGGAACTCGACGAGATCTCGACGAATATTCGTCAGGCC  240
Reverse  :  GAAGCAGCCAATAAGCAGAAGCAGGAACTCGACGAGATCTCGACGAATATTCGTCAGGCC  240
            ************************************************************

Original :  GGCGTCCAATACTCGAGGGCCGACGAGGAGCAGCAGCAGGCGCTGTCCTCGCAAATGGGC  297
Forward  :  GGCGTCCAATACTCGAGGGCCGACGAGGAGCAGCAGCAGGCGCTGTCCTCGCAAATGGGC  300
Reverse  :  GGCGTCCAATACTCGAGGGCCGACGAGGAGCAGCAGCAGGCGCTGTCCTCGCAAATGGGC  300
            ************************************************************

Original :  TTCTGA    303
Forward  :  TTC---    303
Reverse  :  TTC---    303
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Figure 3. The sequencing result of *esxB gene* (304 bp) of *M. tuberculosis* inserted on the pET SUMO plasmid

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CLUSTAL O(1.2.4) multiple sequence alignment

Original   ACAGAGCAGCAGTGGAATTTCGCGGGTATCGAGGCCGCCGGCAAGCGCAATCCAGGGAAAT  60
Forward    ACAGAGCAGCAGTGGAATTTCGCGGGTATCGAGGCCGCCGGCAAGCGCAATCCAGGGAAAT  60
Reverse    ACAGAGCAGCAGTGGAATTTCGCGGGTATCGAGGCCGCCGGCAAGCGCAATCCAGGGAAAT  60
           ************************************************************

Original   GTCACGTCCATTCATTCCCTCCTTGACGAGGGGAAGCAGTCCCTGACCAAGCTCGCAGCG  120
Forward    GTCACGTCCATTCATTCCCTCCTTGACGAGGGGAAGCAGTCCCTGACCAAGCTCGCAGCG  120
Reverse    GTCACGTCCATTCATTCCCTCCTTGACGAGGGGAAGCAGTCCCTGACCAAGCTCGCAGCG  120
           ************************************************************

Original   GCCTGGGGCGGTAGCGGTTCGGAGGCGTACCAGGGTGTCCAGCAAAAATGGGACGCCACG  180
Forward    GCCTGGGGCGGTAGCGGTTCGGAGGCGTACCAGGGTGTCCAGCAAAAATGGGACGCCACG  180
Reverse    GCCTGGGGCGGTAGCGGTTCGGAGGCGTACCAGGGTGTCCAGCAAAAATGGGACGCCACG  180
           ************************************************************

Original   GCTACCGAGCTGAACAACGCGCTGCAGAACCTGGCGCGGACGATCAGCGAAGCCGGTCAG  240
Forward    GCTACCGAGCTGAACAACGCGCTGCAGAACCTGGCGCGGACGATCAGCGAAGCCGGTCAG  240
Reverse    GCTACCGAGCTGAACAACGCGCTGCAGAACCTGGCGCGGACGATCAGCGAAGCCGGTCAG  240
           ************************************************************

Original   GCAATGGCTTCGACCGAAGGCAACGTCACTGGGATGTTCGCATAGGGCAAC    291
Forward    GCAATGGCTTCGACCGAAGGCAACGTCACTGGGATGTTCGCATAGGGCAAC    291
Reverse    GCAATGGCTTCGACCGAAGGCAACGTCACTGGGATGTTCGCATAGGGCAAC    291
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Figure 4. The sequencing result of *esxA gene* (291 bp) of *M. tuberculosis was* inserted into the pET SUMO plasmid.

This study also conducted the sequencing process on the *inserted* genes. The sequence results were analyzed, and the alignment was performed with the ClustalW program, which showed that the encoding genes CFP-10 and ESAT-6 inserts were in the appropriate frame and there was no change in position or exchange between the deoxyadenosine start codon (A) with deoxyadenosine stop codon (A) that was bound within the deoxythymidine (T) on the cloning site TA. Positive bacterial colonies carrying the inserted gene

which were in the appropriate frame, were used for further analysis of recombinant proteins. The sequencing data confirmed that each gene inserted into the pET SUMO plasmid showed a compatible size to the inserted fragment. The cloning method was done by inserting PCR products with amplified genes encoding CFP-10 and ESAT-6 *M. tuberculosis* into the pET SUMO vectors based on the Champion™ pET protocol SUMO Protein Expression System, Invitrogen kits. Plasmid gene in the ligation process was not necessary because the pET SUMO vector has a T overhang sequence design. The Taq polymerase of the PCR product has a non-template-dependent activity that adds a single deoxyadenosine (A) to the 3 'end. Design of pET SUMO vector has a single deoxythymidine (T) residue that would bind to a single deoxyadenosine (A) of the PCR product.

The results in electrophoresis of recombinant protein product with SDS-PAGE and Western blotting using anti-histidine taq monoclonal antibody (6x) are shown in Figures 5 and 6. Recombinant protein obtained from cultures and IPTG induction optimization visualized in SDS-PAGE with gel gradient was 12% for resolving gel and 5% for stacking gel, respectively (Figure 5). The Western blotting examination of recombinant proteins was visualized with PVDF paper (Figure 6).

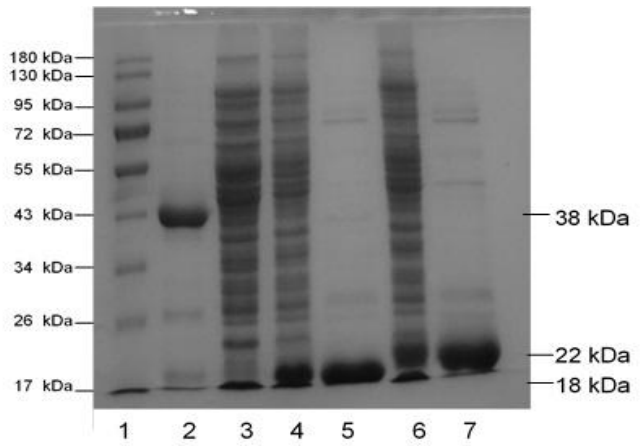

Figure 5. SDS-PAGE of purified CFP-10 and ESAT-6 proteins pET SUMO clone in *E. coli* BL21 (DE3). Line 1: protein ladder, line 2: positive control (38-kDa), line 3: negative control (*E. coli* BL21 without pET SUMO plasmid), line 4: crude ESAT-6 proteins line 5: a purified ESAT-6 proteins (18 kDa), line 6: crude CFP-10 proteins and line 7: a purified CFP-10 proteins (22 kDa).

The bands of recombinant CFP-10 and ESAT-6 fusion SUMO protein obtained from competent cell *E. coli* BL21 (DE3) bacteria pellet induced by IPTG 1 mM were visualized by SDS-PAGE, and these showed that the bacteria were able to express CFP-10 and ESAT-6 recombinant proteins. Visualization of CFP-10 and ESAT-6 recombinant proteins with SDS-PAGE resulted in molecular weights of proteins at 22-kDa and 18-kDa, respectively. The molecular weights of CFP-10 and ESAT-6 proteins were 10-kDa and 6-kDa, respectively (Seifert et al., 2021). The additional molecular weight to the two types of proteins was due to the addition of tag proteins from the pET SUMO plasmid of 12-kDa. Furthermore, to ensure the existence of both proteins, Western blotting using an anti-His tag antibody (mouse monoclonal) was performed. The presence of a further anti-His tag antibody was detected with anti-mouse IgG antibodies. The PVDF paper showed the visualized bands after adding a 1-step NBT/BCIP substrate solution at 22-kDa and 18-kDa positions. Previously, a study conducted by Yindeeyoungyeon et al. (2015**)** using the pET24b vector showed that the molecular weights of CFP-10 and ESAT-6 are about 12,5 kDa and 11,5 kDa, respectively. ESAT-6 (6-kDa an early secretory antigenic target or ESXA) and CFP-10 (10-kDa culture filtrate protein or ESXB) genes were located in the RD1 (Salon et al., 2014). At the RD1 locus, gene esx is part of the gene that encodes a continuous membrane protein. Genes in RD1 include *Rv3871* to *Rv3879c* (Mahmoudi et al., 2013; Anes et al., 2023).

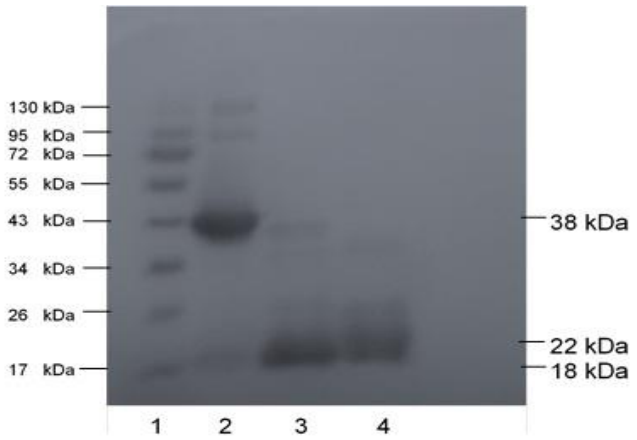

Figure 6. Western blotting of CFP-10 and ESAT-6 proteins by using 6x anti-histidine Tag monoclonal antibody. line 1: protein ladder, line 2: positive control (38-kDa), line 3: a purified ESAT-6 protein (18 kDa), and line 4: a purified CFP-10 protein (22 kDa).

Gene of *CFP-10* and *ESAT-6* are controlled by the same operon. The secretion of these two genes produces proteins that play an important role in the virulence of *M. tuberculosis* (Peng et al., 2016). The CFP-10 and ESAT-6 are proteins encoded by ESX-1 and secreted into the phagosome. The secreted ESAT-6 protein can induce the lysis of the phagosomic membrane from macrophages (Koiri et al., 2025). Proteins secreted early in the onset of TB infection are ESAT-6 and CFP-10, which play a role in adhesion, invasion, and cell lysis in macrophages that

act as virulence factors of granuloma formation or tissue damage and some abnormalities (Mertaniasih et al., 2016; Passos et al., 2024). CFP-10 protein and ESAT-6 were suggested to be the main virulence factors in *M. tuberculosis* (Forreland et al., 2013) because they can modulate the immune response by producing INFγ and then stimulate T cells causing delayed hypersensitivity (DTH) reactions (Abebe et al., 2017; Handayani et al., 2018) and suppressing the production of Reactive Oxygen Species (ROS) in the macrophages. The CFP-10 antigen may also inhibit the mediated TLR-2 signal, playing a role in pore formation, causing infected cell apoptosis and having cytolytic effects (Seghatoleslam et al., 2016; Malakar et al., 2023)

The CFP-10 protein is located in the upstream part of the ESAT-6 antigen, and found in *M. tuberculosis* complex (MTBC), but is not found in *M. bovis* strains and 2 specific *M. tuberculosis* antigens (Wu et al., 2017). Tests using CFP-10 and ESAT-6 recombinant proteins suggest that the combined use of CFP-10 and ESAT-6 may provide a screening strategy for the development of a tuberculosis serodiagnostic test and tuberculosis vaccine (Guo et al., 2023).

Conclusion

The encoding genes CFP-10 and ESAT-6 of *M. tuberculosis* from clinical isolates of patients were successfully inserted after amplification into the pET SUMO vector and transformed into competent cells (*E. coli* BL.21 (DE3)). The amplification results for CFP-10 and ESAT-6 obtained specific bands with nucleotide sizes 304 bp and 291 bp, as well as nucleotide weights, 22 kDa and 18 kDa, respectively. They may provide a screening strategy for the development of candidates for recombinant vaccines against *M. tuberculosis*

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Author Contributions
Led the research, Conceptualization, F.F.; Supervised the overall research work, drafted the manuscript, and revised intellectual content, F.F., N.I., L.U., and M.N.S; Participated in sampling, made available relevant literature, collection of data, executed the experiment, interpretation of the results, and drafted the manuscript, N.I., L,S, S.Z, L.U and M.N.S. All authors have read and approved the final manuscript.

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Conflicts of Interest
The authors declare no conflict of interest.

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