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Cloning, expression, and purification of fusion antigens MPT83 and ESAT6 from the local strain of *Mycobacterium tuberculosis* in *Escherichia coli* as a seed vaccine candidate against tuberculosis

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ABSTRACT

Eradicating tuberculosis (TB) globally is increasingly challenging with the growing number of drug-resistant *Mycobacterium tuberculosis* (*M. tuberculosis*) strains. The development of more potent TB vaccines is critical to complement overall TB control strategies and overcome the growing challenge of drug resistance. In this study, the recombinant plasmid pGEM-T Easy- Rv2873 + Rv3875 has been generated by inserting the Rv3875 gene, which encodes the ESAT6 protein, into the pGEM-T Easy- Rv2873 vector at the *BamHI*/*Hind*III cloning site. Following transformation into *E. coli* JM109, the plasmid was extracted, PCR amplified, and DNA sequencing. The existence of the appropriate recombinant Rv2873 + Rv3875 fusion genes was confirmed through the observation of a band of 948 base pairs in the colony PCR product containing fusion antigens. A band measuring 3966 base pairs was observed in the recombinant plasmid pGEM-T Easy- Rv2873+Rv3875, supporting the presence of the desired fusion genes target. The fusion genes Rv2873+Rv3875 were cloned into the expression vector pTrcHisA. This resulted in the pTrcHisA Rv2873+Rv3875 recombinant fusion plasmid, which was subsequently introduced into the *E. coli* BL21 strain through transformation. The fusion protein, comprising the 6XHis tag, exhibited a molecular mass of around 28 kilo Dalton and was synthesized as an insoluble protein inside *E. coli* BL21. In conclusion, the purified recombinant fusion protein MPT83 and ESAT6 hold promise for TB diagnosis and show potential as vaccine candidates in the future.

INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by *M. tuberculosis* that can affect multiple organs of the body. It mostly attacks the lungs, termed pulmonary TB, and can spread to other organs, also known as extrapulmonary TB. People with pulmonary TB can infect others by coughing up the bacterium [1]. *M. tuberculosis* is resistant to acidity due to the high lipid content of its cell membrane [2, 3]. Additionally, susceptibility to ultraviolet (UV) light and radiation allows the bacteria to spread more easily at night [4-6].

About 10.6 million people fell ill with TB in 2022, with 1.3 million of them dying, including those with HIV [1]. Indonesia contributes 10% of TB incidents globally, ranking second after India [7]. The death toll in Indonesia due to TB reached more than

144,000, which is higher than in previous years [8]. Eighty percent of Indonesia's TB patients are adults of employment age, which has devastating social and economic consequences [8]. The conditions favor TB bacteria to thrive, contributing to the disease's widespread distribution. Coinfection of TB/HIV, which requires a complex combination of therapies and often causes drug-drug interaction, and the emergence of multidrug-resistant TB only adds fuel to the fire [9]. To overcome this issue, developing cost-effective vaccines and rapid as well as rapid and accurate diagnostic technologies is essential. TB infection occurs when individuals inhale droplet nuclei carrying TB germs [10], leading to four possible outcomes: a negative tuberculin skin test, active TB, latent TB, or reactivation of latent TB into active TB within months to years [11].

Diagnostic tests and immunizations based on MPT83 (Rv2873) and Rv3875 have potential [12,13]. *M. tuberculosis* complex (MTC) species have these proteins. The *M. tuberculosis* Region of Difference 2 (RD2) region contains the Rv2873 gene and the Rv3875 gene, which codes for MPT83 and a 6-kilo Dalton secretory protein bearing the ESAT6 antigen, respectively. ESAT6 antigen has been reported specific to healthy and dividing cells and not found in most other mycobacterium types including *M. bovis* BCG. Several studies confirm that MPT83 and ESAT6 elicit a strong hypersensitivity reaction, and increase interferon release in TB patients, thus potentially enhancing the immune response against TB [14,15]. Both MPT83 and ESAT6 antigens serve as virulence markers in *M. tuberculosis* strains. They have been found only in complex *M. tuberculosis* strains and could be used in T-cell-based TB vaccines and diagnostics [16-18]. In addition, ESAT-6 has the ability to drive and stimulate protective immune responses, protecting against long-term chronic infections or post-exposure, and can induce immunity against *M. tuberculosis* pathogens, thus having great potential for use in TB vaccine development [19]. Previous studies have shown that mice treated with MPT83 homologous monoclonal antibodies exhibited improved outcomes when infected with *M. tuberculosis* [20]. Therefore, both these antigens are appropriate highly immunogenic TB seed vaccine candidates as well as for T-cell-based TB diagnostic tests.

In this study, a fusion antigen protein made of the MPT83 and ESAT6 genes through recombinant DNA technology was developed. To produce the fusion antigen protein, we constructed the fusion plasmid pGEM-T Easy-Rv2873 plus Rv3875, by inserting the Rv3875 gene encoding the ESAT6 protein into the pGEM-T Easy-Rv2873 vector at the *BamH*I/*Hind*III cloning site [21]. Subsequently, the fusion genes for Rv2873 and Rv3875 were subcloned into the *Nhe*I/*Hind*III cloning site of the expression vector pTrcHisA to create pTrcHisA-Rv2873 + Rv3875. Recombinant 6XHis-tagged fusion protein MPT83 plus ESAT6 was produced by expressing these genes in the *Escherichia coli* (*E. coli*) BL-21strain, making it a potential addition to improved TB vaccines. By successfully expressing this fusion protein as a seed vaccine candidate, we are at a promising initial stage in the development of an effective TB vaccine. This progress paves the way for further research to evaluate the ability of this fusion protein to stimulate a strong and protective immune response, thereby contributing significantly to global efforts to control and eliminate TB.

MATERIALS AND METHODS

Materials

JM109 and BL-21 *E. coli* cells were purchased from Promega, USA. pTrcHisA vector was purchased from Thermo Ficher Scientific. *BamH*I and *Hind*III restriction enzymes were purchased from NEB New England Biolabs. PCR kits with Master Mix GoTaq green were purchased from Promega, USA. Ni^{+2}-NTA affinity chromatography was

purchased from GoldBio St. Louis, Missouri. LB media, Ampicillin, IPTG, X-gal, imidazole, PBS buffer, and Tris-HCl were purchased from Sigma.

DNA of *M. tuberculosis* sample preparation

M. tuberculosis isolate was obtained from the culture collection of Wahidin Sudirohusodo Hospital Makassar, Indonesia [22]. Any procedure dealing with human subjects was conducted in compliance with ethical guidelines and had been approved by the Ethics Committee at the Faculty of Medicine Hasanuddin University, Makassar, Indonesia, with an ethics approval number. 24/UN4.6.4.5.31/PP36/2024. The DNA of *M. tuberculosis* was extracted using the method described previously [22]. DNA extract was stored at -20 °C until used in PCR.

Primer design and Rv3875 gene amplification

Rv3875 gene of extracted *M. tuberculosis* DNA was amplified by PCR with Master Mix GoTaq green (Promega, USA, Cat No. M7122), and primers: Rv3875-F (5′-gcgggatcc atgacagagcagcagtgg-3′) and Rv3875-R (5′-ccgaagcttctatgcgaacatcccagtg -3′), which were designed to introduce *BamH*I restriction sites before the start codon and *Hind*III restriction sites after the end codon, respectively. Denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 30 seconds was repeated 30 times after a 5-minute pre-denaturation at 90 °C. The longest period of extension occurred at 72 °C for seven minutes. The PCR result was then run electrophoresis on 1.0% agarose gel [21, 22].

Purification of PCR products

The PCR products of the Rv3875 gene were purified using an ez-10 column in the Geneaid kit (Qiagen). Reagents for DNA binding, washing, and elusion were included in the kit, all of which were applied in the purification procedure. The main objective of PCR product purification was to acquire contaminant-free DNA fragments appropriate for ligation into the pGEM-T vector. The agarose gel was sliced and homogenized, and then the PCR findings were placed in Eppendorf tubes with around 50 microliters of PB buffer solution to begin the purification process. The supernatant was obtained by centrifuging the remaining mixture at 13,000 rotations per minute for two minutes at ambient temperature. Following the removal of the supernatant, a volume of 700 microliters of PEP washing buffer was introduced into the spin column. Afterward, the sample was spun at a speed of 13,000 rpm for two minutes as an additional wash step for the PCR products. In the last step, 35 microliters of Elution buffer were added to the spin column and centrifuged again to collect the eluate. Electrophoresis was performed on a 1% agarose gel to verify the high purity level.

ESAT6 protein-encoding Rv3875 gene ligation into pGEM-T easy-Rv2873 plasmid

After properly mixing 50 µL of *E. coli* JM109 competent cells with 10 µL of the ligation product that had been inserted Rv3875 gene, the final volume was 100 µL. To serve as positive control, we cultivated competent *E. coli* JM109 cells with and without antibiotics (ampicillin, 100 µg/mL). The ligation product and *E. coli* JM109 competent cells were placed in three separate tubes and stored at 4 °C for 30 minutes. The samples were then exposed to a heat shock at 42°C as described previously [23]. After that, the

culture tubes spent 3 hours in a shaker incubator set at 37 °C and 150 rotations per minute. The samples were centrifuged for two minutes at 13,000 rpm. The vacuum centrifuged ligation products reached 100 microliters. After adding 0.8 mg 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), ampicillin (100 μg/mL), and Isopropyl β-D-1-thiogalactopyranoside (IPTG) (40 micromolar), the cells were grown at 37°C for 15-18 hours.

Sequencing of the recombinant plasmid pGEM-T Easy- Rv2873 +Rv3875

DNA sequencing was performed to validate the nucleotide sequence. Bigdye Terminator sequencing was utilized on ABI PRISM 310 Biosystem hardware on this recombinant plasmid, pGEM-T Easy- Rv2873 +Rv3875. The sequencing results were visualized and evaluated by using Bioedit v.7.0.10.

Expression of recombinant protein fusion of MPT83 and ESAT6

To make the recombinant plasmid pTrcHisA-Rv2873+Rv3875, the Rv2873 and Rv3875 genes were fused to produce a fusion protein and then introduced into the pTrcHisA expression vector [24]. In order to produce recombinant fusion proteins consisting of MPT83 and ESAT6, the pTrcHisA-Rv2873+Rv3875 plasmid was introduced into *Escherichia coli* BL-21cells through transformation methods. The transformed cells were incubated overnight in a shaker incubator maintained at a temperature of 37 °C. After incubation, 4 mL of the bacterial recombinant culture was utilized as a sample without IPTG (MPT83 + ESAT6 non-induction, -IPTG), and the rest of the culture was diluted and added to fresh Luria-Bertani media. To reduce the amount of inclusion body proteins and maximize the production of MPT83 and ESAT6 proteins, 40 micromolars of IPTG was added to the final 5 mL of bacterial recombinant culture, and the mixture was incubated at 16 °C for 6-7 h until optical density (OD) of the cell cultures about 2.0-4.0.

After incubation, the *E. coli* BL-21 cultured cells were harvested by 13,000 rpm centrifugation for five minutes at 4 °C. The pellet was next mixed with a nutrient-rich 1X phosphate-buffered saline (PBS) solution containing 1% Triton X-100 with pH 7.5. Sonication was performed intermittently for 30 seconds using a 20 kHz frequency to disrupt the bacterial cell walls, allowing for the release of biomolecules from the *E. coli* BL-21 strain cells into the clear liquid [20]. To isolate this recombinant protein from the bacterial cellular debris, the material had to be centrifuged at 13,000 rpm for a minute while keeping the temperature at approximately 4°C.

Protein expression was assessed through the utilization of 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) [16, 25], employing the recombinant proteins MPT83 and ESAT6 as reference standards in comparison to a conventional protein ladder (Tiagen, Biotech Beijing).

Purification of recombinant protein fusion of MPT83 and ESAT6

Recombinant protein fusion of MPT83 and ESAT6 was purified using nickel-nitrilotriacetic acid (Ni^{2+}-NTA) affinity chromatography (GoldBio St. Louis, Missouri) with a His Tag-agarose matrix. Following column washing, this recombinant protein was eluted using elution buffer 0.5 M Tris-HCl buffer containing imidazole in varied concentrations (100-300 mM). SDS-PAGE gel electrophoresis was performed to evaluate

the purified recombinant protein, which was then stained with Coomassie Brilliant Blue from Merck [25].

RESULTS

Amplification of ESAT6-encoding Rv3875 gene

The amplification of the Rv3875 gene was successfully achieved and showed a band of 288 bp (Figure 1A). The PCR product size matched that reported by GenBank (Access Number: KJ095583 for Gene ID: 886657), indicating that the Rv3875 gene, which includes the ATG start codon, is 288 bps in length and codes for the ESAT6 protein. In the absence of DNA, the negative PCR control showed no bands. DNA purification was successful because, as shown in Figure 1B, agarose gel electrophoresis revealed the presence of a 288 bps DNA band free of dimers and dimerization products.

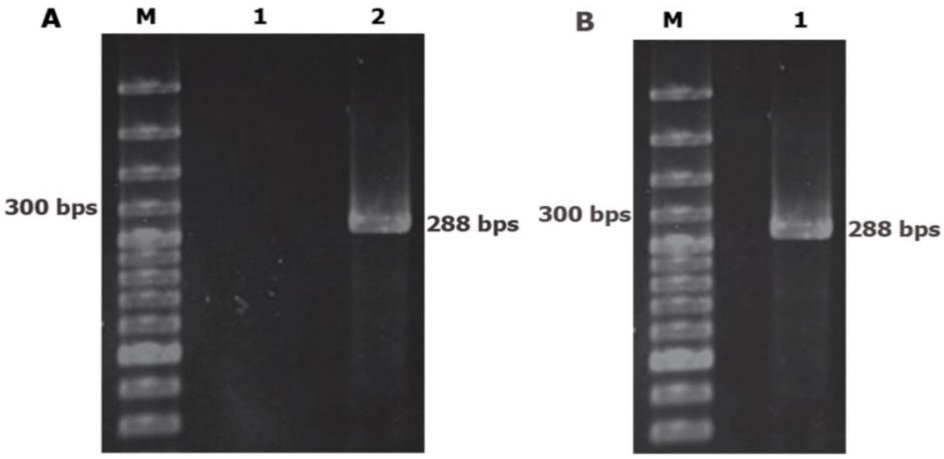

Figure 1. A) DNA amplification by polymerase chain reaction visualized on a 1% agarose gel, and B) Purification of PCR-amplified DNA encoding the ESAT6 protein from the Rv3875 gene using a Purification Kit (Qiagen).

Constructing the Rv3875 gene in the pGEM-T Easy-Rv2873 subcloning plasmid and transformation

In this study, the Rv3875 gene was cloned for further investigation and analysis. The pGEM-T Easy- Rv2873 +Rv3875 vector was constructed by inserting the target gene (gene of interest) into the pGEM-T Easy- Rv2873 cloning plasmid. We determined that the 660-bp long Rv2873 gene encodes the MPT83 protein and that the 288-bp long Rv3875 gene encodes the ESAT6 protein.

E. coli recombinant white colony transformation and screening results are shown in Figure 2. As depicted in Figure 2A, the absence of bacterial colonies was observed on Luria-Bertani agar plates when utilized as a negative control, specifically with competent *E. coli* JM109 cells lacking plasmids. This observation indicated no growth of *E. coli* JM109 cells without plasmids either autonomously or due to contamination. Figure 2B shows the blue colony which carries the vector pGEM-T Easy as transformation control. The experimental procedure included the introduction of pGEM-T Easy- Rv2873 into *E. coli* JM109 competent cells are depicted in Figure 2C. A collective sum of 396 bacterial colonies was seen on the Luria-Bertani plates. The utilization of the pGEM-T Easy- Rv2873 vector for transfection has yielded positive outcomes. The *E. coli* JM109 competent cells were subjected to transfection using the

recombinant vector pGEM-T-Easy-Mpt83+Rv3875, as depicted in Figure 2D. A transformation effectiveness of 62.9% is inferred from the detection of 249 white bacterial colonies. Transformation of the recombinant plasmid encoding Rv3875 has been successful, as evidenced by the appearance of these white colonies.

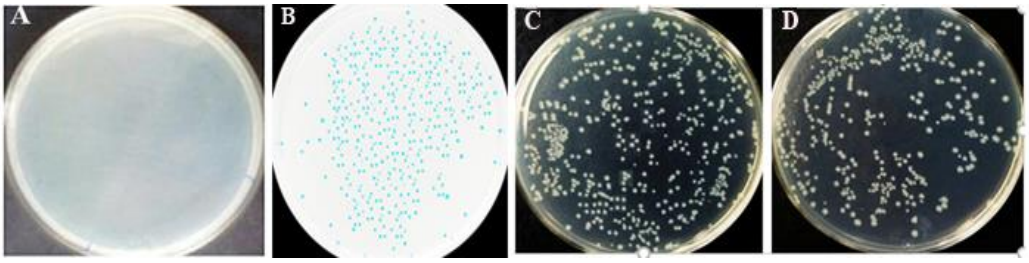

Figure 2. *E. coli* recombinant white colony transformation and screening (- control), there is no growth in plasmid-free competent cells of the *E. coli* strain JM109 (A). Blue colony, which harbors the vector pGEM-T Easy alone as control of transformation (B). Many white colonies, which harbor the vector pGEM-T Easy-Rv2873, were seen following the transformation of *E. coli* JM109 competent cells (C). The recombinant plasmid pGEM-T Easy- Rv2873 +Rv3875 is responsible for the proliferation of white colonies in *E. coli* cells (D).

Isolation and characterization of the recombinant plasmid pGEM-T Easy-Rv2873 +Rv3875

The recombinant vector pGEM-T Easy-Rv2873+Rv3875 exhibited DNA fragments of 288 bp (from Rv3875 gen alone) and 3678 bp (from the pGEM-T Easy- Rv2873) upon restriction with *BamH*I and *Hind*III enzymes, respectively (Figure 3). The pGEM-T Easy-Rv2873 vector has a length of 3678 base pairs, while the Rv3875 gene that has been inserted into it has 288 base pairs in length. The construction of the recombinant vector, pGEM-T Easy- Rv2873+Rv3875, was achieved through the insertion of the DNA of the Rv3875 gene into the pGEM-T Easy- Rv2873 vector, as seen in Figure 3.

White *E. coli* colonies harboring the MPT83 and ESAT6 fusion protein-encoding Rv2873 and Rv3875 genes were identified using the PCR colony technique (Figure 2C). The PCR result showed a single DNA band of 1,371 bps. Insert DNA from white colonies harboring the Rv2873 and Rv3875 genes measures 948 bps (columns 1-2) in Figure 4A. However, neither the Rv2873 nor the Rv3875 DNA inserts were found (Figure 4A, column 3).

Re-inoculation was conducted on a solid Luria-Bertani medium supplemented with 100 micrograms/mL of ampicillin subsequent to the identification of the converted white and isolated colony. Four colonies with white pigmentation were cultured in liquid Luria-Bertani media, supplemented with 100 micrograms/mL of ampicillin. The vectors were subsequently isolated from these colonies through the utilization of the QIAprep Spin Miniprep Kit manufactured by Qiagen, USA. The extraction resulted in the successful isolation of the recombinant vector pGEM-T Easy- Rv2873 +Rv3875, as confirmed by inspecting and displaying the electrophoresis results on a 1.5 % agarose gel (Figure 4B). Overall, the study demonstrated the successful isolation of the recombinant vector containing the Rv2873 and Rv3875 genes, which encode the MPT83 and ESAT6 fusion proteins, respectively, through the PCR colony method and subsequent vector extraction using the QIAprep Spin Miniprep Kit. The electrophoresis results confirmed the desired recombinant fusion plasmids in the three white colonies measure 948 bps (columns 1-3) in Figure 4B.

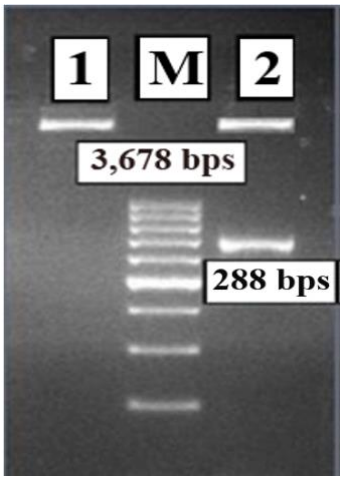

Figure 3. After running the Rv3875 gene via an agarose gel electrophoresis, the gene is ligated into the pGEM-T Easy- Rv2873 vector. Column 1: *BamH*I and *Hind*III enzymes can only cut the pGEM-T Easy- Rv2873 vector to produce one DNA band with size 3678 bps. Column 2: Two DNA bands of 3678 and 288 base pairs are produced from the *BamH*I / *Hind*III-restricted product of pGEM-T Easy- Rv2873 +Rv3875 vector/plasmid—specifically, 100 bps in Column M Genetic identifier.

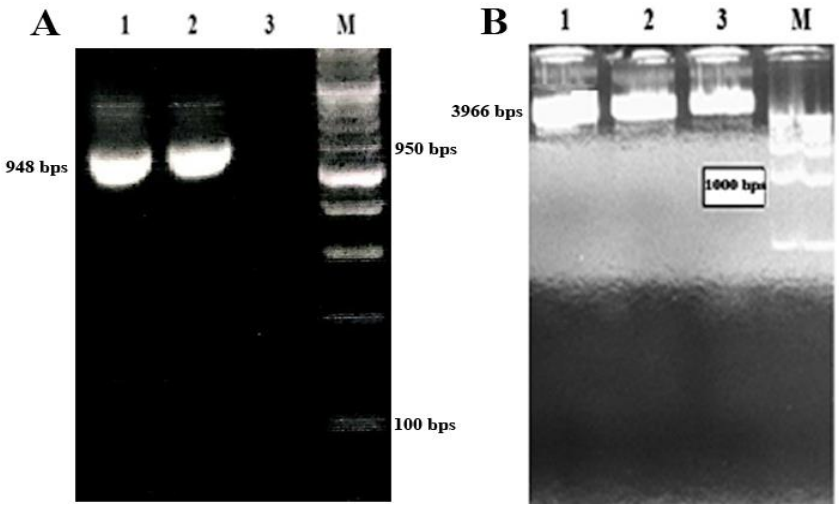

Figure 4. (A) The PCR product of the fusion genes Rv2873 and Rv3875 codes for MPT83 and ESAT6 fusion proteins. Columns 1-2 show the PCR results for the white colonies from two independent clones, indicating that their PCR product had a length of 948 bps, whereas column 3 served as a negative control. Isolated white colonies were inoculated onto a PCR kit mix and subjected to the PCR procedure before being analyzed on an agarose gel electrophoresis device at 1.5 percent. (B) Mini-preparations of recombinant plasmid pGEM-T Easy-Rv2873 +Rv3875 (3966 bps in length) from three separate and independent white bacterial colonies on LB plate in Figure 2D.

Recombinant plasmid sequencing

As depicted in Figure 5, the sequencing results showed conformity with the combined nucleotide sequences of the Rv2873 and Rv3875 genes encoding MPT83 and ESAT6 proteins, respectively. Combining these two genes produces a band of 948 bps, which begins with the start codon ATG and stop codon TGA. This sequence encodes 315 amino acids from the methionine (M) residue to the leucine (L). These sequencing results confirmed that fusion plasmid pGEM-T Easy- Rv2873 + Rv3875 was successfully created, which also provided crucial information regarding the encoded MPT83 and ESAT6 proteins.

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>MPT83+ESAT6
1     ATG ATC AAC GTT CAG GCC AAA CCG GCC GCA GCA GCG AGC CTC GCA   45
1     Met Ile Asn Val Gln Ala Lys Pro Ala Ala Ala Ala Ser Leu Ala   15

46    GCC ATC GCG ATT GCG TTC TTA GCG GGT TGT TCG AGC ACC AAA CCC   90
16    Ala Ile Ala Ile Ala Phe Leu Ala Gly Cys Ser Ser Thr Lys Pro   30

91    GTG TCG CAA GAC ACC AGC CCG AAA CCG GCG ACC AGC CCG GCG GCG   135
31    Val Ser Gln Asp Thr Ser Pro Lys Pro Ala Thr Ser Pro Ala Ala   45

136   CCC GTT ACC ACG GCG GCA ATG GCT GAC CCC GCA GCG GAC CTG ATT   180
46    Pro Val Thr Thr Ala Ala Met Ala Asp Pro Ala Ala Asp Leu Ile   60

181   GGT CGT GGG TGC GCG CAA TAC GCG GCG CAA AAT CCC ACC GGT CCC   225
61    Gly Arg Gly Cys Ala Gln Tyr Ala Ala Gln Asn Pro Thr Gly Pro   75

226   GGA TCG GTG GCC GGA ATG GCG CAA GAC CCG GTC GCT ACC GCG GCT   270
76    Gly Ser Val Ala Gly Met Ala Gln Asp Pro Val Ala Thr Ala Ala   90

271   TCC AAC AAC CCG ATG CTC AGT ACC CTG ACC TCG GCT CTG TCG GGC   315
91    Ser Asn Asn Pro Met Leu Ser Thr Leu Thr Ser Ala Leu Ser Gly   105

316   AAG CTG AAC CCG GAT GTG AAT CTG GTC GAC ACC CTC AAC GGC GGC   360
106   Lys Leu Asn Pro Asp Val Asn Leu Val Asp Thr Leu Asn Gly Gly   120

361   GAG TAC ACC GTT TTC GCC CCC ACC AAC GCC GCA TTC GAC AAG CTG   405
121   Glu Tyr Thr Val Phe Ala Pro Thr Asn Ala Ala Phe Asp Lys Leu   135

406   CCG GCG GCC ACT ATC GAT CAA CTC AAG ACT GAC GCC AAG CTG CTC   450
136   Pro Ala Ala Thr Ile Asp Gln Leu Lys Thr Asp Ala Lys Leu Leu   150

451   AGC AGC ATC CTG ACC TAC CAC GTG ATA GCC GGC CAG GCG AGT CCG   495
151   Ser Ser Ile Leu Thr Tyr His Val Ile Ala Gly Gln Ala Ser Pro   165

496   AGC AGG ATC GAC GGC ACC CAT CAG ACC CTG CAA GGT GCC GAC CTG   540
166   Ser Arg Ile Asp Gly Thr His Gln Thr Leu Gln Gly Ala Asp Leu   180

541   ACG GTG ATA GGC GCC CGC GAC GAC CTC ATG GTC AAC AAC GCC GGT   585
181   Thr Val Ile Gly Ala Arg Asp Asp Leu Met Val Asn Asn Ala Gly   195

586   TTG GTA TGT GGC GGA GTT CAC ACC GCC AAC GCG ACG GTG TAC ATG   630
196   Leu Val Cys Gly Gly Val His Thr Ala Asn Ala Thr Val Tyr Met   210

631   ATC GAT ACG GTG CTG ATG CCC CCG GCA CAG ATG ACA GAG CAG CAG   675
211   Ile Asp Thr Val Leu Met Pro Pro Ala Gln Met Thr Glu Gln Gln   225

676   TGG AAT TTC GCG GGT ATC GAG GCC GCG GCA AGC GCA ATC CAG GGA   720
226   Trp Asn Phe Ala Gly Ile Glu Ala Ala Ala Ser Ala Ile Gln Gly   240

721   AAT GTC ACG TCC ATT CAT TCC CTC CTT GAC GAG GGG AAG CAG TCC   765
241   Asn Val Thr Ser Ile His Ser Leu Leu Asp Glu Gly Lys Gln Ser   255

766   CTG ACC AAG CTC GCA GCG GCC TGG GGC GGT AGC GGT TCG GAG GCG   810
256   Leu Thr Lys Leu Ala Ala Ala Trp Gly Gly Ser Gly Ser Glu Ala   270

811   TAC CAG GGT GTC CAG CAA AAA TGG GAC GCC ACG GCT ACC GAG CTG   855
271   Tyr Gln Gly Val Gln Gln Lys Trp Asp Ala Thr Ala Thr Glu Leu   285

856   AAC AAC GCG CTG CAG AAC CTG GCG CGG ACG ATC AGC GAA GCC GGT   900
286   Asn Asn Ala Leu Gln Asn Leu Ala Arg Thr Ile Ser Glu Ala Gly   300

901   CAG GCA ATG GCT TCG ACC GAA GGC AAC GTC ACT GGG ATG TTC GCA   945
301   Gln Ala Met Ala Ser Thr Glu Gly Asn Val Thr Gly Met Phe Ala   315

946   TAG   948
316   End   316
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Figure 5. The predicted polynucleotide (cDNA) and amino acid (AA) sequences for the fusion proteins MPT83 and ESAT6 were encoded by the Rv2873 and Rv3875 genes, respectively.

Transformation and culture of *E. coli* Strain BL21(DE3) cells with pTrcHisA-Rv2873 +Rv3875 to generate fusion MPT83 Plus ESAT6 recombinant protein

Introducing the plasmid pTrcHisA-Rv2873 +Rv3875 into *E. coli* strain BL-21 cells yielded the results in Figure 6A; the bacteria multiplied and formed white colonies on

the Agar Luria-Bertani culture. Subcloning is complete when white colonies form, proving that the fusion genes Rv2873 and Rv3875, which code for the MPT83 and ESAT6 proteins, respectively, have been achieved through the insertion into the pTrcHisA vector. Errors in expression in the lacZ gene of the pTrcHisA vector generated the white coloration of the *E. coli* colonies.

The *E. coli* strain BL-21 containing this recombinant fusion protein was then cultured in liquid LB medium with and without 40 micromolar IPTG induction for six hours (Figure 6B), and then the pellets were collected. The results were then analyzed on SDS-PAGE, as shown in Figure 6C. The cultivation of the cells with the pTrcHisA-Rv2873+Rv3875 plasmid by induction with 40 micromolar IPTG resulted in a significant protein band with molecular weight 28 kDa (Figure 6C column 1 and 3), but not found in the absence of IPTG (Figure 6C column 2 and 4). This result confirmed that MPT83 and ESAT6 are soluble proteins presented inside the cells.

Figure 7 shows the purified recombinant MPT83-ESAT6 fusion protein. Figure 7 (lanes 1-2) compares cells without and with IPTG, similar to Figure 6C. The next lanes show the success of purifying the MPT83 plus ESAT6 fusion protein, demonstrating a single protein band of 28 kDa of molecular weight.

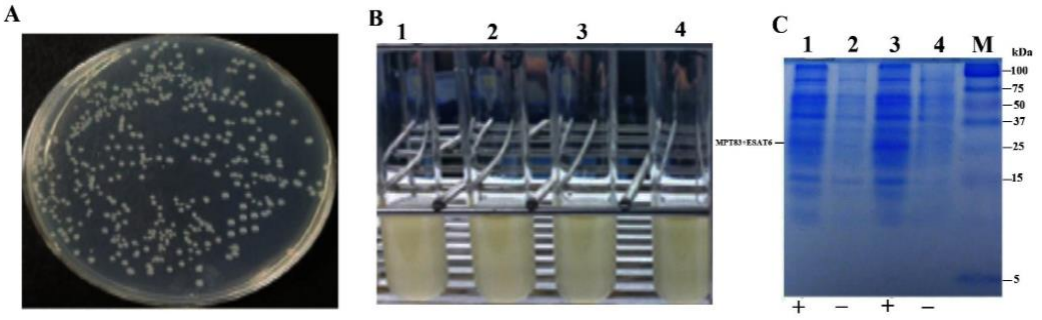

Figure 6. The recombinant plasmid pTrcHisA-Rv2873 +Rv3875 was introduced into the *E. coli* BL-21 strain. Subsequently, the transformed strain was evaluated on Luria-Bertani agar plates (A) and liquid culture media to express recombinant proteins with and without IPTG (B). Recombinant protein expression and growth of MPT83 plus ESAT6 in the absence and presence of 40 micromolar IPTG on SDS-PAGE analysis (C).

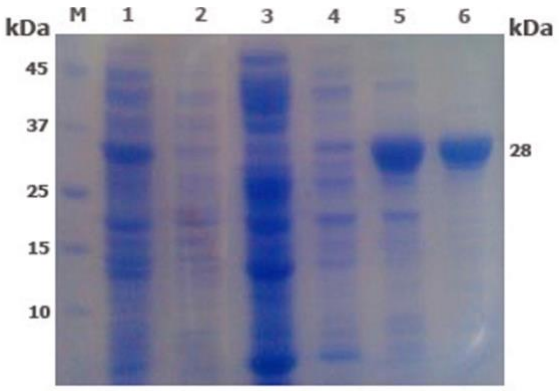

Figure 7. Expression, purification, and SDS-PAGE (10%) analysis of purified recombinant MPT83 plus ESAT6 fusion proteins. It was possible to separate and purify 6XHis-tagged MPT83 and ESAT6 using affinity chromatography with a His Tag-agarose matrix. Proteins were isolated from cell lysates and various chromatographic fractions by electrophoresis in a 10% SDS-PAGE. The proteins could be seen thanks to CBB staining. Molecular weight proteins marker (M), induced whole cell lysates (Lane 1), un-induced whole cell lysates (Lane 2), wash flowthrough (Lane 3), flowthrough of elution with 0.5 M Tris-HCl buffer with 100 mM imidazole pH 7.3 (Lane 4), 200 mM imidazole pH 5.9 (Lane 5), and 300 mM imidazole pH 4.5 (Lane 6), MPT83 plus ESAT6 fusion proteins bands at 28 kDa are shown.

DISCUSSION

In this study, we produced MPT83 and ESAT6 fusion antigen proteins by cloning the Rv3875 gene encoding the ESAT6 protein into the previously created pGEM-T Easy-Rv2873 vector [21] to produce the pGEM-T Easy- Rv2873 + Rv3875 fusion plasmid. Similar to previous research, we determined that the 660-bp long Rv2873 gene encodes the MPT83 protein [14] and the 288-bp long Rv3875 gene encodes the ESAT6 protein. Overhanging bases (T) can be found at both ends of the linear pGEM-T Easy- Rv2873 vector, facilitating a more efficient PCR product ligation procedure by preventing self-ligation at the target insertion site with T-overhangs (X). To enhance the ligation process, a larger ratio of inserts to vectors—three to one—was employed. At thirty degrees Celsius, T4 DNA ligase is at its most active.

The Rv3875 gene ligation product and the pGEM-T Easy-Rv2873 cloning vector were then transformed into *E. coli* JM109 competent cells and the subsequent blue-white screening were selected because it has the ampicillin resistance (Amp^r) site and the lacZ gene, as reported in previous studies [24, 26]. To introduce recombinant DNA into *E. coli* cells, scientists used a thermal shock technique that briefly opened the cell membrane [26]. The rapid inflation and deflation of the cell wall brought about by the alternating cold and hot shocks allowed outside plasmid DNA to pass through the cell wall and enter the cell.

During the transformation and screening process of *E. coli* recombinants, different outcomes were observed for the transformed cells. Competent cells of *E. coli* strain JM109 that did not carry the plasmid could not grow. As a null hypothesis, this finding verifies that the presence of the altered plasmids is responsible for the growth seen in the other samples. *E. coli* JM109 containing the pGEM-T Easy plasmid without genes were grown with blue colony (Figure 2B). Whereas the presence of white colonies harboring the vector pGEM-T Easy- Rv2873 (Figure 2C) and pGEM-T Easy-Rv2873+Rv3875 recombinant plasmid (Figure 2D) was confirmed. The white colonies demonstrated that the corresponding vector had successfully transformed and was present in the *E. coli* JM 109 competent cells. These findings support the successful cloning and transformation of the pGEM-T Easy- Rv2873 vector and the recombinant plasmid pGEM-T Easy-Rv2873 +Rv3875 into *E. coli* JM109 competent cells.

Blue-white screening identified bacterial cells that successfully contained recombinant plasmids with gene inserts. The lacZ gene in the pGEM-T Easy vector allows blue-white screening to identify successful cloning of the RV2873 and Rv3875 fusion target genes. Transcription of the lacZ gene is initiated by IPTG [27]. LacZ encodes the enzyme β-galactosidase, breaking lactose into sugar components such as glucose and galactose. Blue bacterial colonies are formed when the enzyme β-galactosidase is present, which breaks down X-gal into galactose and 5-bromo-4-chloro-3-hydroxyindole, which produces a blue color. When blue colonies appear, the β-galactosidase enzyme works, meaning the lacZ gene is activated, thus indicating that DNA insertion into the vector has not occurred. In contrast, when Rv2873 and Rv3875 DNA were inserted into the multi-cloning site (MCS) vector, the lacZ gene was disrupted in the recombinant vector, preventing hydrolysis of the galactose substrate in the growth medium and causing the colonies to remain white. White bacterial colonies form when cells lack the enzyme β-galactosidase. Blue-white screening effectively identified successful transformants after cloning and transfecting the pGEM-T Easy-Rv2873 vector and the pGEM-T Easy-Rv2873 +Rv3875 recombinant plasmid into *E. coli* JM109 competent cells.

The construction of the recombinant vector, pGEM-T Easy- Rv2873+Rv3875, was achieved through the insertion of the DNA of the Rv3875 gene into the pGEM-T Easy-Rv2873 vector. The recombinant vector pGEM-T Easy-Rv2873+Rv3875 exhibited DNA fragments of 288 bp (from Rv3875 gen alone) and 3678 bp (from the pGEM-T Easy-Rv2873) upon double restriction with *BamH*I and *Hind*III enzymes. PCR colony technique and sequencing analysis also confirmed Rv2873 and Rv3875 genes insertion into pGEM-T Easy on the correct site and position in the cloning vector.

In this study, recombinant proteins MPT83 and ESAT6 were successfully produced in the *E. coli* BL-21 strain, forming white colonies. White colonies containing this recombinant fusion protein product were observed after *E. coli* B-L21 cells were transformed with the pTrcHisA-Rv2873+Rv3875 and cultured. The growth and synthesis of the recombinant protein were demonstrated by the visual change in the Luria-Bertani liquid medium from transparent to cloudy, which confirmed the success of the recombinant protein synthesis [19].

Cell walls, including cell membranes, can be efficiently disrupted by sonication. Biomolecules such as proteins, lipids (fats), and monosaccharides are released from the *E. coli* BL-21 strain cells during sonication and enter the clear liquid outside the cells. The presence of these biomolecules in the liquid would raise the fluid viscosity. Overall, the sonication process was employed to disrupt the bacterial cell walls and cell membranes, leading to the release of biomolecules from the *E. coli* BL-21 strain cells into the clear liquid [28]. This step is crucial for obtaining the desired proteins, MPT83 and ESAT6.

The use of IPTG as an inducer significantly enhances recombinant protein expression. In previous studies, the induction of recombinant proteins was found to be highly dependent on cell growth when inducers like IPTG were used. Higher IPTG concentrations could lead to increased recombinant protein production, but excessively high inducer concentrations could harm cell viability [30]. Another study reported that lesser doses of IPTG were preferable at high temperatures. The inducible expression of recombinant proteins and other target proteins in *E. coli* cells has led to IPTG's widespread use as an inducer in the scientific community [31]. Our result demonstrated that MPT83 and ESAT6 are both soluble proteins found inside cells. The protein was found to be quite prevalent in the supernatants but at lower concentrations in the pellet cells. This result showed that the *E. coli* successfully created a 28-kilo Dalton recombinant protein composing 6XHis-tagged MPT83 and ESAT6.

MPT83 has been demonstrated to elicit a significant and specific immunological response against *M. tuberculosis*. MPT83 could stimulate immune cells, including macrophages and dendritic cells, which in turn release cytokines like TNF-α and IFN-γ to initiate the adaptive immune response. So far, research on MPT83 as a seed vaccine candidate has been tested in the form of an RNA or DNA vaccine in mouse models and showed a specific MPT83 CD8+ T cell response [32]. Nonetheless, nucleic acid-based vaccines for TB have yet to be substantially examined, yet they may offer a framework for further vaccine design [33]. Besides, MPT83 and ESAT6 incorporated in Vaccine subunit H107, along with six other antigens not found in the BGC strain, have also been developed and tested in mouse models [34]. This vaccine's administration, along with BCG, enhances the adaptive response to *M. tuberculosis* infection, specifically in inducing a subset of under-differentiated CD4 Th1 cells that accumulate at the site of infection and persist post-Mtb challenge. Th1 with less differentiation may have a greater potential to develop memory T cells, which are essential for establishing sustained protection.

The achievement of creating this recombinant fusion protein offers new avenues for investigation. The limitation of this study is that it is only at the stage of fusion protein expression, and no research has yet been conducted to study the antibody response to the produced fusion antigen, either *in vitro* or *in vivo*. Future studies may use antigens obtained from this recombinant fusion protein to create TB vaccines, including the antibody response. This could be a promising direction for advancing TB vaccine research and development.

CONCLUSIONS

The PCR successfully amplified DNA bands from the genomic DNA of *M. tuberculosis*' Rv2873 and Rv3875 genes, which codes for the MPT83 plus ESAT6 fusion protein. These DNA fragments measured 948 bps in length and comprised the entire coding sequence for the MPT83 plus ESAT6 fusion protein down to its stop codon, TGA. Insertion of the Rv3875 gene, which codes for the ESAT6 protein, into the pGEM-T Easy Mpt83 plasmid yielded the pGEM-T Easy- Rv2873 +Rv3875 vector. As a result, the ESAT6 protein became fused with the MPT83 protein. Successful subcloning of the pGEM-T Easy- Rv2873 +Rv3875 plasmid into the pTrcHisA vector was achieved by employing nucleotide and amino acid sequence predictions of the 6XHis-tagged MPT83 plus ESAT6 recombinant proteins. *E. coli* BL-21 strain cells produced a 28-kilo Dalton fusion protein. The 28-kilo Dalton fusion protein was successfully produced in *E. coli* cells following amplification of the Rv2873 and Rv3875 genes from *M. tuberculosis*, construction of the pGEM-T Easy- Rv2873+Rv3875 vector, and subcloning of the fusion protein into the pTrcHisA vector. This study may advance our knowledge of MPT83 and ESAT6 fusion proteins, opening up their potential applications in future research, notably in vaccine development or other diagnostics and therapeutic approaches.

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AUTHOR CONTRIBUTIONS

RBL, AA, AK, RA, RA, and NH collaborated to conduct practical research and laboratory techniques, gather and analyze data, and draft and edit the manuscript. MNM, AN, HK, and IH contributed to the critical reviewing and proofreading of this manuscript. All the authors have reviewed and approved the final version of the paper.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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