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In-Vitro Construction of Virus-Like Particles by Purified Sars-Cov-2 Structural Proteins

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Abstract

COVID-19 is an illness caused by the coronavirus known as Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). This virus was first discovered in Wuhan, China, in December 2019. It has since become a huge health crisis in the world, as it has resulted in massive suffering and death. In order to fight these killer viruses, one needs to know their construction, life cycle, in which they infect the host cells and reproduce viral particles. An effective and efficient system of studying the virus without involving its infectious genetic material is also needed to develop appropriate therapeutic drugs and vaccines. The perfect system to be used in such studies is Virus-Like Particles (VLPs). VLPs are non-infectious particles that resemble the morphology and assembly characteristics of natural viruses without having viral nucleic acids. This renders them harmless tools to investigate virus assembly, entry and immune response mechanisms, and drug and vaccine development. In this paper, we concentrated on two significant structural proteins of SARS-CoV-2, which are the Membrane (M) protein and the Nucleocapsid (N) protein. The proteins are involved in the assembly of viruses and stabilization of their structure. M and N protein genes were cloned and produced with the help of a bacterial expression system. The purified proteins were extracted using Ni-NTA affinity chromatography, which is the selective binding and purification of the protein of interest. After purification, dialysis of the proteins against the urea buffer was done to re-establish the natural structure of the proteins. Refolded M and N protein was then incubated under set conditions in-vitro to promote Virus-Like Particles assembly. The fact that M and N proteins allowed the formation of SARS-CoV-2 VLPs successfully revealed that they can self-assemble and offers a great opportunity in future studies. They can be used in examining different viral components and functions, screening antiviral agents, and aiding in the establishment of successful treatment and prevention methods against SARS-CoV-2 and other coronaviruses.

Keywords: SARS-CoV-2, Virus-Like Particles (VLPs), M Protein, N Protein, Cloning, Expression, Ni-NTA Affinity Chromatography, Therapeutic Development.

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Introduction

Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) first appeared in Wuhan, China, in December 2019 and quickly spread worldwide. By October 2020, it had reached 189 countries, infecting over 43 million people and causing more than one million deaths. Like the earlier SARS-CoV-1 and MERS-CoV, this virus is highly contagious and dangerous. However, there are still no FDA-approved drugs for its treatment. Hence, it is essential to study this virus in detail to develop effective therapeutics, and this can be supported using virus-like particles (VLPs), lab-made particles formed by the structural proteins of SARS-CoV-2. Coronaviruses are among the largest RNA viruses, having a single-stranded, positive-sense RNA genome of about 26–32 kilobases. They have a helical structure and are around 120 nm in diameter. Under the electron microscope, they appear crown-like, which is why they are named "coronaviruses" (corona = crown). There are about 40 known types, infecting both humans and animals. Among the four genera alpha (α), beta (β), gamma (γ), and delta (δ), only alpha and beta coronaviruses infect humans. SARS-CoV-2, responsible for COVID-19, belongs to the beta coronavirus group. Structure of SARS-CoV-2 The virus has a 29.7 kb RNA genome that codes for four main structural proteins: Spike (S), Membrane (M), Envelope (E), and Nucleocapsid (N), along with 16 non-structural proteins (nsp1–nsp16). Spike (S) protein: Helps the virus attach to host cell receptors and fuse with the cell membrane. Membrane (M) protein: Most abundant; plays a key role in viral assembly. Nucleocapsid (N) protein: Binds to the RNA genome and helps form the nucleocapsid. It also interacts with the M protein during virus formation. Envelope (E) protein: Involved in virus assembly, release, and pathogenesis. Together, these proteins enable the virus to infect host cells and multiply effectively. Life Cycle of SARS-CoV-2 The virus attaches to the ACE2 receptor on human cells through the spike protein. A host enzyme called TMPRSS2 helps the virus enter the cell. Once inside, the viral RNA is released and used to produce two large polyproteins (pp1a and pp1b), which are then cut into non-structural proteins (Nsps), forming the replication complex. Structural proteins (S, E, M) are made in the endoplasmic reticulum (ER) and gathered into vesicles. The N protein binds to the viral RNA to form the nucleocapsid. All these components come together to form new virus particles, which are then transported through the Golgi apparatus and finally released from the cell through exocytosis. Membrane (M) Protein The M protein of SARS-CoV-2 has 223 amino acids, a molecular weight of 24.5 kDa, and spans the viral membrane. Its short N-terminal part faces outside, while the long C-terminal end faces inside the virus. It binds to all other structural proteins: With the N protein, it stabilises the RNA–protein complex. The S protein, it helps in viral entry and assembly. The E protein, it assists in virus budding and release. Because of these interactions, the M protein is crucial for the formation of Virus-Like Particles (VLPs). Virus-Like Particles (VLPs) VLPs are non-infectious, self-assembling structures made from viral proteins but without genetic material. They resemble real viruses in shape and size (100–1000 nm). VLPs can trigger strong immune responses without causing infection, making them ideal for use in vaccines, drug delivery, and gene therapy. Studying the interactions of structural proteins, especially the M protein, helps scientists design effective VLP-based vaccines and therapeutics against SARS-CoV-2.

MATERIALS AND METHODS

Procedure

The nucleotide sequence of the SARS-CoV-2 M gene was used for designing primers for PCR amplification. Restriction enzyme sites were selected using the NEBcutter tool by choosing zero cutters for the insert and single cutters for the pRSET expression vector. The BamHI (GGATCC) restriction site was added to the forward primer, and the EcoRI (GAATTC) restriction site was added to the reverse primer. Suitable adaptor sequences were added to satisfy primer design conditions. The forward primer sequence was 5′-CGCGGATCCATGGCGGATTCTAAT-3′, and the reverse primer sequence was 5′-CCGGAATTCTCATTGCACGAGGAG-3′.

The PcDNA3.1-SARS-M glycerol stock was used for plasmid isolation. From the isolated plasmid, the M gene was amplified using 0.4 µl (100 ng/µl) of template DNA. PCR was carried out in a total reaction volume of 10 µl containing 5.39 µl DEPC water, 1.0 µl Taq buffer, 0.2 µl dNTPs, 0.2 µl forward primer, 0.2 µl reverse primer, 0.2 µl Taq polymerase, and 0.41 µl template DNA. PCR was performed using an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at temperatures of 50°C, 54°C, 58°C, 60°C, and 64°C for 30 seconds, and extension at 72°C for 1 minute. Final extension was carried out at 72°C for 4 minutes, followed by a hold at 4°C. The PCR product was analysed on a 1% agarose gel, and the amplified M gene was extracted using the Thermo Scientific Gel Extraction Kit.

The extracted M gene and pRSET vector were subjected to double digestion using BamHI and EcoRI restriction enzymes. The digestion mixture for both insert and vector consisted of DEPC water, CutSmart buffer, BamHI, EcoRI, and the respective DNA, with a final volume of 15 µl. Digestion was carried out at 37°C for 3 hours. The digested products were resolved on a 0.8% agarose gel, and the desired bands were gel-extracted using the Thermo Scientific Gel Extraction Kit.

The digested M gene insert and pRSET vector were ligated using T4 DNA ligase in a vector-to-insert ratio of 1:7. The ligation reaction contained DEPC water, 5X ligase buffer, ligase enzyme, pRSET vector, and M gene insert in a total volume of 20 µl. The ligation mixture was incubated at 37°C for 3 hours and stored at −20°C until further use.

Chemically competent DH5α cells were prepared using the calcium chloride method. An overnight primary culture of DH5α was grown in LB medium and further inoculated into fresh LB medium until the optical density reached 0.5. The cells were chilled on ice, centrifuged at 8000 rpm at 4°C, and resuspended in 0.1 M $MgCl_2$. After a second centrifugation, the pellet was resuspended in 0.1 M $CaCl_2$ and incubated at 4°C. The cells were finally resuspended in 0.1 M $CaCl_2$ containing 15% glycerol, aliquoted into sterile Eppendorf tubes, and stored at −80°C.

The ligation product was transformed into DH5α competent cells by the heat shock method. Competent cells were thawed on ice, and 10 µl of ligation mixture was added. After incubation on ice, the cells were heat-shocked at 42°C for 90 seconds and immediately transferred back to ice. Fresh LB medium was added, and the cells were incubated at 37°C with shaking for 1–2 hours. The cells were centrifuged,

resuspended, and plated on LB agar plates containing ampicillin. The plates were incubated overnight at 37°C.

Two transformant colonies were randomly selected and screened by restriction digestion. Plasmid DNA from the selected colonies was digested with BamHI and EcoRI using a total reaction volume of 15 µl and incubated at 37°C for 3 hours. The digested products were analysed on a 0.8% agarose gel to confirm the presence of the M gene insert.

Plasmid DNA was isolated from confirmed positive clones using the GeneJET Plasmid Miniprep Kit according to the manufacturer's protocol. The isolated plasmid was quantified, stored at −20°C, and analysed by agarose gel electrophoresis.

BL21 competent cells were prepared using the same calcium chloride method as described for DH5α cells and stored at −80°C. The recombinant pRSET-M plasmid was transformed into BL21 competent cells by the heat shock method. The transformed cells were recovered in LB medium and plated on LB agar plates containing ampicillin. Positive colonies were further patched onto fresh antibiotic plates.

For protein expression, a single BL21 colony containing pRSET-M was grown overnight in LB medium with ampicillin. The culture was sub-cultured and grown until the optical density reached 0.5–0.6 at 600 nm. Protein expression was induced by adding IPTG to a final concentration of 1 mM, and the culture was incubated overnight at 30°C. Cells were harvested by centrifugation and lysed using lysis buffer, followed by sonication. The lysate was centrifuged, and both pellet and supernatant were mixed with loading dye, boiled, and analysed on a 12% SDS-PAGE gel to confirm overexpression of the M protein.

Large-scale expression was performed similarly for protein purification. The harvested cells were lysed by sonication and centrifuged to obtain the clarified lysate. The lysate was incubated with Ni-NTA beads overnight at 4°C for binding of the His-tagged M protein. The beads were loaded onto a chromatography column, washed sequentially with wash buffers, and the bound protein was eluted using increasing concentrations of imidazole ranging from 100 mM to 500 mM. Eluted fractions were analysed by SDS-PAGE.

Western blotting was performed to confirm the identity of the purified M protein. The protein was transferred from the gel to a membrane, stained with Ponceau, blocked with 5% skimmed milk, and incubated with primary antibody followed by secondary antibody. Detection was carried out using a chemiluminescent substrate, and band intensity was analysed using a chemidoc imaging system.

The purified M protein was dialysed for refolding. The protein was initially dialysed against a buffer containing 8 M urea and 20 mM Tris for 24 hours, followed by gradual dilution with Tris buffer. Final dialysis was carried out in buffer containing 150 mM NaCl and 20 mM Tris. The refolded protein was stored at −80°C and analysed by SDS-PAGE.

For in-vitro formation of virus-like particles, the M protein was mixed with the N protein and dialysed in PBS buffer (pH 7.4) for 36 hours. The protein mixture was then stored at −80°C and analysed by SDS-PAGE and Western blotting. Additionally, M and N proteins were mixed in different ratios of 1:1, 1:2, 1:3, 2:1, and 3:1 and incubated at room temperature for 24 hours. The samples were analysed by Western blotting to assess virus-like particle formation.

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Result

Molecular cloning:

PCR amplification of M from pCDNA3.1 SARS-CoV-2 M:

The amplification was done according to the procedure described above, and the PCR product was run on 1% agarose gel. As we can observe in figure (a), there is an around a 669bp band which could be seen on the gel which corresponds to the M of SARS- CoV- 2.

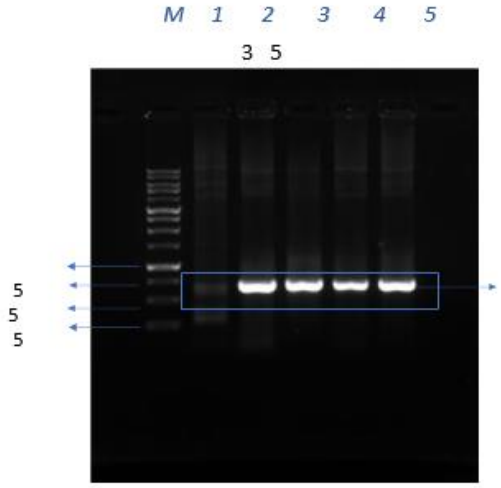

Fig 6: PCR AMPLIFICATION OF M GENE

6.1.2. Double digestion of M amplicon and pRSET vector with BamHI and EcoRI:

The PCR amplified product of M was gel extracted and subjected to double digestion with BamHI and EcoRI. The pRSET vector was also double-digested with the AboveMentioned enzymes. There was an insert in the pRSET vector previously, so that when double digestion is done, if the insert is seen, then the double digestion of the pRSET vector would be done properly. As we can observe in the figure, the digested M and pRSET vector can be seen at around 670bp and 2.9kb, respectively.

M	1	2	3	4	5

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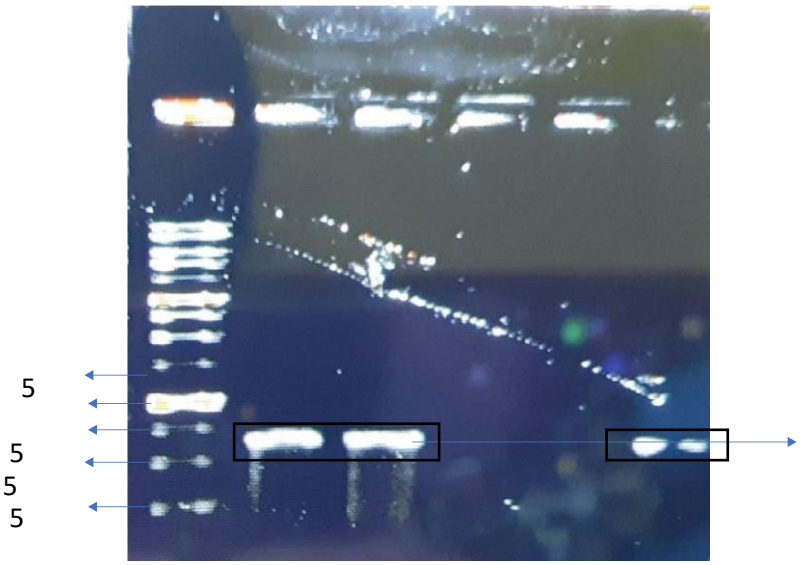

Fig 7: DOUBLE DIGESTION OF M GENE

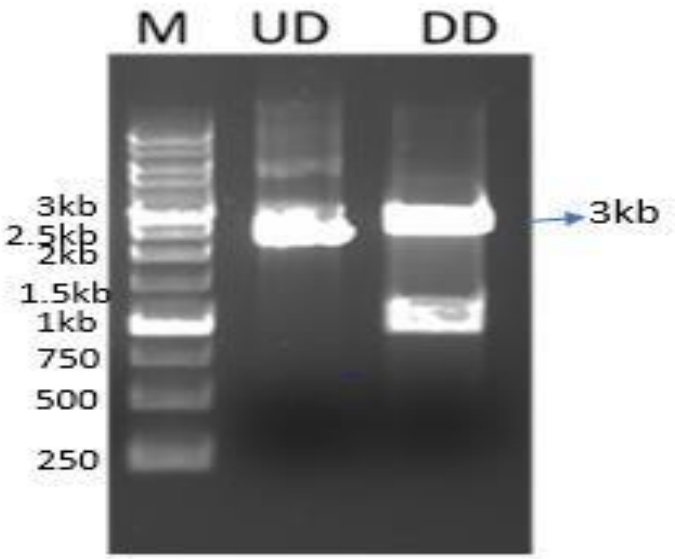

Fig 8: DOUBLE DIGESTION of pRSET

UD–Undigested Vector

DD–Double digested Vector

Ligation of the M insert into the pRSET vector and Transformation:

The digested M and pRSET in the above gels were gel eluted and ligated by using the reaction mixture

mentioned previously. The ligated mixture was transformed into DH5α cells. The colonies obtained were patched onto the ampicillin LB plate.

Plasmid isolation and double digestion of pRSET-M:

The primary culture was kept for the two randomly selected colonies from the abovepatched plate, and the plasmid was isolated and double digestion was done using BamHI and EcoRI enzymes. The agarose gel in the figure shows that there is a 669bp insert coming out from the vector due to the double digestion, which corresponds to the M insert.

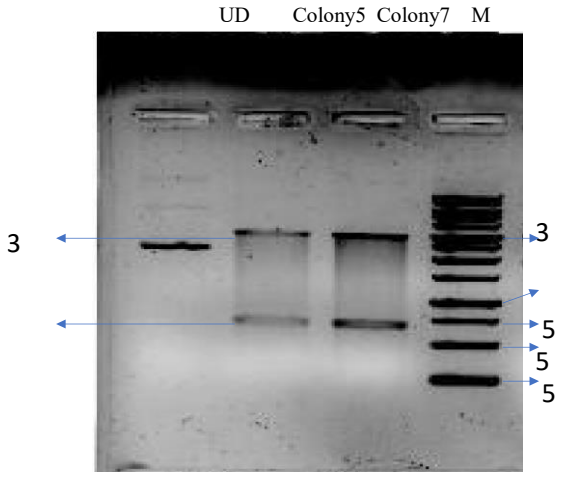

Fig 9: Double digestion of pRSET-M

Expression and Purification of M protein:

Overexpression of pRSET-M in BL21 cells:

The plasmid was then transformed into BL21 cells, and the colonies were obtained on the LB ampicillin plate. The colonies were picked, and induction was given according to the procedure mentioned above, and an SDS gel was run. The SDS gel shown in the figure showed a band at 31kDa, which corresponds to the size of the M protein.

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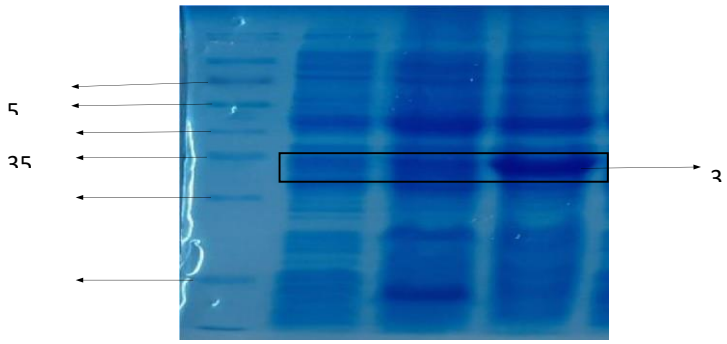

pRSETFig 10: -M Protein Expression

Purification of M protein:

The protein was purified according to the protocol mentioned above in the materials and methods section. The SDS gel of the flowthrough, washes and elutions was run, and this gel was stained and detected. A 31kDa band can be seen in this gel, which shows purified protein corresponding to M.

The washes and Elutions were given with the following Imidazole Concentrations.

W1 and W5 – 10Mm Imidazole
E1 – 100mM Imidazole
E2 – 200mM Imidazole
E3 – 200mM Imidazole
E4 – 300mM Imidazole
E5 – 400mM Imidazole
E6 – 500mM Imidazole

W1-Wash 1
W2-Wash 2
E1 – Elution 1
E2 – Elution 2
E3 – Elution 3
E4 – Elution 4
E5 – Elution 5 E6 – Elution 6

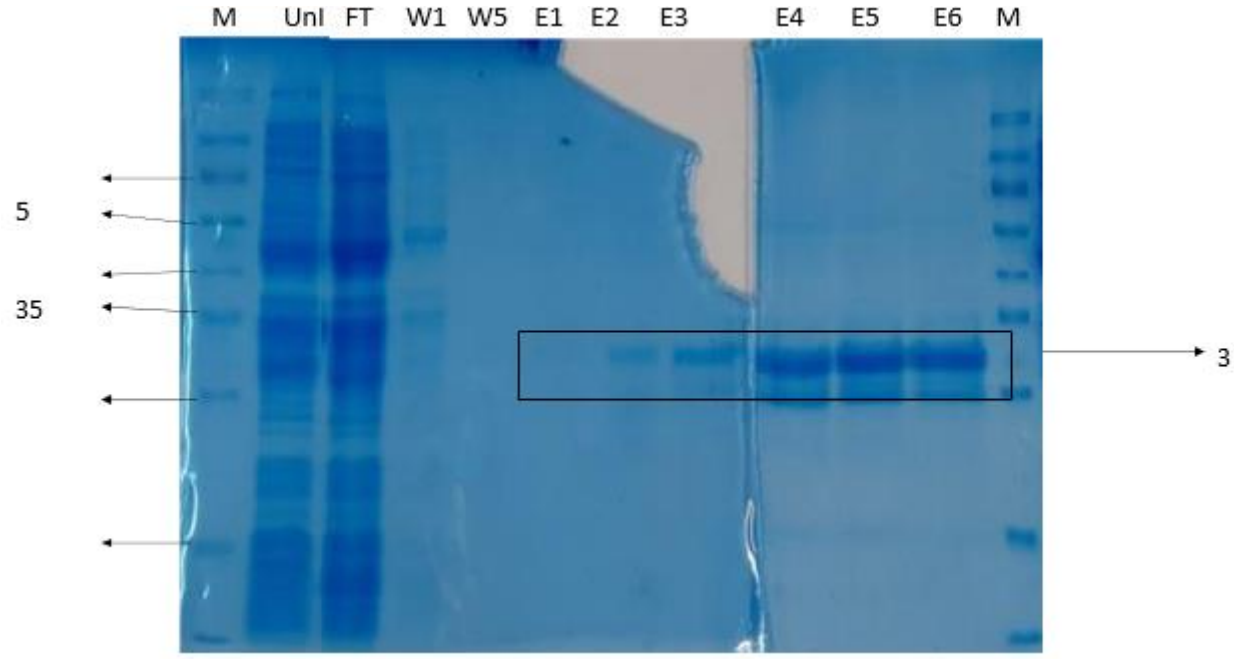

Fig 11: pRSET -M P ro tein purification

Western Blotting:

The purified protein was subjected to western blotting as discussed in the materials and methods. A band at 31KDa confirms the Protein M.

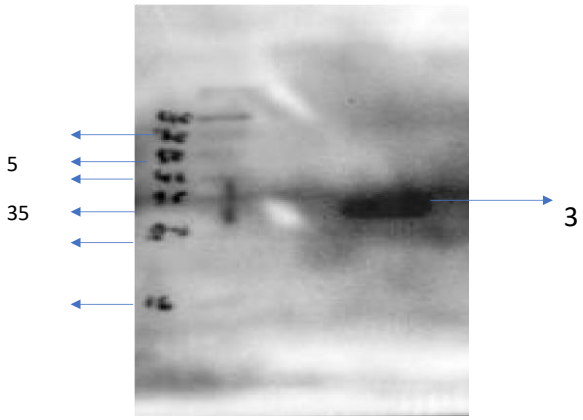

Fig 12: Western Blot of M Protein

In-Vitro formation of Virus-Like Particles:

In-Vitro Formation of VLPs using PBS buffer:

The purified protein is then combined with Nucleocapsid(N) Protein in a 1:2 ratio and dialysed in PBS buffer as mentioned in the Materials and Methods section. The SDS gel of the dialysed M, Incubated M and N fraction and dialysed N was run, and this gel was stained and detained which is represented in the figure. A 31kDa band and a 47KDa band can be seen in this gel, corresponding to M and N, respectively and in the M and N incubated fraction also. But no significant bands of increased molecular weight were observed.

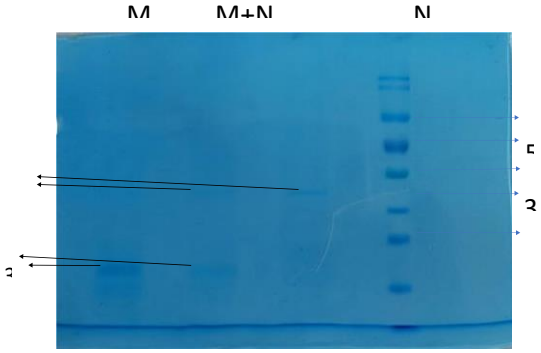

Fig 13: SDS-PAGE of incubated M+N

Western Blotting:

The same samples were then subjected to Western blotting to check for the presence of VLPs. Yet no increased molecular weight bands were observed.

Bl21	M+N	Control	N

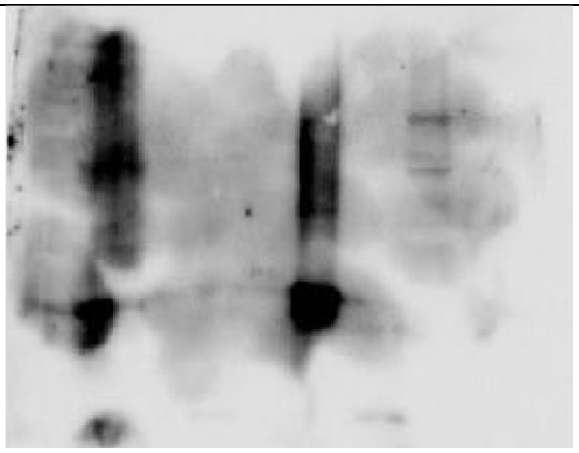

Fig 14: Western blotting of Incubated M+N

The Western blotting was again done by incubating different concentrations of M and N at Room temperature (24°C). A band at around 72KDa may correspond to the VLPs of SARS-CoV-2 structural proteins.

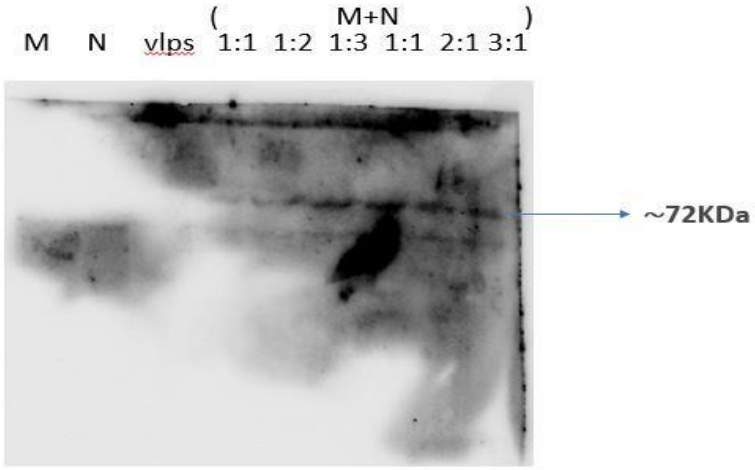

Fig 15: Western blotting of Incubated M+N

Conclusion

Virus-like particles (VLPs) play an important role in studying the structure of viruses, developing vaccines, and creating nanoparticle delivery systems. They can be produced by both in vivo (inside living cells) and in vitro (outside living cells) methods. However, the in vitro method is preferred because it avoids contamination from host cell materials. Different expression systems, such as bacterial, yeast, or mammalian, can be used to produce VLPs. Among these, the bacterial expression system is commonly used because it provides high levels of protein expression, is cost-effective, and has well-established purification methods. Earlier studies showed that the M protein alone cannot form VLPs. The combination of M and N or M and E proteins is required for their basic formation, while all four structural proteins (S, E, M, and N) are needed for efficient VLP production. Hence, in this study, M and N proteins were selected for VLP formation. The genes were cloned into the pRSET bacterial expression vector, which allows high protein expression through the T7 promoter and easy purification due to the 6X His-tag. The proteins were purified using Ni-NTA affinity chromatography. The refolding of M and N proteins was done using urea buffer, followed by dialysis in PBS buffer (pH 7.5) at 4°C. Although some proteins precipitated, no VLPs were observed initially through SDS-PAGE or Western blot. As temperature can influence VLP formation, different M: N ratios (1:1, 1:2, 1:3, 2:1, 3:1) were tested at 24°C. A band around 72 kDa was seen in Western blot analysis, which may represent the combined mass of the M and N proteins (31 kDa + 47 kDa). The increasing band intensity with higher M concentration indicates that the M protein is the key factor driving VLP assembly. To confirm the formation of VLPs, further testing under various pH and temperature conditions is required. Additional characterisation methods such as Dynamic Light Scattering

(DLS), Atomic Force Microscopy (AFM), and Transmission Electron Microscopy (TEM) will help confirm their structure. Once confirmed, these SARS-CoV-2 VLPs can be valuable tools for studying the virus in detail and for developing VLP-based vaccines and therapeutics in the future.

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