infections. Researchers have since applied *in silico* and clinical research techniques, particularly on structural proteins: spike (S), envelope (E), membrane (M) and nucleocapsid (N) (Figure 1.1) and also non-structural/accessory proteins in the human coronavirus genomes to conclude that human coronaviruses are phylogenetically similar (Graham, Donaldson, & Baric, 2013; Shi et al., 2015).

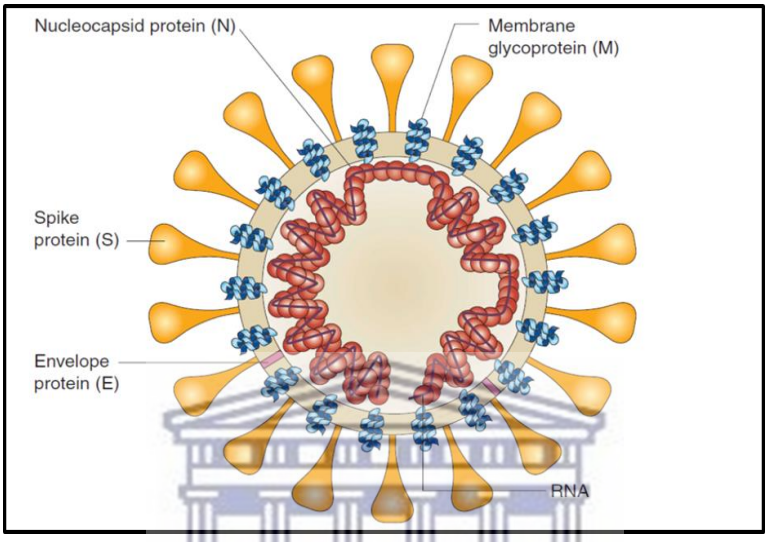

Figure 1.1: Structural proteins of HCoV-NL63

Schematic diagram of the coronavirus structure - Membrane (M), envelope (E), spike (S) and nucleocapsid (N). The viral surface proteins (spike, envelope, and membrane) are embedded in a lipid bilayer envelope derived from the host cell. The single-stranded positive-sense viral RNA is associated with the nucleocapsid protein. *Source*: (Peiris, Guan, & Yuen, 2004)

HCoV Genome Mutations

The coronavirus genome displays high frequencies of mutation with approximately 1 mutation occurring with every genome replication cycle (Moya, Holmes, & González-Candelas, 2004). Categorically RNA viruses display a high rate of recombination, which subsequently contributes to their overall genetic diversity (Pérez-Losada et al., 2015). These factors are what permit RNA-viruses to adapt relatively quickly to changes in their environment and ensuring virus propagation. Recombination is thought to be controlled by a "copy choice mechanism" whereby the RNA polymerase enzyme switches to a different copy template during RNA synthesis: -1 frameshift slippage

CHAPTER 2

LITERATURE REVIEW

HCoV-NL63

Viral Genome and Replication

Viral Genome

HCoV-NL63 genome is 27553 base pairs in size (GenBank: AY567487.2) which is capped at 5'-end and polyadenylated at 3'-end (Abdul-Rasool & Fielding, 2010). The genome order of HCoV-NL63 is graphically shown in Figure 2.1 (Pyrc, Berkhout, & Van Der Hoek, 2007). Replicase genes account for the first two thirds of HCoV-NL63 genome which codes the polyprotein replicase translated from 1a and 1b (Woo et al., 2010).

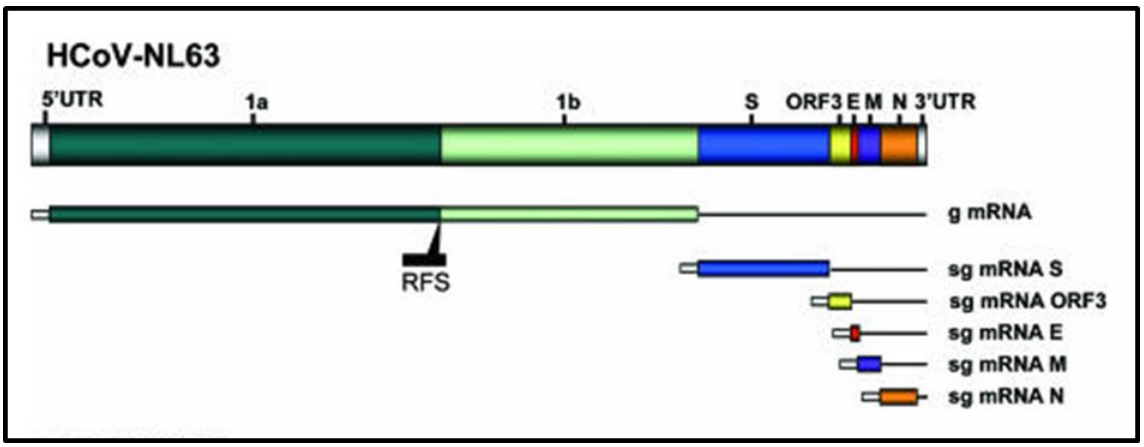

Figure 2.1: Schematic organization of the HCoV-NL63 genome

Corresponding subgenomic (sg) mRNAs generated during the discontinuous transcription process are shown (Pyrc, Berkhout, & Van Der Hoek, 2007).

White and blue colony growth were present on serial dilution spread plates 10^{-1} and 10^{-2}, no growth occurred on 10^{-3}. White colony growth is the qualitative confirmation of successful HCoV-NL63 ORF3 transposition into the Bacmid.

DH10Bac *E. coli* Midiprep

A total of 4 well isolated white colonies were picked and cultures underwent a miniprep which yielded DNA concentrations of 0.45 µg/µL and 0.49 µg/µL, respectively. Based on nanodrop sample purity readings, the Bacmid-ORF3 of 0.45 µg/µL sample was deemed more acceptable for downstream experiments

PCR Confirming Recombinant Bacmid-ORF3 DNA

DNA isolated from white colonies (0.45 µg/µL midiprep sample) generated an amplicon size of 2.3 kB + the size of the insert of 741 bp, thus equalling a band of ≈3 kB as a positive result for successful transposition of Bacmid-ORF3 (Figure 4.8). Lane 1 shows amplification using M13 forward and reverse primers. The product size is 3041 bp, which was the calculated expected size of the amplicon. A combination of the primers was used to further confirm correct orientation and successful transposition of the pFastBac-ORF3 gene. This is demonstrated in Lanes 2 and 3; In Lane 2, M13 forward primer was used in combination with the ORF3-specific reverse primer. In Lane 3, M13 reverse primer was used in combination with ORF3-specific forward primer. Furthermore, the results show successful generation of recombinant Bacmid DNA that contains HCoV-NL 63 ORF genes. The manual states a band of 300 bp is indicative of no gene transposed into the Bacmid.

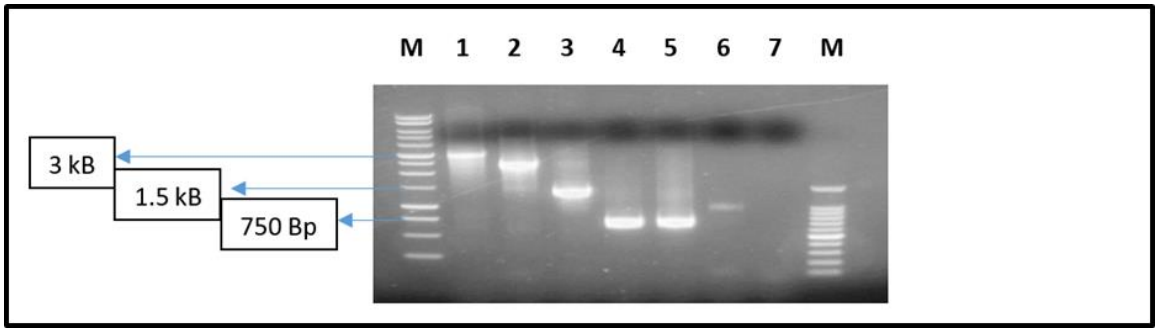

Figure 4.8: RT-PCR confirming recombinant Bacmid-ORF3 in midiprep sample

Agarose gel figure labelled from left to right. **Lane M:** 1 kB DNA Marker; **Lane 1:** shows amplification using M13 forward and reverse primers with a 3041 bp product; **Lane 2:** shows amplification using M13 forward primer with the ORF3-specific reverse primer with a 2390 bp product; **Lane 3:** shows amplification using ORF3-specific forward primer and M13 reverse primer with a 1320 bp product; **Lane 4:** shows amplification ORF3-specific forward and reverse primers with a 741 bp product; **Lane 5:** Positive (+) control. The positive (+) control used was pFastBac-ORF3 midiprep amplified with ORF3-specific forward and reverse primers with a 741 bp product; **Lane 6:** Positive (+) transposition control. The transposition control used was Bacmid-GUS midiprep amplified using M13 forward and reverse primers with a 4200 bp product; **Lane 7:** Negative (-) control containing all PCR reagents except reference template (Bacmid-ORF3); **Lane M:** 100 bp marker.

Producing Recombinant HCoV-NL63 ORF3 Baculovirus

Transfection Generating ORF3-Baculovirus

Production and Collection of P0 Transfection Viral Stock

Transfection with 1 µg Bacmid-ORF3 DNA displayed the most favourable results, cell supernatant was the P0 viral stock. Fresh SF9 cells were infected with the P0 transfection stock and P1 stock was collected at 48 hours post-infection.

Viral Plaque Assay

Quantification to Establish Viral Titre of P1 Stock

The P1 stock showed definite CPE to cells post-infection, although the recombinant virus required the titre to be determined for infection at ΔMOI. The initial viral plaque assay after MTT staining

determined the titre of the P1 ORF-Baculovirus stock was concentration of $6.5x10^6$ pfu/mL was too low. According to the *Bac-to-Bac™ Baculovirus Expression System* manual for protein expression studies a viral stock titre of minimum of $10x10^6$ pfu/mL is required.

Viral Plaque Purification and Amplification Generating P2 Plaque Purified Stock

Amplification and Generation of P2 Stock Viral Stock

P1 viral stock was amplified via plaque picking and infection. A total of 5 plaque purified P1 viral stocks were generated, with each originating from a single viral plaque to enhance ORF3-Baculovirus purity. The 5 viral plaque P1 stocks were ranked in levels of virulence based on CPE caused to infected cells. Plaque purified P1 stocks originating from viral plaque assay labelled as plaques 1-5 were amplified by independently infecting fresh SF9 cells over a 72-hour time course. Harvested P2 viral stocks were expected to have a far higher viral. The P2 viral stock originating from plaque 1 of the plaque purified P1 stock showed the higher infectivity compared to the plaque purified P1 stocks originating from viral plaques 2–5.

Final Viral Plaque Assay to Quantify Titre of P2 Viral Stock

Final Viral Plaque Assay to Establish Viral Titre of P2 Stock

SF9 insect cells were infected with the P2 viral stock ranging from lowest viral concentration 10^{-14} to the highest 10^{-4}. Viral plaques were present in experimental wells 3 days post-infection. On day 7 post-infection the cells-only control and viral infection experimental wells were stained for ORF3-Baculovirus plaques (Figure 4.9).

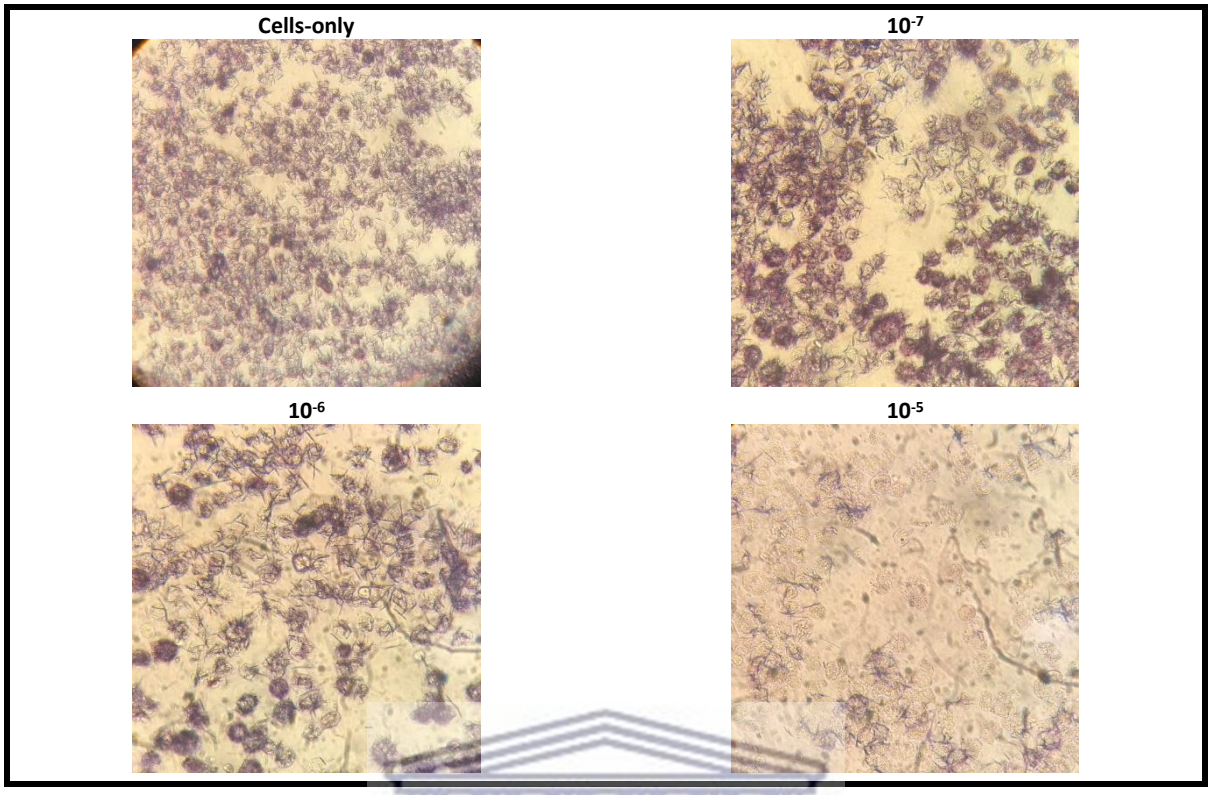

Figure 4.9: Final viral plaque assay quantifying P2 viral stock

MTT stained ORF3-Baculovirus plaques 7 days post-infection. Image displays cells-only control well and P2 viral stock infection for wells with viral infection concentrations 10^{-7} to 10^{-5} which was determined to be the optimal range for calculating the P2 stock titre. Cells viewed under inverted light microscope.

Living SF9 cells displayed a blue/black stain and the definitive clear areas in the cell monolayer were indicative of non-viable/infected SF9 cells, these were the ORF3-Baculovirus plaques.

Quantified P2 High Titre ORF3-Baculovirus Stock

The viral plaque assay after MTT staining determined that the titre of the viral plaque 1 P2 plaque purified ORF-Baculovirus stock was $15x10^6$ pfu/mL, as 15 plaques were counted in the dilution well and calculated according to the equation listed in the methodology. This plaque purified ORF3-Baculovirus P2 viral stock titre is well above the minimum threshold of $10x10^6$ pfu/mL, according to the *Bac-to-Bac™ Baculovirus Expression System* manual for protein expression studies.

ORF3-Baculovirus Protein Expression

ORF3-Baculovirus SF9 cell ΔMOI Assay for Varying HCoV-NL63 ORF3 Protein Expression Levels

SF9 Cell Infection

The known titre concentration plaque purified P2 viral stock originating from viral plaque 1 was used to infect SF9 cells at the ΔMOI range set of 0.1; 1; 2.5; 5 and 10 pfu/mL. After the fixed time course incubation of 72 hours, apart from MOI of 0.1 and 1, CPE was viewed in infected SF9 cells.

ORF3-Baculovirus Lysate Collection and Quantification

Infected Cell Lysate Collection

The SF9 ORF3-Baculovirus infected SF9 cell lysates were quantified via spectrophotometry at 562 nm and generated an R^2 value of 0.9885.

HCoV-NL63 ORF3 Target Protein Detection

SDS-PAGE ORF3-Baculovirus Lysates

Sample Loading

SF9 cell lysates generated from ΔMOI 72-hour infection containing Baculovirus expressing HCoV-NL63 ORF3 protein was denatured and loaded at a constant total protein concentration of 30 µg per Lane on SDS-PAGE.

Coomassie Stain SDS-PAGE ORF3-Baculovirus 72-hour ΔMOI Lysates

SDS-PAGE

SDS-PAGE gels containing ΔMOI lysates underwent Coomassie stain and de-stain. SDS gel (Figure 4.10) contains 30 µg total protein per Lane for all lysates loaded - cells-only and experimental ΔMOI lysates. The cells-only control is separated by an empty Lane and the ΔMOI range was loaded in ascending order (0.1; 1; 2.5; 5 and 10 pfu/mL). Only ~55 kDa band was visible in all sample lanes.

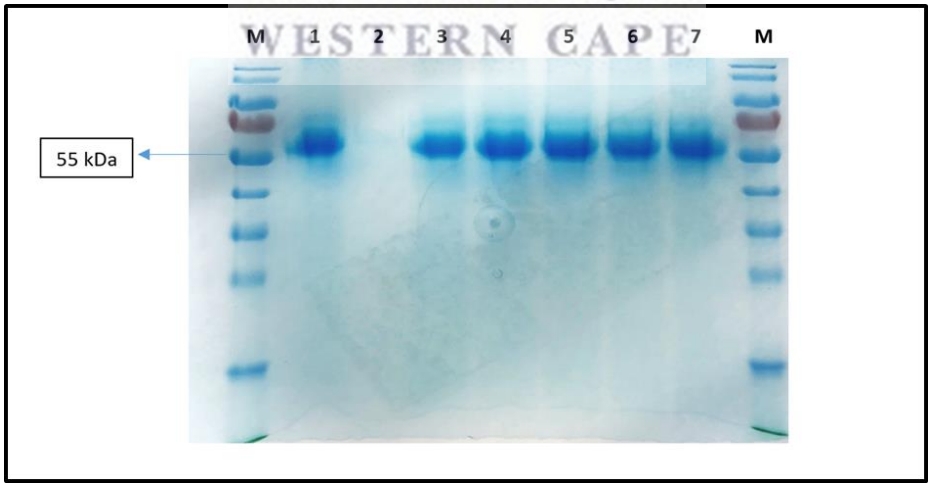

Figure 4.10: Coomassie stain SDS-PAGE ORF3-Baculovirus 72-hour ΔMOI lysates

SDS-PAGE labelled left to right. **Lane M**: Prestained PAGE ruler; **Lane 1:** Cells-only control; **Lane 2:** Empty; **Lane 3:** MOI 0,1 pfu/mL; **Lane 4:** MOI 1 pfu/mL; **Lane 5:** MOI 2,5 pfu/mL **Lane 6:** MOI 5 pfu/mL; **Lane 7:** MOI 10 pfu/mL.

Ponceau Stain ORF3-Baculovirus 72-Hour ΔMOI Lysates

Ponceau Stain

The SDS gel containing ΔMOI lysates underwent a wet transfer for total protein migration to a PVDF membrane, which was subsequently Ponceau stained. The PVDF membrane (Figure 4.11) contains 30 µg total protein for all lysates loaded - cells-only and experimental ΔMOI lysates. The cells-only control is separated by an empty Lane and the ΔMOI range was loaded in ascending order (0.1; 1; 2.5; 5 and 10 pfu/mL). Again, only a ~55 kDa band visible.

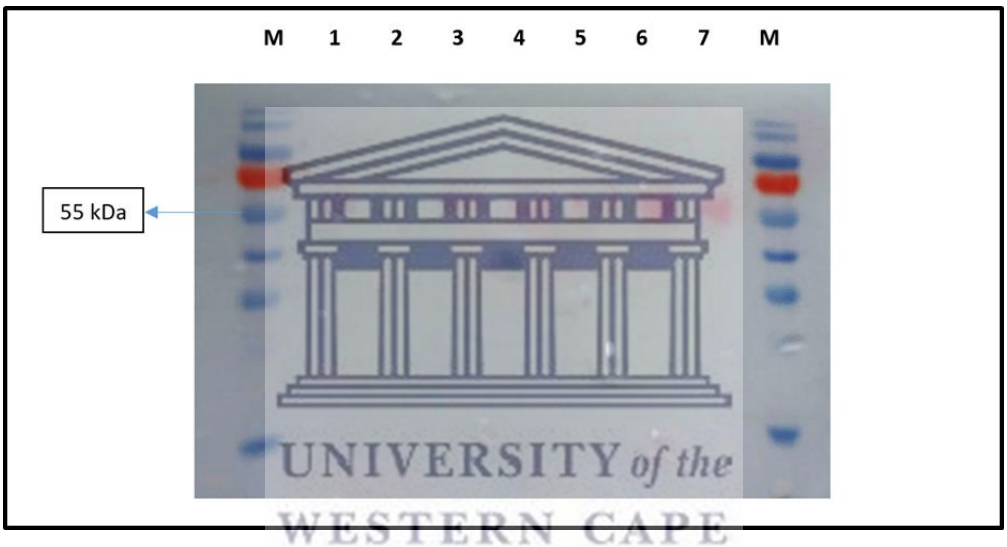

Figure 4.11: Ponceau stain ORF3-Baculovirus 72-hour ΔMOI lysates

Ponceau stained PVDF membrane labelled left to right. **Lane M:** Prestained PAGE ruler; **Lane 1:** Cells-only control; **Lane 2:** Empty; **Lane 3:** MOI 0,1 pfu/mL; **Lane 4:** MOI 1 pfu/mL; **Lane 5:** MOI 2,5 pfu/mL; **Lane 6:** MOI 5 pfu/mL; **Lane 7:** MOI 10 pfu/mL.

Western Blot ORF3-Baculovirus 72-Hour Infection with Plaque 1-5 P2 Viral Stocks

Western Blot

The membrane underwent Western blot as discussed in methodology, using lab synthesised polyclonal mice serum as primary antibody probing and secondary antibody probing with m-IgGκ bp-HRP recombinant commercial antibody. The PVDF membrane (Figure 4.12) contains 30 µg total protein loaded for all samples loaded; cells-only and experimental plaque purified P2 stocks. Now a

~25.6 kDa band was visible in lanes 2,3, 4, 5 and 6.

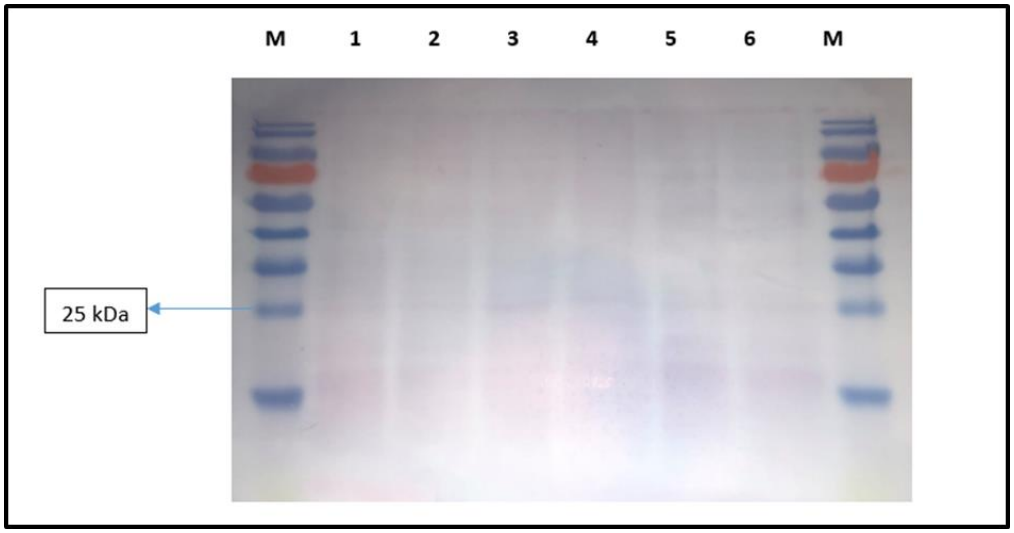

Figure 4.12: Western blot ORF3-Baculovirus 72-hour infection SF9 cell supernatant P2 stocks of viral plaques 1-5

Western blot using lab synthesised polyclonal mice serum as primary antibody probing and secondary antibody probing with m-IgGκ bp-HRP recombinant commercial antibody. Membrane labelled left to right. **Lane M:** Prestained PAGE ruler; **Lane 1:** Cells-only control; **Lane 2:** Viral plaque 1 P2 stock; **Lane 3:** Viral plaque 2 P2 stock; **Lane 4:** Viral plaque 3 P2 stock; **Lane 5:** Viral plaque 4 P2 stock; **Lane 6:** Viral plaque 5 P2 stock.

Conclusion

Viruses from the *Coronaviridae* family have some of the largest viral genomes and are characterised to contain (+) ssRNA which is a major contributing factor causing genetic recombination; as the "copy-choice" mechanism which permits the loss and gaining of coding domains (Figure 2.1) leads to the emergence of novel strains (Van Boheemen et al., 2012). The physical structure of coronaviruses, shown in Figure 1.1 display a great similarity by having the same structural proteins (spike, envelope, membrane and nucleocapsid); which have been of great value to researchers who have used various *in silico* and bioinformatics modelling techniques to draw inferences on lethality amongst HCoVs (Peiris, Guan, & Yuen, 2004). Similarly, the importance of the non-structural/accessory ORF proteins

are of great significance as they contain the TRS motif which aid in transcription regulation of viral structural proteins and the colocalization of structural proteins in the ERGIC (Müller et al., 2010; Yount et al., 2006).

This study aimed to clone and express the HCoV-NL63 ORF3 protein using the *Bac-to-Bac® Baculovirus Expression System;* as the use of recombinant proteins have been shown to be of importance in vaccine development and as antigens for detection and screening of antibodies (Ren et al., 2004).

The molecular cloning and subsequent expression of individual proteins assist to elucidate the functioning of the protein. By expressing the ORF3 protein in an expression vector, a greater understanding of the functional and molecular properties can be determined.

The recombinant protein produced with the Baculovirus system can be used for gene delivery of therapeutics which include; vaccine therapy, diagnostics, and medical research sectors involving public health (van Oers, Pijlman, & Vlak, 2015).

Promega restriction enzymes *BamH* I and *Xho* I along with *Promega* Buffer B, only having a 75% digestion efficiency, therefore agarose gels of digested ORF3 constructs display bands for the vector, the gene of interest and a third band of partially undigested vector.

The rationale of obtaining the purified HCoV-NL63 ORF3 proteins expressed with the *Bac-to-Bac® Baculovirus Expression System* was that conformational changes of the transmembrane glycoprotein could be further studied and possibly used in vaccine research (Pyrc et al., 2004). As the ORF3 protein boosts the relative amounts of the proteins encoded by the downstream reading frames (Cann, 2016) and the inclusion of the ORF3 protein in virions supports the viral assembly and budding processes of HCoV-NL63 (Müller et al., 2010).

However, the ΔMOI protein lysates harvested, which were denatured with Laemli's incubation buffer,

loaded at a fixed total protein concentration of 30 µg/µL on SDS-PAGE indicated a protein product of 57 kDa present in the control and experimental Lanes (Figure 4.10). Therefore, apart from the protein being present in the cells-only control lysates and experimental ΔMOI protein lysates the outcome does not align with published literature which states the size of HCoV-NL63 ORF3 is 25.6 kDa (Pyrc et al., 2004). The 55 kDa protein seen via SDS-PAGE and Coomassie stain is most likely a cellular protein with no relation to ORF3 and the experiment was repeated numerous times at multiple denaturation temperatures for validation (figures included in APPENDIX 2).

However after secondary antibody probing and incubation with a chemiluminescent substrate the Western blot in Figure 4.12 indicate proteins transferred at the expected size of approximately 25.6 kDa. Subsequent repeated attempts with ΔMOI protein lysates (figures included in APPENDIX 2), did not detect the target size protein with the listed antibodies.

In conclusion, these results are indicative that the gene of interest HCoV-NL63 ORF3 was successfully cloned into the *Bac-to-Bac® Baculovirus Expression System* which was the main objective of this Masters research project. The RT-PCR containing the Bacmid-ORF3 DNA which amplified the Bacmid vector at its M13 binding sites and gene-specific sites within the vector were a clear indication that HCoV-NL63 ORF3 was successfully cloned into this BEVS, based on the calculated amplicon size contained in the manufacturer's instructions. Furthermore, the Bacmid-ORF3 DNA was used to successfully transfect SF9 cells and subsequently generate ORF3-Baculovirus particles. Two successful viral plaque assays further confirmed the production of Baculoviral plaques which were quantified for HCoV-NL63 ORF3 expression studies. There is no commercially available HCoV-NL63 ORF3 antibody, further research is required to fully assess serological responses to the expressed ORF3 protein. Unfortunately, protein expression indicated weak binding of the primary antibody to proteins expressed.

Future Perspectives

Possible areas of future research include the synthesis of a new HCoV-NL63 ORF3 antibody for protein expression studies. The antibody produced could further be used for immunofluorescence microscopy assays to indicate the host cellular localisation of HCoV-NL63 ORF3 proteins. Finally, as the expression of the protein of interest falls under a greater umbrella project within the Molecular Biology and Virology Lab, the ORF3-Baculovirus could be used in transfection studies to gain a greater understanding of the ORF3 accessory protein role in viral assembly and packaging HCoV-NL63 within host cell lines.

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APPENDIX 1

Hcov-Nl63 Orf3 Gene Sequence Alignment

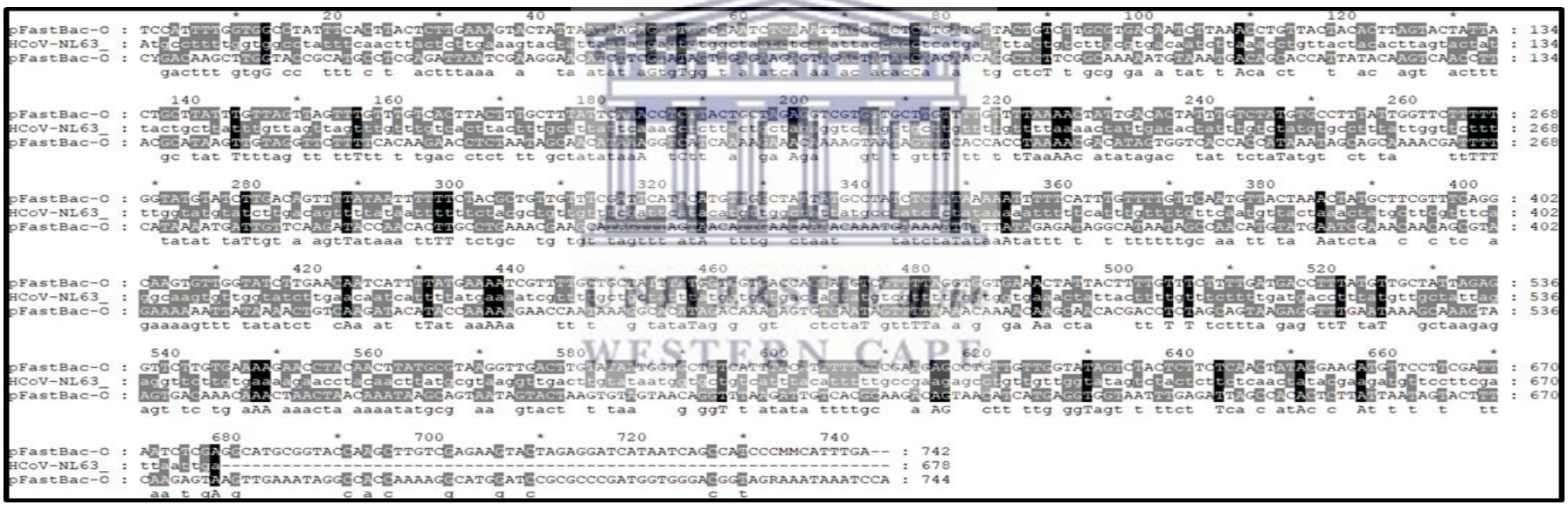

Image above depicts sequencing alignment result of pFastBac-ORF3 Forward (Top), HCoV-NL63 ORF3 NCBI (Middle) and pFastBac-ORF3 Reverse (Bottom).

APPENDIX 2

SDS-PAGE

Image above contain SDS PAGE repetitions where ORF3-Baculovirus protein lysates of 30 µg per Lane were linearized at room temperature, 40°C and 80°C respectively.

Western Blot

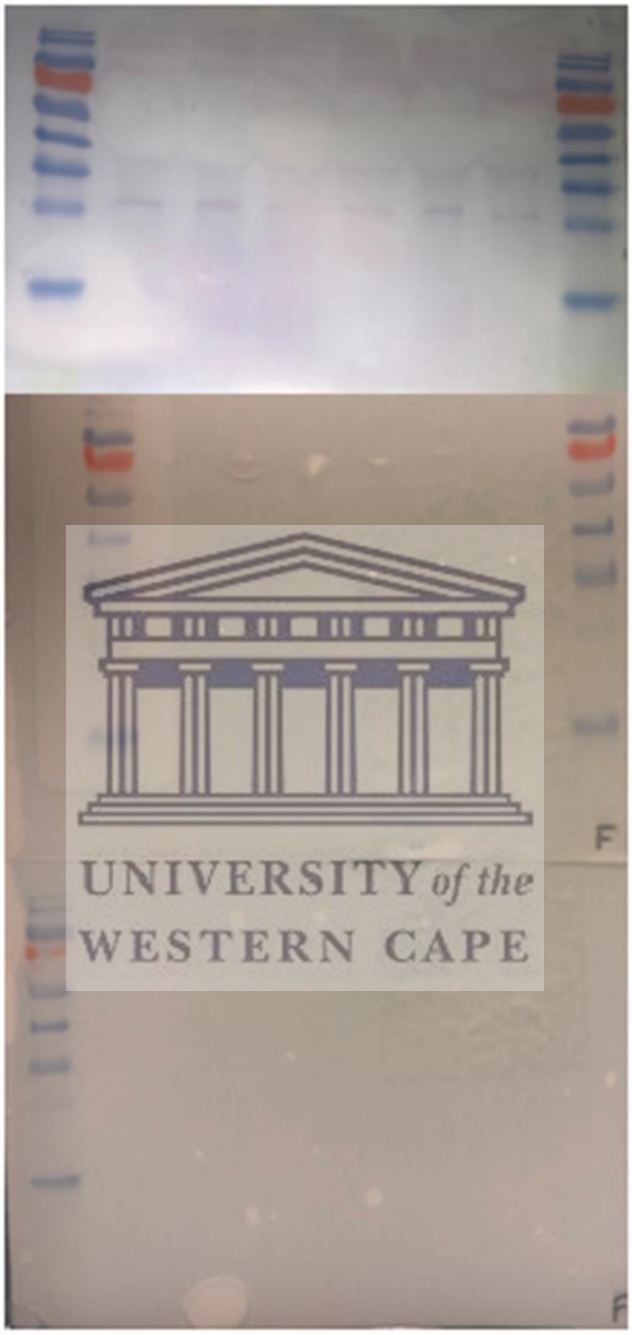

Image above contain Western Blot repetitions where ORF3-Baculovirus protein lysates were probed with the primary antibody at the dilution factors of 1:500, 1:200 and 1:100 respectively.