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CLONING, EXPRESSION AND T CELL EPITOPE PREDICTION OF *FBPA* AND *FBPB* GENES OF *MYCOBACTERIUM TUBERCULOSIS* CLINICAL ISOLATES

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ABSTRACT

The effective treatment and accurate diagnosis of tuberculosis (TB) are not established yet. The Bacillus Chalmette-Guerin vaccine did not provide significant results in the prevention of TB and had only 0-80% efficacy. The *fbpA* and *fbpB* genes of *M. tuberculosis* are antigenic proteins and considered to be virulence factors. They are capable of stimulating immune responses in TB patients. In this study, we observed cloning, expression and T-cell epitope prediction of *fbpA* and *fbpB* genes from clinical isolates. The isolates of MultiDrug-Resistant (MDR-TB) were cultured and extracted. The fresh Polymerase Chain Reaction (PCR) products of the *fbpA* and *fbpB* genes were inserted into pET SUMO plasmids and transformed into *Escherichia coli BL21 (DE3)*, then expressed in LB medium induced by 1.0 µM of IPTG. Sample sequences were analyzed by ClustalW and NCBI BLAST programs. The T-cell epitope prediction was analyzed by GENETYX vers 8.0. The PCR results were 1071 bp (*fbpA* gene) and 978 bp (*fbpB* gene). The SDS-PAGE and Western blotting weighed 48-kDa (*fbpA* gene) and 46-kDa (*fbpB* gene). We obtained seven specific T-cell epitopes based on IAd Pattern Position on both genes. Based on Rothbard/Taylor Pattern Position, we discovered twenty-three and sixteen specific T-cell epitopes for *fbpA* and *fbpB* genes, respectively. The *fbpA* and *fbpB* genes that encode Ag85A and Ag85B proteins have epitopes that are recognized by lymphocyte T-cells and are potentially subunit TB vaccine candidates in the future.

Keywords: *fbpA* gene, *fbpB* gene, T cell epitopes prediction, *M. tuberculosis*

INTRODUCTION

Tuberculosis (TB) is an infectious disease that causes high rates of mortality and morbidity worldwide, especially in developing countries and continues to be a challenging burden on public health. Globally, there were 6.3 million new cases of TB in 2017 and the mortality rate reached 1.3 million, making TB the second leading cause of death from infectious diseases after Human Immunodeficiency Virus (HIV) Infection. Indonesia has the second largest burden of TB cases in the world after India. In 2017, there were 425,089 TB cases in Indonesia, indicating a dramatic increase compared to the previous year of 360,565 cases with a mortality rate of 42 per 100,000 population (**Wang *et al.*, 2017; WHO 2018; Ministry of Indonesia Health 2018**). The problem of preventing and controlling TB is very complex because the effective TB treatment and accurate diagnosis are not established yet (**Guginno *et al.*, 2015**). *Mycobacterium tuberculosis* has unique characteristics, such as slow bacterial growth, diverse strains, and virulence due to multidrug-resistant tuberculosis (MDR-TB), which increases human susceptibility to HIV, and manifests latent infections that reach 40-50% of cases. Meanwhile, there are decreasing effectiveness of TB vaccines and less optimal diagnostic methods (**WHO 2014; Yuen *et al.*, 2014**). To date, TB treatment and prevention protocols still apply the Directly Observed Treatment Short-Course (DOTS) program and the administration of the Bacilli Culmette-Guerin (BCG) vaccine. Unfortunately, both the DOTS program and BCG vaccine are not proceeding optimally. The conventional BCG vaccine is still the major vaccine in early protection against *M. tuberculosis* infection. It cannot provide optimal protection to prevent the transmission of MDR-TB and the current epidemic of TB cases. The BCG vaccine is only effective in preventing miliary TB or meningitis prevention for children, but does not provide protection in pulmonary TB for adults with latent and reactivation of TB (**Piubelli *et al.*, 2013**). The development of better diagnostic tools and the discovery of new vaccine candidates are the two main ways to solve the TB problems (**Dillon *et al.*, 2000; WHO 2013**). Ag85 complex is a main fraction of the excretory protein of *M. tuberculosis* and *M. bovis* BCG culture filtrate that has the potential to increase protection against TB. This protein is present on the cell wall surface and needed for the survival of *M. tuberculosis* in macrophages (**Kuo *et al.*, 2013**). Antigen 85 complex (Ag85A, Ag85B, Ag85C) has a *mycolyl transferase* enzyme activity that is involved in mycolic acid pathway to arabinogalactan from cell walls and required for biogenesis in cord factors (*trehalose-dimycolate*). These proteins contribute to the attachment of *M. tuberculosis* to host cells and can maintain *M. tuberculosis* survival in the intracellular parts of host cells. (**Launois et al., 2011; Piubelli *et al.*, 2013: Zarif *et al.*, 2013**). The proteins of Ag85A and Ag85B are encoded by the *fbpA* and *fbpB* genes, respectively. Secreted antigen 85 protein is immunogenic and expressed continuously by *M. tuberculosis* and can stimulate B cell and T cell responses. T cells can activate the cellular and humoral immunity system through release of cytokines and activating macrophage cells, NK cells, DC cells and Tc cells to destroy intracellular *M. tuberculosis*-infected cells (**Jiang *et al.*, 2015; Rizzi *et al.*, 2012; Metcalfe *et al.*, 2016**). Recombinants of Ag85A (32 kDa) and Ag85B (30 kDa) proteins are promising for TB vaccine candidates because they are the major proteins secreted by *M. tuberculosis* and show strong immunogenic properties (**Yuk *et al.*, 2016**). This study aimed to clone, express and predict the specific T cell epitopes of the *fbpA* and *fbpB* genes of *M. tuberculosis* from clinical isolates.

MATERIAL AND METHODS

Extraction and measurement concentration of DNA

In this study, isolates of *M. tuberculosis* were obtained from sputum of patients who were diagnosed with MDR-TB in Dr. Sutomo Hospital Surabaya. TB Ag MPT 64 Rapid test (SD Bioline) was used for detection of bacterial antigens in serum. The isolates of *M. tuberculosis* were subcultured (refreshed) to elicit isolates in 2-3 weeks of log phase growth. The DNA of isolates were extracted using the DNeasy Blood and Tissue Kit (Qiagen). Resistance test and DNA extraction of isolates were performed in Balai Besar Labaratorium Kesehatan (BBLK) Surabaya and used *M. tuberculosis* strain H37Rv as a control. Identification of MDR-TB was done on Lowenstein Jensen (LJ) medium with BACTEC MGIT 960 System (BD) methods and confirmed by GeneXpert® System (Cepheid USA) test. The concentration of bacterial DNA extract was measured by a NanoDropTM2000/ 2000c Spectrophotometer (Thermo Fisher Scientific, USA).

Design of primers and Polymerase Chain Reaction (PCR)

The nucleotide sequence of the primers used in this study was determined by the genome sequence of *M. tuberculosis* H37Rv (ATCC 27294) which was obtained from GenBank and designed by BLAST pick primers software (www.ncbi.nlm.nih.gov/tools/primer-blast/index). The Primary forward used is: 5'- *GGA TGC GTT GAG ATG AGG ATG AG* -3 ', reverse primer: 5'- *GTT TCC TAA ATC CCG TCC CTA GCT* -3', (*fbpA* gene) and forward 5'- ACA GAC GTG AGC CGA AAG ATT C -3 'and as a reverse primer are: 5'- *TCA GCC GGC GCC TAA CGA ACT CT* -3' (*fbpB* gene). The PCR was performed in a total volume of 25 µl, that contained 12.5 µL PCR mix (GoTaq Green Master Mix Promega), 2 µl primary forward, 2 µl primary reverse, 6.5 µl ddH_2O and 2 µl DNA template. An initial denaturation of 5 min at 95^{0}C was followed by 35 cycles of denaturation at 95^{0}C for 5s, annealing at 56^{0}C for 1s, and extension at 72ºC for 1 min, followed by a final extension at 72ºC for 10 min, then the tube was cooled for 10 minutes at 12ºC. The PCR product was analyzed by electrophoresis on 1% agarose gel in Tris/boric acid/EDTA buffer and observations were conducted in transilluminator UV light.

Cloning, transformation and expression of Ag85 proteins

The fresh PCR product was mixed with pET SUMO plasmid (Champion™ pET SUMO Protein Expression System, Invitrogen) for ligation process by T4 DNA ligase in a microtube. Then, it was incubated at 15ºC for at least 4 hours or (15ºC/overnight) in a water bath and transformed into *E. coli* competent cells (One Short®Mach1TM-T1R) with heat shocked method at 42ºC for 30 seconds. The results of transformation were then spread on LB medium containing 50 µg/ml kanamycin and incubated at 37ºC for 24 to 48 h. The plasmids were purified using a commercial kit (PureLinkTM HQ Mini Plasmid Purification Kit) and transformed into *E. coli* BL21(DE3) competent cells (One Short® cells). The transformed bacteria were grown in 5 ml Luria-Bertani (LB) broth medium containing 50 µg/ml kanamycin, and incubated at 37^{0}C 180 rpm for overnight. Then, 5% of overnight culture was removed to 100 mL LB broth medium, incubated with shaking at 37^{0}C for 4 h (optical density ± 0.6), and supplemented with 1 mM IPTG (Amresco, USA). The incubation was continued for 3 h, then the cell pellet was collected by centrifugation at 4^{0}C, 10,000 rpm for 5 min. The bacterial cells were then re-suspended three time in Phosphate Buffer Saline (PBS) and degraded by sonicator. The supernatants were collected by centrifugation at 4^{0}C, 10,000 rpm for 5 min.

Purification and SDS-PAGE

The Ag85A and Ag85B recombinant proteins were purified using nickel column chromatography and the purified lysate was loaded into a 10 ml Ni-NTA column (Ni-NTA Purification System, NOVEX by Lab Technologies Cat No. K950-01). Initially, the column was washed with wash buffer (250 mMNaH_2PO_4, 2.5 M NaCl and 100 µL 3 M imidazole). The proteins were eluted with elution buffer (250 mMNaH_2PO_4, 2.5 M NaCl and 1.25 mL 3 M imidazole). The purified recombinant proteins and supernatant of cell pellets were analyzed using 15% sodium dodecyl sulfate-polyacrylamide gels. The protein samples were taken and then added with loading dye with a ratio of 1: 4 (10 µl loading dye: 40 µl sample), then heated at 80ºC for 5 min. Electrophoresis was performed at 120 volts for 2 hours. The proteins were visualized by staining with Coomassie blue for 1 hour using a shaker. The gel was washed with aquabidest, then washed with a solution of destining (50% methanol, 10% acetic acid glacial, and 40% aquabidest) for 30 min until the band was clearly visible.

Western blotting of recombinant proteins of Ag85A and Ag85B

The protein bands from the SDS-PAGE were transferred to polyvinylidene fluoride paper (PVDF) 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 anti-histidine Tag monoclonal antibody and secondary antibody (Goat IgG anti-mouse) were added. After the washing process, this reaction was ended by the adding of NBT/BCIP substrate for 2-5 minutes.

T cell epitope prediction of recombinant proteins of Ag85A and Ag85B

PCR products of the *fbpA* and *fbpB* genes were sent to 1st BASE for sequencing processes. 1st BASE used ABI PRISM 370xl Genetic Analyzer (Applied Biosystem). Sample sequences were then analyzed using ClustalW (www.genome.jp/tools-bin/clustalw). The T cell epitopes' prediction was analyzed by GENETYX version 8.0.

RESULTS

Amplification of the *fbpA* and *fbpB* genes of *M. tuberculosis* which encode the Ag85A and Ag85B proteins was found to be of 1071 bp and 978 bp, respectively (Figure 1). In this study, we used twenty-five (25) MDR-TB clinical isolates, with all samples showing the properly positioned band of the *fbpA* and *fbpB* genes. The alignment of the sequencing of those genes was analyzed by the ClustalW program which was compared to the original sequence of *M. tuberculosis H37Rv*. The alignment results showed a high similarity of sample sequences with *M. tuberculosis* H37Rv which was 99%. We also analyzed the sample sequences using the NCBI BLAST program and showed that all sample sequences had a 100% homologous sequence with various strains of *M. tuberculosis* found in the NCBI data base (Table 1). The fresh PCR product from the amplified *fbpA* and *fbpB* genes which have the similar sequence with *M. tuberculosis* H37Rv were then inserted into the pET SUMO plasmids and transformed to *E. coli* competent cells. The *E. coli* bacteria carrying plasmids were grown in LB agar medium, otherwise those that do not contain plasmids cannot grow. The growth of each *E. coli* bacteria that were carrying different plasmids in the medium showed different numbers of colonies. The colonies of *E. coli* competent cells carrying the inserted *fbpA* and *fbpB* genes that grew on the LB agar medium were 5 and 18 colonies, respectively (Figure 2).

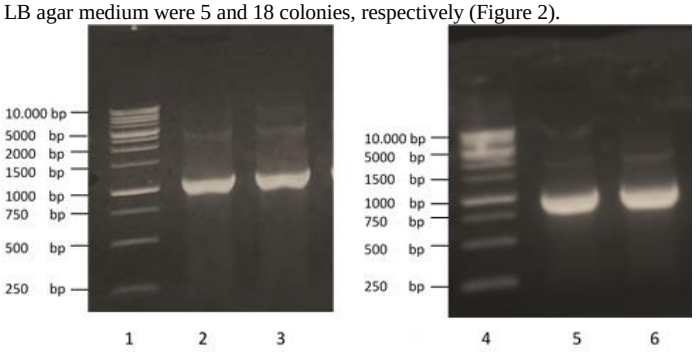

Figure 1 Results of PCR samples of clinical isolate. Line 1: DNA ladder, line 2 and 3: *fbpA* gene (1071bp), line 4: DNA ladder, line 5 and 6: *fbpB* gene (978 bp).

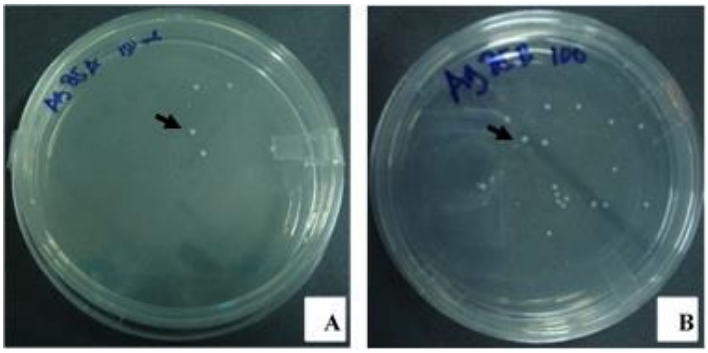

Figure 2 The growing of *E. coli* competent cells on LB agar. The colonies of *E. coli* competent cells containing a pET SUMO plasmid inserted with *fbpA* gene (A) and *fbpB* gene (B) of *M. tuberculosis*.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the purified Ag85A and Ag85B recombinant proteins in Figure 3 showed that the molecular weights were 48 kDa and 46 kDa, respectively. The Western blotting examinations of recombinant proteins performed with PVDF paper elicited the similar molecular weights of Ag85A and Ag85B proteins with SDS-PAGE of 48 kDa and 46 kDa, respectively. The results of SDS-PAGE and Western blotting recombinant protein product using anti-histidine taq monoclonal antibody (6x) are shown in Figures 3 and 4.

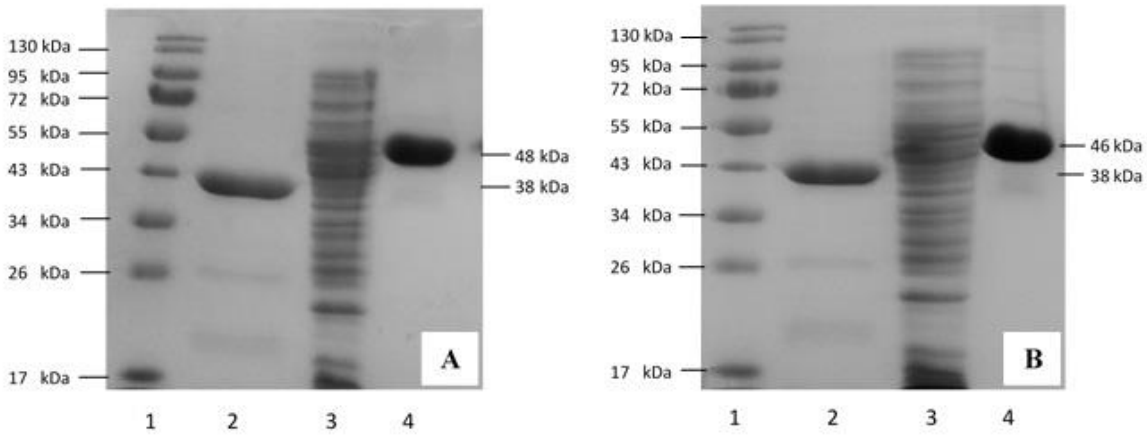

Figure 3 SDS-PAGE of purified Ag85A and Ag85B proteins pET SUMO clone in *E. coli* BL21 (DE3). Line 1: protein ladder, line 2: positive control (38-kDa), line 3: negative control (*E. coli* BL21 without pET SUMO plasmid), line 4: a purified Ag85A proteins (A). Line 1: protein ladder, line 2: positive control, line 3: negative control (*E. coli* BL21 without pET SUMO plasmid), and line 4: a purified Ag85B protein (B).

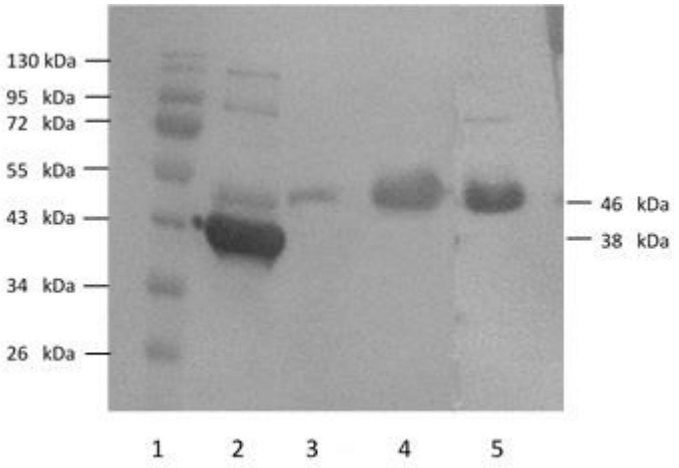

Figure 4 Western blotting of Ag85A and Ag85B proteins by using 6x anti-histidine Tag monoclonal antibody. line 1: protein ladder, line 2: positive control (38-kDa), line 3 and 4: a purified Ag85A protein (48 kDa), and line 5: a purified Ag85B protein (46 kDa).

The *fbpA* and *fbpB* genes of *M. tuberculosis* each had seven specific epitopic T cells prediction based on the IAd Pattern Position, while the prediction of specific T cell epitopes based on the Rothbard/Taylor Pattern Position shows that the *fbpA* gene has 23 specific T cell epitopes and *fbpB* gene has 16 specific T cell epitope positions (Table 2).

Table 1 Homology of samples isolate with bacteria in NCBI database.

No	Bacteria species	Query (%)	Coverage E.value	Identity (%)
1.	M. tuberculosis strain DKC2	100	0.0	100
2.	M. tuberculosis variant bovis	100	0.0	100
	BCG strain BCG-S48	100	0.0	100
3.	M. tuberculosis strain	100	0.0	100
4.	TBMENG-03	100	0.0	100
5.	M. tuberculosis strain H107	100	0.0	100
6.	M. tuberculosis strain H83	100	0.0	100
7.	M. tuberculosis strain GG-	100	0.0	100
8.	229-10	100	0.0	100
9.	M. tuberculosis strain GG-	100	0.0	100
10.	186-10	100	0.0	100
	M. tuberculosis strain GG-137-10			
	M. tuberculosis strain GG-134-11			
	M. tuberculosis strain GG-129-11			

Table 2 Prediction of T cell epitopes of Ag85A and Ag85B recombinant proteins of *M. tuberculosis*.

Gene	T cell epitope			
	IAd Pattern Position		Rothbard/Taylor Pattern Position	
	Amino acid position	Sequence	Amino acid position	Sequence
fbpA	18-23 39-44 46-51 93-98 179-184 183-188 225-230	VRGAVT VSGLVG VGGTAT LDGLRA LSMETA ASSALT AGGYKA	20-23 28-32 32-35 41-44 44-47 53-56 59-63 158-161 161-164 201-204 205-208 219-223 232-235 239-243 253-257 260-264 253-257 281-284 285-288 306-309 309-312 334-337 343-347	GAVT RLVVG GAVG GLVG GAVG GAFS GLPVE ELPG GWLQ GAMS GLLD GLAMG DMWG DPAWQ DPLLN KLIAN RVWVY KFLE GFVR GVFD DFPD RALG GPAPQ
fbpB	16 - 21 31 - 36 78 - 83 148 - 153 156 - 161 185 - 190 210 - 215	IGTAAA AGGAAT LDGLRA LSANRA PTGSAA AGSLSA AGGYKA	8-11 13-17 26-29 29-32 33-36 38-41 44-48 143-146 152-155 204-208 214-217 217-220 224-228 238-242 245-249 266-269	RAWG RLMIG GLVG GLAG GAAT GAFS GLPVE ELPQ RAVK GLAMG KAAD DMWG DPAWE KLVAN RLWVY EFLE

DISCUSSION

In this study, the *fbpA* and *fbpB* genes of *M. tuberculosis* encoding the Ag85A and Ag85B proteins were successfully amplified using specific primers designed from the *M. tuberculosis* genome strain H37Rv. The results of amplification of the genes encoding Ag85A and Ag85B *M. tuberculosis* clinical isolates obtained specific bands with nucleotide sizes of 1071 bp and 978 bp, respectively. Analysis of sequencing results of the *fbpA* and *fbpB* genes conducted by the ClaustalW program showed that the gene had a 99% similarity to the original sequence of the *M. tuberculosis* gene H37Rv. Sequencing of the *fbpA* and *fbpB* genes was done to confirm that there were no wrong gene sequences nor errors in the frame position of the gene to be inserted in the pET SUMO plasmids. The cloning process in this study was done by mixing fresh PCR products from the amplification results of the *fbpA* and *fbpB* genes into pET SUMO vectors based on the SUMO Protein Expression System Champion ™ pET protocol, using Invitrogen kits. The digest using restriction enzyme of the insertion sequencing gene or plasmid in the ligation process were not done because pET SUMO vector has a T overhang sequence design. Taq polymerase from PCR products has a non-template dependent activity that adds a single deoxyadenosine (A) to end 3'. The pET SUMO vector design used has an overhanging deoxythymidine (T) at end 3' that can bind to a single deoxyadenosine (A) from PCR products. The growth of *E. coli* BL21 (DE3) competent cells that contain gene insertion was observed in LB Agar medium (containing kanamycin 50 µg / mL). The colonies of *E. coli* BL21 (DE3) competent cells can be grown on media containing kanamycin because bacteria carry pET SUMO vectors that have a marker for the resistant gene to kanamycin. The PCR results of several colonies of *E. coli* BL21 (DE3) using specific primers were in accordance with the band size expected. Amplification of deoxyribonucleic acid (DNA) plasmids carrying the *fbpA* and *fbpB* genes was also done using forward SUMO and T7 reverse primers to identify the insertion gene in the right frame. Results of some amplified colonies showed that *fbpA* and *fbpB* genes inserted in the pET SUMO were transformed to competent cells accurately. Alignment sequences using the ClustalW program indicate that the gene encoding Ag85A and Ag85B proteins insertion was in the right frame and there was no change in position or exchange between deoxyadenosine (A) start codon and deoxyadenosine (A) stop codon which binds to deoxythymidine (T) in the TA cloning site. SDS-PAGE of Ag85A and Ag85B recombinant proteins obtained from the sonication of *E. coli* BL21 (DE3) bacteria induced by 1 mM IPTG showed that the bacteria were able to express *fbpA* and *fbpB* genes, with their molecular weights of 48-kDa and 46-kDa, respectively. The molecular weights of Ag85A and Ag85B proteins are 36-kDa and 34-kDa, respectively. The addition of molecular weight in both types of protein is due to the addition of protein tags from pET SUMO plasmids of 12-kDa. The expression of recombinant proteins fused with SUMO (small ubiquitin-related modifiers) shows a significant increase of proteins in the yield whose expression is very difficult to find in *E. coli* (**Lee, 2008; Gopal and Kumar, 2013; Ceylan and Erdogan, 2017**). Western blotting on PVDF paper using a monoclonal anti-histidine tag antibody was done to ensure the SDS-PAGE results were similar with the expected proteins. Monoclonal antibodies will bind with histidine sequences in taq SUMO of pET SUMO plasmids specifically. Western blotting results showed that the band was visualized after adding 1-step NBT/BCIP substrate solution according to SDS-PAGE results. Based on the analysis of the *fbpA* and *fbpB* gene sequences using the NCBI BLAST program results showed that all samples had 100% homology with several strains of *M. tuberculosis* and no similar sequencing was found with non-tuberculosis mycobacteria (NTM). The results of this homology indicate that the *fbpA* and *fbpB* genes of *M. tuberculosis* are highly conserved genes **(Hugen 2014).** The study conducted by **Jiang et al. (2015)** about single nucleotide polymorphism in Ag85 genes of *Mycobacterium tuberculosis* complex from samples of clinical isolates with several strains showed that only three of 180 samples had mutations with changes in amino acids, which were found in bases 47, 245, 312 for Ag85A proteins and in bases 44 and 140 for Ag85B proteins. The results of this study indicate that low amino acid changes occur in these proteins and show the highly conserved genes that encode both proteins (**Jiang *et al.*, 2015**). We did analysis of specific T cell epitopes using GENETYX software ver. 8.0 and found seven specific T cell epitope positions based on the IAd Pattern position on both Ag85A and Ag85B proteins and twenty-three specific T cell epitopes based on the Rothbard/Taylor Pattern position for Ag85A proteins, whereas for Ag85B protein we found sixteen specific T cell epitopes based on the Rothbard/Taylor Pattern position. The prediction of T cells epitope carried out by **D'Souza et al.** (2003) using EpiMer Program obtained five T cell epitopes for each Ag85A and Ag85B proteins. The position of T cell epitopes for Ag85A protein is present in the amino acid sequence 101-105, 121-134, 148-171, 198-207 and 270-280, whereas for Ag85B proteins it is present in positions 65-70, 101-105, 128-133, 146 -154 and 270-280. The study conducted by **Zhang et al. (2016)** found a different position of T cell epitopes on the Ag85B proteins. The results of their study revealed that the position of T cell epitopes was present in the amino acid sequence 45-58, 77-88, 98-107 and 191-206. In our study the results we obtained for the prediction of T cell epitopes were different with the studies conducted by **D'Souza et al. (2015)** and **Zhang et al. (2016)** who found five T cell epitopes on both Ag85A and Ag85B proteins. The difference in the number and position of T cell epitopes obtained by previous researchers with our research was probably caused by differences in the primers design used for amplification of the *fbpA* and *fbpB* genes, resulting in differences in the number of sequences of target genes obtained. This difference in results may also be due to the different programs used to predict T cells epitopes. The research conducted by **D'Souza et al. (2015)** predicted the T cell epitopes of Ag85 proteins using the EpiMer program, while Zhang et al. predicted T cell epitopes by software Proped, BIMAS and SYFPEITHI. Using GENETYX software, we predicted the Ag85A and Ag85B proteins T cell epitopes to understand the immune response to *M. tuberculosis* infection. Some studies state that the cellular immune system mediated by CD4+ T-cells and CD8+ T cells is very important for controlling latent and active TB (**Dewi et al. 2018**). Based on the T cell epitopes' prediction that we did, the results showed that the Ag85A and Ag85B proteins can be identified by T lymphocyte cells, which show that the protein has the potential as a subunit vaccine candidate and material for serodiagnostics for tuberculosis in the future (**D'Souza et al. 2003**).

CONCLUSION

In this study we succeeded in observing the cloning and expression of *fbpA* and *fbpB* genes of *M. tuberculosis* obtained from samples of clinical isolates in *E. coli* BL21 (DE3) competent cells, with molecular weights of 48-kDa and 46-kDa respectively. We discovered seven specific T cell epitope predictions based on IAd Pattern positions of Ag85A and Ag85B proteins, twenty-three specific T cell epitopes of Ag85A proteins and sixteen specific T cell epitope of Ag85B proteins predictions based on the Rothbard/Taylor Pattern positions.

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