Cloning and expression of recombinant protein CFP21 from *Mycobacterium tuberculosis* as a tuberculosis vaccine candidate

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**Abstract.** Increased resistance to TB drugs, may render vaccine development a more effective approach to stop or reduce TB epidemics. The antigen Culture Filtrate Protein Filtrat 21 (CPF21) is an immudominant protein encoded in RD 2 region of the *Mycobacterium tuberculosis* genome, capable of obtaining a strong hypersensitivity reaction and to induce very high interferon-gamma (IFN-\(\gamma\)) responses in patients with tuberculosis. In order to construct the recombinant plasmid pGEM-T Easy-CFP21 and express it in *E. coli* BL21, the CFP21 gene was amplified from *M. tuberculosis* H37Rv genomic DNA using PCR *in vitro*, and inserted into the pGEM-T Easy cloning vector. The recombinant plasmid was then transformed into *E. coli* JM109, followed by plasmid extraction, PCR amplification, and DNA sequencing. The correct recombinant CFP21 gene was subcloned into expression vector pGEX-2TK and transformed into *E. coli* BL21 strain. The white recombinant colony was selected, cultured, induced with 50 \(\mu\)M IPTG, and identified using SDS-PAGE electrophoresis method. These results demonstrated that CFP21 gene has been constructed and expressed successfully. The molecular weight was about 47 kDa as the fusion protein GST-CFP21 and expressed as insoluble protein. In conclusion, the target gene CFP21 has been cloned into host *E. coli* BL-21 strain and expressed successfully. In the future, the purified recombinant fusion protein GST-CFP21 paves the way for TB diagnosis and vaccine development.

1. **Introduction**

Tuberculosis (TB), malaria and AIDS are the of the three major infectious diseases globally, with 9 million new cases and 2 million deaths per year. In Indonesia, 70% of TB cases occur in the productive age that causes enormous social and economic burden. *Mycobacterium tuberculosis* has infected about one third of the population worldwide. Every year, around 8 million infected people...
develop active disease and 2 million of them die from tuberculosis. Around 10% of TB cases in the world are found in Indonesia and this puts Indonesia at third highest TB burden country after India and China[1].

Management of TB is increasingly difficult with the emergence of drug resistant strains. Multi-drug resistant \textit{M. tuberculosis} (MDR-TB) strain is known to be resistant to two main antibiotics that are often used, namely isoniazid and rifampicin. Furthermore, \textit{M. tuberculosis} strains that show resistance to additional TB drugs (Extensive drug-resistant \textit{M. tuberculosis}, XDR-TB) were found. Besides isoniazid and rifampicin, XDR-TB strains are also resistant to fluoroquinolones and injection drugs e.g. kanamycin, amikacin, or capreomycin [2]. The emergence of these strains has caused new issues in TB management globally and made WHO proclaim TB as a worldwide crisis. Due to increased pathogen resistance to TB drugs, the development of more effective vaccines is believed to be a necessary solution to stop or reduce the TB epidemic. Some criteria for an effective TB vaccine are the capabilities to provide better immunity than BCG; to stimulate a quick response for protection against TB; to reduce pain in people affected by TB; and to prevent TB reactivation [2,3].

The formation of antibodies to the \textit{M. tuberculosis} antigen takes a long time because the tuberculosis bacterial infection is a slow type hypersensitivity reaction and involves the cellular immune responses more than the humoral immune responses in its pathogenesis. Thus, antibody-based examination cannot detect TB disease at the initial stage of infection [4]. At present, several types of antigens from \textit{M. tuberculosis} are being examined as TB vaccine candidates. ESAT-6 is an antigen that is widely used in several previous studies as TB vaccine candidates. In its development, the ESAT-6 antigen is an important protein used in the diagnosis to distinguish between non-TB and TB patients (who have been vaccinated with BCG). Thus, ESAT-6 is no longer used in the development of TB vaccines [5,6,7]. In our previous study, we successfully cloned and produced MPT83 recombinant antigen using the \textit{E. coli} BL-21 cell as a host [8]. Besides the MPT83 antigen, another antigen, CFP21, is found in the \textit{M. tuberculosis} genome. CFP21 antigen is an immunodominant protein encoded in the RD2 region in the \textit{M. tuberculosis} genome and proven capable of inducing IFN-\gamma released from cytotoxic T lymphocytes of TB patients, so that the antigen is very potential as a vaccine candidate [9,10].

Based on the description above, a study of cloning and expression of CFP21 antigen with recombinant DNA technology has been carried out as a candidate for an effective TB vaccine in the future. Thus the antigen is expected to decrease the cost of making TB vaccines, provide immunity against active TB patients in productive age, which in turn can reduce the morbidity and mortality caused by TB.

\section{Materials and methods}
\subsection{Materials}
The materials used in this study were \textit{E. coli} strain JM109 and BL21, plasmid pGEM-T Easy and pGEX2TK, LB medium (Luria Bertani), ampicillin, IPTG, NaCl, KCl, Na$_2$HPO$_4$, KH$_2$PO$_4$. Loading buffer, Acrylamide / bis solution (Bio-rad), Tris Base (Calbiochem), SDS (Sigma), Ammonium Persulfate (Sigma), Tetramethylthylenediamine (Sigma), Glisine (Sigma), protein marker (Thermo Scientific), Methanol (Merck), Glacial Acetic Acid, Geneaid Kit, Qiagen Kit, master mix go Tag green Kit, loading dye, DNA marker 100 bp, Bacto trypton, Bacto agar, Bacto yeast, comma brilliant blue (Bio-rad), Filter tip (Genfollower), and Barrier tips (Multiguard).

\subsection{Instruments}
The instruments used in this study were thermo cycle PCR machines, shakers incubator (Heidolph Duomax 1030), centrifuges (Profuge), sonicators, Mini Protean II Bio Rad, Electrophoresis (Bio-rad), autoclaves (Hirayama), laminar air flow (Esco Ductless Fume Cabinet), Erlenmeyer (Pyrex), scale (Kern 440-47N), micropipette (Bio rad), beaker (Pyrex), eppendorf tube (Axygen), Hot plate (Memmert), and waterbath (Memmert).
2.3 Methods

2.3.1 Isolation of M. tuberculosis DNA genome. Isolation of M. tuberculosis H37Rv strain genomic DNA was carried out by the modified Boom method. Into 100 μl of sample, 900 μL of L6 lysis buffer was added, and centrifuged at 12,000 rpm for 10 minutes. Then the supernatant was added with 20 μL cell, followed by centrifugation at 10,000 rpm for 15 seconds. The precipitate was washed with 1 mL of L2 buffer twice. Then washed with 1 mL of 70% ethanol, and 1 mL of acetone twice. The genomic DNA deposition is dissolved in buffer TE pH 7.4 [11].

2.3.2 Amplification of CFP21 gene coding 21 kDa antigen. Amplification of the CFP21 gene was carried out with the PCR technique using a pair of specific primers. The primary sequence used in this study was CFP21-F: 5'-GCCGAGATCCATGCATTTCG-3' and CFP21-R: 5'-GGCAAGCCTTCTAAGTGTCCTG-3' [9,10] BamHII restriction enzyme sites (bold underline) are added before the ATG start codon in the primary forward and HindIII (bold underline) sites are added after the codon stops at the reverse primer. Components of the PCR reaction of 25μL were made by reacting 1 μL of the forward primer (10pmol), 1 μL of reverse primer (10pmol), and 3μL of isolated genomic DNA and fulfilled with Master Mix go Taq green. The PCR conditions used were pre-denaturation of 94°C for 5 minutes, initial denaturation at 94°C for 30 seconds, primer attachment (annealing temperature) at 55°C for 30 seconds, and elongation at 72°C for 30 seconds. Lengthening the final DNA fragment at 72°C for 7 minutes and the PCR reaction was carried out in 30 cycles.

2.3.3 Cloning CFP21 gene to cloning vector of pGEM-T Easy. The amplified CFP21 gene was cloned to the pGEM-T Easy cloning vector. PCR products were isolated from agarose gel with GFX column KIT and then cut with BamHII / HindIII restriction enzyme, ligated into the pGEM-T Easy cloning vector after being cut with the same restriction enzyme. The ligation results were transformed into E. coli JM109 and selected by selection media containing ampicillin, X-Gal and IPTG. Colonies containing pGEM-T Easy with inserted CFP21 DNA should be white in color and confirmed with colony PCR technique by Qiagen plasmid kit [12]. Determination of the nucleotide sequence of the CFP21 gene as a DNA insertion was performed using a DNA sequencer (Applied Biosystems ABI 310 Model). The DNA sequencing results were then homologized with the order in the data bank to determine the homology level with the help of the BLAST program on the NCBI website (http://www.ncbi.nlm.nih.gov) and Bioedit application v.7.0.9 (https://bioedit.software.informer.com/7.0.9/).

2.3.4 Expression of recombinant protein CFP21 in E. coli strain BL21. The CFP21 gene fragments that had been confirmed by the DNA sequencing technique were subcloned into the pGEX2TK expression vector to produce plasmid pGEX2TK-CFP21. E. coli BL21 (DE3) cells were transformed with the recombinant plasmid pGEX2TK-CFP21 containing the CFP21 gene to produce fusion protein GST-CFP21. Recombinant E. coli BL21 clones that had carried plasmid constructs pGEX2TK-CFP21 were inoculated into 15 mL of LB media containing 100 μg/mL ampicillin and then incubated for 10 hours in a shaker incubator at 37°C at 250 rpm. Then 2% (300 μl) of E. coli BL21 culture was transferred into 10 mL of new LB media which was added with 100 μg/mL ampicillin, incubated for 2 hours in a shaker incubator at 37°C. A total of 5 mL of bacterial culture was transferred to a test tube that did not contain IPTG (CFP21 non-induction, - IPTG). The remaining bacterial culture (5 mL) underwent protein expression by 50 μM IPTG induction for 6 hours (CFP21 by induction, + IPTG). E.coli BL21 culture in each test tube was centrifuged at a 14,000 rpm for 5 minutes, to separate the supernatant from the cell pellet. Proteins were extracted from cell pellets which were resuspended in PBS 1X buffer pH 7.4 containing (0.8 gr NaCl, 0.02 gr KCl, 0.144 gr Na2HPO4, 0.024 gr KH2PO4 and 100 mL ddH2O) and sonicated. Bacterial walls were ruptured with a 30-second recurring sonication technique 3 times with a power of 20 kHz. The fractured bacterial cell suspension was centrifuged at a speed of 14,000 rpm at 4°C for 1 minute to separate recombinant proteins from bacterial cell debris. Analysis of recombinant protein expression in the supernatant and cell pellet used sodium dodecyl
sulphate-polyacrilamide gel (SDS-PAGE) electrophoresis technique. The results of CFP 21 recombinant protein were analyzed by comparing the results of protein bands on polyacrylamide gels with protein markers. Then the data was presented in the form of images and descriptively narrated.

3. Results and discussion

3.1 Isolation of M. tuberculosis DNA genome

The CFP21 antigen was isolated from M. tuberculosis genomic DNA H37Rv using the modified Boom method with a heating-centrifugation/boiling technique using DNA extraction kit with consideration of purity and primary specific attachment specificity at the amplification stage of the target gene region. The number of samples used were 4 colonies from M. tuberculosis, strain H37Rv. Of the four samples, each DNA concentration was measured using a nanodrop spectrophotometer at absorbance of 260 nm (data not shown). Data on agarose electrophoresis analysis of M. tuberculosis strain H37Rv genomic DNA and negative control samples are shown in Figure 1.

![Figure 1](image.png)

**Figure 1.** Electrophoresis results of genomic DNA isolated from M. tuberculosis strain H37Rv (line 1-4) and negative control (line 5).

3.2 Amplification of CFP21 gene encoding 21 kDa antigen

The results of amplification of CFP21 gene with PCR using specific primer are shown in Figure 2.

![Figure 2](image.png)

**Figure 2.** The results of PCR amplification (column 2), purification of CFP21 gene product DNA using Purification Kit (Qiagen) (column 3), negative control (column 1), and DNA marker (column M)
From Figure 2, it is shown that the PCR product is in the same migration position with the 600 pbs DNA marker, precisely measuring 608 bps. This is in accordance with the data from GenBank (No. access: NC_000962.3) that the CFP21 gene encodes 21 kDa antigen consisting of 608 bps. The PCR product then becomes the target gene to be ligated into the pGEM-T Easy cloning vector.

3.3 Construction of CFP21 gene into cloning vector pGEM-T Easy
Cloning was performed in order to obtain CFP21 genes in large quantities. In this study we used the pGEM-T Easy cloning vector because it has the Origin of Replication (ORI) for insertion DNA fragments. In addition, the pGEM-T Easy vector has an ampicillin (Amp) gene and lacZ gene site that plays a role in blue-white screening during transformation into competent cells of E. coli JM 109. DNA ligation result from CFP21 gene encoding 21 kDa antigens insertion into pGEM-T Easy cloning vectors to obtain recombinant plasmids (Figure 3).

![Figure 3. The result of electrophoresis of agarose gel in ligation process of CFP21 gene into pGEM-T Easy cloning vectors. Column 1: pGEM-T Easy vector; Column 2: recombinant plasmid pGEM-T Easy-CFP21, each had been restricted by BamH I/HindIII restriction enzyme. Column 3: DNA marker](image)

From the Figure 3 above, it appears that the CFP21 gene insertion has been successfully ligated to the pGEM-T Easy cloning vector. This is evident in column 2 where pGEM-T Easy-CFP21 which has been restricted with BamH I and HindIII produced two bands of DNA fragments of 3015 pbs and 608 bps, so it can be concluded that the plasmid pGEM-T Easy has been easily inserted with the CFP21 gene.

3.4 Transformation of pGEM-T Easy-CFP21 into competent cell (E. coli JM109)
The transformation was carried out using the competent cell E. coli JM109 which functioned as a cell host that would multiply the recombinant plasmid pGEM-T Easy-CFP21. The transformation resulted in white bacterial colonies and blue colonies on agar LB plate medium containing X-gal, IPTG and ampicillin (Figure 4). White colonies indicate that the insertion DNA has been successfully inserted
into the vector, while the blue colony means that the insertion DNA has not been successfully inserted to vector (self-ligation). Agar LB plate medium that has been added with ampicillin antibiotics, isopropyl β-D-1-thiogalactopyranoside (IPTG), and X-Gal, can be used as a selection medium for transformed competent cell growth. Ampicillin serves as the first selection medium, because the bacteria that will grow are bacteria that have ampicillin resistance genes, namely E. coli containing vector pGEM-T Easy. The addition of IPTG was intended as a transcription inducer in the lac operon gene lacZ.

![Figure 4](image)

**Figure 4.** *E. coli* cells growth where blue colony is self-ligated and white colony contains pGEM-T Easy-CFP21 recombinant plasmid.

The lacZ gene will encode an β-galactosidase enzyme which functions to break down lactose into glucose and galactose. The presence of the β-galactosidase enzyme in this method is detected by the breakdown of the colorless X-gal substrate (5-bromo-4chloro-3-indolyl-β-D-galactoside) to blue galactose and 5-bromo-4-chloroindigo [13]. The CFP21 insertion into pGEM-T Easy cloning vector will disrupt the expression of β-galactosidase enzyme, and therefore, bacterial colonies successfully transformed with the pGEM-T Easy-CFP21 plasmid will appear white. Cells not transformed with this particular plasmid will remain blue.

### 3.5 Isolation and characterization of pGEM-T Easy-CFP21 recombinant plasmid

Analysis of CFP21 gene insertion DNA was carried out by colony PCR technique. The PCR product shows that there is one DNA band with a size of 608 bps. The PCR results prove that plasmid DNA from the white colonies contained insertion DNA in the form of the CFP21 gene (Figure 5A columns 4-6), whereas the blue colony PCR results this band did not appear (Figure 5A columns 1-3). Recombinant plasmid isolation from three white colony in Figure 5A, was carried out based on the QIAprep Spin Miniprep Kit from Qiagen (USA). The results obtained can be explained by electrophoresis technique and visualized on agarose gel (Figure 5B column 1-3).
3.6 Sequencing of pGEM-T Easy- CFP21 Recombinant Plasmid

The results of sequencing of the genetic code of cDNA fragments from CFP21 cloned into the pGEM-T Easy plasmid resulted in recombinant plasmid pGEM-T Easy-CFP21 using the Bigdye Terminator method with the Applied ABI PRISM 310 Biosystem sequencing tool, which was then processed with Bioedit application. v. 7.0.9. The sequencing result is shown in Figure 6. The size of the cDNA in full sequencing of the gene encoding the CFP21 protein is 654 bps (Figure 6), with 217 amino acid residues starting from the amino acid methionine (M) to stop codon.

Figure 5. A. Colony PCR CFP21 gene products as insert DNA, column 1-3 were derived from blue colonies and column 4-6 were derived from white colonies. White colonies were picked up and cultured in LB liquid media followed by CFP21 gene amplification by PCR and then analyzed on agarose gel electrophoresis. B. Visualization of recombinant plasmid isolation from three white colony in A panel.
Figure 6. Nucleotide sequence and amino acid prediction from CFP21 gene

3.7 Expression of CFP21 gene to E. coli BL21 cell
This study started by growing E. Coli BL21 cells carrying recombinant plasmid pGEX2TK-CFP21. E.
Coli BL21 cells were taken from the cloning of the previous CFP21 recombinant protein which was
characterized by the growth of white colonies on LB (Luria Bertani) media. Blue colonies show no
DNA insertion in the vector plasmid. White colonies are cells that do not have the activity of the
enzyme β-galactosidase. This is due to the insertion of DNA fragments located between the lac Z gene so that DNA fragments will activate the expression of the lac Z gene [14]. In order to confirm that bacteria grown on ampicillin media were successfully inserted into the CFP21 gene, the transformation process was carried out. The white colonies formed indicate that the bacterial clones were successfully inserted in the CFP 21 gene. The white colonies were then used as candidates for the production of CFP21 recombinant protein as future TB vaccine candidates.

The growth of *E. coli* BL21 pGEX2TK-CFP21 recombinant plasmid carrier was characterized by the occurrence of discoloration in the media to become cloudy. According to [15] *E. coli* is a prokaryote organism that is most widely used as a host cell in research. *E. coli* bacteria have the ideal characteristics as host cells which can reproduce themselves in a short and stable time, having high expression levels, non-pathogenic and most importantly can be introduced with various foreign genes that encode target proteins.

**Figure 7.** Growth of *E. coli* strain BL21 containing recombinant plasmid pGEX2TK-CFP21 in (A) Agar LB plate medium and (B) liquid LB culture medium. (1) Growth and expression of GST-CFP21 recombinant fusion induced by IPTG. (2) Expression of recombinant proteins not induced by IPTG. (3) Control medium without transformed by plasmid.

Growth of *E. coli* BL21 that contain recombinant plasmid pGEX2TK-CFP21 in liquid LB media containing ampicillin caused turbidity in culture media, as shown in Figure 7B line 1 and 2. Cell lysis was carried out by ultrasonication method. The physical cleaning method using ultrasonic waves is commonly known as the sonication method. The Sonication method is a cell-breaking method that utilizes vibrations from sound waves with high frequency to lyse cells. Proteins are extracted from deposits of cells resuspended in the lysis buffer and are sonicated until dissolved. Breaking bacterial walls with a one minute recurring sonication technique. Sonication is carried out in lysis buffer solution at 20 kHz until the liquid looks clear. Then centrifugation was performed to separate recombinant proteins and bacterial cell walls. Cell deposition as an insoluble fraction and supernatant containing insoluble protein was analyzed using SDS-PAGE method.

The SDS-PAGE method is a technique for separating polypeptide chains in proteins based on charged molecular weight under the influence of an electric field [12]. Before SDS-PAGE is carried out, proteins are first converted from globular structures into linear structures with denaturation. This analysis with SDS-PAGE uses polyacrylamide gel consisting of stacking gel and separating gel. Stacking gel has a well that serves as a place to place the sample while separating gel is where the protein will move towards the anode.

The results of the electrophoresis were then immersed in a staining solution (comassie brilliant blue R-250). Comassie brilliant blue R-250 functions as a protein blue giver in electrophoresis gel. The positively charged Comassie brilliant blue R-250 will bind to negatively charged proteins through electrostatic forces.

Recombinant protein GST-CFP21 expression with IPTG induction for 6 hours clearly shows the
blue fusion protein band on SDS-PAGE with a molecular weight of 47 kDa derived from a combination of GST (26 kDa) and CFP21 (21 kDa), whereas expression without IPTG induction does not show this band (Figure 8). This proves that the inducer (IPTG) greatly determines the success of recombinant protein expression. IPTG (isopropyl-β-D-thiogalactopyranoside) is a common inducer used for the induction of expression of recombinant proteins as well as induction materials that can increase the expression of target proteins in E. coli cells.

The formation of GST-CFP21 protein when induced with IPTG proved the reaction between protein samples and loading buffer. This is influenced by the content of glycerol and bromophenol blue in the loading buffer. Glycerol serves to stabilize the weight of the sample to settle at the bottom of the sample well, while blue bromophenol serves as tracking dye to mark the farthest limit of the movement of protein samples at electrophoresis so that the protein band appears blue. This is consistent with [15] statement that IPTG compound cannot be metabolized by cells, so the induction rate is always constant even though there is an addition of IPTG. The results of the analysis by SDS-PAGE electrophoresis method in samples induced with 50 µM IPTG showed that GST-CFP21 recombinant protein was expressed in the insoluble (pellet cell) fraction. The formation of this insoluble protein may occur because the GST-CFP21 fusion protein has a molecular weight of 47 kDa. The study of Hasegawa et al (2002) [16] comparing the molecular weight of several recombinant proteins with their level of solubility, shows that proteins with a size below 30 kDa require fusion with solubelitas and protein enhancers tend to be expressed in soluble. Whereas the insoluble form is due to above 30 kDa protein being expressed excessively and heterologically in E. coli, so that it still allows the inclusion of the body (inclusion body protein) which is an insoluble protein which blends with E. coli cell debris. The next research will be sought methods of expression of fusion protein GST-CFP21 to become a soluble protein which can be separated from E.coli cell debris.

![SDS-PAGE Image](image.png)

**Figure 8.** SDS-PAGE (10%) analysis results (1) Protein Marker (M), which is arranged from top to bottom: 212kDa; 116 kDa; 97.4 kDa; 66.2 kDa; 45 kDa; 31 kDa; (2) Expression of GST-CFP21 recombinant fusion protein induced by 50 µM IPTG for 6 hours (+ IPTG); (3) Expression of GST-CFP21 recombinant fusion protein without induced by IPTG (- IPTG).
4. Conclusion
From the results and discussion described above it can be concluded that: (1) CFP21 antigen from the bacterium *M. tuberculosis* strain H37Rv was successfully amplified by PCR technique, which produced a partial band with the size of 608 bp start ATG codon. (2) At the cloning stage of CFP21 gene into pGEM-T Easy vector, it produces pGEM-T Easy-CFP21 recombinant plasmid with a formed band length of 3623 bps. (3) The CFP21 gene successfully sub-cloned into the expression vector pGEX-2TK produces plasmid pGEX-2TK-CFP21 and is expressed in *E coli* BL-21 cells to produce fusion proteins with a molecular size of 47 kDa as a fusion protein GST-CFP21.

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