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CLONING AND EXPRESSION OF MPT83 PLUS MPT64 FUSION PROTEIN FROM *Mycobacterium tuberculosis* IN *Escherichia coli* BL21 (DE3) STRAIN AS VACCINE CANDIDATE OF TUBERCULOSIS

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ABSTRACT

Mycobacterium tuberculosis drug-resistant strains have emerged, creating a new difficulty in the treatment of tuberculosis around the world and prompting the World Health Organization to declare TB an international emergency. With TB treatment resistance increasing, developing more potent vaccinations can ensure the TB epidemic is stopped or reduced. The MPT64 gene was amplified from the genomic DNA of the *M. tuberculosis* local strain using polymerase chain reaction (PCR) *in vitro* and then transferred into the pGEM-T Easy-Mpt83+Mpt64 vector and expressed in the *E. coli* BL21 (DE3) strain. Plasmid DNA was isolated and amplified using PCR, followed by DNA sequencing. Subcloned into the expression vector pTrcHisA on *Nhe*I/*Hind*III cloning site before being converted into the *E. coli* BL21 (DE3) strain were the correct recombinant MPT83 and MPT64 gene fusions. The white recombinant colony was propagated and maintained using 40 µM IPTG, then cells and protein recombinants were harvested, and SDS-PAGE electrophoresis was used to identify it. The protein was then purified with a 6XHis tagged-agarose beads purification kit. The results showed that the MPT83 and MPT64 gene fusion was successfully produced, expressed, and purified. The fusion protein of MPT83 and MPT64 with a His tag from the expression vector had a molecular weight of about 46 kDa and was expressed as a soluble protein. Cloned from the host strain of the *E. coli* BL21 (DE3) strain cell, the fusion genes were successfully produced in the bacteria. Pathways to tuberculosis diagnoses are laid by the purified recombinant fusion proteins, MPT83 and MPT64, and may be more successful in the future as a vaccine candidate.

Keywords: Tuberculosis, Cloning, Recombinant, Plasmid, MPT83 Plus MPT64.

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INTRODUCTION

Pulmonary tuberculosis, the most common manifestation of tuberculosis caused by the bacteria *Mycobacterium tuberculosis (M. tuberculosis)*, is an infectious disease that can potentially affect other body tissues or organs (extrapulmonary TB). When people with pulmonary tuberculosis cough up *M. tuberculosis* bacteria, the disease spreads through the air.[1] The substantial lipid content of the cell membrane of *M. tuberculosis* renders it acid resistant.[2,3] Since the bacterium that causes tuberculosis is susceptible to Ultra Violet (UV) light and radiation,[4,5] it is more likely to spread at night.[6] Tuberculosis kills an estimated 8 million people annually, with 1.5 million deaths occurring in the developing world. In terms of TB incidence, Indonesia comes in third place, accounting for 8.4% of all cases worldwide.[1] In Indonesia, around

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80% of TB patients are of working age, increasing both the disease's economic and social consequences. Since most people in Indonesia live in areas where TB germs can thrive and proliferate, there are a lot of people with tuberculosis in the country. As the number of HIV patients grows and *M. tuberculosis* develops multi-drug resistance (MDR), the situation has become even worse. Hence, developing a cheap and efficient vaccine and a rapid and adequate diagnostic procedure is necessary to overcome this challenge. Droplet nuclei harboring tuberculosis germs are inhaled, resulting in TB infection. Exposure to TB germs has four possible outcomes: no infection, infection but *M. tuberculosis* is quickly eliminated, active TB, or dormant TB infection which may reactivate and becomes active shortly or even years.[7] MPT83 (Rv2873) and MPT64 (Rv1980c) appear suitable for immune system-based diagnostic testing and vaccinations. *M. tuberculosis* complex (MTC) species have both proteins. MPT83 antigen is encoded by the Rv2873 gene in the *M. tuberculosis*' RD2 region. Rv1980c encodes a secretory protein MPT64 antigen, which is 24 kDa. This antigen is not found in *M. leprae*, *M. bovis*, and other types of mycobacteria. Only healthy and dividing cells contain the MPT64 antigen. MPT83 and MPT64 antigens are virulence markers in MTB. MPT83 and MPT64 antigens are exclusively detected in complex *M. tuberculosis* strains. MPT83 and MPT64 are good candidates for a TB vaccine and kit of diagnostic tests.[8–10] According to a prior study, mice infected with *M. tuberculosis* fared better when treated with MPT83 homologous monoclonal antibodies. *M. tuberculosis*'s MPT83 and MPT64 antigens are the only ones of their kind.[11] This work used recombinant DNA techniques to construct an MPT83 and MPT64 fusion antigen protein. The Rv1980c gene, which makes the MPT64 protein, was cloned, and inserted into vector pGEM-T Easy-Mpt83 at the *Bsr*I/*Hind*III cloning site, to produce pGEM-T Easy-Mpt83+Mpt64 recombinant vector. Next, from this recombinant plasmid, the Mpt83 plus Mpt64 genes fusion was subcloned into expression vector pTrcHisA on *Nhe*I/*Hind*III cloning site to yield pTrcHisA-Mpt83+Mpt64, then expressed into *Escherichia coli* (*E. coli)* BL21 (DE3) strain cells to produce recombinant 6XHis tag fusion protein MPT83 plus MPT64 and can be used as candidates for the tuberculosis vaccine more effectively. Fusion proteins are likely to help safeguard TB patients in their productive years, leading to lower rates of TB-related illness and death in the future.

EXPERIMENTAL

PCR Amplification of the Specific Gene Target, Which Encodes the MPT64 Protein

The Rv1980c gene, which encodes MPT64, was amplified using Polymerization Chain Reaction (PCR) The primer and condition used were similar to the previous study[12]: initial denaturation at 90ºC for 5 minutes, 30 cycles of 94ºC for 30 seconds, 55ºC for 30 seconds, 72ºC for 30 seconds beginning denaturation, and 30 seconds of annealing at 55ºC. The last DNA fragment's 7-minute complete extension at 72ºC. Electrophoresis of the PCR result was carried out using a 1.5% agarose gel electrophoresis system.

DNA Extraction from PCR Product

DNA binding, washing, and elution are all included in the kit's purification steps. PCR product purification was carried out to obtain pure DNA fragments for ligation to the pGEM-T Easy vector (Promega, Cat No. A1380). About 50 µL of PB buffer solution was added to Eppendorf tubes containing PCR products from agarose gel that had been cut and homogenized. The sample was then placed into a spin column tube and spun for 1 minute at 12,000 rpm and ambient temperature. The supernatant was collected, and the spin column was then centrifuged at 12,000 rpm for one minute before adding 700 microliters of PEP washing buffer. The sample was subsequently transferred to a tube of the spin column. After adding 35 µL of EB buffer, the produced liquid was flushed out. The sample was then transferred to a sterile Eppendorf tube. After three minutes, the tube was spun at 12,000 rpm for one minute. Electrophoresis was used to verify the purification results of PCR products on a 1.5 percent agarose gel. After collecting the supernatant, seven hundred microliters of PEP washing buffer were added to the spin column and centrifuged at 12,000 rpm for one minute to collect PCR products.

The Ligation of the Specific Gene Target Expressing MPT64 Protein into the pGEM-T Easy-Mpt83 Vector

To bind the DNA, the enzyme T4 DNA Ligation was used. Purified PCR results-*BamH*I/*Hind*III (4 µL) was used to ligate the Rv1980c gene fragments with the pGEM-T Easy-Mpt83 vector (4 µL). Each reaction needs 1 µL of T4 DNA Ligase, 3 µL of nuclease-free water, 1 µL of *BamH*I/*Hind*III, and 2 µL of 2x Rapid

Ligation Buffer. Then, each mixture was put into a 1.5 mL PCR tube and homogenized. Afterward, the mixtures were incubated for 20 hours at ±4°C to create pGEM-T Easy-Mpt83+Mpt64 fusion plasmid.

JM109 *E. coli* Cell Transformation and Characterization of the Vector pGEM-T Easy-Mpt83+Mpt64

An amount totalling 10 microliters of the ligation product (Rv1980c) was added to an amount equal to 50 microliters of *E. coli* JM109 competent cells and homogenized afterward. For the positive control, competent *E. coli* JM109 cells were cultivated in the presence or absence of antibiotics while, *E. coli* JM109 cells without vectors were cultivated with or without antibiotics and used as a source of a variety of antibiotics. The three tubes were maintained at ±4°C for 30 minutes. The samples underwent a 90-second thermal shock at ±42°C before being put on ice, incubated for an hour, and given 600 µL of Luria-Bertani (LB) liquid media. At 37°C and 150 rpm, the culture tubes were maintained for three hours in a shaker incubator. A minute of 12,000 rpm centrifugal force was applied to the sample. Ligation products were concentrated to 150 µL; then, the cells were subsequently cultured at 37°C for 14-16 hours with 0.8 mg X-gal, 100 µg/mL ampicillin (amp), and 40 µM isopropyl β-D-1- thiogalactopyranoside (IPTG). PCR colony method and sequencing analysis were used to verify the insertion of Rv2873 and Rv1980c genes into pGEM-T Easy plasmid.[10]

MPT83 Plus MPT64 Recombinant Protein Fusion Subcloning and Expression in *E. coli* BL21 (DE3) Strain

A fusion protein encoding the Rv2873 and Rv1980c genes was added and sub-cloning to the pTrcHisA expression vector (Thermo Fisher Scientific, Cat. No. V36020) to form the recombinant plasmid pTrcHisA-Mpt83+Mpt64, which was validated for the nucleotide sequence of its mRNA using DNA sequencing. To create the recombinant fusion proteins MPT83 and MPT64, the Rv2873 and Rv1980c fusion genes from the plasmid pTrcHisA-Mpt83+Mpt64 were transformed into *E. coli* BL21 (DE3) cells. When the white colonies appeared, the *E. coli* strain BL21(DE) bearing the pTrcHisA-Mpt83+Mpt64 plasmid was isolated and incubated at 37 degrees Celsius in a shaker incubator overnight. Once this step was completed and cultured for two hours, the *E. coli* culture was placed into ten milliliters of the new Luria-Bertani medium. Only around five milliliters of bacterial culture were transferred to the Eppendorf tube with no IPTG added (MPT83 plus MPT64 non-induction, -IPTG). The MPT83 and MPT64 protein expression was stimulated by 40 µM IPTG for six hours in the remaining 5 mL of bacterial culture. To separate the supernatant from the pelleted cell, each culture of *E. coli* BL21 (DE3) strain cells was centrifuged at 13,000 rpm for 5 minutes at 4°C. It was necessary to remove proteins from pellet cells and then resuspend them in 1X Phosphate-buffered saline pH 7.4, which contained the following additives: (0.144 grams di-Sodium hydrogen phosphate anhydrous, 0.02 grams potassium chloride, 0.8 grams Sodium chloride, 0.024 grams Monopotassium phosphate, and 100 mL double-distilled water). The cell wall of bacteria was broken three times with a power of 20 kHz using a 30-second repeating sonication procedure. Recombinant proteins were extracted from bacterial cell waste, and the suspension of bacterial cell debris was centrifuged at 13,000 rpm for one minute at ±4°C. The expression of proteins in pellet cells and supernatants was purified by affinity chromatography using the 6XHis Tag-agarose matrix (Thermo Ficher, Scientific) and recombinant proteins had been examined by SDS-PAGE (12%). The recombinant proteins MPT83 and MPT64 were contrasted with a standard protein ladder (Tiangen Biotech, Beijing) on polyacrylamide gels. The findings were shown in photographs or graphically and explained in detail.

RESULTS AND DISCUSSION

Rv1980c Gene Amplification and Purification Encoding MPT64 Protein

A previous investigations[13] found that the Rv1980c gene could be amplified using *M. tuberculosis* genomic DNA and particular primers made according to the materials and techniques, as shown in Fig.-1. The negative PCR control, on the other hand, does not show any DNA. It is possible to see the PCR-resulted DNA band moving in a similar direction as DNA ladder 681 bp, in Fig.-1. The DNA size results were consistent with GenBank data (Access No.: NC_000962.2 with Gene ID: 885925), which demonstrated that the Rv1980c gene's 681 bps length including ATG start codon encoded the MPT64 protein. The target gene (insert) was created from the PCR result and ligated into the pGEM-T Easy-Mpt83 cloning vector to create the pGEM-T Easy-Mpt83+Mpt64 plasmid. This was similar to another study[13], which found that the

MPT83 protein was coded by the Rv2873 gene, which was 660 bp long, and the MPT64 protein was coded by the Rv1980c gene, which was 681 bp long. Agarose gel electrophoresis results showed 681 bp DNA bands and no dimers or dimerization product, proving that the DNA purification procedure was successful (Fig.- 1B).

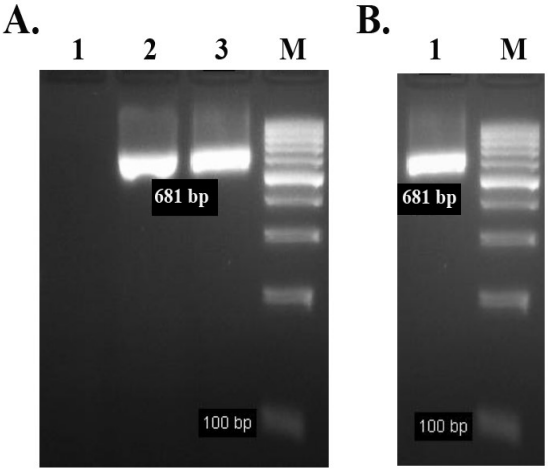

Fig.-1: A. PCR-Amplified DNA Visualization on 1.5% Agarose and B. PCR-Amplified DNA Purification from the Specific Gene Target Encoding MPT64 Protein using a Purification Kit (Qiagen)

Rv1980 Gene Construction and Transformation in pGEM-T Easy-Mpt83 Subcloning Vector

The Rv1980c gene was cloned to be studied and defined in sufficient numbers. Because of its amp resistance site (*Amp*r) and lacZ gene, the pGEM-T Easy-Mpt83 cloning vector was used in this investigation for blue-white screening during the transformation into *E. coli* JM109 competent cells. The linear vector of the pGEM-T Easy-Mpt83 vector has overhanging bases on both ends (T). The PCR product ligation procedure can be more efficient because it eliminates self-ligation due to a T-overhang on the target site of insertion/ligation.[14] The insert-to-vector ratio in this study was 4:1. To boost the likelihood of insert binding to vectors (ligation process), we increased the number of inserts (four-fold) compared to the vectors. Thirty degrees Celsius is optimal for T4 DNA ligase's enzymatic activity. The recombinant pGEM-T Easy-Mpt83+Mpt64 that was produced during the ligation process was then transformed into *E. coli* JM109 competent cells and cultured on agar LB plate media with amp, X-gal, and IPTG (Fig.-2). The thermal shock approach was employed in the transformation process. By this approach, the *E. coli* cell membrane could be opened in a short period, allowing recombinant DNA to enter the cells. Cold and hot shocks to *E. coli* JM109 cells alternately caused the cell wall to inflate and deflate rapidly, allowing DNA from outside the cell to enter. Heat shock of 42 degrees Celsius for 90 seconds was found to be an effective approach for the transformation process.[15]

(A) (B) (C)

Fig.-2: Transformation and Screening Product of White Colony from *E. coli* recombinant. No Competent Cells of *E. Coli* Strain JM109 Were Grown Without a Recombinant Plasmid (negative control, (A). *E. coli* JM109 Competent Cells Grew Largely in a White Colony that Contained the Modified pGEM-T Easy-Mpt83 Vector (positive control, (B). Growth of *E. coli* Cells in a White Colony that Carries the Recombinant Plasmid pGEM-T Easy-Mpt83+Mpt64 (C).

On Luria-Bertani agar plate media containing antibiotic ampicillin, competent *E. coli* JM109 cells lacking plasmids failed to form colonies (Fig.-2A). This result is due to the fact that there was just one amp-resistant *E. coli* JM 109 in the negative control. Fig.-2B shows *E. coli* JM109-competent cell transformation with

the pGEM-T Easy-Mpt83 vector; there were about 950 colonies on agar Luria-Bertani plates. The recombinant vector pGEM-T-Easy-Mpt83+Mpt64 samples were transfected using *E. coli* strain JM109 competent cells. Figure-2C shows that a transformation efficiency of 16.6% was achieved in the presence of 158 white bacterial colonies. The result of the study showed the effectiveness of the prior cloning method (Fig.-2C), which produced white colonies using the vector pGEM-T Easy-Mpt83+Mpt64 after using the previously successful DNA insert (Rv1980c). *E. coli* colonies (transformants) showing the lacZ gene of the pGEM-T Easy-Mpt83 vector have joined to the plasmid's MCS (multi-cloning site) region.[16] As a result, the enzyme cannot digest the galactose substrate that is present in the growth media since the lacZ gene has been inhibited by this insert. Despite the absence of DNA fragment insertion, the bacterial colonies are blue and capable of breaking down the existing galactose substrate. The competent transformed *E. coli* cells were chosen using Luria-Bertani media employed with amp, IPTG, and X-gal as the growth medium. When IPTG is introduced, the lacZ lac operon gene transcription begins. LacZ encodes the β- amyloglucosidase enzyme, which degrades lactose into glucose and galactose by hydrolysis reaction. The breakdown of the X-gal substrate (5-bromo-4-chloro-3-indolyl-D-galactopyranoside, composed of white to galactose and 5-bromo-4-chloroindigo), might reveal the presence of the amyloglucosidase enzyme. The β-amyloglucosidase produces blue bacterial cell colonies. This result indicates that the vector was not successfully ligated with insertion DNA. Cells lacking the β- amyloglucosidase enzyme will produce white bacterial colonies. When DNA fragments are introduced between the lacZ gene, they activate the lacZ gene, resulting in these effects.[17]

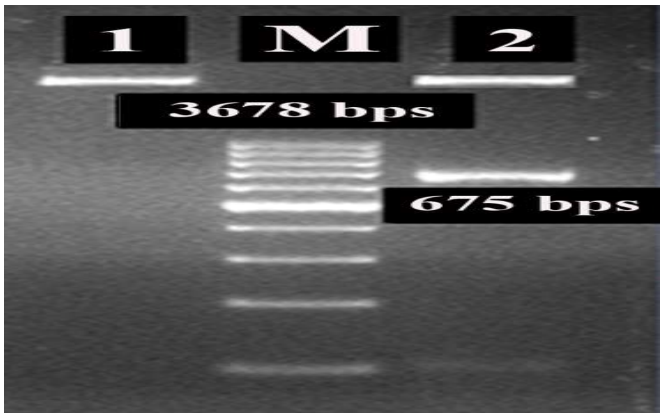

Fig.-3: Electrophoresis of An Agarose Gel Leads to the Ligation of the Specific Gene Target Encoding MPT64 Protein into the pGEM-T Easy-Mpt83 Vector. Column 1: pGEM-T Easy-Mpt83 Plasmid Limited by *BamH*I and *Hind*III Enzymes to Yield One DNA Band 3678 bps. Column 2: The *BamH*I / *Hind*III-Restricted pGEM-T Easy-Mpt83+Mpt64 Vector to Yield Two DNA bands 3678 bps and 675 bps. Column M: 100 bps DNA marker

The foreign DNA from the specific gene target encoding the MPT64 protein was successfully ligated to the pGEM-T Easy-Mpt83 cloning vector, as illustrated in Fig.-3. DNA fragments of 675 bp and 3678 bp may be seen in the second column of pGEM-T Easy-Mpt83+Mpt64 after restriction with *BamH*I and *Hind*III enzymes. Finally, the vector pGEM-T Easy Mpt83 was inserted into the DNA of the Rv1980c gene to produce pGEM-T Easy-Mpt83+Mpt64.

pGEM-T easy-Mpt83 +Mpt64 Recombinant Plasmid Isolation and Characterization

We employed the PCR colony method to determine the white *E. coli* colonies that carried the Rv2873 plus Rv1980c genes, which encode the MPT83 plus MPT64 fusion proteins, in their recombinant *E. coli* colonies (Fig.-2C). The output demonstrated a single 1,329-bp-long DNA band in the final product. As shown in Fig.-4A, columns 1-4, insert DNA from white colonies containing the Rv2873 plus Rv1980c genes, which has a size of 1,329 bps, similar to the *M. tuberculosis* genomic DNA positive control's DNA band (Fig.-4A column +), as shown by PCR findings. However, the blue colony PCR did not detect the Rv2873 plus Rv1980c genes DNA insert (Fig.-4A column -).

The transformed white and solitary colony was re-inoculated with 100 µg/mL amp in a solid Luria-Bertani medium. The QIAprep Spin Miniprep Kit was used to extract vectors from 4 white colonies grown in a liquid Luria-Bertani medium containing 100 µg/mL amp (Qiagen, USA). The recombinant vector pGEM-

T Easy-Mpt83+Mpt64 was successfully isolated as soon as electrophoresis results were inspected and displayed on the 1.5 percent gel (Fig.-4B).

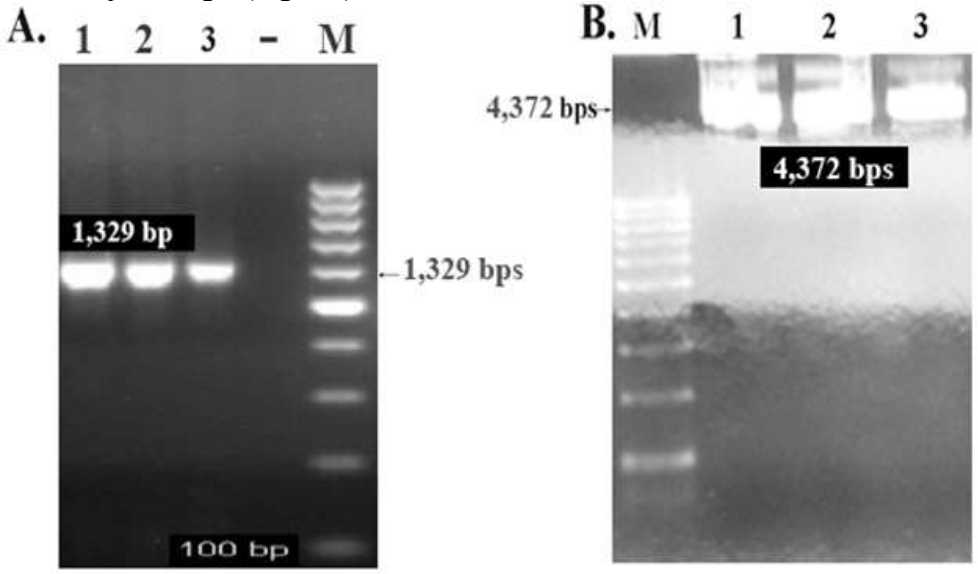

Fig.-4: A: The PCR Results of the Rv2873 Plus Rv1980c Fusion Genes, Which Encodes MPT83 Plus MPT64 Fusion Proteins, as an Insert DNA. Column - Control Was Negative According to the Blue Colony PCR Findings, and Columns 1-3 Were Acquired from the White Colony PCR Product. After Being Isolated, Inoculated onto the PCR Kit Mix, and Put Through the PCR Process, White Colonies Were Then Examined on a 1.5 Percent Agarose Gel Electrophoresis Apparatus. B: The Products of Mini Preparation of Recombinant Fusion Plasmid pGEM-T Easy-Mpt83+Mpt64 with a Length of 4,372 bp Were Isolated from Three Independent Recombinant Colonies on LB Agar Media

Plasmid pGEM-T Easy-Mpt83+Mpt64 Recombinant Sequencing of the Plasmid Was Completed

The fusion plasmid pGEM-T Easy-Mpt83+Mpt64 recombinant was sequenced using the BigDye™ Terminator technique on an ABI PRISM 310 Biosystem sequencing equipment. Bioedit v.7.0.10 was used to analyze the data, and the findings are displayed in Fig.-5. The MPT83 plus MPT64 proteins, with amino acid residues 426 beginning with the methionine residue (M), are encoded by the Rv2873 and Rv1984c genes. The size of the complementary DNA from these genes was 1296 bp, including the initiation ATG codon and end TAG codon (Fig.-5).

Cell Transformation and Cultivation of *E. coli* Strain BL21(DE3) Cells with pTrcHisA-Mpt83+Mpt64 for the Creation of Fusion MPT83 Plus MPT64 Recombinant Protein Products

It was found that the transformation of *E. coli* strain BL21(DE3) cells with pTrcHisA-Mpt83+Mpt64 plasmid resulted in the development of recombinant colonies on Agar Luria-Bertani media (Fig.-6A). Success in subcloning is indicated by white colonies generated, which show that the MPT83 and MPT64 proteins, encoded by Rv2873 and Rv1980c fusion genes, have been successfully inserted into the pTrcHisA vector. An error in the pTrcHisA vector's lacZ gene is to blame for the *E. coli* colonies turning white. For the synthesis of the MPT83 and MPT64 recombinant proteins, *E. coli* white colonies were employed. When the vector-carrying *E. coli* BL21(DE3) strain was present, the color of the liquid Luria-Bertani medium changed from clear to hazy, whereas the control media remained unaffected (Fig.-6C).

The pelleted cell was created following the separation of the supernatant and pellets from the cells in the culture tube (Fig.-6C). Sonication is the best option to remove bacterial cell walls, including membrane cells. Cell membranes are more vulnerable to harm when microbial cell walls are sonicated. An increase in fluid viscosity is seen as a result of *E. coli* BL21(DE3) strain cells being broken down and the release of biomolecules including protein, fat, and glucose from these cells into the clear liquid.

SDS-PAGE Analysis of the Fusion Protein MPT83/MPT64

When recombinant fusion proteins MPT83 and MPT64 are expressed, a blue protein band is generated. Because of the reaction between the loading buffer and the protein sample, this protein band was formed.

Bromophenol blue and glycerol are employed as loading buffers in this experiment. Because glycerol makes a sample heavier, it sinks to the bottom of the sample well rather than floating to the top like water without the substance. When electrophoresing protein samples, the bromophenol blue (3′,3″,5′,5″-tetrabromophenolsulfonphthalein, BPB), was used as a tracking dye to show where the samples have migrated the furthest.

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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     CTG GTC ACG GCT GTC GTT TTG CTC TGT TGT TCG GGT GTG GCC ACG      675
211     Leu Val Thr Ala Val Val Leu Leu Cys Cys Ser Gly Val Ala Thr      225

676     GCC GCG CCC AAG ACC TAC TGC GAG GAG TTG AAA GGC ACC GAT ACC      720
226     Ala Ala Pro Lys Thr Tyr Cys Glu Glu Leu Lys Gly Thr Asp Thr      240

721     GGC CAG GCG TGC CAG ATT CAA ATG TCC GAC CCG GCC TAC AAC ATC      765
241     Gly Gln Ala Cys Gln Ile Gln Met Ser Asp Pro Ala Tyr Asn Ile      255

766     AAC ATC AGC CTG CCC AGT TAC TAC CCC GAC CAG AAG TCG CTG GAA      810
256     Asn Ile Ser Leu Pro Ser Tyr Tyr Pro Asp Gln Lys Ser Leu Glu      270

811     AAT TAC ATC GCC CAG ACG CGC GAC AAG TTC CTC AGC GCG GCC ACA      855
271     Asn Tyr Ile Ala Gln Thr Arg Asp Lys Phe Leu Ser Ala Ala Thr      285

856     TCG TCC ACT CCA CGC GAA GCC CCC TAC GAA TTG AAT ATC ACC TCG      900
286     Ser Ser Thr Pro Arg Glu Ala Pro Tyr Glu Leu Asn Ile Thr Ser      300

901     GCC ACA TAC CAG TCC GCG ATA CCG CCG CGT GGT ACG CAG GCC GTG      945
301     Ala Thr Tyr Gln Ser Ala Ile Pro Pro Arg Gly Thr Gln Ala Val      315

946     GTG CTC AAG GTC TAC CAG AAC GCC GGC GGC ACG CAC CCA ACG ACC      990
316     Val Leu Lys Val Tyr Gln Asn Ala Gly Gly Thr His Pro Thr Thr      330

991     ACG TAC AAG GCC TTC GAT TGG GAC CAG GCC TAT CGC AAG CCA ATC     1035
331     Thr Tyr Lys Ala Phe Asp Trp Asp Gln Ala Tyr Arg Lys Pro Ile      345

1036    ACC TAT GAC ACG CTG TGG CAG GCT GAC ACC GAT CCG CTG CCA GTC     1080
346     Thr Tyr Asp Thr Leu Trp Gln Ala Asp Thr Asp Pro Leu Pro Val      360

1081    GTC TTC CCC ATT GTG CAA GGT GAA CTG AGC AAG CAG ACC GGA CAA     1125
361     Val Phe Pro Ile Val Gln Gly Glu Leu Ser Lys Gln Thr Gly Gln      375

1126    CAG GTA TCG ATA GCG CCG AAT GCC GGC TTG GAC CCG GTG AAT TAT     1170
376     Gln Val Ser Ile Ala Pro Asn Ala Gly Leu Asp Pro Val Asn Tyr      390

1171    CAG AAC TTC GCA GTC ACG AAC GAC GGG GTG ATT TTC TTC TTC AAC     1215
391     Gln Asn Phe Ala Val Thr Asn Asp Gly Val Ile Phe Phe Phe Asn      405

1216    CCG GGG GAG TTG CTG CCC GAA GCA GCC GGC CCA ACC CAG GTA TTG     1260
406     Pro Gly Glu Leu Leu Pro Glu Ala Ala Gly Pro Thr Gln Val Leu      420

1261    GTC CCA CGT TCC GCG ATC GAC TCG ATG CTG GCC TAG                 1296
421     Val Pro Arg Ser Ala Ile Asp Ser Met Leu Ala End                 431
```

Fig.-5: Nucleotide and Amino Acid Sequences from the Fusion Rv2873 plus Rv1980c Genes Encoding MPT83 and MPT64 Fusion Proteins Were Predicted

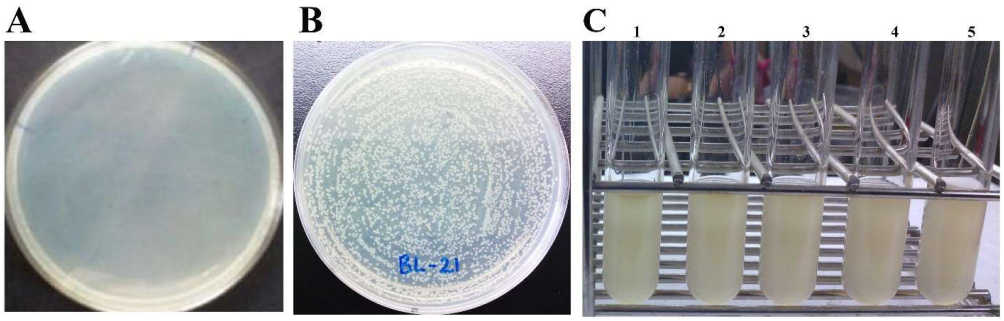

Fig.-6: Luria-Bertani Agar Plate Media and Liquid Culture Media were used to test *E. coli* BL21(DE3) Strain Harboring pTrcHisA-Mpt83+Mpt64 Vector Recombinant Plasmid. IPTG-Independent Growth and Expression of Recombinant Proteins. MPT83 plus MPT64 Recombinant Protein Growth and Expression in the Presence of 40 µM IPTG

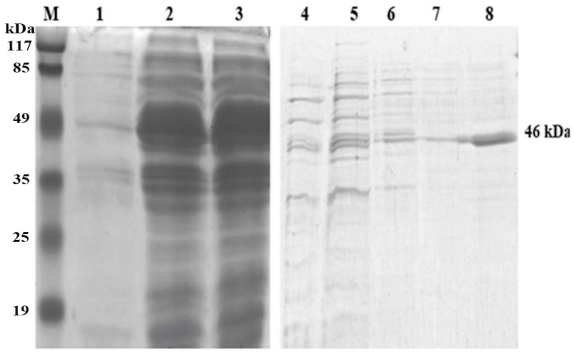

Fig.-7: Purification of the Recombinant MPT83 Plus MPT64 Fusion Protein. 6XHis-Tagged MPT83 Plus MPT64 Were Purified by Affinity Chromatography Using the 6XHis Tag-Agarose Matrix (Thermo Ficher, Scientific). Cell Lysates and Various Chromatographic Fractions Were Analyzed with 12% Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis. Proteins Were Visualized by CBB staining. Column M, Molecular Weight Marker; Column-1, Uninduced Cell Lysates; Column-2, Cell Lysates Induced with 20 µM IPTG; Column-3, cell Lysates Induced with 40 µM IPTG; Column-4, Flow-Through; Column-5, Washed with 0.5 M Tris-Cl Buffer pH 6.3; Column-6, Washed with 0.5 M Tris-Cl Buffer pH pH 5.9; Column-7, 8, Eluate Using 0.5 M Tris-Cl Buffer pH 4.5 and 4.0. The Arrows Indicate the Recombinant MPT83 plus MPT64 Fusion Protein Bands with a Molecular Mass of 46 kDa

We can see in Fig.-7 how thicker the protein band is from the recombinant MPT83 and MPT64 proteins expressed in pellet cells stimulated with 40 µM IPTG for six hours. The 46 kDa protein marker ladder runs across the thickest protein band. The recombinant protein made from MPT83 and MPT64 has a molecular weight of 46 kDa. In terms of molecular mass, MPT83 is 22 kDa and MPT64 is 24 kDa.[8] Protein bands were not seen in pellet cells that had not been treated with IPTG (data not shown). Recombinant protein expression can be considerably improved by the use of inducers (IPTG). Cell growth governed the generation of recombinant proteins when inducers were used in previous studies.[18,19] Because of this, the gene isn't expressed as well and more cells are made per unit of time, but a high concentration of inducer will kill the cells.[20] Another study demonstrated that lower IPTG concentrations were favorable at high temperatures.[21] As an inducer for the development of recombinant proteins and inducers that can boost the expression of target proteins, the IPTG molecule is widely utilized in research. MPT83 and MPT64 are intracellular proteins that are soluble, according to the data from Fig.-7. Despite its limited detection on pellet cells, the protein has a relative dominance in supernatants, allowing us to draw this conclusion. Consequently, a 46 kDa recombinant protein containing 6XHis-tagged MPT83 plus MPT64 in *E. coli* was effectively generated. In the following research series, antigens derived from these recombinant fusion proteins could be employed in developing TB vaccines.

CONCLUSION

DNA bands with an ATG initiation codon and a TAG end codon from a genomic of *M. tuberculosis'* Rv2873 plus Rv1980c genes, which codes for the MPT83 plus MPT64 fusion proteins, were effectively

amplified by PCR and cloning of original DNA. The pGEM-T Easy-Mpt83+Mpt64 vector was created by introducing the Rv1980c gene encoding MPT64 protein into the pGEM-T Easy Mpt83 plasmid. The Mpt83 plus Mpt64 fusion genes were effectively subcloned to the pTrcHisA vector forming pTrcHisA-MPt83+Mpt64 plasmid, capable of expressing the expected 46 kDa fusion protein in *E. coli* BL-21 (DE3) cells.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests existed.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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[RJC-8092/2022]