ABSTRACT

Title of Document: IMPROVED NEWCASTLE DISEASE VIRUS VACCINES AND VECTORS

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Newcastle disease (ND) is an economically important disease of poultry worldwide. The use of vaccines to control ND is necessary because of frequent outbreaks of the disease in enzootic countries. The fusion (F) and hemagglutinin-neuraminidase (HN) proteins of Newcastle disease virus (NDV) are multifunctional proteins that play critical roles during infection. The F protein of NDV is a type I membrane glycoprotein that mediates the fusion of viral envelope to the host cell membrane. The F protein activation initiates a series of conformational changes in the F protein leading to virus-cell membrane fusion, which occurs at the cell surface at neutral pH thus modulating NDV entry and spread.

In the present study, we investigated the role of tyrosine to alanine mutation at amino acid position 524 and 527 in the F protein cytoplasmic tail (CT) of NDV strain LaSota by using reverse genetic techniques. Our results suggest that tyrosine residues at

524 and 527 position of F protein CT domain play a major role in fusogenicity and in replication thus modulating NDV infectivity. The F protein is synthesized as an inactive precursor, F_0, which is functionally activated after cleavage by host cell proteases into F_1 and F_2 polypeptides, linked by disulfide bonds. The amino acid sequence surrounding the F protein cleavage site determines the virulence of NDV. We also studied the role of other avian paramyxovirus fusion protein cleavage site sequences in F protein cleavage of NDV strain Banjarmasin. This study has helped us to understand the requirement of F protein cleavage site in proteolytic processing, plaque formation and virus infectivity. Further, the role of these F cleavage site mutant viruses as genotype-matched vaccines for virulent NDV infection has been explored.

Reverse genetics has also been used to develop NDV strains as a potential vaccine vectors for various human and animal pathogens, such as highly pathogenic avian influenza (H5N1), human immunodeficiency virus, severe acute respiratory syndrome coronavirus, ebola virus, respiratory syncytial virus and human parainfluenza virus type 3. NDV has several characteristics that makes it a suitable candidate for vaccine vector development. It is safe in humans and animals due to natural host range restriction, expresses foreign protein abundantly, infects via intranasal route, produces both humoral and mucosal immune responses, is antigenically distinct from human and animal pathogens, and lack of preexisting immunity to NDV in humans and animals.

In one vaccine trial with non-human primates, the mesogenic NDV strain Beaudette C (BC) replicated to a high titer and induced a substantially higher antibody

response compared to the lentogenic strain LaSota, and thus appeared to be more effective. However, NDV strains that have a polybasic cleavage site in the F protein and an intracerebral pathogenicity index (ICPI) >0.7 have been classified as Select Agents. Most mesogenic strains, including strain BC, fall into this category and therefore cannot be handled in BSL-2 conditions.

In this study, we constructed a series of recombinant (rNDV) vectors containing the cleavage site sequence of avirulent strain LaSota and other avian paramyxoviruses, together with various regions of the F protein exchanged between NDV strains AKO-18 and BC. We used these modified rNDV vectors to express SIV gp160 envelope protein and immunized guinea pigs. Our results showed that rNDV/SIV vaccines were immunogenic and effectively neutralized SIV mac251 strain *in vitro*. These results support the idea of the use of NDV as a vaccine vector for expression of SIV immunogens capable of inducing neutralizing antibodies against diverse SIV strains, thus providing an improved vaccine vector platform for ultimately testing the NDV vectored vaccines in non-human primates and humans.

IMPROVED NEWCASTLE DISEASE VIRUS VACCINES AND VECTORS

By

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Dissertation submitted to the Faculty of the Graduate School of the
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Dedication

I would like to dedicate this work to my parents and my brother for their love and support.

Acknowledgements

I express my sincere gratitude to my major advisor, Dr. Siba K. Samal, for giving me the opportunity to work in his laboratory and for his excellent mentorship. I would like to thank my advisory committee members Dr. Jeffery DeStefano, Dr. Xiaoping Zhu, Dr. Yanjin Zhang and Dr. Georgiy A. Belov for their valuable time and support all through the study. I would like to acknowledge the excellent support I received from Dr. Sunil Khattar, Dr.Shin-Hee Kim and Dr. Anandan Paldurai for my research and writing. I feel indebted to Dr. Laura Sanglas for her assistance, encouragement and emotional support during my graduate research work. I thank my previous lab members, Dr. Sweety Samal and Dr. Sa Xiao and all of my current colleagues Edris Shirvani and Berin Varghese for creating a healthy working environment in the laboratory. Special thanks to Mr. Daniel Rockemann and Ms. Peggy Barott for their love, kindness and encouragement throughout my study.

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Abbreviations

aa	amino acid
APMV	avian paramyxovirus
APMV-2	avian paramyxovirus serotype 2
APMV-7	avian paramyxovirus serotype 7
APMV-8	avian paramyxovirus serotype 8
bp	base pair
BC	Beaudette C
BSL	Bio Safety Level
cDNA	complementary DNA
CPE	cytopathic effect
Da	Daltons
DF-1	chicken embryo fibroblast cell line
DMEM	Dulbecco's modified Eagles medium
DNA	deoxyribonucleic acid
ELISA	enzyme linked immunosorbent assay
F protein	fusion protein
FBS	fetal bovine serum
GE	gene-end
GFP	green fluorescent protein
GS	gene-start
HA	hemagglutination
HI	hemagglutination inhibition
HN	hemagglutinin-neuraminidase
HPIV-2	human parainfluenza type 2
ICPI	intracerebral pathogenicity index
IGS	intergenic sequence
kDa	kilo Daltons
L protein	large polymerase protein
M protein	matrix protein
mRNA	messenger RNA
MDT	mean death time
MVA-T7	recombinant modified vaccinia strain Ankara expressing T7
MOI	multiplicity of infection
NA	neuraminidase
NDV	Newcastle disease virus
nm	nanometer
N protein	nucleocapsid protein
nt	nucleotide
ORF	open reading frame
P protein	phosphoprotein

PBS	phosphate buffer saline
PCR	polymerase chain reaction
PFU	plaque forming unist
PI	Post-infection
RBC	red blood cell
RER	rough endoplasmic reticulum
RNA	ribonucleic acid
RNP	ribonucleocapsid protein
RT-PCR	reverse transcription PCR
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPF	specific pathogen free
$TCID_{50}$	median 50% Tissue culture infectious dose
UTR	untranslated region
VLP	virus like particle

Chapter 1: General Introduction

1.1 Newcastle disease

Newcastle disease (ND) is a highly contagious disease of chickens that affects many of the domesticated and wild avian species, leading to severe economic losses in the poultry industry all over the world (4, 5, 84). This disease is enzootic in Asia, Africa, Middle East, and some countries of North and South America. The severity of ND outbreaks depends on the virulence of the infecting virus and host susceptibility. In the United States, the virulent form of Newcastle disease (vND) is absent and occurrence of the vND is reportable and may result in trade restrictions. Cormorants, pigeons, and imported psittacine species are more commonly infected with vND and have also been sources of vNDV infections of poultry. There was an outbreak of highly vND in commercial poultry during 2002-2003, originated from illegally imported game fowl, which required $180 million in federal funds for eradication by depopulation of infected poultry (47).

ND is caused by Newcastle disease virus (NDV). NDV was first identified in Java, Indonesia, in 1926, and in the same year, in Newcastle, England, where the virus got its name. NDV is a member of the genus *Avulavirus,* in the family *Paramyxoviridae,* in the order *Mononegavirales* (60). The genome of NDV is a nonsegmented, single-stranded, negative-sense RNA. The genome contains six genes, which encode the nucleocaspid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and large RNA-dependent RNA polymerase

protein (L). The NDV genes are arranged on the genomic RNA in the order 3′-N-P-M-F-HN-L-5′ (84).

NDV strains are categorized into three major pathotypes depending on the severity of the disease in chickens; velogenic (highly virulent), mesogenic (moderately virulent) and the lentogenic (avirulent) strains (5). The velogenic strains cause severe disease in chickens with mortality rate reaching upto 100%. The velogenic strains are further classified into viscerotropic velogenic and neurotropic velogenic. The viscerotrpoic velogenic strain causes hemorrhagic lesions in the gastrointestinal tract; whereas the neurotropic velogenic strains causes nervous signs like paralysis and paresis of the legs and torticollis. The mesogenic strains are of intermediate virulence and cause moderate respiratory signs with occasional nervous signs, while the lentogenic strains cause mild to in apparent infections (5).

The genome of the NDV contains a 55 nucleotides (nt) long viral promoter known as leader at its 3’ end and a 114 nt long viral antigenome promoter known as trailer at its 5’ end (57). The leader and trailer regions play an important role in viral replication and packaging of viral RNA (60). There are conserved sequences present at the beginning and end of each gene, referred as gene-start (GS) and gene-end (GE) signal sequences. The GS sequences are recognized as a transcription initiation signal and the GE sequences are recognized as transcription termination signals by the viral RNA polymerase. Between each gene, non coding sequences known as intergenic sequences (IGS) of variable length

from 1 to 53 nt are present which are shown to play a role in regulation of viral mRNA transcription (96).

NDV has an RNA editing mechanism, wherein a RNA editing site in the P gene directs non-templated addition of one or more G residues to P mRNA by viral polymerase stuttering during transcription. This yields mRNA subpopulations with frame shifts and consequent alternate internal ORFs. Two non-structural proteins, V and W, are produced by one and two G insertions, respectively. The V protein has a highly conserved cysteine rich motif and has interferon antagonist activity (43). The nucleocapsid protein (N) and the genomic RNA together form a core structure, to which the phosphoprotein (P) and the large polymerase protein (L) are attached (59). These three proteins form the transcriptive-replicative complex, which is the minimum infectious unit of NDV. The F and HN proteins are surface glycoproteins in the envelope of the virus. The F mediates fusion of the viral envelope with the host cell membrane, thus mediating entry of the viral genome into the host cell cytoplasm. The HN acts like a docking protein that helps the attachment of NDV to sialic acid receptors on the host cell. The V protein of NDV functions as an alpha interferon antagonist (43) and also a major determinant of host range restriction (72) . The function of the W protein is unknown (59). NDV is also a known oncolytic virus that can target and replicate in certain types of tumor cells compared to normal healthy cells. Preclinical and phase I/II studies with NDV as an oncolytic agent have been conducted with human subjects and have shown promising results (31, 58).

Currently, live attenuated lentogenic strains like the Hitchner B1 (41) and LaSota (35) are used as vaccines all over the world. Although these strains are able to prevent the mortality associated with the disease, ND outbreaks occur in different parts of the world depending on the environmental conditions and stress. Hence, there is a need for a highly stable and effective vaccine.

An important application of reverse genetics is the generation of recombinant viruses for vaccine development. NDV has several characteristics that make it suitable for vaccine vector development. It is safe in humans and animals due to natural host range restriction, expresses foreign protein abundantly, infects via the intranasal route, and produces both humoral and mucosal immune responses, is antigenically distinct from human and animal pathogens and lack of preexisting immunity to NDV in humans and animals. Immunogenicity and protective efficacy of lentogenic strains of NDV as vaccine vectors against human and animal pathogens have been tested in various studies

Using the reverse genetic system, we have investigated the role of tyrosine to alanine mutations in the cytoplsamic tail of NDV F protein. We have further studied the effectiveness of other avian paramyxovirus fusion protein cleavage sites (APMV FPCS) in generating genotype matched vaccines for NDV. Additionally, we also evaluated the efficacy of modified rNDVs as vaccine vectors by expressing simian immmno-deficiency virus (SIV) envelope glycoprotein 160 (gp160).

1.2 Research objectives

The specific objectives of the present study were:

1. To understand the role of tyrosine to alanine mutations in the cytoplsmic tail of F protein in NDV strain LaSota.
2. To investigate the effects of other APMV FPCS in NDV to generate genotype matched vaccines.
3. To study the effectiveness of modified rNDVs as vaccine vectors.

Chapter 2: Review of Literature- NDV virology and vaccine vector development

2.1 Classification

NDV is a single-stranded, negative-sense RNA ((-)ssRNA) virus, belong to the order *Mononegavirales*, the family *Paramyxoviridae*, the subfamily *Paramyxovirinae* and the genus *Avulavirus* (84). The genus *Avulavirus* consist of paramyxoviruses particularly isolated from avian species. These viruses have been classified into 13 serotypes (APMV serotypes 1 through 13) based on hemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays (2, 91). All NDV strains come under *Avian avulavirus 1 or* Avian paramyxovirus 1 (APMV-1). Other important members in the family *Paramyxoviridae* are the mumps virus (MuV), measles virus (MeV), peste-des-petits-ruminants virus (PPRV), simian virus 41 (SV-41) and parainfluenza virus (PIV).

2.2 Virion

The NDV particles are pleomorphic and ranges between 100-400 nm in diameter. The virions contain a dense, centrally packed viral genomic RNA tightly associated with N, P and L proteins. The envelope is covered with spike glycoproteins of 8-12 nm. The genome of NDV is a single strand of RNA of negative sense, consists of 15, 186 nucleotides (nt) (4, 95). The viral genomic RNA in association with the N and P proteins alone can act as a template for viral transcription

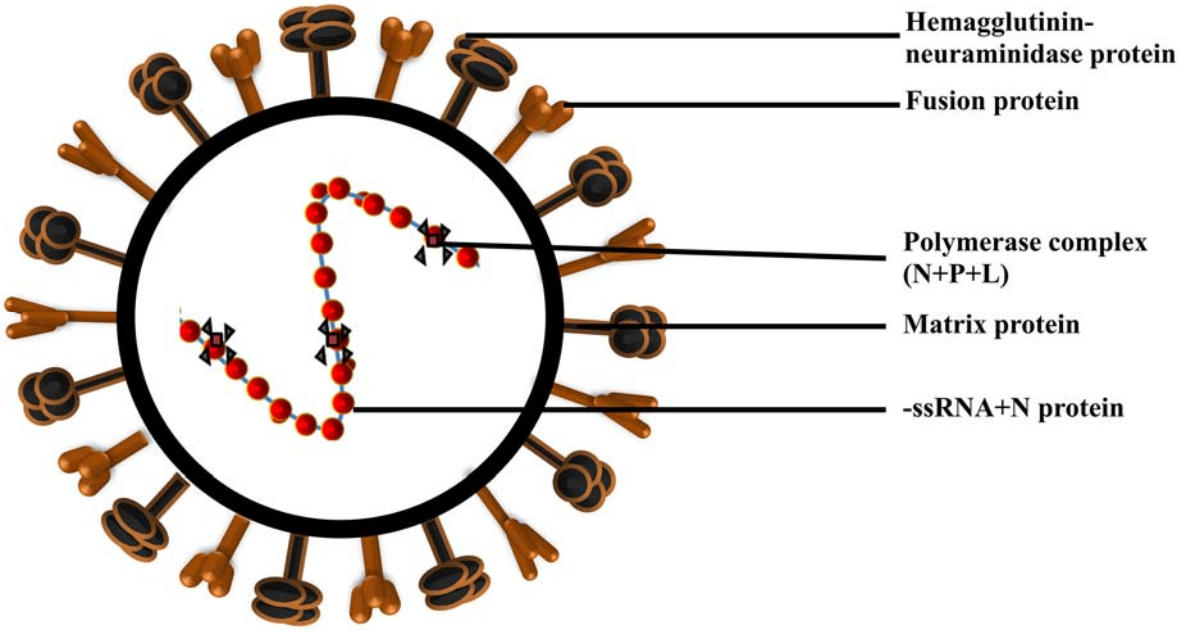

Figure 1. Schematic diagram of Newcastle disease virus (not drawn to scale).

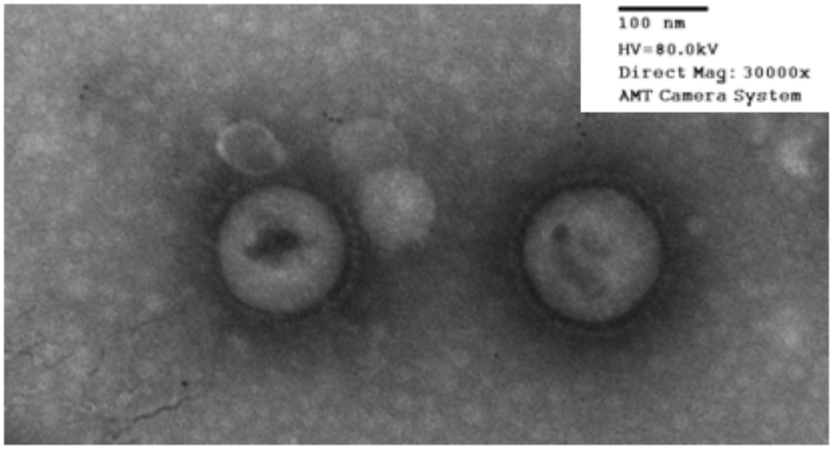

Figure 2. Electron micrograph of negatively stained Newcastle disease virus (strain LaSota) grown in DF-1 cells.

rather than the naked RNA. The nucleocapsid protein (N) and genomic RNA form a core structure, to which the phosphoprotein (P) and the large polymerase protein (L) are attached (60). This core forms the ribonucleocapsid protein (RNP) complex or the transcriptive-replicative complex, which is the minimum infectious unit. The envelope of NDV contains two surface glycoproteins: the fusion (F) protein and the hemagglutinin-neuraminidase (HN) protein. The F protein is required for fusion of the virus into the host cell membrane and HN protein is responsible for attachment of the virus to the sialic acid receptors (84). The F and HN proteins are also the main targets for neutralizing antibodies (63). Internal to the envelope is the matrix (M) protein, which plays an important role in assembly and release of virus particles from the infected cells (74). Figure. 1 shows a schematic diagram of the NDV and Figure. 2 is the electron micrograph of NDV particle.

2.3 Genome organization

The NDV genome consists of six genes (3' N-P/V/W-M-F-HN-L 5') encoding up to eight proteins. The genome at its 3’ end contains a 55 nt long extracistronic region known as the leader and at the 5’ end, 114 nt long region known as the trailer (57). These regions are essential for replication of the genome. At the beginning and the end of each gene are conserved transcriptional control sequences, known as the gene-start (GS) and gene-end (GE) sequences. Between the gene-end and the next gene-start sequences are, the intergenic sequences (IGS), which vary in length from 1- 53 nt (57) (Figure. 3).

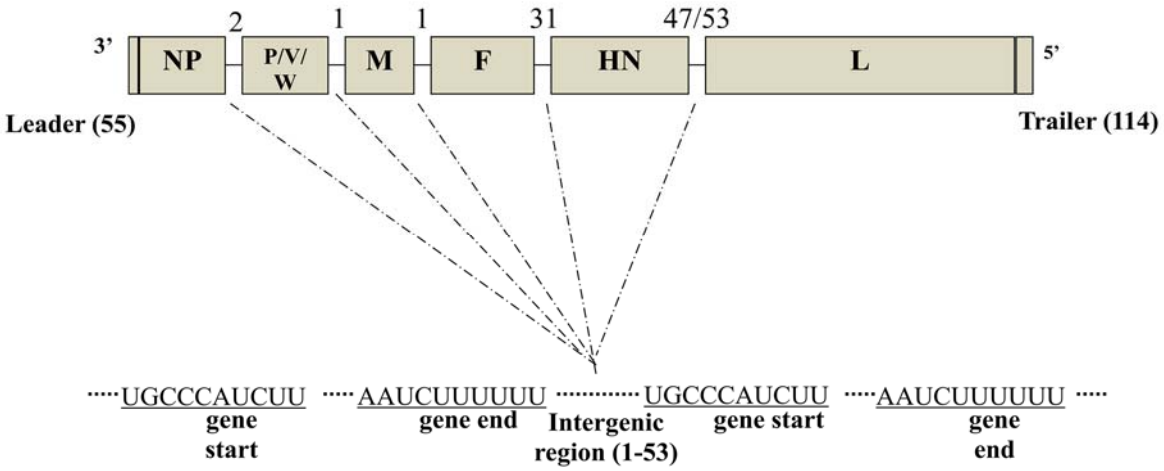

Figure 3. Gene map of NDV: NDV has a single-stranded, negative-sense RNA genome, contains 15,186 nt. Each gene is flanked by conserved gene-start and gene-end sequences. The intergenic sequences present between two genes vary from 1-53 nt in length.

2.4 Viral proteins

The genome of NDV consists of six genes N, P, M, F, HN and L encoding up to eight proteins namely N, P, V, W, M, F, HN and L (84). The two proteins, V and W, are generated by RNA editing during mRNA transcription, where one or more non-templated G residues get inserted into the P gene (88). The genomic RNA together with N, P and L proteins forms the ribonucleocapsid protein complex (RNP), which serves as the template for RNA transcription and replication. On the envelope, two membrane glycoproteins, HN and F, form the spike-like protrusions which help in attachment and infection of the host cells.

2.4.1 Ribonucleocapsid protein complex

The RNA genome of NDV is tightly encapsidated by the N protein to form the N protein RNA complex which serves as the template for transcription and replication. Together with P and L proteins, they form the ribonucleocapsid protein (RNP) complex, which is the minimal transcriptional unit (36).

N protein: The N protein of NDV is 489 amino acids long and has a molecular weight of 55 kDa (84). The N protein serves a major role in viral replication, including encapsidation of the genomic RNA into a ribonucleocapsid (the template for RNA synthesis), association with the P-L polymerase during transcription and replication and interaction with the M protein during virus assembly. The intracellular concentration of unassembled N protein plays an important role in switching from transcription to replication of the viral polymerase (84). The amino terminal region of the N protein is

involved with encapsidation of the viral RNA while the carboxyterminal region binds with the P protein (12).

P protein: The P protein of NDV is 395 amino acids long and has a molecular weight of 50 to 55 kDa depending on the extent of phosphorylation (84). During transcription, there may be three mRNA populations that encode proteins (P, V and W) which have a common N-terminal region, but utilize three different reading frames at their C termini. The unedited version of P gene ORF mRNA, results in formation of the P protein. RNA editing with the addition of one G residue at the editing site (AAAAA↓GGG) near the center of the ORF produces an mRNA which encodes the V protein, whereas addition of two G residues produces an mRNA that encodes the W protein (88). The P protein is essential during transcription and replication. This protein is highly phosphorylated in nature and has been shown to be involved in P-P and P-N interactions (44). The V protein of NDV is 239 amino acids long and has a molecular weight of 36 kDa. It functions as an alpha interferon antagonist by targeting STAT1 for degradation (43). The W mRNA has been found in NDV infected cells but the function of the W protein is still unknown (59, 84)

L (Polymerase) protein: The L protein is the largest viral protein transcribed and it is 2,204 amino acids long with a molecular weight of 250 kDa (84). The L protein is the least available protein, also known as viral polymerase. The L protein does not utilize naked RNA genome as a template, but recognizes only when the genomic RNA is tightly

bound to the N and P protein (36). The L protein also possesses 5' capping and 3' poly (A) polymerase activity required for the generation of nascent viral mRNA (59).

2.4.2 Matrix Protein

The M protein of NDV is 364 amino acids long with a molecular weight of 40 kDa (87). The M protein forms the inner layer of the viral envelope and is responsible for maintaining the viral structural integrity. The M protein interacts with the nucleocapsid and the envelope proteins of the virion. This protein is considered to be the central organizer of viral morphogenesis, interacting with the cytoplasmic tails of the integral membrane proteins, the lipid bilayer, and the ribonucleocapsid and play a vital role in the transport of viral components to the plasma membrane (90).

2.4.3 Envelope glycoproteins

The NDV envelope is made up of two transmembrane glycoproteins,F and HN. They form spike-like protrusions on the outer surface of the virions. The F protein exists on the surface of virus as a trimer, whereas the HN protein exists as a tetrameric spike (65, 82).

F protein: The F protein is synthesized as an inactive precursor protein named F_0 which consists of 553 amino acids. The F_0 is then cleaved by host proteases into F_1 and F_2 subunits, bound by disulphide bonds. The F protein directs fusion of the virion envelope with the plasma membrane of the host cell (81) and is a major immunogenic protein of NDV. The F protein of NDV is a type I transmembrane protein which has its

N-terminus exposed to the extracellular or luminal space. The F protein cleavage is essential for the protein to be functional and biologically active. The F_1 is derived from the carboxyl-terminus and F_2 from the amino-terminus of the F_0 polypeptide (60). The fully glycoslated F protein of NDV contains a 470-amino-acid extracellular domain, a transmembrane domain near the C-terminal, and a 29-amino-acid cytoplasmic tail (15). The molecular weights of F_0, F_1 and F_2 are 67 kDa, 55 kDa and 12 kDa. The amino acid sequence at the cleavage site is the major determinant of virulence in NDV. In the velogenic and mesogenic strains of NDV, the F cleavage site consists of multiple basic amino acids 112(R/K)-R-Q-(R/K)-R↓-F)117, while the lentogenic strains contain one or two basic amino acids 112(G/E)-(K/R)-Q-(G/E)-R↓-L)117 (48, 95). The Presence of multiple basic amino acids in the cleavage site makes them more likely to be cleaved by intracellular proteases like furin which are found in a wide range of cells and tissues, whereas the F protein of avirulent viruses can be cleaved only by extracellular trypsin-like proteases which are only found in the respiratory and intestinal tracts (34).

HN protein: The HN glycoprotein of NDV is a major antigenic determinant of the virus with multiple functions. The HN protein is 577 amino acid residues long with a molecular weight of 74 kDa (84). The HN protein is a multifunctional protein necessary for attachment, fusion, and maturation of NDV. The HN protein is a type II membrane glycoprotein with its N-terminus inside the cytoplasm while the C-terminus is composed of a globular head and ectodomain exposed outside. HN is the attachment protein of the virus (84). The HN protein binds with sialic acid containing receptors and also mediates enzymatic cleavage of sialic acid (neuraminidase activity) from the surface of the virion

as well as from infected host cell membrane. Studies on the crystal structure and mutational analysis of HN protein of NDV have suggested that both hemagglutinin and neuraminidase activity reside at a very close proximity to each other on the protein (18, 19). Along with hemagglutinin and neuraminidase activities, HN also has fusion promotion activity by interacting with F protein of NDV (59).

2.5 Life cycle of NDV

The replication of NDV follows the general pattern of the other nonsegmented negative-strand RNA viruses. The transcription and replication of the NDV genome occurs in the cytoplasm. At the end of the replication cycle, the viral proteins are packaged and the progeny viruses mature by budding through the plasma membrane (Figure. 4).

2.5.1 Virus adsorption and entry

NDV initiates infection by attaching to cell surface receptors followed by the fusion of viral and host cell membranes (60). The HN protein is the viral attachment protein that binds to sialic acid containing receptors, while F protein mediates membrane fusion and viral entry (59). The fusion process is pH independent and thus virus entry occurs at host cell plasma membranes. Upon fusion, disruption of matrix-nucleocaspid occurs and the viral nucleocapsid is released into the host cell cytoplasm.

2.5.2 Transcription

The viral mRNA synthesis begins at the 3′ end of the genome of NDV. The leader sequence contains the regulatory elements needed for gene expression. The first gene, N, is transcribed at the N gene-start (GS) and is terminated at the N gene-end (GE). This results in release of the capped and polyadenylated N mRNA. The transcription of paramyxovirus follows the "start-stop" mechanism until the last mRNA, L mRNA, is synthesized. The polymerase stops at the upstream GE and reinitiates synthesis of the next mRNA at the next GS.

The intergenic sequences (IGS) located in between the genes are not transcribed. Some polymerase falls off at the IGS and some polymerase may bypass the IGS forming a full length positive-strand. This "start-stop" transcription results in a gradient of mRNA relative to the distance of the location of individual gene from the 3' end promoter. Since the N gene is the closest to the 3'end promoter, the N mRNA is produced in higher quantities. The L gene mRNA, located the farthest to the 3'end promoter, is produced in the lowest quantities (Figure. 5). The RNA genome template is copied without dissociation of N from the viral nucleocapsid core during transcription and replication by the polymerase. The genome length of NDV must be in a multiple of six ("Rule of Six") for efficient replication. This rule is most likely related to the finding that each N subunit of the nucleocapsid is associated with exactly six nucleotides (84, 85).

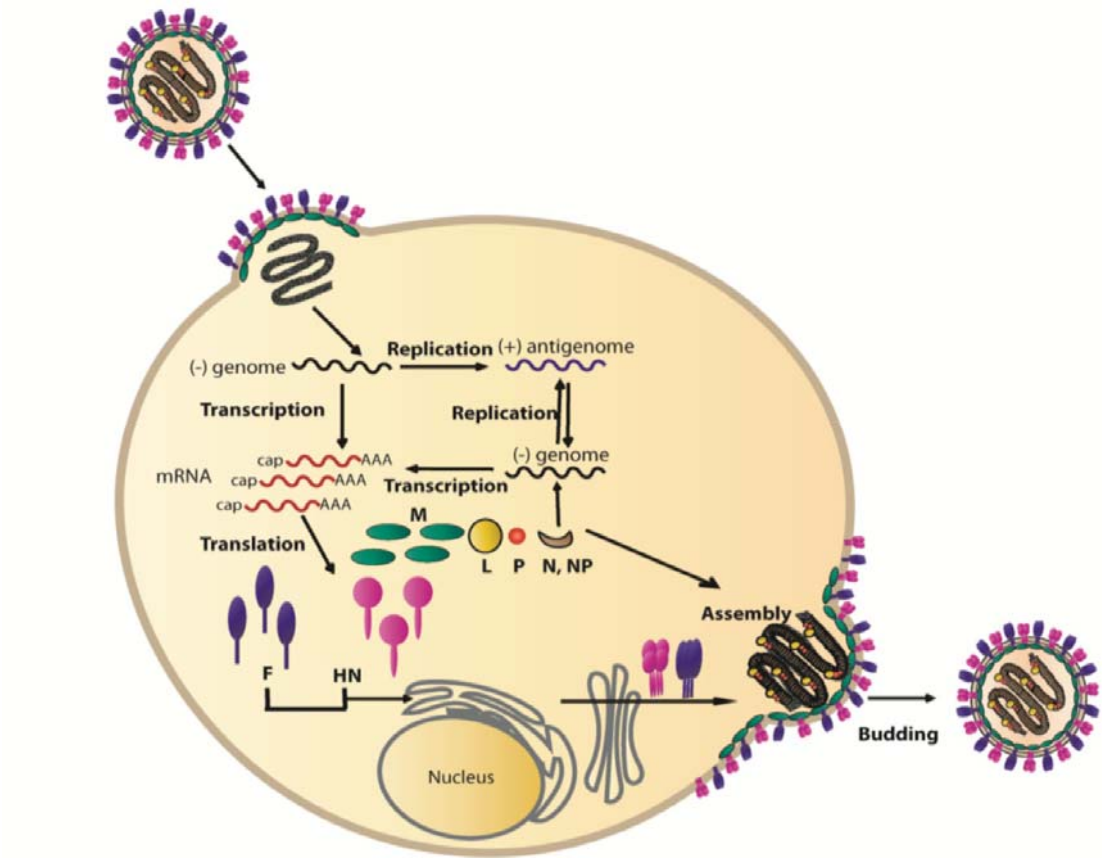

Figure 4. The life cycle of NDV: Modified Image from *El Najjar et al*. (2014) (30).

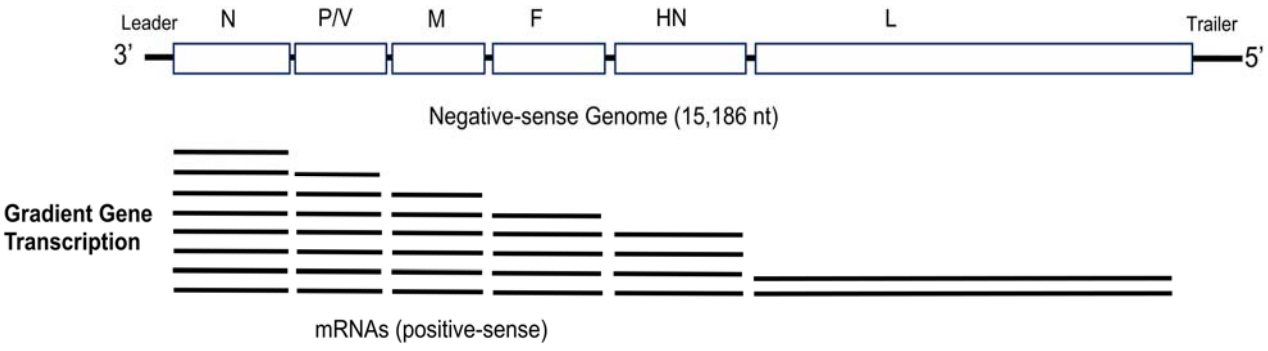

Figure 5. Gradient gene transcription of viral mRNA by viral polymerase (L) protein.

2.5.3 Replication

During replication, the same viral RNA polymerase which was engaged in mRNA synthesis ignores all the junctional signals and synthesizes an exact complimentary copy of the viral genome (7, 93). The (-) sense genome acts as a template for the full-length complimentary copy known as (+) sense antigenome. Both the genome and antigenome are encapsidated by the nucleocapsid protein. The leader and trailer regions of the genome contain specific sequences for initiating encapsidation. The processes of transcription and replication are tightly regulated in paramyxoviruses. The switch from transcription to replication is controlled by the N protein. When unassembled N protein is limiting, the viral RNA polymerase is preferentially engaged in mRNA synthesis, raising the intracellular levels of unassembled N and all other viral proteins. When unassembled N protein levels are sufficient, some viral RNA polymerase activity switches from transcription to replication, thereby lowering the levels of unassembled N protein, as each initiation of encapsidation utilizes many N protein monomers to finish the assembled genome chain. The RNA synthesis of NDV is shown in Figure. 5.

2.5.4 Virus assembly and release

The first step in viral assembly is the encapsidation of genomic RNA into nucleocapsid in the cytoplasm of the infected cell. First, the free N proteins are tightly associated with the genomic RNA to form the ribonucleocapsid protein (RNP) core structure; secondly, the P and L proteins bind to RNP core, forming a complex (27). The assembly of the viral envelope takes place at the cell surface. The F and HN glycoproteins are synthesized in the endoplasmic reticulum (ER) and undergo step-wise

conformational maturation before transported through the secretory pathway. Folding and maturation occurs inside the ER with the help of many molecular cellular chaperones. Only the correctly folded proteins are transported out of the ER to the Golgi apparatus for further post-translation modifications including carbohydrate chain modification of HN protein and cleavage of F_0 protein at the cleavage site to form functional F_1 and F_2 proteins. Cleavage of the F_0 precursor to F_1 and F_2 by host cell proteases is required for progeny virus to become infective (25). The M protein is thought to play the major role in taking the assembled RNP to the plasma membrane to form budding virions. Study on NDV virus-like particle (VLP) suggests that M-HN and M-N interactions are responsible for incorporation of HN and N proteins into VLPs and the F protein is incorporated indirectly due to interactions with N and HN protein (71). During the late stages of infection, the effect of viral replication can result in complete shut off of host macromolecular synthesis and progeny viruses mature by budding through the plasma membrane.

2.6 Reverse genetics

Reverse genetics is a technique that allows the generation of viruses from cloned cDNAs. Genome manipulation of negative-sense RNA viruses is difficult when compared to the positive-sense RNA viruses. This is because the negative-sense RNA viruses require the virion RNA to be assembled into an active transcriptase-replicase complex for the genome to initiate viral infection. Rabies virus was the first negative-sense RNA virus to be rescued by reverse genetics, when plasmids encoding the N, P and L proteins and a full length plasmid coding the entire antigenome under the control of T7

RNA polymerase promoter, were transfected into cells infected with a recombinant vaccinia virus expressing T7 RNA polymerase (86). Rescue of the rabies virus using reverse genetic procedures was followed by recovery of several other viruses such as the vesicular stomatitis virus, human respiratory syncytial virus, SV5, measles virus, Sendai virus, rinderpest virus and parainfluenza virus (8, 16, 28, 32, 39, 61, 76). Reverse genetic systems to recover infectious NDV from cloned cDNAs were first reported in 1999 (75, 80). The availability of reverse genetic technology for NDV and other RNA viruses have provided means to genetically define and study these viruses. The reverse genetic technique for the rescue of NDV is depicted schematically in Figure. 6.

2.7 NDV as a vaccine vector

Viral vector vaccines use live viruses to carry genes that encode foreign antigen when expressed in the infected cells, will elicit an immune response. NDV has several advantages for use as a vaccine vector in humans. NDV is safe and avirulent in humans due to a natural host range restriction. Avirulent NDV strains are also highly safe in avian and non-avian species and a highly desirable candidate as a vaccine vector for the development of human and veterinary

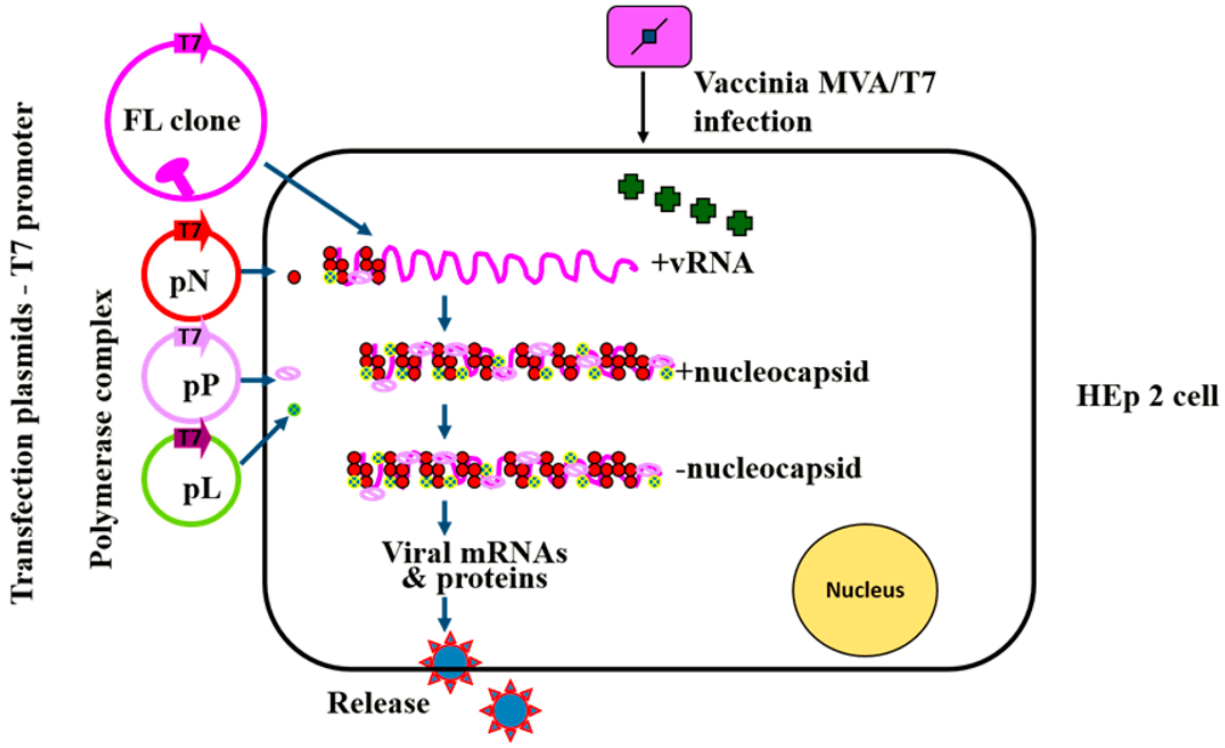

Figure 6. Schematic procedure for the recovery of infectious NDV from cDNA: Intracellular expression of antigenomic full-length cDNA (pNDV) and polymerase complex N, P and L encoded in plasmids were co-transfected into HEp-2 cells. All the plasmids under the control of the T7 RNA polymerase promoter sequence were transcribed by the T7 RNA polymerase, supplied by the recombinant vaccinia MVA/T7. Infectious NDV strain LaSota was generated entirely from cloned cDNA with procedures explained by *Huang et al. (2001*) (42). Image source: Kim and Samal (2016).

vaccines. The use of avirulent NDV strains as a vaccine vector in humans will prevent the possibility of accidental spread of a virulent virus strain from treated patients to birds. NDV replicates in the cytoplasm, does not integrate into the host cell DNA, and does not establish persistent infection. NDV replicates well *in vivo* and induces a robust immune response. NDV can be vaccinated via the intranasal route and has been shown to induce humoral and cellular immune responses both at the mucosal and systemic levels in animal models (13, 20-23). NDV encodes only seven proteins, in contrast to adeno, herpes, and poxvirus vectors whose genomes encode a large number of proteins, thus there is less competition for immune responses between vector proteins and the expressed foreign antigen (55). In non-human primates, the intranasal and intratracheal inoculation with NDV did not cause any disease symptoms and the virus replication was restricted to the respiratory tract (13). In humans, infection by NDV appears to be limited and benign based on clinical studies using NDV as an oncolytic agent (84). NDV shares only a low level of amino acid sequence identity with known human paramyxoviruses and is antigenically distinct from common human and animal pathogens, and thus would not be affected by preexisting immunity in humans. NDV has also been used to express protective antigens of various human pathogens and has shown promising results in non-human primates (13, 20, 21, 23). The establishment of a reverse genetic system for NDV facilitates genetic manipulation and NDV expressing different immunogenic proteins for a variety of viruses can be made for vaccine purposes. NDV is also a strong stimulator of the host immune response, thus providing an adjuvant effect. NDV grows to high titers not only in embryonated eggs (10^9 PFU/mL) but also in Vero cells (10^8 PFU/mL), which is acceptable for human vaccine development (55).

Chapter 3: A Y527A mutation in the fusion protein of Newcastle disease virus strain LaSota leads to a hyperfusogenic virus with increased replication and immunogenicity

3.1 Abstract

Newcastle disease is a highly contagious and economically important disease of poultry. Low-virulence Newcastle disease virus (NDV) strains such as B1 and LaSota have been used as live vaccines, with a proven track record of safety and efficacy. However, these vaccines do not completely prevent infection or virus shedding. Therefore, there is a need to enhance the immunogenicity of these vaccine strains. In this study, the effect of mutations in the conserved tyrosine residues of the F protein of vaccine strain LaSota was investigated. Our results showed that substitution of tyrosine at position 527 to alanine resulted in a hyperfusogenic virus with increased replication and immunogenicity. Challenge study with highly virulent NDV strain Texas GB showed that immunization of chickens with Y527A mutant virus provided 100% protection and no shedding of the challenge virus. This study suggests that the strain LaSota harbouring the Y527A mutation may represent a more efficacious live ND vaccine.

3.2 Introduction

Newcastle disease virus (NDV) causes a highly contagious disease in chickens, resulting in severe economic losses to the poultry industry worldwide (5, 84). NDV isolates are categorized into three pathotypes: lentogenic (low virulence), mesogenic (intermediate virulence) and velogenic (high virulence) based on their pathogenicity in

chickens (1). Several lentogenic strains, such as LaSota and B1, are being used as live vaccines all over the world. Although these strains have been highly successful as live vaccines, they do not completely prevent virulent virus infection or shedding of virulent virus. Newcastle disease (ND) remains a major poultry disease problem in many parts of the world despite repeated vaccination. Therefore, there is a need to enhance the immunogenicity and protective efficacy of current live vaccines. Reverse genetics provides a powerful tool to increase the immunogenicity of current vaccine strains.

NDV belongs to genus *Avulavirus* within the family *Paramyxoviridae*, a family of enveloped, nonsegmented, negative-strand RNA viruses. The genome of NDV contains six genes (3′-N-P-M-F-HN-L-5′), which encode at least seven proteins, namely nucleocapsid (N) protein, phosphoprotein (P), matrix (M) protein, fusion (F) protein, hemagglutinin–neuraminidase (HN) and RNA-dependent RNA polymerase (L). The envelope of NDV contains two transmembrane glycoproteins, the HN protein and the F protein. The HN protein is involved in attachment to host cell receptor and release of progeny virions. The F protein mediates fusion of the viral membrane to the cell plasma membrane, resulting in virus penetration, and also mediates fusion of adjacent cells to form syncytia (84). The NDV F protein is a trimer, and it is synthesized as an inactive precursor, F_0 (66 kDa), which is post-translationally cleaved by host cell proteases into two disulfide-linked subunits, N-terminal F_2 (12.5 kDa) and C-terminal F_1 (55 kDa). The ability of the F protein to be cleaved by proteases is a major determinant of virulence in NDV and a prerequisite for virus entry and cell-to-cell fusion (1).

The NDV F protein is a class I fusion protein that has structural and functional characteristics related to those of the F protein of other paramyxoviruses (60, 68). It is known that the structural features of the ectodomain of the F protein can have a major impact on fusion. Several reports have also indicated a role for the cytoplasmic tail (CT) of F protein in virus entry, F protein cleavage, and fusogenicity (15, 60, 68). It has been found in other enveloped viruses that tyrosine-containing signals, especially Y-X-X-aliphatic/aromatic consensus motifs, in the CT of viral membrane proteins are associated with the transport and delivery of proteins (6, 10, 83, 93). The NDV F CT is 31 aa long and its sequence is highly conserved among different strains (24). We have previously shown that mutations in the two conserved tyrosine (Y524 and Y527) residues in the cytoplasmic domain of the F protein of moderately virulent NDV strain Beaudette C (BC) conferred a hyperfusogenic phenotype, increasing viral replication and pathogenicity (83). The mechanism by which the substitution of tyrosine residues causes hyperfusogenicity is not well understood. It is possible that the substitution may have conferred increased efficiency of synthesis or transport to the cell surface, resulting in higher levels of F protein surface expression that in turn mediated increased cell-to-cell fusion. Alternatively, the substitution might have caused increased incorporation of F protein into virus particles or effects the fusion process.

The effect of tyrosine mutations on immunogenicity of the modified BC strain could not be determined because this virus was highly virulent, causing death of all infected chickens. Conserved tyrosine residues in the CT of the F protein of NDV play an important role in the fusogenicity of the virus, but the role of each tyrosine residue may

vary among strains of NDV. Therefore, it was necessary to determine the effect of tyrosine mutations on virus replication and immunogenicity in the context of a low-virulence strain. The low-virulence strain LaSota was chosen for this study because this strain is widely used as a live NDV vaccine throughout the world.

3.3 Materials and Methods

3.3.1 Cells and Viruses

Chicken embryo fibroblast cell line (DF-1), human epidermoid carcinoma cell line (HEp-2) and African green monkey kidney cells (Vero) were grown in Dulbecco's minimal essential medium (DMEM) with 10% fetal bovine serum (FBS). The modified vaccinia virus strain Ankara (MVA) expressing T7 RNA polymerase was kindly provided by Dr. Bernard Moss (NIH, Bethesda, MD). The low virulent (lentogenic) NDV strain LaSota and its recombinant derivatives were grown in 9-day-old embryonated specific-pathogen-free (SPF) chicken eggs and in DF-1 cells with 10% fresh chicken egg allantoic fluid.

3.3.2 Plasmid construction and rescue of recombinant virus

The construction of plasmid carrying the full length antigenome cDNA (pNDV) of the NDV strain LaSota has been described previously (12). We used site-directed mutagenesis to introduce individual amino acid substitutions into cDNA of the F gene of strain LaSota. Tyrosine residues at positions 524 and 527 were changed to alanine

individually and in combination. The following primer sequences were used for site-directed mutagenesis.

LAS Tyr524F	5’ GCTCTAATGTACAAGCAAAAGGCGC 3’
LAS Tyr524R	5’ GCATGCTAGAATCAGGCTAAG 3’
LAS Tyr527F	5’ GCTAAGCAAAAGGCGCAACAAAAGAC 3’
LAS Tyr527R	5’ CATTAGGTAGCATGCTAGAATCAG 3’
LAS Tyr524&527F	5’ GCTCTAATGGCTAAGCAAAAGGCGCAACAAAAGAC 3’
LAS Tyr524&527R	5’ GCATGCTAGAATCAGGCTAAG 3’

The mutated gene was then inserted into the full-length cDNA clone of the LaSota (Figure. 7). These clones were transfected into HEp-2 cells, and mutant viruses were recovered as previously described (12). The CT mutant viruses were designated as rLas-524, rLas-527, rLas-524+527. The F genes from recovered viruses were sequenced to confirm the mutations. To assay genetic stability, the recovered CT mutant viruses were passaged five times in 9-day-old SPF chicken embryos. From each passage, total RNAs were isolated from infective allantoic fluid using TRIzol reagent (Invitrogen). RT-PCR was performed using the Thermoscript RT-PCR kit (Invitrogen) with gene-specific forward and reverse primers to amplify the F gene. The amplified cDNA fragments were then sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems Inc.) in an ABI 3130*xl* genetic analyzer to confirm the presence of the introduced mutations in the passaged viruses.

3.3.3 RNA extraction and RT-PCR of recovered CT mutant viruses

The recovered recombinant CT mutant viruses rLas-524, rLas-527, and rLas-524+527 were grown in the allantoic cavity of 9-day-old embryonated chicken eggs and after 2 days, the infective allantoic fluid was harvested. Viral RNA was extracted from the recovered viruses using TRIzol (Invitrogen) according to the manufacturer's instructions. Reverse transcription was done with the extracted RNA, using the Thermoscript RT kit (Invitrogen) to synthesize the first strand cDNA. The genomes of recovered CT mutant viruses were sequenced after RT-PCR to confirm the presence of the desired tyrosine to alanine mutations.

3.3.4 Growth characteristics of mutant viruses

Multicycle growth kinetic studies were done in DF-1 cells and in 9-day-old embryonated chicken eggs. Briefly, DF-1 cells grown in six-well plates were infected with each mutant virus, in duplicate, at an MOI of 0.001. After 1 h of adsorption, the cells were washed with PBS and overlaid with Dulbecco's modified Eagle's medium containing 2 % FBS and 10 % fresh allantoic fluid at 37 °C. A sample of the supernatant medium was collected and replaced with an equal volume of fresh medium every 8 h until 64 h post-infection (PI). Similarly, in 9-day-old embryonated chicken eggs, the eggs were inoculated with 100 PFU of each virus, and allantoic fluids were harvested at different time points (24, 48 and 72 h). Virus titers were quantified by $TCID_{50}$ assay on DF-1 cells (77).

3.3.5 Fusion Index assay

Syncytium formation was quantified as described by Kohn (56). Briefly, Vero cells in six-well plates were infected with each virus at an m.o.i. of 0.1. Cells were maintained in 2 % minimal essential medium at 37 °C under 5 % CO_2. At 24h (PI), the medium was removed and the cells were washed with PBS, fixed with methanol for 20 min at room temperature, and then stained with Hematoxylin and Eosin. The fusion index of each mutant virus was calculated by observing 10 fields per well in duplicate. The fusion index is the ratio of the total number of nuclei to the number of cells in which these nuclei are present (i.e. the mean number of nuclei per cell). The fusion index values of all viruses were expressed as a percentage of these values compared to rLaSota, in which the values were considered as 100 per cent.

3.3.6 Plaque assay

Virus containing infective allantoic fluid was serially diluted in ten-folds (10^{-1} to 10^{-7}) in DMEM and 400 µL of each serial dilution was added per well in a 12 well plate of confluent DF-1 cells in triplicates. After 60 min adsorption, cells were overlaid with DMEM (containing 2% FBS, 0.9% methyl cellulose and 10% fresh chicken embryo allantoic fluid) and incubated at 37 °C with 5% CO_2 for 4-7 days. The cells were then fixed with 100% Methanol and stained with 1% crystal violet for enumeration of plaques.

3.3.7 Mean Death Time (MDT) in chicken embryos

The virulence of the recovered CT mutant viruses was determined by the mean death time assay in embryonated SPF chicken eggs (84). A series of 10-fold dilutions of

infective allantoic fluid was made in sterile PBS, and 0.1 mL of each dilution was inoculated into the allantoic cavity of five 9-day-old embryonated eggs. The eggs were incubated at 37 °C and examined four times daily for 7 days. The time of death of each embryo was recorded. The highest dilution at which all embryos died was considered the minimum lethal dose. The MDT was recorded as the mean death time in hours for the minimum lethal dose to kill the embryos. The MDT has been used to classify NDV strains into velogenic (taking under 60 h to kill); mesogenic (taking between 60 h to 90 h to kill) and lentogenic (taking more than 90 h to kill).

3.3.8 Pathogenicity studies in chickens

To test the pathogenicity of the CT mutant viruses *in vivo*, intracerebral pathogenicity index (ICPI) test was performed according to standard procedures (1). For the ICPI test, 0.05 mL of a 1:10 dilution of fresh infective allantoic fluid of each virus was inoculated into a group of ten 1-day-old SPF chicks via intracerebral route. At each observation, the birds were scored 0 if normal, 1 if sick, and 2 if dead. Each experiment had mock-inoculated controls that received a similar volume of sterile PBS. The ICPI is the mean score per bird per observation over the 8 day period. Highly virulent velogenic viruses give values approaching 2, and avirulent or lentogenic strains give values at or close to 0. The OIE Biological Standards Commission similarly recommended that ND vaccines should have an ICPI < 0.7 (70).

3.3.9 Immunization and challenge experiments in chickens

The immunization and challenge studies were performed in chickens at our USDA approved enhanced biosafety level 3 (BSL-3+) containment facility. Two-week-old specific pathogen free (SPF) chickens obtained from Charles River Laboratories, Wilmington, MA, USA were randomly assigned to 5 treatment groups of 10 birds. All birds were housed in separate poultry isolation chambers with *ad libitum* access to feed and water. The birds were vaccinated via the intranasal route with 200 µL of $1x10^6$ 50% egg infective dose (EID_{50}) of rLaSota, rLas-524, rLas-527, rLas-524+527 and PBS. All birds were observed daily for 10 days for any vaccine related clinical signs. Oral and cloacal swabs were collected on 3rd day post vaccination to monitor the vaccine virus shedding. Blood was collected at 1, 2, 3 and 4 weeks post-immunization (WPI) for analyzing NDV antibody response by Hemagglutination Inhibition (HI) assay (16). Four WPI all the groups were challenged by occulo-nasal route with virulent NDV strain Texas GB at 100 chicken lethal dose 50 (CLD_{50}) per bird. All birds were observed 10 days for clinical signs (death, paralysis, and torticollis) of neurotropic velogenic NDV. In order to determine shedding of the challenge virus, oral and cloacal swabs were collected on days 3, 5, and 7 post-challenge from all chickens.

3.4 Results

3.4.1 Construction and recovery of F protein mutant viruses

In the present study, three F protein CT mutant cDNAs of NDV strain LaSota were constructed. These cDNAs were transfected into HEp-2 cells, and mutant viruses were recovered. These viruses were designated as rLas-524, rLas-527, rLas-524+527. The F genes from recovered viruses were completely sequenced which confirmed the presence of each introduced mutation and the lack of adventitious mutations. To determine the stability of each F mutation, the recovered viruses were plaque purified and passaged five times in 9-day-old embryonated chicken eggs. Sequence analysis of the F gene in the mutant viruses after five passages showed that the introduced mutations were stable.

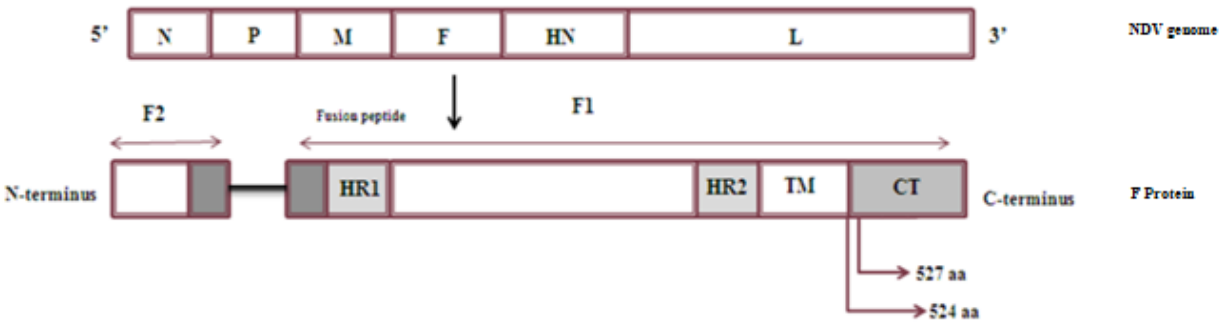

Figure 7. Schematic diagram of the NDV Genome: Gray boxes: heavy shading, fusion peptide cleavage site; light shading, heptad repeats (HR); intermediate shading, cytoplasmic trail (CT); no shading, transmembrane (TM) domain. Schematic representation of the the approximate location of tyrosine to alanine mutation in the cytoplsmic tail of F protein in rLaSota virus are indicated (arrows).

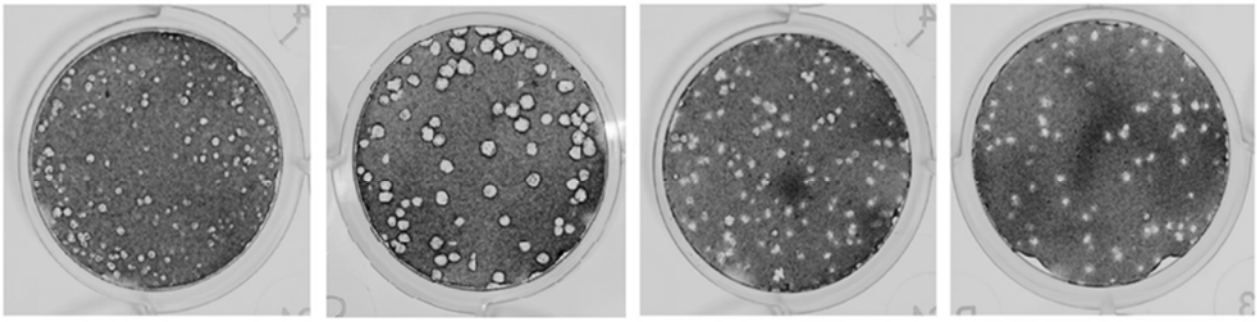

Figure 8. Plaque size and morphology of rLaSota and its cytoplasmic tail (CT) mutant viruses.

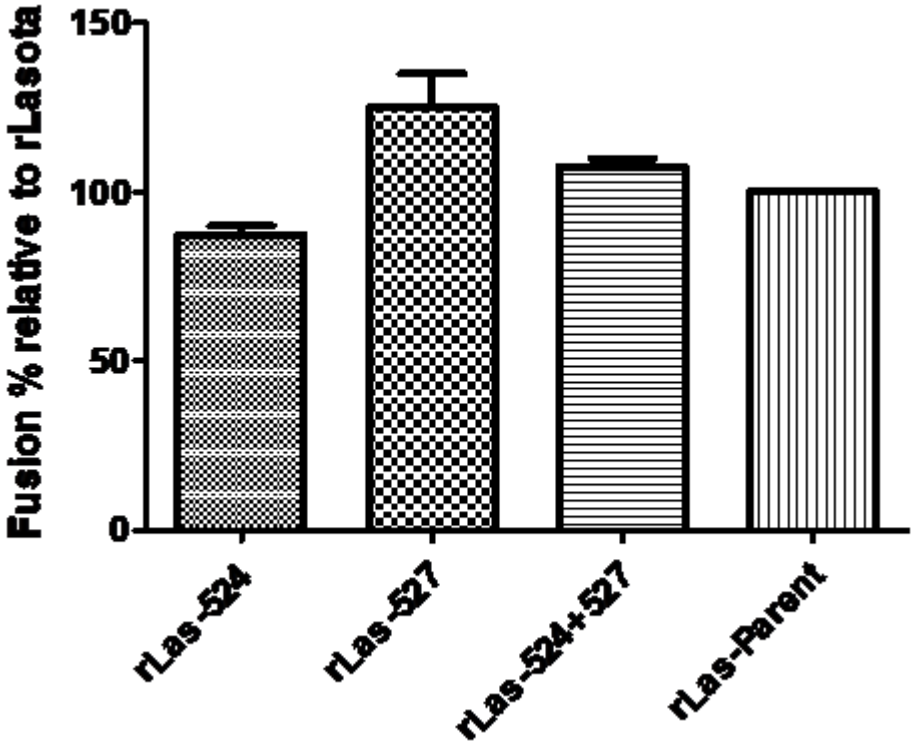

Figure 9. Comparison of the fusogenicity of parental rLaSota and the CT mutant viruses in Vero cells: Relative levels of fusion obtained for the CT mutants compared to parental rLaSota virus are shown. The fusion index was calculated as the ratio of the total number of nuclei in multinuclear cells to the total number of nuclei in the field. Ten fields were counted per group. Fusion levels were normalized to parental rLaSota at 100%. The bars are the means of triplicate wells. Error bars indicate standard error of the mean.

3.4.2 Biological characterization of recombinant viruses

Plaque assay showed that rLas-527 produced slightly larger sized plaques compared to parental and other CT mutant viruses (Figure. 8). Fusion index assay (56) showed that rLas-527 produced more cell fusion compared to parental rLaSota and other CT mutant viruses (Figure. 9). The multi-cycle growth kinetics of the CT mutant viruses was determined in DF-1 cells (Figure. 10) and in embryonated chicken eggs (Figure. 11). The parental LaSota and tyrosine mutants replicated exponentially until 48 hpi, after which they reached a plateau. The growth kinetics of rLas-524 and rLas-524+527 was similar or lower than that of the parental rLaSota, but the growth kinetics of rLas-527 was higher than that of the parental rLaSota. These results indicated that the Y527 mutation yielded to a hyperfusogenic virus that had increased replication *in vitro*.

3.4.3 Pathogenicity of CT mutant viruses

The pathogenicity of rLas-524, rLas-527 and rLas-524+527 was evaluated by ICPI test in 1-day-old chicks and by MDT in embryonated chicken eggs (84). Chicks infected with each CT mutant virus had no apparent clinical signs during the 8-day period of ICPI test (Table 1). These results indicate that all the CT viruses in this study were highly attenuated, and had no discernible effect on the pathogenicity of rLaSota. The ICPI values of the parental and CT mutant viruses were close to zero. The MDT values were more than 90 h, indicating that the mutations in the CT region of F protein did not increase the pathogenicity of the mutant viruses.

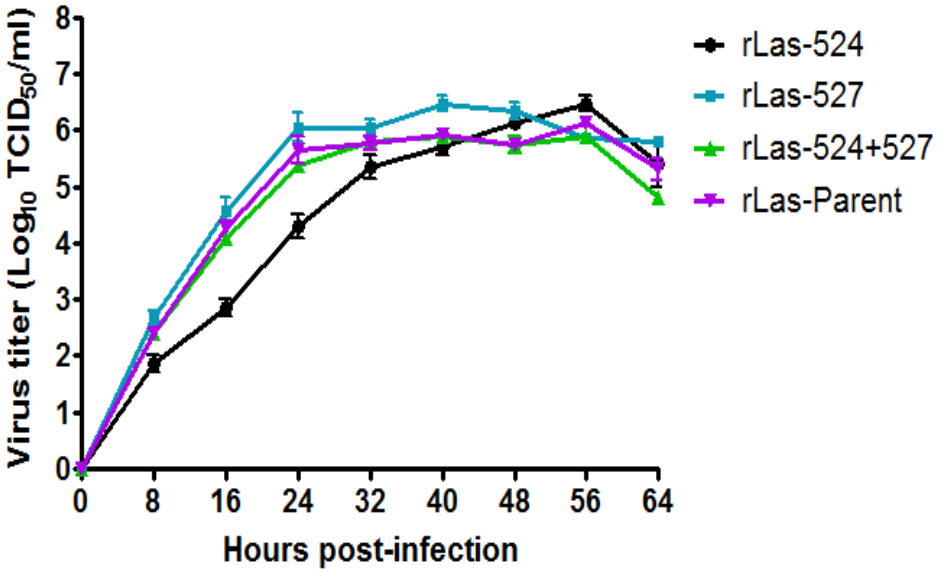

Figure 10. Growth kinetics of parental rLaSota and its CT mutants in DF-1 cells: DF-1 cells were infected at a multiplicity of infection (MOI) of 0.001 of each virus, and the cell culture supernatant was collected at 8 h intervals for 64 h. All virus titers were expressed as mean log $_{10}$ titer ± SEM (standard error of the mean).

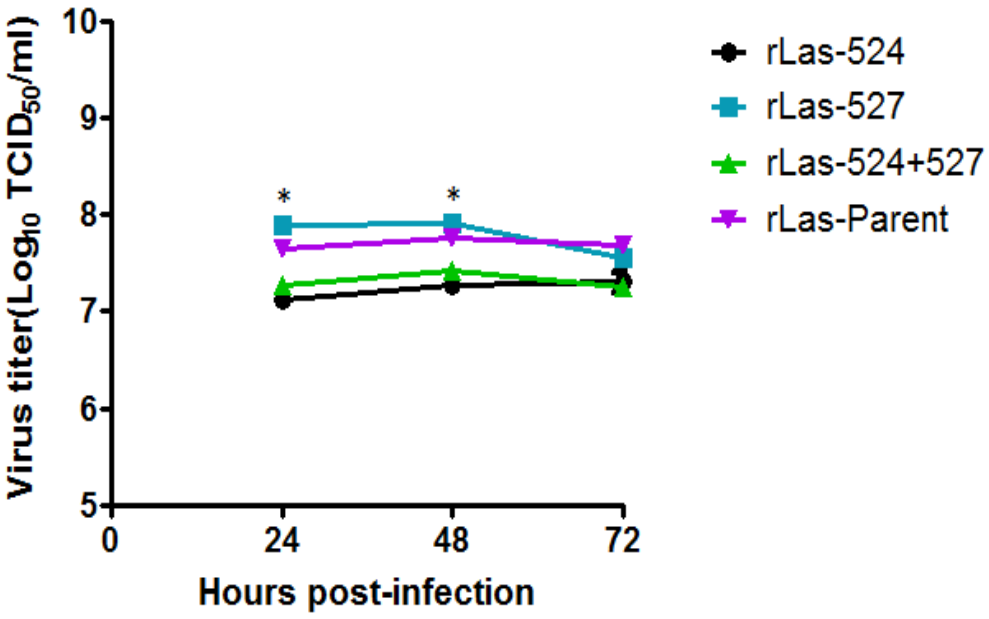

Figure 11. Growth kinetics of parental rLaSota and its CT mutant viruses in 9-day-old embryonated chicken eggs: For each virus, 30 eggs were injected with 10^3 PFU in the allantoic cavity and 10 eggs were chilled at 24 h intervals till 72 h. The virus titer in allantoic fluid was determined by $TCID_{50}$ assay in DF-1 cells**.** All virus titers are expressed as log $_{10}$ titer ± SEM (standard error of the mean). Statistical differences were calculated by two tailed unpaired t test, * is indicated if $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$.

Table 1. Pathogenicity of rLaSota and its CT mutants in embryonated eggs and chicks.

Virus	ICPI	MDT (h)
rLas-524	0.10	98.00
rLas-527	0.20	90.00
rLas-524+527	0.04	95.00
rLas-Parent	0.11	100.00

Table 2. Viral shedding in immunized and challenged birds.

Virus	Vaccine virus shedding		Challenge virus shedding					
	Oral	Cloacal	Oral			Cloacal		
	Day-3	Day-3	Day-2	Day-4	Day-6	Day-2	Day-4	Day-6
rLas-524	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
rLas-527	1/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
rLas-524+527	2/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
rLas-Parent	0/10	0/10	1/10	0/10	0/10	0/10	1/10	0/10

3.4.4 Evaluation of immunogenicity and protective efficacy of the CT mutant viruses in chickens

The immunogenicity of the parental rLaSota and its CT mutant viruses was evaluated in 2-week-old SPF chickens. The birds were randomly assigned into 5 groups of 10 birds each. The birds were infected via occulo nasal route with 200 µL of 10^6 EID_{50} of rLaSota, rLas-524, rLas-527, rLas-524+527 and sterile PBS. Blood samples were collected from all the birds at 1, 2, 3 and 4 WPI for assessing NDV antibodies. None of the chickens infected with the parental or the mutant viruses showed clinical signs, indicating that the mutant viruses were avirulent to chickens. All infected birds were seropositive by 1 WPI as observed by HI test. The birds infected with rLas-527 at 1 WPI had higher HI titer compared to other (Figure. 12). Birds from all the groups were challenged at 4 WPI via oculo-nasal route with virulent NDV strain Texas GB at 100 CLD_{50} per bird. Our results showed all the birds that had been immunized with parental rLaSota or its CT mutant viruses were completely protected from Texas GB challenge without any clinical signs. In contrast, all the birds in the unvaccinated control group died within 5 days after challenge. In order to determine shedding of the challenge virus, oral and cloacal swabs were collected on days 3, 5, and 7 post-challenge from all chickens. Our challenge virus shedding results showed that one of the birds immunized with parental rLaSota, was positive for virus shedding, whereas none of the birds in CT mutant groups showed virus shedding (Table 2). These results suggest that the CT mutant viruses were more effective than regular LaSota in protection against virulent NDV challenge.

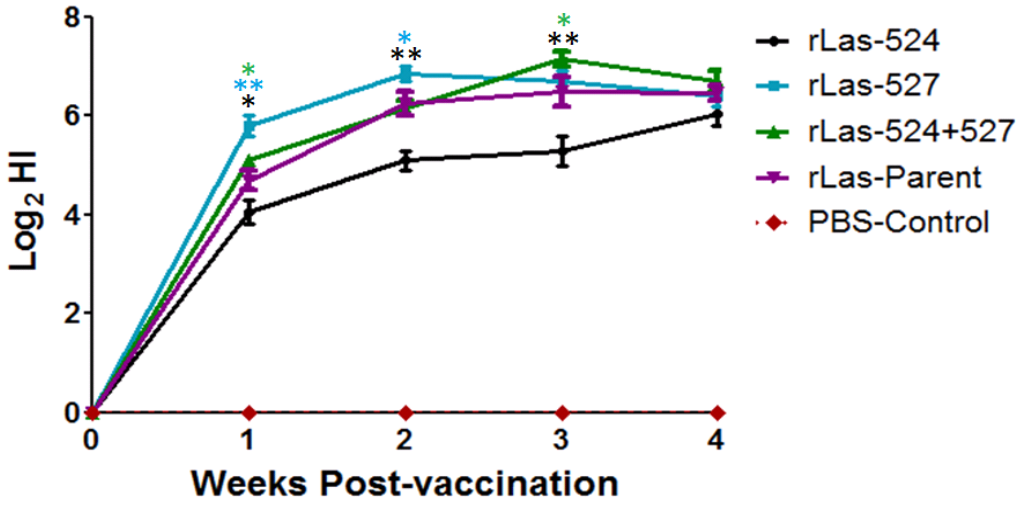

Figure 12. Comparison of humoral immune response in chickens vaccinated with parental rLaSota and its cytoplasmic tail (CT) mutant viruses: Ten 2-week old chickens in each group were immunized with 10^6 PFU of each virus. The blood samples were collected at weekly intervals for 4 weeks. The antibody titer to NDV was measured by hemagglutination inhibition (HI) assay. All antibody titers are expressed as mean reciprocal $\log_2$ titer ± SEM. Statistical differences were calculated by two tailed unpaired t test. * is indicated if $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$

3.5 Discussion

Our results indicate that the currently used vaccine strain LaSota can be modified to induce an elevated antibody response in chickens. We previously showed that mutations in the two conserved tyrosine (Y524 and Y527) residues in the cytoplasmic domain of the F protein of moderately virulent NDV strain BC conferred a hyperfusogenic phenotype, modulating viral replication and pathogenicity (83). However, the effect of tyrosine mutations on immunogenicity of the modified Beaudette C strain could not be determined because this virus was highly virulent, causing death of all infected chickens. Therefore, it was important to determine the effect of tyrosine mutations on immunogenicity in the context of a low virulence strain. The low virulence strain LaSota was chosen for the study because this strain is most widely used as a live vaccine throughout the world.

We found that the Y527A substitution in the CT of F protein of strain LaSota resulted in hyperfusogenic phenotype. The mechanism by which the Y527A substitution causes hyperfusogenicity is not known, but it is possible that this substitution may have conferred increased efficiency of synthesis or transport of the F protein to the cell surface, resulting in higher levels of F protein on the cell surface, that in turn mediated increased cell to cell fusion. For example, in case of parainfluenza virus type 5**,** the F CT is implicated in regulating fusion pore formation (29). Alternatively, the substitution might have caused increased incorporation of F protein into virus particles and effected the fusion process.

In a previous study, the replication of the mesogenic strain BC was increased significantly by a Y524A mutation and decreased by a Y527A mutation (83). But in this study with strain LaSota, the Y527A mutation resulted in significant improvement in the replication and fusogenicity than Y524A mutation. Our results confirmed that the conserved tyrosine residues in the CT of F protein of NDV play an important role in the fusogenicity of the virus, but the role of each tyrosine residue may vary among strains of NDV. Although it is not known why the role of each conserved tyrosine residue varies among strains, we hypothesize that it is due to the differences in the F protein folding which is dependent on amino acid sequence. Substitution of tyrosine residues can enhance or abolish the beta-turn population in the protein structure.

The Y527A mutant virus showed increased virus replication *in vitro*. In our multicycle growth curve experiment, the replication of Y527A mutant virus increased 2 fold at 24h post-infection (PI) than the other CT mutants or the parental LaSota. The increased virus replication may be the result of hyperfusogenic phenotype of the virus, which helps the mutant virus to spread from cell to cell more efficiently and thereby increasing virus replication. Our results showed that Y527A mutation increased the immunogenicity of LaSota virus. The HI titer induced in chicken by Y527A mutant virus was at least 2 fold higher than that induced by the parental LaSota virus at 7 day post-immunization. This result is remarkable considering the fact that LaSota strain is a highly immunogenic vaccine and any further increase in immunogenicity is an added advantage for the virus as a live vaccine candidate. It is particularly important for a NDV vaccine

because NDV is a highly infectious and fast replicating virus and for the vaccine to be efficient it must induce the highest level of immune response possible.

Safety is an important concern for live attenuated vaccines. In our study the Y527A mutation did not increase the pathogenicity of LaSota strain. The major difference between low virulence and high virulence NDV strains is the amino acid sequence at the F protein cleavage site (84). In general, the low virulence strains have monobasic cleavage site, while the virulent strains have multibasic cleavage site. Since our mutation was on the CT, it did not change the F protein cleavage site sequence of strain LaSota. Our virulent NDV challenge study showed that the Y527A mutant virus completely protected all immunized chickens from clinical disease and there was no shedding of challenge virus in any of the immunized chickens. Although all the chickens were protected from disease by LaSota vaccination, there was challenge virus shedding in one of the immunized chickens. This result indicates that the Y527A mutant virus was more effective in preventing virus infection and virus shedding. However larger challenge studies are needed to evaluate the effectiveness of LaSota with Y527A mutation as an improved live vaccine.

In summary, we generated an improved LaSota vaccine candidate that is highly immunogenic and efficacious in the laboratory challenge study. Our results suggest that the modified rLaSota virus may be useful as an improved live NDV vaccine, which will be highly beneficial to the poultry industry throughout the world.

Chapter 4: Evaluation of other avain paramyxovirus fusion protein cleavage site sequences for the generation of genotype matched Newcastle disease virus vaccine

4.1 Abstract

Newcastle disease virus (NDV) causes severe economic losses to poultry industry worldwide. Frequent outbreaks of NDV in commercial chickens vaccinated with live vaccines suggest a need to develop new vaccines that are genetically matched against circulating NDV strains. In this study, the fusion protein cleavage site (FPCS) sequence of strain Banjarmasin (010), a genotype VII virus isolated from an outbreak in Indonesia was individually changed to those of three serotypes of avian paramyxoviruses (APMV serotype-2, -7 and -8). These FPCS mutations modified *in vitro* cell-to-cell fusion activity and made recombinant Banjarmasin virus highly attenuated in chickens. When chickens were immunized with the FPCS mutant viruses and challenged with the virulent Banjarmasin (010) parent virus, there was reduced challenge virus shedding compared to birds immunized with the heterologous vaccine strain LaSota. Among the vaccine candidates, rBanjarmasin containing the avirulent cleavage site sequence of vaccine strain LaSota and APMV-8 induced the highest neutralizing antibody titer and protected chickens with reduced challenge virus shedding. These results show the role of the F protein cleavage site sequence of APMV type 2, 7, and 8 in generating genotype VII matched vaccines and the efficacy of matched vaccine strains to provide better protection.

4.2 Introduction

Newcastle disease (ND) is a highly contagious disease of avian species that cause severe economic losses to the poultry industry. Newcastle disease virus (NDV) is an enveloped virus, a member of the genus *Avulavirus* in the family *Paramyxoviridae.* NDV has a single-stranded, negative-sense, nonsegmented RNA genome that contains six genes, encoding at least seven proteins namely nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), a large polymerase protein (L), and protein V, expressed by RNA editing during synthesis of the P mRNA. NDV strains are classified as highly virulent (velogenic), moderately virulent (mesogenic), or avirulent (lentogenic) on the basis of their pathogenicity in chickens (4).

The envelope glycoproteins, F and HN are the major protective antigens and are main targets for neutralizing antibodies. The F protein is synthesized as an inactive precursor (F_0) that is cleaved by host cell proteases into F_1 and F_2 subunits that are bound by disulphide bonds to be biologically active. The HN protein is responsible for attachment to the sialic acid receptors on the cell membrane and the F protein mediates fusion of the viral envelope with the host cell membrane and injects the viral RNA into the cytoplasm. The F protein cleavage site sequence is well-characterized and the presence of multibasic residues at the protease cleavage site of the fusion (F) protein has been shown to be a primary determinant differentiating virulent versus avirulent strains. The lentogenic avirulent NDV strains typically have basic amino acids at the −1 and −4 positions in the cleavage site (G/E-K/R-Q-G/E-R↓L) and depend on trypsin-like

proteases (added trypsin in cell culture or chicken egg allantoic fluid) for cleavage. This limits the replication of avirulent strains to the respiratory and gastrointestinal tracts, where the secreted proteases are found. Mesogenic and velogenic NDV strains contain a polybasic cleavage site (R/K-R-Q-R/K-R↓F) that has the preferred recognition site for furin R-X-K/R-R↓X, an intracellular protease present in a wide range of cells. All NDV strains belong to a single serotype APMV-1. However, based on the genetic diversity of the F gene, NDV strains have been classified into at least 18 genetic groups known as genotypes. Recently, viruses belonging to genotype V and VII are causing major outbreaks in Asia and Africa whereas the currently used vaccine viruses B1 and LaSota strains belong to genotype II. Although these strains have been highly successful as live vaccines, they do not completely prevent virulent virus infection or shedding.

In our laboratory, we developed reverse genetic system for a highly virulent NDV strain Banjarmasin/010/10 (Ban/010) that was isolated from an outbreak in chickens from Indonesia in 2010 (95). The virulent F protein cleavage site (RRQKR↓F) of the Indonesian virus was modified into avirulent LaSota (GRQGR↓L) cleavage site, named rBanj-AF, and tested as a genotype-matched vaccine virus (95). In this study, we compared the safety and efficacy of other three avian paramyxovirus (APMV) fusion protein cleavage site (FPCS) on protective immunity of genotype VII vaccines developed via reverse genetics. We generated three avirulent recombinant Banjarmasin genotype VII viruses in which the F protein cleavage site was replaced with that of APMV serotypes 2, 7 and 8 viruses. These modified viruses were compared with the recombinant LaSota (rLaSota) virus for replication *in vivo* and *in vitro*, and for

immunogenicity and protective efficacy against challenge with virulent NDV strains GB Texas and Ban/010. These results will be useful for the development of better and efficient genotype-matched NDV vaccine.

4.3 Material and Methods

4.3.1 Viruses and cells

Chicken embryo fibroblast cell line (DF-1) and human epidermoid carcinoma cell line (HEp-2) were grown in Dulbecco's minimal essential medium (DMEM) with 10% fetal bovine serum (FBS). The modified vaccinia virus strain Ankara (MVA) expressing T7 RNA polymerase was kindly provided by Dr. Bernard Moss (NIH, Bethesda, MD). The recombinant NDVs were grown in 9-day-old embryonated specific-pathogen-free (SPF) chicken eggs and in DF-1 cells with 10% fresh chicken egg allantoic fluid.

4.3.2 Plasmid construction and rescue of recombinant viruses

The construction of plasmid pNDV carrying the full length antigenome cDNA of the NDV strain Banjarmasin (rBanj/AF) has been described previously (95). We used overlapping PCR to introduce individual amino acid substitutions into the F gene of strain rBanj/AF. The mutated gene was then replaced into a full-length cDNA clone of the rBanj/AF antigenome using AsiSI and AgeI restriction sites (Figure. 13). The following primer sequences were used for first overlapping fragment.

AsisI-F	5' GCGATCGCTTATAGTTAGCTCAC 3'
APMV-2-R	5'GAACCTCGAGGCAGGTTTTCCTCCGGACGTGGCCACCG 3'

APMV-7-R 5’AAATCTCGATGAGGGGAGTCCTCCGGACGTGGCCACCG 3’

APMV-8-R 5’TAGTCTAGTCTGGGGATATCCTCCGGACGTGGCCACCG 3’

The following primer sequences were used for second overlapping fragment.

Age1-R 5’ TCTTTGGTCGCTTGTAACCGGT 3’

APMV-2-F 5’ AAACCTGCCTCGAGGTTCATAGGTGCCGTTATTGGCAGTG 3’

APMV-7-F 5’ CTCCCCTCATCGAGATTTATAGGTGCCGTTATTGGCAGTG 3’

APMV-8-F 5’ TATCCCCAGACTAGACTAATAGGTGCCGTTATTGGCAGTG 3’

These clones were transfected into HEp-2 cells, and mutant viruses were recovered as previously described (42). The FPCS mutant viruses were designated as rBanj-APMV2, rBanj-APMV7 and rBanj-APMV8. The F genes from recovered viruses were sequenced to confirm the mutations.

4.3.3 Intracerebral Pathogenicity Index Test

All the animals used in this study were housed in isolator cages and cared for in accordance with established guidelines, and the experimental procedures were performed with approval from Institutional Animal Care and Use Committee of the University of Maryland. The pathogenicity of Banjarmasin FPCS mutant viruses was determined by the intracerebral pathogenicity index (ICPI) test in 1-day-old specific pathogen free (SPF) chickens (70). 1-day-old SPF chickens were obtained from Charles River Laboratories, Wilmington, MA, USA. Briefly, for the ICPI test, 0.05 mL of a 1:10 dilution of fresh egg-grown virus was inoculated into group of ten 1-day-old SPF chicks via intracerebral

route. At each observation, the birds were scored 0 if normal; 1 if sick; and 2 if dead. The ICPI is the mean score per bird per observation over the 8-day period. Highly virulent viruses give values approaching 2 and avirulent or lentogenic strains give values close to 0.

4.3.4 Genetic Stability of rBanjarmasin FPCS mutants

The genetic stability of rBanj-APMV2, rBanj-APMV7 and rBanj-APMV8 was confirmed by passaging the viruses at least 10 times in 9-day-old embryonated chicken eggs and five times in the respiratory tract of 1-day-old chickens. For egg passage, diluted virus in PBS containing 10 $TCID_{50}$ was injected into the allantoic cavity of three 9-day-old embryonated SPF chicken eggs. Three days after incubation at 37°C, the passaged virus was harvested from the allantoic fluid and further passaged in new set of eggs. For passage in the respiratory tract of 1-day-old chicks, three chicks per virus were inoculated with 100 µL of a $1x10^5$ $TCID_{50}$ of virus by oculo-nasal route. Three days after inoculation, the trachea and lungs were collected and placed in DMEM containing 10X antibiotics (Invitrogen, USA), homogenized and clarified by centrifugation at 2,500 rpm for 15 min. The supernatants were directly inoculated via occulo-nasal route into a new batch of three 1-day-old chicks. To confirm the presence of the virus in the tissue homogenate, 200 µL of the clarified supernatant was injected into the allantoic cavity of 9-day-old embryonated chicken eggs and tested by HA assay to confirm that virus had been recovered from the tissue samples. From each passage, total RNA was extracted from infective allantoic fluid of 9-day-old SPF chicken embryos, using TRIzol reagent (Invitrogen). RT-PCR was performed using the Thermoscript RT-PCR kit (Invitrogen)

with specific forward and reverse primers to amplify the F gene. The amplified PCR fragments were then sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems Inc.) in an ABI 3130*xl* genetic analyzer to confirm the presence of the introduced mutations in the passaged viruses.

4.3.5 Replication of rBanjarmasin FPCS mutants in 1-day-old chicks

The replication of rBanj-APMV2, rBanj-APMV7 and rBanj-APMV8 virus was compared to rBanj-AF (95) and rLaSota in 1-day-old chickens. Three 1-day-old chickens per virus group were inoculated with rBanj-APMV2, rBanj-APMV7, rBanj-APMV8, rBanj-AF or LaSota by one drop in each eye and nostrils (100 µL/bird) with a titer of $1x10^6$ $TCID_{50}$. All the birds were sacrificed at 3 dpi and tissue samples of brain, lung, trachea and spleen were collected. The tissue samples were weighed and homogenized in media containing 10X antibiotics. The supernatants were titrated in DF-1 cells by $TCID_{50}$ assay.

4.3.6 Growth kinetics in DF-1 cells and 9-day old embryonated eggs

DF-1 cells grown in six-well plates were infected with each mutant virus strain, in duplicates at an MOI of 0.001. After 1 h of adsorption, the cells were washed with PBS and overlaid with DMEM containing 2% FBS and 10% fresh allantoic fluid at 37°C. A 200 µL of supernatant media was collected and replaced with an equal volume of fresh media every 8 h intervals until 64 h post infection (hpi). Virus yield were quantified by $TCID_{50}$ in DF-1 cells by the end-point method of Reed and Muench (77). To study the replication in 9-day-old embryonated eggs, 200 µL of 10 $TCID_{50}$ virus diluted in PBS

was injected into the allantoic cavity of ten 9-day-old embryonated SPF chicken eggs. Three days after incubation at 37°C, the viruses were harvested and titrated by hemagglutination (HA) assay (70).

4.3.7 Immunization and challenge experiment in chickens

The immunization studies were done in our Bio Safety Level-2 (BSL-2) facility and challenge studies were performed at our USDA approved enhanced Biosafety level 3 (BSL-3+) containment facility. 1-day-old SPF chickens were randomly assigned to 6 treatment groups of 20 birds. All birds were housed in separate poultry isolation chambers with *ad libitum* access to feed and water. The birds were vaccinated via occulo-nasal route with 100 µL of $1x10^5$ $TCID_{50}$ of rLaSota, rBanj-AF, rBanj-APMV2, rBanj-APMV7, rBanj-APMV8 or sterile PBS. Serum was collected at 1, 2, 3 and 4 weeks post-immunization (WPI) for analyzing NDV antibody levels by Hemagglutination Inhibition (HI) assay and virus neutralization assay. At 4 WPI, all the groups were challenged by occulo-nasal route with virulent NDV strains Ban/010 and Texas GB at 100 chicken lethal dose 50 (CLD_{50}) per bird. All birds were observed 10 days for clinical signs (death, paralysis, and torticollis). In order to determine shedding of the vaccine and virulent challenge virus, oral and cloacal swabs were collected on day 4 after vaccination and day four after challenge from all chickens.

4.3.8 Serological cross-reaction between Banjarmasin FPCS mutants and LaSota

Serum neutralization activity was measured by the suppression of fluorescence by rBanj-AF and rLaSota expressing enhanced green fluorescent protein (EGFP). These viruses were named rBanj-AF-EGFP and rLaSota-EGFP. Two-fold serial dilutions of complement inactivated serum samples were made in a 96-well plate and incubated with $1x10^3$ $TCID_{50}$ of the recombinant virus at 37 °C for 1 h. The mixtures were then transferred to DF-1 cells in a 96-well plate, incubated for 2 h, and replaced with DMEM containing 2% FBS and 10% fresh allantoic fluid at 37°C. After incubation at 37°C for 48 h, the cells were washed with PBS and fixed with 4% paraformaldehyde. The fluorescence intensity was measured with a microplate reader (Figure.23). The neutralization titer was defined as the reciprocal of the highest serum dilution that resulted in 50% reduction in fluorescence.

4.4 Results

4.4.1 Construction and recovery of rBanjarmasin FPCS mutant viruses

In the present study, three F protein cleavage site mutant cDNAs of NDV strain Banjarmasin containing FPCS of APMV serotypes 2, 7 and 8 viruses were constructed (Figure. 13). cDNAs were transfected into HEp-2 cells and mutant viruses were recovered by procedures described previously (42). The recovered viruses were designated as rBanj-APMV2, rBanj-APMV7 and rBanj-APMV8. The nucleotide sequences of the whole F gene were confirmed for the presence of introduced mutation and also for the lack of adventitious mutations.

4.4.2 Pathogenicity of FPCS mutant rBanjarmasin viruses in 1-day-old chickens

The pathogenicity of F protein cleavage site mutant rBanj-APMV2, rBanj-APMV7 and rBanj-APMV8 viruses were evaluated by intracerebral pathogenicity index (ICPI) test in 1-day-old SPF chicks (70). ICPI values for velogenic strains are close to 2.0 and the lentogenic strains give values close to 0.00. The ICPI values of rBanj-APMV2, rBanj-APMV7 and rBanj-APMV8 were close to 0 (Table 3). These results showed that the FPCS mutant viruses were similar to the vaccine strain LaSota and has the potencial to be used as vaccines for ND.

4.4.3 Genetic Stability of the rBanjarmasin FPCS mutants

The genetic stability of rBanjarmasin FPCS mutants was evaluated in nine-day-old embryonated chicken eggs and in one-day-old chicks. At least ten serial passages were made in embryonated eggs and five passages were made in the respiratory tract of 1-day-old chickens. After the final passage in each regimen, the recovered viruses were subjected to RT-PCR and

sequence analysis. There were no changes found in the nucleotide sequences, indicating a lack of reversion or introduction of adventitious mutations. The passaged viruses were also infected in DF-1 cells (Figures 14 and 15) and 10 individual plaques were picked for RT-PCR and sequencing. No nucleotide changes were detected and all the FPCS mutants were stable.

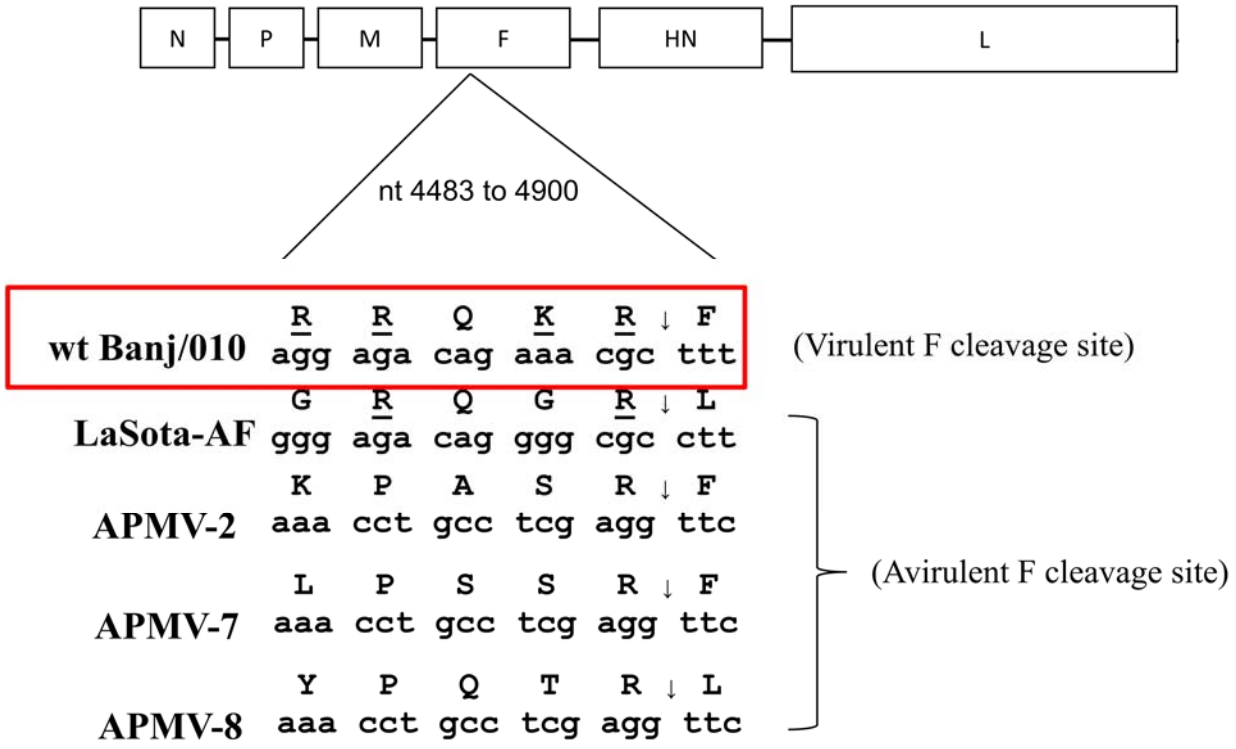

Figure 13. Gene map of a full-length antigenomic cDNA of NDV strain Banjarmasin (Ban/010) with modified FPCS: The arrow indicates the amino acid location where cleavage occurs.

Clevage site mutant viruses	ICPI
rBanj-APMV2	0
rBanj-APMV7	0.25
rBanj-APMV8	0
rBanj-AF	0
rLaSota	0

Table 3. Pathogenicity of rBanjarmasin FPCS mutants in 1-day-old chickens.

4.4.4 Fusion protein cleavage

The cleavage of the F protein was compared in the presence and absence of exogenous protease (10% fresh chicken allantoic fluid) by Western blot analysis using NDV F cytoplasmic tail anti-peptide rabbit serum (Figure. 16). The results showed that the F proteins of rBanj-APMV2, rBanj-APMV7 and rBan-APMV8 were cleaved either in the presence or the absence of exogenous protease (10% fresh chicken allantoic fluid) after 24 h infection in DF-1 cells, whereas the commonly used vaccine strain LaSota can grow well only in the presence of exogenous protease (10% fresh chicken allantoic fluid) (Figure. 16).

4.4.5 Biological characterization of rBanjarmasin FPCS mutant viruses

Plaque assay in DF-1 cells showed that rBanj-APMV2, rBanj-APMV7 and rBanj-APMV8 produced plaques similar to rLaSota (Figure. 15), whereas rBanj-AF produced only

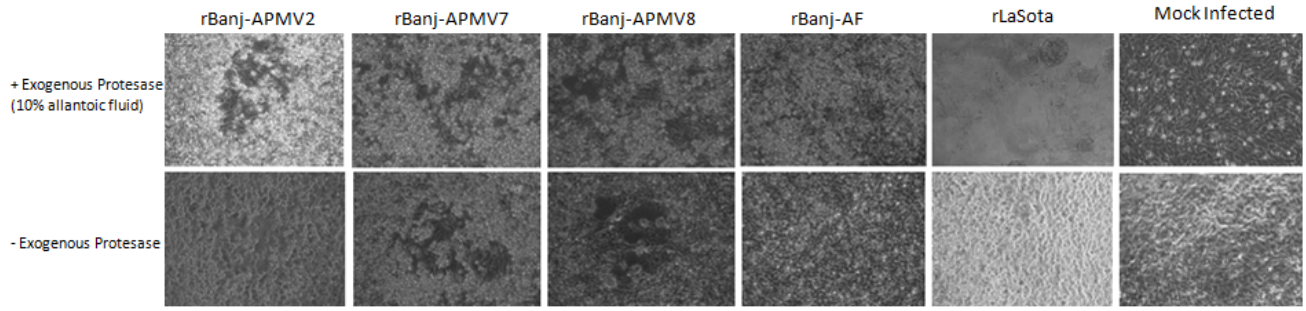

Figure 14. Cytopathogenicity of rBanjarmasin FPCS mutants in DF-1 cells: The cells were infected at an MOI of 1with each of these viruses. After 3 days, the cyotopathic effects (CPE) of each virus infected monolayer was examined under microscope.

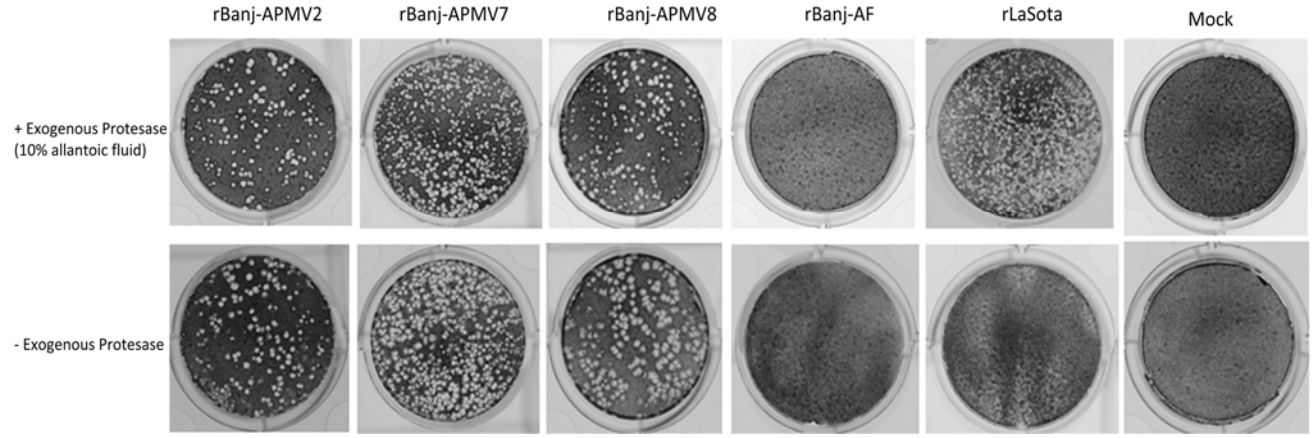

Figure 15. Plaque morphology of rBanjarmasin FPCS mutants in DF-1 cells.

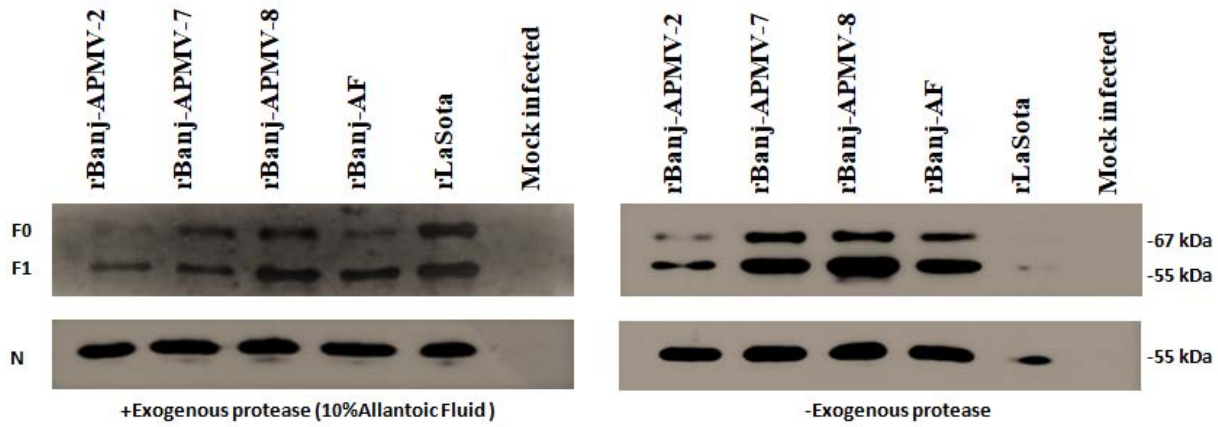

Figure 16. F protein cleavage of rBanjarmasin FPCS mutants and rLaSota virus in DF-1 cells: The cells were infected with respective viruses at an MOI of 1. The cell lysates were collected at 24 h post infection. Western blot was performed with NDV F cytoplasmic tail anti-peptide rabbit serum.

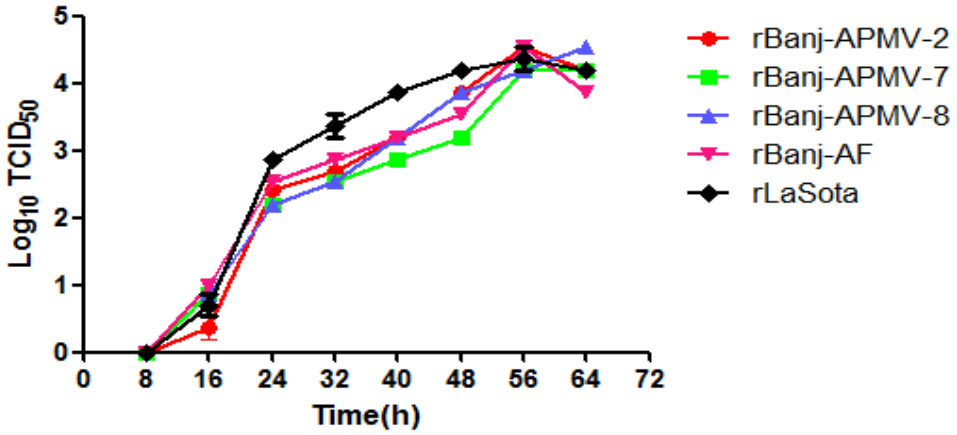

Figure 17. Growth kinetics of rLaSota and rBanjarmasin FPCS mutants in chicken embryo fibroblast (DF-1) cells: Cells were infected at an MOI of 0.001 of each virus and the cell culture supernatant was collected at 8 h intervals for 64 h. All virus titers are expressed as mean $\log_{10}$ $TCID_{50}$/mL ± SEM (standard error of the mean).

single cell infections and no plaques (95). The multi-cycle growth kinetics of the FPCS mutant viruses were determined in DF-1 cells and viral titers were analyzed by the $TCID_{50}$ assay (Figure. 17). The FPCS mutants replicated exponentially until 56 hpi, after which they reached a plateau. The replication of rBanj-APMV2 was slightly lower than other FPCS mutants but reached similar titer at 56 hpi. In 9-day old embryonated eggs, all the viruses reached similar HA titers at 3 days post-inoculation at 37°C (Figure. 18).

4.4.6 Replication and tissue tropism of rBanjarmasin FPCS mutant viruses in one-day-old chicks

We evaluated the replication and tissue tropism of rBanjarmasin FPCS mutant viruses in 1-day-old chickens. Chicks in groups of 3 were inoculated with rBanj-APMV2, rBanj-APMV7, rBanj-APMV8, rBanj-AF or rLaSota by one drop in each eye and nostrils (100 µL/bird) with a titer of $1x10^5$ $TCID_{50}$. All the birds were sacrificed at 3 dpi and tissue samples from brain, lungs, trachea and spleen were collected. The viruses were detected in all organs except brain. Comparison of the viral titers of the tissue samples showed that all mutant viruses replicated well in trachea except rBanj-APMV7, whereas in spleen and lungs all the viruses replicated to similar titers (Figure. 19).

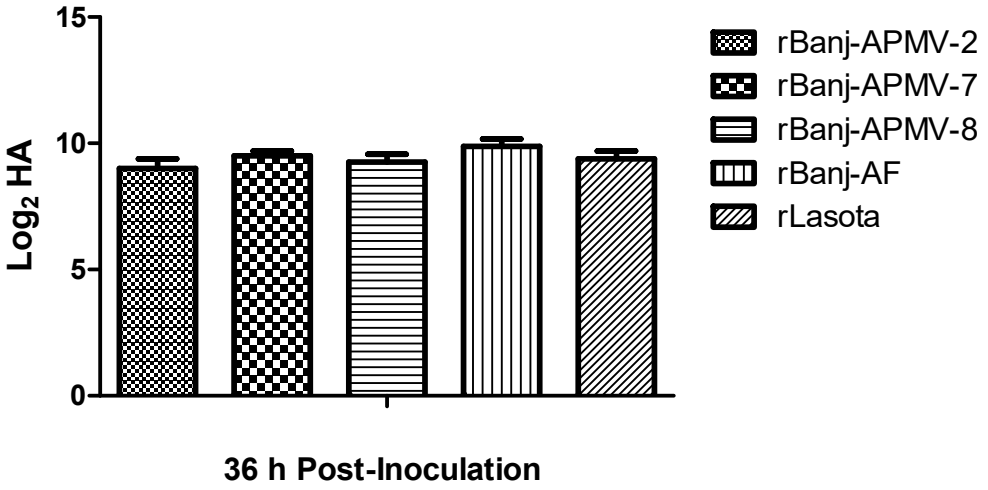

Figure 18. Growth kinetics of rBanjarmasin FPCS mutants and rLaSota in 9-day-old embryonated eggs: 200 µL of 10 $TCID_{50}$ in PBS was injected into the allantoic cavity of ten 9-day-old embryonated SPF chicken eggs. 3 days after incubation at 37°C, the allantoic fluids was harvested and titrated by hemagglutination (HA) assay. The bars are the means of HA titers of triplicate wells. Error bars indicate standard error of the mean.

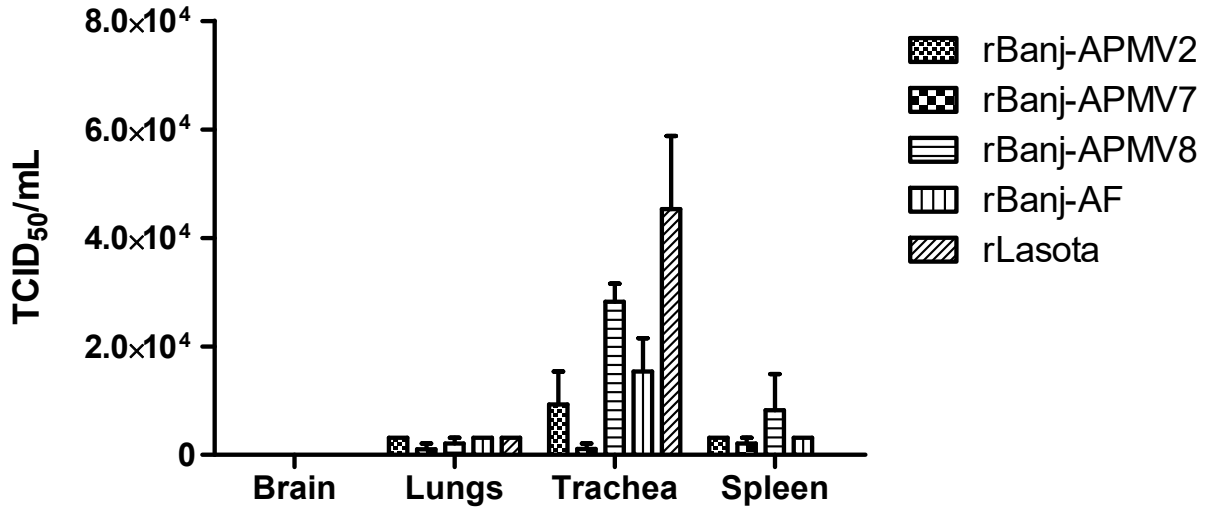

Figure 19. Virus titers and tissue tropism of rBanjarmasin FPCS mutants in one-day-old chickens following oculonasal inoculation: Tissue samples from brain, lungs, trachea, and spleen of 3 chickens (n=3) from each indicated virus group were harvested on day 3 post infection, and virus titers were determined by limiting dilution assay (77). The mean virus titer from 3 chickens per group was given for each tissue sample. Error bars indicate SEM.

4.4.7 Evaluation of immunogenicity and protective efficacy of rBanjarmasin FPCS mutant viruses in chickens

The immunogenicity of the rBanjarmasin FPCS mutant viruses was evaluated in 1-day-old SPF chicks. The birds were randomly assigned into 6 groups of 20 birds. The birds were infected via the occulo-nasal route with 100 µL of 10^5 $TCID_{50}$ of rBanj-AF, rBanj-APMV2, rBanj-APMV7, rBanj-APMV8, rLaSota and PBS. Blood samples were collected from all the birds at 1, 2, and 3 weeks post infection (WPI) to separate serum for assessing NDV antibodies. None of the chickens infected with rLaSota or rBanjaramasin FPCS mutant viruses showed clinical signs, indicating that all viruses were avirulent to chickens. All infected birds were seropositive for NDV antibodies by hemagglutination-inhibition (HI) test. 4 HA units of rBanj-AF or rLaSota were used for HI test. The birds vaccinated with rBanj-AF had higher HI titer to rBanj-AF antigen compared to birds vaccinated with rLaSota. Similarly, birds vaccinated with rLaSota showed higher HI titer to rLaSota antigen, compared to birds vaccinated with rBanj-AF (Figure. 22). Birds from all the groups were challenged at 3 WPI via occulo-nasal route with virulent NDV strains Texas GB (belonging to genotype II) and strain Banjarmasin (belonging to genotype VII) at 100 CLD_{50} (200 µl of 10^4 $TCID_{50}$) per bird. Our results showed all the birds immunized with rLaSota or rBanjarmasin FPCS mutants were completely protected from virulent Texas GB and Banjarmasin challenge without any clinical signs. In contrast, all the birds in the PBS control group had to be euthanized on day 4 or 5 after challenge based on clinical scores. In order to determine shedding of the vaccine and day 4 post challenge virus, oral and cloacal swabs were collected on day 4 post vaccination and challenge from all the chickens.

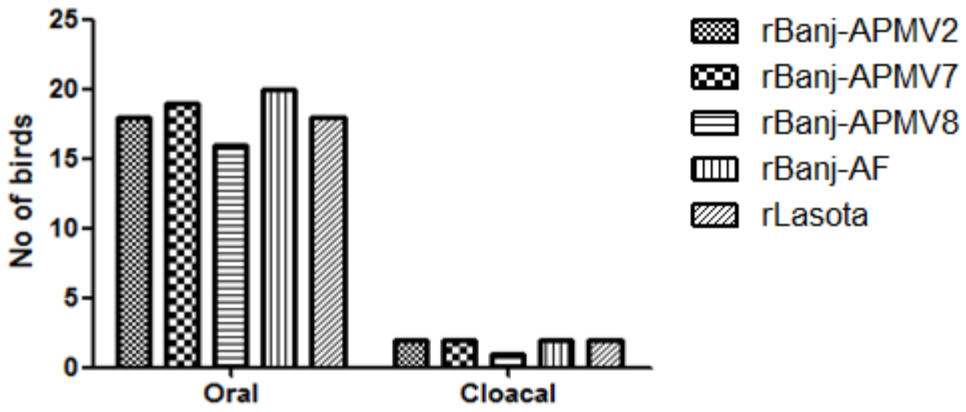

Figure 20. Replication and shedding of vaccine viruses in 1-day-old chickens: Chickens in groups of 20 were infected with rBanjaramasin FPCS mutants via occulo nasal route. At day 4 Post inoculation (PI), oral and cloacal swabs were collected and analysed for the presence of virus by inoculation into 9-day-old embryonated chicken eggs and subsequently tested by hemagglutination (HA) assay.

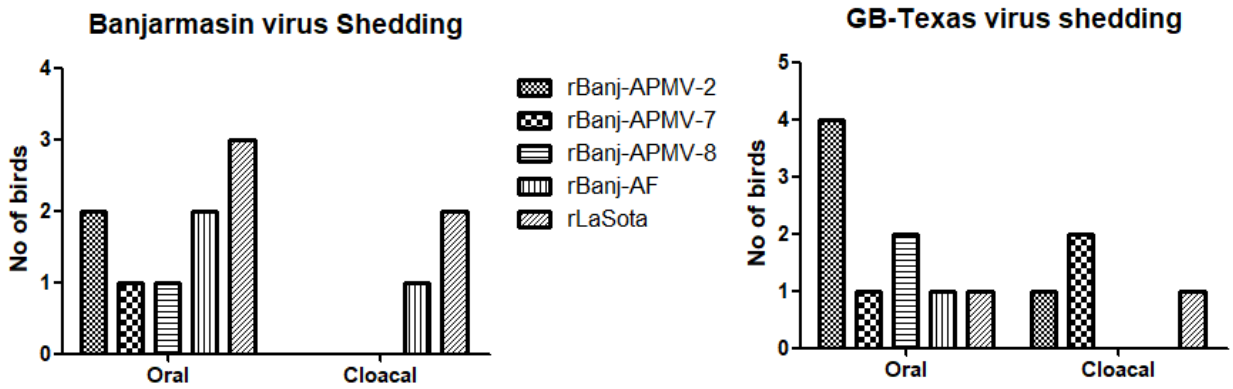

Figure 21. Challenge virus shedding in birds vaccinated at 1-day-old and challenged at 3-week-old age: Groups of 10 birds per virus vaccinated with rBanjarmasin FPCS mutants at 1day-old and challenged with 100 CLD_{50} in 200 µL volume, via occulo nasal route. Oral and cloacal swabs were collected on day 4 post challenge and samples were assayed for virus detection by inoculation into 9-day-old embryonated chicken eggs and subsequently tested by hemagglutination (HA) assay.

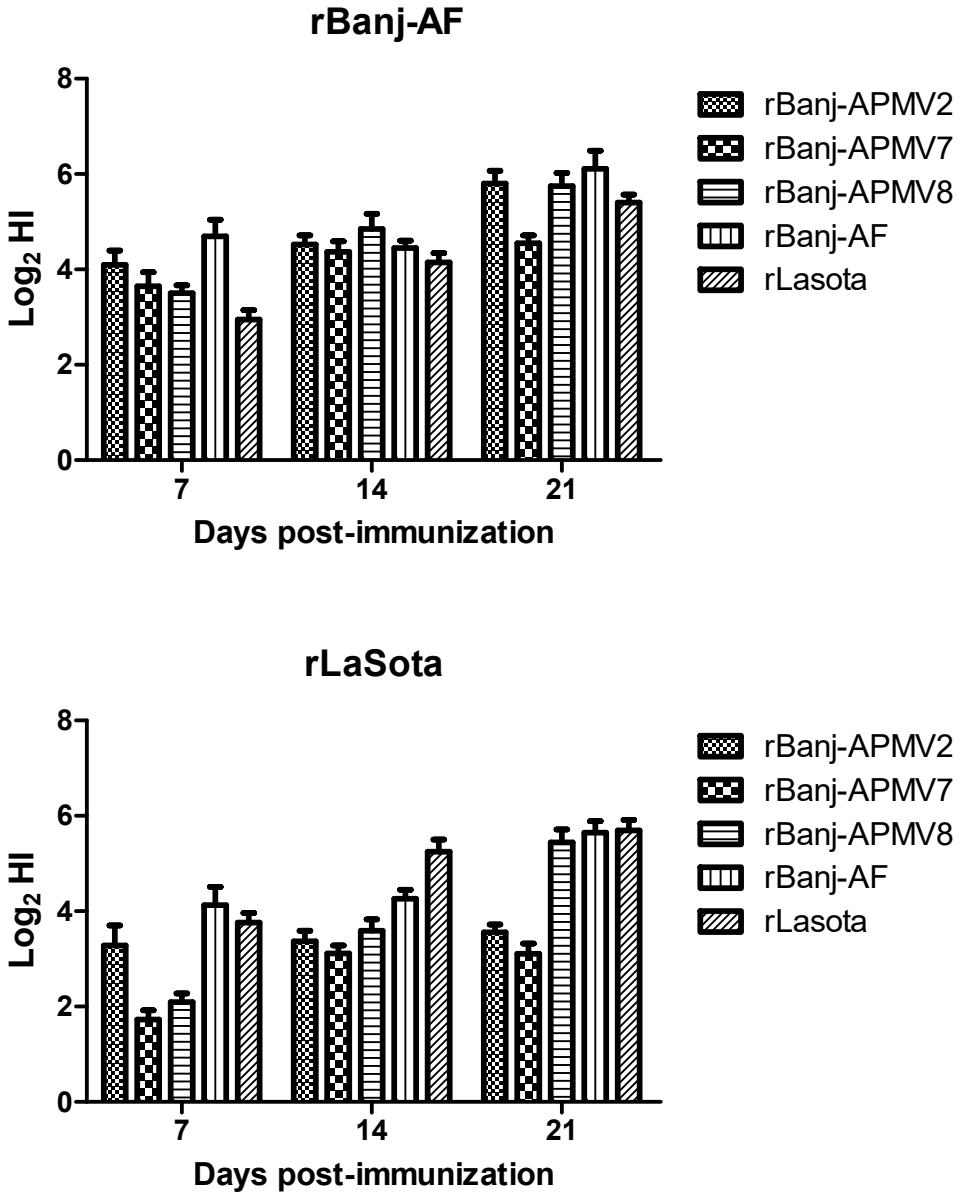

Figure 22. Induction of serum antibodies in 1-day-old chickens in response to vaccination with rBanjarmasin FPCS mutants: 20 Chickens per group were inoculated with 100 µl of ($1x10^5$ PFU) virus via intranasal route. Serum samples were collected at 1, 2, and 3 weeks post infection. Virus specific antibodies were determined by a hemagglutination inhibition assay using 4 HA units of rLaSota and rBanj-AF.

Our vaccine virus shedding results showed the presence of virus from most of the oral swabs and a few of the cloacal swabs (Figure. 20). Our challenge virus shedding results showed that neither of the vaccine gave 100% protection from virulent virus shedding (Figure. 21). But there was at least 50% reduction in challenge virus shedding when the vaccine virus and the challenge virus are from the same genotype and higher shedding was observed when the challenge virus are from a heterologous genotype. Serum samples collected at 7, 14 and 21 dpi and were analyzed by HI and neutralization assays against the two viruses, rBanj-AF and rLaSota. The sera from rLaSota immunized 1-day-old chicks had at least 1 log higher HI titer to the rLaSota virus on day 14, compared to the titers of rBanj-AF mutants. The sera from rBanj-AF immunized chicks had at least 1.5 log higher HI titers to rBanj-AF on day 7, but lower HI titer to the LaSota (Figure. 22). The serum neutralization analysis showed that all the serum samples neutralized the heterologous viruses, but the neutralization titers were at least 2 folds higher to homologous strain than to heterologous strain (Figure. 23 and 24). These results are consistent with the HI results and it was observed that the serum neutralization and HI titers of sera obtained from 1-day-old immunized chicks showed that the genotype matched vaccines induced better neutralizing antibody response.

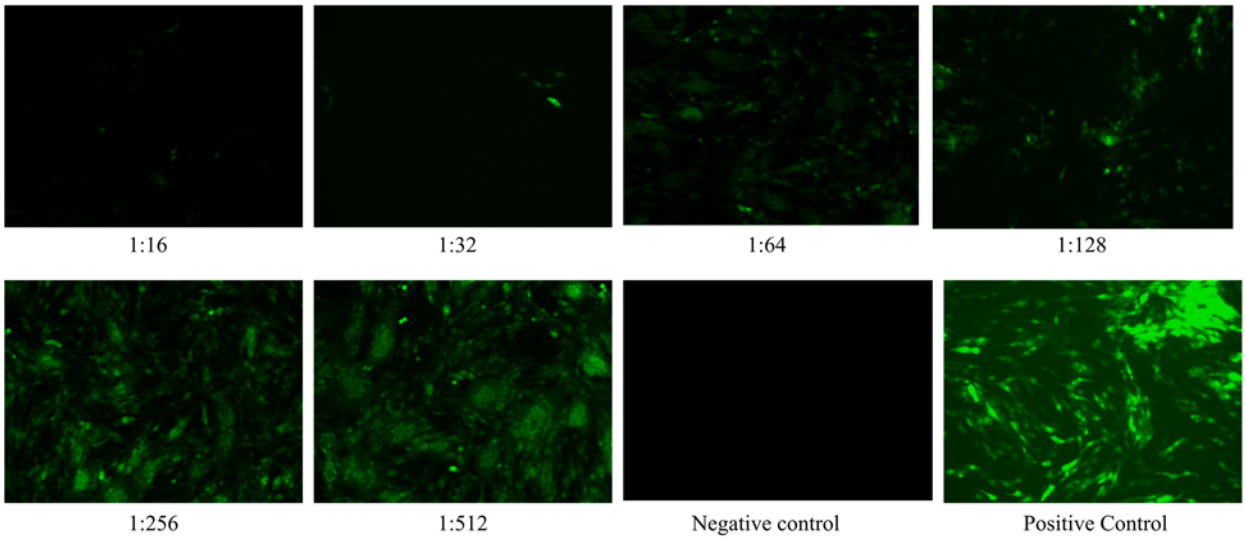

Figure 23. EGFP based fluorescence reduction assay: The figure shown in 20X magnification represents a gradient increase in the green fluorescence intensity by rNDVs expressing EGFP in DF-1 cells, as the complement inactivated serum samples are serially diluted. The fluorescence intensity is then measured with a microplate reader and the neutralization titer was defined as the reciprocal of the highest serum dilution that resulted in 50% reduction in fluorescence.

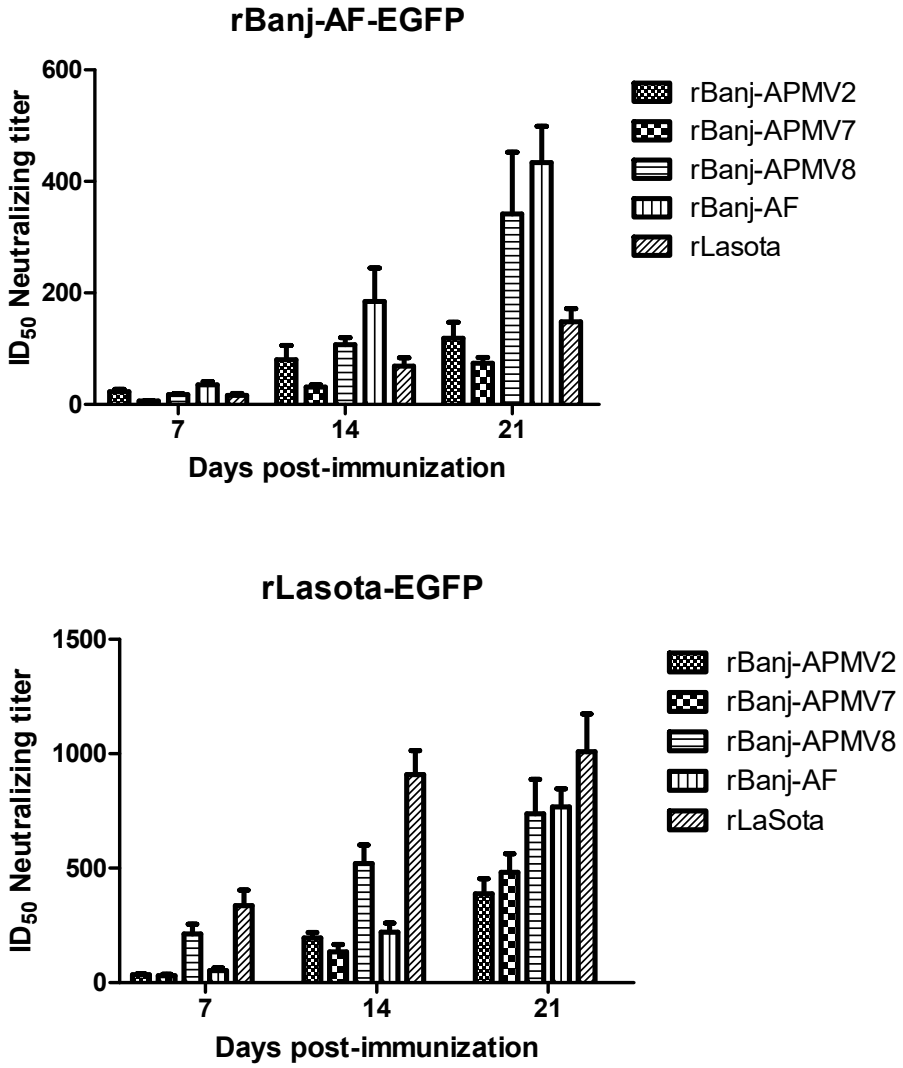

Figure 24. Induction of virus-specific serum neutralizing antibodies: Two-fold serial dilutions of complement inactivated serum samples were made in a 96-well plate and incubated with $1x10^3$ $TCID_{50}$ of the recombinant virus at 37 °C for 1 h. After incubation at 37 °C for 48 h, the cells were washed with PBS and fixed with 4% paraformaldehyde. The fluorescence intensity was measured with a microplate reader. The neutralization titer was defined as the reciprocal of the highest serum dilution that resulted in 50% reduction in fluorescence.

4.5 Discussion

Safety is an important concern for live attenuated vaccines. The major difference between low virulence and high virulence NDV strains is the amino acid sequence at the F protein cleavage site (2). In general, the low virulence strains have monobasic cleavage site, while the virulent strain have multibasic cleavage site. The currently used NDV vaccine strains LaSota and B1 belong to genotype II, whereas the most commonly circulating velogenic NDV strains in Indonesia belonged to genotype VII. The sequence variation between the vaccine strain and currently circulating virulent NDV strain may be the reason for not mounting an effective immune response required to neutralize the field strains, leading to vaccine failure and disease outbreak. Therefore, the goal of this study was to determine an efficient avirulent FPCS for an attenuated, stable, live vaccine virus that could provide better protection against genotype-matched isolates than that provided by the commonly used vaccine strain LaSota. We found that changing the FPCS of the virulent NDV strain Banjarmasin to APMV-2,-7, and -8 FPCS totally attenuated the virus. It also lead to a change in the fusion phenotype, modulating viral replication and pathogenicity. Our virulent NDV challenge study showed that the FPCS mutant virus completely protected all immunized chickens from clinical disease and there was decreased shedding of challenge virus in rBanj-AF and rBanj-APMV8 immunized chickens. Although, all the chickens were protected from disease by rLaSota vaccination, there was more challenge virus shedding when the challenge virus was from a different genotype. However, when the challenge virus was from the same genotype as rLaSota, birds vaccinated with rLaSota was effective in preventing virus infection and shedding. The results presented here indicate that the genotype matched NDV vaccines protect

better than the currently used vaccine strain LaSota given that the challenge virus was from the same genotype as the vaccine virus. However, our results on the effect of FPCS mutations on immunogenicity in birds showed that the rBanj-AF containing avirulent LaSota FPCS and rBanj-APMV8 were better in protecting the birds than rBanj-APMV2 and rBanj-APMV7.

The HI titer induced in chickens by genotype matched mutant virus was at least 2 logs higher than that induced by the LaSota at 7 day post-immunization. This result was remarkable considering the fact that LaSota strain is used all over the world and any further increase in immunogenicity is an added advantage for the virus as a live vaccine candidate. It is particularly important for a NDV vaccine because NDV is a highly infectious and fast replicating virus and for the vaccine to be efficient it must mount highest level of immune response possible. In summary, we evaluated three different APMV FPCS to generate genotype matched vaccine candidates for Indonesian NDV strain Banjarmasin. Our results confirmed that avirulet LaSota cleavage site gave better immune response and neutralizing antibodies which will be highly beneficial to the poultry industry throughout the world.

Chapter 5: Engineered Newcastle Disease virus as an improved vaccine vector against Simian Immunodeficiency virus

5.1 Abstract

SIV infection in macaques is the most suitable model for human immunodeficiency virus (HIV) pathogenesis and vaccine study in humans. As part of an effort to design a safe and effective vaccine against HIV, we examined suitability of naturally-occurring Newcastle disease virus (NDV) strains and modified versions of NDV as vectors for the expression and immunogenicity of SIV envelope protein gp160. All the modified vectors expressed SIV gp160 protein in infected cells and formed higher-order oligomers. The gp160 expressed by these vectors was incorporated into the envelope of NDV. All the modified viral vectors were highly attenuated based on pathogenicity tests in chickens. Immunization of guinea pigs via intranasal route with modified NDV vectors such as rNDV-APMV-2CS/gp160 and rNDV-APMV-8CS/gp160 (NDV strain LaSota containing F protein cleavage site sequences of avian paramyovirus (APMV) serotype 2 and 8, respectively), and rNDV-BC-F-HN/gp160 (NDV strain BC containing LaSota F cleavage site and LaSota F and HN genes) elicited higher humoral and mucosal immune responses compared to other vectors. These modified vectors were also efficient in inducing neutralizing antibody responses to tier 1 SIVmac251.6 and tier 1b SIVmac251/M766 strains. This study suggests that our novel modified vectors are safe and immunogenic and can be considered as an HIV vaccine platform.

5.2 Introduction

An effective and safe vaccine against human immunodeficiency virus-1 (HIV-1) infection is the best option to control HIV pandemic. Efforts to develop a safe and effective vaccine against HIV-1 have been hindered by the lack of a good animal model. High degree of sequence relatedness between macaques and humans and similarities between HIV-1 infection in humans and simian immunodeficiency virus (SIV) infection in rhesus macaques have made macaques as valid animal model to evaluate immunogenicity of different HIV vaccine candidates. Eventually, the creation of SIV-HIV chimeras has led to various simian-human immunodeficiency virus (SHIV) challenge models for HIV-1 infection (69). Protection against experimental infection of macaques with SIV or SHIV has been achieved with a number of different vaccination strategies employing recombinant proteins, peptides, inactivated viruses, DNA and live viral vectored vaccines either alone or in different prime-boost combinations (7, 33, 64). In parallel, there have been six HIV vaccine efficacy trials in humans that have tested four different vaccine concepts but only RV144 trial showed a modest level of efficacy (69). The data from this trial and the data from a macaque challenge study (73) indicated the importance of HIV envelope (Env) protein expressed by recombinant viral vectors and further emphasized the role of antibodies in inducing protective immune responses against HIV.

Among the various vaccines currently evaluated for HIV and SIV, live viral vectored vaccines are the most promising and several of these vectored vaccines are being tested. For safety reasons, majority of these studies have been based on

recombinant viral vectors such as fowl pox virus, vaccinia virus and adenoviruses that are incapable or poorly capable of replication (9, 79). Replication-competent vectors such as vesicular stomatitis virus (VSV) and measles virus are also being investigated (79). However, pre-existing immune responses and safety concerns regarding systemic spread causing viremia and potential neurovirulence were associated with these vectors (17, 46, 79). Therefore, there is a need to evaluate additional viral vectors that are highly immunogenic, have no pre-existing antibodies and have no safety concerns in human population.

Newcastle disease virus (NDV) is a member of the genus *Avulavirus* of the family *Paramyxoviridae*. NDV is a single-stranded, negative-sense RNA virus, containing six genes, which encode at least seven proteins (36). NDV isolates vary greatly in their pathogenicity in chickens and are categorized into three main pathotypes: lentogenic (avirulent), mesogenic (moderately virulent), and velogenic (highly virulent) (1, 36). Lentogenic strains such as LaSota and B1 are being used as live vaccines all over the world (29). NDV has several characteristics that make it suitable for vaccine vector development. It is safe in humans and animals due to natural host range restriction, expresses foreign protein abundantly, infects via intranasal route, produces both humoral and mucosal immune responses, antigenically distinct from human and animal pathogens and there is no preexisting immunity to NDV in humans and animals. Immunogenicity and protective efficacy of lentogenic strains of NDV as a vaccine vector against human and animal pathogens have been tested in various studies (49, 50). In addition, mesogenic strain Beaudette C (BC) has been tested as a vaccine vector and it has been shown to

replicate to a higher titer, did not cause any disease and induced higher antibody responses compared to the lentogenic strain LaSota in rhesus macaques (21). But mesogenic strain BC has a polybasic cleavage site in the fusion (F) protein and has an intracerebral pathogenicity index (ICPI) of more than 0.7 (70). Therefore, it has been classified as a select agent and must be handled only in laboratories containing BSL-3 facility (33). It would be interesting to develop NDV vectors that are more effective in stimulating the immune system than lentogenic strains and yet have the avirulent phenotype with an ICPI of less than 0.7. Hence, we modified the mesogenic strain BC by exchanging either complete or part of genes coding for F and HN proteins and also mutated the polybasic residues at F protein cleavage site to either mono or dibasic residues and developed an array of modified NDV vectors.

In our laboratory, we previously developed NDV as a vaccine vector for human immunodeficiency virus type 1 (HIV-1) (53). We showed that NDV efficiently expressed Env and Gag genes of HIV-1 and produced potent humoral and mucosal immune responses in guinea pigs as well as protective immune responses to challenge with vaccinia viruses expressing HIV-1 Env and Gag in mice (49-53). It has been shown by various laboratories that similar to HIV Env protein, Env protein of SIV is the major viral neutralization and protective antigen. In this study, we investigated the potential of modified recombinant (r) NDVs expressing gp160 (Env protein) of SIV mac 239 strain. We constructed a series of recombinant (rNDV) vectors that express SIV gp160 and used those vectors to immunize guinea pigs. The results of immune response to SIV envelope protein expressed by different modified NDV vectors were evaluated. Our results showed

that rNDV/SIV vaccines were immunogenic and effectively neutralized SIV mac251 strain *in vitro*.

5.3 Materials and Methods

5.3.1 Cells and Viruses

Chicken embryo fibroblast cell line (DF-1), human embryonic kidney cells (HEK 293) and a human epidermoid carcinoma cell line (HEp-2) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The TZM-bl cell line was obtained from the NIH AIDS Research and Reference Reagent Program (NIH ARRRP), Division of AIDS, NIAID, NIH. The cell lines were grown in Dulbecco's minimal essential medium (DMEM) with 10% fetal bovine serum (FBS) and maintained in DMEM with 2% FBS. The modified vaccinia virus strain Ankara (MVA) expressing T7 RNA polymerase was kindly provided by Dr. Bernard Moss (NIH, Bethesda, MD) and propagated in primary chicken embryo fibroblast cells in DMEM with 2% FBS. The NDV strains LaSota and the modified rNDVs were grown in 9-day-old embryonated specific-pathogen-free (SPF) chicken eggs. Viral titers in harvested allantoic fluid were determined by $TCID_{50}$ assay.

5.3.2 Construction of modified versions of NDV vectors

We used plasmid pNDV carrying full-length antigenome cDNA of the NDV strain LaSota (42) and plasmid pBC of strain Beaudette C (BC) that was modified by replacing various regions of the F gene with those of NDV strain AKO-18 (54) and

plasmid pNDV containing a mutation Y527A in the cytoplasmic tail of F protein (63), as described previously. In addition, we also made a few modified versions of recombinant pBC containing the F and HN protein of strain LaSota. All the BC recombinants had the avirulent cleavage site as that of strain LaSota (GRQGR↓L) and hence handled in BSL-2. Two modified pNDV- rLaSota vectors containing the avian paramyxovirus type-2 (APMV-2) fusion protein cleavage site (KPASR↓F) and avian paramyxovirus type-8 (APMV-8) fusion protein cleavage site (YPQTR↓L). They were abbreviated by different names as mentioned in Figure. 25.

5.3.3 Construction and recovery of rNDV and modified versions of rNDV vectors expressing the SIV envelope glycoprotein (gp160)

The plasmid containing codon optimized envelope gp160 gene of SIV mac239 strain was modified by PCR to contain transcriptional signals of NDV and restriction site for *PmeI* restriction enzyme. The following primer sequences were used, (Forward:5'AGCTTTGTTTAAACTTAGAAAAAATACGGGTAGAAGGCCACCATG GGCTGCCTGGGCAAC3', Reverse: 5'GTTTAAACTCACAGCAGGGTCAGTTCC3'). The amplified fragment is cloned into TOPO-TA, sequence confirmed and inserted between the P and M genes of the antigenomic cDNA of LaSota (pNDV) and in all the modified rNDVs (Figure. 25.). Infectious viruses were recovered using reverse genetic system established in our laboratory (42). The recombinant viruses were recovered and propagated in 9-day-old embryonated SPF chicken eggs. To determine the stability of the gp160 genes in the different rNDV vectors, the recovered viruses were passaged 10 times in embryonated chicken eggs and sequenced.

5.3.4 Expression of SIV gp160 in cells infected with rNDV and modified rNDVs

To detect expression of SIV gp160, DF-1 cell monolayers in 6-well plates were infected with 0.1 MOI of rNDVs containing SIV gp160 and the parental rLaSota virus for 24 h. The cell lysates were analyzed for expression of gp160 by Western blot using a 1:100 dilution of SIV gp120-specific monoclonal antibody (VM-18S) obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH. In order to investigate the incorporation of foreign protein into the NDV virion, allantoic fluid of infected embryonated chicken eggs was collected at 3 days post infection (dpi) and centrifuged at 3,000 rpm for 10 min. The viruses were purified by ultracentrifugation through a 30% sucrose gradient and the pellet containing the viruses was collected and analysed by Western blot using SIV gp120-specific monoclonal antibody.

5.3.5 Analysis of SIV gp160 protein oligomers

The oligomeric state of SIV gp160 expressed by the NDV recombinant vectors were analyzed by protein cross-linking, followed by Western blot analysis. Briefly, DF-1 cell monolayers were infected with 0.1 MOI of different rNDVs expressing SIV gp160 and rLaSota. After 24 h, cells were harvested and washed twice in PBS. The proteins were cross-linked using 1mM dithiobis (succinimidyl propionate) (DSP; Pierce), a thiol-cleavable, amine-reactive, and membrane-permeative cross-linker and were incubated at room temperature for 30 min. After cross-linking, samples were prepared in Laemmli's sample buffer (100 mM Tris [pH 6.8], 2% sodium dodecyl sulfate (SDS), 15% glycerol) under reducing conditions containing 5% ß-mercaptoethanol and non-reducing conditions

without 5% ß-mercaptoethanol/ samples were boiled for 5 min and subjected to SDS-PAGE and Western blot with gp120-specific monoclonal antibodies.

5.3.6 Growth kinetics of rNDVs expressing SIV gp160 in DF-1 cells

The multicycle growth kinetics of all the rNDVs was evaluated in DF-1 cells in the presence of 10% chicken egg allantoic fluid. Six-well plates were infected in duplicate with each rNDVs expressing SIV gp160 at an MOI of 0.001. Supernatant (200 µL) was collected at 8 h intervals and replaced with an equal volume of fresh medium until 64 h post infection (hpi). Virus titers in the supernatants were quantified in DF-1 cells by limiting dilution in the presence of 10% allantoic fluid and 2% FBS and expressed as 50% tissue culture infective dose per milliliter ($TCID_{50}$/mL) by the method of Reed and Muench (77).

5.3.7 Pathogenicity of rNDVs expressing SIV gp160 in 1-day-old chicks

The pathogenicity of the different rNDVs expressing SIV gp160 was determined by the intracerebral pathogenicity index (ICPI) test in 1-day-old SPF chicks (3). For the ICPI test, 0.05 mL of a 1: 10 dilution of fresh egg-grown virus was inoculated into group of ten 1-day-old SPF chicks via intracerebral route. At each observation, birds were scored 0 if normal, 1 if sick, and 2 if dead. The ICPI is the mean score per bird per observation over 8 day period.

5.3.8 Immunization of guinea pigs

All the animals used in this study were housed in isolator cages in our Bio Safety Level-2+ facility and cared for in accordance with established guidelines. The experimental procedures were performed with approval from Institutional Animal Care and Use Committee of the University of Maryland. Groups of 5-week-old female guinea pigs (3 animals per group) were immunized individually with the parental vector (rLaSota/gp160) or modified rNDVs expressing SIV gp160 (100 μL each of $1x10^5$ $TCID_{50}$) via intranasal route after xylazine and ketamine anesthesia. The control group was inoculated with rLaSota without any foreign gene. All the animals received one booster dose of immunization after 3-weeks. Blood was collected on day 0 (pre-bleed) and on days 14, 21, 28, 35, 42, 49, 56 and 63. Serum samples were prepared and stored at −70°C. Vaginal wash samples were collected in parallel with the blood samples. To collect vaginal wash samples, animal feeding needles (Fisher Scientific) were used to flush 100 μL of PBS containing protease inhibitor cocktail (Sigma), 4 to 6 times into vaginal cavity. Vaginal wash samples were spun at 10,000 rpm for 15 min to remove cellular debris and supernatants were collected and stored at −70°C.

5.3.9 Serum and mucosal antibody response

The SIV gp160 specific antibody response were analyzed using enzyme-linked immunosorbent assays (ELISAs). 96-well maxisorp ELISA plates (Nunc, Denmark), were coated overnight with a purified recombinant gp130 protein of SIV mac239 obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH (40), at a concentration of 1 μg/mL in 100 μL volume for each well, in sodium caronate-

bicarbonate buffer (pH 9.8). The plates were then blocked with 3% skim milk in PBS for 30 s and then with 2% sucrose in water for 30 s and dried for 2 h at 37°C. Dilutions of serum samples or vaginal wash samples from immunized guinea pigs were prepared in dilution buffer (Synbiotics Carporation, San Diego, CA), added to the plates, and incubated for 2 h at room temperature. The plates were washed three times with plate-washing solution (Synbiotics Carporation) and incubated for 1 h with a 1:1000 dilution of an isotype-specific secondary antibody, namely, horseradish peroxidase (HRP)-conjugated goat anti-guinea pig IgG (KPL, Gaithersburg, MD), goat anti-guinea pig IgG1, goat anti-guinea pig IgG2a (Novus Biologicals, Littleton, CO), or sheep anti-guinea pig IgA (Immunology Consultants Laboratory, Newberg, OR). The plates were washed three times and developed with ABTS (2,2′-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) peroxidase substrate solution (Synbiotics Carporation), development was stopped by the addition of peroxidase stop solution, and analysis was performed at 405 nm using an ELx800 ELISA plate reader (BioTek, Winooski, VT).

5.3.10 Virus neutralization assay

Neutralizing antibody levels were measured using a safe pseudovirus based assay. Virus neutralization was measured as a function of reductions in *tat*-regulated luciferase reporter gene expression after a single round of infection in TZM-bl cells. Envelope pseudotyped viruses of tier 1 SIVmac251.6, tier 1b SIVmac251/M766 strains and tier 2 SIVmac239CS.23 were made by transfecting the appropriate plasmids in 293T/17 cells (66). Briefly, all serum samples were heat inactivated at 56°C for 1 h before use and initially diluted 1:4 in serum free DMEM followed by 2-fold serial dilutions performed in

duplicates (96-well flat-bottom plate) in DMEM (10 µL/well). Pseudovirus (200 $TCID_{50}$) was added to each well in a volume of 40 µL, and the plates were incubated for 1 h at 37°C. TZM-bl cells were then added ($1.5x10^4$/well in 150 µL volume of DMEM containing 10% heat-inactivated FBS and DEAE-dextran (Sigma, St. Louis, MO) at a final concentration of 15 µg/mL. Assay controls included replicate wells of TZM-bl cells alone (cell control) and TZM-bl cells with virus and no antibody (virus control). Cells were incubated for 48 h at 37°C. The medium was removed from each well and replaced with 50 µL of RPMI 1640 medium (Invitrogen) and 50 µL of Bright-Glo luciferase assay reagent (Promega, Madison, WI). The cells were allowed to lyse for 2 min, then 100 µL of each cell lysate was transferred to a 96-well black solid plate, and luminescence was measured using a Victor 3 luminometer (Perkin Elmer). The 50% inhibitory dose (ID_{50}) titer was calculated as the serum dilution that caused a 50% reduction in relative luminescence units (RLU) compared to the level in the virus control wells after subtraction of the cell control RLU value.

5.4 Results

5.4.1 Generation of rNDVs expressing SIV gp160

The transcription cassette containing the ORF of SIV mac239 gp160 gene was inserted as an additional gene-cassette into the antigenomic cDNA of lentogenic NDV strain LaSota and other modified NDV vectors between the P and M genes (Figure. 25). All the rNDVs were recovered by standard reverse genetic technique (42). The designation of each rNDV carrying SIV mac239 gp160 gene is described in Figure.25.

The recovered viruses were passaged at least ten times in 9-day-old embryonated chicken eggs and the presence of the foreign gene was confirmed by sequence analysis.

5.4.2 Expression of SIVgp160 protein by rLaSota and modified rNDVs

To detect expression of the SIV gp160, DF-1 cell monolayers were infected with rLaSota and modified rNDVs containing SIV gp160 gene. Cell lysates were analyzed by SDS-PAGE and Western blot analysis using gp120 specific monoclonal antibody. All the rNDVs expressed the cleaved gp160 Env protein of 120 kDa in size (Figure. 26). The densitometric analysis showed that level of gp160 expressed by different rNDVs varied slightly with modified-rNDV-LaSota-527/gp160 showing higher level of expression followed by modified-rNDV-AKO-331-390/gp160. The foreign envelope protein expressed by rNDV usually gets incorporated into NDV virion (50). To determine the incorporation of SIV gp160 into NDV particle, the allantoic fluid harvested from eggs infected with rNDVs was ultracentrifuged through a 30% sucrose gradient and the purified virus was analyzed by Western blot using gp120-specific monoclonal antibody (Figure. 27). Western blot analysis showed that SIV gp160 is incorporated into NDV

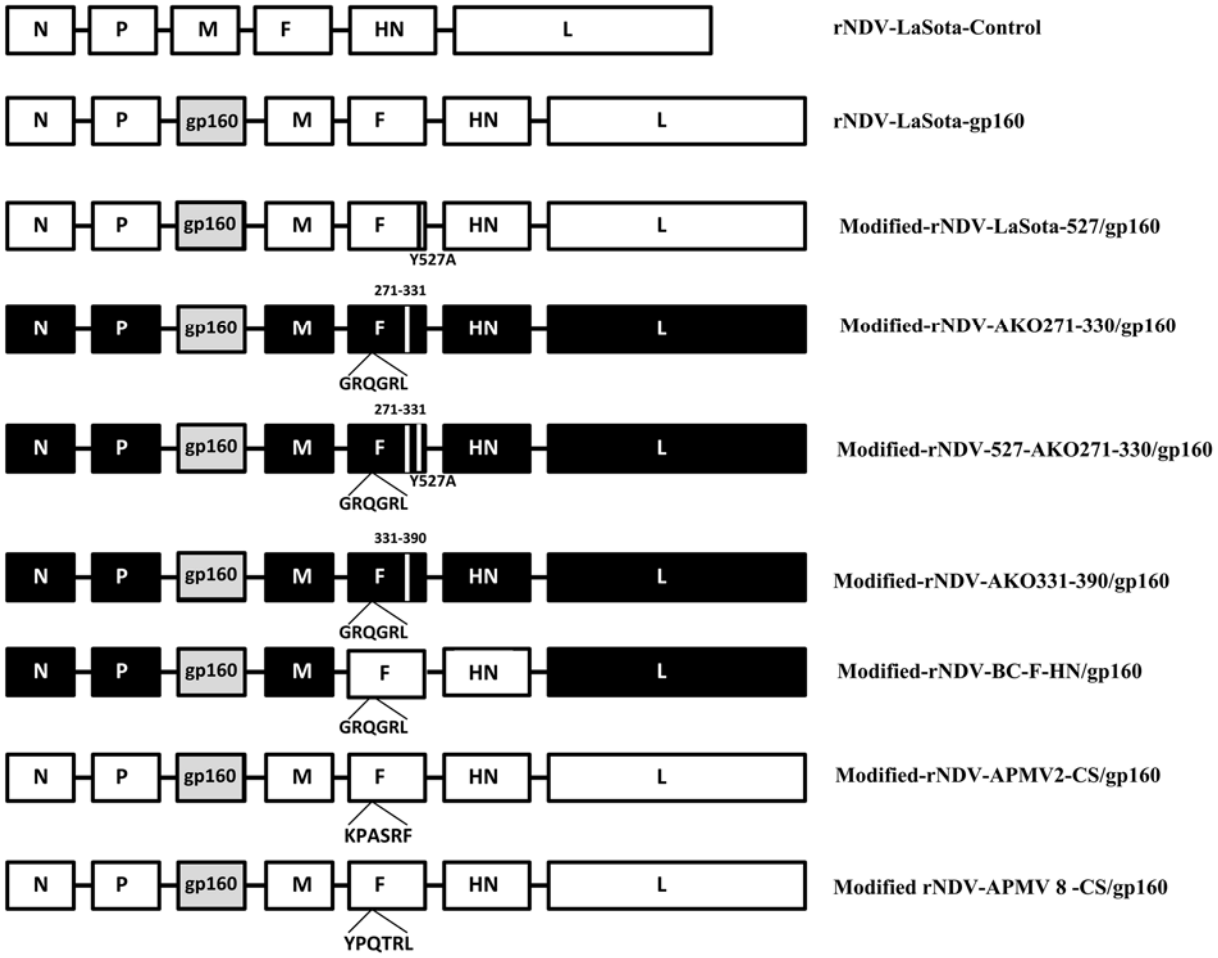

Figure 25. Construction of modified recombinant NDVs expressing the SIV gp160 gene: The SIV gp160 ORF of SIV mac 239 was engineered to be flanked by the NDV gene-start and gene-end signals and was inserted into the intergenic region between the P and M genes in the rNDV vectors. Genes derived from rBC or AKO-18 are shown as black or gray rectangles, respectively. F gene segments derived from the NDV LaSota strain are shown as white bars with designated location. All viruses contain the avirulent F protein cleavage sequence of LaSota, or APMV-2 or APMV-8.

particle. The insertion of SIV gp160 gene into the rNDV genome did not affect expression of HN protein of NDV which is present on NDV envelope, as shown by similar levels of HN protein expression by all the rNDVs.

5.4.2 Oligomeric status of SIV gp160 expressed by rNDV

The envelope protein of SIV assembles to form noncovalently associated oligomers in the endoplasmic reticulum. The oligomeric complex play a role in virus entry and therefore the main target of the neutralizing humoral immune response of the host (62). Hence, we investigated the oligomeric state of the gp160 expressed by different modified NDV vectors in infected DF-1 cells. Cell lysates from NDV infected DF-1 cells were subjected to cross-linking with DSP followed by SDS-PAGE under reducing and non-reducing conditions followed by immunoblotting with gp120-specific monoclonal antibodies. The results of Western blot analysis demonstrated the presence of oligomers of higher molecular weight (>220 kDa) under nonreducing conditions and monomers (120 kDa) under reducing conditions (Figure. 28). These results suggest that rLaSota and modified rNDVs support the expression of SIV gp160 as one predominant oligomer of size greater than 220 kDa.

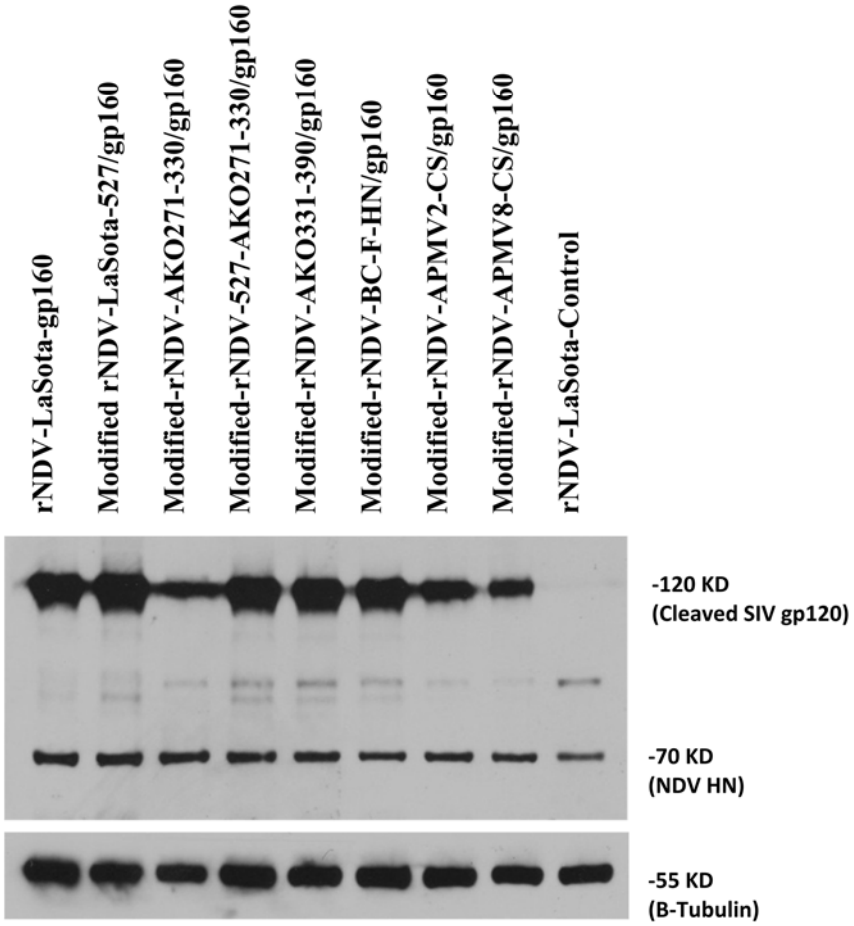

Figure 26. Expression of SIV gp160 *in vitro* by modified rNDV vectors: DF-1 cells were infected with each virus at an MOI of 1, and cell lysates were collected at 24 h post-infection for Western blot analysis using gp120 specific monoclonal antibodies to visualize the SIV gp160, NDV HN and β-tubulin protein.

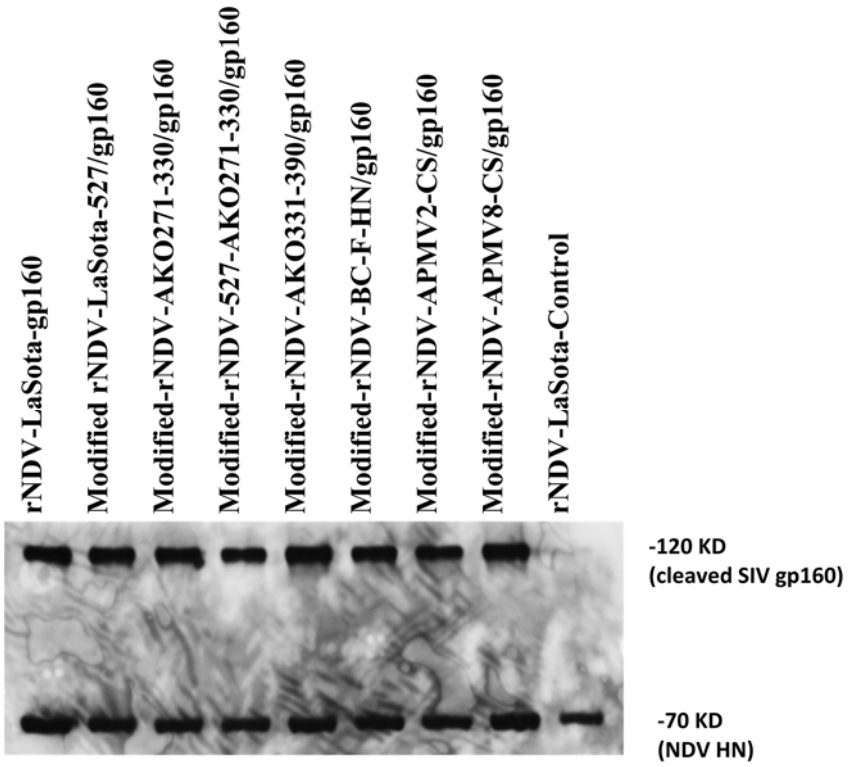

Figure 27. Incorporation of the SIV gp160 into the NDV particles: The viruses were harvested from allantoic fluids of infected eggs at 72 hpi, purified through a 30% sucrose gradient and analyzed by Western blot using monoclonal antibodies to visualize the SIV gp160 and NDV HN protein. Molecular masses of the marker proteins (kDa) are shown in the right margin.

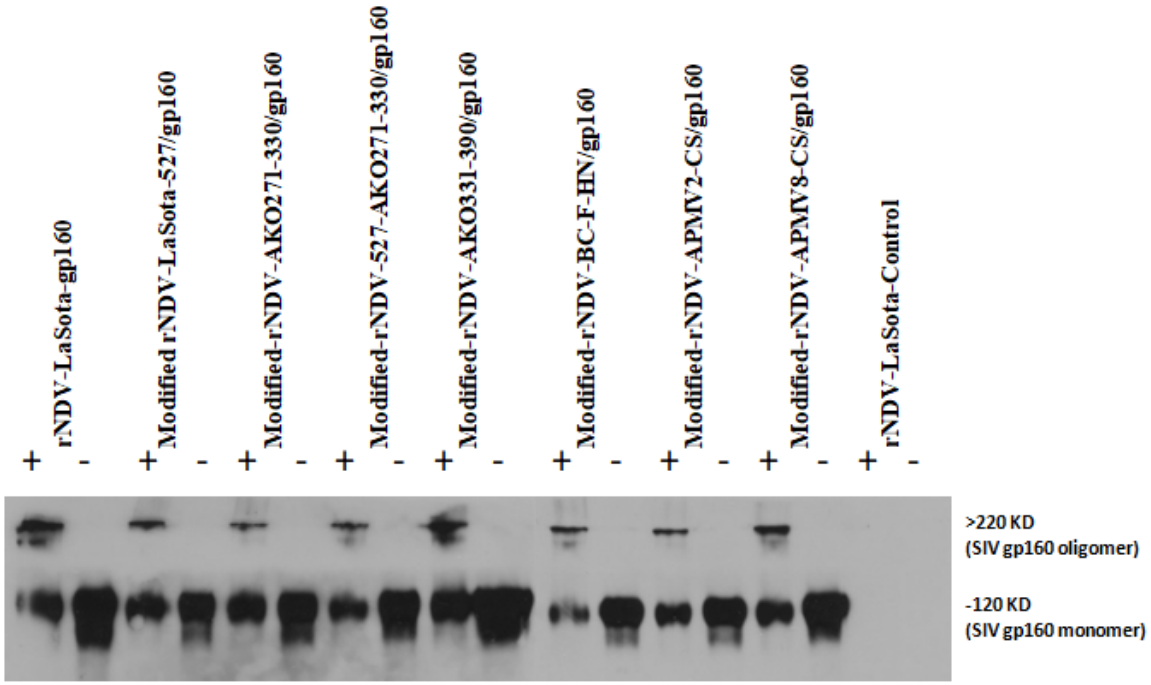

Figure 28. Oligomeric status of SIV-1 gp160 expressed by modified rNDVs: Lysates of DF-1 cells infected with modified rNDVs expressing SIV gp160 were cross-linked with DSP at a final concentration of 1 mM. After the cross-linking, the samples were subjected to SDS-PAGE under reducing (-) or nonreducing (+) conditions and analyzed using Western blot with gp120-specific monoclonal antibodies. The positions of the gp120 monomers and oligomers are indicated by arrows in the right margins. Molecular masses of the marker proteins (in kDa) are shown in the right margin.

5.4.3 Biological characterization of rNDV expressing gp160

The multicycle growth kinetics of the rLaSota and modified rNDVs expressing SIVgp160 were compared in DF-1 cells (Figure. 29). The replication of rLaSota and modified rNDVs containing SIVgp160 gene was reduced compared to that of the parental rLaSota virus with no foreign gene. Although there were differences in the growth kinetics of modified rNDVs, all the viruses containing the SIVgp160 gene insert achieved similar titers at 64 h post infection. The pathogenicity of modified NDVs expressing SIVgp160 and the parental rLaSota virus was evaluated by the ICPI test in 1-day-old chicks. The ICPI values of all rNDVs and modified rNDVs were 0.00, and chicks infected with rNDVs had no apparent clinical signs during the 10-day period of the ICPI test. This suggests that wild type rNDV and modified rNDVs expressing SIVgp160 are avirulent and the insertion of foreign gene had no major effect on the virus growth and pathogenicity.

5.4.4 Evaluation of humoral immune responses in guinea pigs

Nine groups of female Hartley guinea pigs (n=3) were immunized on days 0 and 21 with rLaSota and modified rNDVs expressing SIV gp160 via intra-nasal route. The animals in all the groups did not show any clinical signs of infection or any loss of body weight throughout the study (data not shown), indicating that the rNDVs were avirulent in guinea pigs. The induction of SIV Env-specific total IgG, IgG1 and IgG2a in serum was measured in all the groups on days 14, 21, 28, 35, 42, 49, 56, and 63 by ELISA (Figure. 30). Env-specific antibody responses were detected on day 14 following the initial immunization, except in the rNDV-LaSota control group, where no response was

detected. The total IgG, IgG1 and IgG2a responses increased between days 28 and 42 in all the groups. Total IgG response decreased on day 49 in all the groups and peaked on day 56 before decreasing on day 63. The IgG1 responses decreased on day 49 in all the groups except in rNDV-LaSota-gp160 and in modified-527-AKO271-330/gp160 groups and peaked on day 56 before decreasing on day 63. Similarly, IgG2a responses decreased on day 49 and increased on day 56 except wild type rNDV group. The gp160-specific IgG titer observed with the modified-rNDV-APMV2-CS/gp160**,** modified-rNDV-BC-F-HN/gp160 and modified rNDV-APMV8-CS/gp160 groups were significantly higher than rNDV-LaSota gp160 group. The highest IgG titer was observed in modified-rNDV-APMV2-CS/gp160 group. The antibody titers in other groups were slightly higher than the rLaSota group. These results indicated that SIV Env specific IgG responses could be enhanced significantly by using modified rNDVs in which either NDV strain LaSota F protein cleavage site was replaced with cleavage site of APMV-2 and APMV-8 or NDV strain LaSota F and HN proteins was replaced with NDV strain BC F and HN proteins.

5.4.5 Evaluation of mucosal immune responses in guinea pigs

Vaginal wash samples were collected from each animal at the same time points of blood collection and evaluated by ELISA using SIV gp130-coated plates for antibody responses (Figure. 31). Very low total IgG, IgG1 and IgG2a responses were detected on day 21 following the initial immunization, but the boost on day 21 increased immune responses significantly in all the groups except in the rNDV-LaSota control group. The titer of total IgG, IgG1 and IgG2a peaked between days 28 and 63. Similar to IgG

responses in serum, the response was highest in the modified-rNDV-APMV2-CS/gp160 group.

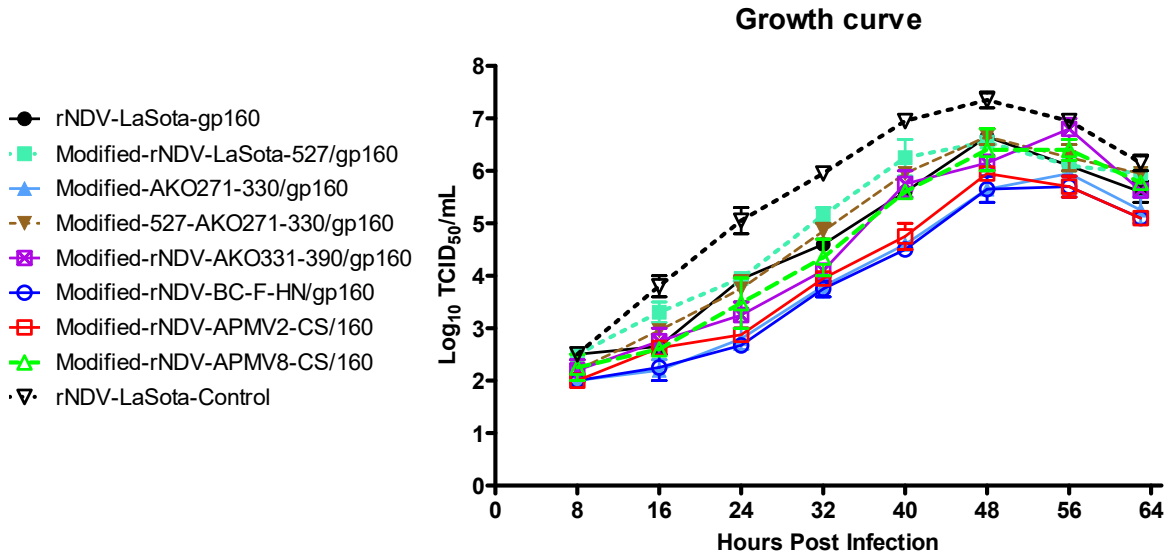

Figure 29. Comparison of multicycle growth kinetics of rLaSota and modified rNDV vectors expressing SIV gp160 in DF-1 cells: DF-1 Cells were infected with each virus at an MOI of 0.001, and cell culture media supernatant aliquots were harvested at 8-h intervals until 64 h postinfection. The virus titers in the aliquots were determined using $TCID_{50}$ asssay in DF-1 cells. Values represent averages of the results from three independent experiments, and error bars show standard deviation.

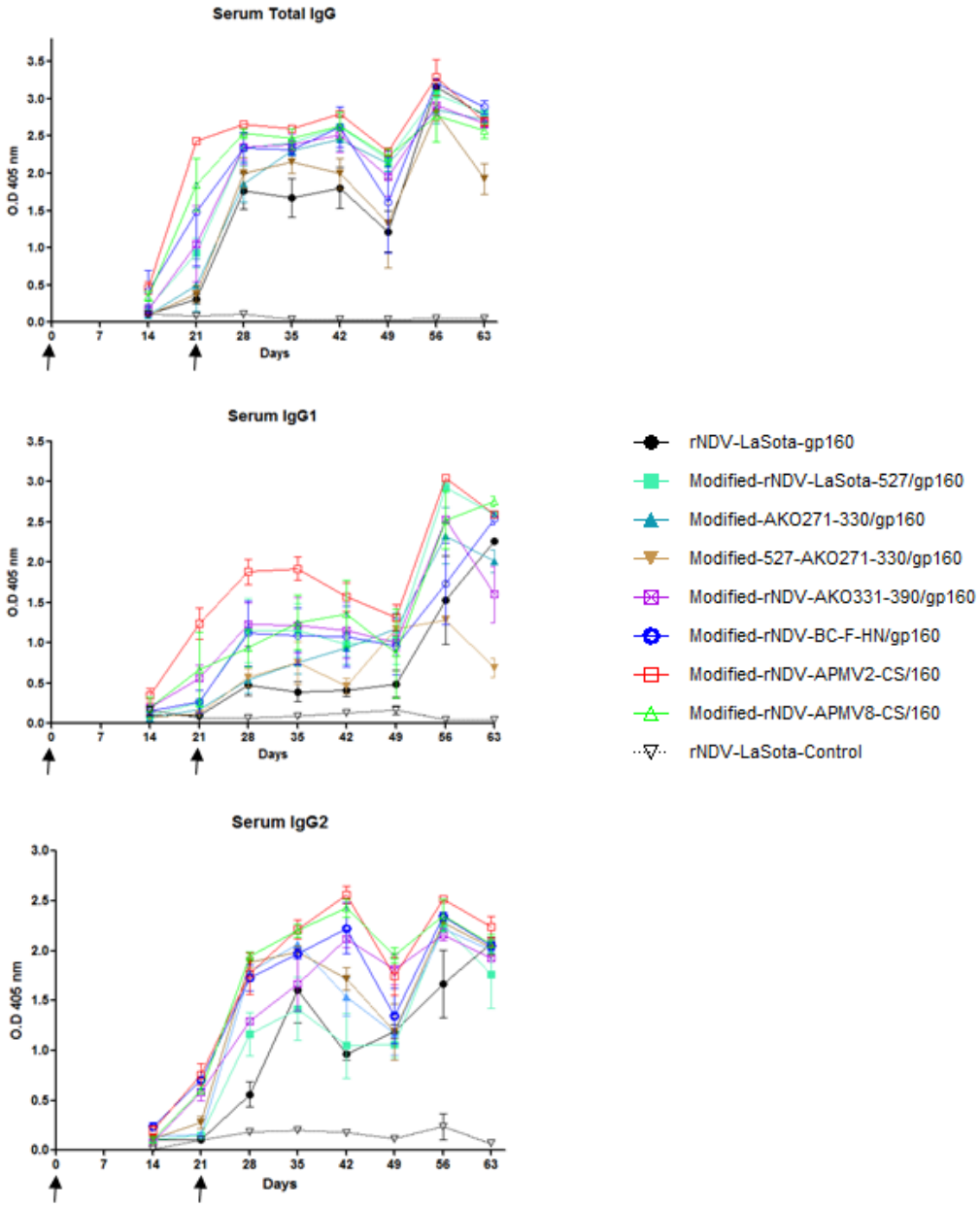

Figure 30. SIV gp160-specific serum antibody responses in guinea pigs. The guinea pigs were immunized with rNDVs: Serum samples (1:1000) from blood collected at the indicated days post immunization were analyzed for anti-SIV gp120 antibodies by ELISA. Arrows indicate times of rNDV immunizations on days 0 and 21. The graph shows the geometric mean values ± standard errors of the mean (SEM) for the 3 animals in each group.

5.4.6 Evaluation of neutralizing antibody responses in guinea pigs

Serum samples from animals immunized with rNDVs expressing SIV gp160 on days 35, 49, and 63 were evaluated by the TZM.bl assay for their ability to neutralize tier 1 SIVmac251.6, tier 1b SIVmac251/M766 and tier 2 SIVmac239CS.23 strains (Figure. 32). Neutralizing antibody (NAb) activity (expressed as ID_{50} values) was detected against SIVmac251.6 in all the groups with the highest titer observed in modified-rNDV-APMV2-CS/160 group on days 35, 49 and 63 followed by modified-rNDV-BC-F-HN/gp160 group on days 35 and 49 and modified rNDV-APMV8-CS/160 group on day 63. The neutralisatizing antibody response in modified-rNDV-APMV2-CS/160 group peaked on day 35. The NAb activity detected against SIVmac251/M766 was higher in rNDV-APMV8-CS/160 group followed by modified-rNDV-APMV2-CS/160 and modified-rNDV-BC-F-HN/gp160. A very low response to tier 2 SIVmac239CS.23 was noticed and this SIVmac239CS.23 isolate was known to be highly neutralization resistant virus from previous studies (45). The NAb response to SIVmac251.6 in immunized guinea pigs was significantly higher than the response to SIVmac251/M766 in all the groups.

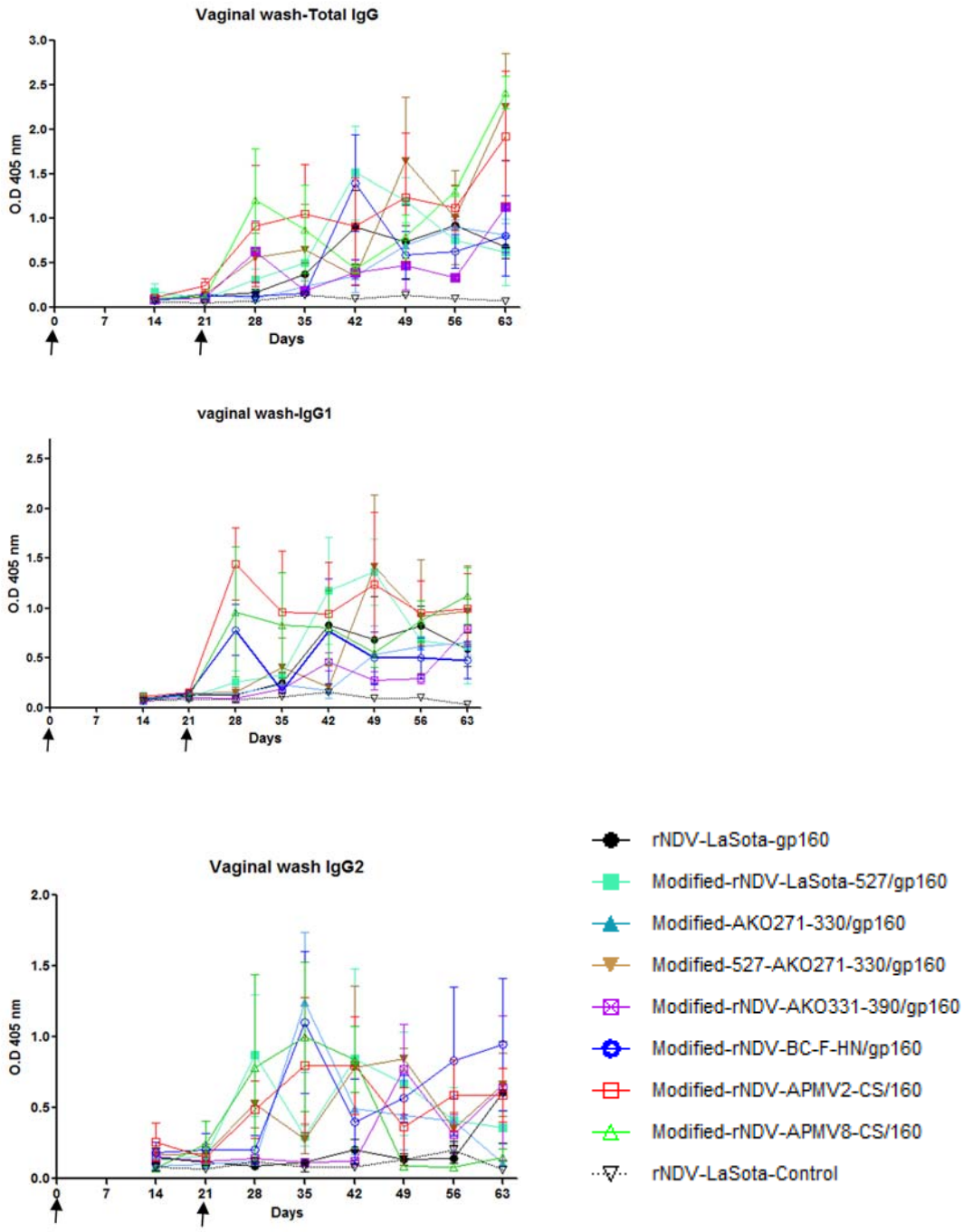

Figure 31. SIV gp160-specific mucosal antibody responses in guinea pigs: The guinea pigs were immunized with rNDVs. Vaginal wash samples (1:10) diluted with PBS, collected at the indicated days post immunization were analyzed for anti-SIV gp130

antibodies by ELISA. Arrows indicate times of rNDV immunizations on days 0 and 21. The graph shows the geometric mean values ± standard errors of the mean (SEM) for the 3 animals in each group.

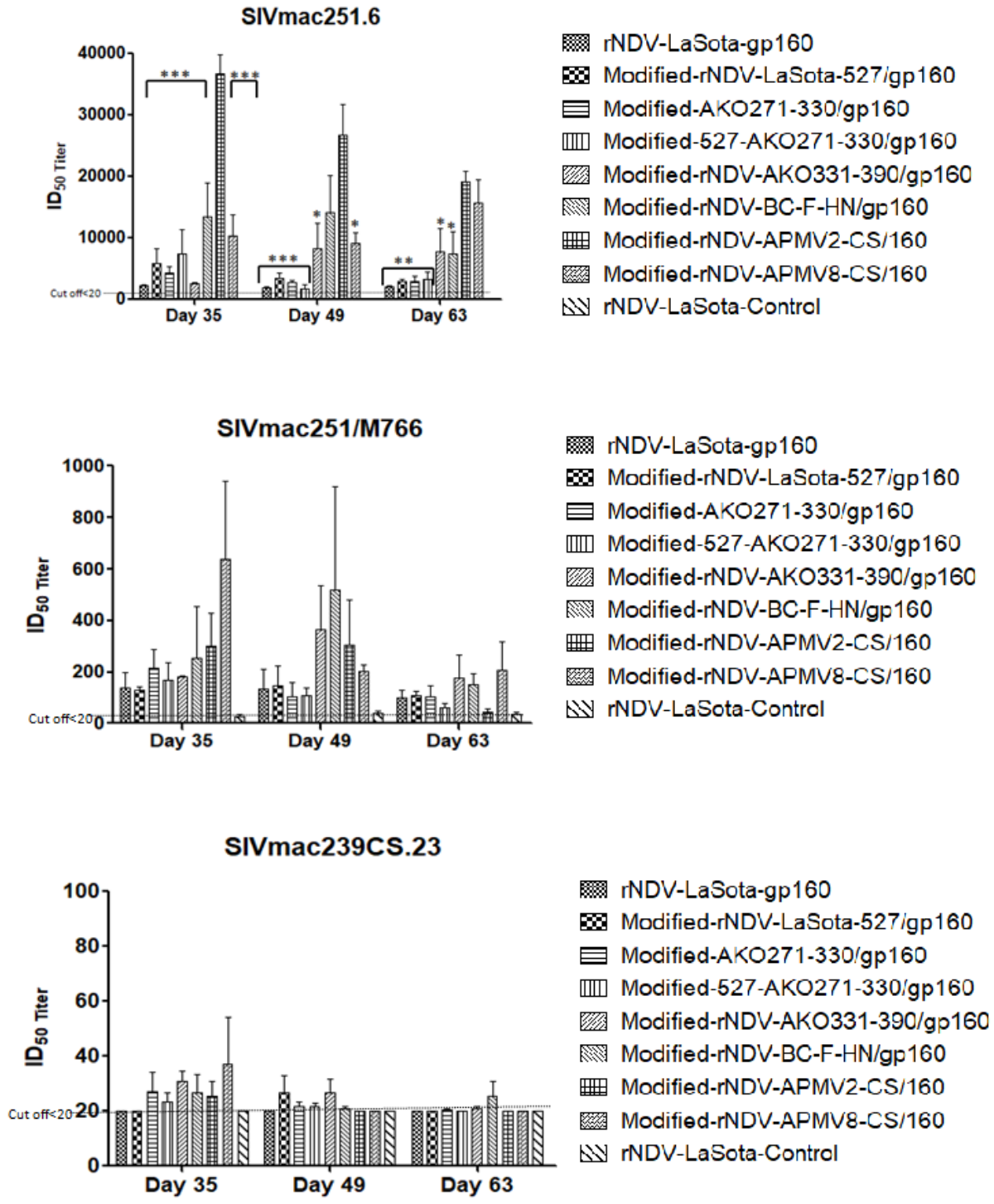

Figure 32. Virus neutralizing antibody activity: 50%-inhibitory-dilution [ID_{50}] titers against heterologous tier 1 SIV mac251.6, tier 1B SIV mac 251/M766 and homologous

tier 1 strain SIV mac239cs.23 from serum samples of guinea pigs immunized with the indicated rNDVs vectors expressing SIV gp160. Guinea pig serum samples were obtained on days 35, 49 and 63 were tested with pseudoviruses by the TZM-bl assay. Preimmune serum samples were used to establish the baseline neutralizing activity in each individual guinea pig, and these values were subtracted from the values shown. The dashed lines denote background titers of <20 against the murine leukemia virus (MLV) negative-control pseudovirus or pooled preimmune serum samples.

5.5 Discussion

NDV has shown promising results as a potential vaccine vector for human use (55). NDV is not a human pathogen due to its natural host restriction and it is antigenically distinct from common human pathogens. Most human beings are not exposed to NDV and hence there is no problem of pre-existing immunity. NDV can be grown to high titers in embryonated eggs or in cell culture and it is one of the most widely used vaccines in poultry industry all over the world. Vaccines derived from NDV vectors can be given nasally and can induce an effective systemic and mucosal immune response. Although both lentogenic and mesogenic NDV strains can be used as vaccine vectors, mesogenic strains have been found to mount an effective immune response in non-human primates compared to lentogenic strains (21). Mesogenic strains are used as vaccines in NDV enzootic countries. In USA, mesogenic strains are classified as select agents and must be handled in BSL-3 facility which makes development and testing of mesogenic NDV vectors challenging. Therefore, we modified the polybasic F cleavage site of mesogenic strain BC to the dibasic F cleavage site of lentogenic strain LaSota. Changing the F cleavage site made the mesogenic virus low virulent and therefore fulfilling the criteria for a vaccine candidate that can be handled in BSL-2 facility. Changing the cleavage site of lentogenic LaSota to APMV-2, 7 and 8 cleavage sites also eliminated the need for exogenous protease supplementation, while still containing avirulent F protein cleavage site. This was done because the fusion activity of NDV can affect virus replication *in vitro* and *in vivo*, and its modification can enhance the potential of NDVs for use as vaccine vectors. All the modified viruses were found to be avirulent by ICPI test and were more immunogenic than rLaSota in guinea pigs.

In our laboratory, we previously reported that rNDV vectors could grow to high titers in cell culture and in embryonated chicken eggs while expressing HIV Env and Gag antigens (51). A single rNDV vector co-expressing Env and Gag can also be self-assembled into HIV-1 virus-like particles (VLPs) (50). Stimulation of an effective neutralizing antibody response is essential for conferring protection against HIV-1. Env-specific antibodies contributed to the modest decrease in the incidence of HIV-1 infection observed among participants in HIV-1 vaccine phase III clinical trial of a prime-boost regimen based on a recombinant canarypox vector and soluble gp120 protein (78). As an experimental vaccine approach designed to elicit broad humoral immune response, we developed different modified rNDV vectors expressing SIVgp160 mac239 envelope protein. The expression of SIV gp160 in DF-1 cells was higher in modified-rNDV-LaSota-527/gp160 followed by the modified-NDV-rBC vectors. Since the envelope protein is expressed in its native form, we could see that they are also inserted into the envelope of NDV particle when the virions are released via budding through the host cell membrane. An important advantage of the SIV envelope protein incorporated in NDV particles is that they can effectively activate key aspects of the immune response to mount potent immune stimulation and provide immunological memory.

SIVmac239CS.23 and its closely related derivatives SIVmac251.6 and SIV mac251/M788 vary greatly in their susceptibility to neutralization with the same antisera (94). rNDV vectors expressing SIVmac239 gp160 induces neutralizing antibodies to strain SIVmac251.6 and SIVmac251/M788 whereas the same antibodies rarely neutralize

infectivity of SIVmac239CS.23. This might be due to a subset of those antibodies that bound with an affinity too low to neutralize the SIVmac239CS.23 virus. The variations in the neutralization titer might be due to the diversity among SIVs infecting different host species and is the result of complex factors in which the virus evolves having its own neutralizing determinants (45). SIVmac239CS.23 was remarkably resistant to neutralization even with homologous antisera induced by our rNDV vectors. The molecular determinants for neutralization of this virus were distinct from those responsible for neutralization of SIVmac251.6 and SIV mac251/M788 (67). The serum antibody levels were increasing from day 28-56 as evident by ELISA results, but the neutralizing antibody titers were decreasing over time. The reduction in neutralizing antibody titer over time might be due to the absence of persistent infection by a replicating virus, which is essential for the clonal selection of high affinity antibodies. Antibodies present in mucosal secretions were measured due to their potential importance in preventing sexual transmission of HIV-1. Immunization with rNDV vectors expressing SIVgp160 elicited gp120-specific IgG that was detectable in the vaginal wash samples of all of the animals and appeared to be in equilibrium with the serum antibody level in most cases. In addition, NDV vectored vaccines have been shown to provide protection against different strains of SIV other than those for which the vaccine was formulated.

In summary, we compared different modified rNDV vectors expressing SIVgp160 by prime and boost strategy, for induction of serological and mucosal antibody responses to tier 1 SIVmac251.6, tier 1b SIVmac251/M766 and tier 2 SIVmac239CS.23 strains. These rNDV vectors can replicate and induce higher levels of neutralizing antibodies in

guinea pigs, without causing any clinical signs. Our results showed that rNDV modified-rNDV-APMV2-CS/gp160 stimulated highest NAb response to SIVmac251/M766 at a higher magnitude than other modified vectors in guinea pigs. However, previous studies have shown that SIVmac239CS is highly neutralization resistant which explains the low neutralizing titers by all the rNDV vectors (45). Our study has identified three improved rNDV vectors that induced higher levels of immunogenicity and protective efficacy against SIV. Whether these rNDV vectors would also provide increased immunogenicity and protection against SIV in higher mammalian species remains to be evaluated.

Chapter 6: Conclusions and Prospects

Newcastle disease (ND) is a highly infectious and fatal viral disease that affects almost all species of birds. The causative agent of the disease is the Newcastle disease virus (NDV). ND is an economically important avian virus that can cause huge economic losses to the poultry industry (84). NDV is an enveloped, negative-sense, single-stranded RNA virus, belonging to the family *Paramyxoviridae* and genus *Avulavirus* (1, 3, 84). The clinical signs reported in birds infected with NDV vary widely depending on the virulence of the virus. In some circumstances, with extremely virulent viruses, the disease may result in sudden death (11). Other factors determine the outcome of the disease such as the host species, age, immune status, co-infection with other organisms and environmental stress (37, 38, 92).

Reverse genetic techniques allowed us to manipulate and introduce site-specific mutations into the genomes of NDV. This is crucial for the study of the functions and the interactions of viral proteins, for the investigation of viral pathogenicity and for the development of novel vaccines. With the help of the reverse genetics, we can also introduce immuno-dominant and protective antigens of other unrelated pathogens into the NDV and engineer novel NDV vectored vaccines for human and animal pathogens.

Live and attenuated vaccines are currently available for NDV and provide certain level of protection in poultry, but outbreaks of virulent NDV have been reported even in commercial farms with a strict vaccination regimen (26). Moreover, depending on the type of NDV vaccine strains used, the environmental conditions, and immune system of individual birds, disease signs may occur (92). Our studies not only prove the need to

improve the existing vaccines, but also provides new ways to attenuate the pathogenicity of circulating NDV strains and use them as improved genotype matched vaccines. Our results on Y527A mutation in NDV F protein demonstrated that function of fusion protein is crucial for the development of live attenuated vaccines. By changing the tyrosine residues to alanine in the F protein cytoplasmic tail (CT), we generated a hyperfusogenic phenotype which showed increased spread of the virus and infectivity. The F protein is the main target for immune response and possesses neutralizing epitopes. The result of this study can be useful to design effective NDV vaccine, by increasing the vaccine virus replication and immunogenicity in chickens.

In our study on the role of three avianparamyxovirus (APMV) types 2, 7, and 8 fusion protein cleavage site (FPCS) sequences in NDV genotype matched vaccines were evaluated. We were able to confirm that the rBanj-AF vaccine was stable and was more efficient in protection against homologous challenge. Our study also supported the idea that an important factor in the recurrent outbreaks of ND in vaccinated birds was antigenic mismatch between the commercial vaccine strains and the currently circulating virulent field strains. We also found that a robust immune response with high neutralizing antibody titer is necessary to prevent viral shedding, which is more effectively induced by a genotype-matched live vaccine.

NDV is an attractive vaccine vector for both human and animal pathogens. NDV vectored vaccine for highly pathogenic avian influenza virus (HPAIV) H5N1 for poultry have been used successfully in China, which played an important role in reducing the

incidence of H5N1 infection in poultry (14, 89). NDV vectors induce robust humoral, cellular and mucosal immune responses. The live attenuated vaccine strains used as vaccine vectors have been used by poultry industry all over the world and have been proved to be safe in avian and non-avian species. The ability of NDV to infect a wide variety of mammalian species makes it a potential vaccine vector for humans and animals.

Our present study with modified NDVs expressing SIV gp160 indicates that the development of NDV vectors by modifying fusion protein cleavage site and other F protein regions between strains are feasible and may broaden the immune responses in host species. Although the efficacy of NDV-vectored SIV vaccine tested in guinea pigs were encouraging, the efficacy of a similar vaccination regimen against defined mucosal simian-human immunodeficiency virus (SHIV) challenge in a non-human primate (NHP) model needs to be evaluated. In summary, our findings suggest that modified rNDVs were safe and can be used as a live vaccine and as a vaccine vector with enhanced replication, expression, and protective efficacy in avian species and potentially in other species including humans.

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