Evaluation of the potential functions of Avian paramyxovirus Accessory proteins

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ABSTRACT

Avian paramyxoviruses (APMVs) consist of twelve distinct serotypes (APMV-1 to -12) isolated from a wide variety of domestic and wild birds. APMV-1/Newcastle disease virus (NDV) is the most characterized and globally important avian pathogen, because of the huge economic loss associated with the disease. However, very little information is known about the pathogenicity of APMV 2-12. APMV expresses six structural and two accessory proteins. The functions of APMV accessory proteins (V and W) are not fully established. Only the function of V protein in NDV is studied so far. V protein was found to be an IFN antgonist and a major virulent determinant of NDV. In this study, we tested for the potential functions of W protein in NDV and fuctions of V protein in other APMV serotypes. Vaccination failure is a major cause for NDV outbreak in developing and tropical countries, because of thermolabile nature of vaccine strains. Thermostable and thermolabile NDV strains exhibit difference in W protein length. In the first part of our study, we mutated the genome of a thermolabile NDV strain to express W protein of different lengths, rescued recombinant viruses by reverse genetics system and tested for thermostability. Our results showed that W protein does not confer thermostability to NDV. In the second part of study, we constructed plasmids expressing APMV -2, -3 and -6V proteins and tested for IFN antagonism by a dual luciferase

reporter assay. Our results showed that APMV-3V acts as IFN antagonist by blocking IFN induction and thereby may play an important role in the evasion of innate immunity.

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ABSTRACT (Public)

Avian paramyxoviruses (APMVs) are RNA viruses with 12 different serotypes (APMV 1-12) infecting a large number of wild and domestic birds. APMV-1/Newcastle disease (NDV) is a highly contagious disease of poultry with severe economic losses and also affects global trade of poultry and poultry products. However, the details of the infectious nature of other APMV serotypes are not fully known. APMV produces six major proteins and two accessory proteins. The accessory proteins V and W are produced by a unique mechanism of RNA editing during virus replication. NDV V protein counteracts the host cell immune response by antagonizing Interferon (IFN). This mechanism helps in the virus replication and directly correlates with the NDV virulence. However, the potential functional roles of the W protein in NDV and V protein in other APMVs are not known. Despite having a good modified live NDV virus vaccine, the poultry industry periodically suffers from vaccine failures. This is due to the temperature sensitivity of the vaccine virus and failure to maintain the cold-chain for vaccine transport and delivery to the birds. We found that naturally occurring temperature resistant (thermostable) NDV strains produce a W protein of shorter length than that in temperature sensitive (thermolabile) strains. Therefore, we hypothesized that W protein

has multiple functions in NDV life cycle and could be exploited for potential vaccine purpose. We modified the NDV genome to produce W proteins of differing lengths and recovered recombinant NDV entirely from cloned DNA copy of the virus by a method called reverse genetics. We tested the rescued virus for the temperature stability by standard hemagglutinin and virus infectivity tests. The results revealed that, the rescued recombinant NDV virus expressing different lengths of W did not show difference in their thermal stability nature, indicating that W protein does not play a significant role in conferring thermostability to the virus. For the second part of the study, we tested the IFN antagonism of APMV -2, -3 and -6 V proteins in chicken cells using dual luciferase reporter assay. The results showed that the V protein of APMV-3 but not APMV-2 and APMV-6 V acts as IFN antagonist counteracting the host immune response, which could help in the replication of the virus in the host cell.

Dedicated to my parents Venkatachalam and Ramakiri,

mentor Dr. Subbiah,

brother Murugaraja, friend Jagadeeswaran for their love,

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ATTRIBUTION

This thesis is composed of four chapters: Literature review, two chapters and general conclusion. These chapters are prepared in a manuscript format.

Elankumaran Subbiah, MVSC, PhD, was an Associate Professor of Molecular Virology at Department of Biomedical Sciences and Pathobiology, Biomedical and Veterinary Sciences, Virginia Tech. Dr. Subbiah helped to design the experiments, provided the resources and guidance for the research work.

Xiang-Jin Meng, M.D., Ph.D (University Distinguished Professor, Department of Biomedical Sciences and Pathobiology, Biomedical and Veterinary Sciences, Virginia Tech) is the Committee Chair and provided guidance for the research work.

Jagadeeswaran Deventhiran was a former graduate student in Dr.Subbiah's group and he contributed to the research project by helping the author with construction of full-length recombinant NDV plasmids.

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Adria Allen, was a former graduate student in Dr.Subbiah's group. She contributed to the research work with the construction of expression plasmids described in chapter 3 of the thesis.

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List of abbreviations

Avian paramyxovirus (APMV)

Baby hamster kidney fibroblast (BHK21)

Caspase-recruiting domain (CARD)

Central Nervous System (CNS)

Chicken embryo fibroblast (DF1)

Double-stranded RNA (dsRNA)

Exotic ND (END)

Fusion protein (F)

Hemagglutination inhibition (HI)

Hemagglutinin – neuraminidase (HN)

Interferon (IFN)

IFN-stimulated gene factor 3 (ISGF3)

IFN-stimulated response element (ISRE)

Inhibitor of κB kinase ε (IKKe)

Laboratory of genetics and physiology-2 (LGP2)

Large polymerase protein (L)

Matrix protein (M)

Messenger RNA (mRNA)

Melanoma differentiation-associated gene 5 (MDA5)

Neuraminidase activity (NA)

Neuraminidase inhibition (NI)

Newcastle disease (ND)

Newcastle disease virus (NDV)

Nucleocapsid protein (N)

Nucleotide oligomerization domain (NOD)-like receptors (NLRs)

Office International des Epizootics (OIE)

Open reading frame (ORF)

Pathogen associated molecular patterns (PAMP)

Phosphoprotein (P)

Ribonucleic acid (RNA)

Ribonucleoprotein (RNP)

Retinoic acid-inducible gene I (RIG-I)

(RIG-I)-like receptors (RLR)

Reverse transcriptase polymerase chain reaction (RT-PCR)

Single-stranded RNA (ssRNA)

Small hydrophobic gene (SH)

Signal transducer and activator of transcription (STAT)

TANK-bindng kinaes 1 (TBK1)

Toll-like receptors (TLRs)

General Introduction

Avian paramyxoviruses (APMV) are a group of viruses that belongs to the genus *Avulavirus* and family *Paramyxoviridae* (Alexander et al, 2000). Twelve distinct serotypes of APMV (APMV-1 to APMV-12) are known till date (Gogoi et al., 2015). APMV-1 includes the highly contagious and globally important avian pathogen, Newcastle disease virus (NDV). NDV is the most well-characterized APMV serotype, because of the huge economic loss associated with the disease. Strains of NDV show a spectrum of virulence from mildly pathogenic to highly lethal, where virulent NDV infections in a flock may result in 100% mortality (Samal, 2011). Vaccination failure is a major cause of ND outbreaks. Thermolabile nature of NDV strains used in vaccine preparation requires uninterrupted cold chain maintenance to maintain the vaccine potency during storage and transport. In developing and tropical countries, where adequate cold chain maintenance facilities are not feasible, ND outbreaks are common due to vacccine failure. The possible solution for the problem could be the production of the thermostable vaccines, which can retain its potentcy sustaining the termperature fluctuations independent of cold chain (Spradbrow, 1993).

APMVs have a negative sense, nonsegmented, RNA genome of 13-19 kb length and has 6 genes in the order of 3'-NP-P-M-F-HN-L-5', coding for six structural proteins (Gogoi et al., 2015). P gene encodes for two accessory proteins V and W by RNA editing mechanism. RNA editing is a unique feature displayed by all the paramyxoviruses. The V and W proteins are expressed from P gene by specific insertion of non-templated 'G' nucleotides by the RNA polymerase at the RNA editing site. Insertion of one 'G'

nucleotide produces V protein while two ‘G’ nucleotides produces W protein. The V and W proteins share the amino co-terminus with the P protein up to RNA editing site but differ in their carboxyl termini (Vidal et al., 1990; Steward et al., 1993). Based on the amino acid sequences of different NDV W protein, researchers suggested that difference in the length of W protein could be an important determinant in the thermostability of NDV strains (Wen et al., 2013). However, it was not studied before.

The disease potential of APMV-2 to APMV-12 is not well established as many of them are isolated from hunter-killed birds or wild birds dying in quarantine or while screening of domestic birds for other poultry pathogens (Samal, 2011). Experimental infection studies demonstrate that APMV 2-9 infects chickens and turkeys. Serosurveillance study conducted in USA has shown that all the serotypes of APMVs are prevalent in commercial poultry flock except APMV-5 (Warke et al., 2008). In general, accessory proteins of paramyxoviruses plays an important role in antagonising interferon (IFN), blocking apoptosis, inhibiting viral mRNA sysnthesis and assisting in viral budding from plasma membrane (Chambers and Takimoto, 2009). Cosnserved cysteine rich C-terminal of NDV V protein of NDV acts as an interferon-antagonist and is one of the important virulence determinants of NDV (Huang et al., 2003). Based on the amino acid sequence alignment, we found that V proteins of APMV 2-9 serotypes have conserved cysteins rich C-terminal similar to that of NDV V protein. However, the functions of V protein in these APMVs serotypes are not fully understood.

Hypothesis:

We hypothesize that

- Difference in the length of W protein influences the thermostability of NDV, which could be exploited for potential translational purpose.
- The V protein of APMV-2, -3 and -6 acts as IFN antagonist and helps in innate immune evasion.

Objectives:

The main objective of the proposal was to decipher the role of accessory proteins (V and W) in APMV. In order to achieve this objective we set up two specific aims:

- **Aim 1:**
- To introduce mutations in the antigenome of thermolabile NDV strain to express W protein of varying lengths and recover the recombinant viruses by reverse genetics.
- To evaluate the potential functions of W protein in thermostability.
- **Aim 2:**
- To clone APMV-2, -3 and -6 V proteins into a mammalian expression vector and demonstrate their expression in chicken cell line (DF1).
- To determine the potential functions of APMV-2, -3 and -6 V proteins in IFN antagonism

Chapter 1

Literature Review

1.1 Introduction:

The family *Paramyxoviridae* includes a large number of important disease causing viruses of humans [Measles virus, Mumps virus, Human respiratory syncytial virus], animals [Rinderpest virus, Newcastle disease virus, Peste des pestis ruminants virus, Canine distemper virus], and zoonotic importance [Hendra and Nipah virus] (Lamb and Parks, 2007). The family *Paramyxoviridae* are enveloped, negative sense, non-segmented RNA viruses and includes two subfamilies namely *Paramyxovirinae* and *Pneumovirinae* (Lamb and Parks, 2007). Based on antigenic cross reactivity, hemagglutination and neuraminidase activity, the Subfamily *Paramyxovirinae* is divided into seven genera namely *Respirovirus, Avulavirus, Rubulavirus, Morbillivirus, Henipavirus, Aquaparamyxovirus* and *Ferlavirus* and the subfamily *Pneumovirinae* into two genus namely *Metapneumovirus* and *Pneumovirus* (King et al., 2011; Audsley et al., 2013).

1.1.1 Genus *Avulavirus*:

Genus *Avulavirus* includes all the paramyxoviruses of avian species except Avian metapneumovirus, which is grouped under the genus *Metapneumovirus* and subfamily *Pneumovirinae*. Avian paramyxoviruses (APMV) have the genome organization that closely resembles with other members of subfamily *Paramyxovirinae.* Based on the hemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays, APMVs are further classified into twelve distinct serotypes: APMV-1 to APMV-12 (Gogoi et al, 2015). APMV-1 serotype includes all strains of Newcastle disease virus (NDV). NDV is the most characterized pathogen among APMVs, because of the severity of the disease and the economic impact caused by the virus in domestic birds. NDV includes strains of

varying pathogenicity from mildly pathogenic to highly lethal. Newcastle disease (ND) caused by virulent strains of NDV is classified by the Office International des Epizootics (OIE) as notifiable List-A disease (Jeong et al., 2013).

1.1.2 APMV morphology and structure:

APMV virion particles are generally spherical in shape with a diameter ranging from 150-250 nm (Samal, 2011; Gogoi et al., 2015). The virion has an lipid bilayer envelope that is derived from the plasma membrane of the host cell and contains two transmembrane glycoproteins, the hemagglutinin – neuraminidase (HN) and fusion (F) protein inserted into the envelope. Matrix (M) protein, a non-glycosylated membrane protein lies beneath the viral envelope. Polymerase complex formed by the nucleocapsid protein (N), phosphoprotein (P) and large polymerase protein (L) along with the viral genomic RNA represents ribonucleoprotein complex, which is the minimal infectious unit present inside the virion (Lambs and Parks, 2007).

1.1.3 Genome organization of APMV:

APMVs have a negative sense, single stranded (non-segmented) RNA genome of nucleotide length ranging from 14,904 to 17,412. APMVs follow the ‘rule of six’ [having their genome length as an exact multiple of 6], a characteristic feature of the subfamily *Paramyxovirinae.* This is found to be required for the precise encapsidation of the genomic RNA by N monomers and efficient RNA replication (Calain and Roux, 1993; Hausmann et al., 1996; Kolakofsky et al., 1998).

The genomic RNA has 3’ and 5’ extracistronic region called the leader and trailer, flanking six genes (3’-N-P-M-F-HN-L-5’) that encodes for the six structural proteins and two accessory proteins. The gene order is highly conserved among the family members

with the exception of APMV-6. APMV-6 has an additional gene SH, between the F and HN gene, encoding for the small hydrophobic protein whose function is not known (Chang et al., 2001; Lambs and Parks, 2007; Samal, 2011).

Leader and trailer regions have a high level of sequence complementarity and are essential for transcription, replication and encapsidation of the newly synthesized genomic RNA. Conserved transcriptional sequences at the beginning and end of each gene sequences are called Gene start (3'-UGCCCAUCU/CU-5') and Gene end (3'-AAUU/CC/UU_{5-6}-5') (Samal, 2011). Between each gene are the highly conserved noncoding 'intergenic sequences', which may vary in length between 1-47 nt. Intergenic sequences control the polyadenylation, transcription termination and reinitiation (Yan and Samal, 2008; Kim and Samal, 2010).

1.1.4 Viral proteins:

All the APMV genome encodes eight proteins namely N, P, M, F, HN, L, V and W, with the exception of APMV-6, which encodes an additional SH protein. Each gene of APMV encodes for a single structural protein except the P gene, which encodes for two additional accessory proteins namely V and W (Steward et al., 1993). Knowledge on the APMV protein structure and functions are mostly derived from studies made on the other members of the family *Paramyxoviridae* namely Sendai virus and Parainfluenza virus and few on NDV (Buchholz et al., 1993; Kingston et al., 2004; Kho et al., 2004; Lamb and Parks, 2007).

1.1.4.1 Nucleocapsid protein:

The N protein, being the first gene transcribed from the viral genome, is the most abundant protein in APMV infected cells and virus particles. It is essential for the

encapsidation of the full-length genomic (-) sense and antigenomic (+) sense RNA making the nucleocapsid RNAse resistant and biologically active (Myers et al., 1999; Samal, 2011). N protein also interacts with other proteins, P and L during transcription and replication, and with M during the virus assembly (Kho et al., 2003).

1.1.4.2 Phosphoprotein:

The P protein is the unedited transcript from P gene and is the non-catalytic subunit of viral RNA polymerase complex. It prevents the uncontrolled encapsidation of the cellular RNA by forming a soluble complex with the N protein. It also acts as a bridge between the N-RNA template and the L protein (Lambs and Parks, 2007; Samal, 2011).

1.1.4.3 Matrix protein:

M, a non-glycosylated membrane protein, is seen underneath the viral lipid bilayer and is associated with the membranes peripherally (Li et al., 1980). It interacts with the nucleocapsid, lipid bilayer and cytoplasmic region of HN protein. This helps in the transportation of viral components to the plasma membrane, assembly of virion and budding of the virus (Stricker et al., 1994; Takimoto and Portner, 2004).

1.1.4.4 Fusion protein:

The F protein is an envelope glycoprotein that facilitates the viral entry into the host cell by fusing the viral envelope with the host cell plasma membrane in a pH independent manner. The F protein undergoes extensive structural change during the fusion process resulting in the delivery of the viral nucleocapsid into the host cell cytoplasm (Lambs and Parks, 2007). The F protein expressed on the plasma membrane of infected cell promotes fusion with the neighboring cells resulting in formation of syncytia or multinucleated giant cell, which is an important mechanism for virus spread

and virulence. The F protein is a type I integral membrane protein, synthesized as an inactive precursor F_0, that needs to be cleaved by host cell proteases to become biologically active subunits F_1 and F_2 linked by disulfide bond (Morrison, 2003).

1.1.4.5 Hemagglutinin-Neuraminidase protein:

The HN protein of APMV is a multifunctional glycoprotein possessing the sialic acid receptor binding activity, neuraminidase (NA) activity and the fusion promotion activity (Lamb and Parks, 2007; Morrison et al., 1991; Samal, 2011). HN protein is a type II integral transmembrane protein having an N terminal cytoplasmic domain, followed by a transmembrane domain, a stalk domain and a C-terminal globular head domain with the receptor binding, NA activity and the antibody binding sites (Hiebert et al., 1985; Mirza et al., 1993). The HN protein has been shown to be associated with the tissue tropism and virulence of APMV (Huang et al., 2004; Panda et al., 2004).

1.1.4.6 Large protein:

The L protein is the largest structural and least abundant protein seen in APMV infected cells and virions. It is an active component of the polymerase complex. All the enzymatic activities related to the synthesis of viral mRNAs and replication of viral genomic RNA and antigenomic RNA, including nucleotide polymerization, mRNA capping, methylation and polyadenylation are found to be associated with the L protein (Hercyk et al., 1988; Grdzelishvili et al., 2005). Interaction of L protein with the P protein makes it functional and P protein helps in tethering the L protein to the nucleocapsid. Pathogenicity studies have shown that L protein is associated with virulence of NDV. (Rout and Samal, 2008).

1.1.4.7 Accessory proteins expressed from P gene:

In general, paramyxoviruses express accessory proteins from their P gene, by two distinct mechanisms - 1) by RNA editing, which result in the expression of V/W/D/I transcripts and 2) by alternate open reading, which result in the expression of the C proteins (Vidal et al., 1990; Hausmann et al., 1999). Expression of accessory proteins by RNA editing is conserved among all members of paramyxoviruses. RNA editing occurs when a specific motif on the P gene directs the RNA polymerase to add one or more non-templated 'G' nucleotides to the P gene transcripts. Frame shift occurring in mRNA transcripts results in the expression of V/W/D proteins (Vidal et al., 1990; Steward et al, 1993). The number and frequency of the G nucleotides inserted depends on the sequences surrounding and within the RNA editing site (Hausmann et al., 1999).

In all paramyxoviruses except Rubulavirus, P protein is an unedited transcript of the P gene. The edited transcripts with addition of one and two 'G' nucleotides result in the expression of V and W/D/I proteins respectively. In Rubulavirus, V protein is expressed from the unedited transcript and addition of two 'G' nucleotides results in the expression of P protein. P, V, and W proteins share the common amino terminus but differ in their carboxyl terminus (Thomas et al., 1988). The products of P gene vary in their relative abundance, where V and W were relatively lesser compared to the P mRNAs (Mebatsion et al., 2001). In addition to RNA editing mechanism, members of the *Respirovirus, Morbillivirus* and *Henipavirus,* utilize alternative open reading frame (ORF) to express family of C proteins and the ORFs are located upstream of the RNA editing site (Bellini et al., 1985; Wang et al., 2001). Accessory proteins expressed from the P gene are involved in a variety of functions such as antagonizing Interferon

production (IFN), anti-apoptotic, inhibition of mRNA translation, and in viral budding (Sakaguchi et al., 2005). The most important function among them is the IFN antagonistic activity by V protein (Curran et al., 1992; Horikami et al., 1997; Hason et al., 2000; Huang et al., 2000; Koyama et al., 2003; Audsley et al., 2013).

V protein of NDV and other paramyxoviruses contain highly conserved carboxyl-terminal domain (CTD) with seven-cysteine residue and a single histidine residue that can form a zinc finger domain. This CTD is essential for the oligomeric structure formation and IFN antagonistic function of V protein (Liston et al., 1994). IFN antagonistic activity of V protein correlates with the virulence of NDV strains (Alamares et al., 2010). Mutations of the residues in the CTD of V protein are shown to disrupt the IFN antagonistic functions. V protein deleted mutants of NDV viruses has impaired growth in cell culture and chicken embryos, and are attenuated in virulence to chickens, which reveals their important role in pathogenicity of the virus (Mebatsion et al., 2001; Huang et al., 2003).

The functional role of W protein is known with Nipahvirus, where it acts as an IFN antagonist targeting Signal transducer and activator of transcription (STAT) proteins (Shaw et al., 2004; Ciancanelli et al., 2009). However the functional role of W protein is not known in other paramyxoviruses (Samal, 2011).

1.1.5 Replication cycle of APMV:

APMV employs the same replication strategy as that of other paramyxoviruses, which occurs in the cytoplasm of the infected cell (Lambs and Park, 2007). The initial requirement is the attachment of the viral glycoprotein HN to its specific sialic acid containing cell surface receptors. Adsorption of HN to the receptors induces a

conformational change, which in turn triggers change in the F protein leading to the fusion of the viral and the host cell membranes and release of the nucleocapsid into the cytoplasm (McGinnes et al., 2002). Viral entry occurs via two main pathways: a) by fusion of the viral envelope with the host cell membrane (pH independent) and b) by receptor mediated endocytosis (Hoekstra and Kok., 1989). An alternative route, caveolae–mediated endocytosis have also been shown for the NDV infection (Cantin et al., 2007). Viral polymerase transcribes the viral genome into mRNAs for the synthesis of viral proteins. Accumulation of viral proteins switches the viral polymerase from transcription to replication mode leading to synthesis of full length positive sense antigenomic, which serves as a template for synthesis of the negative sense progeny RNA genome. Interaction of P and L with the N-RNA template results in the nucleocapsid formation. The assembly and release of APMV infectious virions occurs at the membrane lipid rafts, where the proteins HN, F, and N accumulate (Laliberte et al., 2006). The interaction of M protein with other components of viral particle results in the transport and assembly of virus particle at the plasma membrane. Virus particle is released from the cell by the neuraminidase activity of the HN protein (Pantua et al., 2006).

1.1.6. Reverse genetics:

Reverse genetics is the method by which APMV infectious viruses can be generated entirely from cloned cDNA. Reverse genetics has facilitated the introduction of desired mutations into the viral genome and to rescue recombinant virus (Palese et al., 1996). Reverse genetics of NDV is accomplished by co-transfection of a cell with plasmid encoding for full-length viral anti-genome together with supporting plasmids encoding for N, P, and L proteins (which constitute the polymerase complex) under the

control of T7 RNA polymerase promoter (Palese et al., 1996; Peeters et al., 1999; Nagai, 1999). Source of T7 polymerase is usually supplemented by 1) recombinant vaccinia virus expressing T7 polymerase (MVA-T7) or 2) by a stable cell line constitutively expressing the T7 polymerase (Conzelmann, 1996; Nagai et al., 1999).

1.2 Newcastle disease virus:

In 1926, Kraneveld first recorded ND on the island of Java (Indonesia). Doyle reported a disease with similar characteristic on a farm near the seaport town of Newcastle-upon-Tyne (England) in 1927 and named the disease temporarily as "Newcastle disease" (Samal, 2011). In a brief time period, ND spread rapidly to multiple countries including Korea (1926), India (1927), Sri Lanka (1927), Philippines (1927), Japan (1929) and Australia (1930) and recognized as a new poultry disease. Even though the disease was given local names, 'Newcastle disease' remained popular and is still used (Samal, 2011).

NDV, a major pathogen of the poultry is distributed worldwide and is endemic in Africa, Asia, the Middle East, Central America and parts of South America. In USA, outbreaks caused by virulent strains are called the exotic ND (END), as the disease is not endemic and usually occurs by introduction of virulent strains during importation of exotic avian species.

1.2.1 Host range:

NDV strains have been reported to infect more than 240 species of birds representing 27/50 orders of the birds (Kaleta and Baldauf, 1988). The pathogenicity of ND varies depending on the host species and virus strains. Though avian species are the natural host for NDV, natural and experimental infection of non-avian species has been

reported in calves, swine, sheep, mice, guinea pigs, rabbits, ferrets, hamsters and monkeys. NDV infection is recognized as an occupational hazard to humans working in poultry related operations, and laboratory personnel and usually causes mild, transient conjunctivitis or flu-like symptoms in humans (Reagan et al., 1947; DiNapoli et al., 2007; Subbiah et al., 2008).

1.2.2 NDV genome:

NDV strains genome length exist in 3 categories - a) 15,186 nucleotides (nts) found in strains isolated before 1960 (Krishnamurthy and Samal, 1998), b) 15,192 nts found in isolates from china (Huang et al., 2004), and c) 15,198 nts found in an isolate from Germany (Czegledi et al., 2006) all following the 'rule of six'. Based on the phylogenetic analysis of the genome sequence, NDV strains are divided into 2 classes: Class I are viruses of low virulence isolated from waterfowls and Class II includes majority of virulent and avirulent strains isolated from the poultry, pet and wild birds (Samal, 2011).

1.2.3 Pathogenicity of NDV:

In chickens, NDV strains cause a broad range of clinical signs, which can vary from an asymptomatic form to a highly virulent form causing mortality up to 100%. Based on the virulence, the strains are classified into three major pathotypes: a) Lentogenic – low virulent strains causing mild respiratory disease or subclinical infection, b) Mesogenic – moderately virulent strains causing severe respiratory disease and mortality only in young birds, and c) Velogenic – highly virulent strains causing severe disease with high mortality. Velogenic strains are further classified into Viscerotropic – causing mortality with hemorrhagic lesions in the intestinal tract of the

birds and Neurotropic – causing mortality with predominant neurological and respiratory signs.

1.2.4 Determinants of Virulence:

Viral envelope glycoproteins, F and HN are considered to be the major virulence determinants of NDV (Zohari and Munir, 2013). The amino acid sequence at the precursor F_o protein cleavage site determines the substrate specificity of the cellular proteases, allowing the viral replication restricted to the respiratory or gastrointestinal sites or causing systemic infection (Peeters et al., 1999; Panda et al., 2004). The HN protein having the receptor recognition activities has been shown to contribute to the tissue tropism and virulence (Huang et al., 2004). V protein has been shown to antagonize the IFN response and play an important role in viral pathogenicity (Mebatsion et al., 2001). Experimental results have also shown the contribution of the viral polymerase complex (N, P, and L) proteins to the viral virulence (Samal, 2011).

1.2.5 Transmission and Spread:

NDV is a highly infectious virus, which can rapidly spread among the susceptible population either by inhalation or ingestion of contaminated feed particles. Infected birds shed high amount of virus in its secretion and excretion, which acts as a primary source for the virus spread and infection (Samal, 2011).

1.2.6 Clinical signs:

Clinical signs may vary widely in NDV infected birds depending on the virulence of the virus, host species, age, immune status of the bird, route of exposure and environmental conditions. The incubation period for ND varies from 2-15 days and young birds are the most susceptible. Depending on the predilection site

(respiratory/digestive/nervous system) of the virus strains, the birds may exhibit signs such as nasal discharge, gasping for air, greenish watery diarrhea, depression, muscular tremors, drooping wings, twisting of head and neck, circling and complete paralysis. Thin-shelled eggs, partial to complete drop in egg production, edema of eyelids and neck usually accompany the symptoms and sometimes lead to sudden death. Mortality is highly variable and can be up to 100% with velogenic form of infection.

1.2.7 ND outbreak and economic impact:

Despite the availability and usage of vaccination worldwide since 1950, ND still continuous to be a global threat because of the virus circulation among the reservoir population in domestic and wild bird species (Tolf et al., 2013; Mahmood et al, 2014; Nath et al., 2015). According to Global Animal Health Data report (2006-2009), ND is the second most wide spread animal diseases in many countries around the world. ND is also ranked fourth in terms of the number of livestock units lost due to the disease outbreak (Kapczynski et al., 2013). Backyard poultry represents a major source of income for the people in the rural areas of developing countries. Virulent ND outbreaks will result in complete devastation of the flocks causing a severe economic impact on the life of people. A large number of ND outbreaks has occurred world wide in the recent years, with isolation of new strains of NDV (Dortmans et al., 2012; Kumar et al., 2015). 1,211 ND outbreaks have occurred in 2010 in vietnam alone. Between 2005-2013, 18 NDV strains were isolated from domestic duck samples from different parts of china (Liu et al., 2013; Wu et al.,2015). Reports of isolation of velogenic strains have been reported from many parts of the world; 12 from vietnam between 2007 and 2012, 3 from

Cambodia during 2012-13 and some from egypt between 2011 and 2012, indicating its global threat (Choi et al., 2013; Choi et al., 2014; Radwan et al., 2013).

In USA, velogenic ND strains (END) affecting Cormorants and gulls have been reported from the states of Minnesota, Massachusetts, Maine, New Hampshire and Maryland in 2010 (Diel et al., 2012). END outbreaks could potentially lead to huge economic impact for the poultry industry. For example, 1971 END outbreak in California resulted in depopulating almost 12 million birds and costed $56 million to fight. Last outbreak of END among domestic chicken occurred in the year 2002-03 in California. The outbreak response costed around $161 million and resulted in depopulating 3.16 million birds (Samal., 2011).

1.2.8 Vaccination:

Vaccination is an effective measure for the prevention and control of ND. Lentogenic strains (Hitchner B_1, LaSota, V4, I2) or mesogenic strains (Roakin, Mukteshwar, Komarov) of NDV elicits adequate immune response in birds and are most commonly used in commercial vaccines (Samal., 2011). Live vaccines are usually prepared using lentogenic strains by harvesting the allantoic fluids from infected embryonated fowl eggs and are administered by aerosol sprays or by mixing in drinking water or feed. Inactivated vaccines are prepared with addition of formaldehyde or beta–propiolactone to the infective virus stock and are administered to the poultry flock by either intramuscular or subcutaneous route. Recombinant viral vectors expressing HN gene or F gene or both are also in use against the control of ND (Morrison et al., 1990; Samal, 2011). Several vaccination strategies are followed depending on the requirement such as age, immune status of the birds, disease potential and the national requirement.

Layers are usually vaccinated with a live vaccine followed by boosters with inactivated vaccines at sufficient intervals to maintain adequate immunity.

1.2.9 Pitfalls of current vaccination and possible solution:

Commercially available ND vaccines are heat sensitive and require a uninterrupted cold chain maintenance during transport and storage to maintain the quality of vaccines. Cold chain maintenance is the major cost component of NDV vaccination and accounts for around 80% of the total cost (Das, 2004; Wen et al., 2015). The reliability of cold chain maintenance is questionable due to inappropriate cold chain equipments, temperature fluctuations, power outrages and human error. Inadequate cold chain maintenance may result in loss of vaccine potency and inadequate protection against the disease. The available commercial vaccines are relatively successful in the commercial poultry flocks. However, NDV vaccination is less effective among small scale rural flocks because of inadequate cold chain facilities and prohibitive cost associated with vaccination programme (Aini et al., 1990; Adwar and Lukesova, 2008). The situation is particularly worse in developing and tropical countries due to lack of continuous supply of electricity, and the lack of refrigeration facilities, and equipments. The possible solution to the problem would be the development of a thermostable vaccine, which can sustain the tropical climate without the requirement for the cold chain supply.

1.2.10 Research on NDV thermostability:

The ability of a substance to retain its activity at a particular level after treatment at a specified temperature and time is called thermostability (Orsi et al., 2009). Thermostability of vaccine is determined by the length of time the vacccine retains

infectivity titer at a particular temperature to induce protective immune response (Orsi et al., 2009).

Research on thermostability of NDV generated considerable interest since 1930 (Hanson et al., 1949; Goldman and Hanson, 1955; Tolba and Eskarous, 1959; Cole and Hutt, 1961). Studies have shown that NDV strains lost their infectivity upon heat treatment for 30 minutes at 50-55 ^{0}C (Lomniczi, 1975). Hanson and other researchers have conducted thermostability studies on the hemagglutinin of NDV strains. They proposed that the virulence of NDV is related to its thermostability; where Lentogenic strains of NDV were found to be heat labile and mesogenic, velogenic strains were heat stable maintaining their activity at 56 ^{0}C for at least 30 minutes (Hanson et al., 1967; Lomniczi, 1975). However, it was proved to be false in the year 1971 when McFerran and Nelson isolated a lentogenic strain of NDV with relatively higher thermostabile hemagglutinin than other velogenic strains (McFerran and Nelson, 1971) .

In 1975, Lomniczi conducted experiments for the first time on the thermostability of infectivity along with hemagglutinin activity of NDV strains in correlation to their virulence. He found that the thermoresistance and virulence are not correlated to each other in NDV (Lomniczi, 1975). He proposed the following criteria to define thermostable NDV strains. Strains that show less than 2 logarithmic reductions in their hemagglutinin and infectivity titer after heat treatment at 56 ^{0}C for 30 min and 10 min, respectively, are considered as thermostable. Rapid strides in sequencing technology over the last two decades have resulted in increased availability of complete genome sequences of NDV strains (15.2 kb length). Many researchers have attempted to analyze the genotype contributing to the thermostable nature of the NDV strains (Wen et al.,

2013; Rani et al., 2014). Possibility of the alterations in the HN and L gene contributing to the thermostability were tested with no conclusive results (Tan et al., 1995; Yusoff et al., 1996; Kattenbelt et al, 2006; Uthrakumar et al., 2013). Wen et al., (2013) suggested that difference in the length of W protein could be a important determinant in the thermostability of NDV strains (Wen et al., 2013). However this hypothesis was not tested before.

1.3 Avian paramyxovirus serotypes:

The pathogenicity or disease potential of APMV-2 to APMV-12 is not well established, as most of these serotypes are isolated from domestic, pet and wild birds dying in quarantine or hunter-killed birds or while surveillance for other poultry pathogens or from apparently healthy birds (Samal, 2011). Recent evidences have shown an increased incidence of APMV outbreaks in wild and migratory birds posing significant threat to the domestic bird population (Fornells et al., 2013; Umali et al., 2014). Knowledge on the molecular and biological characteristics of APMVs is increasing, because of the availability of the complete genome sequence and the reverse genetics system. Among the APMV serotypes, APMV -2, -3,-6 and -7 have been shown to be associated with diseases in chickens and turkeys causing respiratory disease and drop in egg production (Tumova et al., 1979; Bankowski et al., 1981; Redmann., 1991; Alexander and Senne., 2008; Kumar et al., 2010).

1.3.1 Avian paramyxovirus type 2:

APMV-2 was first isolated in the year 1956 (Yucaipa, California) from a diseased chicken infected with infectious laryngeotracheitis virus (Bankowski et al., 1960). APMV-2 has been isolated from chickens, turkeys and wild birds across the world (North

America, Europe, Africa and Asia). Frequent isolation of APMV-2 from passerine and psittacine species suggests that these bird species could be potential reservoirs. In turkeys, APMV-2 infection has been shown to affect the hatchability and poult yield (Lipkind et al.,1979). Experimental infecion of chickens and turkeys with strains of APMV-2 suggested that chickens are comparitively more susceptible than turkeys (Subbiah et al., 2010). Strains of APMV-2: Yucaipa, Bangor, England and Kenya have genome lengths of 14904, 15024, 14904 and 14916 nts respectively, with the genome order conserved as that of other APMVs (Gogoi et al., 2015).

1.3.2 Avian paramyxovirus type 3:

APMV-3 was first isolated from turkeys in Ontario (Canada) in 1967 and Wisconsin (USA) in 1968 (Tumova et al., 1979). APMV-3 has been isolated from variety of domestic and wild birds, such as turkeys and ostrich indicating a wide host range (Kaleta et al., 2010). APMV-3 infection has been associated with encephalitis and high mortality in caged birds, severe respiratory disease in turkey, and acute pancreatitis and CNS symptoms in psittacine and passeriformes birds (Tumova et al., 1979; Beck et al., 2003). Experimental infection of young chickens resulted in virus replication in brain leading to intracerebral infection and stunted growth (Alexander and Collins, 1982; Kim et al., 2012). Among APMV serotypes, APMV-3 has been considered as an important pathogen to domestic poultry next to NDV in terms of its pathogenicity. Inactivated vaccines against APMV-3 are in-use for turkey breeder flocks. Netherlands and Wisconsin strains of APMV-3 have genome lengths of 16272 and 16182 nts respectively, with the genome order conserved as other APMVs. Prior infection of chickens with

APMV-3 conferred cross-protection against challenge with virulent NDV (Alexander et al., 1979).

1.3.3 Avian paramyxovirus type 6:

APMV-6 was first isolated in 1977 (Hong Kong) from an apparently healthy domestic ducks. Subsequently, it was isolated from ducks, geese and turkeys around the world (Shortridge et al., 1980; Stanislawek et al., 2002; Warke et al., 2008). Experimentally infection of APMV-6 have shown their apathogenic nature in chickens, but still causes mild respiratory disease, and drop in egg production in turkeys. Genome length of APMV-3 strains varies between 16230 and 16236 nts (Chang et al., 2001; Xiao et al., 2010). The genome consists of 7 genes in the order of 3'-N-P-M-F-SH-HN-L-5'), which differs from other APMVs by having an additional gene which codes for the small hydrophobic protein (SH). The functional role of SH protein in the lifecycle of APMV-6 is not known (Chang et al., 2001).

1.4 Innate immunity:

The germ line encoded innate immune mechanism in a cell is the first line of defense against any invading pathogen, which acts by recognizing the pathogen associated molecular patterns (PAMPs). Innate immune mechanism protects the cell against the invading viruses by the production of IFN, whose main function is to interfere with the viral replication and also plays important role in immunomodulatory functions (Honda et al., 2005; Abbas et al., 2011).

1.4.1 Receptors of Viral infection:

Innate immunity is activated by the detection of viral components such as genomic DNA, single-stranded RNA (ssRNA), double-stranded RNA (dsRNA), RNA

with 5'-triphosphate ends and viral proteins through three types of pattern recognition receptors (PRRs) namely Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors, and nucleotide oligomerization domain (NOD)-like receptors (NLRs) (Seth et al., 2006; Takeuchi and Akira, 2008).

Recognition of the viral components by the PRRs such as TLRs and RLRs activates the intracellular signaling cascade leading to the expression of type I IFNs, proinflammatory cytokines, chemokines and large number of co-stimulatory molecules. The expression of type I IFN further activates the intracellular signaling through the type I IFN receptor resulting in the expression of antiviral genes such as protein kinase R, 2'5'-oligoadynylate synthase which results in the control and elimination of the viral infection (Sadler and Williams, 2008).

TLR family of receptors have been involved in the detection of viral nucleic acid in the extracellular and endosomal compartments: TLR3 recognizes dsRNA; TLR7 and 8 recognize ssRNA while TLR9 recognizes the dsRNA of the viruses (Barton, 2007). TLR 2 and TLR 4 have been shown to detect the presence of viral glycoproteins (Xagorari and Chlichlia, 2008; Gaudreault et al., 2007).

The members of RLRs such as Retinoic acid–inducible gene 1 (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology-2 (LGP2) act as cytoplasmic sensors of viral infection. RIG-I and MDA-5 are the two main RLRs involved in the detection of cytoplasmic viral nucleic acids such as dsRNA and ssRNA (Gitlin et al., 2010). dsRNA is the replicative intermediate for the RNA viruses and ssRNA is formed during the transcription of the viral genomes (Kato et al., 2006; Abbas et al., 2011; Loo and Gale., 2011).

1.4.2 Intracellular detection of Paramyxovirus infection and IFN induction:

Paramyxoviruses are cytosolic replicating RNA viruses, which are recognized by the RIG-I/MDA-5 receptors. RIG-I and MDA-5 consists of carboxyl-terminal DExD/H RNA helicase domain and two amino-terminal caspase-recruiting domain (CARD)-like regions, which are involved in the recognition of dsRNA and recruiting the downstream signaling molecules respectively. Binding of the viral RNA to the helicase domain triggers a conformational change, allowing the CARD domain to interact with the downstream adaptor protein Cardif/MAVS/IPS-1, that leads to the activation of the transcription factors IRF-3 and NF-κB in the cytosol (Kawai et al., 2005; Meylan et al., 2005; Loo et al., 2008). Upon activation, IRF-3 and NF-κB are translocated to the nucleus forming an enhanceosome required for the induction of the IFN-β gene. IFN-β production results in the up-regulation of the transcription factor IRF-7 through a positive feed back mechanism and further enhance the production of IFN inside the host cell (Chambers and Takimoto., 2009).

1.4.3 IFN signaling: Jak/STAT pathway:

Interferons bind to IFN-α/β receptors and induce intracellular signaling pathway, which results in the expression of antiviral proteins. The type I IFN receptors are trans-membrane glycoproteins expressed on the cell surface. Extracellular domain of the receptor binds with the IFN, whereas the cytoplasmic domain binds with the Jak, STAT and other signaling molecules. The receptor consists of two subunits namely IFNAR1 and IFNAR2. In the resting state, the cytoplasmic tail of IFNAR1 is bound to tyrosine kinase 2 (Tyk2) and IFNAR2 is associated with Janus kinase (Jak 1). Binding of IFN promotes dimerization of the receptor and induces a conformational change that results in

the phosphorylation of the tyrosine residues on the cytoplasmic portion of the IFNAR1 by Tyk2. The phosphorylated tyrosine residue on IFNAR1 serves a docking site for STAT2. Then Tyk2 and Jak1 phosphorylate tyrosine 690 on STAT2 and tyrosine 701 on STAT1 respectively (Darnell et al., 1994; Takaoka and Yanai., 2006). Phosphorylated STAT1 and STAT2 form a heterodimer complex and translocate to the nucleus, where it binds with IRF-9 to form the heterotrimer, IFN-stimulated gene factor 3 (ISGF3). The ISGF3 complex binds to the IFN-stimulated response element (ISRE) on the 5' regulatory regions of the IFN-responsive gene promoters resulting in the expression of antiviral proteins such as protein kinase R, 2'5'-oligoadenylate synthetase, Mx GTPase (Randall and Goodbourn., 2008).

1.4.4 Antagonism of innate immunity by the accessory proteins:

In general, viruses exhibit a variety of mechanism to counteract and interfere with the IFN response. Paramyxoviruses antagonize IFN response with the help of accessory proteins such as V, W, and C proteins (Gotoh et al., 2001; Kato et al., 2001). Paramyxoviruses utilize various strategies to inhibit both IFN production and signalling by targeting various cellular proteins (Horvath, 2004; Audsley et al., 2013).

1.4.4.1 Antagonizing IFN production :

Paramyxoviruses utilize their V proteins to bind MDA5 and inhibit the IFN induction. It is the most common mechanism and is reported in atleast 13 paramyxoviruses (Andrejeva et al., 2004; Childs et al., 2007; Childs et al., 2009; Parisien et al., 2009; Ramachandran and Horvath, 2010). V proteins of paramyxoviruses unfold ATP-hydrolysis domain of MDA5 and prevent the RNA bound MDA5 filament

formation, thereby inhibiting the antiviral signaling (Motz et al., 2013; Wu and Hur., 2013).

V proteins also target the downstream signalling components and nuclear translocation to prevent activation of IRF3. Interaction of Mumpsvirus, human parainfluenza-2, parainfluenzavirus-5 V protein with the signaling kinases [TANK-bindng kinases 1 (TBK1) and inhibitor of κB kinase ε (IKKe)] prevent the phosphorylation of IRF3 and facilitate the polyubiquitination and degradation of the kinases and there by interferes with downstream signaling (Lu et al., 2008). Sendai virus and NDV V protein has also been shown to interact with IRF3 and prevent its activation and nuclear translocation (Irie et al., 2012).

1.4.4.2 Antagonizing IFN signalling :

V protein of paramyxoviruses form complexes with STAT proteins, and components of E3 ubiquitin ligase [DDB1 and Cul4A], which results in the proteosomal mediated degradation of STAT proteins. (Andrejeva et al., 2002; Li et al., 2006; Ulane and Horvath, 2002). Some of the paramyxoviruses (*Rubulavirus*), utilize their V protein to target either STAT1 or STAT2 for degradation, with the help of the nondegraded STAT as a co-factor. For example, Simian virus 5 V targets STAT1 for degradation, whereas human parainfluenza virus type-2 V targets STAT2 for degradation (Huang et al., 2003; Precious et al., 2005). Mapeuravirus V protein binds both STAT proteins and and affect their phosphorylation and translocation, without inducing the degradation of STAT proteins (Hagmaier et al., 2007). V protein of Measles virus interacts with JAK1 and STAT2, and inhibit STAT1 phosphorylation, and thereby inhibits the IFN signaling (Takeuchi et al., 2003; Palosaari et al., 2003; Caignard et al., 2007; Caignard et al., 2009).

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Chapter 2

Evaluation of the potential role of Newcastle Disease Virus W protein in virus thermostability

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2.1 Introduction:

Newcastle disease (ND) is a highly contagious and globally important viral disease of avian species. ND is considered to be one of the important pathogens of poultry because of the severity of the disease and the economic losses associated with the disease outbreak. Newcastle disease virus (NDV), the causative agent, infects more than 246 species of birds and shows a spectrum of virulence from asymptomatic to highly lethal (1, 2). Based on the virulence of the disease in chickens, NDV is classified into 3 major pathotypes: a) Lentogenic or avirulent pathotype causing asymptomatic or mild respiratory disease, b) Mesogenic or mildly pathogenic pathotype causing severe respiratory signs with mortality only in very young birds, and c) Velogenic or virulent pathotype causing high mortality up to 100% (3). ND is classified by the Office International des Epizootics (OIE) as notifiable List-A disease when caused by virulent strains of NDV (3, 4).

NDV, a non-segmented, enveloped, negative-stranded RNA virus, belongs to the genus *Avulavirus* under the family *Paramyxoviridae* (5). The genomic RNA of NDV is approximately 15.2 kb in size with six genes in the order of 3'-NP-P-M-F-HN-L-5', coding for six structural proteins namely nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN) and the large polymerase protein (L). HN and F are envelope glycoproteins mediating the viral attachment and fusion with the host cell membrane (6). M protein underlying the viral envelope interacts with other viral proteins and mediates assembly and budding of the virus particle. NP, P and L proteins are found inside the virion forming the viral polymerase complex (5). RNA editing during transcription of P gene results in two

accessory proteins, V and W. RNA editing occurs by stuttering of RNA polymerase at the editing site of P gene which results in insertion of non-templated ‘G’ nucleotides leading to frame shift (7-9). Addition of one and two non-templated ‘G’ nucleotides results in the expression of the V and W proteins, respectively (8). V protein acts as an interferon-antagonist and is one of the important virulence determinants of NDV (10-12). However, the functional importance of W protein is not known yet (2).

Vaccination is an effective measure for the prevention and control of ND. Effective vaccines are available since 1950’s and vaccination program against ND is in practice through out the world (13, 14). The available commercial vaccines are heat sensitive, because of the thermolabile nature of vaccine strains, which requires uninterrupted cold chain maintenance during transport and storage, to maintain the quality of vaccines (15). Cold chain maintenance is the major cost component of NDV vaccination and accounts for around 80% of the total cost (16, 17). The reliability of cold chain maintenance is questionable due to inappropriate cold chain equipment, temperature fluctuations, power outrages and human error. Poor cold chain maintenance may result in loss of vaccine potency and inadequate protection against the disease.

ND vaccination is relatively successful in commercial poultry flocks. However, it is less effective among small-scale rural flocks because of inadequate cold chain facilities and poor cost effectiveness associated with vaccination programme (15, 18). The situation is particularly worse in developing and tropical countries due to lack of continuous supply of electricity and the lack of refrigeration infrastructure. Because of this ND outbreak still occurs, particularly in developing and tropical countries (19, 20). The possible solution for the problem could be production of thermostable vaccines,

which can sustain temperature fluctuation and retain its potency independent of cold chain (21).

Though naturally occurring thermolabile and thermostable strains of NDV are isolated from around the world, the molecular mechanism conferring thermostability to NDV is not known. Rapid strides in sequencing technology over the last two decades have resulted in an increased availability of complete genome sequences of NDV strains (15.2 kb length). Many researchers have attempted to analyze the genotype contributing to the thermostable nature of the NDV strains (22). Possibility of the alterations in the HN and L gene contributing to the thermostability were tested with no conclusive results (23-26). Wen et al (2013) suggested that difference in the length of W protein could be a important determinant in the thermostability of NDV strains (22). However this was never experimentally tested before.

When we compared the amino acid sequences of W protein of different NDV strains, we found that thermostable strains contain truncated W protein (147 aa length) when compared to thermolabile strains (>175 aa length). We hypothesized that the difference in the length of W protein influences the thermostability of NDV. In this study, therefore we introduced mutations in the antigenome of the most commonly used thermolabile LaSota vaccine strain to rescue recombinant NDV viruses with differing lengths of W protein and tested for their thermostability.

2.2 Materials and Methods:

Cells and Virus strain:

Chicken embryo fibroblast (DF1) and Baby hamster kidney fibroblast (BHK21) cells were obtained from American Type Culture Collection (ATCC). Cells were grown

in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% heat inactivated Fetal Calf Serum (FCS) (Thermo scientific) and 1% Penicillin-Streptomycin (Invitrogen). Lentogenic NDV strain, LaSota was used in this study.

Cloning and construction of recombinant plasmids:

The plasmid pLaSota-eGFP (pLaS) expressing the full-length anti-genome of LaSota with an extra cistron insertion for the expression of the enhanced green fluorescent protein (eGFP) as described previously (27, 28) was used for the construction of mutant W plasmid (pLaS147W) and rescue recombinant viruses. The AscI-SacII fragment encoding for the portion of P gene was amplified from pLaS plasmid using specific primer pairs flanked with XbaI and HindIII restriction sites (FP: 5'-ATATATTCTAGATCGGCGCGCCTAATACGACTCAC-3' and RP:5'-ATATAAGCTTCCGCGGCTGGGTTGACTC-3') and subcloned into pcDNA3.1(+) vector between XbaI and HindIII as described previously (27). Subsequently, the proline residue at position 147 was mutated to leucine to ensure the expression of W protein of 147 amino acid length using QuikChange II site directed mutagenesis kit (Agilent Technologies) (FP: 5'GGAATCACCAACGTCTGACTCAACAGCAGGGG-3' and RP: 5'CCCCTGCTGTTGAGTCaGACGTTGGTGATTCC-3'). The introduced mutation was silent regarding the P and V reading frame. The mutated AscI-SacII portion was digested from subclone and ligated to the full-length pLaS plasmid to generate pLaS147W. The mutated sequence of pLaS147W was confirmed by sequencing at Virginia bioinformatics institute (VBI) sequencing facility at Virginia Tech.

Rescue of recombinant viruses:

Recombinant rLaS-eGFP and rLaS147W-eGFP viruses were rescued by reverse genetics system as described previously (29). Briefly, BHK21 cells were seeded in a 6-well plate, grown to 70-80% confluency, and infected with Vaccinia MVA-T7 virus. Later, the cells were transfected with plasmids encoding the full-length pLaS and pLaS147W antigenome, respectively, along with support plasmids encoding NP, P and L proteins. Transfection was done using Lipofectamine® LTX Reagent with PLUS™ Reagent according to manufacturers protocol (Invitrogen). Transfection media was removed 6 hours post-transfection and the cells were washed with Phosphate Buffered Saline (PBS) and replaced with DMEM, 2% FCS, 1% Penicillin-Streptomycin, and 0.2 μg/ml of tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich). After 72 hours, supernatants were harvested and propagated in 10-day old embryonated specific pathogen free (SPF) chicken eggs. Allantoic fluids were collected and tested for the virus specificity by hemagglutination assay and hemagglutination inhibition assay with specific NDV antisera. The recovered recombinant viruses were plaque-purified prior to amplification and further characterization.

Identification of the recombinant viruses by RT-PCR:

The introduced mutations in the P gene segment were confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) and sequencing of the P gene. Briefly, genomic RNA was extracted from rescued recombinant viruses by TRIzol reagent (Invitrogen) as previously described (30). Single-stranded complementary DNA (cDNA) was prepared by High Capacity cDNA Reverse Transcription kit (Applied Biosystems) with viral gene-specific primers. Using P gene specific primers, PCR was performed and

the amplified product was confirmed by sequencing at Virginia bioinformatics institute (VBI) sequencing facility at Virginia Tech.

Thermostability test:

For the thermostability test, 1.0 ml of undiluted allantoic fluids of the recombinant viruses-eGFP and rLaS147W-eGFP were taken in airtight vials and incubated at 56 ^{0}C in a water bath for varying time intervals. After the incubation period, the respective vials were transferred on to ice to stop heat treatment, and samples were subsequently tested for stability by hemagglutinin assay and infectivity titration.

Hemagglutinin Assay:

Hemagglutination assay was performed in V-bottomed micro-titer plates as per the standard micro-hemagglutination procedure using 2-fold serial dilution of the virus sample in 50 μl of PBS. Equal volume of washed 1% chicken RBC was added to each well and incubated at room temperature for 45 minutes. The agglutination titers were calculated as the reciprocal of the highest dilution of the sample giving 100% agglutination of chicken RBC (4, 31).

Titration of Virus Infectivity:

Infectivity titers of the samples were determined by plaque-titration assay. Titration was done using DF1 cells on a 12-well plate as described before (32, 33). Approximately 200 μl of the 10-fold serially-diluted samples were added on to DF1 cell monolayer. After one hour of infection, supernatant was removed and cells washed with PBS. An overlay consisting of DMEM with 0.8% methylcellulose, 1% Penicillin-Streptomycin and 0.2ug/ml of TPCK-trypsin were then added. After 5 days of incubation,

overlay was removed and fixed with 0.01% crystal violet in formalin, and plaques were quantified.

2.3 Results:

Generation of rLaS-eGFP and rLaS147W-eGFP viruses:

Amino acid sequence analysis of selective avirulent thermostable and thermolabile strains of NDV revealed the presence of a truncated W protein in thermostable strains (147aa) (Figure 2.1). The pLaS plasmid expressing the full-length W protein tagged with GFP was used as the backbone to create mutant pLaS147W plasmid expressing truncated W protein (147aa). The AscI-SacII fragment from pLaS was introduced into pcDNA3.1(+) subclone. By using site-directed mutagenesis, proline (CCG) at position 147 in the P gene is mutated to leucine (CTG). This mutation results in the introduction of a stop codon at 148th aa during mRNA transcription of the W protein by RNA editing mechanism, but is silent in the P gene segment with regard to the expression of the P and V proteins. The mutated AscI-SacII segment from the subclone was digested and ligated back to the full-length anti-genome to create the pLaS147W and was subsequently confirmed through sequencing of the P gene segment.

Using reverse genetics system, the recombinant viruses rLaS-eGFP and rLaS147W-eGFP were rescued by transfection of support plasmids expressing NP, P and L proteins along with the full-length anti-genome pLaS and pLaS147W, respectively, in BHK21 cells. Replication of the virus was visualized using tagged GFP protein under the UV-microscope (Figure 2.2) and the supernatant was subsequently amplified in 10-day old embryonated chicken eggs. The sequences of W gene segments were confirmed by

RT-PCR and sequencing (Figure 2.3). Both recombinant viruses contain critical sequences necessary for efficient W protein translation.

Stability of Hemagglutinin and Infectivity following exposure at 56 ^{0}C:

Thermostability of NDV strains is assessed by the stability of hemagglutinin and infectivity following exposure at a higher temperature, usually at 56 ^{0}C (26, 33). The criteria for the classification of NDV strains as thermostable is the time taken for 2 logarithmic reduction in HA activity and viral infectivity to be >30 and >10 minutes respectively (33).

The hemagglutinin stability of recombinant viruses rLaS-eGFP and rLaS147W-eGFP were tested at 56 ^{0}C for different time points (0, 5, 10, 15, 30, and 60 minutes) and both the viruses had a 2 $\log_2$ decrease in HA activity within 5 minutes. By 10 minutes both the viruses lost their ability to hemagglutinate the cRBC's. The loss of infectivity of the recombinant viruses rLaS-eGFP and rLaS147W-eGFP were tested at 56 ^{0}C for different time points (0, 5, 10, and 15 minutes) using plaque assay and both the viruses showed 2 logarithmic reduction in viral titers within 10 minutes. In summary, Hemagglutination stability and virus infectivity tests conducted after heat treatment at 56 ^{0}C showed that both the recombinant viruses rLaS-eGFP and rLaS147W-eGFP were thermolabile in nature with no significant difference in titers (Table 2.1).

2.4 Discussion:

ND is a highly pathogenic viral disease of avian species causing huge economic loss because of the high morbidity and mortality associated with the disease. Even though commercial vaccines are available, ND continues to be a threat worldwide and is endemic in many developing and tropical countries (19). One of the major reasons is the use of

thermolabile NDV strains vaccine preparation, which requires uninterrupted cold chain maintenance.

Experiments have shown that treatment with different solvents did not have any drastic effect on the stability of NDV, suggesting the minimal effect of pH and solvent on viability. However temperature had a major effect on the viability of the virus (34, 35). So the effective solution for the development of a thermostable NDV vaccine would be the use of a thermostable strain in vaccine preparation, which can withstand the effect of temperature.

Thermostability of NDV is tested by their hemagglutinin stability and viral infectivity at 56 ^{0}C. Hemagglutination is a sole property of HN protein, whereas infectivity is a property of an intact virion requiring the envelope proteins and the polymerase complex for the effective viral replication and infection (35, 36). Any internal or external protein, which could alter the composition and organization of the virion structure and envelope glycoproteins, may be a possible reason contributing for the thermostability of the virus. The function of the accessory protein W is unknown. Difference in the length of W protein between thermostable and thermolabile NDV strains suggests that W protein could contribute to the thermostability of the virus.

Our results with hemagglutination stability and viral infectivity of rescued recombinant NDV viruses with differing W protein lengths showed that rescued rLas147W retained their property of hemagglutinin and infectivity as its parental rLaS and the shorter length of W protein did not affect its thermolabile nature. Our experimental results show that the difference in the length of W protein does not impact the thermostability of NDV. We introduced a single stop codon to truncate the W protein

for a shorter length but we did not study the mutations in the N-terminal regions between the strains, which could also be a possible reason for retention of their thermolabile character.

A recent study showed that chimeric NDV LaSota strain bearing the HN protein from a thermostable virus exhibited thermostable phenotype, suggesting that HN is a crucial determinant for thermostability of NDV (37). But the precise mechanism of HN-mediating thermostability is not yet known. So further research should be done on understanding the basic mechanism of HN in NDV thermostability. Future studies should also be directed at determining which amino acids in HN protein are critical for viral thermostability. Whether HN acts alone or in concert with other proteins, such as W protein needs to be studied. Improved knowledge about the genetic basis of thermostability in NDV could aid the rational design of thermostable NDV vaccines with better vaccine efficacy and reduced dependence on cold chain, especially for the developing and tropical countries.

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Figure 2.1: Sequence comparison of thermostable and thermolabile strains of NDV. Genome sequences of thermostable NDV strains such as V4, I2 and TS09C were compared with those of thermolabile strains such as B1 and LaSota using DNASTAR (Lasergene) software. All thermostable strains had a truncated W protein with a stop codon at 148th aa position.

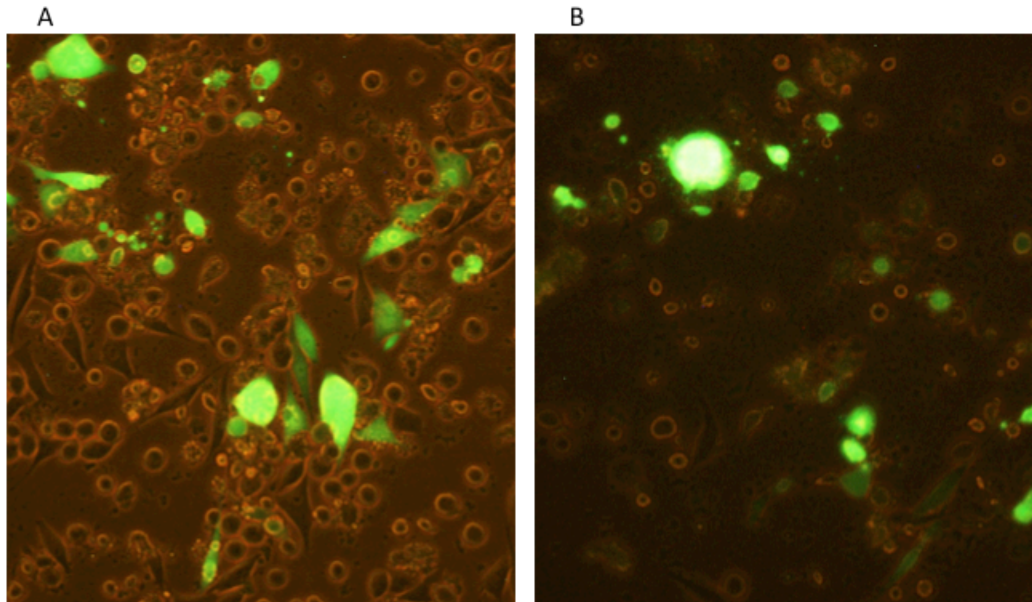

Figure 2.2: Rescue and Replication of recombinant rLaS147W-eGFP and rLaS-eGFP viruses. Using reverse genetics system, the recombinant viruses rLaS-eGFP and rLaS147W-eGFP were rescued. BHK-21 cells pre-infected with Modified vaccinia virus expressing T7-polymerase were co-transfected with support plasmids expressing NP, P and L proteins along with full-length anti-genome pLaS147W and pLaSW, respectively. After 72 hours of transfection, replication of the viruses rLaS147W-eGFP (A) and rLaS-eGFP (B) were visualized under UV-microscope.

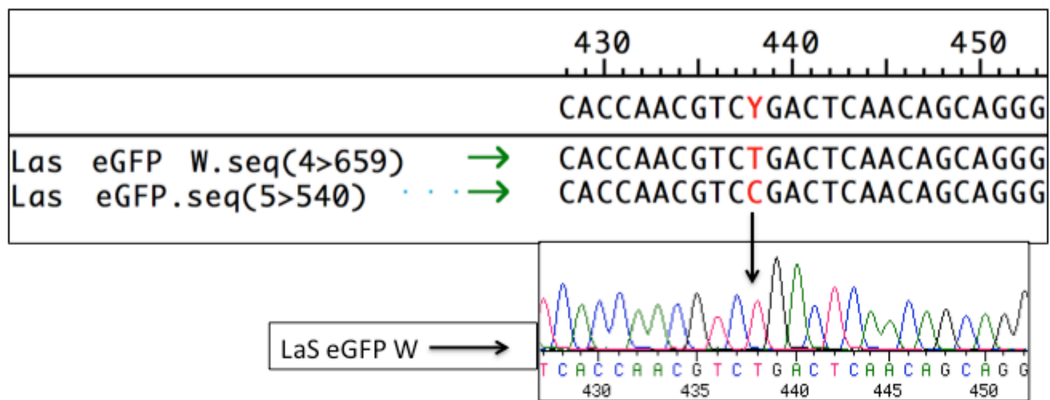

Figure 2.3: Confirmation of introduced mutation in rLaS147W-eGFP virus by RT-PCR. Presence of the introduced mutation in the rescued virus rLaS147W-eGFP was confirmed by RT-PCR and sequencing. The sequencing result showed the presence of an introduced TGA stop codon to express truncated W protein.

Heat treatment time (At 56 ^{0}C)	Log$_2$ decrease in HA activity		Log$_{10}$ decrease in infectivity	
	rLas-eGFP	rLas147W-eGFP	rLas-eGFP	rLas147W-eGFP
5 min	2	2	1	1
10 min	8	8	2	2

Table 2.1: Stability of Hemagglutinin and Infectivity following exposure at 56 ^{0}C.

The rescued recombinant viruses rLaS-eGFP and rLaS147W-eGFP were tested for stability of hemagglutinin and infectivity at 56 ^{0}C. Both the viruses had 2 logarithmic reductions in their respective values within 10 minutes confirming their thermolabile nature.

Chapter 3

Evaluation of the potential role of V protein of APMV-2, -3 and -6 in interferon antagonism

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3.1 Introduction:

Avian paramyxoviruses (APMV) are a group of viruses that belongs to the genus *Avulavirus* and family *Paramyxoviridae*. Based on hemagglutinin inhibition (HI) and neuraminidase inhibition (NI) assays, these viruses are classified into twelve distinct serotypes: APMV-1 to APMV-12 (1). Newcastle disease virus (NDV), a highly contagious and globally important pathogen belongs to APMV-1. APMV-1 is the most well-characterized serotype, because of the huge economic losses associated with the disease. Strains of NDV show a spectrum of virulence from mildly pathogenic to highly lethal. Virulent NDV infection in a flock may result in 100% mortality. (2). On the other hand, the pathogenicity or disease potential of APMV-2 to APMV-12 is not well established, since most of these serotypes are isolated from domestic, pet and wild birds dying in quarantine or hunter-killed birds or while surveillance for other poultry pathogens or from apparently healthy birds (2).

Among the APMV 2-12 serotypes, APMV-2, -3, -6 and -7 have been reported to be associated with diseases in domestic poultry (3, 4). APMV-2 infection causes mild respiratory disease with drop in egg production, and infertility in chickens and turkeys (5-7). APMV-3 infection causes encephalitis with high mortality in caged birds, respiratory disease and decreased egg production in turkeys, and stunted growth in young chickens (8, 9). APMV-6 & -7 infections in turkeys, ducks and migratory birds are associated with mild respiratory disease, and decreased egg production (10, 11). Other APMVs, though isolated from poultry, the frequency of isolation was very low and did not show any clinical signs (2).

The APMV viruses have a non-segmented, negative sense RNA genome of nucleotide length ranging from 14,904 to 17,412 (1). APMV genome has 6 genes (3'-NP-P-M-F-HN-L-5') encoding for the 6 structural proteins: nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) protein and a large polymerase protein (L) (12). P gene encodes for two accessory proteins V and W by RNA editing mechanism. APMV-6 contains an additional gene, SH encoding small hydrophobic protein of unknown function (13). RNA editing is a unique feature displayed by all the paramyxoviruses (14, 15). The V and W proteins are expressed from P gene by specific insertion of non-templated 'G' nucleotides by the RNA polymerase at the RNA editing site. Insertion of one 'G' nucleotide produces V protein while two 'G' nucleotides produces W protein. The V and W proteins share the amino co-terminus with the P protein up to RNA editing site but differ in their carboxyl termini (14, 15).

Interferon (IFN) is the first line of innate immune defense mechanism used by the host cell in response to a viral infection, and the secreted IFN establishes an antiviral state, preventing the replication and spread of viral infection. Over time, viruses have evolved many strategies to evade this innate host defense mechanism (16). RNA editing mechanism and the production of accessory proteins is highly conserved among the members of the family *Paramyxoviridae* (17, 18) and paramyxoviruses utilize their accessory proteins such as V, W and C proteins to overcome the host antiviral effects by antagonizing the IFN response (17-19). Among them, V protein was shown to be a major IFN antagonist. Most paramyxoviruses utilize their V protein to inhibit IFN induction or signaling or both through targeting of cellular proteins such as MDA5, IRF-3 and STATs

(19). V proteins have a highly conserved cysteine rich C-terminal domain and are essential for the formation of the oligomeric structure of the V-protein and IFN antagonistic activity (17, 19).

In recent years, the frequency of isolation of APMVs from domestic and wild birds has increased and these viruses impose a significant and constant threat to domestic poultry. Hence it is important to study the molecular biology of these viruses and the role of their individual proteins. APMV 2-12 serotypes express V protein of varying lengths but has the highly conserved C-terminal cysteine residues as that of the APMV-1 and other paramyxoviruses. With V protein being an IFN antagonist and contributing to the virulence, it is important to study the function of V protein in these viruses. We constructed expression plasmids encoding V protein of the corresponding APMVs and tested for their potential IFN antagonistic activity by dual luciferase reporter assay.

3.2 Materials and Methods:

Cells and Virus:

Chicken embryo fibroblast (DF1) cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Invitrogen) with 10% heat inactivated Fetal Calf Serum (FCS) (Thermo scientific) and 1% Penicillin Streptomycin (Invitrogen). Lentogenic NDV strain, LaSota, tagged with green fluorescent protein (rLaSota-eGFP) was used in this study.

Construction of plasmids expressing APMV V proteins:

The APMV-2, -3, -6 V sequences corresponding to the respective GenBank Accession numbers HM159993.1, EU403085.1, and EU622637.2 along with LaSota V were used in this study. Cloned sequences of APMV V genes were custom gene

synthesized from Integrated DNA Technologies (IDT). Specific gene primers with EcoRI and XbaI sites were used to amplify the regions of V protein by PCR and cloned into a CMV-driven mammalian expression vector pcDNA3.1 between EcoRI and XbaI restriction sites. A total of four plasmids pLaS V, pAPMV-2 V, pAPMV-3 V, pAPMV-6 V expressing the APMV serotypes 1 (LaSota strain), 2, 3 and 6 V proteins with N-terminus flag tag were constructed. The cloned expression plasmids were confirmed by sequencing performed at Virginia Bioinformatics Institute (VBI) sequencing facility at Virginia Tech. Expression of the flag-tagged APMV V proteins were confirmed by immunoblotting using anti-flag mouse monoclonal antibody (GenScript).

Transfection of DF1 cells with APMV V plasmids:

DF1 cells were seeded in a 6-well plate, grown to 70-80% confluency, and transfected with 2 μg of respective APMV V expression plasmid or empty vector in each well. Lipofectamine® 2000 (Invitrogen) Transfection Reagent was added according to manufacturers instruction and incubated at 37 ^{0}C for 24 hrs.

Western blot analysis:

For western blot analysis, whole cell lysates were harvested from the APMV-V expression plasmid-transfected cells at 24 hrs post-transfection and lysed with 100 μl of 10x radioimmunoprecipitation assay (RIPA) lysis buffer containing 100x Halt protease and phosphatase inhibitor cocktail (Thermo Scientific) for 30 min at 4 ^{0}C. Supernatants were then collected by centrifugation at 10,000 X g for 30 min. Quantification of the total protein content of the samples was done using the Bradford assay (Bio-Rad). Equal amounts of protein samples were loaded onto sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) (Bio-Rad) gel, electrophoretically separated and transferred on to a

polyvinylidene fluoride (PVDF) membrane (Bio-Rad). Then blocking was done with 5% non-fat dry milk for 1 hr at room temperature and incubated with a mouse anti-flag antibody (GenScript) or a goat anti-actin antibody (Santa Cruz). The blot was washed after overnight incubation with a primary antibody and then incubated with HRP-conjugated anti-IgG secondary antibodies (Santa Cruz). The myECL imager (Thermo Scientific) was used for taking the images.

Luciferase Reporter Assay:

For the dual luciferase reporter assay, pGL3 firefly luciferase plasmid under IFN-β promoter was used as the reporter plasmid and pRL-TK expressing the renilla luciferase was used as an internal control to standardize the efficacy of transfection. DF1 cells were co-transfected with 250ng of pGL3 -IFNβ firefly reporter plasmid, 10ng of pRL-TK and 0.5ug of plasmids expressing LaS V, APMV-2, 3 & 6 V or empty vector pcDNA3.1. At 24 hours post-transfection, cells were infected with 0.001 MOI of rLaSota-eGFP virus. After twenty hours of infection, whole cell lysates were examined for luciferase activity using Dual-Luciferase® Reporter Assay System (Promega) with a luminometer, GloMax® Discover: Multimode Detection System (Promega).

Statistical Analysis:

Experiments were done in triplicates and the quantitative values expressed as mean ± standard deviation. One-way ANOVA was used to analyze data sets. $p < 0.05$ was considered significant. The data was evaluated with the help of JMP (JMP Software, SAS Institute) and the graphs were prepared using Graph Pad Prism 5 (GraphPad Software Version 5.0).

3.3 Results:

Conservation of cysteine rich C-terminal domain of APMV 1-9 V protein:

The IFN antagonistic activities of the V protein of paramyxoviruses were mapped to their conserved cysteine rich C-terminal domain. The amino acid sequence alignment of APMV 1-9 V proteins showed the presence of a conserved C-terminal domain rich in cysteine residues similar to that of other paramyxoviruses (Fig 3.1). So we constructed plasmids expressing LaS, APMV-2, -3 and -6 V proteins to study the IFN antagonistic activity.

Construction of the LaS V, APMV-2 V, 3 V and 6 V plasmids:

For the construction of APMV-V expression plasmids, we used specific primers with EcoRI and XbaI restriction sites, amplified the coding sequence of APMV V protein from the custom-synthesized plasmid. The PCR-amplified product was cloned between EcoRI and XbaI restriction sites of mammalian expression vector pcDNA 3.1 having an N-terminus flag tag for the construction of pLaS V, pAPMV-2 V, pAPMV-3 V and pAPMV-6 V. All the constructed APMV-V expression plasmids were verified by sequencing and the plasmids were amplified using DH10B^{TM} E.Coli strains.

Expression of LaS V, APMV-2 V, 3 V and 6 V proteins in DF1 cells:

To verify the expression of the APMV V proteins in chicken cell line, DF1 cells were transfected with the constructed APMV V protein expression plasmids. After 24 hours, whole cell lysates were collected and subjected to immunoblot analysis using anti-flag antibody. Equal amounts of proteins were loaded onto the gel to verify and compare the expression of the different V proteins. Our results showed that all the APMV serotypes tested expressed the V protein on DF1 cells, although the level of expression

varied between the different serotypes. The APMV-2 V protein was more efficiently expressed when compared to other serotypes. On the other hand, APMV-3 V was expressed the least (Fig 3.2).

APMV-3 V inhibits activation of IFN-β promoter:

The V proteins of paramyxoviruses were shown to be an IFN antagonist blocking the IFN at various levels of induction or signaling or both (18, 19). We therefore tested the APMV-V plasmids for IFN antagonism at levels of IFN induction by dual luciferase reporter assay. We used firefly luciferase reporter under IFN-β promoter. DF1 cells were co-transfected with plasmids encoding the V proteins of LaSota, APMV -2, 3 and 6 serotypes or empty vector with IFN-β reporter luciferase plasmid and an internal control renilla luciferase plasmid. LaS-V was used as a positive control for the experiment. Our results showed that the plasmid encoding APMV-3 V and LaS V blocked the IFN-β reporter gene activation when compared to the vector control. However, APMV-2 V and APMV-6 V failed to block IFN-β reporter activation (Fig 3.3). This suggests that the V protein of APMV-3 acts as an IFN antagonist blocking at levels of induction.

3.4 Discussion:

The number of outbreaks and isolation of APMV serotypes from wild and domestic bird population have increased over the recent years (1). Experimental infection showed that APMV serotypes are able to infect chickens with low pathogenicity profile (7, 20, 21) and a sero-surveillance study conducted in the United States has shown that all the serotypes of APMVs are prevalent in commercial poultry flocks (22). Since avian species are the natural host for these RNA viruses, there is always greater chance for APMV serotypes to evolve by gaining pathogenicity and expanding their host range (23).

Hence, it is important to determine the molecular mechanism and role of individual proteins in the pathogenicity of APMVs.

F protein cleavage site is a major virulent determinant of NDV and plays important role in tissue tropism and virulence (24). However, experiments done in APMV-2, -4 and -7 F protein cleavage site did not modulate virus replication or tropism or the virulence of these APMVs (25-27). V proteins of paramyxoviruses contain conserved C-terminal cysteine residues and are shown to antagonize the IFN response by blocking IFN induction or signaling or both (33). Several studies have demonstrated the importance of conserved C-terminal residues of V protein in IFN antagonism and their role in conferring the host tropism and virulence to the virus (28, 29, 34, 35).

The amino acid sequence analysis of APMV 1-9 V protein reveals the conserved cysteine rich carboxyl-terminal similar to that of NDV and other paramyxoviruses, which were contributing to the IFN antagonistic activity of these viruses. We constructed plasmids encoding for the APMV-2, -3 and -6 V proteins and confirmed the protein expression in chicken fibroblast cells. We demonstrated that APMV-3 V but not APMV-2 and -6 V significantly inhibits the induction of IFN-β promoter, suggesting that V protein of APMV-3 can block the IFN induction. Previous research has shown that APMV-3 replicates better in DF1 cells when compared to other APMV serotypes and its replication efficiency is comparable to APMV-1 (21). Experimental infection studies have shown that APMV-3 had an intracerebral pathogenicity index (ICPI) value, a factor used to determine the pathogenicity, was greater than that of LaSota strain of NDV (21). Based on our experimental results, it is plausible to propose that the ability of APMV-3 V protein to antagonize IFN confers replication advantage in chickens. This is by far the

first experiment done on V proteins of AMPV serotypes -2, -3 and -6 towards understanding their potential function as an IFN antagonist.

V protein antagonizes the IFN response by blocking at levels of IFN induction or signaling or both (Audsley et al., 2013). V proteins utilize their highly conserved cysteine rich C-terminal region to bind MDA-5, thereby blocking viral dsRNA recognition or target phosphorylation and nuclear translocation of IRF3 to inhibit the IFN induction (9, 30-32). So future research should be focused on understanding the mechanism by which APMV-3V protein antagonizes IFN at the induction level.

Though all the APMV serotypes V proteins have conserved C-terminal cysteine residues, there are differences in the amino acid groups surrounding them. This could be a possible reason for the absence of observed IFN antagonistic activity of other APMVs. Also there is a possibility of APMV-2, -3 and -6 V to antagonize IFN signaling pathways. We tested the IFN antagonism at the induction level, so future research should be directed at understanding the IFN antagonistic activity of these proteins at the signaling level.

Further, recombinant mutant viruses of APMV serotypes lacking the expression of V proteins should be constructed and tested for viral growth and pathogenicity to have a complete understanding on the functional role of these V proteins. In summary, we found that APMV-3 V protein can act as an IFN antagonist by blocking at the IFN induction level and thereby plays an important in innate immune evasion.

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Majority        HRREYSISWSXGT--XXXISWCNPVCSPIXAEPRXXKCXCGXCPXXCILCRNDX-----------------------
                           240       250       260       270       280       290       300
LaSota V.pro    HRREHSISWTMGG--VTTISWCNPSCSPIKAEPRQYPCICGSCPATCRLCASDDVYDGGDITESK             239
APMV-2 V.pro    HRREYSFISRDGR--LEVTSWCNPVCSPIRSEPRREKCTCGTCPESCILCRQPN                        232
APMV-3 V.pro    HRREYSISWSPGSGTFQTETWCNPACSPVTAFPKQYKCACRQCPRFCDLCFNPADYGGIIDLSSVKVVGPSLADTEV 241
APMV-4 V.pro    HRREMAIVTDKATGDIELVEWCNPGCTAVRIEPTRLDCVCGHCPTICSLCMYDD                        224
APMV-5 V.pro    HRREYSLFFSDGR--CSITEWCNPTCRPITAIPSVQRCTCGECPRRCSMCWNDS                        277
APMV-6 V.pro    HRREFSISWRHGQ--CILAEWCNPVCAPVTPEPRTFKCICGRCPRVCINCRNDSGDSEICS                 268
APMV-7 V.pro    HRREYSFAWSAGTNKFIVSSWCNPTCAPIRPYPTVERCRCGNCPKFCPGCQSALGCNQSATQDADKPTKSSNKDH   250
APMV-8 V.pro    HRREYSFTTFHGT--TRVISWCNPQCTPIRARPIYDECRCGECPTTCIMCRDDK                        238
APMV-9 V.pro    HRREHSMVWNANG--IVTISWCNPVCSPVTYEPREFTCSCGSCPTECRLCAGSHRDIRKHSAESE             263
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Figure 3.1: Sequence comparison of V protein of APMV 1-9. The amino acid of sequences of APMV 1-9 V proteins aligned by Clustal W method using DNASTAR (Lasergene) software reveals the conserved cysteine rich C-terminal region.

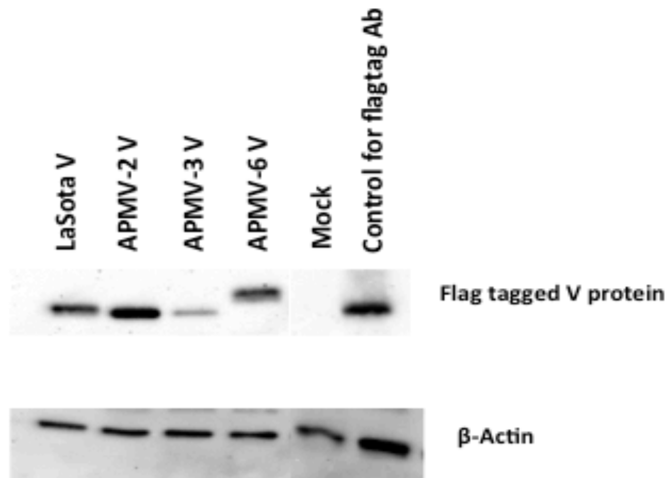

Figure 3.2: Expression of LaSota V, APMV-2 V, -3 V, and -6 V in DF1 cells. DF1 cells were transfected with LaSota, APMV-2, -3, and -6 V expression plasmids. At 24 hours post-transfection, whole cell lysates were collected and immunoblot analysis was performed using anti-flag antibody. β-Actin was used as an internal loading control in the experiment.

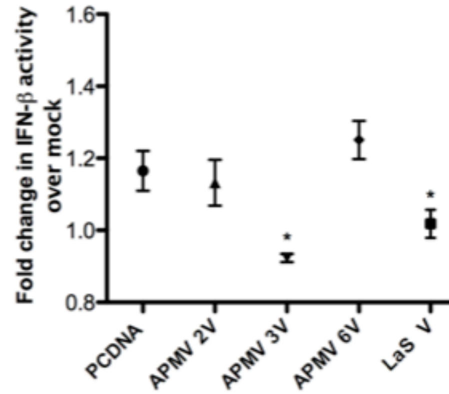

Figure 3.3: APMV-3 V inhibits activation of IFN-β promoter. DF1 cells were co-transfected with plasmids encoding V proteins of LaS, APMV -2, 3 and 6 serotypes or empty vector with IFN-β reporter luciferase plasmid. Cells were infected with 0.001MOI of LaS-eGFP post-transfection. Whole cell lysates were collected 20 hours post-infection to measure IFN- β promoter activity. Firefly luciferase levels were normalized to internal control renilla luciferase and the average per condition was used to determine the fold change in IFN- β promoter activity over the mock. Error bars indicate standard deviation from the three replicates. (* = $p<0.05$).

Chapter 4

General Conclusions and Future Directions

In this study on APMV, we attempted to explore the role of W protein in thermostability of NDV and V protein of APMV-2, -3 and -6 in IFN antagonism. For the first phase of our study, we mutated the antigenome of a thermolabile NDV strain (LaSota) to express the W protein of varying lengths and rescued the recombinant NDV viruses by reverse genetic system. Thermostability tests on the rescued recombinant NDV viruses did not show any difference in HA stability and viral infectivity. Based on our experimental results, we conclude that the difference in the length of W protein does not impact thermostability of NDV. Besides that, a recent study showed that chimeric NDV LaSota strain bearing the HN protein from a thermostable virus exhibited thermostable phenotype, suggesting that HN is a crucial determinant for thermostability of NDV (Wen et al., 2016). But the precise mechanism of HN mediating thermostability is not yet known. So further research should be done on understanding the basic mechanism of HN in NDV thermostability. Future studies should also be directed at determining which amino acids in HN protein are critical for viral thermostability, and whether HN acts alone or in concert with other proteins.

In the second phase of our study, we explored the role of APMV-2, -3, and -6V proteins in IFN antagonism. We constructed plasmids expressing respective APMV V proteins with an N-terminal flag tag. Transfection of chicken fibroblasts with these plasmids and immunoblot analysis of the cell lysates confirmed the expression of these proteins in chicken cells. Based on the dual luciferase assay, we found that APMV-3V significantly inhibited the induction of IFN-β over control. However, APMV-2V and -6V

did not show a significant difference in IFN-β induction. Based on our experimental results, we conclude that APMV-3V antagonizes the IFN induction pathway among the tested APMV-V proteins. V proteins of paramyxoviruses are known to be IFN antagonistic and antiapoptotic in function. V protein antagonizes the IFN response by blocking at levels of IFN induction or signaling or both (Audsley et al., 2013). We tested the IFN antagonism at induction level, so future research could be directed at studying the IFN antagonistic activity of these proteins at signaling level. Further, mutant viruses of APMV serotypes lacking the expression of V proteins should be constructed and tested for viral growth and pathogenicity to have a complete understanding on the functional role of these V proteins.