

Rational design of rotavirus NSP1 and NSP4 to improve the safety profile of rotavirus vaccine candidates

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ABSTRACT

Rotaviruses cause acute gastroenteritis and are the leading cause of diarrhoea-related hospitalisations and deaths worldwide in children under the age of 5. Currently available live-attenuated rotavirus vaccines have significantly alleviated the burden of disease. However, these vaccines are less effective in Sub-Saharan Africa and South-East Asia countries, and there is a need for a better vaccine in some regions. As novel live-attenuated vaccine candidates are being developed in our research group, we must ensure that the vaccines are safe. The non-structural proteins NSP4 and NSP1 contribute to viral pathogenesis. NSP4 is an enterotoxin that increases intracellular calcium levels leading to diarrhoea, while NSP1 inhibits the innate immune response. Understanding how to attenuate these proteins without affecting their other functions in the replication cycle could be the key to increasing the safety profile of novel vaccine candidates. The plasmid-only based reverse genetics system has made it possible to study rotavirus proteins and the impact of altering specific domains. This study's focus was to rationally design NSP1 and NSP4 to rescue rotaviruses that cannot inhibit the nuclear accumulation of phosphorylated STAT1 and STAT2 or increase intracellular calcium levels.

Controls are needed to test the vaccine candidates developed in our research group to ensure that they are safe. The attenuated and virulent NSP4 of the porcine OSU rotavirus can be used as such controls. The proteins were previously expressed using the baculovirus expression system, and it was hypothesised that codon optimising the ORFs would increase protein yield. Two pFastBac-HTA donor plasmids containing the Sf9 insect cell codon optimised OSU-a NSP4 and OSU-v NSP4 ORF were designed and used in the Bac-to-Bac baculovirus expression system to generate recombinant baculoviruses expressing His-tagged OSU-a NSP4 and OSU-v NSP4. Although there were signs of CPE in cells infected with the recombinant baculoviruses, no expression of His-tagged OSU-a NSP4 and OSU-v NSP4 could be observed using SDS-PAGE or immuno-fluorescence monolayer assay.

Studies of the amino acid sequences of OSU-a NSP4 and OSU-v NSP4 showed that there were six amino acid differences between the proteins. Three of the six alterations in OSU-a NSP4 clustered in the signal-transducing domain. Altering these amino acids in OSU-v NSP4 attenuated its ability to increase intracellular calcium and cause diarrhoea. The plasmid-only based reverse genetics system was used to investigate if OSU-a NSP4 could be incorporated into SA11-L2 and if altering aa 135-141 of SA11-L2 to those of OSU-a NSP4 would attenuate the virus. Rotaviruses with OSU-a NSP4 and SA11-L2 NSP4 with aa 135-141 of OSU-a NSP4 were successfully rescued. Replication kinetics showed that the presence of OSU-a NSP4 or alterations in SA11-L2 NSP4 did not hinder viral replication, and both rSA11_OSU-a_NSP4 and rSA11_OSU-a_135-141 grew at a rate similar to rSA11-L2. The calcium flux assay

showed that rSA11_OSU-a_NSP4 could increase intracellular calcium in infected cells, while rSA11_OSU-a_135-141 was partially attenuated. The next step will be to investigate which alternations can be incorporated into the enterotoxin and viroporin domain of GS10(NSP4) of rSA11_OSU-a_135-141 to attenuate the virus completely.

NSP1 anchors to the cytoskeleton using its cytoskeleton domain (aa 84-176) and then inhibits the production of interferons and nuclear accumulation of phosphorylated STAT1 and STAT2. We hypothesised that deleting the cytoskeleton binding domain would allow NSP1 to translocate to the nucleus allowing phosphorylated STAT1 and STAT2 to translocate to also the nucleus. SA11-L2 with GS5(NSP1) lacking the cytoskeleton domain was successfully rescued using the SA11-L2 reverse genetics system. Plaque assays and replication kinetics showed that rSA11_NSP1-tr formed smaller plaques than rSA11-L2, but they replicated at a similar rate. These results showed that there could be a higher interferon response in MA104 cells infected with rSA11_NSP1-tr which prevents effective cell-to-cell spread. Further investigations need to be done to determine the localisation of NSP1 lacking the cytoskeleton binding domain and phosphorylated STAT1 and STAT2 in infected cells.

This is the first report of the rescue of SA11-L2 with OSU-a NSP4, SA11-L2 with SA11-L2 NSP4 containing aa 135-141 of OSU-a NSP4 and SA11-L2 with NSP1 lacking a cytoskeleton binding domain. These study results show that rationally altering NSP1 and NSP4 could lead to the rescue of viruses that lack some of their pathogenic qualities but can replicate well. Further studies need to be conducted in animal models to evaluate if the rescued viruses do not cause diarrhoea and if there is a greater innate immune response during infection with recombinant rotaviruses. These experiments would be proof of principle that these alterations can increase the safety profile of vaccine candidates.

Keywords: Rotavirus, Rotavirus NSP4, Rotavirus NSP1, Intracellular calcium, Innate immune response, Baculovirus expression, Reverse genetics

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ABBREVIATIONS

aa	amino acid
ADP	adenosine 5'-diphosphate
AGE	agarose gel electrophoresis
AHSV	African horsesickness virus
BAC	bacterial artificial chromosome
BTV	bluetongue virus
bp	base pairs (nucleic acids)
Ca²⁺	calcium
CPE	cytopathic effect
DLP	double-layered particle
DMEM	Dulbecco's modified medium
DNA	deoxyribose nucleic acid
dsRNA	double-stranded Ribose nucleic acid
EDTA	ethylenediamine tetraacetic acid
ER	endoplasmic reticulum
EtBr	ethidium bromide
FAST	fusion-associated small transmembrane protein
FBS	foetal bovine serum
GS	genome segment
HDV	hepatitis delta virus
hpi	hours post-infection
IAD	interferon regulatory factor associated domain
IFMA	immunofluorescence monolayer assay
IFN	interferon
IκB	inhibitor of nuclear factor-κB
IKK	inhibitor of nuclear factor-κB kinase
IPTG	isopropylthio-β-galactoside
IRF	interferon regulatory factor
ISG	interferon-Stimulated Gene
ISGF3	interferon-stimulated gene factor 3

JAK	Janus kinase
kDA	kiloDalton
LB	Luria broth
MAVS	mitochondrial Antiviral-Signaling Protein
MCS	multiple cloning site
MDA5	melanoma Differentiation-Associated protein 5
MOI	multiplicity of infection
MRV	mammalian orthoreovirus
MW	molecular weight
NEAA	non-essential amino acids
NLR	nucleotide oligomerisation domain-like receptor
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NSP	nonstructural protein
OAS	oligoadenylate synthetase
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKR	protein Kinase R
PRR	pattern Recognition Receptor
RbCl	rubidium chloride
rBV	recombinant baculovirus
RG	reverse genetics
RIG-1	retinoic Acid-Inducing Gene 1
RLR	retinoic Acid-inducing Gene 1-like Receptor
RNA	ribose nucleic acid
RNaseL	latent ribonuclease
RRV	rhesus Rotavirus
RV	rotavirus
SF	soluble fraction
SA11	simian agent 11

SDS-PAGE	sodium-dodecyl-sulphate polyacrylamide gel electrophoresis
Sf9	Spodoptera frugiperda 9
ssRNA	single-stranded RNA
STAT	signal transducer and activator of translation
TEV	tobacco etch virus
TF	total fraction
TLP	triple-layered particle
TLR	toll-like receptor
UTR	untranslated region
VLP	virus-like particle
VP	viral protein (structural protein)
VV	vaccinia virus
WHO	World Health Organisation
WT	wild-type
w/v	weight per volume

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CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Throughout history, diarrhoeal diseases have been a health problem in young children worldwide. In 2016 diarrhoea was the cause of 1.66 million deaths, the eighth leading cause of morbidity (Naghavi *et al.*, 2017). According to the World Health Organisation, diarrhoea related deaths are the second leading cause of death in children under 5. Ninety percent of diarrhoea related deaths in children under five occur in Sub-Saharan Africa and South Asia (Naghavi *et al.*, 2017). There has been a decline in diarrhoea related deaths over the years due to better sanitation, hygiene, and living conditions.

Historically, bacteria were the agents attributed to diarrhoeal disease because methods to detect non-bacterial pathogens were not yet advanced (Bishop, 2009). In 1946 there were reports of acute diarrhoea in suckling mice (Pappenheimer & Enders, 1946). Although a bacterial agent was suspected, the researchers identified intracellular inclusions in the small intestinal epithelium of infected mice (Pappenheimer & Enders, 1946). This led to the researchers reasoning that a virus was the cause of acute diarrhoea in the mice. In 1958 while producing polio vaccines, Malherbe and colleagues cultured a virus named Simian Agent 11 (SA11), which was obtained from the rectum of a healthy vervet monkey. They observed that during infection inclusion bodies formed in the cell's cytoplasm and this virus did not infect mice (Malherbe & Harwin, 1963). In 1973 using electron microscopy, Bishop and colleagues identified a 70 nm virus with a wheel-like structure in duodenum biopsy tissue from children (Bishop *et al.*, 1973). Thus, the virus was named after the Latin word "rota", meaning wheel (Flewett & Woode, 1978). After malaria and *Streptococcus pneumoniae*, rotavirus (RV) is the third leading cause of pathogen related morbidity in children under the age of 5 (Naghavi *et al.*, 2017).

Nowadays, reverse genetics is a powerful tool used to study the function of genes by altering the genes and analysing the effect on the phenotype. This tool is applied in virology to generate live viruses by transfecting cells with cDNA copies of the viral genome. Mutations can be generated in the viral genome to study the function of different viral genes to help understand viral pathogenesis, the replication cycle, and the development of novel vaccine candidates. A plasmid-based rotavirus reverse genetics system became available in 2017 and has gained momentum to study the virus and develop novel vaccine candidates (Kanai *et al.*, 2017). The use of reverse genetics is starting to improve understanding of rotavirus pathogenesis, but many questions remain unanswered. Some questions are related to the

mechanisms and roles of rotavirus non-structural protein (NSP) 1 and NSP4 in pathogenesis. NSP1 inhibits the innate immune response and NSP4 increases intracellular calcium levels which is associated with diarrhoea in infected individuals (Morelli et al., 2015; Seo et al., 2008). A better understanding of these non-structural proteins could lead to the development of safer rotavirus vaccine candidates.

1.2 Burden of Disease

The main symptoms of rotavirus infection are watery diarrhoea, vomiting and fever (Estes & Greenberg, 2013). There is no specific medication to treat rotavirus infection. Oral rehydration therapy is used to prevent death from dehydration (Nalin *et al.*, 1979; Taylor *et al.*, 1980). Rotavirus was found to be the most prevalent pathogen linked to diarrhoea cases that lead to hospitalisation and death in young children (Tian *et al.*, 2016). More than half of the countries in the world have included rotavirus vaccination in their immunisation programmes. Since 2006 two RV vaccines, Rotarix™ (GlaxoSmithKline Biologicals, Belgium) and RotaTeq® (Merck & Co., Inc., USA), have been commercially available. Vaccine introduction in countries has led to a decline in rotavirus related hospitalisations and deaths.

Rotavirus remains the leading cause of worldwide diarrhoeal related deaths (Tate *et al.*, 2016; Troeger *et al.*, 2018). Rotavirus infection accounts for 128 500 deaths of children under 5 years; making it the leading cause of diarrhoea in young children (Esona & Gautam, 2015; Troeger *et al.*, 2018). Rotavirus infections account for 1.76 million hospitalisations of children under the age of 5 (Hallowell *et al.*, 2022). Analysis of global statistics by the World Health Organisation (WHO) indicates that rotavirus infection accounts for 27% of cases of severe diarrhoea (Burnett *et al.*, 2020b). More than 80% of rotavirus related deaths occurred in Sub-Saharan Africa (Troeger *et al.*, 2018). Rotavirus hospitalisations pose an economic problem to countries, as substantial funds are used to treat infected individuals annually. Rotaviruses affect the infected individuals and the caregivers' quality of life (Wittenberg *et al.*, 2019). Household transmission of the virus may occur when someone is infected, leading to a further burden on caregivers (Lopman *et al.*, 2013; Wikswo *et al.*, 2020).

Since rotavirus can also infect animals, it impacts the agricultural sector as well. Animals important in the agricultural sector that are infected by group A rotaviruses include cattle and pigs (Matthijnssens *et al.*, 2011; Papp *et al.*, 2013). Bovine and porcine rotavirus infections lead to significant losses in calves and piglets, which have an economic impact (Martella *et al.*, 2010).

1.3 Rotavirus Classification

Rotaviruses are a group of viruses with a segmented double-stranded RNA (dsRNA) genome and belong to the *Reovirales* order (Matthijnssens *et al.*, 2022). There are two families in the *Reovirales*, the *Sedoreoviridae* and the *Spinareoviridae* (Matthijnssens *et al.*, 2022). The two families consist of 15 genera. Rotaviruses belong to the Rotavirus genus in the *Sedoreoviridae* family.

Rotaviruses are classified using their serological reactivity and genotyping. Group, subgroup, and serotypes are three significant antigenic specificities of rotaviruses (Hoshino & Kapikian, 2000). Serologically, rotaviruses are grouped into 9 serogroups (A-D, F-J) mainly based on their antigenic reactivity to monoclonal antibodies against VP6 (Hoshino & Kapikian, 2000; Yandle *et al.*, 2020). Enzyme-linked immunosorbent assay (ELISA) is commonly used to identify the serogroup using VP6 monoclonal antibodies (mAb). Group A, B, C, and H rotaviruses infect humans and animals but group A RV (RVAs) account for most human infections. Group D, E, F, and G rotaviruses infect only animals (Kuang *et al.*, 2020; Matthijnssens *et al.*, 2010). Group A rotaviruses are further divided into four subgroups; subgroup I, subgroup II, subgroup I+II, non-subgroup I and non-subgroup II using mAb against VP6 (Estes & Cohen, 1989; Greenberg *et al.*, 1983).

RVAs are divided into G and P serogroups based on structure and neutralisation reactions against VP7 (the glycoprotein) and VP4 (protease-sensitive protein) (Kuang *et al.*, 2020). Currently, 58 P-types and 42 G-types have been identified (Rotavirus Classification Working Group, 2022).

Advances in sequencing methods have allowed the development of a genome-based classification system (Matthijnssens *et al.*, 2008). Whole genome sequencing is used to classify the genome segments into genotypes based on the primary function of the encoded protein (Matthijnssens *et al.*, 2008). The shortened form Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx is used to group strains, where the upper-case letter is the genotype and x is the number of the genotype (Figure 1.1). The genome segments are classified based on the primary function of the proteins they encode, followed by an x indicating the number of the genotype (Table 1.1). Based on RNA-RNA hybridization studies and whole genome phylogenetic analysis, human rotaviruses are grouped into two prominent constellations, WA-like, DS-like, and the rare AU-like constellation (Figure 1.1) (Matthijnssens *et al.*, 2008). The SA11 strain is widely used as a prototype to study rotaviruses because it is non-infectious to humans and propagates well in cell culture (Estes *et al.*, 1979).

			VP[7]	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5										
Wa	Hu	Wa-like	G1	-	P[8]	-	I1	-	R1	-	C1	-	M1	-	A1	-	N1	-	T1	-	E1	-	H1
KU	Hu	Wa-like	G1	-	P[8]	-	I1	-	R1	-	C1	-	M1	-	A1	-	N1	-	T1	-	E1	-	H1
Dhaka16-03	Hu	Wa-like	G1	-	P[8]	-	I1	-	R1	-	C1	-	M1	-	A1	-	N1	-	T1	-	E1	-	H1
D	Hu	Wa-like	G1	-	P[8]	-	I1	-	R1	-	C1	-	M1	-	A1	-	N1	-	T1	-	E1	-	H1
P(rice)	Hu	Wa-like	G3	-	P[8]	-	I1	-	R1	-	C1	-	M1	-	A1	-	N1	-	T1	-	E1	-	H1
ST3	Hu	Wa-like	G4	-	P[6]	-	I1	-	R1	-	C1	-	M1	-	A1	-	N1	-	T1	-	E1	-	H1
IAL28	Hu	Wa-like	G5	-	P[8]	-	I1	-	R1	-	C1	-	M1	-	A1	-	N1	-	T1	-	E1	-	H1
WI61	Hu	Wa-like	G9	-	P[8]	-	I1	-	R1	-	C1	-	M1	-	A1	-	N1	-	T1	-	E1	-	H1
B3458	Hu	Wa-like	G9	-	P[8]	-	I1	-	R1	-	C1	-	M1	-	A1	-	N1	-	T1	-	E1	-	H1
RMC321	Hu	Wa-like	G9	-	P[19]	-	I5	-	R1	-	C1	-	M1	-	A1	-	N1	-	T1	-	E1	-	H1
Dhaka12-03	Hu	Wa-like	G12	-	P[6]	-	I1	-	R1	-	C1	-	M1	-	A1	-	N1	-	T1	-	E1	-	H1
Matlab13-03	Hu	Wa-like	G12	-	P[6]	-	I1	-	R1	-	C1	-	M1	-	A1	-	N1	-	T2	-	E1	-	H1
B4633-03	Hu	Wa-like	G12	-	P[8]	-	I1	-	R1	-	C1	-	M1	-	A1	-	N1	-	T1	-	E1	-	H1
Dhaka25-02	Hu	Wa-like	G12	-	P[8]	-	I1	-	R1	-	C1	-	M1	-	A1	-	N1	-	T1	-	E1	-	H1
DS-1	Hu	DS-1-like	G2	-	P[4]	-	I2	-	R2	-	C2	-	M2	-	A2	-	N2	-	T2	-	E2	-	H2
TB-Chen	Hu	DS-1-like	G2	-	P[4]	-	I2	-	R2	-	C2	-	M2	-	A2	-	N2	-	T2	-	E2	-	H2
B1711	Hu	DS-1-like	G6	-	P[6]	-	I2	-	R2	-	C2	-	M2	-	A2	-	N2	-	T2	-	E2	-	H2
DRC86	Hu	DS-1-like	G8	-	P[6]	-	I2	-	R2	-	C2	-	M2	-	A2	-	N2	-	T2	-	E2	-	H2
DRC88	Hu	DS-1-like	G8	-	P[8]	-	I2	-	R2	-	C2	-	M2	-	A2	-	N2	-	T2	-	E2	-	H2
69M	Hu	DS-1-like	G8	-	P[10]	-	I2	-	R2	-	C2	-	M2	-	A2	-	N2	-	T2	-	E2	-	H2
L26 ^a	Hu	DS-1-like	G12	-	P[4]	-	I2	-	R2	-	C2	-	M1/M2	-	A2	-	N1	-	T2	-	E2	-	H1
N26-02	Hu	DS-1-like	G12	-	P[6]	-	I2	-	R2	-	C2	-	M2	-	A2	-	N1	-	T2	-	E6	-	H2
RV176-00	Hu	DS-1-like	G12	-	P[6]	-	I2	-	R2	-	C2	-	M2	-	A2	-	N2	-	T2	-	E6	-	H2
RV161-00	Hu	DS-1-like	G12	-	P[6]	-	I2	-	R2	-	C2	-	M2	-	A2	-	N2	-	T2	-	E1	-	H2
Au-1	Hu	AU-1-like	G3	-	P[9]	-	I3	-	R3	-	C3	-	M3	-	A3	-	N3	-	T3	-	E3	-	H3
T152	Hu	AU-1-like	G12	-	P[9]	-	I3	-	R3	-	C3	-	M3	-	A12	-	N3	-	T3	-	E3	-	H6
NCDV-Lincoln	Bo		G6	-	P[1]	-	I2	-	R2	-	C2	-	M2	-	A3	-	N2	-	T6	-	E2	-	H3
BRV033	Bo		G6	-	P[1]	-	I2	-	R2	-	C2	-	M2	-	A3	-	N2	-	T6	-	E2	-	H3
RF	Bo		G6	-	P[1]	-	I2	-	R2	-	C2	-	M2	-	A3	-	N2	-	T6	-	E2	-	H3
Uk	Bo		G6	-	P[5]	-	I2	-	R2	-	C2	-	M2	-	A3	-	N2	-	T7	-	E2	-	H3
WC3	Bo		G6	-	P[5]	-	I2	-	R2	-	C2	-	M2	-	A3	-	N2	-	T6	-	E2	-	H3
KJ44	Bo		G5	-	P[1]	-	I1	-	R1	-	C1	-	M2	-	A1	-	N1	-	T1	-	E1	-	H1
KJ75	Bo		G5	-	P[5]	-	I1	-	R1	-	C1	-	M2	-	A1	-	N1	-	T1	-	E1	-	H1
A131	Po		G3	-	P[7]	-	I5	-	R1	-	C2	-	M1	-	A1	-	N1	-	T1	-	E1	-	H1
Gottfried	Po		G4	-	P[6]	-	I1	-	R1	-	C1	-	M1	-	A8	-	N1	-	T1	-	E1	-	H1
OSU	Po		G5	-	P[7]	-	I5	-	R1	-	C1	-	M1	-	A1	-	N1	-	T1	-	E1	-	H1
A253	Po		G11	-	P[7]	-	I5	-	R1	-	C2	-	M1	-	A1	-	N1	-	T1	-	E1	-	H1
YM	Po		G11	-	P[7]	-	I5	-	R1	-	C1	-	M1	-	A8	-	N1	-	T1	-	E1	-	H1
PO-13	Av		G18	-	P[17]	-	I4	-	R4	-	C4	-	M4	-	A4	-	N4	-	T4	-	E4	-	H4
SA11g4"O" (5N)	Si		G3	-	P[1]	-	I2	-	R2	-	C5	-	M5	-	A5	-	N5	-	T5	-	E2	-	H5
SA11-5S	Si		G3	-	P[1]	-	I2	-	R2	-	C5	-	M5	-	A5	-	N5	-	T5	-	E2	-	H5
SA11-30/19	Si		G3	-	P[1]	-	I2	-	R2	-	C5	-	M5	-	A5	-	N5	-	T5	-	E2	-	H5
SA11-30/1A	Si		G3	-	P[1]	-	I2	-	R2	-	C5	-	M5	-	A5	-	N5	-	T5	-	E2	-	H5
SA11-H96	Si		G3	-	P[2]	-	I2	-	R2	-	C5	-	M5	-	A5	-	N5	-	T5	-	E2	-	H5
SA11-Both	Si		G3	-	P[2]	-	I2	-	R2	-	C5	-	M5	-	A5	-	N2	-	T5	-	E2	-	H5

Figure 1.1: Whole genome classification of selected rotavirus strains. The three main genome constellations are grouped by colour. Green represents the Wa-like genotypes, red the DS-1-like genotypes and orange the AU-1-like genotypes. Image used with permission from Matthijnssens et al., 2008.

Table 1.1: Genome coding assignment of group A rotaviruses. Adapted from Matthijnssens et al. (2008) and RCWG (2022).

Genome segment	Size (bp)	Protein product	Molecular weight (kDa)	Genotype	Number of genotypes	Main protein function
1	3302	VP1	125	R	28	RNA-dependent RNA polymerase (RdRp); ssRNA binding; forms a transcription complex with VP3
2	2690	VP2	102,4	C	24	Core protein; RNA binding, required for VP1 activity
3	2591	VP3	98,1	M	24	Methyltransferase; guanylyltransferase; ssRNA binding, forms a transcription complex with VP1; 2',5'-phosphodiesterase
4	2362	VP4	86,8	P	58	Protease-sensitive; cell-attachment; determines virulence; P-type neutralisation antigen; trypsin cleavage into VP5* and VP8*
		VP5*	60			VP5* allows initial attachment to the plasma membrane
		VP8*	28			VP8* allows secondary attachment
5	1611	NSP1	58,7	A	39	Interferon Antagonist; attaches to the cytoskeleton
6	1356	VP6	44,8	I	32	Inner capsid structural protein; species determinant
7	1104	NSP3	34,6	T	28	Translation enhancer; viral 3'-mRNA binding; inhibits host translation
8	1059	NSP2	36,7	N	28	NTPase; non-specific ssRNA binding; important for viroplasm formation; interacts with NSP5 and VP1
9	1062	VP7	37,4	G	42	Outer capsid Glycoprotein; G-type neutralisation antigen; Ca ²⁺ sensitive
10	751	NSP4	20,3	E	32	Enterotoxin; viroporin; modulates intracellular Ca ²⁺ ; receptor for DLPs
11	667	NSP5	21,7	H	28	Phosphoprotein; essential for viroplasm formation; interacts with NSP2
		NSP6	11			Interacts with NSP5

1.4 Rotavirus Genome, Proteins, and Particles

1.4.1 Genome structure and encoded proteins

Rotaviruses have an 11-segment dsRNA genome (Figure 1.2A) encapsulated in a triple-layered capsid. The genome segments range between 3302 bp and 677 bp in size (Table 1.1) (Estes & Cohen, 1989). Each genome segment is monocistronic, except for genome segment eleven, which is bicistronic (Desselberger, 2014; Mitchell & Both, 1988). The proteins encoded by the viral genome are six structural proteins, which form the viral capsid, and six non-structural proteins (Figure 2A). The structural proteins are VP1-VP4 and VP6; VP5* and VP8* are proteolytic fragments of VP4 (Table 1.1). The non-structural proteins (NSP1-NSP6) are essential for virus replication.

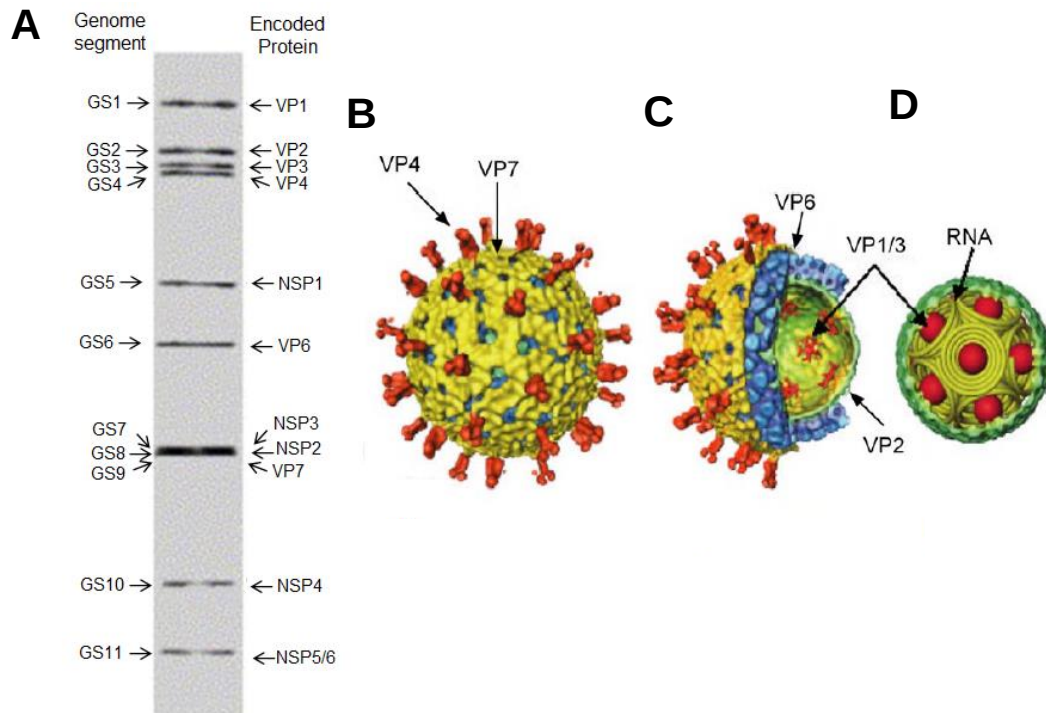


Figure 1.2: Rotavirus genome segments, proteins and particles. (A) Polyacrylamide gel of double-stranded RNA genome segments and the proteins they encode. On the left are the genome segments and the encoded proteins are shown on the right. (B) Structure of rotavirus TLP with VP7 (yellow) and VP4 spikes (red). (C) Cut-way image of RV-TLP showing the middle layer composed of VP6 and the core layer composed of VP2 (green). (D) The transcriptional enzymes, VP3 and VP1 (red), and packaged viral RNA genome within the core particle. Used with permission from Jayaram et al. 2004.

The rotavirus genome segments have three general features. Each genome segment has an open reading frame (ORF) flanked by untranslated regions (UTRs) at the 5' and 3' ends. The 5' UTR at the start of the genome segment is between 9-48 bp and starts with a 5'-GCC-3' followed by a conserved sequence that is part of the non-coding region (Figure 1.3) (Estes & Cohen, 1989). The 5' UTR is followed by the ORF encoding the viral protein and a 3' conserved UTR with two 3' cytidines at its terminus (Figure 1.3) (Estes & Greenburg, 2013). The 3' UTR is between 17-182 bp, and the 5'-UGUGACC-3' is found in most of the genome segments (Chen *et al.*, 2001). During the replication cycle (discussed in Section 1.5), the transcribed positive sense ssRNA, (+)ssRNA, is posttranslational modified. The (+)ssRNA is capped at the 5' end (5'm⁷GpppG(m)) but not polyadenylated at the 3' end (Estes & Greenberg, 2013).

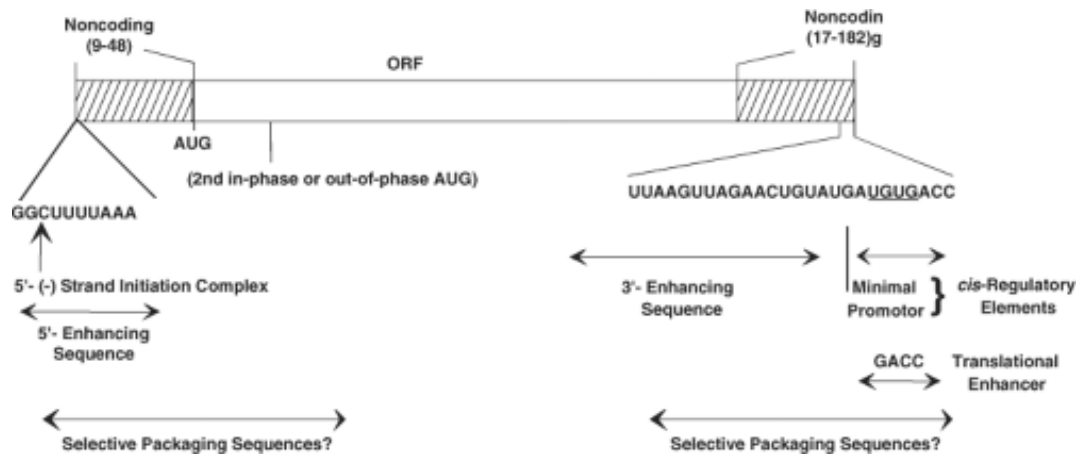


Figure 1.3: Structure of rotavirus genome segments. Rotavirus genome segments have 5' and 3' non-coding regions flanking the ORF. The strand initiation complex is the 5'-GCC-3' at the start of the 5' UTR. The conserved sequence 5'GACC-3' is found at the 3' end of the 3' UTR. The 5' and 3' UTR are needed for (+)ssRNA synthesis and genome packaging. Image used with permission from Estes and Greenberg 2013.

1.4.2 Rotavirus particles

Mature virions are complex triple-layered particles (TLPs) that are approximately 1000 Å in diameter (Li *et al.*, 2009). The inner core of the TLP consists of 120 VP2 proteins arranged into 90 dimers encapsulating the viral genome, the RNA-dependent RNA polymerase (VP1) and VP3, the capping enzyme (Figure 1.2D) (McClain *et al.*, 2010). The core-shell is covered by 60 VP6 dimers (Figure 1.2C), forming a non-infectious, transcriptionally active double-layered particle (DLP) (Li *et al.*, 2009; Mathieu *et al.*, 2001). The DLP is covered by 260 VP7 trimers with 60 VP4 spike trimers protruding from it, forming an infectious TLP (Mathieu *et al.*, 2001).

1.5 Rotavirus replication

In vivo rotaviruses exhibit a particular cell tropism and preferentially infect the mature enterocytes of the small intestine villi (Lopez & Arias, 2006). Rotavirus replication is a complex, multi-step cycle that can be summarised in the following steps: virus particle attachment, internalisation, and uncoating, (+)ssRNA synthesis, protein synthesis, viroplasm formation, (+)ssRNA packaging, dsRNA synthesis, DLP formation, virion maturation and release (Figure 1.4).

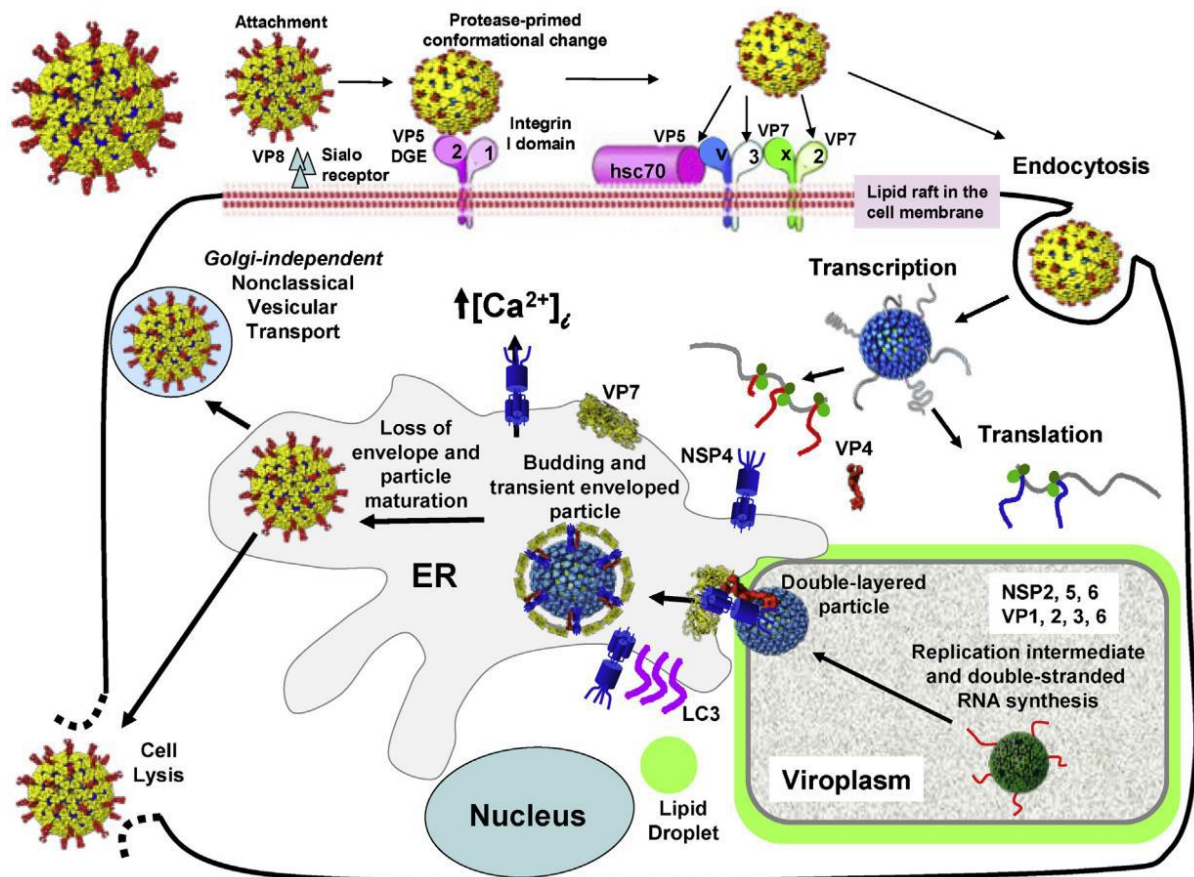


Figure 1.4: The Rotavirus replication cycle. RV-TLPs initially bind to sialoglycans or histo-blood group antigens on the host cell's surface. Subsequently, they interact with other cellular receptors, such as integrins and Hsc70. The RV particle is then internalised through receptor-mediated endocytosis. The outer layer is uncoated due to reduced calcium concentrations in the endosome, releasing transcriptionally active DLPs into the cytoplasm. These DLPs initiate rounds of mRNA transcription, which are subsequently translated into viral proteins. Once sufficient viral protein synthesis has occurred, the RNA genome is replicated and packaged into newly formed DLPs in the viroplasms, which interact with lipid droplets. NSP4 acts as an endoplasmic reticulum (ER) receptor, and binds to the newly generated DLPs, which bud into the ER and are transiently enveloped. Additionally, NSP4 releases calcium from intracellular stores, increasing the intracellular calcium, which stabilises the VP7 trimers that attached on the DLPs to form mature TLPs. Mature virions are released via cell lysis or a non-lytic method. Image used with permission from Desselburger 2014.

1.5.1 Attachment, internalisation and uncoating

Virus tropism is determined by the presence of specific cell surface receptors which the virus interacts with (Bass *et al.*, 1991). Most viruses bind to at least one attachment receptor and entry receptor (co-receptor). Binding to the attachment receptor enables the virus to attach to the cell surface and co-receptors facilitate viral entry into the cell.

Depending on the strain, rotaviruses use either sialic acid receptors or integrin $\alpha 2\beta 1$ to attach to the cell surface (Figure 1.4) (Lopez & Arias, 2004). The protease-sensitive VP4 mediates viral binding to the specific attachment receptor. After the proteolytic cleavage of VP4, VP8* binds to sialic acid receptors using its sialic acid binding site (Dormitzer *et al.*, 2002). Strains that do not use sialic acids to attach to the cell surface use VP5* to bind to integrin $\alpha 2\beta 1$ using its DGE integrin recognition site (Zarate *et al.*, 2000). After initial attachment, the virus binds co-receptors, hsc70 and several integrins using either VP5* or VP7 (Lopez & Arias, 2004). Following attachment, the TLP is internalised by directly penetrating the membrane or via receptor-mediated endocytosis (Fukuhara *et al.*, 1987; Ludert *et al.*, 1987).

1.5.2 Positive-sense ssRNA transcription and protein synthesis

Uncoating of the outer capsid layer due to a change in calcium concentration initiates conformation changes to the DLP and the activation of VP1 and VP3, making it transcriptionally active (Ruiz *et al.*, 2000). Hydrophobic interactions between VP6 and VP2 are essential for forming stable transcriptionally active DLPs (Charpilienne *et al.*, 2002).

The negative-sense strand of the viral genome is used as a template by VP1 to transcribe viral (+)ssRNA that is capped by VP3 at the 5' end (Patton, 1996; Patton & Chen, 1999). The (+)ssRNA transcripts are synthesised inside the DLP and released from the virion core through class I channels (Lawton *et al.*, 2000). The released (+)ssRNA serves as a template for protein synthesis or the synthesis of dsRNA genomes (Chen *et al.*, 1994).

Normal cellular mRNA has a 3' poly-A tail recognised and bound by poly-A binding protein (PABP) that interacts with eukaryotic initiation factor 2- α (eIF2- α), initiating translation. Rotavirus (+)ssRNA lacks the poly-A tail and cannot be bound by eukaryotic PABP. NSP3 recognises the GACC motif at the non-polyadenylated 3' of the viral mRNA and binds it (Gratia *et al.*, 2015). NSP3 competes with PABP for binding to eIF2- α , causing the viral (+)ssRNA to circularise, increasing viral protein expression (Gratia *et al.*, 2015; Groft & Burley, 2002).

1.5.3 Viroplasm formation, (+)ssRNA packaging and DLP formation

Membrane-less electron-dense inclusion bodies called viroplasms form in rotavirus infected cells 2-3 hours post-infection (Carreño-Torres *et al.*, 2010; Eichwald *et al.*, 2004). Replication of the viral dsRNA genome and DLP assembly occur in the viroplasm. The interaction between NSP2 and NSP5 is essential for viroplasm formation (Fabbretti *et al.*, 1999). Viral structural proteins VP1, VP2, VP3, VP6, and (+)ssRNA are also found in the viroplasm (Cheung *et al.*, 2010). Viroplasms are organised into five concentric layers, with NSP4 and NSP2 surrounding

NSP5 found in the centre of the viroplasm. The viral proteins VP1, VP2, and VP6 form the intermediate zone and outer zones are comprised of VP4 and VP7 (Garces Suarez *et al.*, 2019). Viroplasms also associate with cellular protein tubulin and lipid droplets (Crawford & Desselberger, 2016; Criglar *et al.*, 2014).

The mechanism rotavirus uses to assemble a complete set of all 11 (+)ssRNA into the core particles is poorly understood. It is hypothesised that VP1 recognises (+)ssRNA of each genome segment, the complex interacts with VP2 and VP3, and NSP2 interacts with the (+)ssRNA remodelling their secondary structure (Berois *et al.*, 2003; Borodavka *et al.*, 2017; Lu *et al.*, 2008). Once the viral (+)ssRNA is packaged, it is replicated, and VP6 coats the core particles forming DLPs.

1.5.4 Virion maturation and release

Newly formed DLPs are believed to transfer from the viroplasm to the ER by interacting with NSP4. The VP6 binding domain on NSP4 facilitates DLP transport (Meyer *et al.*, 1989). In the ER, DLPs acquire a transient envelope (Estes & Greenberg, 2013). The transient envelope is removed when VP7 and VP4 bind to the DLP in a calcium rich and acidic environment to form TLPs (Trask & Dormitzer, 2006). Rotavirus TLPs exit non-polarised cells via cell lysis. Rotavirus exits polarised cells using a different mechanism; however this mechanism is poorly understood (Gardet *et al.*, 2006; McNulty *et al.*, 1976).

1.6 Role of NSP4 in rotavirus pathogenesis

Rotaviruses are mainly transmitted through the oral-faecal route. However, recent studies have shown that it can also be transmitted through saliva as the virus replicates well in salivary glands (Gasparinho *et al.*, 2014; Ghosh *et al.*, 2022). Diarrhoea is the main clinical sign of rotavirus infection. Other symptoms include vomiting and fever (Staat *et al.*, 2002). The virus uses several mechanisms to induce diarrhoea. Rotavirus affects calcium signalling in infected cells, an important factor in pathogenesis, replication, and morphogenesis (Greenberg & Estes, 2009). Paracrine purinergic signals that manifest as intracellular calcium waves are produced by rotavirus-infected cells and dysregulate calcium signalling in uninfected cells (Chang-Graham *et al.*, 2020). The intracellular calcium waves are caused by the release of adenosine 5'-diphosphate (ADP) that activates the purinergic receptors on nearby cells (Chang-Graham *et al.*, 2020).

Non-structural protein 4 (NSP4) is a 28kDa protein composed of 175 amino acids encoded by genome segment (GS) 10 (Horie *et al.*, 1999; Taylor *et al.*, 1996). NSP4 is a complex,

pleiotropic, multifunctional glycoprotein essential for viral RNA transcription, morphogenesis, and replication (Hu *et al.*, 2012; Lopez *et al.*, 2005). NSP4 functions both intracellularly and extracellularly during viral replication. NSP4 is an endoplasmic reticulum (ER) transmembrane protein with multiple domains (Figure 1.5) (Hu *et al.*, 2012).

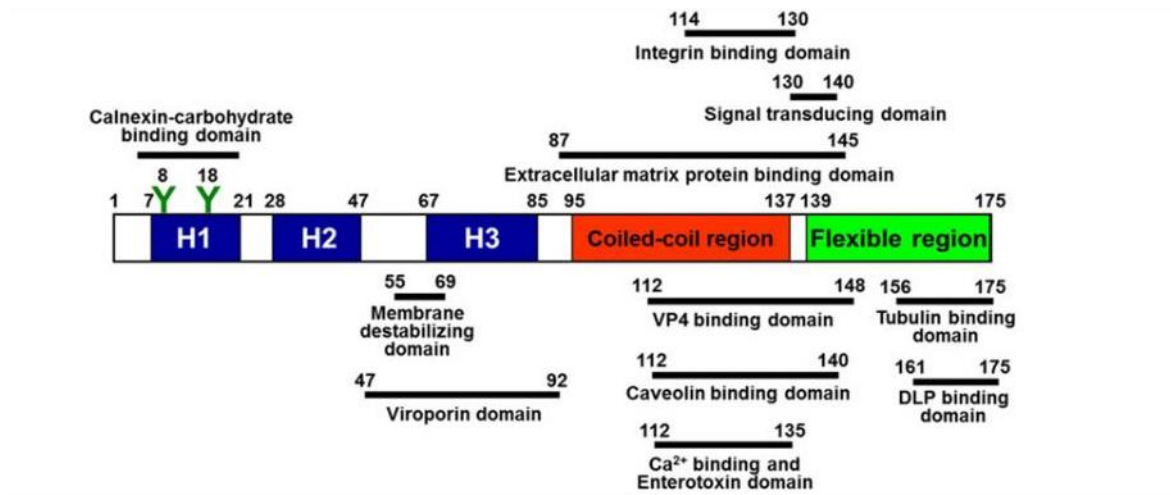


Figure 1.5: A schematic representation of the functional domains of NSP4. NSP4 is composed of three regions, the hydrophobic region, coiled-coil region, and flexible region. The hydrophobic region contains glycosylation sites, the viroporin domain and the membrane destabilisation domain. The hydrophobic region is followed by the coiled-coil region composed of the extracellular matrix binding domain, the VP4 binding domain, the caveolin binding domain, the integrin binding domain and the signal transduction domain. The flexible region is comprised of the DLP binding domain and tubulin binding domain. Image from Hu *et al.*, 2012, with permission.

NSP4 has three hydrophobic regions (H1-H3) located in the N-terminal domain. The H1 region has two N-linked glycosylation sites (Bergmann *et al.*, 1989). The N-glycosylation motif is comprised of asparagine-X-serine/threonine, X can be any amino acid excluding proline (Nurdin *et al.*, 2023). Using rotavirus reverse genetics (Section 1.9) it has been shown that N-glycosylation is important for the protein's localisation and viral replication. Replication was found to be at least 10-fold less in different cell lines when both glycosylation sites are defective (Nurdin *et al.*, 2023).

Intracellular NSP4 functions as a viroporin. Viroporins are small hydrophobic proteins encoded by viruses that form hydrophilic pores by oligomerising in the host cell's membrane, altering the host cell's cellular functions (Nieva *et al.*, 2012). Amino acids 47-92 (Figure 1.5) form the viroporin domain that oligomerises in the ER (Hyser *et al.*, 2010; Hyser *et al.*, 2012). NSP4 is

considered a three-pass transmembrane protein, Figure 1.6 (Hyser *et al.*, 2010). Several lysine amino acids of NSP4 interact with the ER phospholipids promoting the insertion of the viroporin domain into the ER (Hyser *et al.*, 2010). The C-terminal domain of NSP4 remains on the cytoplasmic side of the ER membrane. This region possibly forms two helix hairpin structures in the C-terminal domain, allowing NSP4 to oligomerise, forming an aqueous pore (Hyser *et al.*, 2010). Once the viroporin has successfully formed NSP4 can affect calcium homeostasis, increasing cytosolic calcium levels. The NSP4 viroporin domain causes Ca^{2+} efflux from the ER, activating STIM1 (an ER calcium sensor), which activates the plasma membrane's Ca^{2+} influx channels (Hyser *et al.*, 2013). The role of the viroporin domain in inducing diarrhoea during rotavirus infection has yet to be confirmed.

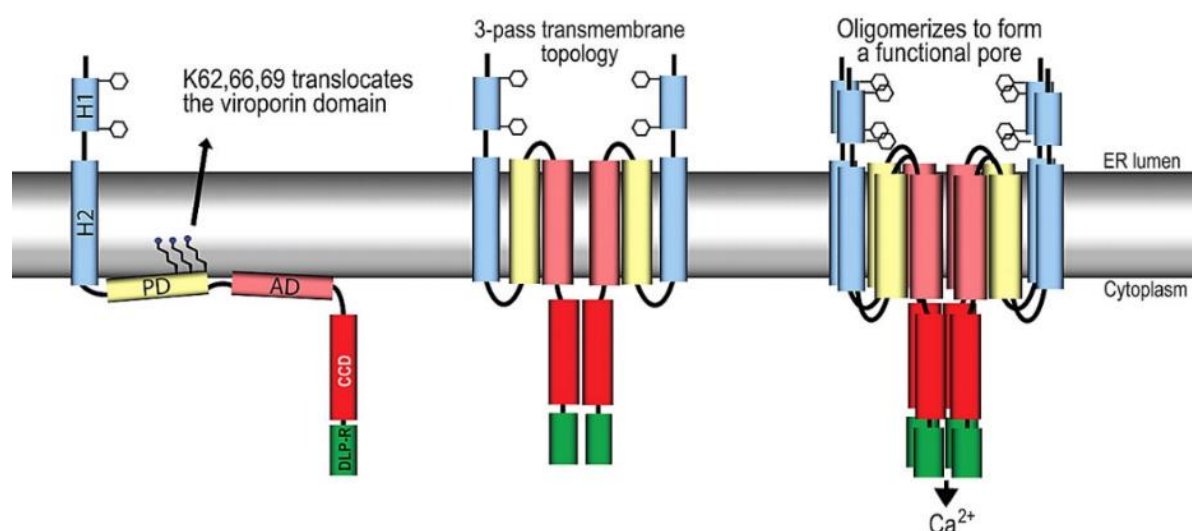


Figure 1.6: Proposed three-pass membrane topology of the NSP4 viroporin. The H2 domain is inserted into the ER membrane allowing the lysine residues to interact with the phospholipids in the ER membrane. This interaction promotes the insertion of the viroporin domain into the ER membrane leading to the oligomerisation of NSP4, forming an aqueous pore. Image taken with permission from Hyser *et al.* 2010.

Following the hydrophobic region is the cytoplasmic coiled-coil domain (CCD) (Figure 1.5), composed of multiple domains. Depending on the pH, the CCD is either a tetramer or pentamer. At neutral pH, the CCD has a high affinity for calcium ions and forms a tetramer, while at low pH, it has no affinity for calcium and forms a pentamer (Sastri *et al.*, 2014). It is proposed that the changes in calcium levels and pH during rotavirus replication promote the

structural changes in NSP4, which promotes multifunctional uses during replication (Sastri *et al.*, 2014).

Extracellular NSP4 is released from the cell and functions as an enterotoxin that enters intestinal cells. While attempting to produce NSP4 antiserum in mice, researchers found that NSP4 induced diarrhoea (Ball *et al.*, 1996). This was the discovery of the first viral enterotoxin. Extracellular NSP4 binds to integrins $\alpha 1\beta 1$ and $\alpha 2\beta 2$ to enter cells using the integrin binding domain (aa114-130) located in the enterotoxin domain, Figure 1.5 (Seo *et al.*, 2008). Integrin binding is mediated by glutamic acid (E120) located in the integrin binding domain (Deepa *et al.*, 2007). A cleavage product of NSP4 (aa112-175) is secreted, activating the calcium signalling pathways in infected intestinal cells to cause diarrhoea (Zhang *et al.*, 2000). The enterotoxin domain is comprised of amino acids 112-135 (Figure 1.5). Via its enterotoxin activity, NSP4 induces diarrhoea by different mechanisms.

1.6.1 Pathophysiology of NSP4

As mentioned in Section 1.5, rotaviruses infect the enterocytes in the villi of the mature small intestine. Infected cells are damaged during viral replication, decreasing the surface area of the intestinal lumen because of the decrease in villous tips (Figure 1.7A) (Leung *et al.*, 2005; Marthaler *et al.*, 2013). Due to the destruction of the absorptive villous cells, there is net secretion since the crypt cells (progenitor cells of the villous cells) are secretory (Leung *et al.*, 2005; Ramig, 2004). The net secretion leads to diarrhoea due to malabsorption because chloride ions (Cl^-) are secreted, leading to the osmotic movement of water into the intestinal lumen (Ramig, 2004).

Another mechanism rotaviruses use to induce diarrhoea is expressing NSP4, which functions as an enterotoxin. One of the mechanisms is targeting proteins associated with the tight junction, disrupting the tight junction and causing paracellular leakage (Tafazoli *et al.*, 2001). A study by Tian and coworkers proved that NSP4 increases intracellular calcium levels by activating the phospholipase C (PLC) signalling transduction pathway (Figure 1.7B) (Tian *et al.*, 1995). When NSP4 activates phospholipase C, inositol-1,4,5-trisphosphate (IP3) is increased in the cell, causing an increase in intracellular Ca^{2+} (Lorrot & Vasseur, 2007; Tian *et al.*, 1995). Only amino acids 114-135 in the enterotoxin domain are necessary to activate the PLC signalling pathway (Tian *et al.*, 1995).

The increase in intracellular calcium induces the release of neurotransmitters such as serotonin that activate the enteric nervous system (ENS), Figure 1.7B (Crawford *et al.*, 2017; Hagbom *et al.*, 2011). In turn, the activation of the ENS causes an increase in cellular cAMP

levels (Crawford *et al.*, 2017). This activates the anoctamin Cl^- channel causing excess chloride secretion (Cl^-) and inhibition of sodium (Na^+) absorption leading to the movement of water into the intestinal lumen causing secretory diarrhoea (Crawford *et al.*, 2017; Lundgren *et al.*, 2000; Ousingawatt *et al.*, 2011).

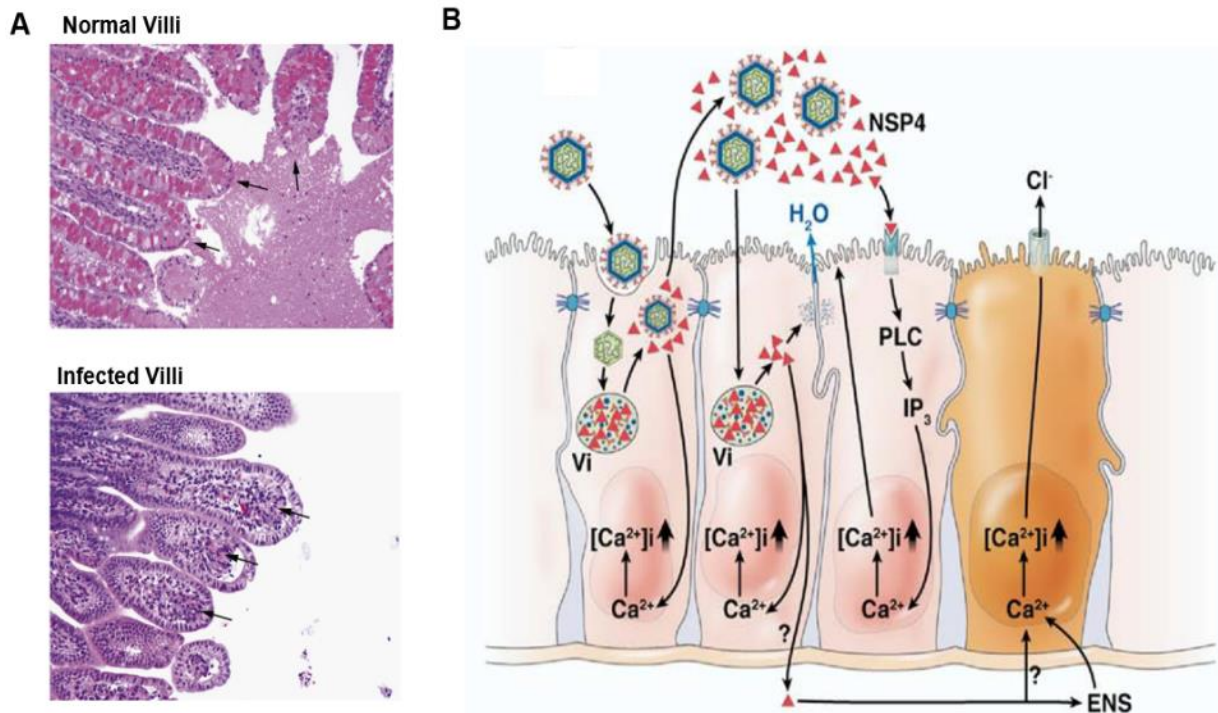


Figure 1.7: Mechanism of rotavirus-induced diarrhoea in infected enterocytes. (A) Normal villi and damaged rotavirus infected villi. The atrophy of infected enterocytes causes the villi to become blunt. (B) NSP4 also causes paracellular leakage in infected cells by disrupting tight junctions. Extracellular NSP4 released from RV infected cells activates the PLC signalling pathway to increase intracellular calcium. An increase in intracellular calcium leads to the activation of the ENS, leading to increased chloride secretion and vomiting. Images used with permission from Marthaler *et al.* 2013 and Greenberg & Estes 2009.

1.6.2 Mutations in rotavirus NSP4 associated with altered virus virulence

A study published in 1998 showed that virulent and tissue culture attenuated rotaviruses of two porcine strains (OSU and Gottfried) have differences in pathogenicity (Zhang *et al.*, 1998). They analysed the amino acid sequences of NSP4 of the virulent and attenuated viruses to determine if there were any differences (Figure 1.8A). They found six mutations, and three of the six mutations clustered together between amino acids 131-140 (Figure 1.8A). Amino acids

131-140 form part of the signal-transducing domain of NSP4, the region of interest for this project.

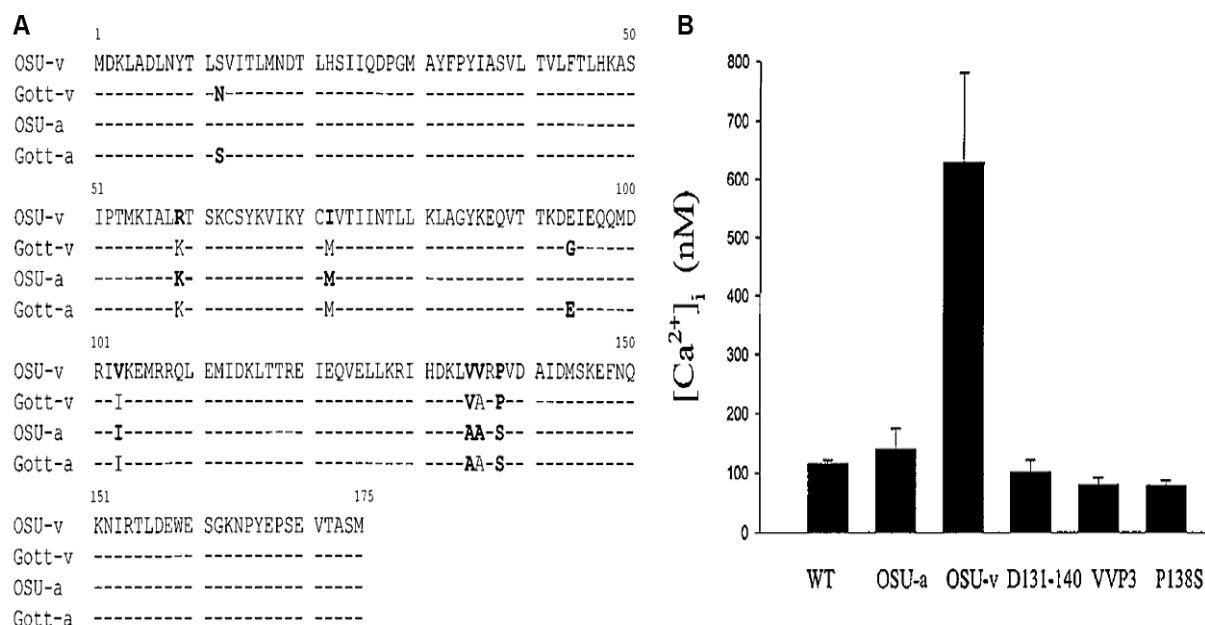


Figure 1.8: NSP4 sequence variation in attenuated and virulent porcine RVs and its effect on intracellular calcium. A) Comparison of attenuated and virulent NSP4 amino acid sequences from OSU and Gottfried rotavirus. The amino acids are shown in single-letter format. Dashes indicate the same residue as in the first line and residues that differ are bolded. Highest sequence variation clustered between aa135-141. B) Intracellular calcium levels in Sf9 cells expressing OSU-a NSP4, OSU-v NSP4 and OSU-v mutants. Cells infected with wild-type baculovirus (WT) were used as a control. Sf9 cells were infected with recombinant baculovirus expressing OSU-a NSP4, OSU-v NSP4, OSU-v with an aa 135-141 deletion, OSU-v with a substitution mutation at 135,136 &138 (VVP to AAS) and OSU-v NSP4 with a P135S point mutation. Mutations in the signal transduction domain (aa 135-141) altered OSU NSP4's ability to mobilise intracellular calcium. Used with permission from Zhang et al. 1998.

NSP4 is known to affect calcium signal transduction to increase intracellular calcium levels, which in turn causes diarrhoea. During rotavirus infection, intercellular calcium levels increase by more than 3-fold (Michelangeli *et al.*, 1991). Zhang and colleagues determined that baculovirus expressed NSP4 of attenuated and virulent OSU (OSU-a and OSU-v) NSP4 mobilised calcium differently. While OSU-v NSP4 can cause an increase in intracellular calcium and diarrhoea, OSU-a NSP4 does not increase in intracellular calcium or cause diarrhoea (Figure 1.8B) (Seo *et al.*, 2008; Zhang *et al.*, 1998). Deleting aa 131-141 of OSU-v

NSP4 or changing amino acids 135,136 and 138 of OSU-v to those of OSU-a attenuates the protein's ability to increase intracellular calcium (Figure 1.8B).

1.7 Antagonism of the innate immune response by rotavirus

1.7.1 Detection of rotavirus by innate immune receptors

Living organisms utilise different mechanisms to protect themselves from foreign microorganisms. The innate immune response is the first defence mechanism. It uses several germline-encoded cytosolic and membrane-bound receptors, called pattern recognition receptors (PRRs), to recognise conserved regions in different pathogens to provide protection against invading pathogens (Medzhitov & Janeway, 1997). These PRRs include cytosolic viral DNA sensors, retinoic acid-inducing gene 1-like receptors (RLRs), Toll-like receptors (TLRs), protein kinase R and nucleotide oligomerisation domain-like receptors (NLRs) (Takeuchi & Akira, 2009; Thompson *et al.*, 2011). Detection of viral nucleic acids by these PRRs leads to different signalling cascades. NLRs are primarily responsible for activating caspase1, which leads to the maturation of interleukin (IL) 18 and IL-1 β (Thompson *et al.*, 2011). On the other hand, TLRs, RLRs and DNA sensors are essential in cytokine and interferon production (Thompson *et al.*, 2011).

Viruses are known to use different strategies to subvert the innate immune response. Rotavirus employs NSP1 and VP3 to evade the innate immune response. The newly synthesised viral dsRNA genome is hidden in the viroplasm, where genome replication and DLP formation occur to protect it from triggering the innate immune response (Trask *et al.*, 2012). However, during rotavirus infection, viral dsRNA is detected by the RLRs (RIG-1 and MDA-5) and TLR3. During rotavirus infection, the PRRs retinoic acid-inducing gene I (RIG-I) or melanoma differentiation-associated protein 5 (MDA-5) in the cytoplasm or toll-like receptor 3 (TLR3) in the endosome recognise viral dsRNA (Figure 1.9A) (Broquet *et al.*, 2011; Zhou *et al.*, 2007). The exact mechanism of how RIG-1 and MDA-5 detect rotavirus dsRNA is poorly understood since viral dsRNA synthesis occurs in the viroplasm during replication. Silencing RIG-1 and MDA-5 using siRNA or infecting cells lacking RIG-1 and MDA-5 showed that RIG-1 and MDA-5 signalling is activated during infection to activate the production of IFN- β (Broquet *et al.*, 2011; Sen *et al.*, 2011). During the transcription of viral mRNA, VP3 caps the transcripts to protect them from detection from the innate immune response, Section 1.5.2. However, VP3 does not efficiently cap all transcripts and these partially capped and uncapped viral transcripts activate RIG-I and MDA-5 (Uzri & Greenberg, 2013).

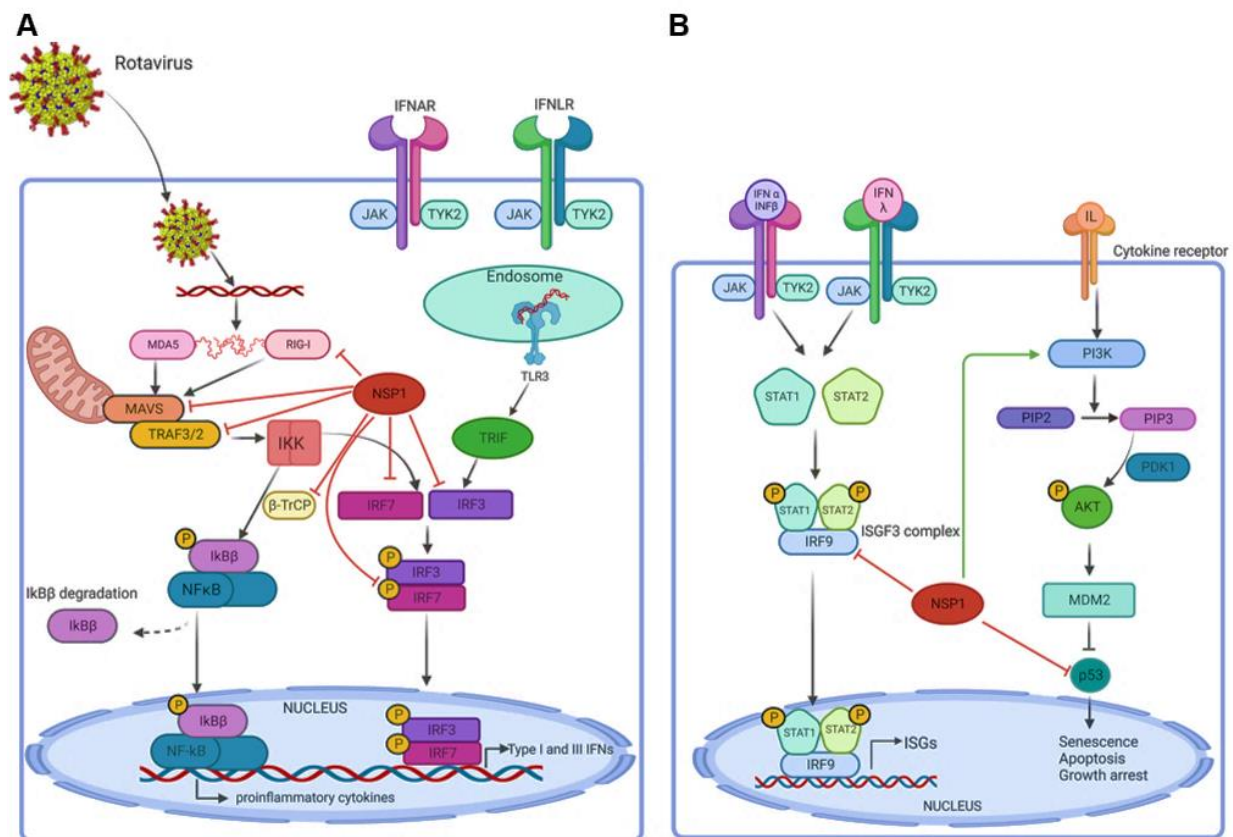


Figure 1.9: Antagonism of the innate immune response by NSP1. A) Detection of viral RNA by MDA-5, RIG-I, and TLR3 activates downstream pathways to activate IRF3, IRF7, and NFκB to induce transcription of proinflammatory cytokines and interferons. RVA NSP1 inhibits the innate immune response by targeting proteins activated by RIG-I, MAVS, and TLR3. B) Activation of the JAK-STAT pathway and p53 by IFNs and interleukins from infected neighbouring cells. NSP1 inhibits IFR9 and p53 to prevent transcription of ISGs and cell death. Image created with Biorender, adapted from Morelli et al. 2015.

1.7.2 Induction of interferon signalling and JAK-STAT pathway by rotavirus

When PRRs identify viral ligands, a complex signalling cascade is activated, triggering the activation of transcription factors. The transcription factors translocate to the nucleus and bind DNA, increasing the expression of proinflammatory molecules. A specific set of secreted macromolecules that interfere with the replication of live influenza virus was identified and were termed interferons (INFs) (Isaacs & Lindenmann, 1957). Interferons are cytokines that can inhibit viral replication by inducing an antiviral state (Fensterl & Sen, 2009). Interferons are grouped into three classes (type I-III). Type I and III INFs are produced in direct response to viral infection, Figure 1.9A (Randall & Goodbourn, 2008). There is an extensive range of type I INFs, but only IFNα and IFNβ are produced in response to viral infection (Randall & Goodbourn, 2008). Type II interferons are not made in direct response to viral infection but

are released by mitogen-activated natural killer cells and T-lymphocytes (Randall & Goodbourn, 2008). Detecting viral nucleic acid PRRs stimulates the production of $\text{INF}\alpha$, $\text{INF}\beta$, and proinflammatory cytokines.

During rotavirus infection, activation of the RIG-I and MDA-5 signalling cascade activates the nuclear transcription factor NF κ B. The activation of MAVS by RIG-I and MDA-5 activates the inhibitor of nuclear factor- κ B (I κ B) kinase (IKK), which initiates the phosphorylation, ubiquitination and degradation of I κ B that is bound to NF κ B (Figure 1.9A) (Liu *et al.*, 2017).

Inhibitor of nuclear factor- κ B inhibits NF κ B from translocating into the nucleus. When it is degraded, NF κ B translocates to the nucleus, initiating transcription of proinflammatory cytokines. Activation of IKK by MAVS also activates IRF3 and IRF7 (Figure 1.9A). In the endosome, ssRNA and dsRNA activate TLR7 and TLR3, respectively (Kawasaki & Kawai, 2014). Recognition of dsRNA by TLR3 initiates downstream signalling to MyD88, causing the phosphorylation of IRF7 (Kawasaki & Kawai, 2014). Activated TLR7 induces the phosphorylation of IRF3 by recruiting TRIF, which activates IKK α that phosphorylates IRF3 (Kawasaki & Kawai, 2014). Phosphorylated IRF3 can form homodimers that translocate to the nucleus inducing transcription of $\text{INF}\alpha$, or heterodimers with phosphorylated IRF7 inducing the transcription of $\text{INF}\alpha/\beta$ (Figure 1.9A) (Honda *et al.*, 2005; Jefferies, 2019). Homodimers of phosphorylated IRF3 translocate to the nucleus and initiate transcription of $\text{INF}\beta$ (Jefferies, 2019).

1.7.3 Rotavirus mediated inhibition of the innate immune response

1.7.3.1 Inhibition of the interferon signalling pathway

NSP1 of RVA inhibits the signalling cascade activated by RIG-I, MDA-5 and TLR3 by inhibiting the downstream proteins in the pathway. It is hypothesised that NSP1 functions as an E3 ubiquitin ligase that polyubiquitinates target proteins, causing their degradation (Morelli *et al.*, 2015). Davis and colleagues showed that NSP1 from OSU, Wa and DS1 strains have an I κ B-like degron (IKD) in their phosphorylated C-terminal domain (Davis *et al.*, 2017). Phosphorylation of this domain is essential for NSP1 to interact with β -TrCP marking it for degradation and preventing it from ubiquitinating I κ B, thereby inhibiting nuclear translocation of NF κ B, Figure 1.9A (Davis *et al.*, 2017). Rotavirus strains such as SA11 lack the IKD domain and cannot degrade β -TrCP (Davis *et al.*, 2017). Rotavirus OSU and SA11 with NSP1 with a naturally truncated IRF binding domain have been shown to down-regulate RIG-I by binding it and causing proteasomal degradation (Qin *et al.*, 2011). NSP1 inhibits the production of $\text{INF}\beta$ by inhibiting TRAF2 (Figure 1.9A). It is hypothesised that the N- and C- terminus of NSP1 are needed to degrade TRAF2 because the C-terminal can bind TRAF2 but cannot degrade

it; only full-length NSP1 can cause ubiquitin-mediated degradation of TRAF2 (Bagchi *et al.*, 2013).

The conserved N-terminal DNA binding domain of mammalian IRFs binds IFN-stimulated response elements (ISREs) in the promoter region of IFN genes and ISGs (Morelli *et al.*, 2015). Interferon regulatory factors (IRF) 3-9 have a type 1 C-terminal IRF association domain (IAD), and IRF1-2 has a type 2 C-terminal IRF association domain (Morelli *et al.*, 2015). Rotavirus SA11 inhibits the upregulation of type I interferons in infected cells by likely binding the conserved N-terminal DNA binding domain in IRF3, IRF5 and IRF7, causing their proteasomal degradation (Barro & Patton, 2007). NSP1 also inhibits the induction of type III interferons by inhibiting IRF1 (Iaconis *et al.*, 2021). Human rotaviruses inhibit IFR-1 better than IFR-3 or IFR-7 compared to other rotavirus strains (Iaconis *et al.*, 2021). The mechanism used by NSP1 to inhibit IRF1 is unknown, as the protein is not degraded in infected cells, unlike IRFs that contain the type 1 C-terminal domain IAD.

1.7.3.2 Inhibition of the JAK-STAT pathway

Interferons released from virus-infected cells induce the expression of interferon-stimulated genes (ISGs) in neighbouring uninfected cells via the Janus kinase–signal transducer and activator of transcription (JAK-STAT) pathway (Fensterl & Sen, 2009). Interferon-stimulated genes cause an antiviral state impeding viral replication at any stage (Goubau *et al.*, 2013). The JAK-STAT pathway regulates systemic and local inflammation during viral infection (Ezeonwumelu *et al.*, 2021). During viral infection, secreted type I interferons (IFN α and IFN β) bind the heterodimeric receptor IFNAR (Fensterl & Sen, 2009). Interferon λ , a type III interferon, activates the JAK-STAT pathway by binding the receptor IFNLR, Figure 1.9B (Majoros *et al.*, 2017). Receptor binding recruits the tyrosine-phosphorylated JAK proteins JAK1 and TYK2, which phosphorylate STAT1 and STAT2 in the SH domain (Decker *et al.*, 2002). STAT1 is phosphorylated at tyrosine 701 and STAT2 phosphorylated at tyrosine 692 (Decker *et al.*, 2002). A complex called interferon-stimulated gene factor 3 (ISGF3) forms between phosphorylated STAT1/STAT2 heterodimers and IRF9 (Figure 1.9B). The transcription complex translocates to the nucleus and recognises interferon-stimulated response elements in the promoters and enhancers of ISGs, activating their transcription, Figure 1.9B (Decker *et al.*, 2002).

Rotavirus inhibits the JAK-STAT pathway to inhibit the expression of ISGs that can inhibit viral replication. Rotavirus uses three different mechanisms to inhibit the STAT-mediated expression of ISGs. These include inhibiting nuclear accumulation of STAT1 and STAT2, degrading IFR-9, and inhibiting the oligoadenylate synthetase (OAS)-latent ribonuclease

(RNaseL pathway). In the absence of IFN, STAT1 and STAT2 are not phosphorylated and localise in the cytoplasm of the cells (Figure 1.10). In the presence of IFN, phosphorylated STAT1, and STAT2 localise to the nucleus (Figure 1.10). In contrast, when IFN-stimulated cells were infected with RRV rotavirus, it was found that STAT1 and STAT2 localised in the cytoplasm, Figure 1.10 (Holloway *et al.*, 2009). Rotavirus did not inhibit IFN-stimulated phosphorylation of STAT1 and STAT2 at 6 hours post-infection (hpi), but inhibited STAT1 phosphorylation at late stages of infection (16-20 hpi) in IFN- β stimulated HT-29 cells (Holloway *et al.*, 2009).

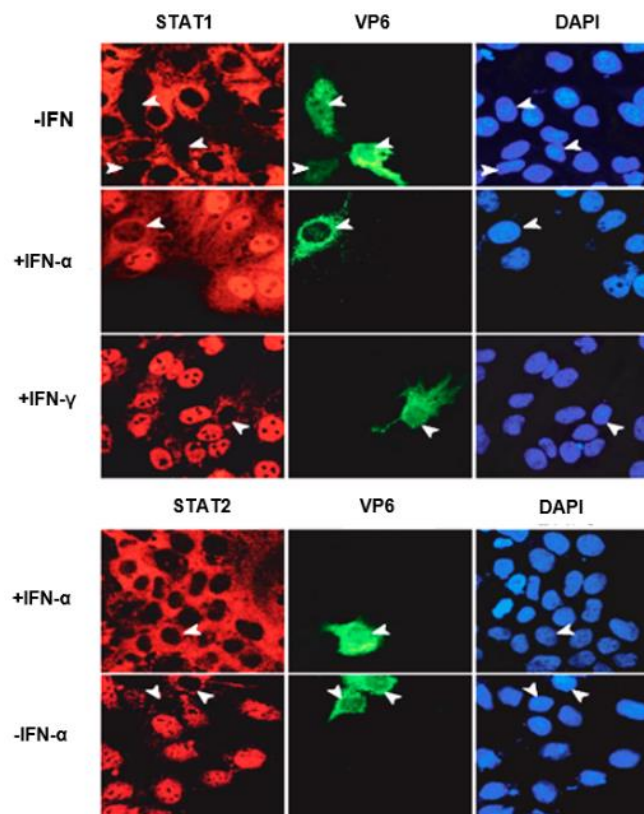


Figure 1.10: Rotavirus inhibition of nuclear translocation of STAT1 and STAT2. In the absence of interferon stimulation, STAT1 and STAT2 localise in the cytoplasm of uninfected and infected MA104 cells. Stimulating cells with INF allows the translocation of STAT1 and STAT2 into the nucleus. Nuclear accumulation of STAT1 and STAT2 is inhibited in rotavirus infected cells. Rotavirus infected cells were visualised using monoclonal antibodies against VP6, DAPI was used to visualise the nucleus and STAT1, and STAT2 were visualised with antibodies against STAT1 and STAT2. Used with permission from Holloway *et al.* 2009.

As mentioned in Section 1.7.4.1, NSP1 targets the type 1 C-terminal IAD present in IRF 3-9. NSP1 of SA11-4F and human Wa can recognise IAD1 in IRF-9 and induce proteasomal degradation (Arnold *et al.*, 2013). The degradation of IRF9 prevents the formation of the ISGF3

complex, which could be a mechanism used by NSP1 to prevent nuclear translocation of activated STAT1 and STAT2. When stimulated by IFN- γ phosphorylated STAT1 can form homodimers that do not need to bind IRF9, meaning that degradation of IRF9 is not the only mechanism used by rotavirus to inhibit STAT1 nuclear accumulation (Holloway *et al.*, 2014). Phosphorylated STAT1 and STAT2 interact with importin α 5, which transports them to the nucleus (Sekimoto *et al.*, 1997). Rotavirus does not interfere with this interaction in early stages of infection and inhibits the translocation of activated STAT1 and STAT2 using an unknown mechanism (Holloway *et al.*, 2014).

During viral infection OAS, a dsRNA sensor that activates RNaseL, is one of the ISGs that is expressed (Zhang *et al.*, 2013). Viral ssRNA and cellular RNA are cleaved by activated RNaseL, and the cleaved RNA activates RIG-I and MDA-5, Figure 1.11 (Morelli *et al.*, 2015; Ogden *et al.*, 2015). SA11 NSP1 inhibits the OAS-RNaseL pathway by causing ubiquitination-dependent degradation of RNaseL (Figure 1.11), the domain responsible for this activity is located upstream of the IRF binding region, Figure 1.12A (Dai *et al.*, 2022). VP3 also inhibits the OAS-RNaseL pathway by cleaving 2',5'-oligoadenylates, using its 2',5'-phosphodiesterase domain, preventing activation of RNaseL, Figure 1.11 (Song *et al.*, 2020).

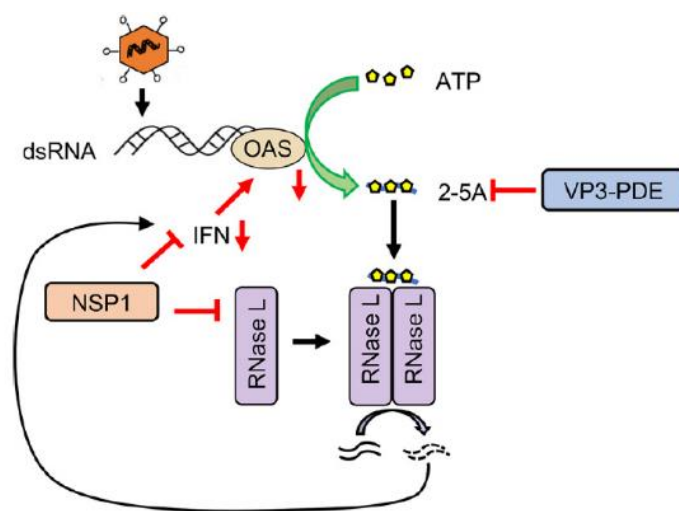


Figure 1.11: Mechanisms used by rotavirus to evade OAS-RNase L signalling. NSP1 inhibits interferon signalling, in turn inhibiting OAS production. The second strategy used by NSP1 is inducing the degradation of RNaseL. VP3 degrades 2'-5'-oligoadenylate, signalling molecules produced by OAS, preventing the activation of RNaseL. Image used with permission by Dai et al. 2022.

During infection, virus spread is limited by apoptosis of infected cells via activation of p53. NSP1 of the RV strain A5-13 prevents premature apoptosis by activating the P13K/AKT

pathway leading to the phosphorylation of AKT, which causes the inhibition of p53 (Bagchi *et al.*, 2010). NSP1 of RVA A5-13 induces proteasome-dependent degradation of p53 to inhibit apoptosis, Figure 1.9B (Bhowmick *et al.*, 2013).

1.7.4 Domain organisation of NSP1

Rotavirus NSP1 is an innate immune response antagonist approximately 58kDa in size (Hua *et al.*, 1994; Morelli *et al.*, 2015). It is the least conserved protein compared to other RVA proteins because of its varying lengths between (486-496 aa) and high sequence variation in the C-terminal region, Figure 1.12A (Mitchell & Both, 1990). Rotaviruses with naturally truncated NSP1, such as bovine RVA, propagate well in permissive cell lines (Hua *et al.*, 1994). However, intact NSP1 is essential for effective virus transmission, replication, and pathogenesis *in vivo* (Hou *et al.*, 2021a). Analysis of NSP1 from different strains has helped to identify the important structural and functional domains (Morelli *et al.*, 2015). The N-terminus of NSP1 of group A and group A-like rotaviruses is a conserved cysteine/histidine-rich zinc-binding RING domain (Hua *et al.*, 1994). The RING-domain, which has the following conserved sequence: C-X2-C-X8-C-X2-C-X3-H-X-C-X2-C-X5-C (Figure 1.12B), X being any other amino acid (Arnold & Patton, 2009). The RING domain mediates NSP1 binding to the 5' end of viral mRNA (Brottier *et al.*, 1992; Hua *et al.*, 1994). Binding viral mRNAs could be a protective measure to prevent the mRNA from being detected by PRRs (Hua *et al.*, 1994).

The region of interest in this study is the cytoskeleton binding region of NSP1 which spans amino acids 84-176. It was identified using deletion mapping in 1994 (Hua *et al.*, 1994). Truncated forms of NSP1 were expressed in CV-1 cells infected with the vaccinia virus (VV) strain vTF7-3. The location of the expressed proteins was determined using immunofluorescence. NSP1 with a deletion of aa 9-83 localised similarly to wildtype NSP1 from infected or transfected cells (Figure 1.13). This showed that the cysteine-rich protein region is not necessary for cytoskeleton binding. The C-terminal domain is also not essential for localising the protein, as the mutants with deletions between aa 117 and 476 localised similarly to wild-type NSP1. However, NSP1 with a deletion at aa 82-476 translocated to the nucleus indicating that the cytoskeleton binding domain had been deleted (Figure 1.13). The cytoskeleton binding domain was determined to span aa 84 to 176.

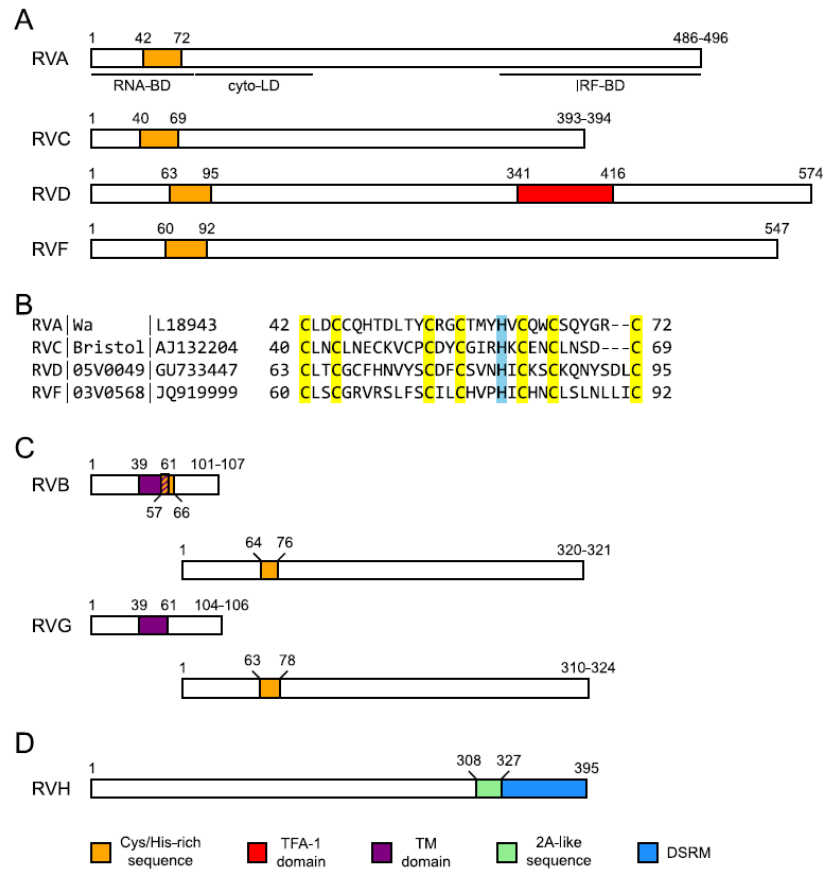


Figure 1.12: Schematic representation of RVA-RVH NSP1 domains. (A) Rotavirus A and RVA-like NSP1 contain an N-terminal Cys/His rich sequence (orange) within the RNA binding domain, followed by a cytoskeleton binding domain and IRF binding domain at the C-terminus. A TFA-1 domain (red) is located in the C-terminus of RVD NSP1. (B) Alignment of the NSP1 ring domain of group A and group A-like rotaviruses. (C) RVB NSP1 overlapping reading frames, ORF1 (purple) has a TM-like central domain and ORF2 (orange) contains a Cys-His RING domain. (D) A DSRM (blue) and 2A-like sequence (green) are found in RVH NSP1. Image used with permission from Morelli et al. (2014).

NSP1 from non-RVA have sequences that are not present in RVA NSP1. Genome segment (GS) 5 of RVB has two overlapping ORFs (Figure 1.12C). The ORF2 product is frequently detected in *in-vitro* translation systems (Eiden, 1994; Morelli et al., 2015). A Cys/His rich sequence is present at the N-terminus of the protein product of ORF2 (Kobayashi et al., 2001). A transmembrane (TM) domain is present in RVB and RVG, and RVH NSP1 contains a dsRNA binding motif (DSRM) in its C-terminal domain, Figure 1.12D (Diller et al., 2019; Yang et al., 2004).

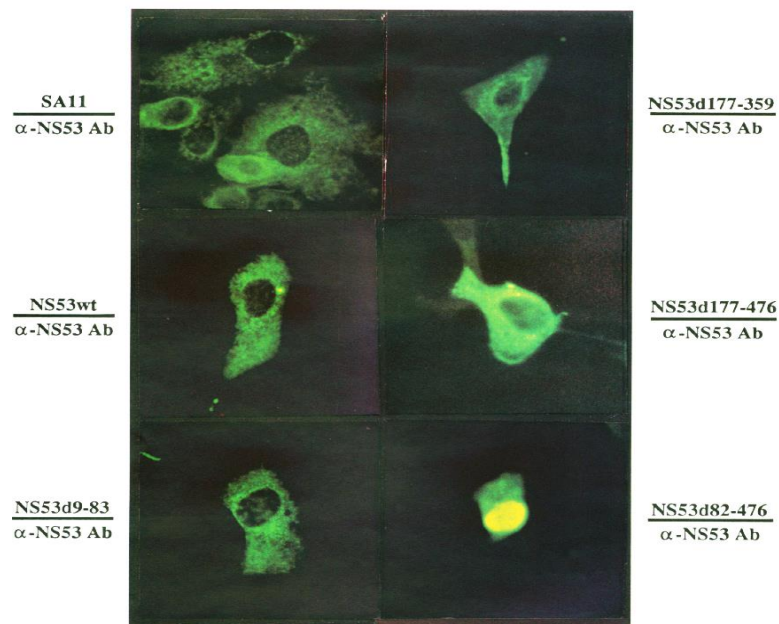


Figure 1.13: Immunofluorescence showing localisation of expressed wild-type and truncated NSP1. IF staining of NSP1 (previously NS53) in rotavirus infected cells or cells transfected with plasmids expressing full-length or truncated NSP1. SA11 NSP1 localised in the cytoplasm in MA104 cells during natural infection. Wild-type SA11 NSP1, NSP1 with a deletion at aa9-83(NS53d9-86) or a deletion at aa117-476 (NS53d117-359 and NS53d117-476) expressed in CV-1 cells localised in the cytoskeleton. NSP1 with deletion at aa82-476 (NS53 D82-476) conjugated in the nucleus. Used with permission from Hua et al. 1994.

1.8 Rotavirus Vaccines

In 1757, Dr Edward Jenner pioneered the concept of vaccination. He previously observed that young girls who milked cows with cowpox did not contract smallpox. He then inoculated an 8 year old boy with cowpox to prevent him from contracting smallpox (Dunn, 1996). Many vaccines have been developed over the years to protect people from disease.

Research into developing a rotavirus vaccine began in the 1970s using the Jennerian approach to vaccination. Researchers showed that calves infected with bovine rotavirus were protected against the strain they were inoculated with and human rotavirus (Vesikari *et al.*, 1983). Bovine rotavirus strain RIT4237 was the first human candidate live oral rotavirus vaccine tested in 1983 (Vesikari *et al.*, 1986; Vesikari *et al.*, 1983). A live candidate vaccine of rhesus origin was also tested, but the trial results were inconsistent (Vesikari *et al.*, 1986).

The first licensed rotavirus vaccine was a tetravalent vaccine called RotaShield. It is comprised of four strains, three human-rhesus reassortment strains that had a rhesus rotavirus (RVV) backbone with VP7 representing G1, G2, and G4 human rotavirus strains and a strain with

the RRV G3 VP7 (Kapikian *et al.*, 1996). The vaccine was used in the USA but removed from the market nine months after cases of intussusception were reported in some immunised children two weeks after immunisation (Control & Prevention, 1999; Simonsen *et al.*, 2001).

1.8.1 Current rotavirus vaccines

The World Health Organisation (WHO) has prequalified four RV vaccines since the removal of RotaShield from the market. These vaccines are licenced internationally (Table 1.2). The two widely used live oral rotavirus vaccines, Rotarix and RotaTeq, were licensed in 2006 and helped alleviate the disease burden. Rotarix (Figure 1.14A) is a two-dose attenuated monovalent vaccine of a human G1[P8] strain (Angel *et al.*, 2012; Glass *et al.*, 2021). RotaTeq is a three-dose pentavalent human-bovine reassortment vaccine; the WC3 bovine backbones have mono-reassortments and contain the capsid proteins of human G1, G2, G3, G3 and P[8] rotaviruses, Figure 1.14B (Glass *et al.*, 2021; Glass *et al.*, 2006). The WHO later prequalified Rotavac and RotaSiil; both vaccines are used in India, and Rotavac is used in Palestine (Burke *et al.*, 2019).

Table 1.2: WHO prequalified and nationally licensed live oral rotavirus vaccines. Table adapted from Burke *et al.* 2019 and Glass *et al.* 2021.

Name	Manufacturer	Formulation	Doses
WHO prequalified			
Rotarix	GlaxoSmithKline Biologicals	G1P[8]	3
RotaTeq	Merck	G1, G2, G3, G4, P[8]	2
Rotavac	Bharat Biologicals	G9P[11]	3
RotaSiil	Serum Institute of India	G1, G2, G3, G4, G9	3
Nationally licensed			
Lanzhou Lamb rotavirus vaccine	Lanzhou Institute of Biological Products	G10P[15]	4
Rotavin-M1	Polyvac	G1P[8]	3

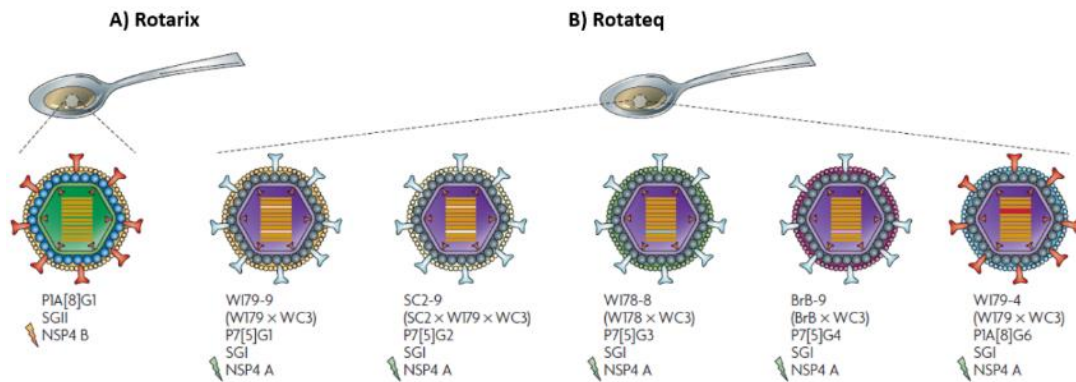


Figure 1.14: Composition of Rotarix and RotaTaq vaccines. A) Rotarix is a monovalent vaccine composed of a human P1A[8]G1, subgroup II VP6 and genogroup B NSP4. B) RotaTaq is a multivalent human-bovine reassortment vaccine comprised of five strains containing VP7 from different serotypes, subgroup I VP6, genogroup A NSP4 and VP3 and VP7 are from a human rotavirus strain. Image used with permission from Angel et al. 2007.

The Lanzhou Lamb rotavirus vaccine and Rotavin have not been prequalified by the WHO but are used in China and Vietnam, Table 1.2 (Burke *et al.*, 2019). These vaccines are locally produced and are similar to Rotarix as they are based on a common circulating strain within the country. The Lanzhou lamb rotavirus vaccine is a monovalent live attenuated vaccine based on a G10P [15] strain which is a lamb rotavirus that was isolated in 1985 (Fu *et al.*, 2007; Fu *et al.*, 2012). Rotavin-M1, licensed in Vietnam, is derived from a G1P[8] isolate from a Vietnamese child and passaged multiple times to attenuate its virulence (Burke *et al.*, 2019; Dang *et al.*, 2012).

Although potential correlates of protection against rotavirus infection have been studied, no clear correlates of protection have been found (Velázquez *et al.*, 2000). Both mucosal and serum antibodies could be associated with protection against RV infection. The currently available vaccines have significantly lowered the number of rotavirus-related deaths and hospitalisations (Burnett *et al.*, 2020a). However, the vaccines have low efficacy in low-income countries (42%-63%) compared to the effectiveness of 72%-98% in high-income countries (Sadiq *et al.*, 2018). The reasons for lower efficacy in low-income countries are still unknown. The proposed reasons for this phenomenon include environmental, host, and pathogen factors (Omatola & Olaniran, 2022). These include the composition of the gut microbiota, malnutrition, co-administration with the oral polio vaccine, differences in circulating rotavirus strains, and the presence of rotavirus antibodies acquired from breastmilk (Babji & Kang, 2012; Lopman *et al.*, 2012; Velasquez *et al.*, 2018). Understanding the reasons for lower efficacy in developing countries will help develop better vaccines for these regions.

1.8.2 Dead-subunit and non-replicating vaccine candidates

Concerns over the reduced efficacy of live oral RV vaccines in developing countries has led to researchers looking into alternative vaccine candidates that can be administered parenterally (Burke *et al.*, 2019). These include rotavirus virus-like particles (VLPs), inactivated viral particles and recombinant VP6 proteins expressed in bacteria (Ward *et al.*, 2008). The rationale for developing parenterally administered vaccines is that they can circumvent the challenges associated with orally administered vaccines. Moreover, dead subunit vaccines can be used as booster vaccines for partially protected individuals.

Virus-like particles are self-assembled multi-subunit viral protein structures that lack genetic material, therefore are non-infectious. They have antigenic proteins and do not replicate, making them a viable vaccine candidate. Rotavirus VLPs have been evaluated as possible subunit vaccines and were found to be immunogenic. Baculovirus-expressed VLPs were found to confer a high level of immunogenicity when administered parenterally in mice and rabbits (Conner *et al.*, 1996; Jiang *et al.*, 1999). However, VLPs only induced high levels of rotavirus specific IgG and low levels of IgA (Conner *et al.*, 1996; Jiang *et al.*, 1999). Similarly, baculovirus-expressed group A rotavirus VP2/6 VLPs were administered intranasally in neonatal gnotobiotic pigs and induced an IgA response when co-administered with mutant *E. coli* heat-labile toxin LT-R192G as an adjuvant (Yuan *et al.*, 2000). However, the VP2/6 VLP did not provide protection when challenged with orally administered virulent Wa.

Other possible rotavirus subunit vaccines that are being investigated include VP8* subunit vaccines. One of the VP8* subunit vaccines includes a parenterally administered Wa VP8* subunit fused to the P2 epitope of the tetanus toxin (Fix *et al.*, 2015). During clinical trials, this subunit vaccine induced an IgA response in all vaccine recipients but an IgG response in only one recipient (Fix *et al.*, 2015).

1.9 Reverse genetics

Classical forward genetics is used to determine the genetic basis of an observed change in phenotype. However, classical genetics can make it difficult to pinpoint the exact mutations responsible for a phenotypic change when multiple mutations are present (Lander & Kruglyak, 1995). With advances in molecular biology, a reverse genetics approach was developed. The reverse genetics (RG) approach includes altering the gene and observing the change in phenotype (Gurumurthy *et al.*, 2016). Viral reverse genetics is a powerful tool used to study the viral replication cycle, pathogenesis, functions of specific proteins, virus-host interactions, and vaccine development. This is achieved by introducing changes in the viral DNA or complementary DNA (cDNA) and generating viruses using the modified DNA.

Reverse genetics for positive sense RNA viruses was easier to achieve because the viral RNA acts as mRNA and can be directly transcribed when transfected into cells (Pekosz *et al.*, 1999). In comparison, reverse genetics for negative-sense RNA viruses proved to be more challenging because negative-sense RNA first needs to be converted to (+)ssRNA. The rescue of Q β bacteriophage by transforming bacteria with the viral cDNA was the advent of RNA virus reverse genetics (Taniguchi *et al.*, 1978). The first mammalian RNA virus reverse genetics system was then developed when infectious poliomyelitis virus was rescued from mammalian cells transfecting with cloned vectors containing viral cDNA (Racaniello & Baltimore, 1981). Subsequently, infectious polio virus could also be rescued from negative-sense RNA transcripts produced from viral cDNA (Kaplan *et al.*, 1985). Later, influenza virus with 8 (-)ssRNA genome segments was rescued by transfecting an *in vitro* transcribed (-)ssRNA into cells infected with a helper virus (Luytjes *et al.*, 1989). Moreover, the rabies virus, a negative-sense RNA virus, was rescued using cDNA in 1994 (Schnell *et al.*, 1994). This was followed by the development of the plasmid-based influenza A reverse genetics system (Pleschka *et al.*, 1996). The rescue of bacteriophage Φ 6, a dsRNA virus with a 3-segment genome, was the first breakthrough in the reverse genetics of dsRNA viruses (Olkkonen *et al.*, 1990).

1.9.1 *Reovirales* reverse genetics

The development of reverse genetics systems for the *Reovirales* family lagged behind other viral reverse genetics systems. Over time three different reverse genetics methods were developed for *Reovirales*. These are the helper-virus dependent reverse genetics, transcript-based reverse genetics, and plasmid-only reverse genetics (Figure 1.15). In 1990 there was a significant breakthrough in *Reovirales* reverse genetics when mammalian orthoreovirus (MRV) was rescued using a helper-virus dependent approach (Roner *et al.*, 1990). In this system, they transfected the viral (+)ssRNA and dsRNA of the ST3 strain of MRV. The ST2 strain was then used as a helper virus (Roner *et al.*, 1990).

The first plasmid-only reverse genetics was developed for MRV in 2007 (Kobayashi *et al.*, 2007). This system used 10 transcription plasmids containing the viral cDNA flanked by a 5' T7 promoter and hepatitis delta virus (HDV) at the 3' terminus. The plasmids were transfected into L929 cells infected with an attenuated vaccinia virus rDIs-T7pol that expresses T7 RNA polymerase (Kobayashi *et al.*, 2007).

Bluetongue virus (BTV) and African horsesickness virus (AHSV) have been rescued using transcript-based reverse genetics systems. The transcript-based reverse genetics system for BTV was described in 2008 and this system used synthetic *in vitro* transcribed RNA (Boyce *et*

al., 2008). In this system, plasmids with the T7 promoter upstream of the cDNA encoding the 10 genome segments of BTV are used to *in vitro* transcribe (+)ssRNA. The T7 plasmids contain BsmBI, Bpil, or BsaI restriction sites downstream of the ORF used to generate transcripts with the correct 3' ends (Boyce *et al.*, 2008). Thereafter, a similar approach was used to rescue AHSV from transcripts (Matsuo *et al.*, 2010).

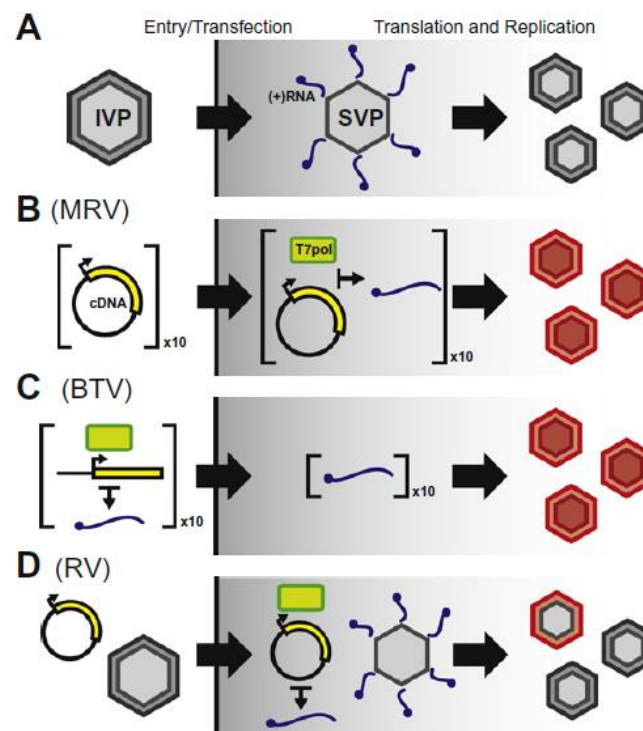


Figure 1.15. Schematic of Reovirales reverse genetics methods. A) Natural viral infection resulting in transcription of viral (+)ssRNA and assembly of progeny virus during replication. B) Plasmid-only reverse genetics system, plasmids encoding the viral proteins are transfected into eukaryotic cells allowing the expression of viral proteins and assembly of virions. C) Transcript-based reverse genetics system, capped (+)ssRNA of the viral genome segments are transfected and serve as templates for protein synthesis, allowing the formation of virions. D) Helper-virus dependent reverse genetics, a plasmid encoding a recombinant form of one of the viral proteins is transfected into cells; following transfection, the cells are infected with a helper virus and the virus with the recombinant protein is selected. Image used with permission Trask *et al.* 2013.

1.9.2 Rotavirus reverse genetics

1.9.2.1 Helper-virus dependent reverse genetics

The first RV reverse genetics system (Figure 1.16) was developed in 2006 by Komoto and colleagues, which was a helper-virus based reverse genetics system based on the KU strain

(Komoto *et al.*, 2006). This rescue approach was based on the influenza virus helper-virus RG method (Komoto *et al.*, 2020).

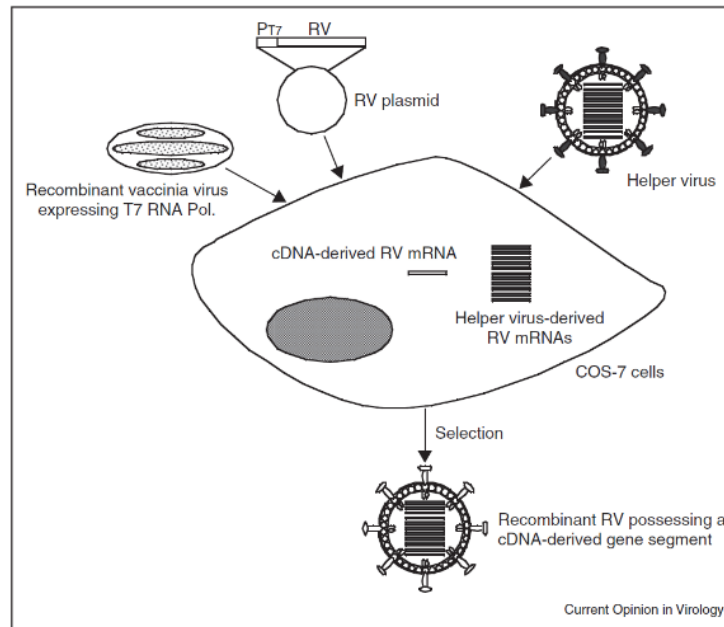


Figure 1.16: Schematic of RV helper-virus dependent reverse genetics system. Eukaryotic cells infected with vaccinia virus expressing T7 RNA polymerase are transfected with a T7 plasmid expressing a recombinant RV protein. Transfected cells are infected with the rotavirus KU helper virus. Strong selection procedures are then used to select recombinant progeny helper viruses. With permission. With permission Taniguchi & Komoto 2012.

COS-7 cells are infected with recombinant vaccinia virus expressing T7 RNA polymerase to rescue recombinant virus using this system. Thereafter, a plasmid containing recombinant cDNA of a specific genome segment flanked by T7 promoter and hepatitis delta virus ribozyme followed by a T7 RNA polymerase terminator is transfected into the infected cells (Figure 1.16). The transfected plasmid allows the expression of (+)ssRNA that can be transcribed into the recombinant protein and packaged into new virions. Subsequently, the transfected cells are infected with a helper virus and during the virus replication cycle (+)ssRNA and viral proteins are produced. The helper virus has a genome segment with an element that will aid in selection (Trask *et al.*, 2013). During the assembly of new virions, wild-type and recombinant helper viruses will form (Figure 1.16). The recombinant virions are then selected after successful assembly. They utilised neutralising monoclonal antibodies against VP4 to select for the recombinant virus (Komoto *et al.*, 2006).

Later a modified helper-virus dependent reverse genetics system was developed which used two selection methods. They utilised a helper-virus with a temperature sensitive mutant RV that had defective NSP2 when cultured at 39°C. There also was a RNAi cleavage site in genome segment 8 (NSP2), allowing RNAi degradation of the (+)ssRNA (Taniguchi & Komoto, 2012; Trask *et al.*, 2010).

1.9.2.2 Plasmid-based reverse genetics

Major advances in RV reverse genetics were made when the first plasmid-only based reverse genetics was developed in 2017, Figure 1.17 (Kanai *et al.*, 2017). This system opened avenues to having multiple recombinant genome segments, allowing the study of many aspects of the virus that could not be studied with single genome segment changes.

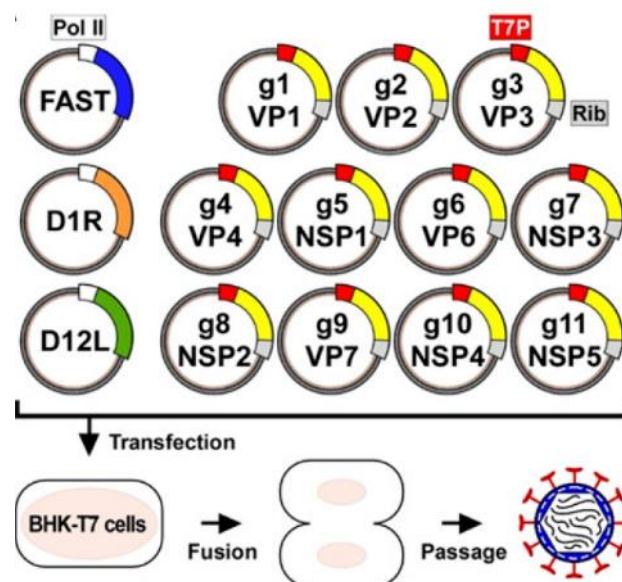


Figure 1.17. First rotavirus plasmid-only based reverse genetics approach. The system consists of 14 plasmids, 11 transcription plasmids with the cDNA of the 11 genome segments, 2 plasmids encoding subunits of the vaccinia virus capping enzyme and 1 encoding the FAST protein. The 14 plasmids are transfected into BSR-T7 cells to allow protein expression and viral assembly. Image used with permission Kanai *et al.* 2017.

This T7-RNA polymerase dependent system uses 14 plasmids (Figure 1.17). Eleven of these plasmids are transcription plasmids with the cDNA of the 11 genome segments of the SA11-L2 strain flanked by a T7 promoter and genomic HDV ribozyme. The ribozyme generates (+)ssRNA with precise 3' ends, which is important for packaging during replication. The other

plasmids are helper plasmids, two encoding the subunits (D1R and D12L) of the vaccinia virus capping enzyme and the final plasmid has an ORF encoding a fusion-associated small-transmembrane protein (FAST) (Figure 1.17). FAST allows cell-to-cell fusion helping to ensure that all 14 plasmids are in one cell. FAST has been shown to improve the replication and pathogenesis of vesicular stomatitis virus (Brown *et al.*, 2009). An equimicrogram ratio of the 14 plasmids is transfected into BHK-T7 cells and the transfected cells are co-seeded with MA104 cells after 48 hours. Once cytopathic effect (CPE) is observed on the co-seeded cells, the virus is further passaged on MA104 cells to increase the viral titre.

Since the development of this system, it has been proved that RV rescue can be achieved only using the 11 plasmids with the cDNA of the 11 genome segments. The research group that achieved this modified the system by transfecting three-fold more of the plasmids encoding NSP2 and NSP5 (Komoto *et al.*, 2018).

1.9.3 Applications of *Reovirales* reverse genetics in vaccine development

With the development of a plasmid-only based rotavirus reverse genetics system came the possibility of developing better and safer rationally designed rotavirus vaccine candidates. Due to the differences in circulating rotavirus strains in different regions developing vaccines based on regional circulating strains could be beneficial. Using reverse genetics, veterinary vaccine candidates were produced for BTV by creating a tissue culture-adapted universal backbone where VP2 and VP5 of different serotypes can be readily reassorted (Nunes *et al.*, 2014). The authors rescued multiple reassortment viruses and showed that a reassortment with VP2 BTV-8 or VP2/VSP5 of BTV-8 protected sheep from disease (Nunes *et al.*, 2014). Creating such a platform for rotavirus vaccines would open an avenue to generate RV vaccines based on regional strains.

1.10 Problem Identification

Rotavirus NSP4 and NSP1 contribute to viral pathogenesis. NSP4 increases cytosolic calcium levels causing diarrhoea, and NSP1 inhibits the nuclear translocation of phosphorylated STAT1 and STAT2, inhibiting the innate immune response. Currently, live wild-type attenuated or reassortment rotaviruses are used as vaccines for protection against rotavirus infection. However, their efficacy is lower in Sub-Saharan African and South-East Asian countries. Therefore, there is a need for new safe vaccines in these countries. The entirely plasmid-based rotavirus reverse genetic system empowers scientists to rationally design, study and

incorporate novel features that improve safety and allow differentiation from wild-type viruses into next-generation vaccine candidates.

1.11 Aims and Objectives

This study aims to rationally alter rotavirus GS10(NSP4) and GS5(NSP1) to rescue attenuated rotaviruses that can be possible vaccine candidates. The hypothesis of this project is that the signal transduction domain of NSP4 and the cytoskeleton binding region of NSP1 can be rationally modified to improve the safety of rotavirus vaccine candidates.

The following are the specific aims and objectives of this study:

1. To generate baculovirus expressed OSU-a NSP4 and OSU-v NSP4 to study the calcium ion (Ca^{2+}) flux in tissue culture (Chapter 2).
 - 1.1. Transform the pFastBac-HTA_OSU-a NSP4 and pFastBac-HTA_OSU-v NSP4 constructs into $\Delta\Delta\text{AccBac}$ bacterial cells to generate recombinant bacmid DNA.
 - 1.2. Transfect Sf9 insect cells with recombinant bacmid DNA to generate recombinant baculoviruses expressing His-tagged OSU-a NSP4 and OSU-v NSP4.
 - 1.3. Treat Sf9 and MA104 cells with OSU-a NSP4 and OSU-v NSP4 and measure the Ca^{2+} flux to determine if there is a decrease in calcium flux in cells treated with attenuated NSP4.
2. To incorporate plasmids with full-length OSU-a NSP4 ORF with SA11 UTRs and modified SA11 NSP4 ORF with OSU-a amino acids 135-141 into the SA11-L2 rotavirus reverse genetics system (Chapter 3).
 - 2.1. Incorporating the plasmid constructs (OSU-a GS10 and SA11 GS10 with aa135-141 of OSU-a GS10) in the SA11-L2 reverse genetics system and determine if viral rescue is possible.
 - 2.2. Measure the calcium flux in MA104 cells infected with the rescued viruses to determine if there is a decrease in Ca^{2+} flux.
3. To incorporate a plasmid construct with GS5 that encodes NSP1 with a deletion of aa 84-176 into the SA11-L2 reverse genetics system and evaluate the localisation of NSP1 and nuclear translocation of phosphorylated STAT1 and STAT2 in MA104 cells infected with rescued rotavirus with truncated NSP1 (Chapter 4).
 - 3.1. Design and incorporate a pUC57 construct with truncated NSP1 into the SA11-L2 rotavirus reverse genetics system.
 - 3.2. Use the rescued virus to infect MA104 cells and determine if NSP1 without aa 84-176 translocates to the nucleus.
 - 3.3. Determine if phosphorylated STAT1 and STAT2 translocate to the nucleus in cells infected with the rotavirus virus with NSP1 with a deletion of aa 84-176.

CHAPTER 2

BACULOVIRUS EXPRESSION OF NSP4 OF VIRULENT AND ATTENUATED PORCINE ROTAVIRUSES

2.1 Introduction

Rotaviruses cause acute gastroenteritis that is life-threatening in children under the age of 5. NSP4 is the viral enterotoxin that causes diarrhoea during rotavirus infection (Ball *et al.*, 1996). The main clinical symptom of rotavirus infection is watery diarrhoea, leading to dehydration. NSP4 dysregulates calcium signal transduction and increases intracellular calcium (Ball *et al.*, 2005). NSP4 functions as an intracellular and extracellular protein during the replication cycle. Extracellular NSP4 released from infected cells can enter uninfected intestinal epithelial cells and increase intracellular calcium.

Two porcine rotavirus strains were attenuated by passaging in tissue culture attenuated, and mutations were found in NSP4 of these attenuated viruses (Zhang *et al.*, 1998). These mutations in the signal-transducing domain attenuated NSP4's ability to increase intracellular calcium and cause diarrhoea. However, the protein's ability to bind integrins was not affected (Seo *et al.*, 2008).

The main goal of our research group is to rationally design rotavirus vaccine candidates. While designing these vaccines, we have to ensure that they do not cause diarrhoea. The experiments to test vaccine safety will need controls. The attenuated and virulent forms of porcine rotavirus NSP4 can be used for this purpose. Attempts to express full-length NSP4 in bacterial or eukaryotic cells have been unsuccessful. However, OSU-a and OSU-v NSP4 have been successfully expressed in Sf9 insect cells (Zhang *et al.* 1998).

The experiments described in this Chapter aimed to express His-tagged NSP4 OSU-a and OSU-v. NSP4 is a glycoprotein. The baculovirus expression system was the method used to achieve the aim. This system was developed independently by Dr Summer and Dr Miller in the early 1980s to generate recombinant viruses for protein expression (Summers & Smith, 1987 and Miller, 1988). Since the 1980s, this method has been improved to allow greater expression of heterologous proteins (Martínez-Solís *et al.*, 2018). This system is also used for expressing glycoproteins since insect cells have an N-glycosylation pathway (Shi & Jarvis, 2007).

2.2 Materials and Methods

2.2.1 Bac-to-bac baculovirus expression system

The baculovirus expression system utilises baculovirus' natural ability to infect insect cells. The *Autographa californica* multiple-capsid nuclear polyhedrosis virus (AcMNPV) is the commonly used baculovirus in laboratory settings (Felberbaum, 2015). Today, the Invitrogen Bac-to-Bac system (Figure 2.1), which uses *E. coli* cells that propagate bacmid DNA is widely used to generate recombinant proteins (Felberbaum, 2015). The baculovirus genome was modified by inserting sequences of the bacterial artificial chromosome, kanamycin resistance gene, and transposition target site (Luckow et al., 1993). To improve the integrity of the expressed proteins, the chitinase and v-cathepsin genes of the AcMNPV bacmid were deleted, generating the AcBac Δ CC *E. coli* cells (Kaba et al., 2004).

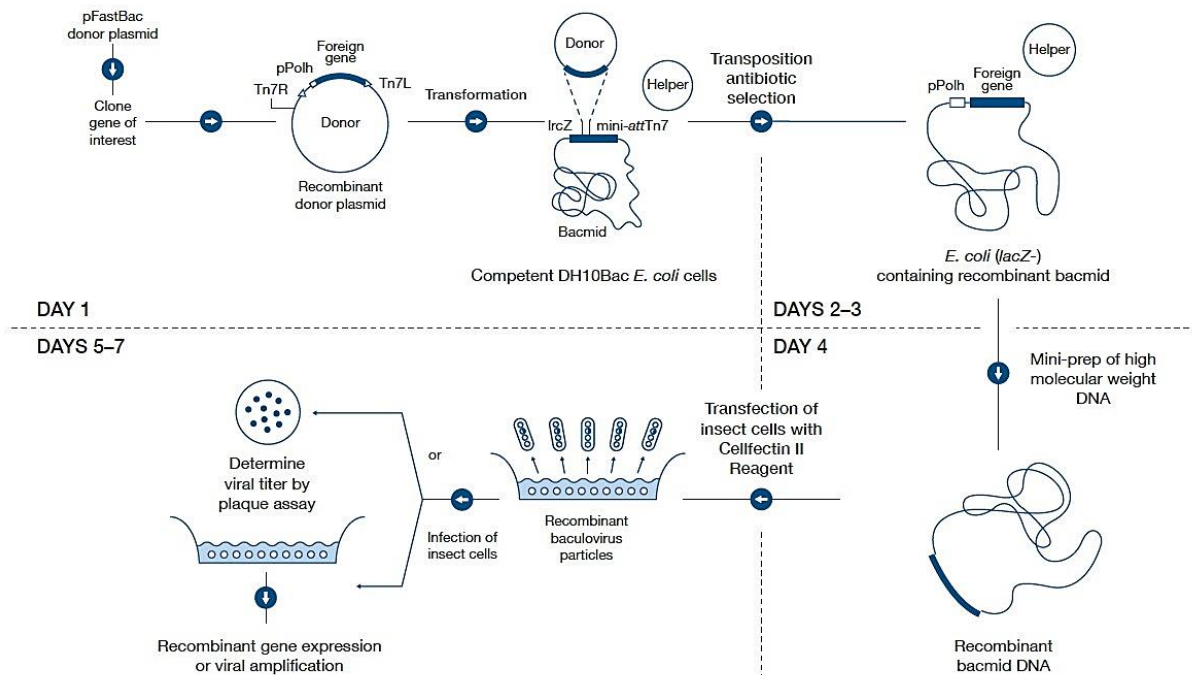


Figure 2.1: Schematic of the Bac-to-Bac expression system. The generation of recombinant baculoviruses and expression of proteins using the baculovirus expression system is depicted in the figure. The system uses site-specific transposition to generate recombinant bacmid DNA. The recombinant bacmid DNA is transfected into insect cells generating recombinant baculoviruses that express proteins of interest. Image from Thermofisher.com public domain.

In the Bac-to-Bac baculovirus expression system (Figure 2.1), the donor plasmid has the mini-Tn7 site containing a gentamicin resistance gene, polyhedrin promoter and the foreign gene in the multiple cloning site (MSC). These donor plasmids are transformed into AcBac Δ CC *E.coli* cells. After successful transformation, the mini-Tn7 site is transposed into the mini-attTn7 site of the bacmid DNA with the help of a transposase enzyme encoded by the helper plasmid (Figure 2.1). The helper plasmid also contains a tetracyclin resistance gene. Successful transposition of the mini-Tn7 site disrupts the lacZ α gene in the mini-attTn7 site. The lacZ α gene encodes for β -galactosidase, which uses X-gal as a substrate and Isopropyl β -D-1-thiogalactopyranoside (IPTG) is used to induce the expression of β -galactosidase. Without transposition, lacZ α produces β -galactosidase that cleaves X-gal producing blue pigmentation (Sherwood, 2008). When the lacZ α gene is interrupted by transposition, the lack of β -galactosidase will yield white colonies, and recombinant and non-recombinant bacmid selection is possible.

2.2.2 Design, codon optimisation and construction of donor plasmids

The two NSP4 proteins selected for the study are from the two OSU strains (Table 2.1). The pFastBac-HTA donor plasmids were designed using Snapgene software (GSL Biotech LLC). The ORF of OSU-v GS10 was placed in the multiple cloning site (MSC) of the pFastBac-HTA backbone using the NcoI and KpnI restriction sites. The NcoI and KpnI restriction sites were also used to allow the cloning of other ORFs into the pFastBac-HTA backbone. The OSU-v GS10 ORF was inserted downstream of the polyhedron promoter, His-tag, and tobacco etch virus (TEV) site (Figure 2.2A). The His-tag was placed at the N-terminal (Figure 2.2A) because NSP4's hydrophobic region is on the N-terminal, and this should ensure better protein solubility. Mr LJC Theart designed the pFastBac-HTA donor plasmid (Figure 2.2B) with the OSU-a NSP4 insert similarly to the pFastBac-HTA plasmid with OSU-v NSP4 ORF.

The designed plasmid constructs were sent to GenScript (Piscataway, New Jersey, USA) for Sf9 insect cell optimisation and synthesis. The plasmids arrived in TOP10 bacterial stab cultures and were archived at -80°C.

Table 2.1: NSP4 of porcine rotavirus strains used to design pFastBac-HTA donor plasmids.

RVA source of genome segment 10 ORF	GenBank accession number
RVA/Pig-tc/USA/1975/OSU/G5P7/virulent NSP4	MT025924.1
RVA/Pigtc/USA/1975/OSU/G5P7/attenuated NSP4	MT025925.1

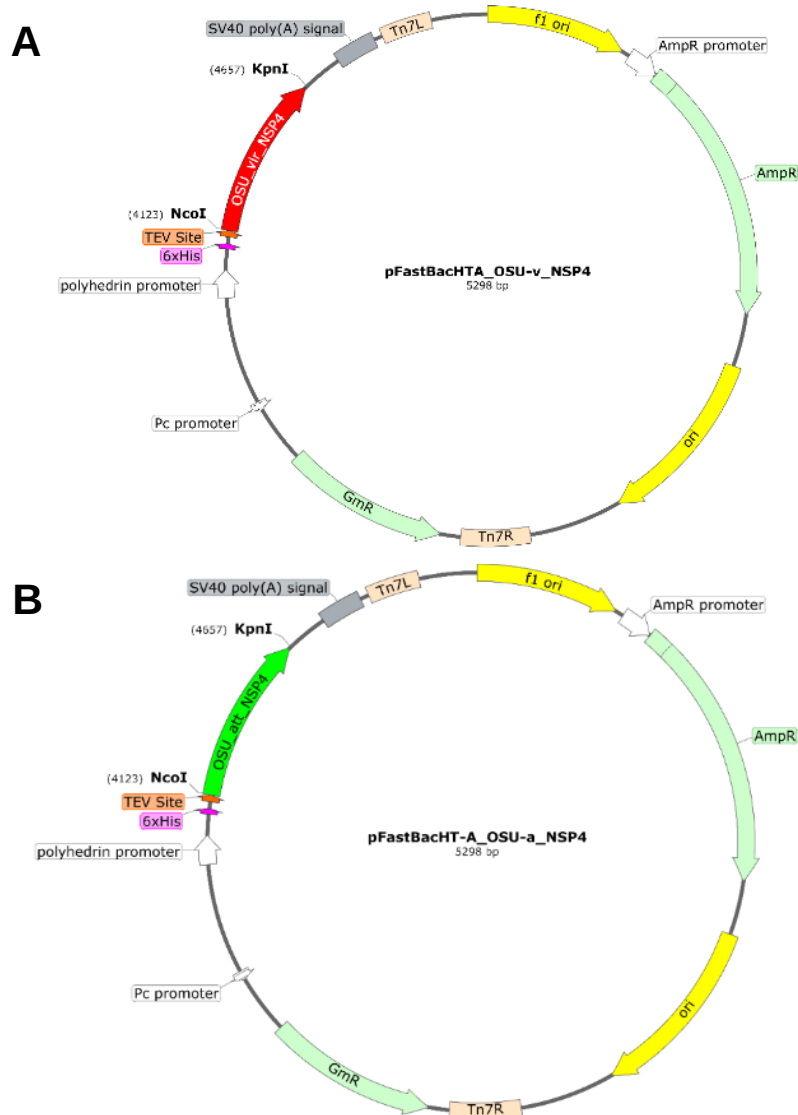


Figure 2.2: Map of pFastBac_OSU-v_NSP4 donor plasmid. The pFastBac-HTA containing the polyhedrin promoter, 6xHis tag (pink) and TEV site (orange) was modified by inserting porcine rotavirus NSP4 ORFs in the MSC using the NcoI and KpnI restriction sites. A) pFastBac-HTA containing rotavirus OSU-v NSP4 ORF (red). B) pFastBac-HTA containing rotavirus OSU-a NSP4 ORF (green).

2.2.3 Preparation and transformation of chemically competent JM109 bacterial cells

T0P10 glycerol stocks containing pFastBac-HTA_OSU-a_NSP4 could not be found in the -80°C storage, but purified pFastBac-HTA_OSU-a_NSP4 was available to use for the experiments. The purified plasmid had to be transformed into JM109 bacterial cells for long-term storage and to purify enough of the plasmid needed for the study.

There is an electrostatic repulsion between the cell membrane and plasmid DNA. Treating bacterial cells with chemicals such as calcium chloride has been used for years to make bacterial cells competent enough to take up the plasmid DNA that needs to be introduced into the cells. The calcium chloride ions bind to the cell surface, neutralising the charge, which allows the foreign plasmid DNA to bind to the cell membrane.

Escherichia coli (*E. coli*) JM109 cells were made chemically competent using the rubidium chloride (RbCl) method (Hanahan, 1983). *E. coli* JM109 cells have a mutation in their *RecA* and *endA* genes which minimises the rate of recombination and increases plasmid stability.

Glycerol stocks of JM109 cells stored at -80°C were streaked onto a Luria Bertani (LB) agar plate (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) and incubated overnight at 37°C. To prepare a starter culture, a single colony was selected and inoculated into 5 ml SOC media (20 g/L tryptone, 5 g/L yeast extract, 0.5 g/L NaCl, 250 mM KCl and 20mM glucose). The starter culture was incubated at 37°C, shaking at 225 rpm. The starter culture was inoculated into 50 ml of LB broth at a ratio of 1:100 (LB broth: starter culture) and incubated for 5 hours while shaking at 37°C until it reached an OD₆₀₀ of 0.4 to 0.6. Once the bacterial culture reached the appropriate OD₆₀₀, the culture was spun down at 5000 xg at 4°C for 10 minutes. The supernatant was removed, and the pellet was resuspended in 20 ml of ice-cold TFB1 (100 mM RbCl, 50 mM MnCl, 30 mM potassium acetate, 10 mM CaCl₂, 15% (v/v) glycerol, pH 5) and incubated on wet ice for 5 minutes. Thereafter, the resuspended cells were pelleted at 5000 xg for 5 minutes. The cell pellet was resuspended in 2 ml ice-cold TFB2 (10 mM MOPS, 10 mM RbCl, 75 mM, CaCl₂, 15% (v/v) glycerol, pH 6.5) and incubated on ice for 60 minutes. Then 50 µl of the competent bacterial cells were then aliquoted into 1 ml microcentrifuge tubes and snap-frozen in liquid nitrogen. The aliquots were stored at -80°C for long-term storage.

2.2.4 Transformation of chemically competent JM109 bacterial cells

Transformation is the process in which bacteria take exogenous DNA from their environment. The heat shock transformation was used to transform competent JM109 bacterial cells during this project. When there is a drastic change in temperature in the environment of chemically

competent cells, pores form on the cell membrane allowing the plasmid DNA to enter the cells. The cells are then returned to low temperatures to stabilise the cell membrane.

Frozen competent JM109 cells were thawed on ice and 50 ng of pFastbac-HTA_OSU-a NSP4, donor plasmid DNA, was added, and the tube was gently flicked to mix. The tube was incubated on ice for 30 minutes, and then the cells were heat shocked for 30 seconds at 42°C and immediately placed on ice for 2 minutes. A volume of 900 µl SOC medium preheated to 37°C was added to the cells and incubated for an hour while shaking at 37°C. The cells were then spread onto a LB agar plate containing 50 µg/ml carbenicillin and incubated overnight at 37°C. Single colonies selected from each plate were streaked onto the master LB agar plate containing 50 µg/ml carbenicillin and used to make glycerol stocks.

Glycerol protects the bacterial cell membrane from damage by ice crystals when they are frozen for long-term storage. Glycerol stocks of all bacterial cells were prepared by adding glycerol to a final concentration of 15% to bacterial cells grown overnight and stored at -80°C.

2.2.5 Purification of donor plasmids

Small-scale plasmid extractions were performed using a Qiagen mini-prep extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The kit is based on the biochemical principle that an alkaline buffer can selectively denature genomic DNA (Bimboim & Doly, 1979). At a pH of 12, the genomic DNA is denatured however, because of its closed circular structure, plasmid DNA is not denatured. During neutralisation, the plasmid can quickly renature. However, the genomic DNA cannot renature fully and complementary strands cannot reanneal due to its size, and it precipitates out of solution. The plasmid DNA can be isolated from other cellular components by binding to a silica column at a high salt concentration.

Using the mini-prep kit, the pFastBac-HTA donor plasmids were extracted from previously prepared glycerol stocks. The bacterial cells were previously cultured and stored as glycerol stocks for future plasmids extractions. pFastBac_OSU-a_NSP4 was extracted from JM109 glycerol stocks and pFastBac_OSU-v_NSP4 was extracted from TOP10 glycerol stocks. Overnight bacterial cultures were prepared by inoculating 5 ml of LB broth containing 50 mg/ml carbenicillin with a scoop of the glycerol stocks and incubated overnight while shaking. The cells from the overnight cultures were centrifuged for 5 minutes at 6800 g. The supernatant was discarded, and the cells were resuspended in 250 µl buffer P1 (Tris, EDTA, glucose, RNase-A, and LyseBlue). EDTA destabilises the cell membrane by chelating divalent metal ions and inhibits DNases, while glucose maintains the osmolarity of the Tris buffer, and

RNase-A denatures released RNA. Then, 250 µl of chilled buffer P2, the alkaline lysis buffer containing NaOH and SDS, was added and mixed by gently inverting. The LyseBlue in the solution turns the solution blue once the pH increases. Then 350 µl of neutralising buffer (N3) was added to neutralise the solution by rapidly decreasing the pH, turning the solution colourless. The cultures were centrifuged at 17900 g for 10 minutes; the pellet contained genomic DNA, proteins, and RNA. Thereafter, 800 µl of the supernatant was transferred to a silica spin column, centrifuged for 1 minute at 17900 g, and the flow through was discarded. The column membrane was then washed with 750 µl buffer PE and centrifuged for 1 minute at 17900 g, the flow through was discarded, and the column was centrifuged again to remove any remaining buffer. The column was then placed in a clean 1.5 ml micro-centrifuge tube and the plasmid was eluted from the column membrane by adding 50 µl nuclease-free water (Merck and Co., Inc, New Jersey, U.S) preheated to 75°C. The column was then centrifuged for 1 minute at 17900 g to elute the plasmids. The purified plasmids were sent for Sanger sequencing at Stellenbosch University's Central Analytical Facilities.

2.2.6 Spectrometric analysis of nucleic acids

Spectrometric analysis of plasmid DNA was done to determine the concentration and purity of extracted plasmid DNA using a Nanodrop One spectrophotometer (Thermo Scientific, Massachusetts, USA). Nucleic acids and proteins absorb light at different wavelengths. Nanodrop spectrophotometry is based on the Beer-Lambert law, which states that the absorbance of a solution is directly proportional to its concentration. Nucleic acids absorb light at 260 nm. To ensure that the sample consists of pure DNA and there is no RNA contamination, the absorbance ratio between A₂₆₀ and A₂₈₀ is determined. At A₂₆₀ of 1 the concentration of double-stranded DNA is 50 µg/ml. Pure DNA is signified by an A₂₆₀/A₂₈₀ between 1.7 and 1.9. The stage of the Nanodrop One spectrophotometer was cleaned with nuclease-free water and then blanked with the buffer the DNA was resuspended in. Thereafter, the sample was loaded, and the concentration and purity of the purified plasmid DNA were determined.

2.2.7 Agarose gel electrophoresis (AGE)

Nucleic acid samples were analysed using agarose gel electrophoresis. AGE was used to determine the size of the nucleic acid fragments. The method uses an agarose gel and an electric current to separate nucleic acids based on their size. During electrophoresis, a current is run across an agarose gel, allowing the nucleic acids in the gel to migrate from the anode to the cathode because of the negatively charged phosphodiester backbone. When agarose

is heated in a buffer and cooled, it forms a porous gel matrix. The smaller nucleic acids can migrate faster through the gel because they face less resistance. Ethidium bromide (EtBr), which intercalates with the nucleic acids, allows their visualisation under ultraviolet (UV) light.

1% agarose gels (w/v) were prepared by dissolving agarose in 1X TAE buffer (40 mM Tris, 20 mM acetate and 1 mM EDTA) by heating the mixture (Sambrook & Russell, 2001). The dissolved agarose was cooled to 45°C-55°C before adding EtBr to a final concentration of 0.5µg/ml. The gels were cast in the appropriate-sized casting tray with a well comb at room temperature. GeneRuler™ 1 Kb DNA ladder (Thermo Scientific, Massachusetts, USA) was loaded in the first well to serve as standard and nucleic acids samples mixed with 6X loading dye (Thermo Scientific, Massachusetts, USA) according to the manufacturer's instructions and were loaded in the remaining wells. The loading dye is composed of two dyes (bromophenol blue and xylene cyanol) and glycerol, the dyes help track the nucleic acids in the gel, and the glycerol helps keep the nucleic acids in the gel by increasing their density. Two dyes are used in the loading dye to ensure all bands are visualised. Bromophenol blue migrates quicker through the gel and helps visualise shorter fragments, while xylene cyanol migrates slower through the gel and helps visualise larger fragments. The gels were run for 60 minutes at 70V in TAE buffer and visualised using a BioRad Chemidoc™ imaging system (BioRad, California, USA).

2.2.8 Generation of recombinant baculoviruses using the Bac-to-Bac system

2.2.8.1 Transformation of competent cells with donor plasmids

To generate recombinant bacmid DNA, the pFastBac-HTA donor plasmids (pFastBac_OSU-v_NSPI and pFastBac_OSU-a_NSPI) were transformed into chemically competent AcBACΔCC *E.coli* cells. The bacterial cells were made competent using the calcium chloride method (Higa & Mandel, 1970). Using the heat shock method, 100 ng of donor plasmid DNA was transformed into competent AcBACΔCC *E.coli* cells. Thereafter, the cells were plated on LB agar plates containing 10 µg/ml gentamycin, 50 µg/ml kanamycin, 50 µg/ml tetracycline, 32 µg/ml X-gal and 4% isopropylthio-β-galactoside (IPTG). The plates were incubated overnight at 37°C, and single white colonies were selected. The selected colonies were used to make overnight cultures by inoculating them into 5 ml of LB broth containing antibiotics (10 µg/ml gentamycin, 50 µg/ml kanamycin, 50 µg/ml tetracycline) and incubated overnight at 37°C while shaking at 225 rpm. Bacmid DNA was extracted from 3 ml of the culture, and the remaining bacteria were used to prepare glycerol stocks.

2.2.8.2 Isolation of bacmid DNA

Recombinant and wild-type bacmid DNA were extracted from AcBAC Δ CC *E.coli* cells using a protocol from the MSc study of Petrus Jansen van Vuren (Jansen van Vuren, 2006). This method is a modified version of the alkaline plasmid extraction method by Birnboim and Doly (1979). 3 ml bacterial cultures were grown overnight and centrifuged for 1 minute in microcentrifuge tubes at 8000 xg. The supernatant was discarded, and the cells were resuspended in 200 μ l GTE buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris/HCl pH 8 and 0.1 mg/ml RNase-A). Then 400 μ l of 0.2 M NaOH/1%SDS solution was mixed with the samples and incubated for 5 minutes. Following incubation, 300 μ l of chilled 5 M KAc pH 5.2 was gently mixed with the sample. The samples were then incubated on ice for 5 minutes. Subsequently, the samples were centrifuged at 14000 xg for 8 minutes, and the supernatant was transferred to clean microcentrifuge tubes. 800 μ l of ice-cold isopropanol was added to the samples and then incubated on ice for 5-10 minutes to precipitate the DNA. The mixture was centrifuged again for 14000 xg for 5 minutes, and then supernatant was discarded. The pellets were washed with 500 μ l of 70% ethanol and then air dried. The dried bacmid DNA pellets were resuspended in nuclease-free H₂O and quantified on a Nanodrop One spectrophotometer (Section 2.2.6).

2.2.8.3 Polymerase chain reaction (PCR)

Successful transposition of the expression cassette from the donor plasmid to the bacmid DNA was determined using PCR. PCR analysis also confirms if the bacmid DNA from white colonies is pure recombinant or if there is wild-type contamination. The method uses DNA polymerase and primers to amplify a strand of DNA. Because of the double-stranded nature of DNA, it needs to be denatured using high heat. The primers which anneal to the single-stranded DNA provide a 3'-OH allowing the next nucleotide to bind via phosphodiester bonds.

The Q5 High fidelity DNA polymerase (New England Biolabs, Massachusetts) was used. M13 sequencing primers (Table 2.1) purchased from TIB MolbioTM were used for the experiments. The PCR was set up as follows: 0.02 U/ μ l Q5 high fidelity DNA polymerase, 1X Q5 reaction buffer, 0.5 μ M M13F primer, 0.5 μ M M13R primer, 200 μ M dNTPs, 100 ng bacmid DNA and nuclease-free water to a final volume of 50 μ l.

The following PCR cycle conditions were used on the thermocycler (T100 Thermal Cycler, Biorad, California, USA): initial denaturation for 10 minutes at 94°C, followed by 25 cycles of denaturation for 45 seconds at 94°C, primer annealing for 45 seconds at 55°C and elongation for 5 minutes at 72°C, final elongation for 10 minutes at 72°C and held at 4°C.

The PCR amplicons were analysed on a 1% agarose gel and visualised using a BioRad Chemidoc™ imaging system (BioRad, California, USA). The size of the expected amplicons (Figure 2.2) was calculated as shown in Figure 2.3.

Table 2.1: M13 forward and reverse sequencing primers.

Primer	Primer sequence	No. of bases	Tm
M13F	5'GTTTCCAGTCACGAC3'	17 bases	55°C
M13R	5'CAGGAAACAGCTATGAC3'	17 bases	55°C

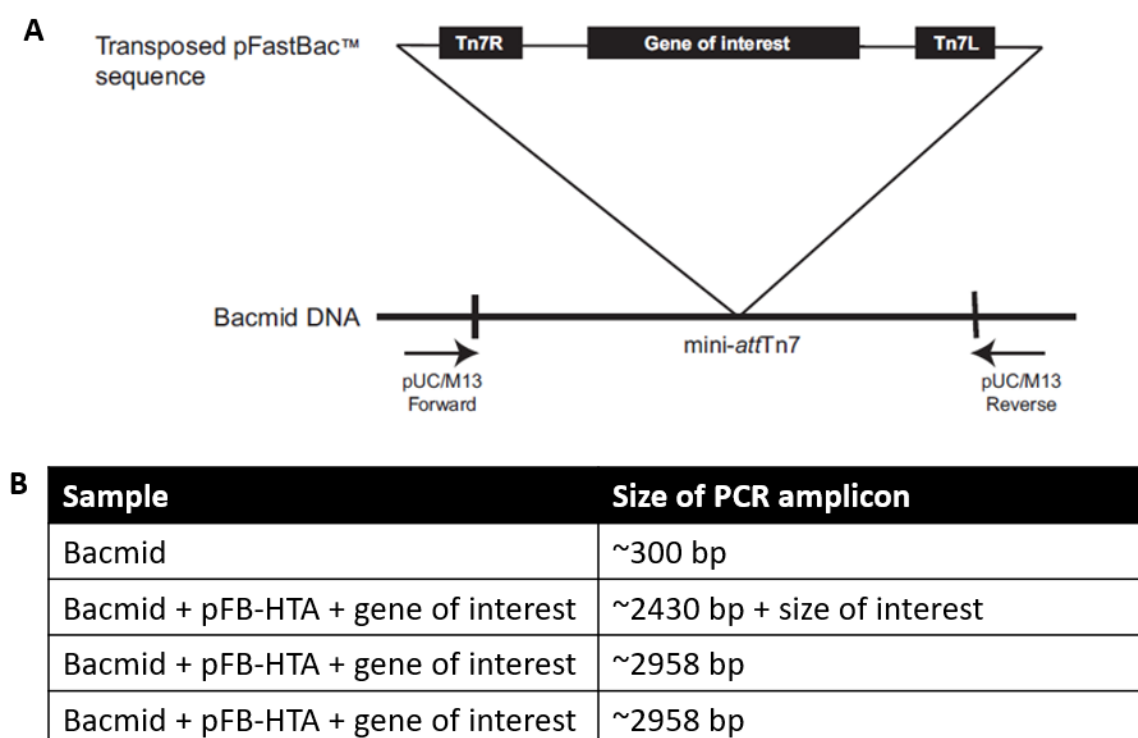


Figure 2.3: Schematic of the mini-attTn7 transposition region and expected PCR amplicons. A) The gene of interest is cloned into the bacmid mini-Tn7 site. During transposition, the gene of interest is transposed into the bacmid DNA's mini-attTn7 site for PCR of the transposition region primers binding the Tn7R and Tn7L sites. B) The expected amplicon size after successful transposition. Adapted from the Invitrogen Bac-to-Bac baculovirus expression system manual (Life Technologies, Grand Island, NY) and KC Jere's PhD thesis (2012).

2.2.8.4 Culturing of Sf9 insect cells

Sf9 cells were maintained as monolayers in 75cm³ flasks. The cells were grown in TC-100 insect cell medium (Gibco Life Technologies, California, USA) supplemented with 1% antibiotic/antimycotic (Gibco Life Technologies, California, USA) and 10% foetal bovine serum (FBS, Gibco Life Technologies, California, USA) and incubated at 27°C. Cells were allowed to grow to 80% confluency and then detached by gently pipetting before being split at a 1:10 ratio.

2.2.8.5 Generation and amplification of recombinant baculoviruses

To generate recombinant baculovirus expressing His-tagged OSU-a and OSU-v NSP4, pure recombinant bacmid DNA was transfected into Sf9 insect cells. The bacmid DNA was transfected using FuGene® HD transfection reagent (Promega Corporation, Wisconsin, USA), a non-liposomal reagent, according to the manufacturer's instructions. Transfection reagents form a barrier around the nucleic acid being introduced into the eukaryotic or insect cells to overcome the repulsive negative charge between the cell membrane and nucleic acids backbone.

Insect cells were seeded in 6-well plates at a density of 1x10⁶ cells/ml per well the day before the transfection. Transfection complexes were prepared in 15 ml sterile polystyrene tubes by combining 2.5 µl transfection reagent with 60 µl OptiMEM (Gibco Life Technologies, California, USA) and 1 µg of bacmid DNA.

The ratio of FuGene® HD to bacmid DNA was 3:1. The contents were mixed and incubated for 30 minutes at room temperature. The transfection complex was added dropwise onto Sf9 insect cells seeded previously and incubated at 27°C until complete CPE was observed (approximately 5 days post-transfection). Once complete CPE was observed, the contents of each well were transferred to a microcentrifuge tube and centrifuged for 5 minutes at 500 xg. The transfection supernatants (P0) were transferred to clean microcentrifuge tubes and stored at 4°C.

The generated (P0) recombinant baculoviruses were used to infect Sf9 cells to increase the viral titre and recombinant protein yield. Cells were seeded at a density of 1x10⁶ cells/ml per well in a 6-well plate and infected with 200 µl of the transfection supernatants. After 30 minutes, 1.5 ml of TC100 insect cell medium (Gibco Life Technologies, California, USA) supplemented with 1% antibiotic/antimycotic (Gibco Life Technologies, California, USA) and 10% foetal bovine serum (FBS) (Gibco Life Technologies, California, USA) was added to each

well. The cells were incubated at 27°C until complete CPE was observed. Thereafter, the cells were detached from each well and each well's contents were transferred to a 5 ml tube, centrifuged for 5 minutes at 500 xg and then the supernatant was transferred to a new tube and stored at 4°C. The cell pellet from each well was used to analyse whether the recombinant baculoviruses expressed his-tagged OSU-a and OSU-v NSP4. The recombinant viruses were passaged 4 times before determining if there was expression of His-tagged OSU-a NSP4 and His-tagged OSU-v NSP4 using SDS-PAGE (Section 2.2.9) and IFMA (Section 2.2.10).

2.2.9 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE, developed by Ulrich Laemmli (Laemmli, 1970), was used throughout the study to confirm the expression of His-tagged OSU-a NSP4 and His-tagged OSU-v NSP4. After cell lysis, the proteins were heated in the presence of a reducing agent. β -mercaptoethanol (a reducing agent) breaks the disulphide bridges that hold the protein in its more complex conformation. When the proteins are denatured, they can be separated based on their molecular weight when an electric current is applied.

Harvested Sf9 cells that were infected with recombinant baculovirus (Section 2.2.8.5) were lysed in 200 μ l of RIPA buffer (0.1% SDS, 50 mM Tris-HCl pH8, 150 mM sodium chloride, 0.5% sodium deoxycholate, 1% Triton X-100) containing Pierce protease inhibitor mini tablets (Thermo Scientific, Massachusetts, USA). The cells were lysed for an hour before proteins were denatured. The proteins were mixed with 4X Laemmli sample buffer (BioRad, California, USA) and 5% β -mercaptoethanol at a ratio of 3:1 (Protein: reducing agent). The samples were then heated for 5 minutes at 95°C, cooled, and subjected to SDS-PAGE. The PageRuler Prestained Protein Ladder (Thermo Scientific, Massachusetts, USA) or Precision Plus Protein Dual Color Standards (BioRad, California, USA) was also loaded in the wells at each end of the gel to serve as a molecular weight marker.

SDS-PAGE gels were prepared as described by Sambrook and Russel (2001). The gels comprised of a 12% resolving gel (12% acrylamide/bisacrylamide mix, 0.25 M Tris pH 8.8, 1% (w/v) SDS, 1% (w/v) ammonium persulphate (APS) and 0.04% TEMED) and a 5% stacking gel (5% acrylamide/bisacrylamide mix, 126 mM Tris pH 6.8, 1% (w/v) SDS, 1% (w/v) APS and 0.1% TEMED). The gels were electrophoresed at 120V in TGS buffer (25 mM Tris, 250 mM glycine, 0.1% (w/v) SDS, pH 8.3), and the gels were run until the bromophenol blue dye front migrated to the end of the resolving gel.

The gels were stained with 0.1% (w/v) Coomassie stain (0.1% Coomassie Brilliant Blue in 50% methanol, 10% glacial acetic acid and 40% H₂O) and destained in destaining solution

(50% methanol, 10% glacial acetic acid and 40% H₂O). The proteins were visualised using a BioRad Chemidoc™ imaging system (BioRad, California, USA).

2.2.10 Immunofluorescent monolayer assay (IFMA)

Immunofluorescent monolayer assay was used to verify if there was expression of the desired proteins. The biochemical principle of this method is to fix cell monolayers using an organic solvent. Methanol/acetone is used to precipitate the viral particles and cellular proteins and increase the cell membrane's permeability to allow the antibodies to enter the cell. The organic solvent dissolves cellular lipids making the cell membrane permeable (Jamur & Oliver, 2010). The fixed cells are incubated with primary antibodies that bind the proteins of interest, washed, and then incubated with secondary antibodies, which bind the constant region of the primary antibody. The secondary antibodies have fluorescent tags conjugated to them, which emit light when viewed using a fluorescent microscope.

The growth medium on Sf9 cells infected with wild-type or recombinant baculovirus was removed and replaced with an ice-cold fixing solution (methanol/acetone, 1:1 ratio) 72 hours post-infection (hpi). Then the cells were fixed at -20°C for 30 minutes. The fixing solution was discarded, and the cells were washed twice with wash buffer (1x PBS and 0.05% Tween-20). After that, the wash buffer was discarded, and blocking buffer (1x PBS, 1% (w/v) tryptone, 0.05% Tween-20) was added and incubated for 30 minutes at 37°C. After incubation, the blocking buffer was removed, and rabbit anti-his tag monoclonal primary antibodies (GenScript, Piscataway, New Jersey, USA) diluted 1:2000 in the blocking buffer were added and incubated at 37°C for 60 minutes. Following incubation, the primary antibodies were discarded, and the fixed monolayer was washed three times with wash buffer. Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, Waltham, USA) diluted 1:2000 was added to the fixed monolayer and incubated for 60 minutes at 37°C for the secondary antibody to bind. After removing the secondary antibody solution, the cells were washed twice with 1X PBS and visualised using a Nikon eclipse TE2000-S fluorescent microscope (Nikon Instruments Inc., NY, USA). Alex Fluor 488 is excited at 495 nm and has an emission range of 519 nm.

2.3 Results and Discussion

2.3.1 Sequence analysis of pFastBac_OSU-a_NSP4 and pFastBac_OSU-v_NSP4 donor plasmids

To express His-tagged OSU-a NSP4 and His-tagged OSU-v NSP4 two donor pFastBac-HTA donor plasmids (pFastBac_OSU-a_NSP4 and pFastBac_OSU-v_NSP4) were *in silico* designed and purchased from GenScript. The two plasmids were designed to contain the His-tag, TEV site and Sf9 insect cell codon optimised OSU-a GS10(NSP4) ORF or OSU-v GS10(NSP4) ORF (Figure 2.2). His-tag, TEV site are helpful for protein purification. The his-tagged proteins can be separated from other proteins using nickel affinity chromatography. TEV protease recognises and cleaves the TEV site; this cleavage will separate the NSP4 proteins from the 6xHis-tag. The plasmids were Sf9 codon optimised in an attempt to have better protein expression. The plasmids arrived in TOP10 bacterial stab cultures and were stored at -80° as glycerol stocks. pFastBac_OSU-v_NSP4 was purified from TOP10 overnight cultures as described in Section 2.2.5. The glycerol stocks of TOP10 cells with pFastBac_OSU-a_NSP4 could not be found, but purified pFastBac_OSU-a_NSP4 was found in 4°C storage and transformed into JM109 cells and purified from overnight cultures.

The pFastBac_OSU-a_NSP4 and pFastBac_OSU-v_NSP4 donor plasmids were sent for Sanger sequencing to ensure the ORF was correct and had no mutations. Both donor plasmids contain N-terminal 6XHis-Tag and TEV site, which is helpful for protein purification. Sequencing analysis was done over the mini-Tn7 region where the polyhedrin promoter, His-tag, TEV site and ORF of the gene of interest (OSU-a and OSU-v GS10(NSP4)) are located. The M13 forward and reverse primers were used, which bind at the polyhedrin promoter and downstream of the ORF (Figure 2.2 and Figure 2.3). The forward sequences, reverse complement sequences, and chromatograms were analysed using MEGA7 software. The sequence from the designed pFastBac_OSU-a_NSP4 and pFastBac_OSU-v_NSP4 (Section 2.2.1) was used as the references.

Sequence analysis of the sequencing data and chromatograms showed that the OSU-a NSP4 ORF (Figure 2.4) and OSU-v NSP4 ORF (Figure 2.5) had no mutations and no mutations were found in the His-tag and TEV sites of both plasmids. Therefore, the purified plasmids could be used to generate recombinant bacmid DNA.

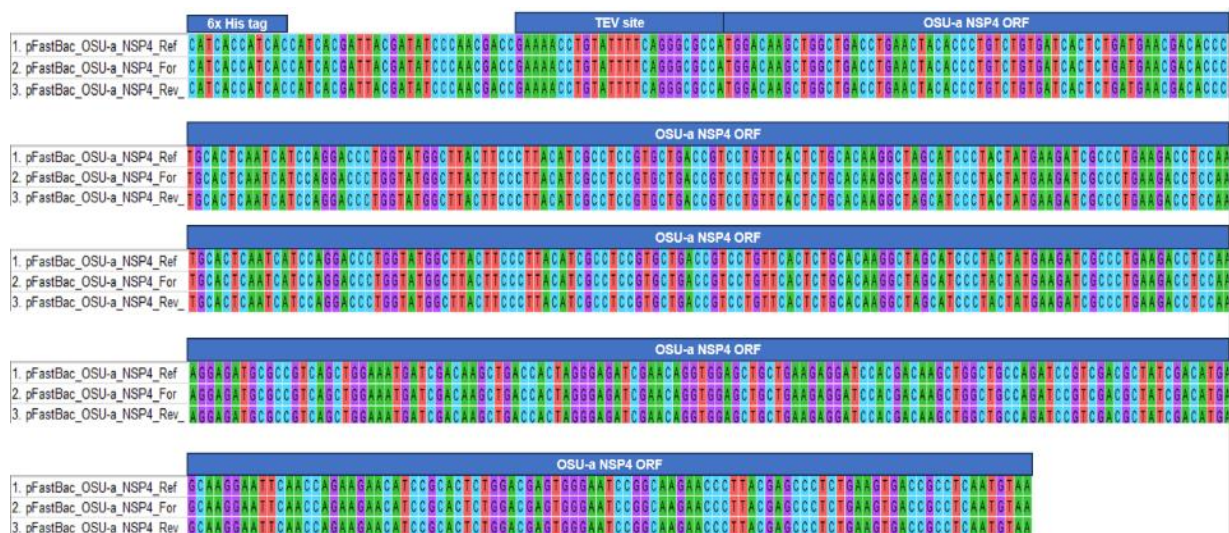


Figure 2.4: Sequence alignment of the forward and reverse Sanger sequencing data of the ORF of pFastBac_OSU-a_NSP4. The forward and reverse sequences aligned correctly with the reference sequences. No mutations were present in the His-tag, TEV site and OSU-a NSP4 ORF.

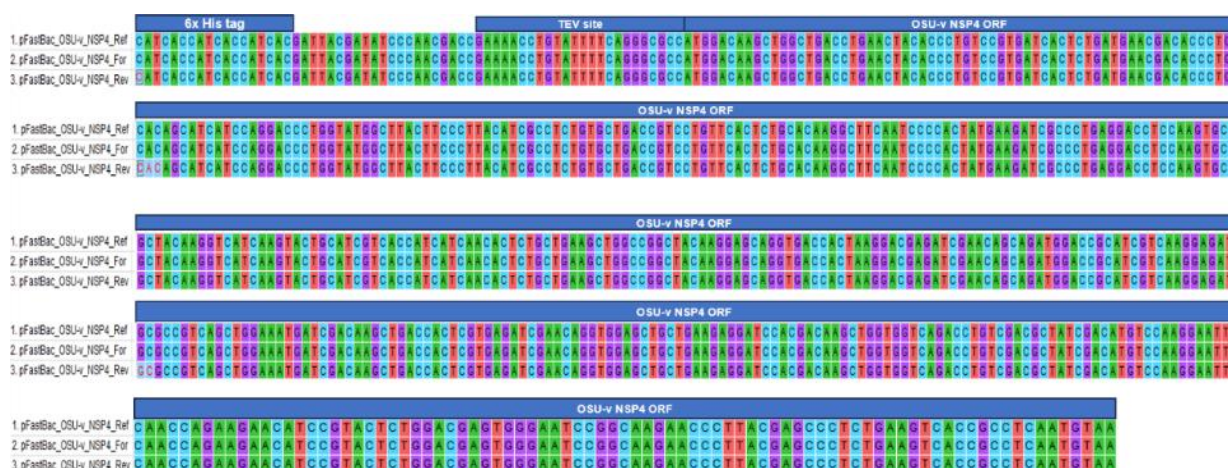


Figure 2.5: Sequence alignment of the forward and reverse Sanger sequencing data of the ORF of pFastBac_OSU-v_NSP4. The forward and reverse sequences aligned correctly with the reference sequences. There were no mutations present in the His-tag, TEV site and OSU-v NSP4 ORF.

2.3.2 Generation of recombinant bacmids containing the ORFs encoding OSU-a and OSU-v GS10(NSP4)

To generate bacmid DNA containing the ORFs encoding His-tagged OSU-a and His-tagged OSU-v GS10(NSP4) pFastBac_OSU-v_NSP4 and pFastBac_OSU-a_NSP4 were transformed into AcBACΔCC *E.coli* cells. In the AcBACΔCC *E.coli*, transposase allows the open reading frame in the pFastBac-HTA donor plasmids to be transposed into the mini-T7-

att site, disrupting the LacZ gene (Figure 2.1). Colonies with successful transposition should be white when grown on agar plates with gentamicin, kanamycin, tetracycline, X-gal and IPTG. The antibiotics ensure that each of the pFastBac-HTA donor plasmids, bacmid and helper plasmids are propagated, and only bacteria with all three will grow.

The transformation and transposition efficiency was low. Eight of 32 colonies were white of the AcBAC Δ CC cells transformed with pFastBac-HTA_OSU-a-NSP4. Thirty-seven white colonies formed on agar plates with cells transformed with pFastBac-HTA_OSU-v-NSP4. The white colonies were streaked onto agar plates supplemented with antibiotics, X-gal and IPTG to confirm that they were not mixed with wild-type AcBAC Δ CC *E.coli* cells. Four of the 8 selected colonies of cells transformed with pFastBac-HTA_OSU-a-NSP4 were truly white. After streaking the 37 colonies of cells transformed with pFastBac-HTA_OSU-v_NSP4, only 10 were truly white. The truly white colonies were grown overnight, as described in Section 2.2.7.1, and their bacmid DNA was extracted. Two blue colonies were used as wild-type bacmid controls.

To determine if the white colonies had authentic recombinant bacmid DNA, PCR analysis was done using M13 forward and M13 reverse primers. Using bacmid DNA with wild-type contamination will generate a mixture of wild-type and recombinant baculovirus. Wild-type baculovirus is known to out-compete recombinant baculovirus viruses and will affect the expression of the protein of interest.

The wild-type bacmid DNA had amplicons of 300 bp (Figure 2.6), which was the expected amplicon size indicating that no ORF was inserted in the mini-T7-att site (Figure 2.1). If an amplicon of 300 bp is generated from recombinant bacmid DNA, it indicates a mixture of wild-type and recombinant bacmid DNA. The four amplicons from selected white colonies from AcBAC Δ CC *E.coli* cells transformed with pFastBac-HTA_OSU-a_NSP4 (OSU-a NSP4 ORF col 2 ,4, 5 and 7) were all 2958 bp, which was the expected amplicon size (Figure 2.6, Table2.1). Colony 7 of AcBAC Δ CC *E.coli* transformed with pFastBac-HTA_OSU-a_NSP4 had two amplicons, an amplicon of ~2958 bp and a larger amplicon of ~5000 bp. Due to the presence of an unknown amplicon bacmid from colony 6 of AcBAC Δ CC *E.coli* transformed with pFastBac-HTA_OSU-a_NSP4 was not used for further experiments.

Eight of nine amplicons from selected white colonies from AcBAC Δ CC *E.coli* cells transformed with pFastBac-HTA_OSU-v_NSP4 (OSU-v NSP4 ORF Col 3, 5, 7, 11, 12 and 13) plasmids were 2958 bp (Figure 2.6). They all had the correct amplicon size and were all pure recombinant bacmid DNA. No amplicon was generated from recombinant bacmid containing from colony 3 of AcBAC Δ CC *E.coli* cells transformed with pFastBac-HTA_OSU-v_NSP4. This could be due to the pellet being smaller due to losses encountered when washing the pellet

with ethanol during the bacmid DNA extraction. Two of the ten selected white colonies (Col 16 and 36) from AcBAC Δ CC cells transformed with pFastBac-HTA_OSU-v NSP4 donor plasmids were not pure recombinants and had a 300bp amplicon corresponding to wild-type bacmid (Figure 2.6).

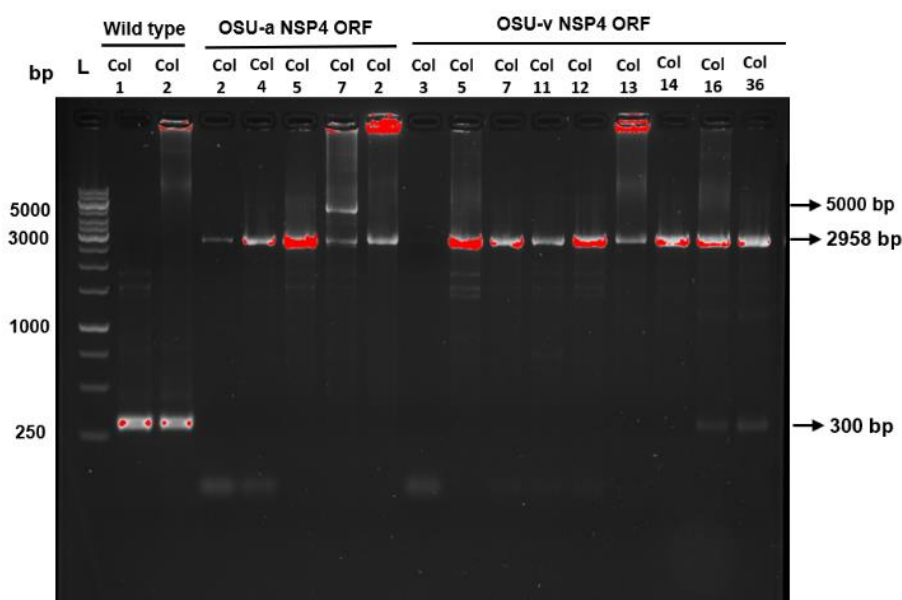


Figure 2.6: PCR analysis of bacmid DNA extracted from white AcBAC Δ CC bacterial colonies (col). Bacmid DNA from four bacterial colonies transformed with pFastBac-HTA containing OSU-a NSP4 ORF and nine bacterial colonies transformed with pFastBac-HTA with OSU-v NSP4 ORF were analysed. GeneRuler™ 1 Kb DNA ladder (Thermo Scientific, Massachusetts, USA) labelled L.

2.3.3 Baculovirus expression of His-tagged OSU-a NSP4 and OSU-v NSP4 in Sf9 insect cells

To generate recombinant baculoviruses expressing His-tagged OSU-a NSP4 and OSU-v NSP4, the recombinant bacmid DNA was transfected into Sf9 insect cells (Section 2.2.7.6). Only the bacmid found to be purely recombinant that had a 2958 bp amplicon and did not have a 300 bp wild-type bacmid amplicon (Figure 2.6, OSU-a NSP4 ORF col 2, 4, 5 and OSU-v NSP4 ORF col 5, 7 and 12), were used to express His-tagged OSU-a and OSU-v NSP4. The wild-type bacmid (Figure 2.6, wild-type col 1 and 2) was also transfected to generate a wild-type baculovirus used as controls. The generated baculoviruses were used to infect Sf9 cells to increase the viral titre to get higher protein expression. When complete CPE was observed (5 days post-infection), infected cells were lysed, and the proteins in the cells were analysed using SDS-PAGE.

Unglycosylated OSU-a NSP4 and OSU-v NSP4 have a molecular weight of 20 kDa and 28 kDa when fully glycosylated. The expression of OSU-v NSP4 as a His-tagged fusion protein should yield proteins that have a MW between 21 kDa and 29 kDa because the MW of the His-tag is 0.8 kDa. Analysis of the total protein fraction (TF) and soluble protein fraction (SF) extracted from Sf9 cells infected with recombinant baculoviruses (rBV) expressing His-tagged OSU-a (rBV OSU-a-NSP4_col2, rBV OSU-a-NSP4_col4 and rBV OSU-a-NSP4_col5) showed that there was no expression of OSU-a NSP4 (Figure 2.7). His-tagged OSU-v NSP4 was also not expressed in Sf9 cells infected with rBV OSU-v-NSP4_col5, rBV OSU-v-NSP4_col7, rBV OSU-v-NSP4_col12 (Figure 2.7).

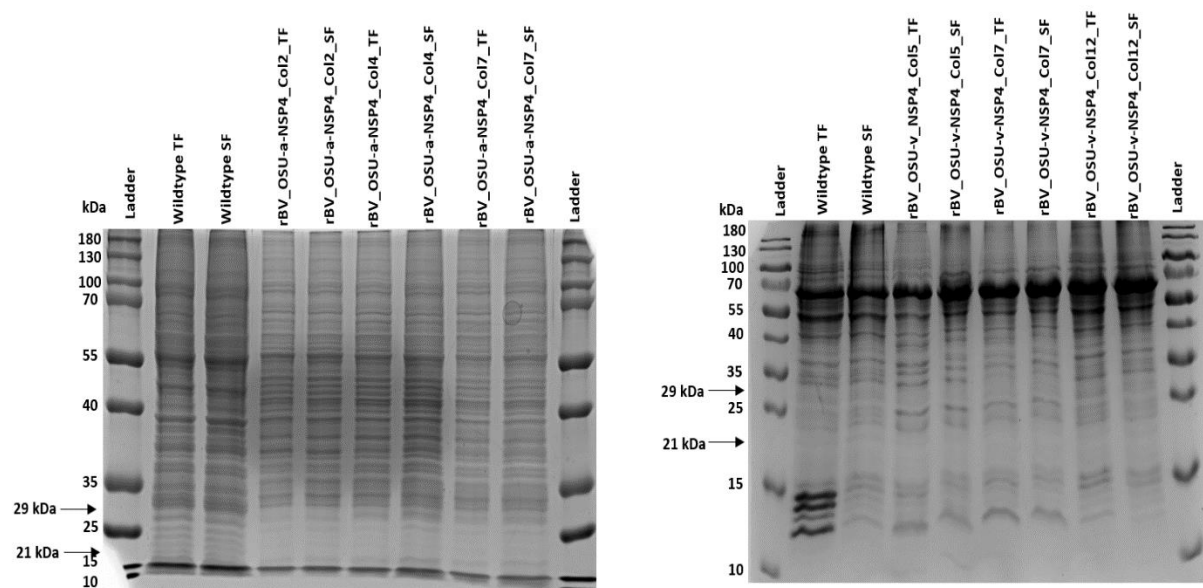


Figure 2.7: SDS-PAGE analysis of infected Sf9 cells. Infected cells were infected with wild-type (WT) or recombinant baculoviruses (rBV). No expression of OSU-a or OSU-v NSP4 is observed from Sf9 cells infected with recombinant baculoviruses generated from recombinant bacmid DNA from different bacterial colonies. TF=total fraction, SF= soluble fraction and Ladder= PageRuler Prestained Protein Ladder (Thermo Scientific, Massachusetts, USA).

Although there was no evidence of baculovirus expression of His-tagged OSU-a and OSU-v NSP4 on SDS-PAGE gels, there was evidence of CPE in Sf9 cells infected with rBV. Therefore, IFMA was performed to assess if there were any signs of His-tagged OSU-a NSP4 and OSU-v NSP4 expressed in Sf9 cells infected with rBV. For this experiment, Sf9 insect cells were infected with rBV and wild-type baculovirus and incubated for 72 hours. The infected cells were fixed at 72 hpi and used for IFMA (Section 2.2.10). IFMA (Figure 2.8) was performed using His-tag antibodies that should bind the His-tag at the N-terminus of OSU-v NSP4 and

OSU-a NSP4. Alexa Fluor 488 goat anti-rabbit IgG secondary antibodies were used, and green fluorescence was expected if there was expression of His-tagged OSU-a NSP4 or OSU-v NSP4.

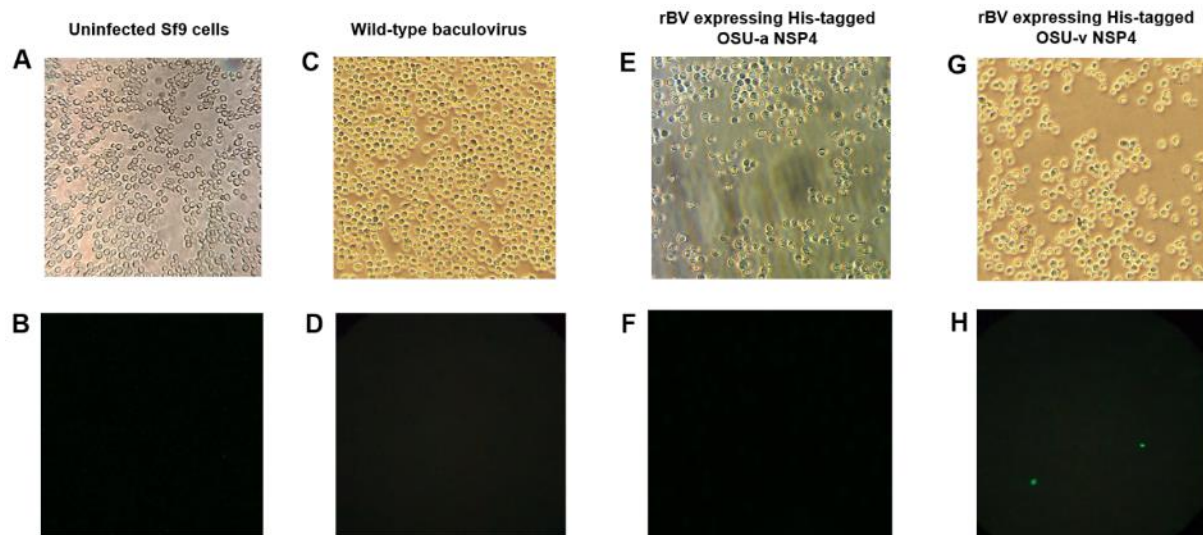


Figure 2.8: IFMA of Sf9 insect cells infected with wild-type and recombinant baculoviruses. Uninfected Sf9 insect cells A) brightfield B) fluorescence. Sf9 cells infected with wild-type baculovirus C) brightfield and D) fluorescence. Sf9 cells infected with rBV expressing His-tagged OSU-a NSP4 E) brightfield and F) fluorescence. Sf9 cells infected with rBV expressing His-tagged OSU-v NSP4 G) brightfield and G) fluorescence.

No CPE or fluorescence was observed in uninfected cells, which was expected since uninfected cells do not have His-tagged proteins (Figure 2.8A and Figure 2.8B). CPE was observed in Sf9 monolayers infected with wild-type baculovirus (Figure 2.8C). There was no fluorescence in the wild-type monolayers, indicating that there was no expression of His-tagged proteins by wild-type baculovirus (Figure 2.8D). CPE was observed on monolayers infected with rBV expressing His-tagged OSU-a NSP4 (Figure 2.8E) and monolayers infected with rBV expressing His-tagged OSU-v NSP4 (Figure 2.8F). There was no fluorescence when the monolayers infected with rBV expressing OSU-a NSP4 were visualised under UV light (Figure 2.8G). This indicated that there was no expression of His-tagged OSU-a NSP4. Two sites of foci were seen on a monolayer infected with rBV expressing his-tagged OSU-v NSP4 (Figure 2.8H). This was autofluorescence and could have been due to issues during the monolayer's fixing. Although CPE was observed in Sf9 cells infected with rBV, there was no expression of His-tagged OSU-a NSP4 or His-tagged OSU-v NSP4. The observed CPE in infected Sf9 cells could have been due to wild-type baculovirus contamination. However, this

could not be further pursued due to time constraints, and the focus was shifted to the rotavirus reverse genetics approach.

2.4 Summary

This study aimed to generate a recombinant baculovirus expressing His-tagged OSU-a NSP4 and OSU-v NSP4. As rotavirus vaccine candidates are being developed in our research group, OSU-a and OSU-v NSP4 can be used as controls in future studies. The Bac-to-Bac baculovirus expression system was used to try and achieve this aim because it has previously been used to express OSU-a NSP4 and OSU-v NSP4.

Two pFastBac-HTA donor plasmids containing his-tagged OSU-v NSP4 and OSU-a NSP4 ORFs were successfully transformed into AcBACΔCC *E.coli* to generate recombinant bacmid DNA. Blue-white screening and PCR analysis were utilised to determine pure recombinant bacmid DNA. Pure recombinant bacmid DNA was transfected into Sf9 cells to generate recombinant baculoviruses expressing the proteins of interest. None of the recombinant bacmid DNA expressed either His-tagged OSU-a or OSU-v NSP4. CPE was observed on Sf9 cells infected with recombinant baculovirus but, IFMA analysis showed that there was no expression of His-tagged OSU-a or OSU-v NSP4. The presence of CPE but no expression of his-tagged OSU-a and OSU-v NSP4 could be an indication of wild-type baculovirus contamination, but this was not confirmed due to time constraints.

The OSU-a and OSU-v NSP4 ORFs were insect cell codon optimised during this study, but codon optimisation of the OSU-a and OSU-v NSP4 ORFs could have negatively impacted their expression. The GS10(NSP4) ORFs were codon optimised to increase the expression levels of these proteins in an organism that they are not normally expressed in. However, Zhang and colleagues (Zhang *et al.*, 1998) did not codon optimise the ORFs when they expressed OSU-a NSP4 and OSU-v NSP4. Codon optimisation has been found to have a negative impact on expression levels of some proteins. One example is the expression of codon optimised PAS_chr2-2_0616 and PAS_chr2-2_0788 in *P. pastoris* showed that codon optimisation decreased the mRNA levels and proteins levels of PAS_chr2-2_0616 and PAS_chr2-2_0788 (Liu *et al.*, 2022). Due to time constraints, the focus of the study was shifted to the reverse genetics approach.

CHAPTER 3

INVESTIGATING THE EFFECT OF RECOMBINANT SA11-L2 WITH OSU-a NSP4 AND SA11/OSU-a (aa 135-141) NSP4 ON INTRACELLULAR CALCIUM LEVELS

3.1 Introduction

The commercially available rotavirus vaccines are less effective in countries in Africa and South-East Asia. This could be due to the differences in circulating strains in these regions. The two widely used vaccines RotaTeg and Rotarix are based on strains from Europe and North America. The focus of our research is to rationally design rotavirus vaccine candidates and vaccine safety is an important factor. When designing these vaccines, we must ensure that they will be non-pathogenic.

Signal transduction is a biochemical process where a ligand binds which initiates a cascade of biochemical signalling reactions. NSP4 is a viral enterotoxin and has been identified as the protein that causes calcium flux and diarrhoea in rotavirus infected individuals (Ramig, 2004). Diarrhoea is caused by an increase in intracellular Ca^{2+} that causes water to osmotically move into the intestinal lumen (Crawford *et al.*, 2017). Most of the currently available rotavirus vaccines (Section 1.8.1) were attenuated by passaging the viruses multiple times. Our research group has taken a different approach in that we rationally alter specific rotavirus proteins to attenuate them. It is important to understand which regions of NSP4 need to be mutated to attenuate it without affecting its other functions such as DLP transport. Sequence analysis of GS10(NSP4) of attenuated and virulent NSP4 of OSU and Gottfried porcine rotaviruses showed that a cluster of amino acids (aa 135,136 and 138) is important for attenuation. These amino acid changes in OSU-a NSP4 lead to it losing its ability to increase intracellular Ca^{2+} and cause diarrhoea (Seo *et al.*, 2008; Zhang *et al.*, 1998). This cluster of altered amino acids was found in the signal transducing domain which spans aa 135-140 (Figure 1.5).

Since its advent, the SA11-L2 plasmid-only based rotavirus reverse genetics system has been used to study different rotavirus proteins. The aim of the study described in this Chapter was to incorporate plasmids with full-length OSU-a NSP4 ORF with SA11 UTRs and modified SA11 NSP4 ORF with OSU-a amino acids 135-141 into the SA11-L2 rotavirus reverse genetics system. This was done to try and rescue SA11 rotavirus that cannot increase intracellular Ca^{2+} , thereby increasing the safety profile of vaccine candidates.

3.2 Materials and Methods

3.2.1 Plasmid design

Mr J Herbert designed the two pUC57-T7 plasmids used to rescue recombinant rotaviruses with OSU-a GS10(NSP4) and SA11_L2 GS10(NSP4) with aa 135-141 of OSU-a NSP4. Two pUC57-kan-T7 transcription plasmids were designed and ordered from GenScript (Piscataway, USA). The first plasmid was designed to have a SA11-L2_GS10 (NSP4) ORF containing aa 135-141 of OSU-a_GS10 (NSP4) downstream of the T7 promoter (Figure 3.1A). The second plasmid was designed to contain the OSU-a_GS10 (NSP4) ORF flanked by the SA11 5' and 3' UTRs (Figure 3.1B). Flanking the OSU-a_GS10 ORF with SA11 UTRs was done to improve genetic incompatibility with SA11-L2. Both plasmids were designed to have an anti-genomic HDV ribozyme downstream of the 3' UTR. The ribozyme is needed to generate (+)ssRNA with precise 3' ends. The plasmids arrived in TOP10 bacterial stab cultures. The plasmids were purified from the TOP10 bacterial stab cultures, transformed into EPI400 cells, and stored at -80°C.

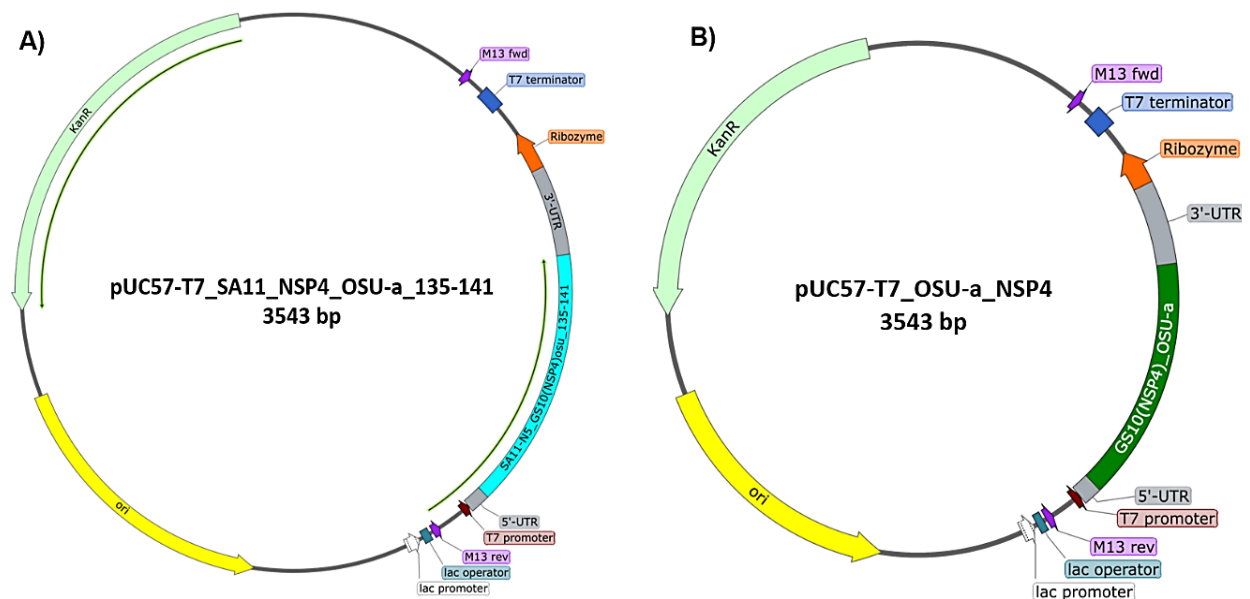


Figure 3.1: Plasmid maps of pUC57-T7 transcription plasmids used to rescue SA11-L2 with recombinant NSP4. A) The SA11-L2_GS10 containing aa 135-141 of OSU-a GS10(NSP4) (light blue) flanked by SA11 5' and 3' UTRs (grey) was placed under the control of the T7 promoter (brown) in a pUC57 backbone. The anti-genomic HDV ribozyme (orange) was placed downstream of the 3' UTR. B) The full-length OSU-a GS10(NSP4) ORF (green) flanked by SA11 5' and 3' UTRs (grey) was placed under the control of the T7 promoter (brown) in a pUC57 backbone. The HDV ribozyme (orange) was placed downstream of the 3' UTR.

The plasmid containing the SA11-L2_GS10 (NSP4) ORF containing aa 135-141 of OSU-a_GS10 (NSP4) was purified using a Qiagen mini-prep extraction kit as described in Section 2.2.4. The purified plasmid was sent for Sanger sequencing at Stellenbosch University's Central Analytical Facilities. The plasmid with the OSU-a_GS10 (NSP4) ORF was sent for Sanger sequencing by Mr J Herbert. The endotoxin-free purified (Section 3.2.2) plasmids were transformed into JM109 cells (as described in Section 2.2.2) and grown overnight for endotoxin-free plasmid purification (Section 3.2.2).

3.2.2 Endotoxin-free plasmid purification

Bacterial endotoxins, also known as lipopolysaccharides (LPS), are the predominant components of the outer layer of gram-negative bacterial cell walls (Rietschel *et al.*, 1994). Endotoxins are responsible for host interactions and virulence (Rosenfeld & Shai, 2006). During plasmid extractions, bacterial endotoxins typically contaminate the isolated nucleic acids. This occurs because endotoxins are released during the cell lysis step and since endotoxins are negatively charged, they co-purified with the DNA during DNA isolation. Some mammalian cell lines are more sensitive to endotoxins because they have certain pathophysiological effects such as activation of the immune system, changing the morphology of the cell or cell death (Butash *et al.*, 2000; Rietschel *et al.*, 1994). All these effects impact transfection and viral rescue efficiency during reverse genetics. Therefore, this necessitates the removal of endotoxins during plasmid purification. The Machery-Nagel NucleoBond Xtra Midi Plus endotoxin-free kit was used for endotoxin-free plasmid isolation. This kit is based on the plasmid purification method by Birnboim & Doly (Section 2.2.4), but a low salt concentration is used to bind the DNA on an anion exchange resin. The DNA is eluted from the column at a high salt concentration.

Endotoxin-free plasmid purification was performed according to the manufacturer's instructions. Firstly, 5ml of starter culture was prepared by inoculating LB broth with appropriate antibiotics (50 µg/ml carbenicillin for pT7 plasmids and 50 µg/ml kanamycin for the pUC57-kan-T7 plasmids) with bacteria from glycerol stocks and grown overnight at 37°C with shaking at 300 rpm for 8 hours. The starter culture was then diluted 1:1000 in 100 ml LB broth with antibiotics and grown overnight with shaking at 37°C. The cells were harvested by centrifugation at 6000 g for 10 minutes at 4°C and resuspended in resuspension buffer RES-EF containing RNase A. The cells were lysed in LYS-EF lysis buffer for 5 minutes at room temperature. While lysis was taking place, the nucleobond® Xtra columns were equilibrated by applying 15 ml buffer EQU-EF. After cell lysis, 8 ml of neutralisation buffer NEU-EF was added to the lysate, and then the lysate was incubated on ice for 5 minutes. After that, the

lysate was loaded into the equilibrated column and the column was allowed to empty via gravity flow. Then the column filter was washed with 5 ml of buffer FIL-EF and after the buffer had completely flowed through the filter containing the precipitate was removed and discarded. The column was then washed twice, firstly with 35 ml and then with 15 ml of buffer ENDO-EF and the column was emptied via gravity flow. The plasmid bound to the column was eluted with elution buffer (ELU-EF) and the plasmid yield and purity was determined using a Nanodrop One Spectrophotometer (Section 2.2.6).

3.2.3 Reverse genetics

3.2.3.1 Mammalian cell culture

For successful viral rescue transfection, transcription of (+)ssRNA, protein expression, viral formation, and propagation must occur. During the project, two cell lines were used for reverse genetics, one for transfection and another for viral propagation. Baby hamster kidney epithelial cells (BSR-T7/5) were used for transfection and African Green Monkey kidney epithelial cells (MA104) were used for viral propagation. BSR-T7/5 cells express T7 polymerase, which is needed to transcribe the transcription cassettes used for rotavirus reverse genetics. For a virus to propagate a cell line needs to be susceptible and permissive. Although rotavirus proteins can be expressed in BSR-T7/5 cells the virus cannot be propagated in those cells as they are not susceptible to SA11-L2 rotavirus. BSR-T7/5 cells were provided by Prof AC Potgieter (Deltamune, Pty Ltd). Prof HG O'Neill (University of the Free State) provided the MA104 cells.

The cells were cultured in 25 cm³ or 75 cm³ cell culture flasks or 6 well plates (TPP Techno Plastic Products AG, Trasadingen, Switzerland). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco Life Technologies, California, USA) supplemented with 5% FBS (Gibco Life Technologies, California, USA), 1% non-essential amino acids (NEAA, Gibco Life Technologies, California, USA) and 1% antibiotic/antimycotic (Anti-Anti, Gibco Life Technologies, California, USA). Geneticin (Gibco Life Technologies, California, USA) was added to the growth medium to a final concentration of 1 mg/ml in the medium used to maintain BSR-T7/5 cells. Geneticin is used for the selection of BSR-T7/5 cells expressing T7 RNA polymerase. The cells were grown to a confluence of 90% before subculturing. To subculture each cell line, the growth medium was removed, and the cells were washed twice with 5 ml of phosphate-buffered saline (PBS) (Gibco Life Technologies, California, USA). After washing, the PBS was removed and 2 ml of 0.05% trypsin-EDTA was added onto the monolayer and incubated for 1-3 minutes in a humidified incubator at 37°C. The trypsin was removed and 1 ml of FBS was used to inhibit further trypsinisation and detach the monolayer. The detached

cells were added to a new flask containing complete growth medium depending on the subculturing ratio.

3.2.3.2 Rescue protocol

The rescue protocol used by Komoto and colleagues (Komoto *et al.*, 2018) was adapted to rescue wild-type SA11-L2 (RVA/Simian-tc/ZAF/SA11-L2/1958/G3P[2] [SA11]) and recombinant SA11-L2 during this project. Equimicrogram plasmid mixtures enzyme were prepared for transfection (Table 3.1). Each mixture comprised of the 11 transcription plasmids containing the ORFs of the 11 viral genome segments downstream of a T7 promoter and two expression plasmids expressing the two subunits of the VV capping enzyme (Table 3.1).

To prepare the transfection mixtures, the concentration of each plasmid was determined using a NanoDrop One spectrophotometer and the correct volume required for the mixture was determined using the following formula: $Required\ plasmid\ volume = \frac{Required\ ng\ plasmid\ DNA}{DNA\ concentration}$. After mixing the plasmids the final concentration of the plasmid mixture was determined using a NanoDrop One spectrophotometer. 4 µg of the plasmid mixture was transfected per well in a 6-well plate.

Table 3.1: Amounts of each plasmid used to prepare equimicrogram reverse genetics plasmid transfection mixtures.

Plasmid	Ratio	Amount of plasmid (µg)
pT7_GS1	1	0.25
pT7_GS2	1	0.25
pT7_GS3	1	0.25
pT7_GS4	1	0.25
pT7_GS5	1	0.25
pT7_GS6	1	0.25
pT7_GS7	1	0.25
pT7_GS8	3	0.75
pT7_GS9	1	0.25
pT7_GS10	1	0.25
pT7_GS11	3	0.75
phCMVDream_D1R	1	0.125
phCMVDream_D12L	1	0.125
Total		4

TransIT-LT1 (Mirus Bio, Madison, USA) was the transfection reagent used for reverse genetics experiments. It was selected because it has high transfection efficiency and low toxicity to the cells. Transfection complexes were prepared according to the manufacturer's instructions. To prepare transfection complexes a volume equating to 4 µg of the prepared plasmid mixture was added to 250 µl OptiMEM in a DNA lo-bind tube and mixed by gently pipetting. Thereafter TransIT-LT1 was added to a ratio of 2.5 µl per 1 µg of plasmid DNA. The mixture was vortexed for 2-3 seconds and incubated for 15-30 minutes at room temperature to allow the DNA-lipid complexes to form.

Transfections were performed in 6-well plates (TPP Techno Plastic Products AG, Trasadingen, Switzerland) seeded with BSR-T7/5 cells grown to 75%-90% confluence at 37°C in a 5% CO₂ humidified incubator. At the selected confluency, transfection is optimal because the cells are still dividing and metabolically active. Before transfecting the cells, the complete medium on the monolayer was discarded and the monolayer was washed three times with 1 ml 1x PBS. Washing the monolayer removed any cellular debris. Following this, 2 ml of fresh complete growth medium (DMEM supplemented with 1% NEAA and 1% Anti-Anti and 5% FBS) and the cells were transfected by adding the transfection complexes drop-wise to each well. Thereafter, the cells were incubated overnight at 37°C in a 5% CO₂ humidified incubator. The following day the complete medium was removed, and the monolayer was gently washed three times with 1 ml 1X PBS and FBS-free DMEM supplemented with 1% NEAA and 1% Anti-Anti was added. The medium was changed the following day because it was observed that the transfection reagent caused cell damage and negatively affected rescue efficiency. FBS-free DMEM was added because trypsin which is used to activate rotaviruses, is inactivated by FBS. After this incubation period, the cells were incubated for 24 hours at 37°C in a 5% CO₂ humidified incubator and co-seeded with MA104 cells. Co-seeding was done to ensure that any rotavirus virions that form and burst out of BSR-T7/5 cells would immediately have cells that they can infect to help increase virus titre. To co-seed, MA104 cells were treated with 0.5% trypsin-EDTA after washing twice with 1X PBS. The cells were then detached with 1 ml FBS, and the suspended cells were pelleted at 500 xg. The supernatant was removed, and the cells were washed twice by resuspending them in 1 ml of DMEM warmed to 37°C to wash any excess FBS. After the final wash step, the cells were resuspended in 1 ml of DMEM warmed to 37°C and the cells were stained with 0.4% trypan blue (Gibco Life Technologies, California, USA), using an equal ratio of cells to trypan blue, and counted using a stage haemocytometer. After counting the cells, the 1 ml cell suspension was used to co-seed the wells with MA104 cells at a density of 2×10^5 per well. Once the co-seed MA104 cells had adhered, porcine trypsin type IX (Sigma-Aldrich, St.Louis, Missouri) was added to each well to a final concentration of 0.5 µg/ml. Porcine trypsin is needed to

cleave VP4 into VP8* and VP5* for VP5* to interact with integrins for infection to occur. After three days of incubation at 37°C in a 5% CO₂ humidified incubator, the co-seeded monolayers were freeze-thawed.

To store or propagate the rescued viruses, the transfected and co-seed monolayer was freeze-thawed three times. The cells were frozen at -20°C and thawed at room temperature. Once thawed, the whole lysate was pipetted to be homogenised before it was refrozen. After the last freeze-thaw cycle, the whole lysate was transferred to a labelled 2 ml tube and stored at 4°C until the virus needed to be passaged. This lysate was labelled as P0.

3.2.3.3 Viral propagation

To increase the rescued viruses so that they can be used for further experiments, they were propagated on MA104 cells. To activate the inoculum, porcine trypsin type IX was added to a final concentration of 10 µg/ml to the required volume of whole lysate and mixed by vortexing for 3 seconds. Thereafter, the whole lysate was incubated at 37°C for 30 minutes.

The viruses rescued during this MSc were propagated on MA104 monolayers seeded in 25cm³ or 75cm³ flasks. To propagate the virus the monolayer was washed with 1X PBS and the activated lysate was added to the monolayer and incubated at room temperature for 60 minutes with gentle shaking. Thereafter, the activated lysate was removed and stored at 4°C and then FBS-free DMEM supplemented with 1% NEAA, 1% Anti-Anti and porcine trypsin type IX to a final concentration of 0.5 µg/ml was added to the infected monolayer. The infected cells were incubated at 37°C in a 5% CO₂ humidified CO₂ incubator until CPE was observed. Once 80%-100% CPE was observed, the remaining cells were detached by pipetting. The cell debris was pelleted via centrifugation at 2500 xg for 10 minutes and used for rescue verification.

3.2.4 Rescue verification

3.2.4.1 dsRNA extraction

Double-stranded RNA (dsRNA) extraction was done to verify that the intended virus was successfully rescued. The TRIzol (Thermo Scientific, Massachusetts, USA) based dsRNA extraction protocol described by Potgieter and colleagues (Potgieter *et al.*, 2009) was used during this study. This method is based on a phenol-chloroform RNA extraction method. TRIzol is a chemical solution composed of phenol and guanidinium chloride which denatures proteins, cell membranes, and other cellular material. Adding chloroform helps separate the

lysate into an organic and aqueous phase. The proteins will be in the bottom organic phase, DNA in the middle interface and RNA in the top aqueous phase.

After infecting MA104 cells with activated inoculum and 80%-100% CPE was observed, the remaining attached cells were detached by pipetting and transferred to a 15 ml Falcon tube. The whole lysate was centrifuged at 2500 xg for 10 minutes to pellet the cells. The supernatant was transferred to properly labelled Falcon tubes and stored at 4°C. The cell pellet was resuspended in 750 µl TRIzol, transferred to a 2 ml microcentrifuge tube and incubated for 10 minutes at room temperature. After incubation, 250 µl of chloroform was added and the sample was vortexed to mix and then incubated for 2 minutes at room temperature. Thereafter, the sample was centrifuged for 30 minutes at 16000 xg. The RNA containing aqueous phase was transferred to a clean 1.5 ml microcentrifuge tube and isopropanol was added at a 1:1 ratio. The sample was incubated for 10 minutes at room temperature, followed by 30 minutes of centrifugation at 16000 xg to pellet the RNA. The supernatant was removed, and the pellet was resuspended in 30 µl LiChrosolv HPLC water (Merck, Darmstadt, Germany) and the extracted RNA was analysed using AGE (Section 2.2.6).

3.2.4.2. Genome segment-specific cDNA synthesis and amplification

Genome segment-specific cDNA synthesis was performed to send cDNA samples for Sanger sequencing to confirm that the rescued viruses differ from the wild-type virus. The SuperScript III One-step RT-PCR Platinum kit (Invitrogen, Waltham, USA) was used for this project to produce cDNA. This kit has two main steps: synthesis of cDNA from RNA and PCR amplification of the cDNA. The kit uses the Moloney murine leukaemia virus (M-MLV) reverse transcriptase (RT) to convert RNA to cDNA and Taq DNA polymerase for PCR amplification. M-MLV RT lacks DNA endonuclease activity, has reduced RNase H activity and has high thermal stability (Hagen *et al.*, 2013).

To synthesise and amplify GS10 (NSP4) cDNA, the reaction was set up as per Table 3.2. The extracted dsRNA was added to a PCR containing forward and reverse primer (Table 3.3) diluted to the required concentration in Nuclease free water needed for each reaction. The samples were then heated for 30 seconds at 95°C and then immediately incubated on ice. At high temperatures, dsRNA will denature to ssRNA and the primers can anneal to the ssRNA when the sample is cooled on ice (Prof AC Potgieter, personal communication). This was done in an attempt to ensure that any possibly contaminating plasmid DNA, introduced during transfection will not be amplified. Following this step, the required volume of 1U polymerase mix and 2X reaction mix was added to the sample, Table 3.2.

The following reaction conditions were used: 1 cycle of cDNA synthesis at 50°C for 30 minutes followed by 2 minutes of incubation at 94°C. The cDNA was synthesised in 40 cycles that had a 94°C denaturation step for 15 seconds, followed by annealing at 55°C for 30 seconds and elongation for 1 minute 30 seconds at 68°C. This was followed by final elongation at 68° for 5 minutes.

Table 3.2: SuperScript III One-step RT-PCR Platinum reaction mixture.

Contents	Final concentration or volume
2X Reaction Mix	25 µl
Forward primer	0.5 µM
Reverse primer	0.5 µM
1U SuperScript RT/Platinum Taq mix	2 µl
RNA template	1 µl
Nuclease free H ₂ O	to 50 µl

Table 3.3: Primers used for GS10 (NSP4) cDNA synthesis.

Primer	Primer sequence	Length	T _m
SA11corGS10F	5'TAATACGACTCACTATAGGCTTTTAAAAGTTC3'	32 bases	42°C
SA11GS10iNR	5GGGACCATGCCGCGCGTACATTAAGAC3'	30 bases	43°C

3.2.4.3. PCR clean-up

PCR clean up steps were performed on the amplified samples using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) as per the manufacturer's guidelines. This was done to remove impurities, primers, enzymes, excess nucleotides, and salts from the cDNA samples. The kit is used to purify PCR products that are larger than 100 bp. The method uses columns with a silica-gel membrane that selectively bind DNA at high salt concentrations and does not bind primers or other impurities. The impurities are removed using buffers and centrifugation and pure DNA can be eluted at low salt concentrations.

To remove the impurities from the amplified cDNA, 5 volumes of buffer PB was mixed with 1 volume of the sample. The sample was then transferred to a QIAquick spin column that was placed in a 2 ml collection tube. The sample was centrifuged at 17900 xg for 60 seconds to allow DNA to bind to the column membrane. Thereafter, the flow-through was discarded and the column was placed back in the tube. The column was washed by adding 750 µl buffer PE, centrifuging the sample for 60 seconds, and discarding the flow-through. The column was

centrifuged for another 60 seconds at 17900 xg to remove any residual buffer and then transferred to a clean 1.5 ml microcentrifuge tube. The DNA was eluted from the column membrane by adding 50 µl nuclease-free H₂O and centrifuging the column for 60 seconds at 17900 xg. Aliquots of the purified cDNA were sent to Inqaba Biotech for Sanger sequencing using the primers listed in Table 3.3.

3.2.5 Plaque assay

Plaque assays were done in this project to determine the titre of the rescued rotaviruses. To perform plaque assays, a confluent monolayer of permissive cells is infected with the virus and an overlay is applied on the infected monolayer. The overlay helps limit viral spread ensuring that virions that are released via cell lysis from infected cells only spread to the immediate neighbouring cell. Traditionally agarose is used as an overlay. However, during this project, Avicel which is a low-viscosity microcrystalline cellulose, was used for overlays. Agarose overlay has some pitfalls, such as difficulty removing the overlay without disturbing the monolayer and agarose killing the cells if the temperature is too high for the cell type. Avicel is easier to remove with minimal disturbance of the monolayer (Yin *et al.*, 2015).

Plaque assays and multi-step growth kinetics were performed on MA104 monolayers. The MA104s cells were seeded in 12 well plates and allowed to grow to 100% confluency. To perform plaque assays viral stocks were activated with 1 mg/ml porcine trypsin type IX to a final concentration of 0.5 µg/ml and incubated for 30 minutes at 37°C. The activated virus was serially diluted (10^0 - 10^{-10}) in DMEM and added to the monolayers, which were then incubated while shaking for 60 minutes at room temperature. The semi-solid overlay was prepared by combining 2.4% Avicel RC-581 (FMC biopolymer, Philadelphia, USA) with 2X FBS-free DMEM (supplemented with 2 µg/ml porcine trypsin type IX, 2% NEAA, 2% Anti-Anti) in a 1:1 ratio. The final concentrations of the overlay components were as follows: 1.2% Avicel RC-581, 1x DMEM, 1 µg/ml porcine trypsin type IX 1% NEAA, 1% Anti-Anti. Following incubation, the activated inoculum was removed from the monolayers and replaced with 1 ml of the semi-solid overlay. The plates were incubated for 3 days at 37°C in a 5% CO₂ humidified incubator. The overlay was removed after the 3-day incubation period by pipetting, and after that, the cells were fixed for 30 minutes with 4% formaldehyde at room temperature. The fixed cells were stained with 0.25% crystal violet (Sigma-Aldrich, St-Louis, USA) in 20% methanol and the plaques were counted to determine the titre.

3.2.6 Multi-step growth kinetics

Multi-step growth curves were performed to compare the replication kinetics of the recombinant viruses with that of the wild-type SA11-L2 virus. The multi-step growth curves were performed on 100% confluent MA104 cells seeded 25 cm³ flasks. For each virus, 7 flasks were infected with activated viral lysates at a multiplicity of infection (MOI) of 0.001 and incubated at 37°C in a 5% CO₂ humidified incubator. Seven-time points, each 12 hours apart (0, 12, 24, 36, 48, 60 and 72 hours), were selected as the period at which the infection would be halted. At each time point, one flask with infected cells would be transferred to -20°C. Once all seven time point incubations had occurred each flask was freeze-thawed thrice. The whole lysate was used to determine the titre of the virus at each time point by performing plaque assays as described in Section 3.2.5.

The obtained data was normalised by dividing the titre at each time point with the titre at time point zero. The normalised data was log transformed to provide data with a normal distribution. Two-way analysis of variance (ANOVA) of the normalised data was performed using IBM SPSS (IBM, Armonk, USA). Each virus was compared to wild-type SA11-L2 and p-values less or equal to 0.05 was considered statistically significant.

3.2.7 Calcium flux assay

Calcium flux assays were conducted to determine the levels of intracellular Ca²⁺ after infection with recombinant rotaviruses to determine if they could increase intracellular Ca²⁺ levels. The Fura-2AM calcium indicator was used for this experiment. The Fura-2AM assay is a ratiometric assay that is used to measure intracellular Ca²⁺ levels in cells. Fura-2AM is the membrane-permeant derivative of the amino polycarboxylic acid dye that binds free intracellular Ca²⁺. When the dye enters the cell the acetoxymethyl ester is removed by cellular esterases. Without the ester group, the charged dye cannot diffuse out of the cell (Oakes *et al.*, 1988). The excitation wavelength Fura-2 changes when it is bound to calcium, unbound dye excites at 380 nm and bound dye is excited at 340 nm (Grynkiewicz *et al.*, 1985).

The calcium flux assay was conducted on infected MA104 monolayers seeded in a 96-well black plate with a black bottom (SPL Life Sciences, Kyonggi-do, South Korea). MA104 cells were seeded at a seeding density of 4x10⁴ cells per well and allowed to adhere overnight in FluoroBrite™ DMEM (phenol red free medium) supplemented with 5% FBS, 1% Anti-Anti, and 1% NEAA. Phenol red free medium was used for this experiment because phenol red affects the fluorescence readings as it increases the background fluorescence. The seeded cells were incubated for 16 hours at 37 °C in a 5% CO₂ humidified incubator. A 96-well plate (TPP Techno Plastic Products AG, Trasadingen, Switzerland) was seeded the same way as the black 96-

well plates to use as a duplicate plate to track what is happening in the black plate. After the overnight incubation, the seeded cells were washed twice with 1X PBS and infected with activated whole lysate at an MOI of 1 for 1 hour at room temperature. The whole lysate was removed and replaced with the FluoroBrite™ DMEM supplemented 1% Anti-Anti and 1% NEAA with DMEM infected cells were incubated for 16 hours and at 37 °C in a humidified incubator. To serve as a positive control, a monolayer was treated with 1 µM Ionomycin one hour before loading the monolayer with Fura-2AM. Ionomycin is a calcium ion ionophore and increases intracellular Ca^{2+} levels. Following the incubation period, the cells were washed twice with Hepes Buffered Saline (HBS pH7.2, 10 mM Hepes, 140 mM NaCl, 4.7 mM KCl, 10 mM D-glucose, 1.2 mM CaCl_2 , 1 mM MgCl_2) warmed to 37°C. The Fura-2AM indicator was prepared for loading by diluting 1 mM Fura-2AM in FluoroBrite™ DMEM mixed with an equal volume of HBS to a final concentration of 5 µM. The washed cells were loaded with the prepared 100 µl of the Fura-2AM indicator and incubated at 37°C for 60 minutes. Afterward, the indicator was removed, and the Fura-2AM loaded cells were washed for 30 minutes in HBS with 1 mM CaCl_2 . The excess CaCl_2 added to the HBS serves to remove any extracellular dye. After washing, the HBS was discarded and replaced with 50 µl 1XPBS. To chelate intracellular Ca^{2+} infected MA104 cells were treated with 50 µM BAPTA-AM. BAPTA is a chelator that selectively binds Ca^{2+} and has been shown to decrease intracellular calcium in rotavirus infected cells (Collatz *et al.*, 1997; Pérez *et al.*, 1998). The acetoxymethyl ester (AM) group helps BAPTA enter the cell. Once cellular esterases remove the AM group the chelator cannot diffuse out of the cell because BAPTA is negatively charged. The level of intracellular Ca^{2+} was measured using a SpectraMax®iD3. The fluorescence for each plate was read from the top using the following settings: fluorescence wave-length excitation 340 nm and 380 nm, emission at 510 nm for 30 minutes at 5 minutes intervals. Two-way analysis of variance (ANOVA) of the data was performed using IBM SPSS (IBM, Armonk, USA). The intracellular Ca^{2+} levels in the cells infected with each virus were compared to intracellular Ca^{2+} levels in cells infected with wild-type SA11-L2. p-values less or equal to 0.05 was considered statistically significant.

3.3 Results and Discussion

3.3.1 Sequence analysis of pUC57-T7_SA11_NSP4_OSU-a_135-141 and pUC57-T7_OSU-a_NSP4

Experiments described in this Chapter aimed to generate SA11-L2 that had GS10(NSP4) of OSU-a rotavirus and rSA11-L2 that had SA11-L2 GS10(NSP4) that contained amino acids 135-141 of OSU-a NSP4. Introducing these genome segments into the reverse genetics

system was done to attempt to rescue SA11-L2 rotaviruses that replicate but cannot increase intracellular Ca^{2+} levels. Mr J Herbert designed the two NSP4 plasmids used in the study. pUC57-T7_SA11_NSP4_OSU-a_135-141 was designed by replacing aa 135-141 of GS10(NSP4) of SA11 with those of OSU-a GS10(NSP4) (Section 3.2.1). The cluster of amino acids from OSU-a NSP4 that were introduced into SA11-L2 NSP4 is found in the signal-transducing domain of OSU-a NSP4 and did not affect the protein's ability to bind integrins (Seo *et al.*, 2008). pUC57-T7_OSU-a_NSP4 contained full length OSU-a GS10(NSP4) flanked by SA11 UTRs (Section 3.2.1). Both plasmids were archived in EPI400 cells at -80°C .

Both plasmids were purified from overnight cultures using a Qiagen mini-prep kit and sent for Sanger sequencing to evaluate the transcription cassette before incorporating the plasmids into the reverse genetics system. The sequencing data was analysed using Mega 7 software. The forward sequences and the reverse complement of the reverse sequences were aligned with their respective reference sequences (Figure 3.2). Sequence analysis showed that the transcription cassette of pUC57-T7_SA11_NSP4_OSU-a_135-141 aligned correctly with the reference (Figure 3.2). The transcription cassette has a T7 promoter and terminator flanking the 5' UTR, ORF, 3'UTR and HDV ribozyme. The GS10(NSP4) ORF contained the nucleotides that encode aa 135-141 of OSU-a NSP4. This showed that the transcription cassette of pUC57-T7_SA11_NSP4_OSU-a_135-141 was correct, and the plasmid could be used for transfection to rescue recombinant SA11. Sequence analysis showed that the transcription cassette of pUC57-T7_OSU-a_NSP4 aligned correctly with the reference (Figure 3.2). The transcription cassette has a T7 promoter and terminator flanking the 5' UTR, ORF, 3'UTR and HDV ribozyme. The GS10(NSP4) ORF was that of OSU-a NSP4 and the ORF was flanked by SA11 5' and 3' UTRs (Figure 3.2). This showed that the transcription cassette of pUC57-T7_OSU-a_NSP4 was correct, and the plasmid could be used for transfection to rescue recombinant SA11-L2 containing OSU-a NSP4.

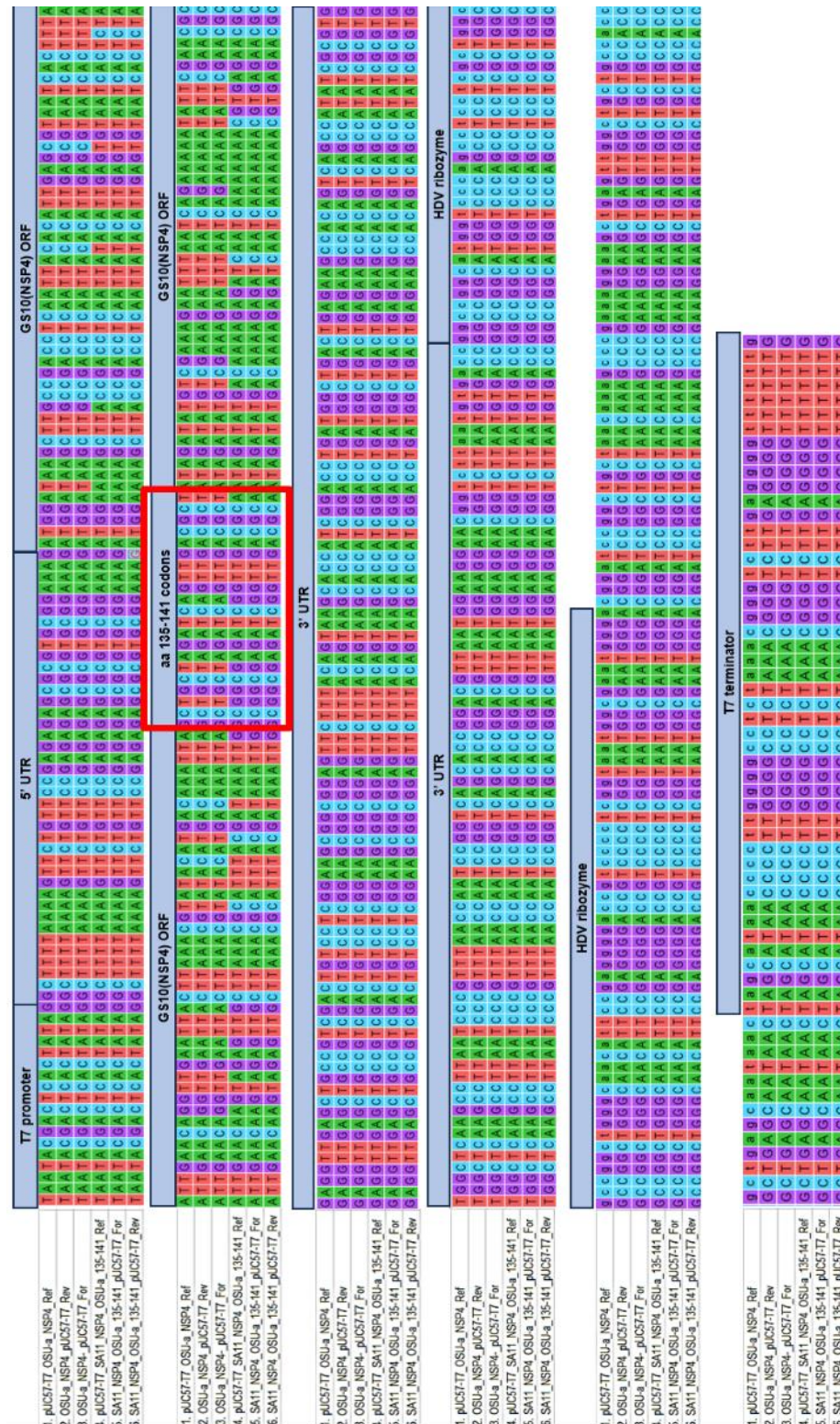


Figure 3.2: Sequence alignment of the forward and reverse sequence of the transcription cassette of pUC57-T7_SA11_NSP4_OSU-a_135-141 and pUC57-T7_OSU-a_NSP4. The transcription cassette of the Sanger sequenced plasmids had a T7 promoter and terminator, 3' and 5' UTRs flanking the ORF and a HDV ribozyme downstream of the 3' UTR. Nucleotides encoding aa 135-141 indicated in red.

3.3.2 Rescue of recombinant SA11-L2 with GS10(NSP4) of OSU-a and SA11-L2 GS10(NSP4) with aa 135-141 of OSU-a NSP4

To determine if the rescue of SA11-L2 rotavirus with SA11-NSP4 containing aa 135-141 of OSU-a NSP4 and SA11-L2 with OSU-a NSP4 was possible, pUC57-T7_SA11_NSPP4_OSU-a_135-141 and pUC57-T7_OSU-a_NSPP4 were used in place of pT7-GS10(NSP4) when preparing the transfection mixtures protocol as described in Section 3.2.3.2. Virus rescue was performed as described in Section 3.2.3.2 by transfecting 4 µg of the respective transfection mixtures into BSR-T7/5 monolayers. The transfected cells were co-seeded with MA104 cells after 24 hours to ensure that any virus released from the BSR-T7/5 cells had susceptible cells to infect. The viruses were blind passaged on MA104 cells after three days to increase the viral yield. The first sign of CPE was observed 1 day after infection in monolayers infected with the P0 viral stocks of each rescued virus. Viral dsRNA was extracted from infected MA104 monolayers to confirm virus rescue after complete CPE was observed. The dsRNA was analysed on a 1% agarose gel (Figure 3.3).

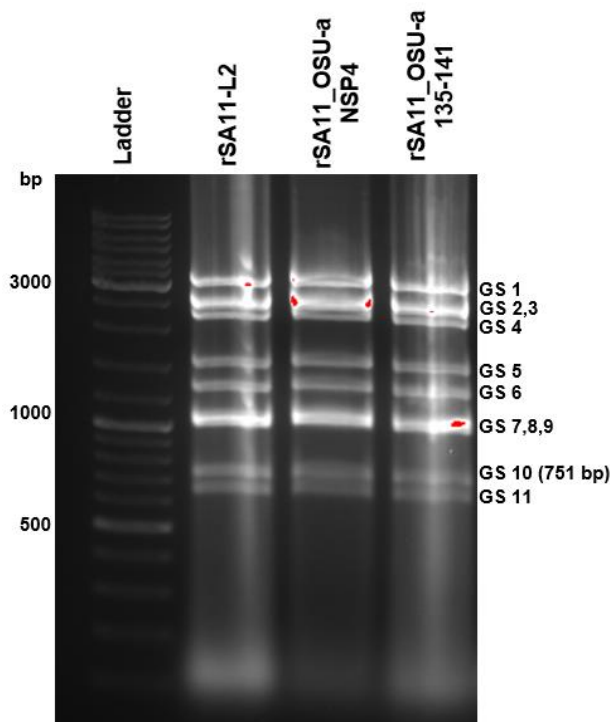


Figure 3.3: Agarose gel of dsRNA extracted from MA104 cells infected with rSA11-L2, rSA11_OSU-a_NSPP4 and rSA11_OSU-a_135-141. The dsRNA of rSA11-L2, rSA11_OSU-a_NSPP4 and rSA11_OSU-a_135-141 was extracted from MA104 cells infected with P0 whole lysates. The GeneRuler™ 1 kb DNA Ladder (Thermo Scientific) was used as a reference.

The rescued viruses all had the typical dsRNA profile of SA11-L2. GS2(VP2) and GS3(VP3) migrate at the same rate and are visualised at ~ 2400 bp; GS7(NSP3), GS8(NSP2) and GS9(VP7) also migrate at the same rate and migrate to ~1000 bp on an agarose gel. All three rescued viruses had GS10(NSP4) that migrated at the same rate and the GS10(NSP4) band correlated to 751 bp was visualised on the gel (Figure 3.4). The extracted RNA was not treated with LiCl to remove ssRNA and the smears present in each lane could be ssRNA.

GS10(NSP4) specific cDNA synthesis was performed to verify that the GS10(NSP4) of the three rescued viruses differed. The cDNA was generated using a SuperScript III One-step RT-PCR Platinum kit and GS10(NSP4) specific primers listed in Table 3.3 (Section 3.2.4.2). The primers bind the 5' and 3' UTRs of GS10(NSP4). To prevent amplification of plasmid DNA that could be present, the extracted dsRNA was mixed with GS10(NSP4) specific forward and reverse primers and denatured at 95°C before being cooled on ice to allow the primers to bind the denatured dsRNA. The cDNA generated from denatured and undenatured dsRNA of all three viruses migrated at the same rate and had the expected band size of 751 bp. There was no difference in the intensity of the bands generated from denatured and undenatured dsRNA. Therefore, in future the extracted dsRNA will be treated with DNase to remove any possibly contaminating plasmid DNA.

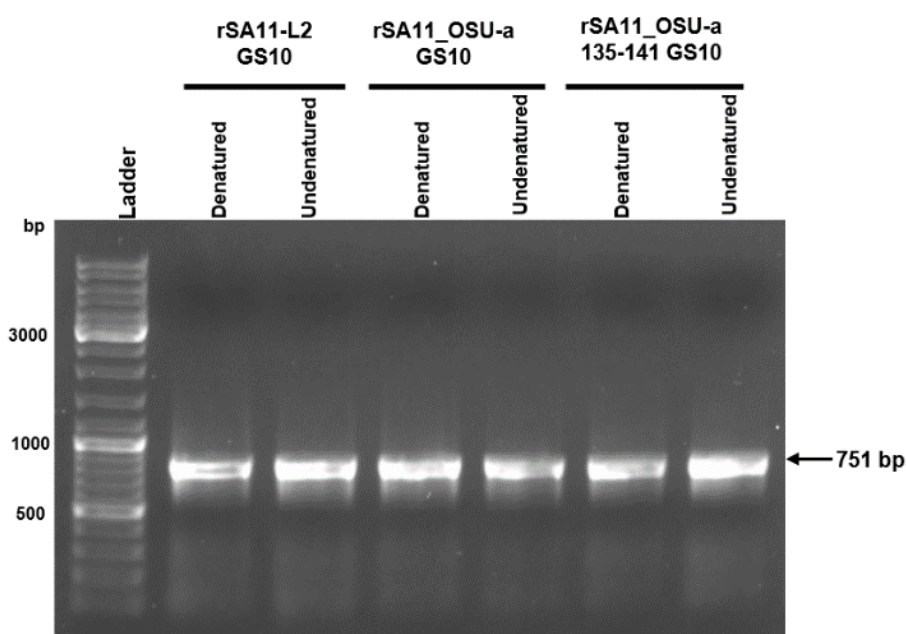


Figure 3.4: Agarose gel of GS10(NSP4) cDNA amplicons. Amplicons generated from denatured and undenatured sample dsRNA was visualised on a 1% agarose gel. The GeneRuler™ 1 kb DNA Ladder (Thermo Scientific) was used as a reference.

There were no restriction enzyme sites specific to each GS10 cDNA product that could be used to verify that the GS10(NSP4) from rSA11_OSU-a_NSP4 and rSA11_OSU-a_135-141 differed from GS10(NSP4) of rSA11-L2. GS10(NSP4) from rSA11_OSU-a_NSP4, rSA11_OSU-a_135-141 and rSA11-L2 were subject to PCR clean-up using a QIAquick PCR purification kit (Section 3.2.4.3) to remove impurities from the samples. The samples were sent for Sanger sequencing and the primers listed in Table 3.3 were used for sequencing.

Each sample's forward and reverse complement sequence was aligned with the correct reference sequence (Figure 3.5). cDNA from GS10(NSP4) of rSA11-L2 had the correct GS10(NSP4). GS10(NSP4) of rSA11_OSU-a_NSP4 aligned with the OSU-a GS10(NSP4) reference. GS10(NSP4) of rSA11_OSU-a_135-141 aligned with the SA11 GS10(NSP4) reference sequence as expected, except for the nucleotides 403-422 encoding aa 135-141. GS10(NSP4) of rSA11_OSU-a_135-141 contained the correct mutation at the nucleotides that encode for aa 135-141 and that region of GS10(NSP4) of rSA11_OSU-a_135-141 aligned correctly with the OSU-a GS10(NSP4) reference. These results confirmed that wild-type rSA11-L2, rSA11-L2 with OSU-a NSP4 and rSA11-L2 with SA11-L2 NSP4 with aa 135-141 of OSU-a NSP4 were successfully rescued. Analysis of the translated amino acid sequence showed that the aa 135-141 of GS10(NSP4) of rSA11_OSU-a_NSP4 and rSA11_OSU-a_135-141 were the same and differed from aa 135-141 of rSA11-L2 (Figure 3.5).

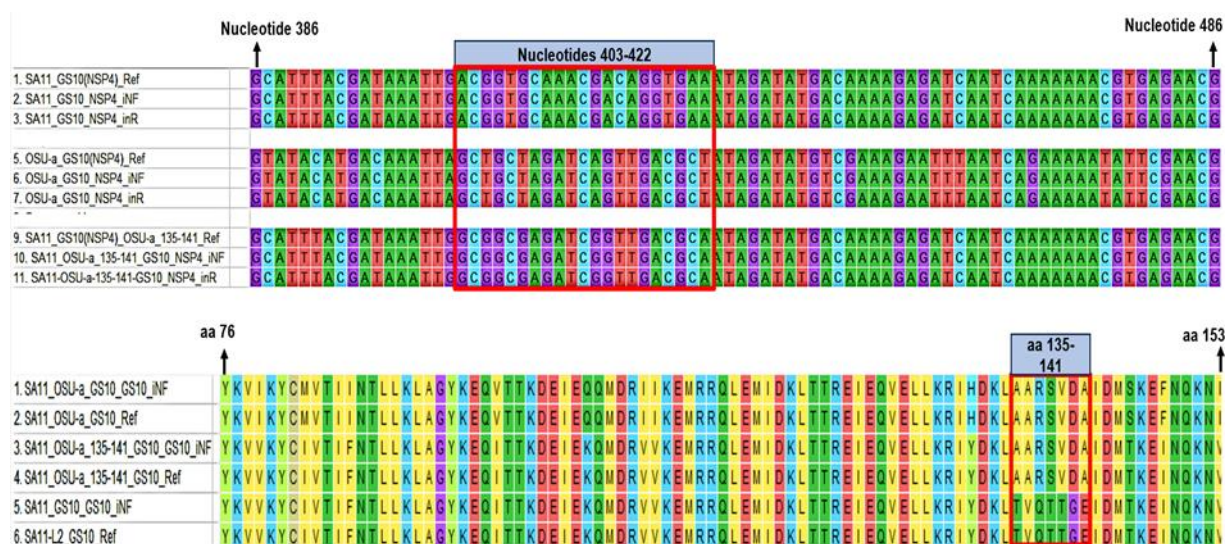


Figure 3.5: Multiple sequence alignment of GS10(NSP4) cDNA generated from MA104 cell pellets infected with rSA11-L2, rSA11_OSU-a_NSP4, rSA11_OSU-a_135-141. Nucleotides 403-422 which encode aa 135-141 in the GS10(NSP4) ORF are shown. Amino acids 135-141 of the translated nucleotide sequence are indicated in red.

3.3.3 Replication kinetics of rescued rotaviruses

Multi-step growth curves were performed to determine whether the replication kinetics of the recombinant rescued viruses, rSA11_OSU-a_NSP4 and rSA11_OSU-a_135-141, were affected by changing GS10(NSP4). Multi-step growth curves were performed over 72 hours at a MOI of 0.001, as described in Section 3.2.6. MA104 cells seeded in 12 well plates were infected with P1 viral stocks from a dilution of 10^0 to 10^{-10} and the infected 12 wells plates were incubated for 7 different periods (0 hours, 12 hours, 24 hours, 32 hours, 48 hours, 60 hours and 72 hours). The growth curve experiments were done in triplicate. The obtained data was normalised, and the statistical difference was determined with two-way ANOVA with LSD post-hoc.

The growth curve of rSA11_OSU-a_NSP4 and rSA11_OSU-a_135-141 was compared to that of rSA11-L2 (Figure 3.6). All three rescue viruses had similar growth patterns. The viruses replicated at a similar rate, rSA11-L2 had a final titre of $1,77 \times 10^8$ PFU/ml, rSA11_OSU-a_NSP4 reached a final titre of $6,6 \times 10^8$ PFU/ml and rSA11_OSU-a_135-141 reached a final titre of $2,00 \times 10^7$ PFU/ml. Although the final titre of rSA11_OSU-a_135-141 was lower than the titre of rSA11-L2 and rSA11_OSU-a_NSP4, there was no statical difference between the replication kinetics of the three viruses.

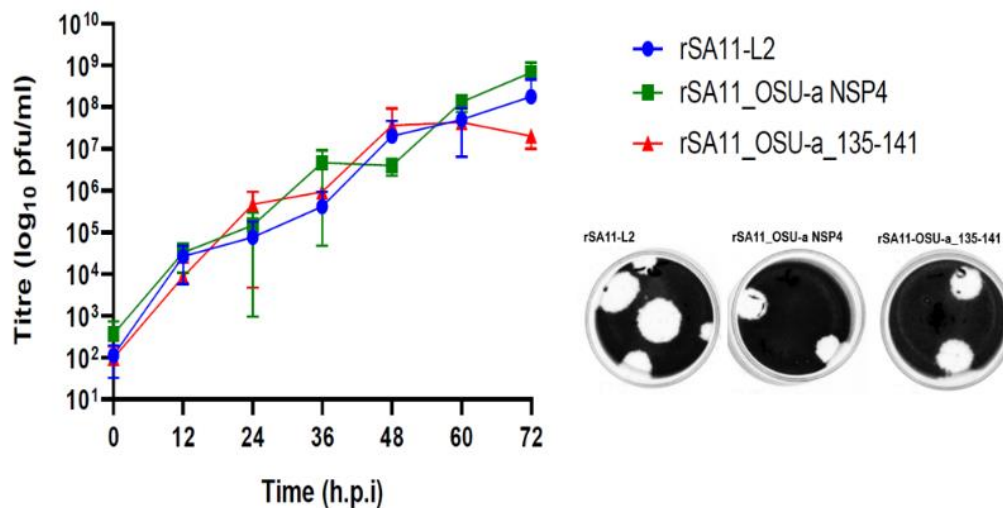


Figure 3.6: Growth kinetics and plaque morphology of rSA11-L2, rSA11_OSU-a_NSP4 and rSA11_OSU-a_135-141. The titre of each virus is shown at every 12-hour interval. The mean value of each triplicate was plotted on each time point and the error bars indicate the standard deviation of the experiments done in triplicate. There was no statistical difference in the growth rates of rSA11-L2, rSA11_OSU-a_NSP4 and rSA11_OSU-a_135-141. The plaque sizes of all three rescued viruses were similar.

The size of the plaques formed by rSA11_OSU-a_NSP4 and rSA11_OSU-a_135-141 were similar to those formed by rSA11-L2. These results show that rSA11_OSU-a_NSP4 and rSA11_OSU-a_135-141 can still replicate well and their replication has not been affected by the changes in GS10(NSP4). rSA11_OSU-a_NSP4 and rSA11_OSU-a_135-141 can still spread from cell to cell at a similar rate to rSA11-L2. Substituting aa 135-141 of the SA11-L2 NSP4 with those of OSU-a NSP4 did not affect the replication kinetics of rSA11_OSU-a_135-141. SA11-L2 has a DS-1-like GS10(NSP4) and OSU-a GS10(NSP4) is Wa-like (Figure 1.1). The Wa-like NSP4 DLP binding domain did not affect the binding of the DS-1-like VP6, the outer layer of the rSA11_OSU-a_NSP4 DLP.

3.3.4 Assessment of intracellular calcium levels in MA104 cells infected with rescued rotaviruses

To determine the effect of the rescued viruses on intracellular Ca^{2+} levels Ca^{2+} flux assays were conducted. MA104 cells were infected at a MOI of 1 16 hours prior to conducting the Ca^{2+} flux assay. The Ca^{2+} flux was only evaluated 18 hpi because it has been shown that at a MOI of 1, a significant increase in intracellular Ca^{2+} is seen after 16 hpi (Chang-Graham *et al.*, 2019). The Ca^{2+} flux assay was performed using Fura-2AM fluorescence dye as described in Section 3.2.7. Cells treated with ionomycin, which increases the intracellular Ca^{2+} , served as a positive control. BAPTA-AM decreases intracellular Ca^{2+} and can also decrease intracellular Ca^{2+} levels in rotavirus infected cells. Infected cells were treated with BAPTA-AM to determine its effect on intracellular Ca^{2+} levels in cells infected with rSA11_OSU-a_NSP4 and rSA11_OSU-a_135-141. The levels of bound and unbound Ca^{2+} were determined at 340 nm and 380 nm every 5 minutes for 30 minutes. The 340 nm/380 nm ratio was determined for each time point, and statistical analysis was performed. The 340 nm/380 nm ratio indicates the amount of Ca^{2+} at each point and is the ratio of Fura-2AM dye that is bound to Ca^{2+} and unbound Fura-2AM. The 340 nm/380 nm ratio is directly proportional to intracellular Ca^{2+} . Two-way ANOVA with LSD post-hoc was used to determine the statistical difference of the intracellular Ca^{2+} levels of cells infected with rSA11-L2, rSA11_OSU-a_NSP4 and rSA11_OSU-a_135-141.

The intracellular Ca^{2+} levels in uninfected cells, ionomycin-treated cells and cells infected with rSA11-L2, rSA11_OSU-a_NSP4 and rSA11_OSU-a_135-141 were determined and compared (Figure 3.7A). There was a significant difference in intracellular calcium levels in uninfected MA104 cells compared to cells treated with ionomycin. Uninfected MA104 cells had low levels of intracellular Ca^{2+} and had a mean 340 nm/380 nm of 0.65, while cells treated with ionomycin had a significant increase in intracellular Ca^{2+} and had a mean 340 nm/380 nm

of 1.12. These were the expected results since ionomycin is an antibiotic that facilitates the entry of Ca^{2+} ions through the plasma membrane (Morgan & Jacob, 1994). The mean 340 nm/380 nm was 1.13 in cells infected with rSA11-L2 and was significantly different to intracellular calcium levels in uninfected cells. This was the expected result because SA11-L2 is known to increase intracellular Ca^{2+} levels in infected cells. rSA11_OSU-a_NSP4 did not cause an increase in intracellular Ca^{2+} levels, and the mean 340 nm/380 nm was 0.64 in cells infected with this virus. The intracellular Ca^{2+} levels in cells infected with rSA11_OSU-a_NSP4 were similar to those of uninfected cells. These results were similar to those seen by Zhang and coworkers in Sf9 cells infected with baculovirus expressing OSU-a NSP4 (Zhang *et al.*, 1998).

The mean 340 nm/380nm was 0.87 in MA104 cells infected with rSA11_OSU-a_135-141. This mean value is significantly different from the mean intracellular calcium in cells infected with SA11-L2. Although there was an increase in intracellular Ca^{2+} in cells infected with rSA11_OSU-a_135-141, the increase in intracellular Ca^{2+} was lower than the Ca^{2+} flux caused by rSA11-L2. This could be because only aa 135-141 OSU-a NSP4 were introduced into SA11-L2. Therefore, only three (aa 135, 136 and 138) of the six mutations (aa 59, 72, 103, 135, 136 and 138) found in OSU-a GS10(NSP4) were introduced into SA11-L2 (GS10)NSP4. Although substituting aa 135, 136 and 138 of OSU-v NSP4 with those of OSU-a NSP4 attenuated OSU-v (Zhang *et al.*, 1998), the other three mutations found outside of the signal-transducing domain could be important in attenuating NSP4 from other rotavirus strains. Extracellular NSP4 (aa 112-175) is released from infected cells during infection, increasing intracellular Ca^{2+} in neighbouring cells (Zhang *et al.*, 2000). These amino acids form the enterotoxin and signal transducing domain of NSP4. Only amino acids that form part of the signal-transducing domain were altered during this study, and none of the amino acids in the enterotoxin domain (aa 112-134) of SA11-L2 GS10(NSP4) were changed. Hyser and colleagues showed that aa 70-85, found in the viroporin domain of NSP4, facilitate the increase in cytoplasmic Ca^{2+} . Altering this region attenuated the viroporins domain's ability to increase cytoplasmic Ca^{2+} (Hyser *et al.*, 2010). The enterotoxin and viroporin domains of rSA11_OSU-a_135-141 were not altered; this could be a possible reason why the virus can still increase intracellular Ca^{2+} .

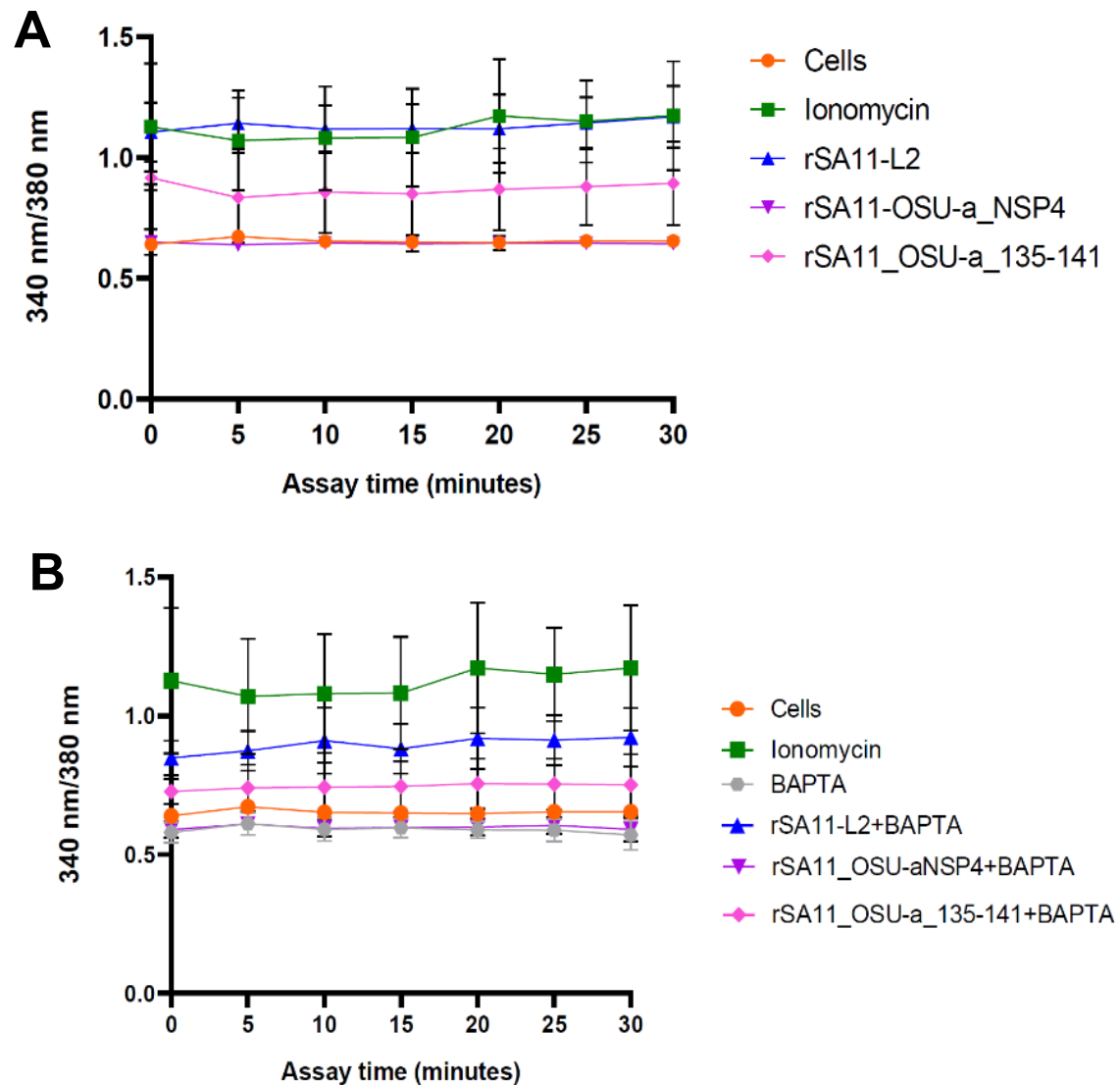


Figure 3.7: Intracellular Ca^{2+} cells treated with ionomycin or BAPTA-AM and infected with rescued viruses. The ratio of bound and unbound Fura-2AM was plotted for each infected monolayer over 7 time points. A) Cells were treated with ionomycin or infected with rSA11-L2, rSA11-OSU-a_NSP4 or rSA11-OSU-a_135-141. B) Uninfected MA104 cells and cells infected with rSA11-L2, rSA11-OSU-a_NSP4 or rSA11-OSU-a_135-141 and treated with BAPTA-AM. Bound and unbound Fura-2AM was measured every 5 minutes. The mean 340 nm/380 nm ratio was plotted for each triplicate and the error bars indicate the standard deviation.

Treating infected cells with BAPTA-AM decreased the intracellular Ca^{2+} levels in the infected cells (Figure 3.7B). Ionomycin and BAPTA-AM had opposite effects on intracellular Ca^{2+} levels. While ionomycin increased intracellular Ca^{2+} (mean 340 nm/340 nm of 1.12), BAPTA-AM decreased intracellular Ca^{2+} levels in MA104 cells and the mean 340 nm/380 nm was 0.59 in cells treated with BAPTA-AM. The mean 340 nm/380 nm in cells infected with rSA11_OSU-a_NSP4 and treated with BAPTA-AM was 0.59. Since rSA11_OSU-a_NSP4 did not increase intracellular Ca^{2+} in infected cells, treatment with BAPTA-AM did not cause a significant difference in intracellular Ca^{2+} . The mean 340 nm/380 nm in cells infected with rSA11-L2 and treated with BAPTA-AM was 0.89. Treating cells infected with rSA11-L2 with BAPTA-AM decreases intracellular Ca^{2+} levels by 21%. This shows that treating the cells infected with rSA11-L2 with BAPTA-AM decreased the Ca^{2+} flux in rSA11-L2 infected cells. The mean 340 nm/380 nm in cells infected with rSA11_OSU-a_135-141 was 0.87 and decreased to 0.74 when treated with BAPTA-AM. Adding BAPTA-AM to cells infected with rSA11_OSU-a_135-141 decreased the intracellular Ca^{2+} levels by 15%. The decrease in intracellular Ca^{2+} was not as significant as the decrease in intracellular Ca^{2+} seen in cells infected with rSA11-L2. Treating infected cells with BAPTA-AM decreased the intracellular Ca^{2+} levels in cells infected with rSA11-L2 and rSA11_OSU-a_135-141. However, there was still a higher Ca^{2+} flux in cells infected with rSA11-L2 than in cells infected with rSA11_OSU-a_135-141.

3.4 Summary

In this Chapter the effect of recombinant SA11-L2 with OSU-a NSP4 and SA11-L2 with SA11 NSP4 with aa 135-141 of OSU-a NSP4 on intracellular Ca^{2+} levels of infected MA104 cells was evaluated. The specific objectives to achieve this aim were to incorporate pUC57-T7_OSU-a_NSP4 and pUC57-T7_SA11_NSP4_OSU-a_135-141 into the SA11-L2 reverse genetics system, replacing pT7-NSP4 and determine if rescue is possible. Lastly, to perform Ca^{2+} flux assays to determine if the recombinant viruses could cause Ca^{2+} flux in infected cells.

The rescue of SA11-L2 with full-length OSU-a NSP4 and SA11 NSP4 with aa 135-141 of OSU-a was successful. The wild-type SA11-L2 was rescued and served as a control for the rescue experiments, growth kinetics and Ca^{2+} flux assay. The two recombinant viruses had similar replication kinetics to SA11-L2. The presence of Wa-like OSU-a NSP4 did not affect DLP transport and rSA11_OSU-a_NSP4 reached a maximum titre that was not statistically

different from the titres of the other two viruses. The presence of OSU-a NSP4 or SA11 NSP4 with OSU-a NSP4 aa 135-141 did not affect cell to cell spread of the recombinant SA11 viruses (rSA11_OSU-a_NSP4 and rSA11_OSU-a_135-141). The plaques formed by the rSA11_OSU-a_NSP4 and rSA11_OSU-a_135-141 were similar to those formed by rSA11-L2. This indicates that the replication kinetics of rSA11_OSU-a_NSP4 and rSA11_OSU-a_135-141 were unaffected by having GS10(NSP4) that differed from SA11-L2 GS10.

rSA11_OSU-a_NSP4 did not increase the intracellular Ca^{2+} levels in infected MA104 cells. Although NSP4 of rSA11_OSU-a_135-141 had aa 135-141 of OSU-a NSP4, it could still increase intracellular Ca^{2+} levels. However, the Ca^{2+} flux caused by rSA11_OSU-a_135-141 was not as high as the Ca^{2+} flux caused by rSA11-L2. This could be because the enterotoxin domain of GS10(NSP4) was not changed to that of OSU-a NSP4. Treating the infected cells with BAPTA-AM decreased the levels of intracellular Ca^{2+} . However, the effect of BAPTA-AM in cells infected with rSA11-L2 was greater than that of BAPTA-AM on cells infected with rSA11_OSU-a_135-141.

This is the first report of OSU-a GS10(NSP4) being incorporated into the SA11-L2 backbone using reverse genetics and shown to attenuate the virus's ability to increase intracellular Ca^{2+} . Only partial attenuation was achieved by changing aa 135-141 of SA11-L2 GS10(NSP4) with those of OSU-a NSP4. In future biological repeats need to be performed to confirm the calcium flux data since only technical repeats were done during this study. The levels intracellular calcium levels can also be measured over a longer time period to determine if rSA11_OSU-a_135-141 causes a more significant increase in intracellular calcium levels at later stage of infection. Further studies need to be done to determine the amino acids that can be altered in the enterotoxin and viroporin domain to fully attenuate SA11-L2 GS10(NSP4). Studies also need to be done in animal models to determine if the decrease in intracellular Ca^{2+} correlates to reduced diarrhoea after infection.

CHAPTER 4

INCORPORATING NSP1 LACKING A CYTOSKELETON BINDING DOMAIN IN THE SA11-L2 REVERSE GENETICS SYSTEM

4.1 Introduction

The innate immune response is the first line of defence against pathogens. Viruses use different strategies to evade the innate immune response, optimizing their replicative capacity (Nelemans & Kikkert, 2019). Rotavirus antagonises the innate immune response during its replication cycle to ensure its survival. NSP1 is one of the proteins that antagonises the innate immune response and has many targets (Morelli *et al.*, 2015). The JAK-STAT pathway, activated by interferons, is inhibited by NSP1 using different mechanisms. These include proteasomal degradation of IRF9 preventing the formation of the ISGF3 transcription complex, inhibiting the nuclear accumulation of phosphorylated STAT1 and STAT2, and inhibiting STAT phosphorylation at late stages of infection (Holloway *et al.*, 2009; Holloway *et al.*, 2014). STAT phosphorylation and nuclear translocation are necessary to transcribe ISGs, which help combat infection. Although NSP1 is not necessary when infecting permissive cell cultures, it is important for viral growth, transmission, and pathogenesis during normal infection of humans and animals (Hou *et al.*, 2021b).

The main goal of this study is to rationally design rotavirus proteins that contribute to pathogenesis to improve the safety profile of rotavirus vaccine candidates. Attenuating proteins that inhibit the innate immune response could limit viral replication and reduce the viral load. Altering viral proteins that inhibit the innate immune response could also enhance viral recognition improving the innate and adaptive immune response to the virus.

The experiments described in this Chapter aim to remove the cytoskeleton binding domain of NSP1 (aa 84-176) to prevent it from anchoring to the cytoskeleton. Truncating NSP1 could prevent it from binding and inhibiting the nuclear translocation of phosphorylated STAT1 and STAT2. The specific objectives for the experiments described in this Chapter were to design a plasmid construct with truncated NSP1 and incorporate it into the SA11-L2 rotavirus reverse genetics system. Thereafter, to infect MA104 cells with rescued virus and determine if truncated NSP1 translocates to the nucleus. Lastly, to determine if phosphorylated STAT1 and STAT2 translocate to the nucleus in cells infected with the rescued virus.

4.2 Materials and Methods

All materials and methods described in Chapter 3 were used in this study as well as those described below.

4.2.1 Plasmid design

The plasmid used to achieve the aim of this Chapter was designed *in silico* using Snapgene. The designed plasmid contains a SA11-L2_GS5 (NSP5) ORF (Genbank accession number: KJ450835.1) that had a deletion of the nucleotides that encode amino acids 84-176 downstream of the T7 promoter in a pUC57-kanamycin backbone (Figure 4.1). The plasmid was synthesised by GenScript and delivered in its lyophilised form. The lyophilised plasmid was diluted in nuclease-free H₂O and transformed into JM109 cells as described in Section (2.2.2). The plasmid was purified from JM109 as described in Section 2.2.4 and sent for Sanger sequencing at Inqaba Biotech.

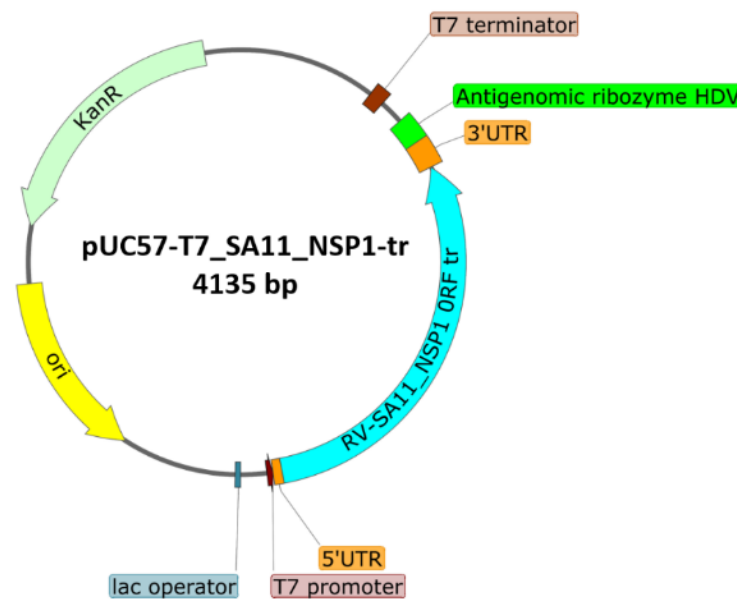


Figure 4.1: Plasmid map of pUC57-T7_SA11_NSP1-tr. The SA11_NSP1 ORF with a deletion of the nucleotides that encode aa 84-176 (blue) flanked by 5' and 3' UTRs (orange) under the control of a T7 promoter (brown) in a pUC57 backbone. The anti-genomic HDV ribozyme is located downstream of the 3' UTR.

4.2.2 Rescue of recombinant virus and Rescue verification

The recombinant virus was rescued as described in Section 3.2.3.2 using the pUC57-T7_SA11_NSP1-tr transcription plasmid. dsRNA genome segment-specific cDNA was generated from purified dsRNA and amplification and Sanger sequencing were performed using GS5(NSP1) specific primers listed in Table 4.1.

Table 4.1: Primers used for GS5 (NSP1) cDNA synthesis, amplification, and Sanger sequencing.

Primer	Primer sequence	Length	Tm
Primers used for cDNA synthesis and amplification			
SA11corGS5F	5'TAATACGACTCACTATAGGCTTTTTTTTGAAAAG3'	34 bases	42°C
SA11GS5iNR	5'GGGACCATGCCGCGCGTTACAGTATTTTG3'	31 bases	43°C
Primers used for Sanger sequencing			
SA11_GS5-rev	5'GGCTTTTTTTTGAAAAGTCTTG TG 3'	21 bases	54°C
SA11_GS5-for	5' GGTTACAGTATTTTGCCAGC3'	24 bases	56°C

4.3 Results and Discussion

4.3.1 Sanger sequence analysis of purified pUC57-T7_SA11_NSP1-tr

To rescue SA11-L2 with NSP1 that lacks the cytoskeleton binding domain, pUC57-T7_SA11_NSP1-tr was designed and purchased from GenScript (Section 4.2.1). The lyophilized plasmid was diluted in nuclease-free H₂O and transformed into competent JM109 *E.coli* cells for propagation and long-term storage. Before incorporating the plasmid into the SA11-L2 reverse genetics system, it was purified from an overnight bacterial culture using a Qiagen miniprep kit (Section 2.2.5) and sent for Sanger sequencing analysis. This was done to verify that the transcription cassette was correct to ensure that no mutations were present that could lead to difficulties in viral rescue.

The forward and reverse complement of the reverse sequencing data (Figure 4.2) were analysed using Mega 7. The sequence data was aligned to the transcription cassette of the pUC57-T7_SA11_NSP1-tr plasmid map. Figure 4.2 shows that the transcription cassette of purified pUC57-T7_SA11_NSP1-tr aligned correctly to the pUC57-T7_SA11_NSP1-tr reference. There were no mutations in the T7 promoter, HDV ribozyme or T7 terminator (Figure 4.2). The ORF lacked the cytoskeleton binding domain, aa 84-176 (276 bp), Figure 4.2. Having established that the transcription cassette of pUC57-T7_SA11_NSP1-tr was correct, the plasmid was purified using an Endotoxin-free kit and incorporated into the SA11-L2 reverse genetics system.

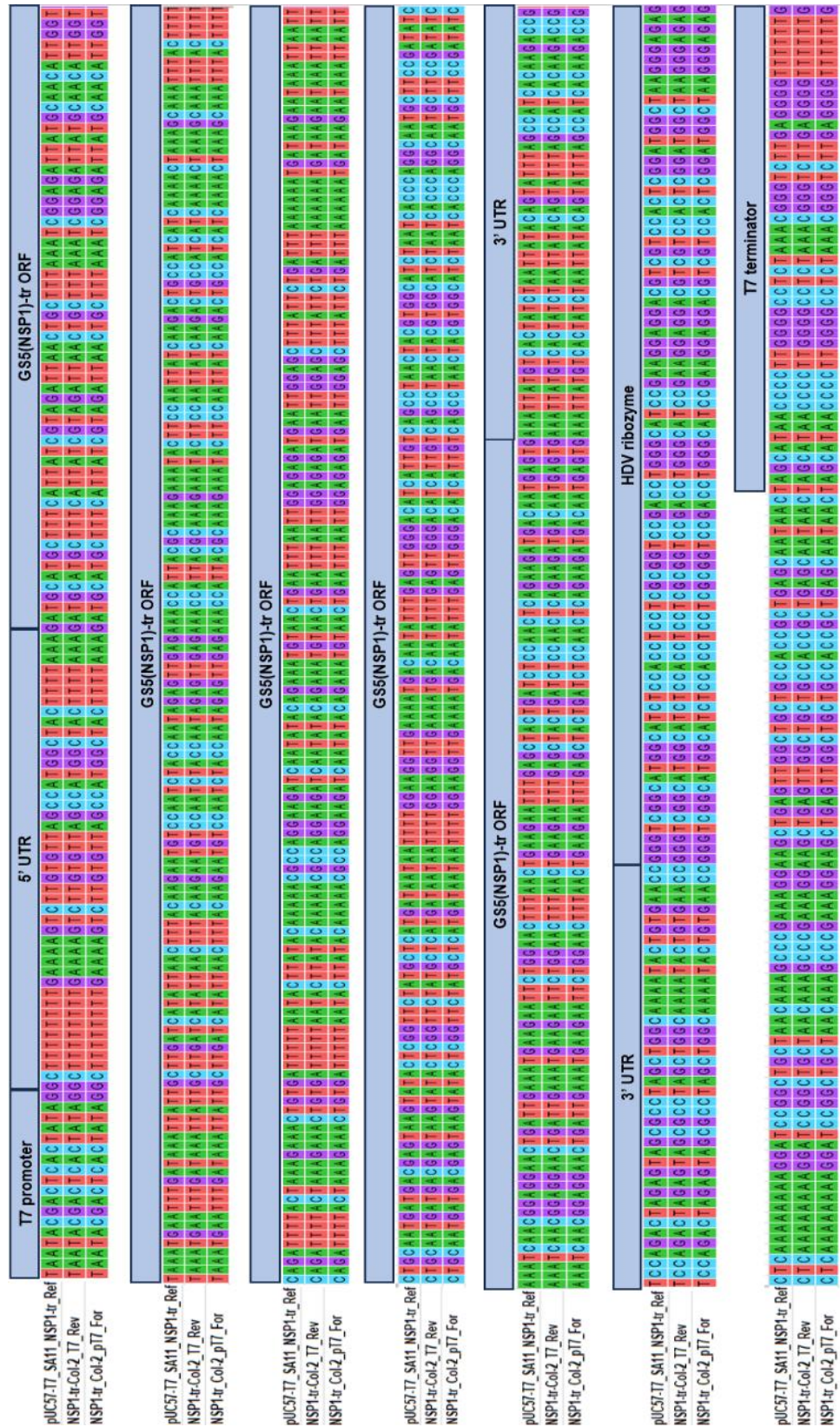


Figure 4.2: pUC57-T7_SA11_NSPI-tr transcription cassette. The transcription cassette contained the GS5(NSP1) that lacked the cytoskeleton binding domain flanked by 5' and 3' UTRs. The anti-genomic ribozyme is located downstream of the 3'UTR.

4.3.2 Rescue of recombinant SA11-L2 with NSP1 which lacks the cytoskeleton binding domain

To rescue SA11-L2 with truncated NSP1, pUC57-T7_SA11_NSPI-tr was incorporated into the SA11-L2 reverse genetics system as described in Section 3.2.3.2. pT7_NSPI-SA11 was exchanged with purified endotoxin-free pUC57-T7_SA11_NSPI-tr when preparing transfection plasmid mixtures. Virus rescue was performed as described in Section 3.2.3.2 using 4 µg of the prepared plasmid mixture per well of a 6-well plate. The rescued P0 virus stock was blind passaged once on a 25 cm³ flask and the infected MA104 cells were used for rescue verification. CPE was observed two days after infection on MA104 monolayers infected with rSA11-L2 and rSA11_NSPI-tr. Viral dsRNA extracted from infected MA104 cells was visualised using AGE (Figure 4.3). The difference in intensity of the dsRNA bands of rSA11-L2 and rSA11_NSPI-tr indicates a higher yield of dsRNA from the monolayer infected with rSA11-L2. This could be due to the viruses not growing to the same titre. The banding pattern of the dsRNA of rSA11-L2 and rSA11_NSPI-tr were both correct. rSA11-L2 had full-length GS5(NSP1) that is 1610 bp which migrated slower than GS6(VP6) which is 1400 bp. Truncated GS5(NSP1) is 1331 bp which is 279 bp shorter than SA11-L2 NSP1 and migrated at the same rate as GS6(VP6).

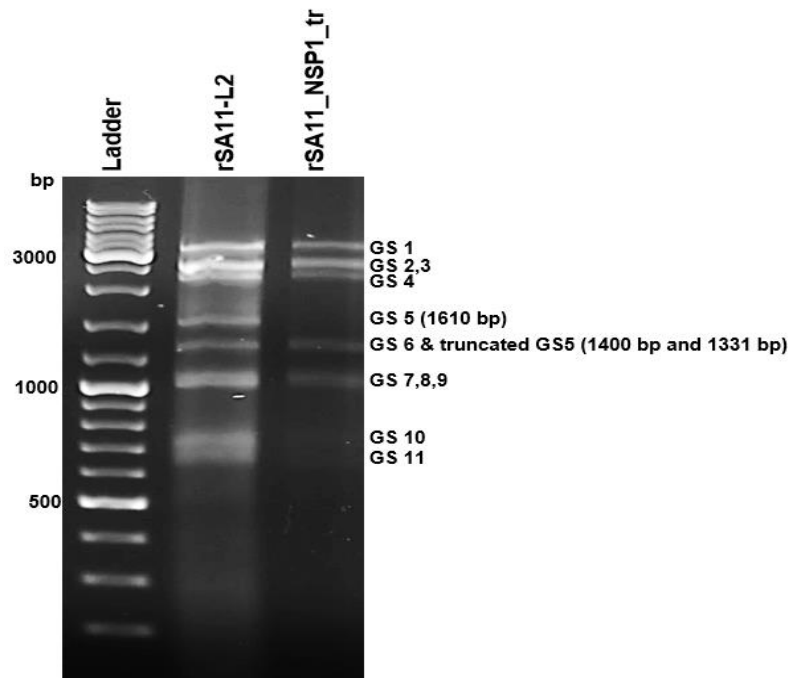


Figure 4.3: Agarose gel of dsRNA extracted from cell MA104 cells infected with P0 viral stocks. Genome segments of rSA11-L2 and recombinant rSA11_NSPI-tr. The GeneRuler™ 1 kb DNA Ladder (Thermo Scientific) was used as a reference.

To confirm that the rescued recombinant virus contained GS5(NSP1) and that the correct truncation was present, GS5(NSP1) specific cDNA synthesis was performed. The generated cDNA was visualised on an agarose gel and sent for Sanger sequencing. The cDNA was generated using the SuperScript III One-step RT-PCR Platinum kit as described in Section 3.2.4.2 using GS5(NSP1) specific primers (Table 4.1). Analysis of the cDNA on an agarose gel showed that GS5 from rSA11_NSPI_tr and rSA11-L2 migrated differently (Figure 4.4). GS5(NSP1) from rSA11_NSPI_tr was 1331 bp while GS5(NSP1) from rSA11-L2 was 1610 bp.

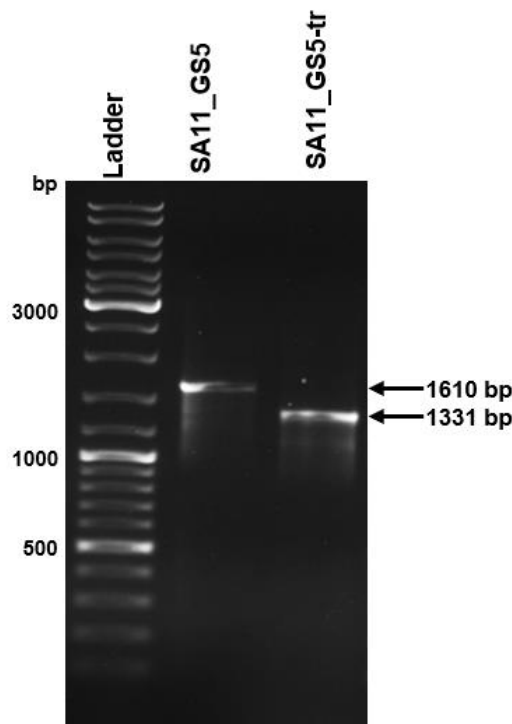


Figure 4.4: Agarose gel analysis of GS5(NSP1) cDNA generated from dsRNA of rSA11-L2 and recombinant rSA11_NSPI_tr. The amplicons generated from rSA11-L2 and rSA11_NSPI_tr were 1610 bp and 1334 bp. The GeneRuler™ 1 kb DNA Ladder (Thermo Scientific) was used as a reference.

Analysis of the Sanger sequencing data of GS5(NSP1) of rSA11-L2 and rSA11_NSPI_tr confirmed that the two viruses harboured GS5(NSP1) that were different (Figure 4.5). GS5(NSP1) from rSA11-L2 aligned correctly with the SA11-L2 GS5(NSP1) reference. GS5(NSP1) generated from rSA11_NSPI_tr aligned correctly with the reference at the nucleotides 1-250, which encode aa 1-83 and nucleotides 530-1491, which encode for amino acid 177-520. Nucleotides 251-529 encoding aa 84-176 were absent therefore, rSA11_NSPI_tr lacked the cytoskeleton binding domain.

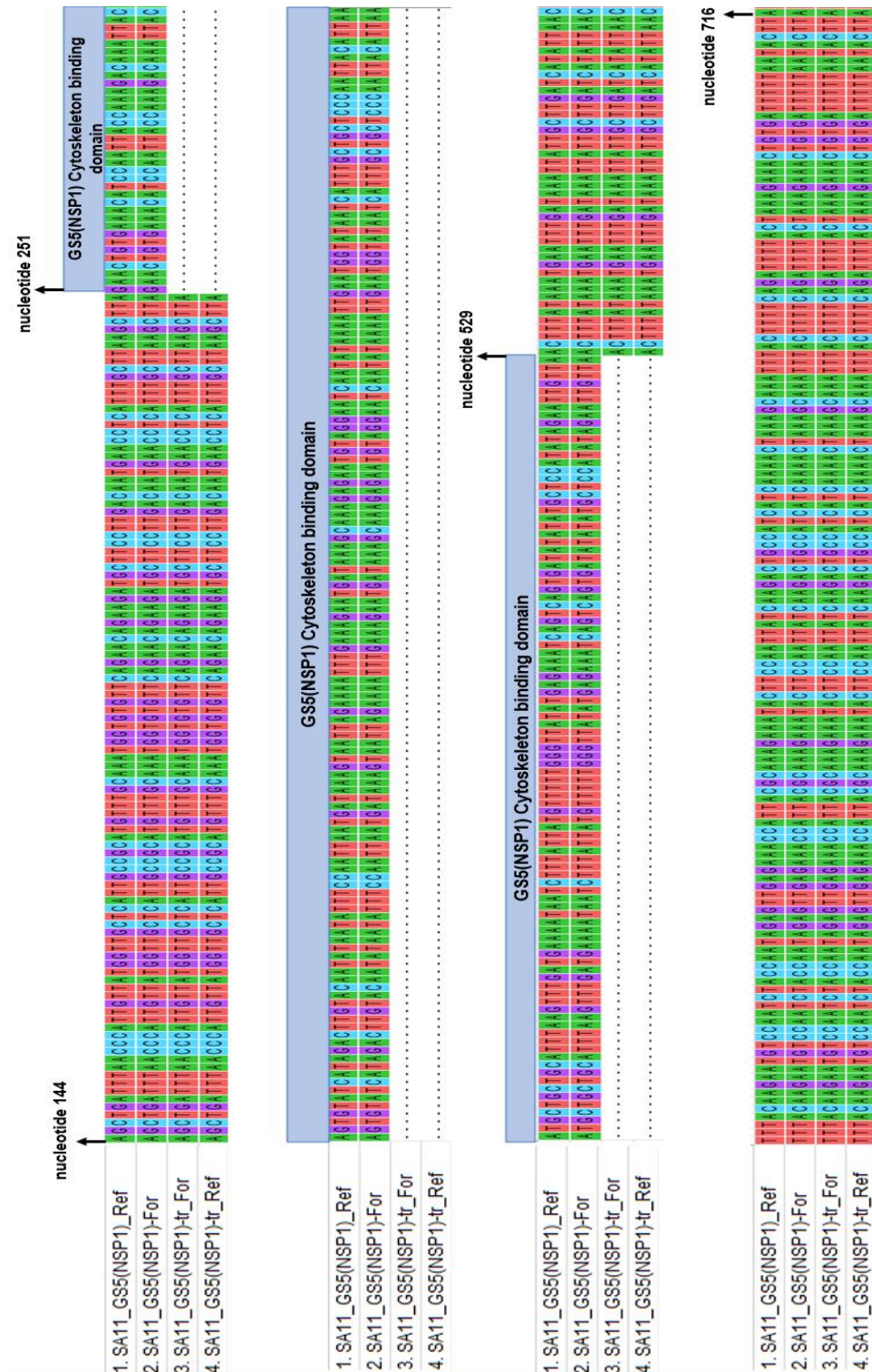


Figure 4.5: Sequence alignment of the forward Sanger sequences of GS5(NSP1) cDNA generated from dsRNA extracted from rSA11-L2 and rSA11_NSP1_tr. The sequences were aligned with SA11 GS5(NSP1) reference sequence and the truncated SA11 GS5(NSP1) reference. The GS5(NSP1)-tr cDNA generated from rSA11_NSP1_tr had a deletion of the cytoskeleton binding domain. Nucleotide 144 to 716 of the aligned sequences are shown and the dashes represent the deleted nucleotides (nucleotides 251-529).

4.3.3 Replication kinetics of recombinant SA11-L2 with NSP1 which lacks the cytoskeleton binding domain

Plaque assays were performed in triplicate on rSA11-L2 and rSA11_NSP1_tr and, as described in Section 3.2.5, using the P1 viral stocks to determine the titres of the viral stocks. The plaques formed by rSA11-L2 were larger than those formed by rSA11_NSP1_tr (Figure 4.6). Smaller plaques have been observed on cells infected with rotaviruses with naturally truncated NSP1 and deletion mutants (Barro & Patton, 2005; Taniguchi *et al.*, 1996). The smaller plaque sizes could be because there is a higher innate immuneresponse when NSP1 is truncated, inhibiting effective cell-to-cell spread (Barro & Patton, 2005).

To compare the replication kinetics of rSA11-L2 and rSA11_NSP1_tr, multi-step growth curves (Section 3.2.6) were performed in triplicate by infecting MA104 cells at a MOI of 0.001 over 72 hours. The data set was normalised, and the statistical difference was determined using two-way ANOVA with LSD post-hoc. The growth curve of rSA11_NSP1_tr was similar to that of rSA11-L2. At 36 hours, rSA11_NSP1_tr had a higher titre (3.70×10^6 PFU/ml) than rSA11-L2, which had a titre of 4.17×10^5 PFU/ml). However, the viruses reached a final titre that was not statistically different (Figure 4.6). At 72 hours, rSA11_NSP1_tr reached a titre of 1.17×10^8 PFU/ml and rSA11-L2 reached a final titre of 1.77×10^8 PFU/ml.

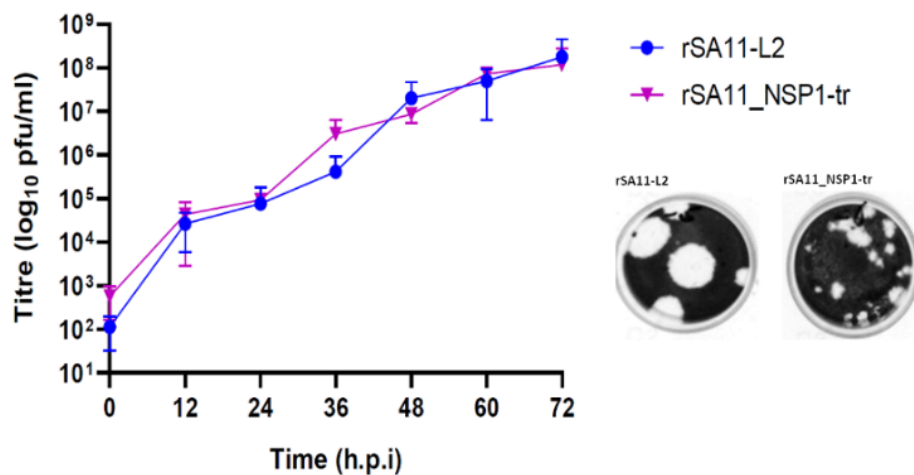


Figure 4.6: Replication kinetics and plaque morphology of rSA11-L2 and recombinant SA11-L2 with GS5(NSP1) without a cytoskeleton binding domain. The growth curve and plaque morphology of rSA11_NSP1_tr were compared to the growth curve of and plaque morphology of rSA11-L2. There was no statistical difference between the final titre of rSA11-L2 and rSA11_NSP1_tr. The experiment was performed in triplicate and the mean value at each time point is plotted on the curve. Error bars indicate the standard deviation.

4.3 Summary

The experiments in this Chapter aimed to incorporate NSP1 which lacks the cytoskeleton binding domain (aa 84-176) into the rotavirus SA11-L2 reverse genetics system. This was to attempt to test the hypothesis that a rotavirus that encodes NSP1 that cannot bind the cytoskeleton binding domain would translocate into the cell's nucleus and will lose its ability to inhibit the nuclear translocation of phosphorylated STAT1 and STAT2.

pUC57-T7_SA11_NSP1-tr was successfully incorporated into the SA11-L2 reverse genetics system. This resulted in the rescue of recombinant SA11 that had GS5(NSP1) that lacked a cytoskeleton binding domain. The replication kinetics of rSA11_NSP1_tr were not significantly different from that of rSA11-L2. However, the plaque size of rSA11_NSP1_tr was smaller than that of rSA11-L2. The smaller plaque size may be due to a higher innate immune response in the cells infected with rSA11_NSP1_tr. This could be because truncated NSP1 could not effectively bind and inhibit interferons, IRF3 and nuclear accumulation of phosphorylated STAT1 and STAT2. The lack of cytoskeleton binding could be preventing NSP1 from effectively inhibiting IFN transcription and pSTAT1 and pSTAT2 nuclear translocation. The presence of more IFNs and ISGs such as IRF3 leads to the activation of antiviral responses in neighbouring cells limiting the effective cell-to-cell transfer of the virus.

Due to time constraints, the localisation of truncated NSP1, phosphorylated STAT1 and STAT2 in cells infected with rSA11_NSP1_tr could not be determined. The location of NSP1 without a cytoskeleton binding domain and phosphorylated STAT1 and STAT2 needs to be determined to establish where NSP1 localises when it is not bound to the cytoskeleton. We should also establish if there are more phosphorylated STAT1 and STAT2 in the nucleus of cells infected with rSA11_NSP1_tr than cells infected with rSA11-L2. This could help to elucidate the mechanism of IFN and STAT inhibition by NSP1.

CHAPTER 5

CONCLUDING REMARKS AND FUTURE PROSPECTS

Despite the use of commercially available vaccines, rotaviruses are still the leading cause of diarrhoeal-related deaths worldwide in children under the age of 5. Rotaviruses are responsible for 128 500 deaths annually and 1.76 million hospitalisations of children under 5 years of age (Hallowell *et al.*, 2022; Troeger *et al.*, 2018). Diarrhoea is the main clinical symptom of rotavirus infection and is caused by the viral enterotoxin NSP4 (Ball *et al.*, 1996). NSP4 dysregulates calcium signalling by activating the PLC signalling transduction pathway, thereby increasing intracellular calcium levels and causing diarrhoea (Lorrot & Vasseur, 2007; Tian *et al.*, 1995). Rotavirus NSP1 also contributes to viral pathogenesis by inhibiting the innate immune response. NSP1 anchors to the cytoskeleton and causes proteasomal degradation of proteins that activate the production of interferons (Hua *et al.*, 1994; Morelli *et al.*, 2015). NSP1 also inhibits the nuclear accumulation of phosphorylated STAT1 and STAT2, which is essential for the transcription of ISGs to induce an antiviral state (Fensterl & Sen, 2009; Holloway *et al.*, 2009).

There is a need for more effective rotavirus vaccines in regions where the currently available vaccines are less effective. Our research group aims to develop regional rationally designed live attenuated rotavirus vaccine candidates using the rotavirus reverse genetics system. Vaccine safety is an important factor during vaccine development, and attenuating the proteins that contribute to pathogenesis could lead to increased safety. The development of the plasmid-only rotavirus reverse genetics system has made it possible to rescue reassortment viruses to study the functions of specific viral proteins and develop vaccines (Kanai *et al.*, 2017). Different reassortment viruses have since been rescued using the reverse genetics system. The focus of this study was to rationally design NSP4 and NSP1 to rescue rotavirus that cannot increase intracellular calcium or inhibit the nuclear accumulation of phosphorylated STAT1 and STAT2.

Baculovirus expression of NSP4 of virulent and attenuated porcine rotaviruses

Mutations were found in NSP4 of two porcine rotavirus strains that were attenuated by passing multiple times in tissue culture (Zhang *et al.*, 1998). Three of the six mutations (aa 135, 136 and 138) clustered together in the signal-transducing domain (aa 130-140) (Seo *et al.*, 2008; Zhang *et al.*, 1998). Zhang and coworkers generated recombinant baculoviruses expressing OSU-a and OSU-v NSP4 and found that OSU-a could not increase intracellular calcium levels in Sf9 cells. As vaccine candidates are being developed in our research group, controls are

needed when evaluating if the vaccine candidates can increase intracellular calcium and cause diarrhoea. Since NSP4 destabilizes the membrane of bacterial and eukaryotic cells, these expression systems are not ideal for expressing full-length NSP4 (Browne *et al.*, 2000; Hyser *et al.*, 2010). Since the baculovirus expression system was successfully used before by Zhang and coworkers, it was selected to express OSU-a and OSU-v NSP4 for this study.

To express His-tagged OSU-a NSP4 and His-tagged OSU-v NSP4, two pFASTBac-HTA donor plasmids containing the OSU-a NSP4 and OSU-v NSP4 ORF downstream of the His-tag ORF were designed. GS10(NSP4) ORFs were Sf9 insect codon optimised to increase protein yield. Both plasmids were transformed into AcBAC Δ CC *E.coli* cells to generate recombinant bacmids. PCR analysis confirmed that recombinant bacmid DNA containing the OSU-a NSP4 and OSU-v NSP4 ORF were generated. Recombinant baculoviruses were generated, and CPE was observed on Sf9 cells infected with the recombinant baculoviruses. However, there was no expression of His-tagged OSU-a NSP4 and His-tagged OSU-v NSP4.

Since CPE was observed in infected Sf9 cells, but there was no expression of His-tagged OSU-a NSP4 and His-tagged OSU-v NSP4, the CPE could have been due to wild-type baculovirus contamination. Another reason for the lack of expression could be that instead of improving yields of the recombinant proteins. Codon optimisation has been found to negatively impact the transcription and translation of certain genes (Liu *et al.*, 2022). Thus, codon optimisation of OSU-a and OSU-v GS10(NSP4) ORF could have negatively impacted their expression. To determine if codon optimization had a negative impact, the non-codon optimised ORF should be used in future experiments to express OSU-a and OSU-v NSP4. An alternative to expressing full-length NSP4 would be to express aa 86-175 peptides of OSU-a NSP4 and OSU-v NSP4 since NSP4 aa 86-175 peptides with a GST fusion tag have been successfully expressed in bacterial cells and could cause diarrhoea in neonatal mice (Horie *et al.*, 1999). These peptides can then be used as controls for experiments when determining the capacity of rationally designed vaccine candidates to cause diarrhoea.

Investigating the effect of recombinant SA11-L2 with OSU-a NSP4 and SA11/OSU-a aa135-141 NSP4 on intracellular calcium levels

While NSP4 increases intracellular calcium levels, causing diarrhoea, it is also involved in DLP transport during rotavirus replication (Meyer *et al.*, 1989; Zhang *et al.*, 2000). To rescue SA11-L2 that has an attenuated NSP4 but retains the ability to replicate, NSP4 must be attenuated without affecting its other functions. This study aimed to rationally design SA11-L2 NSP4 and incorporate OSU-a GS10(NSP4) into SA11-L2 to rescue rotaviruses that could not increase the intracellular calcium of infected cells.

rSA11_OSU-a_NSP4 and rSA11_OSU-a_135-141 was rescued by replacing pT7-NSP4 that expresses SA11 GS10(NSP4) with pUC57-T7 plasmids that had full-length OSU-a GS10(NSP4) ORF and the SA11 GS10(NSP4) ORF containing aa 135-141 of OSU-a NSP4. Sanger sequencing performed on GS10(NSP4) cDNA generated from dsRNA extracted from cells infected with rSA11-L2, rSA11_OSU-a_NSP4, and rSA11_OSU-a_135-141 showed that GS10(NSP4) of the three viruses was different. rSA11_OSU-a_NSP4 had full-length OSU-a NSP4 and GS10(NSP4) of rSA11_OSU-a_135-141 had the correct alteration at aa 135-141. Multi-step growth curves showed that rSA11-L2, rSA11_OSU-a_NSP4 and rSA11_OSU-a_135-141 had similar replication kinetics and formed plaques of similar size. These results showed that OSU-a GS10(NSP4), a DS-1-like genome segment, was successfully reassorted into SA11-L2, which has a Wa-like GS10(NSP4). This reassortment did not hinder the transport of DLPS, and viral replication was not negatively affected.

Intracellular calcium levels were measured in MA104 cells infected with rSA11-L2, rSA11_OSU-a_NSP4 and rSA11_OSU-a_135-141 using Fura-2AM dye that binds cytosolic calcium. The results showed that rSA11-L2 could increase the intracellular calcium in infected cells, as reported in other studies. rSA11_OSU-a_NSP4 did not increase intracellular calcium levels in infected MA104 cells and rSA11_OSU-a_135-141 increased intracellular calcium, but the increase in intracellular calcium was significantly lower than in cells infected with rSA11-L2. These results showed that substituting aa 135-141 of SA11 GS10(NSP4) with those of OSU-a GS10(NSP4) did not fully attenuate the virus's ability to increase intracellular calcium.

In future other regions that contribute to the complete attenuation of SA11 NSP4 need to be determined. In the experiments of this MSc, the enterotoxin domain of SA11-L2 GS10(NSP4) was not altered. Identifying amino acids that can be changed in the enterotoxin domain could help fully attenuate SA11-L2 GS10(NSP4). Murine and Wa rotaviruses have attenuated strains, and specific mutations were identified in GS10(NSP4) of these attenuated strains (Chang *et al.*, 1999). These mutations should also be introduced into SA11-L2 GS10(NSP4) to investigate if incorporating these specific alterations will fully attenuate SA11-L2 GS10(NSP4).

Incorporating NSP1 lacking a cytoskeleton binding domain in the SA11-L2 reverse genetics system

During rotavirus replication, NSP1 is anchored to the cytoskeleton, inhibiting interferon production and nuclear accumulation of phosphorylated STAT1 and STAT2. The cytoskeleton binding domain of NSP1 was mapped to aa 84-176 (Hua *et al.*, 1994). While determining the domains of NSP1 using deletion mapping, Hua and colleagues showed that NSP1 lacking aa

82-476 localized in the nucleus when expressed in CV1 cells. Thus, removing the cytoskeleton binding domain of NSP1 could result in it translocating to the nucleus preventing it from inhibiting the nuclear accumulation of phosphorylated STAT1 and STAT2 and possibly increasing vaccine safety.

A pUC57-T7 plasmid containing NSP1 lacking the cytoskeleton binding domain was incorporated into the SA11-L2 reverse genetics system. Rescue of rSA11_NSPI-tr was achieved, and analysis of dsRNA and GS5(NSP1) cDNA proved that GS5(NSP1) of rSA11_NSPI-tr was 276 bp shorter than full-length SA11-L2 GS5(NSP1). The absence of the cytoskeleton binding domain was verified using Sanger sequencing. The growth kinetics of rSA11_NSPI-tr were compared to that of rSA11-L2. Multi-step growth curves showed that rSA11_NSPI-tr replicated at a similar rate to rSA11-L2. However, rSA11_NSPI-tr formed smaller plaques compared to rSA11-L2 similar to plaques formed by rotaviruses that have naturally truncated GS5(NSP1) (Barro & Patton, 2007; Taniguchi *et al.*, 1996). This could be due to a more significant interferon response in cells infected with rSA11_NSPI-tr, which limits cell-to-cell spread (Barro & Patton, 2007).

In follow up investigations the location of NSP1 lacking the cytoskeleton binding domain (aa 84-176) should be determined in MA104 cells infected with rSA11_NSPI-tr using confocal microscopy. The nuclear translocation of phosphorylated STAT1 and STAT2 should also be determined to evaluate if NSP1 lacking cytoskeleton binding domain can inhibit the nuclear translocation of phosphorylated STAT1 and STAT2. The mRNA levels of the ISGs expressed during viral infection should be evaluated in cells infected with rSA11_NSPI-tr and rSA11-L2 to determine if phosphorylated STAT1 and STAT2 accumulate in the nucleus.

Contribution of study

This is the first study reporting the plasmid-only based reverse genetics system being used to rescue SA11-L2 rotavirus with OSU-a NSP4, SA11-L2 NSP4 with aa 135-141 and NSP1 lacking the signal transducing domain. In previous studies, rotaviruses were attenuated by passaging the virus multiple times in tissue culture. The results of this study showed that the alterations incorporated into NSP4 and NSP1 did not prevent viral rescue or replication. Further studies must be conducted in animal models to investigate if rSA11_OSU-a_NSPI4 and rSA11_OSU-a_135-141 and rSA11_NSPI-tr are less pathogenic.

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APPENDIX

A. Study ethics approval



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North-West University Animal Care, Health and
Safety Research Ethics Committee (NWU-
AnimCareREC)

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studies)

7 March 2022

ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC) on 07/03/2022, the NWU-AnimCareREC hereby approves your study as indicated below. This implies that the NWU-AnimCareREC grants its permission that, provided the general conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: Rational design of rotavirus NSP1 and NSP4 to improve the safety profile of rotavirus vaccine candidates.

Principal Investigator/Study Supervisor/Researcher: Prof AA van Dijk

Student: K Hlongwane – 35914033

Ethics number:

N	W	U	-	0	0	7	5	1	-	2	2	-	A	5
Institution				Study Number						Year			Status	

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation;
A = Authorisation

Application Type: Single study

Commencement date: 07/03/2022

Expiry date: 31/03/2023

Risk:

Category 0

Approval of the study is provided for a year, after which continuation of the study is dependent on receipt and review of an annual monitoring report and the concomitant issuing of a letter of continuation. A monitoring report is required at the end of February annually until completion.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:

- The principal investigator/study supervisor/researcher must report in the prescribed format to the NWU-AnimCareREC:
 - Annually on the monitoring of the study, whereby a letter of continuation will be provided annually, and upon completion of the study; and
 - without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.
- The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the principal investigator/study supervisor/researcher must apply for approval of these amendments at the NWU-AnimCareREC, prior to implementation. Should there be any deviations from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.
- Annually a number of studies may be randomly selected for active monitoring.

- The date of approval indicates the first date that the study may be started.
- In the interest of ethical responsibility, the NWU-AnimCareREC reserves the right to:
 - request access to any information or data at any time during the course or after completion of the study;
 - to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;
 - withdraw or postpone approval if:
 - any unethical principles or practices of the study are revealed or suspected;
 - it becomes apparent that any relevant information was withheld from the NWU-AnimCareREC or that information has been false or misrepresented;
 - submission of the annual monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or
 - new institutional rules, national legislation or international conventions deem it necessary.
- NWU-AnimCareREC can be contacted for further information via Ethics-AnimCare@nwu.ac.za or 018 299 1208

Please note: Due to the nature of the study i.e. (laboratory work involving in vitro analysis of commercial cell lines that have been genetically transformed with recombinant plasmids), this study will be able to proceed during the current alert level, following receipt of the approval letter. No additional COVID-19 restrictions have been placed on the study except that the researcher must ensure that before proceeding with the study that all research team members have reviewed the North-West University COVID-19 Occupational Health and Safety Standard Operating Procedure.

NWU-AnimCareREC would like to remain at your service and wishes you well with your study. Please do not hesitate to contact the NWU-AnimCareREC for any further enquiries or requests for assistance.

Yours sincerely,



Digitally signed by
Christiaan B Brink
(Tiaan)
Date: 2022.03.07
16:52:25 +02'00'

Chairperson: NWU-AnimCareREC

Current details (22854704) G:\My Drive\9. Research and Postgraduate Education\9.1.5.4 Templates\9.1.5.4.2_NWU-AC_EAL.docm
22 February 2022
File Reference: 9.1.5.4.2

B. Conference outputs

J. Herbert, M.J. Visser, **K. Hlongwane**, A.C. Potgieter, A.A. van Dijk. Problems with plasmid stability of transcription cassettes of a rotavirus SA11-N5 reverse genetics system. 14th International dsRNA Symposium in Banff, Canada, 10-14 October 2022.

C. Rotavirus reverse genetics pT7_SA11-L2 plasmid maps

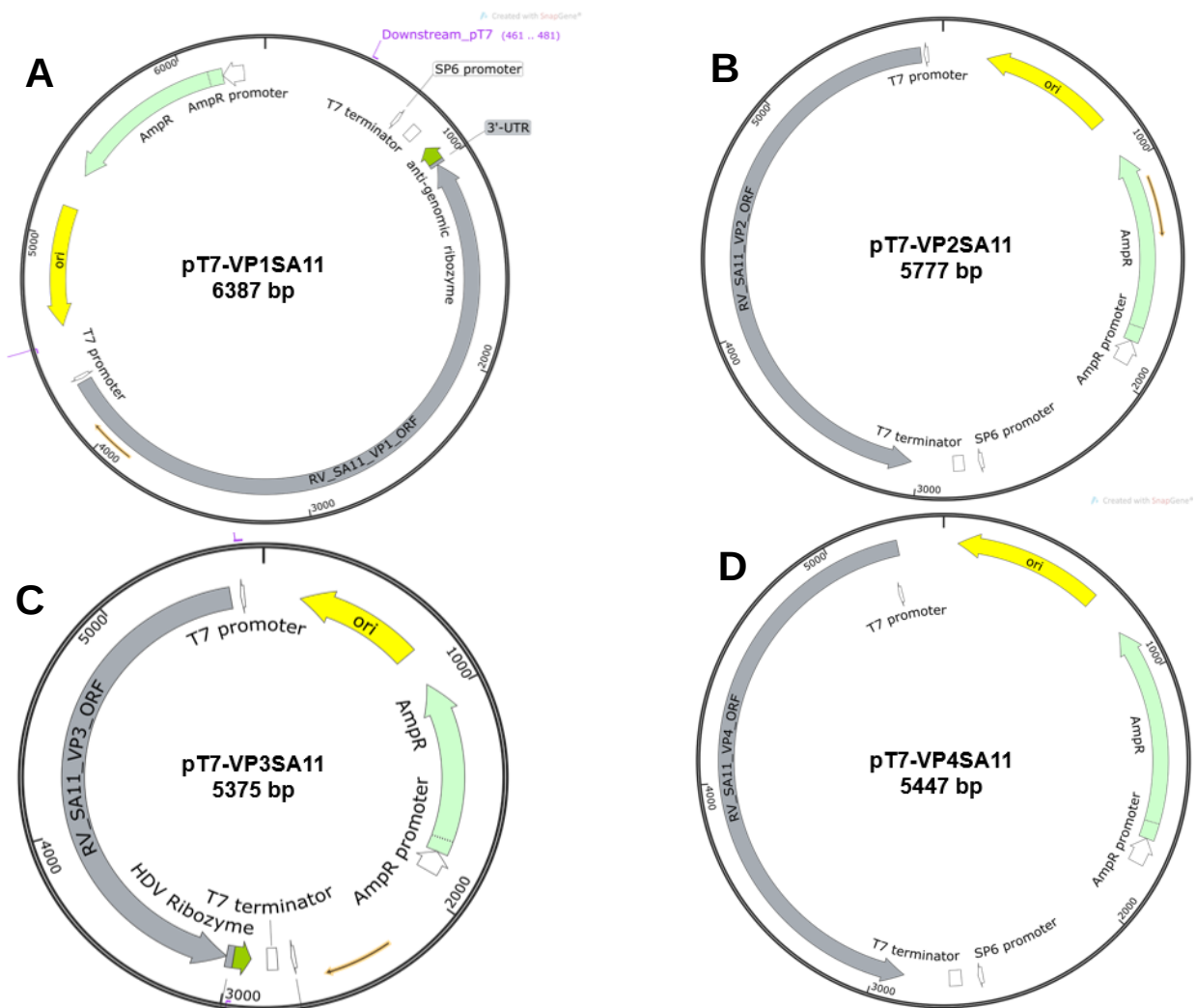


Figure S1: SA11-L2 reverse genetics plasmid maps. A) pT7-VP1SA11, B) pT7-VP2SA11, C) pT7-VP3SA11, D) pT7-VP4SA11.

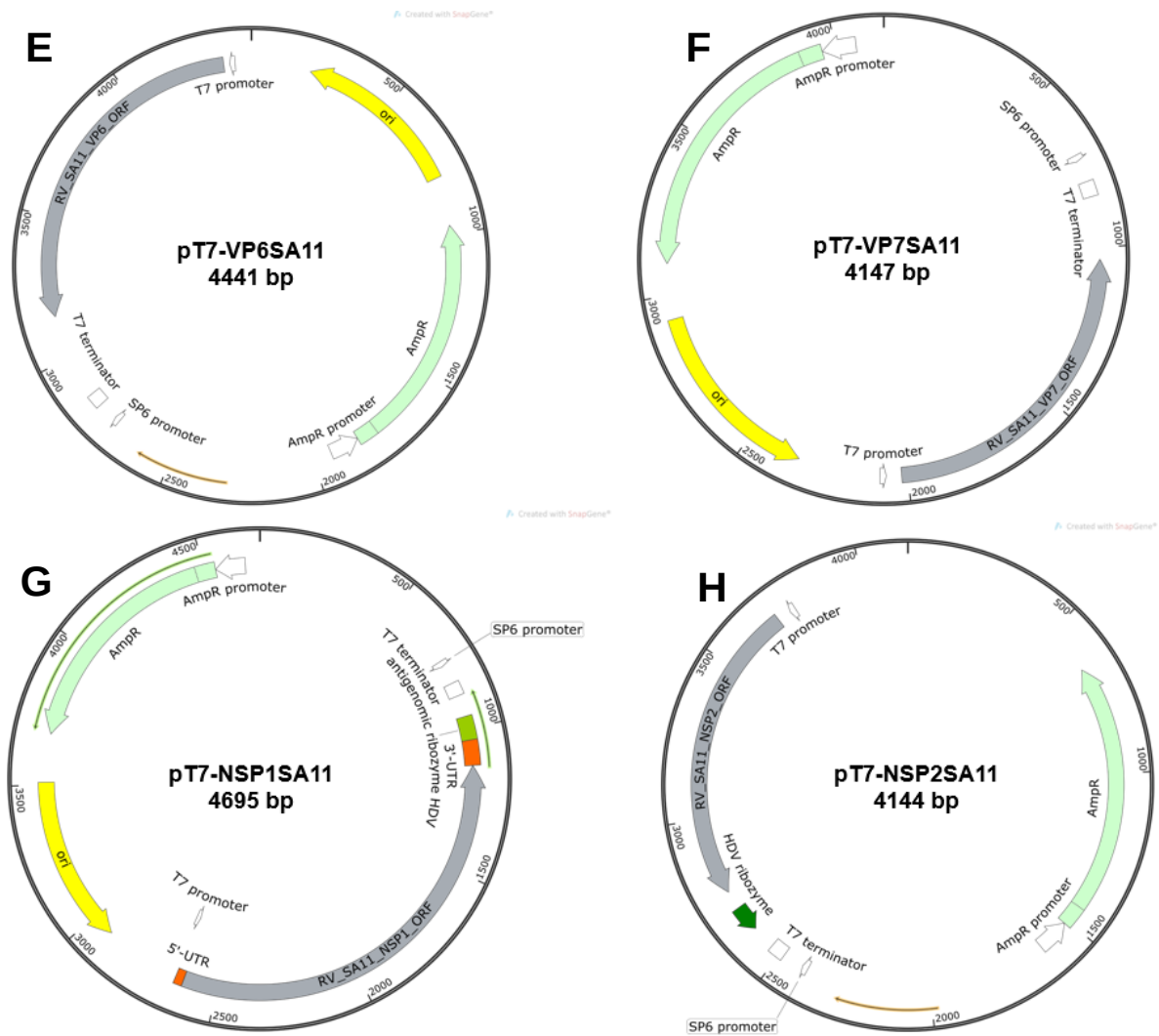


Figure S2: SA11-L2 reverse genetics plasmid maps. E) pT7-VP6SA11, F) pT7-VP7SA11, G) pT7-NSP1SA11, H) pT7-NSP2SA11.

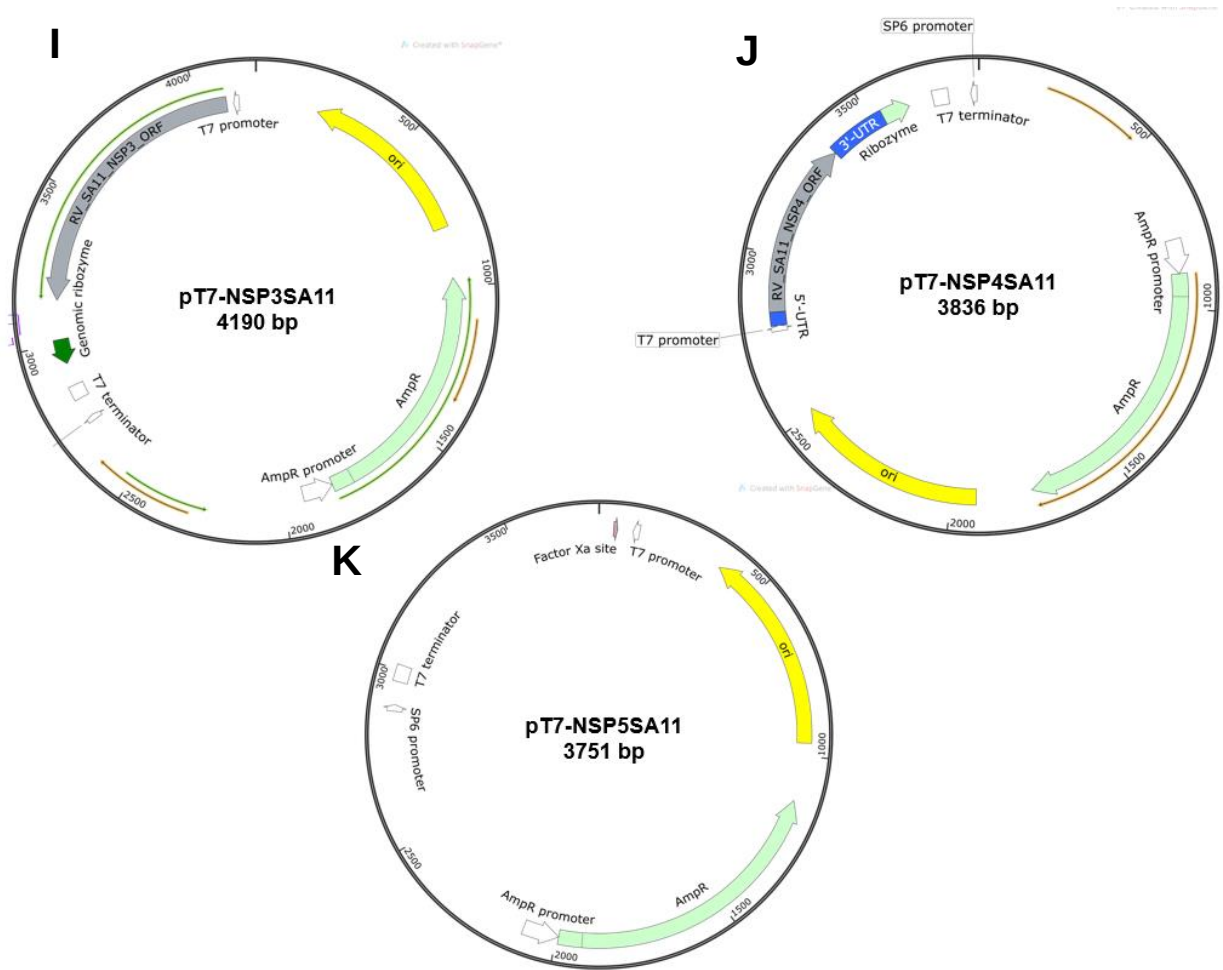


Figure S3: SA11-L2 reverse genetics plasmid maps. I) pT7-NSP3SA11, J) pT7-NSP4SA11, K) pT7-NSP5SA11.

D. First replicate of time-point plaque assays

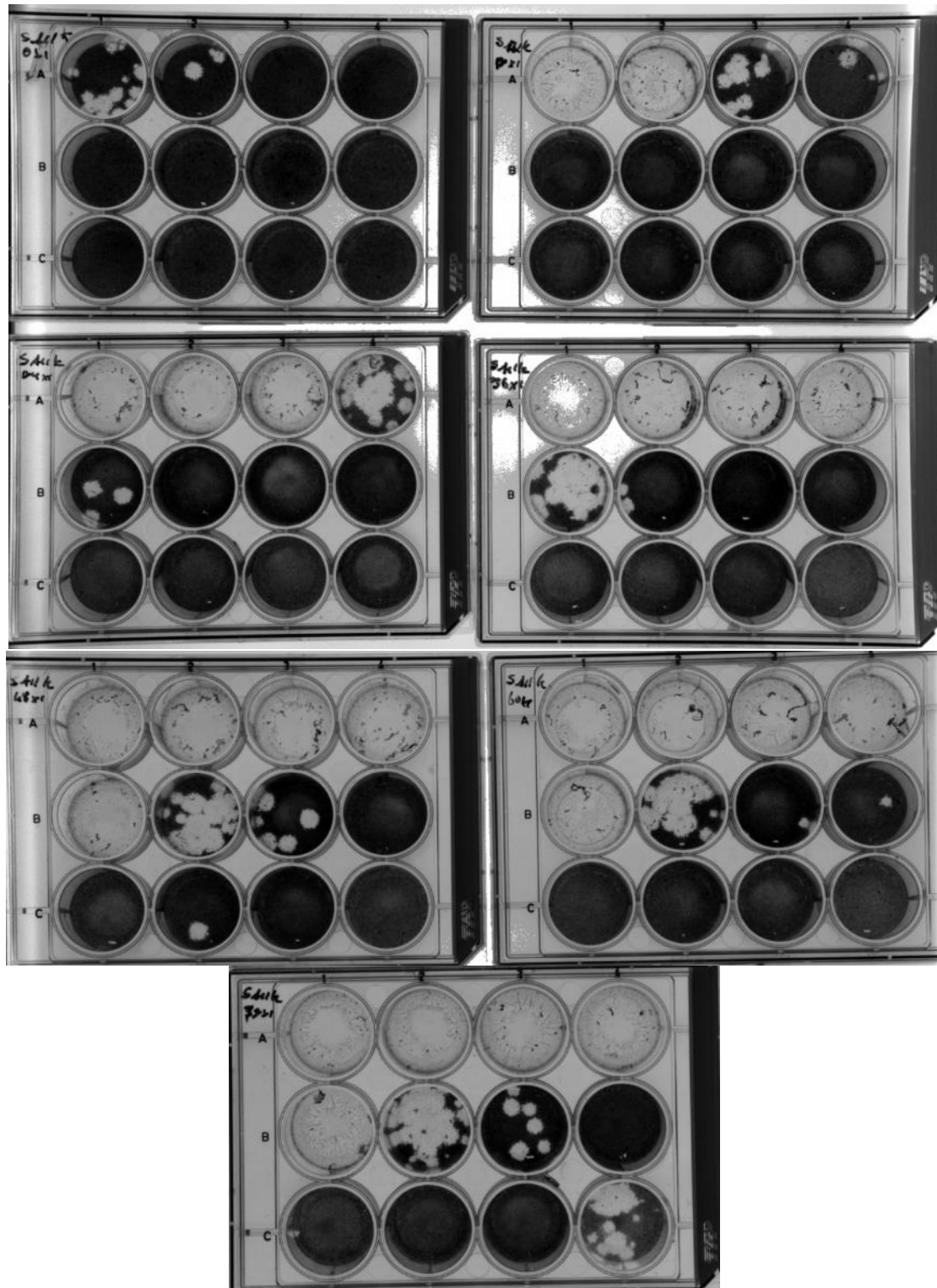


Figure S4: The first replicate of the plaque assays of rSA11-L2 at each time point.

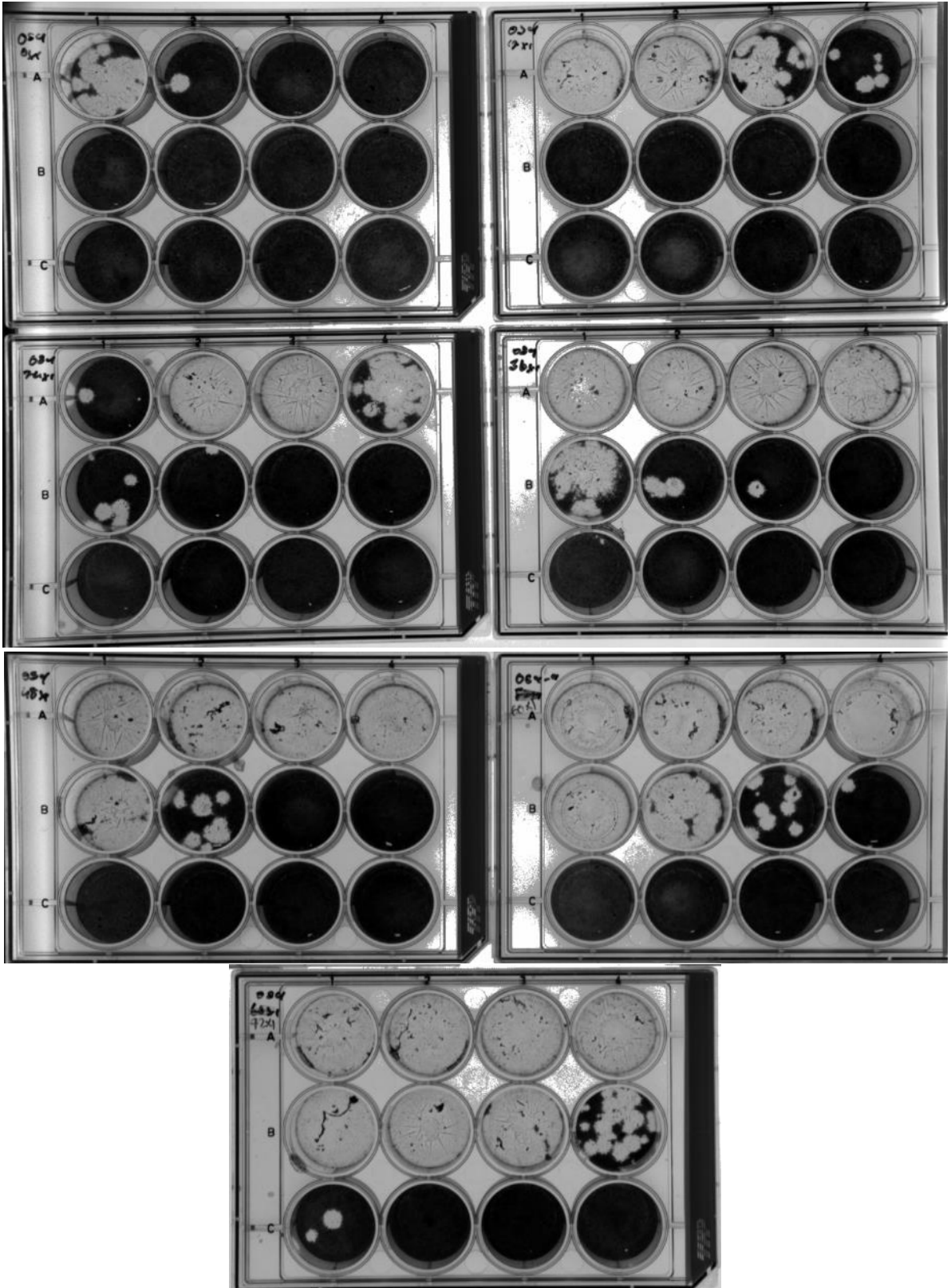


Figure S5: The first replicate of the plaque assays of rSA11-OSU-a at each time point.

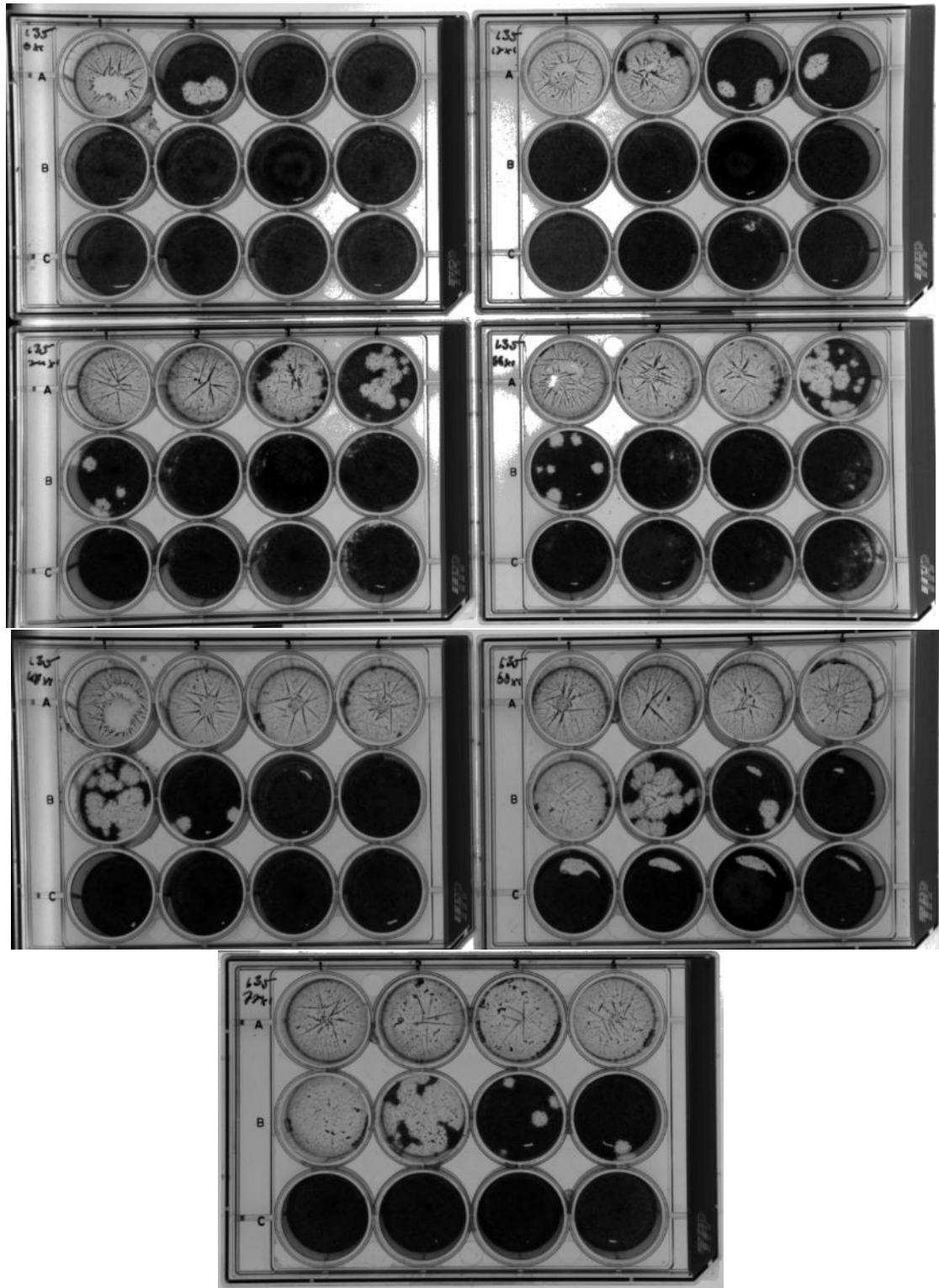


Figure S6: The first replicate of the plaque assays of rSA11-OSU-a_135-141 at each time point.

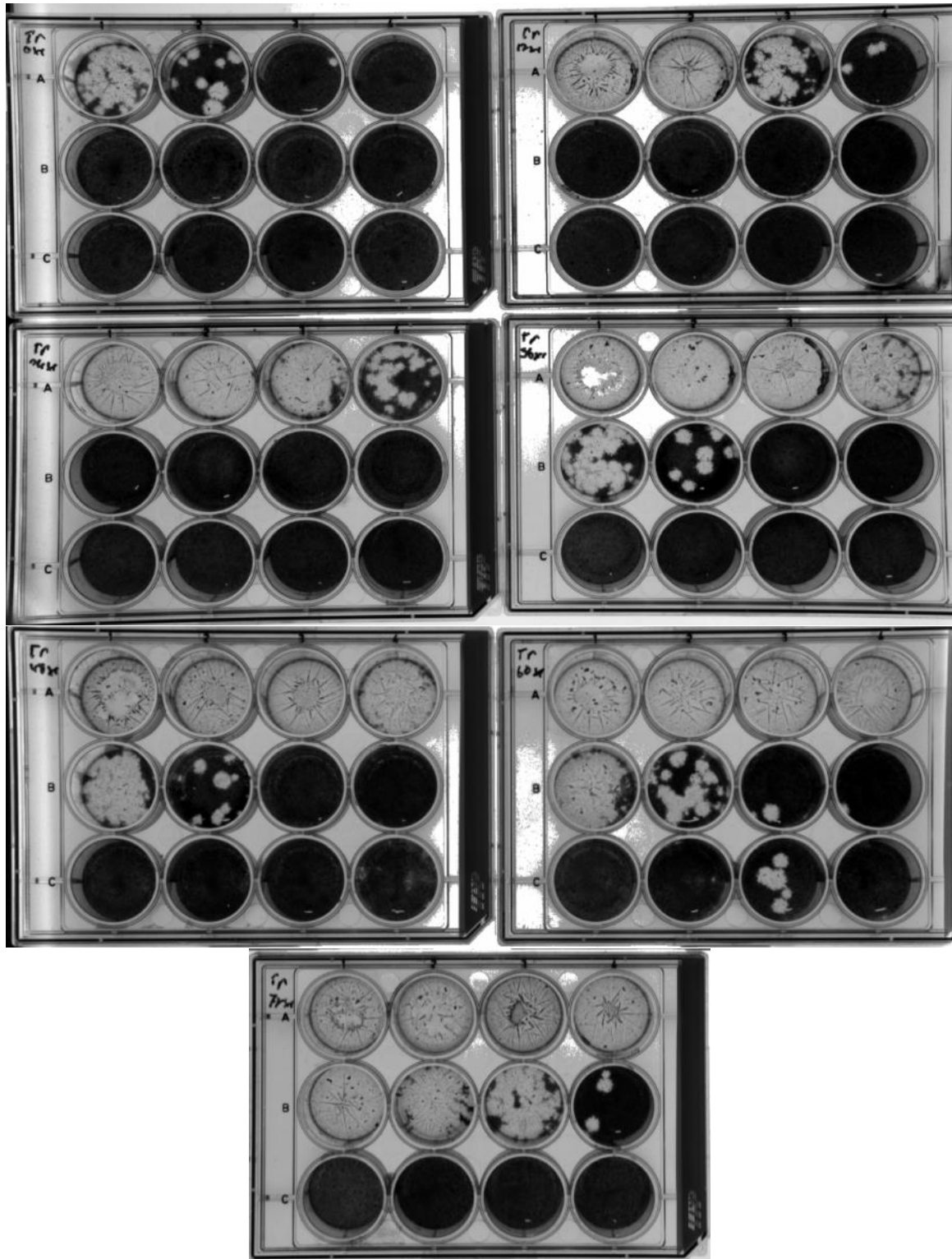


Figure S7: The first replicate of the plaque assays of rSA11-NSP1-tr at each time point.

E. Multi-step growth curves raw data

Table S1: Multi-step growth curves raw titration data.

SA11-L2 (PFU/ml)	0 hpi	12 hpi	24 hpi	36 hpi	48 hpi	60 hpi	72 hpi
Repeat 1	1,00E+02	5,00E+04	2,00E+04	1,00E+06	1,00E+07	2,00E+07	1,00E+07
Repeat 2	2,00E+02	2,00E+04	2,00E+05	2,00E+05	5,00E+07	1,00E+08	5,00E+08
Repeat 3	4,00E+01	1,00E+04	1,00E+04	5,00E+04	7,00E+05	3,00E+07	2,00E+07
Average	1,13E+02	2,67E+04	7,67E+04	4,17E+05	2,02E+07	5,00E+07	1,77E+08
SD	66,00	16996,73	87305,34	416999,87	21387898,97	35590260,84	228667638,48
%CV	58%	64%	114%	100%	106%	71%	129%
SA11_OSU-A GS10 (PFU/ml)	0 hpi	12 hpi	24 hpi	36 hpi	48 hpi	60 hpi	72 hpi
Repeat 1	1,00E+02	5,00E+04	3,00E+05	1,00E+07	5,00E+06	1,00E+08	1,00E+09
Repeat 2	8,00E+02	4,00E+04	1,00E+04	2,00E+06	2,00E+06	2,00E+08	1,00E+08
Repeat 3	2,00E+02	8,00E+03	1,30E+05	2,00E+06	5,00E+06	1,00E+08	9,00E+08
Average	3,67E+02	3,27E+04	1,47E+05	4,67E+06	4,00E+06	1,33E+08	6,67E+08
SD	309,1206	17913,3718	118977,12	3771236,17	1414213,562	47140452,1	402768199,1
%CV	84%	55%	81%	81%	35%	35%	60%
SA11_OSU-A 135-141 (PFU/ml)	0 hpi	12 hpi	24 hpi	36 hpi	48 hpi	60 hpi	72 hpi
Repeat 1	2,00E+02	2,00E+04	2,00E+05	6,00E+05	2,00E+06	1,00E+07	3,00E+07
Repeat 2	6,00E+01	5,00E+03	1,00E+06	1,00E+06	1,00E+08	1,00E+08	1,00E+07
Repeat 3	3,00E+01	1,00E+03	2,00E+05	1,20E+06	6,00E+06	2,00E+07	2,00E+07
Average	9,67E+01	8,67E+03	4,67E+05	9,33E+05	3,60E+07	4,33E+07	2,00E+07
SD	74,09	8178,56	377123,62	249443,83	45284287,19	40276819,91	8164965,81
%CV	77%	94%	81%	27%	126%	93%	41%
SA11_GS5_tr (PFU/ml)	0 hpi	12 hpi	24 hpi	36 hpi	48 hpi	60 hpi	72 hpi
Repeat 1	9,00E+02	2,00E+04	1,00E+05	7,00E+06	5,00E+06	1,00E+08	3,00E+08
Repeat 2	7,00E+02	9,00E+04	1,00E+05	1,00E+06	1,00E+07	6,00E+07	3,00E+07
Repeat 3	1,20E+02	2,00E+04	8,00E+04	1,20E+06	1,10E+07	6,00E+07	2,00E+07
Average	5,73E+02	4,33E+04	9,33E+04	3,07E+06	8,67E+06	7,33E+07	1,17E+08
SD	330,79	32998,32	9428,09	2782484,90	2624669,29	18856180,83	129700509,72
%CV	58%	76%	10%	91%	30%	26%	111%

F. Statistical analysis of growth curves data using Two-way ANOVA

Multiple Comparisons

Dependent Variable: Titre

	(I) Virus	(J) Virus	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	SA11_GS5_tr	SA11_OSU-A 135-141	-53.2857	21.91291	.107	-115.2180	8.6465
		SA11_OSU-A GS10	-29.8571	21.91291	.537	-91.7894	32.0751
		SA11-L2	-49.0000	21.91291	.151	-110.9322	12.9322
	SA11_OSU-A 135-141	SA11_GS5_tr	53.2857	21.91291	.107	-8.6465	115.2180
		SA11_OSU-A GS10	23.4286	21.91291	.712	-38.5037	85.3608
		SA11-L2	4.2857	21.91291	.997	-57.6465	66.2180
	SA11_OSU-A GS10	SA11_GS5_tr	29.8571	21.91291	.537	-32.0751	91.7894
		SA11_OSU-A 135-141	-23.4286	21.91291	.712	-85.3608	38.5037
		SA11-L2	-19.1429	21.91291	.818	-81.0751	42.7894
	SA11-L2	SA11_GS5_tr	49.0000	21.91291	.151	-12.9322	110.9322
		SA11_OSU-A 135-141	-4.2857	21.91291	.997	-66.2180	57.6465
		SA11_OSU-A GS10	19.1429	21.91291	.818	-42.7894	81.0751
Sidak	SA11_GS5_tr	SA11_OSU-A 135-141	-53.2857	21.91291	.145	-117.9930	11.4215
		SA11_OSU-A GS10	-29.8571	21.91291	.717	-94.5644	34.8501
		SA11-L2	-49.0000	21.91291	.209	-113.7073	15.7073
	SA11_OSU-A 135-141	SA11_GS5_tr	53.2857	21.91291	.145	-11.4215	117.9930
		SA11_OSU-A GS10	23.4286	21.91291	.881	-41.2787	88.1358
		SA11-L2	4.2857	21.91291	1.000	-60.4215	68.9930
	SA11_OSU-A GS10	SA11_GS5_tr	29.8571	21.91291	.717	-34.8501	94.5644
		SA11_OSU-A 135-141	-23.4286	21.91291	.881	-88.1358	41.2787
		SA11-L2	-19.1429	21.91291	.950	-83.8501	45.5644
	SA11-L2	SA11_GS5_tr	49.0000	21.91291	.209	-15.7073	113.7073
		SA11_OSU-A 135-141	-4.2857	21.91291	1.000	-68.9930	60.4215
		SA11_OSU-A GS10	19.1429	21.91291	.950	-45.5644	83.8501

Based on observed means.

The error term is Mean Square(Error) = 1680,615.

Figure S8: Multiple comparisons of the mean virus titer of rSA11-L2, rSA11-OSU-a, rSA11-OSU-a_135-141 and rSA11-NSP1-tr using Two-way ANOVA with LSD post-hoc tests.

G. Calcium flux raw data

Table S2: Mean, standard deviation and coefficient of variance of the 340 nm/360 nm values at each time point.

BAPTA														
	0 min	5 min	10 min	15 min	20 min	25 min	30 min							
Repeat 1	1.41656	1.28129	1.30553	1.30127	1.38559	1.23347	1.39903							
Repeat 2	1.05572	1.06221	1.0565	1.04423	1.21231	1.26011	1.17144							
Repeat 3	0.90692	0.86664	0.87868	0.90125	0.9202	0.95452	0.9483							
Average	1.13264	1.07005	1.08024	1.08225	1.1727	1.14936	1.17292							
SD	0.21398	0.16937	0.17506	0.16551	0.19205	0.13821	0.18401							
%CV	19%	16%	16%	15%	16%	12%	16%							
rSA11-L2-BAPTA														
	0 min	5 min	10 min	15 min	20 min	25 min	30 min							
Repeat 1	0.78001	0.91482	1.00438	0.91625	1.00763	0.9458	1.00203							
Repeat 2	0.90154	0.91509	0.95191	0.94898	0.95458	0.9805	0.96473							
Repeat 3	0.86396	0.79158	0.77667	0.77935	0.79492	0.81098	0.8015							
Average	0.8485	0.87383	0.91099	0.88153	0.91905	0.91243	0.92275							
SD	0.05081	0.05816	0.09736	0.07347	0.0904	0.07312	0.08708							
%CV	6%	7%	11%	8%	10%	8%	9%							
rSA11_OSU-aNSP4-BAPTA														
	0 min	5 min	10 min	15 min	20 min	25 min	30 min							
Repeat 1	0.56354	0.59715	0.56219	0.55801	0.56389	0.57167	0.54121							
Repeat 2	0.58333	0.61326	0.60331	0.61352	0.61766	0.61793	0.61073							
Repeat 3	0.6213	0.62108	0.6192	0.62106	0.61593	0.62761	0.62051							
Average	0.59005	0.6105	0.5949	0.59753	0.59916	0.60574	0.59082							
SD	0.02382	0.00996	0.02402	0.02811	0.02495	0.02441	0.03553							
%CV	4%	2%	4%	5%	4%	4%	6%							
rSA11_OSU-a_135-1410 min														
	0 min	5 min	10 min	15 min	20 min	25 min	30 min							
Repeat 1	0.67893	0.64331	0.64066	0.6465	0.65718	0.65613	0.62882							
Repeat 2	0.7343	0.79746	0.7964	0.82478	0.83378	0.83891	0.83915							
Repeat 3	0.76835	0.7798	0.79369	0.76596	0.7756	0.76701	0.78744							
Average	0.72719	0.74019	0.74358	0.74575	0.75552	0.75401	0.7518							
SD	0.03685	0.06888	0.07279	0.07417	0.07349	0.07518	0.08949							
%CV	5%	9%	10%	10%	10%	10%	12%							
rSA11_OS10 min														
	5 min	10 min	15 min	20 min	25 min	30 min								
Repeat 1	0.61186	0.62581	0.63172	0.62038	0.61983	0.6252	0.61985							
Repeat 2	0.70896	0.66456	0.67406	0.67996	0.67829	0.67355	0.67253							
Repeat 3	0.62734	0.62542	0.63381	0.62734	0.63739	0.6357	0.63698							
Average	0.64939	0.6386	0.64653	0.64256	0.64517	0.64482	0.64312							
SD	0.0426	0.01836	0.01949	0.0266	0.02449	0.02076	0.02194							
%CV	7%	3%	3%	4%	4%	3%	3%							
rSA11_OS10 min														
	5 min	10 min	15 min	20 min	25 min	30 min								
Repeat 1	0.89371	0.76236	0.78094	0.78009	0.78406	0.81838	0.82513							
Repeat 2	0.94562	1.0449	1.05	1.0431	1.06296	1.05894	1.08957							
Repeat 3	0.9063	0.69313	0.73725	0.72467	0.75683	0.7594	0.76421							
Average	0.91521	0.83346	0.85606	0.84929	0.86795	0.87891	0.89297							
SD	0.02211	0.15216	0.13829	0.13889	0.13834	0.12956	0.14123							
%CV	2%	18%	16%	16%	16%	15%	16%							

H. Statistical analysis of calcium flux data using Two-way ANOVA

Multiple Comparisons						
Dependent Variable: Calcium						
	(I) Virus	(J) Virus	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound Upper Bound
Tukey HSD	BAPTA	Cells	-62766588.00	85434389.977	.998	-338783606.0 213250430.03
		Ionomycin	-608055656.0*	85434389.977	<.001	-884072674.0 -332038638.0
		rSA11_135-1	-241804819.7	85434389.977	.130	-517821837.7 34212198.315
		rSA11-L2	-616707895.3*	85434389.977	<.001	-892724913.3 -340690877.3
		rSA11-L2+BA	-381647743.3*	85434389.977	.001	-657664761.3 -105630725.3
		rSA11-OSU-a	-47427026.57	85434389.977	1.000	-323444044.6 228589991.46
		SA11_135+BA	-231501105.9	85434389.977	.169	-507518123.9 44515912.173
		SA11_OSU+BA	-84450935.57	85434389.977	.985	-360467953.6 191566082.46
	Cells	BAPTA	62766588.00	85434389.977	.998	-213250430.0 338783606.03
		Ionomycin	-545289068.0*	85434389.977	<.001	-821306086.0 -269272050.0
		rSA11_135-1	-179038231.7	85434389.977	.487	-455055249.7 96978786.315
		rSA11-L2	-553941307.3*	85434389.977	<.001	-829958325.3 -277924289.3
		rSA11-L2+BA	-318881155.3*	85434389.977	.013	-594898173.3 -42864137.26
		rSA11-OSU-a	15339561.429	85434389.977	1.000	-260677456.6 291356579.46
		SA11_135+BA	-168734517.9	85434389.977	.567	-444751535.9 107282500.17
		SA11_OSU+BA	-21684347.57	85434389.977	1.000	-297701365.6 254332670.46
	Ionomycin	BAPTA	608055656.0*	85434389.977	<.001	332038637.97 884072674.03
		Cells	545289068.0*	85434389.977	<.001	269272049.97 821306086.03
		rSA11_135-1	366250836.3*	85434389.977	.002	90233818.256 642267854.32
		rSA11-L2	-8652239.2857	85434389.977	1.000	-284669257.3 267364778.74
		rSA11-L2+BA	226407912.71	85434389.977	.191	-49609105.32 502424930.74
		rSA11-OSU-a	560628629.4*	85434389.977	<.001	284611611.40 836645647.46
		SA11_135+BA	376554550.1*	85434389.977	.002	100537532.11 652571568.17
		SA11_OSU+BA	523604720.4*	85434389.977	<.001	247587702.40 799621738.46
	rSA11_135-1	BAPTA	241804819.71	85434389.977	.130	-34212198.32 517821837.74
		Cells	179038231.71	85434389.977	.487	-96978786.32 455055249.74
		Ionomycin	-366250836.3*	85434389.977	.002	-642267854.3 -90233818.26
		rSA11-L2	-374903075.6*	85434389.977	.002	-650920093.6 -98886057.54
		rSA11-L2+BA	-139842923.6	85434389.977	.780	-415859941.6 136174094.46
		rSA11-OSU-a	194377793.14	85434389.977	.375	-81639224.89 470394811.17
		SA11_135+BA	10303713.857	85434389.977	1.000	-265713304.2 286320731.89
		SA11_OSU+BA	157353884.14	85434389.977	.655	-118663133.9 433370902.17
	rSA11-L2	BAPTA	616707895.3*	85434389.977	<.001	340690877.26 892724913.32
		Cells	553941307.3*	85434389.977	<.001	277924289.26 829958325.32
		Ionomycin	8652239.2857	85434389.977	1.000	-267364778.7 284669257.32
		rSA11_135-1	374903075.6*	85434389.977	.002	98886057.542 650920093.60
		rSA11-L2+BA	235060152.00	85434389.977	.155	-40956866.03 511077170.03
		rSA11-OSU-a	569280868.7*	85434389.977	<.001	293263850.68 845297886.74
		SA11_135+BA	385206789.4*	85434389.977	.001	109189771.40 661223807.46
		SA11_OSU+BA	532256959.7*	85434389.977	<.001	256239941.68 808273977.74
	rSA11-L2+BA	BAPTA	381647743.3*	85434389.977	.001	105630725.26 657664761.32
		Cells	318881155.3*	85434389.977	.013	42864137.256 594898173.32
		Ionomycin	-226407912.7	85434389.977	.191	-502424930.7 49609105.315
		rSA11_135-1	139842923.57	85434389.977	.780	-136174094.5 415859941.60
		rSA11-L2	-235060152.0	85434389.977	.155	-511077170.0 40956866.030
		rSA11-OSU-a	334220716.7*	85434389.977	.007	58203698.685 610237734.74
		SA11_135+BA	150146637.43	85434389.977	.709	-125870380.6 426163655.46
		SA11_OSU+BA	297196807.7*	85434389.977	.026	21179789.685 573213825.74
	rSA11-OSU-a	BAPTA	47427026.571	85434389.977	1.000	-228589991.5 323444044.60
		Cells	-15339561.43	85434389.977	1.000	-291356579.5 260677456.60
		Ionomycin	-560628629.4*	85434389.977	<.001	-836645647.5 -284611611.4
		rSA11_135-1	-194377793.1	85434389.977	.375	-470394811.2 81639224.887
		rSA11-L2	-569280868.7*	85434389.977	<.001	-845297886.7 -293263850.7
		rSA11-L2+BA	-334220716.7*	85434389.977	.007	-610237734.7 -58203698.68
		SA11_135+BA	-184074079.3	85434389.977	.449	-460091097.3 91942938.744
		SA11_OSU+BA	-37023909.00	85434389.977	1.000	-313040927.0 238993109.03
	SA11_135+BA	BAPTA	231501105.86	85434389.977	.169	-44515912.17 507518123.89
		Cells	168734517.86	85434389.977	.567	-107282500.2 444751535.89
		Ionomycin	-376554550.1*	85434389.977	.002	-652571568.2 -100537532.1
		rSA11_135-1	-10303713.86	85434389.977	1.000	-286320731.9 265713304.17
		rSA11-L2	-385206789.4*	85434389.977	.001	-661223807.5 -109189771.4
		rSA11-L2+BA	-150146637.4	85434389.977	.709	-426163655.5 125870380.60
		rSA11-OSU-a	184074079.29	85434389.977	.449	-91942938.74 460091097.32
		SA11_OSU+BA	147050170.29	85434389.977	.731	-128966847.7 423067188.32
	SA11_OSU+BA	BAPTA	84450935.571	85434389.977	.985	-191566082.5 360467953.60
		Cells	21684347.571	85434389.977	1.000	-254332670.5 297701365.60
		Ionomycin	-523604720.4*	85434389.977	<.001	-799621738.5 -247587702.4
		rSA11_135-1	-157353884.1	85434389.977	.655	-433370902.2 118663133.89
		rSA11-L2	-532256959.7*	85434389.977	<.001	-808273977.7 -256239941.7
		rSA11-L2+BA	-297196807.7*	85434389.977	.026	-573213825.7 -21179789.68
		rSA11-OSU-a	37023909.000	85434389.977	1.000	-238993109.0 313040927.03
		SA11_135+BA	-147050170.3	85434389.977	.731	-423067188.3 128966847.74

Figure S9: Multiple comparisons of the mean 340 nm/380nm in cells infected with rSA11-L2, rSA11-OSU-a, rSA11-OSU-a_135-141 or treated with ionomycin or BAPTA-AM. $p < 0.05$ was considered as statistically significant.

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		End Page	3219
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Instructor Name	Prof AA van Dijk	Expected Presentation Date	2023-08-04

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