Evaluation of recombinant Newcastle disease viruses (NDV) as candidate vaccine delivery vectors for rotavirus VP7 and NSP4

By

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Submitted in fulfilment of the requirements in respect of the degree Magister Scientiae majoring in Biochemistry

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ABSTRACT

Rotavirus (RV) is one of the leading causes of neonatal calf diarrhoea (NCD), a disease which has a devastating impact on the agricultural industry due to high morbidity and mortality rates. There is also mounting evidence for interspecies transmission of RV from animals to humans which contribute to strain diversity and stresses the need for a One Health approach in RV control. The development of the Newcastle disease virus (NDV) reverse-genetic system has opened up ways in which attenuated NDV La Sota can be used as a vaccine vector in non-avian species. Natural host-range restrictions and an inability to combat the host's innate immunity, has rendered the use of attenuated NDV in mammals inherently safe. Since NDV is antigenically distinct from common animal pathogens, it will not be recognized by a pre-existing immunity. By utilizing the genome sequence of a South African bovine group A RV, recombinant NDVs were engineered to express RV outer capsid protein, VP7, and enterotoxin protein, NSP4. Protein expression was confirmed by immunofluorescent monolayer assay (IFMA) and western blot analysis. The ability of the recombinant NDVs to elicit humoral immune responses were evaluated in laboratory-bred adult mice. Vaccination was done twice via the oronasal or subcutaneous route and blood samples were collected 3 weeks after each immunization. The serums of the vaccinated mice were analysed for RV-related humoral immune responses by IFMAs and virus neutralization assays. Immune responses induced in mice dosed with rNDV-VP7 were suboptimal and lacked neutralizing ability for either mode of administration. This might be explained by a loss of antigenic determinates resembling those of the native protein when VP7 is expressed in the absence of other RV proteins. Immune responses induced in rNDV-NSP4 vaccinated mice were promising and correlated well with an oronasal route of administration. It has been reported that antibodies directed against NSP4 have the capacity to neutralize the enterotoxicity of the protein and reduce the severity of RV-related diarrhoea during the early stages of infection. In addition, NSP4 has been shown to have adjuvant properties. This study indicates the potential of rNDV-NSP4 in a combination vaccine which might help to prevent diarrhoea in new-born calves and increase the immune responses towards a co-administered antigen.

DECLARATION

I, Larise Oberholster, declare that the Master's Degree research dissertation or publishable, interrelated articles, or coursework Master's Degree mini-dissertation that I herewith submit for the Master's Degree qualification at the University of the Free State is my independent work, and that I have not previously submitted it for a qualification at another institution of higher education.

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Signature **Date**

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RESEARCH OUTPUTS

The following posters were presented at scientific conferences:

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Oberholster, L. Potgieter, C. & O'Neill, H.G. (2018) Removal of rotavirus VP7 signal peptide influences protein folding. **Joint Meeting of South African Society of Biochemistry and Molecular Biology and Federation of African Societies of Biochemistry and Molecular Biology, 2018, Potchefstroom, South Africa, 8-11 July 2018.**

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CHAPTER 1 – LITERATURE REVIEW

1.1 Introduction

Historically, viruses were defined as a filterable, living liquids that are undetectable by light microscopy yet capable of causing disease in higher organisms (Beijerinck, 1898). The concept of a filtrable infectious agent was first reported in 1892 by Dimitri Ivanovski, a young graduate student majoring in botany at the University of St. Petersburg, who noted a filterable agent capable of causing disease in tobacco plants. This infectious agent would later become known as the tobacco mosaic virus (Ivanovski, 1892). Today, a virus is defined as a reproducible entity that consist of genetic material encapsulated by a protective outer coating. These entities are considered lifeless due to the fact that they are deprived of a metabolic system, intrinsic mobility and the potential to respond to external stimuli. However, since viruses have genetic continuity with the potential for mutation, this remains a highly controversial topic. A virus is entirely dependent on the host cell for its translational machinery which allows for the production of multiple copies of the virion and often leads to the destruction of the cell (Goodheart, 1969).

The discovery of bovine rotavirus (RV) resembles that of the tobacco mosaic virus whereby young calves inoculated with a bacteria-free filtrate of diarrhoeic calf faeces were seen to develop neonatal calf diarrhoea (NCD) shortly after (Mebus *et al.*, 1969). NCD has a devastating impact on the agricultural industry as a result of the high morbidity and mortality rates, reduced growth rates, increased susceptibility to co-infections and costs associated with treatment and prophylactic measures against RV infection (Rocha *et al.*, 2017).

The term ‘zoonosis’ (Greek, *zoon* ‘animal’, *nosos* ‘disease’), which is often referred to in the field of virology, can be defined as the natural transmittance of infectious diseases from vertebrate animals to humans. RV is one such infectious agent and was shown to be the main etiological agent responsible for severe dehydrating diarrhoea in young children (Bishop *et al.*, 1973). Human and animal RVs share morphological characteristics and a common group antigen which make interspecies transmission possible (Holland, 1990). There is mounting evidence for animal-to-human RV transmission which contribute to RV strain diversity and emphasises the need for a One Health approach in RV control (Bányai *et al.*, 2009; Ghosh *et al.*, 2011). The One Health concept is recognized by the American Veterinary Medical Association to be “a collaborative effort of multiple disciplines working locally, nationally and globally to attain optimal health for people, animals and the environment” (American Veterinary Medical Association 2008).

Vaccination strategies against RV infection in bovine are based on a passive immunisation approach which aims to increase the level of RV-specific antibodies in the colostrum (Rocha *et al.*, 2017). At present, dams are vaccinated with inactivated RV particles, however, discrepancies exist regarding the efficacy of the vaccine in experimental conditions and double-blind field trials (Kim *et al.*, 2002). To address this problem, research is being done on new-generation vaccines, which include sub-unit proteins, DNA vaccines and viral vectors as carriers of transgene products (Fernandez *et al.*, 1998; Dhama *et al.*, 2009). Collectively these novel efforts might help to reduce the RV strain burden circulating in bovine, thereby limiting animal-to-human reassortment.

1.2 RV genome organization and virion structure

Rotavirus is a double-stranded RNA (dsRNA) virus which forms part of the family *Reoviridae* under the genus *Rotavirus. The* triple-layered icosahedral capsid contains an 11-segmented genome that encodes for six structural proteins (VPs) and six non-structural proteins (NSPs). The virion core contains the RNA-dependent RNA polymerase (RdRp, VP1), the scaffolding protein (VP2), the capping enzyme (VP3) and the viral genome. The viral core together with 260 trimers of VP6 constitute the double-layered particle (DLP), which in turn is surrounded by 260 trimers of VP7 and 60 trimers of VP4 to form the triple-layered particle (TLP) (Fig. 1.1). Proteolytic cleavage of VP4 into VP5* and VP8* subunits results in the conformational transition of the protein from a disordered to an ordered state which enables the virus to enter the host cell. The VP4 proteins constitute the viral spikes and interact with both VP7 and VP6, in which the base of VP5* is half buried (Crawford *et al.*, 2001). The non-structural proteins consist of NSP1, that acts as an antagonist of the cellular interferon response (Graff *et al.*, 2002); NSP2 that is needed for viroplasm formation and contains nucleoside triphosphatase activity (Fabbretti *et al.*, 1999; Taraporewala *et al.*, 1999); NSP3 that has been shown to inhibit host cell protein synthesis (Padilla-Noriega *et al.*, 2002); NSP4, an enterotoxin excreted by the host cell which also functions as a receptor for the budding stage of RV morphogenesis (Au *et al.*, 1989; Ball *et al.*, 1996); NSP5 that is involved in formation and control of the viroplasm (Fabbretti *et al.*, 1999; Criglar *et al.*, 2014) and NSP6 which has RNA binding capability (Gonzalez *et al.*, 1998; Rainsford and McCrae, 2007). The virion contains 132 channels which span the outer layers and connect the inner core with the outer surface. These channels are classified according to their position and size, with 12 type I channels being distributed along the five-fold axis, 60 type II channels along the three-fold axis and 60 type III channels along the two-fold axis (Prasad *et al.*, 1988).

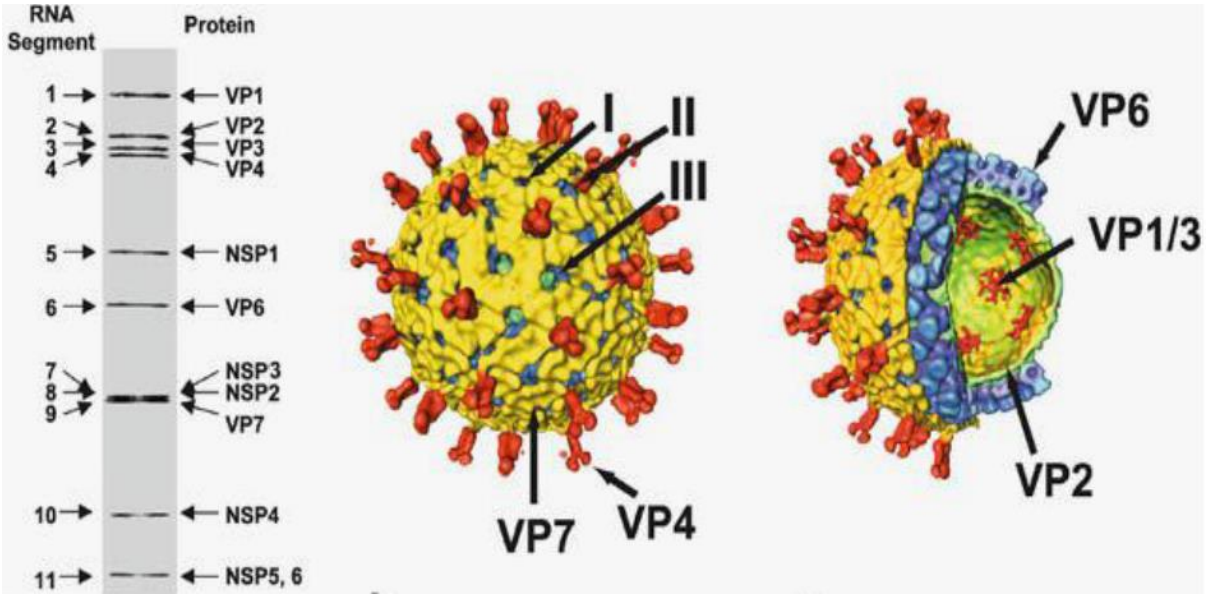


Fig. 1.1. **Schematic representation of the rotavirus genome and structural composition**. Double-stranded RNA segments are numbered according to migration pattern on PAGE which are encoded into the corresponding RV proteins to the right of the gel (left). Representation of rotavirus particle with surface proteins VP7 and VP4 including channels I, II and III (middle). Cross section of the rotavirus particle showing the middle layer, VP6, the inner layer, VP2, and the VP1/3 complex (right) (Taken from Jayaram *et al*., 2004).

1.3 Classification of RV

The *Rotavirus* genus is composed of eight antigenic groups (RVA-RVH) that are classified according to the genetic variability of the coding sequence for VP6 (Matthijnssens *et al.*, 2012). Strains belonging to RVA-RVC and RVH are zoonotic whereas strains belonging to RVD-RVG have to date only been reported in animals (Dhama *et al.*, 2015). Strains belonging to the same group are capable of genetic reassortment. Group A RVs are responsible for the majority of infections in humans and livestock, such as bovine, and can further be subdivided into G and P genotypes. The G types are determined by the nucleotide sequence encoding the glycoprotein, VP7 and the P types are determined by the nucleotide sequence encoding the protease-sensitive protein, VP4 (Estes and Kapikian, 2007). Serotypes of VP7 and VP4 are defined by their reactivity to monoclonal or polyclonal antisera whereas the genotypes are determined by sequence analysis. A variable region on VP8* is used to determine P-type specific epitopes since genotypes and serotypes of P types do not always correlate and is therefore denoted by square brackets (Larralde and Gorziglia, 1992; Matthijnssens *et al.*, 2009). The genotypes and serotypes of type G are synonymous. An all-inclusive nucleotide sequence-based classification system for the complete genome of RVAs was proposed in 2008. In this system the VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 encoding genome segments are differentiated based on specific nucleotide percent cut-off values (Matthijnssens *et al.*, 2008).

1.4 RV replication cycle

Rotaviruses exhibit a natural tropism for absorptive cells in the small intestine, suggesting the presence of RV specific receptors that make virus attachment and penetration possible. Nevertheless, they can replicate in a range of non-intestinal cells, making them less host cell specific than what was previously thought and suggests the presence of additional RV-specific receptors (Ciarlet *et al.*, 2002; Lopez and Arias, 2006). The replication of RV includes the following steps (Fig. 1.2).

1.4.1 Attachment and internalization

While RVs bind a wide variety of cells, the related infection efficiencies have been found to differ between cell lines. This suggests that initial attachment occurs with a common receptor but that co-receptors are critical for post-attachment and virus entry into the cell (Ciarlet *et al.*, 2002). The outer capsid of RV consists of the VP4 spikes and glycosylated VP7 proteins which are involved in the attachment of the virus to host cell. The large icosahedral particles of the *Reoviridae* make it especially difficult for the virus to cross the cell membrane to initiate gene replication and expression. Protease-primed conformational changes in VP4 expose certain domains of VP5*. These domains are lipophilic and normally concealed by VP8*. The conformational changes in the VP4 spike are necessary for the effective penetration of RV into the cell (Fig. 1.2A) (Trask *et al.*, 2010).

1.4.2 Uncoating

Rotavirus VP7 comprises the majority of the viral outer layer of which the integrity of the arranged trimers is dependent on the Ca^{2+} concentration of the surrounding media (Dormitzer and Greenberg, 1992). Each VP7 subunit is held in place by two calcium ions at each subunit interface, equalling six calcium ions per trimer. Upon entering the cell, the outer capsid is removed as a result of the low Ca^{2+} levels in the endosomes which leads to the exposure of the DLPs (Fig. 1.2B) (Aoki *et al.*, 2009).

1.4.3 Transcription and translation

The RV particle possesses its own RNA-dependent RNA polymerase (RdRp), VP1 that acts as both a transcriptase and a replicase (Patton, 1996). RdRp forms part of the transcription complex (TC), which possesses the needed enzyme activity for the formation of capped, non-polyadenylated messenger RNA (Lawton *et al.*, 2000). These positive single-stranded RNA (ssRNA) transcripts are released from the DLP through the class I channel system and are encoded into the six structural proteins or six non-structural proteins (Fig. 1.2C) (Lawton *et al*., 1997).

1.4.4 Replication and DLP assembly in viroplasms

During RV replication electron-dense inclusion bodies, known as viroplasms, are observed in the cytoplasm of the cell (Petrie *et al.*, 1982). The formation of these viroplasmic inclusions is driven by hyperphosphorylation of NSP5 and its interaction with NSP2 (Fabbretti *et al.*, 1999). Viral replication and packaging of (+)ssRNA occur simultaneously in the viroplasm, whereby replication complexes (VP1/3/ssRNA) interact with VP2 decamers to form the viral core (Gallegos and Patton, 1989). The exact mechanism by which the correct set of dsRNA segments are packaged into individual virus particles, remains unclear. Once the core particles are formed, transcapsidation occurs via VP6 resulting in the DLPs (Fig. 1.2D) (Patton *et al*., 2004).

1.4.5 Budding and formation of transient envelope

Rotavirus undergoes a unique morphogenic pathway which involves the acquisition of a transient envelope following budding of the DLP from the viroplasm into the endoplasmic reticulum (ER) (Petrie *et al.*, 1983).The budding process is mediated by NSP4 which functions as a intracellular receptor in the ER membrane (Au *et al.*, 1989). The receptor activity of NSP4 is localized to the C-terminus of the protein which interacts with VP6 on the newly synthesized sub-viral particles (Fig. 1.2E) (Taylor *et al*., 1996).

1.4.6 Loss of envelope and particle maturation

NSP4 is lost from the mature virus particle with the addition of ER-localized VP7 and VP4 to form the TLPs (Chen *et al.*, 2009). Since NSP4 has membrane destabilizing abilities it is believed to a play a key role in the removal of the transient envelope during virus maturation (Tian *et al.*, 1996). The fully assembled RV particles are released from the cell either by a budding process or by cell lysis (Fig. 1.2F) (McNulty *et al.*, 1976; Gardet *et al.*, 2006).

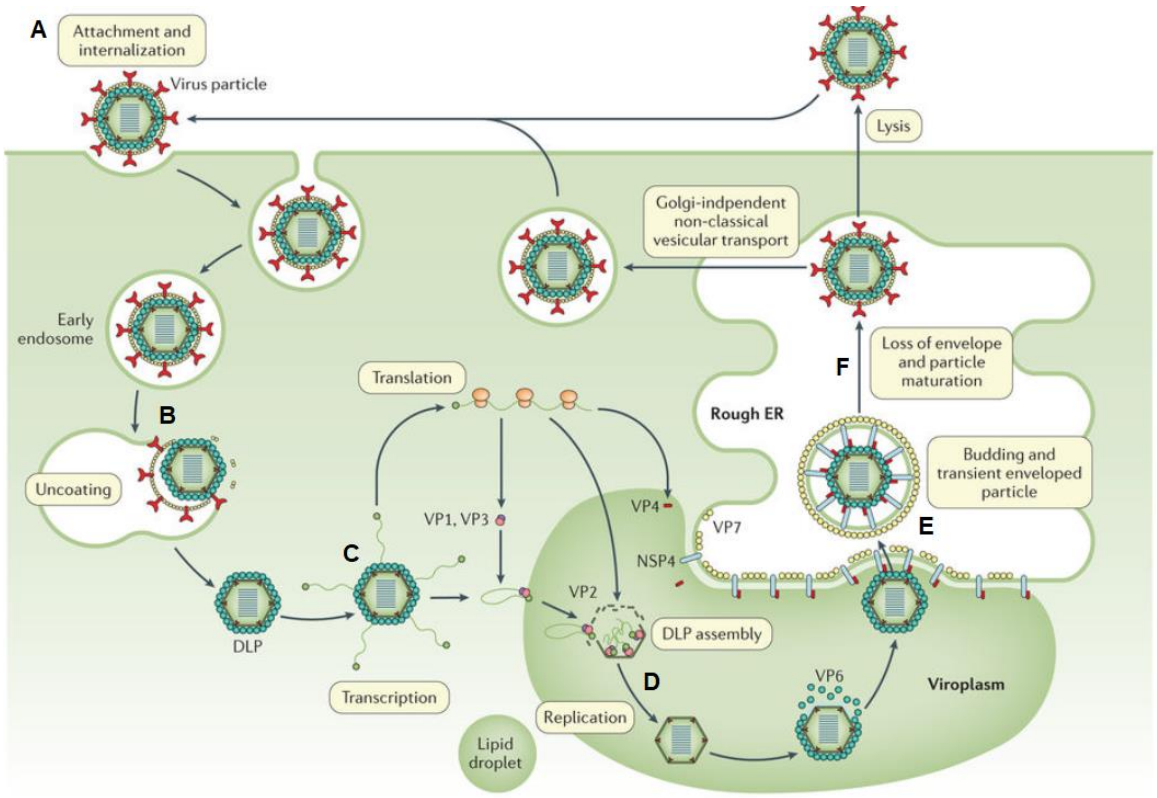


Fig. 1.2. **Schematic representation of the rotavirus replication cycle**. Replication occurs via the following steps: A) attachment and internalization, B) uncoating, C) transcription and translation, D) replication and DLP assembly, E) budding and formation of transient envelope, F) loss of envelope and particle maturation (Taken from Crawford *et al.*, 2017).

1.5 Pathogenicity of RV

The mucosa of the small intestine is topographically arranged into villi and crypts of which the former is extended approximately 1 mm into the intestinal lumen. The apical domains are separated from the basolateral domains by a junctional complex that connects the polarized cells. RV infects and replicates in the mature epithelial cells at the apex of the villi (Fig. 1.3A) (Jourdan *et al.*, 1997). A symptomatic infection results in the destruction of mature enterocytes which in turn are replaced by immature, undifferentiated cells that lack sodium-potassium ATPase activities. The loss in absorptive capacity results in nutrient malabsorption and excessive fluid loss (Argenzio, 1985).

The enterotoxicity of NSP4 also contributes to the pathogenicity of RV. NSP4 is a multifunctional protein, which causes Ca^{2+}- and age-dependent diarrhoea in mice by promoting Cl^- secretion across the intestinal mucosa. The enterotoxicity of NSP4 accounts for the intestinal fluid accumulation in the early stages of RV infection, during which diarrhoea occurs in the absence of significant mucosal disruption (Ball *et al.*, 1996). RV infection results in a three-fold increase of intracellular Ca^{2+} with NSP4 being the sole mediator (Michelangeli *et al.*, 1991; Tian *et al.*, 1994). NSP4 induces the release of Ca^{2+} from the ER and increases the permeability of the plasma membrane which allows for the uptake of extracellular Ca^{2+} into

the cell. The exact mechanism by which internal NSP4 triggers the release of Ca^{2+} from internal stores is not well understood, however, it is clear that this process occurs independent of phospholipase C (PLC) (Tian *et al.*, 1995). In contrast, extracellular NSP4 induces the release of Ca^{2+} from the ER by activating a signalling cascade that involves PLC and 1,3,5-triphosphate inositol phosphatase $(IP)_3$ (Ramig, 2004). In crypt cells, NSP4 induces an increase in intracellular Ca^{2+} and secretion of Cl^-, either directly or through stimulation of the enteric nervous system (ENS) (Lundgren *et al.*, 2000; Ramig, 2004) (Fig 1.3B).

The correlation between Ca^{2+} mobilization and fluid transport in the gut of susceptible animals was determined using mice deficient of cAMP-mediated transport known as cystic fibrosis transmembrane conductance regulator (CFTR) gene knock-out mice. NSP4 was shown to induce age-dependent diarrhoea in CFTR mice irrespective of their inability to perform cAMP-mediated Cl^- secretion. The study also found that Ca^{2+} mobilization is not directly responsible for age-dependent Cl^- secretion related to secretory diarrhoea. In contrast, NSP4 is capable of inducing an iodide influx in crypt cells isolated from CFTR mice which was both age-dependent and Ca^{2+}-dependent. Therefore, NSP4 might induce age-dependent diarrhoea through a pathway that requires the presence of Ca^{2+} and that is regulated by the anionic halide permeability of the apical plasma membrane (Morris *et al.*, 1999).

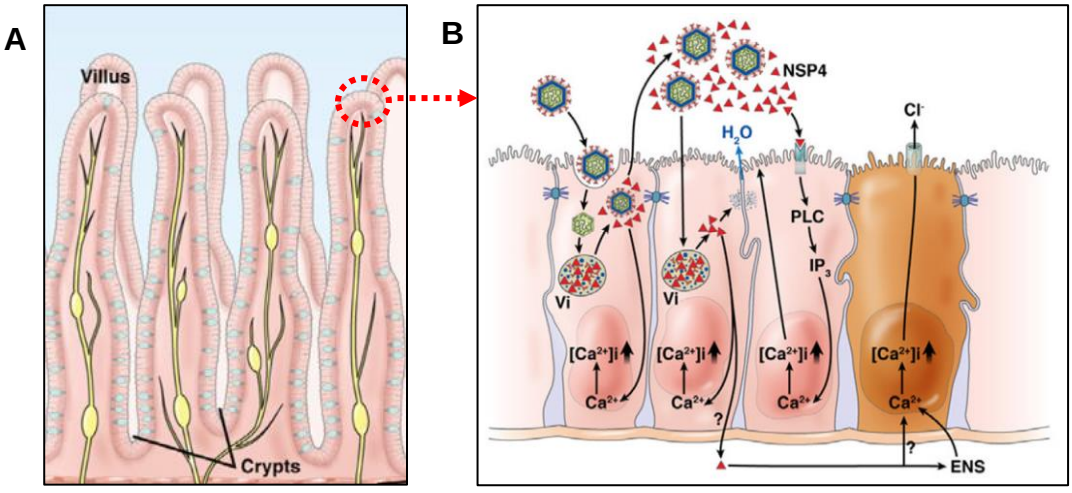


Fig. 1.3. **Mechanism by which rotavirus induces diarrhoea**. A) Rotaviruses infect and replicate in mature enterocytes at the apex of the villi spanning the small intestine; B) The enterotoxin NSP4 accounts for symptoms of diarrhoea in the early stages of RV infection by inducing the release of Ca^{2+} from internal cellular stores. External NSP4 binds to receptors located on the cell surface and triggers a signalling cascade that activates phospholipase C (PLC) and inositol phosphatase $(IP)_3$, thereby increasing internal Ca^{2+} levels. Crypt cells (brown cell) exhibit an increase in intracellular Ca^{2+} and secretion of Cl^- which is induced by NSP4, either directly or via the enteric nervous system (ENS) (Taken from Ramig, 2004).

1.6 Epidemiology of RV infection

To effectively combat RV disease, the One Health approach, which requires the cooperation of multiple disciplines for optimal health in humans and animals, is essential. The necessity of a One Health approach is further emphasized by the fact that RVs are globally distributed and causes great financial loss in clinical and agricultural sectors. Although the absolute number of RV associated deaths have decreased from 453 000 in 2008 to 130 000 in 2016, RV remains the main etiological agent responsible for severe dehydrating diarrhoea in young children (Tate *et al.*, 2012; Troeger *et al.*, 2018). Sub-Saharan Africa accounted for 105 000 RV-related deaths in 2016 with countries such as the Central African Republic, Nigeria, Niger, Chad and Sierra Leone bearing the highest rates of mortality (Fig 1.4) (Troeger *et al.*, 2018). The marked decrease in RV-related deaths can be attributed to the inclusion of globally licensed RV vaccines in the vaccination schedule of numerous countries by recommendation of the World Health Organization (WHO) (The World Health Organization, 2009). These vaccines are, however, live-attenuated and not suitable for use in animals due to the risk of RV reassortment.

In new-born calves, RV diarrhoea is more severe than diarrhoea caused by any other pathogen and results in devastating financial loss for cattle farmers (Chauhan *et al*., 2008). Rotavirus related deaths in neonates can go up to 80% but a mortality rate of 5-20 % is more common. The mortality rate is increased in neonates who obtained insufficient amounts of colostrum postpartum and are confined to barns/sheds due to the accumulation of RV in these spaces (Dhama *et al.*, 2009). Under natural conditions, infection can occur shortly after birth with viral shedding observed within 48 hours of life (McNulty *et al.*, 1976). The infection rate is increased by the high concentration of viral shedding in animal faeces and the small dose of RV needed to cause infection. RVs from calves have been reported in a number of countries including the United Kingdom, France, Italy, Netherlands, Sweden, Switzerland, Finland, Turkey, Bulgaria, Bangladesh, Egypt, India, Sri Lanka, USA, Canada, Brazil, Argentina and Australia, indicating its global distribution in bovine (Chauhan *et al*., 2008).

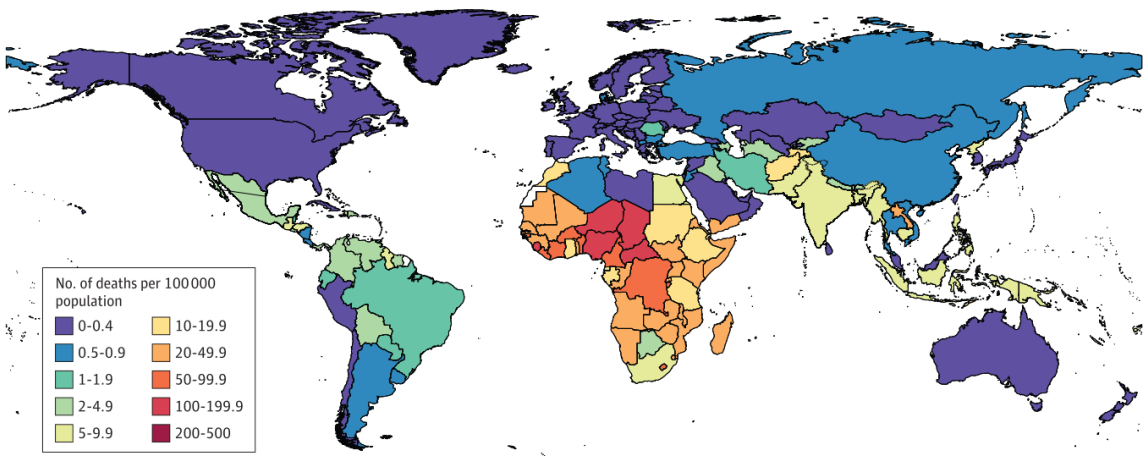


Fig. 1.4. **World-wide map depicting RV-related mortality rate in children under the age of five in 2016.** RV-related deaths are highly prevalent in Sub-Saharan Africa with the highest mortality rates occurring in non-developing countries such as Central African Republic, Nigeria, Niger, Chad and Sierra Leone (Taken from Troeger *et al.*, 2018).

1.7 Reassortment and interspecies transmission

Bovine RVs share a common ancestry with human RVs which makes interspecies transmission possible and adds to the RV strain burden circulating in humans (Matthijnssens *et al.*, 2008). Interspecies transmission can occur by animal-human reassortants, containing genome segments that originated from different species, or non-reassortant animal viruses, in which all the genome segments originated from the same host species (Doan *et al.*, 2013). In bovine, G6, G8 and G10 are the predominant RV serotypes and typically associate with P[1], P[5] or P[11] (Rocha *et al.*, 2017). The G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] combinations are commonly found in humans while other serotypes, such as G8, G10, G11 and G12, are suspected to have originated from animals. Genotype G8 have been isolated form children on a number of occasions and serves as evidence of the direct transmission of bovine RVs to humans (Hasegawa *et al.*, 1984; O'Halloran *et al.*, 2000; Bányai *et al.*, 2009; Ghosh *et al.*, 2011). Zoonotic transmission of animal RVs are often coupled to reassortment in which genomic segments from different strains are interchanged during co-infection of the same cell (Mcdonald *et al.*, 2016). RV reassortants occur widely in nature with an increased risk in sub-Saharan Africa due to a high level of mixed RV infections (Mwenda *et al.*, 2010). Exchange of genome segments can also occur between vaccine strains or vaccine strains and circulating field strains. In a case study, 4 out of 61 infants vaccinated with RotaTeq® developed hospitable diarrhoea as a result of reassortment between vaccine components (Donato *et al.*, 2012). RotaTeq® is a globally licensed, live-attenuated pentavalent vaccine that consist of five bovine-human reassortant strains with a bovine WC3 strain backbone (Heaton and Ciarlet, 2007). Cumulatively, these findings demonstrate the potential of bovine-human

reassortants to add to RV strain diversity and further complicate RV infections in infants and young children.

1.8 RV-induced immunity

1.8.1 Innate immunity

Intestinal epithelial cells (IECs) recognize viral components which include mRNA, genomic RNA and RNA replication intermediates. The IECs detect viral RNA by pattern recognition receptors (PRRs), such as Toll-like receptor-3 (TLR3), melanoma differentiation-associated gene-5 (MDA-5) and retinoic acid-inducible gene-I (RIG-1), depending on chemical structure and localization of the RNA within the cell (Frias *et al.*, 2011). TLR3 primarily detects dsRNA in endosomal compartments, whereas MDA-5 and RIG-I detect viral nucleic acid within the cytoplasm. Both these pathways induce a cellular signalling cascade which results in the phosphorylation and dimerization of IFN-regulatory factor-3 (IRF3) (Meylan and Tschopp, 2006). IRF3 is translocated to the cell nucleus where it triggers the production of IFN genes and IRF-stimulated gene (ISG) products (Meylan and Tschopp, 2006; Takeuchi and Akira, 2008). Type I (IFN-α, IFN-β) and III (IFN-ʎ1, IFN-ʎ2/3) IFNs are key components of the host innate immunity and are secreted from the infected cell to bind to receptors on neighbouring cells, including IFNAR and IFNʎR. Binding induces synthesis of IFN-stimulated proinflammatory gene products (MxA, Mx1, RNAseL, OAS, PKR) through the JAK-STAT pathway (Donnelly and Kotenko, 2010). TLR3 also trigger the activation of the nuclear factor κB (NF-κB) pathway, which allows for the secretion of proinflammatory cytokines and chemokines (CXCL10, IL-6, IL-8, MCP-1) (Fig. 1.5) (Frias *et al.*, 2011). As with other TLRs, TLR3 recognizes pathogen associated molecular patterns (PAMPs) by a leucine-rich repeat (LRR) motif located in its ectodomain. Upon recognition of PAMPs, the cytoplasmic Toll-interleukin-1 receptor (TIR) domain recruits TIR-containing adapters to mediate intracellular responses and induce pro-inflammatory genes which play a key role in the establishment of an antiviral state (Meylan and Tschopp, 2006). RV often evades the host's innate immune response by exploiting non-structural protein NSP1, an important determinant of RV virulence. NSP1 is responsible for the degradation of key components of the cellular signalling cascade, including IRF3 (Arnold et al. 2013).

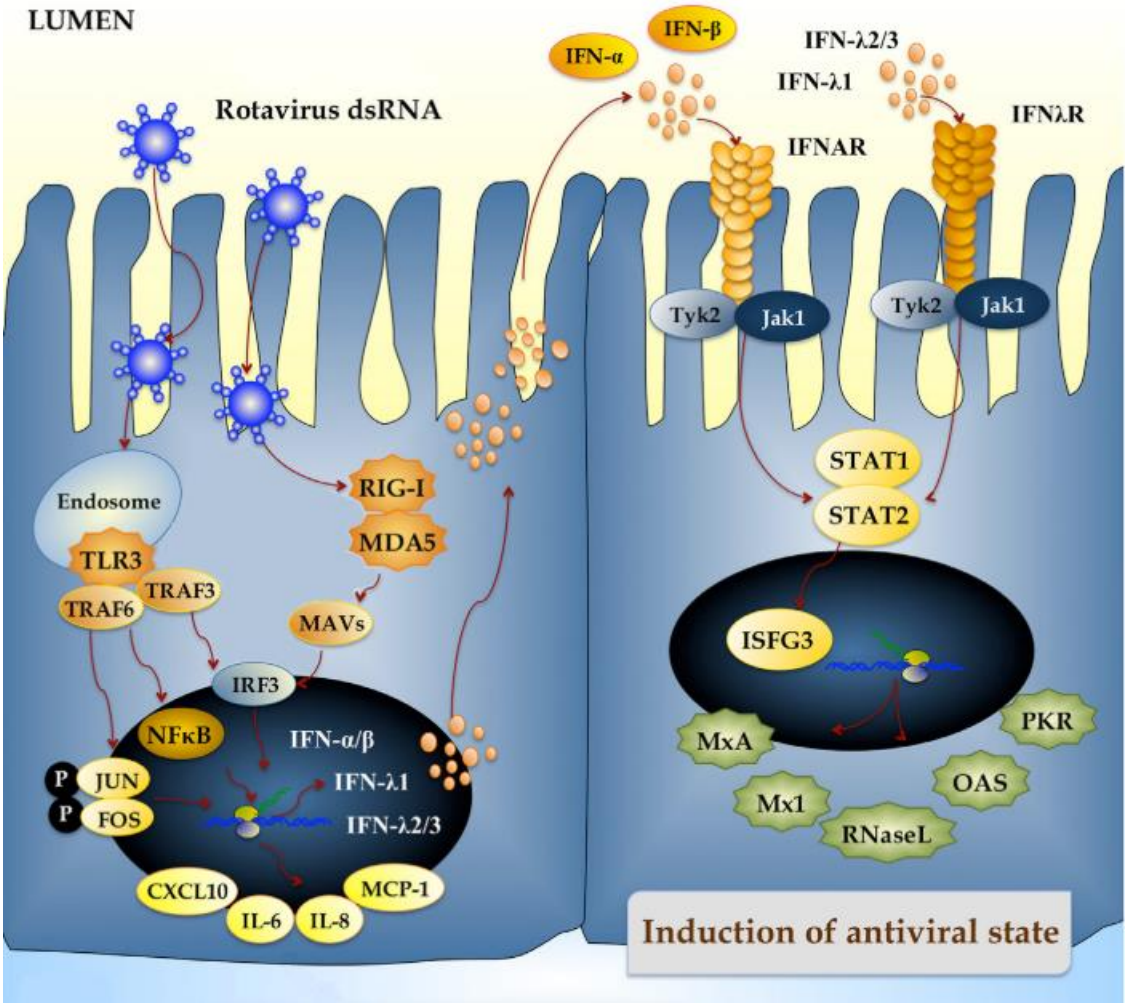


Fig. 1.5. **Innate immune response in intestinal epithelial cell (IEC) following infection with rotavirus**. Upon entrance into the IEC, RV dsRNA is recognized by pattern recognition receptors (PRRs), including Toll-like receptor-3 (TLR3), melanoma differentiation-associated gene-5 (MDA-5) and retinoic acid-inducible gene-I (RIG-I), depending on where the viral nucleic acid is localized. Both pathways converge at the IFN-regulatory factor-3 (IRF3) level which leads to the induction of cellular signalling cascades that upregulate the expression of type I (IFN-α/β) and type III (IFN-ʎ) interferons. This, in turn, induces the synthesis of genes with antiviral properties (MxA, Mx1, RNaseL, OAS and PKR) in neighbouring cells. PRRs also trigger the synthesis and release of cytokines and chemokines (CXCL10, IL-6, IL-8 and MCP-1) through the NF-κB pathway (Taken from Villena *et al*., 2016).

1.8.2 Acquired immunity

1.8.2.1 Cellular

$CD8^+$ T cells, also known as cytotoxic T lymphocytes, play a substantial role in the initial clearance of primary RV infection (Franco and Greenberg, 1995). In severe combined immunodeficient (SCID) mice infected with murine RV, passive transfer of $CD8^+$ T cells allowed for the complete clearance of primary RV infection, even in the absence of RV-specific antibodies. However, this response was short-lived and shown to diminish within 8 months (Dharakul *et al.*, 1990; Estes and Greenberg, 2013). The role of T cells in the control of primary RV infection was studied in gnotobiotic calves depleted of either $CD4^+$ or $CD8^+$ T cells using

monoclonal antibodies. In calves depleted of $CD8^+$ T cells, there was a significant increase in viral shedding while $CD4^+$ T cell depleted calves showed a reduction in serum antibody responses (Oldham *et al.*, 1993). While $CD8^+$ T cells provide short-term protection in knockout mutant mice, it is memory B cells that are necessary for long-term protection from reinfection. Memory B cells also provide protection in the absence of T cells but with a significantly reduced response compared to that seen in wild-type mice (Franco *et al*., 2006).

1.8.2.2 Humoral

Immunity in neonatal calves, during the early stages of life, is completely dependent on them obtaining immunoglobulins from the maternal colostrum. The neonate can respond to antigens, but the response is often delayed and unable to eliminate the infectious agent (Tizard, 1987). When these antibodies are not passively transferred via the colostrum, the new-born calf is predisposed to systemic and gastrointestinal infections (Norcross, 1982). Immunoglobulin synthesis increases shortly after birth causing the development of the gastrointestinal immunity to accelerate. Neutralization of viral particles is a major consequence of colostral antibodies which plays a critical role in the protection of neonatal calves against RV infection (Woode *et al*., 1975; Castrucci *et al.*, 1984). Passively transferred anti-RV IgG antibodies are transported to the small intestine of the neonate which becomes asymptomatically infected to allow the production of antibodies to prevent subsequent disease. (Holland, 1990). This balance is however disrupted by large-scale livestock production systems where the animals are prematurely weaned, restricted to confined, virus-contaminated environments and milk antibodies are diluted in feed supplements (Saif and Fernandez, 1996).

Neutralization is the foundation of immunological protection against RV infection in present vaccination programs. Human case studies indicate that a single RV infection or immunization is sufficient to confer heterotypic protection against subsequent disease (Velazquez *et al.*, 1996; Ruiz-Palacios *et al.*, 2006). Monoclonal antibodies directed against surface proteins, VP7 and VP4, protect against various strains of RV when passively transferred to mice (Matsui *et al.*, 1989). This widespread immunity is governed by heterotypic neutralizing epitopes located on VP7 and VP5* (subunit of VP4) (Nair *et al.*, 2017). VP7 comprises the majority of the virion outer capsid and is 6.5 times more abundant than VP4 (Ghosh *et al.*, 2012). The most efficient neutralizing monoclonal antibodies are directed against VP7 which neutralizes RV by preventing virion decapsidation. Antibodies bound to VP7 inhibit Ca^{2+} chelation and solubilization of the protein, a vital step of the RV replication cycle (Ludert *et al.*, 2002). VP7 is also shown to induce murine B-cell activation in the absence of VP4 and viral RNA (Blutt *et al.*, 2004).

Other immunogenic RV proteins include the structural protein VP6 and non-structural protein NSP4. While secretory IgA directed against VP6 are non-neutralizing *in vitro*, they do provide protection *in vivo* (Burns *et al.*, 1996; Vega *et al.*, 2013; Pastor *et al.*, 2014). VP6-specific antibodies interfere with the RV replication cycle by binding to type I channels on transcriptionally active DLPs, preventing ssRNA transcripts from exiting the virion (Aiyegbo *et al.*, 2013). In RV immune humans, VP6 is shown to have broad binding reactivity across secretory and intestinal antibody secreting cells (ASC), which includes B cell subsets with phenotypes representing the entire B cell pool (Nair *et al.*, 2016).

Mice immunized with NSP4 amino acid peptides spanning residues 114 – 135 had a significant decrease in the severity and duration of diarrhoea when challenged with an infectious dose of simian RV SA11. (Ball *et al.*, 1996). Interestingly, anti-NSP4 antibodies administered during the late stages of diarrhoea not only halt the progression of disease, but also reverse histological changes in the small intestine (Hou *et al.*, 2008). Furthermore, a VP6-NSP4 fusion protein injected into BALB/c mice had an increased immune response compared to that of VP6 administered on its own, indicating the adjuvant properties of NSP4 (Afchangi *et al.*, 2017).These studies indicate the potential of NSP4 to serve as an immunogen in RV vaccine development.

1.9 Bovine RV vaccines

The first bovine RV vaccines consisted of live-attenuated bovine rotaviruses (BRVs) (Scourvax-Reo: Norden Laboratories, Lincoln, Nebraska, USA) that were orally administered to calves shortly after birth (Mebus *et al.*, 1973). This vaccination approach seemed effective in experimental conditions but showed poor efficacy in double-blind field trials (Acres and Radostits, 1976). A probable reason could be the difference between serotypes of circulating field strains and that of the vaccine, or from the interference of maternal antibodies (Acres and Radostits, 1976; Van Zaane *et al*., 1986). It is also possible that the new-born calf might be exposed to virulent BRV strains before a protective immune response has had time to develop (Saif and Fernandez, 1996). This prompted researchers to look at a passive immunisation approach which aims to increase the production of anti-RV immunoglobulins in mammary secretions and consequently prolong their production in milk. At present, pregnant dams are parenterally inoculated with a combination vaccine that includes inactivated BRVs (G6P[5]), inactivated bovine corona viruses and *Escherichia coli* (K99) F5 antigen (ROTAVEC® CORONA, MSD Animal Health, Mpumalanga, SA) (Chauhan, *et al.,* 2008). The efficacy of the vaccine is shown by the difference in morbidity between calves from vaccinated and unvaccinated dams. The clinical manifestation of disease is dependent on factors such as the amount of colostrum consumed, the feeding period and the RV-specific antibody titres present

in the colostrum (Holland, 1990). Good hygienic practices and management procedures help to reduce the risk of infection, which might be improved by the use of antibiotics to prevent secondary bacterial infections (Dhama *et al.*, 2009). While dams vaccinated with inactivated BRVs are shown to have increased antibody titres in milk and colostrum, there are discrepancies regarding the efficacy of the vaccine in field trials (Kim *et al.*, 2002). Research is being done to develop new generation RV vaccines which include virus-like particles, recombinant sub-unit protein, edible plant-based vaccines, DNA vaccines and virus-based recombinant vectors (Dhama *et al.*, 2009).

1.10 Alternative vaccines

1.10.1 Virus-like particles

Virus-like particles (VLPs), obtained from heterologous expression systems, assemble in the absence of a viral genome to form structures that are synonymous to the native virus particle. They are capable of inducing high levels of cellular and humoral immune responses that mediate protection against virus infection (Conner *et al.*, 1996; Agnello *et al.*, 2006). The major structural proteins of RV (VP2, VP4, VP6 and VP7) can be combined and produced in different expression systems to form particles that resemble the native virion. In a study conducted by Crawford and co-workers, the capsid encoding genes of RV were cloned into the Baculovirus expression vector system (BEVS) and subsequently expressed in insect cells. Co-expression of VP2 and VP6 resulted in VLPs that resembled the RV DLP and co-expression of VP2, VP4, VP6 and VP7 assembled into particles resembling the TLP (Crawford *et al.*, 1994). In another study, rotavirus-like particles expressed in yeast were shown to induce cellular immune responses capable of diminishing viral shedding in adult mice (Rodriguez-Limas *et al.*, 2014)

Seropositive dams vaccinated with heterologous VLPs containing BRV RF VP2 and Simian RV SA11 VP4, VP6 and VP7 showed increased antibody titers in colostrum, milk and serum compared to dams vaccinated with inactivated SA11 particles (Fernandez *et al.*, 1998). Calves fed colostrum from VLP vaccinated dams showed complete protection from diarrheic disease whereas calves fed colostrum from dams vaccinated with inactivated SA11, only showed partial protection (Fernandez *et al.*, 1998). The immune responses elicited by RV VLPs have also been evaluated in other animal models such as mice, rabbits and gnotobiotic piglets (Redmond *et al.*, 1993; Ciarlet *et al.*, 1998; Azevedo *et al.*, 2010).

Rotavirus-like particles would be a rational alternative to the live-attenuated vaccines or inactivated viral particles because they do not require inactivation, do not elicit handling of potential pathogens and cannot convert back to their infectious form (Rodríguez-Limas *et al.*, 2011).

1.10.2 Recombinant sub-unit proteins

The expression of recombinant proteins is a key aspect of molecular research and the application thereof in the production of subunits vaccines have been increasing. *E. coli*-expressed VP8* was evaluated for its antibody inducing abilities in pregnant dams and shown to elicit milk antibody titers that remained above a 510 threshold for 10 days postpartum. This indicated that neonates born to VP8* vaccinated dams might be protected from RV-related disease under specific management conditions (Lee *et al.*, 1995). However, as with any viral infection, the protective capacity of the antibody titer in milk might be overcome with a high challenge dose (Snodgrass *et al.*, 1980).

Gram-positive lactic acid bacteria are effective expression systems to produce viral proteins and because of their adjuvant properties they are especially useful for vaccination purposes. RV proteins NSP4, VP7, VP8 and VP6 have been expressed in *Lactococcus lactis* and in every case shown to induce significant humoral immunity in small animal models (Enouf *et al.*, 2001; Perez *et al.*, 2005; Marelli *et al.*, 2011; Temprana *et al.*, 2018).

1.10.3 Edible vaccines

Recombinant immunogens produced in transgenic plants is an attractive alternative to traditional vaccination strategies since the oral administration route might increase the efficacy of the vaccine against enteric pathogens, while also being conveniently included in the diet of the desired animal (Mason *et al.*, 1996). Several bacterial and viral antigens have already been produced in plants and shown to have immunogenic potential. These include *E. coli* heat-labile protein, cholera toxin B subunit and transmissible gastroenteritis coronavirus (TGEV) glycoprotein S (Haq *et al.*, 1995; Arakawa *et al*.,1998; Gomez *et al.*, 2000). The development of a seed-based bivalent vaccine composed of RV NSP4 and VP6 was shown to induce high levels of serum IgG and intestinal IgA when orally administered to mice (Feng *et al.*, 2017). In another study, a fusion between VP4 peptide and reporter enzyme β-glucuronidase was expressed in alfalfa plants and shown to elicit RV-specific immunity when administered to pregnant mice. The offspring of the vaccinated dams were protected from viral infection, demonstrating that immunization induced both humoral and secretory anti-RV responses (Wigdorovitz *et al.*, 2017). Some plant-based RV proteins have the potential to form VLPs as in the case of the co-expression of VP2 and VP6 in *Nicotiana bethamiana* (Pêra *et al.*, 2015).

1.10.4 DNA vaccines

With DNA vaccines, expression of the immunizing protein is done directly in the host cells. This circumvents time consuming and labour-intensive procedures that are associated with recombinant subunit vaccines and VLPs, such as protein purification (Tang, *et al*., 1992). The

endogenous expression of antigens is important for the generation of CTLs which can react to a variety of different strains due to its specificity for conserved viral regions that are associated with major histocompatibility complex (MHC) class I molecules. This enables $CD8^+$ to recognize infected cells which are subsequently killed to prevent further spread of the virus (Doherty and Zinkernagel, 1975). The peptides that associate with the MHC molecules originate from endogenously expressed viral proteins, regardless of the protein's function or location. Therefore, CTLs can provide heterologous protection by recognizing epitopes from internal, conserved viral proteins (Yewdell and Bennink, 1989). On the other hand, exogenous proteins enter the endosomal pathway to be presented by MHC class II molecules that are not as efficient in eliciting $CD8^+$ responses (Holling *et al.*, 2004). In a study done by Ulmer and co-workers, BALB/c mice inoculated with a DNA construct expressing influenza A nucleoprotein induced a nucleoprotein-specific cytotoxic T-cell immune response and subsequent protection from Influenza A virus (Ulmer *et al.*, 1993). Similarly, the direct injection of plasmid DNA carrying human immunodeficiency virus (HIV) type 1 envelope glycoprotein, induced both HIV-specific cellular and humoral immune responses in mice (Wang *et al.*, 1993). Regarding RV DNA vaccines, Chen and co-workers showed VP4, VP7 and VP6-expressing plasmids to elicit RV-specific CTL responses in mice, while also inducing neutralizing antibodies for the VP4 and VP7 DNA constructs (Chen *et al.*, 1997). However, since immune responses induced by DNA vaccines in larger animal models are much weaker compared to that seen in mice, this platform still requires considerable improvement (Kutzler and Weiner, 2015).

1.10.5 Viral vectors as vaccines

The potential of viruses to serve as carriers of transgene products have been exploited in the advancement of subunit vaccines. Viral vectors are among the most effective carriers in providing protective immunity against viral infections in animals (Sharpe *et al.*, 2017). Following the eradication of smallpox, the concept of vaccination was continued using recombinant pox viruses to express genes from heterologous pathogens to elicit an immune response against the same pathogen. Safety concerns regarding pox virus-based vaccines were resolved using replication-deficient constructs. An early example of such a construct was the Copenhagen strain of vaccinia virus expressing rabies virus glycoprotein and consequently shown to protect rabbits against symptoms of rabies (Kieny *et al.*, 1984). The vaccinia virus has also been used to express antigens from the herpes virus (Rooney *et al.*, 1988), hepatitis B virus (Smith et al. 1983a) influenza virus (Smith et al. 1983b) and human immunodeficiency virus (Cooney *et al.*, 1991). However, it was concluded that a pre-existing immunity towards vaccinia virus might significantly decrease the efficacy of the replication-deficient vaccinia virus vaccine.

Another renowned viral vector is the replication-defective adenovirus which was originally developed for application in human gene therapy (Vorburger and Hunt, 2002). The deletion of the E1 locus, a necessity for the initiation of viral replication, rendered the adenovirus replication-defective (Berkner, 1988). In addition to expressing transgenes in most cells, these viruses are readily cultivated and produced in large quantities which make them attractive candidates for gene therapy. The adenoviral recombinants also induced strong cellular and humoral immunity which prompted Xiang and co-workers to examine the capacity of these constructs to serve as vaccine vectors. A replication-defective human adenovirus type 5 (AdH5) was used to express rabies virus G protein and consequently delivered protection against rabies virus (Yang *et al*., 1994; Xiang *et al.*, 1996). However, a significant number of humans are seropositive to AdH5 which drastically decreases or completely abolish the efficacy of the AdH5-based vaccine (Chirmule *et al.*, 1999). To circumvent this problem, replication-deficient adenovirus vectors, based on chimpanzee serotypes, were developed (Farina *et al.*, 2001; Reyes-Sandoval *et al.*, 2004; Roy *et al.*, 2004). Such a construct, chimpanzee adenovirus C7 (AdC7), was evaluated for its ability to induce an immune response against coronavirus in mice with a pre-existing immunity against AdH5. Compared to the AdH5-based vaccine, which was highly attenuated, the presence of anti-AdH5 had little effect on the efficacy of the AdC7-based vaccine. However, in this study, the pre-existing anti-AdH5 immunity was generated using replication-deficient AdH5, which differs from wild-type AdH5 in that it is unable to replicate. The immune responses generated from replication-deficient AdH5 would, therefore, not reflect that of a natural infection which might interfere with the efficacy of a AdC7-based vaccine (Zhi *et al.*, 2006). A recombinant adenovirus expressing RV VP7 and NSP4 was evaluated in for its potential to induce cellular and humoral immune responses in mice and shown to deliver protection upon challenge with RV (Xie *et al.*, 2015). However, adenoviruses are highly prevalent in cattle, and therefore not suitable for use as a candidate vaccine delivery vector in bovine, since a pre-existing immunity will greatly influence the efficacy of the vaccine (Motes *et al.*, 2004).

1.11 Newcastle Disease Virus

1.11.1 Introduction

Newcastle disease virus (NDV) is a non-segmented negative-sense RNA virus which mainly infects avian species and has the potential to cause devastating economic losses in the poultry industry. NDV belongs to the family *Paramyxoviridae* and is further classified under the sub-family *Paramyxovirinae* within the genus *Avulavirus*.The approximate 15 kb genome contains six genes that encode for a nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin neuraminidase protein (HN) and large polymerase protein (L) (Fig. 1.6) (Dortmans *et al.*, 2011).

The NDV virion is surrounded by a lipid envelope which associates with the HN, F and M proteins. The HN and F glycoproteins resemble the viral spikes and are involved in viral attachment and penetration whereas the non-glycosylated M proteins are attached to the inner envelope surface. The N, P and L proteins associate with the genomic and anti-genomic RNA to form the RNP complex which serves as the template for viral replication and transcription (Errington and Emmerson, 1997). The viral genes are arranged in the order 3'-N-P-M-F-HN-L-5' and separated by untranslated regions known as intergenic sequences (IGS) (Millar and Emmerson, 1998). The RNA polymerase is believed to transcribe the six viral genes sequentially from a single transcription start site resulting in an abundance of mRNA from genes that are located closer to the 3' end (Lamb and Kolakofsky, 1996).

The pathogenicity of NDV is attributed to the efficiency of cellular proteases to cleave the F protein in order for the virus to become infectious. Cleavage sites that contain the (R-X-K/R-R) consensus sequence have been found to belong to more virulent strains than those lacking the polybasic motif. The (R-X-K/R-R) serves as the recognition site for the intracellular protease, furin, whereas cleavage sites with fewer arginine/lysine residues can only be cleaved by secretory proteases thereby restricting viral spread (Choppin and Sheid, 1979; Gotoh *et al.*, 1992). The HN protein is responsible for tissue tropism and is therefore considered as an additional contributor to the virulence of NDV (Huang *et al.*, 2004). Strains of NDV can be classified as either lentogenic (non-virulent), mesogenic (intermediate) or velogenic (highly virulent) depending on their pathogenicity in avian species (Beard and Hanson, 1984). The lentogenic NDV strains, La Sota (Goldhaft, 1980) and Hitchner B1 (Hitchner and Johnson, 1948) are used in the poultry industry for vaccination against Newcastle disease.

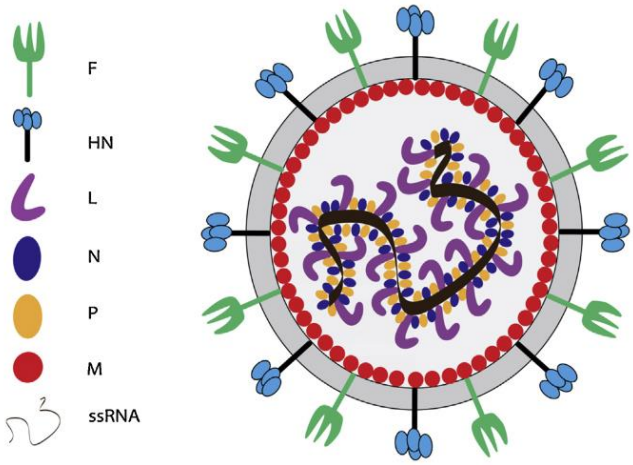


Fig. 1.6. **Schematic representation of Newcastle disease virus structure.** The Newcastle disease virus single-stranded RNA genome encodes for a fusion protein (F), haemagglutinin neuraminidase protein (HN), large polymerase protein (L), nucleocapsid protein (N), phosphoprotein (P) and matrix protein (M) (Taken from Ganar *et al.*, 2014).

1.11.2 NDV as a vaccine vector

The requirements for a viral vaccine vector include the ability to infect and replicate in a desired host species without causing disease. Ideally the host species should be seronegative for the virus which should be antigenically distinct from other pathogens that normally cause infection. A pre-existing immunity might interfere with the efficacy of the vaccine as in the case of vaccinia virus and Adh5 (Cooney *et al.*, 1991; Chirmule *et al.*, 1999). Because of its natural host-range restrictions and inability to counteract the innate immunity, NDV is considered an attractive vaccine candidate for use in mammalian species (Park *et al.*, 2003; Huang *et al.*, 2004). In addition, NDV is antigenically distinct from common animal and human pathogens and will therefore not be affected by existing widespread immunity (Bukreyev *et al.*, 2005). Other paramyxoviruses used as vaccine vectors include Sendai virus that was recombinantly engineered to express the firefly luciferase protein and simian virus 5 (SV5) which was constructed to incorporate and express the green fluorescent protein (GFP) reporter gene (Hasan *et al.*, 1997; He *et al.*, 1997).

A comprehensive study was performed by Zhao and co-workers to evaluate the expression efficiency of a singular foreign gene located at different positions of the recombinant NDV genome. The secreted alkaline phosphatase (SEAP) reporter gene was incorporated at the NP-P, M-F, HN-L junctions or the region directly following the L gene. For all the recombinants, the SEAP gene was flanked by IGS resembling those that surround the M gene. All the recombinants showed high expression levels except for the strain where the SEAP gene succeeded the L gene (Zhao and Peeters, 2003). This is expected since RNA polymerase binds to a single point on the NDV genome and initiates transcription without disruption from the 3' end to the 5' end, thereby producing a gradient in the quantity of transcripts (Hasan et al. 1997; Conzelmann 1998; Sakai et al. 1999).

1.11.3 Animal trials involving recombinant NDV

A full-length cDNA clone of the NDV Hitchner B1 strain was engineered to express the influenza A virus HA protein. The rNDV was evaluated for its ability to elicit an immune response in BALB/c mice and was found to provide complete protection in mice injected with a lethal dose of influenza virus (Nakaya *et al.*, 2001). In a separate study, Nakaya and co-workers constructed a rNDV expressing the simian immunodeficiency virus (SIV) gag protein which was found to elicit a cellular immune response in mice (Nakaya *et al.*, 2004).

The NDV LaSota strain and Beaudette C (mesogenic) strain were used to express the HN gene of the human parainfluenza virus type 3 (HPIV3) and evaluated in African green monkeys (La Sota and Beaudette C) and rhesus monkeys (only Beaudette C). Direct examination of lung tissue from African green monkeys revealed low viral shedding which suggests that NDV

is inherently safe to use as a vaccine vector. The recombinant NDV strains were found to elicit a humoral immune response of which the level of HPIV3 specific immunity was speculated would be highly protective against a HPIV3 challenge (Bukreyev *et al.*, 2005).

Similar NDV strains were engineered to express the spike S glycoprotein of the severe acute respiratory syndrome-associated coronavirus (SARS-CoV). African green monkeys were immunized with two doses of the rNDVs and monitored for neutralizing antibody levels and vaccine efficacy when challenged with SARS-CoV. Both NDV constructs were successful in inducing protective immunity against SARS-CoV. The rNDV LaSota strain resulted in a 236-fold reduction of the viral load in examined lung tissue compared to the control animals and rNDV Beaudette C resulted in an even more significant 1,102-fold reduction (DiNapoli *et al.*, 2007). In addition, several other promising NDV-based vaccines have been used in mammalian species to stimulate an immune response including the Gn and Gc glycoproteins of the Rift Valley fever virus (Kortekaas *et al.*, 2010a) and the Env and Gag proteins of the human immunodeficiency virus type 1 (Khattar *et al.*, 2015).

1.12 Rationale

Rotavirus is a common cause of NCD which has a devastating impact on beef and dairy industries world-wide (Lorenz *et al*., 2011). Vaccination of pregnant dams with inactivated BRVs is shown to elevate levels of anti-RV antibodies in colostrum and milk, however, there are inconsistencies regarding the efficacy of the vaccine in field studies (Saif and Fernandez, 1996). BRVs share common group antigens with human RVs which makes interspecies transmission possible and adds to the RV strain burden in humans (Holland, 1990). Therefore, a One Health approach is needed, which involves the collaboration of multiple disciplines related to human and animal health, to effectively combat RV infection and restrict animal-to-human reassortment.

Current vaccination strategies against RV infection aims to neutralize the virus either by prohibiting viral entry into the cell or by preventing viral decapsidation. Neutralizing antibodies directed against RV outer-capsid protein, VP7, is responsible for the latter and renders the virus inactive by prohibiting Ca^{2+} chelation, a vital step of the RV replication cycle (Ludert *et al.*, 2002). RV VP7 comprises the majority of the outer-capsid of the virus and is shown to elicit memory B-cell activation in the absence of the viral spike protein, VP4, and viral RNA (Blutt *et al.*, 2004).

RV non-structural protein, NSP4, has important immunogenic characteristics worth considering since antibodies directed against NSP4 are shown to reduce the severity and duration of diarrhoeic symptoms in neonatal mice (Ball *et al.*, 1996). In addition, NSP4, was shown to have significant adjuvant properties when administered as a fusion protein with RV

VP6, indicating the potential of NSP4 as a co-administered antigen in a RV vaccination regime (Afchangi *et al.*, 2017).

The development of the NDV reverse genetics system has opened up ways in which NDV can be used as a viral vaccine vector for the expression of foreign antigens (Peeters *et al.*, 1999). NDV is an attractive candidate for a vaccine vector since its replication is restricted in non-avian hosts and it will not be recognized by a pre-existing immunity that might influence the efficacy of the vaccine (Chare *et al.*, 2003; Park *et al.*, 2003).

Our group previously constructed recombinant NDVs containing the NSP4 and VP7 open reading frames (ORFs) of bovine RV strain RVA/Cow-wt/ZAF/1604/2007/G8P[1] (Jere *et al.*, 2012). However, due to the unavailability of commercial antibodies against RV NSP4, it was not possible to detect expression of NSP4 using an immunoperoxidase monolayer assay (IPMA) and western blot analysis. Cells infected with rNDV-VP7, showed sub-optimal expression of VP7 as shown by IPMA and western blot analysis (Aschenbrenner, 2017, available upon request). Rotavirus VP7 exerts a toxic effect when recombinantly expressed in *E. coli*, plant cells and eukaryotic cells (McCrae and McCorquodale, 1987; Emslie *et al.*, 1995; Pêra *et al.*, 2015). It is speculated that this cytotoxicity might be circumvented by the removal of the signal sequence located at the N-terminal region (aa 1 – 50) of the precursor VP7 (pVP7) (Whitfeld *et al.*, 1987). The signal peptide of pVP7 contains two prominent hydrophobic regions, H1 (aa 6 -23) and H2 (aa 32-48). Both H1 and H2 are preceded by in-frame initiation codons and have the potential to direct VP7 transport across the ER membrane (Stirzaker *et al.*, 1987). Despite the rapid cleavage of the signal peptide, VP7 does not enter the secretory pathway but remains membrane bound (Stirzaker and Both, 1989). VP7 increases the Ca^{2+} levels within the ER which results in a disruption of Ca^{2+} homeostasis within the cell (Zambrano *et al.*, 2008). An increase in intercellular Ca^{2+} concentration is associated with cytotoxicity and cell death in RV infected cells (Perez *et al.*, 1998). In the current study, in order to reduce cytopathicity and elevate VP7 expression in rNDV-VP7 infected cells, recombinant NDVs were engineered to contain the VP7 ORF without the signal peptide.

1.13 Aims and Objectives

The aim of this project was to evaluate the efficacy of recombinant NDVs expressing bovine RV proteins, VP7 and NSP4, in a small animal model as a proof-of-concept for the development of a novel vaccine candidate against RV infection in bovine. The rationale for the vaccine is based on a passive immunization approach which aims to increase RV-specific antibodies in the colostrum and milk of vaccinated dams immediately postpartum.

To achieve the aforementioned, the following objectives were formulated:

I. Construction of recombinant NDV containing the open reading frame of VP7 without the signal peptide (VP7-1) (Chapter 2).

II. Evaluation of VP7-1 and NSP4 expression from recombinant NDVs (Chapter 2)

III. Adaption and propagation of bovine RVA strain 1604 to cell culture for utilization in serological studies (Chapter 3).

IV. Evaluate the ability of recombinant NDV-derived VP7 and NSP4 to elicit an immune response in mice as a proof-of-concept (Chapter 3).

CHAPTER 2 – CONSTRUCTION OF NDV CONTAINING TRUNCATED VP7 ORF (VP7-1) AND VERIFICATION OF ROTAVIRUS VP7-1 AND NSP4 EXPRESSION

2.1 Introduction

Reverse genetics can be defined as the modification of a gene in order to investigate the phenotypic alterations related to that modification (Hardy *et al.*, 2010). This molecular tool has provided valuable insights into the pathogenesis and replication of RNA viruses through targeted alterations of the viral genome. Virus reverse genetic systems make use of biological properties that typically feature in RNA viruses and include the production of multiple copies of the viral genome by RNA-dependent-RNA polymerase (RdRp) and the localization thereof in the cytoplasm (Stobart and Moore, 2014). The genomes of positive-sense RNA viruses are directly translated into proteins by cellular ribosomes shortly after infection of the host cell. Besides the addition of plasmids coding for RdRp, reverse genetic systems for positive-sense RNA viruses rarely require the addition of helper-plasmids to assist in protein synthesis. Instead, a full-length cDNA clone of the viral genome, under the control of a T7 promoter, or transcribed genomic RNA is delivered into the cytoplasm of the cell (Shi *et al.*, 2002; Yount *et al.*, 2003; Scobey *et al.*, 2013). On the other hand, reverse genetic systems of negative-sense RNA viruses incorporate helper constructs, which, in addition to RdRp, introduce other proteins essential for replication initiation (Conzelmann, 1998). A common feature of all negative-sense RNA viruses is the arrangement of their genetic information into tight, helical ribonucleoprotein (RNP) complexes. It is these RNP complexes, and not naked RNA, that are suitable for replication and transcription by RdRp. These structures never disassemble and remain unchanged following RNA synthesis (Banerjee, 1987).

Reverse genetics systems of different RNA viruses have provided the necessary platform for their implementation in vaccine development (Stobart and Moore, 2014). The first infectious RNA virus generated from a full-length cDNA clone was that of poliovirus in 1981 (Racaniello and Baltimore, 1981). Since then, cDNA clones that represent all major families of RNA viruses have been established, some of which, have been used for the development of chimeric vector-based vaccines. These include a recombinant alphavirus engineered to contain the coding region for human parainfluenza virus type 3 (HPIV3) hemagglutinin-neuraminidase protein; a chimeric dengue virus (DENV) vaccine based on Japanese encephalitis virus attenuated backbone and yellow fever 17-D vaccine strain containing the Zika virus preM/E proteins (Greer *et al.*, 2007; Li *et al.*, 2013; Touret *et al.*, 2018).

The establishment of the reverse genetics system for NDV has paved the way for using these viruses as carriers of transgene products in the development of sub-unit vaccines. Infectious NDV, generated from a full-length cDNA clone of the NDV La Sota strain, namely pNDFL, was reported in 1999 by Peeters and co-workers (Peeters *et al.*, 1999). This system comprised the pOLTV5 transcription plasmid which contained NDV cDNA fragments cloned between the T7 RNA polymerase promoter region and the hepatitis delta virus ribozyme (Fig. 2.1). Transcription was driven by a T7 RNA polymerase, expressed by a recombinant fowlpox virus, and plasmids encoding the NP, P and L proteins which was co-transfected with the cDNA clone. The NDV reverse genetics system developed by Peeters and co-workers has been implemented in recombinant DNA technology to serve as a vaccine carrier for the antigen-encoding sequences of HPIV3, severe acute respiratory syndrome-associated coronavirus (SARS-CoV); Rift Valley fever virus and human immunodeficiency virus (HIV) (Bukreyev, *et al.*, 2005; DiNapoli *et al.*, 2007; Kortekaas *et al.*, 2010a; Kortekaas *et al*., 2010b; Khattar *et al.*, 2015). Because of the outcomes of these studies, in that a NDV-based vaccine was able to induce effective immune responses *in vivo*, it was decided to utilize this system for the expression of rotavirus (RV) immunogenic proteins.

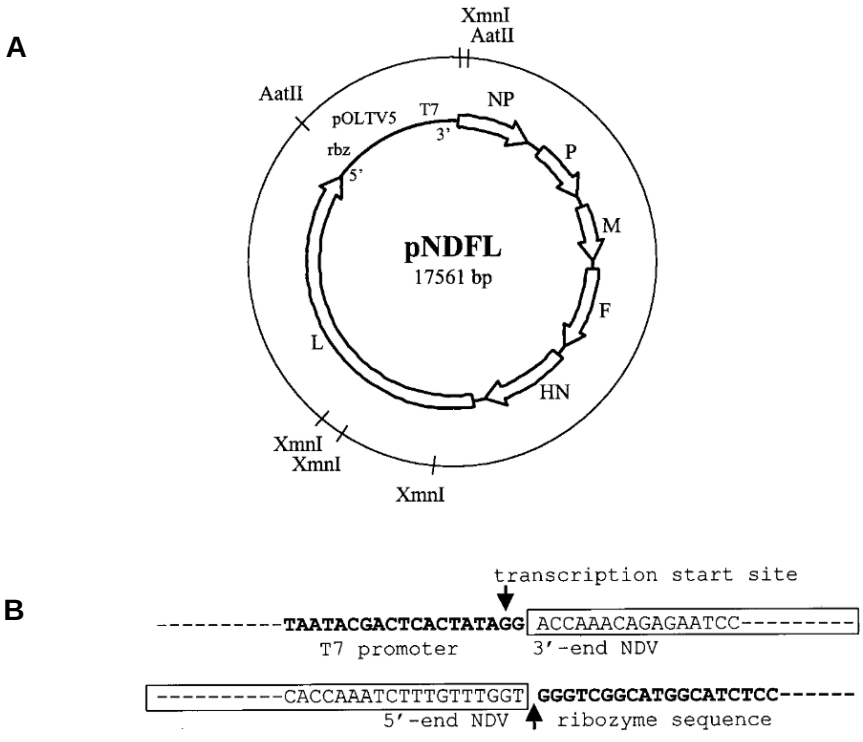


Fig. 2.1. **Schematic overview of the pNDFL plasmid.** (A) Circular map of pNDFL comprising the full-length NDV La Sota cDNA clone, containing the coding regions for NP, P, M, F, HN and L, inserted into the pOLTV5 plasmid between the T7 RNA polymerase promoter region (T7) and the hepatitis delta virus ribozyme (rbz). (B) Enlargement of the terminal ends of the NDV cDNA clone (boxed) shows the nucleotide sequences of the T7 RNA polymerase promoter and hepatitis delta virus ribozyme at the 3'- and 5'-ends, respectively (Taken from Peeters et al. 1999).

As with most members of the *Paramyxovirinae*, NDV replication is dependent on a genome length equalling a multiple of six for efficient viral replication to occur (Phillips *et al.*, 1998). Each NP subunit is attached to precisely six bases of genomic RNA which is exclusively recognized by the viral RdRp (Calain and Roux, 1993). A change in position of the NP subunit, other than a multiple of six bases, results in a shift of the promoter initiation region and inefficient viral replication (Egelman *et al.*, 1989; Kolakofsky *et al.*, 1998). Recombinant DNA technology involving the cDNA clone of NDV La Sota (pNDFL) should adhere to the rule-of-six for successful transcription to occur (Phillips *et al*., 1998; Peeters *et al.*, 1999).

The insertion of a foreign gene into the NDV genome often includes the addition of NDV-specific intergenic sequences (IGS) to the terminal ends of the insert (Millar and Emmerson, 1998). This results in the formation of the expression cassette which is further engineered so that it is flanked by *SapI* restriction sites to facilitate transfer into the pGEM-PM shuttle vector. With the exception of the expression cassette and *SapI* sites, the pGEM-PM plasmid contains the sequence that is located between the *ApaI* and *NotI* sites on the pNDFL plasmid (J. Kortekaas *et al.*, 2010). This region is replaced with the one from the pGEM-PM vector and enables the expression cassette to be inserted, via the pNDFL plasmid, into the P-M gene junction of the viral genome (Fig. 2.2). There the foreign gene is transcribed into additional, separate mRNAs. It is recommended that no more than a single gene be inserted into the NDV genome, since an increase in gene number and genome size runs parallel to viral attenuation (Bukreyev *et al.*, 2006).

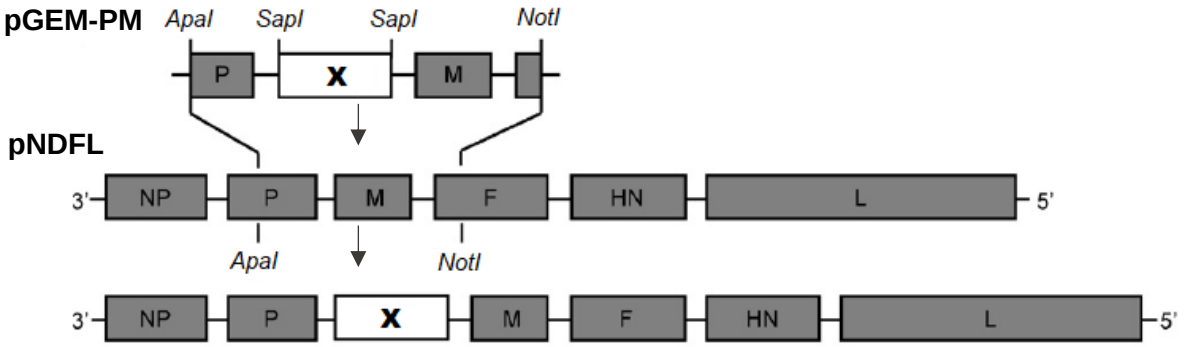


Fig. 2.2. **Construction of pNDFL containing a foreign gene of interest.** The foreign gene (X) is introduced between the SapI sites of the pGEM-PM shuttle vector. The resulting *ApaI/NotI* fragment is exchanged with the one from the pNDFL vector (Taken from Kortekaas *et al.*, 2010a).

The genome sequences of three South African bovine RVA strains from the diarrhoeic faeces of three separate calves on a farm in the Western Cape Province were previously determined. Whole-genome sequencing using the 454® pyrosequencing platform showed RVA/Cow-wt/ZAF/1603/2007/G6P[5] and RVA/Cow-wt/ZAF/1605/2007/G6P[5] to share a G6-P[5]-I2-R2-C2-M2-A3-N2-T6-E2-H3 genotype constellation, whereas RVA/Cow-

wt/ZAF/1604/2007/G8P[1] was genotyped as G8-P[1]-I2-R2-C2-M2-A3-N2-T6-E2-H3 (Jere *et al.*, 2012). The latter was successfully propagated in MA104 cells and sequencing of the adapted strain showed unchanged consensus sequences for VP7 and NSP4 encoding regions (Mlera, *et al*., 2013, personal communication). Prior to the commencement of this study, it was decided to utilize the adapted bovine RVA 1604 strain for the development of recombinant NDVs.

In this chapter, expression in various cell lines of RV VP7 and NSP4 delivered by a NDV vector was evaluated. Although expression of VP7 was previously demonstrated (Aschenbrenner, 2017), toxicity was observed, and subsequent lower-than-expected expression levels were obtained. Since VP7 is targeted to the ER by a signal sequence (aa 1 – 50) located on the N-terminal region, where its retention in the ER interferes with Ca^{2+} homeostasis, recombinant NDV containing the VP7 ORF without the signal sequence was generated, in hopes of elevating VP7 expression. The expression levels of the truncated VP7 (VP7-1) was compared to that of the unaltered protein, in order to select the recombinant with the highest immunogenic potential in a live animal model. To verify expression of the previously undetected NDV-derived NSP4, peptides were designed representing an immunodominant region of the protein and injected into rabbits for the production of polyclonal antibodies.

2.2 Materials and methods

2.2.1 Mammalian cell cultures and maintenance

The mammalian cell lines used during this part of the study, included BSR-T7/5 and L929 cells that were provided by Deltamune (Pty) Ltd. The BSR-T7/5 cell line is a clone of BHK-21 (Baby Hamster Kidney) cells which stably expresses T7 RNA polymerase (Buchholz *et al.*, 1999). The L929 cell line is a sub-clone of the parent L strain which was derived from the adipose and areolar tissue of a C3H/An mouse (Sanford *et al*., 1948). Both cell lines were maintained as monolayers in 25 cm^2 tissue culture flasks (Corning, United States) while undergoing routine passage in Dulbecco's modified Eagle's Medium (DMEM) containing GlutaMAX™, Pyruvate and 4.5 g/L D-Glucose (Gibco™, United States) with added 1x Antibiotic-Antimycotic (Gibco™, United States) and 5 % (v/v) FBS (Gibco™, United States). Between culturing, the cells were incubated at 37°C in a 5 % CO_2-incubator (ESCO, Singapore). Once the cells reached a confluency of 80 %, the supernatant was removed, and the cells were washed with phosphate buffered saline (PBS) (Gibco™, United States). The cells were briefly incubated with 0.05 % trypsin-EDTA (Gibco™, United States) until cell detachment was observed. The cells were resuspended in the appropriate volume of culture media and passaged into new 25 cm^2 tissue culture flasks. To facilitate T7 polymerase expression in BSR-T7/5 cells, the culture

media was supplemented with Geneticin (Gibco™, United States) at a final concentration of 1 mg/ml.

2.2.2 Design of VP7-1 ORF

The coding sequence for the VP7 N-terminal region (aa 1 – 50) of RVA/Cow-wt/ZAF/1604/2007/G8P[1], from now on referred to as RVA 1604, was removed *in silico* and engineered to contain NDV-specific IGS followed by *SapI* restriction sites at either end of the ORF. The expression cassette was designed so that the resulting DNA copy of the NDV-VP7-1 would comply to the rule-of-six. The gene was optimized for expression in *Bos Taurus* (bovine) using the OptimumGene™ codon optimization tool and synthesized by GeneScript®, (United States) (see Appendix 1).

2.2.3 Plasmids

The pGEM-PM shuttle vector and cDNA clone of NDV strain La Sota, pNDFL, were provided by Deltamune (Pty) Ltd along with the pCIneo-NP, pCIneo-P and pCIneo-L helper plasmids (Peeters *et al.*, 1999). As mentioned, the pGEM-PM contains a cassette with a sequence similar to the sequence spanning the region between the *ApaI* and *NotI* sites on the pNDFL plasmid. The pGEM-PM cassette is engineered to contain *SapI* sites at the P-M junction to facilitate insertion of the foreign gene of interest (see Appendix 2 and 3).

2.2.4 Viruses

The recombinant viruses implemented in this study included NDV La Sota strain containing the truncated RVA 1604 VP7 ORF without the signal peptide (aa 1-50) (NDV-VP7-1) prepared in this study. Previously rescued NDV containing the RVA 1604 VP7 full-length ORF (NDV-VP7) or NSP4 ORF (NDV-NSP4) (Aschenbrenner, 2017, available upon request) and a recombinant NDV La Sota control that did not contain a foreign insert were also included.

2.2.5 Antibodies

Antibodies utilized during this study included those that were self-raised at Deltamune (Pty) Ltd animal testing facilities and those that were commercially bought. For the former, polyclonal antibodies (pAbs) specific for NDV La Sota or peptides representing RVA 1604 NSP4 antigenic region, aa 136 – 149 [VRSTGEIDMTKEIN] (anti-NSP4 $_{pep136-149}$) were raised in rabbits. The purified antigen (virus/peptide) was mixed with adjuvant ISA 71R VG (Seppic, France), according to the manufacturer's instructions, and 50-100 µg of the antigen was injected into adult rabbits followed by a booster dose on day 21. The serums of the vaccinated rabbits were collected on day 42. Commercially bought antibodies included goat pAbs raised against the Nebraska calf diarrhoea virus (NCDV) (Abcam, United Kingdom, ab20036) and

rabbit pAbs raised against peptides representing RVA 1604 VP7 antigenic region, aa 177 – 190 [QTDEANKWISMGDS] (anti-$VP7_{pep177-190}$) (GenScript, United States).

2.2.6 General cloning procedures

2.2.6.1 Preparation of competent cells

Escherichia coli JM109 (*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (r_k^-,m_k^+), *rel*A1, *sup*E44, Δ (*lac-pro*AB), [F' *tra*D36, *pro*AB, *laq*I^qZΔM15]) (Promega, United States) competent cells were prepared as described before (Hanahan, 1983; Green and Rogers, 2013). *E. coli* JM109 (Promega, USA) cells were collected from – 80 °C glycerol stocks and streaked onto Luria Broth (LB) agar (5 g/l yeast extract, 10 g/l tryptone, 10 g/l NaCl, 15 g/l agar bacteriological) and incubated overnight at 37 °C. A single colony was selected and inoculated into 5 ml LB and left to incubate at 37 °C overnight while shaking. From the pre-inoculum, 1.5 ml was transferred to a 500 ml Erlenmeyer flask containing 50 ml LB and shaken at 37 °C until an OD_{530} of ~ 0.45 was reached (Unicam, Heλios Υ, United Kingdom). The culture was transferred to a 50 ml centrifuge tube and placed on ice for 15 min. The cells were pelleted at 2 500 x g (Eppendorf Centrifuge 5418, Germany) for 5 min at 4 °C and gently resuspended in 21 ml ice-cold TFB1 (1 M Tris, 0.5 M EDTA, 100 mM RbCl, 50 mM $MnCl_2$, 30 mM potassium acetate, 10 mM $CaCl_2$, 15 % (v/v) glycerol) and incubated on ice for 90 min. The cells were once again pelleted at 2 500 x g for 5 min at 4 °C and resuspended in 3 ml ice-cold TFB2 (10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2$, 15 % (v/v) glycerol). The cell suspension was aliquoted in 1.5 ml microcentrifuge tubes, 200 µl each, and placed in liquid nitrogen, where-after the aliquots were transferred to -80 °C for storage.

2.2.6.2 Transformation of competent cells

For plasmid propagation, competent *E.coli* JM109 cells were transformed as described before (Green and Rogers, 2013). Briefly 1.5 ml microcentrifuge tubes, containing 200 µl of the competent cell line, were retrieved form -80 °C and thawed on ice. The appropriate amount of plasmid DNA was added, and the tubes were incubated on ice for 1 hour. The tubes were placed at 42 °C for 40 sec and transferred to ice for 2 min. The cells were revived with the addition of 800 µl LB followed by incubation at 37 °C for 1 hour while shaking. The transformation reaction was plated on LB agar containing the appropriate antibiotics and incubated overnight at 37 °C.

2.2.6.3 Agarose gel electrophoresis

Analysis of DNA was done by gel electrophoresis on a 1 % agarose gel prepared with 1 x Tris-Acetic-EDTA (TAE) (40 mM Tris, 20 mM glacial acetic acid, 2 mM EDTA) buffer and 5 µg/ml ethidium bromide (Sigma-Aldrich, United States). The samples were combined with DNA Gel

Loading Dye (6x) (Thermo Fisher Scientific, United States) and subjected to electrophoresis at 90 V for 45 min (Bio-Rad Laboratories Inc., United states). The GeneRuler^{TM} DNA Ladder mix (Thermo Fisher Scientific, United States) was used as a DNA marker. The gel was analysed under UV light using the ChemiDoc^{TM} MP Imaging System (Bio-Rad Laboratories Inc., United States).

2.2.7 Cloning of VP7-1 ORF into pGEM-PM

2.2.7.1 Restriction digest of pGEM-PM with *SapI*

DNA concentrations were determined by using a NanoDrop 1000 spectrophotometer (Thermo Scientific, United States). The pGEM-PM shuttle vector was linearized by combining 3 µg of plasmid DNA with 10 U *SapI* (Thermo Scientific, United States), 0.5 x FastDigest Buffer (Thermo Scientific, United States) and Ultrapure water in a final reaction volume of 20 µl. The reaction was left to incubate overnight at 37°C and sequentially analysed by gel electrophoresis on a 1 % agarose gel (refer to section 2.2.5.3). The appropriate DNA fragment was purified using the MinElute® Gel extraction kit (Qiagen, Germany) according to the manufacturer's instructions.

2.2.7.2 In-Fusion® HD cloning of VP7-1 into pGEM-PM

The VP7-1 ORF was amplified from the pUC57 vector (GenScript, United States) using PCR and gene-specific primers with 15 bp extensions homologous to the pGEM-PM vector ends [(forward 5'-CAAGGTCCAGCTCTTCAAGC-3') (reverse 5'-TGGGGCTGAGGAAGAGCTAC'-3)] (Fig. 2.3). The reaction was set up by combining 50 ng template DNA with 0.4 U Phusion High-Fidelity DNA polymerase (Thermo Scientific, United States), 1 x Phusion HF Buffer (Thermo Scientific, United States), 0.2 mM dNTPs (Thermo Scientific, United States) and 0.5 µM of the forward and reverse primers in a final volume of 20 µl. PCR was performed at an initial denaturation step of 98 °C for 30 sec followed by two cycles of 98 °C for 10 min, 35 °C for 30 sec and 72°C for 2 min. This was then followed by 15 cycles of 98 °C for 10 sec, 55 °C for 20 sec, 72 °C for 2 min and a final elongation step of 72 °C for 5 min. The PCR product was analysed by gel electrophoresis on a 1 % agarose gel (refer to section 2.2.5.3). This was followed by excision of the appropriate DNA fragment using the MinElute® Gel extraction kit according to the manufacturer's instructions.

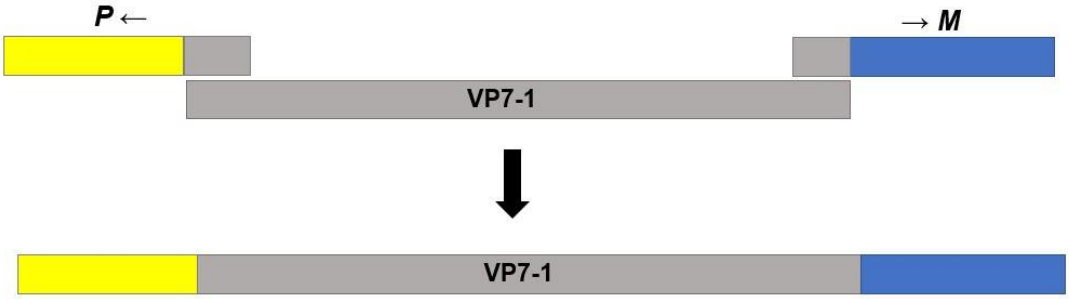


Fig. 2.3. **Schematic diagram depicting amplification of the VP7-1 ORF.** Gene-specific primers were designed with 15 bp overlaps complementary to the ends of the linearized pGEM-PM shuttle vector.

The purified PCR fragment was cloned into the linearized pGEM-PM using the In-Fusion® HD cloning kit (Takara Bio Inc, Japan). The kit is designed to fuse DNA fragments using the In-Fusion enzyme which recognizes 15 bp overlaps at the ends of PCR-generated inserts and linearized fragments (Lestini *et al.*, 2013; Chen *et al.*, 2014). The reaction was set up by combining 50 ng of the purified PCR product with 100 ng linearized vector, 2 µl 5x In-Fusion® HD enzyme premix and filled to 10 µl with deionized water. This was followed by incubation at 50 °C for 15 min. Thawed competent *E. coli* JM109 cells, to which 5 µl of the In-Fusion® reaction was added, were incubated on ice for 15 min. The cells were plated on pre-warmed LB plated containing 100 µg/ml carbenicillin (Novagen, Germany) and incubated overnight at 37 °C. Transformed colonies were picked and inoculated into 4 ml LB containing 100 µg/ml carbenicillin and left to incubate overnight at 37 °C. Plasmid extraction was done using the QIAprep® Spin Miniprep kit (Qiagen, Germany) according to the manufacturer's instructions.

To verify that the VP7-1 ORF was successfully inserted into the pGEM-PM shuttle vector, restriction digest was performed using the *SapI* restriction enzyme. The reactions were set up by combining 900 ng plasmid DNA with 10 U *SapI*, 1 x FastDigest Buffer and filled up to 20 µl with deionized water, which was incubated at 37 °C for 30 min.

2.2.8 Cloning of VP7-1 ORF into pNDFL

2.2.8.1 Restriction digest with *ApaI* and *NotI*

The pNDFL plasmid and pGEM-PM containing the VP7-1 ORF was digested using *ApaI* and *NotI* restriction enzymes. The reactions were set up combining either 2.5 µg of pGEM-PM-VP7-1 or 900 ng of pNDFL with 50 U *ApaI* (New England Biolabs, United States), 10 U *NotI* (New England Biolabs, United States), 2 µl CutSmart® Buffer (New England Biolabs, United States) in a final volume of 20 µl. The reactions were incubated overnight at 37°C and analysed by gel electrophoresis on a 1 % agarose gel (refer to section 2.2.4.4). The

appropriate fragments were excised and purified using the MinElute® Gel extraction kit according to the manufacturer's instructions.

2.2.8.2 Ligation of PM-VP7-1 with pNDFL

The digested pNDFL vector was ligated to the PM insert containing the VP7-1 ORF. The reaction was set up by combining 3 µl of the vector DNA with 6 µl of the insert, 400 U Quick Ligase (New England Biolabs, United States) and 0.2x Quick Ligase Reaction Buffer (New England Biolabs, United States) which was filled to 20 µl with deionized water and incubated for 30 min at 16 °C. *E. coli* JM109 cells were transformed with the total reaction volume (refer to section 2.2.4.2) during which 30 µg/ml kanamycin was implemented for colony screening. Single colonies were inoculated into 50 ml LB containing 30 µg/ml kanamycin and incubated overnight at 16 °C. Plasmid extraction was done using the EndoFree® Plasmid Maxi kit (Qiagen, Germany) according to the manufacturer's instructions. This was followed by plasmid screening for recombinant pNDFL-VP7-1 using *SapI*. The reaction setup was done by combining 900 ng plasmid DNA with 10 U *SapI*, 1 x FastDigest Buffer and filled up to 20 µl with deionized water, which was incubated at 37 °C for 30 min.

2.2.9 Rescue of recombinant NDV

2.2.9.1 Transfection

The concentrations of the NDV transfection plasmids were determined by using a NanoDrop 1000 spectrophotometer. For the transfection mixture, 2.77 µg pNDFL-VP7-1 was combined with 4.44 µg pClneo-NP, 2.22 µg pClneo-P, 2.22 µg pClneo-L and 46.6 µl TransIT-LT1 (Mirus Bio LLC, United States) in 400µl 1x OPTI-MEM Reduced Serum Medium (Gibco™, United States). The reactions were incubated at room temperature for 30 min and sequentially added drop-wise to a confluent monolayer (10 cm^2) of BSR-T7/5 cells. The cells were incubated at 37 °C in a 5 % CO_2-incubator for 48 hours.

2.2.9.2 Infection of embryonic chicken eggs

Specific-pathogen-free (SPF) embryonic chicken eggs (AVI Farms, South Africa) were inoculated according to method described by (Cunningham, 1956). The transfected BSR-T7/5 cells were analysed for cytopathic effect and 100 µl of the overlaying media was injected into the allantoic fluid of six 10-day-old embryonic chicken eggs. For the negative control, 100 µl 1 x PBS was injected into the allantoic fluid of two embryonic chicken eggs. The eggs were incubated at 37 °C for 96 hours and monitored daily for pre-mature embryonic death, not related to NDV infection.

2.2.9.3 Hemagglutination assay

The eggs were placed at 4 °C for 4 hours and analysed to ensure embryonic death. A small volume of the allantoic fluid was removed and combined with 5 % chicken red blood cells in a 1:1 ratio. The samples were carefully mixed on a white surface until blood coagulation was confirmed. The allantoic fluid of the eggs that tested positive for hemagglutination were harvested, aliquoted and kept at -70 °C.

2.2.9.4 Virus passage in embryonic eggs

The harvested allantoic fluid was thawed and used to prepare a ten-fold dilution series from 10^{-6} to 10^{-9} as described by (Cunningham, 1956). The dilutions were used to inoculate 10-day-old SPF embryonic chicken eggs. Six eggs were inoculated per dilution and incubated at 37 °C for 96 hours. The eggs were placed at 4 °C for 4 hours where-after hemagglutination assays were performed (refer to section 2.2.8.3). The 50 % embryo infection dose (EID_{50}) was calculated using the Reed and Muench method (Reed and Muench, 1938).

2.2.10 Sequencing of NDV-VP7-1 genomic RNA

To confirm that the VP7-1 ORF was successfully inserted into the pNDFL plasmid and that no nucleotide changes had occurred during the cloning procedure, the genome of the recombinant NDV-VP7-1 virus was extracted and sequenced using the Illumina MiSeq platform (UFS, Bloemfontein).

2.2.10.1 Virus purification

Allantoic fluid that tested positive for hemagglutination was harvested and clarified at 1000 x g for 10 min. The supernatant was added to a 25 % (w/v) sucrose cushion and centrifuged for 45 min at 25 000 x g (L8-80 Ultracentrifuge BECKMAN, United States). The supernatant was discarded, and the pellet was resuspended in 1 ml 1 x PBS.

2.2.10.2 RNA extraction

RNA extraction of the purified virus sample was performed using the MagNA Pure Compact Nucleic Acid Isolation kit (Roche, Switzerland) and by following the manufacturer's instructions. The purification process is automated and relies on the tendency of nucleic acids to adsorb to glass (silica) in the presence of chaotropic salts (Vogelstein and Gillespie, 1979). The viral proteins were disrupted by Proteinase K in the presence of guanidine HCl, enabling the RNA segments to bind to the magnetic glass beads. Residual DNA was removed by the addition of DNase where-after the magnetic beads, containing the RNA segments, were extracted and washed. The RNA was eluted at 55 °C followed by extraction of the magnetic beads from the solution.

2.2.10.3 Sequencing and analysis

The extracted RNA was submitted for sequencing at the National Institute for Communicable Diseases (NICD) Sequencing Core Facility (NSCF) located in Sandringham, South Africa using the Illumina MiSeq Next Generation Sequencer (Illumina, Inc., United States). Sequencing data, in the form of paired-end reads, was imported into the CLC bio® Genomics Workbench (Version 12, Qiagen, Germany) in FASTQ format. The reads were trimmed and mapped to the NDV-VP7-1 theoretical sequence.

2.2.11 Verification of protein expression

2.2.11.1 Immunofluorescence monolayer assay (IFMA)

BSR-T7/5 and L929 cells were passaged and seeded into 96-well flat-bottomed plates (Nunc™, United States) with ten-fold dilutions of the harvested virus (rNDV La Sota, rNDV-VP7, rNDV-VP7-1 or rNDV-NSP4) in DMEM growth medium containing 1 x Antibiotic-Antimycotic and 5 % (v/v) FBS, totalling 200 µl/well. The plates were incubated at 37 °C for 24 hours in a 5 % CO_2-incubator. The cells were fixed with 4 % (w/v) formaldehyde (Merck, Germany) for 20 min followed by permeabilization with 0.5 % (v/v) Tween-20™ (Sigma-Aldrich, United States) in 1 x PBS for 30 min at room temperature. This was followed by blocking with 1 x PBS, containing 1 % (w/v) tryptone (Biolabs, South Africa) and 0.05 % (v/v) Tween-20™, for 1 hour at 37 °C. The blocking buffer was removed and 100 µl of the primary antibody, anti-NDV (1:5000 in blocking buffer); anti-$VP7_{pep177-190}$ (1:500 in blocking buffer) or anti-$NSP4_{pep136-149}$ (1:2000 in blocking buffer), was added to the cells and left to incubate for 1 hour at 37 °C. The cells were washed three times with 1 x PBS containing 0.05 % (v/v) Tween-20 where-after a 1:3000 dilution of the goat anti-rabbit fluorophore conjugate (Alexa Fluor Pluss 488, Invitrogen, United States) was added and left to incubate for 20 min at 37 °C. Visualization was done using a fluorescence microscope (AXIO Vert.A1 Inverted Microscope, Zeiss, Germany).

2.2.11.2 Western blot analysis

Cell lysates were prepared by incubating a confluent monolayer (75 cm^2) of BSR-T7/5 cells with a 1:10 dilution of the harvested virus (rNDV La Sota, rNDV-VP7, rNDV-VP7-1 or rNDV-NSP4) in supplemented DMEM growth medium for 1 hour at 37°C while rocking. The inoculum was replaced with 20 ml supplemented DMEM growth medium and incubated for 48 hours at 37 °C in a 5 % CO_2-incubator. Following washing with 1 x PBS, the cells were detached using a cell scraper and resuspended in 50 µl 1 x PBS containing 1 % (w/v) complete protease inhibitor cocktail (pH 7.4) (Roche, Switzerland) and 10 µM proteasome inhibitor, MG132 (Sigma-Aldrich, United States). Sodium dodecyl sulphate polyacrylamide electrophoresis

(SDS-PAGE) gels (10 %) were prepared by combining 4 ml distilled water with 10 % (w/v) acrylamide mix (acrylamide/bis-acrylamide) (Sigma-Aldrich, United States), 375 mM Tris (pH 8.8) (Sigma-Aldrich, United States), 0.1 % (w/v) SDS (OmniPur®, United States), 0.1 % (w/v) ammonium persulfate (APS) (Sigma-Aldrich, United States), and 0.04 % (v/v) TEMED (Sigma-Aldrich, United States) for the running gel. The solution was poured between the glass plates and allowed to polymerize for 40 min at room temperature. The stacking gel was prepared by combining 1.72 ml distilled water with 5 % (w/v) acrylamide mix, 130 mM Tris (pH 6.8), 30 µl 0.1 % (w/v) SDS, 0.1 % (w/v) APS and 0.1 % (v/v) TEMED. The solution was poured onto the running gel and allowed to polymerize for 30 min at room temperature. The cell lysates were thawed on ice and combined with the sample buffer [5 % (v/v) Beta-mercaptoethanol (Merck, Germany) and 95 % (v/v) Laemmli Sample Buffer (Bio-Rad Laboratories Inc., United States)] in a 1:1 ratio. The samples were boiled for 10 min at 98 °C and subjected to electrophoresis at 110 V for 40 min, with the Spectra™ Multicolor Broad Range Protein Ladder (Thermo Fisher Scientific, United States) as the protein marker (Laemmli, 1970). The proteins were wet blotted (0.025 M Tris, 0.2 M glycine and 20 % (v/v) methanol (pH 8.4)) onto a BioTrace™ NT pure nitrocellulose blotting membrane (Pall Corporation, United States) at 100 V for 1 hour. The membrane was placed in blocking buffer [5 % (w/v) skimmed milk (Nestle, South Africa), 0.2 % (v/v) Tween-20™ in 1 x Tris-buffered saline (TBS)] and left to incubate overnight at 4 °C. The blocking buffer was removed, and the membrane was washed with 1 x TBS containing 0.2 % (v/v) Tween-20™. The primary antibody, which was either anti-NDV (1:5000), anti-RVA (1:5000); anti-VP7$_{pep177\ -\ 190}$ (1:500) or anti-NSP4 $_{pep136\ -\ 149}$ (1:2000), was added to the membrane and left to incubate for 1 hour at room temperature. The membrane was washed and a 1:5000 dilution of the goat anti-rabbit HRP conjugate (Invitrogen, United States) was added and left to incubate for 1 hour at room temperature. The membrane was washed and a solution of 1 x TBS containing 0.5 mg/ml 4-chloro-1-naphthol (Sigma-Aldrich, United States) and 0.5 % (v/v) hydrogen peroxidase (Sigma-Aldrich, United States) was poured over the membrane. This was incubated in the dark for 20 min and protein bands were visualized with the ChemiDoc™ MP Imaging System.

2.3 Results and discussion

2.3.1 Overview of recombinant pNDFL-VP7-1 construction

The coding sequence for RVA 1604 VP7 was retrieved from GenBank (JN831225) and modified *in silico* (Jere *et al.*, 2012) (Fig. 2.4). The region coding for the signal sequence was removed and replaced with an initiation codon. The ORF was further engineered to contain IGS and *SapI* restriction sites at the terminal ends to facilitate cloning into the pGEM-PM shuttle vector. The VP7-1 ORF was amplified using primers with 15-bp extensions identical to the terminal ends of the linearized vector. The resulting amplicon was cloned into pGEM-PM by In-Fusion® HD Cloning, where-after the *ApaI/NotI* fragment was exchanged with that of pNDFL, introducing the VP7-1 ORF into the cDNA clone of the NDV genome (pNDFL-VP7-1) (Fig. 2.5).

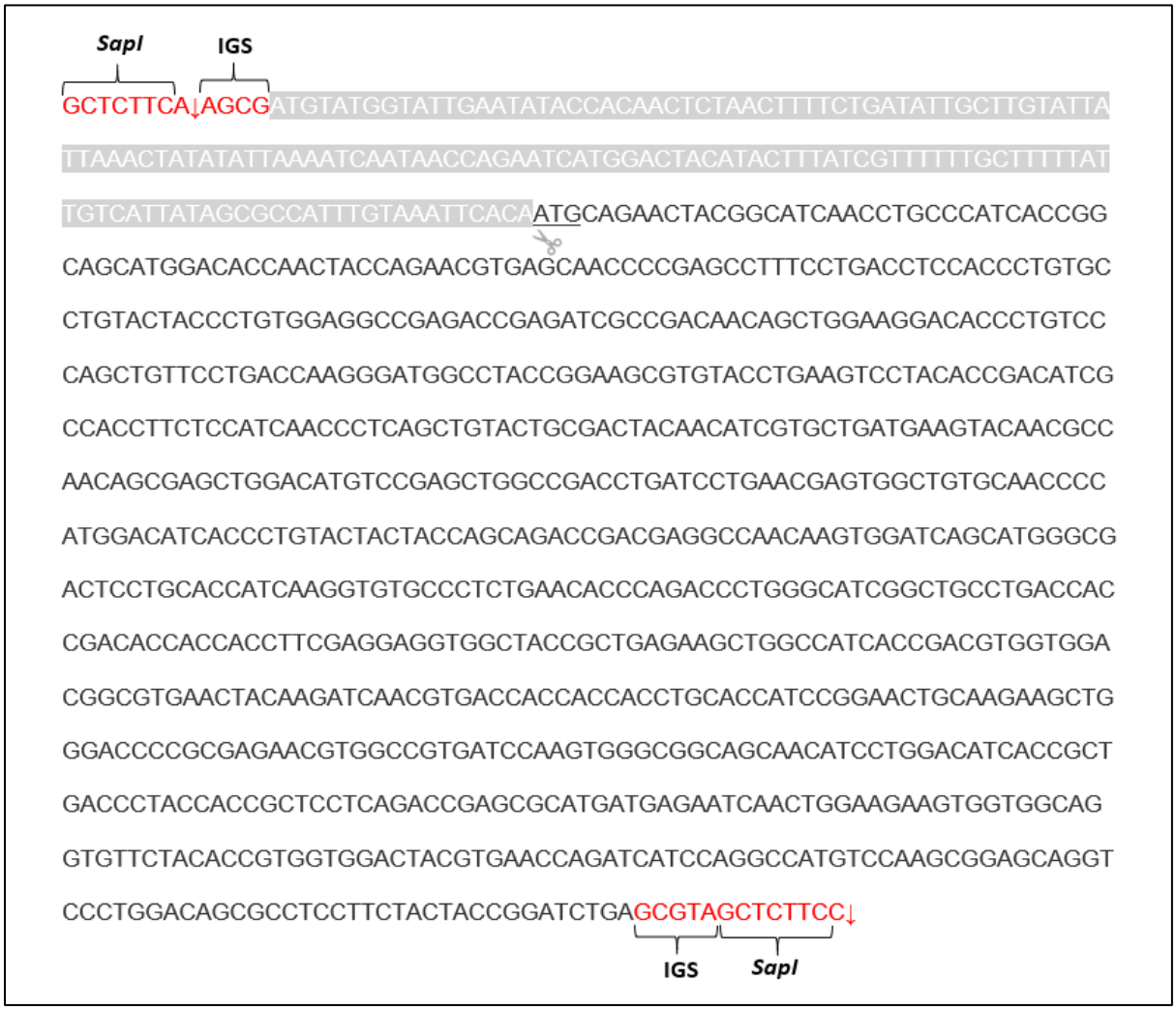


Fig. 2.4. **Virtual design of VP7-1 ORF.** The sequence encoding for the signal peptide was removed (shading) and replaced with a single start site (underlined). The ORF was further engineered to contain IGS followed by *SapI* restriction sites at either end of the coding region (red).

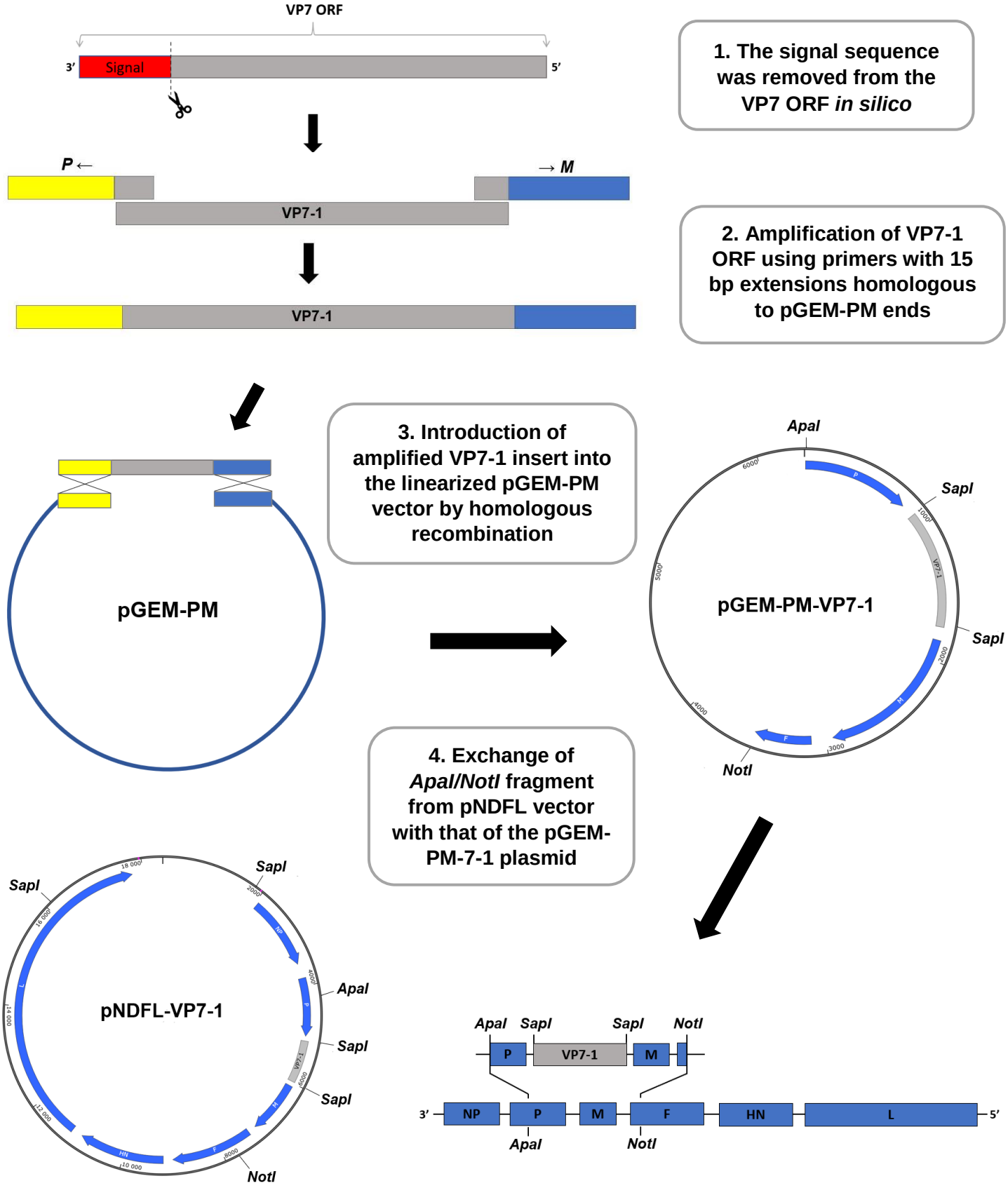


Fig. 2.5. **Schematic outline of the cloning strategy used for the insertion of the VP7-1 ORF into the pNDFL vector.**

2.3.2 Plasmid screening using *SapI*

Insertion of the VP7-1 ORF into the pGEM-PM shuttle vector, followed by sub-cloning into the pNDFL plasmid, was verified by restriction digest using the *SapI* restriction enzyme. Gel electrophoresis of the restriction digests revealed both pGEM-PM-VP7-1 (Fig. 2.6) and pNDFL-VP7-1 (Fig. 2.7), to contain the predicted fragment size of the VP7-1 ORF (860 bp), indicating the successful insertion of the VP7-1 encoding sequence into the pNDFL plasmid via the pGEM-PM shuttle vector. Subcloning of the PM-VP7-1 insert into the pNDFL vector introduced two additional *SapI* sites to the two *SapI* sites contained in the vector, giving rise to the four digested fragments observed on the gel.

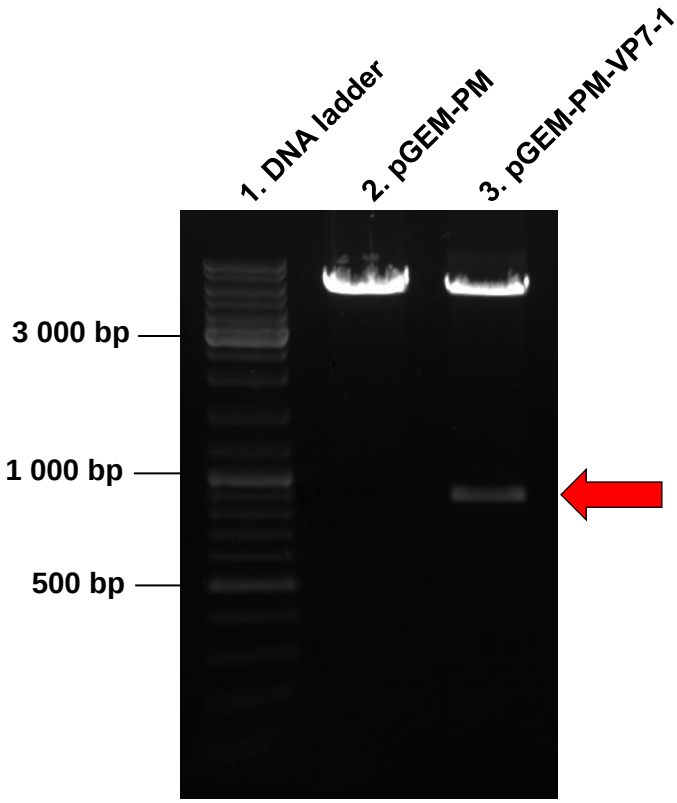


Fig. 2.6. **Agarose gel electrophoresis of *SapI* restriction digests to verify insertion of VP7-1 ORF into pGEM-PM.** 1) GeneRuler™ DNA Ladder mix; 2) negative control (pGEM-PM); 3) pGEM-PM-VP7-1. Restriction digest showed pGEM-PM-VP7-1 to contain a 860 bp fragment (arrow), corresponding to the predicted fragment size of VP7-1

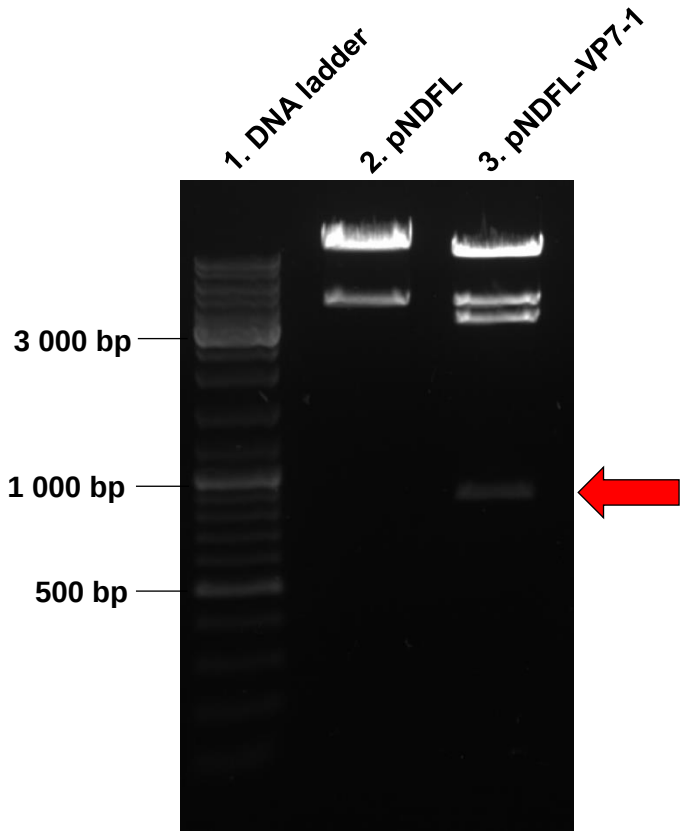


Fig. 2.7. **Agarose gel electrophoresis of *SapI* restriction digests to verify insertion of VP7-1 ORF into pNDFL.** 1) GeneRuler™ DNA Ladder mix; 2) negative control (pNDFL); 3) pNDFL-VP7-1. Restriction digest showed pNDFL-VP7-1 to contain a 860 bp fragment (arrow), corresponding to the predicted fragment size of VP7-1.

2.3.3 Rescue of recombinant NDV

BSR-T7/5 cells transfected with the recombinant pNDFL-VP7-1 and helper plasmids, showed 50 % cytopathic effect (CPE) following a 16-hour incubation period. The remaining cells were resuspended in the overlaying media and inoculated into six 10-day-old SPF embryonic chicken eggs. For the negative control, two 10-day-old SPF embryotic eggs were inoculated with a standard solution of PBS. Following a 96-hour incubation period, successful rescue of the recombinant NDV was confirmed by hemagglutination assays (Fig. 2.8). The six eggs that were infected with the transfection supernatant tested positive for blood coagulation, indicating the presence of NDV. The reliability of the test was confirmed by the little to no blood coagulation observed in the negative controls.

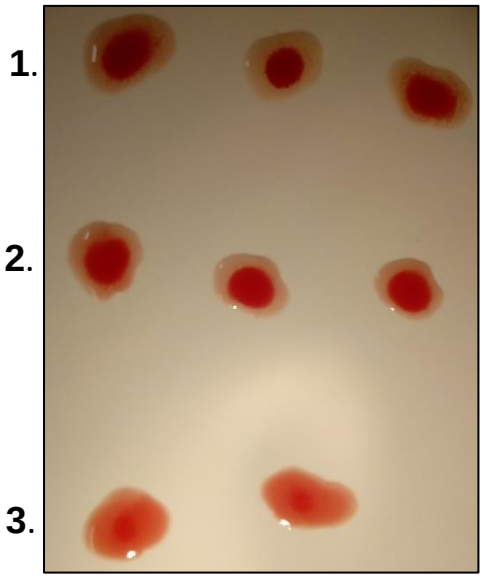


Fig. 2.8. **Hemagglutination assay to verify the rescue of the recombinant NDV**. The six eggs that were infected with the transfection supernatant (row 1,2) tested positive for blood coagulation, verifying the presence of virus. Eggs that received an inoculation with PBS showed little to no blood coagulation, indicating the reliability of the results (row 3).

2.3.4 MiSeq® sequencing of NDV-VP7-1 genomic RNA

To verify the correct insertion of the truncated VP7 ORF at the P-M junction of the NDV genome and ensure viral transcription, the NDV-VP7-1 genomic RNA was extracted and subjected to next generation sequencing (NGS). Sequencing data, in the form of paired-end reads, was successfully generated using the MiSeq® (Illumina, Inc) NGS platform. A total of 1 614 154 reads were generated of which the paired-end reads were trimmed and mapped to the NDV reference sequence (Fig. 2.9) or the NDV-VP7-1 predicted sequence (Fig. 2.10). Reference mapping to the NDV reference sequence showed successful insertion of the VP7-1 ORF at position 3 172 bp (arrow) of the NDV genome. Of the total reads generated, 94 % mapped to the NDV-VP7-1 predicted sequence. Reference mapping revealed the rescued NDV-VP7-1 sequence data to be identical to that of the predicted sequence with no single base changes observed at the terminal ends or within the VP7-1 gene (results not shown). Coverage patterns (Fig. 2.11) showed complete coverage throughout the entire NDV-VP7-1 genome with a minimum threshold of 1000 reads.

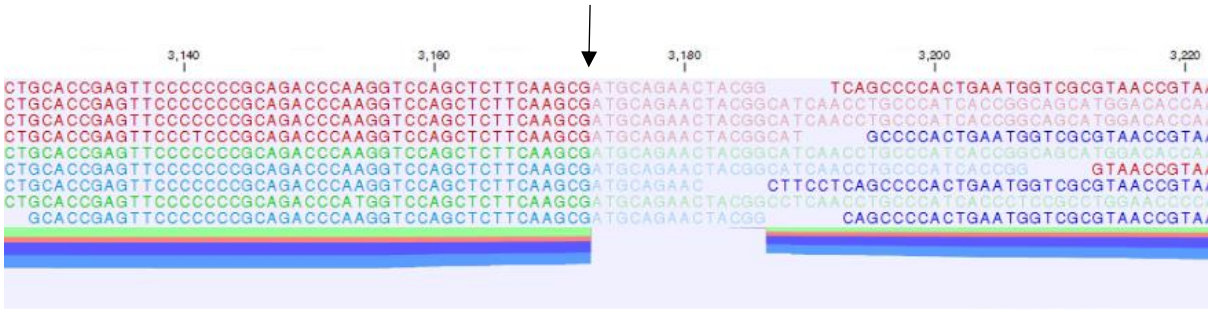


Fig. 2.9. **Representation of the paired-end sequencing reads of NDV-VP7-1 mapped to the reference genome of NDV**. Reference mapping indicates the successful insertion of the VP7-1 ORF at position 3,172 of the NDV genome (arrow).

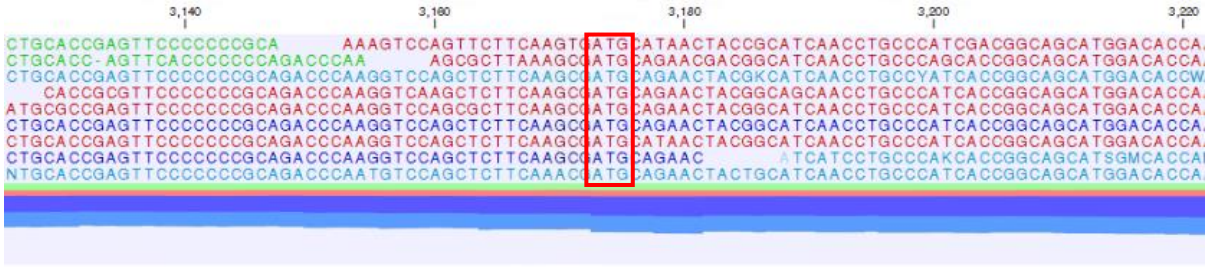


Fig. 2.10. **Representation of the paired-end sequencing reads of NDV-VP7-1 mapped to the predicted sequence of NDV-VP7-1**. The VP7-1 start codon is depicted (red box) with no single base changes observed preceding the VP7-1 gene.

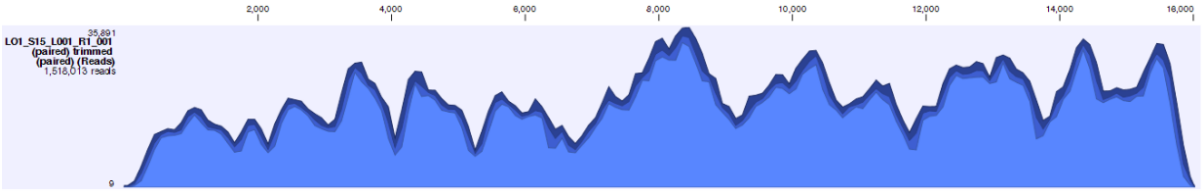


Fig. 2.11. **Coverage graph of paired-end sequencing reads mapped to the NDV-VP7-1 reference sequence**. A total of 1 518 013 reads mapped to the reference sequence and showed sufficient coverage throughout the entire NDV-VP7-1 genome.

2.3.5 Verification of protein expression

Expression of NDV-derived VP7-1 and NSP4 (previously undetected) was verified by IFMA and western blot analysis.

2.3.5.1 Immunofluorescence monolayer assay (IFMA)

Expression of VP7-1 could not be detected in either cell line using the primary goat polyclonal antibody raised against the Nebraska calf diarrhoea virus (NCDV) (results not shown). It was speculated that the removal of the VP7 signal sequence resulted in the misfolding of the protein and therefore a loss of antigenic determinants recognized by anti-RV pAbs. Proteins that are synthesized in the cytosol are often transported to organelles such as lysosomes, the Golgi apparatus or ER. Information regarding the localisation of proteins to these organelles is dependent on the presence of a signal sequence on the nascent polypeptide. The signal sequence is decoded by receptors that transports the partially synthesized protein to the correct intracellular compartment where it is fed through a proteinaceous pore or channel. (Walter and Lingappa, 1986). Translocation of polypeptides across the ER membrane occurs co-translationally and can either be fated for secretion via the Golgi apparatus or remain resident in the ER (Whitfeld *et al.*, 1987; Stirzaker and Both, 1989).

Rotavirus VP7 is an example of a membrane glycoprotein which is targeted to the ER but restricted from entering the secretory pathway. As soon as the hydrophobic signal sequence emerges from the ribosome, it is recognized and bound by the signal recognition particle (SRP) which transports the partially synthesized protein to the ER. The N-terminal region (aa 1 – 50) of precursor VP7, which comprises the signal sequence, is cleaved as it is fed through the aqueous channel that span the translocon (Ericson *et al.*, 1983; Whitfeld *et al.*, 1987). Once the VP7 polypeptide enters the ER lumen it is co-translationally glycosylated to contain a N-linked ‘polymannose’ moiety at Asn-69 (Hanover and Lennarz, 1981; Arias *et al.*, 1982; Whitfeld *et al.*, 1987). This glycosylation step, in addition to an oxidative environment, is a crucial step for correct disulphide bond formation and protein folding. (Mirazimi and Svensson, 1998). Since VP7-1 does not contain a signal sequence identifiable by cellular receptors, its

transport to the ER is prohibited. It is therefore probable that VP7-1 remains localized to the cytoplasm where a highly reducing environment and the absence of N-linked glycosylation processes result in the misfolding of the protein. Rabbit anti-$VP7_{pep177-190}$ (GenScript®) was purchased for the purpose of detecting misfolded VP7-1 in cell culture. However, in NDV-VP7-1 infected BSR-T7/5 cells, that were infected with recombinant NDV, as shown by IFMA (Fig. 2.12A), VP7-1 remained undetected (Fig. 2.12B). The functionality of the antibody was verified by the detection of unaltered VP7 in BSR-T7/5 (Fig. 2.13) and L929 (Fig. 2.14) cells. Unsurprisingly, the expression levels of the protein were poor and corresponded to previous observations using antibodies raised against the whole RV particle (Aschenbrenner, 2017). Detection of VP7 in L929 cells resulted in irregular staining patterns that did not seem to have specificity towards infected cells (Fig. 2.14A). These results were not repeated in the negative control that were subjected to similar conditions using rabbit anti-$VP7_{pep177-190}$ and therefore expression of VP7 in L929 cells remains inconclusive (Fig. 2.14D).

Expression of NSP4 was clearly observed in both BSR-T7/5 (Fig. 2.15) and L929 (Fig. 2.16) cells with the presence of distinctive cytoplasmic vesicular structures, known as puncta (Hyser *et al.*, 2010). These vesicular structures are associated with viroplasms in RV-infected cells where they play a key role in viral replication. The formation of puncta is exclusive to NSP4 and dependent on intracellular Ca^{2+} levels (Berkova *et al.*, 2006; Hyser *et al.*, 2010). NSP4 is shown to co-localize with the autophagosomal marker, LC3, within these structures. It was proposed that NSP4 interferes with the autophagosomal maturation of the cell by decreasing lysosome formation and increasing the size of proximal organelles such as the ER (Berkova *et al.*, 2006).

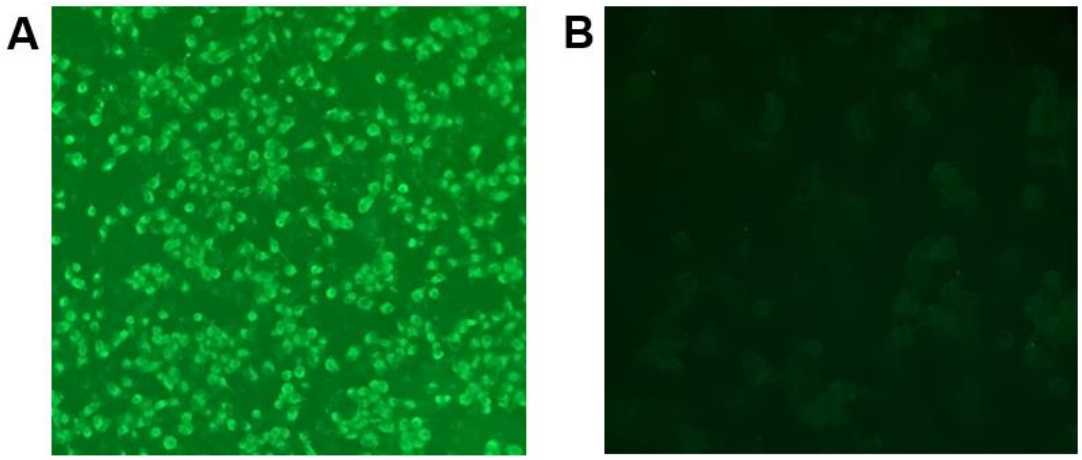


Fig. 2.12. **IFMA of rNDV-VP7-1 infected BSR-T7/5 cells**. (A) Detection of NDV using rabbit anti-NDV pAbs; (B) expression of VP7-1 remained undetected using rabbit anti-$VP7_{pep177-190}$ pAbs.

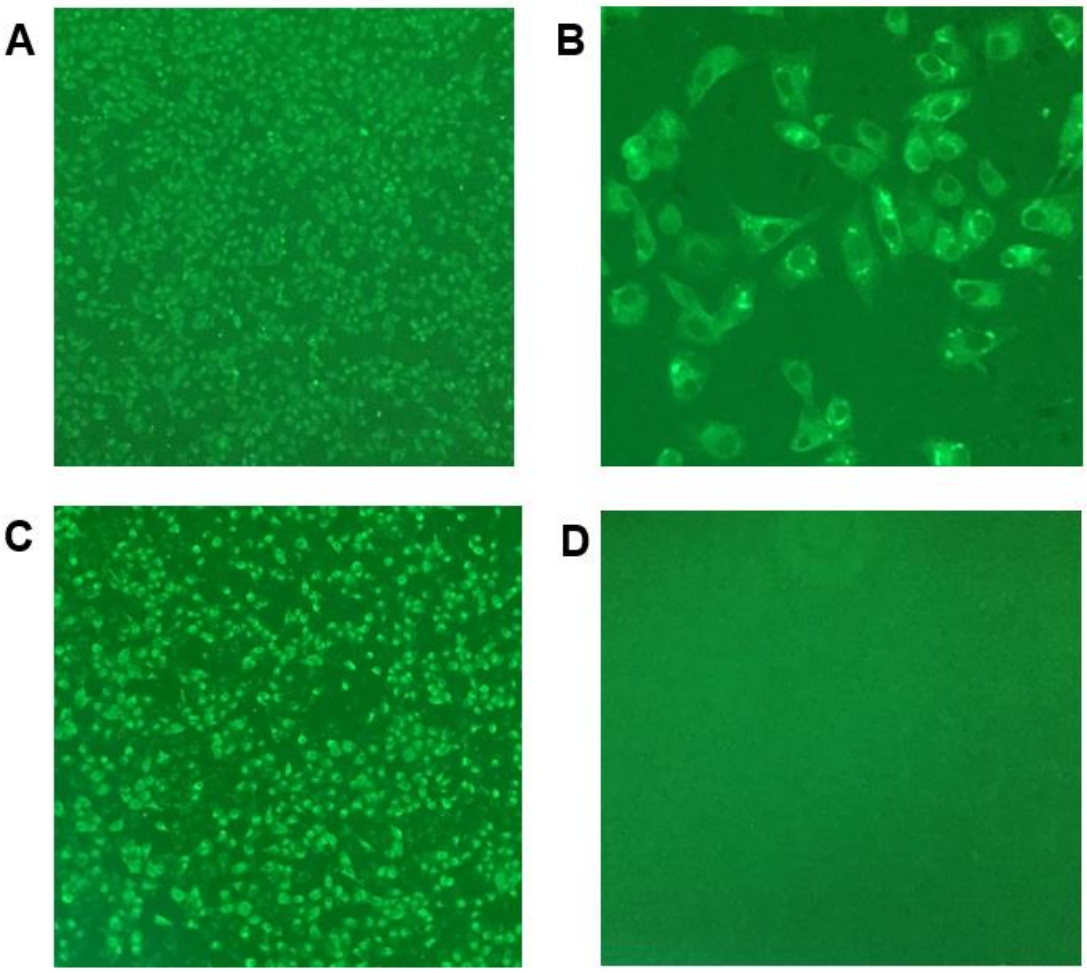


Fig. 2.13. **IFMA of rNDV-VP7 infected BSR-T7/5 cells**. Detection of VP7 using rabbit anti-$VP7_{pep177-190}$ at 10x magnification (A) and 40x magnification (B); detection of NDV using rabbit anti-NDV pAbs (C); uninfected BSR-T7/5 cells (mock) stained with rabbit anti-$VP7_{pep177-190}$ (D).

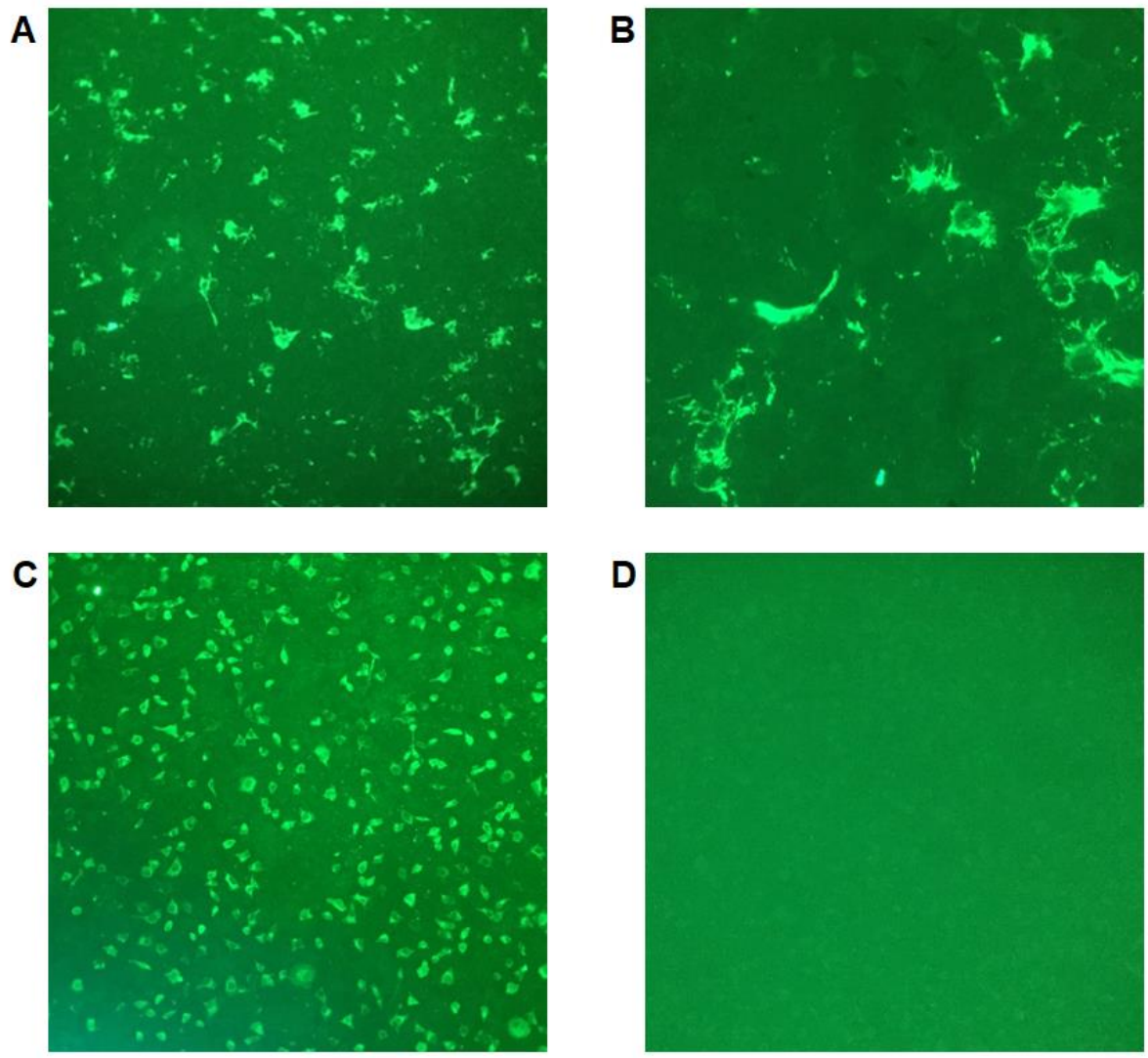


Fig. 2.14. **IFMA of rNDV-VP7 infected L929 cells**. Detection of VP7 using rabbit anti-$VP7_{pep177-190}$ at 10x magnification (A) and 40x magnification (B); detection of NDV using rabbit anti-NDV pAbs (C); uninfected L929 cells (mock) stained with rabbit anti-$VP7_{pep177-190}$ (D).

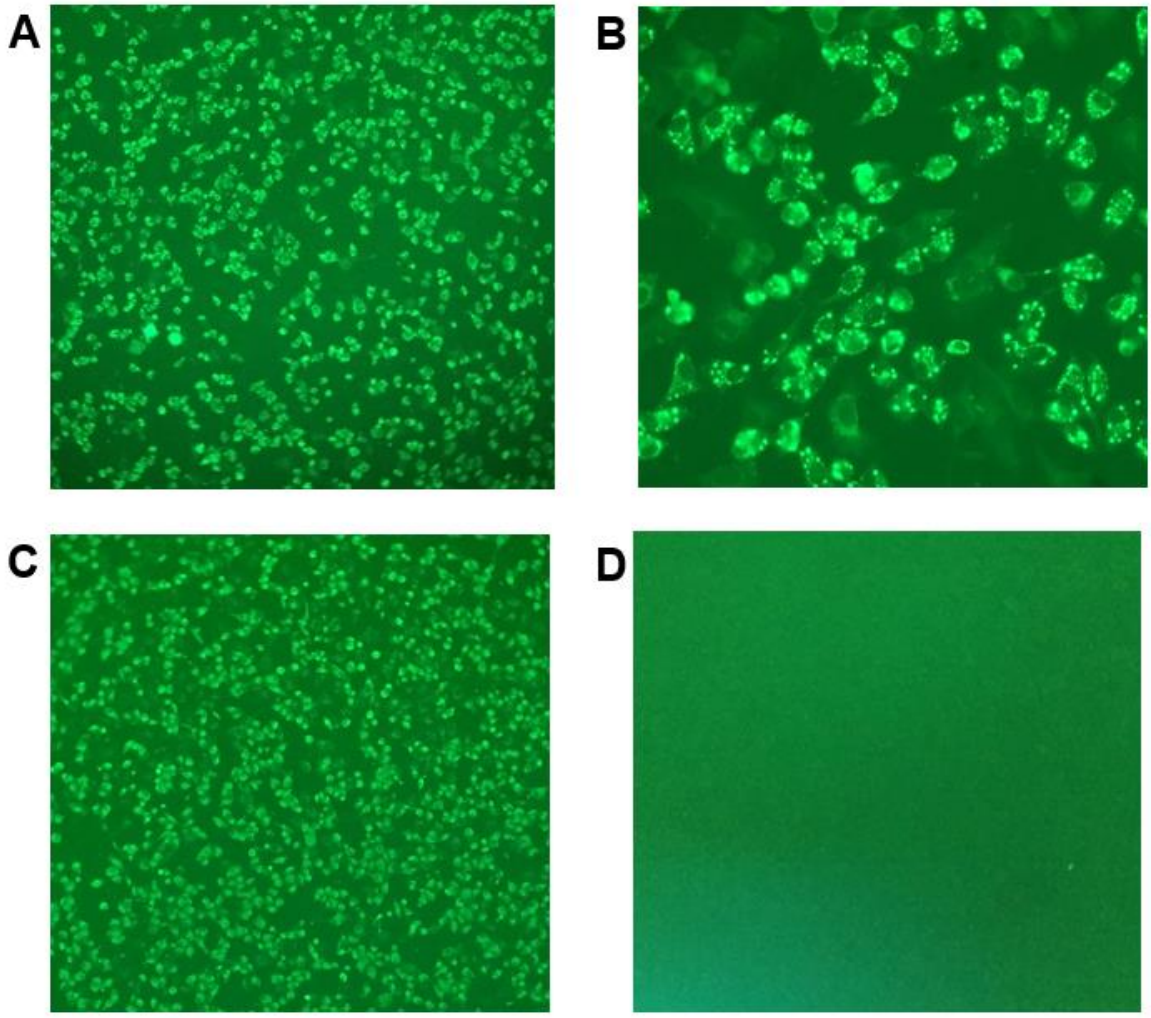


Fig. 2.15. **IFMA of rNDV-NSP4 infected BSR-T7/5 cells**. Detection of NSP4 using rabbit anti-NSP4 $_{pep126–140}$ pAbs at 10x magnification (A) and 40x magnification (B); detection of NDV using rabbit anti-NDV pAbs (C); uninfected BSR-T7/5 cells (mock) stained with rabbit anti-NSP4 $_{pep126–140}$ (D).

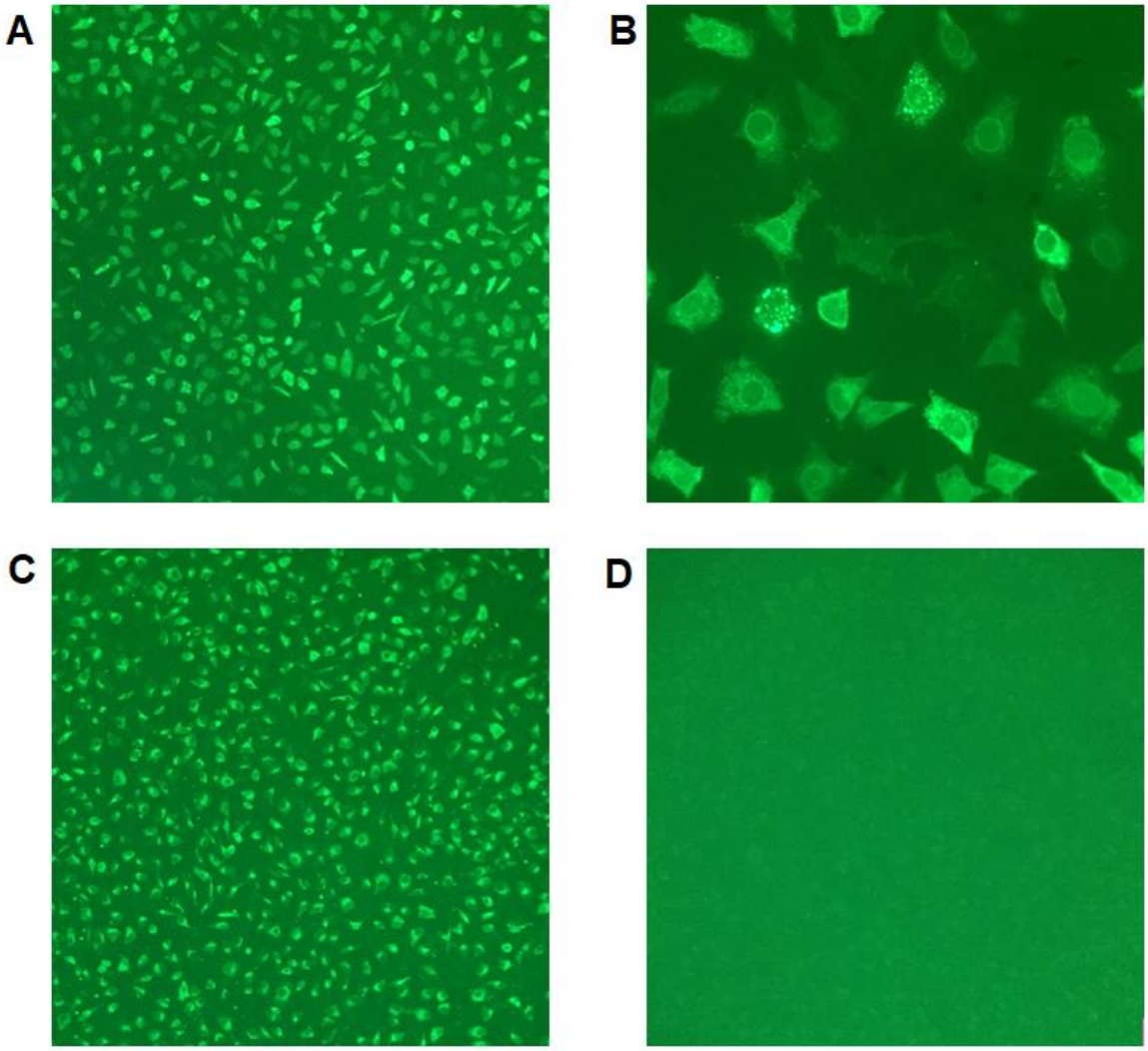


Fig. 2.16. **IFMA of rNDV-NSP4 infected L929 cells.** Detection of NSP4 using rabbit anti-$NSP4_{pep136–149}$ pAbs at 10x magnification (A) and 40x magnification (B); detection of NDV using rabbit anti-NDV pAbs (C); uninfected L929 cells (mock) stained with rabbit anti-$NSP4_{pep136–149}$ (D).

2.3.5.2 Western blot analysis

Similar to IFMA, expression of VP7-1 was undetectable in BSR-T7/5 cells using either anti-RV (Fig 2.17) or anti-VP7$_{pep177-190}$ (Fig. 2.18). NDV-derived VP7 (~ 38 kDa) was detected and showed improved expression levels compared to previous observations (Aschenbrenner, 2017). Non-specific binding was observed with the utilization of anti-VP7$_{pep177-190}$, indicating specificity of the antibodies towards an endogenous protein in BSR-T7/5 cells. It was speculated, that a lack of detection of VP7-1 might be a consequence of its immediate degradation since the protein is no longer translocated to the ER. The majority of intracellular proteins are degraded via the ubiquitin proteasome pathway (UPP) which involves the addition of an ubiquitin chain and targeted degradation by the 26S proteasome. This system is especially useful for eliminating damaged or misfolded proteins that are a consequence of mutations, denaturation or biosynthetic errors (Waxman *et al*., 1987). Another major protein degradation pathway, complementary to the UPP, involves the uptake and hydrolysis of misfolded proteins by lysosomes which constitute membrane-enclosed organelles that contain proteases and other digestive enzymes (De Duve *et al.*, 1953).

To determining whether failure to detect VP7-1 was due to protein degradation or a lack of expression, western blot analysis was performed with the addition of inhibitors, including 1 % (w/v) complete protease inhibitor (PI) cocktail (pH 7.4) (Roche, Switzerland) and 10 µM proteasome inhibitor MG132 (Sigma-Aldrich, United States). The protease inhibitor cocktail was implemented on its own or in combination with MG132. Expression of VP7-1 was confirmed with an expected molecular weight of 34.5 kDa (Fig. 2.19). The expression of VP7-1 was not dependent on the inclusion of MG132, suggesting degradation of VP7-1 by proteases residing in the lysosomes.

Anti-NSP4$_{pep136–149}$ was used to verify expression of NDV-derived NSP4 (Fig. 2.20). Analysis by electrophoresis on a 15 % acrylamide gel showed two distinctive bands with molecular weights corresponding to 28 kDa and 23 kDa. The 28 kDa band agreed with literature regarding the monomeric molecular weight of NSP4 (Ericson *et al.*, 1983). The presence of the 23 kDa band might be attributed to the formation of oligomeric structures between the 7 kDa cleavage products (aa 112 – 175) of NSP4 as reported by (Zhang *et al.*, 2000).

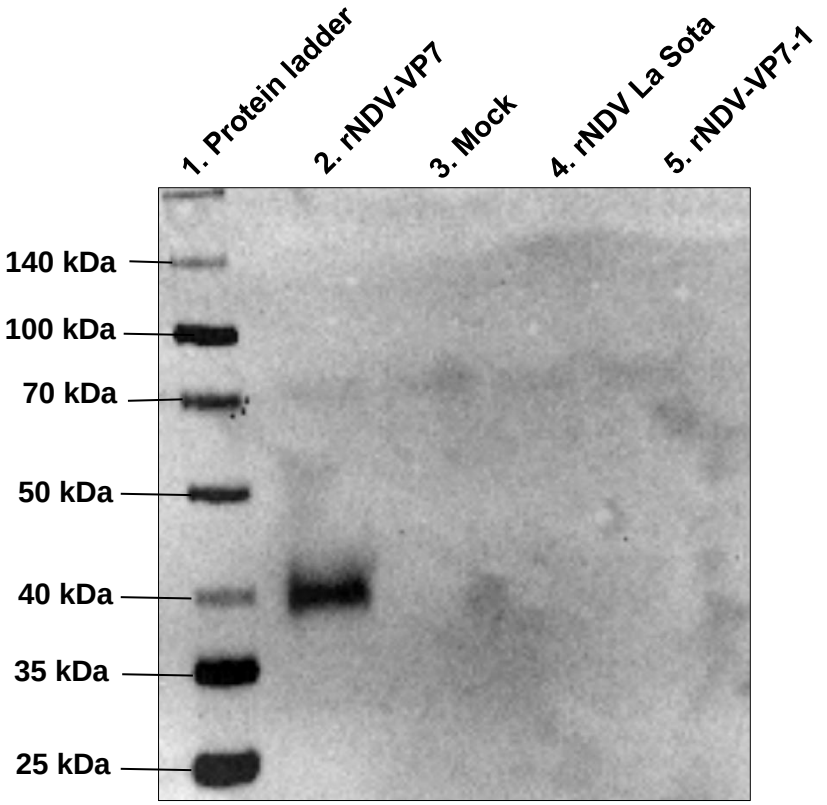


Fig. 2.17. **Western blot analysis of BSR-T7/5 cells infected with rNDV-VP7 variants using goat anti-RV pAbs**. 1) Spectra^{TM} Multicolor Broad Range Protein Ladder; 2) rNDV-VP7 infected cells; 2) Uninfected BSR-T7/5 cells (mock); 3) rNDV La Sota infected cells; 4) rNDV-VP7-1 infected cells.

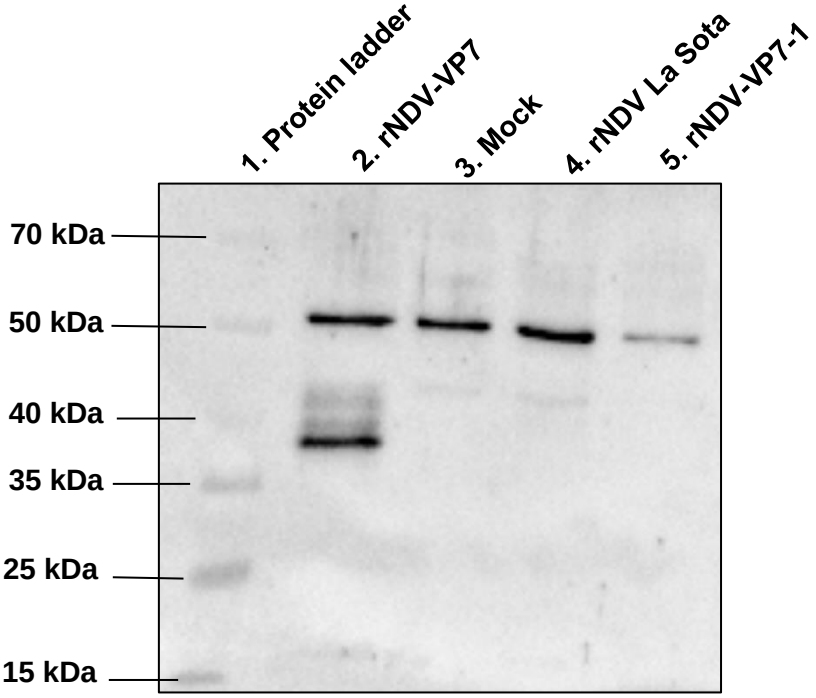


Fig. 2.18. **Western blot analysis of BSR-T7/5 cells infected with rNDV-VP7 variants using rabbit anti-VP7$_{pep177 - 190}$ pAbs**. 1) Spectra^{TM} Multicolor Broad Range Protein Ladder; 2) rNDV-VP7 infected cells; 2) Uninfected BSR-T7/5 cells (mock); 3) rNDV La Sota infected cells; 4) rNDV- VP7-1 infected cells.

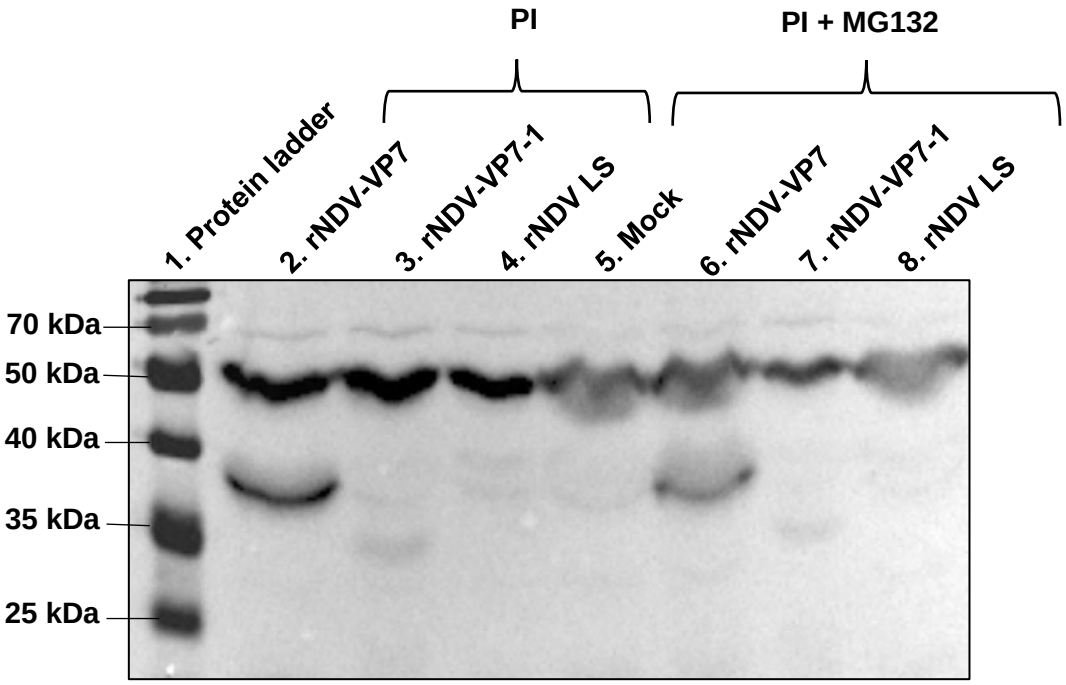


Fig. 2.19. **Western blot analysis of BSR-T7/5 cells infected with rNDV-VP7 variants in the presence of Roche complete protease inhibitor (PI) cocktail (pH 7.4) with/without MG132.** For the primary antibody rabbit anti-VP7$_{pep177-190}$ was used. 1) PageRuler^{TM} Plus Prestained Protein Ladder; 2) rNDV-VP7 infected cells; 3) rNDV-VP7-1 infected cells; 4) rNDV La Sota infected cells; 5) uninfected BSR-T7/5 cells + MG132; 6) rNDV-VP7 infected cells + MG132; 7) rNDV-VP7-1 infected cells + MG132; 8) rNDV La Sota infected cells + MG132.

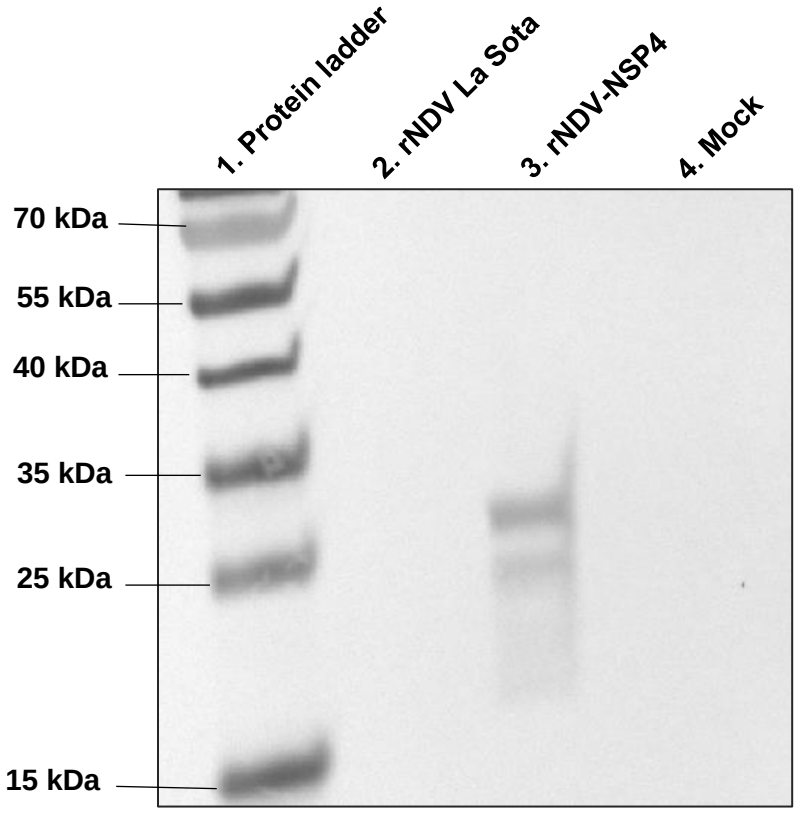


Fig. 2.20. **Western blot analysis of BSR-T7/5 cells infected with rNDV-NSP4 using rabbit anti-NSP4 pAbs**. For SDS-PAGE, cell lysates were run on a 15 % acrylamide gel followed by blot transfer.1) PageRuler^{TM} Plus Prestained Protein Ladder; 2) rNDV La Sota infected cells; 3) rNDV- NSP4 infected cells; 4) Uninfected BSR-T7/5 cells.

2.4 Summary

Expression of previously undetected NDV-derived NSP4 was confirmed by IFMA in BSR-T7/5 and L929 cells. Cytoplasmic vesicular structures were observed in either cell line and corresponded to literature regarding the formation of puncta in RV infected cells. For additional verification of NSP4 expression, western blot analysis was performed and resulted in bands corresponding to the molecular weights of monomeric NSP4 (28 kDa) and oligomeric structures of the 7 kDa cleavage product (23 kDa) (Ericson *et al.*, 1983; Zhang *et al.*, 2000).

The recombinant NDV containing the VP7-1 ORF was successfully constructed and rescued from SPF embryonic chicken eggs as shown by hemagglutination assay. Sequencing of the genomic RNA extracted from allantoic fluid showed successful insertion of the VP7-1 start codon at position 3 172 of the NDV genome with no single base changes observed throughout the coding sequence or at the terminal ends. However, expression of VP7-1 was undetectable in BSR-T7/5 or L929 cells using antibodies raised against the whole RV particle. It was speculated that the removal of the signal sequence resulted in improper folding of VP7-1 and loss of antigenic determinants that are found on the native protein. Nevertheless, antibodies were raised against VP7-1 immunogenic region aa 177 - 190, which was predicted to detect the protein irrespective of its conformational integrity. These antibodies were incorporated into IFMA and western blot analysis but were unable to detect expression of VP7-1. To verify that an inability to detect VP7-1 was due to protein degradation and not a lack of expression, western blot analysis was repeated with the incorporation of a protease inhibitor cocktail and the proteasome inhibitor, MG132. Expression of VP7-1 was detected irrespective of the inclusion of MG132 and was therefore suggested to undergo an alternative degradation pathway to that of the UPP, possibly by proteases residing in the lysosome.

CHAPTER 3 – HUMORAL IMMUNITY ELICITED BY RECOMBINANT NDV EXPRESSING RV PROTEINS IN MICE

3.1 Introduction

The propagation of viruses in cell culture is essential for the *in vitro* evaluation of novel vaccine candidates which require viral stocks for virus neutralization assays and other cell-based immunological studies (Lipkin and Firth, 2015). This sometimes proves problematic since not all viruses have the potential to adapt to well-established cell lines, often due to a lack of appropriate cellular receptors or host factors that support viral transcription and assembly (Ren *et al.*, 1990; Martina *et al.*, 2006). The challenges associated with the adaption of RV to cell culture have greatly been overcome by the incorporation of low concentrations trypsin in the media surrounding the virus. Trypsin specifically cleaves RV spike protein (VP4) resulting in the conformational changes needed for viral entry into the cell (Arias *et al.*, 1996). Once an animal RV strain is adapted to cell culture, it has the capacity to grow to titres of 10^7 and 10^8 PFU/ml (Arnold *et al.*, 2012).

Prior to immune-based studies *in vitro,* it is necessary to select an appropriate animal model in which the immune responses induced will most closely reflect those of the target species. Laboratory-bred mice is one of the most widely studied models and offers a range of advantages compared to larger animal models. These include economic feasibility, potential to incorporate large numbers of animals in a single trial, short gestation periods coupled by multiparous births and the availability of pathogen-specific immunologically naïve animals (Ciarlet *et al.*, 2002). Genetically homogeneous mice can be obtained from selective breeding which enables researchers to identify quintessential characteristics of the innate and adaptive immunity (Franco *et al*., 2006). The only significant drawback of this model is a maximum blood volume of 200 µl, only 10 - 15 % of the total blood volume of a 25 g mouse, can safely be removed without fluid replacement. For the collection of larger samples, euthanasia is essential since removal of more than 15 % of the total blood volume can result in hypovolemic shock (Diehl *et al.*, 2001).

Laboratory-bred mice offer an excellent means of investigating the immune correlates of protection related to RV infection *in vivo*. Infant mice (<15 days) are susceptible to diarrhoeic disease upon RV infection whereas adult mice remain asymptomatic and prone to viral shedding (Knipping *et al.*, 2011). The disease, originally termed epizootic diarrhoea of infant mice, was first characterized in 1948 by Cheever and Mueller and later associated with RV infection following the detection of viral antigens in the intestinal lumen of suckling mice

(Cheever *et al*., 1948; Wilsnack *et al*., 1969). The mouse model is made further attractive for RV-related studies, given its susceptibility to a number of heterotypic RV strains that commonly occur in animals and humans. These include SA11 (simian), RRV (rhesus) and B223 (bovine) which have been shown to induce severe gastroenteritis in suckling mice (Ramig, 1988). Other cases of BRV strains eliciting diarrhoea in neonatal mice have also been reported (Kashyap *et al.*, 2018). The mouse model has been applied excessively in RV vaccine development providing a reliable source to test the efficacy of a novel vaccine candidate (Xie *et al.*, 2015). As proof of concept, recombinant NDVs expressing bovine RVA VP7 and NSP4 were evaluated for their ability to induce humoral immune responses in mice.

3.2 Materials and methods

3.2.1 Whole genome characterisation of RVA 1604

Prior to animal studies, RVA/Cow-wt/ZAF/1604/2007/G8P[1] (RVA 1604) was adapted to MA104 cells to serve as the required antigen for immunological assays. Whole-genome sequencing of the adapted virus and resequencing of RVA 1604 from stool was done using the MiSeq (Illumina, Inc) next-generation sequencing (NGS) platform.

3.2.1.1 RVA 1604 propagation

MA104 cells, a kind gift from Dr. Ulrich Desselberger (University of Cambridge) were used during this part of the study. The cell line was established from the foetal kidney of an African green monkey and is generally used for the propagation and quantification of RV (Whitaker and Hayward, 1985). The cell line was maintained as a monolayer in 75 cm^2 tissue culture flasks (Corning, United States) while undergoing routine passaging in DMEM growth medium containing GlutaMAX™, pyruvate and 4.5 g/L D-Glucose (Gibco™, United States). The media was supplemented with 1 x Pen/Strep Amphotericin B (Lonza, Switzerland), nonessential amino acids (NEAAs, Gibco™, United States) and 2 % (v/v) foetal bovine serum (FBS) (Gibco™, United States). Between passaging, the cells were incubated at 37° C in a 5 % CO_2-incubator (Thermo Scientific Forma® Steri-Cycle® CO_2 Incubators, United States). Once the cells reached a confluency of 80 %, the medium was removed, and the cells were washed with phosphate buffered saline (PBS) (Sigma-Aldrich, United States). The cells were briefly incubated with 0.05 % trypsin-EDTA (Gibco™, United States) until cell detachment was visible. The cells were resuspended in the appropriate volume of fresh DMEM growth media and passaged into new 75 cm^2 tissue culture flasks (Nunc™, United States).

The stool sample, containing bovine RVA 1604, was thawed on ice and 500 µl thereof was combined with 3 ml supplemented DMEM growth media. An equal volume of VERTREL (Sigma-Aldrich, United States) was added where-after the mixture was vigorously shaken and

centrifuged for 10 min at 6 000 x g (YINGTAI TD50, China) to clarify the sample. The VERTREL extraction was repeated. The mixture was vigorously shaken, and the clarification step was repeated. The supernatant was carefully removed and filtered using a 0.2 µm syringe filter (PALL Corporation, United States). The filtrate was incubated with 10 µg/ml porcine trypsin IX (Sigma-Aldrich, United States) for 30 min at 37 °C and added to a confluent monolayer (75 cm^2) of pre-washed MA104 cells. The cells were left to incubate at room temperature for 1 hour while rocking where-after the filtrate was removed and replaced with fresh supplemented growth medium. The cells were incubated at 37 °C in a CO_2-incubator until cytopathic effect (CPE) was confirmed. A confluent monolayer of cells that received similar treatment, without the addition of the purified virus, served as the negative control.

3.2.1.2 RNA extraction

Once the cell monolayer showed 70 % CPE, the remaining cells were scraped, and the suspension was centrifuged for 10 min at 4 000 x g. The pellet was resuspended in 500 µl PBS and thoroughly combined with 900 µl TRI reagent (Sigma-Aldrich, United States) and 100 µl VERTREL (Sigma-Aldrich, United States). Once the solution was homogeneous, 300 µl chloroform (Sigma-Aldrich, United States) was added and the solution was gently mixed by inversion. The solution was centrifuged for 15 min at 20 000 x g (Centrifuge 5804 R, Eppendorf, Germany) where-after the clear supernatant was transferred to a clean microcentrifuge tube containing 750 µl isopropanol (Merck, Germany). The solution was gently mixed by inversion and centrifuged for 15 min at 20 000 x g. The supernatant was decanted, and the pellet was resuspended in 95 µl Elution buffer of the NucleoSpin® Plasmid kit (Macherey-Nagel, Germany). Single-stranded RNA was removed by precipitation with 2 M LiCl (Sigma-Aldrich, United States) overnight at 4 ° C. The sample was centrifuged at 4 °C for 30 min at 20 000 x g where-after the supernatant was transferred to a clean microcentrifuge tube. The MEGAclear™ Transcription Clean-Up Kit (Invitrogen by Thermo Fisher Scientific, United States), which is generally used for the purification of dsRNA from transcription reactions, was used to remove residual single-stranded RNA and LiCl from the sample. Briefly, 350 µl Binding Buffer and 250 µl absolute ethanol (Merck, Germany) was added to the supernatant and mixed by pipetting. The solution was transferred to a MEGAclear™ Filter Cartridge and centrifuged for 1 min at 14 000 x g (Centrifuge 5418, Eppendorf, Germany). The flow-through was discarded and the membrane was washed with 500 µl of the Wash Solution. The Filter Cartridge was placed in a clean Elution Tube and 30 µl of the Elution Solution was applied to the centre of the Filter Cartridge. The tube was incubated for 5 min at 80 °C and centrifuged for 2 min at 14 000 x g. The quality of the dsRNA was evaluated by gel electrophoresis on a 1 % agarose gel prepared with 0.5 x Tris-Borate-EDTA (TBE) (50 mM Tris, 50 mM boric acid, 1 mM EDTA) buffer and 5 µg/ml ethidium bromide (Sigma-Aldrich,

United States). Briefly, 2 µl of each of the samples were combined with 2 µl 6x DNA Gel Loading Dye (Thermo Fisher Scientific, United States) and subjected to electrophoresis for 30 min at 120 V (Bio-Rad Laboratories Inc., United States). The GeneRuler^{TM} DNA Ladder mix (Thermo Fisher Scientific, United States) was used as a DNA marker. The gel was analysed under UV light using the ChemiDoc^{TM} MP Imaging System (Bio-Rad Laboratories Inc., United States).

To ensure that the full-length segments of the RV genome were sequenced, an 'anchor primer', PC3-T7 loop (5' – pGGATCCCGGGAATTCGGTAATACGACTCACTATATTTTTATA GTGAGTCGTATTA–OH – 3'), was synthesized (Integrated DNA Technologies, United States) and ligated to the 3' ends of the dsRNA as described before (Potgieter *et al.*, 2009). Briefly, 200 ng/µl of the anchor primer was ligated to 0.4 – 200 ng of the dsRNA in 50 mM HEPES, pH 8.0 (Sigma-Aldrich, United States of America), 18 mM $MgCl_2$ (Sigma-Aldrich, United States), 0.01 % BSA (Takara Bio Inc, Japan) 3 mM DTT (Roche, Switzerland),1 mM ATP (Thermo Fisher Scientific, United States), 10 % (v/v) DMSO (Thermo Fisher Scientific, United States), 20 % (w/v) PEG_{6000} (Thermo Fisher Scientific, United States), and 30 U T4 RNA Ligase (Takara Bio Inc, Japan) in a final reaction volume of 30 µl. The reaction was mixed and incubated overnight at 37 °C. The ligated dsRNA was purified by using the MinElute Gel Extraction kit (Qiagen, Germany) and by following the manufacturer's instructions.

Synthesis of complementary DNA (cDNA) was performed using the Maxima H Minus Double Stranded cDNA kit (Thermo Fisher Scientific, United States) according to the manufacturer's instructions. Briefly, 13 µl of the ligated dsRNA was denatured at 95 °C for 5 min where-after 1 µl random hexamer primer was added and incubated at 65 °C for 5 min in a thermal cycler (Bio-Rad Laboratories Inc., United States). For first strand synthesis, 5 µl 4x First Strand Reaction Mix was added to the tubes along with 1 µl First Strand Enzyme Mix. The reactions were incubated for 10 min at 25 °C followed by 2 hours at 50 °C and terminated by heating at 85 °C for 5 min. Second strand synthesis included the addition of 55 µl nuclease-free water, 20 µl 5x Second Strand Reaction Mix and 5 µl Second Strand Enzyme Mix with a final volume of 100 µl. This was followed by a final incubation step at 16 °C for 1 hour. The reactions were terminated by the addition of 6 µl 0.5 M EDTA, pH 8.0.

3.2.1.3 Sequencing and analysis

The cDNA was submitted for sequencing at the Next Generation Sequencing Unit at the University of the Free State (NGS-UFS), South Africa using the Illumina MiSeq Next Generation Sequencer (Illumina, Inc., United States). The samples were prepared by using the NextEra XT DNA Library Prep Kit (Illumina, Inc., United States). The NGS library was validated using the Bioanalyzer 2100 (Agilent Technologies, United States) and the

concentration was normalized to 8 pM. Sequencing was performed using the MiSeq Reagent Kit V3 (600 cycles) with 301 bp paired-end reads. The data was imported onto the CLC bio® Genomics Workbench, v. 12 (Qiagen, Germany) in FASTQ format. The reads were trimmed and assembled into *de novo* contigs. Contigs that showed >100 x coverage were identified with BLAST (Basic Local Alignment Search Tool) using the National Centre for Biotechnology Information website (NCBI). The trimmed reads were also mapped to the RVA 1604 reference sequence obtained from GenBank (JN831215 – JN831225) and the consensus sequences for the 11 genome segments were obtained. The consensus sequences were aligned to the corresponding reference sequences and single nucleotide changes were documented.

3.2.2 Vaccination of mice

Ethical clearance for the evaluation of recombinant NDVs expressing RV proteins were obtained from the relevant committees (see Appendix A4 – A6). Adult NMRI (Naval Medical Research Institute) mice (SA vaccine producers, South Africa) were housed in groups of six in standard mouse cages (360 x 200 x 140 mm, Techniplast, United States), which were kept under biosafety level – 3 (BSL3) conditions at Deltamune (Pty) Ltd animal testing facilities. The cages were enriched with shredded paper, wood shavings and toilet rolls. Sanitation was maintained daily by the removal of faecal matter and the replacement of wet bedding with dry wood shavings. Before the commencement of the animal trial, a total of 36 mice tested seronegative for RV. The mice were randomised, six per group, for dosing with recombinant NDV via the oronasal (on) or subcutaneous (sc) route. The mice were vaccinated on day 1 with 1 x 10^7 $TCID^{50}$ rNDV La Sota, rNDV-VP7 or rNDV-NSP4 contained in 100 µl allantoic fluid (Table 3.1). Inoculations were administered by Mr. Roelf Greyling, an animal technologist employed by Deltamune (Pty) Ltd. Four of the mice remained unvaccinated and were kept as controls. The mice were monitored daily for pyrogenic reactions and signs of lethargy. On day 20, half of the mice from each group (3 mice/group) were euthanized by intravenous injection of 0.2 ml pentobarbitone (Euthanase, South Africa), where-after intracardiac bleeds were collected and stored at -70 °C. The remaining mice were administered a booster dose of 100 µl 1 x 10^7 $TCID_{50}$ for the respective recombinant NDVs on the same day. The mice were kept for 18 days at standard conditions followed by euthanasia and bleeding on day 38, according to the aforementioned procedure (Fig. 3.1).

Table 3.1. **Immunization regime indicating vaccine description, inoculation route, number of mice inoculated (n) and dose administered**

Group	Description	Route	n	Dose ($TCID_{50}$)
1	rNDV La Sota	*on*	6	1×10^7
		sc	6	
2	rNDV-VP7	*on*	6	1×10^7
		sc	6	
3	rNDV-NSP4	*on*	6	1×10^7
		sc	6	

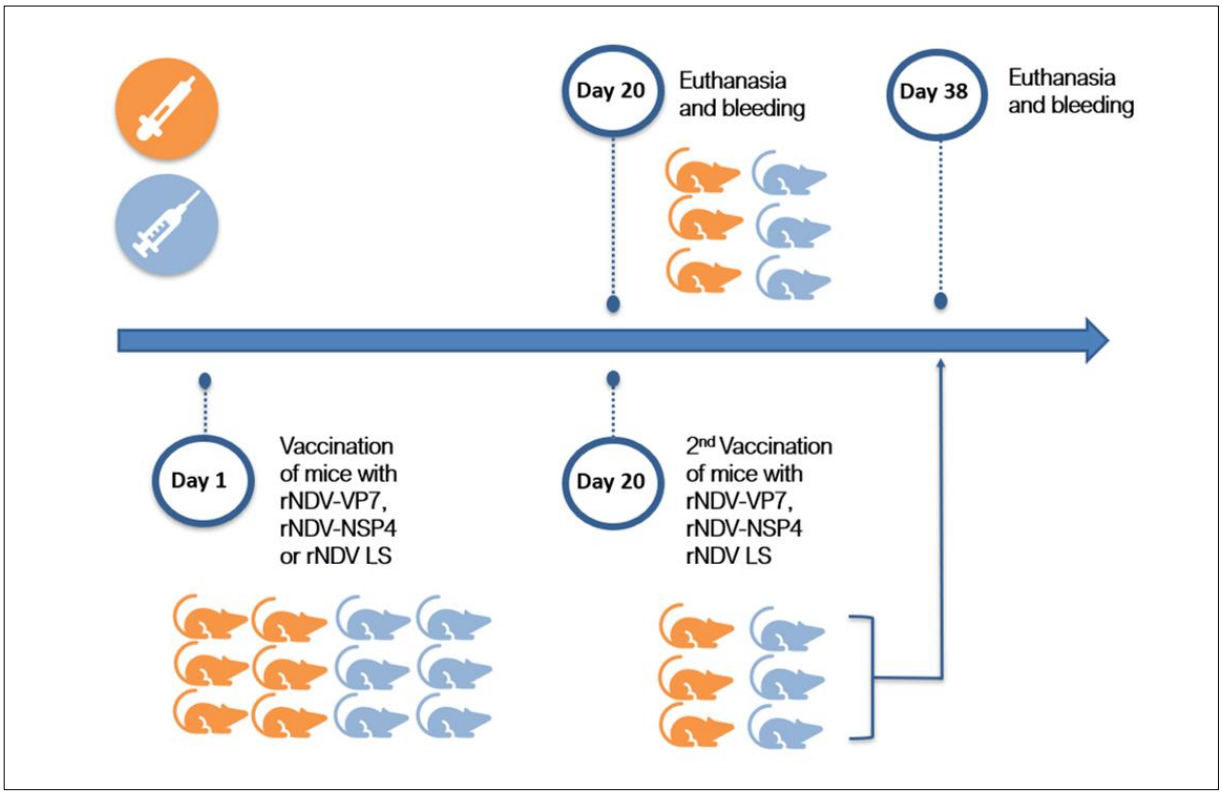


Fig. 3.1. Schematic diagram depicting the timeline of the animal trial. Mice were vaccinated on day 1 with either rNDV-VP7, rNDV-NSP4 or rNDV La Sota via the oronasal (orange) or subcutaneous (blue) route, equalling 6 mice/group. On day 20, half of the mice from each group were euthanised and bled (3 mice/group). The remaining mice received the booster dose on the same day followed by euthanasia and bleeding on day 38.

3.2.3 Immunoassays

3.2.3.1 Immunofluorescent monolayer assay (IFMA)

To determine the antibody responses induced in vaccinated mice, IFMAs were performed as previously described (J Kortekaas *et al.*, 2010). A 25 cm^2 confluent monolayer of MA104 cells were passaged 1:2 and seeded into 96-well flat-bottomed plates (Nunc™, United States) with a 1:100 dilution of trypsin-activated RVA 1604 in DMEM containing GlutaMAX™, Pyruvate and 4.5 g/L D-glucose with added 1 x Antibiotic-Antimycotic (Gibco™, United States) and 5 % (v/v) FBS (Biochrom, Germany), totalling 200 µl/well. The plates were incubated at 37 °C for 16 hours in a 5 % CO_2-incubator. The medium was removed, and the cells were fixed with -20 °C methanol-acetone (Merck, Germany) for 20 min at -20 °C. The methanol-acetone was removed where-after the cells were blocked with 1 x PBS, containing 1 % (w/v) Tryptone (Biolabs, South Africa) and 0.05 % (v/v) Tween-20™ (Sigma-Aldrich, United States) for 1 hour at 37 °C. Two-fold serial dilutions (starting with 1:10) was prepared for each serum sample collected from the vaccinated mice. The blocking buffer was removed from the cells and 50 µl of each serum dilution was added to the cells and left to incubate for 1 hour at 37 °C. The serum was removed, and the cells were washed three times with 1 x PBS containing 0.05 % (v/v) Tween-20. A 1:1000 dilution in blocking buffer of the goat anti-mouse fluorophore conjugate (Alexa Fluor Plus 488, Invitrogen, United States) was added to the cells and left to incubate at 37 °C for 20 min. The cells were washed and visualized with a fluorescence microscope (AXIO Vert.A1 Inverted Microscope, Zeiss, Germany).

3.2.3.2 Immunoperoxidase monolayer assay (IPMA)

To determine whether IgA specific immune responses were induced in vaccinated mice, standard IPMAs were performed as previously described (Kortekaas *et al.*, 2010b). MA104 cells were passaged and seeded in 96-well flat-bottomed plates with a 1:100 dilution of RVA 1604 in supplemented DMEM medium, totalling 200 µl/well. The plates were incubated for 24 hours at 37 °C in a 5 % CO_2-incubator. The medium was removed, and the cells were washed with 1 x PBS. The cells were incubated for 20 min at room temperature with 4 % (w/v) formaldehyde (Merck, Germany). The formaldehyde was removed, and the cells were permeabilized with 1 x PBS containing 0.5 % (v/v) Triton X-100 (Sigma-Aldrich, United States) at 37°C for 30 min. The cells were blocked with 1 x PBS containing 0.05 % (v/v) Triton X-100 and 1 % (w/v) tryptone digest for 1 hour at 37 °C. Two-fold serial dilutions (starting with 1:10) was prepared for each serum sample collected from the immunized mice. The blocking buffer was removed from the cells and 50 µl of each serum dilution was added to the cells and left to incubate for 1 hour at 37 °C. The cells were washed three times with 1 x PBS containing 0.05 % (v/v) Triton X-100 where-after a 1:3000 dilution of goat anti-mouse IgA cross-absorbed

HRP conjugate (Invitrogen, United States) was added to the cells and left to incubate for 30 min at 37 °C. The cells were washed three times and peroxidase activity was detected by using 3-amino-9-ethylcarbazole (AEC) (Sigma-Aldrich, United States) as the substrate. Briefly, 20 mg AEC was dissolved in 2.5 ml dimethyl sulfoxide (DMSO) (Sigma-Aldrich, United States) and combined with 50 mM acetate buffer [60 mM sodium acetate trihydrate (Sigma-Aldrich, United States); 140 mM glacial acetic acid (Merck, Germany), pH 5] and 0.015 % (v/v) hydrogen peroxidase (Merck, Germany). The substrate-buffer was filtered through a 0.2 µM filter and added to the cells until peroxidase activity was observed. The reaction was stopped by washing with 1 x PBS containing 0.05 % (v/v) Triton X-100.

3.2.3.3 Fluorescent focus neutralization assay

Virus neutralization assays for the detection of RV-neutralizing antibodies in vaccinated mice were done as described before (Ciarlet and Connor, 2000). Two-fold serial dilutions of the serum samples from the immunized mice were prepared (starting with 1:10) and combined in a 1:1 ratio with a 1:100 dilution of trypsin-activated RVA 1604 in supplemented DMEM medium. The virus-antibody solutions were incubated for 1 hour at 37 °C. A 25 cm^2 confluent monolayer of MA104 were passaged 1:2 and seeded with the virus-antibody dilutions into 96-well flat-bottomed plates. The plates were left to incubate at 37 °C in a CO_2-inubator for 16 hours. The media was removed, and the cells were fixed and blocked as described in section 3.2.3.1. The blocking buffer was removed where-after a 1:1000 dilution in blocking buffer of rabbit polyclonal antibodies raised against RV SA11 core particles (provided by Deltamune (Pty) Ltd., South Africa) was added to the cells and left to incubate for 1 hour at 37 °C. The primary antibody was removed, and the cells were washed three times with 1 x PBS containing 0.05 % (v/v) Tween-20. A 1:3000 dilution in blocking buffer of the goat anti-rabbit fluorophore conjugate (Alexa Fluor Plus 488, Invitrogen, United States) was added to the cells and left to incubate at 37 °C for 20 min. The cells were washed and visualized with a fluorescence microscope.

3.3 Results and discussion

3.3.1 Whole genome characterisation of RVA 1604

A confluent monolayer of MA104 cells showed complete CPE following a 16-hour incubation with the VERTREL-enriched RVA 1604-containing stool sample. Since the negative control remained confluent for more than 5 days, the observed cytotoxicity suggested the successful adaption of RVA 1604 to cell culture.

Electrophoretic analysis of the extracted dsRNA revealed a 3:3:2 migration pattern for both the stool sample and the adapted virus, with the characteristic co-migration of segments 7, 8 and 9, as well as segments 2 and 3, typically seen for group A RVs (Tam *et al.*, 1986; Chandrashekar *et al.*, 2013). The dsRNA extraction from stool contained residual ssRNA in the form of a light smear and a thick band at the bottom of the gel.

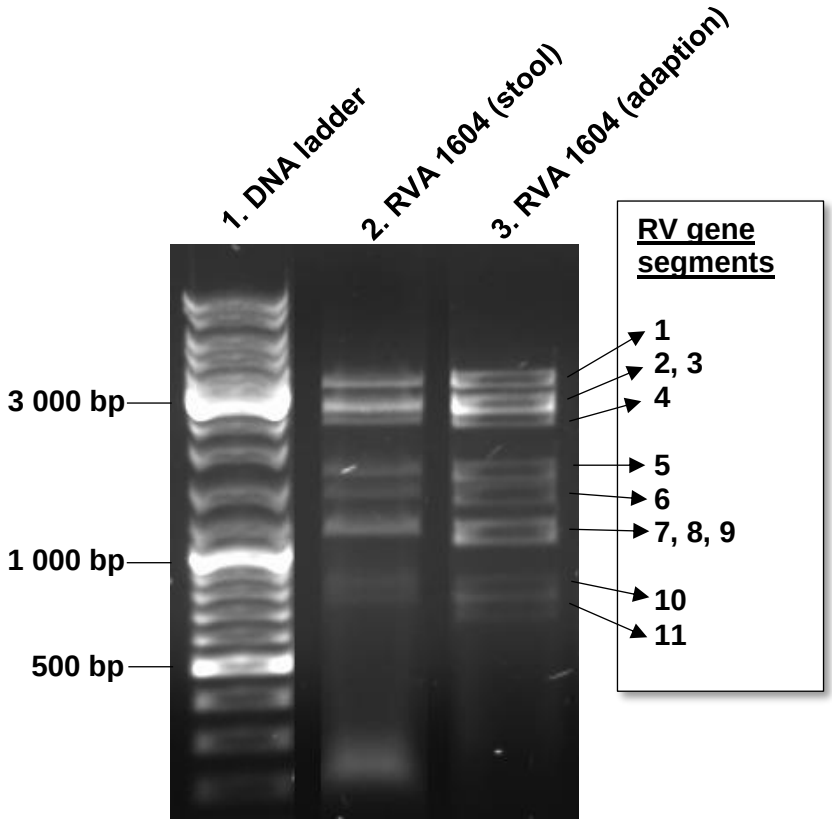


Fig. 3.2. **Agarose gel electrophoresis of dsRNA isolated from RVA 1604-containing stool sample and following adaption to MA104 cells.** 1) GeneRuler™ DNA Ladder mix; 2) RV 1604 (stool); 3) RV 1604 (adaption).

Sequencing data, in the form of paired-end reads, was successfully generated using the MiSeq® (Illumina, Inc) NGS. A total of 640 884 reads were generated for dsRNA isolated from the stool sample. Analysis of the assembled *de novo* contigs using BLAST analysis revealed the complete set of genome segments for RVA 1604 and, unexpectedly, that for RVA 1603. This shows that the diarrhoeic calf from which the faeces was collected had a co-infection of

both RVA 1604 and RVA 1603. Reference mapping of the trimmed reads to the RVA 1604 and 1603 reference sequences (Table 3.2) resulted in total coverage of the full genome sequences for all 11 segments of both viruses. Of the total reads generated, 76 % mapped to RVA 1604 and 21 % to RVA 1603. Lack of detection of RVA 1603 in the stool sample by 454® pyrosequencing combined with sequence-independent genome amplification serves as an indicator of the difference in sensitivity between the two NGS platforms (Jere *et al.*, 2012).

Sequencing of dsRNA extracted from the adapted RVA sample, generated 436 802 reads. Identification of the *de novo* contigs using BLAST analysis indicated the presence of both RVA 1604 and 1603. Of the total reads generated, 72% mapped to RVA 1604 and 23% to RVA 1603 (Table 3.3). All of the genome segments showed complete coverage except for RVA 1603 genome segment 4 (S4, VP4) to which only 112 (0.025%) reads mapped (Fig. 3.3). This might be explained by an event of reassortment that occurred between the two viruses during which S4 of RVA 1603 was replaced with that of RVA 1604. The precise mechanism underlying packaging of the correct set of 11 dsRNA segments into the virion core during RV replication remains to a large extent unknown. It is proposed that the positive-sense ssRNA possess *cis*-acting elements that interact with one another to form a supramolecular complex prior to encapsulation by the viral core protein (Gog *et al.*, 2007). These *cis*-acting elements or packaging signals are unique to each RV segment and thought to exist within the 5' and 3' untranslated regions (UTRs) located at RNA termini of group A viruses (Li *et al.*, 2010). For a hybrid progeny to emerge at detectable levels in a viral population, a reassortment event should, at least to some extent, add to the evolutionary fitness of the virus (McDonald et al. 2016). Previous attempts to propagate RVA 1603 in cell culture were unsuccessful (Mlera, *et al*., 2013, personal communication) and was possibly due to low attachment efficiency mediated by the P[5] VP4 protein of RVA 1603. It is possible that the replacement of RVA 1603 S4 with that of RVA 1604 enhanced the ability of the virus to bind to cellular receptors and facilitate penetration into the host cell. However, whether this exchange of genetic material occurred before or after adaption to cell culture cannot be determined from the available data.

Another reassortment event was suspected by a 40 % increase in the reads that mapped to RVA 1603 S3 (VP3), following adaption to cell culture, and a 44 % decrease in those that mapped to S3 of RVA 1604. Genome segment 3 encodes for the viral capping enzyme, VP3, which has also been implemented as a mechanism of RV to evade the innate immune response (Liu *et al*., 1992; Hoshino *et al.*, 1995). VP3 is shown to antagonize the oligoadenylates synthetase (OAS)-RNase L pathway which is among the most efficient anti-viral effectors induced by IFNs (Zhang *et al.*, 2013). This observation requires further evaluation in the form of serial passaging of the adapted viruses and resequencing of the genomic RNA.

Alignment of the consensus sequences obtained in this study for RVA 1604 and 1603 from stool and the corresponding reference sequences obtained from GenBank (Jere *et al.*, 2012) showed variations in the form of single base changes that occurred throughout the genome. Some of these mutations were non-synonymous, giving rise to amino acid alterations at position 622 (X→Tyr) in VP3 and 294 (Ser→Arg) in NSP3 of RVA 1604. Alterations in the amino acid sequences of RVA 1603 were observed at position 250 (Gln→Lys) in VP7, 463 (Ile→Met) in VP2 and 160 (Glu→Lys) in NSP4 (see Appendix A7).

Alignment of the consensus sequences from stool with that of the virus adaption to cell culture showed a silent point mutation (C→T) at position 422 of S5 (NSP1) for RVA 1604. This is not surprising since the NSP1 coding region have been associated with spontaneous mutations events in the past (Tian *et al.*, 1993; Taniguchi *et al*., 1996). No further nucleotide changes were observed throughout the entire RVA 1604 and 1603 genomes for both the adapted strains and the stool sample. Protein alignment using the Clustal Omega multiple sequence alignment tool (EMBL-EBI) were performed to determine whether the presence of RVA 1603 would interfere with the evaluation of NDV-derived RVA 1604 VP7 and NSP4 immune responses in mice. The NSP4 protein of the two viruses was shown to have 93 % aa sequence identity with the majority of variations occurring outside the immunodominant region (aa 114 – 135) (Fig. 3.4). Despite having different genotypes, VP7 was shown to have 81 % aa sequence identity, however, the majority of these variations occurred within immunodominant regions, I (aa 78 – 160; 257 – 312) and II (161 – 256) (Aoki *et al.*, 2009) (Fig. 3.5).

Table 3.2. **Sequence data of dsRNA extracted from stool mapped to RVA 1604 and RVA 1603 reference sequences available in GenBank** (Jere *et al.*, 2012).

Genome segment	*RVA/Cow-wt/ZAF/1604/2007/G8P[1]*			*RVA/Cow-wt/ZAF/1603/2007/G6P[5]*		
	Length (bp)	**Genotype**	**Mapped reads**	**Length (bp)**	**Genotype**	**Mapped reads**
S9	1 062	G8	34 317	1 062	G6	8 688
S4	2 362	P[1]	74 672	2 362	P[5]	11 119
S6	1 356	I2	40 134	1 356	I2	10 903
S1	3 302	R2	71 299	3 302	R2	16 048
S2	2 687	C2	68 467	2 687	C2	13 851
S3	2 591	M2	50 021	2 591	M2	27 493
S5	1 578	A3	27 104	1 579	A3	11 679
S8	1 059	N2	44 868	1 059	N2	6 545
S7	1 078	T6	29 941	1 074	T6	7 871
S10	751	E2	25 095	751	E2	10 641
S11	667	H3	19 609	667	H3	8 527

Table 1.3. **Sequence data of dsRNA isolated from the virus adaption to cell culture mapped to RVA 1604 and RVA 1603 reference sequences available in GenBank** (Jere *et al.*, 2012).

Genome segment	*RVA/Cow-wt/ZAF/1604/2007/G8P[1]*			*RVA/Cow-wt/ZAF/1603/2007/G6P[5]*		
	Length (bp)	**Genotype**	**Mapped reads**	**Length (bp)**	**Genotype**	**Mapped reads**
S9	1 062	G8	19 898	1 062	G6	8 370
S4	2 362	P[1]	53 007	2 362	P[5]	112
S6	1 356	I2	28 685	1 356	I2	1 682
S1	3 302	R2	53 043	3 302	R2	6 150
S2	2 687	C2	42 199	2 687	C2	5 772
S3	2 591	M2	15 033	2 591	M2	46 581
S5	1 578	A3	43 668	1 579	A3	4 446
S8	1 059	N2	16 803	1 059	N2	2 990
S7	1 078	T6	23 389	1 074	T6	4 635
S10	751	E2	10 668	751	E2	14 340
S11	667	H3	8 249	667	H3	7 882

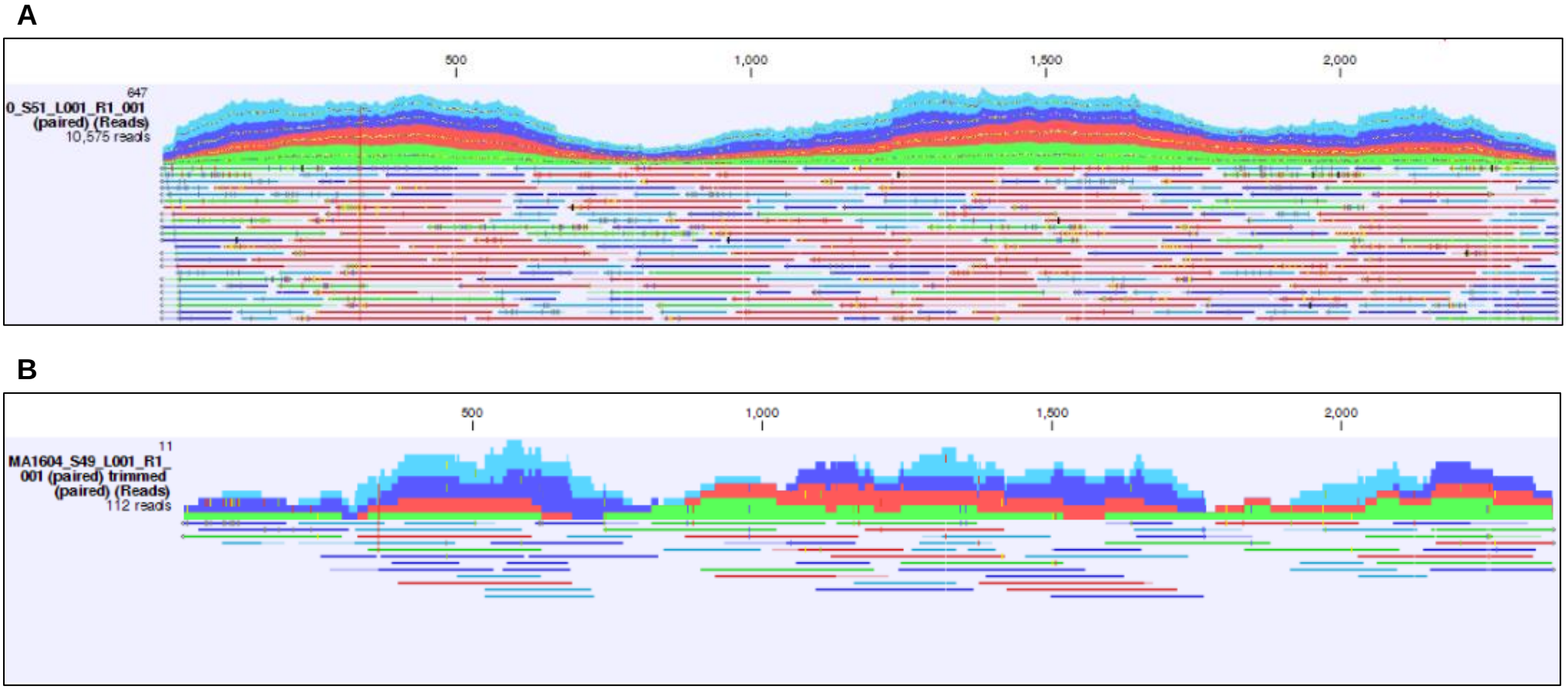


Fig. 3.3. **Mapping graphs depicting the coverage of paired-end reads mapped to RVA 1603 genome segment 4 obtained from GenBank** (Jere *et al.*, 2012) **for dsRNA extracted form stool (A) and from the virus adaption (B).** A total of 10 575 reads mapped to the reference sequence prior to adaption, with > 100 x coverage for the whole genome segment. This number was reduced to a total of 112 reads following adaption to cell culture with insufficient coverage (<10) throughout the genome segment.

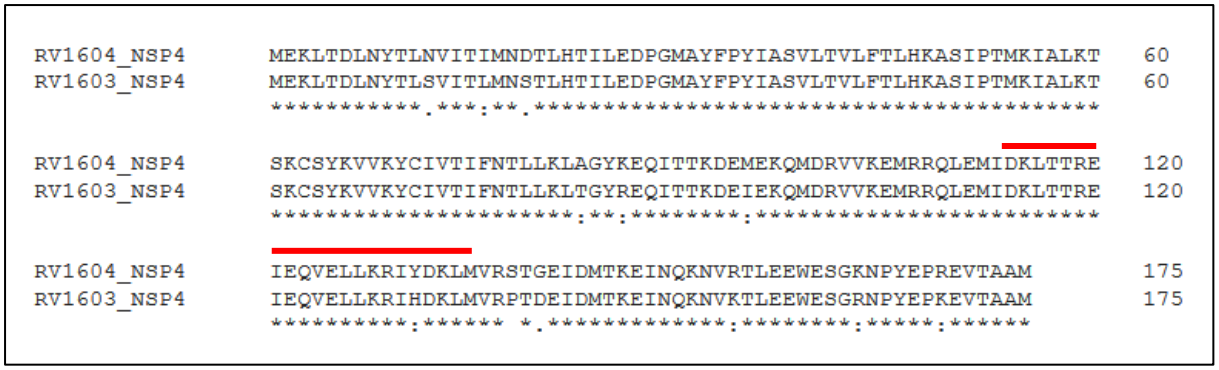


Fig. 3.4. **Sequence alignment of RVA 1604 and 1603 NSP4 using Clustal Omega multiple sequence alignment tool (EMBL-EBI).** The majority of amino-acid variations occurred outside the immunodominant region at position 114-135 (red) of NSP4. The (*) indicates identical residues, whereas (:) and (.) indicates conserved and semi-conserved substitutions, respectively.

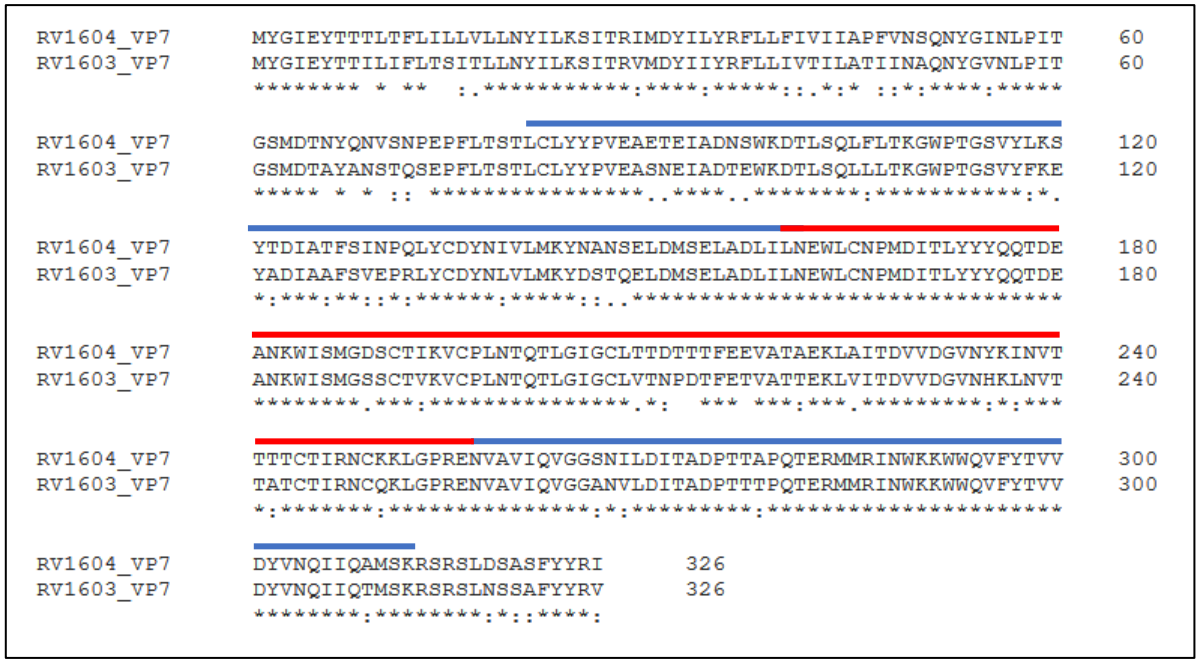


Fig. 3.5. **Sequence alignment of RVA 1604 and 1603 VP7 using Clustal Omega multiple sequence alignment tool (EMBL-EBI).** The majority of amino-acid variations occurred within immunodominant regions I, aa 78-160 and 257-312 (blue), and II, aa 161-256 (red), of VP7. The (*) indicates identical residues, whereas (:) and (.) indicates conserved and semi-conserved substitutions, respectively.

3.3.2 Vaccination of mice

The immunogenicity of rNDV-VP7 and rNDV-NSP4 were investigated in 6-week-old NMRI mice with rNDV La Sota serving as the negative control. Vaccination was done via the oronasal or subcutaneous route where-after a booster dose was administered on day 20. The mice remained asymptomatic throughout the study and displayed no clinical signs associated with Newcastle disease, thereby adding to the safety profile of NDV La Sota in non-avian species (Bukreyev *et al.*, 2005; DiNapoli *et al.*, 2007; Kortekaas *et al.*, 2010a). Euthanasia was

performed on day 20 and 38 via Pentobarbitone overdose where-after blood samples were collected and clarified. The sera from the vaccinated mice (~ 0.5 ml/mouse) were quantitively analysed for overall immunoglobulin responses, IgA-specific responses and virus neutralization capabilities.

3.3.3 Immunofluorescent monolayer assay (IFMA)

To determine the antibody responses induced in vaccinated mice, standard IFMAs were performed. RV-specific antibody titers, irrespective of the isotype of the antibodies, were determined in each mouse as the reciprocal of the corresponding serum dilution where cellular fluorescence was clearly visible. The serum samples (36) were grouped according to vaccine type (rNDV La Sota/rNDV-VP7/rNDV-NSP4), mode of immunization (on/sc) and total number of immunizations received, equalling 3 mice per group. Since the present study was a proof-of-concept which made use of a small number of animals, statistical analysis was not performed. Rather, the potential of rNDV-derived VP7 and NSP4 to induce humoral immunity in the susceptible animal was investigated (Fig. 3.6).

Antibody responses induced by rNDV-VP7 was relatively low compared to that of rNDV-NSP4, irrespective of mode of immunization. This might be explained by the poor expression of VP7 observed *in vitro* or by a lack of antigenic determinants resembling those of the native VP7 protein. Formation of these epitopes and their subsequent recognition by neutralizing and non-neutralizing antibodies are highly dependent on the association of VP7 with other RV proteins. When VP7 was recombinantly expressed by herpes simplex virus-1, it maintained non-neutralizing epitopes while its capacity to induce neutralizing antibodies was lost (Dormitzer and Greenberg, 1992). RV-specific antibody titers induced in rNDV-NSP4 vaccinated mice showed considerable variations within each group but seemed to correlate better with an oronasal mode of administration. A single outlier, belonging to rNDV-NSP4/on, was observed and shown to have an increased antibody titer compared to the rest of the group. It is possible that oronasal administration of the vaccine resulted in its dispersion to pulmonary cavities where it was allowed to spread more efficiently, thereby increasing the expression of NSP4. The F protein of velogenic NDV strains are cleaved by ubiquitous proteases whereas those of lentogenic strains, including NDV La Sota, are cleaved and activated by trypsin-like enzymes that are secreted by a limited number of tissues and organs of avian origin (Fujii *et al.*, 1999). However, such proteases have also been reported in the alveolar fluid of rat lungs (Kido *et al.*, 1992). It is suggested that proteases in the pulmonary cavities of the vaccinated mouse, similar to that found in rat lungs, allowed for the activation and subsequent spread of rNDV-NSP4 *in vivo*. This viral spread would however be self-limiting, since the virus does not have the ability to counteract the innate immunity of mammalian species (Park *et al.*, 2003).

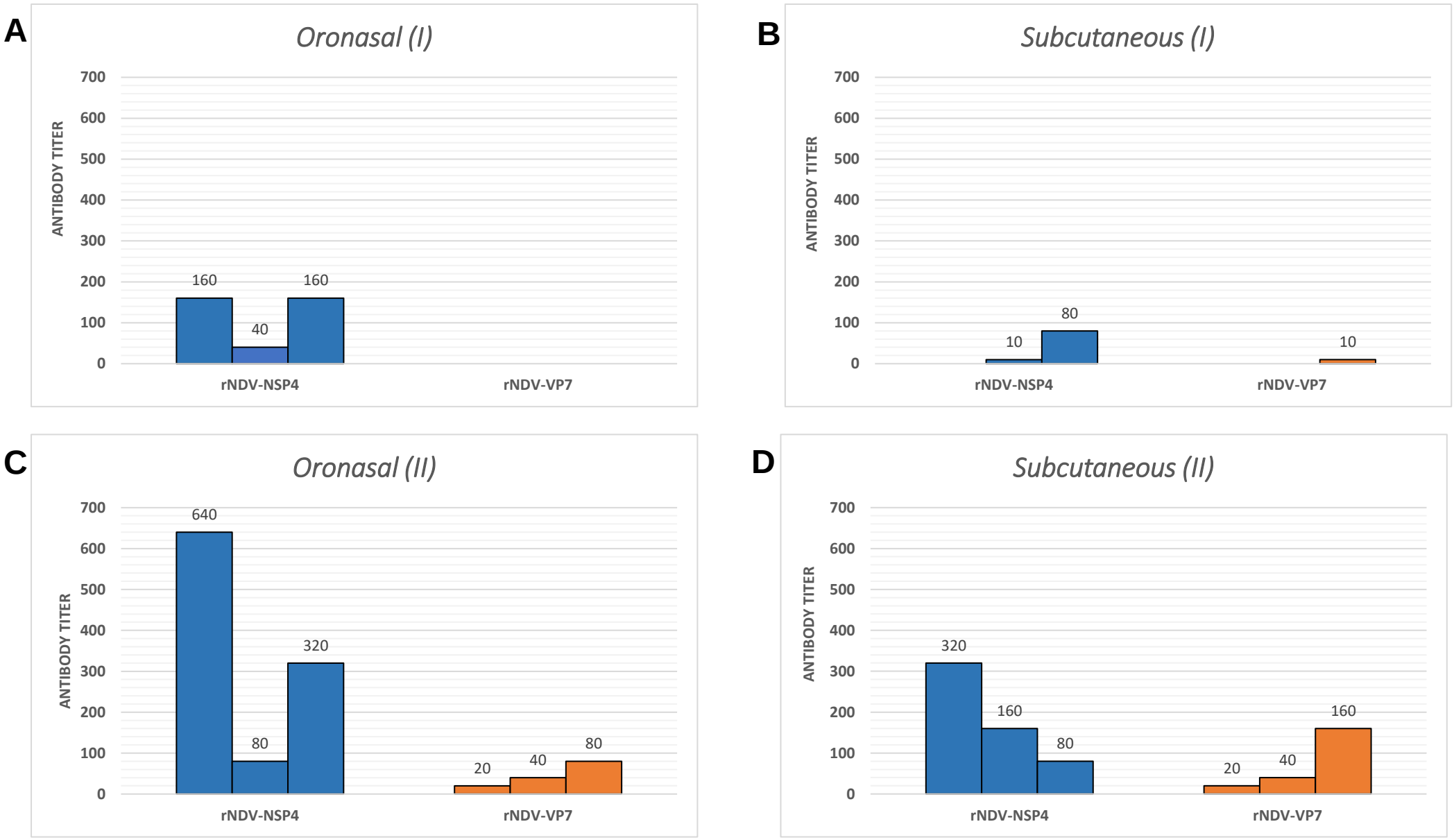


Fig. 3.6. **Evaluation of RV-specific antibody responses induced in mice following first (I) and second (II) immunization with recombinant NDVs, as shown by IFMA.** Mice were divided into groups of three to receive oronasal (A, C) or subcutaneous (B, D) inoculations with rNDV-NSP4 (blue), rNDV-VP7 (orange) or rNDV La Sota. The antibody titer was determined as the reciprocal of the serum dilution where fluorescence was clearly visible. Sera from rNDV LS vaccinated mice were included as the negative control and examined for nonspecific reactions in RV-infected cells (results not shown). Because of the small number of animals utilized during this trial, statistical analysis was not performed.

Interestingly, IFMA showed the serum of vaccinated mice to interact differently with the cell-based antigen, depending on the mode of immunization (Fig. 3.7). It would seem that the serum isolated from mice oronasally injected with the NDV recombinants were not only able to recognize NSP4 localized to the ER but also scattered throughout the cytoplasm, whereas those of mice that received subcutaneous injections only interacted with the protein localized to the ER. However, for the latter, the possibility of nuclear staining can only be excluded with simultaneous staining using DAPI.

NSP4 is a multifunctional protein that has three distinct destinations in a RV-infected cell. These include the ER membrane, the ER Golgi intermediate compartment and the punctate vesicular structures (Au *et al.*, 1989; Zhang *et al.*, 2000; Berkova *et al.*, 2006). In the ER membrane NSP4 functions as a receptor for the budding stage of RV DLPs from the viroplasms into the ER lumen (Au *et al.*, 1989). The transmembrane domain comprises residues 24 – 44 which is oriented in such a way that the N-terminal domain is located in the ER lumen whereas the C-terminal is projected towards the cytoplasm (residues 45 – 175) (Bergmann *et al.*, 1989). The N-terminal half of the protein contains three hydrophobic domains, H1 – H3, with H3 (residues 67 – 80) embedded in the cytoplasmic face of the ER membrane (Bergmann *et al.*, 1989) (Fig. 3.8). Residues 112 – 175, which comprises the enterotoxin domain (residues 144 -135), is cleaved from the protein by an unknown protease and subsequently released from the infected cell via a Golgi-dependent pathway (Srivastava and Jain, 2015). The secreted NSP4 binds to the glycosaminoglycans of neighbouring cells and induces a cellular cascade which is responsible for symptoms of diarrhoea during the early stages of RV infection (Ball *et al.*, 1996; Dong *et al.*, 1997; Didsbury *et al.*, 2011).

It is well documented that NSP4 is cleaved and secreted from RV-infected epithelial cells to induce age-dependent diarrhoea in the gut of a susceptible animal, however, its behaviour in systematic infections is less clear (Ball *et al.*, 1996; Dong *et al.*, 1997). Since antibodies induced in mice subcutaneously vaccinated with rNDV-NSP4 were only able to recognize possible ER-localized NSP4 in RV-infected MA104 cells, it is proposed that the protein behaves differently in different cell types.

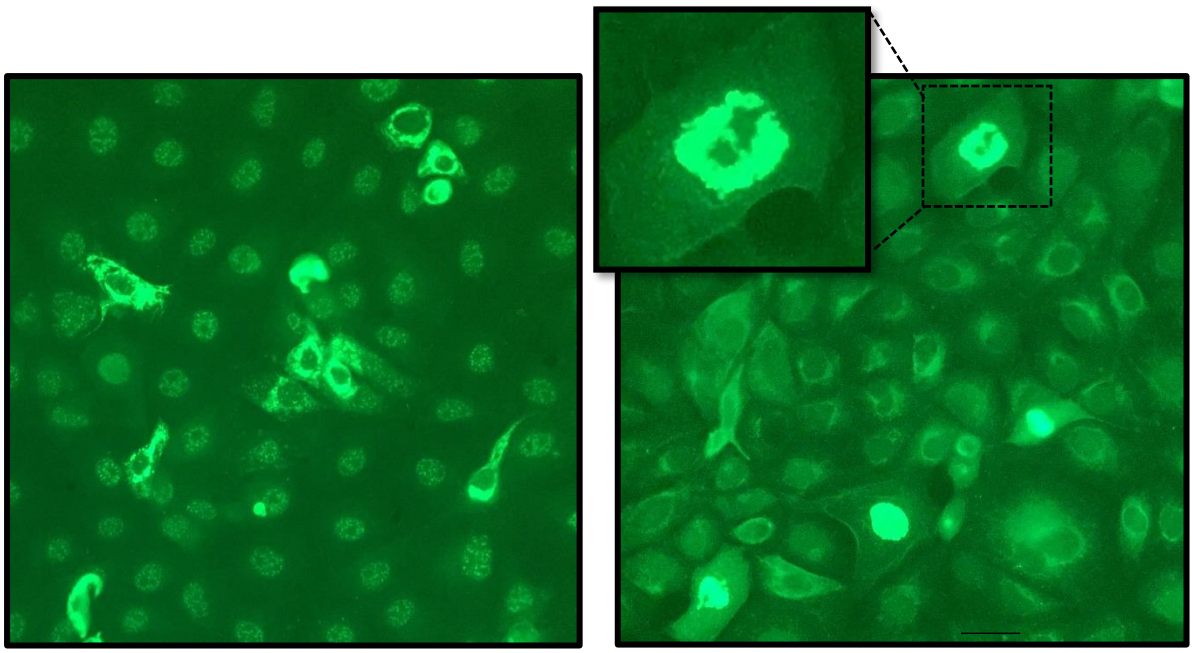


Fig. 3.7. **Immunofluorescent monolayer assay (IFMA) depicting the interaction between RVA 1604/1603-infected MA104 cells and serum obtained from mice vaccinated with rNDV-NSP4 via the oronasal (left) or subcutaneous (right) route.** The enlarged image indicates the strong affinity of anti-NSP4 pAbs from subcutaneously vaccinated mice to ER-localized NSP4.

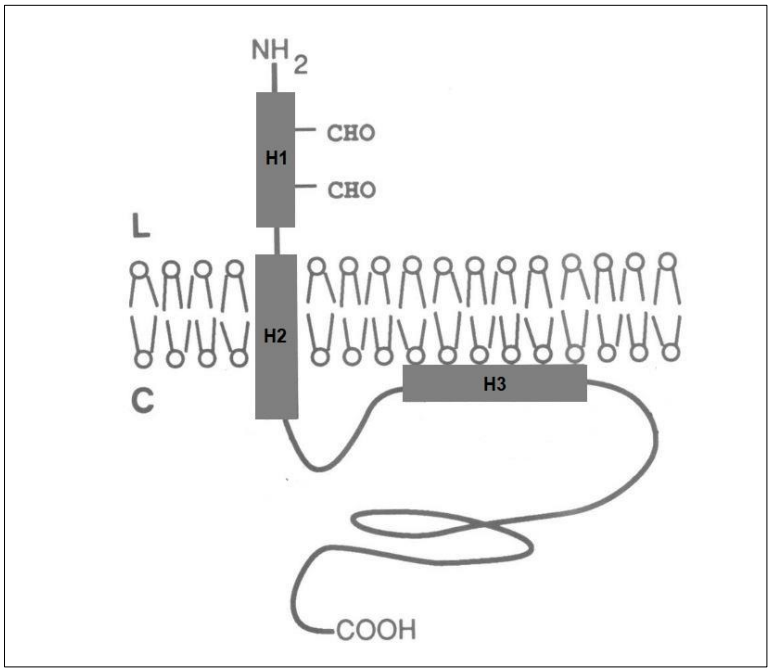


Fig. 3.8**. Schematic diagram illustrating the topology of NSP4 in the ER membrane.** The N-terminal is oriented towards the ER lumen (L) and contains the hydrophobic domains H1, H2 and H3. The latter is associated with the cytoplasmic face of the ER membrane leaving the remainder of the C-terminal residues projecting into the cytoplasm (C) (Taken from Bergmann et al. 1989).

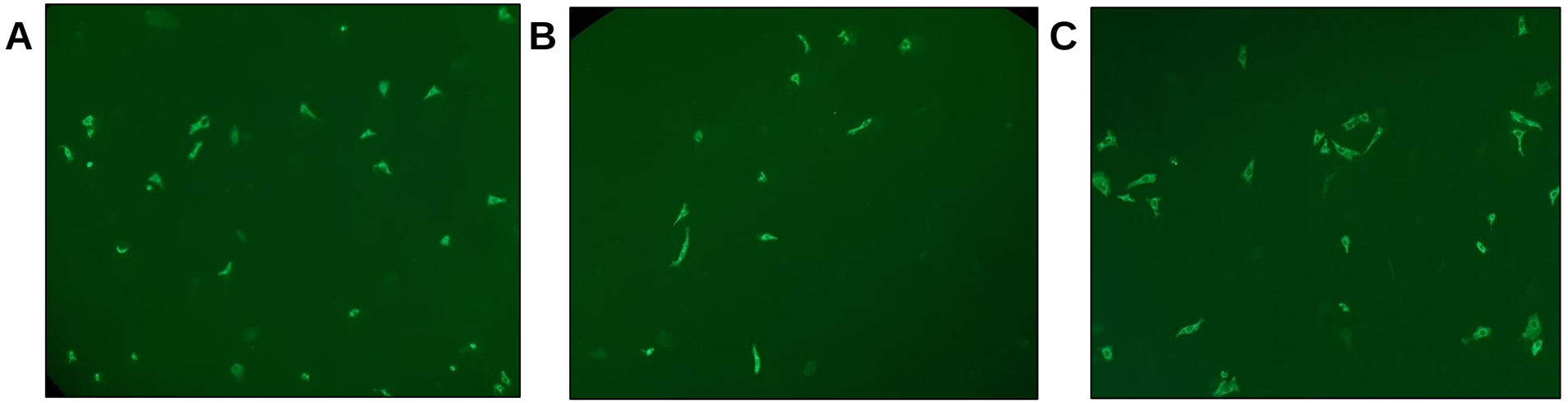
3.3.4 Immunoperoxidase monolayer assay (IPMA)

To determine whether RV-specific IgA antibodies were present in the serum of vaccinated mice, IPMAs were performed using a goat anti-mouse IgA cross-absorbed HRP conjugate. IgA is the most abundant isotype in the body and shown to be critical for protection against RV infection in the intestine (Burns *et al.*, 1995; Blutt *et al.*, 2013). Intestinal IgA can persist for more than a year following initial RV exposure, during which mice are completely protected from reinfection (Burns *et al.*, 1995). However, despite the importance of IgA in the gut of susceptible animals, it plays a minor role in protection against systematic infection. Rather, it is IgG and IgM antibodies that are responsible for clearance of the virus from the bloodstream (Janeway *et al.*, 2001; Blutt *et al.*, 2013). In accordance with the abovementioned, no RV-specific IgA responses were detected in serum of vaccinated mice (results not shown). The results from this study suggest that antibodies directed against NDV-derived NSP4 and VP7 in the serum of vaccinated mice likely consist of IgG and IgM isotypes. For a more comprehensive understanding of the IgA-inducing capabilities of these proteins, future research would need to include evaluation of RV-immune responses in the gut of the vaccinated animal.

3.3.5 Fluorescent focus neutralization assay

Current vaccination strategies against RV infection are aimed at neutralization of the virus by blocking viral attachment to the host cell or by preventing removal of the outer-capsid protein. Antibodies directed against VP7 are capable of both and neutralize the virus either by preventing binding to cellular receptors or by inhibiting Ca^{2+} chelation required for viral uncoating (Ludert *et al.*, 2002; Fleming *et al.*, 2007). Immune responses induced in mice vaccinated with recombinant NDVs were evaluated for their neutralizing capabilities by fluorescent focus neutralization assay (Fig. 3.9). Serum antibodies induced in mice vaccinated with rNDV-VP7 were unable to reduce the rate of RV infection in MA104 cells compared to that of the negative control (rNDV La Sota). This was not surprising considering the low VP7-specific antibody titers induced in rNDV-VP7 vaccinated mice as shown by IFMA. The results also correspond with a loss of neutralizing epitopes when VP7 is expressed in the absence of other RV proteins (Dormitzer and Greenberg, 1992). While antibodies directed against NSP4 are considered to be non-neutralizing *in vitro*, they have been shown to prevent RV-induced diarrhoea by neutralizing the enterotoxicity of the protein (Ball *et al.*, 1996). As was expected, serum antibodies in rNDV-NSP4 vaccinated mice failed to neutralize RV *in vitro*, however, the potential of these antibodies to protect against RV-induced diarrhoea *in vivo* requires further investigation.

Oronasal



Subcutaneous

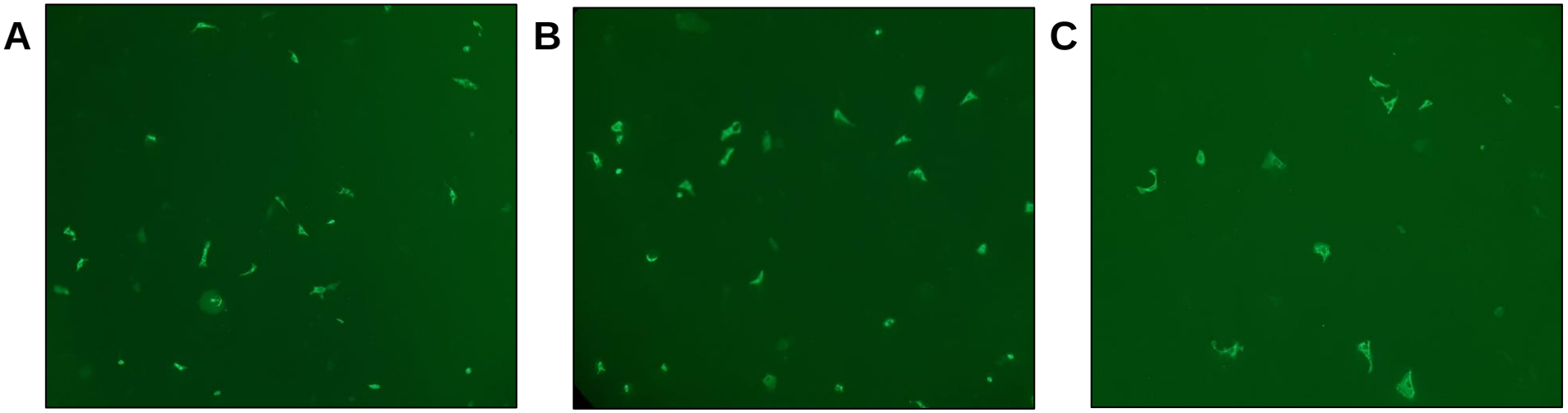


Fig. 3.9**. Evaluation of neutralizing antibodies induced in mice vaccinated with NDV recombinants as shown by fluorescent focus neutralization assay.** Antibodies induced in mice vaccinated with rNDV La Sota (A), rNDV-VP7 (B) or rNDV-NSP4 (C) via the oronasal of subcutaneous route were evaluated for their ability to prevent RV infection of MA104 cells. Neither VP7- or NSP4-specific antibodies were shown to reduce the rate of infection compared to that of the negative control (rNDV La Sota) for either mode of immunization, thereby indicating a lack of neutralizing capability.

3.4 Summary

MA104 cells showed complete CPE following incubation with the VERTREL-extracted RVA 1604-containing stool sample, thereby suggesting the successful adaption of RVA 1604 to cell culture. The quality of the extracted dsRNA from the stool sample and the virus adaption was verified by gel electrophoresis and indicated the characteristic co-migration of genome segments 7, 8 and 9, typically seen for group A rotaviruses.

Sequencing by MiSeq (Illumina, Inc) generated high throughput data, with large numbers of reads generated for both samples. Surprisingly, the sequence data revealed the presence of RVA 1604 and 1603, which was previously undetected by 454® pyrosequencing. The full sets of genome segments for both viruses were detected in stool, however, following adaption to cell culture, a drastic reduction in the number of reads that mapped to S4 of RVA 1603 was observed. This suggests a potential reassortment event in which the genome segment was replaced with that of RVA 1604 during co-infection of the same cell. A silent point mutation was observed in S5 of RVA 1603 following adaption to MA104 cells, whereas the consensus sequences of RVA 1604 remained unchanged. Protein alignment of VP7 and NSP4 of RVA 1604 and 1603 showed a high conservation rate in the immunodominant region of NSP4 but not those of VP7.

Mice vaccinated with recombinant NDVs remained asymptomatic throughout the study with no clinical signs related to Newcastle disease. Antibody responses induced by rNDV-VP7 was significantly lower compared to that of rNDV-NSP4, which might have originated from a loss of antigenic determinants or low expression levels observed for the protein *in vitro*. Whether or not the presence of RVA 1603 had implications on the detection of NDV-derived VP7 and NSP4 immune responses remains to be elucidated. Although antibody responses induced by NDV-derived NSP4 were variable within each group, these titers seemed to be important, especially when administered via the oronasal route. NSP4-specific antibodies responded differently towards RV-infected MA104 cells depending on the route of immunization. Antibodies from mice vaccinated via the oronasal route were able to interact with NSP4 localized to the ER and the cytoplasm whereas those from subcutaneously vaccinated mice only reacted with the former. Whether this was due to a nonconformity of the cleavage of the protein in different cell types, remains unknown. While the results obtained in this study suggested ER and cytoplasmic localisation, localisation to the nucleus can only be excluded through co-staining with DAPI.

It was deduced that the antibodies elicited in the serum of the vaccinated mice likely consisted of IgG and/or IgM isotypes, since no RV-specific IgA antibodies were detected. This was not

surprising since IgA plays a minor role in systematic infections compared to its importance in the gut of a susceptible animal. However, for future studies it would be more beneficial to exam RV-specific IgA responses in the intestine of vaccinated mice. Neither VP7 nor NSP4 had the capacity to induce neutralizing antibodies, which in the case of VP7 can be attributed to a loss of antigenic determinates when expressed in the absence of other RV proteins. NSP4 is not known to induce neutralizing antibodies, however, the potential of rNDV-NSP4 to prevent diarrhoea through neutralization of the enterotoxin, needs to be explored further.

CHAPTER 4 – DISCUSSION AND CONCLUSION

In this study, recombinant NDV was successfully engineered to contain the rotavirus VP7-1 ORF as shown by next-generation sequencing using the Illumina MiSeq NGS platform. The VP7 ORF was inserted as planned at the P-M junction of the NDV La Sota genome, with no single nucleotide changes observed throughout the VP7-1 encoding sequence or at the regions flanking the gene. Regardless, expression of VP7-1 could not be verified by IFMA and western blot analysis using antibodies raised against the whole RV particle. It was suggested that the lack of detection of VP7-1 was due to the misfolding of the protein and its subsequent degradation, since antibodies raised against RV whole virus or peptides resembling VP7 antigenic determinants were able to detect expression of the unaltered VP7 protein.

In a RV infected cell, partially synthesized VP7 is transported to the ER by signal recognition particles (SRPs) which bind to the second hydrophobic region located in first 50 residues of the premature VP7 (pVP7) protein (Whitfeld *et al.*, 1987). Translocation of VP7 across the ER membrane occurs co-translationally. As soon as the hydrophobic signal sequence emerges, it is recognized by the SRPs which also interacts with the ribosome to halt translation. The ribosome-nascent chain-SRP complex is transported to the ER membrane where the SRP interacts with the SRP receptor. Translation recommences while pVP7 is in the process of being translocated across the ER membrane through an aqueous channel that span the membrane (Ng and Walter, 1994). Despite the rapid cleavage of the signal peptide between Ala50 and Gln51, VP7 does not enter the secretory pathway but remains membrane bound (Whitfeld *et al.*, 1987; Stirzaker and Both, 1989).

Recombinant expression of VP7 was previously shown to be highly cytotoxic in cell culture (McCrae and McCorquodale, 1987; Emslie *et al.*, 1995; Pêra *et al.*, 2015; Aschenbrenner, 2017) and speculated to be a result of a rise in intracellular Ca^{2+} levels associated with the retention of VP7 in the ER (Perez *et al.*, 1998; Zambrano *et al.*, 2008). Therefore, in hopes of circumventing the cytotoxic effect of NDV-derived VP7 and consequently elevating its expression in cell culture, the signal sequence of the protein was removed (VP7-1) to prevent transport to the ER. However, from the results of this study, it is clear that prohibition of VP7 translocation to the ER lumen results in misfolding of the protein which might be attributed to the reducing environment of the cytosol and a lack of glycosylation which aids in the protein folding process (Mirazimi and Svensson, 1998). Since VP7-1 was only detected in the presence of protease inhibitors, it was further suspected that the misfolded protein was fated for degradation by lysosomes, and not subjected to the ubiquitin proteasome pathway which constitutes the major degradation pathway of eukaryotic cells (De Duve *et al.*, 1953). Because

the removal of the signal sequence resulted in the misfolding and consequent degradation of VP7, it is clear that rNDV-VP7-1 is an unsuitable vaccine candidate against RV infection and did not require further evaluation *in vivo*. While VP7 was poorly detected by IFMA, western blot analysis showed ample expression levels of the protein in cell culture. This was probably due to the use of antibodies directed against predicted linear epitopes of VP7, which was not exposed on the surface of the folded protein.

Significant levels of NSP4 expression was detected in either cell line and associated with cytoplasmic vesicular structures known as puncta. The formation of puncta is dependent on intracellular Ca^{2+} levels and the co-localization of NSP4 with the autophagic marker, LC3. In RV-infected cells, these NSP4-induced autophagosome-like vesicles form cap-like structures that surround the viroplasms, the primary site of RV replication and assembly (Berkova *et al.*, 2006). NSP4 has been shown to induce autophagy which enables ER-localized VP7 and NSP4 to be transported to the viroplasms. Here, at the viroplasm-NSP4/LC3 interface, an interaction between NSP4 and the immature virus particles triggers budding of the DLPs into the ER and their subsequent acquirement of the outer-capsid protein (Estes and Kapikian, 2007). Interestingly, the formation of puncta is also shown to suppress autophagy maturation and its antiviral function by preventing the fusion of viroplasms with lysosomes (Crawford *et al.*, 2012). Puncta is critical for RV replication and maturation and was shown in the current study to be induced by NDV-derived NSP4 in the absence of other RV proteins.

To generate viral stocks for cell-based immunological studies, RVA/Cow-wt/ZAF/1604/2007/G8P[1], which was concurrently isolated with two other bovine RVA strains from a farm in the Western Cape, was adapted to cell culture (Jere *et al.*, 2012). Sequencing revealed the isolated stool sample and the cell culture adaption to contain RVA 1604 and 1603, which was previously undetected by 454® pyrosequencing. The viruses have similar genome constellations except for S4 (VP4) and S9 (VP7), which are G8P[1] and G6P[5] for RVA 1604 and 1603, respectively. The sequencing data suggested a major reassortment event in which RVA 1603 S4 was replaced with that of 1604. Whether reassortment occurred before or after adaption to cell culture remains unknown. It was suggested that the attachment efficiency mediated by a P[1] type, served more beneficial for propagation in MA104 cells than that of type P[5]. The VP4 subunit, VP8*, is essential for viral attachment to the host cell and is shown to recognize carbon moieties on the cell surface (Dormitzer *et al.*, 2002). The incorporation of sialic acid (SA), including N-acetyl- and N-glycolylneuraminic acid, into cellular receptors is important for RV attachment, since treatment with sialidase has been shown to reduce the infectivity of the virus (Isa, *et al*., 2006). It has been proposed that recognition of SA-containing receptors by VP8* determines, at least to some extent, the age, host and tissue

specificities of RV infection. However, not all RVs are dependent on the presence of SA to mediate successful penetration into the host cell and are termed sialidase insensitive strains (Delorme *et al.*, 2001). It was shown that all RVs, irrespective of origin, belonging to P types [1], [2], [3] and [7] were sensitive to treatment with sialidase and those belonging to other P types were not. This observation did not alter with different VP4-VP7 combinations in different reassortant strains (Ciarlet *et al.*, 2002). Therefore, the attachment efficiency mediated by RVA 1604 type P[1] VP4 might be directly linked to its recognition of SA contained in the cellular receptors of MA104 cells. Adaption to cell culture did not result in nucleotide changes to the genotype of RVA 1604, whereas a silent point mutation was observed in S5 (NSP1) of RVA 1603. Genome segment 10 (NSP4) showed a high conservation rate between the two viruses, whereas, S9 (VP7) showed a number of variations in immunodominant regions. This was not surprising, considering the different G genotypes of the viruses.

Immunological studies of rNDV-VP7 and rNDV-NSP4 was performed in mice which provides a reliable model for studying the correlates of immunity involved in RV infection (Knipping *et al.*, 2011; Xie *et al.*, 2015). Mouse immunized with recombinant NDVs showed no indication of disease, which corresponded with literature regarding the safety of the vaccine in non-avian species (Bukreyev *et al.*, 2005; Kortekaas *et al.*, 2010a). Since the F protein of lentogenic NDV strains can only be cleaved by proteases secreted by a limited number of tissues, the virus undergoes a single round of replication in the vaccinated animal and is therefore prevented from spreading to uninfected neighbouring cells (Gotoh *et al.*, 1992).

The immune responses elicited in rNDV-VP7 vaccinated mice were low for either mode of immunization and correlated with a loss of immunogenic determinants when VP7 is expressed in the absence of other RV proteins. Likewise, rNDV-VP7 failed to induce neutralizing antibodies, which is the cornerstone of current vaccination strategies. These results, in combination with the cytotoxic effect of recombinantly expressed VP7 in cell culture, suggest NDV-derived VP7 to be an unsuitable vaccine candidate against RV infection.

Immune responses induced in rNDV-NSP4 vaccinated mice correlated better with an oronasal route of administration. The recognition of NSP4 in RV infected cells by serum antibodies was different depending on the route of immunization. Antibodies from mice vaccinated via the oronasal route recognized NSP4 localized to the ER and scattered throughout the cytoplasm, whereas antibodies from subcutaneously vaccinated mice only recognized the former. It is proposed that the increased immune response observed in oronasal vaccinated mice was due to the cleavage and secretion of the NSP4 active signal peptide (aa 112 – 175) in the gut of the susceptible animal (Dong *et al.*, 1997). The signal peptide comprises the enterotoxin

domain (aa 114 – 135) which is also shown to be highly immunogenic (Arakawa *et al.*, 1998; Yu and Langridge, 2001).

RV NSP4 is a multifunctional protein which plays a key role in RV morphogenesis and pathogenesis (Au *et al.*, 1989). The protein is responsible for fluid accumulation in the small intestine during the early stages of RV infection, before any histological disruption to the intestinal lining is observed (Ball *et al.*, 1996). Secretory NSP4 induces diarrhoea by binding to apical receptors which triggers a PLC-IP_3 cascade that releases Ca^{2+} from internal stores (Dong *et al.*, 1997). NSP4 is an excellent target for vaccination strategies since antibodies directed against NSP4 aa 114 – 135 has been shown to neutralize the enterotoxicity of the protein, thereby reducing the severity and duration of diarrhoea. The lack of diarrhoeic symptoms in rNDV-NSP4 vaccinated adult mice corresponds with the notion that enterotoxicity of NSP4 is age-dependent (Ball *et al.*, 1996).

Current vaccination strategies against RV infection in bovine are based on a passive immunization approach of pregnant dams with inactivated RV particles for the induction of neutralizing antibodies. The neonate receives the neutralizing antibodies through consumption of colostrum which confers protection from subsequent RV disease (Holland, 1990). While antibody titers induced by inactivated RV particles were promising in experimental conditions, protection was lacking in field trails, which might be attributed to circulating field strains with serotypes different to that of the vaccine (Kim *et al.*, 2002).

While antibodies directed against NSP4 are non-neutralizing, in that they do not prevent viral infection, they are shown to have heterotypic reactivity (Ray *et al.*, 2003). Therefore, anti-NSP4 antibodies might neutralize the enterotoxicity of a broad range of NSP4 serotypes and prevent subsequent diarrhoea. In addition, NSP4 acts as an adjuvant for a co-administered antigen, which includes a fusion protein between NSP4 and VP6 and non-RV proteins such as keyhole limpet hemocyanin (KLH) and tetanus toxoid (Kavanagh *et al.*, 2010; Afchangi *et al.*, 2017). The results of this study indicate the potential for the inclusion of rNDV-NSP4 in a combination vaccine which might reduce severity of RV-related diarrhoea in new-born calves and serve to boost the immune responses to a co-administered antigen. Future considerations should include the examination of immune responses induced by NDV-derived NSP4 in the gut of the susceptible mice. Furthermore, the potential of passively transferred anti-NSP4 antibodies to protect suckling mice from diarrhoea, should also be evaluated.

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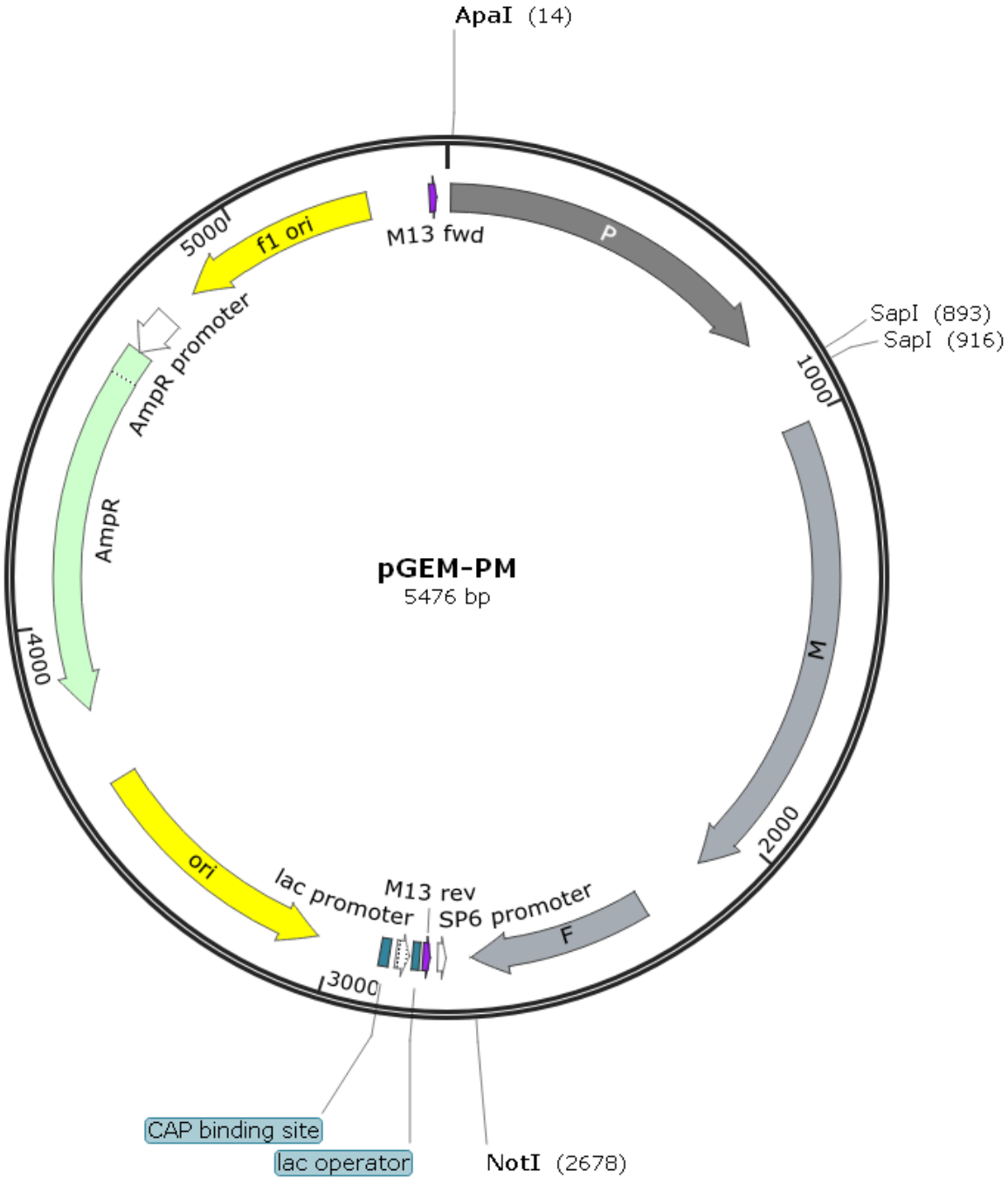
A. APPENDIX

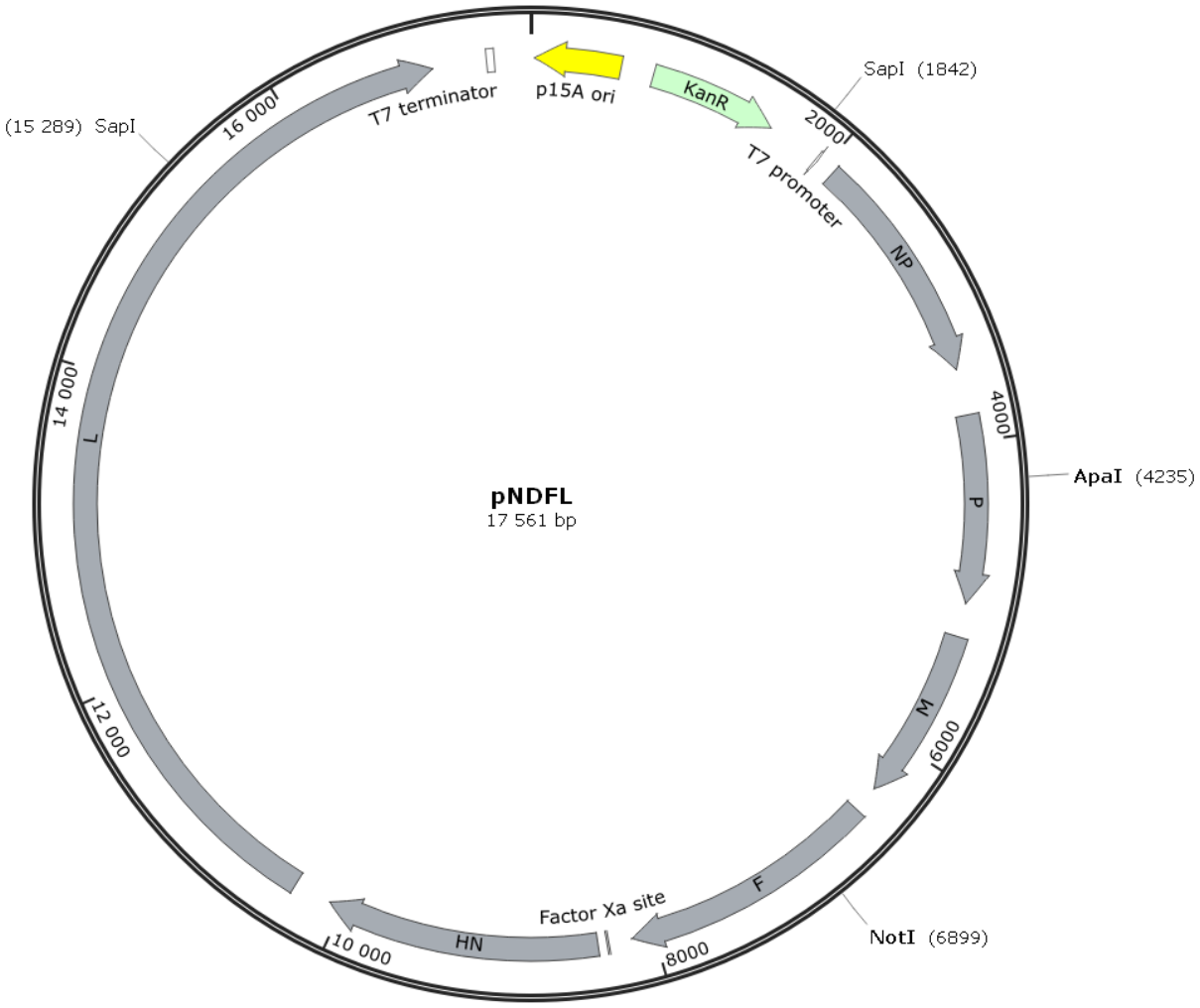
A1. Optimization of VP7-1 ORF in *Bos Taurus* using the OptimumGene™ Codon Optimization Analysis Tool from GeneScript

```
Optimized     1   ATGCAGAACTACGGCATCAACCTGCCCATCACCGGCAGCATGGACACCAACTACCAGAAC
Original      1   ATGCAGAACTACGGCATTAATTTACCGATAACGGGGTCAATGGATACAAATTATCAAAAT

Optimized    61   GTGAGCAACCCCGAGCCTTTCCTGACCTCCACCCTGTGCCTGTACTACCCTGTGGAGGCC
Original     61   GTATCTAATCCAGAACCGTTTTTAACATCAACGCTATGCTTATATTACCCAGTGGAAGCA

Optimized   121   GAGACCGAGATCGCCGACAACAGCTGGAAGGACACCCTGTCCCAGCTGTTCCTGACCAAG
Original    121   GAGACGGAGATAGCAGACAATTCGTGGAAGGATACTTTATCGCAGTTGTTCTTGACAAAA

Optimized   181   GGATGGCCTACCGGAAGCGTGTACCTGAAGTCCTACACCGACATCGCCACCTTCTCCATC
Original    181   GGATGGCCAACTGGTTCCGTTTATCTTAAGAGCTACACAGATATTGCAACTTTTTCAATA

Optimized   241   AACCCTCAGCTGTACTGCGACTACAACATCGTGCTGATGAAGTACAACGCCAACAGCGAG
Original    241   AATCCGCAACTATATTGTGATTATAACATAGTGTTGATGAAATATAATGCCAATTCAGAA

Optimized   301   CTGGACATGTCCGAGCTGGCCGACCTGATCCTGAACGAGTGGCTGTGCAACCCCATGGAC
Original    301   CTGGATATGTCAGAATTAGCCGATTTGATACTTAATGAATGGCTTTGTAACCCAATGGAC

Optimized   361   ATCACCCTGTACTACTACCAGCAGACCGACGAGGCCAACAAGTGGATCAGCATGGGCGAC
Original    361   ATAACATTGTACTACTATCAACAAACGGATGAAGCGAACAAATGGATATCAATGGGAGAT

Optimized   421   TCCTGCACCATCAAGGTGTGCCCTCTGAACACCCAGACCCTGGGCATCGGCTGCCTGACC
Original    421   TCATGTACGATTAAGGTATGTCCGCTGAATACACAGACATTAGGAATTGGTTGTCTTACC

Optimized   481   ACCGACACCACCACCTTCGAGGAGGTGGCTACCGCTGAGAAGCTGGCCATCACCGACGTG
Original    481   ACGGATACTACGACTTTTGAAGAAGTTGCAACAGCTGAAAAATTGGCAATAACGGACGTT

Optimized   541   GTGGACGGCGTGAACTACAAGATCAACGTGACCACCACCACCTGCACCATCCGGAACTGC
Original    541   GTCGATGGAGTAAATTATAAGATAAATGTAACGACAACTACATGTACTATTAGAAATTGT

Optimized   601   AAGAAGCTGGGACCCCGCGAGAACGTGGCCGTGATCCAAGTGGGCGGCAGCAACATCCTG
Original    601   AAGAAATTGGGACCACGAGAGAATGTTGCAGTTATACAAGTAGGTGGTTCAAATATATTA

Optimized   661   GACATCACCGCTGACCCTACCACCGCTCCTCAGACCGAGCGCATGATGAGAATCAACTGG
Original    661   GATATTACGGCAGACCCAACAACAGCACCGCAAACAGAAAGAATGATGAGAATAAATTGG

Optimized   721   AAGAAGTGGTGGCAGGTGTTCTACACCGTGGTGGACTACGTGAACCAGATCATCCAGGCC
Original    721   AAGAAATGGTGGCAAGTTTTTTACACAGTCGTTGACTACGTAAATCAAATAATTCAAGCA

Optimized   781   ATGTCCAAGCGGAGCAGGTCCCTGGACAGCGCCTCCTTCTACTACCGGATC
Original    781   ATGTCCAAGAGATCACGGTCATTGGATTCAGCATCATTTTATTATAGAATT
```

A2. Plasmid map of pGEM-PM shuttle vector



A3. Plasmid map of pNDFL



A4. Ethical clearance from UFS Animal Research Ethics Committee

Animal Research Ethics

02-Jul-2018

Dear Miss Larise Oberholster

Student Project Number: UFS-AED2018/0028

Project Title: Evaluation of recombinant Newcastle Disease Viruses (NDV) as candidate vaccine delivery vectors for rotavirus VP7 and NSP4 proteins in mice

Department: Microbial Biochemical and Food Biotechnology (Bloemfontein Campus)

You are hereby kindly informed that, at the meeting held on , the Interfaculty Animal Ethics Committee approved the above project.

Kindly take note of the following:

1.
A progress report with regard to the above study has to be submitted Annually and on completion of the project. Reports are submitted by logging in to RIMS and completing the report as described in SOP AEC007: Submission of Protocols, Modifications, Amendments, Reports and Reporting of Adverse Events which is available on the UFS intranet.

2.
Researchers that plan to make use of the Animal Experimentation Unit must ensure to request and receive a quotation from the Head, Mr. Seb Lamprecht.

3.
Fifty (50%) of the quoted amount is payable when you receive the letter of approval.

Yours Sincerely

Mr. Gerhard Johannes van Zyl
Chair: Animal Research Ethics Committee

A5. Ethical clearance from Deltamune Ethical committee

APPROVAL IS HEREBY GRANTED BY THE ETHICS COMMITTEE OF DELTAMUNE (PTY) LTD FOR THE USE OF SENTIENT ANIMALS FOR:

Project title/ Study number:	Engineering a recombinant Newcastle Disease Virus (NDV) as a candidate vaccine delivery vector for rotavirus		
Ethics committee protocol number:	O-18-08	**Study category: (A – F)**	C
Project Leader/ Study Director:	H.G O'Neill & Prof. M. Smit		
Experimental starting date:	2018	**Planned completion date:**	2018
Requested by:	H.G O'Neill	**Received by:**	Leoni Smit

ANIMAL INFORMATION

SPECIES	**Mice**				
BREED OF ANIMALS	NMRI	**AGE:**	6 weeks	**WEIGHT**	N/A
TOTAL NUMBER ANIMALS	48	**MALE:**	NA	**FEMALE:**	48

This approval is valid for .....3..... years from the date of commencement and is give, based on the fulfilment of the following additional requirements:

Comments:
Ethics approval from UFS required before commencement of study
.....

APPROVAL BY CHAIRPERSON OF DELTAMUNE ETHICS COMMITTEE

I.M. Wright		05/04/2018
NAME	**SIGNATURE**	**DATE**

APPROVAL BY REPRESENTATIVE FROM NSPCA

L. Smit		2018/04/26
NAME	**SIGNATURE**	**DATE**

A6. Section 20 ethical clearance

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/9

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E-mail: christiaan@Deltamune.co.za; OneillHG@ufs.ac.za

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Dear Prof O'Niell

Your application dated 4 April 2018 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Title of research/study: Evaluation of recombinant Newcastle disease viruses (NCD) as candidate vaccine delivery vectors for rotavirus VP7 and NSP4 proteins in mice.
Researcher: Prof Hester Gertruida O'Neill; Prof Christiaan Potgieter
Institution: DAFF approved BSL 3 laboratory (DAFF-C01) at Deltamune Roodeplaat;
Permit Expiry date: 31 August 2018
Our ref Number: 12/11/1/1/9 (758KL)
Your ref: O-18-08

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2018 -04- 2 6

A7. Nucleotide differences between consensus sequences obtained for RVA 1604 and 1603 from stool and reference sequences obtained from GenBank

A

Gene segment	Nucleotide differences	
	RVA 1604	RVA 1603
S9	—	(T→C)*795; (C→A)*796
S4	—	(G→A)*336
S6	—	—
S1	—	(A→T)*2274; (G→A)*3123
S2	(Y→C)*2245	(A→G)*1405
S3	(Y→T)*1699; (R→A)*1873; (Y→T)*1913; (R→A)*2005; (R→A)*2269; (R→A)*2275	(C→T)*1279
S5	(T→C)*422	DEL: (T)*11
S8	—	—
S7	(T→G)*907	—
S10	DEL: (A)*11	(G→A)*519
S11	—	—

B

Genome segment	Non-synonymous mutations	
	RVA 1604	RVA 1603
VP7	—	(Gln→Lys)*250
VP4	—	—
VP6	—	—
VP1	—	—
VP2	—	(Iso→Met)*463
VP3	(X→Tyr)*622	—
NSP1	—	—
NSP2	—	—
NSP3	(Ser→Arg)*294	—
NSP4	—	(Glu→Lys)*160
NSP5	—	—

- The position of the nucleotide change is indicated by (*n)
- Underlined values in Table A correspond to non-synonymous mutations in Table B