Cloning and Expression of SARS-CoV-2 Membrane Recombinant Protein in Prokaryotic Expression System

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Abstract

Background: The M protein is one of the structural protein of SARS CoV-2. The M protein is relatively conserved and stable than other structural protein. This made immunodominant epitopes of M protein has the advantage to be learned with the aim to understanding its immunogenicity and its antigenicity. Several studies have shown that M protein successfully expressed in several expression system, *E. coli* was one of them. In this study, M gene of SARS CoV-2 was cloned, sequenced, expressed in *E. coli* BL21 (DE3) system, and purified with denature condition. The membrane recombinant protein can be used for development of a SARS CoV-2 antibody diagnostic system.

Methods: the gene encoding the SARS-CoV-2 M protein in the form of gBlocks was cloned into the cloning vector and then subcloned into the pQE80L prokaryotic expression vector. There were three stages of recombinant plasmids verification which were the colony PCR, restriction, and sequencing. The M gene cloned in pQE80L was expressed by using BL21 and purified under denature condition.

Results: The recombinant plasmid pQE80L was confirmed containing M protein using primer that specifically amplify the multiple cloning sites (MCS) of pQE80L and produce 595 bp amplicon that indicating the presence of recombinant gene. Restriction of recombinant plasmid using BamHI and HindIII produced 306 bp and 4709 bp DNA bands. The sequence of M gene in pEQ80L has been confirmed by sequencing. Further to ensure the M gene could be expressed in prokaryotic system, the recombinant plasmid was transformed into BL21 bacteria. The SARS-CoV-2 membrane protein with a size of 11,83 kDa has been successfully expressed and purified using the Ni-NTA agarose purification technique under denature conditions.

Conclusion: the gene encoding the membrane protein of SARS-CoV-2 has been successfully cloned and expressed in the prokaryotic expression system.

Keywords: SARS-CoV-2, membrane protein, cloning

Abstrak

Latar belakang: *Protein M merupakan salah satu protein struktural SARS-CoV-2. Protein M merupakan protein yang relatif lestari dan tidak mudah untuk bermutasi dibandingkan protein struktural lainnya. Hal ini menjadikan protein M memiliki kelebihan untuk dipelajari epitop imunodominannya, dengan tujuan untuk memfasilitasi pemahaman terkait imunogenisitas dan antigenisitasnya. Beberapa penilitian menunjukkan ekspresi protein M telah dilakukan pada beberapa sistem ekspresi, salah satunya pada sistem ekspresi E. coli. Pada penelitian ini dilakukan pengklonaan gen M SARS CoV-2, sekuensing, ekspresi pada E. coli BL21(DE3), dan purifikasi dengan kondisi denatur. Protein rekombinan membran ini kemudian dapat digunakan dalam pengembangan sistem diagnostik antibodi SARS CoV-2.*

Metode: *gen penyandi protein M SARS-CoV-2 dalam bentuk gBlocks diklona ke dalam vector pengklonaan dan kemudian disubklona ke dalam vector ekspresi prokariot pQE80L. Tiga tahapan verifikasi terhadap plasmid rekombinan, yaitu PCR koloni, restriksi, dan sekuensing dilakukan untuk memastikan bahwa gen target telah berhasil diklona. Plasmid ekspresi pQE80L yang membawa gen M kemudian diekspresikan dan dipurifikasi pada kondisi denatur.*

Hasil: *Plasmid rekombinan pQE80L dikonfirmasi mengandung protein M menggunakan primer yang secara spesifik mengamplifikasi multiple cloning site (MCS) pQE80L dan menghasilkan amplicon berukuran 595 bp yang menunjukkan keberadaan gen rekombinan. Restriksi plasmid rekombinan menggunakan BamHI dan HindIII menghasilkan pita DNA berukuran 306 bp dan 4709 bp. Sekuen gen M di pQE80L dikonfirmasi dengan sekuensing. Untuk memastikan bahwa gen M dapat diekspresikan di sistem prokariot, plasmid rekombinan ditransformasi ke dalam bakteri BL21. Protein membran SARS-CoV-2 dengan ukuran 11,83 kDa telah berhasil diekspresikan dan dipurifikasi menggunakan teknik purifikasi Ni-NTA agarose pada kondisi denature.*

Kesimpulan: *gen penyandi protein membrane SARS-CoV-2 telah berhasil diklona dan diekspresikan pada sistem ekspresi prokariot*

Kata kunci: *SARS-CoV-2, protein membran, pengklonaan*

INTRODUCTION

In December 29th 2019, 4 first cases of pneumonia of unknown etiology reported in China, those 4 cases were related to the Huanan Wholesale Seafood Market (South China).[1] Less than two weeks later, in January 10th 2020, first copy of coronavirus genome which is suspected to the cause of the pneumonia of unknown etiology cases was published in Gene bank with access number MN988668. This virus then referred as *severe acute respiratory syndrome coronavirus 2*, etiological agent of coronavirus 2019 disease (Covid-19) (SARS-CoV-2).[2] SARS-CoV-2 virus genome known to be single stranded RNA sized 29,8–30 kb.[3] Two-thirds genome at 5' position is composed with ORF1ab which encodes polyprotein ORF1ab, meanwhile at the end of 3' four main structural protein encoded in the following order: spike glycoprotein (S), envelope (E), membrane (M), and Nucleocapsid (NP).[4] ORF1ab and NP gene were the main targets of development of rapid and accurate detection of Covid-19 at that time, by *real-time reverse transcriptase polymerase chain reaction* (RT-PCR) detection on nasal and nasopharyngeal swabs specimen.[5] Increasing sensitivity and specificity of test remain as an urgent need to overcome the pandemic. Serological testing complements virus detection indication past infection that can be used for therapeutical purposes.[6] Various studies have been using various SARS-CoV-2 structural protein for their studies development. M protein was one of them. The SARS-CoV-2 M protein is relatively conserved protein compared to other structural protein, this becomes researcher's background to carry out further studies about this protein.[7] Studying immunodominant protein was promising as M protein is relatively conserved and mutates slowly compared to other structural protein.[8] This becomes advantage to use immunodominant epitopes of SARS CoV-2 M protein facilitated our understanding of antigenicity and immunogenicity of M protein from other variants.[9] Some studies shown that expression of M protein already performed in many expression systems. Those systems are *Escherichia coli*, baculovirus, and HEK293 expression systems.[1,8] In this study, SARS-CoV-2 M protein gene was optimized, cloned, sequenced, expressed in *Escherichia coli* BL21(DE3) and purified with denature method. The optimization was done for secondary structure of M gene. The optimization of RNA secondary structure was carried out to determines its stability and effect on protein expression.[10,11] The recombinant membrane protein can be used for the development of a SARS-CoV-2 diagnostic system.

METHODS

Cloning gBlocks MDx Gene Fragment into pBluescript II KS(+)

The MDx gene coding immunodominant epitopes of M gene (unpublished data) with length 306 bp was synthetized in IDT Malaysia via local supplier. The gene was purchased as gBlock DNA fragments. The gBlock fragment then cloned into blunt cloning vector pBluescript II KS(+), and plasmid containing MDx gene was name pKS_MDx.

Subcloned Gene Coding SARS-CoV-2 Matrix Protein into pQE80L

Gene coding SARS-CoV-2 Matrix was subcloned from pKS_MDx to pQE80L (Qiagen) inserted in *Bam*HI and *Hin*dIII fragment. Vector and insert used in the subcloned reaction were prepared by restricted 10 µg plasmid of each pQE80L and pKS_MDx. The restriction was performed by adding 10 µg in a tube containing 1x NEB2 buffer, 1xBSA (NEB) and 40,000 Unit *Bam*HI (NEB) and DNAseRNAse free water (Ambion) to volume 100µl. The mixture was incubated for 4 hours at 37°C. After that, the DNA was desalted using QIAEXII gel extraction kit (Qiagen) following the procedure described by the

manufacturer. Further, DNA was restricted by *Hin*dIII. DNA that has been cut with *Bam*HI was added to a tube containing 1x NEB4 buffer, 1xBSA, and 40,000 Unit *Hin*dIII (NEB) and DNAseRNAse free water (Ambion) to volume 100µl. After incubated overnight at 37°C, DNA was run on 0.8% LMA containing crystal violet (Invitrogen). DNA was purified from LMA by using S.N.A.P UV-free DNA isolation kit (Invitrogen). Ligation was performed by using vector: insert comparison = 1:3. Reaction ligation was incubated at 16°C for overnight. Ligation was transformed into chemically competent *Escherichia coli* Top10 by heat shock method.[12]

Modeling Ribo Nucleic Acid (RNA) Secondary Structure

The secondary structure of messenger RNA is generated from the SARS-CoV-2 M sequence (the sequence is unpublished) using the dynamic programming algorithm described in MaxExpect program.[13] The software can be accessed freely at Mathews Lab Home – University of Rochester (https://rna.urmc.rochester.edu).

Selection of Recombinant Cloned

Recombinant bacteria containing recombinant plasmid coding SARS-CoV-2 membrane gene was screened using PCR colony. For pKS_MDx, selection of recombinant cloned was done with primers M13F (5'-GTAAAACGACGGCCAGTG-3') and M13R (5'-GGAAACAGCTATGACCATG-3') that recognizingspecific sites in pBluescript II KS(+) was used to amplify the DNA inserted in multiple cloning sites. For pQE80L_MDx selection of recombinant cloned was done with primers pQEF (5'-GTATCACGAGGCCCTTTCGTCT-3') and pQER (5'-CATTACTGGATCTATCAACAGGAG-3') that recognizing specific sites in pBluescript II KS(+) was used to amplify the DNA inserted in multiple cloning sites. The PCR reaction was performed using DreamTaq DNA polymerase (Thermoscientific) following the manufacturer's instruction. Bacterial colonies were picked up using sterile toothpick, streaked on replica plates, and put into PCR tubes as PCR template. The colonies producing expected DNA amplicons were grown in 4 ml LB broth containing 100 µg/ml ampicillin, incubated at 37°C overnight.[12] Plasmids were isolated using QIAPrep Spin Miniprep Kit (Qiagen) and characterized using enzyme restriction. Plasmid that shown positive result was sent to IDT Malaysia via local supplier for sequencing by using Sanger method to verify the gene sequence. The shipment of DNA was accompanied by Material transfer agreement (MTA) signed by sender and receiver. MTA stated that the receiver was allowed to use the DNA only for method stated in research protocol.

Protein Expression

The verified plasmid was transformed into bacteria *Escherichia coli* BL21 (DE3) chemically competent using heat shock transformation.[12] Three colonies were picked up and cultured in 4 ml LB broth containing 100 µg/ml ampicillin, incubated overnight at 37°C. Overnight culture was grown in Terrific broth (Gliserol 1%, Trypton 1,2%, Yeast extract 1,2%, KH_2PO_4 0,34%, and K_2HPO_4 1,1%) containing 100 µg/ml ampicillin at 1:20 (v/v) for 2 hours at 37 °C. After incubated for 2 hours, IPTG with final concentration 1 mM added, and 1 ml cultures were collected after 0, 1, 2, 3, and 4 hours after induction. The incubation temperature of induction was 37°C.[12]

Protein Purification

Bacteria were lysed under denature condition. Bacteria were lysed using denature buffer (50mM Sodium Phosphate, 6 M Guanidine HCl, 300 mM NaCl (pH 8.0)), then incubated for 1 hour 20 minutes on rocking incubator speed 60 rpm (BioRad) at room temperature. After incubation, samples were centrifuged at 12000 rpm for 5 minutes at 4°C. Protein was purified using Ni-NTA agarose (Qiagen) and the purification was conducted following the manufacturer's instruction. Wash buffer with pH 8.0 was used with the buffer compositions are 1 M Tris-HCl pH 8.0, 500 mM NaCl, 400 mM KCl, 1 M β-mercaptoethanol, 20 mM Imidazole, 5 M $MgCl_2$, 6 M Glycerol, and 1 0M Urea. Protein was eluted using elution buffer that contains the same components as wash buffer but with Tris-HCl pH 5.9. The purified recombinant protein was analyzed by 15% SDS PAGE.

Ethical Clearance

The studies didn't involving human participants and or animal subjects.

RESULTS

The MDx gene was ordered as gBlocks DNA fragment, in attempt for storage, gBlocks fragment was cloned into pBluescript II SK(+) cloning vector which already linearized using *Eco*RV enzyme. Subcloning MDx gene from cloning vector into expression vector was then carried out to produce M protein that can be expressed in *E. coli* system (Figure 1).

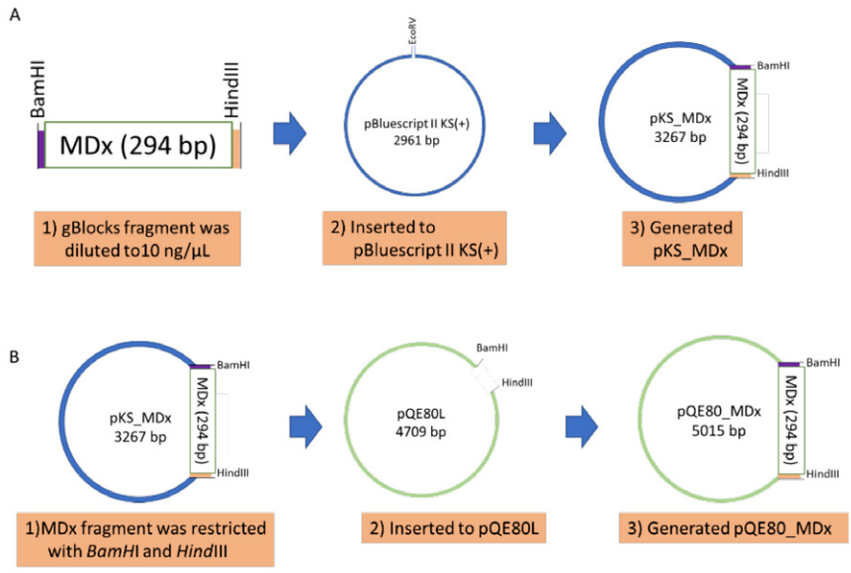

Figure 1. Clone Design of MDx Gene Fragment. (A) To Cloning Vector (B) To Prokaryotic Expression Vector

PCR colony using primer M!3F and M13R was performed on 11 colonies that confirmed by blue-white screening. Electrophoresis gel using agarose 0.8% showed 8 plasmids contain recombinant DNA. Those colonies were isolated and restricted with *Hin*dIII enzyme. Positive plasmids contain recombinant DNA with correct 5' ✍ 3' orientation will produce band sized 3267 bp, while plasmids contain recombinant DNA with 3' ✍ 5' orientation will produce two band sized 310 bp and 2957 bp. The final verification was carried out by sequenced one of the recombinant plasmids which was confirmed has the correct orientation by restriction (Figure 2).

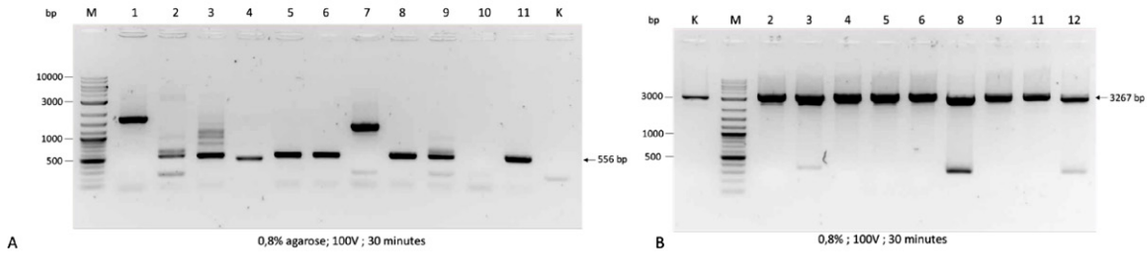

Figure 2. Screening of Recombinant Plasmid. (A) PCR Colony. M: Marker, K: pBluescript II KS(+), 1-11: pKS_MDx 1-11. (B) Restriction Enzyme Analysis pKS_MDx with HindIII, The Arrow Showed Plasmid with Right Orientation

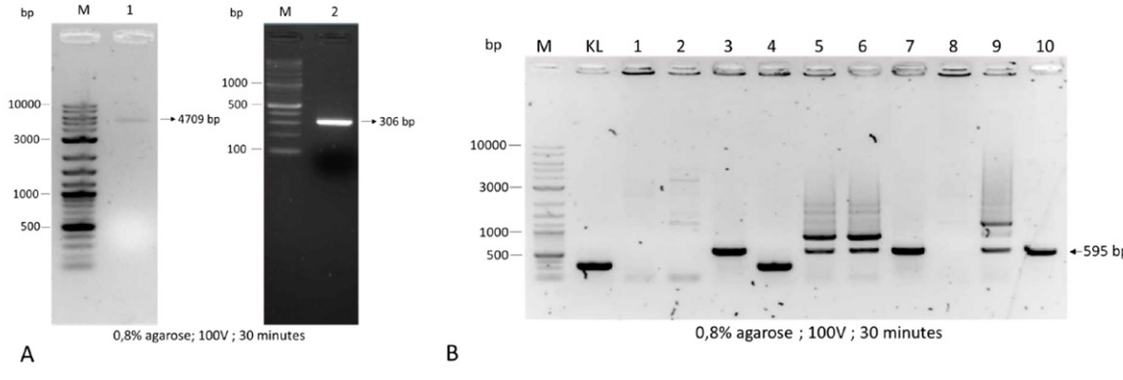

Figure 3. Subcloning of MDx into pQE80L. (A) Low Melting Agarose of DNA Vector and Insert. M : Marker, 1: pQE80L, 2: MDx (B) PCR Colony. M: Marker, KL: pQE80L, 1-10: Recombinant Plasmid pQE80_MDx

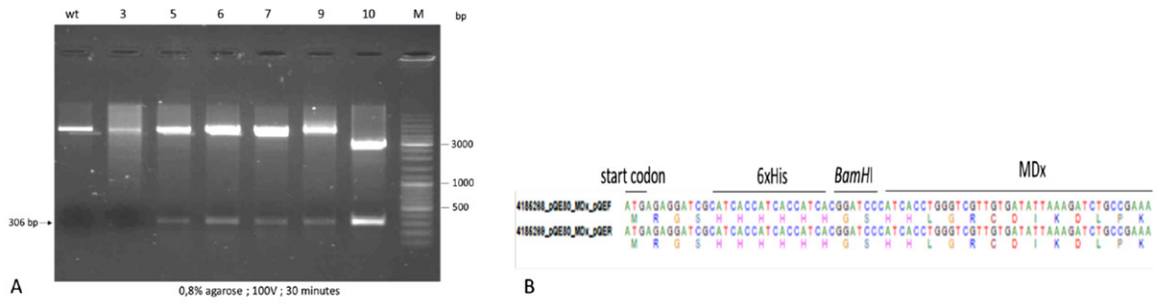

Figure 4. Screening of Recombinant Plasmid. (A) Restriction with BamHI and HindIII. M: Marker, WT: pQE80L, 3,5,6,7,9,10: pQE80_MDx (B) Alignment from Sequencing Result Showed that The Position of MDx Gene was In Frame with 6xHis.

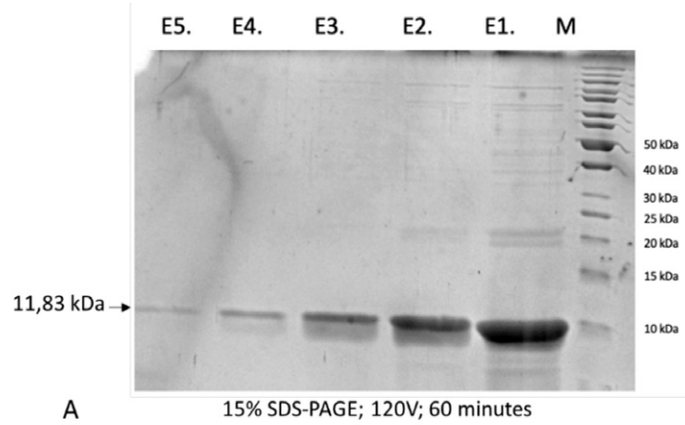

Figure 5. Purification of MDx Recombinant Protein using Ni-Nta, M: Marker, E1-E5: The Elution of MDx Recombinant Protein

The verified recombinant plasmid was subcloned into pQE80L. Linearized vector and MDx gene insert were successfully isolated from LMA, sized 4709 and 306 bp respectively (Figure 3). After ligated, recombinant plasmids were transformed into *E. coli* Top10, the growing colonies were subjected to PCR colony. The colonies produced 595 bp amplicon indicated those colonies possibly contain the DNA of interest. The pQE80L wild type (WT) produced 289 bp DNA that closely migrated to 300 bp marker (Figure 3).

Plasmids were isolated from PCR confirmed colonies. Recombinant plasmids migrated slower than pQE80L WT on 0.8% agarose gel, indicating those plasmids has larger size than pQE80L WT due to the insertion of MDx gene (data was not shown). The recombinant plasmids were further analyzed using restriction enzyme. The recombinant plasmids produced DNA fragments 4709 and 306 bp in length (Figure 4). The alignment of the sequencing result showed the inserted-gene sequences was not mutated and can be expressed in frame with 6xHis Tag (the full sequence of MDx was not shown).

The verified recombinant plasmid was transformed in bacteria that used for recombinant protein production, *E. coli* BL21 (DE3). SDS PAGE result from several trial of protein expression showed overexpressed band sized 11.83 kDa. Protein purified using Ni-NTA purification and showed that MDx protein was successfully purified, even though still no clear single band was visible (Figure 5).

The secondary structures of the mRNA were predicted using web server (https://rna.urmc.rochester.edu/) with maxecpect program (Figure 6). The RNA-binding proteins bind to their target RNA molecules by recognizing specific RNA sequences presented in specific structures.

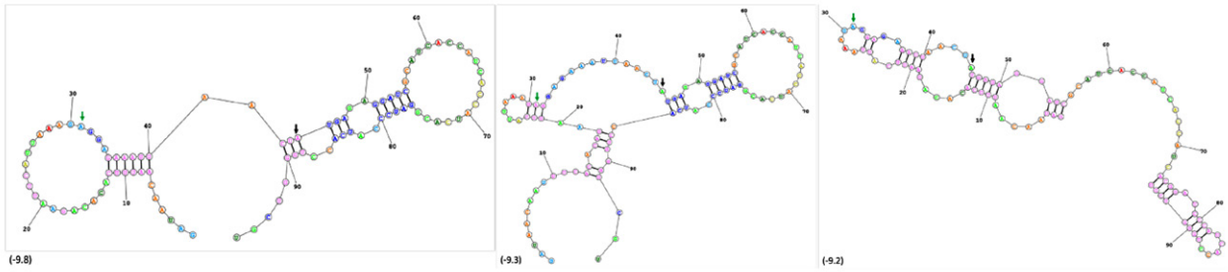

Figure 6. The mRNA Secondary Structure Near the Start Codon AUG Predicted Using Maxexpect Program. Green Arrows Indicate the First Position of the SD (Shine-Dalgarno) Sequence; Black Arrows Indicate the First Position of the Start Codon AUG. The Overall Free Energy of Each Secondary Structure (kcal/mol) is Shown in between Brackets

DISCUSSIONS

One of the fastest way to carry out the recombinant plasmid selection is by perform direct PCR colony from colonies grown on selective agar medium.[12] The primer pairs used in PCR colony could be varies in its attachment sites, either outside the Multiple Cloning Site (MCS), on insert, or on vector.[14] In this studies, we perform PCR colony for both cloning and subcloning using primer pairs outside the MCS, this was done with aim to obtained different size DNA between wild type plasmid and recombinant plasmid. In recombinant plasmid pBluescript II KS(+)_ MDx, M13F and M13R primers used to confirmed the recombinant selection. Plasmid wild type will produce band sized 289 bp and recombinant plasmid will produce band sized 595 bp. This size difference was due the insertion of MDx gene into the MCS of pBluescript II KS(+) plasmid. Further plasmid verification performed by restriction analysis to ensure the correct insert orientation (5' ✍ 3') was cloned, this was done because the MDx gene was inserted at the blunt *Eco*RV restriction site on pBluescript II KS(+). pBluescript II KS(+) plasmid is a cloning plasmid which function as gene preservation. To obtain MDx protein that can be expressed in *E. coli*, the MDx gene need to subcloned in prokaryotic expression vector, in this case pQE80L.

Recombinant membrane protein of SARS-CoV-2 can be expressed in prokaryote system that showed by the overexpression of 11,83 kDa protein band after induction with IPTG, whereas that band could not be found in wild type (data not shown). Based on mRNA modelling, the ribosomal binding site (RBS) was in a single-stranded or unstructured. Modelling was used to predict the translation probability of a mRNA in host cells.[15,16] Protein production in *E. coli* depend on the capability of 16sRNA to bind to a specific segment of mRNA called Shine Dalgarno (SD) or ribosomal binding site (RBS)[17] and the interaction only occurs when the SD/RBS in a single-stranded or unstructured state.[18] mRNA secondary structure prediction encoding recombinant protein shows that of the 3 possible secondary structures, two of them have SD/RBS sequence positioned in internal-loop and stem. The conformational differences of the mRNA caused differences in free energy total produced. The first structure that constructed by the web has total free energy and the biggest possibility than the other two, with the SD sequences in the loop position. The loop structure caused the SD sequence and start codon to be exposed, with the result that gene expression in *E. coli* occurs.[19]

The M protein of SARS-CoV-2 is expressed in *E.coli* cells by IPTG induction. After going through a series of expression optimization stages it is known that protein is excreted in the cell pellet in the form of insoluble protein or inclusion bodies. Inclusion bodies are protein aggregates formed due to protein misfolding.[20] The recombinant SARS-CoV-2 membrane protein could be purified under denature condition, because under native condition the protein retained in pellet. Recovery of active protein from inclusion bodies aggregates remains to be a cumbersome task and requires standardization of solubilization and refolding methods. The major hurdle associated with purification of proteins from inclusion bodies is the sub-optimal refolding of recombinant proteins into native conformation. Poor refolding is often associated to high concentrations of urea or guanidine hydrochloride (GuHCl) used to solubilize the IB proteins.[21]

CONCLUSION

In conclusion, we have succeeded in the molecular cloning of SARS-CoV-2 membrane gene, and have transformed the recombinant plasmid pQE80-MDx into *E. coli* BL21(DE3). The expression and purification procedures in this study have provided a simple method to obtain pure membrane protein of SARS-CoV2. The membrane protein obtained can be used for further study of its structure and function.

ACKNOWLEDGMENT

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Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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