ABSTRACT

Title of Dissertation: DEVELOPMENT OF AVIAN PARAMYXOVIRUS VECTORED VACCINES

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Avian Avulaviruses (formerly Avian Paramyxoviruses, APMV) are important pathogens of avian species and have been used as viral vectors for more than two decades. Among all APMVs, Newcastle disease virus (NDV or APMV-1) has been most extensively used as a vaccine vector for protection against avian and animal diseases, and as an oncolytic agent. For poultry vaccination, the preexisting maternal antibodies against NDV can neutralize APMV-1 vectors resulting in vaccination failure. Hence, there is a need to develop new vaccine vectors that would escape neutralization by the maternal antibodies. In the first part of my study, I created a reverse genetics system for Avian paramyxovirus-3 strain Wisconsin (APMV-3 Wisc.) which was proven to be nonpathogenic for day-old chicks and embryonated chicken eggs. The virus was used as a vector to express the enhanced green fluorescent protein (GFP) as a heterologous antigen. The recombinant APMV-3 Wisc. expressing GFP was compared with similarly constructed APMV-1 strain LaSota and APMV-3 strain Netherlands-based vectors for GFP expression and growth kinetics in

vitro, and for immunogenicity, safety, and tissue tropism in day-old specific pathogen-free (SPF) chicks. APMV-3 strain Netherlands (APMV-3 Neth.) showed the highest growth rate and GFP expression in chicken fibroblast DF-1 cells, followed by APMV-1 LaSota and APMV-3 Wisconsin. In day-old chicks, APMV-3 Neth. spread to different organs, decreased feed intake and caused stunted growth. APMV-3 Wisc. and APMV-1 LaSota were confined to the respiratory tract and did not induce any pathogenic effects. All three constructs induced seroconversion of the vaccinated chicks for the vector antigens. Thus, the reverse genetics system created in this study for APMV-3 Wisc. allows the development of safe APMV vector antigenically different from NDV that can be used for day-old chicks vaccination. In addition, it provides a tool to study the molecular basis of APMV3 pathogenesis. In the second part of my study, I explored a novel approach for the expression of a foreign gene as an uninterrupted open reading frame (ORF) with a cognate gene of NDV vector. This approach is expected to promote the foreign gene expression stability. Avian influenza virus (AIV) hemagglutinin (HA) protein-coding sequence was fused in-frame with various proteins of NDV vector, with a 2A self-cleaving peptide, a furin cleavage site, or both, placed between the AIV and NDV sequences for separation of the two proteins. Among different constructs tested, we only recovered viable viruses with AIV HA fused C-terminally to the NDV HN gene. These viruses demonstrated a higher expression level of AIV HA than the vector constructed according to a traditional scheme of expressing the transgene as a separate transcriptional unit. Also, they showed increased stability of the transgene expression over multiple passages in embryonated chicken eggs. Our results demonstrate the advantages and limitations of

this novel method of foreign gene expression that need to be considered for the development of NDV-based vaccine or therapeutic vectors.

DEVELOPMENT OF AVIAN PARAMYXOVIRUS VECTORS

by

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Dedication

I would like to dedicate my dissertation for scholars working in the viral vector vaccine research and industry.

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List of Abbreviations

2A	2A Peptide of Picornaviruses
aa	Amino Acid
AIV	Avian Influenza Virus
APMV	Avian Paramyxovirus
rAPMV	Recombinant Avian Paramyxovirus
APMV-3 Neth.	Avian Paramyxovirus-3 Strain Netherlands
APMV-3 Wisc.	Avian Paramyxovirus-3 Strain Wisconsin
BSA	Bovine Serum Albumin
BWT	Body Weight
cDNA	Complementary Deoxyribonucleic Acid
DAPI	4′,6-Diamidino-2-Phenylindole
DF-1	Chicken Embryo Fibroblast Cells
DMEM	Dulbecco's Modified Eagle Medium
ECE	Embryonated Chicken Egg
ELISA	Enzyme-Linked Immunosorbent Assay
F	APMV Fusion Protein
F2A	Furin Cleavage Site With 2A Peptide
FBS	Fetal Bovine Serum
FFU	Focal Fluorescent Unit/ Foci Forming Unit
FG	Foreign Gene
FLC	Full Length Clone
FMD	Foot And Mouth Disease
FP	Fowl Pox
GE	Gene End (Transcription Stop Signal)
GFP	Green Fluorescent Protein
GS	Gene Start(Transcription Start Signal)
HA	Hemagglutinin Protein/ Hemagglutination Assay
HDV-Rz	Hepatitis Delta Virus Ribozyme Sequence
HEp-2	Human Epithelial Cell Type-2

HI	Hemagglutination Inhibition
HN	Hemagglutinin Neuraminidase
HPAI	Highly Pathogenic Avian Influenza Virus
HRP	Horseradish Peroxidase
HVT	Herpes Virus Of Turkey
IBDV	Infectious Bursal Disease Virus
IBV	Infectious Bronchitis Virus
IFA	Immunofluorescent Assay
IFN	Interferon
IGS	Intergenic Sequence
ILTV	Infectious Laryngotracheitis Virus
IRES	Internal Ribosomal Entry Site
kb	Kilo Base
kbp	Kilo Base Pair
KDa	Kilo Dalton
L	Large Polymerase Protein
M	Matrix Protein
MDA	Maternally Derived Antibodies
MDV	Marek's Disease Virus
MOI	Multiplicity Of Infection
mRNA	Messenger Ribonucleic Acid
MVA-T7	Modified Vaccinia Ankara Strain Expressing T7 Polymerase
N	Nucleoprotein
ND	Newcastle Disease
NDV	Newcastle Disease Virus
nt.	Nucleotide
ORF	Open Reading Frame
OD	Optical Density
P	Phosphoprotein
P.I.	Post-Infection
PBS	Phosphate Buffered Saline
pN	Nucleoprotein Support Plasmid

pP	Phosphoprotein Support Plasmid
pL	Large Polymerase Support Plasmid
PVDF	Polyvinylidene Difluoride
RDRP	Viral RNA Dependent RNA Polymerase
RE	Restriction Enzyme
rLaSota	Recombinant LaSota Virus
RNA	Ribonucleic Acid
RNP	Ribonucleoprotein
RT-PCR	Reverse-Transcriptase Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
Seq.	Sequence
SFV	Semliki Forest Virus
SH	Small Hydrophobic
SPF	Specific Pathogen Free
TBS	Tris-Buffered Saline
UTR	Untranslated Region
VEE	Venezuelan Equine Encephalitis
wt	Wild Type Virus

Chapter 1: Introduction

Background

Avian Paramyxoviruses (APMVs) are pleomorphic, enveloped viruses with a single-stranded negative-sense RNA genome. Avian Paramyxovirus type 1 (APMV-1), better known as Newcastle disease virus (NDV), is the most characterized member of APMVs as it is the causative agent of Newcastle disease (ND) in chickens. ND is an economically important infectious disease affecting poultry species causing high mortality and drop in egg production (Miller & Koch 2013). Two decades ago, the first reverse genetics (RG) system for APMV-1 was created and recombinant APMV-1 was recovered. That enabled using it as a virus vector for avian and mammalian pathogens and understanding the molecular basis of APMV pathogenicity.

While APMV-1 vector was well studied and evaluated as a recombinant vaccine, the potential of other APMVs remains less explored. The advantage offered by other APMVs as poultry vaccine vectors is the evasion of the preexisting immune response against NDV in newly hatched commercial chicks. Also, antigenically different APMV vectors can be used in a prime boost vaccination strategy to avoid vaccination interference by the anti-vector immune response.

APMV-3 is one of the other APMVs used as a vector vaccine against avian and mammalian pathogens. APMV-3 was isolated from turkeys with respiratory disease, parakeets and psittacine birds with pancreatitis and CNS symptoms. There are two subtypes of APMV-3: APMV-3 strain Netherlands (APMV-3 Neth.) and APMV-3 strain Wisconsin (APMV-3 Wisc.). APMV-3 Neth. is considered the prototype of APMV-3. A reverse genetic system was created for APMV-3 Neth. and it was used as a vector vaccine for avian diseases (e.g. ND, Avian influenza, Infectious Bursal disease) and human Ebola virus infection. Yet, for poultry

vaccination, APMV-3 Neth. was found to cause stunted growth in young chicks and kills the embryo in embryonated chicken eggs (ECE) during propagation. This pathogenicity hindered its application in the poultry vaccine industry. On the other hand, APMV-3 Wisc. (a turkey virus) was not associated with any pathogenic effect in young chicks nor did it kill chicken embryos. Lack of chicken pathogenicity indicated that APMV-3 Wisc. could be a promising potential substitute for NDV vectors. In the current study, we generated a reverse genetic system for APMV-3 Wisc. and evaluated it as a vaccine vector for young chicks. The availability of reverse genetic systems for both APMV-3 Wisc. and APMV-3 Neth. will also allow the investigation of the molecular basis of different pathogenicity between these two strains.

The important concern for all vectored vaccines, including those based on APMVs is the stability of the heterologous antigen expression as there is no selective pressure on the virus to keep expressing the foreign gene. Foreign genes are usually added as separate transcription units into APMV genome. We hypothesized that if the foreign gene was included into APMV own gene transcription unit, it would prevent the selection of mutations interrupting the foreign gene ORF expression.

In the second part of our study, we tried to express avian influenza (AI) HA gene from APMV-1 gene transcription units using 2A peptide and/or furin cleavage site sequences for the separation of the transgene and APMV-1 polypeptides. Viable constructs were compared with the virus vector expressing HA from the corresponding genomic location as a separate transcriptional unit (traditional expression technique) for the HA expression level, stability and incorporation of HA into the recombinant APMV-1 particles.

Research Objectives:

- Development of a reverse genetics system for APMV-3 Wisc. and using it as a virus vector for expression of eGFP.
- *In vitro* and i*n vivo* evaluation of recombinant APMV-3 Wisc. as a vector vaccine for day old chicks in comparison to APMV-3 Neth. and APMV-1 strain LaSota.
- Application of a novel approach for foreign antigen expression (Avian influenza hemagglutinin) by APMV-1 vector in-frame with a vector protein using 2A peptide and/or Furin cleavage site.
- Comparison of in-frame (2A based) expression of Avian influenza hemagglutinin (HA) with separate transcription unit expression (conventional technique) in terms of expression level, stability and incorporation of HA to the recombinant virus.

Chapter 2: Review of Literature

Classification of Avian Paramyxoviruses

Family *Paramyxoviridae* is one of 11 families in the order *Mononegavirales* comprising single-stranded negative-sense RNA viruses sharing the same genomic transcription and replication pattern. Family *Paramyxoviridae* has four subfamilies: *Avulavirinae*, *Metaparamyxovirinae*, *Orthoparamyxovirinae*, and *Rubulavirinae*. In the latest classification by the International Committee on Taxonomy of Viruses (ICTV), all APMVs are placed under the subfamily *Avulavirinae* in three genera: *Metaavulavirus*, *Orthoavulavirus* and *Paraavulavirus*, based on the L-gene amino acid sequence (Amarasinghe et al., 2019). Up to date, there are 22 officially recognized species of avian avulaviruses (AAvV) corresponding to 22 avian paramyxoviruses (APMV-1 through -22) (ICTV 2021). Genus *Orthoavulavirus* includes nine species of Avian orthoavulaviruses (including APMV-1). Genus *Paraavulavirus* includes two species of Avian paraavulaviruses (including APMV-3). Genus *Metaavulavirus* includes 11 species of Avian metaavulaviruses. The detailed classification of *Avulavirinae* is described in (Paldurai and Samal, 2019).

APMV-1 Classification

APMV-1 (Avian orthoavulavirus 1) is the first and most recognized APMV as it causes Newcastle disease in chickens (Kraneveld, 1926) (Suarez et al., 2020). Newcastle disease is a neuro, enteric, respiratory disease caused by NDV in wide range of avian species and it causes devastating economic losses due to mortality and drop in egg production in susceptible poultry flocks (Miller & Koch 2013).

On the antigenic level, all APMV-1 (NDV) strains belong to the same serotype (based on HN neutralization) with the exception of minor antigenic differences detected by monoclonal antibodies (Miller et al. 2007). On the genomic level, NDV isolates are classified into lineages or genotypes based on phylogenetic analysis of the F (fusion) protein amino acid sequence (Aldous et al., 2003; Diel et al., 2012). Till now, there are 21 genotypes of APMV-1 (Dimitrov et al., 2019) and new genotypes emerge through frequent antigenic drift (Aldous et al., 2003; Miller et al., 2009) and/or much less frequent recombination (Han et al., 2008 and Zhang et al., 2010).

The pathogenicity of APMV-1 strains vary from asymptomatic to highly pathogenic. The World Organization for Animal Health (formerly known as Office International des Epizooties, OIE) has classified NDV strains according to their pathogenicity in chickens into five pathotypes: 1) Viscerotropic velogenic and 2) Neurotropic velogenic (high mortality, morbidity and severe pathogenicity), 3) Mesogenic (less pathogenic and low mortality), 4) Lentogenic (mild or subclinical respiratory infection), and 5) Asymptomatic (subclinical enteric infection) (OIE, 2021). Both lentogenic and asymptomatic strains are used as vaccine strains for protection against NDV (Suarez et al., 2020).

Virus Structure

APMV Morphology

APMVs are enveloped pleomorphic viruses of 100-500 nm in size (Samal, 2019). The virus envelope is a lipid bilayer derived from the host cell membrane. The virus envelope is covered by transmembrane glycoproteins, HN and F proteins, which are responsible for virus attachment and entry to the host cell, respectively. Under the virus envelope the matrix protein

encloses the virus helical nucleocapsid. The nucleocapsid is composed of RNA genome tightly wrapped with the nucleoprotein for its protection and associated with phosphoprotein and large polymerase protein, RNA dependent RNA polymerase (RdRP) (Fig.1) (Samal, 2021). The viral nucleocapsid is the minimal infectious unit.

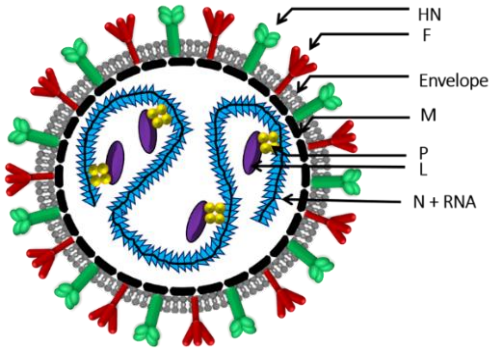


Figure 1: APMV virion composition.: Enveloped APMV particle showing single stranded RNA genome wrapped by nucleoprotein (N) units and closely associated with phosphoprotein (P) and Large polymerase protein (L). Matrix (M) protein is surrounded by envelope lipid bilayer derived from host cell membrane and harboring Fusin (F) and hemagglutinin (HN) (Samal, 2019).

APMV Genome

APMVs genome is a negative sense single-stranded linear non-segmented RNA of about 15-17 kb. Most APMV genomes include six genes encoding six main proteins; three polymerase complex proteins N (nucleoprotein), P (phosphoprotein), L (large-polymerase protein), and three membrane-associated proteins; M (matrix protein), F (fusion protein), HN (hemagglutinin-neuraminidase protein) (Fig.2); APMV-6 has an additional SH (small hydrophobic) gene (Paldurai and Samal, 2019). The P gene encodes two extra nonstructural proteins (V and W proteins) expressed through RNA editing (Steward et al., 1993). APMV genome nucleotide (nt) length is a multiple of six, reflecting the number of nucleotides covered by each nucleocapsid molecule (the "rule of six") (Kolakofsky et al., 1998).

APMV genome termini have 3' and 5' leader and trailer sequences, respectively. The length of the leader is 55 nucleotides in all APMVs, the trailer length varies between different APMVs. The leader and trailer sequences control APMV genome transcription and replication as they contain the RDRP promotor binding site (the first 12 nt) (Samal, 2019). APMV genes are located in separate transcription units flanked by transcription start and stop signals (gene start and gene end) and separated by noncoding intergenic sequences (IGSs). Inside each gene, the ORF is flanked by untranslated regions (UTRs) which are expected to control gene expression (Yan et al., 2009; Kim and Samal 2010). The gene-start and gene-end signals are recognized by viral polymerase during transcription. The IGSs vary in length from 1 nt. (e.g. APMV-1 P/M IGS) to 63 nt. (e.g. APMV-3 P/M IGS) (Samal, 2019). IGS length was shown to affect downstream gene transcription, virus replication and the pathogenicity level (Yan and Samal, 2008).

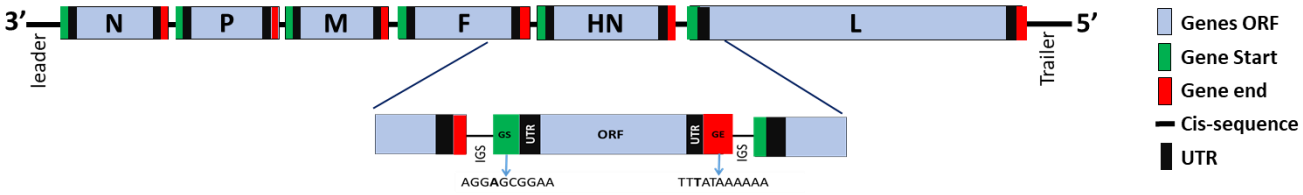


Figure 2: APMV negative sense RNA genome component: APMV have six main genes; three encodes polymerase complex proteins N, P, L genes and three encodes structural proteins M, F, and HN genes. Every gene (transcription cassette) has its own gene start and gene end signals and separated from the other gene by intergenic sequence (IGS).

Viral Proteins and APMV biology

APMV genome encodes eight proteins: N, P, V/W, M, F, HN, and L. The N, P, and L proteins represent the polymerase complex proteins. Together with the viral genome RNA, they form the viral nucleocapsid which is the minimal infectious unit. The M, F and HN proteins are

associated with the virus envelope, where the M underlies the virus envelope, and the F and HN are integral membrane proteins protruding from the APMV envelope (Samal, 2019).

Polymerase complex proteins:

The nucleocapsid protein (N)

N protein is the most abundant protein produced by APMV in the infected cell. APMV-1 N protein is 489 aa long with a molecular weight of 55 KDa. During APMV genome replication the N protein binds to the genomic and antigenomic RNAs forming helical nucleocapsid structures in the cytoplasm. N protein was found to bind nonspecifically to the host cell RNA forming nucleocapsid-like structures when expressed in uninfected cells (Kho et al., 2003). The N protein protects the viral RNAs from RNase digestion and impairs recognition of the viral RNAs by the host innate immune sensors (Lamb and Parks, 2013). In infected cells, once N protein is synthesized as N° (soluble, monomeric N), the P protein binds it forming N°-P complex to prevent N° self-assembly.

The Phosphoprotein (P)

The P protein exists as a tetramer. It is expressed along with two other proteins (V and W) from the same gene. The P protein is heavily phosphorylated at specific serine and threonine residues. In APMV-1, the P protein is about 42 KDa by calculation, but in SDS-PAGE it shows several bands of 50-55 KDa because of its different phosphorylated forms. It was shown that phosphorylation of some P residues regulates APMV-1 transcription and replication (Qiu et al., 2016). During genome transcription and replication, P protein acts as a connection between the nucleoprotein and the RdRP (L) protein to form the N-P-L complex (Horikami et al., 1992).

V protein and W protein

The V and W proteins are nonstructural proteins expressed through RNA editing during transcription of the P gene (Steward et al., 1993). The P protein is produced from the unedited P mRNA, while the RNAs coding for V and W proteins are produced by the insertion of a single or double non-templated G residues, respectively, at a conserved editing site in the P gene. Hence, the 3 proteins share the same N-terminus, but not the C-termini. The ratio of P, V and W mRNAs transcribed from the P gene in APMV-1 was found to be 68%, 29% and 2% respectively (Mebatsion et al., 200I).

The V is a multifunctional protein and plays critical role in NDV replication and pathogenesis (Alamares et al.,2010). It interferes with IFN signaling in avian cells by targeting STAT1 for degradation (Huang et al., 2003; Qiu et al., 2016c) and interacting with MDA5 leading to inhibition of IFN regulatory factor 3 (Park et al., 2003). The V protein exclusive activity in avian cells defines APMV restriction to the avian hosts (Park et al., 2003). Deletion of V or both V and W proteins resulted in attenuated mutant viruses with severely impaired replication in vitro and in vivo (Mebatsion et al., 200I; Huang et al., 2003; Park et al., 2003). The V protein was found in a very small amount in purified NDV particles. The W protein role for APMV replication is still unknown (Samal, 2019).

L (large-polymerase protein)

The polymerase protein is the largest and the least abundant protein in APMV virions. In APMV1, L protein is 250 kDa and only 50 molecules are present per virus particle (Samal, 2019). It has six conserved domains similar to the polymerases of other linear negative-sense RNA viruses (Poch et al., 1990). The L protein conserved domains were shown to be responsible

for its different enzymatic activities during transcription and genome replication, e.g. nucleotide polymerization, mRNA capping, methylation, polyadenylation (Lamb and Parks, 2013, Sleat and Banerjee, 1993; Grdzelishvili et al., 2006). Being conserved among different virus families, those domains are considered potential targets for effective anti-viral drugs. The L protein interacts with P protein, which in turn interacts with the N protein in complex with the viral RNA thus connecting the polymerase to the nucleocapsid which is required for viral RNA synthesis (Lamb and Parks, 2013).

Membrane-associated proteins

M (Matrix protein)

The M protein is the most abundant protein in the virus particle. It acts as a shunt between the viral envelope and the nucleocapsid. APMV-1 M protein is 364 aa long and has a molecular weight of 40 KDa (Seal et al., 2000). The M protein forms dimers and expression of M protein in cells is sufficient for assembly, budding and exit of virus-like particles (VLP) from the cell (Pantua et al., 2006). During virion budding the M protein binds with the HN N-terminal cytoplasmic tail and N protein C-terminus in the RNP (Pantua et al., 2006; Schmitt et al., 2010). Interaction of M and N proteins helps to direct the nucleocapsid to the virus budding sites and is expected to facilitate the virus release (Schmitt et al., 2002). The M protein surface positively charged residues promote binding with the negatively charged cell membrane. Although APMV replication occurs in the cytoplasm, the M protein was reported to localize in the cell nucleus during early stages of infection (Peeples, 1988) and it is expected to inhibit the host cell transcription and protein expression (Duan et al., 2014).

F (Fusion protein),

Fusion protein is responsible for the virus entry, through fusion of the viral envelope with the cell plasma membrane, and cell to cell transmission by fusing adjacent cell membranes at later stages of infection (Samal et al., 2013).

APMV-1 F protein is a type I membrane protein with a cleavable signal sequence at the N-terminus, a transmembrane domain and a cytoplasmic tail at the C terminus. F polypeptide is initially synthesized as inactive precursor, F0, which is directed to the endoplasmic reticulum (ER) by the N-terminus signal sequence and activated by endoprotease cleavage at the 116 amino acid residue yielding F2 and F1 subunits connected by a disulfide bond (Morrison, 2003). The molecular weights of APMV-1 F0, F1 and F2 are 66, 55 and 12.5 kDa, respectively (Samal, 2019). In the ER, F monomers are glycosylated and packed as homotrimers then transported through the Golgi complex to the virus assembly sites in the cell membrane. F0 cleavage exposes a hydrophobic fusion peptide in the F1 N-terminus responsible for virus envelop fusion with the cell membrane. The F1 C-terminus has the TM domain which anchors the F protein in the virus envelope or the infected cell membrane and two heptad repeat motifs. APMV-1 F2 has a third heptad repeat motif. Heptad repeat motifs have strong affinity and assemble together to form six-helix coils that promotes membranes fusion (Chen et al., 2001; Swanson et al., 2010). APMV F protein-mediated fusion is pH-independent, it proceeds in both the acidic pH of endosomes and the neutral pH on cell surface.

F protein cleavage is critical for APMV infectivity and is the main determinant of APMV virulence (Peeters et al., 1999). The F protein cleavability depends on the amino acid sequence at the F protein cleavage site residues (112-117). Virulent APMVs are characterized by a

multibasic amino acid stretch at the F protein cleavage site with a phenylalanine residue at position 117, which is cleavable by the ubiquitous furin proteases localized in the trans-Golgi. The F proteins of low pathogenic or non-pathogenic APMVs have a monobasic amino acid motif in their cleavage site which is processed extracellularly by trypsin-like proteases available only in certain tissues, e.g. respiratory and intestinal tissues (de Leeuw et al., 2003; Suarez et al., 2020). The antibodies induced against F protein are neutralizing and protect against APMV infection (Kim et al., 2013).

HN (Hemagglutinin-Neuraminidase protein)

HN protein is an integral type II transmembrane protein responsible for virus attachment to the host cell sialic acid receptors. It contains the N-terminal cytoplasmic tail with uncleaved signal sequence, transmembrane domain and C- terminal extracellular mushroom-shaped domain composed of a stalk region and a large globular head. The globular head has a receptor-binding domain, neuraminidase activity and neutralizing antibody binding sites (Mirza et al., 1993), while the stalk region interacts with the F protein during virus entry to promote envelope fusion with the cell membrane (Mirza and Iorio, 2013). The HN protein exists as a homotetramer (two disulfide bond-linked dimers) (Crennell et al., 2000; Zaitsev et al., 2004). HN is directed to the cellular secretory pathway by the N-terminal signal peptide, where it is oligomerized, glycosylated and is transferred to the virus budding sites in the cell surface (El Najjar et al., 2014).

APMV-1 HN protein has 14 cysteine residues which are important for HN folding and keeping its structural integrity through disulfide bonds (McGinnes and Morrison, 1994) and most are conserved across different strains (McGinnes et al., 1987). APMV-1 HN has six possible N-

linked and one O-linked glycosylation sites (Pegg et al., 2017) which are important for structural folding, stability, trafficking to the cell surface and receptor binding (McGinnes and Morrison, 1995, Panda et al., 2004b).

APMV-1 HN molecular weight is about 74 kDa, but different APMV-1 lineages and pathotypes express HNs of 12 different sizes determined by the positions of the stop codons. It was found that HN C-terminus length affects APMV-1 pathogenesis, immunogenicity and thermostability (Huang et al., 2004; Jin et al., 2017; Wen et al., 2016). Virulent APMV-1 strains have 571 aa HN proteins, while less virulent strains have 577 aa and 616 aa HN proteins (Jin et al., 2016; Zhang et al., 2014). Only HN proteins of 616 aa length require post-translational cleavage activation to be functional in virion attachment (Nagai et al., 1976). It is suggested that APMV strains with shorter HNs may have evolved from those with longer HNs through the introduction of stop codons that result in optimal HN length for APMV pathogenesis. The HN protein neuraminidase activity cleaves the sialic acid between the HN and cell receptors to release progeny viruses from infected cells, and it prevents progeny virions from self-aggregation (Lamb and Parks, 2013).

The crystal structure of HN extracellular domain revealed a globular head and a stalk region. The globular head is composed of four monomers, each with a sialic acid-binding site (site I) and NA activity (Crennell et al., 2000) and in the head dimer interface there is another sialic acid binding site (site II) (Zaitsev et al., 2004). All sialic acid binding sites are involved in receptor binding (Iorio et al., 2001). The strong binding affinity of site I is important to maintain a virus-receptor attachment during the fusion process (Mahon et al., 2011). Mutations in site I

residues were shown to attenuate APMV1 virulence while mutations in site II abolished HN receptor binding and fusion activity (Khattar et al., 2009; Bousse et al., 2004).

APMV-1 HN stalk crystal structure reveals a four-helix bundle between two globular head dimers (Yuan et al., 2011). The HN stalk has specificity determinants for homotypic F and HN interaction for fusion activation (Deng et al., 1995, Deng et al., 1999). After receptor binding, the HN globular head transmits a fusion signal to the stalk region which triggers the F protein fusion activity for cell entry (Porotto et al., 2012). The HN cytoplasmic tail interacts with the M protein during virus assembly at the cell surface (Garcia-Sastre et al., 1989).

Molecular basis of APMV virulence

F protein is the main determinant of APMV pathogenicity because its cleavage site sequence affects the F cleavability (activation) in different tissues. Replacement of F cleavage site of a virulent NDV strain with that of a vaccine strain significantly decreased the pathogenicity index and vice versa (Panda et al., 2004a; Hu et al., 2009). Still, the F cleavage site is not always the sole determinant for APMV virulence, it was shown that different F proteins with the same cleavage site can change the virus virulence (Paldurai et al., 2014).

Among components of the polymerase complex, L protein is a major contributor to APMV virulence (Rout and Samal, 2008). The L-protein was found to be the second determinant of APMV-1 virulence after the F protein (Paldurai et al., 2014; Yu et al., 2017). Although the mechanism of L protein effect on APMV virulence was not investigated, it was shown that the polymerase complex proteins (N, P and L) activity is directly related to the NDV strain virulence (Dortmans et al., 2010). Although, the P gene codes for V protein which is important for APMV

pathogenesis and virulence, different P genes were reported to have little effect on the varying pathogenicity between different NDV strains (Paldurai et al., 2014).

APMV life cycle

APMVs can infect a wide variety of avian cells. Paramyxoviruses replication cycle occurs in the cell cytoplasm and it ranges from 14-30 hours, virulent strains of NDV complete it in 10 hours (Plemper and Lamb, 2020). The replication cycle is composed of three main stages:

Virus attachment and cell entry

APMV cell entry occurs by virus envelope fusion with the cell membrane. Sialic acid binding domains in the HN globular head upon engagement with sialic acid transmit a fusion signal to the stalk region of nearby F proteins which triggers a cascade of F protein conformational changes resulting in the fusion of the virus envelope with the cell membrane. During virus attachment and entry, the M protein dissociates from the nucleocapsid and surface proteins, and the nucleocapsid is released to the cell cytoplasm. Unlike influenza virus, APMV fusion with the cell membrane is pH-independent, although acidic pH promotes the virus entry by endocytosis (San Román et al., 1999).

Genome expression and replication

The virion-associated L protein with the help of P protein (polymerase complex) starts viral genome transcription (Hamaguchi et al., 1983). The polymerase complex binds to the promotor sequence in the 3’ leader and starts sequential transcription of the viral genes according to the transcription start and stop signals (gene start and gene end). During virus transcription, the polymerase molecule occasionally dissociates from the viral genome resulting in gradient

expression of the viral genes, so that the genes located closer to the 3' end are expressed at a higher level than those closer to the 5' end. The polymerase adds 5' cap and 3' polyAs to the synthesized viral mRNAs, the latter due to polymerase stuttering over the gene end sequence containing repeated U nucleotides. The HN and F proteins are synthesized at the ER and are directed to the secretory pathway to be delivered to the cell membrane.

The viral polymerase shifts from genome transcription to genome replication by skipping gene start and stop signals and producing positive full-length antigenomic RNA. Antigenomic RNA acts as a template for production of negative viral progeny genomes. All viral genomes and antigenomes are fully encapsidated by the nucleoprotein during virus replication. The integrity of the leader and trailer sequences is critical for virus replication since they have the polymerase promotor sequences at their ends. It is believed that polymerase shifting from transcriptase into replicase happens after the accumulation of a sufficient amount of viral nucleoprotein (Baker and Moyer, 1988; Horikami et al., 1992). The viral genomic replication and transcription levels are controlled by *cis*-elements in the genome leader, trailer, IGS and UTRs (Samal, 2019).

Virus assembly and release

The viral proteins are synthesized in different subcellular compartments in the infected cell. The F and HN are synthesized, glycosylated, and oligomerized in the ER. They interact to form a protein complex to be trafficked to the cell membrane (Stone-Hulslander and Morrison, 1997). Co-trafficking of F and HN ensures their constant ratio in the progeny virions. The helical nucleocapsid interacts with the P and L proteins to form a ribonucleoprotein (RNP) which binds to the M protein through the N protein tail. The M protein is trafficked to the virus budding sites in the cell membrane where it interacts with the cytoplasmic tail of HN proteins (Battisti et al.,

2012). The F protein in the envelope interacts with the HN but not the M protein. Co-immunoprecipitation has shown that F protein interacts with the N protein and it is expected to assist the RNP targeting to the virus budding sites at the cell membrane (Pantua et al., 2006). The M protein is the main driver of the virus assembly and budding from the infected cell. APMV can also spread to the adhering cells through a direct fusion of cell membranes creating a syncytium, without being exposed to the extracellular milieu. The ability of APMV to form syncytia is related to its virulence and pathogenesis.

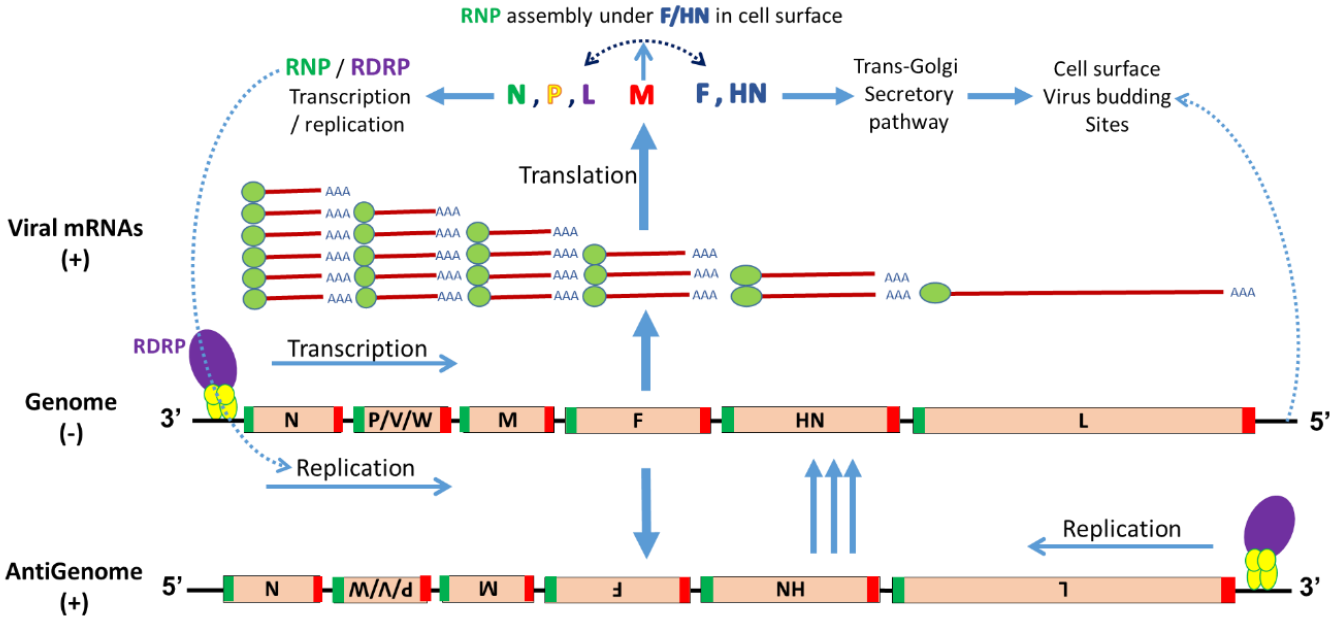


Figure 3 Replication and transcription of APMV genome. During transcription, the viral RNA polymerase forms 5' capped, polyadenylated mRNAs starting from the the 3' end. The transcription starts at the gene start and stop at the gene end of each gene. During transcription, the RNA polymerase detach from the genome and restart from the beginning yielding the gradient pattern of APMV mRNAs. At replication, the RNA polymerase ignores the transcription stop signal and produce positive full length antigenomic RNA which acts as a template for further production of negative sense viral genomic RNA copies.

Reverse genetics

A reverse genetic system was initially created for APMV-1 two decades ago (Krishnamurthy et al., 2000), then it was created for other APMVs (reviewed in (Samal, 2019)). In reverse genetic systems, an infectious virus is recovered from its cDNA expressing plasmid which allows manipulation of the viral phenotype from its genomic sequence. The cDNA serves as a template for synthesis of the viral RNA to initiate the replication cycle. For non-segmented negative-stranded RNA viruses, viral genome or antigenome wrapped by N protein and polymerase complex (P and L proteins) are minimum requirements for initiation of a virus replication cycle (Arnheiter et al., 1985). The main applications of APMV reverse genetic systems is: 1) Understanding the molecular basis of APMV pathogenicity, 2) Using APMVs as a genetically modified or vector vaccines, 3) The development of APMVs as oncolytic agents (Kim and Samal 2016; Samal, 2019).

Reverse genetic techniques

The commonly used reverse genetic system for APMVs relies on co-transfection of mammalian cells with a mixture of plasmids encoding for virus anti-genomic RNA and N, P, and L proteins under the control of a T7 RNA polymerase promotor. The N, P and L plasmids are used in a ratio equivalent to their natural expression levels by APMVs. The T7 RNA polymerase is usually expressed from a helper virus, Modified Vaccinia strain Ankara (MVA), upon infection of the transfected cells (Krishnamurthy et al., 2000). The N-protein encapsidate the full length anti-genomic RNA. The polymerase complex (P and L proteins) binds the promotor sequence in the 3' anti-genome trailer and synthesizes negative-sense viral genomic RNA completing the first APMV replication cycle.

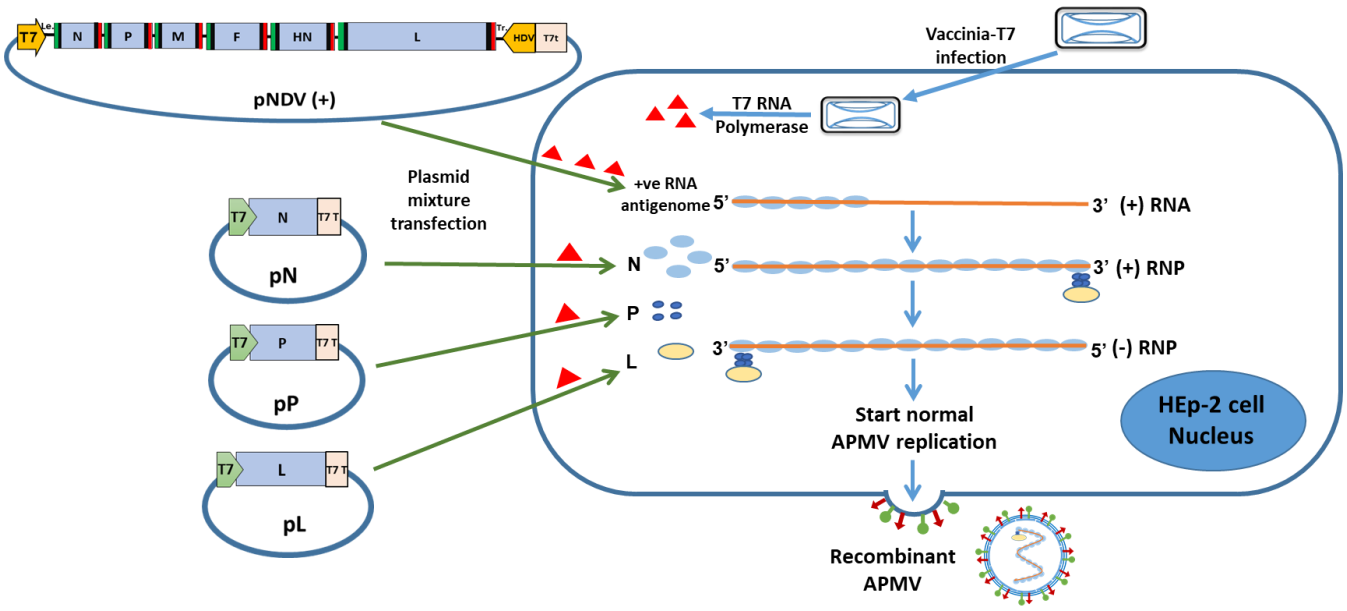


Figure 4. APMV reverse genetics system: Plasmid mixture of helper plasmids (pN, pP, pL) and FLC (pNDV(+)) plasmids are used to transfect Hep-2 cells. The cells are co-infected by modified vaccinia virus expressing T7 RNA polymerase which starts transcription of the transfected plasmids yielding positive antigenomic RNA and mRNAs of the polymerase complex proteins (N, P, and L). The mRNAs are translated into viral RNA polymerase which transcribes the antigenomic RNA (+) yielding full length viral genomic RNA(-) to start the normal APMV replication cycle.

Different modifications of this general scheme were developed including a two plasmid recovery system (Liu et al., 2017). In that system N, P and L proteins were expressed from one plasmid instead of three different plasmids. In another approach, the MVA as a source of T7 polymerase is replaced by stable cell lines or a plasmid construct expressing T7 polymerase (Yeong et al., 2021; Jiang et al., 2009), or even replacing the T7 RNA polymerase by a eukaryotic transcription driven by cytomegalovirus (CMV) promoter- (Li et al., 2011). That eliminates the cytopathic effect induced by MVA, the requirement for the purification of the recovered APMV from vaccinia virus, and improves the recovery efficiency.

APMV Vectored Vaccines

APMV-1 (NDV) Vectors:

NDV was the first APMV to be used as a vector vaccine for protection against avian and mammalian pathogens and as an oncolytic agent. NDV vectored vaccines targeted different avian pathogens including infectious laryngeotrachitis (ILT) virus, infectious bronchitis (IB) virus, avian influenza virus, infectious bursal disease virus, avian meta-pneumovirus, and human pathogens including SARS coronavirus, human influenza, Ebola virus, measles virus, HIV, human parainfluenza virus and respiratory syncytial virus (reviewed in Kim and Samal, 2016; Vilela et al., 2022). A vectored vaccine based on LaSota strain of NDV against avian influenza was successfully authorized and commercialized for poultry vaccination in China and Mexico (Sarfati-Mizrahi et al., 2010; Lozano-Dubernard et al., 2010). Recently, intranasal administered NDV vectored vaccine against SARS CoV-2 was submitted for phase-I clinical trial (Warner et al., 2021).

Advantages of NDV vaccine vectors:

NDV has special characteristics that make it an attractive vaccine vector for avian and mammalian use. First, it has naturally available non-pathogenic strains (e.g. LaSota and Hitchner B1 strains) which can be used safely for avian and human vaccination (Suarez et al., 2020). Second, it exhibits a reasonable degree of genetic stability with rare to no evidence of recombination (Chare et al., 2003; Bukreyev and Collins 2008). Third, it replicates in the respiratory tract inducing mucosal and systemic immune responses (Cornax et al., 2012). Fourth, it replicates in the host cell cytoplasm, with no phases in the nucleus, minimizing the risk of viral

genome incorporation into the host genome. Fifth, unlike other virus vectors, e.g. Adeno, Herpes, and Pox viruses, APMVs are small and code for six viral proteins only, offering less competition with the expressed foreign protein for the host immune response. Sixth, an NDV vector was reported to accommodate up to 5 kb foreign gene fragment (Samal, 2019), which is sufficient to encode protective antigens of several viruses. Seventh, APMVs genome has a modular organization of well-separated genes, that facilitates introduction of a transgene. Eighth, in humans, NDV is attenuated due to its natural host restriction (Bukreyev and Collins, 2008; Bukreyev et al., 2005), Ninth, NDV induces high levels of type I IFN in mammalian cells, promoting a B-cell response against the vector and the expressed foreign antigen (Honda et al., 2003; Grieves et al., 2018). Lastly, NDV is an avian pathogen that can be used for human vaccination without the concern for preexisting neutralizing antibodies (Samal, 2019).

APMV vectors other than APMV-1

Other APMVs were consequently used as vector vaccines in chickens and other animals. APMV-3 grows efficiently in mammalian and avian cells and it was shown to induce robust mucosal and systemic immune responses in chickens (Kumar, 2010; Paldurai and Samal, 2019; Shirvani et al., 2020b) and in non-human primates (Kattar et al., 2013). APMV-3 strain Netherlands was successfully used as a vector vaccine for protection of chickens against NDV, infectious bursal disease virus (IBDV) and highly pathogenic avian influenza virus H5N1 (HPAIV) (Kumar, 2010; Shirvani et al., 2020b; Varghese, 2021) and as a vector vaccine against Ebola virus in guinea pigs (Yoshida et al., 2019). APMV-2, APMV-6, and APMV-10 were evaluated as vector vaccines against HPAIV (H5N1), yet they conferred limited protection (up to 50%) (Tsunekuni et al., 2017).

The main advantage of other APMVs over APMV-1 vector is the evasion of the preexisting (maternal) antibodies against NDV in chickens. These antibodies are transferred to commercial chicks from NDV immunized breeders limiting the efficacy of APMV-1 vectored vaccines (Tsunekuni et al., 2014; Bertran et al., 2018). Also, other APMV vectors can be used in combination with an NDV vector in a prime-boost vaccination strategy to limit the development of the immune response against the vector proteins.

Expression of heterologous antigens by APMV vectors

Foreign genes are usually added to APMV vectors as separate transcription units in the intergenic regions of APMV genome (Samal, 2021). The foreign gene expression follows the gradient transcription level of APMV genes. Thus for higher expression levels, the transgene is placed closer to the 3'end in the APMV genome. The optimal insertion site for the highest level of transgene expression varies according to the protein and the APMV vector (Huang et al., 2001; Yoshida et al., 2019; Yoshida and Samal 2017). In APMV-1, the usual site for optimum expression of a transgene is the P/M junction. A Kozak sequence is added before the foreign gene ORF for improved transcription. The full genome nucleotide length, including the added gene fragment, should follow the rule of six (Calain and Roux, 1993, Kolakofsky et al., 1998).

Recently, novel approaches were developed allowing the foreign gene expression from the transcription units of APMV genes. This was achieved by either encoding both genes as one ORF with a self-cleaving 2A sequence separating the two polypeptides, or by placing an internal ribosomal entry site (IRES) between the ORFs coding for a viral vector and a foreign protein

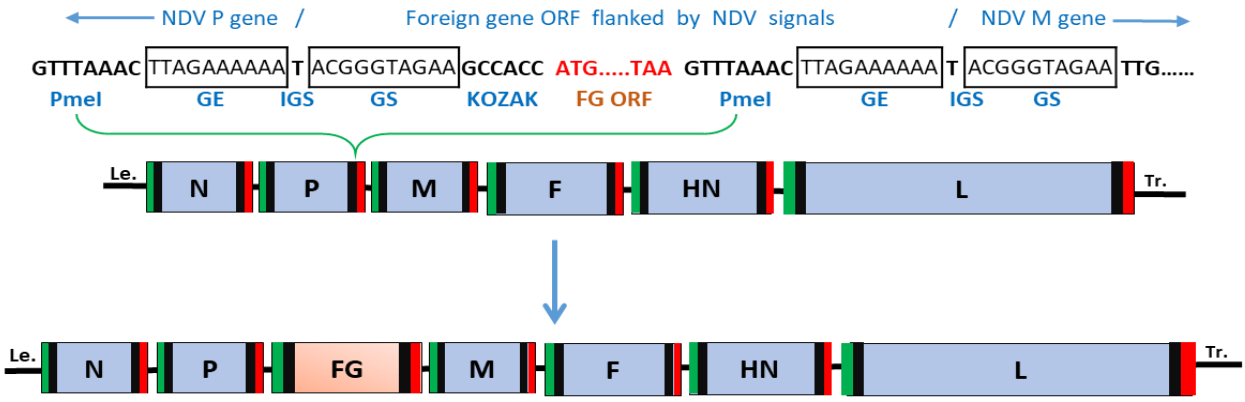


Figure 5 Expression of a Foreign gene (FG) by APMV-1 as a separate transcription unit in the P/M gene junction. The whole added gene cassette nucleotide length should be a multible of six and a Kozak sequence is included upstream the foreign gene ORF for improved expression.

(Wen et al., 2015; Zhang et al., 2015). Self-cleaving 2A peptides are short elements (18-22 amino acids) found in polyproteins of some picornaviruses (and other viruses) and which during their translation prevent with high frequency the formation of a peptide bond between the last two amino-acids of 2A peptide (Donnelly et al., 2001). That results in the separation of the connected polypeptides with most of the 2A sequence connected to the C-terminus of the upstream protein, and the last amino acid of 2A (proline) connected to the N-terminus of the downstream protein. The genes connected by a 2A peptide are expressed equimolarly which may be important for specific applications like the production of antibody heavy and light chains (Lin et al., 2018). The efficacy of 2A-dependent peptide separation is not perfect, it depends on the 2A peptide sequence, the nature and the order of the surrounding proteins (Donnelly et al., 2001b; Anderson et al., 2012; Minskaia et al., 2013; Kim et al., 2011). It was shown that adding a GSG linker before the 2A peptide enhances its cleavage efficiency (Chng et al., 2015). For the expression of proteins that are processed in the secretory pathway a furin cleavage site can be added before the 2A peptide to remove the 2A remnants from the upstream polypeptide. A 2A

peptide was used to express GFP from the NDV N, M and L transcription units. Expression of GFP using 2A peptide from the M gene transcription unit yielded higher expression levels than when the protein was expressed from a separate transcription unit in the P/M junction (Wen et al., 2015).

Internal ribosome entry site (IRES) is an RNA element present in viral genomes (e.g, picornaviruses) and some eukaryotic mRNAs that mediate the ribosome binding and the initiation of mRNA translation independent of the 5' end signals. Viral IRES commonly used to create polycistronic vectors are over 500 nucleotides in length (Wang and Marchisio, 2021). IRES-driven translation is usually less efficient than 5' cap-dependent translation. Thus, expression of a foreign gene using an IRES was lower compared to the expression of the same protein from an independent transcription unit in the corresponding site (He et al., 2020). Still, IRES allows the co-expression of two proteins with authentic C- and N- termini from the same RNA, which cannot be achieved by 2A peptides. One of the essential parameters for recombinant vector vaccines is the stability of the transgene expression (Han et al., 2008, Wertz et al., 2002). Since there is no selective pressure for the virus vector to keep the foreign gene during replication (if it is not required for virus infectivity), it is important to assess the heterologous antigen expression after serial passaging in a relevant propagation or application system (Bukreyev et al., 2006).

Antigen incorporation into recombinant APMV vectors

Incorporation of the expressed antigens into the recombinant vaccine virions helps to improve the immune response against the foreign protein (Park et al., 2006; Bukreyev et al., 2006). Heterologous surface antigens expressed by APMV vectors usually show little

incorporation to the recombinant APMV virions. They sometimes can be detected by a sensitive western blot assay but not upon commassie staining of the virion proteins resolved in a gel (Yoshida et al., 2019). The replacement of the cytoplasmic tail of HA of AIV with the cytoplasmic tail of APMV F significantly increased the incorporation of HA into APMV virions, and this approach can likely be implemented for other cell-surface-exposed antigens (Ingrao et al., 2021; Hu et al., 2017).

Other viral vectors used for poultry vaccination

After the advancement of recombinant DNA technology, different viruses were used as vector vaccines for poultry protection against different pathogens as an alternative to the conventional live or inactivated vaccines. Virus vector vaccines offer important advantages over inactivated and attenuated vaccines. They retain the capacity of induction of strong cellular immunity like live vaccines, but the production cycle of vectored vaccines does not rely on propagation of pathogenic viruses and they cannot revert to virulence. Moreover, they provide the possibility of differentiating infected and vaccinated birds, which is important in epidemiological surveys. Lastly, they usually escape neutralization by the MDA against the target pathogen.

Poxviruses

Avipoxviruses are large DNA viruses that infect birds causing two forms of clinical infection: dry form with proliferative skin lesions and wet form with diphtheritic membrane in oropharyngeal mucosa (Tripathy and Reed, 2013). Tissue culture attenuated strains of fowl pox virus (FPV) and canary pox viruses have been used for poultry vaccination against poxvirus

infection either by wing-web stabbing after 4 weeks of age, or by subcutaneous injection of day-old chicks, or by *in ovo* injection (Romanutti et al., 2020). Avipoxviruses, especially FPV-vectored vaccines, were used for dual protection against poxvirus infection and diverse avian pathogens such as NDV, infectious laryngotrachitis (ILT) virus, Avian influenza virus (AIV), IBDV and Mycoplasma gallisepticum (Hein et al., 2021; Skinner et al., 2005).

Avipoxviruses have attractive features that promoted their success and commercialization as vaccine vectors: 1) They have large genomes (260-340 kbp) with several non-essential sites that facilitate incorporation of a large gene or multiple gene fragments for a multivalent-vector vaccine by homologous recombination (Sharma et al., 2002). 2) Unlike other DNA viruses, poxviruses replicate in the cytoplasm minimizing the risk of host genome alteration by the viral genome. 3) They induce efficient cellular and humoral immune response against the expressed foreign antigen simulating natural infection (Smith et al., 1983). 4) They can be prepared in a lyophilized form stable at a temperature of 4-5C , i.e. they don't require liquid nitrogen or a deep freeze system in the distribution chain.

A significant limitation for Avipoxviruses vectored vaccines is the tedious work required for their mass application by wing-web stabbing. Also, their efficacy can be impaired by subsequent FPV vaccination or infection, i.e. rFPV vectors are not recommended to be reused for multiple vaccinations (Zhao et al., 2014; Swayne et al., 2000). It was found that Avipoxviruses vectored vaccine for ILT did not protect against ILT virus shedding (Vagnozzi et al., 2012; Johnson et al., 2010) which may be due to the lack of mucosal immune response.

Herpesviruses (Marek's disease virus)

Marek's disease (MD) is an oncogenic lymphoproliferative disease of chickens caused by an alpha-herpesvirus from the genus Mardivirus. The main feature of this disease is the ability to cause long-term latent infection by delivering the viral genome to the cell nucleus and escaping the host immune system (Robinson and Mahony, 2021). There are three different species of Marek's disease viruses (MDV), formerly known as MDV serotype 1, which is oncogenic and causes MD in chickens, serotype 2 (non-oncogenic, affects chickens) and serotype 3 which is non-oncogenic and affects turkey, named as herpesvirus of turkeys (HVT) (Kannaki and Gowthaman, 2020). The three serotypes were used for chicken vaccination against MD (Gimeno, 2008).

MDV and HVT vaccines are administrated by subcutaneous injection of day-old chicks or by *in ovo* injection of 18 day old embryos. Recombinant MDV and HVT have been used as viral vectors for the expression of protective antigens from AIV, NDV, ILTV and IBDV (Romanutti et al., 2020). The main advantage of using herpesvirus-vectored vaccines in chickens is the long-term protection they confer against the targeted antigen along with protection against MD (30-60 weeks) (Hein, 2009). Their safety for in ovo injection enables their large scale mechanical administration . MDV vectors were used to express two different antigens from two pathogens, so that they could be used as multivalent vaccines for protection against three avian viruses including the MDV (reviewed in Romanutti et al., 2020). Another advantage of MDV vectored vaccines is the evasion of MDA against MD and the targeted pathogen (Sakaguchi et al., 1998).

The main downside of MDV-vectored vaccines is the delayed onset of protective immunity against the expressed antigen (3-4 weeks) (Esaki et al., 2013; Palya et al., 2014), thus it is always recommended to combine MDV vectored vaccines with a conventional live vaccine against the targeted pathogen if an early protection is required, as in the case of NDV. Also, MDV virions are cell-associated that require liquid nitrogen for the distribution and preservation of MDV-based vaccines , increasing their cost and limiting availability in remote places (Baigent, 2006).

Adenoviruses

Adenoviruses are non-enveloped double-stranded DNA viruses which infect different vertebrate species including birds. Avian adenoviruses include non-pathogenic and pathogenic strains. Avian adenoviruses are associated with different diseases in poultry such as egg-drop syndrome, inclusion body hepatitis and hydropericardium syndrome, and hemorrhagic enteritis in turkeys (Hafez, 2011).

Different serotypes of fowl adenovirus and replication-defective human adenoviruses were used as vector vaccines for protection of chickens against several pathogens such as NDV, AIV, IBV, IBDV and MDV (Romanutti et al., 2020; Kerstetter et al., 2021; Ferreira et al., 2021; Francois et al., 2004). Adenoviruses can infect different cell types and deliver their genome to the nucleus as an episome stable for weeks to months (Adam et al., 1994; Ramos et al., 2011).

The advantages of Adenovirus vectors include: 1) They can be easily prepared and quickly produced on large scale for newly emerging pathogens (Zhu et al., 1999). 2) Recombinant adenoviruses express the transgene in large quantities with efficient stimulation of specific CD4 and CD8 T-cells (Maeda et al., 2005). 3) The non-pathogenic strains with known

genomic sequence and transcriptional pattern are available (Payet et al., 1998). 4) They are genetically stable and have low toxicity as virus vectors, the vector proteins show low inflammatory response (Avakian et al., 2007). 5) Human adenovirus vectors can be safely administered to chickens by different routes, and chickens do not have preexisting immunity against human adenoviruses.

Although different Adenoviruses have been developed as vector vaccines for avian pathogens, none of them were authorized for commercial use due to their limited efficacy. Some strains of fowl adenovirus have shown incomplete protection against IBDV when used as a vector vaccine by oro-nasal route (Francois et al., 2004). One reason may be the reduced *in vivo* replication of the left end-deleted recombinant fowl adenoviruses used as a vector (Deng et al., 2013). Human Adenovirus vectors have shown efficient protection against the pathogens whose antigens were expressed, but they cannot protect against avian adenovirus infection.

Alphaviruses

Alphaviruses are enveloped positive sense single-stranded RNA viruses infecting different animal and bird species. Two alphaviruses, Semliki Forest virus (SFV) and Venezuelan equine encephalitis virus (VEE), have been used as vector vaccines for chicken immunization against IBDV and AIV, respectively (Phenix et al., 2001, Schultz-Cherry et al., 2000). The main advantages of alphavirus vectors, are: 1) Their ability to infect different cell types. 2) Alphaviruses can be used as recombinant virus vectors or self-amplifying RNA. 3) They can afford up to 8 kb gene inserts (Lundstrom, 2019). 4) High level of transient transgene expression. 5) RNA replication cycle proceeds in the cytoplasm minimizing the risk of host genome modification by viral sequences.

Although VEE virus-vectored vaccine received a conditional USDA approval in 2015 for immunization against H5N8 AIV (Bertran et al., 2017,Pantin-Jackwood et al., 2019), alphaviruses were not commercialized for poultry use. The main drawback of alphavirus replicons was their low immunogenicity sufficient for only partial protection (Kapczynski et al., 2017, Schultz-Cherry et al., 2000). Another limitation of alphavirus vectors is the lack of an efficient packaging system for mass production (Lundstrom, 2019).

Chapter 3: Recovery of Recombinant APMV-3 Strain Wisconsin by Reverse Genetics and Its Evaluation as a Vaccine Vector for Chickens.

Abstract

A reverse genetic system for avian paramyxovirus type-3 (APMV-3) strain Wisconsin was created and the infectious virus was recovered from a plasmid-based viral antigenomic cDNA. Green fluorescent protein (GFP) gene was cloned into the recombinant APMV-3 genome as a foreign gene. GFP expression by the recovered virus was confirmed for at least 10 consecutive passages. APMV-3 strain Wisconsin was evaluated in comparison with APMV-3 strain Netherlands and APMV-1 strain LaSota as a vaccine vector. The three viral vectors expressing GFP were compared for GFP expression level, growth rate in chicken fibroblast cells, and tissue distribution and immunogenicity in specific pathogen-free (SPF) day-old chicks. APMV-3 strain Netherlands showed the highest growth rate and GFP expression level among the three APMV vectors in vitro. APMV-3 strain Wisconsin and APMV-1 strain LaSota vectors were mainly confined to the trachea after vaccination of day-old SPF chickens without any observable pathogenicity, whereas APMV-3 strain Netherlands showed wide tissue distribution in different body organs (brain, lungs, trachea, and spleen) with mild observable pathogenicity. In terms of immunogenicity, both APMV-3 strain-vaccinated groups showed HI titers two to three fold higher than that induced by APMV-1 strain LaSota vaccinated group. This study offers a novel paramyxovirus vector (APMV-3 strain Wisconsin) which can be used safely for young chicks vaccination as an alternative for APMV-1 strain LaSota vector.

Introduction

The family *Paramyxoviridae* contains pleomorphic, enveloped viruses with a non-segmented, negative-sense RNA genome. Members of this family have been isolated from a wide variety of avian and mammalian species around the world, which includes many important human, animal and avian pathogens (Samal, 2011). The family *Paramyxoviridae* is divided into four subfamilies; *Avulavirinae*, *Orthoparamyxovirinae*, *Metaparamyxovirinae* and *Rubulavirinae*. Avian paramyxoviruses (APMVs) are placed in subfamily *Avulavirinae* in three genera: *Orthoavulavirus*, *Metaavulavirus*, and *Paraavulavirus*. In the latest ICTV classification, APMV-1 was placed in genus *Orthoavulavirus* and APMV-3 was placed in genus *Metaavulavirus* (Amarasinghe et al., 2019)

APMV-1 is the best characterized member among APMVs because its virulent strains, known as Newcastle disease virus (NDV), cause a highly contagious disease with major economic importance in chickens worldwide (Samal, 2019). However, our knowledge about replication and pathogenicity of other APMVs is very limited. The complete genome sequences of one or more representative strains of other APMVs have been reported (Paldurai and Samal, 2019). The genome lengths of all APMVs range from 15 to 17 kb. Most APMV genomes consist of six genes: N (nucleocapsid), P (phosphoprotein), M (matrix protein), F (fusion protein), HN (hemagglutinin-neuraminidase protein), and L (large polymerase protein); except APMV-6 which has an additional SH (small hydrophobic) gene (Paldurai and Samal, 2019).

To date, reverse genetics systems have been developed for APMV-1, APMV-2, APMV-3, APMV-6, APMV-7, and APMV-10; “reviewed in (Paldurai and Samal, 2019)”. The reverse genetics system of APMV-1 has greatly advanced our understanding of its replication and

pathogenesis. In addition, it has been used as a vaccine vector for animal and human pathogens (Samal, 2021). However, the potential of reverse genetics systems of other APMVs has not been fully evaluated.

The disease potential of APMV-1 has been well studied (Samal, 2019). APMV-2, APMV-3, APMV-6 and APMV-7 have been associated with mild respiratory disease in poultry (Paldurai and Samal, 2019). APMV-3, was first isolated from turkeys with respiratory tract disease in Ontario, Canada, in 1967 and then in Wisconsin, USA, in 1968 (Tumova et al., 1979). Since then, APMV-3 strains have been isolated from turkeys in England, France and Germany (Samal, 2011). However, most APMV-3 isolations have been from psittacine and passerine birds held in quarantine (Alexander et al., 1982). There are two distinct strains of APMV-3 with varying pathogenicity in chickens. APMV-3 strain Netherlands is mildly pathogenic to young chickens, whereas APMV-3 strain Wisconsin is non-pathogenic to young chickens (Alexander and Chettle, 1978; Kumar et al., 2010a,b; Kumar et al., 2008).

The complete genome sequences have been determined for APMV-3 strain Netherlands and APMV-3 strain Wisconsin. Both strains share 67% nucleotide identity and 78% amino acid identity. Antigenic analysis by cross-HI and cross-neutralization tests showed that both strains belong to the same serotype but represent two antigenic subgroups (Kumar et al., 2010). The F protein cleavage site of APMV-3 strain Netherlands has a multi-basic amino acid motif, similar to that of virulent APMV-1 (NDV) strains, whereas APMV-3 strain Wisconsin has a monobasic amino acid motif at its F protein cleavage site, similar to that of avirulent APMV-1 strains (Table.1) (Kumar et al., 2010b; Kumar et al., 2008, Suarez et al., 2020).

Table 1: F protein cleavage site motif in APMVs.

APMV-1 (avirulent)	111	G-G-**R**-Q-G-**R**-L	117
APMV-3 (wisconsin)	96	P-**R**-P-S-G-**R**-L	102
APMV-3 (Netherlands)	101	A-**R**-P-**R**-G-**R**-L	107
APMV-1 (virulent)	111	G-**R**-**R**-Q-**K**-**R**-F	117

Basic amino acids (R=arginine, K=lysine) are in bold, mentioned numbers indicate amino acids position in the F protein

A reverse genetics system has been developed for APMV-3 strain Netherlands and the recombinant virus has been used as a vaccine vector to evaluate the role of NDV F and HN proteins in the protective immunity (Kumar, 2010). Recently, APMV-3 strain Netherlands was used successfully as a vaccine vector for protection of chickens against HPAI (H5N1) (Shirvani et al., 2020b). It was also found that the P-M gene junction is the optimal insertion site in the genome of APMV-3 strain Netherlands for foreign gene expression (Yoshida and Samal, 2017). APMV-3 strain Netherlands expressing Ebola virus glycoprotein was found to elicit mucosal and humoral immune responses against the Ebola virus glycoprotein in guinea pigs (Yoshida et al., 2019). These results indicate that the recombinant APMV-3 strain Netherlands has great potential as a vaccine vector for veterinary and human uses. The advantage APMV-3 vector is expected to have over APMV-1 vaccine vectors is the minimal cross-reactivity with NDV MDA in commercial chickens and systemic spread to different organs (Kumar, 2010). It was shown to induce a better immune response than lentogenic strains of APMV-1 (Shirvani et al., 2020b; Varghese, 2021) without being a select agent as moderately pathogenic strains of APMV-1 (e.g. BC-strain) which enable their use as vector vaccine. In addition, APMV-3 strains are frequently isolated from turkeys; therefore, they may be successful vaccine vectors for turkey vaccination.

APMV-3 strain Netherlands is considered the prototype of APMV-3 (Alexander and cheetle, 1978), and it has some pathogenic effects in day-old chickens (Alexander and Collins, 1982). It causes stunted growth (especially in young broiler chickens) and kills the embryo during propagation in embryonated chicken eggs (ECE) (Alexander and Collins, 1982; Kumar et al., 2010a). By contrast, APMV-3 strain Wisconsin was reported to be non-pathogenic to young chickens and does not kill chicken embryos during propagation in ECE (Kumar et al., 2010a). Therefore, we hypothesized that APMV-3 strain Wisconsin may offer a safer vaccine vector for day-old chickens or *in ovo* vaccination. To test this hypothesis, we developed a reverse genetic system for APMV-3 strain Wisconsin and recovered the recombinant virus. Then it was tested as a virus vector expressing the green fluorescent protein (GFP) as a foreign protein and compared with recombinant APMV-3 strain Netherlands and APMV-1 strain LaSota vectors expressing GFP in vitro and in vivo.

Materials and Methods

Viruses, Cells and Animals

Chicken embryo fibroblast (DF-1) and human epidermoid carcinoma cells type-2 (HEp-2) were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells infected by APMVs were maintained in DMEM supplemented with 2% FBS and 10% chicken egg allantoic fluid (as a source of exogenous protease). The viruses used in this study were: APMV-3 strain turkey/Wisconsin/68 (APMV-3 Wisc.), obtained from national veterinary services laboratory, Ames, Iowa; and recombinant APMV-1 strain LaSota (rLaSota) and recombinant APMV-3 strain parakeet/Netherlands/449/75 (rAPMV-3 Neth.) expressing GFP were prepared in our laboratory with the GFP gene cloned into their P-M gene

junctions. Modified vaccinia virus strain Ankara expressing T7 polymerase (MVA-T7) was obtained from Dr. Bernard Moss (National Institute of Allergy and Infectious Diseases). The avian paramyxoviruses were grown in 10-day-old specific pathogen-free (SPF) ECE by intra-allantoic inoculation. The SPF chickens and ECE were obtained from Charles River Laboratories, Manassas, VA, USA.

Construction of Avian Paramyxovirus 3 Strain Wisconsin (APMV-3 Wisc.) Antigenomic Full-Length Plasmid

APMV-3 Wisc. RNA was isolated from purified virus using Trizol reagent (Invitrogen) following the manufacturer's protocol. A complete virus antigenomic cDNA was created using the viral genomic RNA, superscript reverse transcriptase IV (Invitrogen) and short random primers (hexamers) following the manufacturer's protocol. The viral genome was divided into six major fragments (I-VI) (corresponding to the six virus genes) using restriction enzyme (RE) sites. All RE sites were introduced in the downstream un-translated region of each gene except for the L-gene where three RE sites, naturally present in its open reading frame (ORF), were used to sub-divide the L-gene into four smaller fragments (Fig. 6A). Three RE sites, two RsrII and one SacII, in the ORF of N, P, and F genes, respectively, were deleted by silent mutagenesis, thus they can be used for other genome fragments cloning. For that reason, fragments I, II and IV were synthesized in two pieces and connected by overlapping polymerase chain reaction (PCR) to delete RsrII and SacII sites. Plasmid pBR322/dr was used as a backbone vector to clone the virus full length antigenome. Plasmid pBR322/dr was previously prepared by modifying of low-copy-number plasmid pBR322 to include the T7 RNA polymerase promoter, a polylinker and the hepatitis delta virus (HDV) ribozyme sequence (Collins et al., 1995).

A multiple cloning site oligonucleotide (polylinker) was designed to contain RE sites used for cloning of the complete virus genome. The polylinker was cloned into the pBR322/dr plasmid between AscI and RsrII sites. This cDNA was used as a template to create the virus subgenomic fragments by PCR using primers bearing the RE sites and high-fidelity platinum pfx polymerase enzyme (Invitrogen). The subgenomic fragments were cloned sequentially into the prepared vector. The L gene was cloned first in a reverse order (Fig. 6A; starting from VIa to VId) followed by insertion of other fragments to form the antigenomic full-length clone (FLC) of APM3 strain Wisconsin. Each fragment was confirmed for the absence of any unintended mutation by sequence analysis using a big dye terminator kit. A T7 RNA polymerase promotor sequence was inserted before the virus anti-genomic leader and a the HDV ribozyme sequence was inserted after the virus anti-genomic trailer. The resulting APMV-3 Wisc. full-length expression plasmid was termed “pAPMV3 Wisc. FLC.” (Fig. 6A).

Construction of APMV-3 Wisc. Support Plasmids

Reverse transcription PCR (RT-PCR) was used to create APMV-3 Wisc. N and P genes ORF cDNA, which was cloned into the expression plasmid pTM (pTM-N and pTM-P) (Fig. 6B). RE used for cloning the N gene were NcoI and SpeI, and for the P gene were NcoI and XhoI. APMV-3 Neth. L gene was used instead of APMV-3 Wisc. L gene for its recovery. APMV-3 Neth. L gene cDNA was previously cloned into the expression plasmid PcDNA3.1 using XbaI and NheI RE [7] (Fig. 6B). Both pTM and PcDNA3.1 plasmids had T7 polymerase promotor sequence. The cloned genes were sequence confirmed before being used in virus recovery.

Table 2: Nucleotide changes involved for pAPMV-3 Wisc. FLC fragments construction:

Fragment order		Nucleotide no.	Old nt.	New nt.	Original seq.	New seq.	RE site
I		Before(1)	GGCGCGCCTAATACGACTCACTATAGGG				AscI + T7 pr
		1590	A	C	caagcg 1588-1593	caCgTg	PmlI site
II		1592	C	T			
		3074	A	G	attttatc 3074-3081	GtttAaAc	PmeI site
		3078	T	A			
III		3080	T	A			
		4391	C	G	gccgccag 4389-4396	gcGgccGC	NotI site
		4395	A	G			
IV		4396	G	C			
		6517	C	G	accagt 6515-6520	acGCgt	MluI site
V		6518	A	C			
		8577	A	G	ccacgt 8575-8580	ccGcgG	SacII site
VI	VId	8580	T	G			
	VIc		---	---	aggcct 10896 -10901		StuI site
	VIb		---	---	tacgta 11916 -11921		SnabI site
	VIa		---	---	ggtacc 13925 -13930		KpnI site
		After (16182)	GGGTCGGCATGGCATCTCCACCTCCTCGCGGTCCG				HDV-Rz. + RsrII

Table 3: Nucleotides changes involved for restriction enzyme sites deletion:

Deleted RE site	Original seq.	Corrected seq.
SacII	ccgcgg 6386-6391	TcgAgg
RsrII	cggaccg 1291-1297	cCgaTcg
RsrII	cggaccg 2682-2688	TggCccg

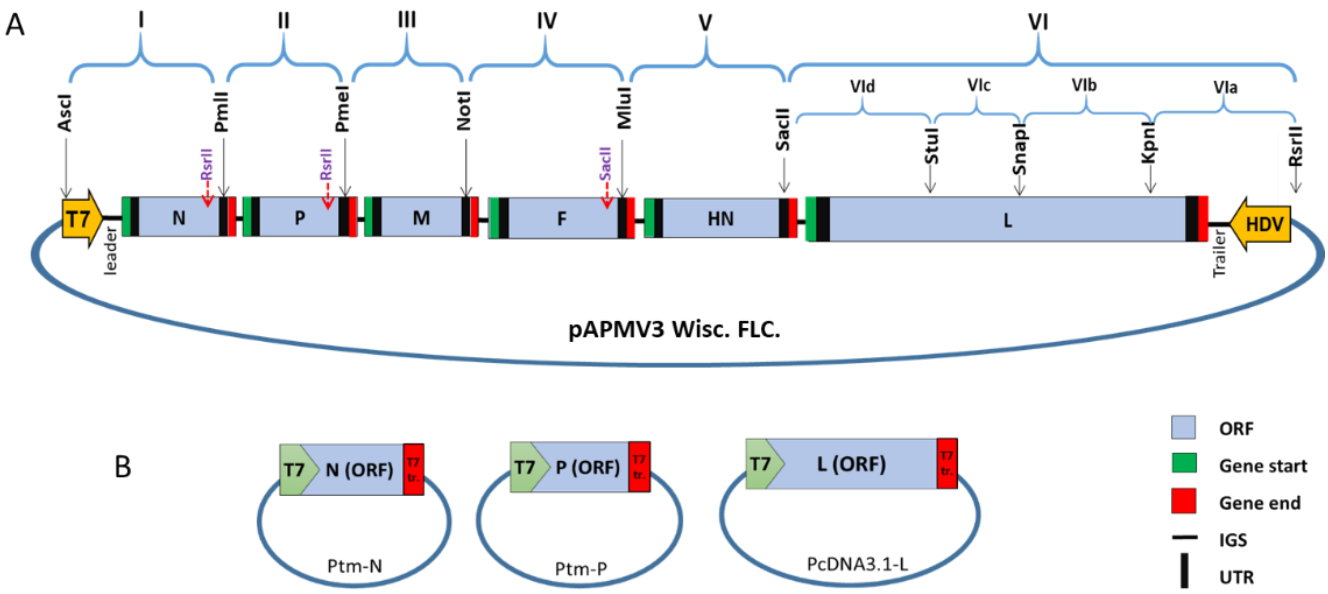


Figure 6. Construction of avian paramyxovirus 3 strain Wisconsin full-length clone (pAPMV-3 Wisc.) and support plasmids (A) The full-length cDNA clone was constructed by assembling six sub-genomic fragments into pBR 322/dr using a 116 nucleotide long oligonucleotide linker to form an antigenomic full-length cDNA clone (pAPMV-3 Wisc.). (B) Three support plasmids were constructed by individually cloning the N, P, and L genes into T7 polymerase expression plasmids (pTM for the N and P genes and pCDNA 3.1 for the L gene).

Recovery of Recombinant APMV-3 Wisc.

Recovery of infectious recombinant APMV-3 Wisc. (rAPMV-3 Wisc.) was carried out using the constructed plasmids following our established protocol (Krishnamurthy et al., 2000). Briefly, HEp2 cells were co-transfected with the plasmids pAPMV3 Wisc. FLC (5 µg), pTM-N (3 µg), pTM-P (2 µg) and PcDNA3.1-L (1 µg) using 15µL of Lipofectamine 2000 transfection reagent (Invitrogen). The plasmid mixture was mixed in 1 mL reduced-serum medium Gibco Opti-MEM™ containing 1 focus-forming unit per cell of modified vaccinia virus expressing T7 RNA polymerase (MVA-T7). The whole plasmid mixture in Opti-MEM medium was used to transfect HEp2 cells in a six-well plate for six hours. After transfection, the cells were washed twice and incubated in DMEM containing 10% allantoic fluid and 2% FBS. Three days later, the

whole cell culture was frozen until being injected into 10-day-old SPF ECEs for virus recovery. Eggs allantoic fluid was collected three days post-injection and the virus recovery was determined by HA assay. Positive samples were further propagated in 10-day-old SPF ECE and the genome of the recovered virus was sequenced in its entirety. Presence of the newly introduced and deleted RE sites from the viral genome were used as genetic markers to confirm the recovery of the rAPMV3-Wisc. virus.

Construction and Recovery of Recombinant APMV-3 Wisc. Expressing Green Fluorescent Protein (GFP)

The cDNA of enhanced GFP gene was inserted at the PmeI site in the P-M gene junction of the pAPMV-3 Wisc. FLC. The GFP ORF was flanked by the M gene-start and P gene-end sequences of APMV-3 Wisc. A Kozak sequence was inserted before the GFP ORF for enhanced translation. The length of the inserted gene cassette (822 nucleotides) was adjusted to a multiple of six by adding four nucleotides after the GFP ORF following the rule of six (Calain and Roux, 1993, Kolakofsky et al., 1998) (Fig. 7A). Recombinant APMV-3 Wisc. expressing GFP (rAPMV-3 Wisc.\GFP) was recovered using the same procedure mentioned above. GFP expression upon the recovered virus infection was observed in DF-1 cells (Fig. 7B). In order to ensure consistent and efficient expression of GFP by the recombinant virus, the recovered virus was plaque purified twice in DF-1 cells and passed in eggs for eight serial passages before being tested again for the presence of GFP gene by RT-PCR and GFP expression in DF-1 cells. APMV-3 Wisc. did not produce visible plaques in DF-1 cells under methyl cellulose overlay like APMV-3 Neth. Hence, rAPMV-3 Wisc.\GFP was purified by infecting DF-1 cells at high dilutions and covering it with 0.8% methyl cellulose overlay medium containing 10% allantoic

fluid and 2% FBS. Two days post-infection, single fluorescent foci of rAPMV-3 Wisc.\GFP were picked and propagated in 10-day-old SPF ECE.

Measuring Multicycle Growth Kinetics of the Constructed Recombinant Viruses and Wild-Type APMV-3 Wisc.

Multicycle growth kinetics of wild type APMV-3 Wisc., rAPMV-3 Wisc. and rAPMV-3 Wisc.\GFP were determined in DF-1 cells. Eighty percent confluent DF-1 cells in six well plate were infected with a multiplicity of infection (MOI) of 0.01 of each virus, then 200µl cell supernatants were collected at 12-hour intervals for three days. The viruses used for infection and the collected cell supernatants were titrated in DF-1 cells by immunostaining to count the virus fluorescent foci (Khattar et al., 2011). The focal fluorescent unit count (FFU/ml) was obtained by infecting DF-1 cells in 24-well plates with 10-fold serially diluted virus and covering it with 0.8% methylcellulose overlay medium containing 10% allantoic fluid and 2% FBS. Two wells were infected for every dilution and the average count was calculated. Two days post-infection, the overlay was removed and the cells were fixed and permeabilized by methanol for 30 minutes. Fixed cells were washed twice by phosphate-buffered saline (PBS), 5 minutes each, followed by blocking using 3% goat serum for 30 minutes. Immunostaining was done using rabbit anti-APMV-3 N protein primary antibody for two hours. The cells were then washed four times by PBS and incubated for 1 hour with Alexa flour-labelled goat anti-rabbit secondary antibody. Cells were then washed three times in PBS, the virus fluorescent foci were counted under a fluorescent microscope and the virus titer was calculated as FFU/ml. Titration of virus aliquots for growth kinetics experiments of rAPMV-3 Wisc.\GFP, rAPMV-3 Neth.\GFP and rLasota\GFP was done by counting virus foci expressing GFP without immunostaining (fig. 8A).

In Vitro Analysis of GFP Expression by the Recombinant APMV Vectors

GFP expression by the three recombinant viruses was measured in DF-1 cells using Western blot. DF-1 cells in a twelve-well plates were infected by 0.5 MOI of each virus. Cell lysate from each well was collected at 24 and 48 hours post-infection using 120 µL Radio immunoprecipitation assay lysis buffer (RIPA). The collected lysates were kept on ice for 15 mins then centrifuged at 15,000× g for 15 mins. The lysate supernatants were separated and mixed with 6x protein loading dye, boiled for 10 mins and subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). GFP amount was measured by Western blot using polyclonal rabbit anti-GFP and normalized against the cellular protein β-tubulin, measured by mouse anti β-tubulin (from Santa Cruz Biotechnology, Inc.) as a loading control.

Evaluation of rAPMV-3 Wisc.\GFP, rAPMV-3 Neth.\GFP and rLasota\GFP as Vaccine Vectors in Chickens

Four groups of day-old SPF chickens (nine chickens per group) were housed in negative pressure isolators in biosafety level 2 animal facility. Feed and water were provided *ad libitum*. Three groups of one-day-old chickens were intraocular vaccinated by 10^6 FFU of each virus vector per bird using fresh allantoic fluid. The remaining group was mock infected by PBS as a negative control. Back titration of the viruses used in vaccination showed the actual dose of (1–3)*10^5 FFU of each virus per bird. Three days post-infection, three chickens were euthanized from each group and different organs (Brain, Lung, trachea and spleen) were collected for virus detection and titration. Half of the brain, both lungs and trachea of each chicken were separately homogenized in 1.5 mL DMEM containing 5x antibiotic (Pen Strep) while the spleen was homogenized in 1 mL DMEM. The organ homogenates were clarified and the supernatants were

titrated for the vaccinating viruses in DF-1 cells. Duplicate wells in 24-well plates were infected by 250 µL of (2^{-1}, 10^{-1} ,10^{-2}) dilutions of organ homogenates and virus titers were calculated as focal fluorescent unit per organ. The remaining chickens were observed daily for any clinical signs of illness for 10 days and weighed two weeks post-infection. Two weeks post immunization, serum samples were collected from the six chickens in each group to evaluate the induced antibody immune response against the used APMV vector and the expressed foreign protein (GFP) (fig. 9A). Animal experiments were done following guidelines and after approval of the Animal Care and Use Committee (IACUC) and Institutional Biosecurity Committee (IBC), University of Maryland.

Detection of The Induced Antibody Response in Chickens

Collected serum samples were used to measure the induced humoral immune response against the used virus vector by haemagglutination inhibition (HI) assay and against the expressed GFP by a homemade ELISA. HI assay was performed using 4 haemagglutinating (HA) units of the homologous APMV vector following the standard protocol (OIE, 2021). Our homemade ELISA for detection of sera anti-GFP titer was prepared using purified GFP™ obtained from Applied Biological Materials (abmCat.No.:000033P). The purified protein was dissolved in 10mM PBS to make a final GFP concentration of 1µg/µl. High binding ELISA plate was coated with 100ul of GFP solution (10ug/ml PBS) overnight at 4C, then it was covered directly by 200ul of the blocking buffer (PBS with 2% BSA and 10% sucrose) overnight at 4C. Serum samples were diluted 1:100 in sample dilution buffer and incubated for 30 min at room temperature then the plate was washed with washing buffer (PBS with 0.05% tween-20) for seven times. Goat anti-chicken HRP-conjugated antibodies were diluted 1:100 in sample diluent buffer and 100µl were

added and incubated for 30 min at the room temperature, the plate was washed for six times with water then 100µl of TMB (3,3',5,5'-Tetramethylbenzidine) was added and incubated for 5 min. Optical density was measured at 630nm in a (plate reader model).

Experiments and Results

Recovery of rAPMV-3 Wisc. by a Reverse Genetics System

Antigenomic cDNA fragments of APMV3-Wisc. were synthesized by RT-PCR from genomic RNA and cloned into the pBR322/dr using the designed polylinker. The cDNA fragments were tested for the presence and the absence of the introduced and deleted RE sites, respectively, (Table.2), and sequenced. The sequences were compared with the wild-type APMV-3 Wisc. sequence (GenBank accession number, EU782025) to ensure the absence of unintended mutations. Three G residues were included at the 5' end of T7 promotor to improve the transcription efficiency. APMV-3 Wisc. L-gene was cloned into three different expression plasmids: pTM, pCDNA3.1 and pGEM7zf, however none of them was able to recover the virus. Hence, APMV-3 Neth. pCDNA3.1-L was used instead, with APMV-3 Wisc. pTM-N and pTM-P support plasmids to recover the rAPMV-3 Wisc. in HEp-2 cells. The recovered virus was grown in ECE (virus recovery was confirmed by ECE Allantoic fluid HA activity). The recovered virus was serially passed three times in 10-day-old SPF ECE to remove the vaccinia virus. The virus genomic RNA was isolated from HA-positive allantoic fluid and confirmed to have the introduced genetic markers of the rAPMV-3 Wisc. construct by complete genome sequencing.

Construction and Recovery of rAPMV-3 Wisconsin Expressing GFP

Enhanced GFP gene was cloned at the PmeI site in the P-M gene junction of rAPMV-3 Wisc. The inserted GFP cassette was confirmed by sequencing. The rAPMV-3 Wisc.\GFP was recovered using the same protocol as for rAPMV-3 Wisc. and was used to infect DF-1 cells. GFP expression was visualized under a fluorescent microscope (Fig. 7B). The virus was passaged twice in SPF ECE, plaque purified in DF-1 cells and passed for eight serial passages in SPF ECE to evaluate the stability of GFP expression. GFP gene stability was confirmed by RT-PCR and GFP expression in DF-1 cells until the last passage (data not shown). Multicycle growth kinetics of wild-type APMV-3 Wisc., rAPMV-3 Wisc. and rAPMV-3 Wisc.\GFP in DF-1 cells are shown in (Fig. 7C). Recombinant APMV-3 Wisc. has shown compromised growth rate especially after adding GFP. Two days post infection, rAPMV-3 Wisc.\GFP showed an average of one log10 lower titer than that of rAPMV-3 Wisc. and about two log10 less titer than that of the wild-type APMV-3 Wisc. By the end of the third day, rAPMV-3 Wisc.\GFP and rAPMV-3 Wisc. reached similar titers to the w.t. virus.

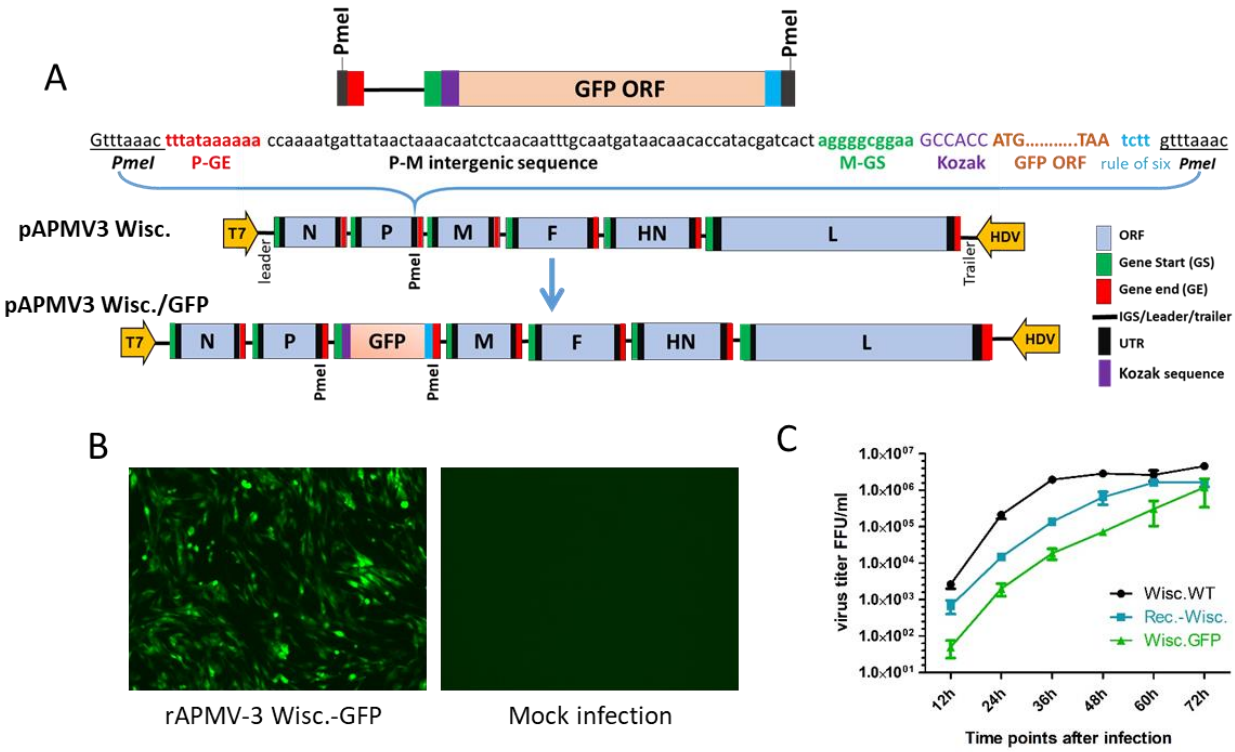


Figure 7. Construction of recombinant APMV-3 Wisc. expressing green fluorescent protein (GFP). (A) GFP gene cassette was constructed by including P gene-end, P-M intergenic sequence, M gene-start, and Kozak sequence for enhanced translation. The GFP cassette was cloned between P and M genes at the PmeI site. The full length cDNA clone was termed pAPMV3 Wisc.\GFP. (B) GFP expression by recombinant APMV-3 Wisc.\GFP (rAPMV-3 Wisc.\GFP) in DF-1 cells two days post-infection. (C) Multicycle growth kinetics of wild type APMV-3 Wisc., rAPMV-3 Wisc., and rAPMV-3 Wisc.\GFP in DF-1 cells using 0.01 multiplicity of infection for each virus. The virus titers were calculated as a mean of two different growth kinetics experiments in FFU/mL.

Multicycle Growth Kinetics and GFP Expression of rAPMV-3 Wisc.\GFP, rAPMV-3 Neth.\GFP and rLasota\GFP in Vitro

Viral fluorescent foci phenotype and multicycle growth kinetics of the three APMV vectors expressing GFP showed that rAPMV-3 Neth.\GFP had the highest growth rate with about 10 and 100 fold higher virus titer than that of rLasota\GFP and rAPMV-3 Wisc.\GFP 36 h post-infection, respectively (Fig. 8A-8B). GFP expression in the DF-1 infected cells was compared among the three virus vectors at 24 and 48 hours post-infection using western blot (Fig. 8C). One-day after infection, rAPMV-3 Wisc.\GFP showed the lowest expression of GFP

followed by rLasota\GFP (about two times that of rAPMV-3 Wisc.\GFP), while rAPMV-3 Neth.\GFP showed the highest expression (about three times that of rAPMV-3 Wisc.\GFP). Two days post-infection, rAPMV-3 Wisc.\GFP and rLaSota\GFP showed similar levels of GFP expression, while rAPMV-3 Neth.\GFP remained to be the highest GFP-expressing vector (1.5X that of rAPMV-3 Wisc.\GFP). Cellular β-tubulin was used to normalize the amount of the expressed GFP to the amount of the loaded cell lysate per well.

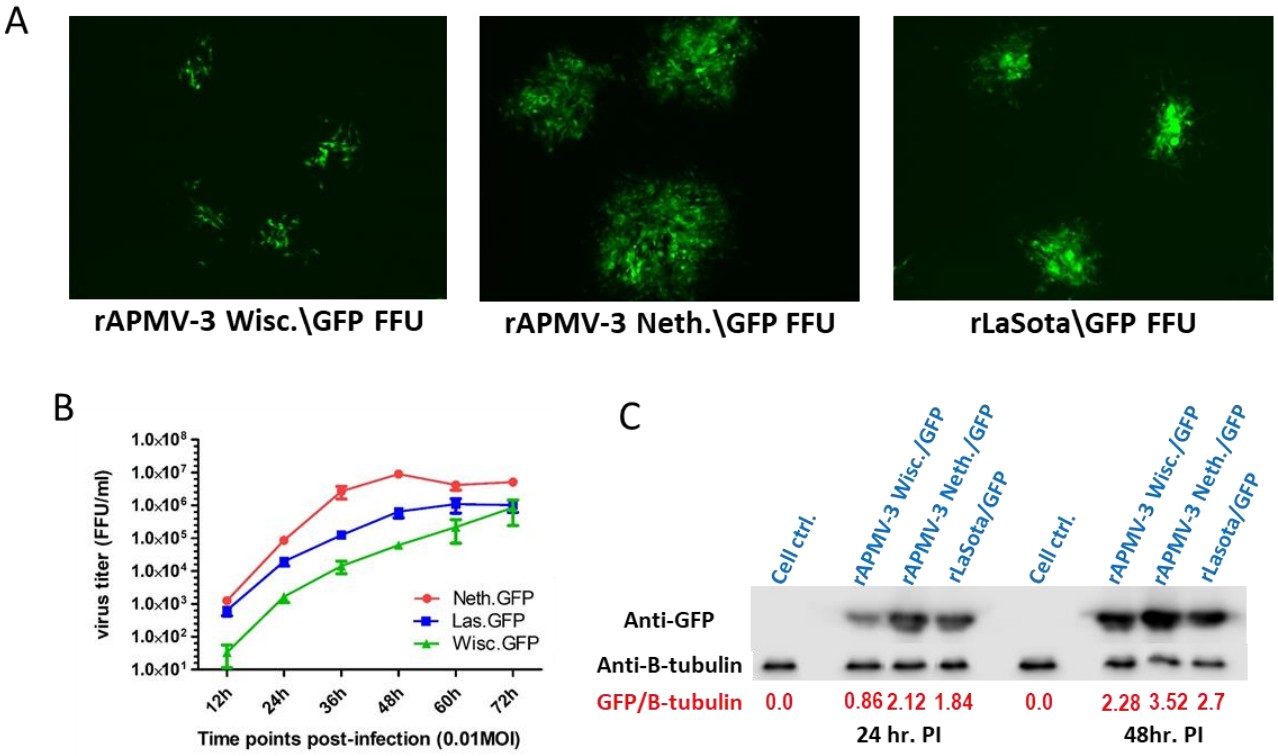
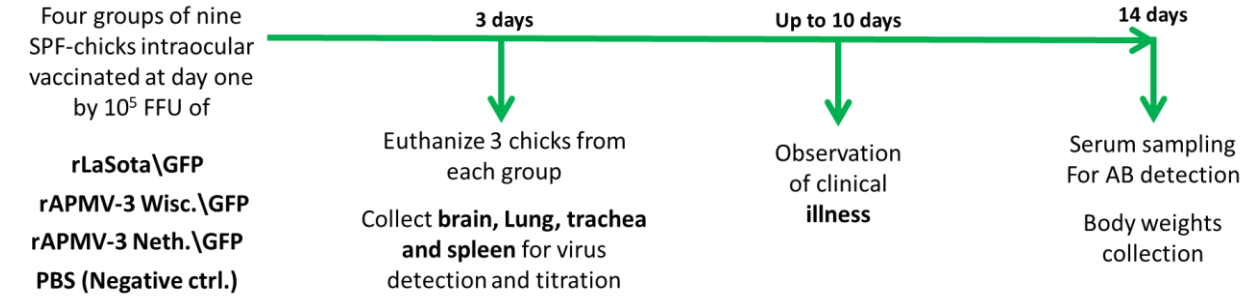


Figure 8. Characterization of rLaSota\GFP, rAPMV-3 Wisc.\GFP and rAPMV-3 Neth.\GFP in DF-1 cells. (A) Fluorescent microscopy of fluorescent foci of the three recombinant vectors expressing GFP in DF-1 cells two days-post-infection. (B) Multicycle growth kinetics of the three viruses in DF-1 cells at 12 hour intervals using 0.01 MOI of each virus. (C) Western blot for GFP expressed by the three viral vectors using 0.5 MOI of each virus in DF-1 cells. Cell lysate was collected 24 and 48 hours post-infection and analyzed by Western blot using polyclonal rabbit anti-GFP antibodies and monoclonal mouse B-tubulin antibodies.

Growth Characteristics, Safety and Immunogenicity of rAPMV-3 Wisc.\GFP, rAPMV-3 Neth.\GFP and rLasota\GFP in Chickens

One-day-old SPF chickens in groups of nine were inoculated by (1-3)*10^5 FFU of each virus per bird (by the ocular route). Brain, lung, trachea and spleen were collected separately from three birds of each group, three days post-infection, homogenized and titrated for the corresponding viruses. rLaSota\GFP showed the highest mean virus titer in the trachea (10^4–10^5 FFU/organ) with no evidence of the virus in the brain or spleen (Fig. 9C). rAPMV-3 Neth.\GFP showed high virus titers in all examined organs: trachea, brain, spleen and lungs (with mean virus titer of 10^3–10^4 FFU/organ). rAPMV-3 Wisc.\GFP was detected only in the trachea with a titer of (10^2 FFU/organ) (Fig. 9C). The remaining chickens in each group were observed daily for signs of illness for 10 days and body weight was cheched for two weeks post-infection. No clinical signs were observed in all groups except for chickens infected with the rAPMV-3 Neth.\GFP. They showed early decrease in food consumption, stunted growth and abnormal feathering after vaccination (Fig. 9B). Their average body weight was 20% lower than that of the control group. On the other hand, both rLaSota\GFP and rAPMV-3 Wisc.\GFP groups did not show a significant change in body weight compared to the control group (Fig. 9D). The serum samples were collected 14 days post infection and analyzed for the antibodies against the corresponding viruses used for vaccination by HI assay and for the antibodies against GFP by an ELISA. All chicks in the three groups were seroconverted when compared to the control group. The mean HI titer of the chicks infected with rLaSota\GFP, rAPMV-3 Wisc.\GFP, and rAPMV-3 Neth.\GFP were $2^{3.5}$, 2^6, 2^7 respectively (Fig. 9E). Our homemade ELISA detected only anti-GFP antibodies in chicks vaccinated by rAPMV-3 Neth.\GFP vector, but not in other groups (Fig. 9F).

a



b

rLaSota\GFP

rAPMV-3 Wisc.\GFP

rAPMV-3 Neth.\GFP

Mock infected (-ve ctrl.)

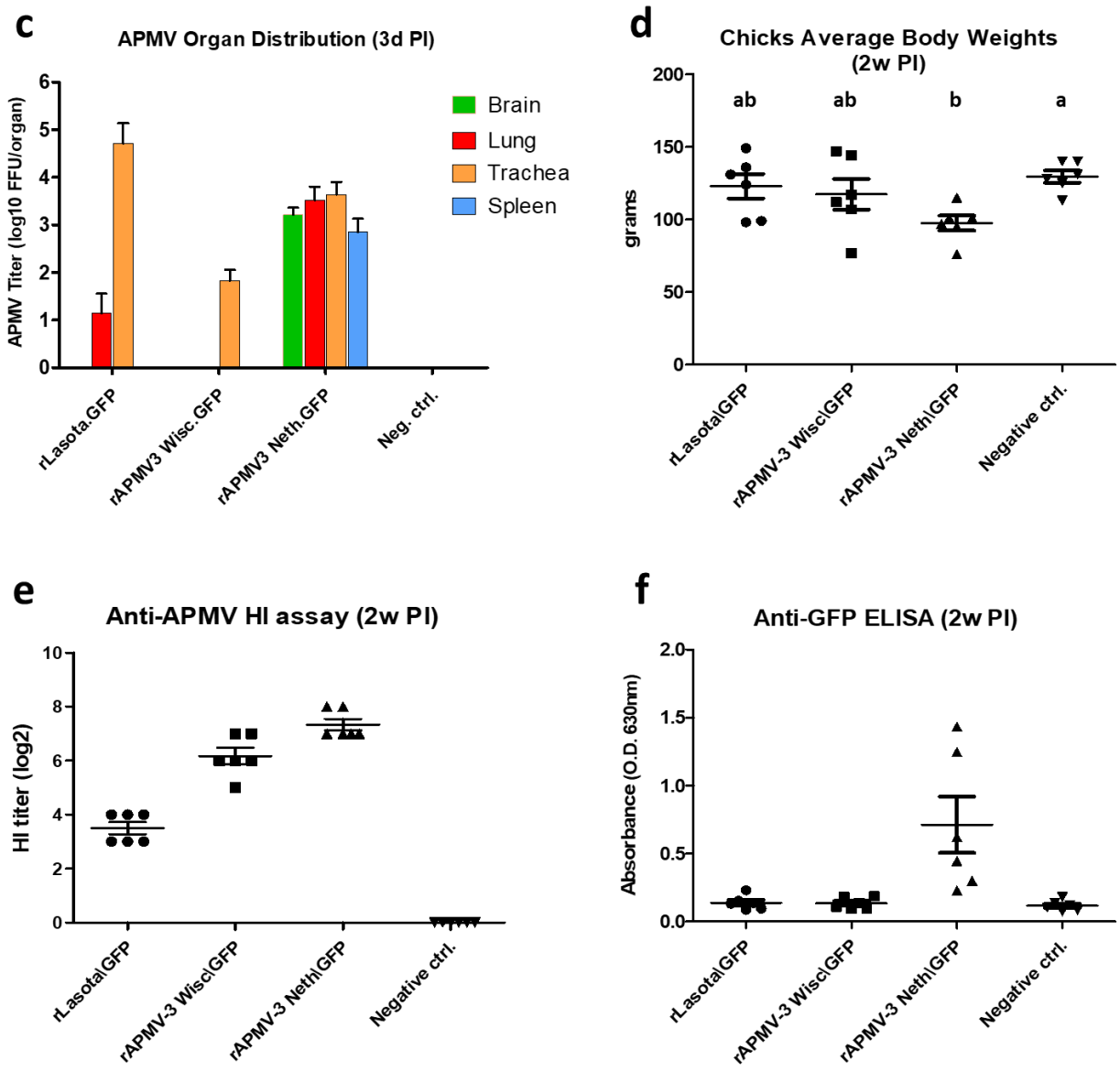


Figure 9: In vivo evaluation of rAPMV-3 Wisc.\GFP, rAPMV-3 Neth.\GFP and rLasota\GFP as Vaccine Vectors in Chickens (A) Schematic diagram of the in-vivo experiment for APMV vectors evaluation in SPF chicks. (B) Pathological changes observed in vaccinated chicks (two weeks post immunization): Chicks vaccinated by rAPMV-3 Neth.\GFP are showing ruffled-abnormal feathering and stunted growth. All other groups were similar to mock (PBS) vaccinated group with no signs of illness. (C) Three birds of each immunized group were sacrificed 3 days P.I. and the brain, lung, trachea, and spleen of each individual bird was titrated for the used APMV vector in DF-1 cells using the FFU count. The plotted virus titers are expressed as mean FFU per organ. (D) Average body weight of chicken groups in (gm) two weeks post vaccination. (E) Serum samples of the immunized chickens were analyzed two weeks P.I. for HI activity against 4 HAU of the vaccinating virus. Results represent the mean log2 HI titers of six chicks from each immunized group. (the error bar represents SEM). (F) Serum samples of immunized groups were analyzed by ELISA for anti-GFP antibodies using ELISA plates coated by purified GFP, absorbance was recorded at 630nm wave length. The graph shows mean optical denisty values of each group with SEM.

Discussion

Avian paramyxoviruses have been used as vaccine vectors against different avian and human pathogens for more than two decades (Kim and Samal, 2016; Krishnamurthy et al., 2000). Although APMV-1 is the most broadly used virus vector for poultry vaccination, there is always a concern regarding potential neutralization by anti-NDV MDA circulating in young commercial chicks (Bertran et al., 2018). To overcome that obstacle, chimeric NDV constructs (Steglich et al., 2013; Kim et al., 2017) or antigenically different APMVs vectors are used (Xiao et al., 2012; Shirvani et al., 2020b).

Among other APMVs, APMV-3 has shown promising results as virus vector for poultry vaccination. It induces higher immune response in chickens than lentogenic strains of APMV-1 and propagates systemically in different organs (Kumar, 2010; Shirvani et al., 2020b; Varghese, 2021). Up to date, there are two strains of APMV-3 that were fully characterized: APMV-3 strain Wisconsin and APMV-3 strain Netherlands. Although both APMV-3 strains were considered nonpathogenic (Kumar et al., 2010a), APMV-3 Neth. was reported to show mild pathogenicity in newly-hatched chicks and to kill the embryos during propagation in ECE. This safety concern has prevented its authorization as a poultry vaccine vector. APMV-3 Wisc., on the other hand, was completely safe in young chicks and did not kill embryos during ECE propagation (Alexander and Collins, 1982; Kumar et al., 2010a). For those reasons, APMV-3 strain Wisconsin was expected to offer a safer vector for young chick vaccination.

In our study, we created a plasmid-based reverse genetics system for APMV-3 Wisc. and evaluated it as a virus vector for day-old chicks vaccination. For APMV-3 Wisc. recovery, we used the polymerase L gene of APMV-3 Neth. since the cognate L gene failed to recover the

APMV-3 Wisc. That can be explained by a more robust replication of the APMV-3 Neth. so that its L gene would be more efficient in APMV-3 Wisc. recovery. We also noticed that APMV-3 Wisc. can be recovered more efficiently using all three support plasmids (N, P, and L) of APMV-3 Neth. rather than a mix of the APMV-3 Wisc. N and P plasmids with APMV-3 Neth. L plasmid. This finding suggests the lower polymerase activity of APMV-3 Wisc. L protein compared to that of APMV-3 Neth. and demonstrates the vital role played by the L protein in APMV recovery. It also indicates that the polymerase complex proteins (N, P and L) of APMV-3 Neth. are capable of recognizing the cis-acting regulatory sequences (leader and trailer) of APMV-3 Wisc. and support its replication.

Similar findings where the polymerase complex proteins of one virus can support replication of a closely related virus belonging to the same genus were shown previously (De Graaf et al., 2008; Yunus et al., 1999). It should be noted that the polymerase protein genes of APMV-1 (LaSota strain) did not support the replication of APMV-3 strain Wisconsin (our unpublished results). The development of a reverse genetics system for paramyxoviruses is challenging and some APMVs are non-recoverable (Samal, 2021). Our study confirms this observation, as APMV-3 Wisc. was non-recoverable using its own L gene in the support plasmids. Hence, the low activity of the polymerase protein of some APMVs may be a reason for their unsuccessful recovery.

Multicycle growth kinetics showed one log10 lower virus titer of the recovered APMV-3 Wisc. than its parental wild-type virus. Similar growth retardation in other recombinant APMVs compared to their parental viruses was observed previously (Xiao et al., 2012). In our

recombinant virus, the reason for its lower growth rate could be the 18 nucleotide changes introduced in its genomic UTRs (Table 2).

The recovered virus was successfully used as a vector to express GFP as a foreign protein and the stable GFP expression was confirmed for at least 10 consecutive passages in ECE. The growth rate of the rAPMV-3 Wisc.\GFP was about 1 log10 lower than that of the parental recombinant virus and 2 log10 lower than that of the wild-type virus during the first 48 hours post-infection. Although similar growth retardation was observed in other APMV vectors expressing foreign genes (Abozeid et al., 2019; Kim et al., 2014), the significant decrease of rAPMV-3 Wisc./GFP growth rate suggests that the vector is unlikely to effectively express larger trangenes (GFP gene is only 700 nucleotides) without further modifications.

Growth kinetics and GFP expression of rAPMV-3 Wisc.\GFP was compared with rAPMV-3 Neth.\GFP and rLaSota\GFP in DF-1 cells. The rAPMV-3 Neth.\GFP showed the highest growth rate with about 10 and 100-fold higher virus titers than rLasota\GFP and rAPMV-3 Wisc.\GFP, respectively at 36 h post-infection. In agreement with growth kinetics, the GFP expression was the highest in rAPMV-3 Neth.\GFP followed by rLaSota\GFP and rAPMV-3 Wisc.\GFP. This finding is in accordance with difference of the three vectors fluorescent foci sizes at 48 hours post-infection. The remarkable difference observed in the growth patterns of both APMV-3 strains is possibly due to the difference in their polymerase complex activity and F proteins cleavage site sequences .

An *in vivo* study was performed to compare tissue distribution and humoral anti-body response of the three APMV vectors in day old chicks. Our results showed that rAPMV-3 Neth./GFP propagated in different body organs: brain, lung, trachea, and spleen after intraocular

vaccination of day-old chicks, whereas rAPMV-3 Wisc./GFP and rLaSota/GFP were confined only to the respiratory tract by the third day post-infection. The systemic propagation of APMV-3 Neth./GFP came at the expense of mild pathogenicity observed in young chickens shortly after vaccination. APMV-3 Neth./GFP vaccinated group showed decreased feed intake, early retarded growth, and abnormal feathering. Similar observation of stunted growth was previously reported in young chicks experimentally infected by APMV-3 strain Netherlands (Alexander and Collins, 1982). On the other hand, both rAPMV-3 Wisc. and rLaSota vaccinated groups did not show any visible pathogenicity.

Recombinant LaSota virus replication was the highest in the respiratory tract among the three virus strains used in this study. Hence, the rLaSota may be a suitable vaccine vector for protection against poultry respiratory viruses that require strong mucosal immunity, e.g. infectious laryngotrachitis virus. The broad tissue tropism of APMV-3 Neth. could be due to the multi-basic amino acid sequence in its F protein cleavage site. This allows it to be cleaved by the ubiquitous intracellular furin protease available in different tissues, whereas rAPMV-3 Wisc. and rLaSota were restricted to the respiratory tract as their F protein cleavage site has single basic amino acid (Arginine) which makes it cleavable only by extracellular trypsin-like protease restricted to the respiratory tract and intestine (Fujii et al., 1999; Xiao et al., 2012).

As rAPMV-3 Neth. replicated systemically in different organs, it induced three-fold higher HI titer than that induced by rLaSota. Therefore, rAPMV-3 strain Netherlands may be a better vector for protection against pathogens requiring a strong systemic immune response, e.g., avian influenza or infectious bursal disease virus. Recent studies have also shown that APMV-3 strain Netherlands vector induced a higher immune response against the expressed foreign

antigen (Ebola virus glycoprotein, avian influenza HA, IBDV VP2) than that induced by rLaSota vector (Yoshida et al., 2019; Shirvani et al., 2020b; Varghese, 2021). Although all constructs have induced neutralizing antibody response against the vector proteins, only rAPMV-3 Neth.\GFP vector showed detectable antibody response against the expressed foreign antigen (GFP) by ELISA. The capacity of LaSota vectors to induce antibody response against the expressed transgenes was demonstrated in multiple studies (Abozeid et al., 2019; Dey et al., 2017). This suggests that the sensitivity of our ELISA used for detection anti-GFP antibodies was low and calls for an evaluation of the protective efficacy of antigens expressed from APMV-3 Wisc. in a real vaccination challenge study.

Chapter 4: Application of a novel technique for expression of Avian influenza (H5N1) hemagglutinin (HA) from Avian Paramyxovirus vector

Abstract

Newcastle disease virus (NDV) is extensively explored as a vector for vaccine and oncolytic therapeutic development. In conventional NDV-based vectors, the transgene is arranged as a separate transcription unit in the NDV genome. Here, we expressed hemagglutinin protein (HA) of an avian influenza virus using an NDV vector design where the transgene ORF is encoded in-frame with the ORF of an NDV gene. This arrangement does not increase the number of transcription units in the NDV genome, and imposes a selection pressure against mutations interrupting the transgene ORF. We placed the HA ORF upstream or downstream of N, M, F and HN ORFs of NDV so that both proteins are encoded in-frame and are separated by either a self-cleaving 2A peptide, furin cleavage site, or both. Only constructs where HA was placed downstream of the NDV HN were recoverable. These constructs expressed the transgene at a higher level than the NDV vector expressing the same transgene from the same genomic position as a separate transcription unit. More importantly, the HA gene expressed in fusion with the NDV vector protein appeared to be more stable over multiple passages. Thus, this design may be useful for applications where the stability of the transgene expression is highly needed in the recombinant NDV vector.

Introduction

Newcastle Disease virus (NDV) is a single-strand negative sense RNA virus infecting poultry and other avian species (OIE, 2021; Kaleta, 1988; Suarez et al., 2020). NDV outbreaks are a major concern for commercial poultry operations and some avirulent (lentogenic) strains of NDV have long been used worldwide as live vaccines (Thornton et al., 1980).

The NDV genome codes for six individual genes N, P, M, F, HN, and L (from the 3' end of the negative strand RNA genome). Each gene encodes one open reading frame (ORF) flanked by transcription start and stop signals, and individual genes are separated by short intergenic sequences (Sato et al., 1987). N (nucleocapsid) protein forms a complex with the viral RNA, P (phosphoprotein) is essential for the viral RNA-dependent RNA polymerase (RDRP) activity, M (matrix) is a structural protein forming the inner layer of the virion (Battisti et al., 2012), F (fusion) and HN (hemagglutinin-neuraminidase) are glycoproteins exposed on the surface of the NDV virion that mediate its attachment to and penetration of the cellular membrane (Morrison et al., 1991, Heminway et al., 1994), and L (large protein) is the enzymatic subunit of the RDRP. The fully functional RDRP activity also requires N and P proteins (Horikami et al., 1992). Due to a phenomenon of RNA editing, when the polymerase co-transcriptionally inserts additional nucleotides (Vidal et al., 1990), the P gene in addition to the mRNA coding for P protein is transcribed into two other mRNAs coding for proteins V and W, the antagonists of the anti-viral response and the major determinants of NDV species restriction (Poole et al., 2002, Andrejeva et al., 2004, Park et al., 2003, Yang et al., 2021). The viral polymerase transcribes NDV genes starting from the 3' end of the genome RNA so that it starts and stops the transcription of each gene individually. Since the re-initiation of transcription is not absolutely efficient, this strategy

results in the transcriptional gradient, so that the genes located closer to the 3’ end of the negative-strand RNA genome generate more mRNAs and therefore express a higher level of the corresponding proteins than those located closer to the 5’ end (Samal, 2019, Wignall-Fleming et al., 2019, Collins and Wertz, 1983, Iverson and Rose, 1981).

The modular genome organization of NDV makes it relatively easy to insert additional transcription units arranged with NDV-derived transcription start and stop signals. Moreover, the flexible pleomorphic NDV virions do not impose a strict limit on the length of the RNA that can be packaged. These features and the long experience with the non-pathogenic NDV strains as vaccines made NDV a promising vector platform. Multiple vaccine candidates for animal and human diseases have been developed using NDV as a vector for the expression of protective antigens (DiNapoli et al., 2007a, DiNapoli et al., 2007b, DiNapoli et al., 2010a, DiNapoli et al., 2010b, Freeman et al., 2006, Bukreyev et al., 2005). In addition, NDV has been actively explored as an oncolytic agent and demonstrated safety and efficacy in numerous clinical trials (reviewed in (Hu et al., 2020, Bello et al., 2020, DiNapoli et al., 2007a, Kim and Samal, 2016, Huang et al., 2020, Burman et al., 2020)).

While the insertion of an additional gene in the NDV genome is relatively straightforward, such a strategy is not without its setbacks. Due to the gradient nature of NDV transcription, the extra transcription unit inevitably results in a decrease of the expression of viral genes located further downstream in the genome. Moreover, there is no selection pressure for the virus to keep the insert, on the contrary, the deletion of the extra sequence may provide a replication advantage to the mutants. While NDV proved to be a rather stable platform for the expression of different transgenes (Viktorova et al., 2018, Yu et al., 2020, Manoharan et al.,

2018a), it is an RNA virus with a relatively low fidelity RDRP (Barr and Fearns, 2010), and the insert stability is a major concern for all practical applications of NDV vectors.

The goal of this study was to investigate the possibility of expressing avian influenza virus (AIV) hemagglutinin (HA) protein in-frame (fused) with a NDV vector protein using a self-cleaving 2A peptide sequence and/or a furin protease cleavage site for co- and/or post-translational separation of the connected proteins. The expression of the transgene in fusion with a cognate NDV protein (as single ORF) is expected to increase the transgene expression level as the number of transcription units in the NDV genome remains the same. Moreover, such an arrangement should theoretically safeguard against nonsense mutations in the transgene ORF including the insertions and deletions that result in the ORF interruption. If the extra ORF precedes the NDV ORF, the nonsense mutation would directly block the translation of the NDV sequence, while if such ORF is placed downstream of the NDV ORF, the nonsense mutation would result in an mRNA with an extended 3' non-coding region subject to the cellular nonsense-mediated decay mechanisms (Lejeune, 2022).

Previously, NDV vectors where the ORF of eGFP was placed in-frame with the N, M or L proteins ORFs were shown to be viable (Wen et al., 2015). However, we could recover viable viruses that tolerated the AIV HA inserts only downstream of the HN gene of NDV, i.e. before the last gene (L) in the genome. Accordingly, these constructs expressed HA to a significantly lower level than NDV vectors designed previously where the HA gene is placed closer to the 3' end of the genome (Shirvani et al., 2020a), so their use as vaccine candidates against AIV is not currently practical. We also found that the expression of AIV HA may interfere with the incorporation of NDV HN protein into the virions, likely explaining the decreased infectivity of

the recombinant constructs. Nevertheless, the transgene expression by these constructs was considerably higher than by a control vector where the same transgene was placed between HN and L as a separate transcription unit, and multiple passage experiments confirmed higher stability of the vectors expressing AIV HA in fusion with NDV HN. Thus, our results demonstrate the advantages of the NDV vectors expressing foreign proteins in-frame with NDV HN protein, the limitations imposed by a specific insert, and offer additional insights into NDV replication.

Materials and Methods

Cells:

DF-1 and HEp-2 (Chicken embryo fibroblast and human epidermoid carcinoma) cells were obtained from the ATCC (American Type Culture Collection, Manassas, VA, USA). HeLa cells were kindly provided by Dr. Ehrenfeld (NIH). All cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with L-glutamine, sodium pyruvate and 10% fetal bovine serum (FBS). Infected cells were incubated in DMEM supplemented with 2% FBS and 10% allantoic fluid (exogenous protease source) except for the HeLa cells for the analysis of primary infections where allantoic fluid was omitted.

Viruses

All NDV constructs were generated and rescued using a reverse genetic system of a non-pathogenic LaSota strain essentially as described in (Huang et al., 2001). At least three independent attempts of rescue were performed before the constructs were deemed non-viable. Fragments encoding codon-optimized hemagglutinin of AIV H5N1 strain

A/Vietnam/1203/2004AIV HA ORF (Genbank: AAW80717.1) connected to NDV genes were synthesized by Genscript (Piscataway, NJ, USA) or Genwiz (South Plainfield, NJ, USA) and cloned into the full-length backbone plasmid using standard molecular cloning procedures. Cloning details are available upon request. Viruses were propagated in specific pathogen-free embryonated chicken eggs at 10-day age by intra-allantoic injection. P/M-HA NDV construct with AIV HA introduced as a separate transcription unit between P and M genes was essentially as described in (Shirvani et al., 2020a) with the 5' and 3' UTR sequences of NDV LaSota M gene added to the HA insert.

Antibodies

Polyclonal chicken anti-LaSota serum used in immunostaining was prepared by prime-boost vaccination of specific pathogen-free chicks with NDV LaSota strain. Rabbit anti-influenza A H5 serum was obtained from BEI Resources, NIAID, NIH (NR-4487). Anti-NDV N and anti-NDV M protein antibodies were prepared in house by double immunization of rabbits with the gel purified protein bands of NDV BC strain according to the protocol described in (Samuel et al., 2011). Monoclonal mouse anti-NDV HN antibody was obtained from Dr. Ron Iorio (University of Massachusetts Medical School, USA). Polyclonal chicken anti-AIV H5N1 serum was described in (Shirvani et al., 2020b). Horseradish peroxidase (HRP)-conjugated anti-β-actin monoclonal antibody was from Millipore Sigma (St Lois, MO, USA). Goat anti-rabbit and goat anti-chicken Alexa secondary antibody conjugates were from ThermoFisher Scientific (Waltham, MA, USA). HRP-conjugated anti-rabbit, anti-mouse, and anti-chicken secondary antibodies were from Cell Signaling (Danvers, MA, USA), SeraCare (KPL brand) (Gaithersburg, MD, USA) and Zoetis (Synbiotics brand) (Parsippany-Troy Hills, NJ, USA), respectively.

Virus titer, growth kinetics and foci phenotype assays

Virus titers were determined by a fluorescent foci assay as described in (Elbehairy et al., 2021). Briefly, DF-1 cells grown in a 24-well plate at 80-90% confluency were infected with serial virus dilutions and covered by 0.8% methylcellulose overlay with DMEM supplemented with 2% FBS and 10% allantoic fluid. Two days post-infection, the overlay was removed, cells were fixed, permeabilized with methanol and stained with rabbit anti-NDV N polyclonal antibodies. Virus titers were calculated as fluorescent focus forming units (FFU)/ml. For the fluorescent foci phenotype assay, area of at least 60 fluorescent foci for each virus in at least 15 randomly chosen fields of view was measured using Carl Zeiss ZEN software. For multicycle growth kinetics, DF-1 cells were infected with an MOI of 0.01 of each virus and aliquots of cell culture supernatant were collected at 12 hours intervals for three days and the amount of infectious virus was determined. The results from three independent experiments were used for statistical calculations.

Immunostaining

HeLa cells grown on coverslips in 12-well plates at 70-80% confluence were infected and incubated in DMEM with 1% FBS and no allantoic fluid. The cells were fixed 18-24 hours post-infection with 4% formaldehyde for 15 minutes, washed 3X by PBS, permeabilized with 0.2% Triton X-100 for 5 min and stained with rabbit anti-AIV HA and chicken anti-NDV sera. The coverslips were mounted using Fluoromount-G medium (VWR, (Radnor, PA, USA)) for fluorescent microscopy. For HA expression stability assessment, at least 300 single well-separated infected cells in at least six randomly chosen fields of view were analyzed per sample.

Virion purification and analysis

NDV virions were purified from allantoic fluid from the fifth passage for all constructs except for HN/L-HA which was purified from the second passage material because the loss of HA expression was detected for this construct over multiple passages. Allantoic fluid (30 ml) was clarified by centrifugation at 12,000 xg for 30min and the virions were sedimented through 30% (w/v) sucrose cushion in PBS for 2.5h at 140,000 xg at 4°C . The purified virus pellets were resuspended in 400 µl of PBS.

Western blot.

Cells and purified NDV virions were lysed in RIPA buffer supplemented with a protease cocktail inhibitor (Millipore Sigma, (St Lois, MO, USA)). Cellular lysates were sonicated on ice to disrupt the nuclear DNA. Samples containing 40 µg and 10 µg of protein of cellular lysates or purified NDV virions, respectively, were denatured in Laemmli buffer and resolved on a 4-15% gradient polyacrylamide gel (BioRad, (Hercules, CA, USA)). The proteins were transferred on a PVDF membrane and incubated sequentially with the primary antibodies and the corresponding secondary antibodies conjugated to HRP for 1 h each in TBS buffer (50 mM Tris-HCl, pH 7.6; 150 mM NaCl) supplemented with 0.1% Tween 20 (Millipore Sigma, (St Lois, MO, USA)) and 2% Amersham Western blot blocking agent (Cytiva, (Little Chalfont, UK)) with three 5 min washes in TBS after each antibody incubation. The blots were developed with ECL Select luminescent reagent (Cytiva, (Little Chalfont, UK)), and the digital images were recorded with Azure 500 bioimager (Azure Biosystems, (Dublin, CA, USA)).

Data processing and statistical analysis

The data were analyzed using GraphPad Prism statistical package, unpaired (two-sample) t-test analysis was performed to compare pairs of experimental and control conditions, differences with $p<0.05$ were considered significant. Graphs show average values with standard deviation bars. For the comparison of the level of expression of AIV HA by different constructs, the western blot band densities were quantified using Image Studio Lite software (Li-Cor, (Lincoln, NE, USA)). The HA0 and HA1 densities were combined. The graph shows AIV HA expression adjusted to the level of N protein for each construct, normalized relative to the control construct HN/L-HA.

Experiments and Results

Construction and recovery of NDV vectors expressing avian influenza hemagglutinin from the authentic NDV transcription units.

We first investigated if NDV genes can be modified so that both the NDV protein and the AIV HA can be expressed from the same mRNA. Our strategy was to place the AIV HA sequence in-frame with the NDV gene ORFs separated by either a 2A self-cleaving sequence, or a furin cleavage site, or both 2A and furin cleavage sites together (Fig. 10). The 2A sequences are 18-22 amino-acid elements found in polyproteins of some picornaviruses (and some other viruses) that upon translation prevent the formation of a peptide bond between its last two amino-acids with high frequency (Donnelly et al., 2001a, Donnelly et al., 2001b) (Fig 10). For our constructs, we chose the Porcine Teschovirus 2A sequence that was reported to be highly efficient in cells of diverse vertebrate species (Kim et al., 2011). Furins are membrane-associated

proteases residing in the trans-Golgi network and the endosomal system with the protease domain facing the lumen of the membranous compartments which cleave proteins transiting the cellular secretory pathway at the polybasic sequences (Thomas, 2002, Shiryaev et al., 2013). Placement of a furin cleavage site before the 2A should result in the removal of the 2A sequence, leaving only four amino-acids from the furin site attached to the C-terminus of upstream ORF (Fig. 10).

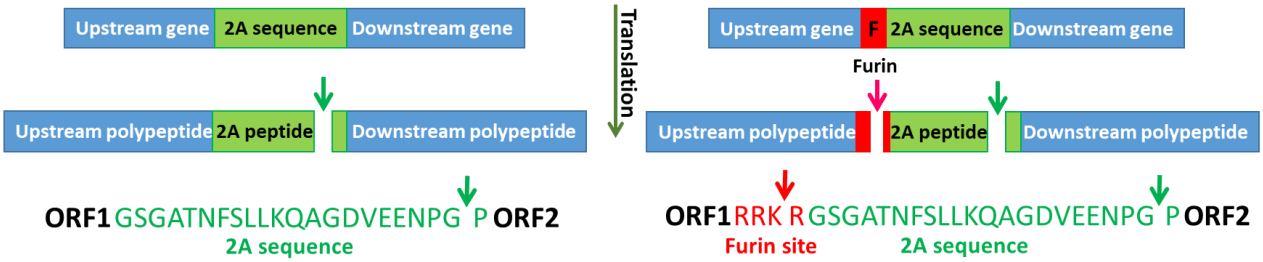


Figure 10: Schematic diagram of porcine teschovirus 2A peptide and furin with 2A peptide post and cotranslational cleavage of the connected polypeptides. Arrows indicate 2A and furin cleavage positions.

Since the introduction of 2A and/or furin cleavage site inevitably modifies the NDV protein sequences, we targeted only genes coding for four structural proteins N, M, F, and HN. We reasoned that since transcription of the P gene must sustain a tightly regulated co-transcriptional mRNA editing, and L protein is the enzymatic subunit of the viral RDRP, their extensive modifications are unlikely to generate efficiently replicating viruses.

For the N and M genes, AIV HA was placed upstream or downstream of the NDV ORFs separated by the 2A sequence (Fig. 11). For the glycoproteins F and HN, AIV HA was placed at the C-termini of the NDV sequences to prevent interference with the N-terminally located signal sequences targeting these NDV glycoproteins to the cellular secretory pathway. In the case of HN, the NDV and AIV sequences were connected by either the 2A sequence alone, or a furin

cleavage site followed by the 2A, or a furin cleavage site alone. For F, only a construct with both a furin cleavage site and a 2A sequence between the NDV and the transgene ORFs was investigated (Fig. 11).

We could rescue the two constructs where NDV HN was connected to AIV HA by either 2A or a furin cleavage site followed by 2A (HN.2A.HA and HN.F2A.HA), but not any other construct, in spite of multiple rescue attempts. As a control for the constructs where AIV HA is expressed from the same RNA as NDV HN, we generated a construct where the AIV HA was introduced as a separate transcription unit between the HN and L genes (HN/L-HA), i.e. in the same relative position in the NDV genome (Fig. 12). This construct was successfully rescued as expected since multiple studies demonstrated that recombinant NDVs carrying an insert in this position are viable (Viktorova et al., 2018, Yu et al., 2020, Manoharan et al., 2018a).

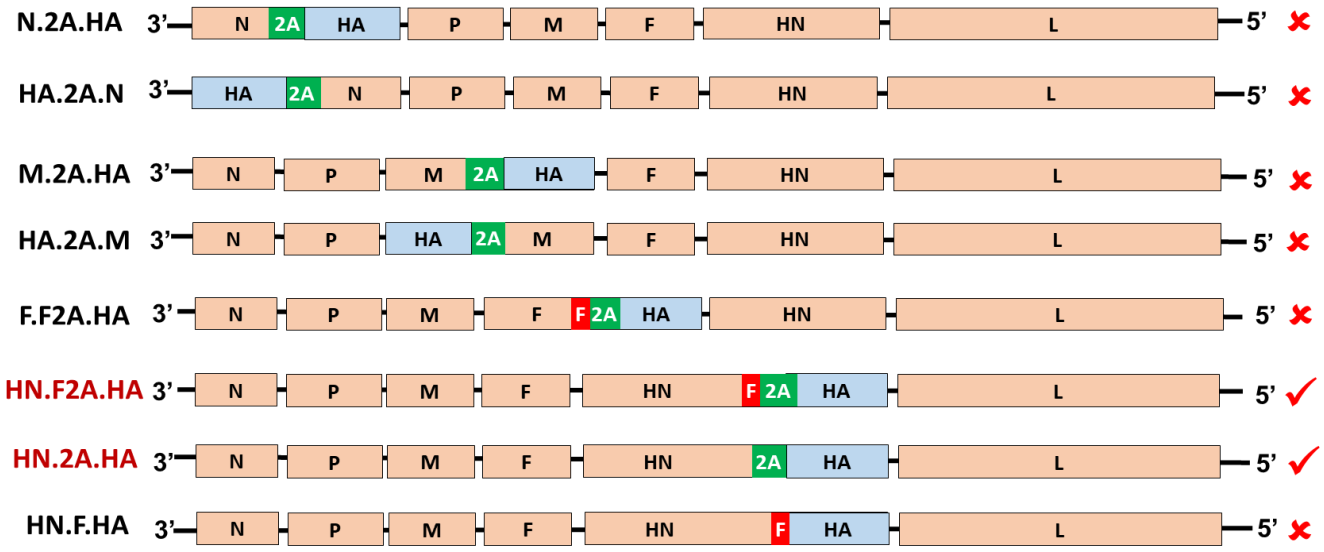


Figure 11. Construction and recovery of recombinant NDVs expressing avian influenza virus hemagglutinin (AIV HA) in-frame with NDV authentic genes. Schemes of recombinant NDV genomes with the AIV HA inserts (blue) constructed. The rescued constructs are indicated with red right mark on the right.

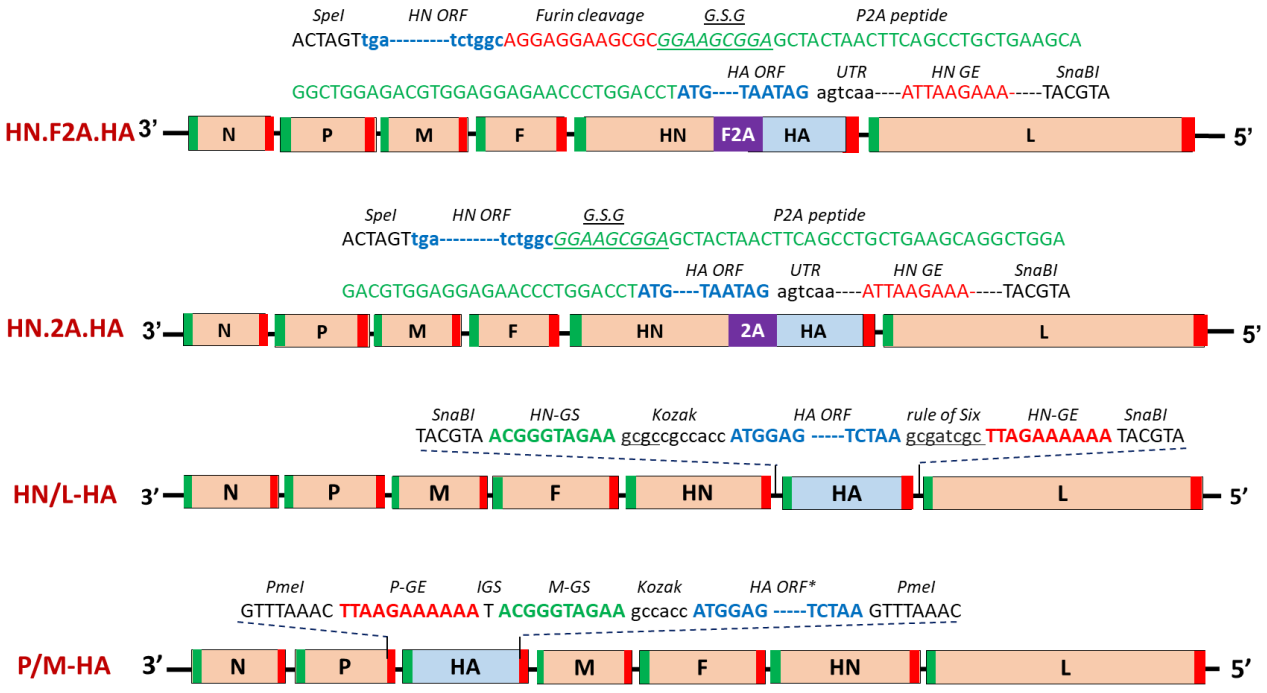
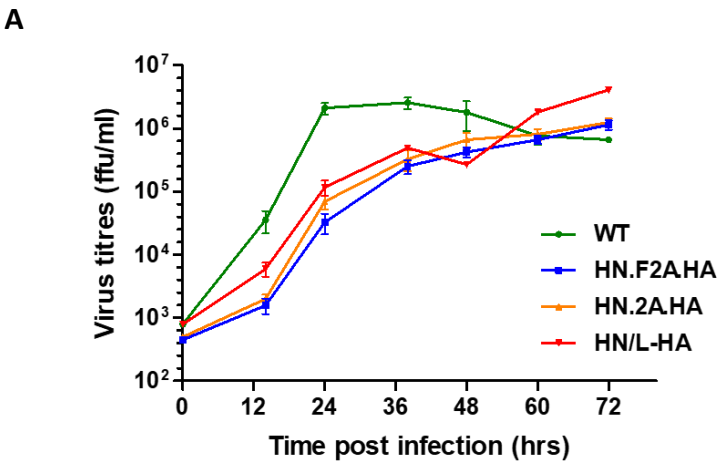


Figure 12: Nucleotide sequences of the cloned gene fragments in positively recovered rLaSota constructs. (*): Highly pathogenic Avian Influenza HA cleavage site was replaced by that of low pathogenic Avian Influenza in the P/M-HA construct and surrounded by 5' and 3' UTR of NDV M-gene.

Characterization of the growth properties of recombinant NDV viruses expressing AIV HA from the HN transcription unit.

We compared the growth kinetics of HN.2A.HA, HN.F2A.HA, HN/L-HA, and the wt virus in a multi-cycle growth experiment in a chicken DF-1 cell line. In this experiment, a small number of cells is infected at the beginning, so the kinetics of the virus yield reflects their replication efficiency. All three recombinant constructs showed similar growth kinetics which was significantly slower than that of the wt virus. The wt virus titer peaked at 36 h p.i., while the recombinant constructs reached similar titers (about 10^6 FFU/ml) by 72 h p.i (Fig. 13A).

We also analyzed the efficacy of the viral spread in a fluorescent foci phenotype assay. As can be seen in Fig. 13B, all of the recombinant constructs generated foci significantly smaller than the wt control. However, the foci of HN.F2A.HA construct were somewhat larger than those of other recombinant constructs. These data show that the insertion of AIV HA significantly compromised the infectivity of the NDV vector, whether the recombinant protein is expressed from a separate transcription unit, or from the HN gene of NDV.



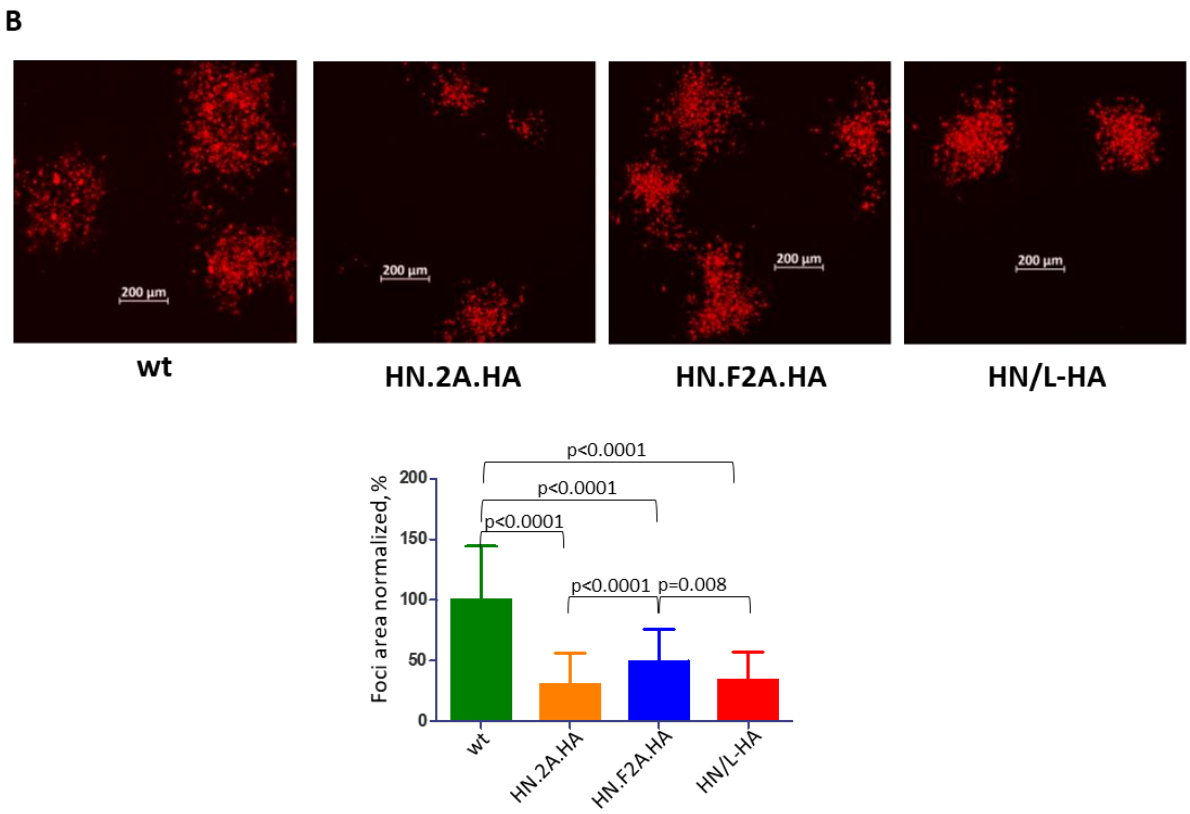


Figure 13. Characterization of recombinant NDV viruses growth properties of. **A**. Multicycle growth kinetics. DF-1 cells were infected at an MOI of 0.01 of each virus and the virus titer in the medium was detected at 12-hour intervals in three independent experiments except for the control HN/L-HA which was repeated twice. **B.** Fluorescent foci phenotypes of recombinant NDVs in DF-1 cells. DF-1 cells were infected by serially diluted constructs and covered by 0.8% methylcellulose overlay. The cells were fixed and immuno fluorescence stained for NDV antigen 48 h p.i.. Individual fluorescent foci areas were measured using Carl Zeiss ZEN software, and the data were normalized to the average foci area of the wt virus. Scale bar is 200µm.

Characterization of the AIV HA transgene expression from the NDV HN transcription unit.

We first analyzed the expression of AIV HA in a western blot. Together with our new recombinant constructs, we also included the P/M-HA virus, a previously developed construct where HA is placed as a separate transcription unit between the P and M genes, a site close to the 3' end of the NDV genome that is usually utilized for a high level of expression of recombinant proteins from NDV vectors (Shirvani et al., 2020a). AIV HA is expressed as a precursor (HA0)

which is cleaved by subtilisin-like proteases into HA1 and HA2 subunits connected by a disulfide bond in the mature HA protein (Bertram et al., 2010). It should be noted that the P/M-HA construct codes for an AIV HA with a mutated HA1/HA2 cleavage site, so only the uncleaved HA0 precursor could be detected (Shirvani et al., 2020a). Control cells were infected with a wt virus without the insert, or mock-infected. All the recombinant constructs, including P/M-HA, replicated to a similar level as judged by the accumulation of the NDV N protein (Fig. 14A). The level of AIV HA expression was the highest from the P/M-HA construct, as expected. Both HN.2A.HA and HN.F2A.HA expressed AIV HA at a higher level than the control HN/L-HA construct, with HN.F2A.HA being particularly efficient (Fig. 14A). The HA0 precursors expressed by HN/L-HA, HN.2A.HA, and HN.F2A.HA were properly processed, even though the smaller HA2 subunit was not always detected on western blots. In the case of HN.2A.HA and HN.F2A.HA, a noticeable amount of uncleaved HN-HA fusions was observed, indicating that the 2A-driven peptide separation and processing of the furin site are not completely efficient in this system (Fig. 14A).

We next examined the cellular localization of AIV HA expressed from the recombinant constructs. For all constructs, the major HA signal was observed at the plasma membrane, indicating the efficient trafficking of the protein expressed from the recombinant NDV vectors. At the same time, in cells infected with the P/M-HA, a significant amount of HA signal was observed in intracellular structures, likely corresponding to the ER and the Golgi, although we did not perform a detailed analysis. This may indicate that either the lack of HA0 processing prevents efficient HA trafficking through the cellular secretory pathway, or may just reflect a higher level of HA expression from this construct (Fig. 14B).

The trafficking of AIV HA to the plasma membrane where it extensively co-localized with the NDV glycoprotein signal suggests that AIV HA may be incorporated in NDV virions. Such incorporation of a vaccine antigen into NDV virions was shown to increase the efficacy of NDV-vectored vaccines (Bukreyev et al., 2006, Park et al., 2006). We analyzed the presence of AIV HA in purified virions in a western blot. We detected the HA2 subunit only but no specific signals for HA1 or unprocessed HA0. The level of HA2 incorporation generally correlated with the level of HA expression and processing by different constructs (Fig. 15, western blot). There was also a trace HA2 signal in P/M-HA virions suggesting that some processing of the HA0 with mutated HA1/HA2 cleavage site still occurs. In HN.F2A.HA virions a signal for the unprocessed HN-HA fusion was also present. In a coomassie stained gel of purified virions no additional bands were detected in recombinant constructs compared to the control, indicating that any HA fragments constituted a minor fraction compared to the structural proteins of NDV virions (Fig. 15). Interestingly, in the virions of all constructs expressing AIV HA, the amount of the NDV HN relative to M or N proteins was significantly lower compared to that in wt virions, indicating a specific interference of HA AIV with the trafficking and/or incorporation of HN in the virions (Fig. 15).

Together, these data demonstrate that one ORF strategy could increase the expression of a foreign protein from NDV vectors, and that AIV HA expressed from the NDV constructs is correctly processed inside the cells, but it is not incorporated in the NDV virions in a properly assembled form, and that the interference of AIV HA with the trafficking of NDV HN may underlie the impaired infectivity of recombinant viruses.

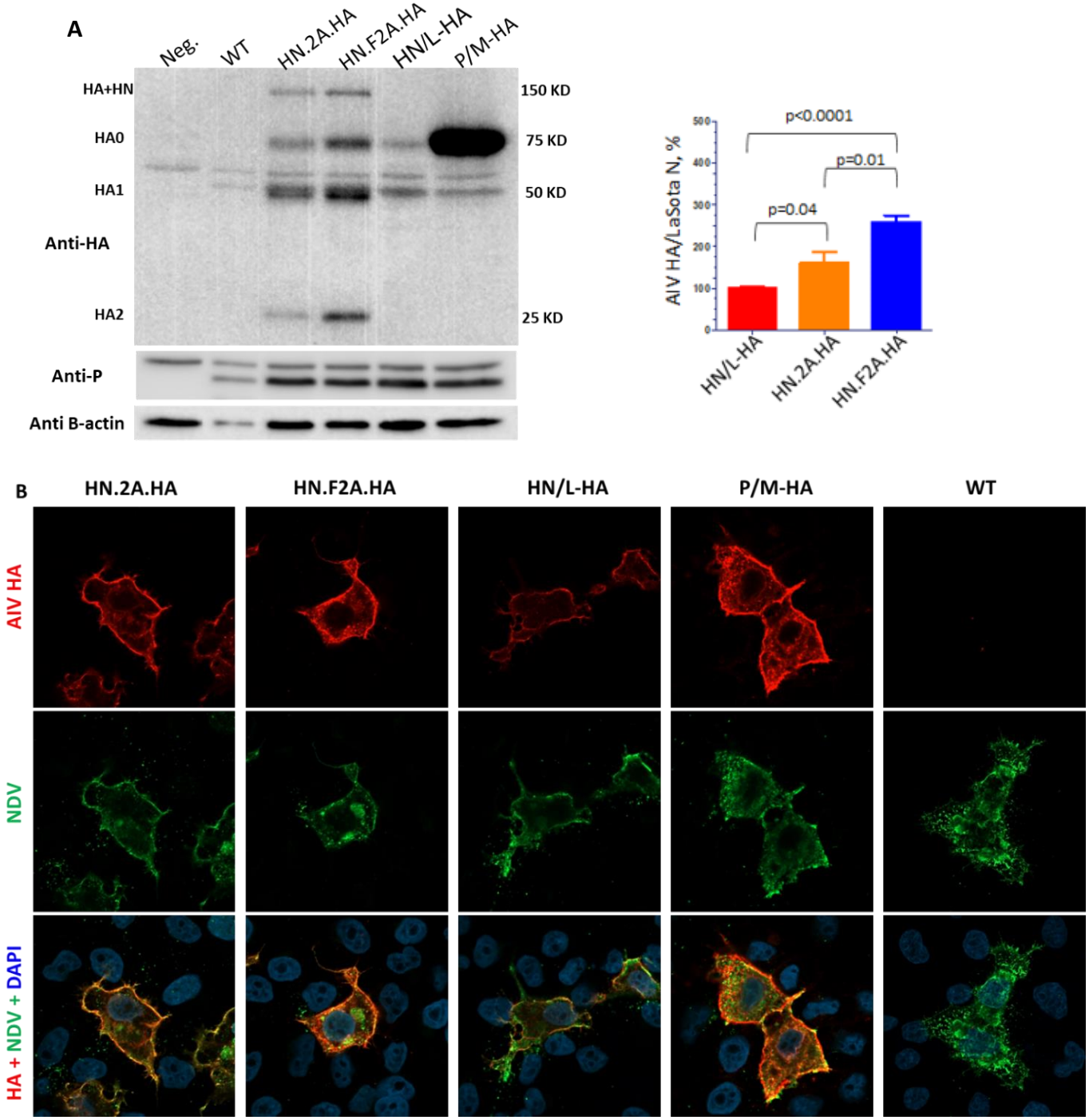


Figure 14. Characterization of expression of avian influenza virus hemagglutinin by recombinant NDVs. **A.** DF-1 cells were infected with an MOI of 1 of each construct, incubated for 48h and processed for the western blot analysis. The same membrane was sequentially probed with the indicated antibodies. The graph shows the quantitation of HA expression relative to N protein normalized to the control construct HN/L-HA from three independent experiments. **B.** HeLa cells infected at a low MOI were incubated without exogenous proteases to prevent virus spread from the originally infected cells. Cells were fixed and stained at 24 h p.i.. All images were taken under the same conditions.

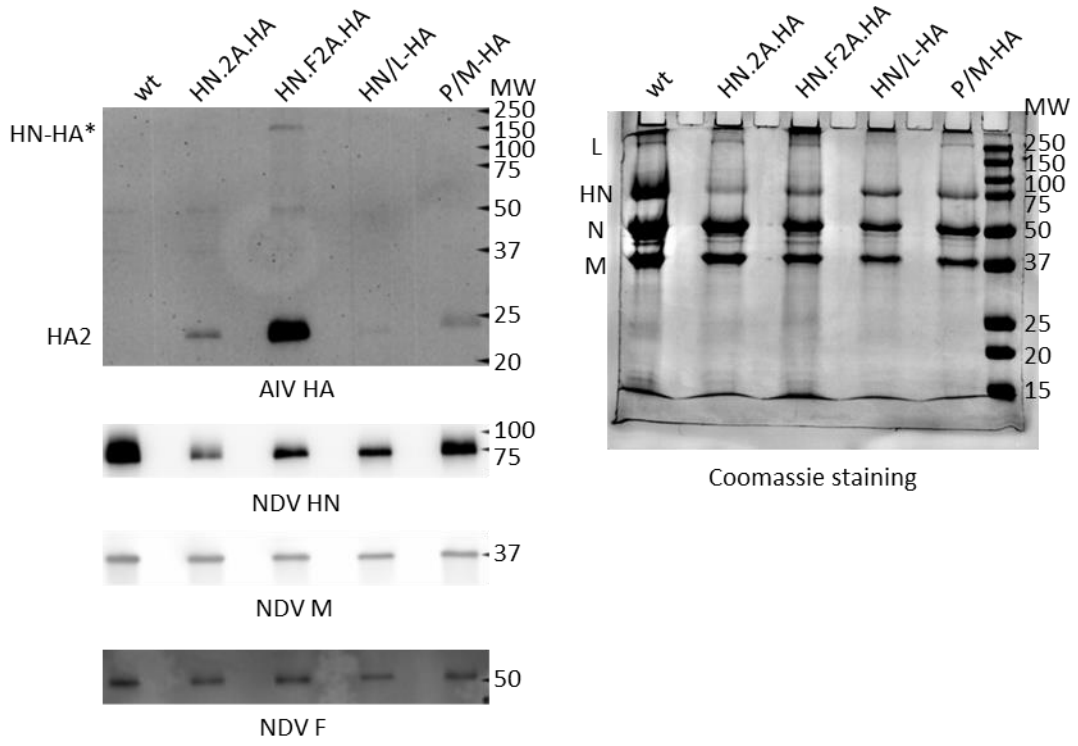


Figure 15. Incorporation of Avian Influenza Virus Hemagglutinin into Recombinant NDVs. Purified NDV virions were analyzed in western blots for the NDV structural protein and AIV HA content (left) and for total protein composition using Coomassie staining (right).

Recombinant NDV vectors expressing AIV HA from the NDV HN transcription unit are stable over multiple passages.

The stability of recombinant constructs expressing foreign proteins is a major concern for all viral vector applications. We analyzed the expression of AIV HA from the recombinant constructs that express HA from the NDV HN transcription unit (HN.2A.HA and HN.F2A.HA), and those that express HA as a separate transcription unit (HN/L-HA and P/M-HA) over 10 passages in embryonated chicken eggs. It should be noted that one passage in an egg comprises multiple cycles of infection. The expression of AIV HA was evaluated in cells infected with a low MOI and incubated without exogenously added proteases necessary for the formation of infectious virions of a non-pathogenic LaSota strain of NDV used as a backbone for the

recombinant constructs. In these conditions, the virus may spread only to a few nearby cells likely due to a limited processing of the F protein, cell division and/or direct intercellular contacts (Chu et al., 2019, Manoharan et al., 2018b, Sattentau, 2008, Duprex et al., 1999), allowing analysis of protein expression after primary infections. Fig. 16A provides an example of the microscopy images showing the infected cells positive for both NDV and AIV HA antigens (arrows), or those positive for only the NDV antigen (arrowheads), used for the analysis of the stability of HA expression. Almost all cells infected with each construct from passage one expressed AIV HA. In the material from passage four, a noticeable drop in HA AIV expression was observed for the constructs where HAIV HA is introduced as a separate transcription unit in the genome (84% and 92% for HN/L-HA and P/M-HA, respectively). This trend continued in the material from passage 11, where the number of HA positive cells further dropped to 78% and 90% for HN/L-HA and P/M-HA, while the expression of HA was still detected in almost all cells infected with HN.2A.HA and HN.F2A.HA (Fig. 16B).

These data demonstrate that the expression of a transgene from the same ORF as an NDV protein results in more stable recombinant constructs than a traditional approach of placing separate transcription units in the NDV genome.

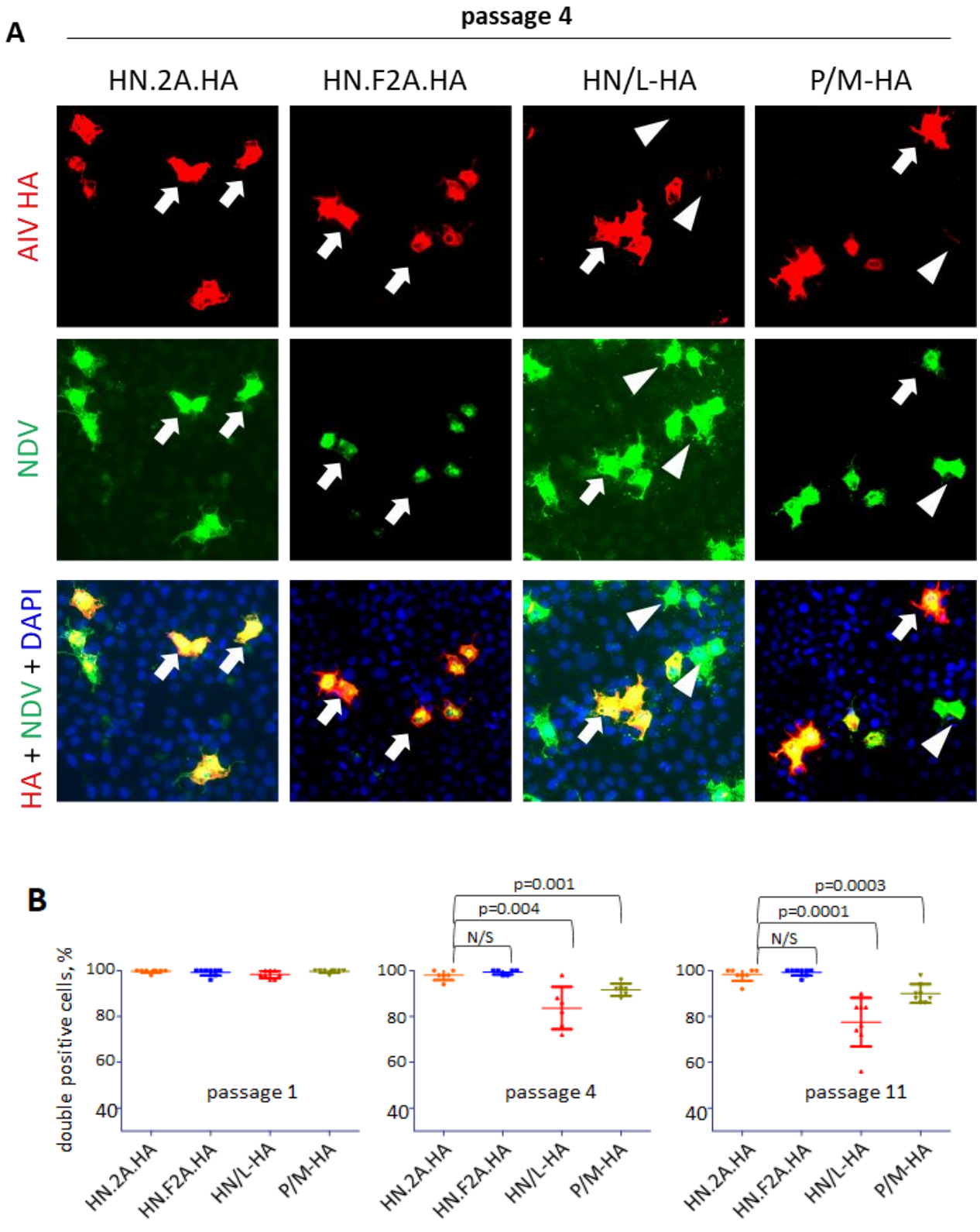

A
passage 4
HN.2A.HA
HN.F2A.HA
HN/L-HA
P/M-HA
AIV HA
NDV
HA + NDV + DAPI
B
double positive cells, %
100
80
60
40
passage 1
passage 4
passage 11
p=0.001
p=0.004
N/S
p=0.0003
p=0.0001
N/S
HN.2A.HA
HN.F2A.HA
HN/L-HA
P/M-HA

Figure 16. Analysis of the stability of expression of avian influenza virus hemagglutinin by recombinant NDVs. All recombinant NDVs were passaged 10 times in embryonated chicken eggs and the percent of infectious viruses expressing AIV HA was analyzed by immunofluorescence. **A.** Images of HeLa cells infected at a low MOI with the viruses from passage four and incubated without exogenous proteases to prevent virus spread from the originally infected cells. The cells were fixed and stained at 24 h p.i. with anti-NDV polyclonal chicken antibodies and anti-HA polyclonal rabbit antibodies. The images are intentionally overexposed to make the differences in the transgene expression easily detectable. Arrows indicate cells positive for both NDV and AIV HA antigens (i.e. infected with a recombinant virus expressing the transgene), arrowheads indicate cells positive only for NDV antigens (i.e. infected with a virus that lost the transgene expression). **B.** Scatter plots showing quantitation of AIV HA expression by recombinant NDVs upon serial passages using the immunofluorescence assay as in (A). Each dot represents data from a randomly chosen field of view, at least 300 total infected cells were counted for each construct in each passage.

Discussion

The optimization of a transgene expression from a viral vector should address several criteria, including, but not necessarily limited, to the level of expression of a recombinant protein, its proper posttranslational processing, and stability of the recombinant vector over multiple replication cycles. Here we investigated a novel approach to the construction of NDV vectors, a popular viral platform for the development of vaccines and oncolytic therapeutics. The modular genome organization of NDV prompts for an intuitive design where a foreign insert is arranged as a separate gene in the NDV genome with its own transcription start and stop signals. However, alternative approaches to the construction of NDV vectors are possible, which theoretically may be advantageous over the traditional scheme. In this work, we explored the possibility of expressing a protective antigen of AIV in-fusion with a NDV protein. In this approach, there is no introduction of a separate transcription unit in the NDV genome that could negatively affect the expression of downstream genes. Moreover, this cloning scheme should theoretically apply a selective pressure against nonsense mutations in the foreign sequence as it would inevitably prevent the translation of the NDV ORF, or affect the stability of the mRNA if the foreign sequence is placed upstream or downstream of the NDV ORF, respectively.

However, this design requires the introduction of sequences mediating either co-translational or posttranslational separation of the NDV protein from the added polypeptide. The remnants of such sequences may remain attached to the C- and N-ends of the cleavage site.

Previously, a proof of principle for such an approach was demonstrated for NDV vectors expressing eGFP as an ORF placed upstream of N, M or L proteins, with the eGFP and NDV proteins separated by a self-cleaving 2A sequence of foot and mouth disease virus followed by a ubiquitin monomer (Wen et al., 2015). While we designed constructs where AIV HA was placed upstream or downstream of ORFs of different NDV genes, we could rescue only those where HA was fused downstream of the NDV HN protein. One possible explanation that sites that could tolerate eGFP insertions could not accept AIV HA is that the proteins are significantly different. eGFP is ~27KDa, while AIV HA is ~63KDa, and while eGFP is a cytoplasmic protein lacking specific targeting signals, HA is targeted to the cellular secretory pathway, so that the mRNA coding for HA would be co-translationally recruited to the ER. Thus, the translation of mRNAs containing eGFP or HA ORFs would likely proceed differently, affecting the expression of the fused NDV ORFs. Both the NDV HN and AIV HA are glycoproteins targeted to the cellular secretory pathway which may facilitate their compatibility within the same transcription unit. It also cannot be excluded that the difference in the efficacy of the sequences used for protein separation contributed to the different viability of the recombinant constructs. Interestingly, sequence analysis of different NDV strains shows that HNs have the most variable C-termini among all NDV proteins (Jin et al., 2016), which may explain why this protein tolerated the modification of the C-terminus by the remnants of 2A or furin site sequences.

The only difference between the two viable constructs where the AIV HA is encoded downstream of NDV HN is that in HN.2A.HA the two proteins are separated by a self-cleaving 2A peptide, while in HN.F2A.HA additional four amino-acids constituting a furin cleavage site are placed before the 2A. Yet, the HN.F2A.HA construct showed a larger fluorescent foci phenotype and expressed HA to a higher level than HN.2A.HA. While the efficiency of the processing of this furin cleavage site does not seem to be particularly high, since the construct HN.F.HA where the same proteins are separated by the furin site alone was not viable, it is possible that even a limited processing of this site may explain the higher infectivity of HN.F2A.HA. The cleavage of the furin site in this construct would remove the 2A polypeptide from the C-terminus of HN, leaving only four extra amino-acids instead of 21 as in the case of HN.2A.HA, thus providing a more authentic HN. Alternatively, the extra sequence coding for the furin site may somehow stabilize the now much longer RNA coding for both NDV HN and AIV HA.

We observed a negative correlation between the expression of AIV HA and the incorporation of NDV HN in the virions. This correlation could not be explained by a direct competition at the virion assembly sites since only traces of the HA2 subunit but not the properly assembled HA was found in the virions generated by the recombinant viruses, even though the HA precursor was properly processed. Likely, AIV HA competes with NDV HN during the earlier transiting through the cellular secretory pathway. These data also demonstrate a high specificity of trafficking and incorporation of structural glycoproteins into NDV virions. That suggested that addition of transmembrane domains and cytoplasmic tail sequences of the NDV

glycoproteins to the foreign gene should be exploited if incorporation of a recombinant protein in the NDV virions is desirable as previously reported in (Park et al., 2006).

Both constructs expressing AIV HA from the same RNA as the NDV HN were noticeably more stable over multiple passages compared to both constructs expressing this protein from separate transcription units, whether located close to the 3' or to the 5' end of the NDV genome. This observation is in accordance with the theoretical considerations underlying the design of this series of recombinant vectors. This approach may be specifically useful for the expression of the proteins with a negative effect on NDV infectivity, such as AIV HA which interfered with the proper assembly of NDV virions.

In our study, the tolerated position for AIV HA insertion in-frame with a NDV protein was located closer to the 5’ end of the NDV genome, which lowers the expression level of the transgene and making it unpractical for vaccine development. Even though, the improved stability of the transgene can be accepted as a tradeoff for applications requiring lower expression level of a transgene. For example, NDV is actively pursuit as an oncolytic agent (Song et al., 2019, Zamarin and Palese, 2012, Cuoco et al., 2021), and it is often advantageous to enhance the oncolytic properties by expression of a cytokine such as granulocyte-macrophage colony-stimulating factor, which may not be safe if expressed at very high levels, but would be beneficial provided long term low level of expression with higher stability (Grossardt et al., 2013, Cerullo et al., 2010, Kohno et al., 2007). In order to improve the expression of a transgene in-frame with the NDV proteins, different approaches should be explored for improved cleavability of the connected proteins with least number of extra amino acid residues. That would allow expressing the transgene from proximal transcription units in NDV genome, and

thus improving expression level without compromising their stability as when expressed as separate transcription unit. Overall, NDV and related viruses are a versatile vector platform for a variety of applications, and investigation of different arrangements of foreign gene expression cassettes is important for realizing the full potential of this promising biotechnology tool.

Chapter 5: Conclusion and Future Prospects

Avian paramyxoviruses are promising vectors for the development of human and veterinary vaccines and oncotherapeutic agents. Their natural tropism for the respiratory epithelium makes them particularly attractive for the development of vaccines against established and emerging respiratory pathogens. Still, the full potential of this versatile viral vector platform is not realized. While multiple APMV-based vectored vaccine prototypes have been developed, few if any found their way into the broad medical or veterinary practice. That may be due to their structural instability being enveloped RNA viruses.

The poultry industry is a highly attractive field for application of APMV-based vaccines, yet their efficacy in commercial flocks is hindered by the routine vaccination against APMV1 (Newcastle Disease virus), which is also the most popular platform for the development of APMV-vectored vaccines. In the first part of my study, I developed and characterized a vaccine vector platform based on APMV3, a virus antigenically distinct from APMV1. While the results demonstrated excellent safety of the new vector in chickens, further optimization of its growth properties and immunogenicity in commercially important poultry species is required.

The two often mutually exclusive requirements for any viral vector system is the high level of expression, and the stability of the transgene over multiple replication cycles during the propagation or application of the recombinant viral agent. In the second part of my study, I explored a novel method of incorporation of a foreign gene into APMV genome. While the traditional approach is to arrange the transgene expression unit as a separate gene in the linear APMV genome, I placed the sequence coding for an avian influenza virus hemagglutinin protein in one ORF with a proper virus vector protein. For the co- and post-translational separation of

the two polypeptides, a self-cleaving picornaviral 2A sequence alone, or in combination with a furin cleavage site, was inserted between the two protein-coding sequences. Among all the APMV1 proteins tested, only HN tolerated such modification. The expression of the transgene from this construct was higher than from a construct where the transgene was encoded as a separate transcription unit, and the fusion design demonstrated an exceptional stability of the transgene. Still, the level of expression of a protective vaccine antigen from the HN ORF was not sufficient for a practical vaccine vector.

Thus, further development of antigenically different APMV vectors and optimization of the transgene expression strategies is required for the full realization of the potential of this promising viral vector platform. Based on my studies I envision the following future directions for the improvement of APMV-based vaccines, especially for the application in poultry industry:

1. Both APMV-3 strains have different pathogenicity and growth characteristics. My created reverse genetic system can be used to understand the molecular basis of their pathogenicity and hence generating a chimeric virus that would combine high growth characteristics and the low pathogenicity in young chicks.
2. The accumulating evidence suggests that different transgenes may perform differently as protective vaccine antigens when expressed from the same vector, thus the optimization of a virus vector should be done for individual vaccine candidates and tested in vivo (animal study) for vaccine protection against virus challenge.
3. While the expression of a transgene in the same ORF as a viral vector protein proved to be advantageous, my current constructs yield suboptimal expression level. Hence, it is possible that alternative cleavage systems, such as the addition of a ubiquitin moiety to the 2A peptide

(Wen et al., 2015), or the addition of a cleavage site for a different cellular protease, may enable the incorporation of the transgene in-frame with NDV proximal genes (P or M) for higher expression, with the improved transgene stability.

4. Exploring the underlying molecular mechanisms behind the transgene different stability when expressed as a separate transcription units versus as one ORF with an essential viral vector protein. This research will not only be important for the improvement of the vectors for practical applications, but will advance the fundamental knowledge of APMV replication.

In conclusion, my study have resulted in the creation of an antigenically different APMV vector which was shown to be safe for young chicks vaccination. Explored the possibility of a transgene expression in-frame with an APMV vector protein and shown its positive impact on the transgene expression level and stability compared to the traditional expression approach.

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