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by Ahyar Ahmad

FILE	6._AHMAD_2019_J._PHYS.__CONF._SER._1341_032010.PDF (1.09M)	WORD COUNT	5709
TIME SUBMITTED	07-DEC-2019 07:00AM (UTC+0700)	CHARACTER COUNT	29947
SUBMISSION ID	1228976856		

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To cite this article: A Ahmad et al 2019 J. Phys.: Conf. Ser. 1341 032010

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Cloning and characterization of Rv1980c gene encoding MPT64 Protein from *Mycobacterium tuberculosis* as a new candidate vaccine of tuberculosis

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Abstract. Pathogenic mycobacteria are one of the major causes of human mortality in the world. *Mycobacterium tuberculosis* is an etiological agent of human tuberculosis. Designing new vaccines including recombinant protein vaccines may be considered as a new approach for preventing or reducing tuberculosis epidemics. In order to construct protein recombinant as candidate vaccine, the Rv1980c gene encoding MPT64 protein was amplified from *M. tuberculosis* H37Rv strain genomic DNA using the PCR method and inserted into the cloning vector pGEM-T Easy. The recombinant plasmid pGEM-T Easy-MPT64 was then transformed into *E. coli* JM109 and cultivated under standard procedure, followed by plasmid extraction, PCR amplification, and DNA sequencing. The correct Rv1980c gene was confirmed by DNA sequencing and subcloned into expression vector pQE30Xa to yield recombinant plasmid pQE30Xa-MPT64, and transformed into *E. coli* BL21 strain. Transformed white recombinant colony was selected, cultured, induced with 40 μ M IPTG, and identified using SDS-PAGE electrophoresis method. The molecular weight was found to be about 24 kDa and identified as recombinant protein MPT64. The target gene has been cloned into host *E. coli* BL-21 strain and expressed successfully as a soluble protein. The recombinant fusion recombinant protein MPT64 paves the way for tuberculosis diagnosis and vaccine development in the future, especially in Indonesia.

1. Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis*, is an infectious disease which usually affects the lungs (pulmonary TB) and may also attack other tissues or organs (extrapulmonary TB). This disease spreads in the air when pulmonary TB patients excrete *M. tuberculosis* bacteria, for example through coughing [1]. *M. tuberculosis* has a high-fat content on the cell membrane which renders it resistant to acid. This bacterium is not resistant to ultraviolet radiation, therefore tuberculosis transmission tends to occur at night [2].

Approximately one-third of the world populace is infected by *M. tuberculosis*. Each year, about 8 million infected individuals develop active tuberculosis and 2 million among them die. Indonesia ranks as the third largest number of TB incidence where 10% of global TB cases occur in Indonesia [1]. About 70% of TB patients in Indonesia are in productive age which results in increase of both

economic and social burdens. A large number of TB cases in Indonesia is due to the fact that most Indonesians live in places with optimum conditions for the development and spread of TB germs. Besides, the increment in the number of HIV cases and the presence of multi-drug resistance (MDR) to *M. tuberculosis* has made the situation worse and more alarming. Tuberculosis cases in Sulawesi were found to be around 22,597 cases, with the highest number of cases found in South Sulawesi Province, which was around 8,297 cases. Data obtained from the Makassar City Disease Prevention and Health Sanitation Development Department shows that in Makassar City in particular, the number of new patients with pulmonary TB BTA 2014 was 76.52% (2,761 patients from 3,608 targets), this number increased from in 2013 with 1,811 cases out of 2,500 targets [3]. Therefore, an effective and affordable vaccine and the development of a fast and appropriate diagnostic method are needed to overcome this problem.

TB infection occurs due to the inhalation of droplet nuclei containing tuberculosis germs. After exposure to TB germs there are four conditions that can occur, namely at first infection does not occur (marked by a negative tuberculin skin test); in the second step infections then become active TB (primary TB); in the third step it becomes latent TB where the immune mechanism prevents the progression of the disease to TB active; and at fourth step the latent TB becomes reactivated and develops into active TB, a few months to several years later [4].

Two proteins in *M. tuberculosis* appear to be possible protein candidates for the development of immune system-based diagnostic tests and vaccines, namely MPT83 (Rv2873) and MPT64 (Rv1980c). Both types of protein are found in the species from the *M. tuberculosis* complex (MTC). **Mycobacterium Protein Tuberculosis (MPT 64) antigen, a 24-kDa secreted protein encoded by the Rv1980c gene, is an antigen from *M. tuberculosis* bacteria not found in BCG, *M. bovis* and *M. leprae* strains and other mycobacterial species.** MPT64 antigen is only found in healthy, normally growing and actively replicating cells. It is found in MTB as a marker for virulence. MPT64 antigen which is encoded by the RD2 region is only found in the complex strains of *M. tuberculosis*. Several studies confirm that MPT64 causes a strong hypersensitivity response to stimulate high levels of IFN in TB patients so that it can improve the immune system of TB patients. Therefore, MPT64 antigens are appropriate for TB vaccine candidates and T-cell-based TB diagnostic tests [5, 6].

Previous studies have suggested that MPT83 homologous monoclonal antibodies can improve the survival of mice infected with *M. tuberculosis* [7]. A study by [8] found that MPT64 is a specific antigen for *M. tuberculosis*. MPT64 is a 24 kDa protein produced by *M. tuberculosis* and encoded by the Rv1980c gene which contains 228 amino acids and has a gene size of 687 bp.

Based on the background above, in this study MPT64 antigen has been produced with recombinant DNA technology. Antigen production can be done by first cloning the Rv1980c gene encoding the MPT64 protein, then expressed into *E. coli* BL21 cells. Biotechnological production of this proteins, is expected to facilitate the development of a vaccine to protect against TB for patients in productive age, which in turn is expected to reduce the morbidity and mortality caused by TB in the future.

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2. Materials and methods

2.1 Materials

The materials used in this study were PCR Kit, go Tag green master mix, genomic DNA from *M. tuberculosis* H37Rv strain, MPT64-F and MPT64-R oligonucleotide primer, *Bam*HI, *Hind*III, agarose, EB buffer, PE buffer, PB buffer, 2x rapid ligation buffer, T4 DNA ligation enzyme, vector pQE30Xa, plasmid pGEM-T Easy, *E. coli* cells strain JM109 and BL21, nuclease-free water, medium Luria Bertani (LB), ampicillin, CaCl₂, IPTG, Tris base, X-Gal, boric acid, EDTA, TBE buffer, EtBr, Geneaid Kit, Qiagen Kit, loading dye, 100 bp DNA marker, protein marker ladder, NaCl, Bacto tryptone, Bacto agar, and Bacto yeast.

2.2 Instrumentations

The tools used in this study were Centrifuge (Profuge), Waterbath (Memmert), Vortex (Heidolph), incubator shaker (Heidolph), 1000 incubator, thermo cycle PCR machine, shaker incubator (Heidolph Duomax 1030), sonicator (Titraxmax 1000), incubator (memmert), electrophoresis machine (bio-rad), gel doc (bio-rad), micropipette (bio-rad), oven (Electrolux), autoclave (Hirayama), refrigerator (LG), laminar air flow (Labconco), freezer (Gen), ice maker (Hoshizaki), Erlenmeyer (Pyrex), test tubes (Pyrex), petridish plates, microcentrifugal tubes, microtube (Eppendorf), power supply, and heatshock plates.

2.3 Amplification of Rv1984c gene encoding MPT64 protein with PCR

The polymerase chain reaction (PCR) aims to multiply the Rv1980c gene encoding MPT64 protein. The PCR technique uses a pair of specific primers for Rv1980c gene. The primary sequences used in this research were MPT64-F (5'- GCGGATCCATGCTGGTCAC-3') and MPT64-R in sequence of (5'-GCCGAAGCTTAGGCCAGC-3') which added *Bam*HI restriction site (bold underline) before start codons on the forward primer and *Hind*III restriction site (bold underline) after stop the codon on the reverse primer [9]. The 25 μ L PCR mixture consists of 1 μ L of forward primer (10pmol), 1 μ L of reverse primer (10pmol), 3 μ L of genomic DNA from the previous research [10] and sufficient volume with Master Mix go Taq. Thermo cycle condition was: pre-denaturation of 90°C for 5 minutes followed by 30 cycles of initial denaturation at 94°C for 30 seconds, primer attachment (annealing) at 55°C for 30 seconds, and elongation at 68°C for 30 seconds. The extension of the final DNA fragment at 72°C for 7 minutes. The 2% agarose gel electrophoresis was used to visualize the PCR product.

2.4 Purification of DNA from PCR product

Purification of PCR products using Geneaid Kit (Qiagen) which has EZ-10 column. The kit has stages of purification namely gel dissociation, DNA binding, Wash and DNase I elution. The purification of PCR products aims to obtain pure DNA fragments that will be ligated to the pGEM-T Easy cloning vector. Cut PCR products from agarose gel was mixed and dissolved in 50 μ L PB buffer solution inside an Eppendorf tube. Next it was transferred to a spin column tube and centrifuged for one minute at 13,000 rpm at room temperature. The supernatant formed was discarded and 700 μ L of PE washing buffer was added to the spin column, then centrifuged at a speed of 15,000 rpm for 1 minute. The formed liquid was removed, after that it was transferred to the sterile eppendorf tube and 35 μ L EB buffer was added. The mixture was then left for 3 minutes and then centrifuged at 13,000 rpm for 1 minute. PCR product purification results were confirmed by electrophoresis method on 2% agarose gel.

2.5 Ligation of Rv1980c gene encoding MPT 64 protein into cloning vector of pGEM-T Easy

The ligation process was carried out using the T4 DNA Ligation enzyme. The composition of the ligation reaction consisted of fragments of the Rv1980c gene of purified PCR product-*Bam*HI / *Hind*III (6 μ L), pGEM-T Easy-*Bam*HI/*Hind*III (1 μ L), 2x rapid ligation buffer (2 μ L), T4 DNA ligase enzyme (1 μ L) and nuclease-free water (3 μ L). Then each composition is inserted in a 1.5 mL PCR tube. After being homogeneous, all the mix solutions are incubated at 4°C for 24 hours.

2.6 Transformation and characterization of pGEM-T Easy-MPT64 plasmid into E.coli cell JM109

The method used in this transformation was based on the heat shock method[11]. A total of 10 μ L of ligation product containing the inserted gene (Rv1980c) was added into 50 μ L suspension of competent cells of *E. coli* JM109 and then mixed until homogeneous. As a positive control, 2 μ L of plasmid pGEM-T Easy without insert were used to transform competent *E. coli* cells. Competent cells of *E. coli* JM109 without plasmids were used as a negative control. The three tubes were incubated on ice for 1 hour. The heat shock process was carried out at 42°C for 90 seconds, then incubated on ice for 1 hour. Next, 600 μ L of liquid LB media was added. The culture tubes were incubated using a shaker

incubator at 37°C at 150 rpm for 3 hours. Then centrifuged at 12,000 rpm for 1 minute. The ligation products of 600 µL were concentrated to 150 µL, then spread aseptically as much as 50 µL each on agar LB plates media containing 100 µg/mL ampicillin, 0.8 mg X-gal and 40 µM IPTG, then incubated at 37°C for 14-16 hours. To confirm the inserted gene (Rv1980c) ligation process into pGEM-T Easy plasmid, PCR colony technique analysis was performed [11].

2.7 Sub-cloning and expression of recombinant protein MPT64 in *E. coli* strain BL21

The Rv1980c gene fragment encoding the MPT64 protein which has been confirmed for its nucleotide sequence with the DNA sequencing technique was subcloned into the pQE30Xa expression vector to produce recombinant plasmid pQE30Xa-MPT64. The competent cell of *E. coli* BL21 (DE3) was transformed with the recombinant plasmid pQE30Xa-MPT64 containing Rv1980c gene to produce recombinant protein MPT64 and grown on LB plate at 37°C for overnight. Then the *E. coli* BL21 clone carrying recombinant pQE30Xa-MPT64 plasmid was picked and grown into 500 µL LB media containing 100 µg/mL ampicillin, then incubated overnight in the shaker incubator at 250 rpm at 37°C. Then 250 µL of *E. coli* BL21 culture was transferred into 10 mL of new LB media, incubated for 2 hours. The bacterial culture was pipetted as much as 5 ml and transferred to the Eppendorf tube without the addition of IPTG (MPT64 non-induction, -IPTG). The remaining bacterial culture was 5 ml and used for the protein expression, by induction with 40 µM IPTG for 6 hours (IPT64 by induction, + IPTG). *E. coli* BL21 culture in each culture tube was centrifuged at a speed of 14,000 rpm for 5 minutes at 4°C, to separate the supernatant from the pelleted cell. Proteins were extracted from pellet cell, resuspended in lysis buffers namely 1X PBS pH 7.4 containing (0.8 gr NaCl, 0.02 gr KCl, 0.144 gr Na2HPO4, 0.024 gr KH2PO4, 100 ml ddH2O) and sonicated. The bacterial cell wall was ruptured with a 30-second recurring sonication technique 3 times with a power of 20 kHz. The bacterial cell debris suspension was spin in a centrifuge at 14,000 rpm of 1 minute at 4°C to separate the supernatant containing recombinant proteins from bacterial cell debris. Analysis of recombinant protein expression in supernatants and pellet cells was performed by using sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE, 8%) electrophoresis technique. The results of MPT64 recombinant protein were analyzed by comparing it with the protein marker ladder on polyacrylamide gels. The results were presented in images and distinctly described.

3. Results and discussion

3.1 Amplification and purification of Rv1980c gene encoding MPT64 protein

The results of the amplification of the Rv1980c gene by using the PCR technique using *M. tuberculosis* genomic DNA were obtained from previous studies [10] and specific primers designed as shown in the materials and methods obtain DNA bands as shown in Figure 1. From Figure 1A, the band of PCR-produced DNA is seen in the same movement position with DNA marker 688 bps, while negative PCR control does not show DNA bands. The results of the DNA size matched with the data from GenBank (Access No.: NC_000962.3) showing that the Rv1980c gene encoding the MPT64 protein consisted of 688 bps as had also been confirmed by [10]. The PCR product then became the target gene (insert) which will be ligated into the pGEM-T Easy cloning vector. The formation of DNA bands measuring 688 bp and the absence of dimers in the visualization on agarose gel electrophoresis indicated that the DNA purification performed was successful (Figure 1B).

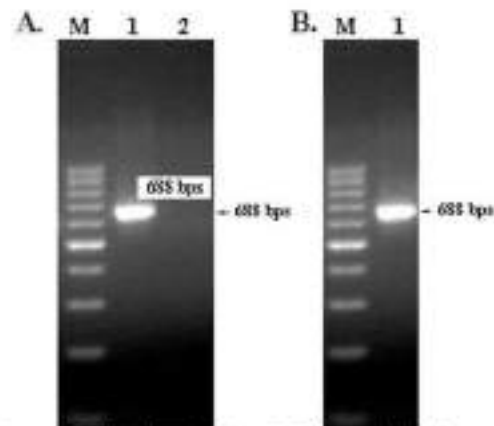


Figure 1. A. Visualization of DNA based from PCR amplification on an agarose gel and B. Purification of PCR produced DNA from Rv1980c gene using Purification Kit (Qiagen)

3.2 Construction and transformation of Rv1980 gene in cloning vector pGEM-T Easy

Cloning was carried out to amplify the Rv1980c gene in large quantities so that there are enough numbers to be analyzed and characterized. In this research, the pGEM-T Easy cloning vector was used because it had the Origin of Replication (ORI) area, had the site of ampicillin (*Amp^r*) resistance and *lacZ* gene which had a role in blue-white screening during transformation to the competent cell of *E. coli* JM109. According to [12] the pGEM-T Easy vector is a linear plasmid that has an overhanging sequence (T) at both ends. The T-overhang on the target DNA insertion/ligation site can increase the efficiency of the PCR product ligation process because it prevents self-ligation. In this study, the insert to vector ratio 6:1 was used. Adding the insert six-fold of the vector was intended to increase the chance of insert to bind to the vector (ligation process) catalyzed by T4 DNA ligase enzyme. The optimum temperature for T4 DNA ligase enzyme activity is at 30°C. The product of the ligation process was then transformed into competent cells of *E. coli* JM109 and then grown on agar LB plate media with added ampicillin, IPTG, and X-gal (Figure 2).

The transformation method used is the heat shock method. This is a simple method that can cause pores from the *E. coli* cell membrane to open in a short time and be ready to accept the recombinant vector that will enter. In this method, *E. coli* JM109 cells are given a shock at cold and hot temperatures alternately so that the cell wall expands and deflates rapidly to allow DNA from outside to enter the cell. This result is similar to the research conducted by [11] who found that the transformation process was carried out by the heat shock method at 42°C for 90 seconds.

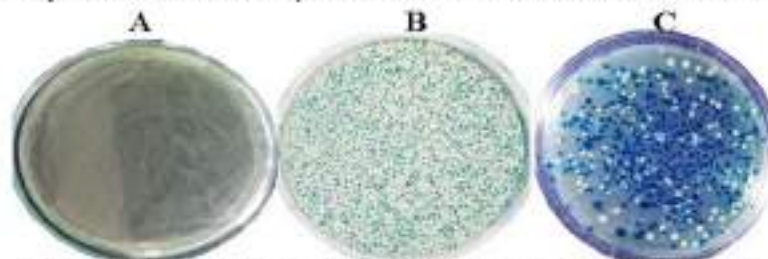


Figure 2. Transformation and screening results of the blue and white colonies from *E. coli* recombinant. A. (negative control), *E. coli* JM109 competent cells without plasmid B (positive control) *E. coli* cells growth where blue colony containing plasmid pGEM-T Easy without insertion DNA transformed, and C. *E. coli* cells growth where white colony contains pGEM-T Easy-MPT64 recombinant plasmid and the blue colony is self-ligated.

In Figure 2A (negative control), *E. coli* JM109 competent cells without plasmids did not grow colonies on agar LB plate media. This is because in the negative control there were only *E. coli* JM109 did contain the plasmid coding for ampicillin resistance. Figure 2B (positive plasmid control) shows *E. coli* JM109 competent cells transformed with pGEM-T Easy vector without DNA insertion. In this culture, about 1340 colonies were blue in agar LB plate media. In comparison, *E. coli* JM109 competent cells transformed with the recombinant plasmid pGEM-T Easy-Mpt64 grown on agar LB plate media containing X-gal and IPTG obtained 749 blue bacterial colonies and 59 white colonies (Figure 2C), which meant the efficiency of the transformation process was 7.3%.

The white colonies formed (Figure 2C) showed the success of the cloning process, namely the successful DNA insertion (Rv1980c Mpt64) into the pGEM-T Easy plasmid resulting in the recombinant plasmid pGEM-T Easy-Mpt64. While the blue colonies are bacterial colonies that did not contain DNA insertion in the transformed plasmid vector, but only contain self-ligating plasmids instead. This result is similar with the research conducted by [13] that white *E. coli* colonies (transformant cells) show that DNA encoding MPT64 has been ligated in the MCS (multi-cloning site) region contained in the lacZ gene of the pGEM-T Easy plasmid. This DNA fragment insert inhibited the lacZ gene from encoding the subunit of β -galactosidase so that the enzyme becomes non-active and unable to degrade galactose in the growth media. On the other hand, blue bacterial colonies maintained the Lac Z gene intact due to the lack of DNA insertion, and thus allow the breakdown the existing galactose substrate in the growth media.

The growth media for selecting transformed *E. coli* competent cells used was LB media with ampicillin addition, X-Gal, and isopropyl β -D-1-thiogalactopyranoside (IPTG). The addition of IPTG serves to initiate transcription in the lacZ lac operon gene. The lacZ gene will encode a β -galactosidase enzyme which functions to degrade lactose into glucose and galactose. The existence of the β -galactosidase enzyme can be identified by the degradation of the X-gal substrate (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) which is white to blue galactose and 5-bromo-4-chloroindigo. The β -galactosidase enzyme will make bacterial cell colonies in blue colour. This shows the absence of insertion DNA that was successfully ligated into the plasmid. On the contrary, cells that do not have the β -galactosidase enzyme will generate white cells bacterial colonies. These results is due to the insertion of DNA fragments sited between the lacZ genes so that the inserted DNA fragments will switch on the activation of the lacZ gene [14].

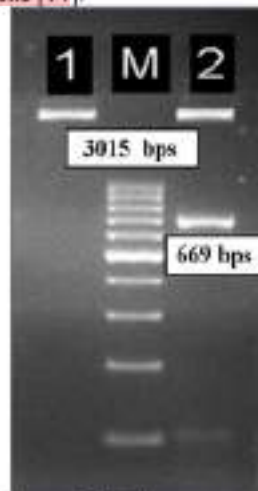


Figure 3. Agarose gel electrophoresis results in the process of ligating the Rv1980c gene into the pGEM-T easy-Mpt64. Column 1: pGEM-T easy vector that is restricted with the *Bam*HI / *Hind*III enzymes, column 2 of the pGEM-T Easy-Mpt64 vector which is restricted with the *Bam*HI / *Hind*III enzymes, column M: DNA marker.

Column 2 of the agarose gel electrophoresis in Figure 3, shown that pGEM-T Easy-Mpt64, restricted with *Bam*HI and *Hinc*III, produced 2 bands of 3015 bps and 19 bps DNA fragments (19 bps were deleted from the artificial nucleotide in primer pairs). Thus, it can be concluded that the plasmid pGEM-T Easy had been successfully inserted with the DNA of Rv1980e gene encoding the MPT64 protein, resulting in the pGEM-T Easy-Mpt64 recombinant plasmid (Figure 3). Result reveals that the DNA insert from the Rv1980e gene had been successfully ligated to the pGEM-T Easy cloning vector as shown in Figure 3.

2.4 Isolation and characterization of recombinant plasmid pGEM-T easy-Mpt64

To confirm the presence or absence of insert DNA of the Rv1980e gene encoding the MPT64 protein, colony PCR technique was performed on the recombinant *E. coli* cell clones that grew on agar LB plates medium (Figure 2C). The PCR products showed that there was one DNA band measuring 688 bps. The PCR results demonstrate that plasmid DNA from white colonies contained insert DNA in the form of Rv1980e gene with a size of 688 bps (Figure 4A column 1-4), which is similar to the DNA band in positive control of *M. tuberculosis* genomic DNA (Figure 4A column +). Whereas the blue colony did not show the DNA insertion of the Rv1980e gene after analysis with colony PCR (Figure 4A column -).

The results of the transformation in the single white colonies were inoculated again into agar LB media containing 100 µg/mL ampicillin. The isolation of plasmids from four replicas of white colonies cultured 1 mL liquid LB media containing 100 µg/mL ampicillin was performed according to the QIAprep Spin Miniprep Kit (Qiagen, USA). The results of the isolation of recombinant plasmid pGEM-T Easy-Mpt64 obtained were analyzed by electrophoresis and visualized on 2% agarose gel (Figure 4B).

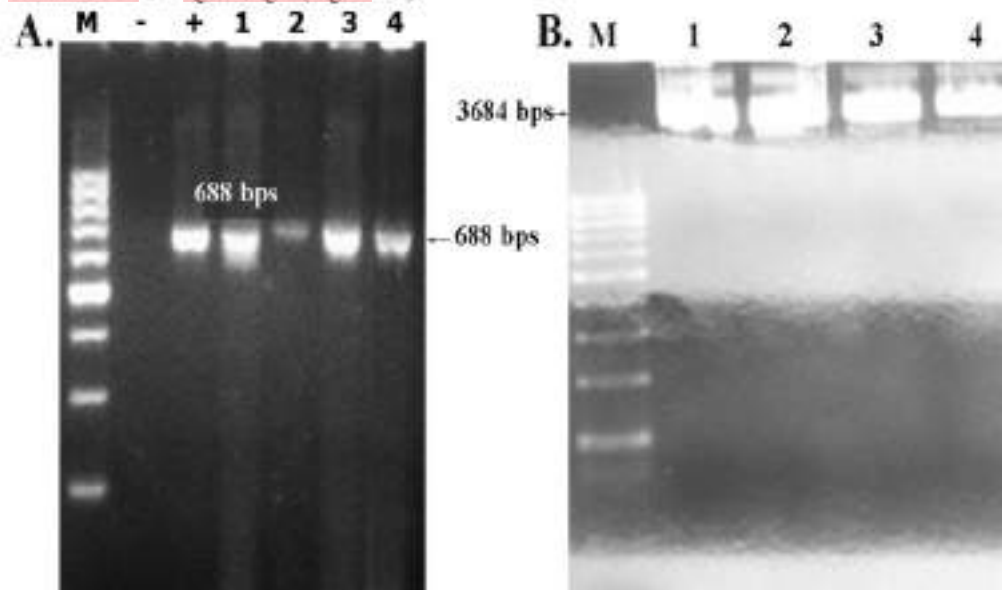


Figure 4. A. The colony PCR product of Rv1980e gene encoding the MPT64 protein as insert DNA. Column - control was negative from the results of blue colony PCR, column + control positive from the results of *M. tuberculosis* H37Rv strain genomic DNA, and columns 1-4 derived from the results of white colony PCR. Blue and white colonies were picked and inoculated on the PCR kit mix followed by the PCR process and then visualized on 2% agarose gel electrophoresis. B. The results of the isolation of the recombinant plasmid pGEM-T Easy-Mpt64 from four white bacterial colonies on agar LB plate media.

3.4 Sequencing of plasmid pGEM-T Easy-Mpt64 recombinant

The isolated recombinant plasmid from pGEM-T Easy-Mpt64 positive colonies were sequenced by Applied ABI PRISM 310 Biosystem sequencing device using the BigDye Terminator method. The data was then analyzed with Bioedit v.7.0.9 application, and the results are shown in Figure 5.

>MPT64

1	ATG CTG GTC ACG GCT GTC GTT TTG CTC TGT TGT TCG GGT GTG GCC	45
1	Met Leu Val Thr Ala Val Val Leu Leu Cys Cys Ser Gly Val Ala	15
46	ACG GCC GCG CCC AAG ACC TAC TGC GAG GAG TTG AAA GGC ACC GAT	90
16	Thr Ala Ala Pro Lys Thr Tyr Cys Glu Glu Leu Lys Gly Thr Asp	30
91	ACC GGC CAG GCG TGC CAG ATT CAA ATG TCC GAC CCG GCC TAC AAC	135
31	Thr Gly Gln Ala Cys Gln Ile Gln Met Ser Asp Pro Ala Tyr Asn	45
136	ATC AAC ATC AGC CTG CCC AGT TAC TAC CCC GAC CAG AAG TCG CTG	180
46	Ile Asn Ile Ser Leu Pro Ser Tyr Tyr Pro Asp Gln Lys Ser Leu	60
181	GAA AAT TAC ATC GGC CAG ACG CGC GAC AAG TTC CTC AGC GCG GCC	225
61	Glu Asn Tyr Ile Ala Gln Thr Arg Asp Lys Phe Leu Ser Ala Ala	75
226	ACA TCG TCC ACT CCA CGC GAA GCC CCC TAC GAA TTG AAT ATC ACC	270
76	Thr Ser Ser Thr Pro Arg Glu Ala Pro Tyr Glu Leu Asn Ile Thr	90
271	TCG GCC ACA TAC CAG TCC GCG ATA CCG CCG CGT GGT ACG CAG GCC	315
91	Ser Ala Thr Tyr Gln Ser Ala Ile Pro Pro Arg Gly Thr Gln Ala	105
316	GTG GTG CTC AAG GTC TAC CAG AAC GCC GGC GGC ACG CAC CCA ACG	360
106	Val Val Leu Lys Val Tyr Gln Asn Ala Gly Gly Thr His Pro Thr	120
361	ACC ACG TAC AAG GGC TTC GAT TGG GAC CAG GCC TAT CGC AAG CCA	405
121	Thr Thr Tyr Lys Ala Phe Asp Trp Asp Gln Ala Tyr Arg Lys Pro	135
406	ATC ACC TAT GAC ACG CTG TGG CAG GCT GAC ACC GAT CCG CTG CCA	450
136	Ile Thr Tyr Asp Thr Leu Trp Gln Ala Asp Thr Asp Pro Leu Pro	150
451	GTC GTC TTC CCC ATT GTG CAA GGT GAA CTG AGC AAG CAG ACC GGA	495
151	Val Val Phe Pro Ile Val Gln Gly Glu Leu Ser Lys Gln Thr Gly	165
496	CAA CAG GTA TCG ATA GCG CCG AAT GCC GGC TTG GAC CCG GTG AAT	540
166	Gln Gln Val Ser Ile Ala Pro Asn Ala Gly Leu Asp Pro Val Asn	180
541	TAT CAG AAC TTC GCA GTC ACG AAC GAC GGG GTG ATT TTC TTC TTC	585
181	Tyr Gln Asn Phe Ala Val Thr Asn Asp Gly Val Ile Phe Phe Phe	195
586	AAC CCG GGG GAG TTG CTG CCC GAA GCA GCC GGC CCA ACC CAG GTA	630
196	Asn Pro Gly Glu Leu Leu Pro Glu Ala Ala Gly Pro Thr Gln Val	210
631	TTG GTC CCA CGT TCC GCG ATC GAC TCG ATG CTG GCC TAG	669
211	Leu Val Pro Arg Ser Ala Ile Asp Ser Met Leu Ala End	223

Figure 5. The nucleotide sequence and amino acid prediction from Rv1980c gene encoding the MPT64 protein.

As shown in Figure 5, the size of the cDNA from the Rv1984e gene was 669 bp including the start ATG codon and stop TAG codon (Figure 5), that encodes the MPT64 protein with amino acid residues 222 starting from the amino acid methionine (Met).

3.5 Transformation and culture of *E. coli* BL21 cells carrying recombinant plasmid pQE30Xa-Mpt64 for the expression of recombinant MPT64 protein

E. coli BL21 cells were transformed with the recombinant plasmid pQE30Xa-Mpt64, characterized by the growth of white colonies on agar LB plate media (Figure 6A). The formed white colonies indicate successful sub-cloning procedure, namely the insertion of the Rv1980e gene into the pQE30Xa. White colonies of *E. coli* were used as a cloning agent for the production of MPT64 recombinant protein, a TB vaccine candidate. The presence of *E. coli* BL21 cell carrying recombinant plasmid pQE30Xa-Mpt64 was marked by the change in the colour of the liquid LB media to become cloudy, while the control media that did not carry the plasmid remained clear (Figure 6B).

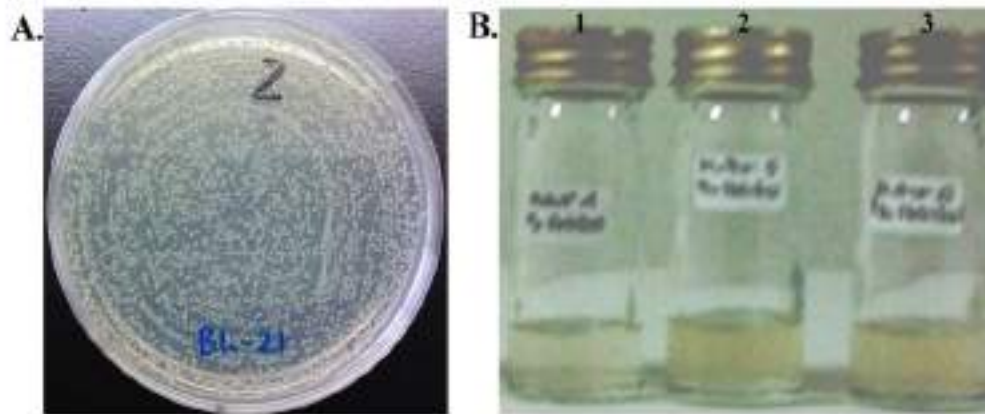


Figure 6. Growth of *E. coli* strain BL21 containing recombinant plasmid pQE30Xa-MPT64 in (A) agar LB plate media and (B) liquid LB culture media. (1) Control of LB liquid media. (2) Growth and expression of recombinant proteins not induced by IPTG. (3) Growth and expression of MPT64 recombinant protein induced by 40 µM IPTG.

After the recombinant cells in the culture tube (Figure 6B) were separated for their supernatant and pellets, the pelleted cell was synthesized. Sonication treatment is the most effective method for destroying microbial cell walls. Sonication causes the microbial cell walls to thin out, as a result, the cell membrane becomes easily damaged. Sonication is carried out until the initially cloudy liquid turns clear accompanied by increased fluid viscosity due to the breakdown of *E. coli* cells accompanied by the release of cell contents such as biomolecules, especially proteins, fats, and carbohydrates.

3.6 Characterization of Protein with SDS-PAGE Method

The presence of a blue protein band formed as a result of MPT64 recombinant protein expression. This protein band was the result of a reaction between the sample and the loading buffer. The loading buffer used contains bromophenol blue and glycerol. The function of glycerol is to increase the specific gravity of the sample so that the sample does not float but was deposited at the bottom of the sample well. The bromophenol blue serves as a tracking dye to mark the farthest limit of the protein samples migration during electrophoresis as shown in Figure 7.

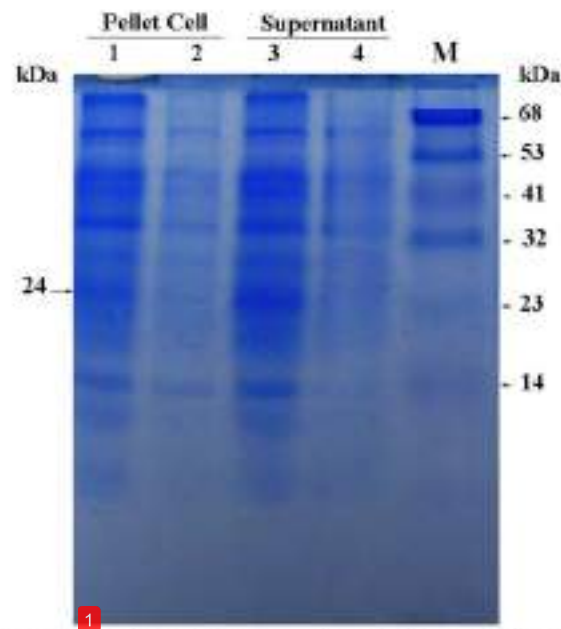


Figure 7. The results of the analysis of the recombinant MPT64 protein expression with the SDS-PAGE method (8%). (1) pellet of cells induced with 40 μ M IPTG for 6 hours; (2) pellet of cells are not induced by IPTG; (3) supernatant induced with 40 μ M IPTG for 6 hours; (4) supernatant not induced by IPTG; and (M) protein marker ladder.

From the results of the recombinant MPT64 protein expression in Figure 7, it is clear that the protein band from the pellet of cells induced with 40 μ M IPTG for 6 hours without blue was thicker. The thickest protein band is located parallel to the 23 kDa protein marker ladder. This indicates that the molecular weight of MPT64 recombinant protein is 24 kDa. This is consistent with the study of Yindeeyoungyeon, *et al.*, 2018 which found that MPT64 protein is a 24 kDa. Whereas in the pellet of cells that were not induced by IPTG there was no visible protein band. This proved that the addition of inducers (IPTG) greatly determines the optimization of recombinant protein expression. The same results in several previous studies showed an increase in recombinant protein production influenced by cell growth when induced by inducers [15,16]. However, low inducer concentration would result in non-optimal expression along with the addition of the number of cells per unit of time while a high inducer concentration would cause cell death [17]. IPTG is an inducer that is commonly used to induce the expression of recombinant proteins as well as inducers which can increase the expression of target proteins in *E. coli* cells. From the data in Figure 7, the MPT64 protein is a soluble intracellular protein. This can be concluded because the protein is dominantly found in supernatants in comparison to the cell pellets, where it is minorly detected. Thus, the MPT64 recombinant protein in *E. coli* BL21 bacteria was successfully produced with a molecular weight of 24 kDa. This recombinant protein is in the form of crude protein (crude extract). This recombinant protein can be used as a candidate antigen raw material for TB vaccine production after further purification is carried out in the upcoming research series.

4. Conclusion

The Rv1980c gene from the local strain of *M. tuberculosis* which encodes MPT64 protein was successfully amplified by PCR technique, which produced 688 bp DNA band including ATG start codon and TAG stop codon. At the Rv1980c gene cloning stage into the pGEM-T Easy vector, it

produced a recombinant fusion pGEM-T Easy-Mpt64 plasmid with a band length of 3684 bps. Based on the prediction of amino acid sequences encoding MPT64 protein in recombinant pGEM-T Easy-MPT64 plasmid, the gene was successfully subcloned into pQE30Xa into the recombinant plasmid pQE30Xa-Mpt64 and expressed in *E. coli* BL-21 cells to produce MPT64 recombinant protein with a molecular size of 24 kDa as shown on a polyacrylamide gel.

Acknowledgements

We would like to show our gratitude to Mrs. Rufika Shari Alim and Mr. Yusriadi for the editorial reading of this manuscript. We are also immensely grateful to Mrs. Rina for her valuable help in technical support and preparation of DNA samples and recombinant plasmid in Hasanudin University Medical Research Centre (HUM-RC), Hasanuddin University Hospital, Makassar, Indonesia. This research was supported in part by a Grant-in-Aid by National Basic Research Project 2019 of Directorate of Career and Competency of Human Resources of the Ministry of Research, Technology, and Higher Education of the Republic of Indonesia (Grant number 1740/UN4.21/PL.01.10/2019).

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