

ABSTRACT

Title of Document: GENETICS OF AVIAN PARAMYXOVIRUS
SEROTYPE 2.

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Avian Paramyxovirus (APMV) serotype 2 is one of the nine serotypes of APMV that infect a variety of bird species around the world. In chickens and turkeys, APMV-2 causes respiratory illness and drop in egg production. To understand the molecular characteristics of APMV-2, the complete genome sequences of prototype strain Yucaipa and strains Bangor, England and Kenya were determined. The genome lengths of APMV-2 strains Yucaipa, Bangor, England and Kenya are 14904, 15024, 14904, 14916 nucleotides (nt), respectively. 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. Sequence comparison of APMV-2 strains England and Kenya with prototype strain Yucaipa show 94–98% nt and 90–100% aggregate amino acid (aa) identities. However, strain Bangor shares low level of nt and predicted aa sequence identities with the other three strains. The phylogenetic and serological analyses of all four strains indicated the existence of two subgroups: strains Yucaipa, England and Kenya represented one subgroup and strain Bangor represented the other subgroup.

All four strains were found to be avirulent for chickens by mean death time and intracerebral pathogenicity test.

To further study the molecular biology and pathogenicity of APMV-2, a reverse genetics system for strain Yucaipa was established in which infectious recombinant APMV-2 was recovered from a cloned APMV-2 antigenomic cDNA. The recovered recombinant virus showed *in vitro* growth characteristics and *in vivo* pathogenicity similar to wild type virus. Recombinant APMV-2 expressing enhanced green fluorescent protein was also recovered, suggesting its potential use as a vaccine vector. Furthermore, generation and characterization of mutant viruses by replacing the fusion protein (F) cleavage site of APMV-2 with those of APMV serotypes 1 to 9 demonstrated that the amino acid composition at F protein cleavage site does not affect the pathogenicity of APMV-2. Overall, the study conducted here has several downstream applications. The complete genome sequence of APMV-2 is useful in designing diagnostic reagents and in epidemiological studies. The reverse genetics system for APMV-2 would be of considerable utility for introducing defined mutations into the genome of this virus and develop vaccine vector for animal and human pathogens.

GENETICS OF AVIAN PARAMYXOVIRUS SEROTYPE 2

By

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Dedication

I dedicate this work to my parents, Dr. G. Subbiah and Mrs. S. Nirmala, for their unconditional love and moral support, my PhD advisor, Dr. Samal for this opportunity to work in his lab and a friend and an angel, Ms. Ireen Dryburgh-Barry for her kindness

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List of Abbreviations

aa	amino acid
APMV	avian paramyxovirus
APMV-2	avian paramyxovirus serotype 2
bp	base pair
BC	Beaudette C
BSL	Bio Safety Level
cDNA	complementary DNA
CEF	chicken embryo fibroblast
CPE	cytopathic effect
Da	Daltons
DAB	Diaminobenzidine
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	message RNA
MDT	mean death time
MOI	multiplicity of infection
MV	measles virus
MVA-T7 polymerase	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-2	recombinant APMV-2
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
VSV	vesicular stomatitis virus

Chapter 1

1.1 Title

General Introduction

1.2 Introduction

Avian Paramyxovirus serotype 2 (APMV-2) belongs to the genus *Avulavirus* within the subfamily *Paramyxovirinae* in the family *Paramyxoviridae*. This virus family is large and diverse whose members have been isolated from many species of avian, terrestrial, and aquatic animals around the world (Lamb and Parks, 2007; Wang and Eaton, 2001). 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, 2003). 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 not much was known about APMV-5 (Lipkind and Shihmanter, 1986). Among them, 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-2 is associated with mild respiratory illness in chickens and turkeys that gets exaggerated with secondary bacterial infections (Alexander, 1980). The virus was first isolated in 1956 at Yucaipa, California, from a chicken (Bankowski *et al.*, 1960). Since then many APMV-2

strains have been isolated from chickens, turkeys and feral birds across the globe (Alexander *et al.*, 1982; Asahara *et al.*, 1973; Collings *et al.*, 1975; Fleury and Alexander, 1979; Goodman and Hanson, 1988; Lang *et al.*, 1975; Lipkind *et al.*, 1979, 1982; Mbugua and Karstad, 1985; Nymadawa *et al.*, 1977; Shihmanter *et al.*, 1997; Weisman *et al.*, 1984; Zhang *et al.*, 2006, 2007). Serological survey of chickens and turkeys has shown a wide prevalence of APMV-2 in the United States. APMV-2 is more prevalent in turkeys than chickens (Bankowski *et al.*, 1968) and affects hatchability and poult yield (Bankowski *et al.*, 1981). Surveillance of wild birds has indicated that APMV-2 is more frequently seen in passerines (Alexander, 1986; Senne *et al.*, 1983).

The genome of APMV-2 has six genes arranged in tandem in the order of 3' -N-P-M-F-HN-L- 5'. 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 length called intergenic sequences (IGS) are present, which are shown to regulate the transcription efficiency of downstream genes in NDV (Yan and Samal, 2008). In addition, the genome of APMV-2 contains a 55 nt long viral promoter known as leader at its 3' end and a 154 nt long viral antigenome promoter known as trailer at its 5' end (Subbiah *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 Kolakofsky, 1996). APMV-2 genome encodes internal proteins: major nucleocapsid protein (N), a phosphoprotein (P) and a large polymerase protein (L); surface glycoproteins: fusion protein (F) and hemagglutinin-neuraminidase (HN) protein and a matrix protein M. 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 one and two G insertions, respectively. The V protein has a highly conserved cysteine motif and has interferon antagonist activity in NDV (Huang *et al.*, 2003).

The outer surface glycoproteins, F and HN, mediate viral penetration and attachment, respectively. The F protein is synthesized as an inactive precursor (F₀) which is cleaved at F protein cleavage site by host-cell proteolytic enzyme(s) into biologically active F₁ and F₂ 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; Ogasawara *et al.*, 1992). Many studies have shown that the F protein cleavage site determines virulence of NDV. Accordingly, the World Organization of Animal Health considers the presence of multiple basic amino acids and phenylalanine at the F protein cleavage site as one of the important criteria to categorize NDV strains under virulent pathotype (OIE, Chapter 2.1.15). The F protein cleavage site of APMV-2 has two basic amino acids and a phenylalanine at F1 N terminus and is avirulent in chickens (Subbiah *et al.*, 2008). Furthermore, each APMV serotype has a unique F protein cleavage site and does not abide to the general rule seen with NDV strains. Hence in this study, F protein cleavage site mutants (each representing a particular APMV serotype) were generated in APMV-2 backbone using reverse genetics to understand the role of amino acid composition at the F protein cleavage site in viral biology and pathogenesis.

Pathogenesis and molecular characteristics of APMV-2 have rarely been studied; only limited sequences of F and HN genes of APMV-2 (GenBank AF422844) and a partial L gene sequence, 2042 bp (GenBank AF515835) were previously accessible in GenBank. The complete genome sequences of none of the strains of APMV-2 were available. Therefore, in this study, the complete genome sequences of four strains of APMV-2 were determined and compared with those of other paramyxoviruses to understand the viral biology at molecular level. The knowledge of complete genome sequence is essential for genetic manipulation of the virus. The most successful system for genetic manipulation of negative-sense RNA viruses is a plasmid based system called reverse genetics. 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 (rabies virus, Schnell *et al.*, 1994; vesicular stomatitis virus, Lawson *et al.*, 1995; human respiratory syncytial virus, Collins *et al.*, 1995; measles virus, Radecke *et al.*, 1995; Sendai virus, Garcin *et al.*, 1995; SV5, He *et al.*, 1997; rinderpest virus, Baron and Barrett, 1997; parainfluenza virus, Hoffman and Banerjee, 1997; bovine respiratory syncytial virus, Buchholz *et al.*, 1999 and Yunus *et al.*, 2001; Newcastle disease virus, Peeters *et al.*, 1999 and Krishnamurthy *et al.*, 2000; human metapneumovirus, Biacchesi *et al.*, 2004a and Herfst *et al.*, 2004; and avian metapneumovirus, Naylor *et al.*, 2004 and 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-2 was established which was then used to study the role of F protein cleavage site in virus biology and pathogenesis.

1.3 Research Objectives:

1. Determination and comparison of complete genome sequences of APMV-2 prototype strain Yucaipa and strains Bangor, England and Kenya.
2. Comparison of the pathogenicity of APMV-2 strains Yucaipa and Bangor in chickens and turkeys.
3. Establishment of a reverse genetics system for APMV-2 and recovery of infectious recombinant APMV-2/Yuc expressing a foreign gene.
4. Study of the role of amino acid composition at the fusion protein cleavage site in APMV-2 biology and pathogenesis.

Chapter 2

2.1 Title

Review of Literature

2.2 Classification

APMV-2 belongs to the genus *Avulavirus* within the subfamily *Paramyxovirinae* in the family *Paramyxoviridae* under the order *Mononegavirales* (Lamb and Parks, 2007). Paramyxoviruses are divided into two subfamilies, *Paramyxovirinae* and *Pneumovirinae*, based on structure, genome organization, and sequence relatedness (Lamb and Parks, 2007). Subfamily *Paramyxovirinae* comprises five genera: *Respirovirus* (i.e., Sendai virus [SeV] and human parainfluenza virus types 1 and 3 [HPIV-1 and -3]), *Rubulavirus* (i.e., simian virus type 5 [SV5], mumps virus [MuV], and human parainfluenza virus types 2 and 4 [HPIV-2 and -4]), *Morbillivirus* (i.e., measles [MeV] and canine distemper [CDV] viruses), *Henipavirus* (i.e., Hendra [HeV] and Nipah [NiV] viruses), and *Avulavirus* (comprising the nine serotypes of avian paramyxoviruses [APMV-1 to -9]). Subfamily *Pneumovirinae* contains two genera, *Pneumovirus* (comprising human respiratory syncytial virus [HRSV] and its animal counterparts) and *Metapneumovirus* (comprising human metapneumovirus [HMPV] and its avian counterpart [AMPV]). The genus *Avulavirus* consists of paramyxoviruses particularly isolated from avian species except for avian metapneumovirus. These viruses have been classified into nine serotypes (APMV serotypes 1 through 9) based on hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests (Alexander 2003). The economically important poultry pathogen, Newcastle disease virus (NDV) belongs to serotype 1.

2.3 Virion

APMV-2 is a pleomorphic and enveloped virus with diameter ranging between 100 to 300 nm, which is similar to NDV (Fig. 2.1, 2.2). The paramyxoviral envelope is derived from host cell membrane and encloses a helical nucleocapsid. The outer surface of the envelope consists of two viral spike glycoproteins, namely fusion (F) and hemagglutination neuraminidase (HN) proteins. The F protein aids in the fusion of virion with the host cell membrane in a pH-independent mechanism while the HN protein is required for the attachment of the virion to host cell. Under the envelope lies the matrix protein (M) which is shown to play major roles in virus assembly and budding in NDV. The envelope encloses the ribo-nucleocapsid core structure or the transcriptive-replicative complex, formed by nucleocapsid protein (N) bound to the genomic RNA; also attached to this structure are the phosphoprotein (P) and large polymerase (L) protein (Lamb and Parks, 2007). The viral RNA genome is single stranded, non-segmented and negative-sensed.

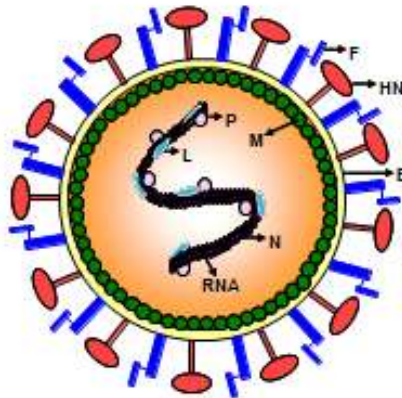


Fig. 2.1 Schematic diagram of a virion of Avian Paramyxovirus serotype 2 (not drawn to scale).

N, Nucleocapsid protein; P, Phosphoprotein; M, Matrix protein; L, Large polymerase protein; F, Fusion glycoprotein; HN, Hemagglutinin-Neuraminidase glycoprotein; E, Envelope.

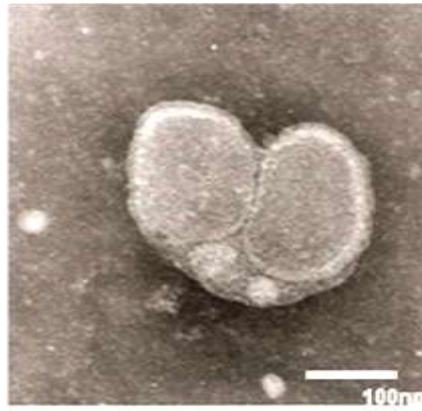


Fig. 2.2 Electron micrograph of negatively stained pleomorphic Avian Paramyxovirus serotype 2 (strain Yucaipa).

The supernatant of APMV-2 strain Yucaipa infected chicken embryo fibroblast cells were collected four days post infection and following clarification at 4000 rpm for 10 min, negative staining was performed and observed under electron microscope.

2.4 Genome organization

The APMV-2 genome contains six genes in tandem and encodes upto eight proteins. These genes are arranged in a linear order, 3'-NP-P-M-F-HN-L-5' as shown in Fig. 2.3. On each end of the genome is a 3' and 5' extracistronic sequence, known as the leader and trailer, respectively. The leader and trailer regions are cis-acting regulatory elements involved in replication, transcription and packaging of the genomic and antigenomic RNAs. Each gene is flanked by conserved transcriptional control sequences, known as the gene start (GS) and gene end (GE), respectively which are followed by 5' and 3' untranslated regions (UTR). Non-coding intergenic sequences (IGS) are located between each gene boundary.

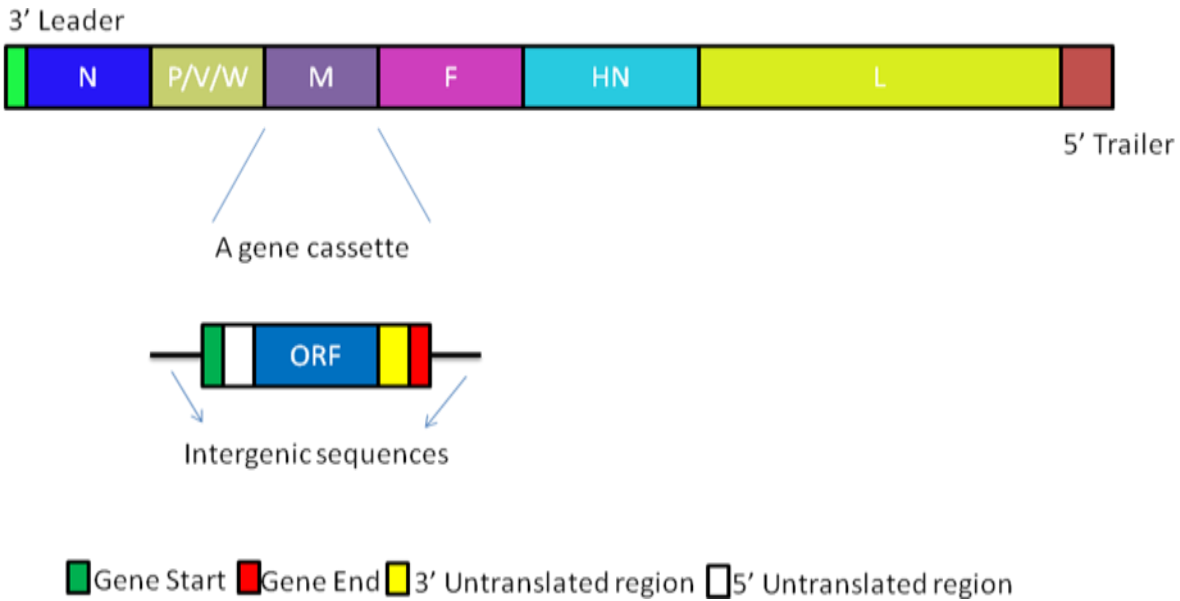


Fig. 2.3 Genome organization of APMV-2 and schematic diagram of a gene cassette.

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 gene start and gene end and has untranslated sequences at 3' and 5' regions of the ORF between each gene boundary.

2.5 Viral proteins

The genome of APMV-2 consists of six genes N, P, M, F, HN and L encoding upto eight proteins: N, P, V, W, M, F, HN and L (Subbiah *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-2 are known to be similar to those of APMV-1 and other paramyxoviruses; hence, the knowledge about the APMV-2 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 tightly bound with viral genomic RNA and forms the nucleocapsid core. Two other viral proteins, the phosphoprotein (P) and the large polymerase protein (L) are loosely attached to this core and together form the transcriptive-replicative complex or the active polymerase complex which is the minimum infectious unit of the virus (Lamb and Parks, 2007). The N protein binds to viral genomic RNA and makes it RNase resistant. The N protein interacts with P and L proteins during transcription and replication and with M protein during virus assembly. The intracellular level of unassembled N protein regulates the switching of viral transcription to replication of the viral genome (Blumberg and Kolakofsky, 1981; Blumberg *et al.*, 1981).

P, V and W proteins: The P protein produced from unedited P mRNA is acidic and the heavily phosphorylated viral protein (McGinnes *et al.*, 1988; 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 proteins. 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 (Lamb and Parks, 2007; 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 least abundant in the virion core (about 50 copies per virion). It is the major component of the viral RNA dependent RNA polymerase (Banerjee, 1987; Tordo *et al.*, 1988). In general, the L protein has 5' capping and 3' polyadenylation activities on viral mRNAs. Further, the L protein has been shown to be a virulence determinant in 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 proteins and its affinity to interact with the nucleocapsid might be the driving force for forming a budding virus particle (Peeples, 1991).

2.5.3 Envelope glycoproteins

APMV-2 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 aids in the attachment of the virus to host cell membrane.

F protein: The F protein mediates fusion between cells causing giant cell formation (syncytia) and also fusion between the virus envelope and host cell plasma membrane, in a pH-independent manner. This forms pores on plasma membrane through which the viral nucleocapsid is delivered into the host cell. The fusion and syncytia formation caused by F protein of the virus is one of the important factors for virulence as well as viral spread within infected host. The F protein is a type I integral membrane protein and is synthesized as an inactive precursor (F_0), which is cleaved by using host-cell proteolytic enzyme(s). This cleavage results in biologically active protein that has two subunits F_1 and F_2 interconnected by disulfide bond (Scheid and Choppin, 1974). In NDV, F protein is synthesized on rough endoplasmic reticulum and targeted towards host cell membrane for expression. The NDV strains that have multiple basic amino acids at their F protein cleavage site are cleaved by intracellular subtilisin-

like proteases, whereas, those NDV strains that have a single basic amino acid at their F protein cleavage site require exogenous proteases for cleavage activation (Ortmann *et al.*, 1994; Scheid and Choppin, 1974).

Several studies have shown that the amino acid sequence at F protein cleavage site is the major virulence determinant of NDV; the avirulent strains of NDV have a monobasic amino acid residue at F protein cleavage site and a leucine at the F1 N terminus, while virulent NDV strains possess multiple basic amino acids at F protein cleavage site and a phenylalanine at the F1 N terminus (Nagai *et al.*, 1976; Ogasawara *et al.*, 1992). Accordingly, the World Organization of Animal Health considers the presence of multiple basic amino acids and phenylalanine at the F protein cleavage site as one of the important criteria to categorize NDV strains under virulent pathotype (OIE, Chapter 2.1.15). The F protein cleavage site of APMV-2 has two basic amino acids and a phenylalanine at F1 N terminus but is still avirulent in chickens (Subbiah *et al.*, 2008). The recently available complete genome sequences of other APMV serotypes show that each APMV serotype has unique F protein cleavage site and does not seem to follow the general rule as seen with NDV strains.

HN protein: The HN protein is a type II integral membrane protein. The C-terminus end is the globular head which is the site for the attachment of virus to the host cells via sialic acid containing host receptors. The HN protein also cleaves sialic acid enzymatically (neuraminidase activity), thus assisting virus release from surface (Crennell *et al.*, 2000). In addition it has been shown to have fusion promoting activity (Lamb and Parks, 2007).

2.6 Stages of APMV-2 replication

The replication of APMV-2 is very similar to that of other paramyxoviruses (Fig. 2.4).

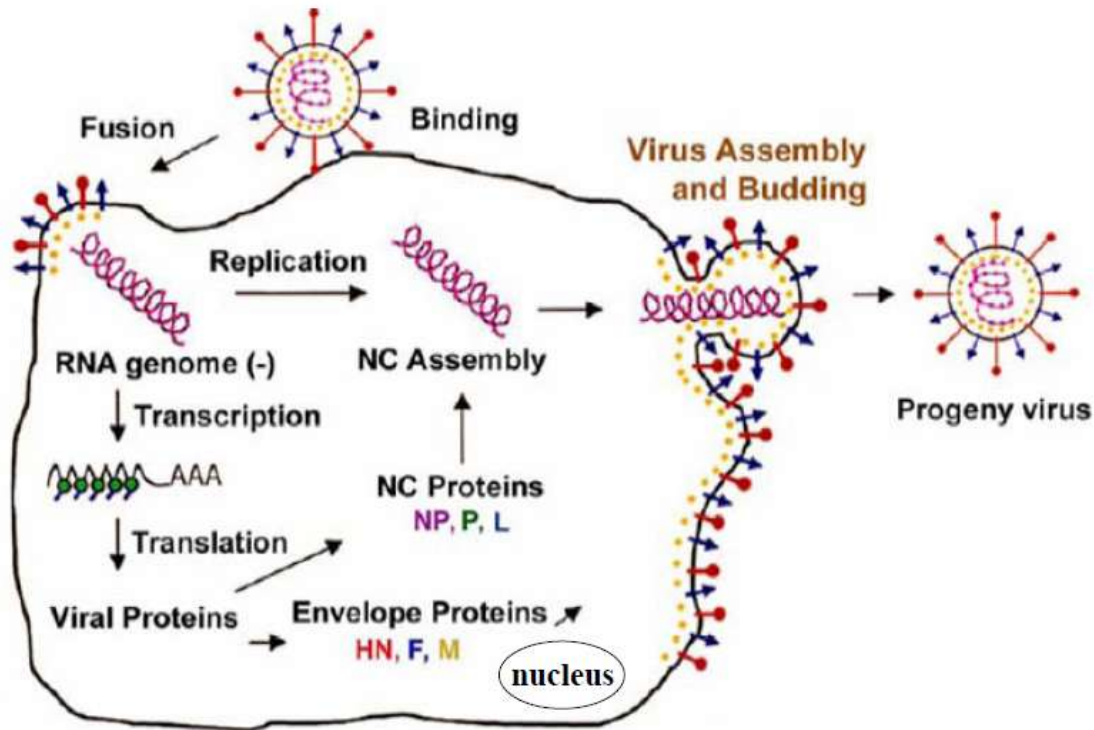


Fig. 2.4 Stages of replication in a Paramyxovirus.

Schematic diagram of stages of replication in a paramyxovirus, adapted from <http://www.urmc.rochester.edu/SMD/mbi/education/courses/MBI456files/ParamyxovirusII.pdf>

2.6.1 Virus attachment, fusion and entry

The initial step of APMV-2 infection is the attachment of the virus to host cell receptor mediated by HN protein (Huang *et al.*, 1980). The next step is fusion of viral envelope with the host cell plasma membrane at neutral pH mediated by F protein and subsequently the release of the viral nucleocapsid into the host cell cytoplasm.

2.6.2 Transcription

The negative-sense RNA genomes of paramyxoviruses are non-infectious, because uninfected host cells lack RNA-dependent RNA polymerase (RDRP) activity. The viral mRNA transcription occurs in cytoplasm. The viral 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 continue to transcribe the downstream genes. 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). The mRNA are capped and polyadenylated. The intergenic regions located between each gene are not transcribed. The optimal length of the intergenic sequences has been shown to be required for the efficient transcription of each downstream gene (Yan and Samal, 2008).

2.6.3 Genome replication

The concentration of viral proteins, particularly, N protein determines the switch of RDRP from transcription mode to replication of viral genome. A full length complimentary copy known as (+) antigenome is first produced by ignoring all the start-stop signals (Blumberg and Kolakofsky, 1981; Nagai, 1999). These (+) antigenomes are used as templates for synthesis of (-) genome for packaging into the new viral progenies. It has been shown that the leader and trailer regions contain specific signals for encapsidation (Blumberg and Kolakofsky, 1981). The RNA synthesis of a paramyxovirus is shown in Fig. 2.5.

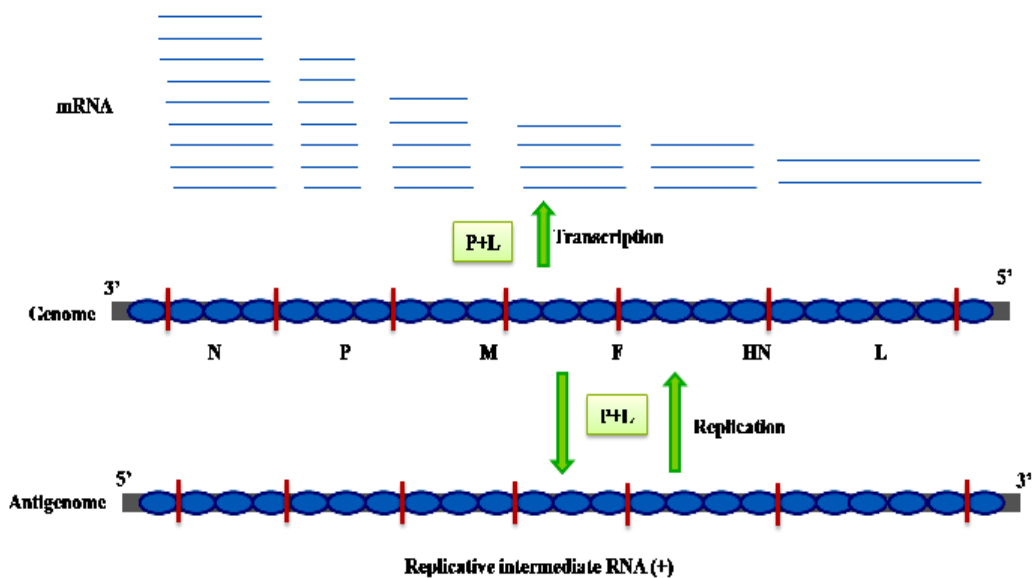


Fig. 2.5 Schematic diagram of RNA replication and transcription in paramyxovirus.

Schematic diagram showing transcription and replication of a paramyxovirus. Genome and antigenome are shown encapsidated by nucleocapsid protein subunits (ovals). 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 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.

2.6.4 Virus assembly and release

The nucleocapsid core assembly occurs in the cytoplasm of host cell. Initially, free 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 *et al.*, 1978). The membrane glycoproteins (F and HN) synthesized on rough endoplasmic reticulum (ER) undergo conformational maturation before getting transported to the cell surface. Folding and maturation occur in the ER and the correctly folded proteins

reach the Golgi apparatus for post translational modifications, such as modification of carbohydrate chains of HN protein and cleavage of multiple basic cleavage sites of F protein. The glycoproteins are transported to the cell surface via vesicles where the assembly of the virus envelope occurs (Doms *et al.*, 1993; 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 proteins, which in turn associate with the nucleocapsid thus helping the process of budding of the mature virions.

2.7 Epidemiology of APMV-2 infections

APMV-2 was first isolated from a diseased chicken in Yucaipa, California (Bankowski *et al.*, 1960). Subsequently, many APMV-2 infections have been reported in chickens, turkeys, racing pigeons and feral birds across the globe (Alexander *et al.*, 1982; Asahara *et al.*, 1973; Collings *et al.*, 1975; Fleury *et al.*, 1979; Goodman *et al.*, 1988; Lang *et al.*, 1975; Lipkind *et al.*, 1979; Lipkind *et al.*, 1981; Lipkind *et al.*, 1982; Mbugua *et al.*, 1985; Nymadawa *et al.*, 1977; Shihmanter *et al.*, 1997; Weisman *et al.*, 1984). APMV-2 strains are endemic among passeriformes and psittacines in Senegal and on the island of Hiddensee, GDR, in the Baltic Sea (Alexander, 1993, Fleury & Alexander, 1979, Nymadava *et al.*, 1977). The prevalence of APMV-2 antibodies has been studied in different avian species including commercial poultry (Ley *et al.*, 2000; Zhang *et al.*, 2007; Warke *et al.*, 2008). Furthermore, APMV-2 strains have been isolated from chickens; broilers and layers in USA, Canada, Russia, Japan, Israel, India, Saudi Arabia, Great Britain and Costa Rica, and from turkeys in the USA, Canada, Israel, France and Italy (Wood *et al.*, 2008). Serological surveys of poultry in the USA indicated that this virus was widespread and more frequently infected turkeys (Bankowski *et al.*, 1968, Bradshaw &

Jensen, 1979). APMV-2 infection induced more severe symptoms in turkeys than in chickens (Alexander, 1993). APMV-2 has been shown to affect the hatchability and poult yield of turkeys (Bankowski *et al.*, 1981). However, more serious disease has been reported, especially in turkeys during secondary bacterial infections (Lang *et al.*, 1975 and Yegana *et al.*, 1985). Virus isolation and the presence of antibodies have also indicated the presence of APMV-2 infections in turkey flocks without causing any clinical disease (Bradshaw & Jensen, 1979). APMV-2 isolated from commercial layer farms and from broiler breeder farms in Scotland was suspected to be the cause for drop in egg production (Wood *et al.*, 2008).

2.8 Reverse genetics

In negative-sense RNA viruses, the naked viral genome is non-infectious because the uninfected cells do not have RDRP required for the synthesis of viral proteins. The RDRP complex is virally encoded and packaged into the virion particles. Reverse genetics is a method of generation of infectious virus from entirely cloned cDNA of the viral genome. In this system plasmids expressing viral proteins N, P and L and full length antigenome are transfected in cells. All these plasmids are under the control of T7 RNA polymerase promoter. The T7 RNA polymerase is either provided by a recombinant vaccinia virus expressing bacteriophage T7 RNA polymerase or constitutively expressed in the cell line like BHK T7. This technique helps to manipulate and genetically engineer viruses.

The rabies virus was the first successfully recovered virus using this approach (Schnell *et al.*, 1994). Several other viruses such as the vesicular stomatitis virus (Lawson *et al.*, 1995; Whelan *et al.*, 1995), simian virus 5 (He *et al.*, 1997), human respiratory syncytial virus (Collins *et al.*, 1995), sendai virus (Garcin *et al.*, 1995; Kato *et al.*, 1996), rinderpest virus (Baron and

Barrett, 1997), parainfluenza virus (Durbin *et al.*, 1997; Hoffmann and Banerjee, 1997), measles virus (Radecke *et al.*, 1995) and Nipah virus (Yoneda *et al.*, 2006) have been recovered by similar approach. The recoveries of infectious NDVs from cDNA using reverse genetics system were first reported in 1999 (Romer-Oberdorfer *et al.*, 1999; Peeters *et al.*, 1999). The reverse genetics systems available for NDV are: lentogenic strain LaSota (Huang *et al.*, 2001; Romer Oberdorfer *et al.*, 1999 ; Peeters *et al.*, 1999), B1 (Nakaya *et al.*, 2001) , mesogenic strain Beaudette C (Krishnamurthy *et al.*, 2000) and velogenic strain Hert/33 (de Leeuw *et al.*, 2005). The reverse genetics system has been used to study of molecular determinants of viral pathogenesis and to develop vaccines and vaccine vectors against emerging pathogens.

Chapter 3

3.1 Title

Determination of the complete genome sequences of four strains of avian paramyxovirus serotype 2: Evidence of existence of two subgroups within serotype 2

(Complete genome sequence of APMV-2 strain Yucaipa was previously published as: Subbiah *et al.*, 2008. *Virus Res* 137: 40-48.)

3.2 Abstract

The complete consensus genome sequences of avian paramyxovirus (APMV) serotype 2 strains Bangor, England and Kenya were determined and the sequences were compared with those of APMV-2 prototype strain Yucaipa and other paramyxoviruses. The complete genome sequence of strain Yucaipa was initially determined and published (Subbiah *et al.*, 2008. *Virus Res* 137: 40-48). The genome lengths of APMV-2 strains Yucaipa, Bangor, England and Kenya are 14904, 15024, 14904, 14916 nucleotides (nt), respectively. Each genome consists of six non-overlapping genes in the order of $3'N-P/V/W-M-F-HN-L5'$, with a 55-nt leader at the 3' end. The lengths of trailer at the 5' end of the strains Yucaipa, Bangor, England, Kenya are 154, 173, 154 and 154 nt, respectively. In general, sequence comparison of APMV-2 strains England and Kenya with strain Yucaipa show 94–98% nt and 90–100% aggregate amino acid (aa) identities. However, strain Bangor shares low level of genome and predicted aa sequence identities with the other three strains. Furthermore, strain Bangor has a single basic aa residue ($^{101}TLPSAR\downarrow F^{108}$) at the fusion protein cleavage site compared to the dibasic aa ($^{93}DKPASR\downarrow F^{100}$) found in those of other three strains. Reciprocal cross-hemagglutination inhibition (HI) and cross-neutralization assays using post-infection chicken sera indicated that strain Bangor is antigenically related to

the other APMV-2 strains, but with 4- to 8-fold lower HI titers. Taken together, our results indicated that these four APMV-2 strains represent a single serotype with two subgroups based on nt and aa sequence analyses and by cross-HI and cross-neutralization assays.

3.3 Introduction

The family *Paramyxoviridae* is large and diverse and includes members that have been isolated from many species of avian, terrestrial, and aquatic animals around the world (Lamb and Parks, 2007; Wang and Eaton, 2001). Paramyxoviruses are pleomorphic, enveloped, cytoplasmic viruses with a non-segmented negative-strand RNA genome. Paramyxoviruses are divided into two subfamilies, *Paramyxovirinae* and *Pneumovirinae*, based on structure, genome organization, and sequence relatedness (Lamb *et al.*, 2005). Subfamily *Paramyxovirinae* comprises five genera; *Respirovirus* (including Sendai virus [SeV] and human parainfluenza virus types 1 and 3 [HPIV-1 and -3]), *Rubulavirus* (including simian virus type 5 [SV5], mumps virus [MuV], and human parainfluenza virus types 2 and 4 [HPIV-2 and -4]), *Morbillivirus* (including measles [MeV] and canine distemper [CDV] viruses), *Henipavirus* (including Hendra [HeV] and Nipah [NiV] viruses), and *Avulavirus* (comprising the nine serotypes of avian paramyxoviruses [APMV-1 to -9]). Subfamily *Pneumovirinae* contains two genera, *Pneumovirus* (comprising human respiratory syncytial virus [HRSV] and its animal counterparts) and *Metapneumovirus* (comprising human metapneumovirus [HMPV] and its avian counterpart [AMPV]).

The genome lengths of paramyxoviruses range from 15 to 19 kb and contain 6–10 genes arranged in tandem (Lamb and Parks, 2007). All paramyxoviruses examined to date encode a major nucleocapsid protein (N) that binds the entire length of the genomic and antigenomic RNAs, a nucleocapsid phosphoprotein (P) that is a polymerase co-factor, a large protein (L) that

is the major polymerase subunit and bears catalytic domains, a matrix protein (M) that lines the inner surface of the envelope, a fusion glycoprotein (F) that is a surface antigen that mediates viral penetration and syncytium formation and a major glycoprotein (G) or hemagglutinin-neuraminidase (HN) glycoprotein that is a second surface antigen and mediates attachment. Most members of subfamily *Paramyxovirinae* engage in RNA editing, whereby a specific motif in the P gene directs non-templated addition of one or more nucleotides to a proportion of P transcripts. This produces mRNA subpopulations containing frameshifts generating one or more alternate internal ORFs to produce chimeric proteins in which the upstream end is encoded by the P ORF and the downstream end is encoded by the alternative ORF. The insertion of a single G residue at the P editing site alters the reading frame and produces V protein, in which the downstream domain contains a highly conserved cysteine motif. 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). Alternatively, the insertion of two G residues shifts the reading frame to access an internal ORF that leads to production of the W protein, whose function is not yet understood (Steward *et al.*, 1993).

The APMVs have been classified into nine different serotypes based on hemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays (Alexander, 2003). Newcastle disease virus (NDV) belongs to serotype 1. NDV is the most characterized member among all the APMV serotypes because it produces severe disease in chickens (Alexander, 1980). The APMV-2 was first isolated in 1956 in Yucaipa, California from a diseased chicken that was also infected with infectious laryngotracheitis virus (Bankowski *et al.*, 1960). Since then many APMV-2 strains have been isolated from chickens, turkeys and feral birds around the world (Alexander *et al.*, 1982; Asahara *et al.*, 1973; Collings *et al.*, 1975; Fleury and Alexander, 1979; Goodman and

Hanson, 1988; Lang *et al.*, 1975; Lipkind *et al.*, 1979, 1982; Mbugua and Karstad, 1985; Nymadawa *et al.*, 1977; Shihmanter *et al.*, 1997; Weisman *et al.*, 1984; Zhang *et al.*, 2006, 2007). APMV-2 strain Bangor was isolated from a finch during a routine quarantine evaluation and the biological and serological characterization suggested placing strain Bangor as a separate serotype or as a subgroup within serotype 2 (McFerran *et al.*, 1973; McFerran *et al.*, 1974).

As noted, APMV-2 strains have been isolated from a wide variety of avian species from different parts of the world. But little is known about the serological and genetic relationships among these strains. The information is important for understanding virus evolution and epidemiology and for development of vaccines against these viruses. To date, the complete genome sequences of only the prototype strain Yucaipa is available (Subbiah *et al.*, 2008). As a first step towards understanding the serological and genetic relationship among APMV-2 strains, we have determined the complete genome sequences of three other strains of APMV-2; Bangor, England and Kenya, isolated from a finch, a chicken and a gadwell, respectively, and describe comparison with the complete genome sequence of prototype strain Yucaipa and other paramyxoviruses. Our sequence and antigenic analyses suggested that APMV- 2 strains can be classified into two genetic subgroups under a single serotype.

3.4 Materials and methods:

3.4.1 Virus and cells. APMV-2/Chicken/Yucaipa/Cal/56 (APMV-2 Yucaipa) and APMV-2/Finch/N.Ireland/Bangor/73 (APMV-2 Bangor) were received from the National Veterinary Services Laboratory, Ames, Iowa, USA and APMV-2/Chicken/England/7702/06 (APMV-2 England) and APMV-2/Gadwell/Kenya/3/80 (APMV-2 Kenya) were obtained from Veterinary

Laboratories Agency, Weybridge, UK. The viruses were grown in 9-day-old embryonated, specific pathogen-free (SPF) chicken eggs. Hemagglutination (HA) titers were determined using 0.5% chicken RBC at room temperature. The ability of the viruses to replicate in cell culture was examined in two established cell lines- DF1, chicken fibroblast and Vero, African green monkey kidney. Both the cell lines were grown in Dulbecco's MEM containing 10% fetal bovine serum (FBS) in a 37°C incubator with 5% CO₂.

3.4.2 Replication of viruses in cell cultures. The cell monolayers (DF1 and Vero) were infected with a 10⁻³ dilution of 2⁸ HA units of egg-grown APMV-2 strains Yucaipa, Bangor, England and Kenya and, after 1 h of adsorption, the viral inoculum was replaced with maintenance medium containing 2% FBS with or without the supplementation of exogenous protease (10% allantoic fluid). The cells were observed daily for cytopathic effects (CPE) and the supernatants of the infected cells were collected every 24 h until fifth day post-infection (dpi). Virus titers were determined by serial end-point dilution on monolayers of DF1 cells in 96-well plates. The infected cells were immunostained using polyclonal antisera raised against the viruses in chickens. Virus titers (TCID₅₀/ml) were calculated using Reed & Muench method (Reed & Muench, 1938). The ability of the viruses to produce plaques was tested in both the cell lines under various conditions, including 1% methylcellulose, 1% low melting agar, and 0.8% noble agar with or without magnesium sulfate (25 mM) and 1% diethylaminoethyl dextran (30 µg/ml). Plaques were visualized by staining with either crystal violet or neutral red.

3.4.3 Serological analysis. Antisera against APMV-2 strains Yucaipa, Bangor, England and Kenya were prepared by single infection of 2-week-old chickens via the intraocular (IO) and

intranasal (IN) routes mimicking natural infection. Briefly, groups of three 2-week-old chickens of each group were infected with each virus (2^8 HAU) at separate times to avoid cross-infection. Two weeks after infection the chickens were bled and sera were collected and stored at -20°C . HN-specific antibody titers in the serum samples were determined by HI assay using chicken RBC as described previously (Alexander, 1997). The cross-reactivity of immunized chicken sera was determined by HI assay against heterologous APMV-2 strains. The ability of immunized chicken sera to cross-neutralize heterologous APMV-2 strains was determined by focus reduction microneutralization assay using standard procedures (Borisevich *et al.*, 2007). Briefly, different dilutions of sera were mixed with constant titer of virus (10^3 TCID₅₀/ml), incubated for 2 h at room temperature and transferred to monolayer of DF1 cells in 96-well plates. The plates were incubated for three days at 37°C with 5% CO₂. Each plate included both uninfected and infected cell controls. On the third day, the culture medium was removed and cells were fixed with methanol for 30 min and washed with PBS three times. The fixed cells were immunostained and a 50% focus reduction was considered as the end point of the titration.

3.4.4 Pathogenicity tests. The virulence of the APMV-2 strains was determined by two standard pathogenicity tests for APMV-1: mean death time (MDT) in 9-day-old embryonated SPF chicken eggs and intracerebral pathogenicity index (ICPI) test in 1-day-old SPF chicks (Alexander 1989). Briefly, for MDT, a series of 10-fold (10^{-6} - 10^{-9}) dilutions of fresh infective allantoic fluid in PBS was made and 0.1 ml of each diluent was inoculated into the allantoic cavities of five 9-day-old SPF embryonated chicken eggs (BEE eggs company, PA), which were incubated at 37°C . The eggs were candled 3 times a day for the next 7 days and the time of embryo death, if any, were recorded. The minimum lethal dose (MLD) is the highest virus

dilution that kills all the embryos. The MDT is the mean time in hours for the MLD to kill all the inoculated embryos. The MDT has been used to classify APMV-1 strains into the following groups: velogenic strains (taking less than 60 h to kill); mesogenic strains (taking 60-90 h to kill); and lentogenic strains (taking more than 90 h to kill).

For ICPI, 0.05 ml (1:10 dilution) of fresh infective allantoic fluid of each virus was inoculated into groups of ten 1-day-old SPF chicks via the intracerebral route. The inoculation was done using a 27-gauge needle attached to a 1 ml stepper syringe dispenser that was set to dispense 0.05 ml of inoculum per bird. The birds were inoculated by inserting the needle up to the hub into the right or left rear quadrant of the cranium. The birds were observed for clinical symptoms and mortality once every 8 h for a period of 10 days. At each observation, the birds were scored: 0, if normal, 1, if sick and 2, if dead. ICPI is the mean score per bird per observation over the 10-day period. Highly virulent (velogenic) viruses give values approaching 2, and avirulent (lentogenic) viruses give values close to 0.

3.4.5 Virus RNA isolation and complete genome sequencing. The viral RNA was isolated from the allantoic fluid of virus-infected eggs using RNeasy kit according to the manufacturer's instructions (QIAGEN, USA). The complete genome sequence exclusive of the termini of strain Yucaipa was determined using a combination of three different strategies (Subbiah *et al.*, 2008). First, the nucleotide sequences of the N genes of all the available rubulaviruses and avulaviruses were aligned to identify a consensus sequence that was used to design the forward primer N-451 (5_-GAAGATGATGCACCAGAAGA, numbered according to the consensus sequence). Similarly, L genes of rubulaviruses and avulaviruses were aligned to design two reverse degenerate primers in the conserved regions of L gene; L-5544r, 5_-

NGGNCCRAARTGNCKYTGNGGNGGRTT (N = A/C/G/T, R = A/G, K= G/T, Y = C/T) and L-6960r, 5'- NSWRTARTANCCYTTNGCNGCRTTNCCDATNGT (N= A/C/G/T, S = G/C, W= A/T, R = A/G, Y = C/T, D= G/A/T). A reverse primer from the APMV-2 F gene sequence available in GenBank (accession no. AF422844) (F-127r, 5'-ACTGCGATGGTCCCTGTGAG, numbered according to the Yucaipa strain F gene sequence) and APMV-2 L genes specific forward primers from the partial sequence available in GenBank (accession no. AF515835) were designed. Second, a gene-start forward primer (5'GGAAAACTTGGGGGCGACA) containing the presumptively conserved gene-start sequence at its 3' end (underlined) and a reverse primer (5'TTTTTTCTTAAACCAGGCTTC) with the presumptively conserved gene-end sequence at its 5' end (underlined) were designed. RT-PCR with these primers yielded different regions of all the viral genes. Finally, as the third strategy, most of the L gene was sequenced by primer walking. Briefly, cDNA synthesized from an RT reaction with an L gene-specific forward primer was tailed using 3' poly-C tail with terminal deoxynucleotidyl transferase (Invitrogen). The dC-tailed cDNA was amplified by PCR using an L gene-specific forward primer and a poly dG-containing reverse primer. The PCR-amplified products were cloned and sequenced. Most parts of genomes of the other APMV-2 strains except the 3' and 5' termini were amplified into cDNAs using primers designed from the published APMV-2 strain Yucaipa (Table 3.1). The sequences of the 3' and 5' genomic ends were determined from cDNA prepared by rapid amplification of cDNA ends (RACE) as described previously (Subbiah *et al.*, 2008, Troutt *et al.*, 1992). Briefly, To determine the 3' end of viral RNA, a 5' phosphorylated and 3' blocked RNA oligonucleotide (5'^{phos}CCAAAACGCCAUUCCACCUUCUCUUC 3'^{blocked}), was ligated to viral RNA and to determine the 5' end, cDNA derived using L gene specific forward primer was ligated with the RNA oligo. The following protocol was followed for ligation; 8 µl of viral RNA (1–5 µg) and 1

µl of RNA oligonucleotide (50 pmol) were denatured at 65°C for 5 min and snap frozen on dry ice. The ligation reaction was carried out overnight at 16°C with 4 µl of 10× T4 RNA ligase buffer, 10 units of T4 RNA ligase (Promega, USA), 1mM hexamine cobalt chloride, 10 µg/ml BSA, 25% (w/v) of PEG 8000 and RNase-free water to make a 40 µl reaction mixture. Ligation was terminated by heating to 65°C for 20 min. The adaptor primer (5'-GAAGAGAAGGTGGAAATGGCGTTTTGG) complimentary to the RNA oligo, was then used in the downstream steps to obtain the viral 3' and 5' ends (Subbiah *et al.*, 2008; Li *et al.*, 2005).

Primer name	position within APMV-2 strain Yucaipa genome	Primer sequence
Gene Start forward	Gene start consensus	*NNNNNNNNGGGGGCGA
Gene End reverse	Gene end consensus	*NNNNNNNNNNNTTTTTTCTTAA
N Forward	388-415	ACATGCGAGCTCACGCAACCCTTGCAGC
N Reverse	1019-1044	GCCTGATCAAGGACGACATCTTCTTC
P Forward	1758-1781	CGAAGTCAAGGGCCCGCAAACAAC
P Reverse	2464-2484	CTGACTAATCTCATTCTTTAT
M Forward	3135-3157	CCAAAGAGTTGCAGCAGCAAATC
F Forward	5217-5242	AGTGTCACTACACCAAAGGAGAAGG
HN Forward	6698-6719	CCAGTATGTATATCTCTCTGGG
L1 Forward	8869-8890	ATGCTAGTGAGACACACGCAGG
L1 Reverse	10422-10441	GAATACACAAAGAATGATTG
L2 Forward	11967-11986	ATATATCAGCAAATCATGCT
L2 Reverse	13314-13332	CAGCATACTTGTACCAGCT
L3 Forward	14170-14186	TCACCCTATTCGGACAG

Table 3.1. Primers used to amplify APMV-2 genome based on previously available genome sequence of APMV-2 prototype strain Yucaipa.

*N=A/T/C/G

3.4.6 Virus genome sequence alignment and phylogenetic analyses. Sequence compilation and prediction of ORFs were carried out using the SeqMan and EditSeq programs in the Lasergene 6 (DNASTAR) software package (www.dnastar.com). The search for matching protein sequences in GenBank was done using the blastp program of the same package. The bootstrap values in phylogenetic tree were calculated using 1000 replicas and the construction of phylogenetic trees was performed by maximum parsimony method using MEGA 4 software (Tamura *et al.*, 2007).

3.4.7 Database accession numbers. The complete genome sequences of APMV-2 strains Bangor, England and Kenya were submitted to GenBank (accession number HM159995, HM159993 and HM159994, respectively). Accession numbers for other paramyxovirus sequences used in this study were: Avulaviruses: APMV-1, AF077761; APMV-2 strain Yucaipa, EU338414; APMV-3, EU403085; APMV-4KR, EU877976; APMV-4HK, FJ177514; APMV-6TW, NC_003043; APMV-6HK, EU622637; APMV-6FE, EF569970; APMV-7, FJ231524; APMV-8DEL, FJ215863; APMV-8WAK, FJ215864; APMV-9, EU910942. Rubulaviruses: hPIV-2, NC_003443; PIV5 (also known as SV-5), NC_006430; MuV, NC_002200; simian virus 41 (SV41), NC_006428. Respiroviruses: hPIV-1, NC_003461; hPIV-3, NC_001796; SeV, NC_001552, BPIV-3, NC_002161. Henipaviruses: NiV, NC_002728; HeV, NC_001906. Morbilliviruses: CDV, NC_001921; MeV, AF266288; phocine distemper virus (PDV), NC_006383; rinderpest virus (RPV), NC_006296; peste des petits ruminants virus (PPRV), NC_006383; dolphin morbillivirus (DMV), NC_005283; other paramyxovirus: Atlantic salmon paramyxovirus (ASPV), EF646380; Beilong virus (BeV), NC_007803; Fer-de-Lance virus (FDLV), NC_005084; J virus (JV), NC_007454; Menangle virus (MenV), NC_007620;

Mossman (MoV), NC_005339; Tupaia paramyxovirus (TpV), NC_002199; Pneumoviruses: HRSV, NC001781; BRSV, NC001989. Metapneumoviruses: AMPV, NC007652; HMPV, NC004148.

3.5 Results

3.5.1 *In vitro* growth characteristics of APMV-2 strains Yucaipa, Bangor, England and Kenya. APMV-2 strains Yucaipa, Bangor, England and Kenya yielded titers of 2^{10} – 2^{12} HA units in 9-day-old embryonated SPF chickens eggs at 4 dpi. The inclusion of exogenous protease, either 10% allantoic fluid or trypsin, was not necessary for replication of these viruses in cell culture indicating a lack of requirement of external proteases for efficient cleavage of the F protein. The viruses grew more efficiently in DF1 cells than in Vero cells. Viral CPE involved rounding and detachment of the cells. The growth kinetics and the CPE of all the three strains were similar to those of APMV-2 prototype strain Yucaipa. None of the strains produced syncytia or formed plaques but caused single cell infections similar to that of APMV-2 strain Yucaipa (Fig. 3.1).

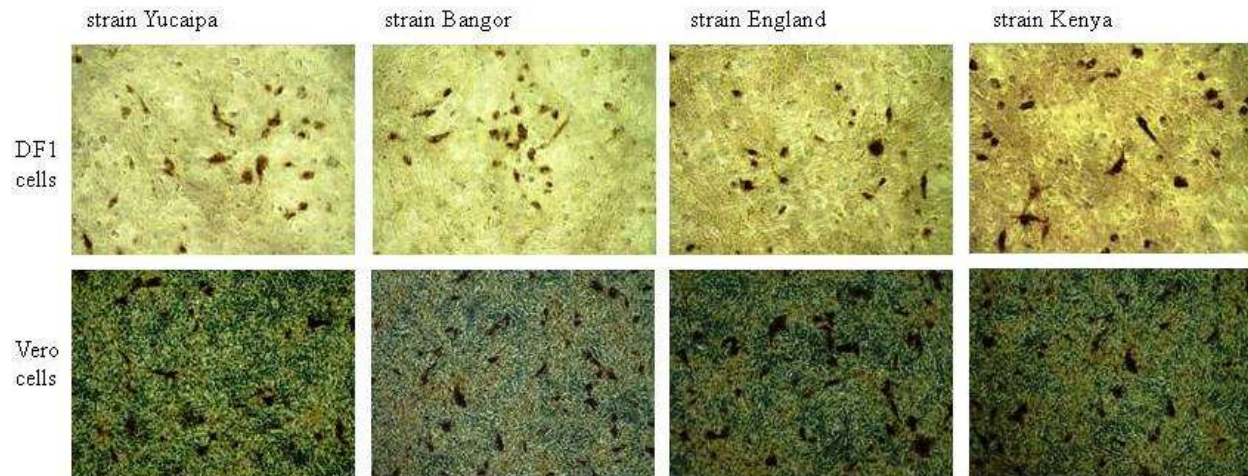


Fig. 3.1. Single cell infection caused by APMV-2 strains Yucaipa, Bangor, England and Kenya in DF1 and Vero cells, three days post infection. The infected cells were immunostained using single-infection sera raised specifically against each of these strains in chickens.

3.5.2 Antigenic relationship among APMV-2 strains. The antigenic relationship among APMV-2 strains Yucaipa, Bangor, England and Kenya was evaluated by reciprocal HI tests using strain specific convalescent sera raised by a single infection of chickens via the IN/IO route. Each of the antiserum exhibited a 2- to 16-fold difference in HI titer between the homologous and heterologous strains (Table 3.2). The HI titer of the APMV-2 prototype strain Yucaipa was 8-, 4-, and 4-fold higher against homologous strain Yucaipa than against strains Bangor, England and Kenya, respectively. Conversely, the HI titer of antisera specifically against strains Bangor, England and Kenya were 4-, 4- and 8-fold higher against the homologous strains than against the prototype strain Yucaipa. The antiserum against strain Bangor showed 2-fold higher HI titer against strain Bangor than against strains England and Kenya. The antiserum specific for strain England showed 4-fold higher titer against strains England and Kenya than

against strain Bangor. The antiserum specific for strain Kenya showed 16- and 2- fold higher titers against homologous strain Kenya than against strains Bangor and England, respectively.

The ability of antisera to neutralize homologous and heterologous APMV-2 strains was assessed by microneutralization assay in DF1 cells. The antiserum specific for strain Yucaipa showed 4-fold higher neutralization titer against homologous strain Yucaipa and strains England and Kenya than against strain Bangor. On the contrary, antisera specific for strain Bangor showed 4-fold higher neutralization titer against homologous strain Bangor than against prototype strain Yucaipa and 2-fold higher neutralization titer against homologous strain Bangor than against strains England and Kenya. The antisera specific to strains England and Kenya showed 4-fold higher neutralization titers against their homologous strains compared to those against strains Yucaipa and Bangor, while showing 2-fold difference between either of the strains. (Table 3.2). These reactions indicated existence of a low level of antigenic differences among APMV-2 strains. Our results suggested that the strains Yucaipa, England and Kenya represented one subgroup while strain Bangor represented the second subgroup.

APMV-2 antiserum	APMV-2 strains	Cross HI titer ^a	Neutralization titer ^b
strain Yucaipa	Yucaipa	160	40
	Bangor	20	10
	England	40	40
	Kenya	40	40
strain Bangor	Yucaipa	20	10
	Bangor	80	40
	England	40	20
	Kenya	40	20
strain England	Yucaipa	40	20
	Bangor	40	20
	England	160	80
	Kenya	160	40
strain Kenya	Yucaipa	80	20
	Bangor	40	20
	England	320	40
	Kenya	640	80

Table 3.2. Antigenic analyses of APMV-2 strains Yucaipa, Bangor, England and Kenya using antisera from chickens infected with individual strains.

^a Cross HI titer is the reciprocal of the highest dilution of antisera that inhibited 4 HA units of the virus.

^b Neutralization titer was defined as the reciprocal of highest dilution of antisera that caused 50% reduction in the focus compared to the positive control wells.

3.5.3 The pathogenicity of APMV-2 strains. The pathogenicity of APMV-2 strains Yucaipa, Bangor, England and Kenya were evaluated by MDT in 9-day-old embryonated SPF chicken eggs and ICPI test in 1-day-old chicks. The MDT and ICPI values for all four APMV-2 strains were more than 168 h and zero, respectively. These results indicated that APMV-2 strains are avirulent in chickens, similar to lentogenic NDV strains.

3.5.4 Determination of the complete genome sequences of APMV-2 strains Yucaipa, Bangor, England and Kenya. We determined the complete genome sequences of APMV-2 strains Yucaipa, Bangor, England and Kenya. Initially the complete genome sequence of strain Yucaipa was determined using three strategies; using consensus primers designed from the existing sequences of other paramyxoviruses, secondly, using primers with gene-start and gene-end sequences and finally by primer walking method as described in materials and methods section. A number of the initial cDNAs in this analysis was synthesized using primers derived from the published sequence of APMV-2 strain Yucaipa (Table 1). Every nt in the complete sequence was confirmed in uncloned cDNA, indicating that it is a consensus sequence. The genome of strain England is similar in length (14904 nt) to that of strain Yucaipa. However, the genome lengths of strains Bangor (15024 nt) and Kenya (14916 nt) are slightly larger than that of strain Yucaipa (14904 nt). The nt lengths of the genome of all four strains are multiple of six. Thus all four strains conform to the rule of six, which is a characteristic of the genome of all members of subfamily *Paramyxovirinae* (Kolakofsky *et al.*, 1998). All three APMV-2 strains have the gene order of ^{3'}N-P/V/W-M-F-HN-L^{5'}.

The complete genome of strain Bangor has 70.4% nt and 75.3% aggregated aa sequence identities with those of strain Yucaipa. The strains England and Kenya are very closely related to strain Yucaipa with a nt sequence identity of 94.5% and 88%, respectively, and an aggregate aa sequence identity of 96.1% and 92.4%, respectively. However, both the strains have 69.4% and 70.8% nt and 76.15% and 76.3% aggregate aa sequence identities, respectively, with those of strain Bangor. Thus, strains Yucaipa, England and Kenya are genetically closely related whereas,

strain Bangor is somewhat distinct. This is consistent with the finding noted before that strain Bangor is distinct antigenically.

The 3' leader sequences of APMV-2 strains consist of 55 nt which is conserved among almost all the members of the subfamily *Paramyxovirinae*. The nt sequences of the leader region of strains Bangor and Yucaipa shows differences at 9 out of 55 nt positions, while those of strains England and Kenya are 100% identical to strain Yucaipa (Fig. 3.2A). The lengths of trailer regions of APMV-2 strains England and Kenya are 154 nt each, same as strain Yucaipa. But the length of trailer region of strain Bangor is 173 nt (Fig. 3.2B). The sequence of trailer region of strains England and Kenya are 100% identical to strain Yucaipa, but the sequence of strain Bangor showed only 51.3% nt identity with the other three strains.

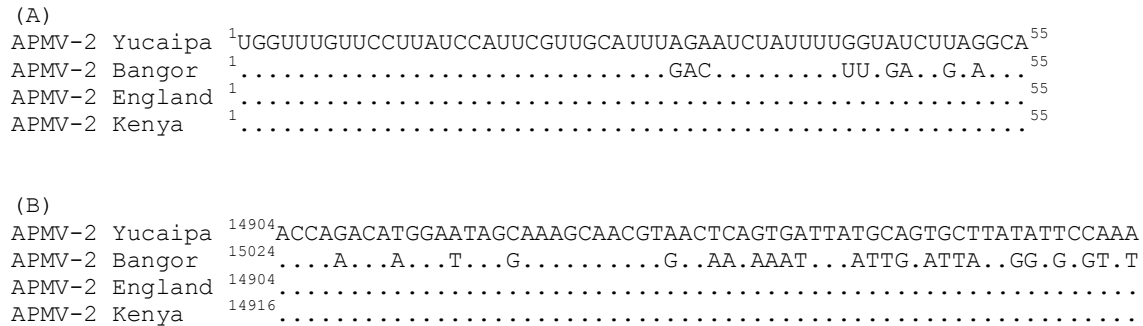


Fig. 3.2. The nucleotide (nt) sequence alignment of the leader (A) and 5' 60 nt of trailer (B) regions of the indicated APMV-2 strains. Dots indicate identity with strain Yucaipa. Sequences are in negative-sense. Numbers indicate nt position.

The proposed gene-start (GS) and gene-end (GE) signal sequences are highly conserved among APMV-2 strains (Table 3.3). In general, the conserved GS and GE sequences of all the four strains are 3'-C5GCUG(U)U(C/A) and 3'-A(U)AAUUC(G)U6, respectively. In strain

Bangor, the GS and GE sequences show single nt variations compared to the other three strains (Table 3.3).

The intergenic sequences (IGS) of APMV-2 strains vary in length from 3 to 23 nt and are mostly conserved between genes N, P, M and F (Table 3.3). The IGS sequences of strain England are 100% identical in length and sequence to strain Yucaipa. The IGS sequences of strain Kenya are also 100% identical in length and sequence to strain Yucaipa except between HN and L genes. However, the IGS sequences of strain Bangor show less than 50% nt identity with that of strain Yucaipa. In particular, the IGS between F and HN in strain Bangor is only 4 nt in length compared to 9 nt in length in the other three strains and the IGS between HN and L is 8 nt in length in strains Bangor and Kenya compared to 3 nt in length in the other two strains.

The nucleocapsid protein (N) gene. The N gene of APMV-2 strains Yucaipa, Bangor, England and Kenya is 1547 nt in length and encodes a N protein of 457 aa (Table 3.3). The N protein of strains Bangor, England and Kenya has 90.4%, 99.3% and 94.5% aa sequence identity, respectively, with that of strain Yucaipa (Table 3.4). An amino acid sequence motif that is highly conserved in the N proteins of members of subfamily *Paramyxovirinae* and known to be involved in N–N self assembly, F-X4-Y-X3-ϕ-S-ϕ-A-M-G, where X represents any amino acid residue and ϕ represents an aromatic amino acid residue (Morgan, 1991) is present within the central domain of N protein of all the four strains (³²⁴FAPANFSTLYSYAMG³³⁸).

Gene	Strain	mRNA features (nt)					Intergenic sequence (nt)	Deduced protein (aa)
		Gene-start	5' UTR	ORF	3' UTR	Gene-end		
N	Yucaipa	GGGGGCGACA	75	1374	77	TTAAGAAAAAA	7	457
	Bangor	GGGGGCGACA	75	1374	77	TTAAGAAAAAA	7	457
	England	GGGGGCGACA	75	1374	77	TTAAGAAAAAA	7	457
	Kenya	GGGGGCGACA	75	1374	77	TTAAGAAAAAA	7	457
P	Yucaipa	GGGGGCGAAG	61	1200	97	TTAACAAAAAA	7	399
	Bangor	GGGGGCGAAT	61	1200	97	<u>TTAAG</u> AAAAAA	7	399
	England	GGGGGCGAAG	61	1200	97	TTAACAAAAAA	7	399
	Kenya	GGGGGCGAAG	61	1200	97	TTAACAAAAAA	7	399
P/V	Yucaipa	GGGGGCGAAG	61	699	599	TTAACAAAAAA	-	232
	Bangor	GGGGGCGAAT	61	699	599	<u>TTAAG</u> AAAAAA	-	232
	England	GGGGGCGAAG	61	699	599	TTAACAAAAAA	-	232
	Kenya	GGGGGCGAAG	61	699	599	TTAACAAAAAA	-	232
P/W	Yucaipa	GGGGGCGAAG	61	624	675	TTAACAAAAAA	-	207
	Bangor	GGGGGCGAAT	61	462	837	<u>TTAAG</u> AAAAAA	-	153
	England	GGGGGCGAAG	61	624	675	TTAACAAAAAA	-	207
	Kenya	GGGGGCGAAG	61	624	675	TTAACAAAAAA	-	207
M	Yucaipa	GGGGGCGAAG	32	1110	117	TTAAGAAAAAA	23	369
	Bangor	GGGGGCGAAT	38	1110	135	TTTAGAAAAAA	23	369
	England	GGGGGCGAAG	32	1110	117	TTAAGAAAAAA	23	369
	Kenya	GGGGGCGAAG	32	1110	117	TTAAGAAAAAA	23	369
F	Yucaipa	GGGGGCGACA	44	1611	31	TTAAGAAAAAA	9	536
	Bangor	GGGGGCGAAA	20	1635	84	TTAAGAAAAAA	4	544
	England	GGGGGCGACA	44	1611	31	TTAAGAAAAAA	9	536
	Kenya	GGGGGCGACA	44	1611	31	TTAAGAAAAAA	9	536
HN	Yucaipa	GGGGGCGACA	66	1743	69	TTAAGAAAAAA	3	580
	Bangor	GGGGGCGAAA	60	1752	61	TTAATAAAAAA	8	583
	England	GGGGGCGACA	66	1743	69	TTAAGAAAAAA	3	580
	Kenya	GGGGGCGACA	72	1749	64	TTAATAAAAAA	8	582
L	Yucaipa	GGGGGCGAAT	11	6729	73	TTAAGAAAAAA	-	2242
	Bangor	GGGGGCGAAT	11	6729	102	TTAAGAAAAAA	-	2242
	England	GGGGGCGAAT	11	6729	73	TTAAGAAAAAA	-	2242
	Kenya	GGGGGCGAAT	11	6729	73	TTAAGAAAAAA	-	2242

Table 3.3 APMV-2 Molecular features of genes and their deduced protein products. Differences relative to the most conserved sequence are underlined.

The phosphoprotein (P) gene and P/V/W editing. The P gene of APMV-2 strains Yucaipa, Bangor, England and Kenya is 1379 nt in length and encodes a P protein of 399 aa (Table 3.3). The P protein of strains Bangor, England and Kenya has 55.8%, 87.7% and 99.5% aa sequence identity, respectively, with that of strain Yucaipa (Table 3.4). The P gene of strains Bangor, England and Kenya contain a putative P gene editing site (3'-UUUUUCCCC (negative-sense), located at nt position 2092–2100 in the viral RNA genome. The addition of a single G residue to the editing site would yield a predicted V protein and the addition of 2 G residues would yield a predicted W protein, as is the case with NDV (Steward *et al.*, 1993). The addition of a single G residue into the encoded mRNA would produce a V mRNA encoding a 232 aa V protein. For all four strains, the V protein domain contains the conserved cysteine rich motif that is characteristic of most members of subfamily *Paramyxovirinae* (Fig. 3.3). The addition of two G residues into the encoded mRNA of the P gene editing site would produce a W mRNA encoding a W protein. The W protein of strains England and Kenya is 207 aa, similar to strain Yucaipa, while that of strain Bangor is only 153 aa (Table 3.3).

APMV-2 Yucaipa	¹⁸¹ HRREYSFISRDGRLEVTSWCNPVCSP ^{IRSEPRREKCTCGT} CPESCILCRQPN ²³²
APMV-2 Bangor	¹⁸¹AC.....I.....I.T...A.....V.K..K..I.....C.SQ ²³²
APMV-2 England	¹⁸¹ ²³²
APMV-2 Kenya	¹⁸¹ ²³²

Fig. 3.3 Amino acid sequence alignment of the C-terminal domain of the V proteins of the indicated APMV-2 strains. Conserved cysteine (C) residues are underlined; dots indicate identity with strain Yucaipa. Numbers indicate the amino acid position.

The matrix protein (M) gene. The M gene of APMV-2 strains England and Kenya is 1280 nt in length similar to that of strain Yucaipa. But the M gene of strain Bangor is 1304 nt in length (Table 3.3). The increased length found in strain Bangor is due to the longer 5' and 3'

untranslated regions. The M gene of all four strains encodes a M protein of 369 aa. The M protein of strains Bangor, England and Kenya has 85.1%, 99.7% and 98.4% aa sequence identity, respectively, with that of strain Yucaipa (Table 3.4).

Strains	N			P			M		
	Bangor	England	Kenya	Bangor	England	Kenya	Bangor	England	Kenya
Yucaipa	90.4	99.3	94.5	55.8	87.7	99.5	85.1	99.7	98.4
Bangor		89.9	89.7		60.8	55.3		84.8	85.1
England			94.1			87.2			98.1
Kenya									

Strains	F			HN			L		
	Bangor	England	Kenya	Bangor	England	Kenya	Bangor	England	Kenya
Yucaipa	79.1	99.8	98.1	75	96	76.2	66.5	94.2	87.8
Bangor		78.9	77.6		75.2	85.1		67.4	68.2
England			97.9			76.4			86.1
Kenya									

Table 3.4. The amino acid percentage identity between APMV-2 strains Yucaipa, Bangor, England and Kenya.

The fusion protein (F) gene. The F gene of APMV-2 strains Yucaipa, Bangor, England and Kenya is 1707 nt in length and encodes a F protein of 536 aa (Table 3.3). The F protein of strains Bangor, England and Kenya has 79.1%, 99.8% and 98.1% aa sequence identity, respectively, with that of strain Yucaipa (Table 3.4). In APMV-1, the cleavage sequence of the F protein has been shown to be a critical factor for viral entry and pathogenesis. For APMV-2 strains England, Kenya and the prototype strain Yucaipa, the aa sequences spanning the F protein cleavage site and adjacent upstream end of the F1 subunit are identical (DKPASR↓F) and

contain dibasic aa residues. Whereas, in strain Bangor, the cleavage site contains only one basic aa residue (TLPSAR↓F). A similar difference in the number of basic amino acids at cleavage site between strains of same serotype has been reported in APMV-6 (Xiao *et al.*, 2010). However, all the APMV-2 strains contain phenylalanine residue at the F1 amino terminal end as seen in virulent APMV-1 strains (Fig. 3.4) (Lamb and Parks, 2007). All the APMV-2 strains sequenced to date contain one or two basic aa residues at the F protein cleavage site and a phenylalanine at the F1 N-terminal end but do not require exogenous protease supplementation for growth in cell cultures.

APMV-2 (Yucaipa)	⁹³ <u>D</u> K PAS <u>R</u> ↓ F ¹⁰⁰
APMV-2 (Bangor)	¹⁰¹ TLPS <u>A</u> R ↓ F ¹⁰⁸
APMV-2 (England)	⁹³ <u>D</u> K PAS <u>R</u> ↓ F ¹⁰⁰
APMV-2 (Kenya)	⁹³ <u>D</u> K PAS <u>R</u> ↓ F ¹⁰⁰
APMV-1 (Avirulent)	¹¹¹ G <u>R</u> R Q <u>R</u> ↓ L ¹¹⁷
APMV-1 (Virulent)	¹¹¹ G <u>R</u> R Q <u>KR ↓ F¹¹⁷</u>
APMV-3 (Netherland)	¹⁰¹ A <u>R</u> P R <u>GR ↓ L¹⁰⁷</u>
APMV-3 (Wisconsin)	⁹⁶ P <u>R</u> P S <u>GR ↓ L¹⁰²</u>
APMV-4	¹¹⁵ ADIQ <u>PR ↓ F¹²¹</u>
APMV-5	¹⁰⁴ G <u>K</u> R K <u>KR ↓ F¹¹⁰</u>
APMV-6 (Hong Kong)	¹¹³ PAPE <u>PR ↓ L¹¹⁹</u>
APMV-6 (IT4524-2)	¹⁰³ SI <u>R</u> E P <u>R ↓ L¹⁰⁹</u>
APMV-7	¹⁰¹ TLP <u>S</u> S R ↓ F ¹⁰⁷
APMV-8	⁹⁸ TYPQ <u>TR ↓ L¹⁰⁴</u>
APMV-9	¹⁰⁵ I <u>R</u> E G <u>RI ↓ F¹¹¹</u>

Fig. 3.4 Alignment of the F protein cleavage site sequence of APMV-2 strains with those of other APMVs. Basic amino acids (R=arginine and K=lysine) are underlined and in bold. Numbers indicate amino acid position.

The hemagglutinin-neuraminidase (HN) gene. The HN gene of APMV-2 strain England is 1899 nt long, similar to strain Yucaipa, while the length of HN gene of strains Bangor and Kenya are 1894 nt and 1906 nt, respectively. The lengths of HN protein of strains Yucaipa and England are 580 aa, while those of strains Bangor and Kenya are 583 and 582 aa, respectively (Table 3.3). The HN protein of strains Bangor, England and Kenya has 75%, 96% and 76.2% aa sequence identity, respectively, with that of strain Yucaipa (Table 3.4). In addition, all the four strains have the hexapeptide (NRKSCS) known to form part of the sialic acid binding site (Mirza *et al.*, 1994).

The large polymerase protein (L) gene. The L gene of APMV-2 strains England and Kenya is 6834 nt long same as that of strain Yucaipa. The L gene of strain Bangor is 6863 in length. The L genes of all four strains encode a L protein of 2242 aa (Table 3.3). The L protein of strains Bangor, England and Kenya has 66.5%, 94.2% and 87.8% aa sequence identity, respectively, with that of strain Yucaipa (Table 3.4). In addition, all four strains have the conserved motif GDNQ in the L protein domain III as seen in all non-segmented negative strand RNA viruses and known to be involved in L protein transcriptional activity (Schnell and Conzelmann, 1995).

3.5.5 Phylogenetic analysis. The phylogenetic tree was generated from alignments of the complete nt sequences of the genome of APMV-2 strains Bangor, England and Kenya with those of the representative paramyxoviruses (Fig. 3.5). The resulting phylogenetic tree shows APMV-2 strains clustering together on a branch that is distinct from other paramyxoviruses. Also, strains Yucaipa, England and Kenya are more closely related to each other than strain Bangor.

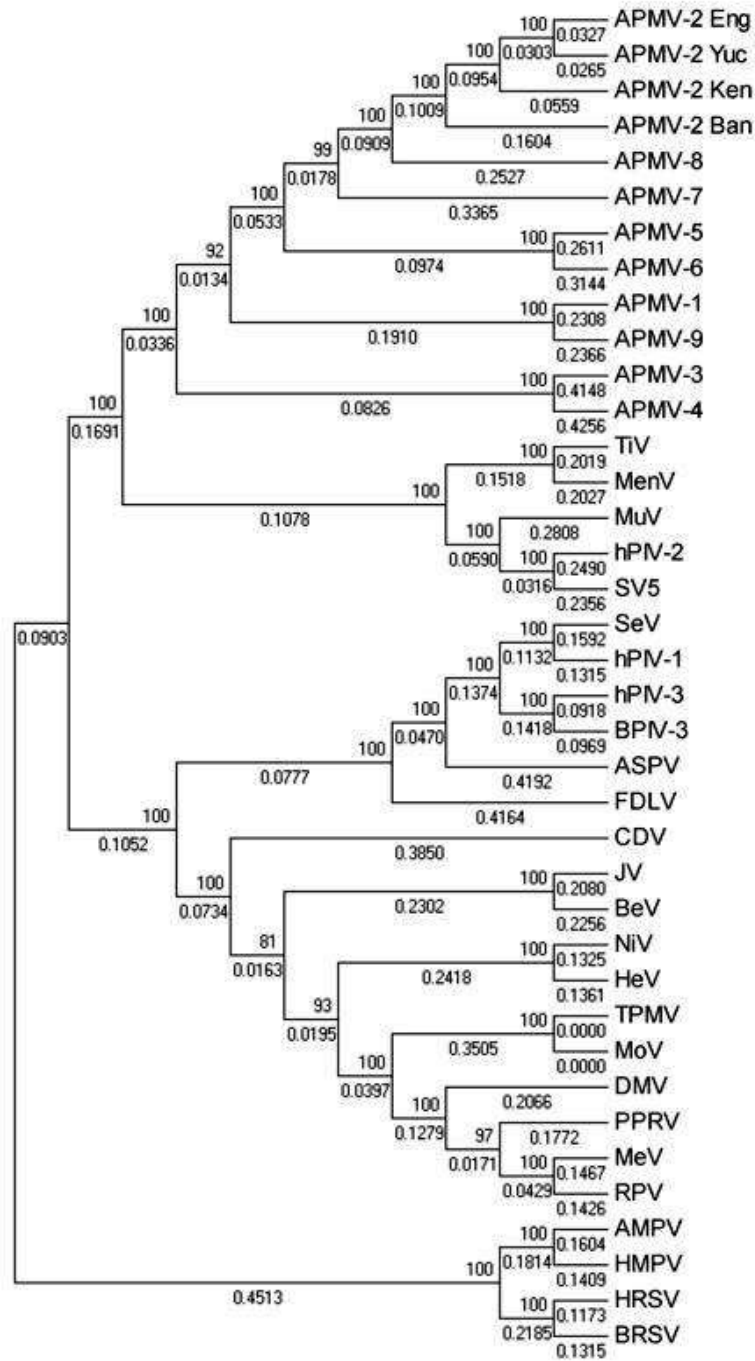


Fig. 3.5 Phylogenetic tree of representative members of the family *Paramyxoviridae*. The phylogenetic tree of representative members of the family *Paramyxoviridae* was constructed with the complete genome sequences and using MEGA 4.1, Molecular Evolutionary Genetics Analysis software. The numbers represent the bootstrap values among different viruses.

3.6 Discussion

Avian paramyxoviruses are classified into nine serotypes based on their serological relationships in HI and NI tests (Alexander, 2003). Among these serotypes, APMV-1 causes severe disease in poultry; hence, a great deal of information is available on the antigenic and genetic relationships among APMV-1 strains isolated from different parts of the world (Alexander, 1988). Recently we and others have reported complete genome sequences for representative strains of APMV-2 to -9 (Subbiah *et al.*, 2008; Kumar *et al.*, 2008; Nayak *et al.*, 2008; Samuel *et al.*, 2010; Chang *et al.*, 2001; Xiao *et al.*, 2009; Paldurai *et al.*, 2009; Samuel *et al.*, 2009). However, very little information is available about the antigenic and genetic relationships among the strains of the serotypes 2 through 9 (Alexander, 2003). In this study we have determined the antigenic and genetic relations among APMV-2 strains Yucaipa, Bangor, England and Kenya isolated from a chicken, finch, chicken and gadwell, respectively. Furthermore, these strains were isolated from different parts of the world and in different years. Therefore, it was interesting to know the antigenic and genetic variations among these strains. The antigenic relationships among these four strains were evaluated using cross-HI and cross-serum microneutralization assays. This information will have implications for studies in pathogenesis, epidemiology and for the development of vaccines against APMV-2.

To evaluate the antigenic relationship among the four APMV-2 strains described in the present study, we raised chicken antisera against strains Yucaipa, Bangor, England and Kenya individually by respiratory infection mimicking a natural route of infection. Since serological responses tend to broaden over time, and with repeated antigenic exposure, we limited the immunization to a single infection and collected serum samples at an early time point (14 dpi). Our results showed that the HI titer of the APMV-2 prototype strain Yucaipa was 8, 4, and 4 -

fold higher against homologous strain Yucaipa than against strains Bangor, England and Kenya, respectively. The antisera specific for strains Bangor, England and Kenya showed 4-, 4- and 8-fold higher against their homologous viruses than against prototype strain Yucaipa. The antiserum specific for strain Bangor showed 2-fold higher HI titer against strain Bangor than against strains England and Kenya. The antiserum specific for strain England showed 4-fold higher titer against strains England and Kenya than against strain Bangor. The antiserum specific for strain Kenya showed 16- and 2- fold higher titers against homologous strain Kenya than against strains Bangor and England, respectively. Our cross-HI results suggested that all four APMV-2 strains represent a single serotype but two antigenic subgroups.

Our results from the microneutralization tests in cell culture showed that the antisera cross-neutralized the heterologous strain, but the neutralization titers were 2- to 4-fold higher against the homologous strain than against the heterologous strains. These results again suggested an antigenic dimorphism that would be consistent with the existence of two antigenic subgroups within APMV-2, with strains Yucaipa, England and Kenya belonging to one antigenic subgroup and with strain Bangor belonging to the second antigenic subgroup, as seen with APMV-3 and -6 strains (Kumar *et al.*, 2010; Xiao *et al.*, 2010). It was previously suggested that strain Bangor be classified as a separate serotype or as a subtype of serotype 2 (McFerran *et al.*, 1974) based on distinct differences in neuraminidase activities (Alexander *et al.*, 1974) and cross serum neutralization tests between strains Bangor and Yucaipa. Our data also suggests the classification of strain Bangor as a separate subgroup within serotype 2. In this respect, it will be interesting to include additional strains to evaluate the antigenic relationships of APMV-2 strains.

The genome lengths of strains Yucaipa, Bangor, England and Kenya are 14904, 15024, 14904 and 14916 nt, respectively. Among APMV-1 (NDV) strains, there are three genome sizes: (1) 15,186 nt in early (>1930s) isolated strains, 2) 15,192 nt in late (>1960s) isolated strains (due to a six nt insertion in the upstream of the N gene), and (3) 15,198 nt (12 nt insertion in the P gene ORF) (Czeglédi *et al.*, 2006). These different genome sizes of NDV strains did not relate to the viral virulence, but seem to be related to the time (year) of virus isolation with the genomes becoming progressively longer (Miller *et al.*, 2009; Czeglédi *et al.*, 2006). However, in APMV-2, the genome length does not seem to be decided by the year of isolation but rather by the host species. The strains Yucaipa and England were both isolated from chicken and have the same genome length (14904 nt). Despite the difference in the genome length, all four strains follow the “rule of six” indicating that this rule is a requirement for virus replication and survival.

The genome sequence of strain Bangor showed 70.4% nt and 75.3% aggregated aa sequence identities with the previously reported prototype strain Yucaipa. The strains England and Kenya are closely related to strain Yucaipa, with a nt sequence identity of 94.5% and 88%, respectively, and an aggregate aa sequence identity of 96.1% and 92.4%, respectively. However, both these strains show 69.4% and 70.8% nt, respectively, and 76.1% and 76.8% aggregate aa sequence identity, respectively, with strain Bangor. The strains England and Kenya show 86.1% nt and 89.9% aggregated aa sequence identities between each other. Our results indicate that strains Yucaipa, England and Kenya are closely related genetically, but strain Bangor is somewhat distinct. This is consistent with the finding noted before that strain Bangor is distinct antigenically.

Comparison of aa sequence relatedness of cognate proteins between the APMV-2 strains showed values ranging from 55.8 to 99.8% aa identity. The P and L proteins of strain Bangor

were more divergent (55.8 and 66.5% aa identity, respectively) than other strains. The extent of variability in the APMV-2 P proteins is similar to that observed among APMV-6 strains (Xiao *et al.*, 2010) but in contrast to that of the P proteins of the two subgroups of HMPV and HRSV, which are more highly conserved (85 and 90% aa identity, respectively) (Biacchesi *et al.*, 2003). The V protein of strain Bangor showed only 57% aa identity with that of prototype strain Yucaipa, while the V protein of strains England and Kenya showed 99.1% and 100% aa identity, respectively, with that of strain Yucaipa. In addition, it is interesting to note that the W protein of strain Bangor was smaller in length, 153 aa compared to a length of 207 aa of the other three strains. Similar difference in W protein size between strains of same serotype has been reported in APMV-8 (Paldurai *et al.*, 2009). Since the role of W protein is not known, the functional significance of the W protein size difference remains to be studied. It is also interesting to find that the F and HN proteins of strain Bangor exhibited more divergence (79.1% and 75% aa identity, respectively) with those of the prototype strain Yucaipa while the F and G proteins of the HMPV subgroups show 95% and 73% aa identity, respectively, and the HRSV subgroups show 89% and 37% aa identity, respectively (Biacchesi *et al.*, 2003).

Another difference between strain Bangor and the other three strains was observed in the fusion protein cleavage site which plays a major role in NDV pathogenesis (Lamb and Parks, 2007). Virulent NDV strains have a multiple basic aa cleavage site R-X-K/R-R↓F, which is cleaved intracellularly by ubiquitous cellular furin-like proteases and a phenylalanine (F) residue at the beginning of the F1 subunit, which also may play a role in facilitating cleavage (Morrison *et al.*, 1993). The avirulent NDV strains have one or a few basic residues at the cleavage site and do not conform to the furin motif and have a leucine (L) residue at the first position of F1 subunit. Interestingly, the putative cleavage sites of other APMV serotypes showed that the

cleavage site sequences of some serotypes are not necessarily predictive of the protease activation phenotype (Samuel *et al.*, 2010). The putative F protein cleavage site (DKPASR↓F) of the strains England and Kenya resembled that of prototype strain Yucaipa and contained dibasic residues and a phenylalanine residue at the F1 terminal end, while that of strain Bangor (TLPSAR↓F) contained only one basic amino acid. However, none of the sites conform to the furin cleavage site. Each of these strains replicated in a trypsin-independent manner in both the cell lines that we tested and the addition of trypsin did not substantially increase virus replication, similar to the prototype strain Yucaipa (Subbiah *et al.*, 2008). On the basis of cleavage site, it will be difficult to predict the virulence of these strains unlike in the case of APMV-1 strains. Our results of MDT in chicken eggs and ICPI in day-old chicks provided evidence of an avirulent phenotype for each of these strains in chickens.

In conclusion, the complete genome sequences were determined for APMV-2 strains Yucaipa, Bangor, England and Kenya. Comparison of the nt and predicted protein aa sequences among four APMV-2 strains showed the existence of divergence between strains Yucaipa, England, Kenya and Bangor, suggesting the subgrouping of the APMV-2 strains as reported with APMV-3 and -6 strains. This grouping based on sequence relatedness and phylogenetic tree was also consistent with the antigenic analysis. This indicated that APMV-2 strains represent two APMV-2 subgroups and we propose that the prototype strain Yucaipa and strains England and Kenya represent one subgroup while strain Bangor represents a second subgroup. It will be interesting in future to look at the antigenic and genetic analyses of other APMV-2 strains isolated from different avian species.

Chapter 4

4.1 Title

Pathogenesis of two strains of Avian Paramyxovirus serotype 2, Yucaipa and Bangor, in chickens and turkeys.

4.2 Abstract

Nine serological types of avian paramyxovirus (APMV) have been recognized. Newcastle disease virus (NDV) belongs to APMV-1 and is the most extensively characterized virus; while relatively little information is available about the other APMV serotypes. In the present study, we examined the pathogenicity of two strains of APMV-2, Yucaipa and Bangor, in 9-day-old embryonated chicken eggs, 1-day-old Specific Pathogen Free (SPF) chicks, and 4-week-old SPF chickens and turkeys. The mean death time (MDT) in 9-day-old embryonated chicken eggs was more than 168 h for both strains and their Intra Cerebral Pathogenicity Index (ICPI) was zero, indicating that these viruses are nonpathogenic in chickens. When inoculated intracerebrally in 1-day old chicks, neither of the strains caused disease nor replicated detectably in the brain. This suggests that the zero ICPI value of APMV-2 reflects the inability of the virus to grow in neural cells. Groups of twelve 4-week-old SPF chickens and turkeys were inoculated oculonasally with either strain, and three birds per group were euthanized on days 2, 4, 6, and 14 post-inoculation for analysis. There were no overt clinical signs of illnesses, although all birds seroconverted by day 6. Virus isolation and quantification was performed from a wide variety of tissue samples as well as from oral and cloacal swabs. Tissue sections were examined by immunohistochemistry. The viruses were

isolated predominantly from the respiratory and alimentary tracts. Immunohistochemistry also showed the presence of large amount of viral antigens in epithelial linings of respiratory and alimentary tracts. There also was evidence of systemic spread even though the cleavage site of the viral fusion glycoprotein does not contain the canonical furin protease cleavage site.

4.3 Introduction

The family *Paramyxoviridae* includes a large group of viruses isolated from a wide variety of animal species. Many members of this family are well known human (measles, mumps and respiratory syncytial viruses) and animal (Newcastle disease virus [NDV], canine distemper and rinderpest viruses) pathogens, while the disease potential of other members is not known. The viruses belonging to family *Paramyxoviridae* are pleomorphic and enveloped with a non-segmented negative-sense RNA genome. This family is divided into two subfamilies, *Paramyxovirinae* and *Pneumovirinae*, based on their structure, genome organization, and sequence relatedness (Lamb *et al.*, 2005). The subfamily *Paramyxovirinae* comprises five genera- *Respirovirus*, *Rubulavirus*, *Morbillivirus*, *Henipavirus* and *Avulavirus*, while the subfamily *Pneumovirinae* contains two genera, *Pneumovirus* and *Metapneumovirus* (Mayo, 2002).

All Paramyxoviruses that have been isolated to date from avian species segregate into genus *Avulavirus*, representing the avian paramyxoviruses (APMV), and genus *Metapneumovirus*, representing the avian pneumoviruses. The APMV comprising genus *Avulavirus* have been organized into nine serotypes (APMV 1 through 9) based on hemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays (Alexander, 2003). 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. The complete genome sequences and the molecular determinants of virulence have been determined for representative NDV strains (Krishnamurthy & Samal, 1998; de Leeuw & Peeters, 1999; Panda *et al.*, 2004; Huang *et al.*, 2004; Rout & Samal, 2008). As a first step in characterizing other APMV serotypes, complete genome sequences of APMV serotypes 2 to 9 were recently determined, expanding our knowledge about these viruses (Chang *et al.*, 2001; Subbiah *et al.*, 2008; Kumar *et al.*, 2008; Nayak *et al.*, 2008; Jeon *et al.*, 2008; Samuel *et al.*, 2009; Paldurai *et al.*, 2009; Xiao *et al.*, 2009; Samuel *et al.*, 2010).

NDV (APMV-1) strains segregate into three pathotypes: highly virulent (velogenic) strains that cause severe respiratory and neurological diseases in chickens; moderately virulent (mesogenic) strains that cause milder disease, and nonpathogenic (lentogenic) strains that cause inapparent infection and can serve as live vaccines against NDV disease. NDV remains the most important poultry pathogen. In contrast, little is known about the disease potential of APMV-2 to APMV-9. APMV-2 infections have been associated with severe respiratory disease and drop in egg production in turkeys (Bankowski *et al.*, 1981; Lipkind *et al.*, 1979). APMV-3 infections have also been found to cause stunted growth in young chickens (Alexander and Collins, 1982). APMV-6 and -7 were reported to cause a mild respiratory disease in turkeys (Alexander, 1997, Shortridge *et al.*, 1980; Saif *et al.*, 1997). APMV-5 caused severe mortality in budgerigars (Nerome *et al.*, 1978). APMV-4, -8, and -9, isolated from ducks, waterfowl, and other wild birds did not produce any clinical signs of viral infection in chickens (Alexander, 1982; Gough & 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-2 appears to be circulating widely and has been isolated from a wide variety of birds, and especially from chickens and turkeys. APMV-2 infections have been reported in chickens, turkeys, racing pigeons and feral birds across the globe (Alexander, 1982; Asahara *et al.*, 1973; Collings *et al.*, 1975; Goodman *et al.*, 1988; Lang *et al.*, 1975; Lipkind *et al.*, 1979; Lipkind *et al.*, 1981; Lipkind *et al.*, 1982; Mbugua *et al.*, 1985; Nymadawa *et al.*, 1977; Shihmanter *et al.*, 1997; Weisman *et al.*, 1984). APMV-2 strains are endemic among passeriformes and psittacines in Senegal and on the island of Hiddensee, GDR, in the Baltic Sea (Fleury & Alexander, 1979, Nymadawa *et al.*, 1977). The prevalence of APMV-2 antibodies has been studied in different avian species including commercial poultry (Ley *et al.*, 2000; Zhang *et al.*, 2007; Warke *et al.*, 2008). APMV-2 strains have been isolated from chickens, broilers and layers in USA, Canada, Russia, Japan, Israel, India, Saudi Arabia, Great Britain and Costa Rica, and from turkeys in the USA, Canada, Israel, France and Italy. Serological surveys of poultry in the USA indicated that this virus was widespread (Bankowski *et al.*, 1968, Bradshaw & Jensen, 1979). APMV-2 infections affected the hatchability and poult yield in turkeys (Bankowski *et al.*, 1981). However, more serious disease has been reported during secondary infections (Lang *et al.*, 1975). Virus isolation and the presence of antibodies have also revealed APMV-2 infections in turkey flocks without causing any clinical disease (Bradshaw & Jensen, 1979). APMV-2 isolated from commercial layer farms and from broiler breeder farms in Scotland was suspected to be the cause for drop in egg production (Wood *et al.*, 2008). Experimental infection of APMV-2 in chicken by intramuscular and intratracheal route failed to produce any detectable respiratory illness (Bankowski & Corstvet, 1961). Similar results were observed in turkeys infected by intratracheal route (Bankowski *et al.*, 1968).

Currently, it is not known whether there is any variation in pathogenicity among APMV-2 strains. The purpose of this study was to evaluate the pathogenicity of APMV-2 strains Yucaipa and Bangor, both of which were completely sequenced recently (Subbiah *et al.*, 2008 and our unpublished data). APMV-2 strain Yucaipa was first isolated in 1956 from a diseased chicken (Bankowski *et al.*, 1960) and is considered the prototype strain of the APMV-2. APMV-2 strain Bangor was isolated in 1973 from a finch by routine examination during quarantine (McFerran, 1973). Initially these two viruses were considered as separate antigenic groups due to their four-fold difference in the serum cross neutralization test, but they are now grouped together as two different strains of APMV-2 (McFerran, 1974). In this study, we studied infection of APMV-2 strains Yucaipa and Bangor in 9-day-old embryonated chicken eggs, 1-day-old chicks, and 4-week-old chickens and turkeys in order to investigate their tropism and pathogenicity. The 1-day-old chicks were infected intracerebrally to evaluate the potential for neurotropism. The older 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.

4.4 Materials and Methods

4.4.1 Viruses and cells. APMV-2 strains Yucaipa (APMV-2/chicken/USA(Ca)/Yucaipa/1956) and Bangor (APMV-2/finch/N.Ireland/Bangor/1973) were obtained from National Veterinary Services Laboratory, Ames, Iowa. APMV-1 lentogenic strain LaSota and mesogenic strain Beaudette C (BC) were used for comparison purposes in pathogenicity tests and for studying virus replication in the brain of 1-day-old chicks, respectively: the former was performed in our

Bio Safety Level (BSL)-2 facility and the latter study was performed in our BSL-2+ animal facility. The viruses were grown in 9-day-old specific pathogen free (SPF) embryonated chicken eggs via allantoic route of inoculation. The allantoic fluids from infected embryonated eggs were collected 96 h post-inoculation and titer of the virus was determined by hemagglutination (HA) assay with 0.5% chicken RBC. The virus titers in the tissue samples were determined by 50% tissue culture infectivity dose (TCID₅₀) assay in DF1 cells (chicken embryo fibroblast cell line), calculated by the method of Reed and Muench (Reed and Muench, 1938).

4.4.2 Mean Death Time (MDT) in 9-day-old embryonated SPF chicken eggs. 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, 1989).

4.4.3 Intracerebral Pathogenicity Index (ICPI) in 1-day-old chicks. Briefly, 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, 1989).

4.4.4 Replication and viral growth kinetics in brain tissue of 1-day-old chicks. To compare the replication of APMV-2 strains Yucaipa and Bangor in chick brains, groups of twelve 1-day-old SPF chicks were inoculated with 0.05 ml of a 1/10 dilution of 2^8 HA units of fresh infected allantoic fluid via the intracerebral route. APMV-1 strain BC was included for comparison purposes. Brain tissue samples were collected by sacrificing three birds from each group on 1, 2, 3 and 4 days post inoculation (dpi), or when any birds died of infection. The samples were snap-frozen on dry ice and homogenized. The virus titers in the tissue samples were determined by 50% tissue culture infectivity dose (TCID₅₀) in DF1 cells (chicken embryo fibroblast cell line) by Reed and Muench method (Reed and Muench, 1938).

4.4.5 Pathogenicity assessment in chickens and turkeys. Two groups of twelve 4-week-old SPF chickens (Charles River, Maryland, USA) were housed in negative pressure isolators in our BSL-2 facility and were provided with food and water ad libitum. Birds in group one were inoculated with a total volume of 0.2 ml of 2^8 HA units of APMV-2 strain Yucaipa contained in freshly-harvested infected-egg allantoic fluid via the intranasal and intraocular routes, and the birds in group two were inoculated with the same dose of APMV-2 strain Bangor by the same routes. The inoculations were performed on separate days to avoid cross infection between the groups. Similarly two groups of twelve 4-week-old Midget White turkeys (McMurray Hatchery, Iowa, USA) were infected with the two strains of APMV-2 using the same dose and the same routes. The birds were monitored every day for clinical signs. Three birds from each group were

ethanized on 2, 4 and 6 dpi by placing them directly inside a CO₂ chamber. The birds were swabbed orally and cloacally just before euthanasia. The following tissue samples were collected on dry ice, both for immunohistochemistry (IHC) and for virus isolation: eyelid, trachea, lung, liver, spleen, brain, colon, caecal tonsil, bursa and kidney. Serum samples were also collected. On day 14, the three remaining birds from each group were euthanized and serum samples were collected. Seroconversion was evaluated by hemagglutination inhibition (HI) assay (Alexander, 1996).

4.4.6 Virus detection and quantification from tissue samples and swabs. Infectious virus was detected by inoculating homogenized tissue samples in 9-day-old embryonated SPF chicken eggs and testing for HA activity of the infected allantoic fluids 4 dpi. All HA positive samples were considered as virus-positive tissue samples. The virus titers in the HA-positive tissue samples were determined by TCID₅₀ method in DF1 cells (Reed and Muench, 1938).

The oral and cloacal swabs were collected in 1 ml of PBS containing antibiotics. The swab containing tubes were centrifuged at 1000x g for 20 min, and the supernatant was removed for virus detection. Infectious virus was detected by infecting this supernatant into 9-day-old embryonated SPF chicken eggs. Positive samples were identified by HA activity of the allantoic fluid harvested from eggs 4 dpi.

4.4.7 Immunohistochemistry. Sections of all the frozen tissue samples were prepared at Histoserve, Inc. (Maryland, USA). The sections were immunostained to detect viral nucleocapsid (N) protein 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 min at -80°C and then washed three times with 2% BSA in PBS and blocked with the same for 1 h at room temperature. The sections were then incubated with a 1:500 dilution of the primary antibody (hyperimmune sera raised against the N protein of APMV-2 strain Yucaipa in rabbit) 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 an *immunofluorescence* microscope.

4.4.8 Preparation of hyperimmune antiserum against the viral N protein in a rabbit.

APMV-2 strain Yucaipa virions were purified on a sucrose gradient and the virion proteins were separated on a 10% SDS-Polyacrylamide gel and negatively stained using E-Zinc TM reversible stain kit (Pierce, Rockford, IL, USA). The N protein band was excised from the gel and destained with Tris-glycine buffer pH 8. The excised gel band was minced in a clean pestle and mixed with elution buffer (50 mM Tris-HCl buffer pH 8, 150 mM NaCl, 0.5 mM EDTA, 5 mM DTT and 0.1% SDS) and transferred to the upper chamber of a Nanosep centrifugal device (Pall Life Sciences, Ann Arbor, MI, USA). After centrifugation two times, the eluted protein in the supernatant was quantified and 0.2 mg of protein was mixed in complete Freund's adjuvant and injected subcutaneously into a rabbit. After two weeks a booster immunization was given with 0.2 mg of protein in incomplete Freund's adjuvant and 2 weeks later the hyperimmune sera was collected. This serum was tested by western blot and was found to recognize specifically the N protein of APMV-2 strains Yucaipa and Bangor.

4.5 Results

4.5.1 The pathogenicity index tests. The pathogenicity of APMV-2 strains Yucaipa and Bangor was evaluated by MDT in 9-day-old embryonated SPF chicken eggs and ICPI in 1-day-old chicks. The lentogenic NDV strain LaSota was included in the pathogenicity test for comparison. The MDT for both of the APMV-2 strains was more than 168 h. The ICPI value was zero for both the strains. The MDT and ICPI values of NDV strain LaSota were 110 h and zero, respectively, consistent with a lentogenic virus. These results indicate that APMV-2 strains Yucaipa and Bangor are probably nonpathogenic to chickens, similar to lentogenic NDV strains.

4.5.2 Virus growth in the chick brain. The ability of the APMV-2 strains Yucaipa and Bangor 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 zero ICPI value of APMV-2 strains was due to the inability of the virus 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 a 1:10 dilution of 2^8 HA units of each virus, strains Yucaipa, Bangor and BC, into the brains of twelve 1-day-old SPF chicks. Three birds from each group were sacrificed on 1, 2, 3 and 4 dpi and virus titers in brain tissue were assayed and expressed as TCID₅₀ per gram of the brain in DF1 cells (Fig. 4.1). Neither of the two APMV-2 strains produced any clinical signs nor did they kill the chicks by 4 dpi. Neither of the two APMV-2 strains was isolated from the brain homogenate of any of the chicks on 1 to 4 dpi, indicating lack of growth in neural tissue. In comparison, the chicks that

were infected with NDV strain BC were either killed or sacrificed by 3 dpi and reached a titer of 2.5×10^5 TCID₅₀/g of brain on day 3.

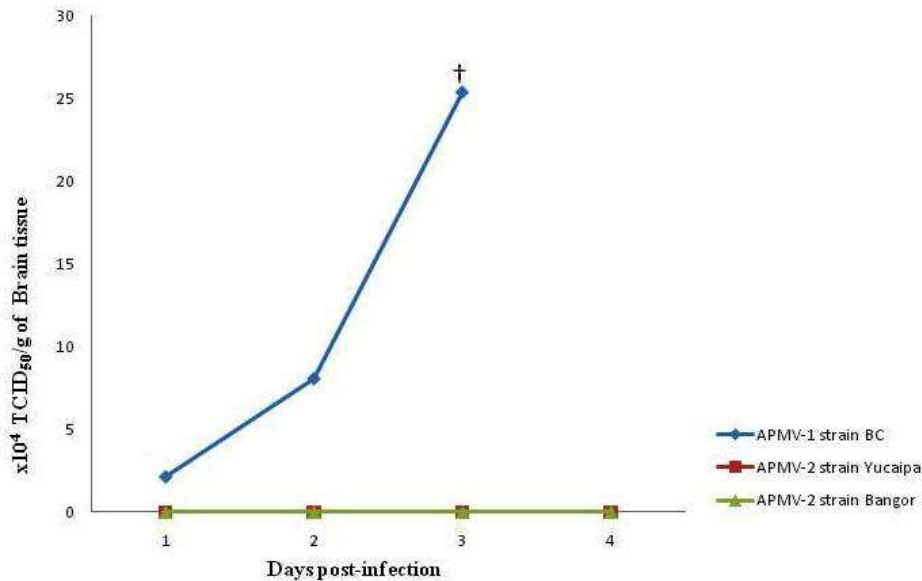


Fig. 4.1 Growth kinetics of APMV-2 strains Yucaipa and Bangor in the brain of 1-day-old chicks and comparison with that of APMV-1 strain Beaudette C.

Twelve 1-day-old chicks were inoculated with 0.05 ml of 1:10 dilution of 2^8 HA units of APMV-1 strain Beaudette C (BC), APMV-2 strains Yucaipa and Bangor via intracerebral route. Brain tissues were collected by sacrificing three birds from each group on 1, 2, 3 and 4 days post inoculation (dpi) or as and when any birds died of infection. Each time point represents the geometric mean of the individual virus titration from the brains of three birds.

† All birds infected with APMV-1 strain BC were either dead or sacrificed by 3 dpi.

4.5.3 Experimental infection of 4-week-old SPF chickens and turkeys. Groups of twelve 4-week-old chickens were inoculated by the intranasal and intraocular routes with 2^8 HA units of

either APMV-2 strain Yucaipa or Bangor. None of the chickens or turkeys displayed any overt clinical signs, and none of the birds died of disease. Further, there were no gross visceral pathological lesions in any of the birds at 2, 4, 6 and 14 dpi.

4.5.4 Virus detection in tissues and swabs. Three birds from each of the four groups were euthanized on 2, 4 and 6 dpi. The following tissue samples were collected for virus detection by inoculation in embryonated chicken eggs: eyelid, trachea, lung, liver, spleen, brain, colon, caecal tonsil, bursa and kidney. Samples that were positive for virus, as measured by HA assay of egg allantoic fluid, were analyzed for virus quantitation using the TCID₅₀ method in DF1 cells.

Table 4.1 shows the distribution and titers of the viruses in various organs on different dpi of chickens. Strain Yucaipa was isolated from eyelids, respiratory tract (trachea and lungs) and alimentary tract (colon and caecal tonsils) in chickens. Although the virus was isolated from bursa in one of the chickens on 4 dpi, the titer of retrieved virus was very low. Strain Yucaipa was not detected in the brain or heart. Strain Bangor was isolated from the same tissues, although the number of virus-positive samples was somewhat less than for strain Yucaipa. In general, the titers in virus-positive tissue samples were similar for the two viruses. In addition, strain Bangor also was detected in the brain and heart in one bird each, but the titers were very low.

In infected turkeys (Table 4.2), strain Yucaipa was isolated from the respiratory tract (trachea and lungs) and eyelids, but not from the alimentary tract. The virus titers in these organs were low compared to those from infected chickens. Strain Bangor was isolated from the respiratory tract (trachea and lungs) and the alimentary tract (caecal tonsils), and the virus titers were higher than those obtained from strain Yucaipa-infected turkeys. No virus of either strain was detectable on 6 dpi from any of the tissues harvested from the infected turkeys. For both

strains, the number of virus-positive samples from all days was considerably less for turkeys than for chickens.

The results of virus detection in oral and cloacal swabs are also presented in Tables 4.1 and 4.2. In chickens, strain Yucaipa was detected in oral swabs on day 4 and in cloacal swabs on days 4 and 6. In comparison, strain Bangor was not detected in oral swabs from chickens but was detected in cloacal swabs like strain Yucaipa on days 4 and 6. In turkeys, strain Yucaipa was not detected in oral swabs but was detected in cloacal swabs on day 4. In comparison, strain Bangor was detected in oral swabs on day 6 and in cloacal swabs on days 4 and 6. In general, strain Yucaipa was detected less frequently in swabs from turkeys than from chickens, whereas the frequency of isolation of strain Bangor between the two species was similar. Virus detection in the swabs with either strain was most frequent in cloacal swabs, and was frequently detected on day 6.

Day of infection	Virus	Chicken No.	Trachea	Lung	Brain	Eyelid	Colon	Caecal Tonsil	Bursa	Heart	Oral Swab	Cloacal Swab
2	Yucaipa	1	-	-	-	1.7X10 ⁴	-	-	-	-	-	-
		2	-	-	-	-	-	-	-	-	-	-
		3	-	2.6X10 ³	-	-	-	-	-	-	-	-
Bangor	1	-	-	-	-	-	-	-	-	-	-	-
	2	7.3X10 ³	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	5.3X10 ³	-	-	-	-	-
4	Yucaipa	1	2.9X10 ³	-	-	1.2X10 ³	4.4X10 ²	-	2.1X10 ²	-	+	-
		2	-	-	-	-	4.3X10 ³	5.8X10 ³	-	-	+	+
		3	-	3.4X10 ³	-	-	-	3.6X10 ³	9.2X10 ³	-	-	-
Bangor	1	-	-	-	-	-	-	-	-	3.5X10 ¹	-	-
	2	-	-	4.1X10 ¹	-	-	7.8X10 ³	4.5X10 ³	-	-	-	+
	3	1.5X10 ⁴	3.8X10 ³	-	-	2.0X10 ³	-	-	-	-	-	+
6	Yucaipa	1	-	-	-	-	-	-	-	-	-	+
		2	-	-	-	-	-	-	-	-	-	+
		3	-	-	-	-	-	-	-	-	-	-
Bangor	1	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	+
	3	-	-	-	-	-	1.3X10 ⁴	-	-	-	-	+

Table 4.1. Viral distribution in tissues (measured by TCID₅₀^{*}) and virus isolation from oral and cloacal swabs (by inoculation in 9-day-old embryonated chicken eggs) in 4-week old chickens inoculated oculonasally with APMV-2 strains Yucaipa and Bangor on days 2, 4 and 6 post infection.

*TCID₅₀ , 50% Tissue Culture Infective Dose

+ = Virus was isolated

- = virus was not isolated

Day of infection	Virus	Turkey No.	Trachea	Lung	Brain	Eyelid	Colon	Caecal Tonsil	Bursa	Heart	Oral Swab	Cloacal Swab	
2	Yucaipa	1	-	2.4X10 ²	-	-	-	-	-	-	-	-	
		2	-	-	-	1.5X10 ³	-	-	-	-	-	-	
		3	-	-	-	-	-	-	-	-	-	-	
	Bangor	1	-	-	-	-	-	-	-	-	-	-	-
		2	-	-	1.7X10 ³	-	-	-	-	-	-	-	-
		3	-	-	1.9X10 ⁴	-	-	-	-	-	-	-	-
4	Yucaipa	1	3.4X10 ²	-	-	-	-	-	-	-	-	-	
		2	-	-	-	-	-	-	-	-	-	-	
		3	-	3.1X10 ²	-	-	-	-	-	-	-	-	+
	Bangor	1	-	-	9.8X10 ³	-	-	-	-	-	-	-	-
		2	-	-	4.7X10 ³	-	-	-	1.2X10 ⁴	-	-	-	+
		3	6.7X10 ³	-	-	-	-	-	1.9X10 ³	-	-	-	-
6	Yucaipa	1	-	-	-	-	-	-	-	-	-	-	
		2	-	-	-	-	-	-	-	-	-	-	
		3	-	-	-	-	-	-	-	-	-	-	
	Bangor	1	-	-	-	-	-	-	-	-	-	-	-
		2	-	-	-	-	-	-	-	-	-	-	+
		3	-	-	-	-	-	-	-	-	-	+	+

Table 4.2. Viral distribution in tissues (measured by TCID₅₀^{*}) and virus isolation from oral and cloacal swabs (by inoculation in 9-day-old embryonated chicken eggs) in 4-week old turkeys inoculated oculonasally with APMV-2 strains Yucaipa and Bangor on days 2, 4 and 6 post infection.

*TCID₅₀ , 50% Tissue Culture Infective Dose

+ = Virus was isolated

- = virus was not isolated

4.5.5 Immunohistochemistry. The frozen sections of all the virus-positive tissue samples and some of the viral-negative control samples were immunostained using monospecific antibodies against N protein of APMV-2 strain Yucaipa. Large amounts of viral N antigens were detected consistently in all the tissue samples that were positive by virus isolation; no viral antigen was detected in tissue samples that were negative by virus isolation. However, no viral N antigens could be detected in the brain of a chicken infected with strain Bangor that was positive by virus isolation (Fig. 4.2).

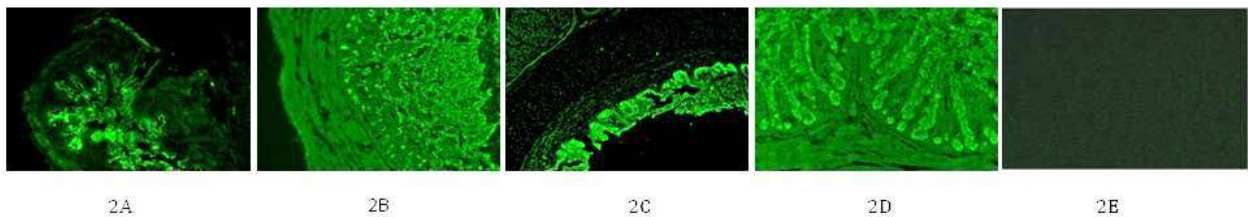


Fig. 4.2 Immunofluorescence of frozen tissues samples using monospecific antibody raised against APMV-2 nucleocapsid protein in rabbits. 2A: Lung from chicken no. 3 infected with APMV-2 strain Yucaipa, 4 dpi; 2B: Caecal tonsil from chicken no. 3 infected with APMV-2 strain Yucaipa, 4 dpi; 2C: Trachea from chicken no. 1 infected with APMV-2 strain Yucaipa, 4 dpi; 2D: Colon from chicken no. 2 infected with APMV-2 strain Yucaipa, 4 dpi; 2E: Brain from chicken no. 2 infected with APMV-2 strain Bangor, 4 dpi.

* dpi: Days post infection.

4.5.6 Seroconversion. An HI assay using chicken erythrocytes was performed with the sera collected from chickens and turkeys on 0, 2, 4, 6 and 14 dpi. The HI titers of the pre-infection chickens and turkeys were 2 or less. An HI titer of greater than 8 was considered positive. All of the inoculated chickens and turkeys seroconverted from day 6 onwards. The mean HI titers in chickens for strains Yucaipa and Bangor was 1:40 and 1:40 on day 6 and 1:2560 and 1:2560 on day 14, and in turkeys was 1:40 and 1:80 on day 6 and 1:2560 and 1:5120 on day 14, respectively.

4.6 Discussion

The APMVs are frequently isolated from a wide variety of avian species around the world. Currently, nine serological types of APMVs have been recognized, of these, the disease potential of APMV-1 (NDV) is well studied, but the disease potential of APMV-2 to APMV-9 is mostly unknown. Here, we have investigated the clinical disease and pathogenesis of APMV-2 strains Yucaipa and Bangor in chicken eggs, in 1-day-old chicks inoculated intracerebrally, and in 4-week-old chickens and turkeys inoculated via a natural route of infection. In this study, 4-week-old chickens and turkeys were chosen over the other age groups because at this age they are fully susceptible to viral infections.

The APMV-2 strains Yucaipa and Bangor were first characterized by standard pathogenicity tests (MDT and ICPI). Results of MDT test showed that both the APMV-2 strains did not kill any of the chicken embryos even after seven days of inoculation. ICPI values of both APMV-2 strains were zero, indicating an absence of morbidity and mortality. Similar ICPI value for APMV-2 strains Yucaipa and Bangor

has been reported previously (McFerran *et al.*, 1974; Shortridge and Burrows, 1997). Our MDT and ICPI values suggest that both strains are apathogenic to chickens. Since the APMV-2 strains did not kill 1-day-old chicks by intracerebral inoculation, we investigated whether the absence of neurovirulence was due to a lack of virus replication in the brain or whether replication occurred without any notable cell destruction. Our results showed that neither of the APMV-2 strains replicated detectably in the brains of the chicks. In contrast, all of the chicks that were inoculated with the mesogenic NDV strain BC died at 3 dpi, and the virus titers in the brain reached a value of 2.5×10^5 TCID₅₀/g. These results suggest that the absence of neurovirulence of APMV-2 strains was due to a lack of neurotropism rather than nonpathogenic replication.

It has been previously shown that experimental infection of 1-day-old chicks with APMV-2 strain SCWDS ID AI02-1008, via the oculonasal route resulted in mild disease and that virus was isolated from trachea, lungs and gut for 7 dpi and from pancreas up to 28 dpi (Warke *et al.*, 2008). In this study, we have evaluated the disease potential and pathogenesis of APMV-2 strains in 4-week-old SPF chickens and turkeys by the oculonasal route of infection. None of the infected birds showed any clinical signs of illness. In chickens, strain Yucaipa was isolated from tissues from both the respiratory and alimentary tracts while in turkeys the virus was isolated only from tissues from the respiratory tract and the titers of recovered virus were low. Each of the viruses was detected in oral and cloacal swabs from both chickens and turkeys, but strain Yucaipa was isolated less frequently from turkeys. Taken together, these results confirmed that strain Yucaipa replicated better in adult chickens than

turkeys. On the other hand, strain Bangor was isolated from respiratory and alimentary tracts of both chickens and turkeys confirming that the virus replicated well in both the tracts in chickens and turkeys.

Visceral gross lesions were not evident in any infected birds at 2, 4, 6 and 14 dpi. Using IHC, viral N protein was detected in the same tissues that were positive by virus isolation except in a brain tissue that was positive by virus isolation but negative by IHC. It is possible that the virus load in this infected brain tissue was too low to be detected by IHC or that the tissue was contaminated with virus during collection. In contrast, staining of the tissues that were negative by virus isolation was very weak or absent. An interesting finding was the presence of large amounts of viral antigens in epithelial cells, suggesting that these cells are highly permissive to viral replication and that extensive virus replication occurred. Thus, assays for infectious virus were considerably less sensitive than IHC in detecting virus replication in the inoculated birds. Another prominent finding of our IHC study was the presence of viral antigen only in the epithelial surfaces of these organs. There was no evidence of viral antigen in the sub epithelial portion of the tissues. This suggests that these viruses have a tropism for the superficial epithelial cells. Nonetheless, the detection of viral antigen, and in some cases infectious virus, in multiple internal organs of the birds indicates that both viruses were capable of replication in multiple tissues rather than being restricted to the 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 disease.

These results show that APMV-2 strains are capable of infecting adult chickens and turkeys using a possible natural route of infection. Serological titers demonstrated a humoral response in all of the birds inoculated with either APMV-2 strain, a further indication of successful replication. However, our results suggest that chickens are comparatively more susceptible than turkeys to APMV-2 infection.

The fusion F protein cleavage site of NDV is a well characterized determinant of NDV pathogenicity in chickens (Millar *et al.*, 1988; de Leeuw *et al.*, 2003; 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, making it possible for virulent strains 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, whereas the first amino acid of the newly-created F1 terminus is phenylalanine for virulent NDV strains, it is 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-2 strain Yucaipa (DKPASR↓F) and strain Bangor (TLPSAR↓F) have one or two basic residues (underlined), which is similar but not identical to the pattern of avirulent NDV strains. Conversely, the F1 subunit of both the APMV-2 strains begins with a phenylalanine residue, as is characteristic of virulent NDV strains, rather than a

leucine residue, as seen in most avirulent NDV strains (Collins *et al.*, 1993). APMV-2 strains Yucaipa and Bangor replicated in a wide range of cells *in vitro* without the addition of exogenous protease and the inclusion of protease did not improve the efficiency of replication. In the present study, the APMV-2 strains were detected abundantly in various internal organs, suggesting a systemic spread of the virus. These results confirm our *in vitro* findings that APMV-2 is capable of efficient intracellular cleavage in the absence of an apparent furin motif in F protein, and show that this confers the ability to spread systemically.

In conclusion, we have shown that adult SPF chickens and turkeys are susceptible to APMV-2 infection without causing overt signs of clinical disease. 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-2 has an affinity for epithelial linings of respiratory and intestinal tracts and lacks the ability to grow in neural tissues, but does spread systemically. Further studies are needed to understand the potential of the virus to affect commercial poultry.

Chapter 5:

5.1 Title

Establishment of a reverse genetics system for Avian Paramyxovirus serotype 2 (APMV-2) and rescue of recombinant APMV-2 expressing foreign protein, EGFP.

5.2 Abstract

The full-length cDNA clone of APMV-2/Yuc was constructed as six different subgenomic fragments in a transcription plasmid pBR322/dr/Yuc between the T7 RNA polymerase promoter and autocatalytic hepatitis delta virus ribozyme. The plasmid pBR322/dr/Yuc was generated by cloning a 73-nucleotide (nt) oligonucleotide 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 were constructed in pGEM7z(+) and L was cloned into pTM1 vector, all under 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 APMV-2/Yuc. The cotransfection of the support plasmids resulted in replication and transcription of the antigenomic RNA leading to generation of recombinant virus. The recombinant virus thus recovered was amplified in 9-day-old embryonated 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 APMV-2/Yuc were similar to wild-type APMV-

2/Yuc. Furthermore, two recombinant viruses, rAPMV-2/Yuc/EGFP and rAPMV-2/Yuc/_{kozak}EGFP, expressing enhanced green fluorescent protein (EGFP) as foreign protein were also recovered and characterized. The growth pattern of GFP-expressing recombinant viruses was similar to the recombinant parental virus. Both 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 APMV-2/Yuc to express a foreign gene successfully and hence could be used to develop APMV-2 as a potential vaccine vector.

5.3 Introduction

Avian Paramyxovirus serotype 2 strain Yucaipa (APMV-2/Yuc) was first isolated in 1956 at Yucaipa, California, from a diseased chicken (Bankowski *et al.*, 1960). It is associated with mild respiratory illness in chickens and turkeys, which gets exaggerated with secondary bacterial infections. APMV-2 is classified along with eight other APMV serotypes in the genus *Avulavirus* within the subfamily *Paramyxovirinae* in the family *Paramyxoviridae*. APMV-2 has a 14,904-nt long, non-segmented, negative-sense RNA genome, which encodes six genes N, P, M, F, HN and L and at least eight proteins N, P/V/W, M, F, HN and L (Subbiah *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 (reviewed in Collins and Murphy, 2002, Neumann *et al.*, 2002 and Conzelmann, 1998). The most successful reverse genetics system is a

plasmid based approach, where in, 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. The reverse genetics system can be applied for the genetic manipulation of viruses to study their molecular biology and pathogenesis and secondly, for development of vaccine vectors against important and emerging pathogens by engineering viruses to express foreign immunogens (Khattar *et al.*, 2010; Bukreyev *et al.*, 2010; Billeter *et al.*, 2009; Buchholz *et al.*, 2006).

This study describes the recovery of recombinant APMV-2/Yuc entirely from cloned cDNA using a reverse genetics system. The rescued recombinant virus was biologically similar to the wild-type APMV-2/Yuc. Furthermore, we have recovered recombinant viruses expressing enhanced green fluorescent protein (EGFP), with and without kozak sequence, to evaluate potential of APMV-2 as a vaccine vector. 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 that this system could be used to develop vaccine vectors.

5.4 Materials and Methods

5.4.1 Cells and Virus. DF-1 cells (Chicken embryo fibroblast cell line) and HEp-2 cells (Human Epidermoid carcinoma tissue from the larynx) were maintained in

Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS). APMV-2 strain Yucaipa (APMV-2/Yuc) was obtained from the National Veterinary Services Laboratory, Ames, Iowa. The wild-type as well as the recombinant viruses were propagated in the allantoic cavity of 9-day-old embryonated specific pathogen free (SPF) chicken eggs. After 72 h of infection, the allantoic fluids were harvested and titrated by hemagglutination assay (HA) using 0.5% chicken RBC at room temperature. The recombinant modified vaccinia virus strain Ankara expressing the T7 RNA polymerase (a generous gift of Bernard Moss, National Institute of Health) was grown in primary chicken embryo fibroblast cells.

5.4.2 Construction of support plasmids. For constructing the support plasmids, the cDNAs bearing the open reading frame (ORF) of nucleocapsid protein (N) and phosphoprotein (P) were cloned into expression vector pGEM7z(+) (Promega, WI, USA) under T7 promoter between Sph I and Hind III, Eco R I and Sac I, respectively. The ORF of large polymerase protein (L) was subcloned as two fragments into pTM1 vector (possessing the encephalomyocarditis virus internal ribosome entry site (IRES) downstream of the T7 RNA polymerase promoter and using the translation initiation codon contained in the Nco I site of the IRES) between the enzyme sites Nco I, Stu I and Sac I. The Sac I enzyme site was artificially created by G11468C mutation within L ORF without changing any amino acids. Briefly, RNA was isolated from the allantoic fluid of APMV-2/Yuc-infected eggs, 72 h post infection using RNeasy kit (QIAGEN, USA) according to the manufacturer's instructions. The cDNA fragments of ORFs of the N, P and L genes were generated by RT-PCR. All RT reactions were

performed with Superscript II reverse transcriptase (Invitrogen) and gene specific primers. The primers used in the RT-PCR are listed in Table 1. The N, P and L support plasmids (pN, pP and pL) were used for the recovery of the recombinant viruses.

N ORF + 5' ACATGCATGCATGTCTTCTGTGTTTTTCAGAATACCAGG 3'
 - 5' CCCAAGCTTTCACCAATCTAATGAGGCCGCATCATTG 3'

P ORF + 5' CCGGAATTCATGGAGTTCACCGATGATGCCGAAA-
 -TTGCTGAGCTG 3'
 - 5' TGACGAGCTCCTAGGCATTGTATATCTG 3'

L ORF Fragment 1: + 5' CATGCCATGGATCAAACCTCAAGCTGACA 3'
 - 5' CCCCTTGAGGAGCTCTATAGTGTCTGGAGA 3'

Fragment 2: + 5' TCTCCAGACACTATAGAGCTCCTCAAGGGG 3'
 - 5' AAAAGGCCTTTAATTGCTTGCATTTCTGAAC-
 -TTCATACAGC 3'

Table 5.1. The list of oligonucleotide primers used in the synthesis of cDNA fragments of N, P and L ORFs. The restriction enzyme sites artificially created in the primers are underlined.

5.4.3 Construction of full length plasmid. The restriction enzyme profile of the complete genome sequence of APMV-2/Yuc was analyzed by SeqBuilder software (DNASTAR Lasergene 8) to facilitate cloning the full length cDNA into a low copy plasmid pBR322/dr. Plasmid pBR322/dr was a modified form of plasmid pBR322 which contained a 72-nt oligo linker between the EcoR I and Pst I sites and hepatitis delta viral 84-nt antigenome ribozyme sequence and T7 RNA polymerase transcription termination signal between the Rsr II and Fse I sites (Krishnamurthy *et*

al., 2000). A 73-nt oligo linker with unique restriction enzyme sites was synthesized and inserted between Asc I and Rsr II sites of the pBR322/dr vector to generate pBR322/dr/Yuc for cloning the full length APMV-2/Yuc. The antigenomic cDNA of APMV-2/Yuc (14,904 nt) was divided into six fragments and sequentially cloned into pBR322/dr/Yuc plasmid between the T7 promoter and Hepatitis delta ribozyme sequence. A total of five unique restriction enzyme sites were created in the full length by mutating 10 nt without changing any amino acids (Fig. 5.1). For cloning, RNA was isolated from the allantoic fluid of APMV-2/Yuc-infected eggs at 72 h post infection, using RNeasy kit. All RT reactions were carried out using Superscript II reverse transcriptase (Invitrogen). The primers used for RT-PCR of the six fragments are listed in Table 3.2. The unique restriction enzyme sites in the full length were generated by the following 10 mutations: C2923A, G2924A, T2925A, G2926C, G4154C, G5971A, A5973T and T7870C in the untranslated regions (UTRs), A11321G and A11322C within L ORF without changing any amino acids. After ligation into the plasmid, each cDNA fragment was sequenced completely. The APMV-2/Yuc full length plasmid was called pAPMV-2/Yuc and had three non-viral G residues adjacent to the T7 promoter, at the 5' end of the antigenome, to enhance promoter efficiency (Biacchesi *et al.*, 2004a).

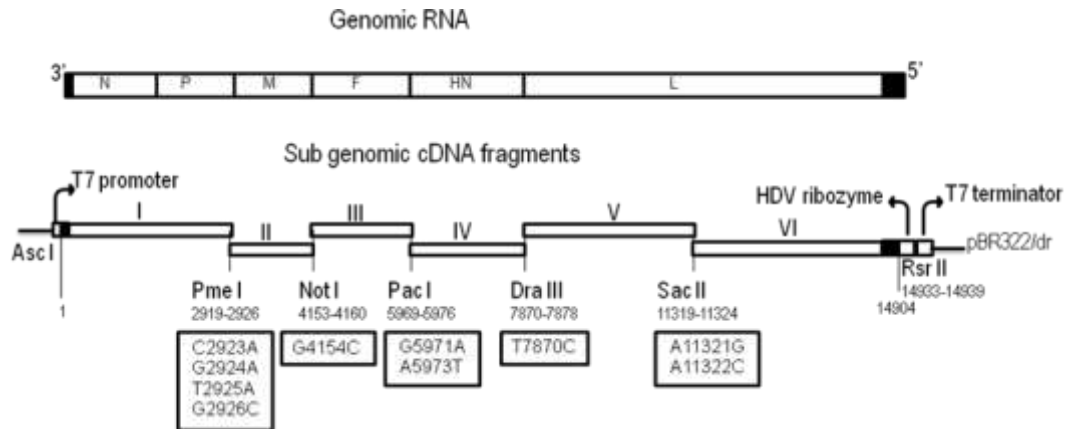


Fig. 5.1 Generation of full length cDNA clone of APMV-2/Yuc.

The full length cDNA clone was constructed by assembling six subgenomic fragments into pBR322/dr/Yuc using a 73-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 AscI and RsrII). The ten nt mutations and their positions, that were made to create the unique restriction enzyme sites in the full length, are represented inside boxes under each enzyme.

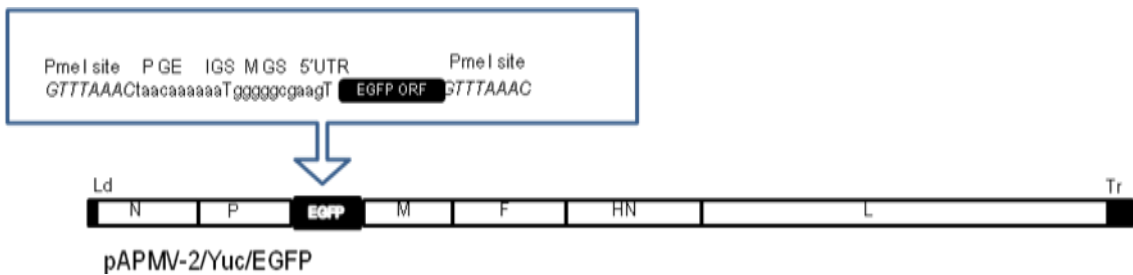
cDNA Fragment	Primers	Order of Cloning
I	+ 5' TCATTGGCGCGCCTAATACGACTCACTATAGGGACC- -AAACAAGG 3' - 5' CATGTGGGTTTAAACTGGTGATATG 3'	1
II	+ 5' TCACCAGTTTAAACCCACATGCTTCCCTGC 3' - 5' GAGGTGTGCGGCCGCACGTGTC 3'	2
III	+ 5' GACACGTGCGGCCGCACACCTC 3' - 5' GTTTAGGCTTAATTAACCTCTCTACA 3'	3
IV	+ 5' GAGAGGTTAATTAAGCCTAAACATGAT 3' - 5' GCTGTTAGACACTACGTGGCTTTTG 3'	4
V	+ 5' CAAAAGCCACGTAGTGTCTAACAGC 3' - 5' TATTTCTTCCGCGGCTCGAATG 3'	5
VI	+ 5' CATTGAGCCGCGGAAGGAAATA 3' - 5' ATGCCAGGTCCGACCGCGAGGAGGTGGAGATG- -CCATGCCGACCACCAGACATG 3'	6

Table 5.2. Oligonucleotide primers used for construction of the full-length cDNA

5.4.4 Construction of full length plasmids expressing EGFP, with and without kozak sequence. The plasmid pAPMV-2/Yuc was modified by the insertion of a transcription cassette containing the ORF for enhanced green fluorescent protein (EGFP) (Clontech, Inc.). The ORF of EGFP was flanked by the Pme I enzyme site, a 10-nt putative P gene-end (TAACAAAAA), 1-nt intergenic sequence (T), 1-nt 5'UTR (T), a 10-nt putative M gene-start (GGGGGCGAAG) upstream and by the Pme I enzyme site downstream. This fragment containing the ORF of EGFP was

cloned between P and M genes in the full length plasmid to generate the pAPMV-2/Yuc/EGFP plasmid (Fig. 5.2). Additionally, plasmid pAPMV-2/Yuc/*kozak*EGFP was constructed by inserting a 6-nt kozak sequence (GCCACC) in front of the start codon of EGFP ORF (Fig. 5.2). The length of the encoded rAPMV-2/Yuc/EGFP and rAPMV-2/Yuc/*kozak*EGFP antigenomes, excluding the non-viral sequences, were 15,654 nt and 15,660 nt, respectively.

A



B

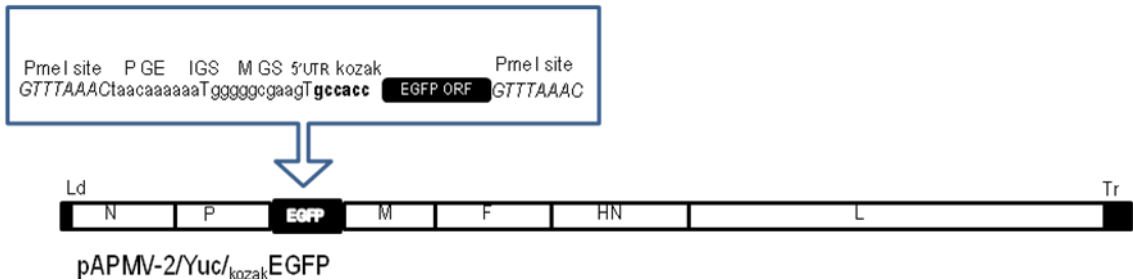


Fig. 5.2 Construction of full length plasmids expressing EGFP, with and without kozak sequence.

The top panel (A) shows the construction of full length plasmid, pAPMV-2/Yuc/EGFP and the bottom panel (B) shows the construction of pAPMV-2/Yuc/*kozak*EGFP along with their respective EGFP cassettes. The EGFP ORF was

inserted as a transcription cassette at the Pme I site (at the putative P gene 5' UTR). This cassette contained the EGFP ORF flanked by a T residue as the 5'UTR, M gene-start (M GS), followed by a T residue as the intergenic sequence (IGS), P gene-end (P GE) and Pme I enzyme site. The EGFP ORF was flanked at the downstream end by another Pme I enzyme site. In the pAPMV-2/Yuc/_{kozak}EGFP, the kozak sequence (GCCACC) was inserted before EGFP ORF.

5.4.5 Transfection and recovery. The recombinant viruses were recovered from the full length plasmids as described previously (Krishnamurthy *et al.*, 2000). Briefly, in a six well plate, HEp-2 cells (80-90% confluent) were infected with MVA-T7 at a one focus forming unit per cell and then transfected with pNP (3 µg), pP (2 µg), pL (1 µg) and pAPMV-2/Yuc (3-5 µg) or the full length plasmids containing EGFP gene. Lipofectamine (Invitrogen, USA) was used for transfection according to the manufacturer's protocol. After 6 h of transfection, the supernatant was discarded and fresh DMEM containing 0% FBS was added. The supernatant was collected after 48 h and passaged in 9-day-old embryonated SPF chicken eggs to remove residual vaccinia virus. The allantoic fluid was harvested at 3 dpi and tested for HA activity (Fig. 3). The recovered viruses were passaged five times in 9-day-old embryonated SPF chicken eggs and RT-PCR and sequencing confirmed the recombinant viruses. The virus stocks were aliquoted and stored at -70 °C until future use.

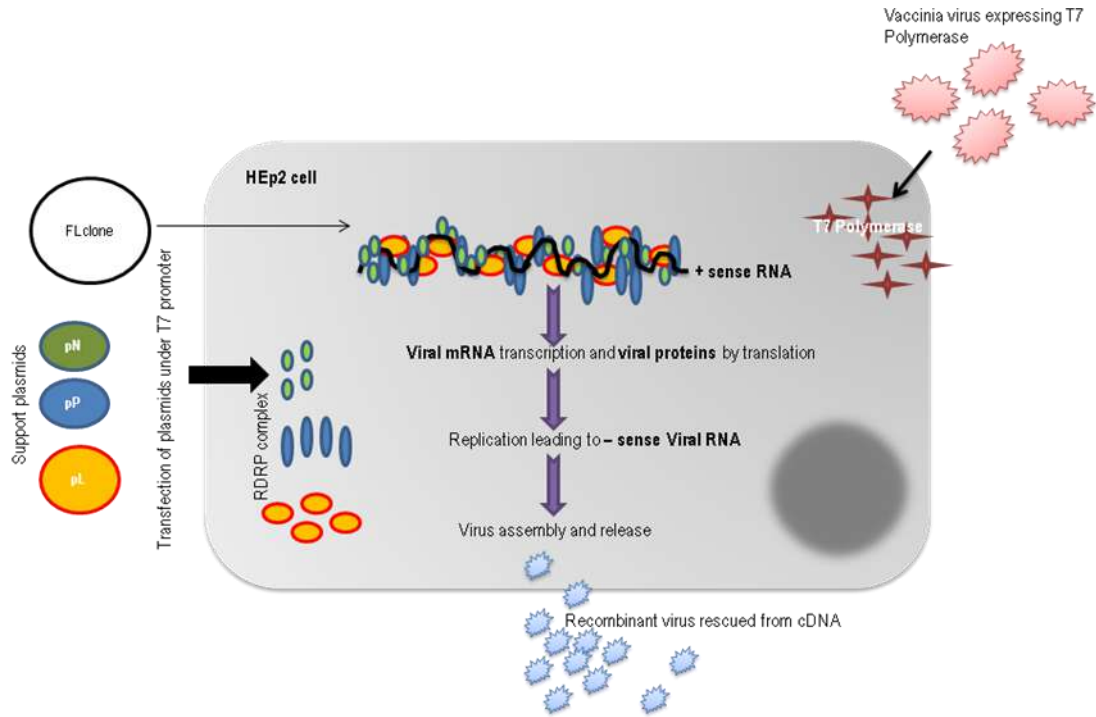


Fig. 5.3 A Plasmid based system for recovery of recombinant APMV-2/Yuc from cDNA.

HEp-2 cells were first infected with recombinant vaccinia virus expressing T7 polymerase and cotransfected with antigenome full length cDNA plasmid pAPMV-2/Yuc 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.

5.4.6 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, P-2629 (5'-CTCCTGAGGTCACAGAAGGAGG-3') and M gene-specific reverse primer, M-3285 (5' CCTGCAGTGACCACTTCTGGCTTTG-3'). The RT-PCR product was

digested using Pme I enzyme and sequenced to confirm the Pme I site. The same primers were used to amplify the GFP gene in the recombinant viruses and DNA sequencing confirmed the presence of the restriction enzyme site, the GFP ORF and the kozak sequence. RNA isolated from wt APMV-2/Yuc 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.

5.4.7 Immuno staining of infected cells. The recombinant viruses were grown in DF1 cells and overlaid with 0.8% methyl cellulose (Sigma) in DMEM without FBS. The infected cells were incubated in 37⁰C incubator. After three days of infection, the overlay was removed and the cells were fixed with methanol at room temperature for 30 min. The cells were then washed and incubated with polyclonal antisera raised against wt APMV-2/Yuc in chickens at 1:500 dilutions for 1 h followed by incubation for 45 min with goat anti-chicken IgG conjugated with horseradish peroxidase (KPL, MD, USA). The virus infected cells were detected under light microscope after staining with DAB substrate (Vector Labs, USA).

5.4.8 Growth kinetics of recombinant viruses and wild-type virus. Briefly, the DF1 cells were grown in six-well plates as monolayer (80% confluency) and infected in triplicates with the following viruses (MOI of 1); wt APMV-2/Yuc, rAPMV-2/Yuc, rAPMV-2/Yuc/EGFP and rAPMV-2/Yuc/_{kozak}EGFP. The supernatants were collected at 24, 48, 72, 96, and 120 h post-infection (p.i). Virus titers in the supernatants were determined by serial end-point dilution in 96-well plates seeded

with DF1 cells. The infected cells were stained by immunoperoxidase staining using polyclonal antibody raised against wt APMV-2/Yuc in chickens. Virus titers ($TCID_{50}/ml$) were calculated using Reed & Muench method (Reed & Muench, 1938).

5.4.9 Pathogenicity tests. The virulence of the recombinant viruses was compared with the wt APMV-2/Yuc by the internationally accepted standard pathogenicity tests: mean death time (MDT) in 9-day-old embryonated SPF chicken eggs and intracerebral pathogenicity index (ICPI) in 1-day-old SPF chicks (Alexander 1989).

Briefly, for MDT, a series of 10-fold (10^{-6} - 10^{-9}) dilutions of fresh infective allantoic fluid in PBS was made and 0.1 ml of each diluent was inoculated into the allantoic cavities of five 9-day-old SPF embryonated chicken eggs (BEE eggs company, PA) and the eggs were incubated at 37°C. The eggs were candled 3 times a day for the next 7 days, and the time of embryo death if any were recorded. The minimum lethal dose (MLD) is the highest virus dilution that kills all the embryos. The MDT is the mean time in hours for the MLD to kill all the inoculated embryos.

For ICPI, 0.05 ml (1:10 dilution) of fresh infective allantoic fluid of each virus was inoculated into groups of ten 1-day-old SPF chicks via the intracerebral route. The inoculation was done using a 27-gauge needle attached to a 1 ml stepper syringe dispenser that was set to dispense 0.05 ml of inoculum per bird. The birds were inoculated by inserting the needle up to the hub into the right or left rear quadrant of the cranium. The birds were observed for clinical symptoms and mortality, once every 8 h for a period of 10 days. At each observation, the birds were scored: 0 if normal, 1 if sick and 2 if dead. ICPI is the mean score per bird per observation over

the 10-day period. Highly virulent (velogenic) viruses give values approaching 2 and avirulent (lentogenic) viruses give values close to 0.

5.5 Results

5.5.1 Construction of support plasmids expressing N, P and L proteins. The support plasmids, pN and pP were generated by inserting the cDNA bearing the ORF of N, P into expression vector pGEM7z(+) between Sph I and Hind III, Eco R I and Sac I, respectively, while pL was obtained by cloning the L ORF as two fragments into pTM1 vector using the enzyme sites Nco I, Stu I and Sac I. The Sac I enzyme site was artificially created by G11468C mutation within L ORF without changing any amino acids. The support plasmids were confirmed by digesting with corresponding restriction enzymes and DNA sequencing of the complete ORF, prior to using them in the recovery of the recombinant viruses (Fig. 5.4).

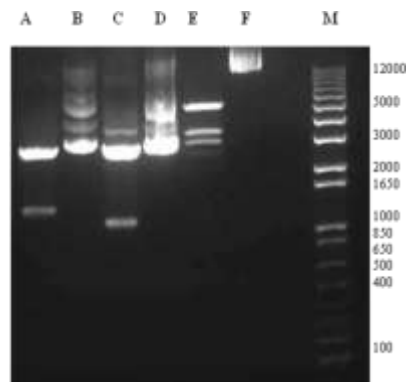


Fig. 5.4 Confirmation of the presence of ORFs inserted in the support plasmids, pN, pP and pL.

The plasmids, pN (B), pP (D) and pL (F) were digested using the restriction enzymes, Sph I and Hind III for N ORF(A), Eco R I and Sac I for P ORF(C) and Nco I, Sac I and Stu I for L ORF (E). Lane M is 1kb plus DNA marker.

5.5.2 Construction of the full length cDNA clone of APMV-2/Yuc. In order to construct the full length cDNA of APMV-2/Yuc, pBR322/dr/Yuc, the whole APMV-2 genome was divided into six fragments and they were sequentially cloned. Each fragment represented one gene except the first fragment that included both N and P genes and fragments 5 and 6 together constituted the large L gene. A 73-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 ten nucleotide mutations, C2923A, G2924A, T2925A, G2926C, G4154C, G5971A, A5973T, T7870C, A11321G and A11322C which were artificially created to generate unique restriction enzyme sites and served as the genetic markers in recombinant viruses.

5.5.3 Construction of full length plasmids encoding EGFP with and without kozak sequence. The full length plasmid encoding the EGFP, pAPMV-2/Yuc/EGFP, was constructed by inserting the EGFP transcription cassette at Pme I 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, additionally, pAPMV-2/Yuc/_{kozak}EGFP, had a 6-nt kozak

sequence in front of the EGFP ORF. The kozak sequence was introduced to determine whether the sequence can enhance the levels of GFP expression. The plasmids were sequenced to confirm the insertion of foreign cassette at the Pme I site.

5.5.4 Recovery of infectious recombinant viruses. The transfection of full length cDNA plasmids pAPMV-2/Yuc, pAPMV-2/Yuc/EGFP and pAPMV-2/Yuc/_{kozak}EGFP along with support plasmids pN, pP and pL in HEp-2 cells infected with MVA-T7, yielded infectious recombinant viruses two days after transfection. The recovered viruses were passaged in 9-day-old embryonated SPF chicken eggs to amplify the recombinant viruses (rAPMV-2/Yuc, rAPMV-2/Yuc/EGFP and rAPMV-2/Yuc/_{kozak}EGFP). RT-PCR of the infective allantoic fluid and DNA sequencing confirmed the presence of genetic markers and the GFP.

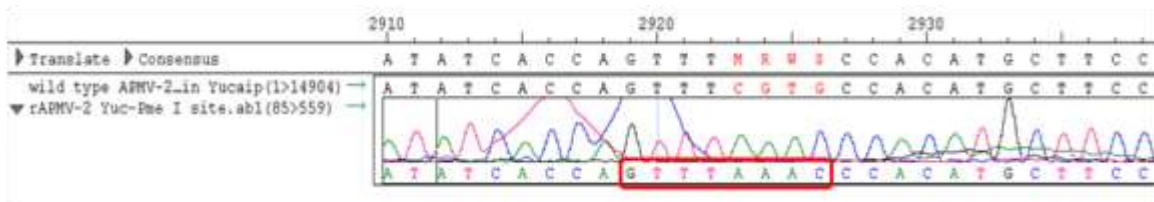


Fig. 5.5 Identification of genetic marker in the recombinant virus. RT-PCR of the region containing the Pme I site was done using primers flanking the Pme I restriction enzyme site, between P and M genes, and the DNA was sequenced to confirm the presence of the mutations, C2923A, G2924A, T2925A and G2926C, that served as the genetic markers. The wt APMV-2/Yuc was used as the control.

5.5.5 *In vitro* characterization of recombinant viruses. The morphological characteristics of recombinant viruses were similar to wild-type virus in DF1 and Vero cells (refer to Chapter 3). None of the recombinants produced plaques but caused single cell infections comparable to wild-type APMV-2 and the maximum CPE was observed on 4 dpi (Fig. 5.6).

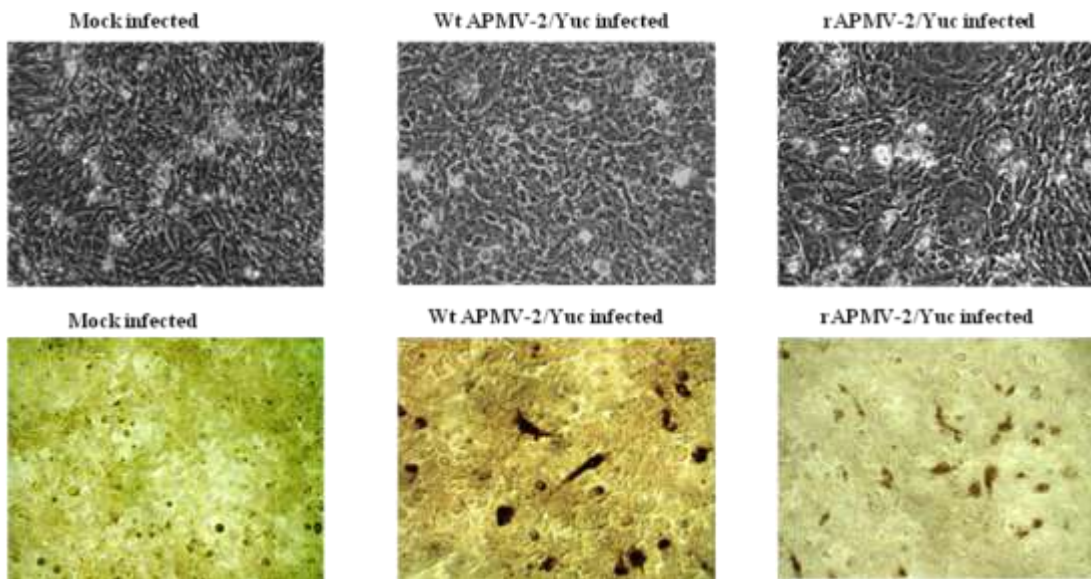


Fig. 5.6 The Cytopathic effects of wild type APMV-2/Yuc and rAPMV-2/Yuc in DF1 cells. The DF1 cells were infected with wild type APMV-2/Yuc and rAPMV-2/Yuc at an MOI of 1 or mock infected with PBS and observed for the cytopathic effects on 3 day post infection (dpi). The lower panel shows immunoperoxidase staining of infected cells on 3 dpi using polyclonal sera against APMV-2/Yuc raised in chickens. The rAPMV-2/Yuc caused single cell infection similar to wild type APMV-2/Yuc.

The GFP expression by the recovered viruses was confirmed by infecting DF1 cells with rAPMV-2/Yuc/EGFP and rAPMV-2/Yuc/_{kozak}EGFP (Fig. 5.7). Both the viruses expressed GFP and caused single cell infections as seen in wild type APMV-2/Yuc.

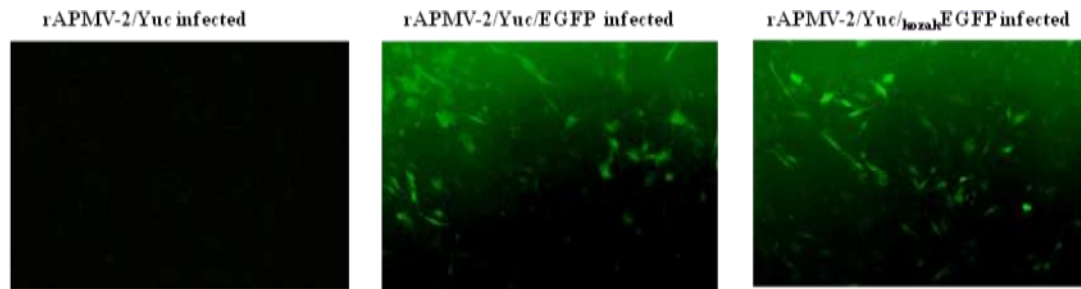


Fig. 5.7 Expression of enhanced GFP by rAPMV-2/Yuc/EGFP and rAPMV-2/Yuc/_{kozak}EGFP. DF1 cells were infected with rAPMV-2/Yuc, rAPMV-2/Yuc/EGFP and rAPMV-2/Yuc/_{kozak}EGFP viruses at an MOI of 1. The infected DF1 cells were observed each day for the expression of GFP under fluorescence microscope. The rAPMV-2/Yuc/EGFP and rAPMV-2/Yuc/_{kozak}EGFP viruses, both stably expressed GFP for at least five serial passages in DF1 cells. The scattered green fluorescent cells were typical of the single cell infection phenotype seen with wt APMV-2/Yuc infection.

The recovered viruses, rAPMV-2/Yuc, rAPMV-2/EGFP, rAPMV-2/_{kozak}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 1 (Fig. 5.8). The kinetics and the magnitude of replication of the recombinant viruses were similar to those of the wild type virus. The virus titers of the recombinant viruses

expressing GFP was 1.5 log lower than those of the wild-type virus, suggesting that the insertion of foreign gene resulted in virus attenuation. The virus titer of wt APMV-2/Yuc, rAPMV-2/Yuc, rAPMV-2/EGFP and rAPMV-2/_{kozak}EGFP were $10^{5.25}$, $10^{4.75}$, $10^{3.25}$ and 10^3 TCID₅₀/ml, respectively, on 4 dpi

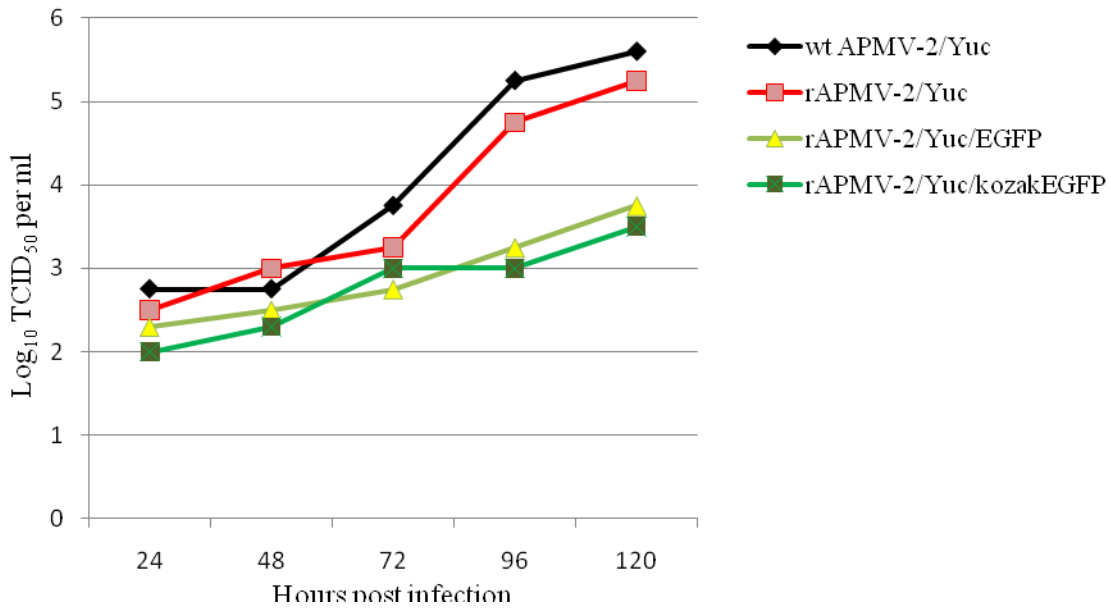


Fig. 5.8 Comparison of growth kinetics of wild type APMV-2/Yuc and rAPMV-2/Yuc, rAPMV-2/Yuc/EGFP and rAPMV-2/Yuc/_{kozak}EGFP.

Briefly, DF1 cells in six-well plates were infected in triplicates with wild type APMV-2/Yuc and the recombinant viruses, rAPMV-2/Yuc, rAPMV-2/Yuc/EGFP and rAPMV-2/Yuc/_{kozak}EGFP, at an MOI of 1 and samples were collected from the culture supernatant at 24 h interval until 120 h post-infection. Virus titers of the samples were determined by serial end-point dilution in 96-well plates seeded with DF1 cells and immunoperoxidase staining using polyclonal antibody against wild

type APMV-2/Yuc, raised in chickens. Virus titres (TCID₅₀/ml) were calculated using Reed & Muench method (Reed & Muench, 1938).

5.5.6 Pathogenicity tests. The virulence of the recombinant viruses were compared with wt APMV-2/Yuc by two internationally accepted tests namely; MDT in 9-day-old embryonated SPF chicken eggs and ICPI in 1-day-old SPF chicks. The recombinant viruses did not kill the chicken embryos even after 7 dpi and had ICPI value of zero, suggesting that the recovered recombinant viruses were avirulent, similar to the wild type APMV-2/Yuc.

5.6 Discussion

This study describes the recovery of infectious recombinant APMV-2 strain Yucaipa from the cloned cDNA by reverse genetics system for the first time. The availability of the complete genome sequence of APMV-2/Yuc 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 system has been used to recover other viruses (rabies virus, Schnell *et al.*, 1994; vesicular stomatitis virus, Lawson *et al.*, 1995; human respiratory syncytial virus, Collins *et al.*, 1995; measles virus, Radecke *et al.*, 1995; Sendai virus, Garcin *et al.*, 1995; SV5, He *et al.*, 1997; rinderpest virus, Baron and Barrett, 1997; parainfluenza virus, Hoffman and Banerjee, 1997; bovine respiratory syncytial virus, Yunus *et al.*,

2001; Newcastle disease virus, Peeters *et al.*, 1999 and Krishnamurthy *et al.*, 2000; AMPV-A, Naylor *et al.*, 2004, AMPV-C, Govindarajan *et al.*, 2006). The growth characteristics of the recombinant virus, rAPMV-2/Yuc generated in this study was similar to that of the wild-type virus. The rAPMV-2/Yuc produced single cell infections in DF1 and Vero cells and was antigenically similar to wild type APMV-2/Yuc, as observed by immunoperoxidase staining of the infected cells. These results indicate the possibility of recovering a wild-type-virus-like recombinant virus entirely from cloned cDNA.

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 (Sakai *et al.*, 1999; Haglund *et al.*, 2000 and Huang *et al.*, 2001 and Biacchesi *et al.*, 2004), stable expression of the inserted foreign genes even after many passages *in vitro* (Bukreyev *et al.*, 1996, Mebatsion *et al.*, 1996; He *et al.*, 1997 and Biacchesi *et al.*, 2004a) and finally, the absence of homologous RNA recombination makes them safe and stable vectors (Lamb and Kolakofsky, 1996 and Palese *et al.*, 1996).

Using the established reverse genetics system, rAPMV-2 expressing foreign protein, enhanced GFP, was recovered. Two full length cDNA constructs were made, one with EGFP transcript cassette alone between P and M gene while the other also had kozak sequence in front of EGFP ORF. 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 gene 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 further behind, also previously it has been shown that the expression of foreign genes are better when placed near the 3' end (Sakai *et al.*, 1999; Wertz *et al.*, 1998). The reason behind using kozak sequence in one of the construct was to see if it improved the GFP expression, as kozak sequence is known to optimize protein translation after mRNA synthesis (Kozak, 1987; Kozak, 1990). The recovered viruses were similar to the parental virus in their growth characteristics but they were 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 and Biacchesi *et al.*, 2004a). There was not much difference in the GFP expression between rAPMV-2/Yuc/EGFP and rAPMV-2/Yuc/_{kozak}EGFP suggesting that the inserted kozak sequence did not affect the expression level of GFP. Both the recombinant viruses stably expressed the foreign protein 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-2 and the recovered recombinant virus showed similar morphological and *in vitro* growth characteristics and pathogenicity to the wild type virus. The reverse genetics system can be used as a tool to understand the APMV-2 molecular biology and pathogenesis. Furthermore, the ability to engineer recombinant APMV-2/Yuc expressing a foreign gene has been demonstrated using enhanced GFP, which has implications in the development of vectored vaccine against emerging pathogens.

Chapter 6

6.1 Title

Effect of fusion protein cleavage site mutations on virulence and pathogenicity of avian paramyxovirus serotype 2

6.2 Abstract

Avian Paramyxovirus (APMV) serotype 2 is one among the nine serotypes of APMV that infect a wide variety of avian species around the world. APMV-2 strains cause mild respiratory illness in chickens and turkeys. To elucidate the molecular basis of the low pathogenicity of these strains we have constructed a reverse genetics system for recovery of infectious recombinant APMV-2 strain Yuc (APMV-2/Yuc) from cloned cDNAs. The rescued virus (rAPMV-2) resembled the parental wild type virus in growth properties *in vitro* and in pathogenicity *in vivo*. We have used the reverse genetics to analyze the role of the putative cleavage site of fusion (F) protein on pathogenesis and virulence of APMV-2. The F protein of APMV-2 is a type I viral glycoprotein and shares features common to other paramyxovirus F proteins. The F protein cleavage site of APMV-2 has two basic amino acid residues and a phenylalanine at the beginning of F1 subunit. APMV-2 does not require exogenous protease supplementation for growth in cell culture and causes single-cell infections without forming syncytia. A total of twelve APMV-2 mutants with F protein cleavage site sequences resembling APMV-2 serotypes 1 to 9 were generated. Our results showed that all the mutants were viable and the mutations were maintained after virus

propagations in embryonated chicken eggs. Any change in the amino acid sequence at the F protein cleavage site resulted in syncytia formation and a slight increase in the virus replication in cell culture, suggesting proteolytic processing of the F protein. However, pathogenicity tests in 9-day-old embryonated chicken eggs and in 1-day-old chicks showed that the mutants were completely non-virulent like the parental and wild type virus. Experimental infection of 2-week-old chickens showed that the mutants were not different from the parental virus in terms of tissue tropism and pathogenesis. These results demonstrated that genetically modified APMV-2 can be recovered from cloned cDNA and suggested that cleavage of the F protein is not a determinant of pathogenicity of APMV-2.

6.3 Introduction

Paramyxoviruses are pleomorphic, enveloped viruses containing a non-segmented, negative-sense, RNA genome. These viruses have been isolated from a great variety of mammalian and avian species around the world (Lamb *et al.*, 2005). All paramyxoviruses that have been isolated to date from avian species segregate into genus *Avulavirus*, representing avian paramyxoviruses (APMV) and genus *Metapneumovirus*, representing the avian metapneumoviruses in the family *Paramyxoviridae*. APMVs have been divided into nine different serotypes (APMV 1 through 9) based on hemagglutination-inhibition (HI) and neuraminidase-inhibition (NI) tests (Alexander, 2003). APMV-1 comprises of all strains of Newcastle disease virus (NDV) and is the most completely characterized serotype due to the severity of disease caused by virulent NDV strains in chickens. The complete genome sequence

and reverse genetics system are available for representative NDV strains: lentogenic strain LaSota (Huang *et al.*, 2001; Romer Oberdorfer *et al.*, 1999 ; Peeters *et al.*, 1999), B1 (Nakaya *et al.*, 2001), mesogenic strain Beaudette C (Krishnamurthy *et al.*, 2000) and velogenic strain Hert/33 (de Leeuw *et al.*, 2005). As a first step in characterizing the other APMV serotypes, complete genome sequences of APMV serotypes 2 to 9 were recently determined, expanding our knowledge about these viruses (Chang *et al.*, 2001; Subbiah *et al.*, 2008; Kumar *et al.*, 2008; Nayak *et al.*, 2008; Jeon *et al.*, 2008; Samuel *et al.*, 2009; Paldurai *et al.*, 2009; Xiao *et al.*, 2009; Samuel *et al.*, 2010).

APMV-2 was first isolated from a diseased chicken in 1956 in Yucaipa, California (Bankowski *et al.*, 1960). Since then many APMV-2 strains have been isolated from domestic poultry, free range, captive and wild birds around the world (Alexander & Senne, 2008). APMV-2 infections have been reported in chickens in United States, Canada, Russia, Japan, Israel, India, Saudi Arabia, Costa Rica and from turkeys in United States, Canada, Israel, France and Italy (Alexander 2000). The infection is more prevalent in turkeys than chickens (Bankowski *et al.*, 1968). APMV-2 infection was shown to affect hatchability and poult yield in turkeys (Bankowski *et al.*, 1981). APMV-2 was found to cause drop in egg production in commercial layer and broiler breeder farms in Scotland (Wood *et al.*, 2008). APMV-2 viruses also have been frequently isolated from passerine and psittacine birds. Surveillance of wild birds has indicated that APMV-2 viruses are more frequent in passerines (Alexander, 1986; Senne *et al.*, 1983).

The genome of APMV-2 strain Yucaipa is 14,904 nucleotides (nt) in length and contains a leader sequence at 3' end of 55 nt length and a trailer sequence at 5' end of 154 nt length. The genome consists of six genes that encode nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN) and large protein (L). The genes are flanked on either side by highly conserved transcription start and stop sequences and have intergenic sequences of varying length. In common with other paramyxoviruses, the P gene contains a putative editing site for the production of V and W proteins (Subbiah *et al.*, 2008).

The F protein of paramyxovirus is synthesized as an inactive precursor (F₀) and is cleaved to two biologically active disulfide bonded F₁-F₂ subunits by host proteases (Lamb and Parks, 2007). The cleavage of F protein is necessary for virus entry and cell to cell fusion. The F protein cleavage site is a well-characterized determinant of NDV pathogenicity in chickens. 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, making it possible for virulent strains 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. This limits the replication of avirulent strains to the respiratory and enteric tracts where the secretory protease is found. The putative cleavage site of strain Yucaipa F protein (⁹³DKPASR↓F¹⁰⁰) has two basic residues (underlined), which is similar but not

identical to the pattern of avirulent NDV strains. Conversely, the F₁ subunit of strain Yucaipa begins with a phenylalanine residue, as is characteristic of virulent NDV strains, rather than a leucine residue, as seen in most avirulent NDV strains (Collins *et al.*, 1993). We found that strain Yucaipa replicated in a wide range of cells without the addition of exogenous protease, and the inclusion of protease did not improve the efficiency of replication (Subbiah *et al.*, 2008). Interestingly, APMV-2 produces single cell infections and does not cause syncytia, a hallmark of paramyxovirus cytopathic effect (CPE). This is incongruent with the observation that the F protein cleavage site is not polybasic and does not conform to the preferred furin motif. Thus, strain Yucaipa is an example of paramyxovirus in which efficient intracellular cleavage occurs in the absence of an apparent furin motif.

To study the effect of mutations at the F protein cleavage site on virulence and pathogenesis, we have developed a reverse genetics system for APMV-2 strain Yucaipa. Infectious APMV-2 was produced for the first time by coexpression of a complete cDNA-encoded antigenomic RNA in the presence of the N, P and L proteins. The full-length APMV-2 cDNA clone was used to generate twelve APMV-2 mutants whose F protein cleavage site resembled those of APMV-1 through -9. Here, we show that all F protein cleavage site mutants were viable and that the mutations were maintained after virus propagation in embryonated chicken eggs. The virulence and pathogenicity of the mutant viruses were evaluated by mean death time in 9-day-old embryonated chicken eggs, by intracerebral pathogenicity test in 1-day-old chicks and by natural route of infection of 2-week-old chickens. Our results showed that the mutations at F protein cleavage site changed the growth characteristics of the viruses

in cell culture, but did not change the virulence and pathogenicity of the viruses in chickens. These results suggest that the cleavability of F protein is not a determinant for virulence of APMV-2.

6.4 Materials and Methods

6.4.1 Virus and Cell lines. APMV-2 strain Yucaipa (APMV-2/Yuc) was obtained from the National Veterinary Services Laboratory, Ames, Iowa. The virus was grown in allantoic cavity of 9-day-old embryonated, specific pathogen-free (SPF) chicken eggs. The allantoic fluid was collected on 3 day post inoculation (dpi) and hemagglutination (HA) titers were determined using 0.5% chicken RBC at room temperature. DF1 (Chicken embryo fibroblast cell line), HEp-2 (Human Epidermoid carcinoma tissue from the larynx) and MDCK (Madin Darby Canine Kidney) were maintained in DMEM with 5% fetal bovine serum (FBS). Vero cells (African green monkey kidney cell line) were maintained in EMEM with 5% FBS. In experiments that required supplementation of exogenous proteases for cleavage of the F protein, either 1ug/ml of trypsin or 5% allantoic fluid was used. The ability of the viruses to produce plaques was tested in DF1, Vero and MDCK cell lines under 0.8% methyl cellulose overlay. Plaques were visualized by staining with 1% crystal violet or by immunoperoxidase method using polyclonal antiserum.

6.4.2 Construction of expression plasmids and full length cDNA clones. For constructing the expression plasmid, the cDNA bearing the open reading frame

(ORF) of N (nt 141- 1514) and P (nt 1681-2880) genes were cloned into expression vector pGEM7Z under T7 promoter between *SphI* and *HindIII*, *EcoRI* and *SacI* enzyme sites, respectively. The ORF of L (nt 7938-14666) gene was subcloned as two fragments into pTM1 vector under T7 promoter using enzyme sites *NcoI*, *StuI* and *SacI*. The site *SacI* was artificially created by G11468C mutation within the L ORF without changing any amino acids. The N, P and L expression plasmids (pN, pP and pL) were used as the support plasmids for the recovery of the recombinant viruses. The full length cDNA of APMV-2 was cloned into vector pBR322/dr/Yuc, a plasmid generated by cloning a 73-nt oligonucleotide linker sequence containing unique restriction enzymes between *AscI* and *RsrII* sites in the pBR322/dr plasmid. The plasmid pBR322/dr is the modified form of plasmid pBR322, designed to include a 72-nt oligonucleotide linker between the *EcoRI* and *PstI* sites, an 84-nt hepatitis delta virus (HDV) antigenome ribozyme sequence and a T7 RNA polymerase transcription termination signal (Krishnamurthy *et al.*, 2000). The full length cDNA clone (pAPMV-2) encoding the complete 14,904 nt long anti-genome of APMV-2/Yuc was divided into six fragments and sequentially cloned into pBR322/dr/Yuc plasmid between the T7 promoter and HDV antigenome ribozyme sequence. A total of five unique restriction enzyme sites were created in the full length cDNA by mutating 10 nt without changing any amino acids, as described in the previous chapter. The third segment containing the F ORF was flanked by *NotI* and *PacI* enzyme sites. The full length cDNA clones of the F protein cleavage site mutants were generated by replacing this fragment with a fragment containing the mutated F protein cleavage site, obtained by overlap PCR. Briefly, the naturally occurring

APMV-2 F protein cleavage site (KPASRF) was replaced with those of the other APMV serotypes 1 through 9 and also one construct, pAPMV-2 (F-L) was created with a leucine residue replacing the phenylalanine residue at the N terminal end of F1 subunit. The mutated fragments were digested with *NotI* and *PacI* enzymes and were used to replace the third segment of pAPMV-2. The following twelve constructs were made; pAPMV-2, the recombinant parental clone of the wild type APMV-2/Yuc, pAPMV-2 (F-L) and pAPMV-2 (Type 1 v), pAPMV-2 (Type 1 av), pAPMV-2 (Type 1 African), pAPMV-2 (Type 3), pAPMV-2 (Type 4), pAPMV-2 (Type 5), pAPMV-2 (Type 6), pAPMV-2 (Type 7), pAPMV-2 (Type 8) and pAPMV-2 (Type 9) with the F protein cleavage site of APMV-1 virulent strains, APMV-1 avirulent strains, APMV-1 African strain (that contains five basic amino acids at the F protein cleavage site), APMV-3, APMV-4, APMV-5, APMV-6, APMV-7, APMV-8 and APMV-9, respectively. The full length clones of all the fusion protein cleavage site mutants were sequenced to their entirety using ABI 3130xl genetic analyzer (Applied Biosystems).

6.4.3 Recovery of recombinant viruses. A recombinant vaccinia virus expressing T7 polymerase-based transfection system was used to recover infectious recombinant APMV-2 from the constructed cDNAs (He *et al.*, 1997; Leyrer *et al.*, 1998; Krishnamurthy *et al.*, 2000). The transfection of full length cDNA clone along with support plasmids was performed in HEp-2 cell monolayer in 6-well plates. Briefly, the HEp-2 cells were grown to monolayer in DMEM at 37⁰C and 5% CO₂. When the cells reached 70% confluency, the medium was changed to opti MEM, 1 h prior to

transfection. The HEP-2 monolayer was transfected with 5 µg of respective full length cDNA plasmids and 3, 2 and 1 µg of pN, pP and pL, respectively in 100 µl of opti MEM per well. 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. Transfection was completed using Lipofectamine 2000 (Invitrogen, USA) according to manufacturer's instructions. The transfection mixture was replaced after 6 h with 1% DMEM. Two days after transfection, the HEP-2 cells were 'frozen and thawed' three times and the resulting supernatant was inoculated into the allantoic cavities of 9-day-old embryonated SPF chicken eggs. The allantoic fluid was harvested 3 dpi and tested for HA activity. Allantoic fluid with a positive HA titer were used for the isolation of the viral RNA genome followed by sequence analysis of the third fragment that included the F protein cleavage site and one of the unique restriction sites (such as *PmeI* site between P and M gene) that served as a genetic marker in the recombinant viruses. The recovered F protein cleavage site mutants were passaged three times in 9-day-old embryonated SPF chicken eggs and their stability was verified by sequencing the F protein cleavage site in each passage.

6.4.4 Growth characteristics of F protein cleavage site mutant viruses. The growth characteristics of the parental and mutant viruses were evaluated in DF1, Vero and MDCK cells with and without 5% allantoic fluid supplementation in the medium. The cells were observed daily for cytopathic effects (CPE) and HA titers were recorded every 24 h until fifth day.

The growth kinetics of the parental and mutant viruses in comparison with the wild type virus was performed in DF1 cells. Briefly, DF1 cells grown in six-well plates were infected with the viruses at an MOI of 1 with or without exogenous protease supplementation. After 1 h adsorption, the infected cells were washed with PBS, the medium was replaced and the cultures were incubated at 37°C in an incubator. At 24, 48, 72, 96, and 120 h post-infection 200 µl of culture supernatants were collected and stored at -70°C for virus titration. Virus titers of the samples were determined by serial end-point assay on DF1 cells in 96-well plates. The infected cells were stained by immunoperoxidase method using the polyclonal antiserum raised against APMV-2/Yuc in chickens. The virus titers (TCID₅₀/ml) were calculated using Reed & Muench method (Reed & Muench, 1938).

The ability of the mutant viruses to produce plaques was tested in DF1, Vero and MDCK cells under 0.8% methylcellulose overlay with and without exogenous supplementation of 5% allantoic fluid. The plaques were stained by 1% crystal violet or immunostained using polyclonal antiserum raised against APMV-2/Yuc in chickens, in DF1 and Vero cells on 3 dpi or in MDCK cells on 7 dpi depending on the onset of plaques.

6.4.5 Pathogenicity tests. The virulence of the parental and mutant viruses was determined by two standard pathogenicity assays: mean death time (MDT) in 9-day-old embryonated SPF chicken eggs and intracerebral pathogenicity index (ICPI) test in 1-day-old SPF chicks (Alexander 1989). Briefly, for MDT, a series of 10-fold (10^{-6} - 10^{-9}) dilutions of fresh infective allantoic fluid in PBS was made and 0.1 ml of

each diluent was inoculated into the allantoic cavities of five 9-day-old SPF embryonated chicken eggs (BEE eggs company, PA) and the eggs were incubated at 37°C. The eggs were candled 3 times a day for the next 7 days, and the time of embryo death if any were recorded. The minimum lethal dose (MLD) is the highest virus dilution that kills all the embryos. The MDT is the mean time in hours for the MLD to kill all the inoculated embryos. The MDT has been used to classify APMV-1 strains into the following groups: velogenic strains (taking less than 60 h to kill); mesogenic strains (taking 60 -90 h to kill); and lentogenic strains (taking more than 90 h to kill).

For ICPI, 0.05 ml (1:10 dilution) of fresh infective allantoic fluid of each virus was inoculated into groups of ten 1-day-old SPF chicks via the intracerebral route. The inoculation was done using a 27-gauge needle attached to a 1 ml stepper syringe dispenser that was set to dispense 0.05 ml of inoculum per bird. The birds were inoculated by inserting the needle up to the hub into the right or left rear quadrant of the cranium. The birds were observed for clinical symptoms and mortality, once every 8 h for a period of 10 days. At each observation, the birds were scored: 0 if normal, 1 if sick and 2 if dead. ICPI is the mean score per bird per observation over the 10-day period. Highly virulent, velogenic viruses give values approaching 2, and avirulent or the lentogenic viruses give values close to 0.

6.4.6 Effect of the number of basic amino acids at F protein cleavage site of mutant viruses on the pathogenesis in 2-week-old chickens. The effect of the number of basic amino acid residues at the F protein cleavage site on viral

pathogenesis and tissue tropism was determined by experimentally infecting 2-week-old SPF chickens with mutant viruses that differed from one another by the number of basic amino acids at their F protein cleavage site: rAPMV-2 (Type 4), rAPMV-2, rAPMV-2 (Type 3), rAPMV-2 (Type 1 v) and rAPMV-2 (Type 5) with one, two, three, four and five basic amino acids at their F protein cleavage site were chosen. Briefly, groups of six 2-week-old SPF chickens were inoculated with 0.2 ml of 2^8 HA units of viruses by oculonasal and intra tracheal route. The birds were observed daily and scored for any clinical signs for 7 dpi. Three birds from each group were euthanized on 3 and 7 dpi and oral and cloacal swabs were taken. The following tissues were collected on 3 dpi on dry ice for virus isolation and immunohistochemistry: brain, trachea, lungs, spleen, kidney and caecal tonsils. On 7 dpi, only brain, trachea and lungs were collected for virus isolation.

6.4.7 Virus isolation from swabs and titration of tissue sample. The oral and cloacal swabs were collected in 1 ml of PBS containing antibiotics (2000 unit/ml penicillin G, 200 $\mu\text{g}/\text{ml}$ of gentamicin sulfate and 4 $\mu\text{g}/\text{ml}$ of amphotericin B; Sigma Chemical Co., St. Louis, MO). The swab containing tubes were centrifuged at 1000x g for 20 min, and the supernatant was removed for virus isolation. Virus isolation was performed by inoculating the supernatant into the allantoic cavity of 9-day-old SPF embryonated chicken eggs and after 3 dpi the allantoic fluid was tested for HA activity. The HA-positive samples were further confirmed by HI tests with APMV-2 specific antiserum. The virus titers in the tissue samples were determined by the following method. Briefly, the tissue samples were homogenized and the supernatant

was serially diluted and used to infect DF1 cells. The 50% end-point tissue culture infectious dose (TCID₅₀/ml) was then calculated using Reed & Muench method after immunostaining the virus infected cells on 4 dpi (Reed & Muench, 1938).

6.4.8 Immunohistochemistry and Histopathology. The frozen tissue samples collected on 3 dpi were sectioned at Histoserve, Inc. (Germantown, Maryland, USA). Briefly, the frozen sections were rehydrated in three 10-min changes of PBS. The sections were fixed in ice cold acetone for 15 minutes at -80°C and then washed three times and blocked with 2% BSA for 1 h at room temperature inside a humidified chamber. The sections were incubated with a 1:500 dilution of the primary polyclonal antiserum for 2 h at RT. After three washes with PBS, the sections were incubated further with the HRP conjugated goat anti-chicken secondary antibody for 30 min. After a final wash cycle, the sections were incubated with DAB (Vector laboratories, USA) for two minutes, washed with distilled water and counterstained with hematoxylin (Vector laboratories, USA). Sections were mounted with mounting medium and examined under light microscope and microphotographs were taken (Zeiss Axiovert 200M). For histopathology, tissue samples were collected from the brain, trachea, lung, spleen, caecal tonsils and kidney collected on 3 dpi were fixed in 10% neutral buffered formalin. The fixed tissues were processed, embedded with paraffin, sectioned, and then stained by hematoxylin and eosin (HE).

6.5 Results:

6.5.1 Construction of full length APMV-2 cDNA clones. The cDNA clone encoding the antigenome of APMV-2 strain Yucaipa was constructed from six cDNA segments that were synthesized by RT-PCR from virion-derived genomic RNA (as described in chapter 5). The cDNA segments were cloned in a sequential manner into a low copy number plasmid pBR322/dr/Yuc between T7 promoter and hepatitis delta virus ribozyme sequence. The resulting APMV-2/Yuc cDNA in the plasmid pBR322/dr/Yuc was a faithful copy of the APMV-2/Yuc genome, except for the following nucleotide changes from the published sequence (Subbiah *et al.*, 2008) that were made to create unique restriction enzyme sites without causing any amino acid changes, necessary to facilitate assembly and to serve as genetic marker: C2923A, G2924A, T2925A, G2926C, G4154C, G5971A, A5973T, T7870C, A11321G and A11322C. The construct contains a T7 promoter which initiates a transcript with three extra G residues at its 5' end. It has been shown in other paramyxoviruses that the presence of extra G residues do not prohibit the rescue of infectious virus from cDNA (Krishnamurthy *et al.*, 2000).

To generate APMV-2 antigenomic cDNAs with mutations in the F protein cleavage site, the third segment (between 4193 to 5976 nt in the genome) that contained the F protein cleavage site was removed using *NotI* and *PacI* sites. Mutations of the F protein cleavage site were performed by overlapping PCR. The resulting cDNA fragments were then used to replace the third cDNA segment in the full length APMV-2 cDNA.

6.5.2 Recovery of infectious parental and F protein cleavage site mutant viruses.

The strategy for producing infectious APMV-2 from cDNA-encoded antigenomic RNA has been described previously for other paramyxoviruses (Krishnamurthy *et al.*, 2000). It involved the coexpression of HEp-2 cells of the four cDNAs encoding the antigenomic RNA and N, P and L proteins, which are necessary for viral RNA replication and transcription. The cDNA transcription was driven by T7 RNA polymerase supplied by a vaccinia –T7 recombinant virus strain MVA. The supernatants from the transfected HEp-2 monolayers were inoculated into the allantoic cavities of 9-day-old embryonated SPF chicken eggs. The allantoic fluid harvested 3 dpi was tested for HA activity. Allantoic fluid with a positive HA titer was used for the isolation of the viral RNA genome followed by sequence analysis of RT-PCR of segment number three that contained the F protein cleavage site. The results indicated that the parental and the F protein cleavage site mutants could be recovered from full-length cDNA clones. None of these recombinant viruses required the addition of exogenous protease during transfection and recovery. Each of these viruses (Table 6.1) was passaged three times in 9-day-old embryonated SPF chicken eggs and the nucleotide sequence of the F protein cleavage site was determined. The sequence data showed that all the mutations introduced in the F protein cleavage site were maintained after three passages in embryonated chicken eggs.

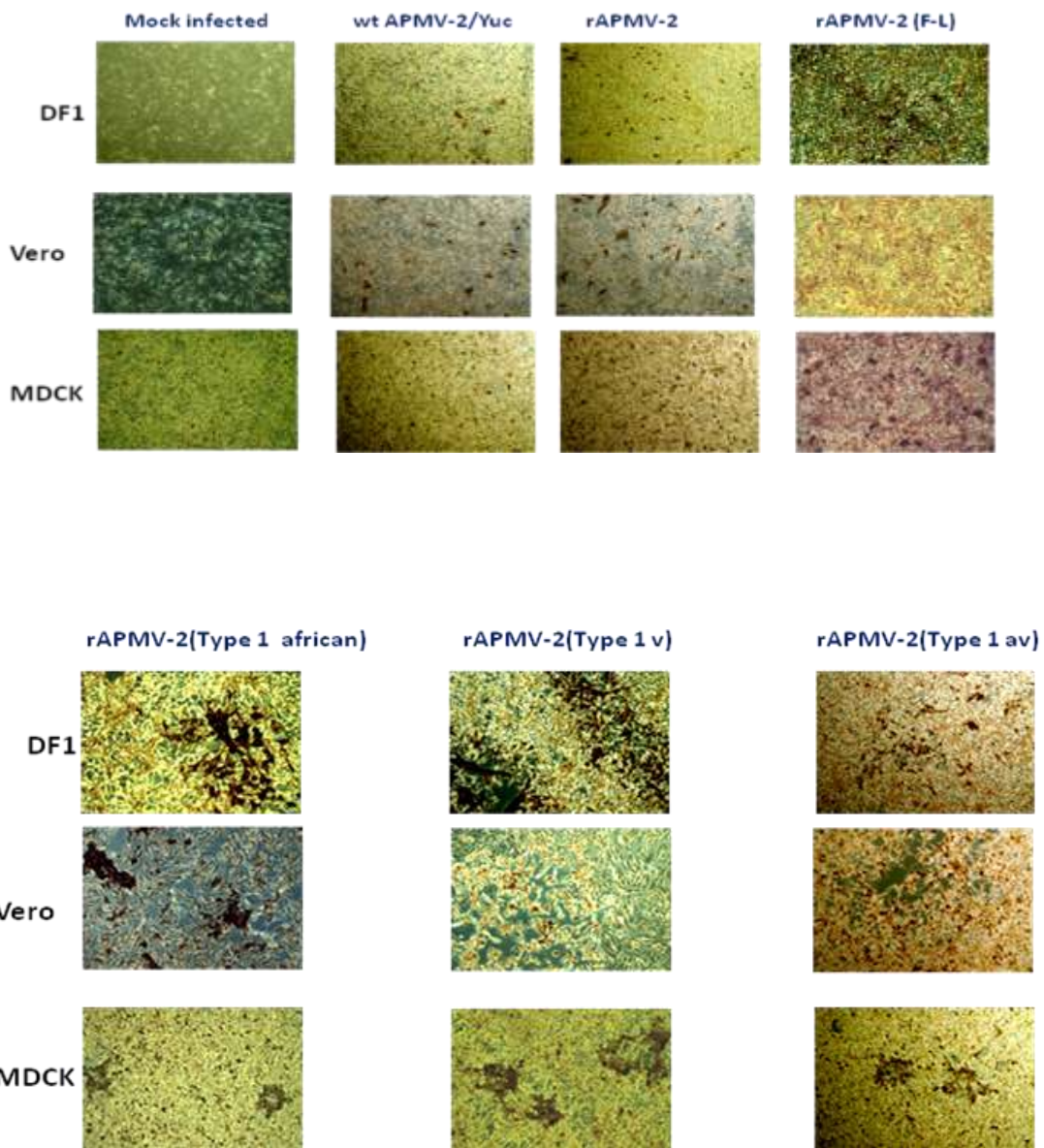
RECOMBINANT VIRUSES	FUSION PROTEIN CLEAVAGE SITE
rAPMV-2	K PASR ↓ F
rAPMV-2 (F-L)	K PASR ↓ L
rAPMV-2 (Type 1 v)	RRQ KR ↓ F
rAPMV-2 (Type 1 av)	GRQ GR ↓ L
rAPMV-2 (Type 1 African)	RRRRR ↓ F
rAPMV-2 (Type 3)	RPR GR ↓ L
rAPMV-2 (Type 4)	DIQ PR ↓ F
rAPMV-2 (Type 5)	KRKKR ↓ F
rAPMV-2 (Type 6)	APE PR ↓ L
rAPMV-2 (Type 7)	LPSSR ↓ F
rAPMV-2 (Type 8)	YPQ TR ↓ L
rAPMV-2 (Type 9)	REGRI ↓ F

Table 6.1. The fusion (F) protein cleavage sites of different APMV serotypes that were incorporated into the F protein cleavage site of APMV-2.

The segment III in pAPMV-2/Yuc was mutated by overlap PCR to change the F protein cleavage site, digested using *NotI* and *PacI* sites and subcloned into the full length cDNA to generate rAPMV-2 F protein cleavage site mutants. The basic amino acids (K and R) at the F protein cleavage site are shown in bold. The arrow indicates site of cleavage.

6.5.3 Growth characterization of parental and F protein cleavage site mutant viruses in cell culture. All the twelve recombinant viruses replicated in DF1, Vero and MDCK cells without requiring the supplementation of exogenous proteases. In general all the viruses replicated well in DF1 and Vero cells, while their growth pattern was comparatively slower in MDCK cells. The parental recombinant virus, rAPMV-2, resembled the wild type virus in its growth characteristics, causing single-cell infections in all three cell lines. However, all F protein cleavage site mutant

viruses except rAPMV-2 (F-L) produced syncytia and plaques under methyl cellulose overlay in the DF1 and Vero cells by 3 dpi and in MDCK cells by 7 dpi (Fig. 6.1). There was no significant difference in the morphology and size of the plaques produced by the F protein cleavage site mutants. The mutant virus, rAPMV-2 (F-L), produced single-cell infections in all the three cell lines, similar to the CPE of wild type virus and rAPMV-2 (Fig. 6.1)



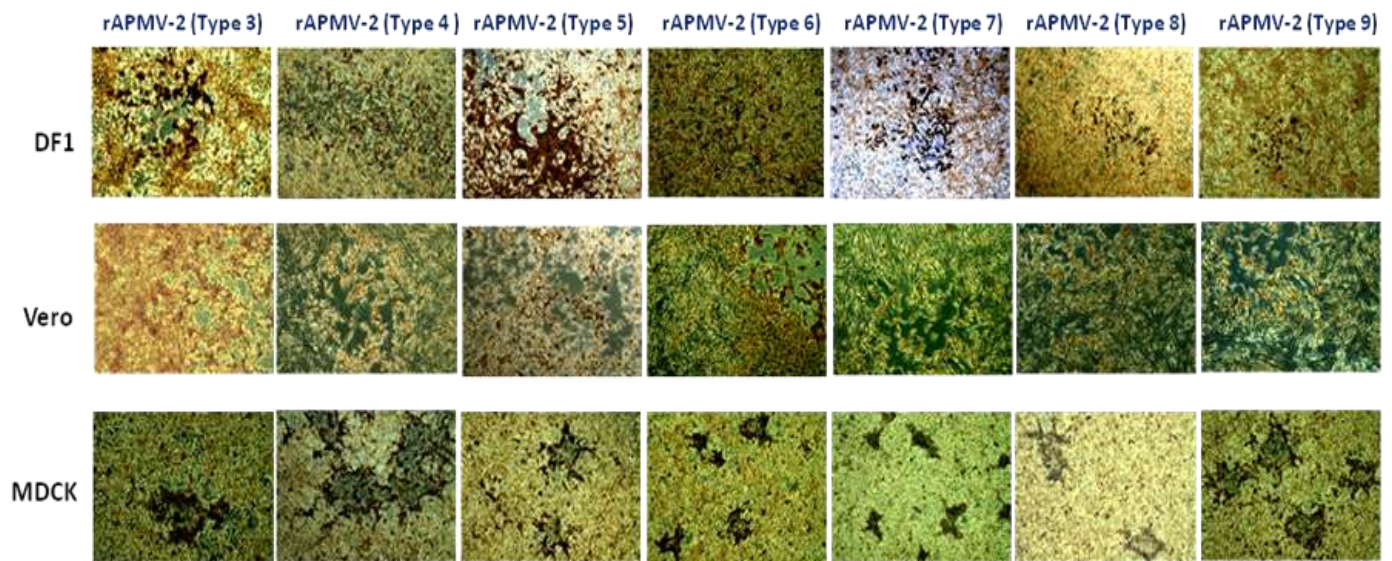


Fig. 6.1 Plaque formation in DF1, Vero and MDCK cell lines on 3, 3 and 7 days post infection, respectively, under methyl cellulose overlay in the absence of exogenous protease supplementation.

The plaques were visualized by immunoperoxidase staining using polyclonal sera raised against APMV-2 strain Yucaipa in chicken.

The efficiency of replication in tissue culture of the parental and F protein cleavage site mutant viruses was compared in a multi-step growth cycle. The monolayers of DF1 cells were infected with the viruses and samples were collected at 24 h intervals and quantitated by TCID₅₀ method. This analysis showed that all the syncytia forming mutant viruses grew to 10-fold higher titer compared to the wild type, rAPMV-2 and rAPMV-2 (F-L) (Fig. 6.2). The kinetics and magnitude of rAPMV (F-L) was similar to that of wild type and rAPMV-2 mutant.

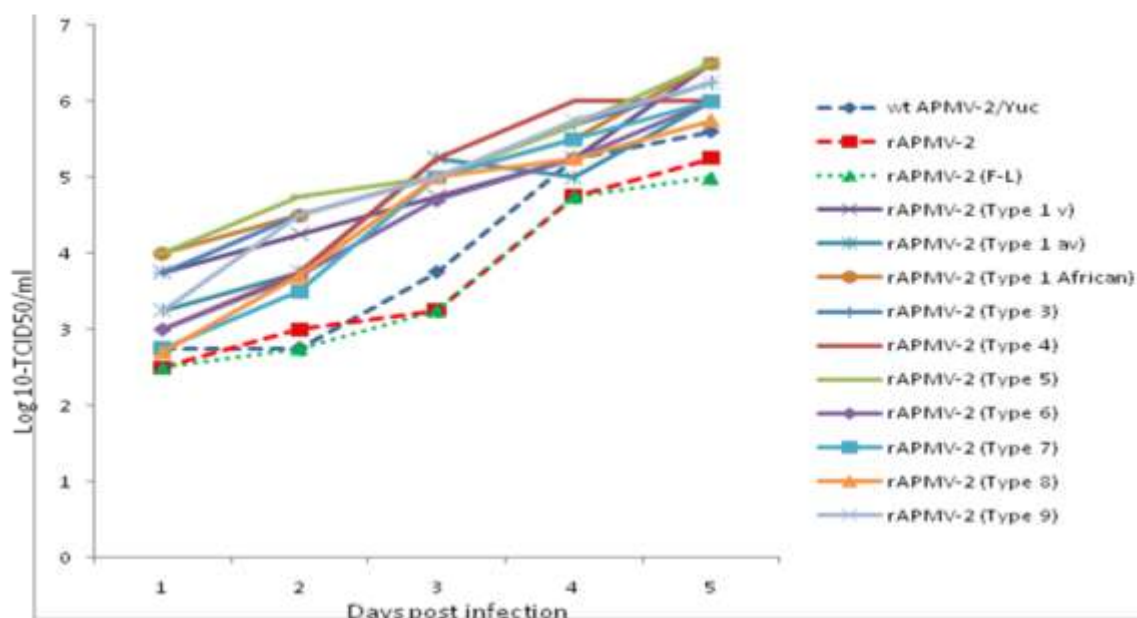


Fig. 6.2 Comparison of kinetics of replication between wild type and fusion cleavage site mutants of APMV-2 in DF1 cells.

DF1 cells in six-well plates were infected in triplicates with wt and recombinant viruses at an MOI of 1 and samples were taken from the culture supernatant at 24 h interval until 120 h post-infection. Virus titers of the samples were determined by serial end-point dilution in 96-well plates seeded with DF1 cells and immunoperoxidase staining using polyclonal antibody against APMV-2 strain Yucaipa raised in chickens. Virus titres (TCID₅₀/ml) were calculated by using Reed & Muench method (Reed & Muench, 1938).

6.5.4 The effect of the F protein cleavage site mutations on virulence. The virulence of the F protein cleavage site mutants was determined by two standard pathogenicity tests namely; mean death time (MDT) in 9-day-old embryonated SPF chicken eggs and intracerebral pathogenicity index (ICPI) in 1-day-old SPF chicks. None of the mutants caused death of chicken embryos similar to wild type virus. The

MDT of the parental and F protein cleavage site mutant viruses was more than 168 h. However, it was observed that the mutant viruses, except for rAPMV-2 and rAPMV-2 (F-L), caused embryo deaths at lower dilutions (10^{-2} to 10^{-3}) between days 7 and 10, which could be due to the rapid rate of replication compared to that of wild type virus in embryonated chicken eggs. All the recombinant viruses had ICPI values of zero resembling wild type APMV-2/Yuc and APMV-1 avirulent strains. Both these tests suggested that the cleavability of the F protein does not influence the pathogenicity of APMV-2 in chickens.

6.5.5 The effect of the number of basic amino acids at F protein cleavage site and pathogenesis in two-week-old chickens. The effect of the number of basic amino acids at the F protein cleavage site on the viral pathogenesis, was studied by experimentally infecting 2-week-old SPF chickens with recombinant viruses that varied from one another in the number of basic amino acids at their F protein cleavage site; rAPMV-2 (Type 4) with one, rAPMV-2 with two, rAPMV-2 (Type 3) with three, rAPMV-2 (Type 1 v) with four and rAPMV-2 (Type 5) with five basic amino acid residues at their F protein cleavage site were chosen for this study. The birds were infected with 0.2 ml of 2^8 HA units of infective fresh allantoic fluid by ocular and intra tracheal route. The birds were observed daily for 7 dpi. There were no apparent clinical signs of illnesses throughout the study period in any of the infected groups. Histopathological examinations of tissue samples collected on 3 dpi revealed similar microscopic findings in all the tested recombinant viruses; the trachea showed mild lymphocytic tracheitis with epithelial attenuation and

regeneration (Fig. 6.3a), in the lungs, mild to moderate multifocal lymphohistiocytic, perivascular and interstitial pneumonia was observed (Fig. 6.3b) and in the spleen, there was minimal lymphoid depletion (Fig. 6.3c) while the other organs were unremarkable.

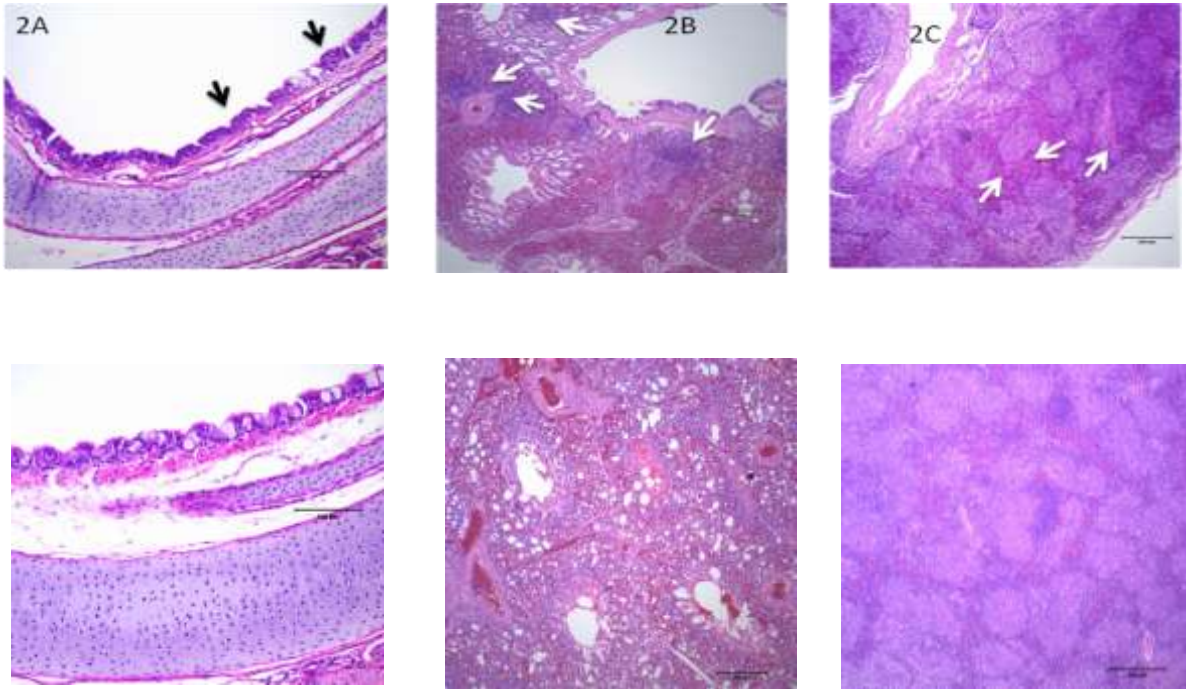


Fig. 6.3 Histopathological features of rAPMV-2 infected chicken. HE stain (A, B and C). The lower panel shows controls of trachea, lung and spleen of uninfected chicken.

2A: In the trachea, minimal to mild attenuation and flattening of the tracheal epithelium with reduction and loss of cilia was observed. There was loss of normal columnar epithelial architecture in these regions with mild epithelial hyperplasia and multifocal replacement by low cuboidal epithelial cells. Low numbers of individually apoptotic cells were seen within the epithelium in these regions. There was mild, multifocal, subepithelial infiltrate of lymphocytes and fewer macrophages in the

lamina propria. In summary, minimal to mild, multifocal, lymphocytic tracheitis with epithelial attenuation and regeneration was noted. 2B: In the lungs, small to moderate numbers of lymphocytes and fewer macrophages were seen infiltrating around blood vessels and within the interstitium. Inflammatory cells formed dense perivascular aggregates that extend into the interstitium, with small numbers of inflammatory cells multifocally infiltrating into the lamina propria subjacent to the airway epithelium. Small numbers of individually apoptotic cells were present in inflammatory aggregates. Mild to moderate, multifocal, lymphohistiocytic, perivascular and interstitial pneumonia was observed. 2C: In spleen, the periarteriolar sheaths and white pulp regions exhibited minimally reduced numbers of lymphocytes. There was also minimal lymphoid depletion.

On 3 dpi comparison of replication of the F protein cleavage site mutant viruses with that of parental rAPMV-2 revealed the following; rAPMV-2 (Type 4) with single basic amino acid at the F protein cleavage site, had comparatively reduced virus titer in brain (by 6 log), in lungs, trachea and spleen (by 5 log), and in kidney (by 3 log) but no significant difference in titer in caecal tonsil. The replication of rAPMV-2 (Type 3) with three unpaired basic amino acids at the FCS, had reduced virus titer in brain (by 3 log) while the virus titer was increased by one log in trachea and spleen and by 0.5 log in lungs and kidney while no significant difference in titer in caecal tonsil. The replication of rAPMV-2 (Type 1 v) and rAPMV-2 (Type 5) mutants each with paired basic amino acids (four and five basic amino acids, respectively at the F protein cleavage site) had reduced titers by one log when

compared to rAPMV-2 in brain and lungs. The rAPMV-2 (Type 1 v) had reduced virus titer in trachea, spleen and kidney by 1.5, 6 and 6 log, respectively, but no significant difference in caecal tonsil. The rAPMV-2 (Type 5) had increased virus titer in trachea by one log, no significant difference in spleen and kidney while the titer in caecal tonsil was reduced by 4 log (Fig. 6.4a).

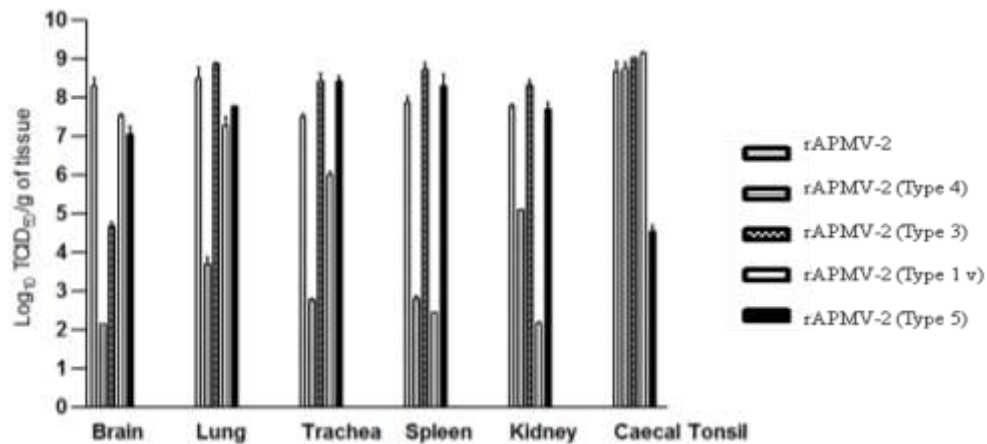


Fig. 6.4a Replication of F protein cleavage site recombinant viruses in different organs of 2-week-old SPF chickens, three days post infection (dpi). Six chickens in five groups were inoculated with 0.2 ml of 2^8 HAU of rAPMV-2, rAPMV-2 (Type 4), rAPMV-2 (Type 3), rAPMV-2 (Type 1 v) and rAPMV-2 (Type 5) by oculonasal and intratracheal route. Three chickens were sacrificed on 3 dpi. The virus titers in brain, lung, trachea, spleen, kidney and caecal tonsil are expressed in \log_{10} TCID₅₀/g of tissue.

On 7 dpi, rAPMV-2 (Type 3) could not be detected in brain tissue of any of the infected birds while the viruses with paired basic amino acids, rAPMV-2 (Type 5) and rAPMV-2 (Type 1 v) had virus titer of 0.5 and 1.5 log more than rAPMV-2

(Type 4) and rAPMV-2/Yuc. In upper respiratory tract (trachea), the virus titer of rAPMV-2 (Type 3), rAPMV-2 (Type 1 v), rAPMV-2 (Type 5) and rAPMV-2 (Type 4) were 0.5, 1, 1.5 and 2.5 log, respectively, lesser than the titer of the parental recombinant virus. In lower respiratory tract (lungs), rAPMV-2 (Type 1 v), rAPMV-2 (Type 5) and rAPMV-2 (Type 4) virus titers were less by 0.5, 1 and 1.5 log, respectively, than that of rAPMV-2 and rAPMV-2 (Type 3) (Fig 6.4b).

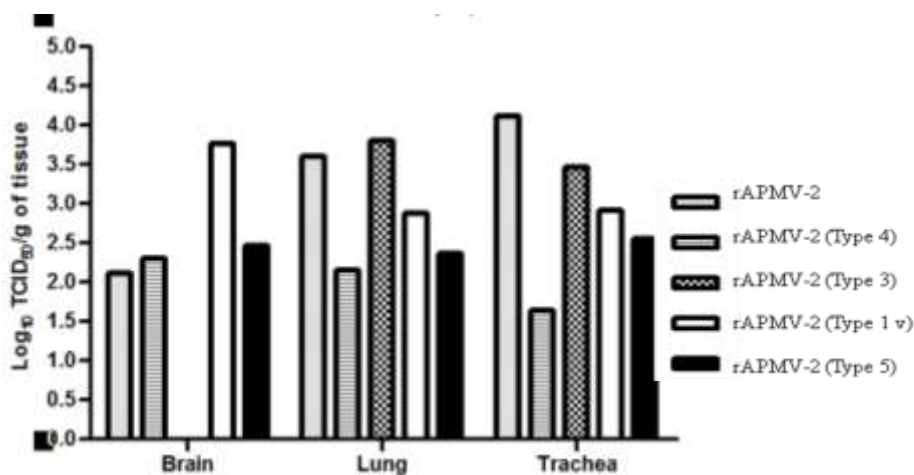


Fig. 6.4b Replication of F protein cleavage site recombinant viruses in different organs of 2-week-old SPF chickens, 7 days post infection (dpi). Six chickens in five groups were inoculated with 0.2 ml of 2^8 HAU of rAPMV-2, rAPMV-2 (Type 4), rAPMV-2 (Type 3), rAPMV-2 (Type 1 v) and rAPMV-2 (Type 5) by oculonasal and intratracheal route. Three chickens were sacrificed on 7 dpi. The virus titers in brain, lung, and trachea are expressed in \log_{10} TCID₅₀/g of tissue.

Viral antigens were detected by immunohistochemistry in tissue samples that were also positive by virus titration study. Immunohistochemistry studies also

showed the presence of large amount of viral antigens in epithelial linings (Fig. 6.5). All the infected birds were seropositive by 7 dpi as observed by HI test. There was no significant difference in oral or cloacal viral shedding between the parental and F protein cleavage site mutant viruses either on 3 or 7 dpi.

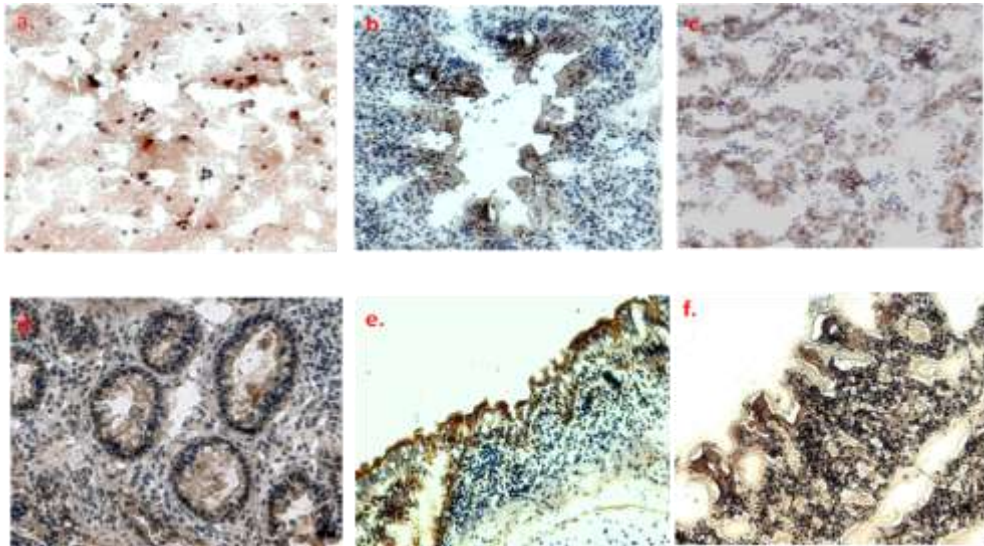


Fig. 6.5 Immunohistochemistry with DAB staining of tissues samples collected three days post infection with rAPMV-2/Yuc. Hematoxylin counterstain.

a. Infected chicken brain, b. Infected chicken kidney, c. Infected chicken spleen, d. Infected chicken caecal tonsil, e. Infected chicken trachea, f. Infected chicken lung.

6.6 Discussion

In paramyxoviruses, proteolytic processing of F proteins is needed for the generation of mature and infectious viruses. The F protein of APMV-2 is a type I viral glycoprotein and shares features common to other paramyxovirus F proteins such as the presence of two heptad repeat domains, a hydrophobic peptide

downstream of cleavage site, five potential N-linked glycosylation sites, a transmembrane domain and a cytoplasmic tail (Fig. 6.6). However, APMV-2 is unique with two basic amino acid residues at F protein cleavage site it does not require exogenous protease supplementation for growth in cell culture. Furthermore, APMV-2 causes single cell infections without forming syncytia.



Fig. 6.6 Schematic representation of APMV-2 Fusion protein (F).

The F protein is proteolytically processed into two fragments, F₁ and F₂, which are disulfide linked. Potential sites of N-linked glycosylation are indicated. The amino acid sequence upstream of the cleavage site is shown along with FP, fusion peptide; HRA and HRB, heptad repeats A and B; TM, transmembrane domain; CT, cytoplasmic tail.

* HR prediction by LearnCoil-VMF program, TM –DAS TM prediction server.

There are nine serotypes of avian paramyxoviruses within the genus *Avulavirus* in the family *Paramyxoviridae* and each serotype possesses a unique F protein cleavage site. In APMV-1 virulent strains have multi basic cleavage site which are recognized and cleaved by furin, a ubiquitous cellular protease. These viruses are able to replicate in different cells and cause systemic infection. The

avirulent strains of APMV-1 have one or occasionally two unpaired basic amino acids that are cleaved by trypsin like exogenous proteases. Hence, these viruses are restricted to areas like respiratory and gastrointestinal tracts and they require supplementation of exogenous proteases for their *in vitro* growth. The F protein cleavage site of other APMV serotypes (2-9) does not seem to follow this general rule applicable to APMV-1.

The F protein cleavage site of APMV-4 (DIQPR↓F) and APMV-7 (LPSSR↓F) contains a single basic amino acid residue, APMV-2 (KPASR↓F) has two unpaired basic amino acids and all these three viruses do not require exogenous protease supplementation for their *in vitro* growth in cell culture (Nayak *et al.*, 2008; Xiao *et al.*, 2009 and Subbiah *et al.*, 2008). It is notable that all three viruses have phenylalanine at their F1 terminal end. APMV-5 strain Kunitachi (KRKKR↓F) does not require exogenous protease supplementation for *in vitro* growth (Samuel *et al.*, 2010). The requirement of exogenous protease supplementation for APMV-6 (APEPR↓L) varies with strains. The other APMV serotypes, APMV-3 (RPRGR↓L), APMV-8 (YPQTR↓L), and APMV-9 (IREGR↓I) require exogenous protease supplementation for *in vitro* growth. It is interesting to note that these three serotypes have a leucine or isoleucine as the first amino acid at the N terminal end of F1 subunit. Each of these APMV serotypes have been isolated from different avian species and are host specific, for example, APMV-2 is endemic among passerines and causes severe respiratory illness in parrots, but only mild respiratory illness in chickens, APMV-5 causes severe mortality in budgerigars, but is apathogenic in

chickens. Hence their behavior could also be contributed in part by the environment provided within their natural host.

The F protein cleavage is required for activating the virus infectivity, and the distribution of activating proteases in the host is a major determinant of tissue tropism and, as such, the pathogenicity (Rott *et al.*, 1995). Furthermore, the F protein cleavage site has been shown to determine the viral pathogenicity in NDV (Peeters *et al.*, 1999; Panda *et al.*, 2004). The World Organization for Animal Health or Office International des Epizooties (OIE) states that “virulent NDV can be defined by the presence of multiple basic amino acids at the C-terminus of the F2 protein and phenylalanine at residue 117, the N-terminus of the F1 protein”. There are some exceptions to this general rule; three isolates from china; SQZ/04, JS05/03, and QE01/99 have lentogenic F protein cleavage site motif (GRQGR↓L) but still are virulent in chickens (Tan *et al.*, 2008). The pigeon paramyxovirus (PPMV-1), an NDV variant has virulent NDV F protein cleavage site motif and yet it is avirulent in chickens.

In this current study we examined the role of amino acid sequence at the F protein cleavage site in the APMV-2 virulence and pathogenesis. In order to determine the molecular basis for APMV-2 virulence, we have developed a reverse genetics system for recovery of infectious recombinant APMV-2 from cloned cDNA for the first time. In this system, recombinant vaccinia virus expressing T7 RNA polymerase was used to synthesize the antigenomic RNA from the full-length cDNA clone and the proteins N, P, and L from the cotransfected expression plasmids. We generated twelve recombinant APMV-2 F protein cleavage site mutants; 11 mutants

resembling the F protein cleavage site of a particular naturally occurring APMV serotype and one mutant where the phenylalanine at the N terminal end of F1 subunit was replaced by a leucine residue.

We were able to recover all the above mentioned F protein cleavage site mutants without exogenous protease supplementation; hence it suggests that no adverse changes happened to affect the F protein folding. All the cleavage site mutations were maintained after several passage in embryonated eggs. Irrespective of the F protein cleavage site or the type of cell line used, none of the rAPMV-2 F protein cleavage site mutants required exogenous protease supplementation for *in vitro* growth in cell cultures, resembling the wild type virus. Similar to wild type APMV-2/Yuc, the recombinants rAPMV-2 and rAPMV-2 (F-L) caused single cell infections, while the other F protein cleavage site mutants produced syncytia and plaques in DF1, Vero and MDCK cell lines. This result suggested that these mutants are able to cause cell to cell fusion leading to syncytia formations. It is possible that the changes in aa sequence at the F protein cleavage site might have produced conformational changes favoring efficient cleavage of F protein, resulting in the syncytia formation. Our growth kinetics results showed syncytia formation increased the replication of the mutant viruses slightly, suggesting that syncytia formation probably causes efficient cell to cell spread of the virus.

A single amino acid change at the N terminal end of F1 subunit did not cause any change in the growth characteristics of APMV-2. Previously, it has been shown in APMV-1 F protein that the phenylalanine residue at F1 amino terminal end is not required for fusion activity but a leucine residue at the same position hampers F

protein cleavage (Morrison *et al.*, 1993). But the F protein cleavage site mutant rAPMV-2(F-L) with a leucine residue at the N terminal end of F1 subunit was similar to wild type APMV-2/Yuc in *in vitro* growth pattern and kinetics and did not require exogenous protease supplementation. This mutant was also avirulent suggesting that the amino acid at this position does not affect the pathogenicity of APMV-2.

The Nipah and Hendra viruses consist of mono basic amino acid at their F protein cleavage site (arginine and lysine, respectively), both cause fatal systemic infections and do not require trypsin supplementation. These viruses are cleaved by cellular cysteine protease, cathepsin L in endosomes unlike other paramyxoviruses that are either cleaved in golgi by furin or extracellularly by trypsin like proteases (Pager & Dutch, 2005; Diederich *et al.*, 2005 and Pager *et al.*, 2006). In Nipah virus F protein, an introduction of multi basic sequence at F protein cleavage site does not allow cleavage by furin but actually prevents activation by any cellular protease. Hence the conformation around the cleavage sites probably determines exclusive activation by cathepsin L, a cysteine protease, or selective cleavage by the serine proteases trypsin and furin (Diederich *et al.*, 2009). Similar phenotype has been observed with WSN strain of influenza A virus that has monobasic cleavage site in Hemagglutinin (HA) protein but does not require exogenous protease supplementation for plaque formation. The type of amino acid and the position also influences the F protein cleavability; In Influenza virus A/turkey/Ireland/1378/85 (H5N8), the lysine replacement of arginine at the carboxy terminal of HA1 abolished the cleavability (Kawaoka & Webster, 1988 and Walker & Kawaoka, 1993).

In F1-R, a Sendai virus mutant, replacement of serine at position -1 by a proline residue changed the phenotype from pneumotropic to pantropic (Tashiro *et al.*, 1988). In SV5, a series of mutant F proteins produced by site directed mutagenesis revealed that the minimum number of arginine residues for cleavage activation of F protein by host cell proteases was four and those with 2 or 3 arginine residues were cleaved by exogenous protease supplementation and the F protein with a single arginine residue was not cleaved. It was also shown that the connecting peptide affected the local conformation of F polypeptide but not the biological activity (Paterson *et al.*, 1989). Apart from the number, position and the type of amino acids at the cleavage site, there are other factors that affect the cleavability and hence the fusion activity, such as a carbohydrate side chain in the vicinity of F protein cleavage site or point mutations at a distance from F protein cleavage site (Rott & Klenk 1988 and Orlich *et al.*, 1990). In NDV, the interaction between the heptad repeat domain (HR 2) of fusion protein is found to be important for the fusion promotion activity through interaction between F and HN proteins (Gravel & Morrison, 2003).

In our study, we found that irrespective of the amino acid sequence at the F protein cleavage site, the MDT of APMV-2 F protein cleavage site mutants were more than 168 h and ICPI values were zero similar to wild type APMV-2/Yuc, suggesting that the F protein cleavage site does not play a role in the virulence of APMV-2. In addition, the role of number of basic amino acids at the F protein cleavage site in pathogenesis was studied in 2-week-old chickens. We chose five mutants that varied from one another in the number of basic amino acid residues at

their F protein cleavage site; rAPMV-2 (type 4), rAPMV-2, rAPMV-2 (type 3), rAPMV-2 (type 1 v) and rAPMV-2 (type 5) with 1, 2, 3, 4 and 5 basic amino acids, respectively, at the F protein cleavage site. Our results demonstrated that monobasic F protein cleavage site containing virus in general replicated inefficiently compared to the parental dibasic recombinant. Our results did not show any significant difference in the viral replication and tissue tropism among the F protein cleavage site, suggesting no direct correlation between the number of basic amino acids at F protein cleavage site and pathogenicity of APMV-2.

We have shown here that the prerequisite for fusion activation in APMV-2 is different from other paramyxoviruses and is dependent on the amino acid sequence at F protein cleavage site. Our results suggest that the F protein of wild type virus is somehow able to initiate infection without causing cell fusion. Any change in the amino acid sequence at the F protein cleavage site causes a local conformational change which leads to cleavage of the F protein and cell to cell fusion or syncytia formation. The cell to cell fusion probably causes slight increase in virus replication. However, this cell to cell fusion does not increase the virulence or pathogenicity of the virus. The pathogenicity of APMV-2 is probably dependent upon other viral or host factors. The availability of a reverse genetics system to recover infectious APMV-2 from cloned cDNA will expand our understanding of not only APMV-2 but other paramyxoviruses in general.

Chapter 7

7.1 Title

Conclusion and Future prospects

7.2 Conclusion and Future prospects:

Family *Paramyxoviridae* includes important human pathogens such as mumps virus, measles virus and Nipah viruses and animal pathogens such as Newcastle disease virus (NDV), rinderpest virus and canine distemper virus. 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 and very little is known about the other serotypes. APMV-2 causes mild clinical illness in chickens and turkeys, which gets exacerbated with secondary bacterial infections. There are also reports of APMV-2 infection causing drop in egg production in chicken and turkey commercial farms. Surveillance studies have shown the endemic existence of APMV-2 among passerines and other wild birds across the globe. Prior to this study, only the epidemiological data of APMV-2 infections were available. In this study four strains of APMV-2 (prototype strain Yucaipa and strains Bangor, England and Kenya) were characterized both *in vitro* and *in vivo*. A sensitive immunoperoxidase method was standardized for titrating APMV-2 strains since none of the viruses produced plaques in cell culture. All four APMV-2 strains were found to be avirulent in chickens by internationally accepted standard pathogenicity tests- MDT in

embryonated chicken eggs and ICPI in day-old chicks. The pathogenesis of strains Yucaipa and Bangor in 4-week-old chickens and turkeys was studied which demonstrated that neither of the strains is virulent in both species of birds and that strain Yucaipa replicated better in chickens than in turkeys.

As a first step towards molecular characterization, the complete genome sequences of all the four APMV-2 strains were determined and compared with other paramyxoviruses. The genome lengths of strain Yucaipa, Bangor, England and Kenya were 14904, 15024, 14904 and 14916 nt, respectively. The genome sequence and phylogenetic analyses justified the classification of APMV-2 under the genus *Avulavirus* within the family *Paramyxoviridae* and also indicated the existence of two subgroups within serotype 2. The concept of subgrouping was further supported by cross HI and cross neutralization tests in cell culture between homologous and heterologous sera. We propose that the strains Yucaipa, England and Kenya form one subgroup while strain Bangor represents another subgroup.

The availability of complete genome sequence enabled construction of a full length cDNA clone for APMV-2 strain Yucaipa (APMV-2/Yuc) and subsequent recovery of infectious recombinant virus by reverse genetics system previously established in our laboratory for avian metapneumo virus, bovine respiratory syncytial virus and NDV 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, two infectious recombinant viruses expressing enhanced GFP were recovered and characterized. The viruses stably expressed GFP

for five consecutive passages in DF1 cells and in chicken embryos suggesting that this system could be used to develop APMV-2/Yuc as a vaccine vector against emerging pathogens.

Previously, it has been shown that the nature and composition of amino acids at the fusion (F) protein cleavage site determines the virulence of APMV-1 (Peters *et al.*, 1999; Panda *et al.*, 2004). Using reverse genetics approach, eleven APMV-2/Yuc recombinant viruses were recovered, each with F protein cleavage site resembling a particular APMV serotype, and one recombinant with leucine residue in place of phenylalanine at the F1 N terminal end. Our results demonstrated that mutations at the F protein cleavage site led to plaque formation in cell culture, but did not affect the pathogenicity of the virus. It was also shown that the F1 N terminal amino acid did not play any role in APMV-2 *in vitro* growth characteristics or *in vivo* pathogenicity.

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-2 strains (ii) The molecular mechanism of cleavage of APMV-2 fusion protein (iii) application of APMV-2 reverse genetics system to develop vectored vaccines against emerging diseases of animals and humans.

Science is fascinating, each and every research finding moves us a step forward in understanding science and nature, only to show us that there is still a vast area to be explored!

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