ABSTRACT

Title of Document: CHARACTERIZATION AND DEVELOPMENT OF REVERSE GENETICS SYSTEM FOR AVIAN PARAMYXOVIRUS TYPE-3 AND ITS EVALUATION AS A LIVE VIRAL VECTOR.

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Avian Paramyxovirus (APMV) serotype 3 is one of the nine serotypes of APMV that infect a variety of avian species around the world. In chickens and turkeys, APMV-3 causes respiratory illness and drop in egg production. To understand the molecular characteristics of APMV-3, the complete genome sequences of prototype strain Netherlands and strain Wisconsin were determined. The genome length of APMV-3 strain Netherlands is 16,272 and for strain Wisconsin is 16,181 nucleotides (nt). Each genome consists of six non-overlapping genes in the order $^{3'}$N-P/V/W-M-F-HN-L$^{5'}$ similar to most of APMVs. Comparison of the APMV-3 strain Wisconsin nt and the aggregate predicted amino acid (aa) sequences with those of APMV-3 strain Netherlands revealed 67 and 78%, identity, respectively. The phylogenetic and serological analyses of APMV-3 strains Netherlands and Wisconsin indicated the existence of two subgroups

within the same serotype. Both the strains were found to be avirulent for chickens by mean death time and intracerebral pathogenicity test.

To further study the molecular biology and pathogenesis of APMV-3, a reverse genetics system for strain Netherlands was established in which infectious recombinant APMV-3 was recovered from a cloned APMV-3 antigenomic cDNA. The recovered recombinant virus showed *in vitro* growth characteristics and *in vivo* pathogenicity similar to parental virus. A recombinant APMV-3 expressing enhanced green fluorescent protein was also recovered, suggesting its potential use as a vaccine vector. Furthermore, generation and characterization of recombinant APMV-3 expressing Newcastle disease virus (NDV) F and HN proteins demonstrated that the F protein plays a major role in protection against virulent NDV challenge. Overall, the study conducted here has several downstream applications. The complete genome sequence of APMV-3 is useful in designing diagnostic reagents and in epidemiological studies. The reverse genetics system for APMV-3 would be of considerable utility for introducing defined mutations into the genome of this virus and developing a vaccine vector for animal and human pathogens.

CHARACTERIZATION AND DEVELOPMENT OF REVERSE GENETICS SYSTEM FOR AVIAN PARAMYXOVIRUS TYPE-3 AND ITS EVALUATION AS A LIVE VIRAL VECTOR.

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Dedication

I dedicate this work to my parents for their selfless sacrifice, my brother Sunil whom I lost during the course of study, my wife Monika for her unconditional love and moral support and my mentor Dr. Siba K. Samal for his ever willing help and constant encouragement.

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To speak gratitude is courteous and pleasant, to enact gratitude is generous and noble, but to live gratitude is to touch Heaven. Traditional words are in plenty but their 'traditional use' has turned this pleasant duty into a formality. This will not project the picture of the volcano of feelings that is rumbling and struggling to burst out of me through words, to vocalize what I owe to my major advisor, Dr. Siba K. Samal, who groomed me at each and every step of the entire course here to be graduate student and made me worth what I can claim to be now. Mere words cannot express the depth of gratitude I owe to him.

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aa	amino acid
APMV	avian paramyxovirus
APMV-3	avian paramyxovirus serotype 3
bp	base pair
BC	Beaudette C
BSL	Bio Safety Level
cDNA	complementary DNA
CEF	chicken embryo fibroblast
CPE	cytopathic effect
Da	Daltons
DF1	Douglas Foster 1
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetate
ELISA	enzyme linked immunosorbent assay
EMEM	essential modified Eagle's medium
F	Fusion protein
FBS	fetal bovine serum
GE	gene-end
GFP	green fluorescent protein
GS	gene-start
HA	hemagglutination

HDV	hepatitis delta virus
HI	hemagglutinin inhibition
HMPV	human metapneumovirus
HN	hemagglutinin-neuraminidase
HPIV-2	human parainfluenza type 2
HPIV-3	human parainfluenza type 3
HRSV	human respiratory syncytial virus
ICPI	intracerebral pathogenicity index
IGS	intergenic sequence
IRES	internal ribosome entry site
IVPI	intravenous pathogenicity index
kDa	kilodaltons
L	large polymerase
M	matrix protein
mRNA	messenger RNA
MDT	mean death time
MOI	multiplicity of infection
MV	measles virus
MVA-T7	recombinant modified vaccinia strain Ankara expressing T7 polymerase
NA	neuraminidase
NDV	Newcastle disease virus
nm	nanometer
N	nucleocapsid protein

nt	nucleotide
NV	Nipah virus
ORF	open reading frame
P	phosphoprotein
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFU	plaque forming unit
PI	post infection
RBC	red blood cell
RER	rough endoplasmic reticulum
RNA	ribonucleic acid
rAPMV-3	recombinant APMV-3
RNP	ribonucleoprotein
RSV	Respiratory syncytial virus
RT-PCR	reverse transcription PCR
SeV	Sendai virus
SDS-PAGE	sodium dodecyl sulfate-poly acrylamide gel electrophoresis
SPF	specific pathogen free
SV5	simian virus 5
$TCID_{50}$	50% Tissue culture infectivity dose
UTR	untranslated region

Chapter 1

1.1 Title

General Introduction

1.2 Introduction

Avian Paramyxovirus serotype 3 (APMV-3) belongs to the genus *Avulavirus* within the subfamily *Paramyxovirinae* in the family *Paramyxoviridae.* This virus family is large and diverse. Its members have been isolated from many species of avian, terrestrial, and aquatic animals around the world (Lamb and Parks, 2007). Paramyxoviruses are pleomorphic, enveloped and have a non-segmented, negative-sense RNA genome. During influenza virus surveillance programs, avian paramyxoviruses were also isolated and were classified into nine serotypes based on hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests (Alexander, 2003a). The cross-HI and –NI tests suggested organizing APMV isolates into two broad subgroups; the first subgroup consisting of APMV-2 and -6 and the second subgroup consisting of APMV-1, -3, -4, -7, -8 and -9, while little was known about APMV-5 (Lipkind and Shihmanter, 1986). Among these serotypes, Newcastle disease virus (NDV) belonging to APMV-1, is an economically important poultry pathogen and hence, the most well characterized virus within this genus. In contrast, little is known about the pathogenesis of other avian paramyxoviruses.

APMV-3 was first isolated from turkeys in Ontario in 1967 and later in Wisconsin in 1968 (Tumova et al., 1979b). Since then, many APMV-3 strains have been isolated from

turkeys in different parts of the world, including England (Macpherson et al., 1983), France (Andral and Toquin, 1984) and Germany (Zeydanli et al., 1988). The APMV-3 strain parakeet/Netherlands/449/75, isolated from parakeets in the Netherlands, is the prototype for the entire serotype (Alexander and Chettle, 1978). APMV-3 was also isolated from non-domesticated species such as *Psittaciformes* and *Passeriformes* (Alexander, 1980). APMV-3 infection has been associated with encephalitis and high mortality in caged birds, and with respiratory disease in turkeys (Tumova et al., 1979b). The virus causes acute pancreatitis and CNS symptoms in psittacine and *Passeriformes* birds (Beck et al., 2003). APMV-3 also infects chickens at an early age, with evidence of stunting growth that may be more marked in broiler chicken breeds (Alexander and Collins, 1982). In terms of pathogenicity in chickens, APMV-3 probably is second in importance only to NDV in chickens.

The genome of APMV-3 has six genes arranged in tandem in the order of 3' -N-P-M-F-HN-L- 5' (Kumar et al., 2008). There are conserved sequences present at the beginning and end of each gene, known as gene start (GS) and gene end (GE), respectively. The GS is recognized as a transcription initiation signal and the GE is recognized as a transcription termination signal by the viral RNA polymerase. Between each gene, non-coding sequences of variable lengths called intergenic sequences (IGS) are present, which are shown in NDV to regulate the transcription efficiency of downstream genes (Yan and Samal, 2008). In addition, the genome of APMV-3 contains a 55 nt long viral promoter known as leader at its 3' end and a 707 nt long viral antigenome promoter known as trailer at its 5' end (Kumar et al., 2008). The leader and trailer regions serve as cis-acting elements in viral genome for replication and packaging of viral RNA (Lamb and Parks,

2007). APMV-3 genome encodes three internal proteins, nucleocapsid protein (N), a phosphoprotein (P) and a large polymerase protein (L), and three surface glycoproteins, fusion protein (F), hemagglutinin-neuraminidase (HN) protein and matrix protein M.

The N protein is the most abundant protein in infected cells and in virus particles. N is an RNA binding protein that coats the full-length genomic (-) sense and anti-genomic (+) sense RNAs to protect them from nuclease digestion and form biologically active templates (Lamb and Parks, 2007). Most members of subfamily *Paramyxovirinae* show RNA editing mechanism, wherein a specific motif within the P gene called RNA editing site directs non-templated addition of one or more G nucleotides to P transcripts by viral polymerase stuttering. This yields mRNA subpopulations with frame shifts causing alternate internal ORFs. Two non-structural proteins, V and W, are produced by insertions of one and two G residues, respectively. The V protein has a highly conserved cysteine motif and has interferon antagonist activity in NDV (Huang et al., 2003). The M is a hydrophobic protein and it is located on the inner face of the virion envelope. In addition, the M protein plays an important role in the assembly of the virion by interacting with the nucleocapsid, the lipid bilayer and the cytoplasmic region of the HN protein.

The outer surface glycoproteins, F and HN, mediate viral penetration and attachment, respectively. The HN protein is responsible for attachment of the virion to sialic acid-containing cell surface receptors. In addition, HN has neuraminidase activity (NA) that cleaves sialic acid form sugar side chains, thereby, releasing progeny virions from the surface of infected cells (Lamb and Parks, 2007). The F protein is synthesized as an inactive precursor (F_0) which is cleaved at its cleavage site by host-cell proteolytic

enzyme(s) into biologically active F_1 and F_2 subunits connected by a disulphide bond (Scheid and Choppin, 1974). The avirulent NDV strains have a monobasic amino acid residue at the F protein cleavage site and a leucine at the F1 N terminus, while virulent NDV strains have multiple basic amino acids at the F protein cleavage site and a phenylalanine at the F1 N terminus (Nagai et al., 1976). The F protein cleavage site of APMV-3 has three basic amino acids and a leucine residue at F1 N terminus and is analogous with the avirulent cleavage site of NDV (Kumar et al., 2008). The neutralizing antibody responses to NDV are directed against both the surface glycoproteins F and HN. Although, antibodies against either F or HN protein provide protection against ND (Heckert et al., 1996), it is still not clear, which surface glycoprotein is the major protective antigen.

Pathogenesis and molecular characteristics of APMV-3 have rarely been studied; only M gene sequence was previously accessible in GenBank (Shihmanter et al., 2005). The complete genome sequences of none of the strains of APMV-3 were available. Therefore, in this study, the complete genome sequences of two strains of APMV-3 were determined and compared with those of other paramyxoviruses to understand its biology at the molecular level. The knowledge of complete genome sequence is essential for genetic manipulation of these viruses. The most successful system for genetic manipulation of negative-sense RNA viruses is a plasmid based reverse genetics system. In this approach, four plasmids – one encoding the viral anti-genome and the others encoding the viral polymerase complex (N, P and L proteins), all under the control of T7 promoter are cotransfected in permissive cells expressing T7 RNA polymerase or in cells infected with recombinant vaccinia virus expressing T7 RNA polymerase. Many

negative-sense RNA viruses have been recovered using this system (Schnell et al., 1994; Collins et al., 1995; Garcin et al., 1995; Lawson et al., 1995; Radecke et al., 1995; Baron and Barrett, 1997; He et al., 1997; Hoffman and Banerjee, 1997; Buchholz et al., 1999; Peeters et al., 1999; Yunus et al., 1999; Krishnamurthy et al., 2000; Govindarajan et al., 2006). There are many downstream applications of reverse genetics technology such as development of vaccines and vaccine vectors and use as a tool to study the molecular biology of viruses. In this study, a reverse genetics system for APMV-3 was established which was then used as a vector to determine the individual contributions of the NDV F and HN proteins in protection and immunity against NDV in chickens.

1.3 Research Objectives

1. Determination of complete genome sequence of APMV- 3 prototype strain Netherlands.

2. Determination of complete genome sequence of APMV- 3 strain Wisconsin and comparison with that of prototype strain Netherlands.

3. Pathogenicity of APMV-3 strains Netherlands and Wisconsin in chickens and turkeys.

4. Establishment of a reverse genetics system for APMV-3 and recovery of infectious recombinant APMV-3 strain Netherlands expressing GFP as a foreign protein.

5. Evaluation of the Newcastle disease virus F and HN proteins in protective immunity using a recombinant avian paramyxovirus type-3 vector in chickens.

Chapter 2

2.1 Title

Review of Literature

2.2 Classification

The family *Paramyxoviridae* includes many species of viruses isolated from avian, terrestrial and aquatic animals isolated worldwide (Lamb and Parks, 2007; Nylund et al., 2008). Members of this family are characterized by pleomorphic enveloped particles that contain a single-stranded, negative sense RNA genome (Lamb and Parks, 2007). The family is divided into two subfamilies, *Paramyxovirinae* and *Pneumovirinae*. Subfamily *Paramyxovirinae* is further divided into five genera: *Rubulavirus* (including mumps virus, human parainfluenza viruses [HPIV] 2 and 4, simian virus type 5 [SV5] and Tioman virus [TiV]), *Respirovirus* (including Sendai virus [SeV] and HPIV-1 and 3), *Henipavirus* (comprising Hendra virus [HeV] and Nipah virus [NiV]), *Morbillivirus* (including measles [MeV] and canine distemper [CDV] viruses), and *Avulavirus* (comprising avian paramyxovirus [APMV] serotypes 1 to 9). Subfamily *Pneumovirinae* is divided into two genera: *Pneumovirus* (comprising human respiratory syncytial virus [HRSV] and its animal counterparts including bovine respiratory syncytial virus [BRSV]), and *Metapneumovirus* (comprising human metapneumovirus [HMPV] and avian metapneumovirus [AMPV]) (Mayo, 2002b; Mayo, 2002a).

2.3 Virion

Examination of partially-purified virus under an electron microscope showed that the APMV-3 particles were enveloped, pleomorphic but mostly spherical in shape, with a

size ranging from 150-250 nm. The viral envelope is derived from the host cell membrane. The outer surface of the envelope consists of two glycoproteins, namely fusion (F) and hemagglutination neuraminidase (HN) proteins. The HN protein mediates attachment by binding to sialic acid receptor, has neuraminidase activity, and plays a role in fusion promotion where as F protein directs the membrane fusion. Matrix protein (M) lies below the envelope and is shown to play a major role in virus assembly and budding of NDV (Fig. 2.1). The viral envelope encloses the ribonucleocapsid core that is formed by nucleoprotein (N), the phosphoprotein (P) and large polymerase (L) protein bound with viral genomic RNA. This viral RNA genome is single stranded, non-segmented and with negative polarity.

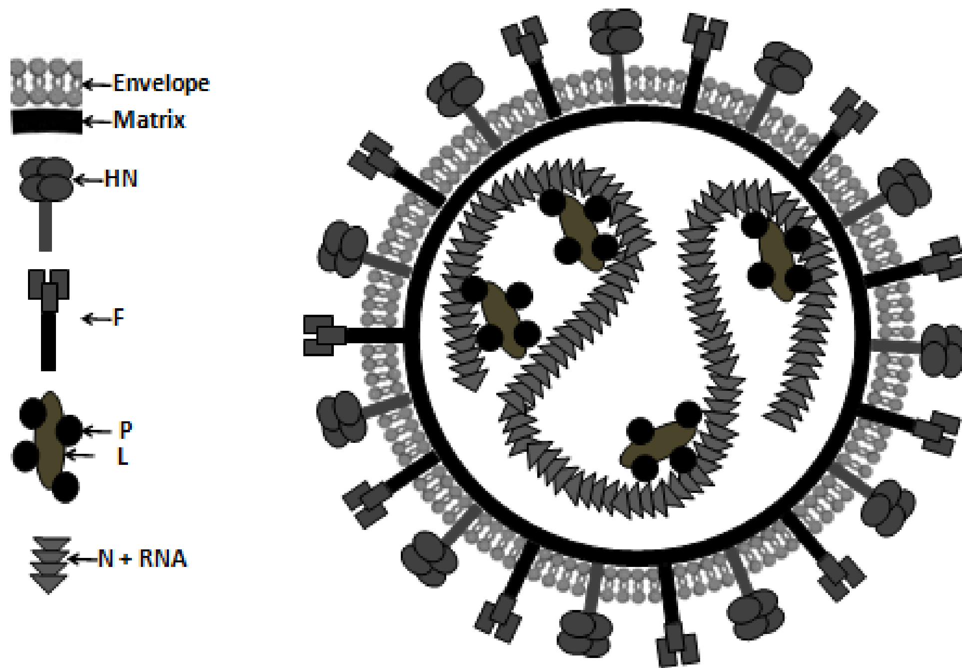


Fig. 2.1. Schematic diagram of an avian paramyxovirus type-3. The lipid bilayer derived from host cell membrane is shown as envelope. The viral matrix protein is shown as a black concentric circle beneath the envelope. The hemagglutinin-neuraminidase (HN) and the fusion (F) glycoproteins are shown imbedded

inside the viral envelope. The inside of virion is made up of negative-strand RNA encapsidated with nucleocapsid protein (N) and associated with the phosphoprotein (P) and the large polymerase protein (L).

2.4 Genome organization

The genome of APMV-3 strain Netherlands consists of 16,272 nt (GenBank accession number **EU403085**). The genome organization of APMV-3 is very similar to that of other members of family *Paramyxoviridae*. The 3' leader sequence of APMV-3 consists of 55 nt while the length of the 5' trailer sequence is 707 nt. They flank the six genes 3'-N-P-M-F-HN-L-5' that encodes for six open reading frames (ORF) corresponding to respective putative proteins. Each gene is flanked by conserved transcriptional control sequences, known as the gene start (GS) and gene end (GE) that are followed by 5' and 3' untranslated regions (UTR), respectively (Fig. 2.2). The GS sequence of APMV-3 strain Netherlands is 3'UCCUCGCCUU and is conserved exactly among the six genes. The GE signal is slightly less conserved: the consensus gene-end sequence is 3'AAUUA$(U)_6$. Non-coding intergenic sequences (IGS) are located between each gene boundary and ranges in length from 31 to 63 nt.

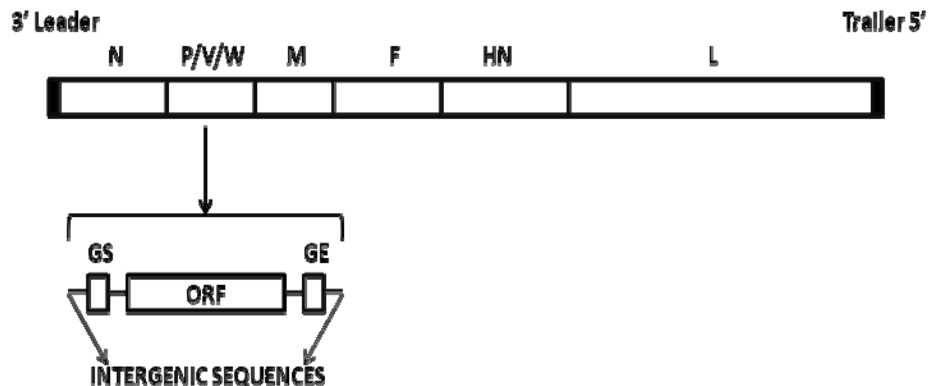


Fig. 2.2. Genome organization of avian paramyxovirus type-3 and schematic diagram of a gene cassette.

The single-stranded, negative-sense genome RNA has leader sequence at 3' end and a trailer sequence at 5' end. The N, P, M, F, HN and L genes are arranged in tandem. Each gene is flanked by conserved GS and GE sequences and has untranslated sequences (UTR) at 3' and 5' regions of the ORF between each gene boundary.

2.5 Viral proteins

The genome of APMV-3 consists of six genes N, P, M, F, HN and L encoding eight proteins: N, P, V, W, M, F, HN and L (Kumar et al., 2008). The two proteins, V and W, are generated by stuttering of the viral RNA polymerase, known as RNA editing, wherein non-templated G residue(s) get inserted into P gene (Steward et al., 1993). The genomic structure and proteins of APMV-3 are known to be similar to those of APMV-1 and other paramyxoviruses; hence, the knowledge about the APMV-3 proteins is derived from studies done with other members of the family *Paramyxoviridae* (Lamb and Parks, 2007).

2.5.1 Nucleocapsid and its associated proteins

The nucleocapsid protein (N): It is the most abundant protein in infected cells and in virus particles. N is an RNA binding protein that coats the full-length genomic (-) sense and anti-genomic (+) sense RNAs to protect them from nuclease digestion and to form biologically active templates. It is able to self-assemble to form herringbone-like structures, which morphologically resemble the authentic nucleocapsid structures (Kho et al., 2003). The N protein forms nucleocapsids only when it is available in a soluble complex with the P protein. The association of the N and P proteins prevents non-specific binding of N protein to cellular RNA and allowing the N protein to bind selectively to the

genome leader and antigenome trailer sequences. It has been shown that the first 25 amino acids of N-terminus of NDV N protein is involved in forming soluble complex with the P protein (Kho et al., 2004). The N protein interacts with P and L proteins during transcription and replication and with M protein during virus assembly.

P, V and W proteins: The P protein produced from unedited P mRNA is acidic and heavily phosphorylated at specific serine and threonine residues (Steward et al., 1993). The P protein along with N and L forms viral polymerase complex. It prevents nonspecific assembly or self aggregation of N protein. The P protein also acts as a chaperone to prevent uncontrolled encapsidation of non-viral RNA by the N protein. The complex of P with unassembled N monomer is believed to regulate the switch from transcription to replication. The non-structural V and W proteins are produced by cotranscriptional insertions of one or two G residues, respectively, at the editing site of the P gene ORF (Steward et al., 1993). The V protein has anti-interferon-alpha activity in NDV, while the function of W protein is still unknown (Huang et al., 2003).

L protein: The L protein is the largest structural protein but the least abundant protein in infected cells or in virions. It is the major component of the viral RNA dependent RNA polymerase (Tordo et al., 1988). The L protein possess all the enzymatic activities necessary for synthesis of viral mRNAs and genomic RNA replication, including nucleotide polymerization, mRNA capping and polyadenylation of mRNAs. Furthermore, the L protein has been associated with the virulence of NDV (Rout and Samal, 2008).

2.5.2 Matrix protein

The M protein is the most abundant protein in the virion. It functions as the central organizer of viral morphogenesis and has been found to interact with the cytoplasmic tails of the integral membrane proteins, the lipid bilayer and the nucleocapsid. The self-association of M protein and its affinity to interact with the nucleocapsid might be the driving force for forming a budding virus particle (Peeples, 1991). In addition, the M protein may also play a role in transport of viral components to the plasma membrane (Takimoto and Portner, 2004).

2.5.3 Envelope glycoproteins

APMV-3 envelope has two integral membrane glycoproteins namely, the fusion (F) glycoprotein that mediates pH-independent fusion of the viral envelope with the plasma membrane of the host cell and the hemagglutinin-neuraminidase (HN) glycoprotein that aides in the attachment of the virus to host cell membrane.

The hemagglutinin-neuraminidase (HN) protein

The HN protein is a multifunctional protein. It is a type II integral membrane protein and is responsible for attachment of the virion to sialic acid-containing cell surface receptors. In addition, HN has neuraminidase activity (NA) that cleaves sialic acid from the surface of the infected cells, thereby, releasing progeny virions (Lamb and Parks, 2007). The HN protein also interacts with the F protein for fusion promotion activity (Morrison et al., 1991). HN exists as homotetramers, the ectodomain consisting of a long stalk and a

terminal globular head that help in attachment. In addition, globular head also has NA activities and all known antibody binding sites (Mirza et al., 1993).

The Fusion (F) protein: The F glycoprotein mediates entry of the virus into the host cell by fusion of the viral envelope to the plasma membrane in a pH independent manner. It is a type I integral membrane protein and is synthesized as an inactive precursor (F_0), which is then cleaved in the trans-Golgi membranes to F_2 and F_1 subunits derived from the amino terminal and carboxyl terminal domains, respectively. The F_1 and F_2 subunits remain covalently linked to each other by disulfide bonds (Morrison, 2003). the F protein is a trimer consisting of a globular head held distal from the viral membrane by stalk and neck regions (Chen et al., 2001).

2.6 Stages of APMV-3 replication

The replication of APMV-3 is very similar to that of other paramyxoviruses (Fig. 2.3).

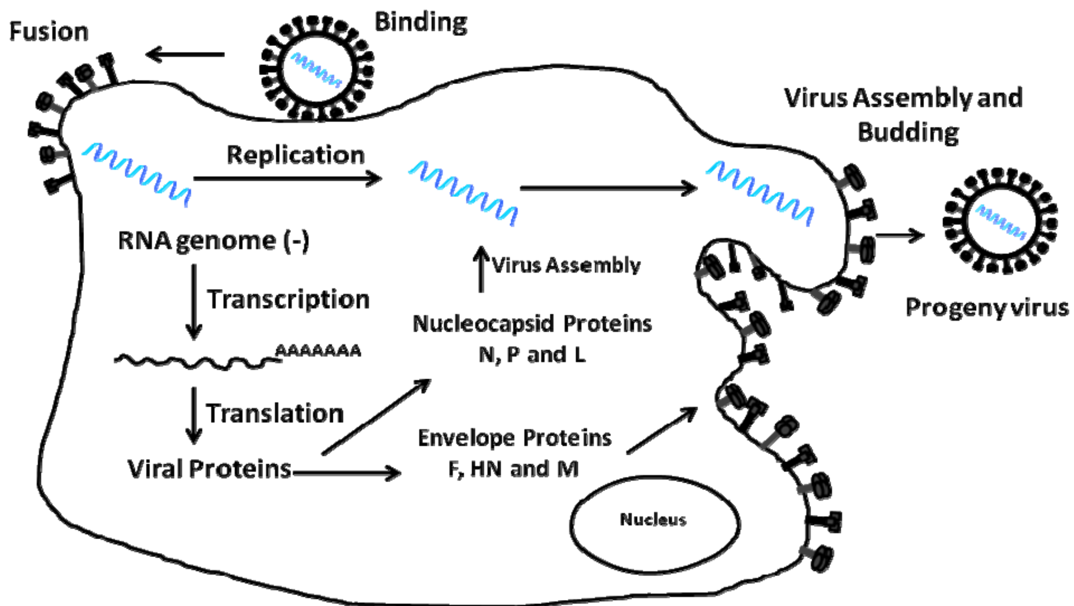


Fig. 2.3. Stages of replication in a avian paramyxovirus. Schematic diagram of different stages of replication in a paramyxovirus.

The initial step of APMV-3 infection is the attachment of the virus to sialic acid containing receptors at the host cell surface that is mediated by HN protein. The next step is fusion of viral envelope with the host cell plasma membrane, which is mediated by F protein and subsequently the release of the viral nucleocapsid into the host cell cytoplasm. In the cytoplasm of the host cell, the viral genome is transcribed into mRNAs and translated to viral proteins. The viral RNA-dependent RNA polymerase (RDRP) complex enters the 3' end of viral genome promoter, i.e. leader, and synthesizes short (+) strand leader RNA followed by re-initiation of N gene mRNA synthesis from N gene start sequence. The transcription in most cases terminates at the GE sequence, but some of RDRP continues to transcribe the downstream genes (Fig. 2.4). The sequential start and stop mechanism results in a gradient mRNA production with higher concentration of the most 3' proximal gene than those of downstream (Cattaneo et al., 1987).

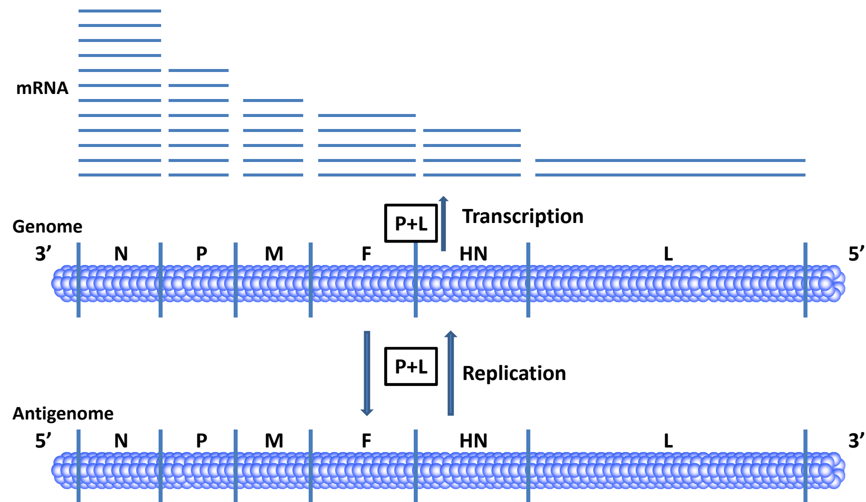


Fig. 2.4. Schematic diagram of RNA replication and transcription in avian paramyxoviruses.

Schematic diagram showing transcription and replication of a paramyxovirus. Genome and antigenome are shown encapsidated by nucleocapsid protein subunits (small spheres). The vertical lines indicate the gene junctions. The polymerase complex (P-L complex) transcribes the genome to yield capped and poly A-tailed mRNAs. When sufficient amount of viral nucleocapsid protein levels are achieved, the viral polymerase switches from its transcription mode to replicative mode to produce antigenome, which serves as the template for the synthesis of the progeny viral genome.

The proteins N, P, and L are required for nucleocapsid assembly. The positive-sense nucleocapsid "antigenome" is then used as template for synthesis of progeny genomic RNAs.

The N protein subunits encapsidate the viral genomic RNA and form a helical ribonucleoprotein (RNP) structure. Subsequently, the P and L proteins loosely bind to the RNP forming transcriptase complex (Kingsbury, 1972). The membrane glycoproteins (F and HN) synthesized on rough endoplasmic reticulum (ER) undergoes conformational

maturation before getting transported to the cell surface following post translational modifications, such as modification of carbohydrate chains of HN protein and cleavage of F protein (Feller et al., 1969). The M protein has a major role in bringing the assembled RNP core to the appropriate place at the plasma membrane (Peeples, 1991). The cytoplasmic tails of F and HN glycoproteins interact with M protein, which in turn, associates with the nucleocapsid thus helping the process of budding of the mature virions.

2.7 Epidemiology of APMV-3 infections

APMV-3 has been isolated from wild and domesticated birds in different parts of the world (Tumova et al., 1979b; Alexander, 1980; Macpherson et al., 1983; Andral and Toquin, 1984; Zeydanli et al., 1988; Alexander, 2003a). The APMV-3 strain parakeet/Netherlands/449/75, isolated from parakeets in the Netherlands, is the prototype for the entire serotype (Alexander and Chettle, 1978). Recently, APMV-3 was isolated from ostrich, indicating a wide host range for the virus (Kaleta et al., 2010). The virus has been isolated from diseased turkeys associated with coughing, nasal discharge and swelling of the infra-orbital sinus (Redmann et al., 1991). APMV-3 has been associated with encephalitis and high mortality in caged birds (Tumova et al., 1979b). The virus causes acute pancreatitis and central nervous system (CNS) symptoms in *Psittacine* and *Passerine* birds (Beck et al., 2003). APMV-3 also infects chickens at an early age, with evidence of stunting growth that may be more marked in broiler chicken breeds (Alexander and Collins, 1982). In terms of pathogenicity to domestic poultry birds, APMV-3 probably is second in importance to NDV.

2.8 Reverse genetics

Reverse genetics is a method by which infectious viruses can be generated entirely from cloned DNA and provides a method for introducing desired changes into the viral genome. Reverse genetics is a useful method for identifying the functions of viral genes. Development of reverse genetics methods for negative-strand RNA viruses has led not only to study the structure and function of viral genes and their proteins, but also to engineer live vaccines and vaccine vectors. Most of the systems for recovery of negative-strand RNA viruses are based on co-transfection of a plasmid expressing full-length antigenomic RNA with three other support plasmids encoding viral N, P and L proteins all under the control of bacteriophage T7 RNA polymerase promoter. The T7 RNA polymerase was provided by either a recombinant vaccinia virus expressing the T7 gene or a cell line constitutively expressing the T7 polymerase.

Many viruses, such as rabies virus (Schnell et al., 1994), vesicular stomatitis virus (Lawson et al., 1995), simian virus 5 (He et al., 1997), human respiratory syncytial virus (Collins et al., 1995), sendai virus (Garcin et al., 1995), rinderpest virus (Baron and Barrett, 1997), parainfluenza virus (Hoffman and Banerjee, 1997), measles virus (Radecke et al., 1995), and Nipah virus (Yoneda et al., 2006) were successfully recovered using this approach. This system has also been used for recovery of different pathotypes of NDV strains: (1) lentogenic strains LaSota (Peeters et al., 1999; Romer-Oberdorfer et al., 1999; Huang et al., 2001) and B1 (Nakaya et al., 2001); (2) mesogenic strains Beaudette C (Krishnamurthy et al., 2000)and Anhinga (Estevez et al., 2007); and (3) velogenic strains Herts 33 (de Leeuw et al., 2005), ZJ1 (Liu et al., 2007) and Texas GB

(Paldurai et al., 2010). The reverse genetics system has been used to study the molecular determinants of viral pathogenesis and to develop vaccines and vaccine vectors against emerging pathogens.

Chapter 3

3.1 Title

Complete genome sequence of avian paramyxovirus type 3 strain parakeet/Netherlands/449/75.

3.2 Abstract

The complete genome sequence was determined for prototype parakeet/Netherlands/449/75 strain of avian paramyxovirus (APMV) serotype 3. The genome is 16,272 nucleotides (nt) in length, consisting of six non-overlapping genes in the order of 3'-N-P/V/W-M-F-HN-L-5', with intergenic regions of 31-63 nt. APMV-3 genome follows the "rule of six" and is the largest among the avian paramyxoviruses reported to date, with a trailer region of 707 nt, the longest in the family *Paramyxoviridae*. The cleavage site of F protein, A-R-P-R-G-R↓L, does not conform to the preferred cleavage site of the ubiquitous cellular protease furin. Therefore, exogenous protease was needed for replication *in vitro*. Alignment and phylogenetic analysis of the predicted amino acid sequences of strain Netherlands proteins with the cognate proteins of viruses of all of the five genera of family *Paramyxoviridae* showed that APMV-3 strain Netherlands is more closely related to APMV-1 than APMV-6.

3.3 Introduction

The family *Paramyxoviridae* includes viruses of many species of avian, terrestrial and aquatic animals isolated worldwide (Lamb and Parks, 2007; Nylund et al., 2008). Members of this family are characterized by pleomorphic enveloped particles that contain a single-stranded, negative sense RNA genome (Lamb and Parks, 2007). The family is

divided into two subfamilies, *Paramyxovirinae* and *Pneumovirinae*. Subfamily *Paramyxovirinae* is further divided into five genera: *Rubulavirus* (including mumps virus, human parainfluenza viruses [HPIV] 2 and 4, simian virus type 5 [SV5] and Tioman virus [TiV]), *Respirovirus* (including Sendai virus [SeV] and HPIV-1 and 3), *Henipavirus* (comprising Hendra virus [HeV] and Nipah virus [NiV]), *Morbillivirus* (including measles [MeV] and canine distemper [CDV] viruses), and *Avulavirus* (comprising avian paramyxovirus [APMV] serotypes 1 to 9). Subfamily *Pneumovirinae* is divided into two genera: *Pneumovirus* (comprising human respiratory syncytial virus [HRSV] and its animal counterparts including bovine respiratory syncytial virus [BRSV]), and *Metapneumovirus* (comprising human metapneumovirus [HMPV] and avian metapneumovirus [AMPV]) (Rima et al., 1995; Mayo and Pringle, 1998; Pringle, 1998; Lamb et al., 2005).

The genomes of members of family *Paramyxoviridae* range in length from 15-19 kb and contain six to ten genes in a linear array (Lamb and Parks, 2007). Transcription begins at a single promoter at the 3' leader end and the genes are copied into individual mRNAs by a start-stop-restart mechanism guided by conserved gene-start and gene-end transcription signals that border the individual genes (Lamb and Parks, 2007). Genome replication involves the synthesis of a complete positive-sense copy of the genome that is called the antigenome and serves as a template for producing progeny genomes. All members of family *Paramyxoviridae* encode a nucleocapsid protein (N), a phosphoprotein (P), a matrix protein (M), a fusion protein (F), an attachment protein called the hemagglutinin (H) or haemagglutinin-neuraminidase (HN) or glycoprotein (G), and a large polymerase protein (L).

Many paramyxoviruses encode additional proteins that play important roles in viral morphogenesis, RNA synthesis, and pathogenesis (Lamb and Parks, 2007). For example, most members of subfamily *Paramyxovirinae* encode additional proteins from the P gene by RNA editing. This involves the introduction of one or more G residues into a subset of the P transcripts by polymerase stuttering at a specific editing sequence motif. This shifts the reading frame to access alternative ORFs in one or both of the alternative frames, resulting in the expression of proteins in which the upstream domain is P-derived and the downstream domain is derived from the alternative frame. In one such protein, called V, the downstream domain contains a cysteine-rich motif that is highly conserved across the subfamily. The V protein has been implicated in the regulation of viral RNA synthesis (Horikami et al., 1996; Lin et al., 2005) and in counteracting host antiviral responses (Goodbourn et al., 2000). All members of subfamily *Paramyxovirinae* follow the "rule of six", whereby efficient RNA replication depends on the genome length being an even multiple of six (Calain and Roux, 1993; Samal and Collins, 1996; Kolakofsky et al., 1998).

The APMVs have been classified into nine different serotypes based on haemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays (Alexander and Collins, 1984). Newcastle disease virus (NDV) belongs to serotype 1. It is the most extensively-characterized APMV and is a major pathogen of poultry worldwide (Alexander, 1980). APMV-2 has been isolated from chickens, turkeys and wild birds, and is responsible for causing decreases in egg production and fertility in turkeys (Lipkind et al., 1979; Tumova et al., 1979a). The pathogenicity of APMV 4-9 is generally unknown except for a few isolated reports (Alexander, 1982). APMV-3 was first isolated from a

turkey in 1968 in Wisconsin. Since then, many APMV-3 strains have been isolated from turkeys in different parts of the world including Canada, the United States (Tumova et al., 1979a) and England (Macpherson et al., 1983). The APMV-3 strain parakeet/Netherlands/449/75, isolated from parakeets in the Netherlands, is the prototype for the entire serotype (Alexander and Chettle, 1978). APMV-3 was also isolated from non-domesticated species such as *Psittaciformes* and *Passeriformes* (Alexander, 1980). APMV-3 has been associated with encephalitis and high mortality in caged birds, and with respiratory disease in turkeys (Tumova et al., 1979a). The virus causes acute pancreatitis and CNS symptoms in psittacine and *Passeriformes* birds (Beck et al., 2003). APMV-3 also infects chickens at an early age, with evidence of stunting growth that may be more marked in broiler chicken breeds (Alexander and Collins, 1982). In terms of pathogenicity, APMV-3 probably is second in importance only to NDV.

An understanding of the molecular and pathological characteristics of APMV-3 is of general interest and is important for developing vaccines against this virus. Here, I report the first complete genome sequence of APMV-3 strain parakeet/Netherlands/449/75. The genome was found to be the longest among the APMVs and has the longest trailer region in family *Paramyxoviridae*. APMV-3 strain Netherlands would be predicted to be avirulent based on its F protein cleavage site and its observed requirement for exogenous protease for replication *in vitro*.

3.4 Materials and Methods

3.4.1 Virus and cells

APMV-3 strain parakeet/Netherlands/449/75 was obtained from the National Veterinary Services Laboratory, Ames, Iowa. Nine-day-old specific pathogen free (SPF)

embryonated chicken eggs and the chicken embryo fibroblast DF1 cells were used to propagate APMV-3 strain Netherlands. Hemagglutination (HA) titers of virus stocks were determined using 0.5% chicken RBC at room temperature. The ability of the virus to replicate in cell culture was examined in nine different established cell lines, each representing a different species of origin: DF1, chicken embryo fibroblast; QT35, Quail fibrosarcoma; MDBK, Madin Darby Bovine Kidney; MDCK, Madin Darby Canine Kidney; Vero, African green monkey kidney; HEp2, Human Epidermoid carcinoma; PK15, Pig Kidney; CEF, Chicken Embryo Fibroblast; and BHK21, Baby Hamster Kidney. All the cells, except DF1 cells, were grown in Eagle's minimum essential media (MEM) with 10% fetal calf serum (FCS), while DF1 cells were grown in Dulbecco's MEM containing 10% FCS. All the cells were grown in a 37°C incubator with 5% CO_2. Cell monolayers were infected with a 10^{-3} dilution of 2^9 HA units of egg-grown APMV-3 strain Netherlands and, after 1 hr of adsorption, the viral inoculum was replaced with maintenance media containing 2% FCS. The growth of virus in each cell type was observed with and without the presence of 10% allantoic fluid, or 1-5 µg/ml of acetyl trypsin/ml (Gibco), or 1-5 µg/ml of α-chymotrypsin/ml (Sigma), each of which provided a source of protease for cleavage of the F protein if necessary. The cells were observed daily for cytopathic effects (CPE) and HA titers were recorded every 24 h until the fifth day post-infection. The virus titer in each cell type was quantified by plaque assay in DF 1 cells as previously described (Krishnamurthy et al., 2000). The cells were overlaid with DMEM containing 2% FCS and 0.9% methylcellulose that were further supplemented with 10% allantoic fluid as a source of proteases. The plaques were visualized by staining with 1% crystal violet after 4 days of infection.

3.4.2 Virus RNA isolation and sequence analysis

Total RNA from virus-infected DF1 cells was isolated using TRIZOL reagent, following the manufacturer's instructions (Invitrogen, USA). Viral RNA also was isolated from allantoic fluid collected from virus-infected eggs, using RNeasy kit according to the manufacturer's instruction (QIAGEN, USA).

Most of the APMV-3 genome, except the 3' and 5' termini, was copied and amplified into cDNA using (i) gene-specific primers designed from the known APMV-3 M gene sequence, and (ii) consensus primers designed by using published *Rubulavirus, Respirovirus* and *Morbillivirus* genome sequences (Table 3.1). All primers were synthesized by a commercial provider (IDT, USA). N 787 forward and N 1022 reverse consensus primers were used to amplify a 235 base pair (bp) fragment that served as one starting point for downstream genome walking (Chang et al., 2001). The primer designed from the available M gene sequence (GenBank Accession number **AY137207**) was used in conjunction with a consensus sequence designed for the F gene, F 350 reverse, to amplify a 800-bp product and F 842 reverse, to amplify a 1500-bp product that were used as a second starting point for downstream genome walking. The HN and L genes were amplified by using gene-start forward (GS forward) primers and consensus reverse primer (Table 3.1). Initial 500 nt in HN gene was amplified by consensus GS forward and consensus HN550 reverse primer (Table 3.1), further downstream was amplified by HN gene-specific forward primer (HN428F correspond to 7047-7068 in antigenome sequence) and consensus HN1568 reverse primer. Furthermore, upstream of L gene was amplified by gene-specific HN forward primer (HN960F correspond to 7579-7603 in antigenome sequence) and consensus L1460 reverse primer (Table 3.1). Subsequently,

downstream of L gene was amplified by L gene-specific forward and consensus L reverse primers listed in table 3.1.

Position in gene	Primer sequences
GS forward	5'WWWWAGGAGCGGAAGG3'
NP787forward	5'AAYRCSGGKMTKRCWSCWTTCTT3'
NP1022reverse	5'GWWSCYAYWCCCATKGCAWA3'
F350reverse	5'GCNGCNGTNGCNACNCCNARNGC3'
P113forward	5'AGGTTCTGAGCAAGCAAAGTATT3'
P137forward	5'CTCCCCTGAACCAAGCACCTCTAC3'
P506reverse	5'ATTGTCTGTGAGTGGGGATTCCG3'
P551reverse	5'GGGGTTACTCCACGTTTCGGTTTG3'
F842reverse	5'GAGTTACCTGWATAMCCAWGAKTT3'
HN1568reverse	5'AGTKGWTGWTGWGTATGCTGCTT3'
HN550reverse	5'ATNACRTTRTGNGTRTARCACCA3'
L1460reverse	5'ATNGCYTTRTCYTTNARRAA3'
L1917reverse	5'CCARTTNARRCARTAYTTYTGNARRTC3'
L2142reverse	5'ATNSWDATCATNGTCCACAT3'
L4400reverse	5'ACNCKNARRTARTANARYTGRTANGC3'
L5325reverse	5'GCNCCNSWNCCYTCNGCNARRTA3'

Table 3.1. Different primers used to amplify APMV-3 genome based on consensus sequences. The primer name represents an individual gene, position from gene-start and forward or reverse from left to right, different unconventional codes represent a mixture of nucleotides (N=A/T/C/G, R= A/G, K= G/T, Y=C/T, S=G/C, W=A/T, D= A/G/T and M=A/C). Sequences used for alignment were taken from published NCBI Genbank NC_003043 for APMV-6, NC_002617 for NDV, NC_003443 for HPIV-2, NC_006430 for SV5, NC_004074 for Tioman virus, NC_001552 for Sendai virus, NC_001796 for HPIV-3, AY988601 for Nipah virus, NC_001906 for Hendra virus NC_001498 for Measles virus, NC_001921 for Canine distemper virus and NC_007454 for J virus.

The sequence of the 3' terminus was determined from cDNA prepared by rapid amplification of cDNA ends (RACE) (Troutt et al., 1992; Li et al., 2005). Genomic RNA obtained from partial purified virus was ligated to adopter1 (5' GGTTTTGCGGTAAAGGTGGAAGAGAAG 3'), using T4 RNA ligase according to the manufacturer's instructions (Invitrogen). The ligated RNA was purified and reverse-

transcribed, using adopter2 (5’ CCAAAACGCCATTTCCACCTTCTCTTC 3’) primer according to the manufacturer’s instructions (Invitrogen). PCR was carried out with adopter 2 and a gene-specific reverse primer (complementary to nucleotides 450 to 472 in the antigenome sequence), using recombinant Taq polymerase (Invitrogen).

The sequence of the 5’ end of the genome was determined from cDNA obtained by 5’ RACE (Invitrogen). Briefly, the downstream end of the L gene and adjoining trailer region were reverse-transcribed using a gene-specific forward primer L6760F representing nucleotides 15411-15433 in the antigenome sequence. The resulting cDNAs were column-purified and tailed with dCTP or, in a parallel reaction, with dGTP, using terminal deoxynucleotide tranferase (TdT, Invitrogen). The cDNAs were then amplified in separate reactions, using an L gene-specific forward primer L7308F representing nucleotides 15959-15981 in the antigenome sequence, and an anchored G primer (provided in the kit) in the case of product tailed with dC, or with oligo dC in the case of product tailed with dG.

PCR-amplified products and plasmid DNAs were sequenced by using BigDye terminator v 3.1 matrix standard kit (Applied Biosystem) and 3130xl genetic analyzer data collection software v3.0 (Applied Biosystem). All sequencing was done in a 3130xl genetic analyzer or 3100 Avant genetic analyzer, using the manufacturer’s instructions. The entire genome was sequenced at least three times, and at least once from uncloned PCR product, to ensure a consensus sequence.

3.4.3 Sequence and phylogenetic tree analysis

Sequence similarity searches were conducted using the basic length alignment search tool (BLAST) from the National Center for Biotechnology Information (NCBI).

DNA pair-wise alignment was done using MagAlign (clustalW) in a Lasergene6 software package. To construct phylogenetic trees and determine divergence among different genus, amino acid sequences were aligned using clustalW multiple alignment algorithm. Modified data sets were generated by bootstrapping samples using a Seqboot program, and modified data were analyzed by Protodist and Fitch program (Phylip 3.67).

3.4.4 Database accession numbers

The complete genome sequence of APMV-3 strain Netherlands has been submitted in GenBank under accession number **EU403085**. Different sequences from GenBank were used for designing primers and comparing the APMV-3 genome. These include NC_003043 for APMV-6, **NC_002617** for NDV, **NC_003443** for hPIV2, NC_006430 for SV5, **NC_004074** for Tioman virus, NC_001552 for Sendai virus, **NC_001796** for hPIV3, **NC_003461** for hPIV1, AY988601 for Nipah virus, **NC_001906** for Hendra virus **NC_001498** for Measles virus, NC_001921 for Canine distemper virus and **NC_007454** for J virus.

3.5 Results

3.5.1 *In vitro* growth characteristics of APMV-3 strain Netherlands

APMV-3 strain Netherlands produced a titer of 2^8-2^{10} HA units in nine-day-old embryonated SPF chickens eggs four days post inoculation. It was necessary to include 10% allantoic fluid for replication of the virus in cell culture, indicating a requirement of external proteases for efficient cleavage of the F protein. Eight out of nine cell lines were found to support growth of APMV-3, as determined by observable CPE and HA activity of the infected cell culture supernatant (data not shown). In each of these cell lines, it was

necessary to include 10% allantoic fluid for replication of the virus in cell culture, indicating a requirement of external proteases for efficient cleavage of the F protein. The virus failed to grow in MDCK cells even after three serial passages. The virus grew most efficiently in MDBK cells, followed by chicken embryo fibroblast, BHK21 and Vero cells. The peak HA titers of the different cell lines tested were: MDBK, 2^7; BHK21, 2^6; CEF, 2^6; Vero, 2^5; PK15, 2^3; DF1, 2^3; QT35, 2^2; and HEp2, 2^2. Viral CPE involved rounding, detachment and syncytia formation. APMV-3 was able to produce distinct plaques under methylcellulose overlay in DF1 cells in the presence of 10% allantoic fluid. The growth of APMV-3 in different cell lines was evident after the addition of allantoic fluid which was not substituted by the addition of either trypsin or chymotyrpsin in the medium.

Examination of partially-purified virus under an electron microscope showed that the particles were enveloped, pleomorphic but mostly spherical in shape, with a size ranging from 150-250 nm (data not shown).

3.5.2 Determination of the complete genome sequence of APMV-3 strain Netherlands

The genome of APMV-3 strain Netherlands consists of 16,272 nt (GenBank accession number **EU403085**). The genome organization is very similar to those of other members of family *Paramyxoviridae*. The genome encodes for six open reading frames (ORF) corresponding to putative proteins N, P, M, F, HN and L, from the 3’ to 5’ end of the genome. In addition, there is evidence of a cysteine-rich V ORF in the P coding region. 84.1% of the genome codes for protein, which is less than the average coding percentage (92%) of other members of subfamily *Paramyxovirinae* (Miller et al., 2003).

The length, position and characteristics of the six genes and their intergenic sequences (IGS) are summarized in Tables 3.2 and 3.3 and described in detail below.

Genes	Hexamer phasing Position at gene start	mRNA characteristics (nt)				Intergenic Sequence nt	Deduced protein characteristics		
		Total length	5'UTR	ORF	3'UTR		Size(aa)	MW (kDa)	p*I*
N	2	1604	50	1374	182	55	457	50.9	5.540
P/V(P)	5	1378	42	1161	175	63	386	41.0	5.660
P/V(V)	5	1379	42	747	590	-	248	26.6	4.749
P/V(W)	5	1380	42	378	960	-	125	13.3	4.451
M	5	1478	89	1191	198	31	396	44.1	9.216
F	2	1922	101	1632	189	34	543	59.5	8.318
HN	2	1974	43	1734	197	59	577	63.8	7.830
L	1	6913	25	6597	291	-	2198	248.7	7.010

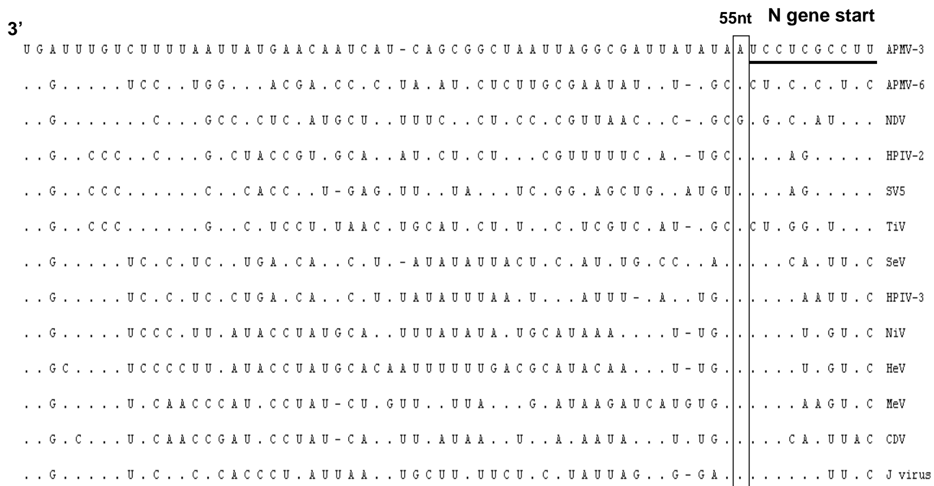
Table 3.2. Genomic features and protein characteristics of APMV-3 strain Netherlands. The table shows hexamer phasing position along with individual genes, coding and non-coding, and intergenic sequences with their protein profiles.

Gene	Gene-start	Gene-end	Intergenic sequence
N	UCCUCGCCUU	AAUUAUUUUUU	CCGAAAGCUCGUGUCUGUCUCUAGGACU GUAUUAAGUCGGCCCCCCUAAGUUGAA
P	UCCUCGCCUU	AA**A**UAUUUUUU	GAGGAUCUAGAUUGUACCUCCGUCUGGC UCGAUUACGCCUCCCUCAUGUGUUCCCC UUAGUU
M	UCCUCGCCUU	AAUUAUUUUUU	GUAACACUCUUACGUUAAAUUCCUGCU UUCA
F	UCCUCGCCUU	A**U**U**A**AUUUUUU	GGAGUUCCACUGGGUAGGACCGGGUAG GCUAACC
HN	UCCUCGCCUU	AAUUAUUUUUU	AGGAGAUAUAUUCUAGGUCUGCUUCCG AUAUAUUCGAUUAUCGAAUCCUACCUU UGCUG
L	UCCUCGCCUU	AAUUA**A**UUUUU	

Table 3.3. Gene-start, gene-end and intergenic sequences of APMV-3 strain Netherlands. The underline nucleotide shows the difference from conserved gene-end sequence.

The 3' leader sequence of APMV-3 consists of 55 nt, a length that is conserved among almost all the members of subfamily *Paramyxovirinae* (Krishnamurthy and Samal, 1998). The similarity of APMV-3 leader sequence to that of NDV and APMV-6 indicated similar promoter recognition patterns for both viruses (Fig 3.1a).

3.1 a)



3.1 b)

APMV-3 5'**ACUAAACAAAAA**GUUAUAAUGAUUUUUAAAUCAGUCUUCAACCUUUUG
NDV 5'**ACCAAACAAAGA**UUUGGUGAAUGACGAGACUACACUCAAGAAUAAUUG
APMV-6 5'**ACCAAACAAGGA**AAUCAUAAGCUUUUUGACUAUGAUCCUUGUCGACUU
SV5 5'ACCAAGGGGAAAACCAAGAUUAAUCCUCUUC-----------------
HPIV-2 5'ACCAAGGGGAAAAUCAAUAUG---------------------------
TiV 5'ACCAAGGGGAAAAUCAAUAUAGG-------------------------

3.1 c)

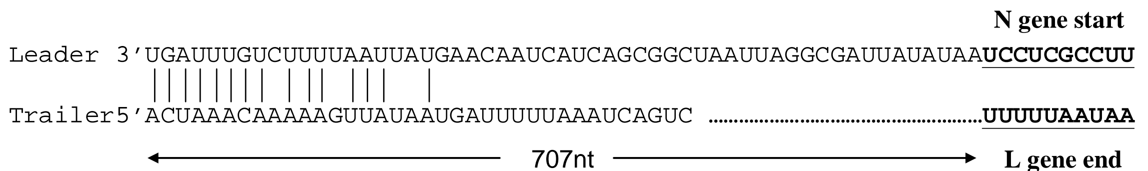


Fig. 3.1. Alignment of the (a) 3' leader and N gene start sequence. Dots indicate identity with the assignment in APMV-3 (Top line). (b) 5' trailer regions of APMV-3 strain Netherlands with other avian paramyxoviruses and Rubulaviruses. (c) Complementary of 3'leader and 5' trailer regions of APMV-3 strain Netherlands. Abbreviations: NDV, Newcastle disease virus(APMV-1); SV5, Simian virus 5; APMV, Avian paramyxovirus; HPIV, Human parainfluenza virus; TiV, Tioman virus; SeV, Sendai virus; NiV, Nipah virus; HeV, Hendra virus; MeV, Measles virus; CDV, canine distemper virus.

Surprisingly, the length of the APMV-3 5' trailer sequence is 707 nt, which is the longest among the members of family *Paramyxoviridae* sequenced to date. Also, the nucleotide assignment at the third position from the 3' leader (3'UGAU) and 5' trailer (ACUA 5') termini was different from those found in other APMVs and other members of subfamily *Paramyxovirinae*. Otherwise, the sequence of the genomic termini of APMV-3 strain Netherlands are closely related to those of APMV-1 and -6, but are different from those of the rubulaviruses, consistent with the classification of avian paramyxoviruses in the genus *Avulavirus* (Fig 3.1b). The sequences of the 3'leader and 5'trailer termini showed a high degree of complementarity (87.5 percent complementarity for the first 16 nt), suggestive of conserved elements in the 3' promoter regions of the genome and antigenome (Fig 3.1c). The gene-start sequence of APMV-3 strain Netherlands is 3'UCCUCGCCUU and is conserved exactly among the six genes (Table 3.3). The gene-end signal is slightly less well-conserved: the consensus gene-end sequence is 3'AAUUA$(U)_6$, but among three of the six signals there is a total of four nt differences involving positions 2, 3, 4, and 6 (Table 3.3). The intergenic sequences of APMV-3 range in length from 31 to 63 nt.

The nucleocapsid (N) gene

The APMV-3 N gene is 1604 nt long and encodes an N protein of 457 amino acid. It contains a highly-conserved sequence motif, 322-FAPGNYSLLYSYAMG-336 (F-X4-Y-X3-α-S- α-AMG, where X is any amino acid and α is any aromatic amino acid), that corresponds to a motif previously identified in the central region of the N protein of other members of *Paramyxovirinae* and which has been implicated in the self assembly of N with RNA or with other N monomers (Yu et al., 1998; Lamb and Parks, 2007). The APMV-3 N protein has 39-41% amino acid sequence identity with that of APMV-1, 38.3% with APMV-6, 32-34% with *Rubulavirus*, 21-22% with *Respirovirus*, 26% with *Henipavirus*, 24% with *Morbillivirus* and 25.4% with J virus.

The phosphoprotein (P) gene

The APMV-3 P gene is 1378 nt long. The unedited version of the P mRNA encodes a P protein of 386 amino acids. The predicted P protein contains an abundance of phosphorylation sites, with 19 serine and 6 threonine residues identified as potential phosphorylation sites using NetPhos 2.0 software. The APMV-3 P protein has 22.8% amino acid sequence identity with that of APMV-1, 19% with APMV-6, 18%-21% with *Rubulavirus*, 9-12% with *Respirovirus*, 13-15% with *Henipavirus*, 12% with *Morbillivirus* and 11.4% with J virus.

The P gene contains a putative editing site, 5'- TTTTTAAAGGGGG (mRNA sense), at positions 362-374 in the P gene (positions 2076-2088 in the complete genome sequence), which showed a high degree of sequence identity with the editing sites in other members of subfamily *Paramyxovirinae* (Fig 3.2). The insertion of a single G residue (mRNA sense) at the editing site would shift the reading frame to encode a 248-

amino acid V protein in which the N-terminal 112 amino acids were derived from P, and the downstream 136 amino acids include seven cysteine residues and one tryptophan residue that are highly conserved within *Paramyxovirinae* (Fig 3.3). Alternatively, the insertion of two G residues would shift the reading frame to access an internal ORF that is open for only 14 additional amino acids, yielding a 125-amino acid W protein.

Virus	Sequence	Genus
APMV-3-	TTAAAGGG	*Avulavirus*
APMV-6-	AAAAAGGG	
NDV-	AAAAAGGG	
SV5-	TAAGAGGG	*Rubulavirus*
HPIV-2-	TAAGAGGG	
TiV	TAAGAGGG	
HPIV-3-	AAAAAGGG	*Respirovirus*
SeV-	AAAAAGGG	
NiV-	AAAAAGGG	*Henipavirus*
HeV-	AAAAAGGG	
CDV-	AAAAAGGG	*Morbillivirus*
MeV-	AAAAAGGG	

Fig. 3.2. RNA editing site in P gene of selected members of the family *Paramyxoviridae*. Sequences are shown in positive mRNA sense. Addition of one and two G residues into the stretch of G residues leads to formation of V and W proteins.

```
                  ☆☆  ☆          ☆ ☆ ☆  ☆ ☆
APMV-3      184  TWCNPACSPVTAFPKQYKCACRQCPRFCDLCF  215
APMV-6      194  SWCNPSWSPIKAEPKQYPCFCGSFPPTCRLCA  225
NDV         194  SWCNPSCSPIRAEPRQYSCTCGSCPATCRLCA  225
HPIV-2      191  EWCNPRCAPVTASARKFTCTCGSCPSICGECE  222
SV5         188  EWCNPSCSPITAAARRFECTCHQCPVTCSECE  219
TiV         181  EWCNPICHPISQFTYRGTCRCGCCPDVCSLCE  212
NiV         425  EWCNPACSRITPLPRRQECQCGECPIECSHCC  456
HeV         423  EWCNPVCSRITPQPRKQECYCGECPTECSQCC  454
CDV         249  KWCNPICTQVNWGIIRAKCVCGECPPTCNECK  280
JVirus      253  EWCNPQCAPITVTPTQSRCTCGECPKVCARCI  284
```

Fig.3.3. Sequence alignment of C terminal end of V proteins of different members of subfamily *Paramyxovirinae*. Stars indicate conserved cysteine and tryptophan residues. Numbers in both side of sequence indicates the amino-acid position.

The matrix protein (M) gene

The M gene of APMV-3 is 1478 nt long and encodes a 396-amino-acid M protein. The protein is highly basic with an isoelectric point of 9.3, which may be important for ionic interaction with acidic N protein (Lamb and Parks, 2007). The bipartite cluster, which comprises clusters of basic amino acids—either 'single cluster', containing three to five basic amino acids, or 'bipartite' sequence, containing two interdependent clusters of such amino acids separated by an intervening sequence, is clearly seen in the positions 257-272, which was similar to that described previously (Coleman and Peeples, 1993). The M protein of APMV-3 contains a putative nuclear localization sequence (NLS) at position 371-383 aminoacids, which was previously found in the C terminal of NDV M protein (Coleman and Peeples, 1993; Shihmanter et al., 2005). . The APMV-3 M protein has 26.3% amino acid sequence identity with that of APMV-1, 25.8% with APMV-6, 22%-24% with *Rubulavirus*, 15-18% with *Respirovirus*, 13-14% with *Henipavirus*, 13-15% with *Morbillivirus* and 15.9% with J virus.

The fusion protein (F) gene

The APMV-3 F gene is 1922 nt long and encodes for a 543-amino-acid F protein. The protein is predicted to be a type I transmembrane protein, similar to the F proteins of other members of family *Paramyxoviridae*. It has a predicted signal anchor of residues 1-19 and a predicted transmembrane anchor of residues 491-513. The putative cleavage site of the APMV-3 F protein is A-<u>R</u>-P-<u>R</u>-G-<u>R</u>↓L (corresponding to 101-107 amino-acid position) (Fig 3.4).

Virus	Start	Sequence	End
APMV-3	101	A-**R**-P-**R**-G-**R**-L	107
NDV (Virulent)	111	G-**R**-**R**-Q-K-**R**-F	117
NDV (Avirulent)	111	G-G-**R**-Q-G-**R**-L	117
APMV-6	113	P-A-P-E-P-**R**-L	119
APMV-2	93	D-K-P-A-S-**R**-F	99
NiV	104	L-V-G-D-V-**R**-L	110
SV5	97	T-**R**-**R**-**R**-**R**-**R**-F	103
HPIV-2	101	K-T-**R**-Q-K-**R**-F	107
SeV	111	D-V-P-Q-S-**R**-F	117
HPIV-3	104	N-P-**R**-T-K-**R**-F	110
CDV	219	G-**R**-**R**-Q-**R**-**R**-F	225
MeV	107	S-**R**-**R**-H-K-**R**-F	113

Fig. 3.4. Comparison of the F protein cleavage site of different members of family *Paramyxoviridae*. Number of polybasic residues (R= Arginine) in some of the strains shown represents virulent cleavage site. The number represents the amino acid sequence covering the cleavage site.

The deduced amino acid sequence contains five putative N-linked glycosylation sites at positions 68 and 81 in the F2 subunit, and at positions 356, 435 and 485 in the F1 subunit, predicted using EXPASY software. The amino-acid sequence of APMV-3 contained heptad repeats located between position 105 to 189 similar to those of other fusion glycoproteins (Chambers et al., 1990). The F protein sequence of APMV-3 also contained an ALGVAT fusion peptide motif (amino acid positions 116-121 in F protein), which is conserved among other family members (Lamb and Parks, 2007). The APMV-3 F protein has 31.3% amino acid sequence identity with that of APMV-1, 30.6% with APMV-6, 29% with APMV-2, 24%-29% with *Rubulavirus*, 21-23% with *Respirovirus*, 21-23% with *Henipavirus*, 20-22% with *Morbillivirus* and 23.9% with J virus.

The hemagglutinin-neuraminaidase protein (HN) gene

The HN gene is 1974 nt long and encodes a polypeptide of 577 amino acids. The HN protein is a type II integral membrane protein that spans the membrane once and has a predicted hydrophobic signal anchor domain spanning residues 27-46. Seven potential N-linked glycosylation sites were identified at positions 32, 57, 122, 312, 325, 383 and 502, using the Expasy, NetNGlyc program. The HN protein contains a high amount of acidic amino acids with an isoelectric point (p*I*) of 7.83. The HN proteins of subfamily *Paramyxovirinae* and the HA proteins of influenza viruses contain a conserved sequence, N-R-K-S-K-S, which is thought to contribute to the sialic acid binding site (Varghese et al., 1983). The aminoacid sequence of APMV-3 HN protein contains a similar motif, N-R-K-S-C-S (237-243 amino-acid position in HN protein). The APMV-3 HN protein has 34% amino acid sequence identity with that of APMV-1, 31% with APMV-6, 31% with APMV-2, 40% with APMV-4, 18%-33% with *Rubulavirus*, 21-22% with *Respirovirus*, 16-17% with *Henipavirus*, 10-11% with *Morbillivirus* and 23% with J virus.

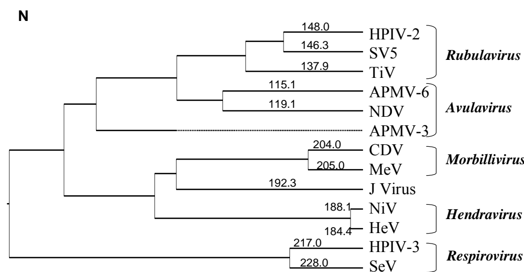
The large polymerase protein (L) gene

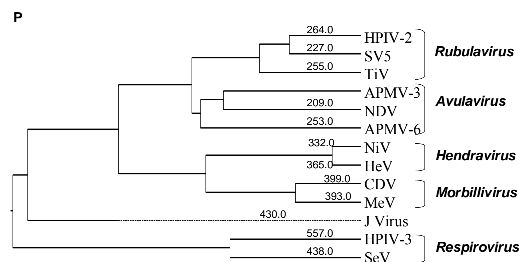
The L gene is 6913 nt long and encodes for 2198 amino-acids L protein. Alignment of L gene sequence of APMV-3 with other members of *Paramyxoviridae* demonstrated six linear conserved domains, which have been described earlier for these family members and represent catalytic domains (Poch et al., 1989). The conserved QGDNQ sequence that is found within domain III involved in transcription activity (Schnell and Conzelmann, 1995) was also found in L protein of APMV-3 at amino-acid positions 744-748. The APMV-3 L protein has 33% amino acid sequence identity with

that of APMV-1, 36% with APMV-6, 32-34% with *Rubulavirus*, 25-26% with *Respirovirus*, 25% with *Henipavirus*, 25-26% with *Morbillivirus* and 24.7% with J virus.

3.5.3 Phylogenetic analysis

Phylogenetic trees were generated from amino acid sequence alignments of N, P, M, F, HN and L proteins of APMV-3 strain Netherlands with the cognate proteins of representative viruses of all five genera of family *Paramyxoviridae* (Fig 3.5). Based on N, P, M, F, HN and L protein phylogenic trees, APMV-3 was phylogenetically closely related to NDV. Phylogenic analysis of HN protein showed that APMV-3 was more closely related to that of APMV-4. All six trees clearly indicated the close genetic relationship between APMV-3 and APMV-1.





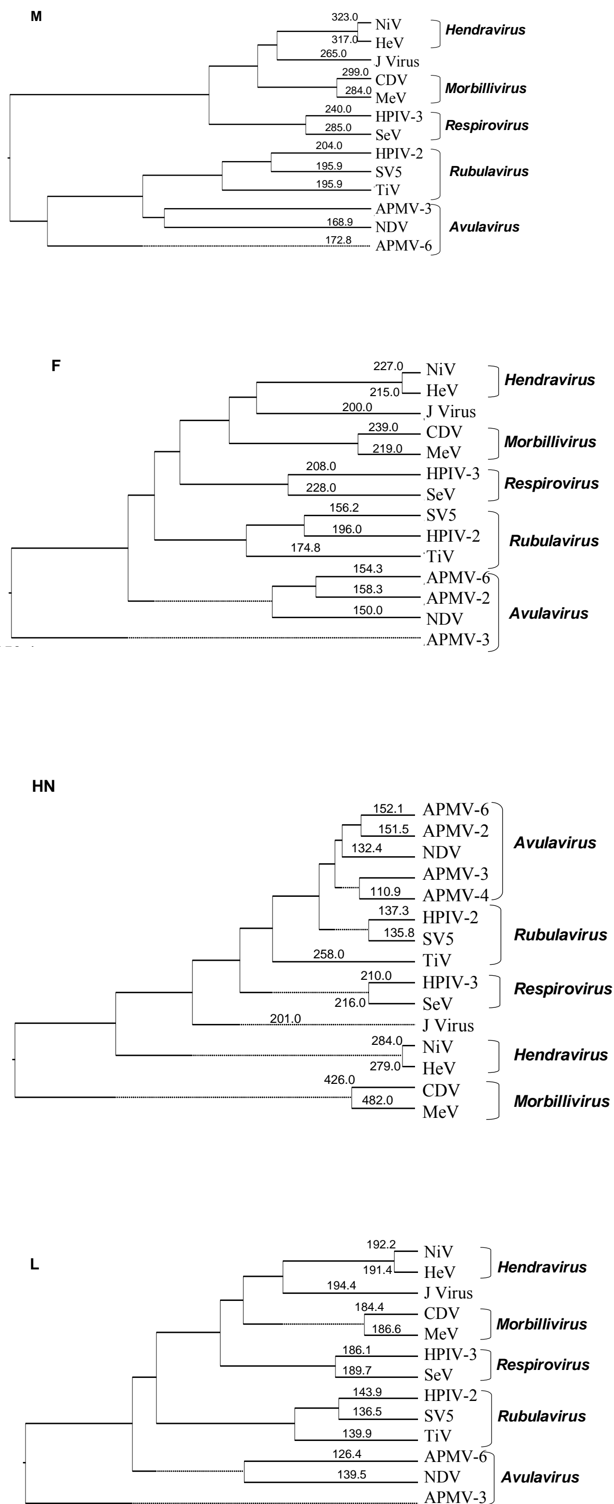


Fig. 3.5. Phylogenic analysis of N, P, M, F, HN and L proteins of members of family *Paramyxoviridae*. The phylogenetic trees were constructed using DNASTAR software package (Lasergene6).

3.6 Discussion

The genome of APMV-3 is 16,272 nt long, which is larger than the other two APMV serotypes sequenced to date, APMV-1 (15,186 nt, 15,192 nt and 15,198 nt) (Krishnamurthy and Samal, 1998; Czegledi et al., 2006; Ujvari, 2006) and APMV-6 (16,232 nt) (Chang et al., 2001). The APMV-3 genome length is also larger than the typical genome size (approximately 15,500 nt) of other members of family *Paramyxoviridae*, but is smaller than the 18,234 nt genome of Hendra virus (Wang et al., 2000) and the 18,954 nt genome of J virus, the largest genome reported to date for *Paramyxoviridae* (Jack et al., 2005). The genome length of APMV-3 was consistent with the "rule of six" that is a characteristic of the subfamily *Paramyxovirinae* (Calain and Roux, 1993). The genome contains six genes (N, P, M, F, HN and L) that are also found in all members of the subfamily. The amino acid sequence relatedness of APMV-3 proteins with the corresponding proteins of APMV-1 and APMV-6 clearly places this virus in the genus *Avulavirus*, consistent with the International Committee on Taxonomy of Viruses statement that amino acid sequence relationships are the main criteria for grouping viruses into genera within the family *Paramyxoviridae* (Lamb et al., 2005). APMV-3 has a 55 nt 3' leader region, which is exactly conserved in length with all other known members of *Paramyxoviridae* (Lamb and Parks, 2007), and its sequence is generally conserved within subfamily *Paramyxovirinae*. For members of subfamily *Paramyxovirinae*, the first 12-13 nt genomic termini are genus-specific and are used in classification of new isolates. Unexpectedly, however, the third nucleotide of the leader region of APMV-3 differed from that of other members of subfamily *Paramyxovirinae*. It

will be of interest to determine whether this difference is consistent in other strains of APMV-3 and might also be found in some of the other serotypes. In most members of family *Paramyxoviridae*, the 5' trailer region is 40-60 nt long (Shioda et al., 1986), although there are a few exceptions where the trailer region is somewhat longer: for example, 155 nt in RSV (Mink et al., 1991) and 159 nt in Mapuera virus (Wang et al., 2007). The trailer region of APMV-3 is unusually long (707 nt), and is the longest trailer among members of family *Paramyxoviridae* sequenced to date. RNA secondary structure analysis using Genebee (www.genebee.msu.su/genebee.html) software provided suggestive evidence of secondary structure in this region (not shown), although it is not clear whether a structure could form with an RNA that is completely encapsidated. Further analysis of the 707 nt trailer region did not reveal any open reading frame, indicating that it is not a part of the L gene which was truncated at some point in the evolution. Whether this unusually long trailer region has any functional significance can ultimately be determined using reverse genetics techniques.

The availability of the first sequence of a representative of APMV-3 described here, together with the recent description of complete sequence of representative of APMV-6 and the availability of complete genome sequences from APMV-1, provides the opportunity to assess the relatedness of these putative serotypes on a genome-wide basis. For example, APMV-3 Netherlands strain shares 41.7% and 42.2% nt sequence identity with representatives of APMV-1 and -6, respectively (a comparison made exclusive of the SH gene of APMV-6 and the long trailer of APMV-3). Amino acid sequence identity between APMV-3 versus -1 and -6 for the N protein was 39-41% and 38.3%, respectively; that involving P was 22.8% and 19%; that involving M was 26.3% and

25.8% respectively; that involving F was 31.3% and 30.6%, respectively; that involving HN was 36% and 31%, respectively (and 41% with HN of APMV-4, the sole reported sequence for that serotype); and that involving L was 33% and 36%, respectively. These values, together with the phylogenic analysis shown in Fig 3.5, show that APMV-3 strain Netherlands is more closely related to APMV-1 than APMV-6. This is consistent with the results of cross HI and NI tests, which suggested that the nine putative serotypes segregated into two broad, larger groupings, the first containing APMV-2 and -6, and the second APMV-1, -3, -4, -7, -8, and -9 (Lipkind and Shihmanter, 1986). Furthermore, the pattern of sequence relatedness outlined above also indicates that the overall extent of divergence among the proposed APMV serotypes is comparable to that among the HPIV serotypes. For comparison, I note the genomes of HPIV-1 and HPIV-3, both of *Respirovirus*, have 60.3% nt identity, and their proteins share the following percent amino acid sequence identities: N, 62; P, 26;M, 63; F, 45; HN, 49%, L, 61 (Newman et al., 2002). HPIV-1 and HPIV-2, representing different genera (*Respirovirus* versus *Rubulavirus*, respectively), share 42.9% nt sequence identity and share the following percent amino acid sequence identities: N, 25; M, 20; F, 24; HN, 28 L, 31 (Newman et al., 2002). Thus, the APMVs appear to be somewhat more divergent than the HPIV-1 and HPIV-3 difference and somewhat less divergent than the HPIV-1 and HPIV-2 difference. This supports the idea that APMV-1 through- 9 are indeed true serotypes. This is offered with the caveat that it will be important to examine cross-reactivity and cross-protection following experimental primary infections of the various APMV serotypes. In addition, it will be important to determine complete sequences for representatives of each of the APMV serotypes and to investigate potential diversity within the serotypes.

The F protein of members of *Paramyxoviridae* is responsible for virus penetration into a host cell and cell fusion. The putative F protein cleavage site of APMV-3 strain Netherlands (A-R-P-R-G-R↓L) does not conform to the preferred furin cleavage site (R-X-R/K-R↓F), which is an important determinant of virulence for NDV and avian influenza viruses. In addition, the first residue of the F1 subunit is leucine, and thus, matches the assignment in avirulent strains of NDV as well as the single strain of APMV-6 sequenced to date, but differs from virulent strains of NDV, which have phenylalanine at this position. The observation that the F protein of APMV-3 strain Netherlands has characteristics consistent with that of an avirulent NDV strain is consistent with its dependence of exogenous protease for replication *in vitro*. However, it is in sharp contrast to the observed virulence of APMV-3 in birds (Alexander and Collins, 1982). It will be interesting to study further the pathogenicity of this virus in chickens and turkeys. The analysis of additional strains of APMV-3, and the development of a reverse genetics system to study viral biology and pathogenesis, and to create live attenuated strains, will be of applied, as well as basic, interest, and should help develop better vaccines for this poultry pathogen.

Chapter 4

4.1 Title

Complete genome sequence of avian paramyxovirus-3 strain Wisconsin: evidence for the existence of subgroups within the serotype.

4.2 Abstract

The complete consensus genome sequence was determined for avian paramyxovirus (APMV) serotype 3 strain Wisconsin. The genome is 16,182 nucleotides (nt) in length, consisting of six non-overlapping genes in the order of 3'-N-P/V/W-M-F-HN-L-5', with a 55-nt leader at its 3' end and a 681-nt trailer at its 5'end. Comparison of the APMV-3 strain Wisconsin nt and the aggregate predicted amino acid (aa) sequences with those of APMV-3 strain Netherlands revealed 67 and 78%, identity, respectively. The nt and aa sequence identities between the two APMV-3 strains were lower than between the two antigenic subgroups of human respiratory syncytial virus (81 and 88% identity, respectively) and the two subgroups of human metapeumovirus (80 and 90% identity, respectively). Reciprocal cross-hemagglutination inhibition and cross-neutralization assays using post infection sera from chickens indicated that strains Wisconsin and Netherlands are highly related antigenically, with only a 2- to 4-fold difference in antibody reactivity between the homologous and heterologous strains. Taken together, the results indicate that the two APMV-3 strains represent a single serotype with two subgroups that differ substantially based on nt and aa sequences, but with only a modest antigenic difference.

4.3 Introduction

The APMVs have been classified into nine different serotypes based on hemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays (Alexander and Collins, 1984). Newcastle disease virus (NDV) composes serotype 1. Naturally-occurring strains of NDV include highly virulent strains capable of causing devastating disease in poultry, as well as strains of moderate or low virulence. NDV is the most highly characterized virus among all APMV serotypes (Alexander, 1980). APMV-2 has been isolated from chickens, turkeys and wild birds, and is responsible for decreases in egg production and fertility in turkeys (Lipkind et al., 1979; Tumova et al., 1979a). The pathogenicity of APMV 4-9 is generally unknown except for a few isolated reports (Alexander, 1982; Warke et al., 2008). APMV-3 was first isolated from a turkey in 1968 in Wisconsin. Since then, many APMV-3 strains have been isolated from turkeys in different parts of the world including Canada, the United States (Tumova et al., 1979a) and England (Macpherson et al., 1983). The APMV-3 strain parakeet/Netherlands/449/75, isolated from parakeets in the Netherlands, is considered the prototype for the entire serotype (Alexander and Chettle, 1978). APMV-3 has also been isolated from non-domesticated species such as *Psittaciformes* and *Passeriformes* (Alexander, 1980). APMV-3 has been associated with encephalitis and high mortality in caged birds, and with respiratory disease in turkeys (Tumova et al., 1979a). The virus causes acute pancreatitis and central nervous system symptoms in psittacine and *Passeriformes* birds (Beck et al., 2003). APMV-3 also infects chickens at an early age, with evidence of stunting growth that may be more marked in broiler chicken breeds

(Alexander and Collins, 1982). In terms of pathogenicity, APMV-3 probably is second in importance only to NDV.

As noted, APMV-3 strains have been isolated from a wide range of avian species and from different parts of the world. But very little is known about the genetic and antigenic relatedness among these strains. This information is important for epidemiological studies and for the development of vaccines against this virus. I recently described a complete consensus sequence of the APMV-3 prototype strain parakeet/Netherlands/449/75 (Kumar et al., 2008), but other strains remain to be characterized. As a first step towards understanding the genetic and serological relationship among APMV-3 strains, I now report the complete genome sequence of APMV-3 strain turkey/Wisconsin/68 and describe comparison with those of prototype strain parakeet/Netherlands/449/75 and other APMV serotypes. The genome of strain turkey/Wisconsin/68 is 16,182 nt long, which is smaller than that of strain parakeet/Netherlands/449/75 by 90 nt. There is 67 % nt and 78% amino acid (aa) sequence identity between the two strains. The cleavage site of F protein of strain turkey/Wisconsin/68 contains a monobasic residue, compared to a dibasic residue in strain parakeet/Netherlands/449/75. Antigenic and sequence analysis suggested that the two strains represent a single serotype with two genetic subgroups.

4.4 Materials and Methods

4.4.1 Virus and cells

APMV-3 strain turkey/Wisconsin/68 was obtained from the National Veterinary Services Laboratory, Ames, Iowa. Nine-day-old specific pathogen free (SPF)

embryonated chicken eggs and the chicken embryo fibroblast DF1 cell line were used to propagate APMV-3 strain Wisconsin. Hemagglutination (HA) titers of virus stocks were determined using 0.5% chicken RBC at room temperature. The ability of the virus to replicate in cell culture was examined in nine different established cell lines, each representing a different species of origin: DF1; MDBK, Madin Darby Bovine Kidney; MDCK, Madin Darby Canine Kidney; Vero, African green monkey kidney; HEp2, Human Epidermoid carcinoma; PK15, Pig Kidney, all the cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). All cells, except DF1 cells, were grown in Eagle's minimum essential media (MEM) with 10% fetal calf serum (FCS), while DF1 cells were grown in Dulbecco's MEM containing 10% FCS. All cells were grown in a 37°C incubator with 5% CO_2. Cell monolayers were infected with a 10^{-3} dilution of 2^9 HA units of egg-grown APMV-3 strain Wisconsin and, after 1 h of adsorption, the viral inoculum was replaced with maintenance medium containing 2% FCS. The growth of virus in each cell type was observed with and without the presence of 10% allantoic fluid, or 1-5 µg/ml of acetyl trypsin/ml (Gibco), or 1-5 µg/ml of α-chymotrypsin/ml (Sigma), each of which provided a source of protease for the cleavage of F protein if necessary. The cells were observed daily for cytopathic effects (CPE) and HA titers were recorded every 24 h until the fifth day post-infection. The virus titer in each cell type was quantified by plaque assay in DF 1 cells as previously described (Krishnamurthy et al., 2000). The cells were overlaid with DMEM containing 2% FCS and 0.9% methylcellulose that were further supplemented with 10% allantoic fluid as a source of proteases. The plaques were visualized by staining with 1% crystal violet after 4 days of infection.

4.4.2 Serological analysis

Antisera against APMV-3 strains turkey/Wisconsin/68 and parakeet/Netherlands/449/75 were prepared at separate times to avoid cross infections. Briefly, groups of six two-week old SPF chicken were inoculated with infective allantoic fluid (2^9 HA units) of each virus through intranasal (IN) and intraocular (IO) routes. Two weeks after inoculation the birds were bled and sera were collected. The sera were heat-inactivated at 56^0C for 30 minutes and stored at -20^0C. The antibody level of serum samples collected from chicken immunized with APMV-3 strains were evaluated by HI assay with chicken erythrocyte as previously described (Alexander, 1988). Cross reactivity of immunized chicken sera were determined by HI assay against the heterologous APMV-3 strains and other APMV serotypes. The ability of chicken sera to cross neutralize heterologous APMV-3 strains was determined by plaque reduction neutralization (PRN) assay in DF-1 cells and virus neutralization (VN) assay in embryonated chicken eggs using standard procedures (Alexander, 1995; Alexander, 2003b).

4.4.3 Virus RNA isolation and sequence analysis

Total RNA from virus-infected DF1 cells was isolated using TRIZOL reagent, following the manufacturer's instructions (Invitrogen, USA). Viral RNA was also isolated from allantoic fluid collected from virus-infected eggs, using RNeasy kit according to the manufacturer's instruction (QIAGEN, USA).

Most of the APMV-3 genome, excepting the 3' and 5' termini, was copied and amplified into cDNAs using (i) primers designed from the published APMV-3 Netherlands genome sequence (Kumar et al., 2008), and (ii) consensus primers designed

using published *Avulavirus, Rubulavirus, Respirovirus*, *Morbillivirus*, and *Henipavirus* genome sequences (Table 4.1). In addition, as APMV-3 Wisconsin sequence became available, it was used to design additional primers. N787 forward and N1022 reverse consensus primers were used to amplify a 235 base pair (bp) fragment that served as one starting point for downstream genome walking (Chang et al., 2001). A primer designed from the available APMV-3 Netherlands P gene sequence (GenBank Accession number **EU403085**) (P 780 forward) was used in conjunction with (i) a consensus sequence designed for the M gene, M350 reverse, to amplify a 800-bp product, and (ii) a second consensus sequence, M1066 reverse, to amplify a 1500-bp product that were used as a second starting point for downstream genome walking. The upstream 850 nt in the HN gene were amplified using the consensus GS forward (GE forward) primer and consensus HN840 reverse primer (Table 4.1). An overlapping region containing the rest of the HN gene was amplified using the consensus forward primer HN490 forward and the consensus gene-end reverse (GE reverse) primer. The upstream end of the L gene was amplified using the consensus primer HN1492 forward primer and consensus L669 reverse primer (Table 4.1). Downstream regions of L gene were amplified by L gene-specific forward and consensus L reverse primers listed in Table 4.1.

The sequence of the 3' terminus was determined from cDNA prepared by rapid amplification of cDNA ends (RACE) (Troutt et al., 1992; Li et al., 2005; Kumar et al., 2008) as described previously (Kumar et al., 2008). The sequence of the 5' end of the genome was determined from cDNA obtained by 5' RACE (Invitrogen) as described previously (Kumar et al., 2008).

To investigate RNA editing of the P gene, DF1 cells were infected with APMV-3 strain Wisconsin at a MOI of 0.1 PFU per cell. mRNAs were isolated from total RNA using an oligo-dT column (Oligotex mRNA mini kit, QIAGEN). RT was performed using a gene-specific reverse primer, P551 reverse (Table 4.1). A 414-bp cDNA fragment spanning the putative editing site in the P gene of APMV-3 strain Wisconsin was amplified using gene-specific primers P137 forward and P551 reverse (identified according to their position in the complete antigenome sequence). The PCR-amplified products were cloned and individual clones were sequenced spanning the putative P gene RNA editing site.

PCR-amplified products and plasmid DNAs were sequenced by using the BigDye terminator v 3.1 matrix standard kit (Applied Biosystems) and 3130xl genetic analyzer data collection software v3.0 (Applied Biosystems). The entire genome was sequenced at least three times, and at least once from uncloned PCR product, to ensure a consensus sequence.

Position in gene	Primer sequences
N787 forward	5′AAYRCSGGKMTKRCWSCWTTCTT3′
N1022 reverse	5′GWWSCYAYWCCCATKGCAWA3′
GS forward	5’WWWWWWAGGGGCGGAA3’
GE reverse	5’WWWWWTTTTTTATTAAW3’
P780forward	5’ CAGGAACATGCAGGCGGC3’
M350reverse	5’CWTAKATTTYGSTTSAAACAAC3’
M1066reverse	5’CNTGDTATYGAAAMATCATGTG3’
HN840reverse	5’CAKTKCAGTSGDGSCTCTTGATC3’
HN490forward	5’GTCGATGATATATAGGAATTTC3’
HN1492forward	5’GCCCTTGACTGAGGGTACAACAC3’
L487forward	5’CATTAATGACTAAGCCTGGG3’
L3020forward	5’GACTCAGCAGGATGAAGAGG3’
L4633forward	5’ GATGTTGGATAGGAGTGATTTGAAG3’
L669reverse	5’AYATTCRGKTGTGGTWATAATTG3’
L2015reverse	5’CAATKGATCMAYTCAARACCATG3’
L3784reverse	5’CGDCRYDGSGCAWAGKGCCTGC3’
L5377reverse	5’GTATATWTWGTMRCTTCTAATG3’

Table 4.1. Primers used to amplify APMV-3 genome based on consensus sequences from *Avulavirus*, *Rubulavirus*, *Respirovirus*, *Morbillivirus,* and *Henipavrus* genome sequences. The primer name indicates the gene name, position from the gene-start, and the forward or reverse direction of priming. N=A/T/C/G, R= A/G, K= G/T, Y=C/T, S=G/C, W=A/T, D= A/G/T and M=A/C. Sequences used for alignment are noted in the Materials and Methods.

4.4.4 Sequence and phylogenetic tree analysis

Sequence similarity searches were conducted using the basic length alignment search tool (BLAST) from the National Center for Biotechnology Information (NCBI). DNA pair-wise alignment was done using MegAlign (clustalW) in a Lasergene 8 software package. Prediction of phosphorylation sites, signal sequences and glycosylation sites in the complete genome sequence was determined by using NetPhos 2.0 software, SignalP 3.0 server and NetNGlyc program respectively (all from ExPASy Proteomics tools). To construct phylogenetic trees and determine divergence among different genera,

aa sequences were aligned using clustalW multiple alignment algorithm. In addition, boot strap values were calculated for maximum parsimony using PHYLIP software.

4.4.5 Database accession numbers

The complete genome sequence of APMV-3 strain Wisconsin has been submitted in GenBank under accession number **EU782025**. Different sequences from GenBank were used for designing primers and comparing the APMV-3 genome. These include NC_002617 for NDV, EU338414 for APMV-2, EU403085 for APMV-3 Netherlands, FJ177514 for APMV-4, NC_003043 for APMV-6, FJ215863 for APMV-8, EU910942 for APMV-9, NC_003443 for hPIV2, NC_006430 for SV5, NC_004074 for TiV, NC_001552 for SeV, NC_001796 for hPIV3, NC_003461 for hPIV1, AY988601 for NiV, NC_001906 for HeV, NC_001498 for MeV, NC_001921 for CDV, NC_006383 for Peste des petits ruminants virus (PPRV), NC_006296 for RPV.

4.5 Results

4.5.1 *In vitro* growth characteristics of APMV-3 strain Wisconsin

APMV-3 strain Wisconsin produced a titer of 2^9-2^{10} HA units per ml of allantoic fluid in 9-day-old embryonated SPF chickens eggs four days post inoculation. It was necessary to include 10% allantoic fluid for replication of the virus in cell culture, indicating a requirement of external proteases for efficient cleavage of the F protein. The virus grew most efficiently in DF1, with the next most efficient growth being in MDBK cells. Viral CPE involved rounding, detachment and syncytia formation. The growth kinetics and plaque morphology in DF-1 cells were similar for APMV-3 strains Wisconsin and Netherlands.

4.5.2 Antigenic relationship between APMV-3 strains Wisconsin and Netherlands

The antigenic relationship between APMV-3 strains Wisconsin and Netherlands was evaluated by reciprocal HI tests using strain specific convalescent sera raised by a single infection of chickens by the IN/IO route. Each of the antisera exhibited a four-fold difference in HI titer between the homologous and heterologous strains (Table 4.2A). As expected, the two APMV-3 strains were serologically distinct from representatives of the other APMV serotypes, although there was a low level of cross-reaction with APMV-8 (Table 4.2A).

The ability of antisera to neutralize homologous and heterologous APMV-3 strains was assessed by PRN test in cell culture and by VN test in embryonated eggs. The results showed that both the antisera cross neutralized the heterologous strain, but the neutralization titers were two-fold higher against the homologous strain than with the heterologous strain (Table 4.2B). These reactions indicated existence of a low level of antigenic difference between the two APMV-3 strains.

(A)

Antiserum	virus								
	APMV-1	APMV-2	APMV-3 Wisconsin	APMV-3 Netherlands	APMV-4	APMV-6	APMV-7	APMV-8	APMV-9
APMV-3 Wisconsin	2	4	32	16	0	0	0	8	2
APMV-3 Netherlands	2	0	8	64	0	0	2	8	0

HI titer are expressed as the reciprocal of the highest dilution of antisera inhibit 4HA units of the virus. Viruses used as antigen were NDV-LaSota, APMV-2/Ck/Yucaipa/56, APMV-3/PKT/Netherlands/449/75, APMV-3/Turkey/Wisconsin/68, APMV-4/DK/HK/D3/75, APMV-6/DK/HK/199/77, APMV-7/Dove/TN/4/75, APMV-8/Goose/DEL/1053/76, APMV-9/DK/NY/22/78.

(B)

Antiserum	Virus	Plaque reduction neutralization titer[b]	Serum neutralization titer (log_2)[a]
APMV-3 (Wisconsin) serum	APMV-3 (Wisconsin)	2048	7.75
	APMV-3(Netherlands)	1024	7.5
APMV-3 (Netherlands) serum	APMV-3 (Wisconsin)	256	6.75
	APMV-3(Netherlands)	512	7.5

[a] The APMV-3 serum neutralizing titer against each strain was determined and is expressed as the reciprocal log2.
[b] Neutralization titer was defined as the titer of serum that reduced plaque number by 60% compared to the positive control wells.

Table 4.2. Antigenic analysis of APMV-3 strains Wisconsin and Netherlands using antisera from chickens infected with the individual strains. (A) Homologous and heterologous hemaggutination-inhibition titers. (B) Homologous and heterologous neutralization titers based on plaque reduction assays in cell monolayers and serum neutralization assays in embryonated eggs.

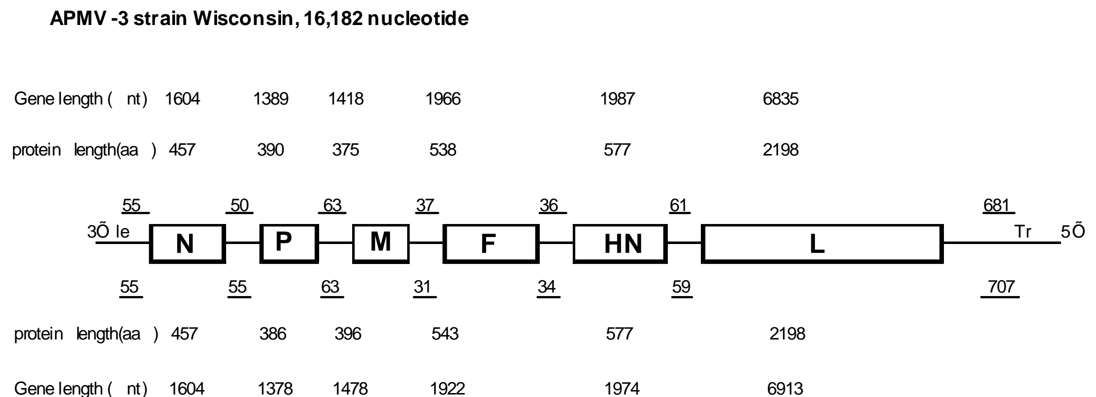
4.5.3 Determination of the complete genome sequence of APMV-3 strain Wisconsin

I determined the sequence of the entire genome of APMV-3 Wisconsin. A number of the initial cDNAs in this analysis were prepared using primers derived from consensus sequences identified by sequence alignment of multiple members of the *Avulavirus*, *Rubulavirus*, *Respirovirus*, *Morbillivirus,* and *Henipavrus* genera (Table 4.1). A few primers were also designed based on the published APMV-3 Netherlands sequence. Every nt in the complete sequence was confirmed in uncloned cDNA, indicating that it is a consensus sequence.

The genome of APMV-3 strain Wisconsin consists of 16,182 nt. Its genome organization is very similar to those of other members of family *Paramyxoviridae*. The

genome codes for six open reading frames (ORF) corresponding to putative proteins N, P, M, F, HN and L, from the 3' to 5' end of the genome (Fig. 4.1A). In addition, there is evidence of a cysteine-rich V ORF in the P coding region. 84% of the genome codes for protein, which is less than the average coding percentage (92%) of other members of subfamily *Paramyxovirinae* (Miller et al., 2003). Comparison of complete genome sequence of APMV-3 strain Wisconsin with that of strain Netherlands showed minor changes in the length of the P, M, F, HN, and L genes as well as the trailer region, whereas the lengths of the N gene and leader region were the same in each strain (Fig. 4.1A and 4.1B).

(A)



(B)

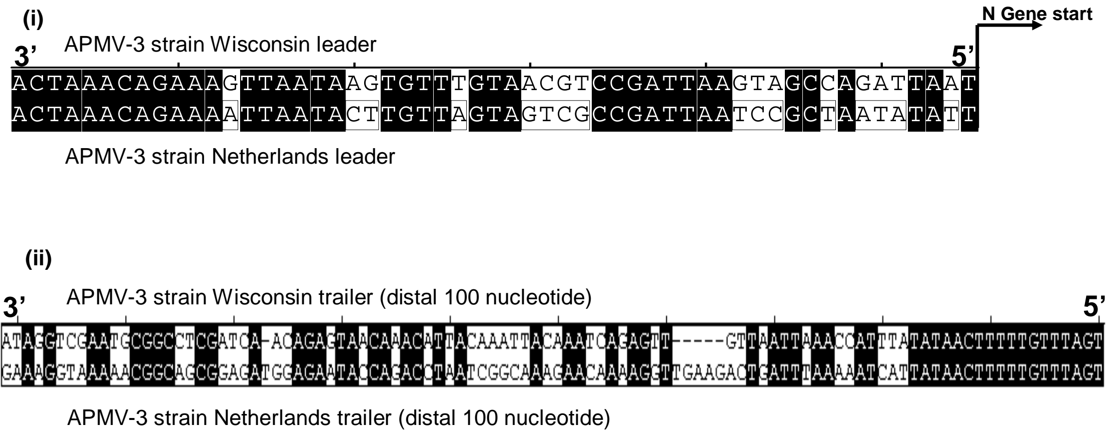


Fig. 4.1. Structure of the APMV-3 genome. (A) Gene maps of APMV-3 strains Wisconsin and Netherlands. Individual genes are indicated by boxes, with gene lengths given in nt together with the predicted amino acid length of the unmodified protein. The nt lengths of the extragenic 3' leader, 5' trailer, and intergenic regions are underlined. (B) Alignment of the sequences (genome-sense) of the leader region (i) or the downstream end of the trailer region (ii) of APMV-3 strains Wisconsin and Netherlands. Shading denotes identity between the strains.

The 3' leader sequence of APMV-3 strain Wisconsin consists of 55 nt, a length that is conserved among almost all the members of subfamily *Paramyxovirinae* (Krishnamurthy and Samal, 1998). The nt sequences of the leader region of the Wisconsin and Netherlands strains differed at 16 out of 55 positions, with the 3'-terminal 12 nt being identical between the two strains (Fig. 4.1B). The length of the 5' trailer region of APMV-3 strain Wisconsin is 681 nt, which is 26 nt shorter than that of APMV-3 strain Netherlands. The sequences of the 3' leader and 5' trailer termini of strain Wisconsin showed a high degree of complementarity, with only a single mismatch in the terminal 16 positions (94% complementarity), suggestive of conserved elements in the 3' promoter regions of the genome and antigenome. The sequence of the genomic termini of

APMV-3 strain Wisconsin showed 59% identity (aggregate of the complete 3' leader and 5' trailer sequence) with that of strain Netherlands.

The boundaries of the individual genes were putatively identified based on the presence of conserved putative gene-start and gene-end transcription signals. The sequence of the APMV-3 strain Wisconsin putative gene-start signal is 3'UCCU/CCGCCUU and is highly conserved among the six genes (Table 4.3). The gene-end signal is slightly less well-conserved: the consensus gene-end sequence is 3'AAU/AUA/U$(U)_6$, but among four of the six signals there is a total of four nt differences involving positions 3 and 5 (Table 4.3). The intergenic sequences of APMV-3 Wisconsin range in length from 37 to 63 nt. The lengths of the intergenic sequences are similar to those in strain Netherlands, although the length is exactly the same only for the P-M gene junction (Fig. 4.1A).

Gene	Gene start	Gene end	Intergenic sequence
N	AGG**A**GCGGAA	TTAA**G**AAAAAA	AGACCTGATGTGTACGAGGAGAAAAATAATTGATGAC AAGCGGAGAAAAT
P	AGG**A**GCGGAA	TT**T**ATAAAAAA	CCAAAATGATTATAACTAAACAATCTCAACAATTTGC AATGATAACAACACCATACGATCACT
M	AGGGGCGGAA	TTAATTAAAAA	TATTAATAATCATTAGCAACATCCGATCGGAATCTTC
F	AGGGGCGGAA	TT**T**ATAAAAAA	ATGCCATGATACTCGTGCGAGTGTAACATAGTAACT
HN	AGGGGCGGAA	TTAA**G**AAAAAA	CTTCACTATCACTCTTTGAGTCGCTGAAGTGAGATTT CAGAAAGGTATGCATCTAAGAAGT
L	AGG**A**GCGGAA	TTAATAAAAAA	

Table 4.3. Putative gene-start, gene-end and intergenic sequences of APMV-3 strain Wisconsin (sequences are mRNA sense). Differences relative to the most conserved sequence are underlined.

The nucleoprotein (N) gene

The N gene of APMV-3 strain Wisconsin is 1604 nt long and encodes an N protein of 457 amino acids. The predicted N protein contains a highly-conserved sequence motif, 322-FAPGNYALLYSYAMG-336 (F-X4-Y-X3-α-S- α-AMG, where X is any aa and α is any aromatic aa), that corresponds to a motif previously identified in the central region of the N protein of other members of *Paramyxovirinae* and which has been implicated in the self assembly of N with RNA or with other N monomers (Yu et al., 1998; Lamb and Parks, 2007). The APMV-3 strain Wisconsin N protein has 88.6% aa sequence identity with that of strain Netherlands (Table 4.4).

Protein	APMV-1	APMV-2	APMV-3(Netherlands)	APMV-4	APMV-6	APMV-7	APMV-8	APMV-9
N	**36.8**	**36.6**	**88.6**	**51**	**37.6**	**36.8**	**34.4**	**36.5**
P	**20.8**	**21.6**	**59.1**	**25.5**	**18.4**	**26.2**	**21.1**	**21**
M	**28.4**	**31.3**	**93.3**	**35.1**	**29.9**	**31.2**	**29.9**	**25.6**
F	**30.8**	**28.9**	**70.1**	**33.1**	**29.8**	**30.6**	**29.7**	**28.3**
HN	**34.7**	**30.6**	**72.6**	**39.3**	**29.7**	**35.9**	**31.5**	**37.2**
L	**33**	**34.9**	**83**	**40.5**	**35.9**	**37**	**34.6**	**33.5**

Table 4.4. Amino acid identity between APMV-3 strain Wisconsin versus strain Netherlands and representatives of the other APMV serotypes. Individual values show the percent identity of N, P, M, F, HN and L proteins among different members of genus *Avulavirus*. The identity was determined by DNASTAR using MegAlign (clustalW) in a Lasergene8 software package.

The phosphoprotein (P) gene

The P gene of APMV-3 strain Wisconsin is 1389 nt long. The unedited version of the P mRNA encodes a P protein of 390 amino acids. The predicted P protein contains an abundance of phosphorylation sites, with 29 serine, 9 threonine and 1 tyrosine residues identified as potential phosphorylation sites using NetPhos 2.0 software. The APMV-3 strain Wisconsin P protein has 59.1% aa sequence identity with that of strain Netherlands (Table 4.4).

The P gene contains a putative editing site, 5'- TTTTTAAAGGGGG (mRNA sense), at positions 374-386 in the P gene (positions 2083-2095 in the complete antigenome sequence), which has a high degree of sequence identity with the editing sites in other members of subfamily *Paramyxovirinae*. The insertion of a single G residue (mRNA sense) at the editing site would shift the reading frame to encode a 252-aa V protein in which the N-terminal 115 amino acids are derived from P, and the downstream 137 amino acids include seven cysteine residues and one tryptophan residue that are highly conserved within the subfamily *Paramyxovirinae* (Fig. 4.2). Alternatively, the insertion of two G residues would shift the reading frame to access an internal ORF that is open for only 12 additional amino acids, yielding a 127-aa W protein. Sequence analysis of cloned cDNAs of mRNAs isolated from infected cell culture showed mRNA with incorporation of one and two G residues at the predicted RNA editing site (Fig. 4.3). The APMV-3 strain Wisconsin V and W proteins have 63% and 55% aa sequence identity respectively with those of strain Netherlands.

```
                        ☆☆   ☆             ☆ ☆  ☆    ☆  ☆
APMV-3(Net)  184 TWCNPACSPVTAFPKQYKCACRQCPRFCDLCF 215
APMV-3(Wis)  188 TWCNPACSPVTALPKQYRCACRQCPKFCDLCF 219
APMV-1       194 SWCNPSCSPIRAEPRQYSCTCGSCPATCRLCA 225
APMV-2       198 SWCNPVCSPIRSEPRREKCTCGTCPESCILCR 229
APMV-4       190 EWCNPGCTAVRIEPTRLDCVCGHCPTICSLCM 221
APMV-6       194 SWCNPSWSPIKAEPKQYPCFCGSFPPTCRLCA 225
APMV-7       195 SWCNPTCAPIRPYPTVERCRCGNCPKFCPGCQ 226
APMV-8       204 EWCNPGCTAVRIEPTRLDCVCGHCPTICSLCM 235
APMV-9       218 SWCNPVCSPVTYEPREFTCSCGSCPTECRLCA 249
```

Fig. 4.2. Sequence alignment of C terminal end of the V proteins of different members of genus *Avulavirus*. Stars indicate conserved cysteine and tryptophan residues. Numbers indicate the amino acid position.

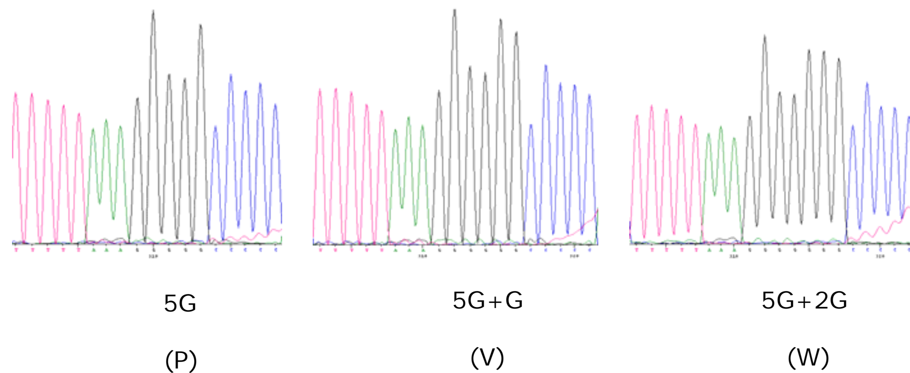


Fig. 4.3. Sequence analysis of the P gene editing site in cDNAs made from polyadenylated intracellular mRNA. This shows sequencing electropherograms across the editing site region of transcripts that were unedited (5G, encoding the P protein), or contained the insertion of one (5G+G, encoding the V protein) or two (5G+2G, encoding the W protein) G residues. The sequence is mRNA-sense.

The matrix (M) protein gene

The M gene of APMV-3 strain Wisconsin is 1418 nt long and encodes a 375-aa M protein. The protein is highly basic with an isoelectric point of 9.1, which may be

important for ionic interaction with the acidic N protein (Lamb and Parks, 2007). The bipartite cluster, which comprises clusters of basic amino acids either 'single cluster', containing three to five basic amino acids, or 'bipartite' sequence, containing two interdependent clusters of such amino acids separated by an intervening sequence is clearly seen in the positions 257-272 (257 KQRRRTPSEITQKVRR 272), which was different in two aa at position 267 and 272 with strain Netherlands (Kumar et al., 2008). The APMV-3 strain M protein has 93.3% aa sequence identity with that of strain Netherlands (Table 4.4).

The fusion (F) protein gene

The F gene of APMV-3 strain Wisconsin is 1966 nt long and codes for a 538-aa F protein. The protein is predicted to be a type I transmembrane protein, similar to the F proteins of other members of family *Paramyxoviridae*. The N-terminus of the F protein contains a predicted signal sequence that would mediate translocation of the nascent protein into the lumen of the endoplasmic reticulum and is predicted by the SignalP 3.0 server to be cleaved between residues 22 and 23 (SQS↓AN). The predicted C-terminal transmembrane domain (residues 484–506) would anchor the protein in the host cell membrane leaving a short cytoplasmic tail of 32 amino acids. The predicted cleavage-activation site of the APMV-3 F protein is R-P-S-G-R↓L (corresponding to 95-102 amino-acid position), which differed at two amino acids with the cleavage site of strain Netherlands (Fig. 4.4). The APMV-3 stain Wisconsin F protein has 70.1% aa sequence identity with that of strain Netherlands (Table 4.4).

```
                                                  ↓
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
APMV-1(Avirulent)        111 G-G-R-Q-G-R-L 117
APMV-2                   93  D-K-P-A-S-R-F 99
APMV-4                   115 V-D-I-Q-P-R-F 121
APMV-6                   113 P-A-P-E-P-R-L 119
APMV-7                   101 T-L-P-S-S-R-F 107
APMV-8                   98  T-Y-P-Q-T-R-L 104
APMV-9                   105 I-R-E-G-R-I-F 111
```

Fig. 4.4. Comparison of the F protein cleavage sites of different members of genus *Avulavirus*. Basic residues (R= arginine, K= lysine) are underlined and in bold. Numbers indicate the amino acid position.

The hemagglutinin-neuraminidase (HN) protein gene

The HN gene of APMV-3 strain Wisconsin is 1987 nt long and encodes a polypeptide of 577 amino acids. The *Paramyxovirinae* HN protein is a type II integral membrane protein that spans the membrane once: the predicted HN protein of APMV-3 Wisconsin has a predicted hydrophobic signal anchor domain spanning residues 23-45. Five potential N-linked glycosylation sites were identified at positions 122, 155, 312, 325 and 342, using the Expasy, NetNGlyc program. The HN protein contains a high amount of acidic amino acids with an isoelectric point (p*I*) of 6.3. The HN proteins of subfamily *Paramyxovirinae* and the HA proteins of influenza viruses contain a conserved sequence, N-R-K-S-K-S, which is thought to contribute to the sialic acid binding site (Varghese et al., 1983). The aa sequence of the APMV-3 HN protein contains a similar motif, N-R-K-S-C-S (positions 237-242, difference underlined). The APMV-3 strain Wisconsin HN protein has 72.6% aa sequence identity with that of strain Netherlands (Table 4.4).

The large polymerase protein (L) gene

The L gene of APMV-3 strain Wisconsin is 6835 nt long and codes for a 2198-aa L protein. Alignment of the L protein aa sequence of APMV-3 Wisconsin with other members of *Paramyxoviridae* demonstrated six linear conserved domains, which have been described earlier for these family members and represent catalytic domains (Poch et al., 1989). The conserved QGDNQ sequence that is found within domain III involved in transcription activity (Schnell and Conzelmann, 1995) was also found in the L protein of APMV-3 at aa positions 744-748. The APMV-3 strain Wisconsin L protein has 83% aa sequence identity with that of strain Netherlands (Table 4.4).

4.5.4 Phylogenetic analysis

Phylogenetic trees were generated from alignments of the complete nt sequence of the genome of APMV-3 strain Wisconsin with the sequences of representative viruses of the other serotypes of APMV excluding only APMV-5, which remains to be sequenced (Fig. 4.5). The resulting phylogenic tree indicates that APMV-3 strains Wisconsin and Netherlands cluster together on a branch that is distinct from those of the other available serotypes.

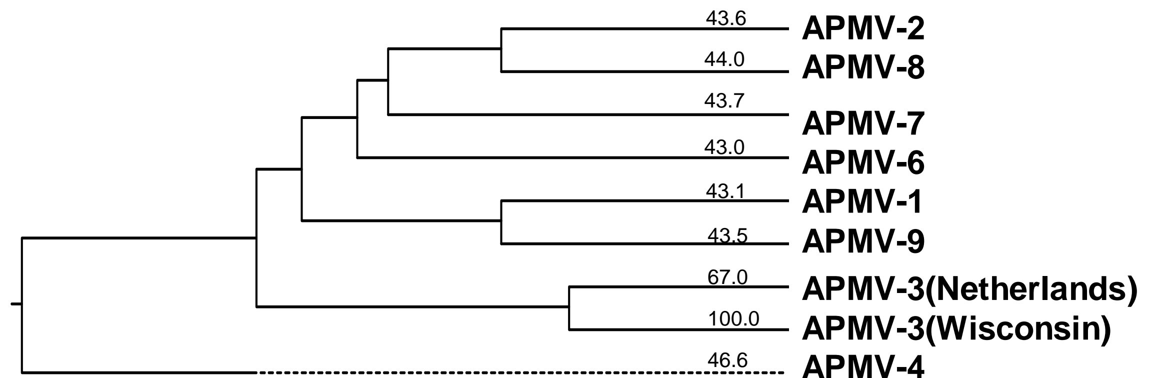


Fig. 4.5. **Phylogenetic tree depicting evolutionary relationships between the members of the family *Paramyxoviridae* based on complete genome nt sequences.**

4.6 Discussion

Avian paramyxoviruses are classified into nine serotypes based on their serological relationships in HI and NI tests. Of these serotypes, a great deal of information is available on antigenic and genetic relationships among APMV-1 (NDV) strains isolated from different parts of the world (Alexander, 1988). Additionally, I and others have recently published complete genome sequences for representative strains of APMV-2, -3, -4, -6, -7, -8, and -9 (Chang et al., 2001; Kumar et al., 2008; Nayak et al., 2008; Subbiah et al., 2008; Paldurai et al., 2009; Samuel et al., 2009; Xiao et al., 2009). However, very little is known about the antigenic and genetic relationships among strains within APMV-2 through -9 (Alexander, 2003b). In this study I compared APMV-3 strain Wisconsin, a virus isolated from a turkey in Wisconsin, and the prototype APMV-3 strain Netherlands, isolated from parakeets in the Netherlands. These two strains differ in their virulence for chickens (unpublished data). APMV-3 strain Wisconsin is avirulent for chickens, whereas APMV-3 strain Netherlands is moderately virulent for chickens leading to paralysis and death occasionally. Therefore, it was of my interest to know the extent of antigenic and genetic variation between these two strains. This information might have implications for studies in pathogenesis and epidemiology and for the development of vaccines.

To evaluate the antigenic relationship between the two APMV-3 strains, I raised chicken antiserum against each virus separately by respiratory infection, mimicking a natural route of infection. Since serological responses tend to broaden over time and with

repeated antigenic exposure, I (i) limited the immunization to a single infection, and (ii) collected the serum samples at an early time point (14 days post infection). The antigenic relatedness between strains Wisconsin and Netherlands was examined by using reciprocal cross- HI and cross neutralization assays based on primary infection sera from chickens. The two APMV-3 strains were found to have a close antigenic relationship in each assay. The differences in homologous and heterologus HI titers between the two strains were 4-fold higher against the homologous virus versus the heterologous virus, indicating a minor antigenic difference. The difference in homologous and heterologous virus neutralization titers between the two strains was 2-fold higher for the homologous virus versus the heterologous virus, similar to the results of the HI tests. These results indicate that the two APMV-3 strains are somewhat more closely related antigenically than the two antigenic subgroups of human respiratory syncytial virus (HRSV). The two serogroups of HRSV exhibit a 3- to 4- fold reciprocal difference in neutralization by polyclonal convalescent serum for homologous versus heterologous strains (Johnson et al., 1987a; Johnson et al., 1987b; Biacchesi et al., 2003). The results suggested that the two APMV-3 strains certainly represent a single serotype, but do not unambiguously represent two antigenic subgroups. In this regard, it will be of interest to evaluate additional strains.

In order to analyze the genetic relationship between the two APMV-3 strains, I determined the complete genome sequence of strain Wisconsin and compared it with the sequence of strain Netherlands that I reported earlier (Kumar et al., 2008). The genome lengths differ slightly: strain Wisconsin was 16,182 nt compared to 16,272 nt for strain Netherlands. Both viruses have identical genome organizations and highly conserved

genome terminal sequences and gene start and stop sequences. The two genomes shared 67% nt identity, compared to 80% nt identity between the two subgroups of human metapneumovirus (HMPV) (Biacchesi et al., 2003) and 81% nt identity between the two subgroups of HRSV (Johnson et al., 1987a). This divergence was the greatest in regions that did not encode protein or constitute cis-acting RNA signals: for example, the intergenic regions between the two APMV-3 strains were 48% identical, similar to the value of 48% identity between two HPMV subgroups and 42% identity between two HRSV subgroups (Biacchesi et al., 2003). When compared with the other serotypes of APMV, the genome of APMV-3 Wisconsin had a nt sequence identity of 43%, 44%, 47%, 43%, 44%, 44%, and 44% versus APMV-1, -2, -4, -6, -7, -8, and -9, respectively. Thus, the divergence of nt sequence between the two APMV-3 strains was substantially more than between the subgroups of HRSV or HMPV, but was substantially less than the divergence between APMV serotypes.

Comparison of nt sequence relatedness of ORFs between the two APMV-3 strains showed 72 to 75% nt identity except for P, F, and HN genes which were more divergent (66.2, 66.7 and 65.7 % nt identity respectively). This pattern is similar to those of the two subgroups of HMPV and HRSV, in which the genes of internal virion proteins are highly conserved, while the genes of outer surface proteins are more divergent (Johnson et al., 1987b; Biacchesi et al., 2003). The most notable exception in the pattern was observed for the P gene of APMV-3 strains. The P gene of the two subgroups of HMPV and HRSV are highly conserved (81 and 85% nt identity respectively), while the P genes are more divergent (66% nt identity) between the two APMV-3 strains.

The total proteome of the two APMV-3 strains shared 78% aa sequence identity compared to 90% aa sequence identity between the HMPV subgroups and 88% aa sequence identity between the HRSV subgroups (Biacchesi et al., 2003). In comparison, the APMV-3 proteome had an aa sequence identity of 31%, 31%, 38%, 30%, 33%, 31%, and 31% when compared to APMV-1, -2, -4, -6, -7, -8, and -9, respectively. Thus, the aa sequence divergence between the two APMV-3 strains was substantially more than between the subgroups of HRSV or HMPV, but was substantially less than the divergence between APMV serotypes.

The aa sequence identity between the cognate proteins of the two APMV-3 strains varied from 59% for P protein to 93% for M protein (Table 4.4). The envelope proteins (F and HN) were more divergent between the two APMV-3 strains (Table 4.4). It was surprising to find that the F protein of two APMV-3 strains exhibited more divergence (70% identity) than the F protein of the HMPV subgroups (95% identity) and the HRSV subgroups (89% identity) (Biacchesi et al., 2003). The HN protein of the two APMV-3 strains had 73% identity, which was higher than between the G proteins of the two HMPV subgroups (55% identity) and the two HRSV subgroups (37% identity). In comparison, the APMV-3 F protein has 31%, 29%, 33%, 30%, 31%, 30%, and 28% when compared to the F protein of APMV-1, -2, -4, -6, -7, -8, and -9, respectively, and the APMV-3 HN protein has 35%, 31%, 40%, 30%, 36%, 32%, and 37% when compared to APMV-1, -2, -4, -6, -7, -8, and -9, respectively (Table 4.4). The P, V and W proteins were more divergence between the two APMV-3 strains (59, 63 and 55% aa identity, respectively) compared to the P proteins of HMPV subgroups (85% aa identity) and HRSV subgroups (90% aa identity) (Biacchesi et al., 2003). Whether the high degree of

divergence in P, V and W proteins is responsible for the difference in the pathogenicity of APMV-3 strains needs further studies.

In conclusion, the complete genome sequence was determined for APMV-3 strain Wisconsin. Comparison of the nt and predicted aa sequences of strains Wisconsin and Netherlands showed that the divergence between the two strains was substantially greater overall than between the two HMPV and HRSV subgroups. This indicated that the two APMV-3 strains represent two distinct genetic subgroups. Given the rather substantial differences in the aa sequences between the APMV-3 strains, it was somewhat surprising that the extent of antigenic difference was modest. This is particularly evident when compared with the HRSV subgroups, where there was substantially greater aa sequence identity overall and yet greater antigenic difference. One factor may be that, while one of the HRSV neutralization antigens (F) was more highly conserved than for the APMV-3 strains, the other HRSV neutralization antigen (G) was much more divergent than its APMV-3 counterpart (HN) (Johnson et al., 1987a). Additional antigenic and genetic analysis involving additional APMV-3 strains is needed to further define the antigenic and genetic variability of APMV-3.

Chapter 5

5.1 Title

Experimental avian paramyxovirus serotype-3 infection in chickens and turkeys

5.2 Abstract

APMVs are divided into nine serotypes. NDV (APMV-1) is the most extensively characterized; while relatively little information is available for the other APMV serotypes. In the present study, I examined the pathogenicity of two divergent strains of APMV-3, Netherlands and Wisconsin, in (i) 9-day-old embryonated chicken eggs, (ii) 1-day-old specific pathogen free (SPF) chicks and turkeys, and (iii) 2-week-old SPF chickens and turkeys. The mean death time in 9-day-old embryonated chicken eggs was 112 h for APMV-3 strain Netherlands and >168 h for strain Wisconsin. The intracerebral pathogenicity index in 1-day-old chicks for strain Netherlands was 0.39 and for strain Wisconsin was zero. Thus, both strains are lentogenic. Both the strains replicated well in brain tissue when inoculated intracerebrally in 1-day-old SPF chicks, but without causing death. Mild respiratory disease signs were observed in 1-day-old chickens and turkeys when inoculated through oculonasal route with either strain. There were no overt signs of illness in 2-weeks-old chickens and turkeys by either strain, although all the birds seroconverted after infection. The viruses were isolated predominantly from brain, lungs, spleens, trachea, pancreas and kidney. Immunohistochemistry studies also showed the presence of large amount of viral antigens in both epithelial and sub-epithelial lining of respiratory and alimentary tracts. The result suggests systemic spread of APMV-3 even though the viral fusion glycoprotein does not contain the canonical furin proteases cleavage site. Furthermore, there was little or no disease despite systemic viral spread and abundant viral replication in all the tissues tested.

5.3 Introduction

Members of the family *Paramyxoviridae* are pleomorphic enveloped particles that contain a single-stranded, non-segmented RNA genome of 13-19 kilobases (Lamb and Parks, 2007). All paramyxoviruses that have been isolated to date from avian species are placed in the genus *Avulavirus* except for avian metapneumoviruses that are placed in the genus *Metapneumovirus* due to antigenic and genomic organization differences. The avian paramyxoviruses (APMVs) that comprise genus *Avulavirus* have been divided into nine different serotypes (APMV-1 through -9) based on haemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays (Alexander and Collins, 1984). APMV-1 comprises all strains of NDV and is the most completely characterized serotype due to the severity of disease caused by virulent NDV strains in chickens (Alexander, 1980). The complete genome sequences and the molecular determinants of virulence have been determined for representative NDV strains (Krishnamurthy and Samal, 1998; de Leeuw and Peeters, 1999; Huang et al., 2004b; Panda et al., 2004; Rout and Samal, 2008). As a first step in characterizing the other APMV serotypes, complete genome sequences of one or more representatives of APMV serotypes 2 to 9 were recently determined, expanding our knowledge about these viruses (Chang et al., 2001; Kumar et al., 2008; Nayak et al., 2008; Subbiah et al., 2008; Paldurai et al., 2009; Samuel et al., 2009; Xiao et al., 2009).

NDV causes respiratory, neurological or enteric disease in birds. Among poultry, chickens are the most susceptible while geese and ducks are the least susceptible (Wakamatsu et al., 2006). NDV strains are classified into low virulent (lentogenic),

moderately virulent (mesogenic), and highly virulent (velogenic) pathotypes based on pathogenicity in chickens. In contrast very little is known on pathogenicity of APMV-2 to -9 in chickens and turkeys. APMV-2 has been shown to cause mild disease and drop in egg production in chickens and turkeys (Warke et al., 2008). APMV-6 and -7 have been associated with respiratory disease in turkeys (Shortridge et al., 1980; Saif et al., 1997). APMV-4, -8 and -9 have been isolated from different species of birds but the clinical signs of the disease in those birds were not apparent (Alexander et al., 1983; Gough and Alexander, 1984; Stallknecht et al., 1991; Maldonado et al., 1995; Capua et al., 2004). Recently, experimental infection of 1-day-old chicks with APMV-2, -4 and -6 showed viral infection in gastrointestinal tract, respiratory tract and pancreas (Warke et al., 2008).

APMV-3 has been isolated from wild and domesticated birds in different parts of the world (Tumova et al., 1979b; Alexander, 1980; Macpherson et al., 1983; Alexander, 2003a). Recently, APMV-3 was isolated from ostrich, indicating a wide host range for the virus (Kaleta et al.). The virus has been isolated from diseased turkeys associated with coughing, nasal discharge and swelling of the infra-orbital sinus (Redmann et al., 1991). APMV-3 has been associated with encephalitis and high mortality in caged birds (Tumova et al., 1979a). The virus causes acute pancreatitis and central nervous system (CNS) symptoms in *Psittacine* and *Passerine* birds (Beck et al., 2003). APMV-3 also infects chickens at an early age, with evidence of stunting growth that may be more marked in broiler chicken breeds (Alexander and Collins, 1982). In terms of pathogenicity to domestic poultry birds, APMV-3 probably is second in importance to NDV. The exact economic impact of APMV-3 infection in poultry industry is not known. This is partly because the pathogenicity of APMV-3 in poultry species is not well

studied. There is a high degree of amino acid sequence variation between APMV-3 and APMV-1 (NDV), but by the HI test there is cross reaction between APMV-3 and APMV-1 serum samples, which often leads to misdiagnosis of APMV-3 as APMV-1.

Currently, it is not known whether there is any variation in pathogenicity among APMV-3 strains in domestic poultry birds. The purpose of this study was to evaluate the pathogenicity of APMV-3 strains Netherlands and Wisconsin in chickens and turkeys. Both of these strains were completely sequenced recently (Kumar et al., 2008; Kumar et al., 2010b). APMV-3 strain Wisconsin was first isolated from a turkey in 1968 in Wisconsin (Tumova et al., 1979b). APMV-3 strain Netherlands was isolated from a parakeet in 1975 in the Netherlands and is the prototype for the entire serotype (Alexander and Chettle, 1978). Initially, these two viruses were considered as two different strains of APMV-3 based on cross HI test using monoclonal antibodies (Anderson et al., 1987; Anderson and Russell, 1988). Recently, reciprocal cross HI and cross neutralization assays using post infection serum from chicken indicated that these two strains are antigenically distinct, although the difference was modest (Kumar et al., 2010b). However, complete genome sequence analysis revealed substantial genome-wide nucleotide and amino acid sequence differences that are consistent with the two strains representing distinct antigenic subgroups (Kumar et al., 2008; Kumar et al., 2010b). In the present study, I studied infection of APMV-3 strains Wisconsin and Netherlands in 9-day-old embryonated chicken eggs, 1-day-old chicks and turkeys, and 2-week-old chickens and turkeys in order to investigate their tropism and pathogenicity. Birds were infected by the oculonasal route and the viral tropism and replication efficiency were evaluated by quantitative virology and immunohistochemistry of a wide range of possible

target organs. In addition, a separate group of 1-day old chicks were infected intracerebrally to evaluate the potential of these viruses to replicate in neural cells.

5.4 Materials and Methods

5.4.1 Viruses and cells

APMV-3 strains parakeet/Netherlands/449/75 and turkey/Wisconsin/68 (obtained from National Veterinary Service Laboratory, Ames, Iowa, USA) and the NDV mesogenic strain Beaudette C (BC) and lentogenic strain LaSota were propagated in nine-day-old specific pathogen free (SPF) embryonated chicken eggs via allantoic route of inoculation. Work with NDV-BC was performed in our Bio Safety Level (BSL) -3 animal facility. The allantoic fluids from infected embryonated eggs were collected 96 h post-inoculation and virus titer was determined by hemagglutination (HA) assay with 0.5% chicken RBC. The virus titer in infected tissue samples was determined by the tissue culture infective dose ($TCID_{50}$) (Reed and Muench, 1938) method and by plaque assay in chicken embryo fibroblast (DF-1) cells (ATCC, Manassas, VA). DF1 cells were maintained in Dulbecco's minimum essential medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C with 5% CO_2 (Kumar et al., 2008). For the plaque assay, DF1 cell monolayers in 12 well plates were infected with different dilutions of tissue homogenates. The tissue homogenates were allowed to adsorb for 1 h at 37^0C, washed with PBS, and then overlaid with 1ml DMEM containing 0.8% (wt/vol) methylcellulose. The virus plaques in the DF1 cell monolayer were visualized 96 h post infection after staining with 1% crystal violet.

5.4.2 Pathogenicity index tests

The pathogenicity of the APMV-3 strains Netherlands and Wisconsin was determined by two standard pathogenicity tests. These included the mean death time (MDT) in 9-day-old embryonated SPF chicken eggs and the intracerebral pathogenicity index (ICPI) in 1-day-old SPF chicks (Alexander, 2009).

MDT value was determined following the standard procedure (Alexander, 1998). Briefly, a series of 10-fold (10^{-6} to 10^{-12}) dilutions of fresh infective allantoic fluid in sterile phosphate-buffered saline (PBS) were made and 0.1 ml of each dilution was inoculated into the allantoic cavities of five 9-day-old embryonated SPF chicken eggs, which were then incubated at 37°C. Each egg was examined three times daily for 7 days, and the times of embryo deaths were recorded. The minimum lethal dose is the highest virus dilution that caused death of all the embryos. MDT is the mean time in hours for the minimum lethal dose to kill all inoculated embryos. The MDT has been used to characterize the NDV pathotypes as follows: velogenic (less than 60 h), mesogenic (60 to 90 h), and lentogenic (more than 90 h) (Alexander, 1980).

For determining ICPI value, 0.05 ml of 1:10 dilution of fresh infective allantoic fluid (2^8 HA units) of each virus was inoculated into groups of ten 1-day-old SPF chicks via the intracerebral route. The birds were observed for clinical symptoms and mortality every 8 h for a period of 8 days. At each observation, the birds were scored as follows: 0, healthy; 1, sick; and 2, dead. The ICPI is the mean score for all of the birds in the group over the 8-day period. Highly virulent NDV (velogenic) viruses give values approaching 2 and avirulent NDV (lentogenic) viruses give values close to 0 (Alexander, 1998).

5.4.3 Replication and viral growth kinetics in brain tissue of 1-day-old chicks

To determine the ability of APMV-3 strains Netherlands and Wisconsin to replicate in chicken brain tissue, groups of fifteen 1-day-old SPF chicks were inoculated with a dose of 0.05 ml of a 1:10 dilution of 2^8 HA units of fresh infected allantoic fluid via the intracerebral route. NDV strain BC and LaSota were included for comparison purposes. Brain samples were collected by sacrificing three birds from each group at 1, 2, 3, 4 and 5 days post inoculation or as and when the birds died of infection. The virus titers in the tissue samples were determined by tissue culture infectivity dose 50 ($TCID_{50}$) in DF1 cells (Reed and Muench, 1938).

5.4.4 Pathogenesis assessment in chickens and turkeys

One-day-old and 2-week-old SPF chickens (Charles River, USA) and 1-day-old and 2-week-old turkeys (Murry-McMurry hatchery, USA) were housed in positive pressure isolators in our BSL-2 facility. Birds confirmed to be negative for APMV-3 specific antibody by HI assay were further isolated for pathogenesis experiments and housed in negative pressure isolators.

The 1-day-old and 2-week-old chickens and turkeys, in groups of 12 for each species and age, were infected with 0.1 ml (10^3 PFU) per bird of APMV-3 strain Netherlands or Wisconsin through the occulonasal route. Infections with the different strains were performed at separate times to avoid cross infections. An additional six birds of each species/age group remained as uninfected controls. Birds were provided with food and water *ad libitum* and monitored daily for any visible signs and symptoms twice

daily. Three birds from each infected group were euthanized on 3, 5, 7 and 14 day post infection (DPI) by rapid asphyxiation in a CO_2 chamber. The birds were swabbed orally and cloacally just before euthanasia. The following tissue samples were collected both for immunohistochemistry (IHC) and for virus isolation: brain, trachea, lung, spleen, kidney and pancreas. In addition serum samples were collected on day 14 when the three remaining birds in each group and the control birds were euthanized. Seroconversion was evaluated by HI assay (Alexander, 1996).

5.4.5 Virus detection and quantification from tissue samples and swabs

Half of the tissue samples containing brain, trachea, lung, spleen, kidney and pancreas were collected aseptically in Dulbecco's minimal essential medium (DMEM) in 10X antibiotic solution containing Penicillin/Streptomycin/Amphotericin B to determine virus content. Briefly, a 10% homogenate of the tissue samples were prepared by using a homogenizer and centrifuged at 420 x g for 10min. The virus content in the supernatants of tissue homogenates was determined by plaque assay in DF-1 cells. Values for each tissue sample were based on average plaque count from two wells. The other half of the tissue samples were flash-frozen on dry ice using chilled 2-methyl butane, held for approximately 24 h, and processed for immunohistochemistry.

The oral and cloacal swabs were collected in 1 ml of DMEM containing antibiotics. The swab containing tubes were centrifuged at 1000g for 20 min, and the supernatant was removed and flash frozen on dry ice for subsequent virus isolation. Virus isolation was performed by infecting the supernatant into 9-day-old SPF embryonated chicken eggs. Positive samples were identified by HA activity of the allantoic fluid harvested from eggs four days post-inoculation. In addition the virus titers in the positive

swab samples were determined using the $TCID_{50}$ method and DF1 cells (Reed and Muench, 1938).

5.4.6 Immunohistochemistry

Tissue samples collected in chilled 2-methyl butane were sectioned by using optimal cutting temperature compound under cryostat (Dolbey- Jamison, USA). The sections were immunostained to detect viral phosphoprotein (P) using the following protocol. Briefly, the frozen sections were thawed and rehydrated in three changes of PBS (10 min each). The sections were fixed in ice cold acetone for 15 minutes at -80° C and then washed three times with 2% BSA in PBS and blocked with the same solution for 1 h at room temperature. The sections were then incubated with a 1:500 dilution of the primary antibody (antipeptide polyclonal antisera raised against the P proteins of APMV-3 strains Netherland and Wisconsin in rabbits) in PBS overnight in a humidified chamber. After three washes with 2% BSA in PBS, sections were incubated with the secondary antibody (FITC conjugated goat anti-rabbit antibody) for 30 min. After a further wash cycle, the sections were mounted with glycerol and viewed under *a fluorescence* microscope.

5.4.7 Preparation of antipeptide polyclonal antisera against the APMV-3 P proteins in rabbits

The antipeptide antisera to the P proteins of the two APMV-3 strains were generated using the following protocol. Peptides DKTPDQGQPSATPS (corresponding

to 60 to 73 amino acid (aa) of P protein of strain Netherlands), SPSQSSSPSPEPST, and FYIPKVNNYHSN (corresponding to 39-52 and 99-110 aa, respectively, of P protein of strain Wisconsin) were custom synthesized (Invitrogen, USA). 0.2 mg of each protein was mixed in complete Freund's adjuvant and injected subcutaneously into a rabbit. After two weeks a booster immunization was given with the respective 0.2 mg of protein in incomplete Freund’s adjuvant and 2 weeks later the hyperimmune serum was collected. The antisera were tested by western blot analysis and were found to recognize specifically the P protein of their respective APMV-3 strain (data not shown).

5.5 Results

5.5.1 Pathogenicity index tests

The pathogenicity of APMV-3 strains Netherlands and Wisconsin was evaluated by MDT in 9-day-old embryonated chicken eggs and ICPI in 1-day-old-chicks. The lentogenic NDV strain LaSota was included in the pathogenicity tests for comparison. The MDT for APMV-3 strain Netherland was 112 hr while that of APMV-3 strain Wisconsin was > 168 hr. The ICPI value for APMV-3 strain Netherlands was 0.39 while the ICPI for strain Wisconsin was zero. The MDT and ICPI values of NDV strain LaSota were 110 hr and zero, respectively, consistent with a lentogenic virus. Although, the ICPI and MDT values of strain Netherlands were higher than those of strain Wisconsin; their values indicate that both the APMV-3 strains are lentogenic viruses. These results indicate that APMV-3 strain Netherlands and Wisconsin are probably nonpathogenic to chickens, similar to lentogenic NDV strains.

5.5.2 Virus growth in the chicken brain

The ability of the APMV-3 strains Netherlands and Wisconsin to grow in the brains of 1-day-old chicks was evaluated in parallel with the mesogenic neurotropic NDV strain BC. This study was performed to determine whether the 0.39 and zero ICPI values of the APMV-3 strains Netherlands and Wisconsin, respectively, was due to the inability of the viruses to grow intracerebrally or if there was virus multiplication without a high degree of cell destruction.

Virus replication was evaluated by inoculating 0.05 ml of 1:10 dilution of 2^8 HAU of each of the four viruses, strains Netherlands, Wisconsin, BC and LaSota, into the brains of fifteen 1-day-old SPF chicks per group. Three birds from each group were sacrificed on days 1, 2, 3, 4 and 5 post inoculation and virus titers in brain tissue were assayed and expressed as $TCID_{50}$ per gram of the brain in DF-1 cells (Fig. 5.1). Neither of the two APMV-3 strains produced any clinical signs nor did they kill any chicks during the 5-day time course. Both of the strains of APMV-3 were isolated from the brain homogenates of all of the chicks on days 1 to 5 day post inoculation, grew with similar kinetics, and reached similar maximum titers of 2x7.9 log_{10} and 2x7.6 log_{10} $TCID_{50}$/g on day 5 for strains Netherlands and Wisconsin, respectively (Fig. 5.1). In comparison, NDV strain BC replicated more rapidly, reached a titer of 2x8.25 log_{10} $TCID_{50}$/g of brain tissue on day 3 post inoculation, and killed all of the chicks by day 3, while strain LaSota showed no replication. The results showed that the efficiency of virus replication of the two APMV-3 strains in brain tissue was similar. Interestingly, while the two APMV-3 strains replicated more slowly than NDV-BC, they achieved a similar final titer compared to NDV-BC but without causing noticeable neurological disease and with no mortality.

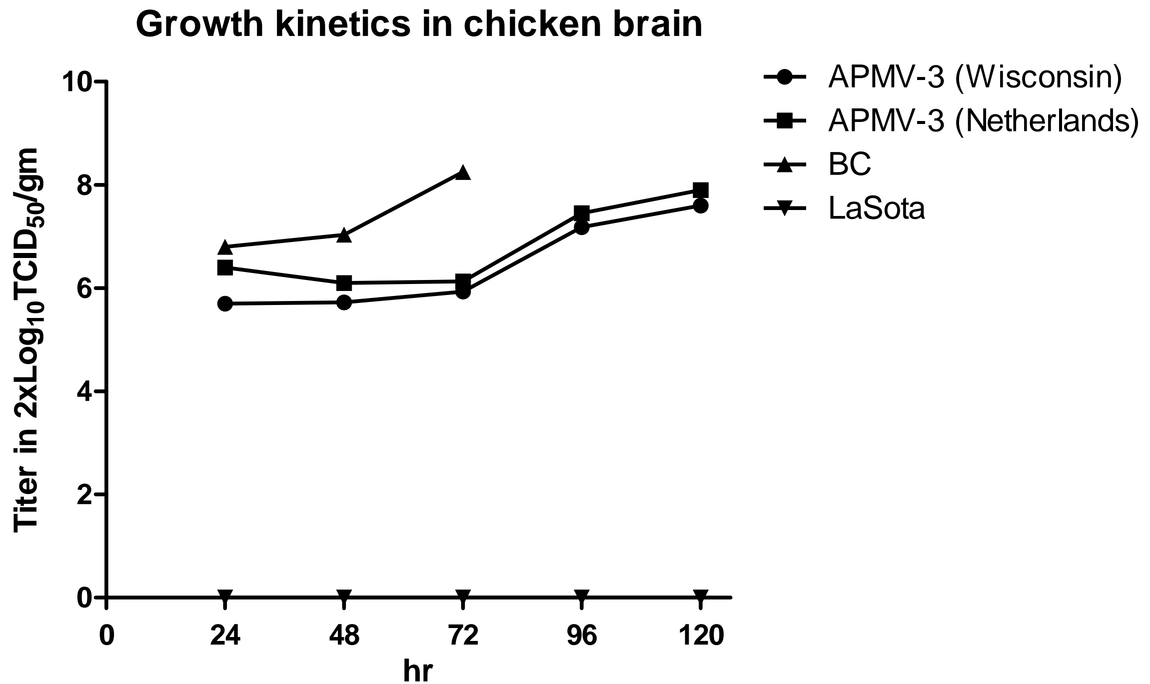


Fig 5.1. Growth kinetics of APMV-3 strain Netherlands and Wisconsin in the brains of 1day-old chicks in comparison with neurotropic APMV-1 strain Beaudette C (BC) and lentogenic strain LaSota.

Fifteen 1-day-old chicks were inoculated with 0.05 ml of 1:10 dilution of 2^8 HA units of APMV-1 strain BC or APMV-1 strain LaSota or APMV-3 strain Netherlands or Wisconsin via the intracerebral route. Brain tissue was collected by sacrificing three birds from each group on 1, 2, 3, 4 and 5 days post inoculation, except that in the case of NDV BC all remaining birds died of infection on day 3 and were assayed at that time point. Each time point represents the geometric mean of the individual virus titers of three birds.

5.5.3 Experimental infection of SPF chickens and turkeys: pathology

1-day-old and 2-week-old chickens and turkeys were inoculated with either APMV-3 strain by the occulonasal route, and birds from each group were sacrificed on days 3, 5, 7, and 14 and evaluated for pathology, virus shedding, and virus replication in selected tissues. Infection of either APMV-3 strain in 1-day-old chickens and turkeys resulted in mild clinical symptoms that included altered gait, respiratory distress, dullness, ruffled-feathers, loss of appetite and weight loss. The birds of the 1-day-old groups of both species generally started showing symptoms at 3 DPI and recovered after 7 DPI. The other visible signs included slight diarrhea that was evident in 1-day-old chickens and turkeys at 4 DPI. The neurological symptoms were more evident in 1-day-old chicks and turkeys infected with APMV-3 strain Netherlands as compared to strain Wisconsin. In contrast, infection of 2-week-old chickens and turkeys with the APMV-3 strains did not result in any clinical signs of disease. APMV-3 infection did not kill any of the 1-day-old or 2-week-old chickens and turkeys (Tables 5.1 and 5.2). The most remarkable finding upon postmortem examination of birds was enlargement of the pancreas with focal necrosis at 5 DPI in the 2-week-old chickens and turkeys (Fig. 5.2). The foci of necrosis were distributed along the entire length of pancreas and the extent of necrosis was similar in both chickens and turkeys. Gross examination of other organs including the brain, trachea, liver, kidney, spleen and lung showed normal tissue morphology with no noticeable gross lesions in either the 1-day-old or 2-week-old birds.

Table-1

1-day-old chickens infected with APMV-3

Virus	Mortality	Virus shedding 3rd day O/C	5th day O/C	7th day O/C	14th day O/C	Mean bodywt(gms)	HI titer log2 14 days p.i
APMV-3 Netherlands	0/12	+/-	+/-	-/-	-/-	70	1:128
APMV-3 Wisconsin	0/12	+/-	+/-	-/-	-/-	75	1:128
Control	0/12	-/-	-/-	-/-	-/-	80	N/D

2-week-old chickens infected with APMV-3

Virus	Mortality	Virus shedding 3rd day O/C	5th day O/C	7th day O/C	14th day O/C	Mean bodywt(gms)	HI titer log2 14 days p.i
APMV-3 Netherlands	0/12	+/-	-/-	-/-	-/-	160	1:512
APMV-3 Wisconsin	0/12	-/-	-/-	-/-	-/-	175	1:256
Control	0/12	-/-	-/-	-/-	-/-	180	N/D

Table. 5.1

Virus shedding and mortality in 1-day-old and 2-week-old chickens infected with APMV-3. Virus shedding in oral secretions and cloacal swabs (C) were measured for 3 birds from each group on the indicated days. Mean body weight also was measured at sacrifice. Sera were collected from three birds in each group on day 14 and mean APMV-3-specific HI titers were determined. The pre-infection and control HI titers were 2 or less.

Table-2

1-day-old turkeys infected with APMV-3

Virus	Mortality	Virus shedding				Mean bodywt(gms)	HI titer log2 14 days p.i
		3rd day O/C	5th day O/C	7th day O/C	14th day O/C		
APMV-3 Netherlands	0/12	+/-	+/-	-/-	-/-	80	1:128
APMV-3 Wisconsin	0/12	+/-	+/-	-/-	-/-	90	1:128
Control	0/12	-/-	-/-	-/-	-/-	120	N/D

2-week-old turkeys infected with APMV-3

Virus	Mortality	Virus shedding				Mean bodywt(gms)	HI titer log2 14 days p.i
		3rd day O/C	5th day O/C	7th day O/C	14th day O/C		
APMV-3 Netherlands	0/12	+/-	-/-	-/-	-/-	140	1:256
APMV-3 Wisconsin	0/12	-/-	-/-	-/-	-/-	170	1:128
Control	0/12	-/-	-/-	-/-	-/-	180	N/D

Table. 5.2

Virus shedding and mortality in 1-day-old and 2-week-old turkeys infected with APMV-3. Virus shedding in oral secretions and cloacal swabs (C) were measured for 3 birds from each group on the indicated days. Mean body weight also was measured at sacrifice. Sera were collected from three birds in each group on day 14 and mean APMV-3-specific HI titers were determined. The pre-infection and control HI titers were 2 or less.

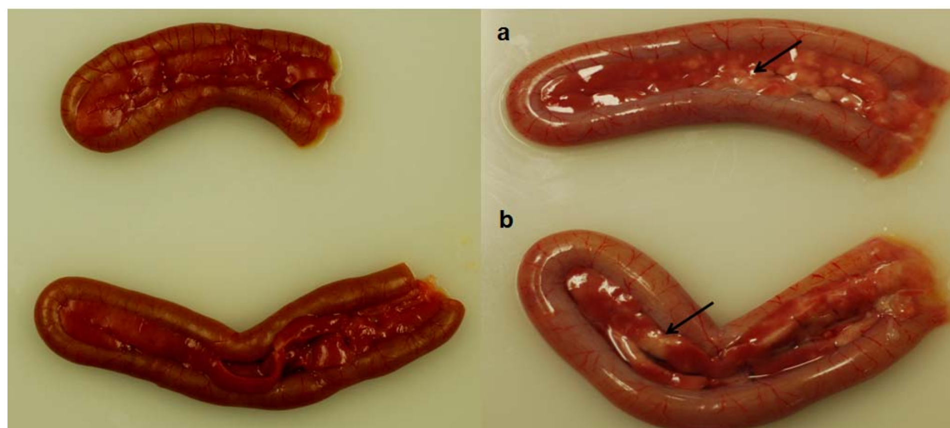


Fig. 5.2

Gross morphology of pancreas collected from 2-week-old turkeys infected with APMV-3 strain Netherlands (a) or Wisconsin (b): comparison with pancreas from uninfected controls (left panels) reveals necrotic foci along the length of the infected organ.

5.5.4 Experimental infection of SPF chickens and turkeys: virus isolation and titration in oral and cloacal swabs

From the experiment above, oral and cloacal swabs were taken when birds were sacrificed on days 3, 5, 7 and 14 DPI. These samples were inoculated into embryonated chicken eggs to detect the presence of infectious virus, which was determined by HA assay of egg allantoic fluid (Tables 5.1 and 5.2). Samples that were positive for virus were analyzed for virus quantitation by plaque assay in DF1 cells (not shown). Neither APMV-3 strain was isolated from cloacal swabs from any of the infected birds. Virus was isolated from oral swabs from 1-day-old chickens on days 3 and 5 for both strains, and from 2-week-old chickens on day 3 for strain Netherlands only (Table 5.1). This same pattern was observed for virus isolated from oral swabs from 1-day-old and 2-week-old turkeys (Table 5.2). Thus, strain Wisconsin was not shed from any of the 2-week-old chickens or turkeys. The highest titers in chickens (2.15 log_{10} $TCID_{50}$/ml) were observed in oral swabs collected on 3 DPI from 1-day-old chicks infected with either strain of APMV-3 (not shown). Similarly, in turkeys, the highest titers (2.12 log_{10} $TCID_{50}$/ml) were observed in 1-day-old poults at 3 DPI with either strain of APMV-3 (not shown).

5.5.5 Experimental infection of SPF chickens and turkeys: virus isolation and titration in tissue samples

In the experiment described above, the following tissue samples were collected from three birds per group on 3, 5, 7 and 14 DPI: brain, trachea, lung, spleen, kidney and pancreas. Homogenates of these tissues were inoculated into embryonated chicken eggs to detect the presence of infectious virus, as described above, and positive samples were titrated by plaque assay in DF1 cells.

Fig. 5.3 shows the distribution and titers of the two APMV-3 strains in various organs on different DPI of chickens. Infection with APMV-3 strains Netherlands and Wisconsin in 1-day-old chicks and 2-week-old chickens resulted in efficient replication of both viruses in the various organs tested. In general, both viruses replicated in most of the tested organs at early time periods of infection and were cleared at 7 DPI. The virus replication titers were higher in 1-day-old chicks than in 2-week-old chickens, and replication usually was the highest in the brain, trachea, and lung. Although APMV-3 strain Wisconsin replicated less efficiently in 2-week-old chickens, the titer of virus in lungs on day 1 was somewhat higher than that of strain Netherlands, suggesting that it might be more respirotropic, whereas strain Netherlands appeared to be more neurotropic on day 1 in the 2-week-old birds.

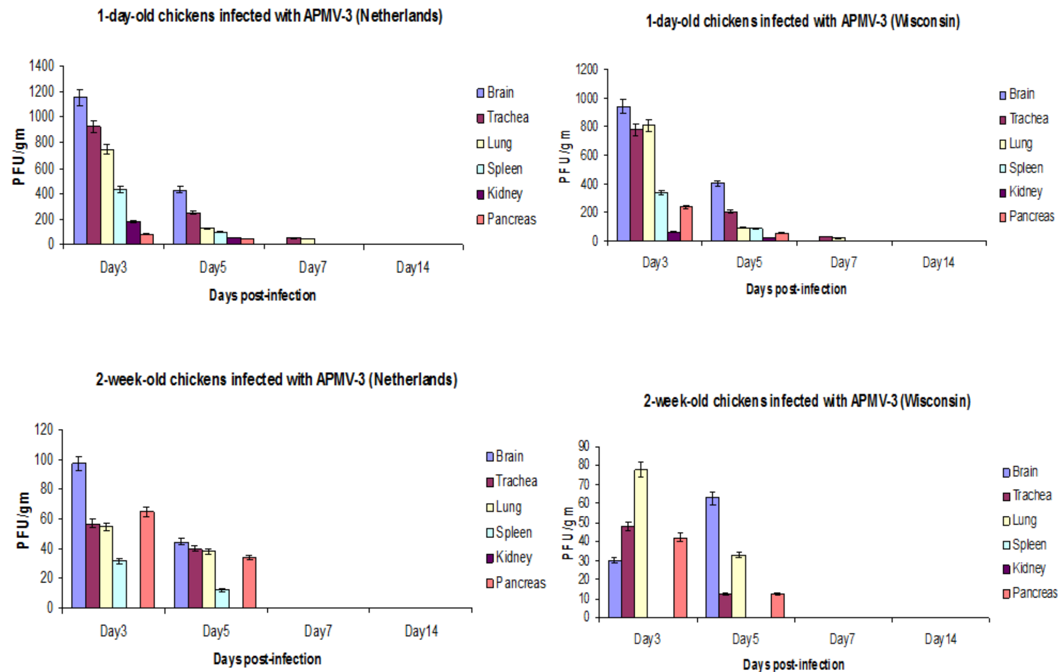


Fig. 5.3

Virus titers from the indicated organs on the indicated days after infection of 1-day-old (upper panels) or 2-week-old (lower panels) chickens with APMV-3 strain Netherlands (left panels) or Wisconsin (right panels). Titers are shown as mean PFU/gm.

In general, infected turkeys had virus replication in fewer organs and for shorter duration than chickens of same age groups (Fig. 5.4). Strain Netherlands was isolated from all of the tested organs on 3 DPI except for the pancreas in 1-day-old turkeys and the kidneys in 2-week-old turkeys. Strain Wisconsin was isolated from all of the tested organs on 3 DPI. Interestingly, APMV-3 strain Wisconsin was not detected at all on 3 DPI in 2-week-old turkeys, but was detected in some of the organs on 5 DPI (Fig. 5.4). No virus of either strain was detectable on 5 and 14 DPI (Fig. 5.4) from any of the tissues

harvested from the infected turkeys, indicating virus clearance, whereas complete clearance in chickens did not occur until 14 DPI. These results suggest that turkeys are less susceptible to infection with either APMV-3 strain compared to chickens.

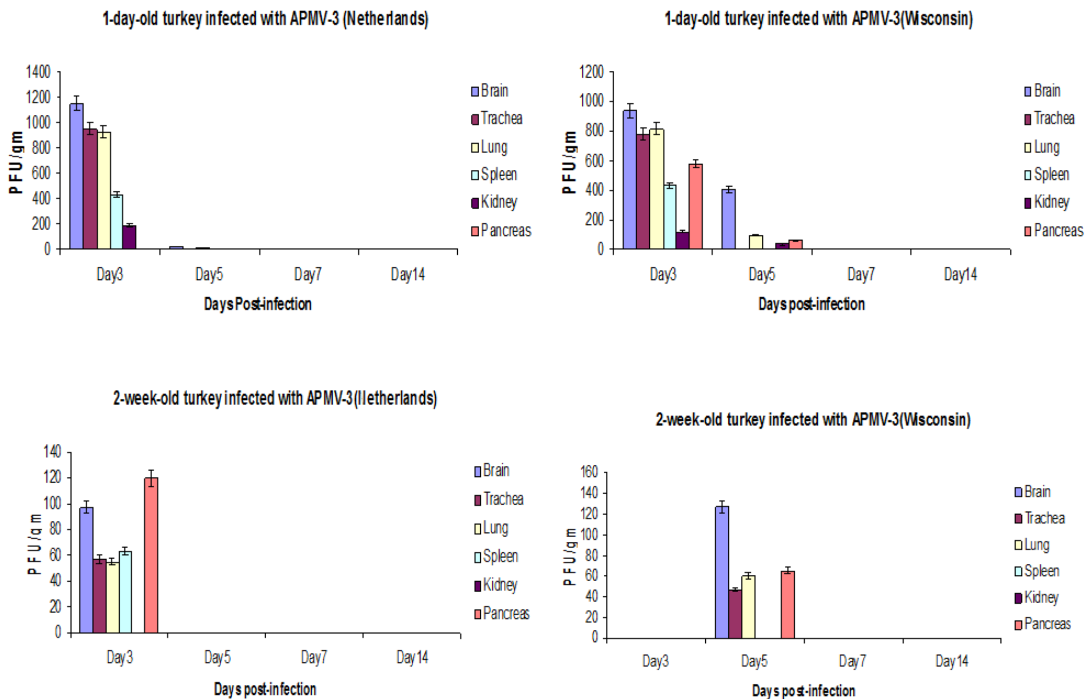


Fig. 5.4

Virus titers from the indicated organs on the indicated days after infection of 1-day-old (upper panels) or 2-week-old (lower panels) turkeys with APMV-3 strain Netherlands (left panels) or Wisconsin (right panels). Titers are shown as mean PFU/gm.

5.5.6 Seroconversion

An HI assay using chicken erythrocytes was performed with the sera collected from chickens and turkeys of both age groups on 14 DPI. The HI titers of the pre-infection and control chickens and turkeys were 2 or less. An HI titer of greater than 8

was considered positive. Each of the 1-day-old and 2-week-old chickens and turkeys infected with either strain seroconverted. The mean HI titers in 1-day-old chicks for both the strains were 1:128; while the mean HI titers in 2-week-old chickens for strain Netherlands and Wisconsin were 1:512 and 1:256 respectively (Table. 5.1). Similarly, the mean HI titers in 1-day-old-turkeys for the both the strains were 1:128, while the mean HI titers in 2-weeks-old turkeys for strain Netherlands and Wisconsin were 1:256 and 1:128 respectively (Table. 5.1).

5.5.7 Immunohistochemistry

The frozen sections of all the virus-positive tissue samples and some of the virus-negative control samples were immunostained using antipeptide polyclonal antibodies against the P proteins of APMV-3 strains Netherlands and Wisconsin. Large amounts of viral P antigen was detected consistently in all the brain, trachea, lung, and pancreas samples that were positive by virus isolation, and no viral antigen was detected in tissue samples that were negative by virus isolation. Extensive immunofluorescence was observed in the brain, trachea, lung and pancreas, suggesting extensive virus replication in these organs (Fig. 5.5). The intensity of staining was greater for strain Netherlands (Fig. 5.3) than for strain Wisconsin (data not shown). The presence of fluorescence around the epithelial lining in the lungs and trachea showed the distribution of viral antigens on respiratory epithelium. The presence of generalized fluorescence in the pancreas and brain showed the uniform distribution of viral antigen. However, no viral P antigen was detected in kidney and spleens that were positive by virus isolation, suggesting that replication was not extensive in these organs.

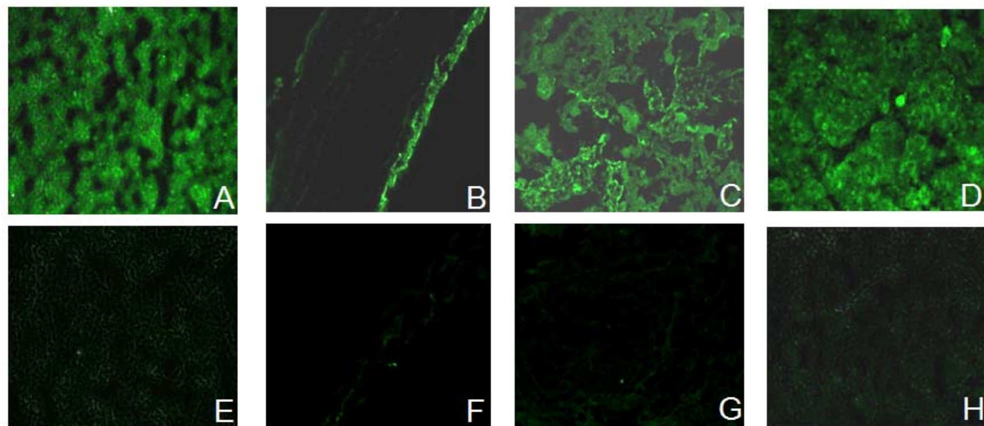


Fig. 5.5

Immunohistochemistry of different organs after three days post infection with APMV-3 strain Netherlands in turkeys. Viral antigen was visualized by indirect immunofluorescence using polyclonal anti-peptide rabbit antibody specific for the APMV-3 P protein as the primary antibody. The fluorescence was observed in brain (a), trachea (b), lung (c) and pancreas (d) while no fluorescence was observed in organs collected from control birds (e-h).

5.6 Discussion

APMVs are frequently isolated from wild and domestic birds around the world and have been placed into nine serotypes based on antigenic relatedness. Of these nine serotypes, APMV-1 (NDV) is the best studied. APMV-2 to -9 are present in both free living and domestic birds, but their disease potential is not known in wild birds nor in domestic birds. It is probable that wild birds can transmit APMV-2 to -9 to domestic birds. APMV-2, -3, -4, -6, -7, -8, -9 have been isolated from domestic poultry birds and antibodies to these viruses have also been detected in domestic poultry. However, the disease potential of APMV-2 to -9 in chickens and turkeys is mostly unknown.

Here, I investigated the clinical disease and pathogenicity of APMV-3 strains Netherlands and Wisconsin in embryonated chicken eggs, in 1-day-old chicks inoculated intracerebrally, 1-day-old chicks and turkeys inoculated oculonasally, and in 2-week-old chickens and turkeys inoculated oculonasally. The oculonasal route was intended to resemble a natural route of infection. In this study, 2-week-old chickens and turkeys were chosen over other possible age groups because at this age they are fully susceptible to viral infection. Furthermore, the pathogenicity in 1-day-old chicks and turkeys was studied to assess the impact of APMV-3 infection in young birds.

APMV-3 strains Netherlands and Wisconsin were first characterized by standard pathogenicity tests (MDT and ICPI). The results of the MDT test showed that APMV-3 strain Netherland was slightly pathogenic (112 hr) compared to strain Wisconsin (>168 hr), which was completely apathogenic. Similar findings were also observed in ICPI test: APMV-3 strain Netherland showed an ICPI value of 0.39, while the ICPI value of strain Wisconsin was zero. Although results of pathogenicity index tests (MDT and ICPI) showed that APMV-3 strain Netherlands was slightly pathogenic to chickens, both strains belong to the lentogenic group of avian paramyxoviruses (Alexander, 1998).

Since the APMV-3 strains did not kill 1-day-old chicks by the ICPI test, I investigated whether the absence of neurovirulence was due to lack of virus replication in brain or whether replication occurred without any notable cell destruction. The growth of APMV-3 strains in chicken brain showed that both the strains are equally competent to grow in brain tissue and the highest virus titer was observed 120 hr post inoculation. However, none of the APMV-3 inoculated chicks died 5DPI, while all chicks inoculated with mesogenic NDV strain BC died 3 DPI. These results suggest that the low ICPI

values of APMV-3 strains were due to nondestructive replication of the virus in brain tissue.

It has been shown previously by experimental infection that APMV-3 strain Netherlands is more virulent than strain Wisconsin (Alexander, 1980). In another study, it was shown that the extent of disease and death in the birds after infection with APMV-3 strain Netherlands depends on dose as well as route of infection (Alexander and Collins, 1982). In this study, I evaluated the pathogenicity of APMV-3 strains Netherlands and Wisconsin in 1-day-old and 2-week-old chickens and turkeys by natural (oculonasal) route of infection. The clinical signs of illness were more evident in 1-day-old birds compared to 2-weeks-old birds. In chickens, both the strains of APMV-3 were isolated from tissues of respiratory, digestive and nervous systems. Similar findings were observed in turkeys that were infected with APMV-3 strains. However, the titers of APMV-3 strain Netherlands in different organs were higher than those of strain Wisconsin in all the infected bird groups. Although both the viruses were detected in oral swabs from both 1-day-old and 2-week-old chickens and turkeys, the shedding of viruses were less in 2-week-old birds. These results confirmed previous findings that chickens and turkeys are susceptible to APMV-3 infection and younger birds are more susceptible than older birds. In addition, my results are in agreement with the previous finding that strain Netherland replicates better than strain Wisconsin in chickens and turkeys (Alexander and Collins, 1982; Russell et al., 1989).

Visceral gross lesions were evident in 2-week-old chickens and turkeys at 3 and 5 DPI. Using IHC, viral P protein was detected in the same tissues that were positive by virus isolation except the spleen and kidney tissues that were positive by virus isolation

but negative by IHC. It is possible that the virus load in infected spleen and kidney was too low to be detected by IHC. An interesting finding was the presence of large amounts of viral antigens at the epithelial cell linings, suggesting that these cells are highly permissive to APMV-3 replication. Another finding of IHC study was the presence of viral antigen in both epithelial and sub-epithelial tissues except for trachea where the antigen was localized only in the epithelial surface. This suggests that this virus has a tropism towards both epithelial and sub-epithelial cells. In addition, the detection of viral antigens, and in most cases infectious virus, in multiple internal organs of the birds indicates that both the viruses are capable of replicating in multiple organs rather than being restricted only to respiratory and alimentary tracts. Presumably, the virus reached the various internal organs through the blood stream. Nonetheless, this extensive amount of virus replication was not accompanied by severe disease in birds. These results show that APMV-3 strains are capable of infecting young and adult chickens and turkeys using an oculonasal route of infection. Serologic assays demonstrated a humoral response in all the birds inoculated with either APMV-3 strain, a further indication of successful replication. However, results suggest that chickens are comparatively more susceptible than turkeys to APMV-3 infection.

The F protein cleavage site of NDV is a well characterized determinant of NDV pathogenicity in chickens (Millar et al., 1988; de Leeuw and Peeters, 1999; Panda et al., 2004). Virulent NDV strains typically contain a polybasic cleavage site that contains the preferred recognition site for furin (R-X-K/R-R↓), which is an intracellular protease that is present in most cells. This provides for efficient cleavage in a wide range of tissues, and increases virulence by making it possible for virus to spread systemically. In contrast,

avirulent NDV strains typically have basic residues at the −1 and −4 positions relative to the cleavage site and depend on secretory protease (or, in cell culture, added trypsin) for cleavage. Also, the first amino acid of the newly-created F1 terminus is phenylalanine for virulent NDV strains and leucine for avirulent NDV strains, an assignment that also reduces the efficiency of cleavage (Morrison et al., 1993). The inability to be cleaved by furin limits the replication of avirulent strains to the respiratory and enteric tracts where secretory protease is available for cleavage. The putative F protein cleavage site of APMV-3 strain Netherlands (ARPRGR↓L) and strain Wisconsin (PRPSGR↓L) have three or two basic residues (underlined), which is similar but not identical to the pattern of virulent and avirulent NDV strains, respectively. Neither APMV-3 strain contains the preferred furin cleavage site. The F1 subunit of both APMV-3 strains begins with a leucine residue, as is characteristic of avirulent NDV strains, rather than a phenylalanine residue, as seen in most virulent NDV strains. The lack of a furin motif in the F protein cleavage site and the presence of leucine as the first residue at the terminus of the F1 subunit predict that both APMV-3 strains should require added protease for replication in cell culture and should be limited to replication in the respiratory or enteric tracts in vivo. However, APMV-3 strains Netherlands and Wisconsin both replicate well in a wide range of cells *in vitro* in the absence of added protease, although each strain grows more efficiently (2 $\log_2$ higher) with the addition of protease (data not shown). Also, in the present study, both APMV-3 strains were detected abundantly in various internal organs, suggesting a systemic spread of the virus. These results indicate that the structure of the F protein cleavage site and F1 terminus are poor predictors of the growth properties of APMV-3 in vitro and in vivo. This suggests (i) that the cleavage phenotype of the

APMV-3 F protein is determined by factors in addition to the F cleavage site and F1 N-terminus, and (ii) that systemic spread is a multifactorial phenomenon. Furthermore, systemic spread was a poor predictor of pathogenesis, since both APMV-3 strains spread systemically and replicated efficiently in the brain, lung, trachea, and pancreas with little or no disease.

In conclusion, I have shown that adult SPF chickens and turkeys are susceptible to APMV-3 infection. However, in commercial chickens and turkeys the disease picture could be quite different depending on management practices, environmental conditions and other concomitant infections. This study has demonstrated that APMV-3 has an affinity for both epithelial as well as sub-epithelial cells of respiratory and alimentary tracts. Finally, dissociations were observed between F protein structure and systemic spread and between systemic spread and disease. Further studies are needed to understand the disease potential of this virus to commercial poultry.

Chapter 6

6.1 Title

Evaluation of pathogenicity of avian paramyxovirus serotypes-3 strain Netherlands infection in hamsters and mice.

6.2 Abstract

Avian paramyxoviruses (APMVs) are frequently isolated from domestic and wild birds throughout the world. The APMVs are separated into nine serotypes (APMV -1 to -9). Only in

the case of APMV-1, also called Newcastle disease virus, has infection of non-avian species been investigated. The APMVs presently are being considered as human vaccine vectors. In this

study, I evaluated the replication and pathogenicity of prototype strains of APMV-3 strain Netherlands in hamsters and mice. The hamsters and mice were inoculated intranasally with APMV-3 and monitored for clinical disease, pathology, histopathology, virus replication, and seroconversion. On the basis of one or more of these criteria, APMV-3 was found to replicate in hamsters and mice. The APMVs produced mild or inapparent clinical signs in hamsters and mice. Gross lesions were observed over the pulmonary surface of both hamsters and mice infected with APMV -3, which showed petechial and ecchymotic hemorrhages. Replication of APMV-3 was confirmed in the nasal turbinates and lungs, indicating a tropism for the respiratory tract. Histologically, infection with the APMVs resulted in lung lesions consistent with broncho-interstitial pneumonia of varying severity and nasal turbinates with blunting or loss of cilia of the

epithelium lining the nasal septa. The majority of APMV-3 infected hamsters and mice exhibited transient histological lesions that self resolved by 14 days post infection. All of the infected hamsters and mice produced serotype-specific HI or neutralizing antibodies, confirming virus replication. Taken together, these results demonstrate that APMV-3 is capable of replicating in hamsters with minimal disease and pathology.

6.3 Introduction

APMV-1 (NDV) is known to replicate in non-avian species including humans (Hofstad, 1950; Nelson et al., 1952; Quinn et al., 1952; Yates et al., 1952), although its only natural hosts are birds. APMV-1 infections in non-avian species are usually asymptomatic or mild. Clinical signs in human infections commonly involve conjunctivitis, which usually is transient and self-limiting. Presently, APMV-1 is being evaluated as a vaccine vector against human pathogens (Bukreyev and Collins, 2008). When administered to the respiratory tract of non-human primates, NDV is highly restricted in replication, but foreign antigens expressed by recombinant NDV vectors are moderately to highly immunogenic. One of the major advantages of this approach is that most humans do not have pre-existing immunity to APMV-1. Pre-existing immunity is a potential drawback for using vectors derived from common human pathogens, and also can be a concern for any vector if two or more doses are necessary to elicit protective immunity. Therefore, I investigated APMV-3 which is antigenically distinct from APMV-1, as alternative human vaccine vectors. Also, APMV-3 will have differences in replication, attenuation, and immunogenicity compared to APMV-1 that may be advantageous. However, the replication and pathogenicity of APMV-3 in non-avian

species has not been studied. As a first step, I evaluated the replication and pathogenicity of APMV-3 in hamsters and mice. In this study, groups of hamsters and mice were infected with a prototype strain of APMV-3 strain Netherlands by the intranasal route and monitored for virus replication, clinical symptoms, histopathology, and seroconversion. The results showed that APMV-3 replicated in hamsters and mice without causing adverse clinical signs of illness, although histopathologic evidence of disease was observed in some cases, and also induced high neutralizing antibody titers.

6.4 Materials and Methods

6.4.1 Virus and cells

APMV-3 strain parakeet/Netherlands/449/75 was obtained from the National Veterinary Services Laboratory, Ames, Iowa. Nine-day-old specific pathogen free (SPF) embryonated chicken eggs and chicken embryo fibroblast DF1 cells were used to propagate APMV-3 strain Netherlands. Hemagglutination (HA) titers of virus stocks were determined using 0.5% chicken RBC at room temperature. DF1 cells were grown in Dulbecco's MEM containing 10% FCS. All the cells were grown in a 37°C incubator with 5% CO_2. The virus titer in each cell type was quantified by plaque assay in DF 1 cells as previously described (Krishnamurthy et al., 2000). The cells were overlaid with DMEM containing 2% FCS and 0.9% methylcellulose that were further supplemented with 10% allantoic fluid as a source of proteases. The plaques were visualized by staining with 1% crystal violet after 4 days of infection.

6.4.2 Preparation of antipeptide polyclonal antisera against the APMV-3 P proteins in rabbits

The antipeptide antisera to the P proteins of the APMV-3 strain Netherlands was generated using the protocol described in chapter 5.4.7.

6.4.3 Experimental infection of hamsters and mice

To study viral replication and pathogenicity, nine each of 4-week-old Syrian golden hamsters and BALB/c mice (Charles River Laboratories Inc, Wilmington, MA) were housed in negative-pressure isolators under Bio Safety Level (BSL)-2 conditions and provided feed and water ad libitum. The animals were cared for in accordance with the Animal Welfare Act and the Guide for Care and Use of Laboratory Animals and the protocols were approved by the institution's IACUC. Six each of hamsters and mice were inoculated intranasally with 100 µl of infectious allantoic fluid containing 2^8HAU of APMV-3, which contained 3 X 10^3 PFU/ml, under isoflurane anesthesia. A group of three each of hamsters and mice served as uninfected controls and were mock infected with normal allantoic fluid. All the animals were observed three times daily for physical activity and for any clinical signs of illness, and were weighed on day 0, 5 and 14 days post inoculation (dpi). Three hamsters and mice from each group were euthanized at 3 (dpi) and the other three (as well as the three animals in the control group) at 14 dpi by rapid asphyxiation in a CO2 chamber. Necropsies were performed immediately postmortem and the following tissue samples were collected for immunohistochemistry (IHC), histopathology, and virus isolation: brain, nasal turbinates, lung, spleen, kidney

and small intestine. In addition serum samples were collected on 14 dpi immediately prior to euthanasia, and seroconversion was evaluated by HI assay (Alexander, 1998).

6.4.4 Virus detection and quantification from tissue samples

Half of each tissue sample was used for virus titration. These samples were collected aseptically in 1 ml of DMEM in 10X antibiotic solution containing 2,000units/ml penicillin, 200ug/ml gentamicin sulfate, and 4ug/ml amphotericin B (Sigma chemical co., St.Louis, MO). They were processed immediately to avoid any reduction in virus titers. Briefly, a 10% homogenate of the tissue samples were prepared by using a homogenizer and clarified by centrifugation at 420 x g for 10min. The virus titers in the clarified supernatants were determined by end-point dilution on DF-1 cells. Ten-fold dilutions of tissue supernatant were inoculated onto DF-1 cells in 96-well plates and incubated for 3 days. The plates were fixed in 10% formalin for 30 minutes and the cells were permeabilized using 2% Triton X-100 for 2 min. The plates were washed five times with PBS to remove any residual formalin in the wells. The cells were blocked using 2% normal goat serum for 60 min and the plates were washed twice with PBS-Tween-20 (PBS-T). The cells were incubated with primary rabbit antiserum raised against the P protein of APMV-3 at room temperature for 1 h. The plates were washed with PBS twice, 5 min each. The cells were incubated with anti rabbit FITC antibody as secondary antibody for 45 minutes and washed finally with PBS twice, 5 min each. The slides were visualized and the virus titers were determined by end point titration method using the Reed and Muench formula, and were photomicrographed using a fluorescent microscope (ZeissAxioshop 2000) (Reed, 1938).

6.4.5 Immunohistochemistry (IHC) and Histopathology

The other half of each tissue sample was used for IHC and histopathology. The tissues were fixed in 10% neutral buffered formalin, held for approximately 7 days, and processed for IHC and histopathology. Paraffin embedded 5-micron sections of all the tissue samples were prepared at Histoserve, Inc. (Maryland, USA). The sections were stained with hematoxylin and eosin for histopathology. Sections were also immunostained to detect viral P protein using the following protocol. Briefly, the tissue sections were deparaffinized in two changes of xylene for 5 minutes each, hydrated in two changes of 100% ethanol for 3 minutes each, changes of 95% and 80% ethanol for 1 minute each, and finally washed in distilled water. The sections were processed for antigen retrieval in a water bath containing sodium citrate buffer (10mM citric acid, 0.05% Tween 20, pH 6.0), at 95-100°C for 40 minutes and then allowed to cool to room temperature for another 60 minutes. The sections were rinsed in PBS-Tween 20, twice for 2 min each. The sections were blocked with 2% BSA in PBS for 1 h at room temperature. The sections were then incubated with a 1:500 dilution of the APMV-3 P-specific rabbit antiserum in PBS for 1 h in a humidified chamber. After three washes in PBS, the sections were incubated with the secondary antibody (FITC conjugated goat anti-rabbit antibody) for 30 min. After a further wash cycle, the sections were mounted with glycerol and viewed under an immunofluorescence microscope.

6.4.6 Serological analysis

Sera were collected from all the hamsters and mice on 14 dpi and evaluated for seroconversion by HI assay (Alexander, 1988).

6.5 Results

6.5.1 Clinical disease and gross pathology

Six four-week-old hamsters and mice were inoculated by the intranasal route with 2^8 HA units of each of the APMV-3. Three animals from each group were sacrificed on day 3 and the remaining three animals were sacrificed on day 14; following sacrifice, the animals were processed for gross pathology, histopathology, IHC, quantitative virology, and seroconversion. Three uninfected animals served as controls and were sacrificed and processed on day 14. All of the animals were observed three times daily and weighed daily.

None of the hamsters infected with APMV-3 showed any visible clinical signs of disease. The uninfected control hamsters appeared healthy and normal. Following sacrifice on days 3 and 14, the followings organs were removed and examined for gross pathology, histopathology, immunohistochemical analysis, and quantitative virology: brain, lungs, nasal turbinates, small intestine, kidney and spleen. Gross pathologic findings were limited to the lungs. There were several diffuse small round shaped red foci on the lungs (Fig. 6.1 marked arrow). There were no gross visceral pathologic lesions in any of the infected hamsters at 14 dpi. No lesions were detected in the uninfected control hamsters.

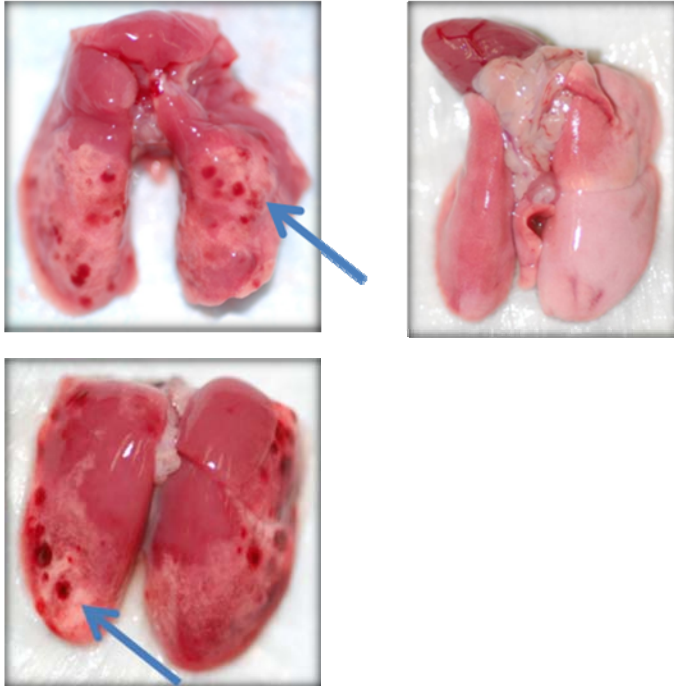


Fig.6.1 Lungs from a hamster infected with APMV-3, showing multifocal areas of consolidation and ecchymotic hemorrhage (arrow marked).

None of the mice infected with APMV-3 displayed any overt clinical signs and loss of weight. Gross examination of lung, nasal turbinate, brain, spleen, kidney and small intestine of all the mice infected with APMV-3 showed normal tissue morphology with no noticeable gross lesions.

6.5.2 Histopathology

Histopathological lesions at 3 dpi were observed in the nasal turbinates and lungs of all infected hamsters and mice. The most predominant histopathological lesions observed in the nasal turbinates were multifocal necrotic/apoptotic epithelial cells and nuclear pyknosis. Along the nasal septum were areas of epithelial necrosis, accumulation of nuclear debris in the mucosal epithelium, vacuolation of epithelial cells, and blunting or loss of cilia. Small numbers of mononuclear cells and neutrophils multifocally infiltrated the nasal mucosa, with transcytosis and exocytosis into the nasal cavity. There

were small accumulations of inflammatory cells and necrotic cellular debris in the nasal cavity. All the lung samples from the infected hamsters and mice exhibited interstitial bronchopneumonia of varying severity at 3 dpi (Fig. 6.2). The inflammatory cell infiltrates were mixed populations of lymphocytes, macrophages, and neutrophils. Additionally, bronchiolar and type II pneumocyte hyperplasia in areas of inflammation with variable degrees of cellular atypia were noticed and the degree of cellular atypia was quite prominent. There were no histopathologic findings for any other organs (brain, small intestine, kidney, and spleen) from any infected hamsters and mice on day 3, or for any of the animals at 14 dpi.

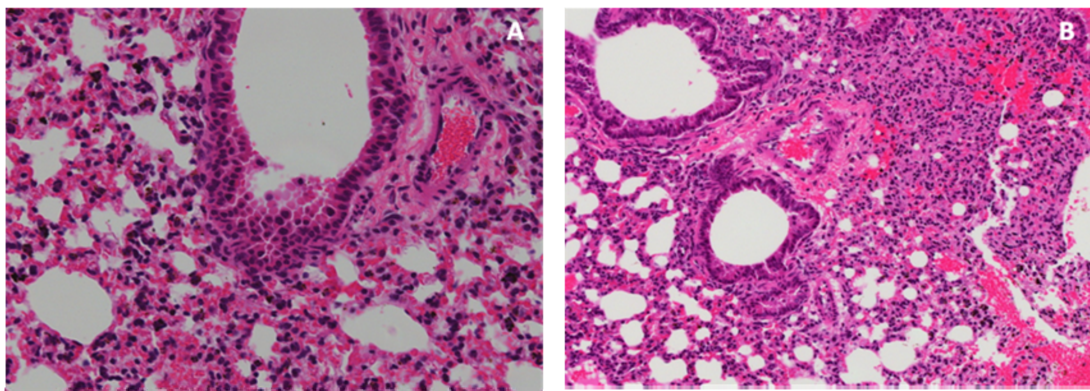


Fig. 6.2 Representative histopathologic lesions in sections of lung from mice 3 dpi with control (A) and APMV-3 (B).

6.5.3 Immunohistochemistry

Deparaffinized sections of the virus-infected and uninfected control tissue (brain, lungs, nasal turbinates, small intestine, kidney, and spleen) were immunostained using polyclonal antisera against the P protein of APMV-3 strain Netherlands. Virus specific

antigens were detected on 3 dpi in the lungs and nasal turbinates (Fig. 6.3). In the nasal turbinates, virus-positive immunofluorescence was noticed throughout the nasal epithelium lining the turbinate bone. In the lungs, the viral antigens were mostly localized in the epithelium surrounding the medium and small bronchi. Viral P antigens were not detected in any additional organs of infected hamsters and mice at 3 dpi, and were not detected in any of the organs at 14 dpi. The organs of uninfected control hamsters and mice were also negative by immunohistochemistry assay.

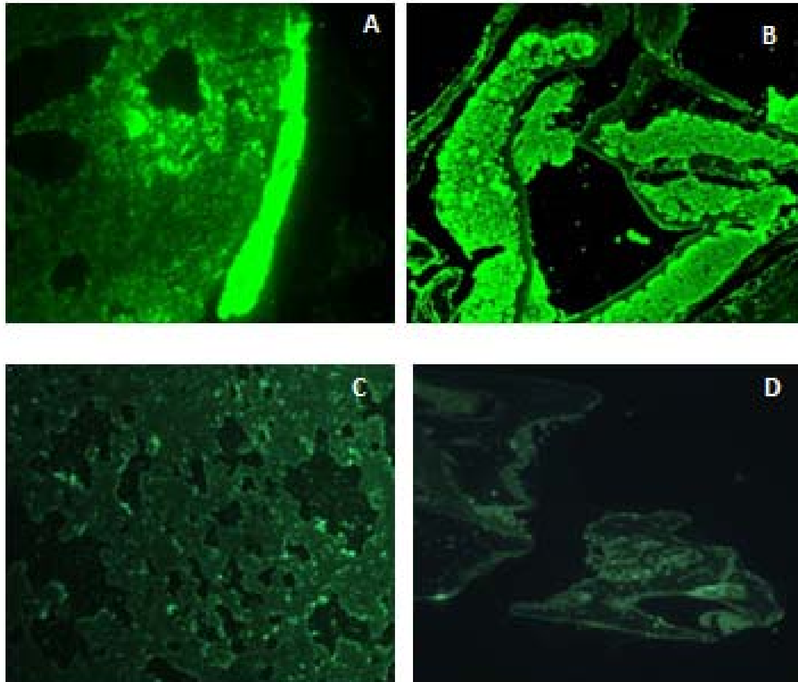


Fig 6.3. Immunofluorescence localization of viral P antigen in sections of lungs (A and C), and nasal turbinates (B and D) from hamster (C and D) and mice (A and B) at 3 dpi. Immunofluorescence was evident in the ciliated epithelium lining the turbinate bone. Moreover, immunofluorescence was also evident primarily at the apical surface of the ciliated epithelial cells and in the cytoplasm (B and D). Immunofluorescence was evident around the bronchial epithelium in the section of the lungs from mice infected with APMVs (A and C).

6.5.4 Virus Isolation and titration in tissue samples

To analyze sites of virus replication, several organs (brain, lungs, nasal turbinate, small intestine, kidney and spleen) were collected on 3 and 14 dpi, for virus isolation and titration. Virus was detected on 3 dpi; no virus was detected in any sample for any serotype on 14 dpi. APMV-3 was isolated from lungs and nasal turbinates of all three hamsters, with mean titers of 5.3×10^2 and 3.6×10^4, respectively. Also, no virus was isolated from the brain of any hamsters infected with APMV-3. Comparatively less titers were detected in mice where the mean titers of APMV-3 in lungs and nasal turbinates were 7×10^1 and 2×10^1, respectively.

6.5.5 Serology

The virus replication in the hamsters infected with APMVs was further investigated by measuring seroconversion 14 dpi. Sera were analyzed by HI assay using chicken erythrocytes The HI titers of the pre-infection hamsters was 2 or less. HI titer greater than 8 was considered positive. The results showed that all of the infected hamsters seroconverted at 14 dpi, indicating replication of APMV-3. The mean HI titer in hamsters for APMV-3 was 1:512. The mean HI titer in serum of mice infected with APMV-3 was 1:64.

6.6 Discussion

APMVs are frequently isolated from a wide variety of avian species and are grouped into nine serotypes based on antigenic reactions (Alexander, 2000). APMV-1 (NDV) is the most extensively characterized member of the APMV serotypes. APMV-3 has been isolated from both wild and domestic birds, but their disease potential in wild or domestic birds is largely unknown. APMV-1 has also been shown to infect a number of non avian species (Hofstad, 1950) and presently is being evaluated as a potential human vaccine vector for human pathogens (Bukreyev and Collins, 2008). Therefore, there is a possibility that APMV -3 could also be used as human vaccine vectors for human pathogens. However, the ability of APMV-3 to replicate in mammalian hosts was unknown. Hence, I investigated the replication and pathogenicity of APMV-3 in Hamsters and mice inoculated intranasally. The intranasal route was intended to resemble the natural route of APMV infection as well as a likely route for vaccine vector administration. APMV-3 produced gross pathological lesions in the lungs, while no gross pathology was evident for any other organs. Virus was recovered from all the animals infected with APMV-3. In all cases, virus was isolated 3 dpi: no virus was detected 14 dpi. The viral titers were moderate and were mostly restricted to the lungs and nasal turbinates.

Using IHC, viral P antigen was detected in the same tissues that were positive by virus isolation. An interesting finding was the presence of large amounts of viral antigens at the epithelial cell linings, suggesting that these cells are highly permissive to APMV-3 replication. In addition, the detection of viral antigens, and in most cases infectious virus,

in nasal turbinates and lungs of the hamsters and mice indicate that APMV-3 replication is mostly restricted to the respiratory tract. These results show that APMV-3 are capable of infecting hamsters and mice using a nasal route of infection and the extensive amount of virus replication in the respiratory tract was not accompanied by severe disease in both the species. Serologic assays demonstrated a humoral response in all the animals infected with APMV-3, a further indication of successful virus replication. The results show that APMV-3 produced HI antibody titers that varied between1:64 to 1:512. The HI test is the most commonly used method to diagnose APMV infections and also is used to measure the antibody response. The replication of APMV-3 in hamsters produced mild rhinitis and mild pathology that was mainly restricted to the respiratory tract. There was a concern that APMV-3 may also replicate in the intestine and shed in feces, which might act as a source of infection for the other animals. But results indicated that none of the APMV-3 infected hamsters and mice showed any replication the intestinal epithelial cells. Importantly, results also suggest that APMV-3 do not cross the blood-brain barrier and do not induce neurological symptoms. Also, previous experimental studies of the APMV-3 in chickens and turkeys showed that they were avirulent. Taken together, these results showed that the APMV-3 replicate moderately in hamsters and mice and produce either mild or no clinical signs, and elicit substantial antibody responses. Therefore, it is possible that APMV-3 might replicate in other mammalian species including humans. In conclusion, this study is the first comparative report on the replication and pathogenicity of prototype strains of APMV-3 in hamsters and mice. My results lay the foundation for a good laboratory animal model for testing the replication and pathogenicity of APMV-3.

Chapter 7

7.1 Title

Development of Avian Paramyxovirus serotype 3 (APMV-3) as a live viral vector: Establishment of reverse genetics system for APMV-3 and use of recombinant APMV-3 (rAPMV3) as a vector to express foreign protein, EGFP.

7.2 Abstract

The full-length cDNA clone of APMV-3 strain Netherlands (pAPMV3fl) was constructed as six different subgenomic fragments in a transcription plasmid pBR322 (pBR322/dr) between the T7 RNA polymerase promoter and autocatalytic hepatitis delta virus ribozyme. The plasmid pAPMV3fl was generated by cloning a 105-nucleotide (nt) long linker sequence containing unique restriction enzymes between *Asc*I and *Rsr*II enzyme sites of pBR322/dr plasmid. The support plasmids expressing N and P proteins were constructed in pTM1 vector and the ORF of L gene was cloned into pcDNA3.1 vector, all under the control of T7 promoter. Transfection of the full length cDNA plasmid into HEp-2 cells and infection with recombinant modified vaccinia strain Ankara expressing the T7 RNA polymerase (MVA-T7) resulted in the synthesis of antigenomic RNA of pAPMV3fl. The cotransfection of the support plasmids resulted in replication and transcription of the antigenomic RNA leading to generation of recombinant virus (rAPMV3). The recovered recombinant virus was amplified in 9-day-old embryonated specific pathogen free (SPF) chicken eggs and was confirmed for the presence of the artificially introduced restriction enzyme site markers using RT-PCR and sequencing. The *in vitro* growth characteristics of the recombinant rAPMV3 were similar to those of

wild-type APMV-3. Furthermore, a recombinant virus, rAPMV3/EGFP expressing enhanced green fluorescent protein (EGFP) as a foreign protein was recovered and characterized. The growth pattern of GFP-expressing recombinant virus was similar to that of the recombinant parental virus. The recombinant viruses stably expressed EGFP for at least five serial passages in 9-day-old embryonated chicken eggs and in DF1 cells. This result shows that the established reverse genetics system can be used to engineer pAPMV3fl and hence could be used to develop APMV-3 as a potential vaccine vector.

7.3 Introduction

APMV-3 was first isolated from turkeys in Ontario in 1967 and Wisconsin in 1968 (Tumova et al., 1979b). APMV-3 has been associated with encephalitis and high mortality in caged birds, and with respiratory disease in turkeys (Tumova et al., 1979b). The virus causes acute pancreatitis and CNS symptoms in psittacine and *Passeriformes* birds (Beck et al., 2003). Experimentally, APMV-3 can infects chickens at an early age with evidence of stunting growth that may be more marked in broiler chicken breeds (Alexander and Collins, 1982; Kumar et al., 2010a), but natural infection of chickens with APMV-3 has never been reported. APMV-3 is classified along with eight other APMV serotypes in the genus *Avulavirus* within the subfamily *Paramyxovirinae* in the family *Paramyxoviridae*. APMV-3 strain Neterlands has a 16,272-nt long, non-segmented, negative-sense RNA genome, which contains six genes N, P, M, F, HN and L and encodes at least eight proteins N, P/V/W, M, F, HN and L (Kumar et al., 2008).

The knowledge of the complete viral genome sequence is essential for genetic manipulation through a reverse genetics system, rendering recovery of recombinant virus

entirely from cloned cDNA (Conzelmann, 1998; Bukreyev et al., 2006). Most of the systems for recovery of NDV are based on co-transfection of cell with a plasmid expressing the full-length antigenomic RNA with three other support plasmids encoding viral N, P and L proteins, all under the control of bacteriophage T7 RNA polymerase promoter. The positive-sense antigenomic RNA instead of negative-sense genomic RNA was used to avoid hybridization with the positive-sense mRNAs transcribed from the support plasmids. The T7 RNA polymerase was provided by either a recombinant vaccinia virus expressing the T7 gene or a cell line constitutively expressing the T7 polymerase. The reverse genetics system has been successfully used for the genetic manipulation of avian paramyxoviruses to study their molecular biology and pathogenesis and also, to develop a vaccine vectors against important and emerging pathogens by engineering viruses to express foreign immunogens (Huang et al., 2004a; Rout and Samal, 2008; Yan and Samal, 2008; Nayak et al., 2009; Yan et al., 2009; Khattar et al., 2010).

This study describes the recovery of recombinant APMV-3 (rAPMV3) entirely from cloned cDNA using a reverse genetics system. The rescued recombinant virus was biologically similar to the wild-type APMV-3. Furthermore, I recovered recombinant viruses expressing enhanced green fluorescent protein (EGFP) to evaluate rAPMV3 as a vector to express foreign gene. The EGFP-expressing recombinant viruses were biologically similar to the parental recombinant and wild-type virus, and stably expressed GFP for at least five consecutive passages, suggesting its potential to be used as a vaccine vector for other animal and human pathogens.

7.4 Materials and Methods

7.4.1 Cells and viruses

HEp-2 and DF1 cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and maintained in DMEM with 5% FBS. APMV-3 strain parakeet/Netherlands/449/75 was obtained from the National Veterinary Services Laboratory, Ames, Iowa. The virus was grown in 9-day-old embryonated SPF chicken eggs by injecting the virus into the allantoic cavity. Four days later, the allantoic fluid was harvested and clarified. The virus was purified as described previously (Kingsbury, 1966).

7.4.2 cDNA synthesis and construction of APMV-3 full-length plasmid

Viral RNA was extracted from the purified virus by using TRIzol (Invitrogen, USA) according to manufacturer's protocol. A total of six cDNA fragments, spanning the entire APMV-3 genome, were generated by RT-PCR (Fig. 7.1). Specifically, each cDNA fragment was primed by a positive-sense oligonucleotide primer (Table 7.1), which carried a recognition sequence for a restriction enzyme that was unique to the APMV-3 genome. The primer corresponding to the fragment I had a T7 promoter overhang 39 nt to the *Asc*I restriction site, and the primer corresponding to fragment VI had a 24-nt 39-end sequence overhang of Hepatitis Delta Virus (HDV) ribozyme sequence. Also, the positive-sense oligonucleotide primer corresponding to fragment IV was modified at nt positions 8610 and 8616 to create an *Mlu*I site tag (at the HN/L intergenic region). The first-strand cDNA was synthesized using Superscript RT-PCR kit (Invitrogen, USA) according to the manufacturer's protocol. Subsequently, PCR was performed using high-

fidelity *Pfx* DNA polymerase (Invitrogen, USA) in a reaction containing the primer used in the RT reaction and the corresponding negative-sense oligonucleotide primer (Table 7.1). The RT-PCR product was then digested with the respective restriction enzymes and ligated to the plasmid pBR322/dr (Fig. 7.1). The fragments were ligated sequentially in the order shown in Table 7.1. Plasmid pBR322/dr was constructed by modification of plasmid pBR322. The modification included a backbone of 105-nt linker between the *Asc*I and *Rsr*II sites and an HDV 84-nt antigenome ribozyme sequence and T7 RNA polymerase transcription termination signal downstream of the polynucleotide linker. After ligation into the plasmid, each fragment was sequenced completely by using BigDye terminator v 3.1 matrix standard kit (Applied Biosystem) and 3130xl genetic analyzer data collection software v3.0 (Applied Biosystem). The resulting APMV-3 full-length expression plasmid was termed pAPMV3fl.

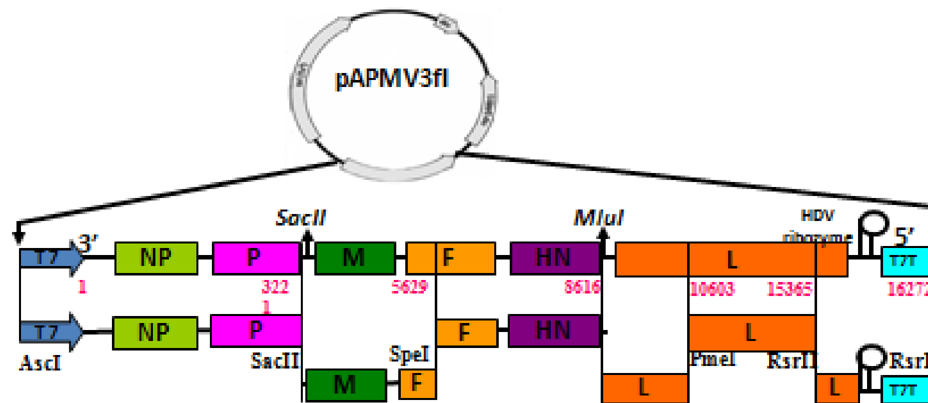


Fig.7.1 Generation of full length cDNA clone of pAPMV3fl.

The full length cDNA clone was constructed by assembling six subgenomic fragments into pBR322/dr using a 105-nt long oligonucleotide linker sequence between T7 RNA polymerase promoter sequence and the hepatitis delta ribozyme sequence, which was followed by T7 terminator sequence (between the restriction enzyme sites Asc*I* and Rsr*II*). The four nt mutations (C6483G to delete an additional *Sac*II

restriction enzyme site and A8616C, A8617G, and G8618T to generate unique *Mlu*I restriction enzyme site) served as the genetic markers in the pAPMV3fl.

cDNA fragment	Primers
I	+ATTCGGCGCGCCTAATACGACTCACTATAGGGACTAAACAGAAAATTAATACTTG
	-GCCTGGGCCGCGGGTTGGGGGAGG
II	+CCCCCAACCCGCGGCCCAGGCTCCATTC
	-ATAAACTAGTGCTCTATCGATCTTC
III	+GAGCACTAGTTTATCAGCCGTATG
	-GACGACGCGTCTGGATCTTATATAGAGGAT
IV	+GACGACGCGTGCTATATAAGCTAATAGCTTAGG
	-CTGGTACCTCCAGTTTAAACAGTAC
V	+AGTACTGTTTAAACTGGAGGTACCAG
	-ACTCCGGACCGAGGTTAGCCTATATTTGC
VI	+AACCTCGGTCCGGAGTATTGAGGTAATGGG
	-GGTCCGGACCGCGAGGAGGTGGAGATGCCATGCCGACCCACTAAACAAAAAGTTA TAATGATTTTTAAA

Table.7.1 The list of oligonucleotide primers used in the synthesis of full length cDNA of APMV-3. The restriction enzyme sites artificially created in the primers are underlined.

7.4.3 Construction of expression plasmids

cDNA fragments bearing the open reading frame (ORF) of N, P, and L genes were generated by RT-PCR. The N gene was cloned in the plasmid pTM-1 (pN) between *Nco*I and *Spe*I sites, while the P gene was cloned (pP) between *Nco*I and *Xho*I sites. The cloned gene was sequenced completely by the dideoxy chain termination method. The L gene was cloned in an expression plasmid pcDNA3.1 (pL) between *Xba*I and *Nhe*I site. The cloned genes were sequenced to entirety by the dideoxy chain termination method.

7.4.4 Construction of full length plasmids expressing EGFP

The plasmid pAPMV3fl was modified by the insertion of a transcription cassette containing the ORF for EGFP (Clontech, Inc.). The ORF of EGFP was flanked by the *Sac*II enzyme site and 18-nt 5' UTR upstream and by 161-nt P gene-end (GE) sequence, 62-nt intergenic sequences, a 10-nt putative M gene-start (GS) (AGGAGCGGAA), 51-nt 3'UTR sequence and *Sac*II enzyme site downstream. This fragment containing the ORF of EGFP was cloned between the P and M genes in the full length plasmid to generate the pAPMV3fl/EGFP plasmid (Fig. 7.2). The length of the encoded pAPMV3fl/EGFP was 17,304 nt, which was an even multiple of six.

The top panel (A) shows the construction of full length plasmid, pAPMV3fl and the bottom panel (B) shows the construction of pAPMV3fl/EGFP along with their respective EGFP cassettes. The EGFP ORF was inserted as a transcription cassette at the *Sac*II site (at the putative M gene 5' UTR).

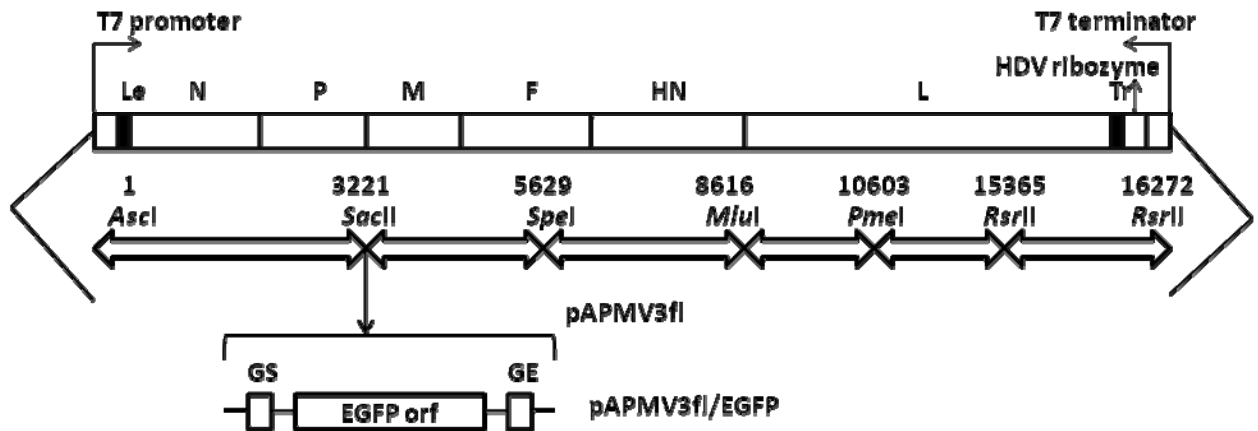


Fig. 7.2 Construction of full length plasmids expressing EGFP.

The EGFP ORF was inserted as a transcription cassette at the *Sac*II site flanked by gene start and gene end sequences.

7.4.5 Transfection and recovery of recombinant viruses

The HEp-2 cells were chosen for transfection experiments because these cells are permissive for APMV-3, transfect efficiently, and are resistant to CPE of vaccinia virus strain MVA. Specifically, cells grown to 80% confluency in six-well plates were washed twice with Opti- MEM just before transfection. The cells were then transfected with 5 µg pAPMV3fl or pAPMV3fl/EGFP, 2.5 µg pN, 1.5 µg pP, and 0.5 µg of pL in a volume of 0.2 ml of Opti-MEM/well. The transfection was carried out with Lipofectamine Plus (Invitrogen, USA), according to the manufacturer's instructions. Along with the transfection mixture, 1 focus-forming unit per cell of recombinant vaccinia virus (MVA/T7) expressing T7 RNA polymerase was added in a volume of 0.8 ml Opti-MEM/well. Six hours later, the medium was replaced with 2 ml Opti-MEM containing 2% FBS. Two days after transfection, the medium was harvested, the cell debris was pelleted by low-speed centrifugation, and the supernatant was used for infecting a fresh batch of HEp-2 cells in 25-cm2 flasks. Alternatively, 100 µl supernatant from transfection was directly used to inject 9-day-old embryonated chicken eggs. Two days later, the eggs were chilled for a period of 12 h, after which the allantoic fluid was harvested. The virus in the allantoic fluid was amplified by further passage in eggs. The recovered viruses were named rAPMV3 and rAPMV3-GFP.

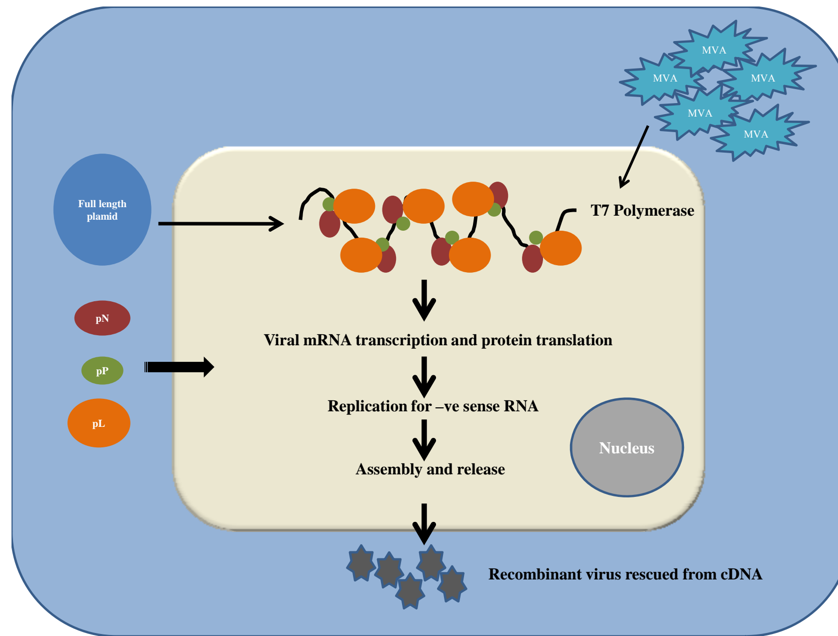


Fig. 7.3. A Plasmid based system for recovery of recombinant rAPMV3 from cDNA.

HEp-2 cells were first infected with recombinant vaccinia virus expressing T7 polymerase and cotransfected with antigenome full length cDNA plasmids pAPMV3fl or pAPMV3fl/EGFP and expression plasmids pN, pP, pL. This cartoon depicts the events that occur in the transfected HEp-2 cell leading to the recovery of recombinant viruses.

7.4.6 Identification of genetic markers in recombinant viruses by RT-PCR and sequencing

RT-PCR was performed on the RNA extracted from recombinant viruses using P gene-specific forward primer and M gene-specific reverse primer. The RT-PCR product was digested using *Sac*II enzyme and sequenced to confirm the foreign gene. RNA isolated from rAPMV3 was included as a control. Furthermore, the GFP expression by the recombinant viruses was determined by monitoring the virus-infected DF1 cells under fluorescence microscope.

7.4.7 Pathogenicity index tests

The pathogenicity of all recombinant viruses was determined by two standard pathogenicity index tests. These included the mean death time (MDT) in 9-day-old embryonated SPF chicken eggs and the intracerebral pathogenicity index (ICPI) in 1-day-old SPF chicks (Alexander, 2009).

MDT value was determined following the standard procedure. Briefly, a series of 10-fold (10^{-6} to 10^{-12}) dilutions of fresh infective allantoic fluid in sterile phosphate-buffered saline (PBS) were made and 0.1 ml of each dilution was inoculated into the allantoic cavities of five 9-day-old embryonated SPF chicken eggs, which were then incubated at 37°C. Each egg was examined three times daily for 7 days, and the times of embryo deaths were recorded. The minimum lethal dose is the highest virus dilution that caused death of all the embryos. MDT is the mean time in hours for the minimum lethal dose to kill all inoculated embryos. The MDT has been used to characterize the NDV pathotypes as follows: velogenic (less than 60 h), mesogenic (60 to 90 h), and lentogenic (more than 90 h) (Alexander, 1998).

For determining ICPI value, 0.05 ml of 1:10 dilution of fresh infective allantoic fluid (2^8 HA units) of each virus was inoculated into groups of ten 1-day-old SPF chicks via intracerebral route. The birds were observed for clinical symptoms and mortality every 8 h for a period of 8 days. At each observation, the birds were scored as follows: 0, healthy; 1, sick; and 2, dead. The ICPI is the mean score per bird per observation over the

8-day period. Highly virulent NDV (velogenic) viruses give values approaching 2 and avirulent NDV (lentogenic) viruses give values close to 0 (Alexander, 1998).

7.4.8 Growth characteristics of the rAPMV3s in DF1 cells

The multicycle growth kinetics of rAPMV3 and rAPMV3-EGFP were determined in DF1 cells. DF1 cells in duplicate wells of six-well plates were infected with viruses at an MOI of 0.01 PFU. After 1 h of adsorption, the cells were washed with DMEM and then covered with DMEM containing 2% FBS and 5% allantoic fluid. The cell culture supernatant samples were collected and replaced with an equal volume of fresh medium at 8-h intervals until 64 h post-infection. The titers of virus in the samples were quantified by 50% tissue culture infectious dose ($TCID_{50}$) assay in DF1 cells.

7.5 Results

7.5.1 Construction of the full length cDNA and expression plasmids

In order to construct the full length cDNA of pAPMV3fl, the whole APMV-3 genome was divided into six fragments and they were sequentially cloned. A 105-nt oligo linker was synthesized to contain unique restriction enzyme sites and was inserted between *Asc* I and *Rsr* II sites of the pBR322/dr vector to clone the full length cDNA. The DNA sequence results of the entire full length cDNA confirmed four nucleotide mutations, C6483G to delete an additional *Sac*II restriction enzyme site and A8616C, A8617G, and G8618T to generate unique *Mlu*I restriction enzyme site and served as the genetic markers in recombinant viruses. The support plasmids, pN and pP were generated

by inserting the cDNA bearing the ORF of N, P into expression vector pTM-1, while pL was obtained by cloning the L ORF as two fragments into pcDNA3.1 (pL) between *Xba*I and *Nhe*I site. Furthermore, the support plasmids were confirmed by digesting with the corresponding restriction enzymes followed by sequencing the complete ORF, prior to using them in the recovery of the recombinant viruses.

7.5.2 Construction of full length plasmids encoding EGFP

The full length plasmid encoding the EGFP, pAPMV3fl/EGFP, was constructed by inserting the EGFP transcription cassette at *Sac*II site between P and M genes. The EGFP ORF was inserted between the genes P and M since this position is known to support stable expression of foreign genes without affecting virus replication. The EGFP cassette contained appropriate viral GS and GE signals along with the EGFP ORF. The plasmids were sequenced to confirm the insertion of foreign cassette at the *Sac*II site.

7.5.3 Recovery of infectious recombinant viruses

The transfection of full length cDNA plasmids pAPMV3fl and pAPMV3fl/EGFP along with support plasmids pN, pP and pL in HEp-2 cells infected with MVA-T7, yielded infectious recombinant viruses two days post transfection. The recovered viruses were passaged in 9-day-old embryonated SPF chicken eggs to amplify the recombinant viruses (rAPMV3 and rAPMV3-EGFP). RT-PCR of the infective allantoic fluid and DNA sequencing confirmed the presence of genetic markers and the GFP. In addition,

green fluorescence in the infected DF1 cells confirms the expression of GFP by the rAPMV3-EGFP which was absent in rAPMV3.

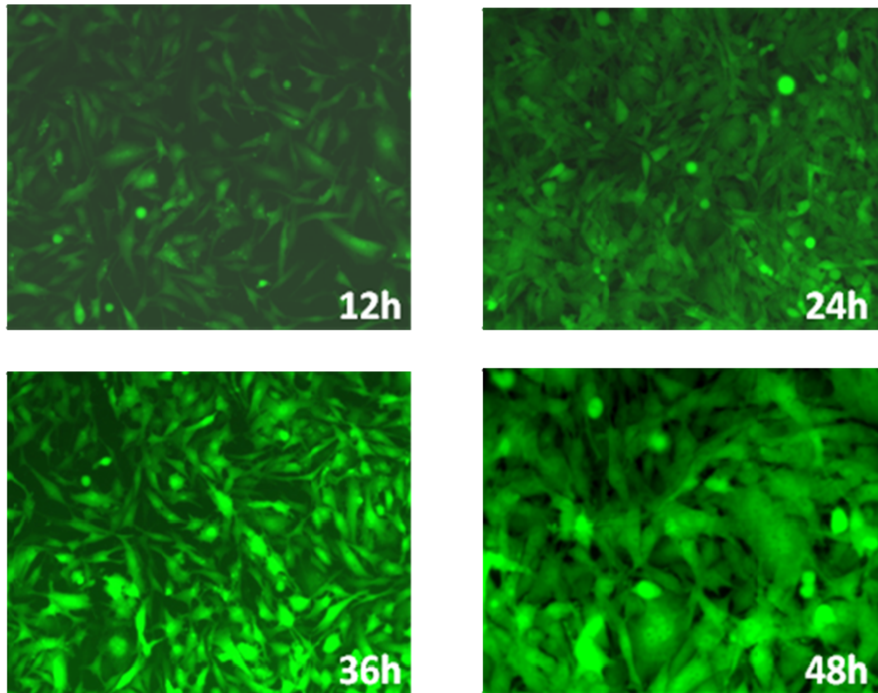


Fig. 7.4. EGFP expression by the rAPMV3-EGFP.

DF1 cells infected with rAPMV3-EGFP showed the GFP expression evident by the green fluorescent which was started 12 hr post-infection and completely saturated the cell culture monolayer 48 hr post-infection.

7.5.4 Biological characterization of recombinant viruses

The cytopathic effects of recombinant viruses were similar to those of parental virus in DF1 cells. The GFP expression by the recovered virus was confirmed by infecting DF1 cells with rAPMV3-EGFP. The recovered viruses, rAPMV3 and rAPMV3-EGFP were compared with the parental wild-type virus for their *in vitro* growth characteristics by multiple-step growth kinetics in DF1 cells at an MOI of 0.01. The

kinetics and the magnitude of replication of the recombinant virus were similar to those of the parental virus. The virus titers of the recombinant virus expressing GFP was 1.5 log lower than those of the parental and recombinant viruses, suggesting that the insertion of foreign gene resulted in slight attenuation.

The pathogenicity of these rAPMV3s was evaluated by the MDT assay in embryonated chicken eggs. The MDTs for these viruses were 120 h (rAPMV3) and 124 h (rAPMV3-EGFP). Furthermore, virulence of these rAPMV3s was evaluated by the ICPI assay in 1-day-old chicks. The ICPI values for these viruses were 0.33 for rAPMV3 and 0.36 for rAPMV3-EGFP compared to 0.39 for parental APMV-3. According to OIE guidelines, an NDV strain with an MDT value of more than 90 h is considered lentogenic or avirulent while for ICPI, virulent NDV viruses give values approaching 2 and avirulent (lentogenic) viruses give values close to 0 (Alexander, 2009). Thus, the two rAPMV3s are avirulent viruses and expression of the EGFP proteins did not increase viral virulence. Indeed, the presence of EGFP gene resulted in the attenuation of the APMV-3.

7.6 Discussion

This study describes the recovery of infectious recombinant APMV-3 strain Netherlands from the cloned cDNA by reverse genetics system for the first time. The availability of the complete genome sequence of APMV-3 strain Netherlands assisted in generating the full length cDNA clone, required for recovery of infectious recombinant virus. In this system, recombinant vaccinia virus expressing T7 RNA polymerase (MVA-T7) was used to synthesize the antigenomic RNA from the full-length plasmid and the

proteins N, P, and L from the cotransfected support plasmids, pN, pP and pL. Similar systems have been used to recover other viruses (Schnell et al., 1994; Yunus et al., 1999; Krishnamurthy et al., 2000; Govindarajan et al., 2006). The growth characteristics of the recombinant virus, rAPMV3 generated in this study was similar to that of the parental virus. These results indicate that it is possible to recover recombinant APMV-2 to -9 entirely from cloned cDNAs.

One of the important applications of reverse genetics system is the development of vaccine vectors by engineering viruses to express foreign immunogens. Paramyxovirus vectors have several advantages as follows; the ability to accommodate large foreign genes without drastic reduction in virus growth, stable expression of the inserted foreign genes even after many passages *in vitro* and finally, the absence of homologous RNA recombination makes them safe and stable vectors.

Using the established reverse genetics system, a rAPMV3 expressing foreign protein, enhanced GFP, was recovered. Full length cDNA constructs were made with EGFP transcript cassette alone between the P and M genes. The enhanced GFP was preferred as the foreign gene mainly because of the small size and the ease of visualization of the expressed foreign protein. The region between P and M genes in the full-length cDNA clone was chosen for insertion of EGFP because paramyxoviruses show gradient transcription pattern wherein the genes located near the 3' end of the genome are transcribed and expressed in higher quantities than those located further behind. This has also been shown in NDV that the expression of foreign genes are better when placed near the 3' end (Huang et al., 2001). The recovered viruses were similar to the parental virus in their growth characteristics but they were slightly attenuated, the

viral titers were 1.5 log lower than the parental virus. The attenuation following the expression of foreign genes has also been reported in other paramyxoviruses (Krishnamurthy et al., 2000). The recombinant virus rAPMV3-EGFP stably expressed the GFP for at least five serial passages in 9-day-old embryonated SPF chicken eggs and in DF1 cells.

In conclusion, a reverse genetics system was established for APMV-3 and the recovered recombinant virus showed similar morphological and *in vitro* growth characteristics and pathogenicity to the parental virus. The reverse genetics system can be used as a tool to understand the APMV-3 molecular biology and pathogenesis. Furthermore, the ability to engineer recombinant rAPMV3 expressing a foreign gene has been demonstrated using enhanced GFP, which has implications in the development of vectored vaccines against emerging pathogens.

Chapter 8

8.1 Title

Evaluation of the Newcastle disease virus F and HN proteins in protective immunity using a recombinant avian paramyxovirus type-3 vector in chickens.

8.2 Abstract

Newcastle disease virus (NDV) is an important pathogen of poultry worldwide. Current vaccination strategies are not completely satisfactory. The NDV surface glycoproteins, namely the fusion (F) and the hemagglutinin-neuraminidase (HN) proteins, are thought to be the major protective immunogens. However, the relative contributions of F and HN to protection in chickens are not well understood. I developed a recombinant version of avian paramyxovirus serotype-3 (APMV-3) by reverse genetics and used this recombinant virus as a vector to determine the individual contributions of the NDV F and HN proteins to protection and immunity against NDV in chickens. Three recombinant viruses, namely the parental (rAPMV3) and rAPMV3 expressing the NDV F (rAPMV3-F) or HN (rAPMV3-HN) protein were constructed. Protective efficacy was analyzed by vaccinating 2-week-old SPF chickens by the oculonasal route and challenging on day 21 post-vaccination with virulent NDV via three different routes of inoculation. Serum antibody responses were measured by hemagglutination inhibition and virus neutralization assays. All three recombinant viruses (rAPMV3, rAPMV3-F, and rAPMV3-HN) protected chickens against NDV challenge via the oculonasal and intramuscular routes, while all unvaccinated birds succumbed to death. This result indicated that rAPMV3 alone can provide substantial cross protection against NDV

challenge. However, immunization with rAMPV3 did not protect chickens against intravenous NDV challenge; whereas, birds vaccinated with rAPMV3-F alone or in combination with rAPMV3-HN were completely protected, and birds vaccinated with rAPMV3-HN alone were partially protected. NDV neutralizing antibody titers induced by rAPMV3-F were greater than those induced by rAPMV3-HN. The result shows NDV F and HN proteins are independent protective antigens, but the F protein contributes more to protection than does the HN protein. Furthermore, rAPMV3, which is avirulent in chickens, can be used as a vaccine vector against NDV, since APMV3 itself provides protection against NDV due to its cross-reactivity, which is further enhanced due to expression of NDV protective antigens.

8.3 Introduction

Newcastle disease (ND) is a highly contagious disease of many avian species, which leads to substantial economic losses in the poultry industry worldwide. The severity of disease depends on the virus strain and the host species. Newcastle disease virus (NDV) is divided into three pathotypes based on the severity of the disease: Lentogenic (apathogenic), mesogenic (moderately pathogenic) and velogenic (highly pathogenic) (Alexander, 1998). Velogenic NDV strains cause acute infections with high mortality, preceded by respiratory and neurological signs with occasional haemorrhagic lesions in the gut, whereas mesogenic NDV strains cause respiratory disease in young birds and decreased egg production in laying flocks (Alexander, 2003a). In contrast, lentogenic NDV strains may produce only mild respiratory signs in chickens.

NDV belongs to the genus *Avulavirus* within the family *Paramyxoviridae (Mayo, 2002a; Mayo, 2002b).* The genome of NDV is a nonsegmented, single-stranded, negative-sense RNA of 15,186 nucleotides (nt) (Krishnamurthy and Samal, 1998; de Leeuw and Peeters, 1999). The genome consists of six genes encoding at least six different proteins in the order of a nucleocapsid protein (N), a phosphoprotein (P), a matrix protein (M), a fusion protein (F), an attachment protein called the hemagglutinin-neuraminidase (HN), and a large polymerase protein (L) (3'leader-N-P-M-F-HN-L-5'trailer). Two additional proteins, V and W, may be produced by RNA editing during P gene transcription (Steward et al., 1993).

The envelope of NDV virions contains two transmembrane glycoproteins, the virus attachment protein, HN, and the fusion protein, F, which form spike-like protrusions on the outer surface of the virion. The HN protein is responsible for the attachment of virus particles to sialic acid-containing receptors on the host cell. It is the largest glycoprotein molecule and carries both haemagglutinating and neuraminidase activities (Scheid and Choppin, 1973; Morrison, 2003). The F protein mediates fusion of the virion envelope with the cellular plasma membrane (Nagai et al., 1989). It has been shown that the polybasic basic amino acid residues at the F protein cleavage site found in virulent NDV strains is a prerequisite for the pathogenic phenotype (Nagai et al., 1976; Glickman et al., 1988; Romer-Oberdorfer et al., 2003). Both F and HN glycoproteins on the surface of NDV are important for virus infectivity and pathogenicity (Nagai et al., 1976; Meulemans et al., 1986; Ogawa et al., 1990; Nagy et al., 1991). Furthermore, F and HN proteins produce virus neutralizing antibody response and are protective antigens (Boursnell et al., 1990b; Cosset et al., 1991; Karaca et al., 1998; Sun et al., 2008). The F

protein is known to play an important role in immunity to the disease and has been shown that this protein alone is sufficient to induce protective immunity to NDV in chickens (Meulemans et al., 1988). The HN protein has also been shown to protect birds from virulent NDV challenge, although the birds showed lower neutralizing antibody titers (Boursnell et al., 1990b; Nagy et al., 1991). It has also been shown that monoclonal antibodies to F protein neutralizes NDV better than monoclonal antibodies to HN protein (Russell, 1988). However, it is still not clear, which surface glycoprotein is the major protective antigen. Although, several vaccine studies have been done with F and HN glycoproteins (Boursnell et al., 1990a; Boursnell et al., 1990b; Romer-Oberdorfer et al., 2003; Lee et al., 2008), their major role in protection against virulent NDV challenge is not well understood.

Several vaccination strategies, including inactivated and live attenuated vaccines, have been evaluated to control NDV. Lentogenic strains are preferentially used for vaccine preparations due to their low pathogenicity for chickens. Although effective live or inactivated ND vaccines are currently available, the virus remains as a major threat to commercial flocks. Inactivated vaccines are used in breeder pullets but not practical because of the high cost and unsuitability for mass vaccination in the field. Although live attenuated vaccines are commonly used in field condition, it is always of the concern that the vaccine viruses may, through either mutation or genetic recombination with circulating strains, become virulent. Furthermore, both current live and inactivated vaccines cannot differentiate infected from vaccinated animals (DIVA). To overcome these difficulties, several strategies have been developed based on *in vivo* expression of NDV surface glycoproteins by DNA vaccines (Loke et al., 2005) or by vectors based on

adenovirus (Perozo et al., 2008), fowl pox virus (Boursnell et al., 1990b; Ogawa et al., 1990; Karaca et al., 1998), baculovirus (Nagy et al., 1991; Lee et al., 2010), pigeon pox (Letellier et al., 1991), herpes virus of turkeys (Morgan et al., 1993; Sakaguchi et al., 1998), Marek's disease virus, and retrovirus (Morrison et al., 1990; Sonoda et al., 2000), and infectious laryngotracheitis virus (ILTV) (Sun et al., 2008) or by transgenic crops (Yang et al., 2007).

The avian paramyxoviruses that comprise genus *Avulavirus* have been divided into nine different serotypes (APMV-1 through -9) based on haemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays. All strains of NDV belong to APMV-1. Although, there is a high degree of amino acid sequence variation between APMV-3 and APMV-1 (NDV), by the HI test there is cross reaction between APMV-3 and APMV-1 serum samples, which often leads to misdiagnosis of APMV-3 as APMV-1. Recently, I sequenced the complete genome of APMV-3 (Kumar et al., 2008; Kumar et al., 2010b). I also studied its pathogenicity study in chickens and turkeys that showed APMV-3 is nonpathogenic to chickens and turkeys (Kumar et al., 2010a).

In the present study, I examined the relative contributions of each of the two NDV surface glycoproteins (F and HN) to induction of neutralizing antibodies and protective immunity in chickens. In order to address this, I generated recombinant avian paramyxovirus type-3 (rAPMV-3) vectors, which individually expressed each of the two surface glycoproteins F and HN of NDV. They were used to immunize chickens either individually or in combinations. Evaluation of the relative neutralization titers of serum antibody, shedding of challenge virus, and protection against lethal NDV challenge conferred by each of the rAPMV-3-vectored NDV surface proteins showed that F

glycoprotein was the major contributor to induction of neutralizing antibodies and protective immunity, followed by HN protein, which conferred partial protection against intravenous challenge.

8.4 Materials and Methods

8.4.1 Cells and viruses

HEp-2 and DF1 cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and maintained in DMEM with 5% FBS. APMV-3 strain Netherlands and NDV strain Texas GB (APMV-1/chicken/U.S.(TX)/GB/1948) were obtained from National Veterinary Services Laboratory (Ames, IA). Recombinant NDV (rNDV) strain LaSota was generated as previously described (Huang et al., 2001). The virus was grown in 9-day-old embryonated SPF chicken eggs by injecting the virus into the allantoic cavity. Four days later, the allantoic fluid was harvested and clarified. Further the virus was purified as described previously (Kingsbury, 1966).

8.4.2 Construction of full-length plasmid containing NDV F and HN gene

Full length cDNA clone (pAPMV3fl) and expression plasmids of APMV-3 were generated as describe in chapter 7.

The restriction enzyme site of *Sac*II at position 3221 of pAPMV3fl was utilized to insert the F and HN genes of NDV between the P and M genes individually (Fig. 8.1). The ORF encoding the F and HN proteins was engineered to contain APMV-3 gene start

and gene end sequences. Specifically, the positive-sense oligonucleotide primers used to amplify the F and HN genes by PCR were modified to contain the *Sac*II site overhang upstream and downstream of the F and HN-specific sequence. PCR was performed by using high-fidelity *Pfx* DNA polymerase. The PCR-amplified fragment of F and HN was inserted at the *Sac*II site of pAPMV3fl and the resulting plasmid was designated as pAPMV3fl-F and pAPMV3fl-HN, respectively. The integrity of the entire foreign sequence was confirmed by sequencing whole F and HN sequences. The insertion of 1662 nt (F orf) and 1734 nt (HN orf) of foreign sequences (a nt multiple of six) resulted in the plasmid encoding an antigenome of 18,252 nt and 18,324 nt, respectively, consistent with the "rule of six" (Calain and Roux, 1993).

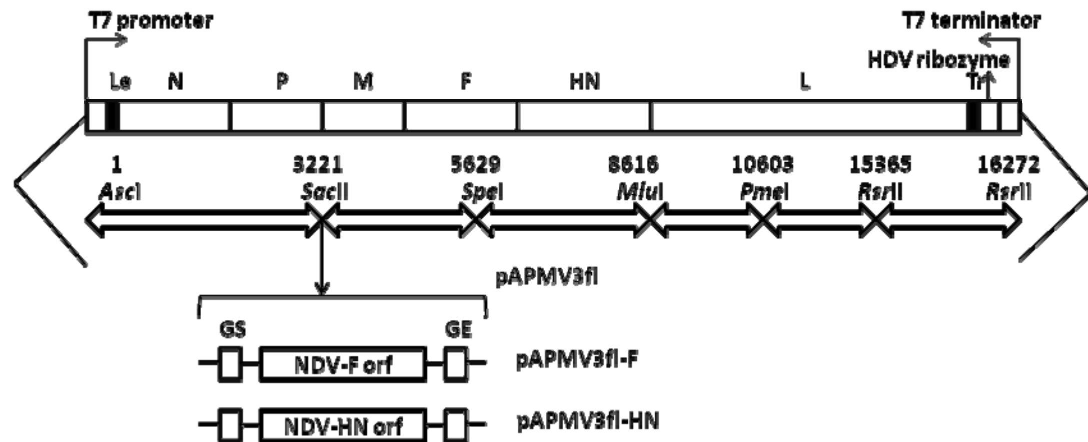


Fig. 8.1 Construction and characterization of rAPMV3s expressing NDV surface protein F and HN. Schematic diagram depicting the rAPMV3 genome with insertion of an added gene engineered to express the NDV surface protein F and HN. The NDV F and HN genes ORF was inserted as a transcription cassette at the *Sac*II site flanked by gene start and gene end sequences.

8.4.3 Transfection and recovery of recombinant viruses

The HEp-2 cells were chosen for transfection experiments because these cells are permissive for APMV-3, transfect efficiently, and are resistant to CPE of vaccinia virus strain MVA. Specifically, cells grown to 80% confluency in six-well plates were washed twice with Opti- MEM just before transfection. The cells were then transfected with 5 µg pAPMV3fl or pAPMV3fl-F or pAPMV3fl-HN, 2.5 µg pN, 1.5 µg pP, and 0.5 µg of pL in a volume of 0.2 ml of Opti-MEM/well. The transfection was carried out with Lipofectamine Plus (Invitrogen, USA), according to the manufacturer's instructions. Along with the transfection mixture, 1 focus-forming unit per cell of recombinant vaccinia virus (MVA/T7) expressing T7 RNA polymerase was added in a volume of 0.8 ml Opti-MEM/well. Six hours later, the medium was replaced with 2 ml Opti-MEM containing 2% FBS. Two days after transfection, the medium was harvested, the cell debris was pelleted by low-speed centrifugation, and the supernatant was used for infecting a fresh batch of HEp-2 cells in 25-cm2 flasks. Alternatively, 100 µl supernatant from transfection was directly used to inject 9-day-old embryonated chicken eggs. Two days later, the eggs were chilled for a period of 12 h, after which the allantoic fluid was harvested. The virus in the allantoic fluid was amplified by further passage in eggs.

8.4.4 Pathogenicity index tests

The pathogenicity of all recombinant viruses was determined by two standard pathogenicity index tests. These included the mean death time (MDT) in 9-day-old

embryonated SPF chicken eggs and the intracerebral pathogenicity index (ICPI) in 1-day-old SPF chicks (Alexander, 2009).

MDT value was determined following the standard procedure. Briefly, a series of 10-fold (10^{-6} to 10^{-12}) dilutions of fresh infective allantoic fluid in sterile phosphate-buffered saline (PBS) were made and 0.1 ml of each dilution was inoculated into the allantoic cavities of five 9-day-old embryonated SPF chicken eggs, which were then incubated at 37°C. Each egg was examined three times daily for 7 days, and the times of embryo deaths were recorded. The minimum lethal dose is the highest virus dilution that caused death of all the embryos. MDT is the mean time in hours for the minimum lethal dose to kill all inoculated embryos. The MDT has been used to characterize the NDV pathotypes as follows: velogenic (less than 60 h), mesogenic (60 to 90 h), and lentogenic (more than 90 h) (Alexander, 1998).

For determining ICPI value, 0.05 ml of 1:10 dilution of fresh infective allantoic fluid (2^8 HA units) of each virus was inoculated into groups of ten 1-day-old SPF chicks via intracerebral route. The birds were observed for clinical signs and mortality every 8 h for a period of 8 days. At each observation, the birds were scored as follows: 0, healthy; 1, sick; and 2, dead. The ICPI is the mean score per bird per observation over the 8-day period. Highly virulent NDV (velogenic) viruses give values approaching 2 and avirulent NDV (lentogenic) viruses give values close to 0 (Alexander, 1998).

8.4.5 Expression of the NDV F and HN proteins in cells infected with rAPMV3s

The expression of the F and HN proteins of NDV by the rAPMV3s was examined by Western blot analysis. Briefly, DF1 cells were infected with rAPMV3, rAPMV3-F,

and rAPMV3-HN at a multiplicity of infection (MOI) of 0.01 PFU. The cells were harvested at 48 h post-infection, lysed, and analyzed by Western blotting using a monoclonal antibody against HN or a rabbit antiserum specific to a C-terminal peptide of the NDV F protein. To examine the incorporation of NDV surface proteins into rAPMV3 particles, Western blot analysis was carried out using partially purified virus from allantoic fluid of rAPMV3s-infected eggs and the same two antisera.

8.4.6 Growth characteristics of the rAPMV3s in DF1 cells

The multicycle growth kinetics of rAPMV3, rAPMV3-F, and rAPMV3-HN were determined in DF1 cells. DF1 cells in duplicate wells of six-well plates were infected with viruses at an MOI of 0.01 PFU. After 1 h of adsorption, the cells were washed with DMEM and then covered with DMEM containing 2% FBS and 5% allantoic fluid. The cell culture supernatant samples were collected and replaced with an equal volume of fresh medium at 8-h intervals until 64 h post-infection. The titers of virus in the samples were quantified by 50% tissue culture infectious dose ($TCID_{50}$) assay in DF1 cells.

8.4.7 Virus titration

Titration of rAPMV3s following *in vitro* or *in vivo* growth was performed by limiting dilution in DF1 cells, using the Reed and Muench method as described previously (Reed, 1938), and the titers were expressed as $TCID_{50}$ units/ml. For both rAPMV3s and NDV, HA titers were determined using chicken red blood cells (RBC). Fifty percent egg infective dose (EID_{50}) values for rAPMVs and NDV were determined

by infecting five eggs per group for each 10-fold serial dilution. Following 24 h of infection, eggs were harvested for allantoic fluid, and the presence of virus was confirmed by HA test. For NDV challenge viruses, the chicken 50% lethal dose (CLD_{50}) was determined by infecting three (5-week-old) chickens per group, and the 50% end point was determined by the Reed and Muench method (Reed, 1938).

8.4.8 Immunization and challenge experiments in chickens

The immunization and challenge experiments were performed in three phases. In first phase of the experiment, 5 groups (n = 13 per group) of 2-week-old SPF chickens were immunized by the oculonasal route with a dose of 10^6 EID_{50} of NDV strain LaSota, rAPMV3 (empty vector), rAPMV3-F, rAPMV3-HN, and a combination of rAPMV3-F and rAPMV3-HN. In addition, 13 birds were kept as an unvaccinated control. Three weeks post-immunization, pre-challenge serum samples were collected for serum antibody response, and the animals were challenged through the intranasal route with 100 CLD_{50} of the highly virulent NDV strain GB Texas. Three chickens from each group were sacrificed on day 3 post-challenge for quantitation of challenge virus replication. Tissue samples were collected from the respiratory tract, including the trachea, nasal turbinates (upper respiratory tract), and lungs (lower respiratory tract), as well as from the lymphoid system (spleen), digestive system (gut), and nervous system (brain). The tissues were homogenized in cell culture medium (1 g/10 ml) and clarified by centrifugation. The challenge virus titers in organs were determined by limiting dilution. The remaining 10 chickens in each group were observed daily for 10 days for disease symptoms and mortality following challenge. To monitor shedding of the challenge NDV, oral and

cloacal swabs were collected on day 3, 5 and 7 post-challenge from all chickens. The NDV challenge virus titers in the swab samples were determined by a limiting dilution assay in DF1 cell monolayers. Post-challenge sera were collected from the surviving birds before they were sacrificed on day 10 post-challenge.

In the second phase, 5 groups (*n* = 5 per group) of 2-week-old SPF chickens were immunized similarly as the first phase. In addition, 5 birds were kept unvaccinated controls. Three weeks post-immunization, the birds were challenged by intramuscular route with highly virulent NDV strain GB Texas. To monitor shedding of the challenge NDV, oral and cloacal swabs were collected on day 3, 5 and 7 post-challenge from all chickens. Post-challenge sera were collected from the surviving birds before they were sacrificed on day 15 post-challenge.

In the third phase, the birds were immunized similarly as the second phase. Three weeks post-immunization, the birds were challenged by intravenous route with highly virulent NDV strain GB Texas keeping all post challenge procedures the same as the second phase.

All challenge experiments were carried out in an enhanced BSL3 containment facility certified by the USDA and CDC, with the investigators wearing appropriate protective equipment and compliant with all protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Maryland and under Animal Welfare Association (AWA) regulations.

8.4.9 Serological analysis

The antibody levels of serum samples collected from chickens vaccinated with rAPMV3s were evaluated by hemagglutination inhibition (HI) and virus neutralization (VN) assays as well as enzyme-linked immunosorbent assay (ELISA) and Western blotting using standard protocols. For the HI assay, twofold serial dilutions of immunized chicken sera (50 µl) were prepared, and each dilution was combined with 4 HA units of rNDV (NDV HI) or APMV-3 strain Netherlands (APMV3 HI). Following 1 h of incubation, 50 µl of 1% chicken RBC was added and incubated for 30 min at room temperature, and hemagglutination was scored.

The virus neutralization test was carried out using post-immunization chicken sera in DF1 cells grown in 96-well tissue culture plates. Briefly, twofold serial dilutions of 50-µl serum samples (complement inactivated) were carried out and incubated for 1 h with 100 $TCID_{50}$ of NDV or APMV-3 in DMEM. Following incubation, cells were infected with virus serum mixture. VN titer was obtained by confirmation of the presence of virus in wells with the highest dilution of serum. Commercial NDV ELISA kits (Synbiotics Corporation, San Diego, CA) were used to detect antibodies against the whole-NDV antigens of rAPMV3s.

8.4.10 Statistical analysis

Statistically significant differences in serological analysis of different immunized chicken groups were evaluated by one-way analysis of variance (ANOVA). The survival

patterns and median survival times were compared using the log-rank test and chi-square statistics. In the log-rank test, survival curves compare the cumulative probability of survival at any specific time and the assumption of proportional deaths per time is the same at all time points. Survival data and one-way ANOVA were analyzed with the use of Prism 5.0 (Graph Pad Software Inc., San Diego, CA) with a significance level of $P < 0.05$.

8.5 Results

8.5.1 Generation of rAPMV3s expressing the NDV F and HN surface proteins

The individual contributions of each of the NDV surface proteins (F, and HN) to immunogenicity and protection were evaluated by separately expressing each protein from an added gene inserted into the genome of APMV3 strain Netherlands by reverse genetics. The F and HN genes were derived from the NDV strain LaSota. Each ORF was placed under the control of a set of APMV3 transcriptional signals and inserted between the P and M genes in the APMV-3 antigenome cDNA. The recombinant viruses were recovered using a previously described reverse genetics method (Krishnamurthy et al., 2000). The orientation of each NDV gene in the genome of the recombinant viruses was confirmed by reverse transcription-PCR (RT-PCR) and nucleotide sequence analysis. The recovered viruses were named rAPMV3 or rAPMV3-F or rAPMV3-HN, respectively.

The expression of the NDV surface proteins by rAPMV3-F and rAPMV3-HN in infected DF1 cells was analyzed using a monoclonal antibody against HN and a rabbit antiserum specific to a C-terminal peptide of the F protein, respectively (Fig. 8.2). Analysis with the monoclonal antibody against HN detected a protein band with an apparent molecular

weight of ~74 KDa in lysates of cells infected with rAPMV3-HN and F-related bands in lysates of cells infected with rAPMV3-F (Fig. 8.2), representing the N-terminally derived proteolytic cleavage product F1, with an apparent molecular weight of ~55 KDa. To examine the incorporation of the NDV surface proteins into APMV3 particles, each of the rAPMV3s was partially purified from infected allantoic fluid by sucrose gradient centrifugation and analyzed by Western blotting. Analysis with the monoclonal antibody against HN detected the HN protein in virus preparations of rAPMV3-HN. In addition, a rabbit antiserum specific to a C-terminal peptide of the F protein detected the F1 protein in the virus preparation of rAPMV3-F (Fig. 8.2). These results indicated that both F and HN were packaged in the APMV3 vector particle. I could not detect the F0 fraction of the NDV F protein because of its processing by the presence of allantoic fluid (exogenous proteases) both in the cell culture and in purified virus preparations.

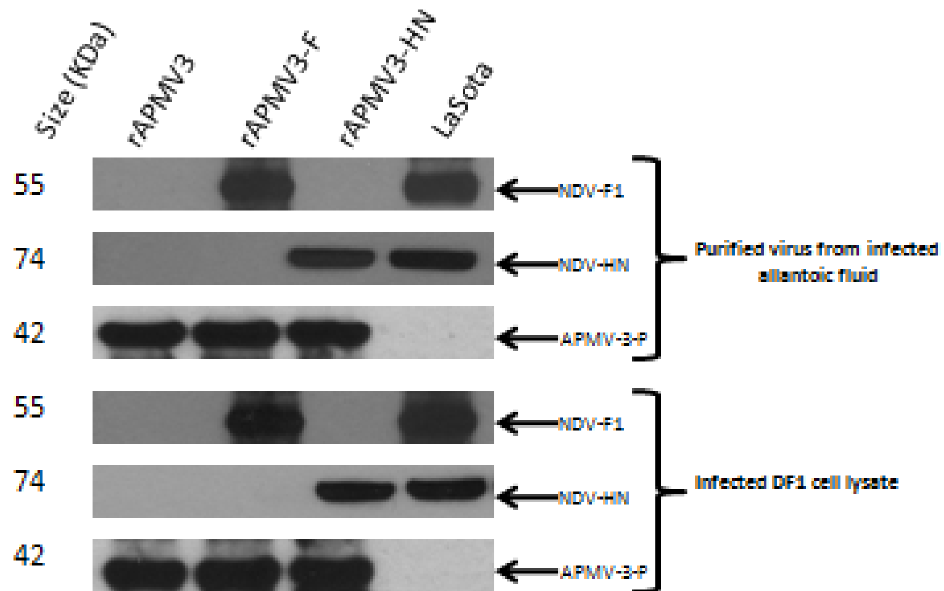


Fig. 8.2 Expression of NDV surface proteins F and HN in DF1 cells and their incorporation into rAPMV3 virions. DF1 cells were infected with the individual rAPMV3 constructs, and 48 h later the cells were

collected and processed to prepare cell lysates. In addition, allantoic fluid from embryonated eggs infected with the individual constructs was clarified and subjected to centrifugation on sucrose gradients to make partially purified preparations of virus particles. These samples were analyzed by Western blot analysis using a monoclonal antibody against the HN protein or a rabbit antiserum specific to a C-terminal peptide of the F protein (bottom). In addition, rabbit antisera specific to an N terminal peptide of the P protein of APMV-3 was also used. Partially purified virus particles of rAPMV3 empty vector (lane 1), rAPMV3-F (lane 2), rAPMV3-HN (lane 3), or rNDV (lane 4) [top 3]. Lysates of DF1 cells that had been infected with rAPMV3 empty vector (lane 1), rAPMV3-F (lane 2), rAPMV3-HN (lane 3), or rNDV (lane 4) [bottom 3].

8.5.2 Biological characterization of rAPMV3 expressing NDV surface proteins

The multicycle growth kinetics of rAPMV3-F and rAPMV3-HN were compared with those of the empty rAPMV3 vector in DF1 chicken fibroblast cells (Fig 8.3). Results demonstrated similar growth pattern between the rAPMV3 empty vector, rAPMV3-F and rAPMV3-HN. However, rAPMV3-F and rAPMV3-HN grew slightly slower than rAPMV3 but achieved maximum titers that were similar with the rAPMV3 empty vector 64 h post-infection.

The virulence of these rAPMV3s was evaluated by the MDT assay in embryonated chicken eggs. The MDTs for these viruses were 120 h (rAPMV3) and 124 h (rAPMV3-F and rAPMV3-HN). Furthermore, virulence of these rAPMV3s was evaluated by the ICPI assay in 1-day-old chicks. The ICPI values for these viruses were 0.33 for rAPMV3 and 0.36 for rAPMV3-F and rAPMV3-HN. According to OIE guidelines, an NDV strain with an MDT value of more than 90 h is considered lentogenic or avirulent while for ICPI, virulent NDV viruses give values approaching 2 and avirulent (lentogenic) viruses give values close to 0 (Alexander, 2009). Thus, the two rAPMV3s expressing the NDV

surface proteins are avirulent viruses and expression of the NDV proteins did not increase their virulence. Indeed, the presence of each NDV gene slightly attenuated the rAPMV3 vectors.

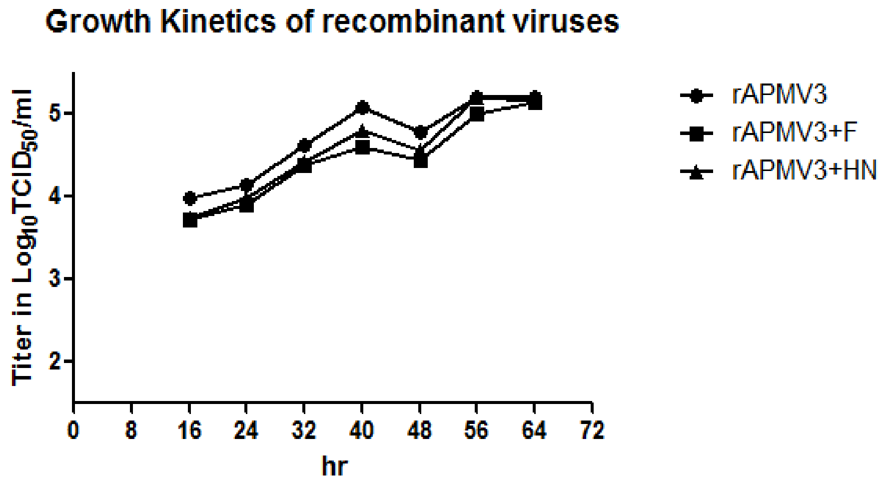


Fig. 8.3 Comparison of multicycle growth kinetics of rAPMV3s in DF1 cells. DF1 cells were infected with 100 PFU (0.01 PFU per cell) of each virus, and the cell culture supernatant was harvested at 8-h intervals. The virus titer in allantoic fluid and cell culture supernatant samples was determined by $TCID_{50}$ assay in DF1 cells.

8.5.3 Evaluation of APMV3 and NDV-specific serum antibody responses following immunization with rAPMV3s expressing NDV surface proteins

To evaluate the immunogenicity of the individual NDV surface proteins, groups of chickens were infected oculonasally with rAPMV3s individually or in combination. APMV3 and NDV-specific serum antibody responses were determined on day 21 post-immunization using an NDV-specific ELISA and an APMV3 and NDV-specific HI assays. High levels of APMV3-specific serum antibodies (~ 9 to 10 log_2 titer) were

detected by HI assays, indicating replication of the rAPMV3s in chickens (Fig. 8.4a). Interestingly, LaSota vaccinated birds also showed 2 log_2 APMV3-specific serum antibodies by HI assay, indicating slight cross reactivity between APMV-3 and NDV.

Moderate NDV-specific serum antibody responses were detected by the rAPMV3-F and rAPMV3-HN measured by NDV-specific ELISA. Surprisingly, relatively high titer of NDV antibody response (~4.5 log_2 titer) was detected in rAPMV3-F and rAPMV3-HN vaccinated birds by HI assays (Fig 8.4b). In addition, the rAPMV3 empty vector vaccinated group also showed~ 1 log_2 HI titer against NDV, again indicating cross reactivity between APMV3 and NDV. The sera collected from rAPMV3-F and rAPMV3-HN vaccinated groups were treated with purified empty vector rAPMV3 to nullify any APMV3 specific background to further confirm the F and HN specific HI antibody response, respectively. The treated sera showed the specific F and HN antibody response in both ELISA and HI assays, indicating F can also induce HI specific antibody (Fig. 8.5). Alternatively, Western blot analysis showed the F and HN-specific antibody responses from rAPMVs groups (Data not shown). The chicken group immunized with rAPMV3-F+ rAPMV3-HN alone had the highest antibody titers, whereas the titer was one-fold less for chickens immunized with rAPMV3-HN and 1/2-fold less for chickens immunized with rAPMV3-F (Fig. 8.4c). Interestingly, the antibody response to the F protein (rAPMV3-F) appeared to be substantially greater than HN (rAPMV3-HN).

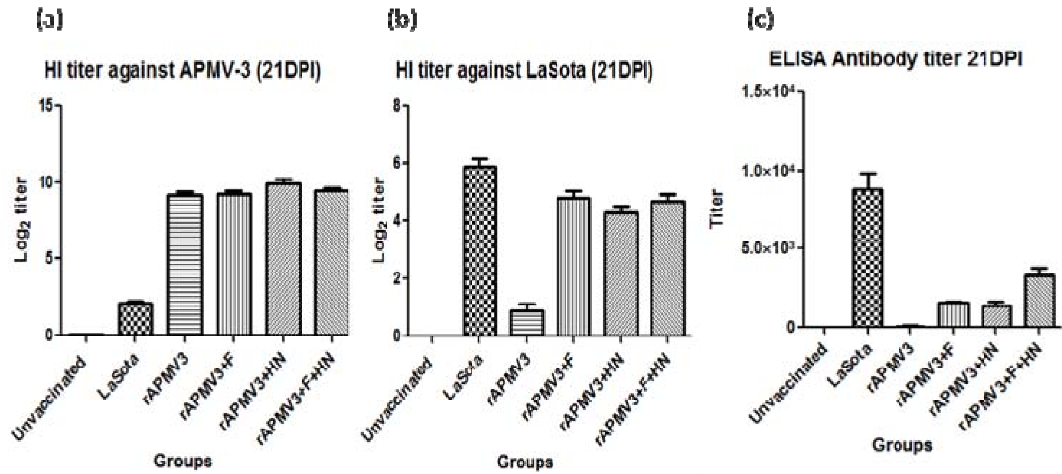


Fig. 8.4 APMV-3 and NDV-specific serum antibody responses in chickens at 21 days following oculonasal immunization with the indicated rAPMV3s administered individually or in combination. APMV-3 specific serum antibody responses were determined by HI assay (a); whereas, NDV-specific serum antibody responses were determined by commercial NDV ELISA (C) and HI assay (B). Titers were expressed as mean reciprocal $\log_2$ titer and statistical differences were calculated by one-way ANOVA with $P < 0.001$.

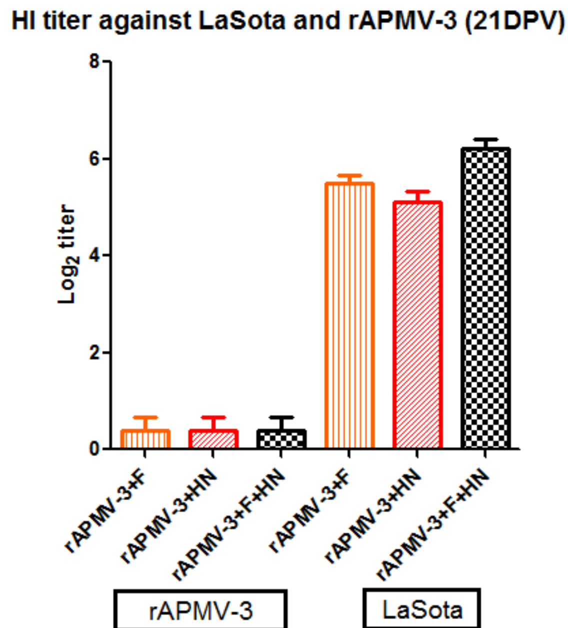


Fig. 8.5 NDV- F and HN specific serum antibody responses in chickens at 21 days following oculonasal immunization. Serum antibody responses were determined by HI assay following treatment of sera with purified rAPMV3 virus.

8.5.4 Evaluation of vaccine virus shedding in rAPMV3s vaccinated chickens

Shedding of rAPMVs was monitored by taking oral and cloacal swab samples on day 3, 5 and 7 post vaccinations from all the birds. The frequency with which animals shed rAPMVs was determined by inoculation of embryonated chicken eggs and DF1 cell monolayers with swab samples, and HA assays were performed to confirm that the isolate was rAPMV. All of the birds immunized with LaSota were positive for both oral and cloacal shedding after 3 days post vaccinations. Two out of 13 in rAPMV3-F and rAPMV3-HN vaccinated chickens and 7 out of 13 in rAPMV3-F+ rAPMV3-HN vaccinated birds were positive for oral shedding after 3 days post-vaccination (Fig. 8.6a). Three out of 13 birds in LaSota vaccinated group were positive for oral shedding with a mean titer of 1.3 $\log_{10}$ even after 5 days post-vaccination, while only one bird showed the cloacal shedding (Fig. 8.6b). No rAPMVs shedding was observed from any bird after 5 days post-vaccination. None of the vaccinated birds showed any shedding of virus after 7 days post-vaccination.

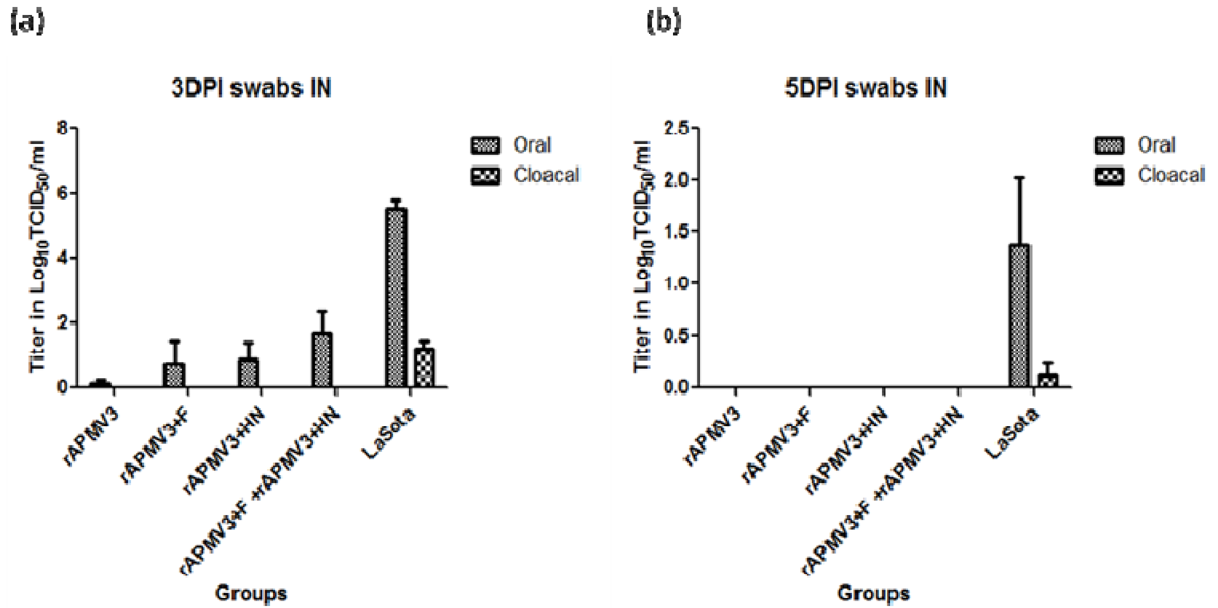


Fig. 8.6 rAPMV3s vaccine virus shedding titer as determined by $TCID_{50}$ assay in oral and cloacal swabs collected from chicken groups immunized with rAPMV3s. No virus was detected in the rAPMV3s vaccinated groups 5DPI.

8.5.5 Evaluation of NDV-neutralizing serum antibody responses in rAPMV3s expressing NDV surface proteins

The ability of sera taken from chickens 21 days after immunization with rAPMV3s individually or in combination to neutralize NDV was assessed by a micro-neutralization assay (Fig. 8.7). Sera from birds immunized with the rAPMV3 empty vector showed detectable neutralizing antibody titers, whereas sera from birds immunized with rAPMV3-F or rAPMV3-HN individually or together induced substantial titers of NDV-neutralizing antibodies, with the titer being one-fold greater for the rAPMV3-F than for the rAPMV3-HN group. The titers for rAPMV3-F + rAPMV3-HN group were

similar to that of NDV LaSota group (positive control) (Fig. 8.7a). Moreover, all the vaccinated group showed high virus neutralizing antibody titers against APMV3 except the NDV LaSota vaccinated group (Fig 8.7b).

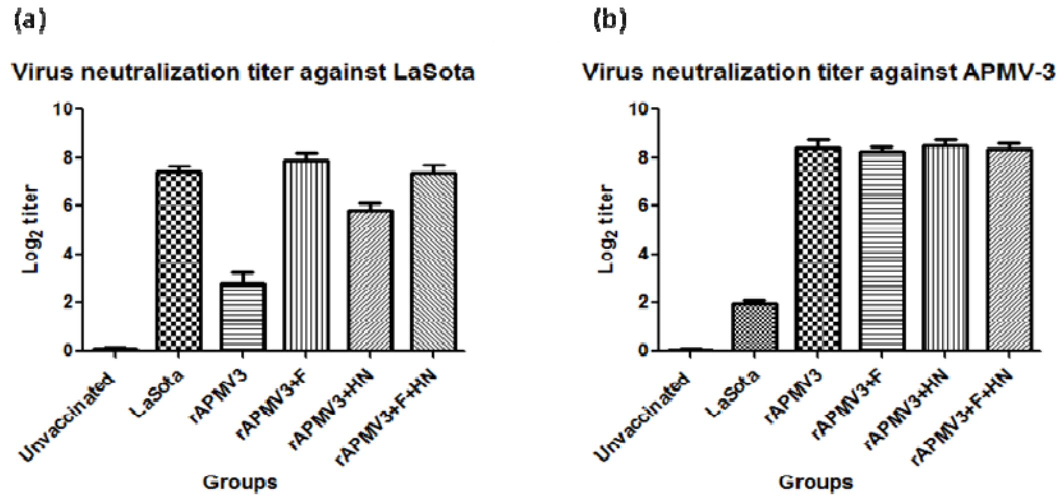


Fig. 8.7 Induction of NDV and APMV3-neutralizing serum antibodies by rAPMV3s vaccine constructs. Chickens were immunized once by the oculonasal route with the indicated rAPMV3s constructs given individually or in combination, and sera were taken 21 days later and analyzed for the ability to neutralize the APMV3 or NDV strain LaSota. The serum-neutralizing antibody titers were expressed as mean reciprocal $\log_2$ titer (means ± SEM). Statistical differences were calculated by one-way ANOVA with $P < 0.001$.

8.5.6 Protective efficacy of rAPMVs against NDV virus and its replication after oculonasal challenge

The chickens that had been immunized with the various rAPMV3s individually or in combination were challenged occulonasaly on day 21 post-immunization with a highly

lethal dose (100 CLD_{50}) of NDV strain Texas GB. On day 3 post-challenge, three chickens from each group were sacrificed and tissues were harvested from the respiratory tract (upper trachea, nasal turbinates, and lungs), lymphoid system (spleen), digestive system (gut) and nervous system (brain) of each chicken, and the NDV challenge virus titers were determined by limiting dilution (Fig. 8.8). All of the chickens in the unvaccinated group died by day 3 post-challenge while chickens in all other groups survived from mortality (Fig 8.11). NDV challenge virus replication was highest in chickens that had been unvaccinated and immunized with the rAPMV3 empty vector, whereas NDV replication in the tissues from chickens immunized with rAPMV3-F or rAPMV3-F+ rAPMV3-HN or LaSota was almost the same (Fig 8.8). There was a substantial reduction in challenge NDV titer compared to the rAPMV3 empty vector group in the lungs (a mean reduction of 5x2$\log_{10}$), trachea (a mean reduction of 13x2 $\log_{10}$), nostrils (a mean reduction of 3x2$\log_{10}$), and spleen (a mean reduction of 6x2$\log_{10}$), whereas replication in the brain was only a 10-fold reduction in mean titer. However, rAPMV3-HN group showed a mean increase of 3x2$\log_{10}$ NDV replication in upper respiratory tract than rAPMV3-F or rAPMV3-F+ rAPMV3-HN or LaSota (Fig 8.8).

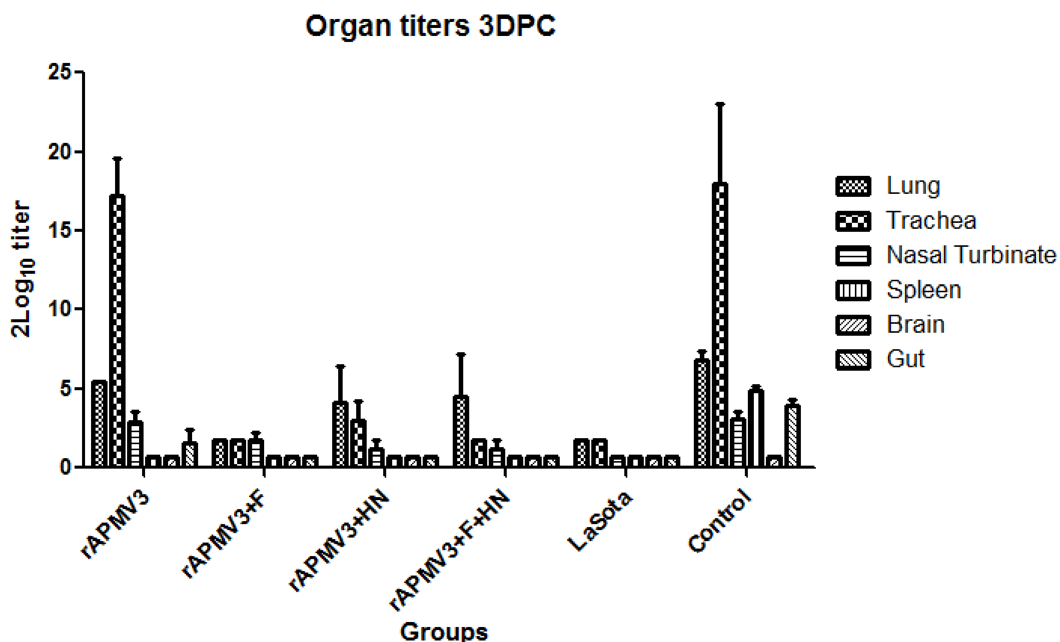


Fig. 8.8 Replication of NDV challenge virus in different organs of chickens infected 21 days after immunization with rAPMV3s. Three chickens from each immunized group were sacrificed 3 days after challenge. Various organs from each chicken were collected and analyzed in DF cells. The NDV titers in different organs of each chicken are expressed in log_{10} $TCID_{50}$ per gram of tissue.

Shedding of NDV challenge virus was monitored by taking oral and cloacal swab samples on day 3, 5 and 7 post-challenge from the remaining 10 animals in each group. All of the birds immunized with rAPMV3, rAPMV3-HN, LaSota, and unvaccinated were positive for oral shedding when the samples were assayed using embryonated eggs. No NDV shedding was observed from any bird in the rAPMV3-F groups. For chickens immunized with rAPMV3-F+rAPMV3-HN, 6 out of 10 birds had NDV oral shedding (Fig. 8.9a). The magnitude of viral shedding was determined by a limiting dilution assay of swab samples (Fig. 8.9). The virus titers in oral shedding were high for unvaccinated chickens as expected, and were not significantly reduced in chickens immunized with

rAPMV3 empty vector. In contrast, no oral shedding was detected in rAPMV3-F group. Low titers of oral shedding were detected in the rAPMV3-F+rAPMV3-HN and rAPMV3-HN groups, indicating that the efficacy of rAPMV3-F was reduced when combined with rAPMV3-HN (Fig. 8.9a). All the challenged groups have no detectable cloacal shedding. Thus, the ability of rAPMV3-F to confer restriction of challenge virus replication against replication near the site of inoculation was substantially greater than rAPMV3-HN.

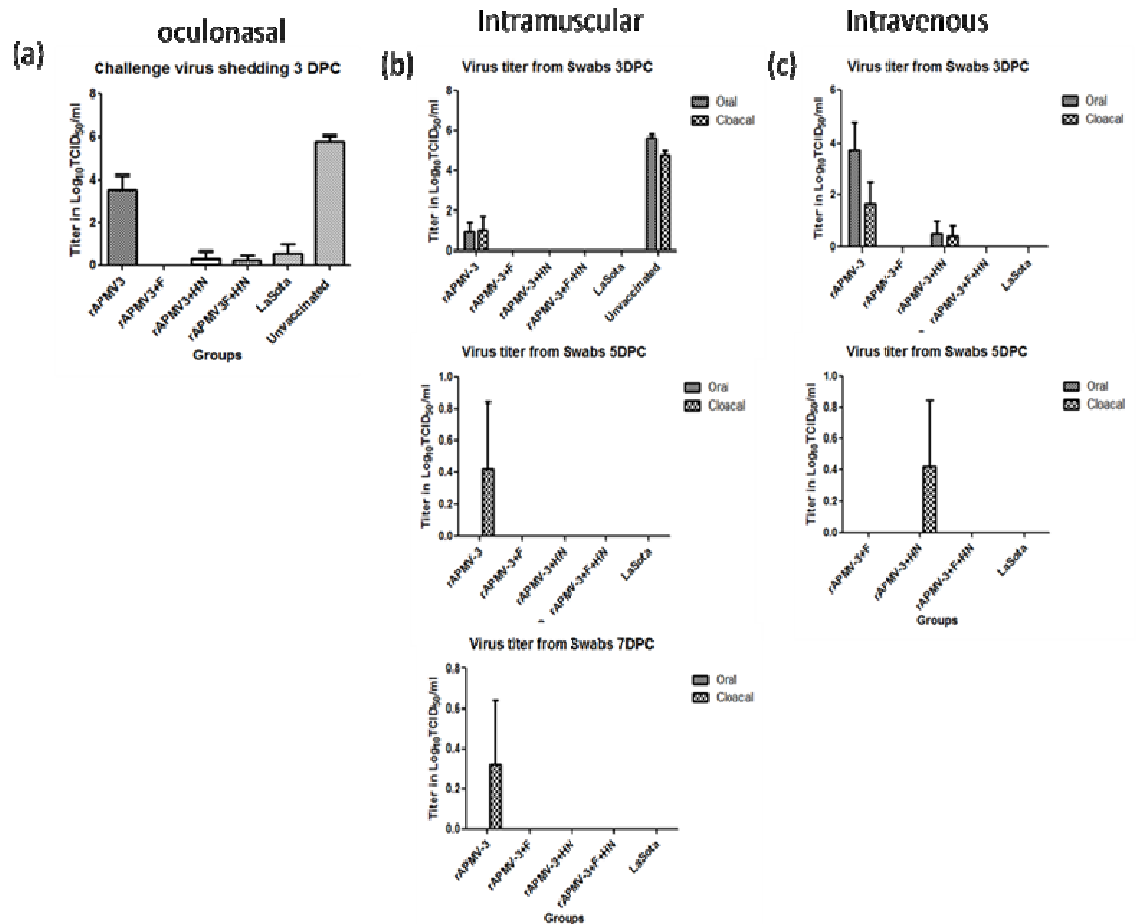


Fig. 8.9 NDV challenge virus shedding titer as determined by $TCID_{50}$ assay in oral and cloacal swabs 3, 5 and 7 DPC after oculonasal (a), intramuscular (b) and intravenous (c) challenge, collected from chicken groups immunized with rAPMV3s. No virus was detected in the rAPMV3-F vaccinated group

3DPC after oculonasal challenge. Furthermore, no virus shedding was observed 5 and 7 DPC after oculonasal challenge. Similarly, no virus shedding was observed 7 DPC after intravenous challenge.

8.5.7 Protective efficacy of rAPMVs against NDV virus and its replication after intramuscular challenge

The chickens that had been immunized with the various rAPMV3s individually or in combination were challenged intramuscularly on day 21 post-immunization with a highly lethal dose (100 CLD_{50}) of NDV strain Texas GB. All of the chickens in the unvaccinated group died by day 4 post-challenge while chickens in all other groups survived from mortality. Shedding of NDV challenge virus was monitored by taking oral and cloacal swab samples on day 3, 5 and 7 post-challenge. All of the birds immunized with rAPMV3 were positive for both oral and cloacal shedding on day 3 post-challenge, while no shedding was observed in rAPMV3-F or APMV3-HN or rAPMV3-F+rAPMV3-HN or LaSota vaccinated groups (Fig. 8.9b). Two out of 5 birds in rAPMV3 vaccinated group shed the NDV challenge virus from oral secretion till day 5 and 7 post-challenge (Fig. 8.9b), while no shedding was evident from cloacal secretion, suggesting inability of rAPMV3 to restrict challenge virus replication in upper respiratory tract.

8.5.8 Protective efficacy of rAPMVs against NDV virus and its replication after intravenous challenge

The chickens that had been immunized with the various rAPMV3s individually or in combination were challenged occulonasaly on day 21 post-immunization with a highly lethal dose (100 CLD_{50}) of NDV strain Texas GB. All the birds in unvaccinated and

rAPMV3 vaccinated group died 3 days post-challenge, while 4 out of 5 birds died on day 4 post-challenge in rAPMV3-HN vaccinated group. Although one of the birds in rAPMV3-HN survived, it showed all the typical neurological signs (head tilting and shaking) till day 8 post-challenge and gradually recovered. None of the birds died in rAPMV3-F, rAPMV3-F+ rAPMV3-HN, and LaSota vaccinated groups. Shedding of NDV challenge virus was monitored by taking oral and cloacal swab samples on day 3, 5 and 7 post-challenge. All of the birds immunized with rAPMV3 and rAMPV3-HN were positive for both oral and cloacal shedding day 3 post-challenge. Although, the titers in rAMPV3-HN were 3 $\log_{10}$ and 1.5 $\log_{10}$ less than rAMPV3 in oral and cloacal shedding respectively, one of the surviving birds shed the virus from the cloaca till 5 days post-challenge (Fig. 8.9c).

The virus replication from birds that died after intravenous challenge showed high challenge virus titers in all the organs (lung, trachea, nasal turbinate, kidney, gut, and brain) tested, while birds that died in rAMPV3-HN group showed clearance of virus from the lower respiratory tract (lung), lymphatic system (spleen), and digestive tract (gut) (Fig. 8.10). High challenge virus replication was observed in the trachea (6.3x2$\log_{10}$), nasal turbinate (19x2$\log_{10}$), and brain (7x2$\log_{10}$) in the rAMPV3-HN group, suggesting that the death was caused by involvement of the upper respiratory tract and nervous system which was evident by the signs of disease in one of the birds that survived (Fig. 8.10).

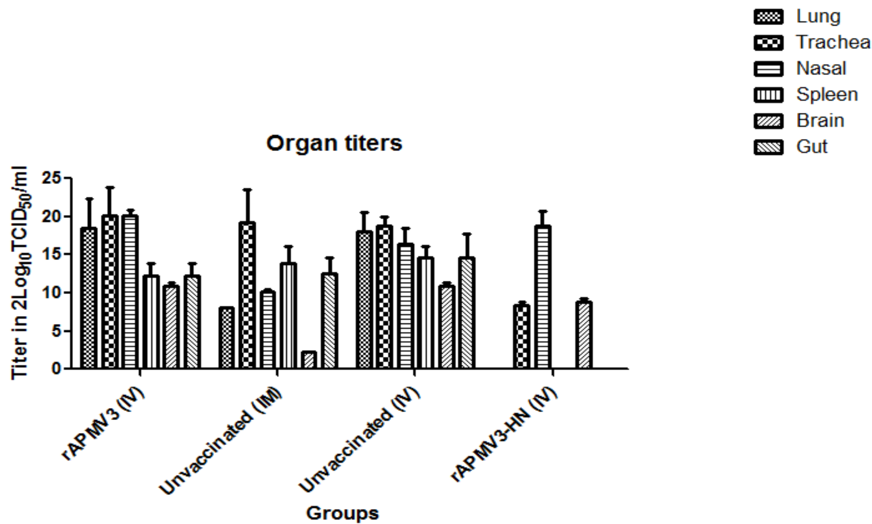


Fig. 8.10 Replication of NDV challenge virus after intramuscular and intravenous challenge in different organs of chickens 21 days after immunization with rAPMV3s. Various organs from each chicken died post-challenge was collected and analyzed in DF1 cells. The NDV titers in different organs of each chicken are expressed in log_{10} $TCID_{50}$ per gram of tissue.

8.5.9 Protective efficacy of rNDVs against death from NDV challenge

In the immunization and lethal challenge experiment described above, the chickens in each group were monitored following challenge to determine the total number of survivors and monitor the kinetics of death. All the chickens survived after intranasal (Fig. 8.11a) and intramuscular (Fig. 8.11b) virulent NDV challenge, while only rAMPV3-F, rAMPV3-F +rAMPV3-HN and LaSota groups survived after intravenous challenge, suggesting the role of rAMPV3+F in protection against intravenous challenge that was absent in rAMPV3+HN (Fig. 8.11c).

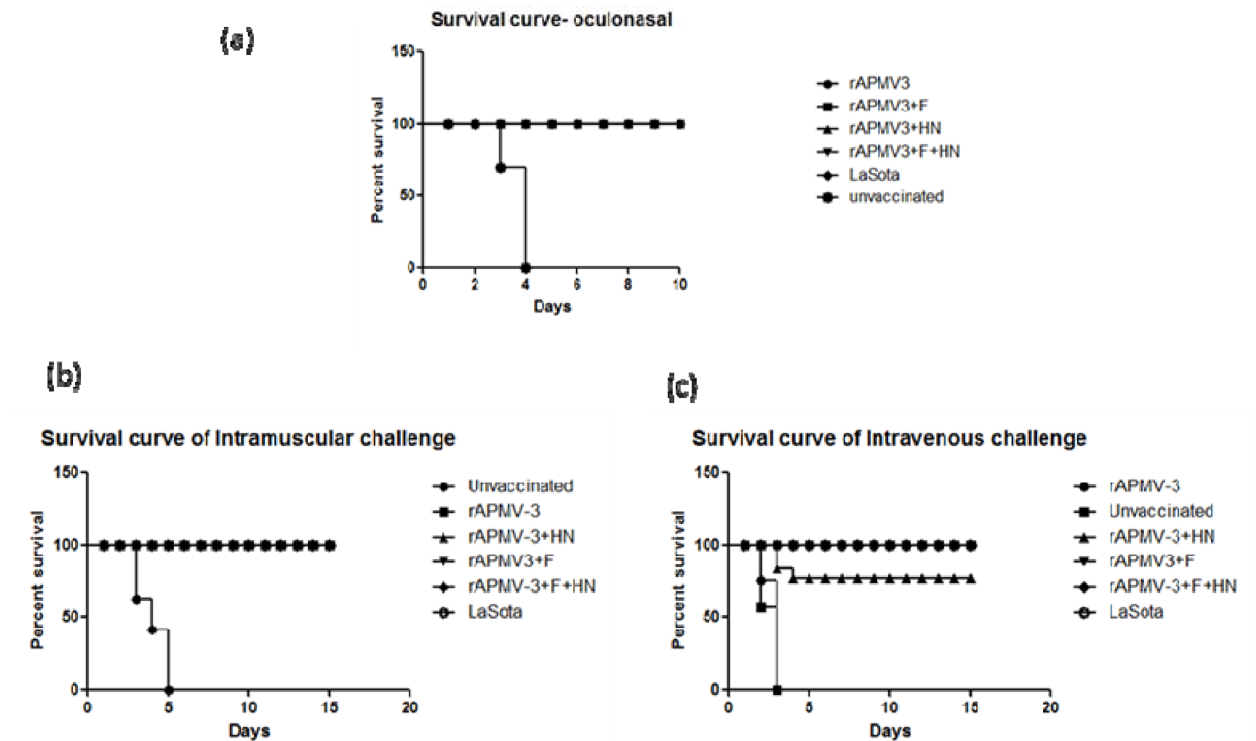


Fig. 8.11 Percent survival of rAPMV3-immunized chickens following NDV challenge by oculonasal (a), intramuscular (b) and intravenous (c) routes. Six chicken groups (10 chickens per group) were immunized with the indicated rAPMV3s either individually (rAPMV3 empty vector, rAPMV3-F, rAPMV3-HN, and LaSota) or in combination (rAPMV3-F+rAPMV3-HN). Twenty-one days after immunization, these groups were challenged with virulent NDV strain GBT. All of the chickens in the unvaccinated control died by day 4 after oculonasal challenge while they died by day 5 after intramuscular challenge. All of the chickens in the rAPMV3 empty vector, rAPMV3-F, rAPMV3-HN, rAPMV3-F+rAPMV3-HN, and LaSota groups survived after both oculonasal (a) and intramuscular challenge (b). All the unvaccinated control, rAPMV3 empty vector, and 4 out of 5 rAPMV3-HN died after intravenous challenge by day 3. All of the chickens in the rAPMV3-F, rAPMV3-F+rAPMV3-HN, and LaSota groups survived after intravenous challenge. Statistical differences between the groups were determined by the log-rank test with a P value of <0.0001.

8.5.10 Comparison of pre- and post-challenge NDV-specific serum antibody responses

I compared the serum antibody responses to NDV proteins in sera collected immediately before challenge (day 21 post-immunization) versus sera collected from survivor chickens on day 10 post-challenge. Commercially available ELISA assay kit specific to NDV was used for determining the sera titers. Pre-challenge sera from rAPMV3-vaccinated birds were negative for NDV specific antibody by ELISA. There were post-challenge increases in mean HI antibody titers of eightfold, twofold, fourfold, fourfold and onefold in the rAPMV3, rAPMV3-F, rAPMV3-HN, rAPMV3-F + rAPMV3-HN and LaSota groups, respectively, consistent with replication of the NDV challenge virus (Fig. 8.12a). Similarly, the mean post-challenge antibody titer increased fourfold, fivefold, twofold, and onefold rAPMV3-F, rAPMV3-HN, rAPMV3-F + rAPMV3-HN and LaSota groups, respectively, consistent with challenge NDV replication (Fig. 8.12b). There were hundred fold increases in serum antibody titer in rAPMV3 empty vector group after intranasal challenge indicating very high replication of challenge virus. This provided further evidence of the high degree of restriction of challenge NDV replication afforded by rAPMV3-F.

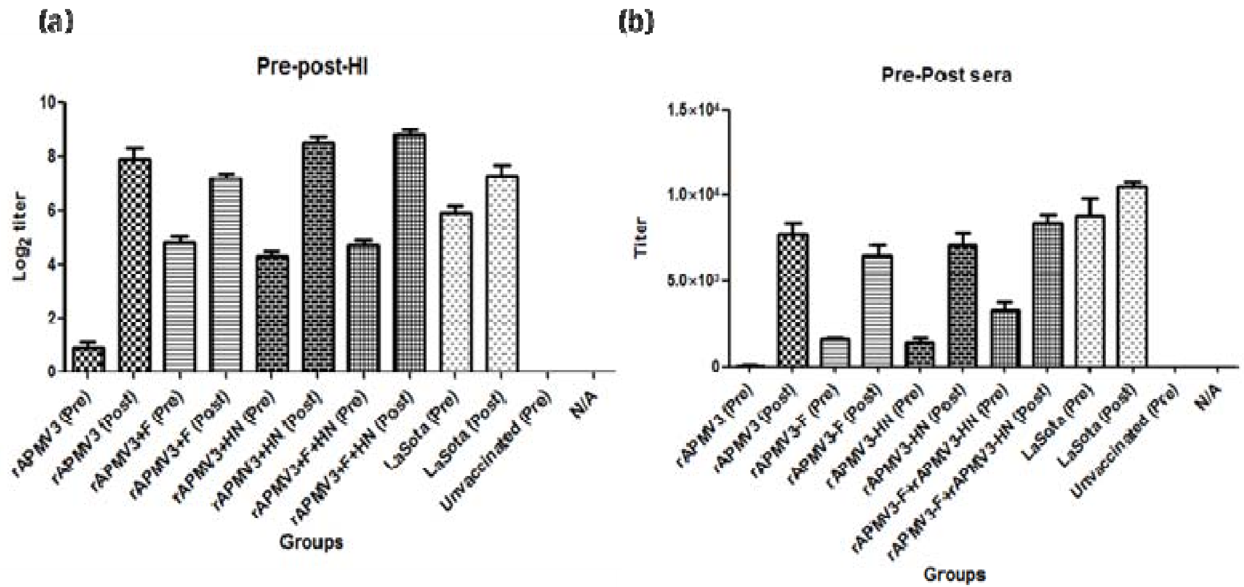


Fig. 8.12 Comparison of pre-challenge (day 21) and post-challenge (day 31) NDV-specific serum antibody titers determined from surviving birds from the following immunization groups: rAPMV3 (n=10), rAPMV3-F (n=10), rAPMV3-HN (n=10), and rAPMV3-F + rAPMV3-HN (n=10). The NDV-specific serum antibody responses titers were measured by HI assay (a) and NDV ELISA (b). The statistical difference between the chicken groups were analyzed by one-way ANOVA, and the *P* values were <0.001 for NDV ELISA.

8.6 Discussion

Newcastle disease is an economically important disease of poultry, and naturally occurring avirulent strains of NDV are widely used as live attenuated vaccines in many countries. Although, many avirulent strains of avian paramyxovirus have been recently sequenced and some of them are characterized for their pathogenicity in chickens, their use as a vector to express protective antigens would provide us a better vaccine against important poultry pathogens. APMV3 is a respiratory pathogen of chickens and has similar cellular and tissue tropisms to that of NDV, and thus is a highly suitable vaccine

vector (Kumar et al., 2010a). Other advantages include an ease of administration, and the ability to distinguish between infected and vaccinated animals by assaying antibody responses to NDV proteins not present in the rAPMV3-vectored vaccine.

The role of F or HN in immunogenicity and protection was not well understood in the context of either natural infection or a vectored vaccine. I prepared rAPMV3s individually expressing the NDV F or HN protein. The recombinant viruses grew efficiently in embryonated eggs and DF1 cells. Western blot analysis showed that both the NDV proteins were expressed in DF1 cells infected with the different recombinant viruses. In addition, both the F and HN proteins were found to be incorporated into the envelope of rAPMV3 virion. Importantly, incorporation of the NDV surface proteins into rAPMV3 particles did not increase the virulence of the vector in standard tests in embryonated eggs and one-day-old chicks, showing that the expression of NDV surface proteins by APMV3 does not pose a bio-safety hazard.

I used rAPMV3 as a vector to compare the relative contributions of each of the two NDV surface proteins (F and HN) to immunogenicity and protection in chickens that were immunized and challenged naturally by the oculonasal route. Moreover, I also used other unnatural routes of challenge infection (intramuscular and intravenous) to cover the broad range of possibilities and to address the role of F and HN surface glycoproteins in protection against NDV. In addition, I also evaluated the immunogenicity and protective efficacy of combinations of two rAPMV3s expressing NDV surface proteins to address whether both of the surface proteins act synergistically and have an added advantage than expressed individually.

In this study, intranasal immunization with rAPMV3-F and rAPMV3-HN alone or in combination induced high levels of NDV-specific HI and neutralizing serum antibodies and completely protected chickens against a potent challenge with NDV. Furthermore, all animals were protected against death including rAPMV3 empty vector group, suggesting cross protection against NDV. This is consistent with the previous study where prior infection with APMV3 showed protection against NDV challenge. Although, APMV3 is serologically distinguishable from NDV, the subtle level of relatedness results in some degree of protection from challenge with virulent NDV (Smit and Rondhuis, 1976; Alexander et al., 1979). Both NDV F and HN proteins are independent neutralization and protective antigens, and a single immunization with a rAPMV3-vectored vaccine expressing F and HN alone induced a strong serum-neutralizing antibody response and complete protection against intranasal challenge with NDV.

DNA vaccines encoding NDV F protein provided protection against NDV challenge in chickens (Sakaguchi et al., 1996). Furthermore, fowlpox virus expressing NDV F protein confers protection against the homologous NDV challenge, while their use in heterologous viral strain was not shown (Taylor et al., 1990). However, the individual contribution of F-specific antibodies to immunogenicity and protection against NDV remained unclear. Monoclonal antibody against both F and HN proteins has a protective effect upon virus infection (Umino et al., 1987). However, it has also been shown that anti-F serum with a marginal neutralizing activity had a comparable protective effect to that of anti-HN serum (Umino et al., 1990). I speculated that the protective effect of F antibody may result from a possible inhibitory effect of cell to cell spread of the virus. In addition, the protection of rAPMV3 expressing F may modulate

the cell mediated immune response against NDV infection thereby protecting bird against the intravenous challenge that was not the case when HN is used as an antigen.

Furthermore, it is thought that F-specific antibodies are not typically associated with HI. However, the titer of HI antibodies induced by rAPMV3-F was only onefold less than that induced by LaSota. Thus, the NDV F protein is a substantial, independent inducer of HI antibodies. It may be that HI by F-specific antibodies is achieved indirectly: specifically, antibodies bound to F on the surface of the NDV particle might cause steric hindrance of HN-mediated attachment and penetration. These findings corroborate our results of influenza NA protein where rNDV expressing the NA protein induced high levels of influenza virus-specific NI and neutralizing serum antibodies, although, NA-specific antibodies are not typically associated with a classical neutralization of virus (Nayak et al., 2010) In the present study, immunization with rAPMV3-F prevents the replication of NDV challenge virus comparable to LaSota. In addition, there was no oral and cloacal shedding of challenge virus, indicating biological containment in absence of potential spread. These results indicate that F-specific immunity reduce NDV replication and confer complete protection in a highly permissive host.

Newcastle disease vaccinations generally protect birds from the more serious consequences of the disease but virus replication and shedding still occur in the infected birds after vaccination, although at reduced levels (Alexander DJ, 1999). A similar result was observed when chickens were immunized with the recombinant glycoprotein, which protected them against clinical signs and mortality but did not prevent virus shedding (Ogawa et al., 1990; Lee et al., 2010). Since APMV3 and NDV are respiratory pathogens

of chickens, with similar cellular and tissue tropisms, an APMV3 vector is probably the most relevant system to analyze the roles of NDV F and HN proteins in immunogenicity and protection. The NDV F and HN proteins are independent protective antigens, but the F protein contributes more to protection than does the HN protein. My result showed that rAPMV3, which is avirulent in chickens, can be used as a vaccine vector against NDV.

Chapter 9

9.2 Title

Conclusion and Future prospects

9.2 Conclusion and Future prospects

Family *Paramyxoviridae* includes important human and animal pathogens. NDV or avian paramyxovirus (APMV) serotype 1 is an important poultry pathogen that causes severe economic loss to poultry industries around the world. There are nine different serotypes of APMV, among which NDV is the most characterized virus but very little is known about the other serotypes. APMV-3 causes mild clinical illness in chickens and turkeys, which gets exacerbated with secondary bacterial infections. APMV-3 also infects chickens at an early age, with evidence of stunted growth that may be more marked in broiler chicken breeds. APMV-3 has been associated with encephalitis and high mortality in caged birds. Moreover, APMV-3 causes acute pancreatitis and central nervous system (CNS) signs in *Psittacine* and *Passerine* birds. Prior to this study, only the pathogenic potential of APMV-3 infections was available. In this study, two strains of APMV-3 (prototype strain Netherlands and Wisconsin) were characterized both *in vitro* and *in vivo*. A method was standardized for titrating APMV-3 strains since the viruses produced plaques in cell culture. The APMV-3 strains were found to be lentogenic in chickens by internationally accepted standard pathogenicity tests- MDT in embryonated chicken eggs and ICPI in day-old chicks. Mild respiratory disease signs were observed in 1-day-old chickens and turkeys when inoculated through oculonasal route with either strain. There were no overt signs of illness in 2-weeks-old chickens and turkeys by either strain, although all the birds seroconverted after infection. In addition, mild respiratory disease

signs were also observed in hamsters and mice inoculated through oculonasal route with APMV-3, suggesting its broad host range. Moreover, both mice and hamsters showed significantly high HI titers against APMV-3, suggesting further the growth of the virus in these laboratory animals.

As a first step towards molecular characterization, the complete genome sequences of the two APMV-3 strains were determined and compared with other paramyxoviruses. The genome lengths of strain Netherlands and Wisconsin was 16,272 and 16,182 nt, respectively. The genome sequence and phylogenetic analyses justified the classification of APMV-3 under the genus *Avulavirus* within the family *Paramyxoviridae* and also indicated the existence of two subgroups within serotype 3. The concept of sub-grouping was further supported by cross HI and cross neutralization tests in cell culture between homologous and heterologous sera. Hence I proposed that the strains represent a single serotype with two subgroups that differ substantially based on nt and aa sequences, but with only a modest antigenic difference.

The availability of complete genome sequence enabled construction of a full length cDNA clone for APMV-3 strain Netherlands (APMV-3/Net) and subsequent recovery of infectious recombinant virus by reverse genetics system previously established in our laboratory using a recombinant vaccinia (expressing T7 polymerase)-based approach. The development of reverse genetics system has several downstream applications such as engineering of vaccines, generation of vaccine vectors and to study molecular biology. In this study, infectious recombinant virus expressing enhanced GFP was recovered and characterized. The virus expressed the GFP for five consecutive

passages in DF1 cells suggesting that this system could be used to develop APMV-3 as a vaccine vector against emerging pathogens.

NDV remains as a major problem for the poultry industry around the world. Previously, it has been shown that both the F and HN glycoproteins on the surface of NDV are important for virus infectivity and pathogenicity. Although several vaccine studies were done with F and HN glycoprotein (Boursnell et al., 1990a; Boursnell et al., 1990b; Romer-Oberdorfer et al., 2003; Lee et al., 2008), their major role in protection against virulent NDV challenge is not well understood. In order to address this, I generated recombinant avian paramyxovirus type-3 (rAPMV3) vectors, which individually expressed each of the two surface glycoproteins F and HN of NDV. Result showed that the NDV F and HN proteins are independent protective antigens, but the F protein contributes more to protection than does the HN protein. Wild-type rAPMV3, which is avirulent in chickens, can be used as a vaccine vector against NDV, since APMV3 itself provides protection against NDV due to its cross-reactivity and in addition can express NDV protective antigens.

This study has opened up new ways to develop vaccines, vaccine vectors and to study molecular biology of paramyxoviruses. It will be interesting to study the following in future: (i) antigenic and sequence analyses of additional APMV-3 strains (ii) The molecular mechanism of cleavage of APMV-3 fusion protein (iii) Application of APMV-3 reverse genetics system to develop vectored vaccines against emerging diseases of animals and humans.

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