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Original Article

Co-Expression of Nucleocapsid (N) and Membrane (M) Proteins of SARS-CoV-2 in Mammalian Cells and Probing with Existing Specific Monoclonal Antibodies.

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Abstract. SARS-CoV-2 is a novel beta-coronavirus with positive-sense, single-stranded ribonucleic acid (ssRNA) encoding 16 non-structural proteins, 9 accessory proteins, and four structural proteins: spike (S), envelope (E), membrane (M), and nucleocapsid (N). Probing immunologically the two structural proteins, N and M, which are essential for the Ribonucleocapsid assembly, using specific monoclonal antibodies (mAb) already developed in house, forms the aim of the study. The results of the study will lead to antibody or assembly-based drug designing and discovery.

Keywords: SARS-CoV-2, Nucleocapsid proteins, Membrane Proteins, Specific Monoclonal Antibodies

1. Introduction

SARS-CoV-2, the virus causing the disease COVID-19, is a beta coronavirus discovered in 2019. The virus is responsible for the recent global pandemic causing deaths and economic losses around the world. Therefore, it is imperative that measures are taken to prevent further spread of the virus (CDC, 2021). The SARS-CoV-2 virus contains the Nucleocapsid (N) protein, and the Membrane (M) protein. The N protein is mainly responsible for identifying and wrapping the virus RNA into a helical symmetrical structure (Wu et al., 2023), while the M protein plays an essential role in viral assembly by organizing other structural proteins through physical interactions and directing them to sites of viral budding. (Dolan et al., 2022) The combination of M and N along with the viral RNA are key to making the ribonucleocapsid, which is one of the steps that can be targeted in combating COVID. The association of SARS -CoV-2 N and M proteins with each other is key to virus assembly and budding (Zhang et al., 2022). Currently, there are antivirals targeting the spike protein or virus entry, but often with little success as the virus is highly mutated at the spike level. The N and M proteins are highly conserved and thus, can be considered, but currently underexplored, as efficient alternative targets. It is essential to have an expression system to screen for antivirals targeting these two proteins or their assemblies to be probed with specific monoclonal antibodies (mAbs). The results of the study will lead to assembly or antibody-based drug designing and discovery. Hence, this study is aiming to establish a mammalian expression system for N by itself and M+N, to screen antiviral inhibitors in future studies especially assembly inhibitors. It is hypothesised that both N and M+N could be expressed in mammalian cells for further structural studies in developing therapeutics to target the highly conserved part of SARS-CoV-2, given that either N associates itself to form a self-assembly or M+N together form a co-assembly. Expression and characterization of the proteins and assemblies would form the basis for a cell-based assay to screen antiviral inhibitors.

The aim of this study is to probe the two structural proteins N and M immunologically through cell transfection, followed by SDS-PAGE, Western Blot, and Immunofluorescence Assay.

2. Experimental

To obtain the cells transfected with N, M and N+M, first, bacterial transformation was carried out to obtain the plasmid constructs of N and M, followed by confirmation using PCR, as shown in Figure 1 below. Next, the mammalian cells were transfected with Lipofectamine, before processing the results.

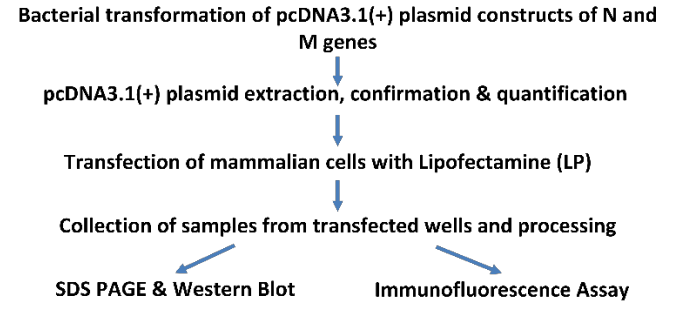

Figure 1. Flowchart of experimental process.

A. Bacterial Transformation

Plasmid pcDNA3.1 with M and N gene inserts were obtained from Addgene, MA. To generate many copies of the plasmids, 80ng of the original plasmid pcDNA3.1 constructs were added into heat competent XL-1 Blue *Escherichia coli (E. coli)* cells and set on ice for 30 minutes, heat shocked at 43ºC in a water bath for 1 min and placed on ice for 2 minutes. A volume of 900µl of Luria Bertani (LB) medium was added and incubated at 37ºC on an orbital shaker for an hour. The cells were then spun at 5000g in a centrifuge for 8 mins. The supernatant was discarded, and 1ml of LB medium was added for resuspension. 100µl of the resuspension was inoculated onto LB agar plate with Ampicillin. The plate was incubated at 37ºC overnight. Next day evening, 3 colonies each from N and M were picked and each added to 5ml of LB broth with 100µg/ml of Ampicillin. The tubes were incubated at 37ºC overnight in an orbital shaker. The next day, cells were spun down at 3100*g* for 8 minutes, supernatant discarded, and the bacterial cell pellet was subjected to plasmid extraction.

B. Plasmid Extraction and Confirmation by PCR

The protocol from FavorPrep Plasmid Extraction Mini Kit (Cat. No.: FAPDE 004, FAPDE 100, FAPDE 300; FavorGen, Taiwan) was followed to extract the two plasmid DNAs each respectively carrying pcDNA3.1 + N and pcDNA3.1 + M inserts. (FavorGen, n.d.) Concentration of plasmid DNA was measured in the UV-Vis Spectrophotometer (Serial No. 5376) nanodrop to calculate the amount of plasmid DNA required for the experiment. Each respective samples of N had concentrations of 564.4ng/µl, 583.8ng/µl, 380.8ng/µl, while that of M had 656.2ng/µl, 198.3ng/µl, 440.6ng/µl. The plasmid DNA were checked for quality by Polymerase Chain Reaction (PCR) to confirm the presence of M and N inserts in the plasmids respectively using standard protocols using CMV forward and pcDNA3.1+ reverse primers. The amplified DNA products were checked by agarose gel electrophoresis for the presence of ~ 1400 base pairs for N and ~ 800 base pairs for M (Fig. 2).

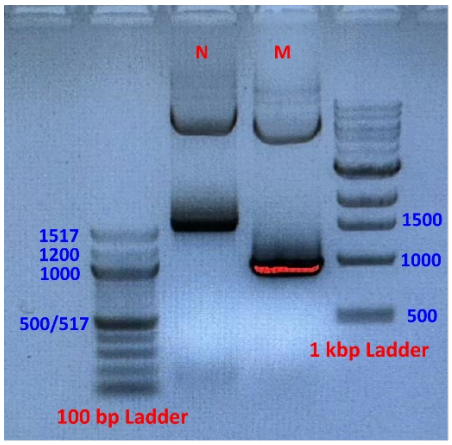

Figure 2. Agarose gel on PCR amplified product to confirm plasmid DNA of M and N after extraction from transformed *E. coli* (XL-1 Blue).

C. Cell Passaging

One vial from the frozen stock of HEK-293 Freestyle cells (Catalog Number R790-07) was thawed in 37ºC sterile water bath. OPTI-MEM (Life Technologies, USA) with 2g Sodium

bicarbonate (Sigma, USA) anti-anti (Gibco, USA) and 10% Foetal Bovine Serum (FBS) (Life Technologies, USA) which form the growth medium, was added to wash cells. The cells were then resuspended in 6ml of OPTI-MEM, transferred to a T25 flask, and incubated at 37ºC, 5% CO_2. To passage the cells, reagents were thawed in 37ºC water bath. The confluence of the cells was ~85%, and a split ratio 1:10 were generally. The medium was aspirated and 3ml of 1x Phosphate Buffer Saline (PBS) was added to remove the dead cells. The PBS was aspirated and 1ml of 1x Trypsin was added and the flask was incubated for exactly 2 mins at 37ºC. 4ml of growth medium was added to inactivate trypsin. The cell suspension was centrifuged at 1,000 rpm for 5 mins at 23ºC. The supernatant was discarded, and the cell pellet was suspended into 3ml of fresh growth medium. After resuspension, 0.3ml was added into a new T25 flask containing 6ml fresh medium to be incubated at 37ºC, 5% CO_2. Cells were observed for 75-80% confluence for further passaging or seeded into 6 well plates to conduct transfection.

D. Protein expression by Mammalian Cell Transfection

HEK-293F cells were counted in an automated cell counter (Bio-Rad TC20 automated cell counter) following the protocol. The total count was $2.70x10^6$ cells/ml, live count $2.54x10^6$ cells/ml. The suspension was diluted appropriately to get 1 million cells per well and added to the 6 well plate and incubated overnight at 37ºC, 5% CO_2. Transfection experiment was performed when cells reached a confluence of 75%. The plasmid DNA with the SFM was prepared first. For each N well, 4µg of pcDNA3.1 + N (5µl as the concentration of plasmid DNA provided were both N and M were 800ng/µl) and 150µl of Serum Free OPTI-MEM Medium (SFM) were required. For each N+M well, 2µg of pcDNA 3.1 + N and 2µg of pcDNA 3.1 + M (2.5µl + 2.5µl) and 150µl of SFM were required. For the mock well, 310µl of SFM and for the EGFP well, 4µg of pcDNA 3.1 + EGFP (5µl) and 150µl of SFM were needed. These DNA + SFM were prepared and left for 5 min at room temperature (RT). Now the Lipofectamine 2000V (LP) is prepared. A total volume of 918µl of SFM and 52µl of LP were mixed and 158µl of this mixture was dispensed to each of the 6 sterile tubes. The DNA prepared were added to the LP + SFM mixture and left for 30 min at RT. The growth medium from the wells of the 6 well plate was aspirated, and the cell surface was gently overlaid with the LP + DNA mixture by rotating the plate gently in a circular fashion. 1ml of growth medium was added to each well and left to the end of the day and later topped up with 2ml of growth medium to be incubated at 37ºC, 5% CO_2 for 96 hours until they were ready to assay for transgene expression, which can be monitored by visualising the green fluorescence in the EGFP well. The expression of both the N protein alone or the M+N together in the cell culture medium and the cell lysate were checked in a 12% SDS PAGE gel for the size followed by a Western Blot and Indirect Immunofluorescent Fluorescent Assay (IFA).

3. Results

A. SDS PAGE and Western Blot of culture supernatant and cell lysate

Once EGFP green fluorescence is almost 100% expressed which needed 96 hours, the medium from the test wells was collected in separate labelled tubes. Thereafter, the manufacturer's protocol for cell lysis buffer II (ThermoFisher, USA) was followed to lyse the cells in respective wells, modified to use the protease inhibitor cocktail (ThermoFisher, USA). A pair of 12% SDS-PAGE gels were casted using standard protocol and saved at 4ºC until

used. All samples from the mock and experiment wells were boiled at 100ºC for 8 mins and added into respective SDS-PAGE gel lanes. Electrophoresis was performed at a constant 90V for 1h 30 mins. One gel was stained with Coomassie Brilliant Blue (CBB) while the other gel underwent a semi-dry transfer (Bio-Rad Trans-Blot Turbo) following the equipment protocol. Following a brief Ponceau S stain and wash to confirm transfer, the membrane was blocked with a blocking buffer containing 0.75g of Bio-Rad Blotting-Grade Blocker dissolved in 25ml of PBS with 0.05% of Tween 20 (PBST) for 1 hour at RT. The membrane was then washed 3 times, 2 min each, with PBST and incubated with 1:200 diluted anti-N specific mAb from the lab for 1hr at RT. After repeating the wash with PBST, the membrane was incubated with 1:3000 dilution of secondary anti-mouse antibody with horseradish peroxide (HRP) conjugate. The blot was developed with chemiluminescent reagents (RPN2109 - Amersham ECL Western Blotting Detection Reagent) following the manufacturer's protocol and capturing the signal in a ChemiDoc Touch Imaging Biorad system.

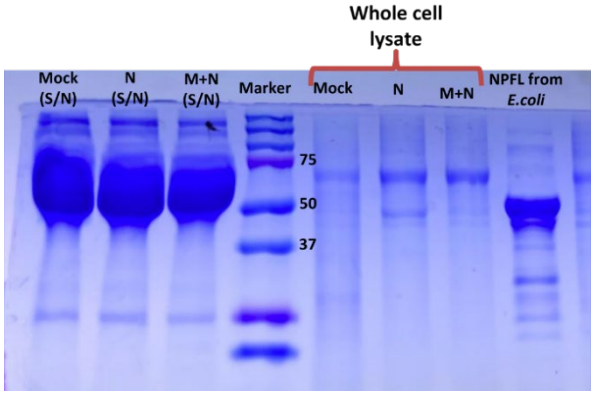

Figure 3. Analysis of Protein expression by Coomassie Brilliant Blue stained 12% SDS PAGE gel.

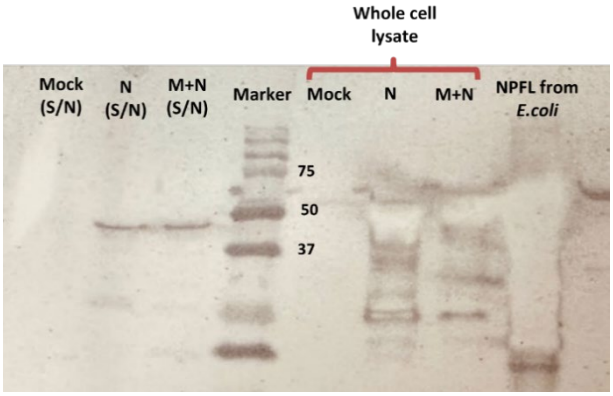

Figure 4. Analysis of immune specificity of the N-Proteins expression by Western Blot with Anti-N specific mABs.

In Figure 3, the mock, N and M+N supernatant (S/N) lanes are smudged due to the presence of copious quantities of Bovine Serum Albumin (BSA) from the growth medium that contains 10% FBS. For N and M+N whole cell lysate lanes, a faint band of N protein can be seen at around 45kDa, while a thick band can be seen on NPFL lane (Positive control) at 50kDa, which is N protein with fusion tags expressed from bacterial system as a reference.

In Figure 4, the western blot of N and M+N S/N lanes showed bands at around 45kDa. For N and M+N whole cell lysates, bands were also seen at around 45kDa, while that of Nucleocapsid from prokaryotic expression is 50kDa since the protein has fusion tags for purification. However, signal from N protein was in excess due to over expression resulting in white patches.

B. Immunofluorescence Assay (IFA)

An IFA was done by fixing the cells in the duplicate plate with 1ml 4% Paraformaldehyde in PBS and incubated at RT for 20 mins. The cells were washed 3 times with 1x PBS for 5 mins each. 1ml of 0.1% Triton-X 100 in PBS was added and incubated at RT for 30 mins to permeabilise the fixed cells. The wash procedure of standard protocols was

followed. 1ml of 5% milk in PBST (PBST-M) was added into each well and incubated for 1 hour on a platform rocker at RT. The wash procedure was repeated, and 1ml of the primary antibody, i.e., Anti-NTD 6C8 with Anti-CTD 3C11 in PBST-M was added into each respective well and incubated for 1 hour on a platform rocker at RT. The primary antibody was removed, and the wash procedure was repeated. An ml of 1:100 diluted of secondary antibody i.e., Polyclonal Rabbit Anti-Mouse Immunoglobulin Fluorescein Isothiocyanate (FITC) conjugate (LOT 41405562) in PBST-M was added and incubated for 1 hour on a platform rocker at RT. After washing, 10µg/µl of Propidium Iodide (PI) in PBS was added and incubated for 5 min on the rocker at RT and washed. The wells were filled with PBS and stored in 4ºC until viewed in fluorescent microscope.

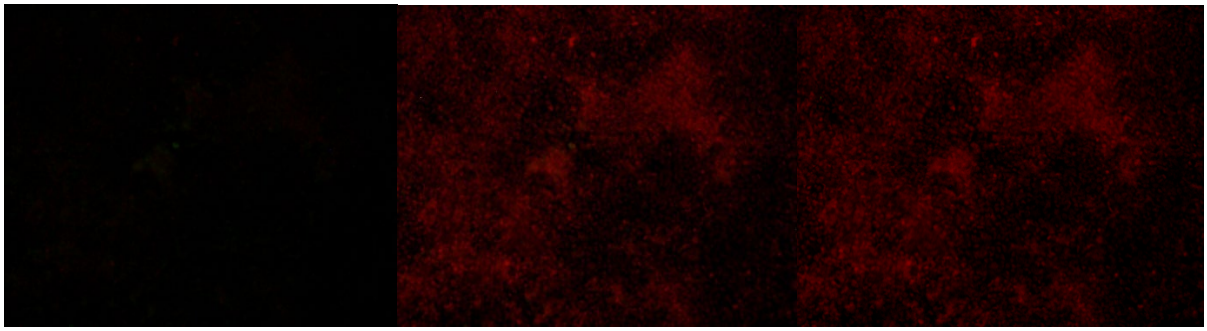

Figure 5. Mock transfected (Left to right: Anti Mouse FITC, PI, Merge).

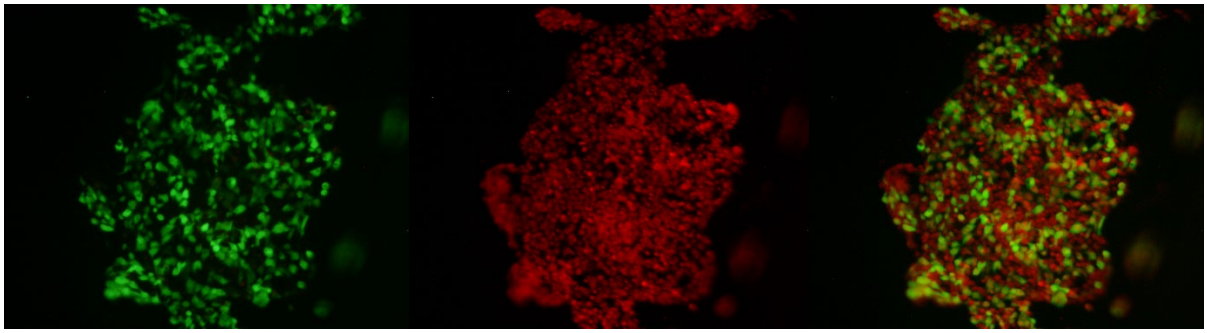

Figure 6. Expression of Nucleocapsid protein (Left to right: Anti Mouse FITC, PI, Merge).

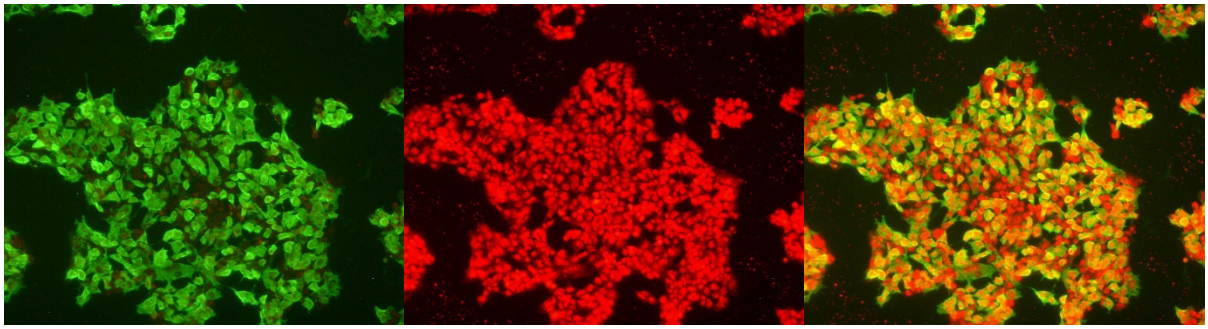

Figure 7. Expression of Nucleocapsid and Membrane protein (Left to right: Anti Mouse FITC, PI, Merge).

IFA of HEK-293F cells were stained with anti-N specific mAbs (primary), anti-mouse FITC (secondary) and PI (nuclear stain). Cells are rounded and no longer spindle shaped. Green fluorescence (Mock transfected control) was not observed in Figure 5, but only in Figure 6 and

Figure 7, where the N was expressed close to the membrane of the cell confirming the expression of N. Red fluorescent (PI) stained nuclei indicate the number of cells present in all three systems. Merged FITC and PI confirm that the fluorescent signal is from cells and not an artefact. The presence of Immunofluorescence confirms the N-protein expression by the mammalian cells. Probing the M protein expression in the saved samples await the sourcing of good quality commercial anti-SARS-CoV-2-M protein antibody.

4. Discussion

The N protein of SARS-CoV-2 as an antigen has distinct advantages over other potential SARS CoV-2 antigens like conservation of the amino acid sequence, expanding knowledge of its genetics and biochemistry, and its strong immunogenicity. Thus, the N protein of SARS-CoV-2 should be strongly considered as a vaccine candidate against COVID-19 (Dutta et al., 2020). The mammalian cell expression of SARS-CoV-2 Nucleocapsid (N) protein is confirmed through IFA and Western blot both employing specific anti N antibody. However, the expression of M protein still requires confirmation with specific anti M antibody. Following this experiment, the self-assembly or co-assembly requires extensive purification through ultracentrifugation of the N and M proteins followed by Dynamic Light Scattering (DLS) and Electron-microscopy (EM) for the actual size and assembly details. With that, we can see how the proteins form structural complex, especially whether N can form a self-assembly or N+M can form a co-assembly (complex). If N+M+RNA together can form an assembly *in vitro*, proven by Ultramicroscopy, we can search for assembly inhibitors targeting any of these assemblies.

In conclusion, this study has formed the test system for downstream research, as future studies to screen antiviral inhibitors no longer need to be done in a Bio Safety Level 3 lab.

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References

Centers for Disease Control and Prevention. (2022, December 19). Coronavirus disease 2019 (covid-19). Centers for Disease Control and Prevention. Retrieved December 26, 2022, from https://www.cdc.gov/dotw/covid-9/index.html#:~:text=COVID%2D19%20is%20a%20respiratory,infected%20may%20not%20have%20symptoms

Cell lysis buffer II. Thermo Fisher Scientific - US. (n.d.). Retrieved December 26, 2022, from https://www.thermofisher.com/order/catalog/product/FNN0021

Dolan, K. A., Dutta, M., Kern, D. M., Kotecha, A., Voth, G. A., & Brohawn, S. G. (2022, October 20). Structure of SARS-COV-2 m protein in lipid nanodiscs. eLife. Retrieved April 26, 2023, from https://elifesciences.org/articles/81702#:~:text=SARS%2DCoV%2D2%20encodes%20four,to%20sites%20of%20viral%20budding.

Dutta, N. K., Mazumdar, K., & Gordy, J. T. (2020, June 16). The nucleocapsid protein of SARS-COV-2: A target for vaccine development. Journal of virology. Retrieved April 26, 2023, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7307180/

FavorPrep plasmid extraction mini kit TM FAPDE 050 FAPDE 200. (n.d.). Retrieved December 26, 2022, from http://www.favorgen.com/favorgen/serv_1/mem_t1/h_1/pdf/plasmid/FAPDE%20004%20050%20200.pdf

Wu, W., Cheng, Y., Zhou, H., Sun, C., & Zhang, S. (2023, January 10). The SARS-COV-2 nucleocapsid protein: Its role in the viral life cycle, structure, and functions, and use as a potential target in the development of vaccines and Diagnostics - Virology Journal. BioMed Central. Retrieved April 26, 2023, from https://virologyj.biomedcentral.com/articles/10.1186/s12985-023-01968-6#:~:text=Biological%20function%20of%20SARS%2DCoV%2D2%20N%20protein&text=It%20is%20mainly%20responsible%20for,form%20ribonucleoprotein%20complex%20(RNP).

Zhang, Z. (2022, August 5). Structure of SARS-CoV-2 membrane protein essential for virus assembly. Nature. https://www.nature.com/articles/s41467-022-32019-3?error=cookies_not_supported&code=7d7d894b-6cf6-4bc7-9ac5-c74a9493956a