ABSTRACT

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                                      VIRUS INTERNAL PROTEINS IN
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Directed By: Dr. Siba K. Samal
                                      Professor and Associate Dean
                                      College of Veterinary Medicine

The internal proteins, nucleocaspid protein (NP), phosphoprotein (P) and large

polymerase protein (L) of Newcastle disease virus (NDV), play an important role in

transcription and replication of the viral genome. However, their role in NDV

pathogenesis has not been explored. In this study, the importance of internal proteins

in NDV virulence was evaluated through a chimeric approach using an established

reverse genetics technique. The L gene between an avirulent NDV strain LaSota and

a moderately virulent NDV strain Beaudette C (BC) was exchanged, recombinant

chimeric viruses were recovered and studied for their pathogenicity in the natural

host, chicken. The results obtained from *in vivo* studies indicated that the L gene of

NDV modulate role in NDV virulence in chickens.

The NP and P genes of NDV were exchanged between BC and LaSota individually as

well as in combination; chimeric viruses were recovered, indicating that heterologous

NP and P genes were functional. *In vitro* replication of chimeric NP and P recombinant viruses in DF-1 cells indicated that the exchange of NP or P gene in NDV did not affect the replication of the chimeric viruses. The *in vivo* studies in chickens showed that the change in pathogenicity of these chimeric viruses was minimal and homotypic interaction between NP and P proteins is necessary for optimum pathogenicity of the virus.

THE ROLE OF NEWCASTLE DISEASE VIRUS INTERNAL PROTEINS IN
PATHOGENESIS

By

Subrat N. Rout

Dissertation submitted to the Faculty of the Graduate School of the
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Advisory Committee:
Professor Siba K. Samal, Chair
Associate Professor Jeffrey J. DeStefano
Associate Professor Daniel R. Perez
Associate Professor Nathaniel L. Tablante
Assistant Professor Xiaoping Zhu

Dedication

This piece of work is dedicated to my parents for their love and support and to those

laboratory birds (chickens) who sacrificed their lives during the study.

ACKNOWLEDGEMENTS

My special thanks go to Ireen Dryburgh-Barry for her kindness, helping nature, tremendous positive spirit and great sense of humor, which has helped to uplift my spirit during the long journey of the graduate life.

Finally, I am indebted to my parents and my brother for their love, moral support and understanding and without their emotional support, this work would not have been possible.

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LIST OF ABBREVIATIONS

BC	Beaudette C
cDNA	complementary DNA
CEF	chicken embryo fibroblast
CPE	cytopathic effect
DF-1	Douglas Foster 1
DMEM	Dulbecco's modified Eagle's medium
EMEM	Essential modified Eagle's medium
FBS	Fetal bovine serum
GS	gene start
GE	gene end
h	hour
HA	hemagglutination assay
HN	hemagglutinin-neuraminidase
hPIV3	human parainfluenza virus 3
ICPI	intracerebral pathogenicity index
IVPI	intravenous pathogenicity index
kD	kilodaltons
L	large polymerase
M	matrix
mRNA	messenger RNA
MDT	mean death time
MOI	multiplicity of infection

NA	neuraminidase
NDV	Newcastle disease virus
nm	nanometer
NP	nucleocapsid protein
nt	nucleotide
ORF	open reading frame
P	phosphoprotein
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PFU	plaque forming unit
PI	post infection
RNA	ribonucleic acid
rNDV	recombinant Newcastle disease virus
RNP	ribonucleoprotein
RT-PCR	reverse transcription-PCR
SPF	specific pathogen free

Chapter 1:

1.1 Title

General Introduction

1.2 Introduction

Newcastle disease virus (NDV) is the causative agent of Newcastle disease (ND) which is a highly contagious viral infection affecting most species of birds worldwide. The virus has many strains with wide spectrum of virulence. Chickens are the most susceptible host, in which the severity of the disease may vary from mild infection with no apparent clinical signs to a severe form causing 100% mortality (Alexander, 1997). The clinical signs and severity of the disease depend on several factors such as virus strains, host species affected, age and immune status of the host and presence of other organisms. Based on the severity of the disease in chickens, NDV strains are grouped into three main pathotypes: lentogenic, mesogenic and velogenic. Lentogenic strains cause mild respiratory infection and are considered to be avirulent. Mesogenic strains are of intermediate virulence causing respiratory infection with moderate mortality while velogenic strains are highly virulent causing 100% mortality in chickens. Velogenic strains are further classified into viscerotropic velogenic and neurotropic velogenic strains. Viscerotropic velogenic strains produce lethal hemorrhagic lesions in the digestive tract whereas neurotropic velogenic strains produce neurological and respiratory disorders (Alexander, 1997).

Newcastle disease is widespread in many countries and remains as a major disease threat to poultry industries. Prophylactic vaccination with live attenuated vaccines is practiced in commercially reared birds throughout the United States as well as in most parts of the world. However, a recent outbreak of exotic Newcastle disease (END) in California, caused by a virulent strain of NDV, led to depopulation of birds at a cost of around $200 million to the poultry industry (Kapczynski and King, 2005). Because ND is a highly infectious disease with potential to cause severe damage to the economy of the poultry industry, NDV is considered as an agro-bioterrorism agent. Currently available vaccines do not provide adequate protection against highly virulent NDV strains. Therefore, better control and prevention of the ND is necessary. This can be achieved by identifying the viral genes involved in pathogenesis.

NDV belongs to the genus *Avulavirus* of the family *Paramyxoviridae* in the order *Mononegavirales* (Mayo, 2002). The virus is enveloped and contains a single linear strand, non-segmented negative sense RNA molecule of 15,186 nucleotides (nt) as its genome (Deleew and BenPeeters, 1999; Krishnamurthy and Samal, 1998). The genomic RNA of the virus is organized into six genes, which encode for at least eight proteins (Peeples, 1988; Steward *et al*., 1993). The genes are arranged in tandem in the order of 3' -NP-P-M-F-HN-L- 5' which encode for nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN) and large polymerase protein (L), respectively. There are conserved sequences present at the beginning and end of each gene, called 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 are present, whose function is unknown. The genome of the NDV contains a 55 nt long viral promoter known as leader at its 3' end and a 114 nt long viral antigenome promoter known as trailer at its 5' end (Krishnamurthy and Samal 1998). The leader and trailer regions serve as cis-acting elements in viral genome for replication and packaging of viral RNA (Lamb and Kolakofsky, 1996). The NP proteins bind to the viral genomic RNA forming nucleocapsid core. The viral genome along with NP, P and L proteins constitutes the transcriptase complex, which is the minimum unit for viral transcription and replication. The M protein forms the inner layer of the viral envelope that provides the driving force for viral assembly and is responsible for maintaining viral structural integrity. F and HN proteins are envelope glycoproteins. The F glycoprotein mediates fusion of the viral envelope with the host cell plasma membrane, thus mediating entry of the viral genome into the host cell cytoplasm. The HN glycoprotein helps in the attachment of virion to host cells, the fusion promotion and the removal of sialic acids from progeny virion particles, thus acts as a neuraminidase. The V protein of NDV functions as an alpha interferon antagonist (Huang *et al*., 2003). The V proteins of other paramyxoviruses have been shown to play an important role in viral pathogenesis (Andrejeva *et al*., 2002). The function of the W protein is unknown (Lamb and kolakofsky, 1996; Steward *et al*., 1993).

The NP protein is the major constituent of the NDV nucleocapsid and is tightly

associated with the viral genome. In paramyxoviruses, the amino terminal region of

the NP protein is involved with encapsidation of the viral RNA while the carboxy-

terminal region binds with the P protein (Buchholz *et al*., 1993). The intracellular

concentration of unassembled NP protein plays an important role in switching from

transcription to replication of the viral genome (Blumberg *et al*., 1981). The L gene,

which constitutes approximately half of the NDV genome, is the last gene to be

transcribed and its encoded L protein is the least abundant viral protein (Poch *et al*.,

1990). The L protein and P protein constitute the active viral polymerase (Lamb and

Kolakofsky, 1996). The active viral polymerase does not utilize naked RNA genome

as a template, but recognizes only when the genomic RNA is tightly bound to the NP

protein (Hamaguchi *et al*., 1983; Horikami *et al*., 1992). The L protein also possesses

5' capping and 3' poly (A) polymerase activity of the nascent viral mRNA (Lamb and

Kolakofsky, 1996). The polymerase gene of the paramyxoviruses plays a major role

in transcription and replication of viral genome.

The transcription and replication of NDV follow the general pattern of the

other nonsegmented negative-strand RNA viruses. The RNA genome template is

copied without dissociation of NP from the nucleocapsid core during transcription

and replication. The polymerases enter the genome through the 3' leader and proceed

along the entire length of the genome by a sequential start-stop mechanism, guided

through conserved GS and GE signals. This generates a free leader RNA and six non-

overlapping sub-genomic RNAs during transcription. There is a polar gradient

transcription of genes in which the most 3' leader proximal genes are transcribed in higher quantities than downstream genes as found in other nonsegmented, negative strand RNA viruses.

In negative strand RNA viruses, the inability of the naked genomic RNA to initiate viral RNA synthesis is a major hindrance for direct genetic manipulations of the virus. However, the development of the reverse genetics system is a major breakthrough, which allows the production of infectious RNA virus from cloned cDNAs. Using this system, manipulation of the negative strand RNA virus genome not only helps us to investigate the functions of the virus genes and proteins but also to insert foreign genes into viral genome. In addition, by introducing genetic mutations into individual viral genes, the function of an individual gene and its role in pathogenesis can be studied in greater detail. Ultimately, the insertion of foreign genes into a viral genome provides a novel way to generate improved viral vaccines and vaccine vectors (Bukreyev *et al*., 1997; Mebatsion *et al*., 1996; Schnell *et al*., 1996).

Several studies on paramyxovirus pathogenesis have led to believe in the involvement of envelope glycoproteins in the viral pathogenesis. However, the involvement of the internal proteins in the pathogenesis of the virus is not clearly understood. Studies from our laboratory as well as from other groups have shown that envelope glycoproteins, F and HN, of NDV play important roles in pathogenesis (Panda *et al*., 2004a, b; Huang *et al*., 2004). The presence of multi-basic amino acids

at the F protein cleavage site of virulent strains plays an important role in pathogenicity. 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 an isolate under the virulent pathotypes (OIE, Chapter 2.1.15). However, there are several NDV stains whose F protein cleavage sites have identical or similar amino acid sequence but produce a wide range of pathogenesis and clinical signs in chickens. For example, the mesogenic NDV strain Roakin has the same fusion cleavage site as the neurotropic velogenic strain, Texas-GB, and the neurotropic velogenic strain, Turkey ND has same fusion cleavage site as that of the mesogenic strain, Anhinga. However, each virus strain shows variety in the degree of pathogenicity and virulence when compared to another (Brown *et al*., 1999). Several researchers have also recovered the mutant NDV in which the virulent cleavage site of the F protein was changed into an avirulent one (Panda *et al*., 2004b) and the avirulent cleavage site was changed into a virulent one (Wakamatsu *et al*., 2006; de Leeuw *et al*., 2003, 2005). However, changing the avirulent cleavage site into a virulent site or virulent cleavage site into an avirulent one, resulted in only partial gain or loss in virulence when compared to the parental-type NDV strains, indicating that other viral genes might also play important roles in the virulence of NDV.

The HN protein of NDV is a major antigenic determinant and also plays an important role in the viral pathogenesis. Our laboratory has developed reverse genetics system for a mesogenic virulent NDV strain Beaudette C (BC) and lentogenic avirulent NDV strain LaSota. The HN genes between BC and LaSota were

exchanged; chimeric viruses were generated and studied for their tropism and virulence (Huang *et al*., 2004). The results showed that chimeric viruses with swapped HN gene were viable and the virulence was altered depending on the strain from which the HN gene was derived. But the gain or loss in virulence was not as that of the parental virus suggesting involvement of other proteins in NDV virulence. We hypothesize that other internal proteins of NDV may play an important role in viral pathogenesis. To date, the role of paramyxovirus internal proteins in pathogenesis has not been examined in detail. Therefore, the overall objective of this study is to determine the roles of N, P and L genes in NDV pathogenesis.

1.3 Research objectives

The specific objectives of the present study were:

(1)   To explore the role of large polymerase protein (L) in NDV pathogenesis.

(2)   To study the role of phosphoprotein (P) in NDV virulence and pathogenesis.

(3)   To investigate the role of nucleocapsid (N) and phosphoprotein (P) in NDV virulence and pathogenesis and

(4)   To develop a temperature sensitive mutant strain of NDV by importation of single amino acid mutation from heterologous paramyxovirus polymerase protein.

Chapter 2 :

2.1 Title

Review of Literature

2.2 Classification

NDV is a member of the genus *Avulavirus* under the subfamily

Paramyxovirinae, family *Paramyxoviridae* and the order *Mononegavirales* (Mayo,

2002, Murphy *et al*., 1995). It is also designated as avian paramyxovirus type 1. Other

important members of this family are measles virus, mumps virus, parainfluenza virus

type 2 (PIV2), simian virus 5 (SV5), nipah virus and hendra virus.

2.3 Virion

The NDV virion particles are large pleomorphic in nature ranging from 150-

400 nm in size. The virions have an envelope which is derived from host cell plasma

membrane. The outer surface of the envelope contains two viral glycoproteins of

about 8-12 nm in length: fusion (F) protein, and hemagglutinin-neuraminidase (HN)

protein. The F protein is required for the fusion of the viral envelope to the host cell

membrane (Homma and Ouchi, 1973) and the HN protein is responsible for the

attachment of the virion to the host cell receptor. The F and HN proteins are the main

immunogenic proteins of the virion (Meulemans *et al*., 1986; Morgan *et al*., 1992).

The core of the virion contains a helical nucleocapsid which is the template for all

RNA synthesis. The core structure is formed by nucleocapsid (NP) proteins tightly

bound to the genomic RNA, to which phosphoprotein (P) and large polymerase (L) proteins are attached (Lamb and Kolakofsky, 1996). The genome of NDV is a negative sense single strand RNA of molecular weight of 5.2 to 5.7×10^6 daltons (Alexander, 1997) consisting of 15,186 nucleotides (Krishnamurthy and Samal, 1998; DeLeeuw and Peeters, 1999). In between the viral envelope and nucleocapsid core is another layer of protein, the matrix (M) protein. This protein is thought to be the driving force for the assembly of the virus particles (Peeples, 1991).

2.4 Genome Organization

The genome of NDV consists of 6 genes arranged in tandem in order of 3' NP-P-M-F-HN-L 5' encoding at least eight proteins (Peeples, 1988; Steward *et al*., 1993). The genome at its 3' end contains a 55nt long extracistronic region known as leader and at 5' end, 114 nt long region known as trailer (Krishnamurthy and Samal, 1998). The leader and trailer are essential for viral genome transcription and replication (Lamb and Kolakofsky, 1996). There are conserved transcriptional control sequences present at the beginning and end of each gene known as gene start (GS) and gene end (GE), respectively. The GS acts as transcriptional promoter and GE acts as transcriptional terminator. Between the genes, there are variable lengths of noncoding nucleotide stretches called as intergenic regions (IGS). The length of the IGS varies from 1-47 nucleotides (Chambers *et al*., 1986b; Krishnamurthy and Samal, 1998) (Fig-3).
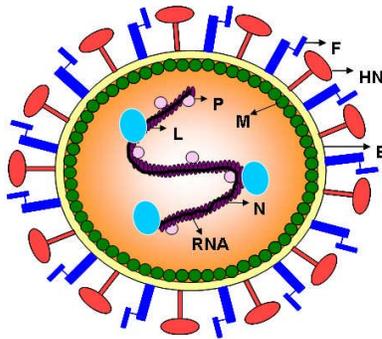
Fig. 1. Schematic diagram of Newcastle disease virus particle (not drawn to scale).

N: Nucleocapsid protein, P: Phosphoprotein, L: Large polymerase protein, M: Matrix

protein, F: Fusion protein, HN: Hemagglutinin-Neuraminidase protein and E:

Envelope
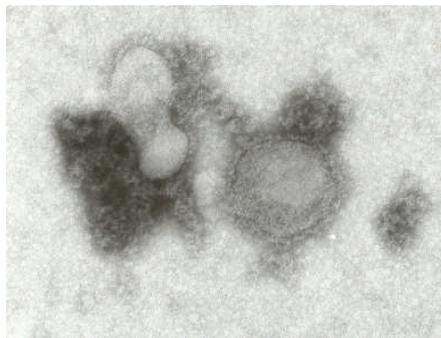
Fig. 2. Electron micrograph of negatively stained pleomorphic Newcastle disease

virus (strain Beaudette C) particles obtained from supernatant of infected chicken

embryo fibroblast cells.
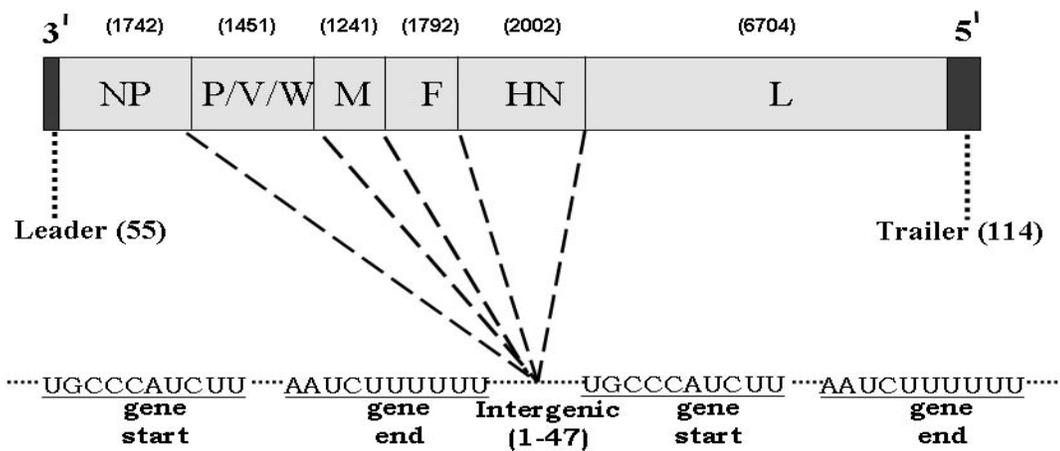
Fig. 3. Genetic map of genomic RNA of NDV. NDV has a single-stranded, negative-sense RNA genome which is 15,186 nucleotides in length. The length of leader, trailer and each gene is shown in parentheses. Each gene is flanked by conserved gene start and gene end sequence. The intergenic sequences present in between two genes range from 1-47 nucleotides in length.

2.5 Viral proteins

The genome of the NDV encodes for at least eight proteins: NP, P, M, F, HN, L, V and W. The V and W proteins are formed by insertion of non-templated G residue(s) into P gene ORF by viral RNA polymerase during P gene transcription by a process called RNA editing (Steward *et al.*, 1993). Most of the knowledge relating to functions of NDV proteins is derived from studies made on other members of the family *paramyxoviridae* (sendai and parainfluenza viruses) or family *rhabdoviridae* (VSV) (Colono and Banerjee, 1976; Iverson and Rose, 1981; Lamb and Kolakofsky, 1996).

2.5.1 Nucleocapsid and its associated proteins

The nucleocapsid protein (NP), which is tightly bound with viral genomic RNA, forms the nucleocapsid core of the virus to which the phosphoprotein (P) and the large polymerase protein (L) are loosely bound (Lamb and Kolakofsky, 1996). These three proteins together constitute the transcriptive-replicative complex, which are the active polymerase complex and the minimum infectious unit of NDV.

NP protein: The NP gene of NDV is 1,747 nt long encoding for a 489 amino acid residues long NP protein. The molecular weight of NP is predicted to be 54 kilodaltons (kD) (Krishnamurthy and Samal, 1998). The functions of NP protein include encapsidation of viral genomic RNA, thus making the nucleocapsid RNase-resistant, association with P and L protein during transcription and replication and

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interaction with M protein during virus assembly. The intracellular concentration of unassembled NP protein plays an important role in the switching of transcription to replication of the viral genome (Blumberg and Kolakofsky, 1981; Blumberg *et al*., 1981).

P protein and P gene editing proteins: The P protein of NDV is the most heavily phosphorylated viral protein. It is highly acidic in nature (McGinnes *et al*., 1988; Steward *et al*., 1993) and is produced from an unedited version of P gene ORF. The length of P gene is 1451 nt long that encodes for 395 aa long P protein. The co-transcriptional insertion of one G nucleotide to the editing site of the P gene ORF produces an mRNA that encodes for V protein, whereas insertion of two G nucleotides produces mRNA that encodes the W protein (Lamb and Kolakofsky, 1996; Steward *et al*., 1993). Sequence analysis of the NDV P gene shows that the protein is rich in serine and threonine residues, acting as potential phosphorylation sites. The P protein along with NP and L forms viral polymerase complex thus acting as a transcriptive and replicative factor. It also forms P-NP^0 complexes with the unassembled NP (NP^0) thus preventing nonspecific assembly or self aggregation of NP^0 proteins.

L protein: The L protein is the largest structural protein with least abundance in the virion core (about 50 copies per virion) and is a major component of the RNA-dependent RNA polymerase in negative-strand RNA viruses (Banerjee, 1987; Tordo *et al*., 1988). The L gene is 6704 nt long and its ORF of 6615 nt codes for a 2204 aa long polypeptide of mass around 242 kD (Yusoff *et al*., 1987). The L protein also

possesses 5' capping and 3' poly (A) polymerase activities on the nascent viral mRNAs.

2.5.2 Matrix protein

M protein is the most abundant protein inside the virion particle. The M gene of NDV is 1241 nt long which encodes for a 364 aa long peptide. The molecular mass of M protein is 40 kD (Chambers *et al*., 1986b). The M protein interacts with the cytoplasmic tails of the integral membrane proteins, the lipid bilayer and the nucleocapsid, and is considered to be the central organizer of viral morphogenesis. The self-association of M proteins and its affinity to interact with the nucleocapsid may be the driving force in forming a budding virus particle (Peeples, 1991).

2.5.3 Envelope glycoproteins

The envelope of NDV contains 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 is responsible in the attachment of the virus to host cell membrane.

F protein: The F gene is 1792 nt long encoding for a 553 amino acid long precursor polypeptide. The F glycoprotein of NDV mediates viral penetration by fusion between virus envelope and host cell plasma membrane, in a pH-independent manner. The fusion creates pores on plasma membrane through which the viral nucleocapsid is delivered into the host cell cytoplasm. The F protein is a type I

integral membrane protein and is synthesized as an inactive precursor (F_0) that requires host-cell proteolytic enzyme(s) for its cleavage. This cleavage of F_0 yields two subunits F_1 and F_2 connected to each other by disulfide link which is biologically active protein (Scheid and Choppin, 1974). F_0 has a predicted molecular weight of around 66 kD whereas F_1 and F_2 are approximately, 55 kD and 12.5 kD, respectively. During viral infection, F gene is transcribed in the cytosol, synthesized on rough endoplasmic reticulum and then, subsequently, targeted towards host cell membrane for its expression. F protein expressed on the infected cell plasma membrane mediates fusion with its neighboring cells thus forming giant multinucleated cells or syncytia which are a hallmark of NDV infection in the host cells. Viruses that have multiple basic amino acids at their cleavage site of F protein are cleaved by intracellular subtilisin-like proteases. However, viruses that have single basic amino acid at their cleavage site of F protein require exogenous proteases for cleavage activation (Ortmann *et al*., 1994; Scheid and Choppin, 1974). The fusion and syncytia formation caused by F protein of the virus is one of the important factors for virulence as well as virus spread.

HN protein: The HN glycoprotein of NDV is a major antigenic determinant of the virus with multiple functions. The HN gene is 1998 nt long that encodes for a 577 amino acid residues long polypeptide. The molecular weight of HN is 74 kD (Chambers *et al*., 1986a). It binds with sialic acid, thus being responsible for binding of virus to sialic acid containing receptor. It also mediates enzymatic cleavage of sialic acid (neuraminidase activity) from the surface of the virion as well as infected host cell membranes. Along with hemagglutinin and neuraminidase activities, it also

has fusion promotion activity by interacting with the F glycoprotein of NDV (Lamb and Kolakofsky, 1996).The HN protein is a type II integral membrane protein with a single hydrophobic domain at N-terminal region that consists of cytoplasmic domain, followed by the transmembrane and the stalk region. The C-terminus end of the HN protein is composed of the globular head or ectodomain which is the main site for the attachment of the virus to the host cells. Detailed studies on the crystal structure and mutational analysis of HN protein of NDV have suggested that both hemagglutinin and neuraminidase activity are resided at a very close proximity to each other inside the protein (Crennell *et al*., 2000; Deng *et al*., 1999; Sheehan and Iorio, 1992).

2.6 Stages of replication of NDV

The replication strategy employed by NDV is very similar to that of other non segmented negative-strand RNA viruses of *paramyxoviridae*. The initial step of the virus infection is the attachment of the virus to the host cell receptor followed by fusion and entry of the viral nucleocapsid. The replication of NDV occurs in the host cell cytoplasm. During the late stages of NDV infection, there is a complete shutdown of host cell macromolecule synthesis.

2.6.1 Virus attachment, fusion and entry

The initial step of NDV infection requires the binding of viral attachment protein, HN protein, to specific cell surface receptors containing sialic acids (Huang

et al., 1980). Sialic acid is found on the most cell surface receptors that are glycoproteins or glycolipids, thus making NDV accessible to a wide range of host cells. Upon adsorption of the virus, the envelope of virus fuses with the host cell plasma membrane at neutral pH. This fusion is mediated by F protein in a pH independent manner. Upon fusion, the disruption of matrix-nucleocapsid occurs and viral nucleocapsid is released into the host cell cytoplasm.

2.6.2 Transcription

Since uninfected host cells lack RNA-dependent RNA polymerase (RDRP) activity, the naked negative-sense RNA genomes of paramyxoviruses are found to be non-infectious. The viral mRNA transcription is intracellular and begins at the 3' end of the genome. Once the nucleocapsid is released into the host cell cytoplasm, the viral RDRP complex enters at 3' end of viral genome promoter, i.e. leader, and synthesizes short (+) strand leader RNA followed by re-initiation of NP gene mRNA synthesis from NP gene start sequence. Majority of RDRP complexes terminates transcription at gene end (GE) sequence, but some of them bypass the GE signal to continue transcription of downstream genes. This sequential start and stop mechanism produces a gradient mRNA production in which 3' proximal gene is higher concentration than those of downstream genes (Cattaneo *et al.*, 1987). The mRNA produced are capped and polyadenylated in nature. The intergenic regions located between each gene are not transcribed. The exact role of these intergenic regions is unknown.

2.6.3 Genome replication

Once the primary transcription produces sufficient mRNAs for translation of viral proteins, there is an increase in the concentration of viral proteins, especially NP proteins that induce replication of viral genome by RDRP. The sufficient amount of unassembled NP proteins renders RDRP to switch from transcription to replication of (-) genome resulting in a full length complementary copy known as (+) antigenome (Kolakofsky and Blumberg, 1982; Nagai, 1999). During this process, all the junctional signals, such as start-stop signals and editing sites, are ignored by RDRP (Blumberg and Kolakofsky, 1981; Nagai, 1999). Then, these (+) antigenomes are used as templates for synthesis of (-) genome for packaging in new viral progenies. Several studies have shown that the leader and trailer regions of the genome contain specific signal for encapsidation (Blumberg and Kolakofsky, 1981). The process of transcription and translation in virus are tightly regulated. The RNA synthesis of NDV is shown in Fig. 4.
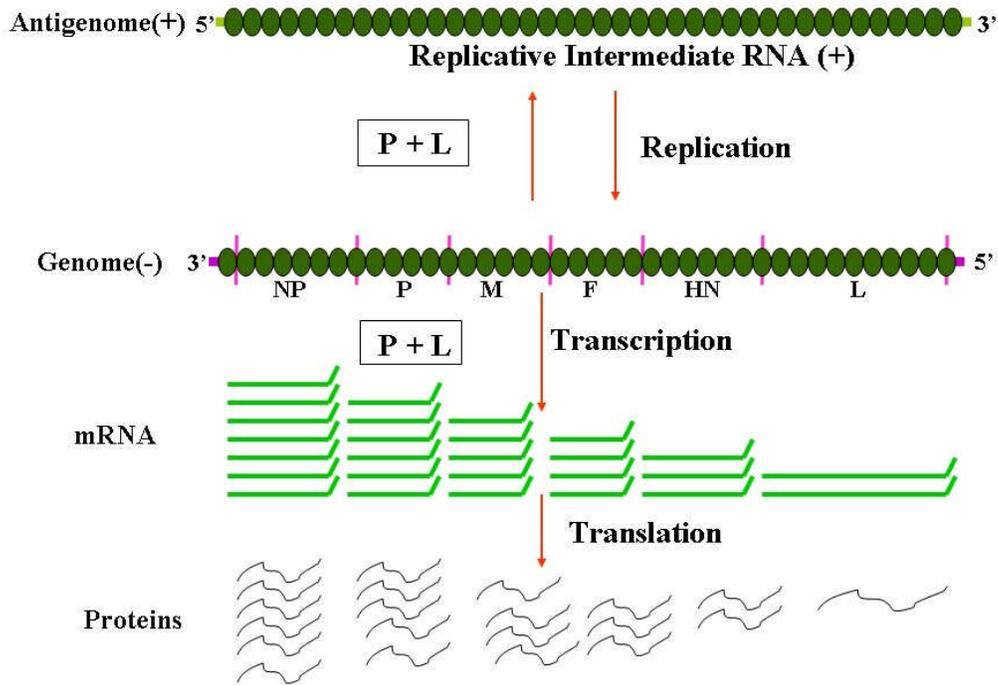
Fig. 4. Schematic diagram of Paramyxovirus transcription and replication. Viral genome and antigenome are depicted as nucleocapsid covered with NP subunits. Transcription occurs in a sequential start-stop fashion during which the polymerases transcribe the genome to produce mRNA. There is a polar attenuation of transcription in which each downstream gene is transcribed less than its upstream neighbor. Vertical lines indicate gene junctions. Oval - Nucleocapsid protein, vertical lines- gene junctions, horizontal lines with slanted side bar lines-mRNA, wavy lines-viral proteins (Figure modified from Collins *et al*., 1996. Respiratory Syncytial virus, In Fields, B.N. Knipe, D.M. and Howley, P.M. (ed). Virology 3rd ed. Raven Press, New York).

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2.6.4 Virus assembly and release

The assembly of nucleocapsid core occurs in the host cell cytoplasm. The nucleocapsids are thought to be assembled in two steps: first, free NP subunits are tightly encapsidated with viral genomic RNA to form helical ribonucleoprotein (RNP) structure, and then P and L proteins are loosely bound to RNP forming transcriptase complex (Kingsbury *et al*., 1978). The membrane glycoproteins of the NDV (F and HN) are synthesized on rough endoplasmic reticulum and then undergo stepwise conformational maturation before being transported to the surface of cell membrane through the secretory pathway. Folding and maturation occur inside the endoplasmic reticulum (ER) with the help of molecular chaperones. Only correctly folded proteins are transported out of ER to Golgi apparatus for further post translational modifications. In the Golgi apparatus, the carbohydrate chains of HN protein are modified and multiple basic cleavage sites of F protein are cleaved. After successful maturation, the glycoproteins are transported to the surface of the cell membrane through vesicles where the assembly of the envelope takes place and subsequently viruses are released through budding (Doms *et al*., 1993; Feller *et al*., 1969). The detailed mechanism of NDV assembly and release at the cell membrane is unknown. The M proteins of the NDV are thought to play a major role for providing driving force that brings the assembled RNP core to the appropriate place at the plasma membrane to form a budding virion particle (Peeples, 1991). The cytoplasmic tails of F and HN glycoproteins make important contacts with M proteins, which in turn associate with the nucleocapsid thus facilitating budding of the mature virions.
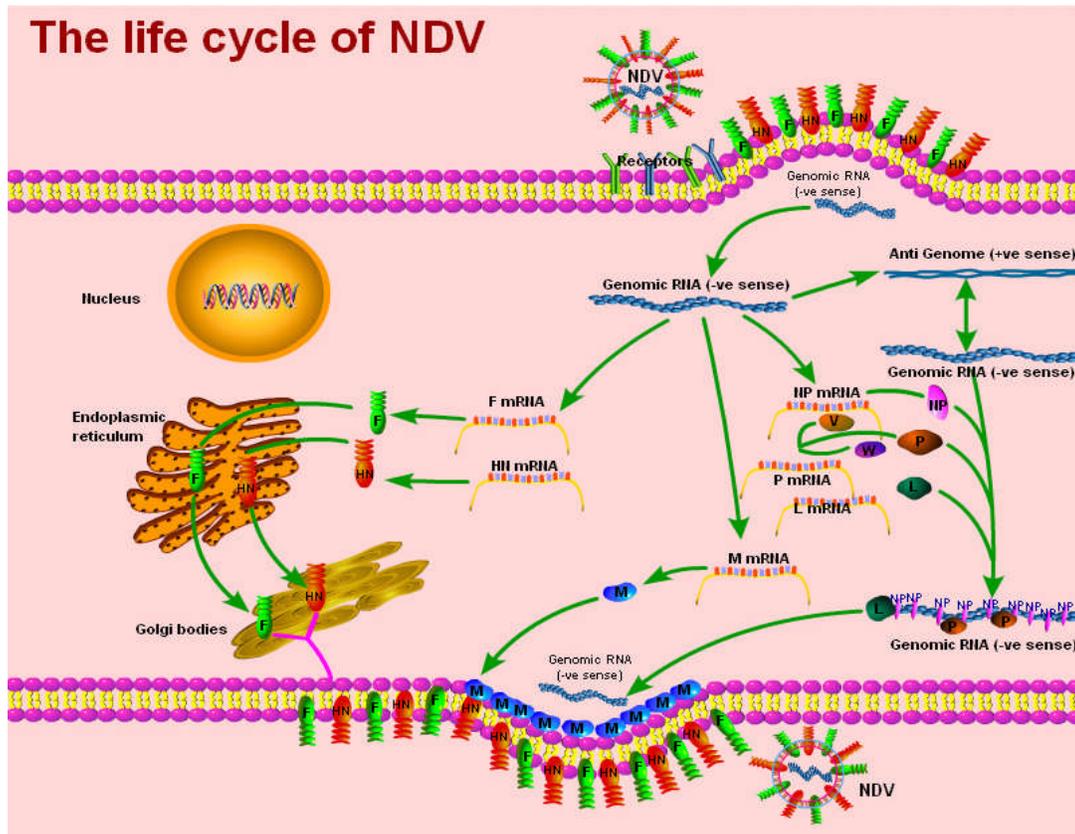
Fig. 5. The life cycle of NDV (Schematic diagram from Field's virology with modification).

2.7 Reverse genetics

Reverse genetics is a method that allows the generation of infectious virus from the cloned cDNA of the viral genome. In nonsegmented negative-strand RNA viruses, the production of infectious virus directly from the viral genome is not possible since the genomic RNA needs to be transcribed into mRNA in order to direct the synthesis of viral proteins in the host cells. This function is mediated by RDRP complex, which is virally encoded and packaged into the virion particles. Therefore, introduction of a reverse genetics system by transfecting plasmids expressing viral accessory proteins such as NP, P and L along with full length antigenome plasmid have made it possible to recover genetically engineered virus (Fig 6). The first virus successfully recovered by using this approach was rabies virus in 1994 (Schnell *et al*., 1994). All these plasmids are under the control of T7 RNA polymerase promoter and T7 RNA polymerase is provided by a recombinant vaccinia virus expressing bacteriophage T7 RNA polymerase. Subsequently, recovery of 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) and measles virus (Radecke *et al*., 1995) have been achieved. 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). Currently reverse genetics systems are available for 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 availability of a reverse genetics system for NDV as well as other viruses has provided essential information and tools to study the viral molecular mechanism in greater detail.
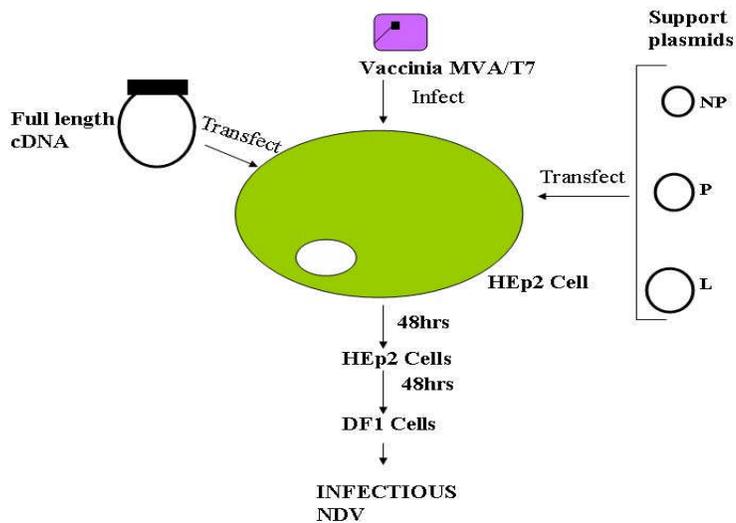
Fig. 6. Schematic Diagram for the recovery of infectious NDV from cDNA. Plasmids encoding for antigenome full length cDNA, NP, P and L mRNA were co-transfected into HEp-2 cells. All the plasmids are under control of the T7 RNA polymerase promoter. The T7 RNA polymerase is provided by the recombinant vaccinia MVA/T7 strain. Infectious NDV was generated entirely from cloned cDNA with procedures explained by Krishnamurthy *et al*., 2000.

Chapter 3 :

3.1 Title

The role of large polymerase protein (L) in NDV pathogenesis

3.2 Abstract

The large polymerase (L) protein of NDV plays a crucial role in transcription and replication. However, the role of L protein in NDV pathogenesis has not been explored. Therefore, the role of L gene in NDV virulence was investigated. In this study, we exchanged the L gene between a virulent recombinant NDV strain, rBeaudetteC (rBC), and an avirulent recombinant NDV strain, rLaSota. The chimeric recombinant viruses were recovered using reverse genetics method and their replication and pathogenicity were characterized both *in vitro* and *in vivo*. The growth characteristics in cell culture and chicken embryos showed that, both chimeric recombinant viruses were able to replicate to similar levels as of their parental strains. The virulence of chimeric recombinant viruses were tested by three standard pathotyping assays, which showed that in virulent chimeric recombinant virus with avirulent L gene, mean death time in 9-day-old chicken embryos was lower compared to its virulent parental type. Intracerebral pathogenicity index in 1-day-old chicks and intravenous pathogenicity index in 6-week-old chickens showed that the virulent virus with avirulent polymerase gene was having higher values when compared to its parental type. In 1-day-old chicken brain, the growth kinetics of the chimeric

recombinant virulent virus with avirulent polymerase was higher compared to its virulent parental strain, which indicated that virulence might be related to the function of the efficiency of L gene for virus multiplication. These results are consistent with the hypothesis that the virulence of NDV is multigenic, and HN or cleavability of F protein alone does not determine the virulence of NDV strains.

3.3 Introduction

Newcastle disease virus (NDV) or avian paramyxovirus type 1 (APMV-1) is a member of the genus *Avulavirus* of the family *paramyxoviridae* under order *Mononegavirales* (Mayo, 2002). It is an important pathogen affecting many species of birds including chickens and also causes significant economic losses to the commercial poultry industry worldwide. The virus is enveloped and contains a negative-sense, single stranded RNA genome of 15,186 nt length (Krishnamurthy and Samal, 1998). The RNA genome of NDV contains six genes encoding the six structural proteins in order from 3' to 5': nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN) and large polymerase protein (L) (Chambers *et al*., 1986b; Wilde *et al*., 1986). In common with several other paramyxovirus, transcriptional editing of the P gene by viral polymerase results in the expression of two nonstructural proteins, V and W proteins (Peeters *et al*., 2004; Steward *et al*., 1993).

NDV infection in birds causes Newcastle disease (ND), which varies from mild to severe form depending on the virus strain, host species, age, immune status, environmental stress, and presence of other organisms (Alexander, 1989). In chickens, the disease may vary from subclinical infection with no mortality to severe infection with 100% mortality. Based on the severity of the disease, NDV strains are categorized into three main pathotypes: lentogenic, mesogenic and velogenic. Lentogenic NDV strains cause inapparent or mild disease and are considered to be avirulent. Mesogenic NDV strains are of intermediate virulence causing respiratory infection with moderate mortality, while velogenic NDV strains are highly virulent causing 100% mortality in chickens. Velogenic NDV strains are further classified into viscerotropic velogenic NDV (VVNDV) and neurotropic velogenic NDV (NVNDV) strains. VVNDV strains produce lethal hemorrhagic lesions in the digestive tract, whereas NVNDV strains cause neurological and respiratory disorders. At present, virulence differentiation among those NDV strains are determined using three *in vivo* tests: 1) mean death time (MDT) in 9-day-old embryonated chicken eggs, 2) intracerebral pathogenicity index (ICPI) in 1-day-old chicks, and 3) intravenous pathogenicity index (IVPI) in 6-week-old chickens. These tests provide convenient end points, thus making it possible to compare the virulence among different NDV strains.

Several studies from different groups have shown that envelope proteins, F and HN, of NDV play important roles in tropism and virulence (Glickman *et al*., 1988; Huang *et al*., 2004; Panda *et al*., 2004a, b). The cleavage of the F protein of

NDV strains by host cell proteases plays an important role in virulence and pathogenesis of the virus (Gotoh *et al*., 1992; Nagai, 1995). The presence of monobasic amino acid at F protein cleavage site of lentogenic strains can only be cleaved by extracelluar trypsin like proteases that are restricted to specific tissues thus making the NDV strains less virulent. Similarly, polybasic amino acids at F protein cleavage site of mesogenic and velogenic strains render the inactive precursor (F_0) easily cleavable by ubiquitous host cell proteases, thus making the virus more virulent and pathogenic. Using reverse genetics, studies on several cleavage site mutants have shown the importance of the amino acid sequence at F cleavage site for NDV in virulence (de Leeuw *et al*., 2003; Panda *et al*., 2004b; Peeters *et al*., 1999) and distribution of the virus in chicken embryos (Al Garib *et al*., 2003). However, the increase in the virulence of these mutants was partial compared to that of the virulent strain, indicating that other viral genes also play important roles in NDV virulence and pathogenesis.

The HN protein, which is a major antigenic determinant of NDV, also plays an important role in viral pathogenesis and tropism. The HN chimera generated by using a low virulent and virulent virus have shown that there is an increase or decrease in pathogenicity depending on the types of the HN gene originated from the strains (Huang *et al*., 2004). However, the gain or loss, in virulence of these NDV recombinant chimeric viruses was partial when compared with their respective parental strains, suggesting involvement of other proteins in NDV virulence. The V protein, an editing product of P gene mRNA also plays an important role in

modulating the virulence in chickens (Huang *et al*., 2003) and embryonated chicken

eggs (Mebatsion *et al*., 2001; Park *et al*., 2003a, 2003b). Although the importance of

F, HN and P genes in the NDV virulence have been demonstrated in many studies,

the role of internal structural protein, such as large polymerase protein (L) in the

NDV pathogenesis has not been well investigated.

The L gene, which constitutes approximately half of the NDV genome, is the

last gene to be transcribed and its translated product is the viral RNA dependent RNA

polymerase (RNAP) or L protein. It is the least abundant protein produced by the

virus (Poch *et al*., 1990). Along with the phosphoprotein, the L protein constitutes the

active viral polymerase (Lamb and Kolakofsky, 1996). This active viral polymerase

does not utilize naked RNA genome as a template, but recognizes it only when the

genomic RNA is tightly bound to the nucleocapsid protein (Hamaguchi *et al*., 1983;

Horikami *et al*., 1992). The L protein also possesses 5' capping and poly (A)

polymerase activity of the nascent viral mRNA (Lamb and Kolakofsky, 1996). The L

gene of paramyxovirus plays a major role in transcription and replication.

Therefore, the objective of this study was to determine the role of the L

protein in NDV virulence and pathogenesis. In this study, the L gene of a moderately

virulent NDV strain Beaudette C (BC) was exchanged with that of an avirulent NDV

strain LaSota. We were able to recover recombinant chimeric viruses, indicating that

polymerases of NDV are functional under heterologous backbone. The replication

kinetics of these recombinant chimeric viruses was studied in DF-1 cells, 9-day-old

chicken embryos, and 1-day-old chicken brains. The pathogenicity of these viruses was evaluated by MDT, ICPI and IVPI assays (Alexander, 1989). Our results demonstrated that the polymerase gene of NDV could contribute to the virulence of the virus in its natural host, chicken.

3.4 Materials and methods

3.4.1 Cells and Viruses

DF-1 cells (Chicken embryo fibroblast cell line; ATCC CRL 12203) were maintained in Dulbecco's modified Eagle's medium (DMEM) and HEp-2 cells were maintained with Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). A moderately pathogenic (mesogenic) NDV strain, BC, and a lentogenic avirulent vaccine strain, LaSota, were received from National Veterinary Services Laboratory (Ames, IA). The recovered recombinants as well as wild-type viruses were propagated into the allantoic cavity of 9-day-old embryonated specific pathogen free (SPF) chicken eggs. After 48 h of infection, the allantoic fluids were harvested and purified (Panda *et al*., 2004). The modified vaccinia virus Ankara recombinant expressing the T7 RNA polymerase (a generous gift of Bernard Moss, National Institute of Health) was grown in primary chicken embryo fibroblast cells.

3.4.2 Construction of plasmids and recovery of chimeric viruses.

Full length antigenomic cDNAs of NDV strains BC and LaSota were cloned into low-copy-number plasmid pBR322 and designated as pBC and pLaSota

respectively. The ORF of nucleocapsid protein (NP) was cloned in the plasmid pGEM7z (+) (Promega, Madison, WI.) between *Eco*RI and *Bam*HI sites, and ORFs of phosphoprotein (P) and large polymerase protein (L) were cloned into an expression plasmid which has an encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) and named as pN, pP and pL, respectively. These full length cDNA clones along with support plasmids pN, pP and pL under T7 promoter were used to recover recombinant viruses rBC and rLaSota (Huang *et al*., 2001; Krishnamurthy *et al*., 2000).

In this study, we generated recombinant chimeric rBC virus containing the LaSota L gene and the reciprocal recombinant chimeric rLaSota virus containing L gene of BC. To exchange L gene between rBC and rLaSota, a unique restriction site, *Pac*I, was created after the L gene ORF on full length plasmid pNDVfl. To prevent any mutations in the trailer region of the pNDVfl, we adopted the cloning strategy given in details in the Fig. 7.

The *Sac*II-*Not*I fragment of pNDVfl containing *Rsr*II site was deleted by inserting a PCR product that was amplified using the AscI F primer (5'-CTGAGGCGCGCCTAATACGACTCACTATAGGACCAAACAGAGAATCCGTG AGTTA-3') and Not/Sac⁻ primer (5'-ACGTGCGGCCGCTGTTTCCGCGGCTG GGTTGACT-3') resulting in the plasmid pNDV-fl (Δ*Rsr*II). The *Afl*II-*Rsr*II fragment of the L gene was amplified from pNDVfl (Both from rBC and rLaSota) in two steps and subcloned into pGEM-7Z (+) between the *Xba*I and *Bam*HI sites. In the first step,

*Afl*II-*Bam*HI fragment was amplified by using the Xba-AflF primer (5'-

GCTCTAGACTTAAGAAACATACGCAAAGAG-3') and BamPac/L⁻ primer (5'-

CTGGATCCATAAT<u>TTAATTAA</u>ATCAACAAGAATACAATTGGCC-3') and then

subcloned between the *XbaI* and *Bam*HI site of pGEM-7Z (+) resulting in pGEM-

7Z(Afl-Pac). The introduced mutation *pac*I site is underlined. The second fragment

was amplified from pNDV-fl using the Pac/B or Pac/L primer (5'-CTGGATCCATA

GT<u>TTAATTAA</u>ATCACCAAGGATACAATTGGCC-3') for rBC and (5'-CTGGA

TCCATAAT<u>TTAATTAA</u>ATCAACAAGAATACAATTGGCC-3') for rLaSota and

the BamRsr⁻ primer (5-'CTGGATCCGGACCGCGAGGAGGTGGAGATG-3') and

then subcloned into the plasmid pGEM-7Z (Afl-Pac) between *Pac*I and *Bam*HI sites.

The resulting plasmids contained *Afl*II-*Rsr*II fragment of both rBC and rLaSota with

an introduced *Pac*I site after the L gene ORF. The mutated *Afl*II-*Rsr*II fragments were

excised from pGEM-7Z and replaced with corresponding counterpart in pNDV-fl of

both rBC and rLaSota. Then *Asc*I-*Sac*II fragment was reintroduced into pNDV

(ΔRsrII) to obtain full length cDNA called as pNDV-fl/PacI. The pNDV-fl/PacI of

rBC and rLaSota were digested with *Age*I and *Pac*I to exchange the L gene. The full

length plasmid of BC with L gene of LaSota was designated as pBCLaSoL, whereas

the full length plasmid of LaSota with L gene of BC was designated as pLaSoBCL.

The chimeric viruses were recovered from these full length plasmids as

described previously (Krishnamurthy *et al*., 2000). Briefly, in a six well plate, HEp-2

cells at 80-90% confluence were infected with MVA-T7 at a one focus forming unit

per cell. Then, transfection was carried out by incubating the vaccinia infected HEp-2

32

cells with 2.5 µg of pNP, 1.5 µg of pP , 1.0µ g of pL along with 5.0 µg of pNDV-fl. Liopectamine^{TM} (Invitrogen) was used for transfection according to the manufacturer's protocol. After 8 h, the supernatant was discarded and fresh DMEM containing 2% FBS was added. Supernatant was collected after 48 h and passaged into fresh HEp-2 cells to remove residual vaccinia virus. After three days infection, the supernatant was harvested, clarified, and infected to 6 well confluent DF-1 cells until the virus specific cytopathic effect or syncytia was developed. The recovered viruses were then plaque purified on DF-1 cells and subsequently, propagated in allantoic cavity of 9-day-old SPF chicken eggs. After 48 h of infection, the allantoic fluid was harvested, centrifuged and the virus stocks were prepared and stored at -70 °C for future use.
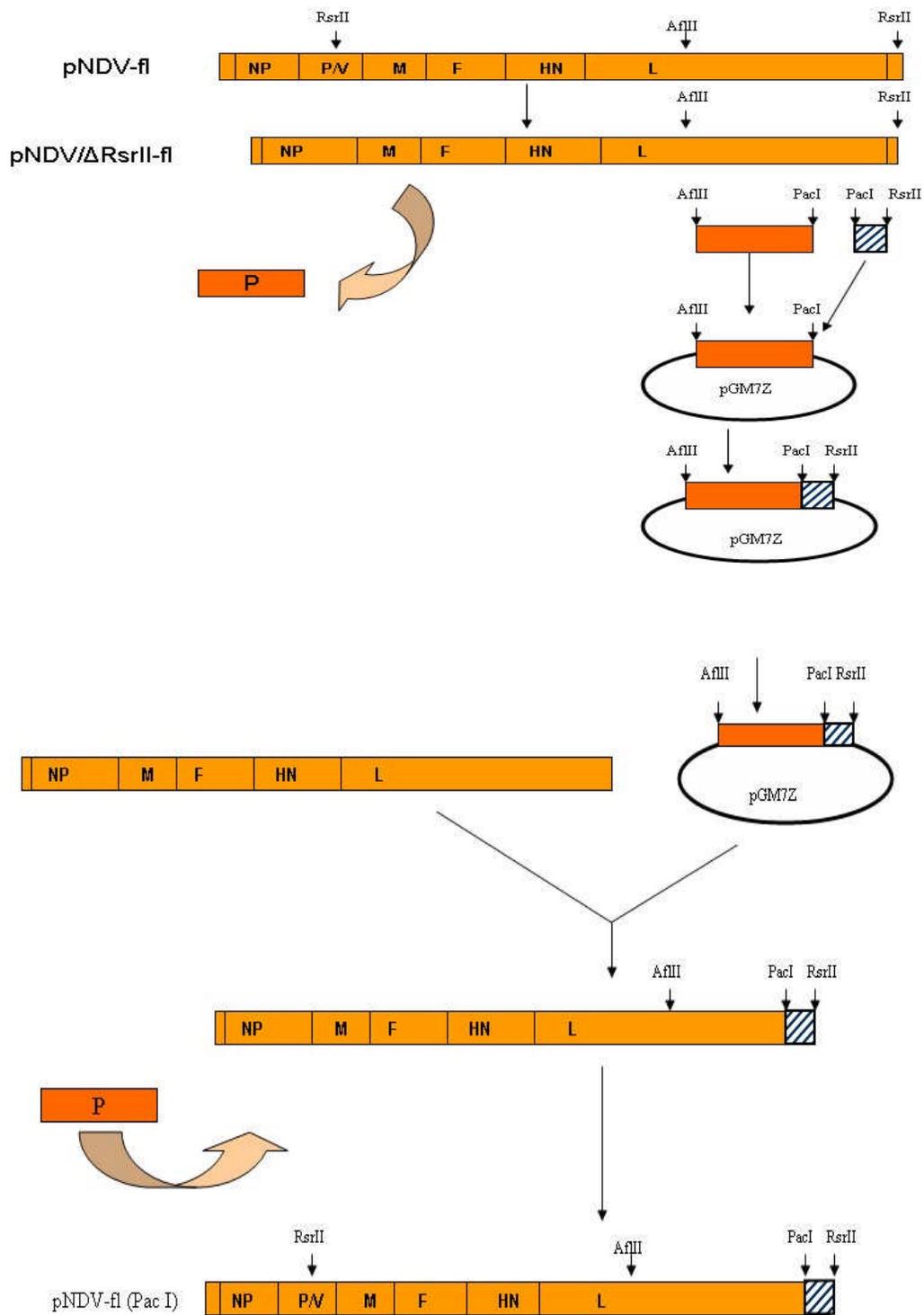
Fig. 7. (A) Diagram of the strategy applied to introduce a unique restriction site *Pac*I to the full length cDNA of BC and LaSota
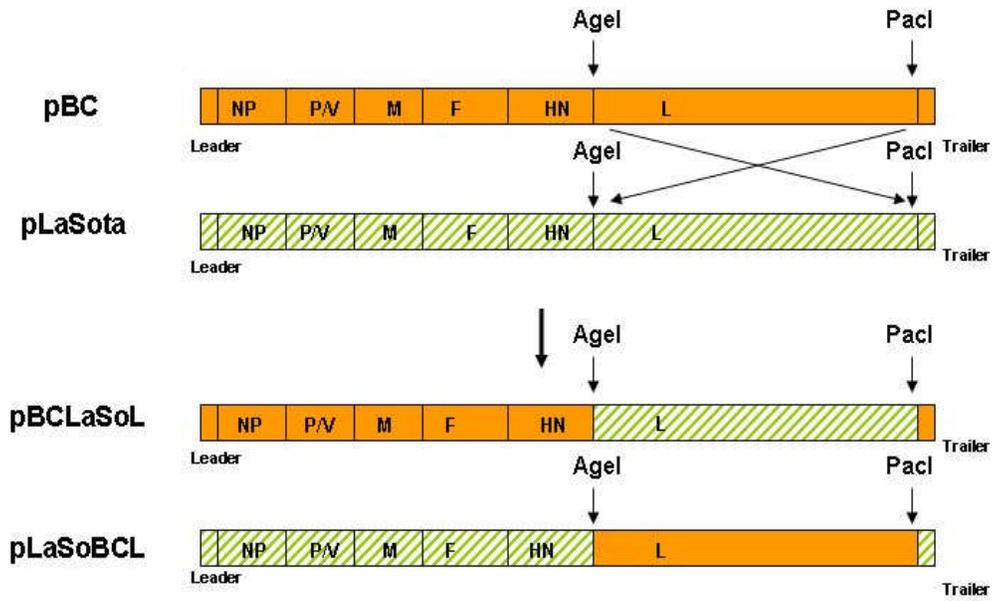
Fig. 7. (B) Schematic representation of the exchange of L gene between the full
length cDNA of BC and LaSota

3.4.3 RNA extraction and RT-PCR of recovered chimeric viruses

The recovered recombinant chimeric viruses were inoculated into the allantoic cavity of 9-day-old embryonated SPF chicken eggs. After 48 h, embryos were collected and chilled. Allantoic fluids were collected, aliquoted, and stored for further characterization. Virus stocks were propagated twice in DF-1 cells before RT-PCR and sequencing. Total RNA from infected DF-1 cells were isolated using TRIzol (Invitrogen, Carlsbad, Calif) according to the manufacturer's instructions. The extracted total RNA was subjected to reverse transcription by the superscript RT kit (Invitrogen) to obtain the first strand cDNAs. The first strand cDNAs were PCR amplified with primers, HN7957 (5'-CGAGTGAGTTCAAGCAGTACCAAAGC-3') and HN/L⁻(5'-TGTCTGCTGAGAATGAGGTG-3') for BC strain; and primers, L6400 (5'-GGGTCTTAGAGTCGAAGATCTC-3') and 15186R (5'-ACCAAACAAAGATTTGG-3'), for the LaSota strain. The PCR products were purified using PCR purification kit (Qiagen, Valencia, CA) and sequenced by ABI 3100 DNA sequencer. The complete L genes of chimeric viruses were also sequenced to confirm the presence of substituted genes.

3.4.4 Growth characteristics of recombinant viruses in DF-1 cells and chicken embryos

The growth kinetics of chimeric recombinant viruses along with their respective wild-type recombinant viruses was determined using single-cycle as well as multi-cycle growth curve in DF-1 cells. For single cycle growth curve and multi-cycle growth curve, the cells were infected at a multiplicity of infection (MOI) of 10

and 0.01, of the virus, respectively. For single cycle growth curve, supernatants were

collected every 4 h up to 32 h post infection (p.i.). For multi-cycle growth curve

supernatants were collected at every 8 h up to 56 h p.i. The virus titers in the samples

were quantified by plaque assay in DF-1 cells. To evaluate replication of recombinant

chimeric viruses in chicken embryo, 9-day-old SPF chicken embryos were infected

with 10^3 PFU of virus particles per embryo through chorio-allantoic route. Every 12 h

interval, allantoic fluid sample was harvested from three embryos and the titer of

virus in the samples was determined by plaque assay. Briefly, supernatants collected

from the virus infected DF-1 cell samples and allanotic fluids were serially diluted

and each dilution (100 μl) was infected to 12 well plate DF-1 cells in duplicates. After

1 h of virus adsorption, supernatants were removed from wells, washed with PBS,

and then overlaid with DMEM supplemented with 0.8% methylcellulose and 2 %

FBS. Cells infected with either rLaSota or rLaSoBCL were added with 1 µg of acetyl

trypsin/ml into the medium. The infected cells were incubated at 37 °C for 3-4 days

until the development of countable plaques. Then, the cells were fixed with methanol

and stained with crystal violet for enumeration of plaques.

3.4.5 Mean death time (MDT) in chicken embryos

The virulence of the recovered chimeric recombinant viruses was determined

by mean death time (MDT) in 9-day-old embryonated SPF chicken eggs (Alexander,

1989). Briefly, a series of 10-fold dilutions of fresh infective allantoic fluild was

made in PBS and 100 µl of each dilution was injected into the allantoic cavity of five

9-day-old embryonated chicken eggs. The remaining samples were kept on ice and

injected into five embryos after 8 h. The eggs were incubated at 37 °C and examined

for embryo mortality four times daily for seven days. The time of each embryo death

was recorded and the highest dilution at which all ten embryos died was considered as

the minimum lethal dose. The MDT was calculated as the mean time in hours taken

by the minimum lethal dose to cause the death of all the embryos.

3.4.6 Pathogenicity studies in chickens

All the animal experiments were performed according to standard animal

protocols approved by Institutional Animal Care and Use Committee at the BSL2+ or

BSL3 facility. The pathogenicity of the recombinant chimeric viruses was studied *in*

vivo using the intracerebral pathogenicity index (ICPI) and intravenous pathogenicity

index (IVPI) as described elsewhere, with modifications (Alexander, 1997). For ICPI,

fresh infective allantoic fluid with HA titer$> 2^4$ were diluted to 1:10 with sterile PBS

and injected into groups of ten 1-day-old SPF chicks, via the intracerebral route. The

inoculation was performed using a 4 mm long 27 gauge needle attached to a 1 ml

stepper syringe dispenser set to dispense 50 µl of the inoculum. The inoculum was

injected into the left or right rear quadrant of the cranium. For IVPI, fresh infective

allantoic fluid with HA titer$> 2^4$, was diluted to 1:10 in PBS and 0.1 ml was

intravenously inoculated into groups of 10 6-week-old SPF chickens. Mock infected

birds were received a similar volume of sterile PBS by the respective route. In both

studies, birds were observed every 8 h for the development of clinical symptoms and

mortality for up to 8 days and 10 days respectively. The scores of ICPI and IVPI

values were calculated according to the method described elsewhere (Alexander, 1997).

3.4.7 Growth kinetics of recombinant viruses *in vivo*

To study the growth kinetics of recombinant chimeric viruses in chicken brain, 1-day-old SPF chicks were inoculated with 10^4 PFU of virus/chick by intracranial route. Brains were collected daily for 7 days and snap frozen. The brain tissues were homogenized and the virus titers in the tissue were determined by plaque assay in DF-1 cells.

3.5 Results

3.5.1 Generation and recovery of chimeric NDVs from cDNA clones

Previously, we reported the recovery of recombinant NDVs from their respective infectious cDNA clones, derived from a mesogenic strain of NDV, BC and a lentogenic NDV strain, LaSota (Huang *et al*., 2001; Krishnamurthy *et al*., 2000). In this current study, we used the established reverse genetics system from our laboratory to explore the possible role of the large polymerase protein in the virulence of NDV. We adopted the strategy of exchanging the L gene from virulent mesogenic strain BC to avirulent lentogenic vaccine strain LaSota by introducing unique restriction site to their respective cDNA clones. This was achieved by introducing *Pac*I site at the end of L gene ORF before the trailer region. Once the L gene was exchanged, the entire cDNA clone of each chimeric virus was sequenced to ensure

the presence of specific gene exchange and the absence of any undesired mutations.

Recombinant chimeric viruses were recovered by transfecting full length cDNA

clones along with support plasmids. To obtain the pure clone of virus, the respective

recombinant viruses were triple plaque purified and amplified in 9-day-old

embryonated eggs. Total cellular RNA was extracted from infected DF-1 cells and

RT was performed to transcribe cDNA fragments, and then PCR was adopted to

amplify the regions covering L gene as well as F gene of NDV. The sequencing of

amplified PCR fragments confirmed the introduction of unique restriction site *Pac*I as

well as exchanged L gene between BC and LaSota. The recovered recombinant BC

with Lasota L gene was designated as rBCLaSoL, whereas the recombinant LaSota

bearing BC L gene was designated as rLaSoBCL. After 5 sequential passages in DF-1

cells as well as 9-day-old embryonated chicken eggs, the chimeric viruses were stable

without any changes in the exchanged region. To rule out that any mutations occurred

in the region of virulent cleavage site, the sequencing of amplified F gene cleavage

site of chimeric viruses was performed. We confirmed the absence of any changes in

the full length backbone of virus.

3.5.2. Cytopathogenicity and plaque morphology of chimeric recombinant viruses

The cytopathic effects (CPE) caused by recombinant chimeric viruses were

studied in DF-1 cells (Fig. 8A). At 24 h PI, the cells infected with recombinant

chimeric viruses, rBCLaSoL and rLaSoBCL, showed similar CPE when compared to

their respective parental virus rBC and rLaSota. However, at 48 h PI, rBCLaSoL

infected cells showed more extensive CPE than rBC and rLaSoBCL. The level of

CPE in rLaSota and rLaSoBCL were comparable both at 24 h and 48 h PI. Similarly,

the average plaque size of rBCLaSoL was slightly larger than that of rBC. There was

no difference in plaque size between rLaSota and rLaSoBCL. As the rLaSota virus

grew 1-log cycle lower than rBC, the plaque sizes of rLaSota and rLaSoBCL were

smaller than that of rBC and rBCLaSoL (Fig. 8B).

3.5.3 Growth of chimeric recombinant viruses in DF-1 cells

The growth characteristics of the wild type as well as chimeric viruses were

assessed by single step as well as multi-step growth curves in DF-1 cells. In multi-

cycle growth kinetics, there was no difference in growth rate between rLaSota and

rLaSoBCL throughout the cycle, whereas rBCLaSoL grew 1-log cycle higher than

rBC virus (Fig. 10). There was no difference in growth pattern between rBC and

rBCLasoL in the early stage of virus replication (up to 24 h), however, the difference

in growth kinetics was observed after 24 h p.i. In one step growth kinetics study,

rLaSota and rLaSoBCL grew at the same rate up to 12 h whereas rBCLaSoL grew 1-

log cycle higher than rBC. After 12 h p.i, the growth pattern of both chimeric viruses

was similar to their respective parental viruses (Fig. 9).

(A)

24hr

48hr

(B)

Mock infected   rBC   rBCBLaSoL   rLaSota   rLaSoBCL
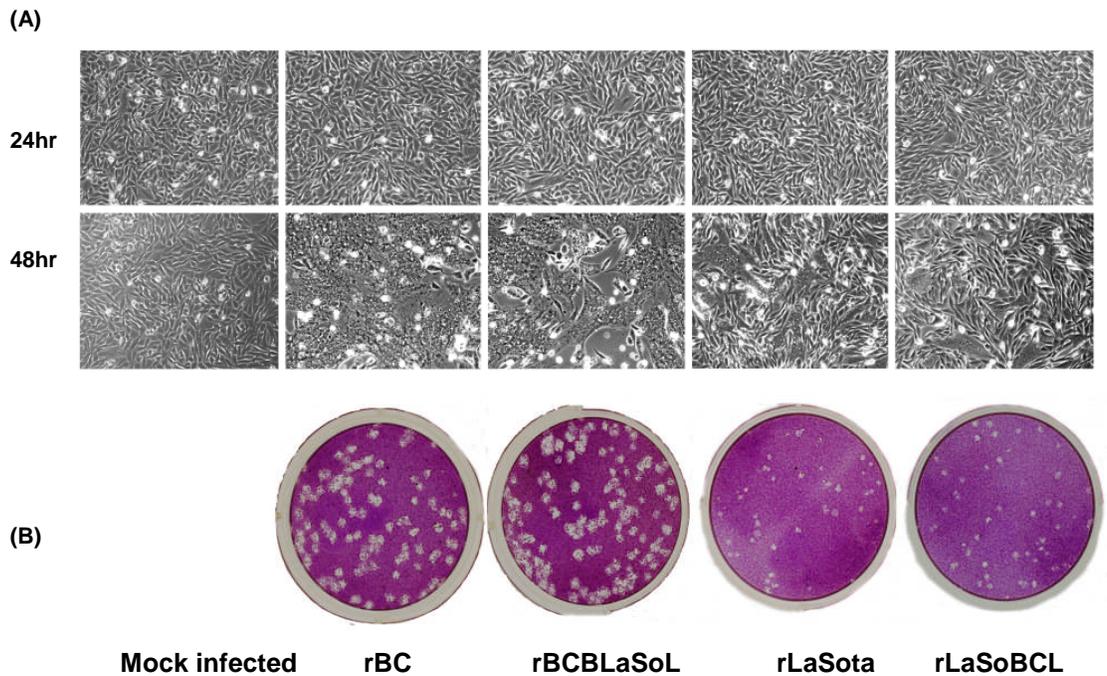
Fig. 8. The cytopathogenicity of parental and chimeric viruses in DF-1 cells. (A) The cells were infected with each virus at a MOI of 0.01. After 24 and 48 h PI, the cytopathic effect (CPE) of each virus-infected monolayer was observed under an inverted microscope.

(B) Plaque morphologies of recombinant as well as parental virus strains. For plaque development, the monolayers of DF-1 cells were infected with each virus for 1 h. Then the cells were washed with PBS and overlaid with 2% DMEM containing 0.8% methyl cellulose. The cells infected with rLaSota or rLaSoBCL were added with 10% fresh allantoic fluid. After 4 days, the plaques were fixed with methanol, stained with crystal violet, and visualized under an inverted microscope.
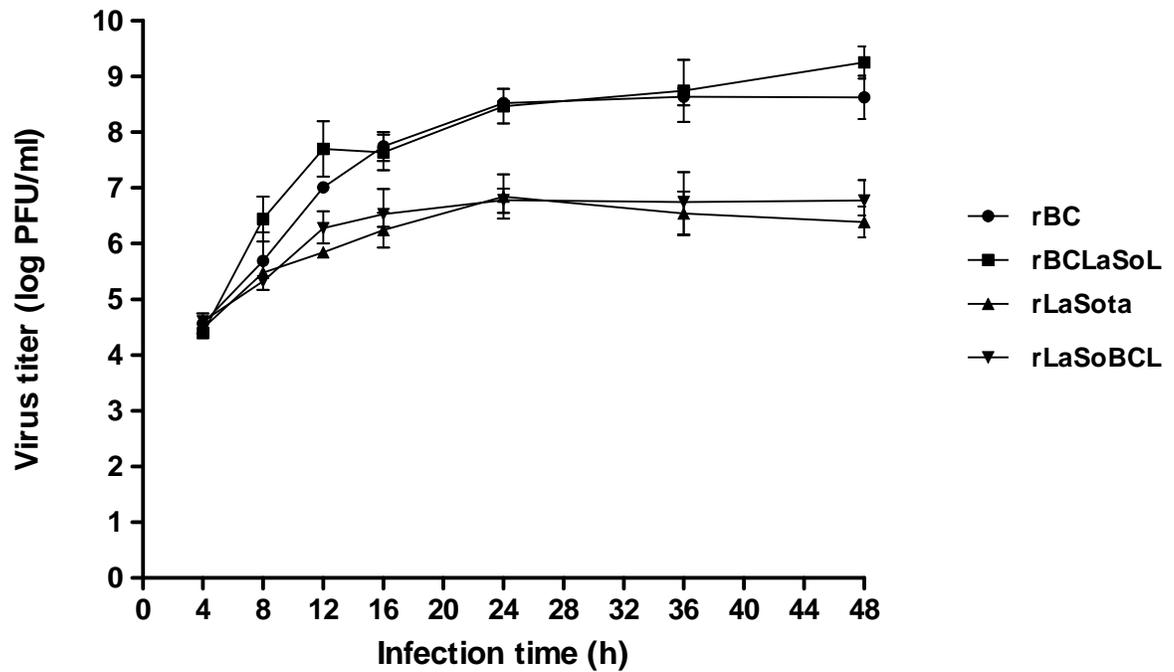
Fig. 9. Single step growth kinetics of rBC, rBCLaSoL, rLaSota and rLaSoBCL in chicken embryo fibroblast (DF-1) cells. Single step growth kinetics study was performed to assess the difference in generation time of the recombinant chimeric viruses compared to wild type viruses. DF-1 cells were infected with each virus at a 10 MOI per cell. At 4 h interval, supernatant samples were collected and replaced by an equivalent volume of fresh medium. The cells infected with rLaSota or rLaSoBCL were added with either 5 µg of trypsin/ml or 10% allantoic fluid. The titers of the viruses in the collected samples were determined by plaque assay in DF-1 cells. The virus infection in DF-1 cells and virus titration were performed in triplicates.

3.5.4 Growth of chimeric recombinant viruses in chicken embryos

The growth rate of rBC, rBCLaSoL, rLaSota and rLaSoBCL viruses were evaluated in 9-day-old embryonated SPF chicken eggs. Embryonated SPF chicken eggs were inoculated with viruses by the allantoic route at a dose of 10^3 PFU/egg. Three embryos were chilled in every 12 h. Allantoic fluid samples were harvested, clarified and titrated on DF-1 cells by plaque assay. Our results showed that the chimeric virus rBCLaSoL grew 1-log higher than rBC up to 36 h post infection and gradually reached to the same level of growth with rBC after 48 h of infection. However, there was no growth difference between rLaSota and rLaSoBCL throughout the kinetic study.
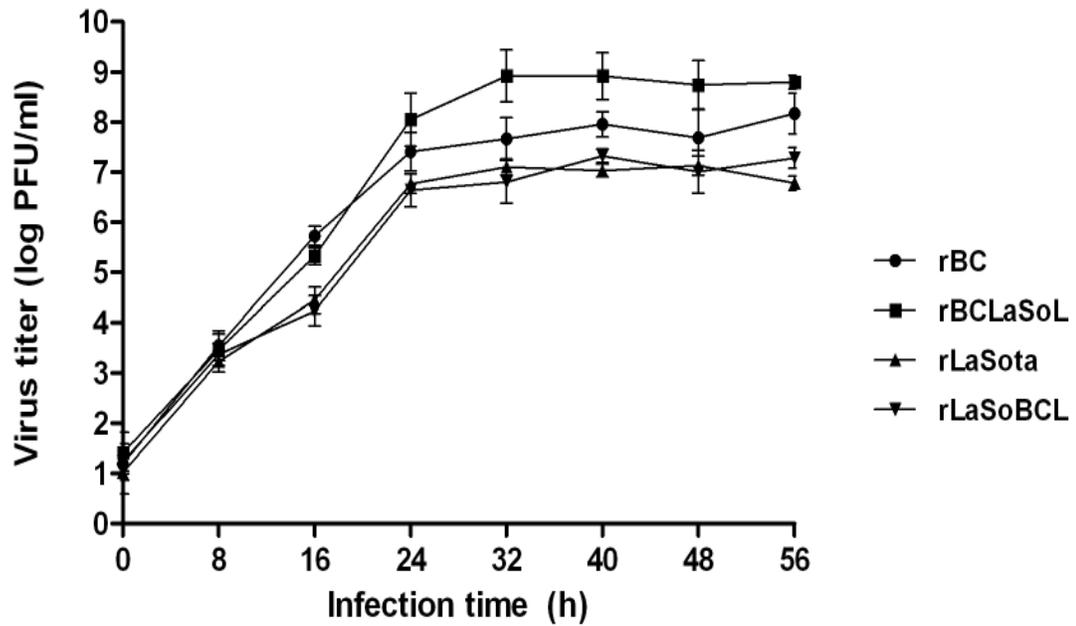
Fig. 10. Multi-step growth kinetics of rBC, rBCLaSoL, rLaSota and rLaSoBCL in chicken embryo fibroblast (DF-1) cells. DF-1 cells were infected with each of these viruses at a MOI 0.01 PFU per cell. Every 8 h, supernatant samples from infected cells were collected and replaced by an equivalent volume of fresh medium. The cells infected with rLaSota or rLaSoBCL were added with either 5 µg of trypsin/ml or 10% allantoic fluid. The titers of the viruses in the collected samples were determined by plaque assay in DF-1 cells. The virus infection in DF-1 cells and the virus titration were performed in triplicates.
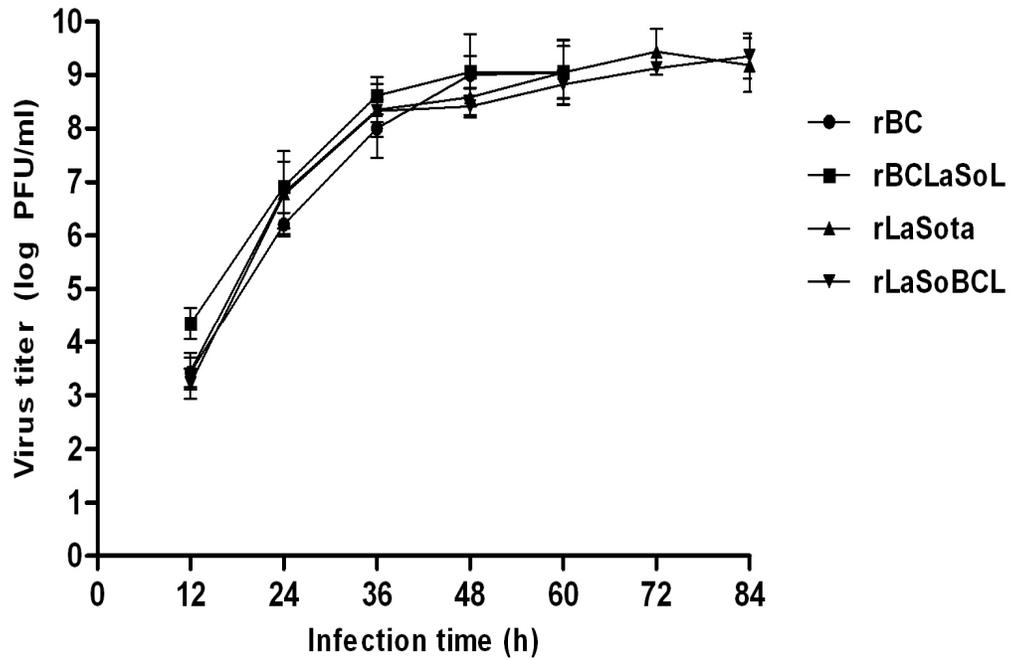
Fig. 11. The growth kinetics of recombinant viruses rBC, rBCLaSoL, rLaSota, and rLaSoBCL in 9-day-old embryonated eggs. The eggs were injected with 10^3 PFU of virus via allantoic route. Three embryos were chilled every 12 h. Allantoic fluids were collected from these eggs and titers of the viruses were determined by plaque assay in DF-1 cells.

3.5.5 Pathogenicity studies of chimeric recombinant viruses

We determined the change in pathogenicity of the chimeric recombinant viruses by standard pathogenicity tests, MDT, ICPI and IVPI. We conducted these tests two times and the results are given in table 1. From our study, MDT results showed that, rBCLaSoL took 53 h and 56 h in test 1 and 2 respectively to kill all the embryos compared to 62 h and 60 h by rBC, whereas, rLaSoBCL took 106 h and 110 h in test 1 and 2 respectively to kill the embryos compared to 106 h and 110 h by rLaSota. In ICPI test, the rBCLaSoL had an ICPI value of 1.70 and 1.80 in test 1 and test 2 respectively, while its mesogenic parental strain, rBC had an ICPI value of 1.20 in test 1 and 1.49 in test 2. However, the rLaSoBCL, showed ICPI value of 0.00 in both test 1 and test 2 as that of its lentogenic parental strain, rLaSota indicating no change in pathogenicity in 1-day-old chicks. In addition, the pathogenicity of these chimeric recombinant viruses was evaluated in 6-week-old chickens by IVPI test. The IVPI value of the rBCLaSoL was 2.26 and 2.33 in test 1 and test 2 respectively when compared to its wild type rBC IVPI value of 2.06 and 2.00 whereas there was no change in IVPI values in the rLaSota and rLaSoBCL.

Table .1. L chimera of NDV and virus pathogenicity *in vivo*

Virus	[a]MDT (h)		[b]ICPI		[c]IVPI	
	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2
rBC	62	60	1.20	1.49	2.06	2.00
rBCLaSoL	53	56	1.70	1.80	2.26	2.33
rLaSota	106	110	0.00	0.00	0.00	0.00
rLaSoBCL	115	114	0.00	0.00	0.00	0.00

The virulence of parental and chimeric recombinant viruses were evaluated by mean death time (MDT) in 9-day-old embryonated SPF chicken eggs, intracerebral pathogenicity index (ICPI) in 1-day-old SPF chicks and intravenous pathogenicity index (IVPI) in 6-week-old SPF chickens.

[a] MDT in hours is the time taken by the minimum lethal dose to kill all 9-day-old embryonated chicken eggs inoculated by allantoic route. Highly virulent viruses take less than 60 h, moderately virulent viruses take 60-90 h, and avirulent viruses take more than 90 h to kill all the chicken embryos.

[b] ICPI was determined by inoculating 1:10 dilution of freshly infective allantoic fluid into a group of 10 one-day-old SPF chicks via intracerebral route. The birds were observed for 8 days for development of clinical signs and scored as 0 (normal), 1 (sick) and 2.0 (dead). The ICPI value was calculated as the mean score per bird per observation.

[C] IVPI value was determined by inoculating 1:10 dilution of fresh infective allantoic fluid into a group of 10 six-week-old SPF chickens via intravenous route. The birds were observed up to 10 days for the development of clinical signs and scored as 0 (normal), 1 (sick), 2 (paralyzed) and 3 (dead). The IVPI value was calculated as mean score per bird per observation.

3.5.6 Growth kinetics and pathogenesis of recombinant chimeric viruses *in vivo*

To evaluate the increase in virulence of the chimeric L virus rBCLaSoL, we conducted the growth kinetics of the recombinants in 1-day-old chicken brain. Chicks were injected intracerebrally with 10^3 PFU of virus per chick, brain samples were collected every 24 h and the replication of chimeric viruses were determined by plaque assay in DF-1 cells. We found that the titers of rBCLaSoL were 2-3 log cycles higher than that of parental rBC in the first three days of post inoculation and continued to show a consistently higher growth titer up to 5 days of infection, indicating an accelerated replication in brain. However, the titer of rLaSota and rLaSoBCL in chicken brain gradually diminished with time due to rapid clearance of viruses from brain over time. This is expected as the NDV strains with avirulent F cleavage sites require trypsin like proteases for their growth found in limited tissues.
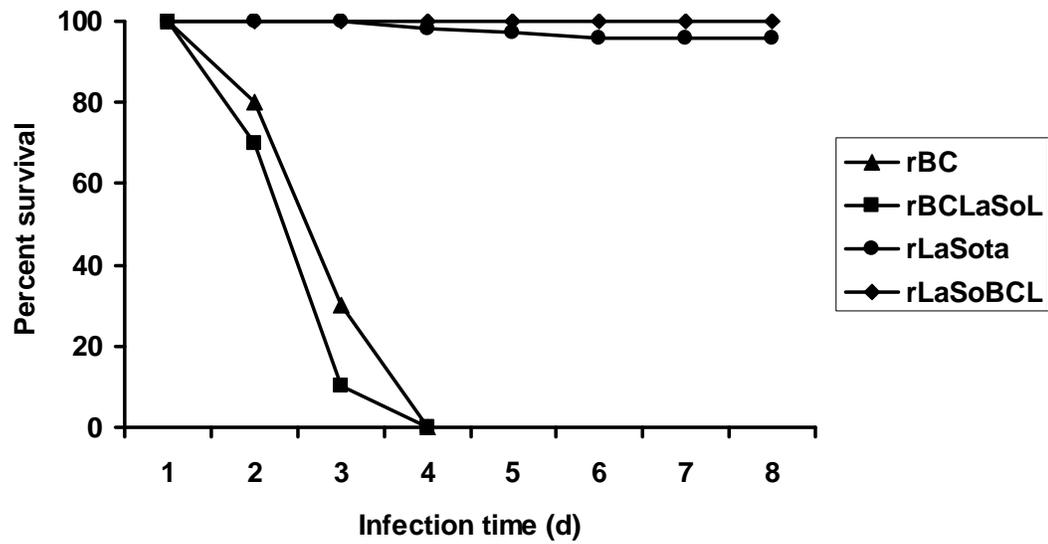
Fig. 12. The survivability of 1-day-old chicks inoculated with the parental or L chimeric viruses. Specific-pathogen-free 1-day-old chicks were inoculated intracerebrally with the 10^5 PFU of parental or L chimeric viruses per chick. Infected chicks were observed daily for 8 days for signs of paralysis and death. Percent survivors of each virus are plotted over time.
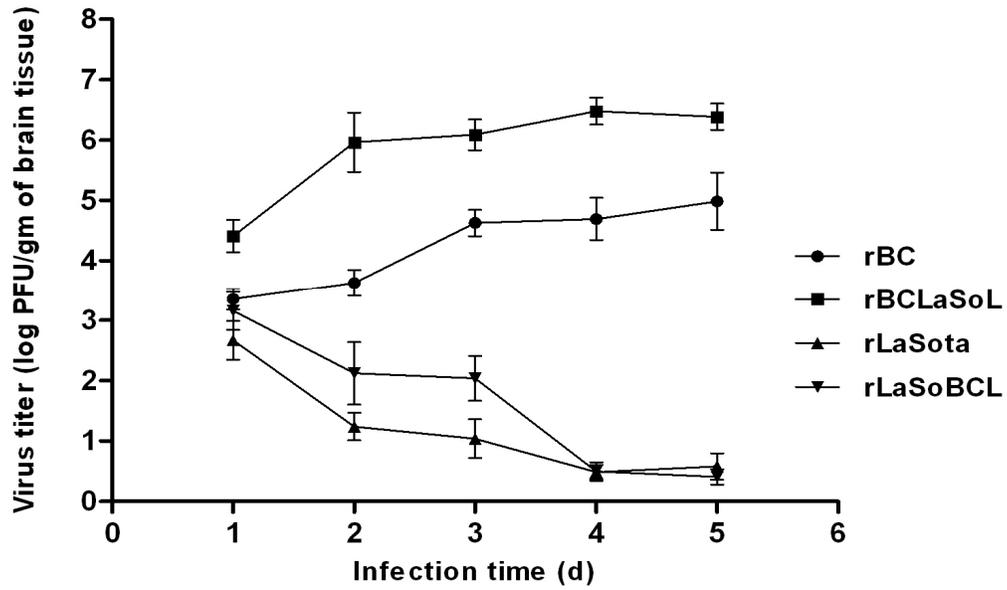
Fig. 13. The growth kinetics of parental and L-chimeric NDV strains in the brains of 1-day-old chicks. SPF 1-day-old chicks were inoculated intracerebrally with 10^3 PFU of virus/chick. Brains from live birds were collected daily, homogenized, and titrated by plaque assay in DF-1 cells.

3.6 Discussion

NDV has shown a wide spectrum of virulence from completely non pathogenic with no clinical signs to highly virulent form with 100% mortality in chickens (Alexander, 1997). In particular, highly virulent velogenic forms of NDV strains are able to reach 2.00 of the ICPI score (the ICPI value ranges from 0.00 to 2.00). Several studies have demonstrated that the HN protein and cleavability of the F protein are major determinants of NDV virulence (Huang *et al*., 2004; Panda *et al*., 2004b). However, several NDV strains, such as Texas-GB, CA-1083 (Fontana) and BC possessing the same F protein cleavage site of virulent strains, show differences in their degree of pathogenicity. Similarly, there are also varieties in the degree of pathogenicity in several avirulent NDV strains, such as B1, Ulster and LaSota containing avirulent F protein cleavage site. These indicate that along with F and HN proteins, other viral proteins might contribute to NDV pathogenesis.

The internal proteins, such as NP, P and L in other paramyxoviruses have been modified to modulate the pathogenesis or to induce attenuation (Whitehead *et al*., 1999; Haller *et al*., 2001; Newmann *et al*., 2004; Bailly *et al*., 2000; Pham *et al*., 2005). However, the contribution of L gene to the pathogenesis of NDV has not been explored in details. In this study, we used a recombinant mesogenic NDV strain, rBC and a recombinant avirulent lentogenic NDV strain, rLaSota to investigate the role of L gene in NDV pathogenesis.

The L gene of NDV encodes for RNA dependent RNA polymerase protein (RDRP) and is the largest gene in the viral genome. Apart from transcription and replication of viral RNA, the RDRP of the virus is a multifunctional viral protein with polyadenylation and capping activities of viral mRNAs (Lamb and Kolakofsky, 1996). Using PCR mutagenesis method, the unique restriction site *Pac*I was introduced after the L gene ORF of both rBC and rLaSota full length cDNA without disturbing the trailer region because any mutations in the trailer region can greatly impair the virus recovery and its function. Using reverse-genetics techniques both the chimeric recombinant viruses were recovered and the pathogenesis of these viruses was studied extensively in its natural host, chickens. Our results indicated that the polymerase gene of NDV might play a role in contributing to pathogenesis in chickens.

Using reverse genetics method, we were able to recover both chimeric virus rBCLaSoL and rLaSoBCL, indicating that the L gene in heterologous backbone is functional. The functionality is probably attributed to the high level of amino acid sequence similarity (99.1%) between phylogenetically related BC and LaSota strains of NDV (Wise *et al*., 2004). However, heterologous polymerase functionality between distantly related other paramyxovirus-1 strains is unclear. To recover all the chimeric viruses, we used support plasmids pNP, pP of BC and LaSota for rBCLaSoL and rLaSoBCL, respectively but only pL of rBC for both chimeric backbone. Therefore, after the recovery, both the recombinant viruses were triple plaque purified to rule out any mixed population of virus. The RT-PCR and sequencing of the L gene

of the chimeric viruses was performed. Sequence analysis of swapped L gene region of chimeric viruses showed the presence of unique restriction site *Pac*I as well as the presence of chimeric L gene. To rule out any possible recombination at plasmid level during transfection, the entire L gene was confirmed by sequencing of the RT product from the L gene mRNA. Differences in growth between parental and chimeric viruses were observed in cells as well as embryos. During multi cycle growth in DF-1 cells, we found the mesogenic NDV strain BC with avirulent NDV strain LaSota polymerase protein grew 1 log titer higher than parental strain. Surprisingly, lentogenic avirulent NDV strain LaSota with mesogenic NDV strain BC L protein showed no difference in growth compared to parental strain LaSota. From MDT, we observed, the mortality in 9-day-old chicken embryos was earlier in case of the virulent NDV with avirulent L gene when compared to its parental strain. In embryonated chicken eggs, when the NDV is injected through allantoic route, the virus grows in cells lining the allantoic cavity, rapidly destroying the cells and releasing to the allantoic fluid. From our kinetics study in embryos, we observed that both virulent as well as avirulent virus reached the highest titer between 24 and 36 h of post injection. However, up to 24 h post inoculation, the virulent virus with avirulent polymerase gene grew 1 log higher compared to its wild type mesogenic as well as lentogenic virus. In NDV infection, no remarkable histopathological changes can be seen in the embryo at any stages of infection, but apoptosis of heart and brain cells is believed to be the major cause of embryo death (Lam *et al*., 1995). Thus, decreased mean death time of the virulent recombinant virus with avirulent L gene

when compared to its parental type, might be due to the accelerated replication and rapid spread of the virus in embryonic tissues.

The pathogenicity of recombinant chimeric viruses was evaluated by ICPI and IVPI tests. In ICPI, the chimeric rBC with L gene of LaSota (rBCLaSoL) has the ICPI value higher than its parental rBC virus, whereas there was no significant change in ICPI value in case of chimeric rLaSota with L gene of BC (rLaSoBCL). The survival rate of 1-day-old chicks injected with rBCLaSoL was lower compared to its parental type rBC whereas in case of rLaSotaBCL, all the chicks survived as in case of rLaSota. In IVPI, the chimeric virus rBCLaSoL had higher value than rBC whereas there was no change in the case of rLaSoBCL. However, compared to the ICPI value between these recombinants, the IVPI value of rBCLaSoL was slightly elevated than the parental type rBC. The mortality rates of 1-day-old as well as 6-week-old chickens were higher in the case of rBCLaSoL when compared to rBC. We performed replication kinetics of these recombinant viruses in the brain of 1-day-old chicks which showed that the growth rate of rBCLaSoL was higher than that of parental type rBC. The MDT in chicken embryos and growth kinetics in chicken brain showed that the faster replication of the virus probably attributes towards the increase in the viral virulence.

In summary, using chimeric approach by reciprocal exchange of the L gene between mesogenic NDV strain BC and lentogenic NDV strain LaSota, we were able to recover both chimeric viruses with heterologous functional polymerase gene. Several *in vivo* studies of these chimeric recombinant viruses demonstrated that in

case of the virulent NDV with avirulent polymerase gene, there is an increase in pathogenicity in chicken embryos as well as chickens. However, chimeric virus bearing virulent L gene in avirulent backbone, there was no significant difference in growth as well as virulence. As there are several NDV pathotypes, it will be worth exploring the role of individual polymerase gene from velogenic and lentogenic NDV strains using both virulent and avirulent backbones. Once the exact role of polymerase is explored, then the function of its individual domains can be further dissected in detail. There are approximately 30 amino acid differences between the L gene of BC and LaSota. These amino acid differences are spread throughout all six domains of polymerase gene. It will be interesting to explore the role of key amino acids in polymerase gene in viral replication and pathogenesis.

Chapter 4 :

4.1 Title

Role of NP and P genes in NDV replication and pathogenesis

4.2 Abstract

The nucleocapsid protein (NP) and phosphoprotein (P) of Newcastle disease virus (NDV) play an important role in viral transcription and replication. However, the role of NP and P in NDV pathogenesis has not been explored. Using reverse genetics system, the role of NP and P genes in NDV virulence and pathogenesis was evaluated. The NP and P genes were exchanged individually as well as in combination between a moderately virulent NDV strain, rBC and an avirulent strain rLaSota. Chimeric NP and P viruses of rBC and rLaSota were recovered, and evaluated for their replication in DF-1 cells as well as in 9-day-old chicken embryos. The pathogenicity of these NP and P chimeric viruses was determined by mean death time (MDT) in 9-day-old chicken embryos and intracerebral pathogenicity index (ICPI) in 1-day-old chicks. The MDT of these chimeric viruses showed little difference when compared to their respective parental strains. Chimeric virulent NDV with avirulent NP or P gene showed a slight decrease in ICPI value indicating a decrease in virulence possibly due to heterotypic interaction. However, the virulence was attained to the same level as parental NDV strain when both NP and P genes of avirulent virus were exchanged to the virulent backbone, suggesting that homotypic

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interaction between NP and P play a major role in NDV pathogenesis. These results indicate that NP and P proteins may play a minor role in NDV pathogenesis and homotypic interaction of these two proteins is necessary for the optimal function of the virus.

4.3 Introduction

Newcastle disease virus (NDV) is an enveloped RNA virus of the genus *Avulavirus* in the family *paramyxoviridae* under the order *Mononegavirales* (Mayo, 2002). The virus is an important avian pathogen affecting most species of birds with worldwide distribution. NDV causes a highly contagious respiratory, neurological or enteric disease in poultry causing significant loss to the poultry industry. There are several types of NDV strains with a wide spectrum of virulence varying from mild respiratory infection to a severe fatal disease with 100% mortality. Based on the severity of disease in chickens, NDV strains are categorized into three main pathotypes. NDV strains that cause mild disease with inapparent clinical signs are considered as lentogenic, whereas strains with intermediate virulence showing respiratory signs are considered as mesogenic. NDV strains that cause severe disease with high mortality are called as velogenic, which are further divided into viscerotropic and neurotropic strains. Viscerotropic velogenic strains cause lethal hemorrhagic lesions in the intestine, whereas neurotropic velogenic strains produce neurological involvement (Alexander, 1997).

NDV has a single stranded, non-segmented negative strand RNA genome of 15,186 nucleotides in length (Krishnamurthy and Samal, 1998). The genome contains six genes in the order 3'-NP-P-M-F-HN-L-5' which encodes for at least eight proteins (Chambers *et al*., 1986a; Wilde *et al*., 1986). The nucleoprotein NP, the phosphoprotein P, the viral RNA-dependent RNA polymerase L and the viral genomic RNA together constitute the ribonucleoprotein complex, which is the minimal transcription/replication unit as found in other members of *Mononegavirales*. The other genes encode matrix protein (M), fusion protein (F) and hemagglutinin-neuraminidase protein (HN). F and HN proteins are important surface glycoproteins found on the envelope of NDV and are major immunogenic proteins. The F glycoprotein mediates fusion of the viral envelope to the host cell plasma membrane (Hernandez *et al*., 1996). The HN glycoprotein binds with sialic-acid containing receptors on host cell membrane surface and promotes the fusion activity of F protein, thereby allowing the virus to attach and penetrate the cell surface. The neuraminidase activities of HN remove the sialic acid from progeny virus particles thus preventing viral self-aggregation and facilitating viral budding process (Lamb and Kolakofsky, 1996). The M protein is the most abundant protein in the virion and is considered to be the central organizer of viral morphogenesis. It interacts with cytoplasmic tails of the envelope glycoproteins, the lipid bilayer and the nucleocapsid thus providing driving force for budding of viral particles (Peeples, 1991).

The genomic RNA as well as its replicative intermediate, a positive sense antigenomic RNA, are tightly encapsidated by the NP proteins. Like other

paramyxoviruses, the NP protein of NDV is a major component of viral nucleocapsid. Improperly self-assembled NP proteins without viral genomic RNA bind phosphoprotein with high affinity and resembles similar to actual viral nucleocapsid (Buchholz *et al*., 1993, 1994). The amino terminal region of the paramyxovirus NP protein interacts with the viral genomic RNA forming nucleocapsid while the carboxyl terminal region interacts with the P protein (Buchholz *et al*., 1994). The NP gene is located at the 3' end of the viral genome next to 53 nucleotide long leader sequence. The NP gene is 1,747 nucleotides (nt) long and its ORF encodes for 489 amino acids peptide with a predicted molecular weight of 54 kD (Errington and Emmerson, 1997; Krishnamurthy and Samal, 1998). The transcriptional activity of the nucleocapsid is abrogated when digested with trypsin by releasing a 12 kD carboxy terminal fragment of NP protein (Heggeness *et al*., 1981).

The P gene of NDV is polycistronic that encodes for a structural polypeptide of 395 amino acids long P protein and two small polypeptides V and W proteins (Steward *et al*., 1993). The V and W proteins are produced by co-transcriptional insertion of one or two non-templated G nucleotides at the edit site of the P gene ORF, respectively, by a process called polymerase stuttering (Hausmann *et al*., 1996; Steward *et al*., 1993). During transcription, 61% of the mRNAs derived from P gene encode for P protein, 27% of the mRNAs encode for the V protein with a single G nucleotide insertion in a +1 frameshift and 8.5% mRNAs encode W protein with two G nucleotide insertion in +2 frameshift (Mebatsion *et al*., 2001). The V protein of NDV plays an important role in host range restriction (Park *et al*., 2003a) and virulence in chicken embryos (Mebatsion *et al*., 2001) and 1-day-old chickens

(Huang *et al*., 2003). The function of the NDV P protein is similar to those of several other paramyxovirus P proteins. It plays an important role in viral genome transcription and replication (Hamaguchi *et al*., 1983; Horikami *et al*., 1992). Together with nucleocapsid protein and viral genomic RNA, P protein constitutes the minimal transcriptional unit that is essential for viral transcription and replication (Curran *et al*., 1993, 1994). The P protein of NDV acts as a chaperone by binding with N protein thus preventing uncontrolled encapsidation of viral RNA by N protein (Errington and Emmerson, 1997).

The role of NP and P proteins in paramyxovirus pathogenesis and virulence is not well understood. The objective of this study was to evaluate the role of NP and P genes in NDV virulence and pathogenesis. In this study, we used full length clones of a moderately virulent mesogenic NDV strain Beaudette C (BC) and an avirulent lentogenic NDV strain LaSota (Krishnamurthy *et al*., 2000; Huang *et al*., 2001). The NP and P genes were exchanged individually as well as in combination to produce chimeric recombinant full length cDNA. Chimeric recombinant viruses were recovered from their respective full length clone and evaluated for their replication in chicken embryo fibroblast cells (DF-1) and pathogenicity by mean death time (MDT) in 9-day-old embryonated chicken eggs and intracerebral pathogenicity index (ICPI) in 1-day-old chicks. Our results showed that all the chimeric viruses were viable, indicating NP or P proteins of heterologous strains in heterologous backbone are functional. The *in vivo* characterization of NP and P recombinant chimeric viruses indicated that the NP or P gene may play a minor role in NDV pathogenesis. This

study also suggests that homotypic interaction between NP and P proteins is necessary for optimum pathogenesis of NDV.

4.4 Materials and Methods

4.4.1 Cells and Viruses

Chicken embryo fibroblast cell line (DF-1) was grown in Dulbecco's Modified Eagle's medium (DMEM) with 10% Fetal Bovine Serum (FBS) (GIBCO) and maintained in DMEM with 5% FBS. HEp-2 cells were grown in Eagle's minimal essential medium (EMEM) containing 10% FBS and maintained in EMEM with 5% FBS. The recombinant Modified Vaccinia Ankara (MVA) strain expressing T7 RNA polymerase (a generous gift of Dr. Bernard Moss, NIH) was grown in primary chicken embryo fibroblast (CEF) cells in DMEM supplemented with 10% FBS. NDV strains rBC and rLaSota were grown in 9-day-old embryonated specific-pathogen-free (SPF) chicken eggs. After 48 h of infection, allantoic fluids in the virus infected SPF eggs were harvested, aliquoted, and stored at -70 °C for future use.

4.4.2 Generation of chimeric full length NDV cDNA

Construction of plasmids pNDVfl carrying the full length of the NDV strain BC and LaSota has been described previously (Krishnamurthy *et al*., 2000; Huang *et al*., 2001). To exchange NP and/or P gene, we introduced unique restriction sites, *Asi*SI between NP and P genes and *Pme*I sites between P and M genes, respectively. The cloning strategy to introduce these two restriction sites into full length cDNA of

rBC and rLaSota is shown in Fig 14. Briefly, to introduce *Asi*SI site between NP and

P gene, *Asc*I-*Sac*II fragments of BC and LaSota were PCR amplified and cloned into

pGEM-7Z (+) (Promega, Madison, WI). Overlapping PCR were performed using the

forward primer, AscI-T7-F (5'-ATTCGGCGCGCCTAATACGAC

TCACTATAGGG-3') and reverse primer 1645ASiSI-R (5'-CGCAAATGCAGC

GATCGCCTACGGGTGAGGATATTGGATGA-3') for the PCR product A and

forward primer, 1645ASiSIF (5'-GGCTAGGCGATCGCTCGATTTG

CGGCCCTATATGACCAC-3') and reverse primer BC/LaSo2400R (5'-

GGGCGGCCTTGACTTGGTTCTGCGGTC-3') for the PCR product B. Then both

PCR products, A and B, were used to generate the final *ASc*I-*Sac*II fragment with

introduced *Pac*I site using the primer AScI-T7F and BC/LaSo2400R. The PCR

product was cloned into pGEM-7Z (+) to yield pGEM-7Z (+) AScI-SacII (*Asi*SI). To

introduce *Pme*I site between P and M genes, *Sac*II-*Not*I fragments of BC and LaSota

were PCR amplified and cloned into pGEM-7Z (+). For BC and LaSota, overlapping

PCR was performed using the forward primer, NP1225 (5'-

GCAGCAAGGAGAGGCCTGGCA-3') and reverse primer, 3210BC-PmeIR (5'-

TAGCTAGTTTAAACACGGTTGCGCGATCATTCAGTGGGG-3') for PCR

product A and forward primer, 3210BC-PmeF (5'-GCGCAACCGTGTTTAAAC

TAGCTACATTAAGGATTAAGA-3' and reverse primer, BC/LaSo 4970R, for PCR

product B. Then both PCR products, A and B, were used to generate the final *Sac*II-

*Not*I fragment with introduced *Pme*I site using primers NP1225 and BC/LaSo4970R.

The PCR product was cloned into pGEM-7Z (+) to yield pGEM-7Z (+) SacII-NotI

(*Pme*I). The resulting plasmids were sequenced to confirm the presence of desired

unique restriction sites, *Asi*SI and *Pme*I, respectively. Then, the *Asc*I-*Sac*II fragment

was incised out from the pGEM-7Z (+) AscI-SacII (*Asi*SI) using restriction enzymes

*Asc*I and *Sac*II, and the *Sac*II-*Not*I fragment was incised out from the pGEM-7Z (+)

SacII-NotI (*Pme*I) using enzymes, *Sac*II and *Not*I. These fragments were

subsequently cloned into full length clone of both pNDV-fl/BC and pNDV-fl/LaSota.

The resulting full length cDNA of pBC and pLaSota contained unique restriction sites

*Asi*SI and *Pme*I. The NP gene was exchanged using *Asc*I and *Asi*SI and P gene was

exchanged using *Asi*SI and *Pme*I sites. The pBC full length plasmid bearing the NP

gene of LaSota instead of its own NP gene was designated as pBCLaSoN, whereas

pLaSota full length plasmid bearing the NP gene of BC instead of its own as

pLaSoBCN. Likewise, the rBC backbone containing P gene of LaSota was designated

as pBCLaSoP and pLaSota backbone containing P gene of BC as pLaSoBCP. In case

of recombinant NDVs where both NP and P genes were exchanged from BC to

LaSota and vice versa, *Asc*I and *Pme*I sites were used and the recombinants were

designated as pBCLaSoP+N and pLaSoBCP+N respectively. The schematic

representation of the cloning strategy is shown in the Fig 15.
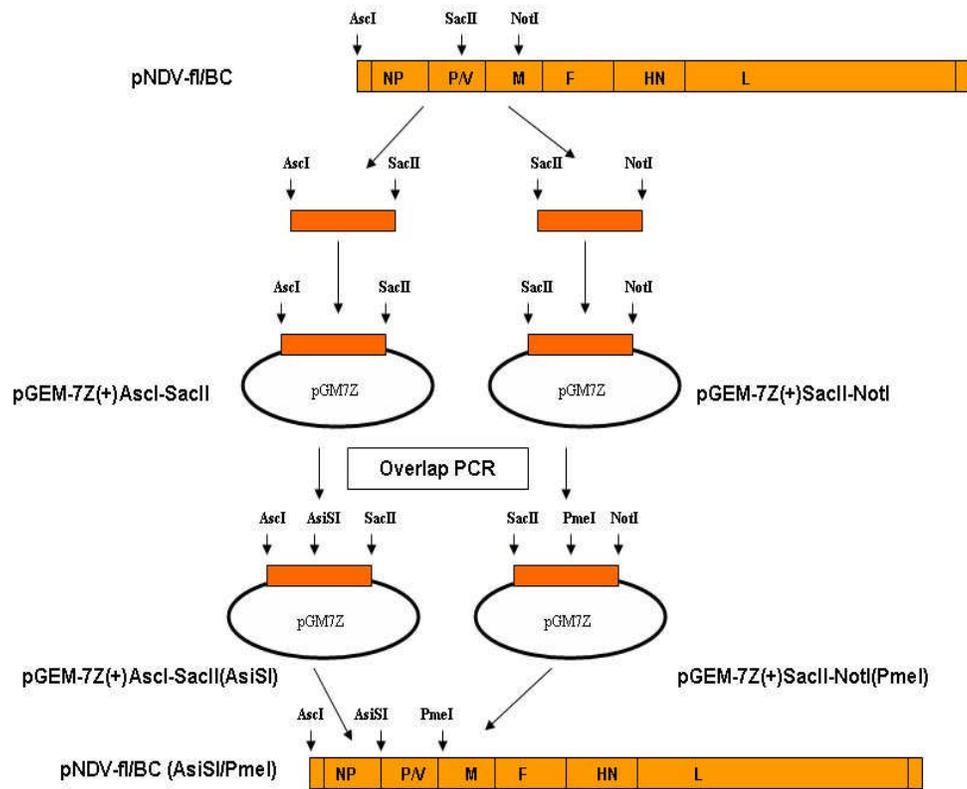
Fig. 14. Cloning strategies adopted to introduce unique restriction sites in the full length cDNA of BC and LaSota. *Asi*SI between NP and P genes and *Pme*I site between P and M genes were introduced by overlapping PCR method.

Fig. 15a
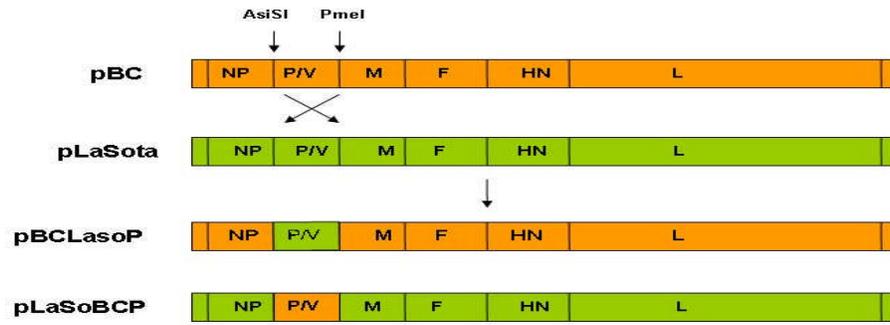
Fig. 15b
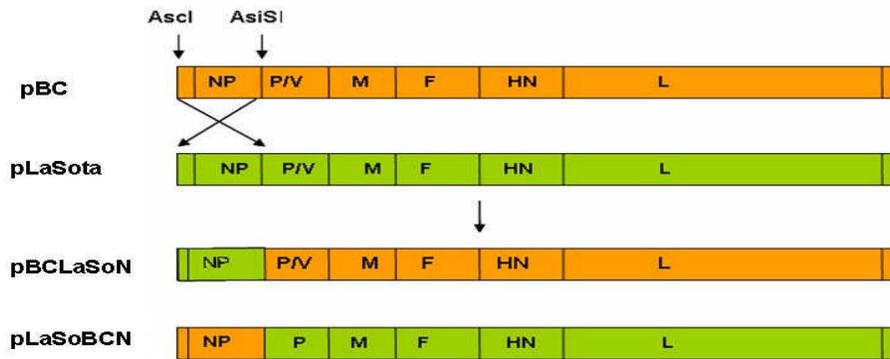
Fig.15c
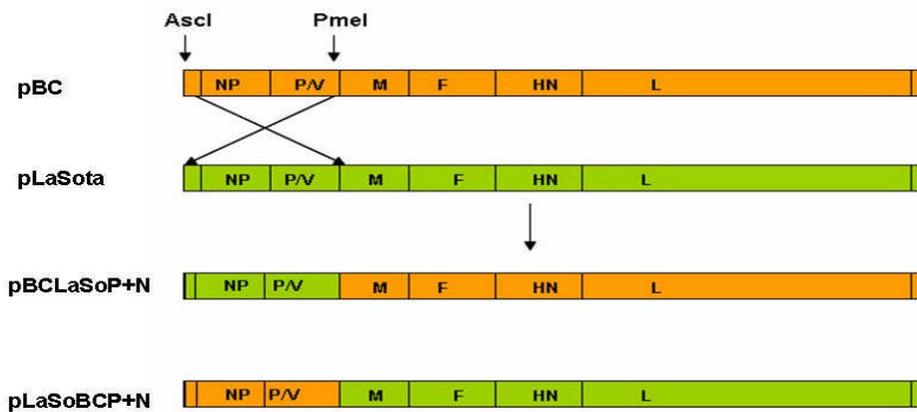
Fig. 15. Schematic representation of the strategies for the exchange of NP, P and both NP+P genes between the rBC and rLaSota full length cDNA. Unique restriction sites, *Asi*SI between NP and P gene and *Pme*I site between P and M gene were (Cont.)

introduced into full length cDNA of NDV strains BC and LaSota, respectively. The P

gene was exchanged as a single gene ORF between rBC and rLaSota using AsiSI and

*Pme*I (Fig. 15a), and the NP gene was exchanged as a single gene ORF between BC

and LaSota by using *Asc*I and *Asi*SI (Fig. 15b). Similarly, both NP and P genes

between rBC and rLaSota were exchanged as single fragment using restriction sites,

*Asc*I and *Pme*I (Fig. 15c)

4.4.3 Transfection and recovery of chimeric recombinant viruses

Transfection and recovery of recombinant NDV viruses were performed as described previously (Krishnamurthy *et al*., 2000). Briefly, HEp-2 cells were grown to 80-90% confluency in 6-well plates and washed twice with PBS just before transfection. The cells were incubated with 500 µl of OptiMEM (Invitrogen) containing 2.5 µg NP, 1.5 µg P and 1.0 µg L support plasmids along with 5 µg of each full length recombinant cDNA clone. Transfection was performed with Lipofectamine^{TM} Reagent (Invitrogen) according to the manufacture's instructions. Simultaneously, HEp-2 cells were infected with recombinant vaccinia virus strain MVA/T7 expressing T7 polymerase at a multiplicity of infection (MOI) of 2.0. After 8 h of incubation at 37 °C, supernatant mixtures were removed and replaced with 2 ml of DMEM containing 5% FBS. Cells were incubated at 37 °C for 48 h and then supernatants were passaged to fresh HEp-2 cells to remove residual vaccinia virus. After 2-3 days, supernatant samples from HEp-2 cells were passaged to DF-1 cells and the cells were examined for development of syncytia, typical of NDV cytopathic effect (CPE). Once the infectious recombinant viruses were obtained, the viruses were plaque purified from DF-1 cells and injected to 9-day or11-day-old embryonated chicken eggs for propagation and stocks preparation.

Table. 2. Different full length and support plasmids used to recover of chimeric

recombinant viruses.

Recovered virus	Full length Plasmid	Support plasmid (pNP)	Support plasmid (pP)	Support plasmid (pL)
rBC	pBC	BC	BC	BC
rBCLaSoP	pBCLaSoP	BC	LaSota	BC
rBCLaSoN	pBCLaSoN	LaSota	BC	BC
rBCLaSoP+N	pBCLaSoP+N	LaSota	LaSota	BC
rLaSota	pLaSota	LaSota	LaSota	LaSota
rLaSoBCP	pLaSoBCP	LaSota	BC	LaSota
rLaSoBCN	pLaSoBCN	BC	LaSota	LaSota
rLaSoBCP+N	pLaSoBCP+N	BC	BC	LaSota

4.4.4 RT-PCR and sequence analysis

Total RNA was isolated from DF-1 cells infected with virus using TRIzol reagent (Invitrogen). Reverse transcription (RT)-PCR was carried out using the superscript RT-PCR kit (Invitrogen) with primers, AscI-T7F (5'-ATTCGGCGCGCCTAATACGACTCACTATAGGG-3') for NP gene, and primers, NP1225 (5'-GCAGCAAGGAGAGGCCTGGCA-3') for P gene. The generated cDNAs were then PCR amplified using primer, NP196 (5'-GACCAGAAGATAGGTGGAAC-3)' and BC/LaSo2400R (5'-GGGCGGCCTTGACTTGGTTCTGCGGTC-3') for NP gene, and primers, NP1225 (5'-GCAGCAAGGAGAGGCCTGGCA-3') and BC/LaSo7970R (5'-TGTATCAGAGCTGCGGCCGCTGTTATTTG-3') for P gene. The NP and P genes amplified by RT-PCR of the recovered viruses were entirely sequenced to ensure that the sequence of the protein remained unchanged. The virulent F protein cleavage site of rBCLaSoN, rBCLaSoP and rBCLaSoP+N and avirulent F protein cleavage site of rLaSoBCN, rLaSoBCP and rLaSoBCP+N were also sequenced to confirm the absence of any undesired mutation at protease cleavage sites.

4.4.5 Growth kinetics of the chimeric recombinant viruses in DF-1 cells

The growth kinetics of rBCLaSoP, rBCLaSoN, rBCLaSoP+N and rLaSoBCP, rLaSoBCN, rLaSoBCP+N along with rBC and rLaSota were determined using multi cycle growth curve in DF-1 cells. For multi cycle growth curve, viruses were infected 0.01 MOI in DF-1 cells. Cells were incubated at 37 °C for 56 h and supernatants were collected every 8 h. The virus titer in the collected samples was titrated by plaque

70

assay in DF-1 cells. Briefly, 10-fold dilutions of supernatant samples were prepared and 100 µl of each diluent was infected to a 12 well plate DF-1 cells in duplicates. After 1 h of incubation, the supernatants were removed from wells, washed with PBS and then overlaid with DMEM containing 0.8% methylcellulose and 2% FBS. The plates infected with either rLaSota or chimeric LaSota virus were added with 1µg of acetyl trypsin/ml or 10% allantoic fluid. The infected cells were incubated at 37 °C for 4 days. Then, the cells were fixed with methanol and stained with crystal violet for enumeration of plaques.

4.4.6 Growth kinetics of the chimeric recombinant viruses in 9-day-old chicken embryos

To evaluate replication of chimeric viruses in chicken embryo, 9-day-old SPF chicken embryos were infected with 10^3 plaque forming unit (PFU) of virus particles per embryo through chorio-allantoic route. Every 12 h, allantoic fluid samples from three chilled embryos were harvested and the titers of virus in the samples were determined by plaque assay. Briefly, supernatants of the collected allanotic fluid were serially diluted and 100 µl of each diluent was infected to a well of 12 well plate DF-1 cells in triplicates. After 1 h of virus adsorption, supernatants were discarded from wells, washed with PBS and then overlaid with DMEM containing 0.8% methylcellulose and 2 % FBS. Cells infected with either rLaSota rLaSoBCP, rLaSoBCN or rLaSoBCP+N were added with 1 µg of acetyl trypsin/ml into the medium. The infected cells were incubated at 37 °C for 4 days until the development

of countable plaques. Then the cells were fixed with methanol and stained with crystal violet for enumeration of plaques.

4.4.7 MDT in chicken embryos

The virulence of the recombinant chimeric viruses was determined by MDT assay in 9-day-old embryonated SPF chicken eggs (Alexander, 1989). Briefly, a series of 10 fold dilutions of fresh infective allantoic fluid was prepared and 100 µl of each diluent was injected into the allantoic cavity of five 9-day-old embryonated chicken eggs. The remaining diluents were kept on ice. After 8 h, another batch of five embryos was injected as described above. The eggs were incubated at 37 °C and examined for embryo mortality three times daily up to 7 days. The time of each embryo death was recorded and the highest dilution at which all ten embryos died was considered as minimum lethal dose. The MDT was calculated as the mean time in hours taken by the minimum lethal dose to cause the death of all the embryos.

4.4.8 Pathogenicity Studies in chickens

All the animal experiments were performed in the USDA approved BSL2+ facility using standard animal protocols approved by IACUC. The pathogenicity of these recombinant viruses was evaluated *in vivo* using intracerebral pathogenicity index (ICPI) test with some modifications (Alexander, 1989). For ICPI, fresh virus infected allantoic fluid (HA titer$> 2^4$) was diluted in sterile PBS (1:10) and injected to a group of ten 1-day-old SPF chicks via intracerebral route. The inoculation was performed into the left or right rear quadrant of the cranium using a 4 mm long 27

gauge needle attached to a 1 ml stepper syringe dispenser set to dispense a fixed

volume of 50 µl of inoculums. Mock infected birds were received similar volumes of

sterile PBS by the same route. Every 8 h, development of clinical signs and mortality

in the infected birds were observed and scored for up to 8 days. The ICPI values were

calculated as mean score per bird per observation (Alexander, 1989).

4.5 Results

4.5.1 Generation and recovery of chimeric recombinant NDVs

The recovery of recombinant NDVs from infectious cDNA clones derived

from a moderately virulent strain BC and an avirulent strain LaSota has been reported

previously (Krishnamurthy *et al*., 2000; Huang *et al*., 2001). Using reverse genetics,

we designed chimeric NDV recombinants in which the NP or P gene of BC was

replaced by the corresponding gene of LaSota, and vice versa. We also designed

chimeric mutants in which both NP and P genes were swapped on both virulent and

avirulent backbone (Fig.15c). The cloning strategy was designed to introduce unique

restriction site *Asi*SI between NP and P genes, and *Pme*I site between P and M genes.

Then NP gene was swapped between rBC and rLaSota using *Asc*I and *Asi*SI

restriction sites and P gene using *Asi*SI and *Pme*I sites. Both NP and P genes were

exchanged using *Asc*I and *Pme*I sites. Sequencing of the entire cDNA clone of each

chimeric virus confirmed the intended gene exchange and the absence of any

undesired mutations. Thus, NP and P genes of NDV were swapped without

introducing any changes in their gene start and gene end signals, noncoding regions or the flanking intergenic regions.

The chimeric viruses were recovered by transfecting full length cDNA clones along with their corresponding support plasmids, pNP, pP and pL. For transfection of rBC with LaSota NP full length clone, we used pNP support plasmid of LaSota instead of BC to prevent potential recombination (Table 2). Similarly, for rLaSota with BC NP gene, pNP of BC was used (Table 2). The transfected supernatants were passaged twice in HEp-2 cells and subsequently, infected to DF-1 cells. After two or three passages in DF-1 cells, we observed the formation of syncytia typical of NDV infections. To obtain the pure clone of virus, the respective recombinant viruses were plaque purified twice and then amplified in 9-day-old embryonated eggs. RT-PCR and sequencing of the amplified NP and P genes from chimeric viruses using gene specific primers verified the introduction of unique restriction sites, *Asi*SI or *Pme*I, as well as exchanged NP or P gene between BC and LaSota. The recovered rBC backbone with LaSota NP gene was designated as rBCLaSoN, whereas rLaSota with BC NP gene was designated as rLaSoBCN. Similarly, rBC with LaSota P gene and rLaSota with BC P gene were designated as rBCLaSoP and rLaSoBCP, respectively. The rBC containing both NP and P gene of LaSota was designated as rBCLaSoP+N, whereas rLaSota containing NP and P gene of rBC was designated as rLaSoBCP+N. We also confirmed no mutations occurred in the exchanged NP and P genes region of the recovered recombinant viruses even after passaging five times both in DF-1 cells and chicken embryos.

4.5.2 Growth of chimeric recombinant viruses in DF-1 cells

To evaluate the ability of growth of recombinant chimeric viruses compared to their parental viruses *in vitro*, multi-step growth kinetics was performed in DF-1 cells. The viruses were infected to DF1 monolayer cells in a six well plate at a MOI of 0.01, and samples were collected at 0 h and then every 8 h for 56 h and titers were evaluated by the plaque assay. The chimeric viruses replicated to similar titers in DF-1 cells when compared to their respective parental strains (Fig. 16). There was no evidence of a delay in replication or significant increase or decrease in the growth pattern. Both recombinant chimeric viruses and rBC reached to 10^8 PFU/ml, suggesting that the introduction of NP or P gene in the heterologous backbone did not affect the growth in DF-1 cells (Fig. 16). We also observed a similar growth trend in the growth of chimeric recombinant LaSota and rLaSota strains (Fig. 16).

4.5.3 Growth kinetics of chimeric recombinant viruses in chicken embryos

The growth characteristics of rBC, rBCLaSoP, rBCLaSoN, rBCLaSoP+N, rLaSota, rLaSoBCP, rLaSoBCN and rLaSoBCP+N viruses were evaluated in 9-day-old chicken embryo by inoculating 10^3 PFU of virus per embryo and determining the virus titer every 12 h by plaque assay. Our results showed that the replication pattern of all these six recombinant chimeric viruses were similar when compared to their respective parental viruses (Fig. 17). These results indicated that the individual as well as combined reciprocal exchange of NP and/or P gene in heterologous genome did not alter the replication of the recombinant chimeric viruses in chicken embryos.
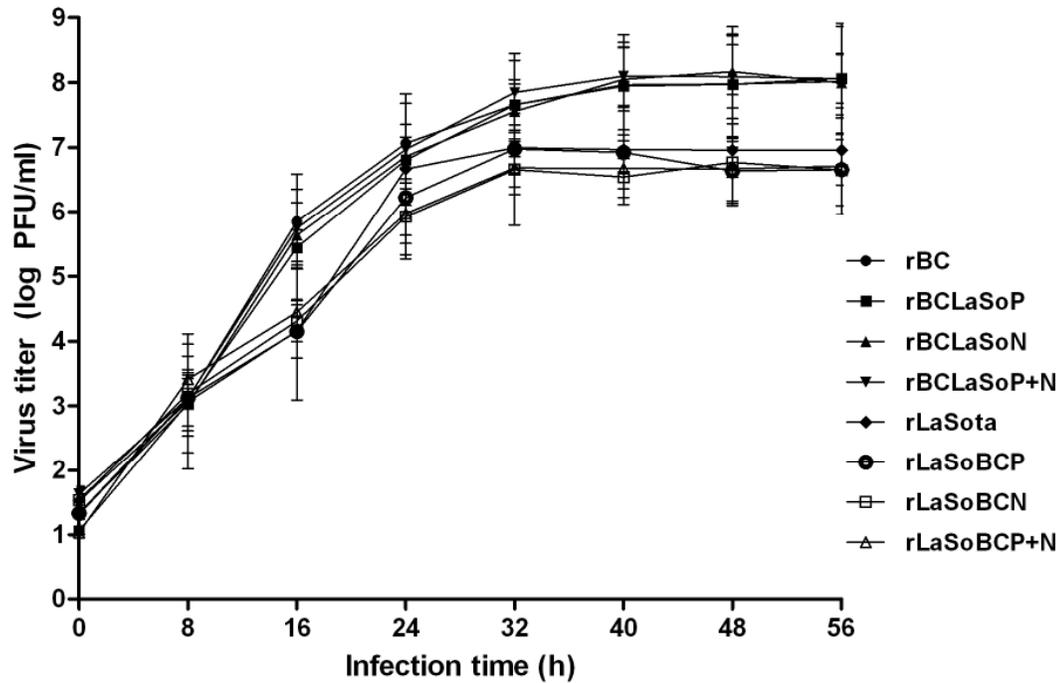
Fig. 16. The multi cycle growth kinetics of parental and chimeric NP and/or P viruses in chicken embryo fibroblast (DF-1) cells. Confluent monolayer of DF-1 cells in six well plates were infected with each of virus at a MOI of 0.01. Supernatant samples were collected at every 8 h and titers of the viruses were determined by plaque assay. The values represented are the means of valued obtained from three independent experiments.
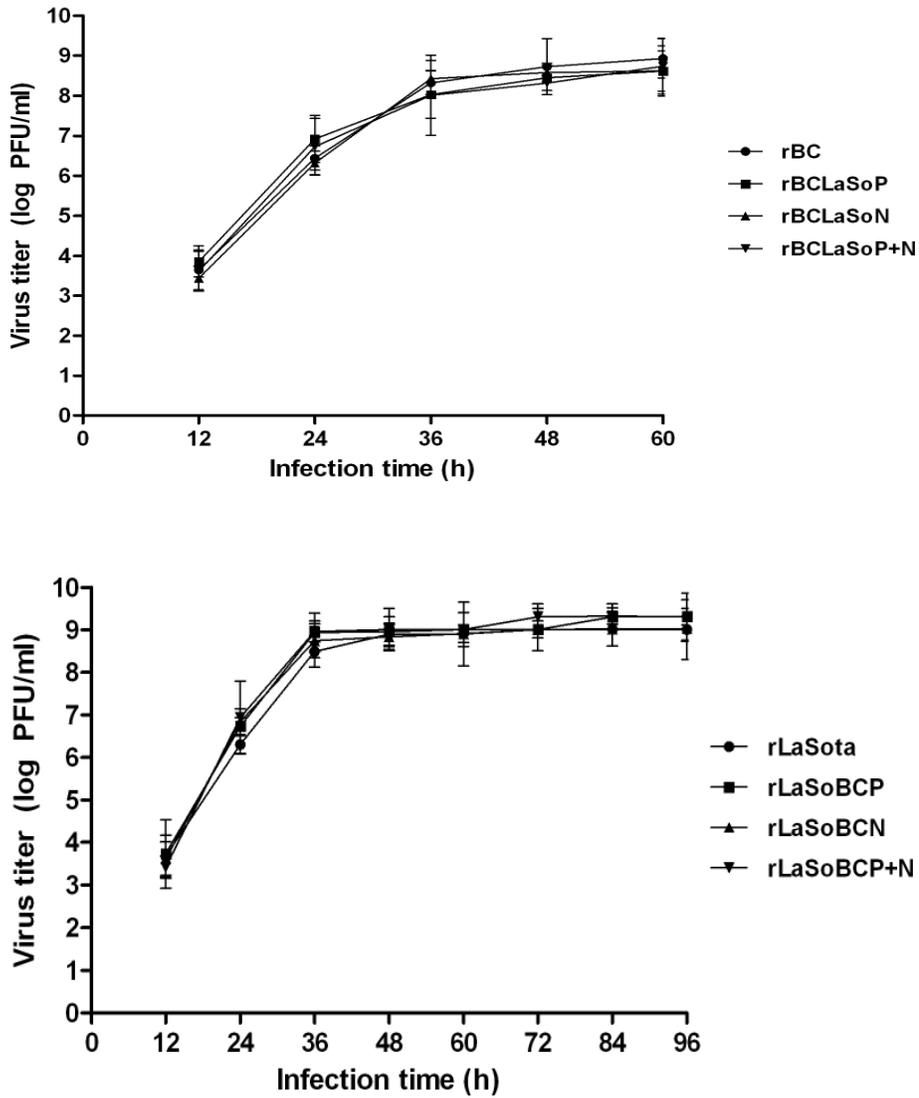
Fig. 17. The growth kinetics of parental and chimeric NP or/and P viruses in chicken embryos. The growth of recombinant viruses (a) rBC, rBCLaSoP, rBCLaSoN, rBCLaSoP+N and (b) rLaSota, rLaSoBCP, rLaSoBCN and rLaSoBCP+N were evaluated in 9-day-old embryonated SPF chicken embryos. The eggs were injected with 10^3 PFU of virus through the allantoic route. Three eggs were chilled every 12 h. Allantoic fluids were harvested, clarified and the virus titers in the samples were determined by plaque assay in DF-1 cells.

4.5.4 MDT in chicken embryos

The pathogenesis of all recombinant viruses was evaluated in 9-day-old embryonated chicken eggs by MDT test (Table 3). The MDT procedure was repeated two times under test 1 and test 2. The MDT of rBCLaSoP was 64 h and 60 h in test 1 and test 2, compared to the parental rBC for which the MDT was 62 h and 60 h respectively. In the test 1 and test 2, the MDT for the BCLaSoN was 60 h and 62 h whereas the MDT of rBCLaSoP+N were 58 h and 61 h respectively. Similarly, the MDT of rLaSoBCP was 116 h and 114 h in test 1 and test 2 respectively, when compared to the parental rLaSota for which the MDT was 106 h and 110 h. The MDT value of rLaSoBCN was 114 h and 109 h whereas the MDT of rLaSoBCP+N were 112 h and 108 h. These MDT values indicated that the exchange of NP or P genes did not affect the pathogenicity of these chimeric viruses compared to their parental strains.

4.5.5 Pathogenicity studies in chickens

The pathogenicity of rBC, rBCLaSoP, rBCLaSoN, rBCLaSoP+N, rLaSo, rLaSoBCP, rLaSoBCN and rLaSoBCP+N viruses in 1-day-old chicks were evaluated by ICPI test (Table 4). The ICPI tests of rBCLaSoP and rBCLaSoN showed a lower ICPI value of 1.24 and 1.31, respectively, compared to its mesogenic parental rBC, which had an ICPI value of 1.49. Interestingly, the ICPI value of rBCLaSoP+N was 1.44 compared to its parental rBC value of 1.49. The ICPI values of rLaSoBCP, rLaSoBCN, rLaSoBCP+N were 0.00, same value as that of their parental strain

rLaSota. Hence irrespective of exchanged NP, P or NP+P gene, the pathogenicity of recombinant chimeric viruses with an avirulent genome was not increased.

Table .3. The *in vivo* pathogenicity of NP and P recombinant chimeric viruses

Virus	[a]MDT (h)		[b]ICPI
	Test 1	Test 2	Test 1
rBC	62	60	1.49
rBCLaSoP	64	60	1.24
rBCLaSoN	60	62	1.31
rBCLaSoP+N	58	61	1.44
rLaSota	106	110	0.00
rLaSoBCP	116	114	0.00
rLaSoBCN	114	109	0.00
rLaSoBCP+N	112	108	0.00

The virulence of recombinant chimeric viruses was evaluated by mean death time (MDT) test in 9-day-old embryonated chicken eggs and intra-cerebral pathogenicity index (ICPI) in 1-day-old chicks.

[a] MDT is expressed in hours taken by the minimum lethal dose of virus to kill all ten inoculated 9-day-old embryonated chicken eggs when injected through allantoic route. Highly virulent velogenic strains of NDV take less than 60 h to kill all the embryos, moderately virulent mesogenic strains take 60-90 h to kill the embryos and lentogenic avirulent strains take more than 90 h to kill all the embryos.

[b]For ICPI test, 50 µl of fresh infective allantoic fluid diluted to 1:10 with PBS was injected to ten 1-day-old SPF chicks via intracerebral route. The inoculation was performed using a 27-gauze needle, attached to a 1 ml stepper syringe dispenser that was set to dispense 50 µl of inoculum per inoculation. Control birds received the same volume of sterile PBS through the same route. The birds were observed daily for 8 days and scored 0, if normal, 1, if sick and 2, if dead. The ICPI value is calculated as mean score per bird, per observation. The ICPI value of highly virulent velogenic strains of NDV approaches towards 2 whereas for avirulent lentogenic strains the value approaches 0.

4.6 Discussion

NDV strains vary in virulence ranging from completely avirulent with in-apparent clinical signs to highly virulent strains causing 100% mortality in chickens. The World Organization of Animal Health considers the NDV isolates having multiple basic amino acids and phenylalanine at the F protein cleavage site as one of the important criteria to categorize under virulent category (OIE, Chapter 2.1.15). However, there are several NDV strains whose F protein cleavage sites have identical or similar amino acid sequences but produce a wide range of pathogenicity and clinical signs in chickens. Similarly, using chimeric approach, HN proteins of virulent or avirulent origin have shown to play an important role in NDV virulence (Huang *et al.*, 2004). However, the exchange of HN protein alone only affected the virulence partially, suggesting that other proteins of NDV may play important roles in NDV virulence and pathogenesis.

The NP and P genes were exchanged individually or in combination using full length cDNA clones of BC and LaSota. The chimeric viruses were recovered and confirmed for the presence of substituted gene by RT-PCR sequencing of the exchanged genes. These recombinant chimeric viruses grew as efficiently as their respective parental NDV strains, indicating heterologous NP and P genes are fully functional. The high level similarities in the amino acid sequence in the NP and P proteins between BC and LaSota (97.9% in NP and 97.5% in P) probably attributes to the heterologous functional ability. Thus, it will be interesting to exchange NP and/or

P gene from distantly-related APMV-1 strains with virulent as well as avirulent NDV backbone, and then study their effects on NDV replication and pathogenesis. The recombinant chimeric viruses were assessed for their replication in DF-1 which showed that all the chimeric viruses grew efficiently when compared with their respective parental viruses, indicating that the replication capability of these viruses is not hampered by heterologous exchange of either NP gene or P gene. Interestingly, when both NP and P gene of avirulent strain were exchanged to virulent strain and vice versa, the replication of all the chimeric viruses remained unchanged.

We also tested the growth of these recombinant viruses *in vivo* and found that all the recombinant chimeric viruses replicated to the same level as their respective parental strain in 9-day-old embryonated chicken eggs. This confirmed that the replication of the chimeric viruses was not significantly changed due to exchange of NP, P or NP+P gene(s). The pathogenicity of the chimeric viruses was assessed by MDT and ICPI tests. The ICPI of the chimeric rBC virus bearing the P gene of LaSota (rBCLaSoP) and the chimeric rBC bearing the NP gene of LaSota (rBCLaSoN) were lower when compared with that of the parental rBC strain. This might be due to heterotypic interaction between NP and P gene. Interestingly, the ICPI value of the chimeric rBC virus bearing both the NP and P gene of LaSota (rBCLaSoP+N) was close to the value of parental rBC strain. It is well known that the NP and P proteins of paramyxovirus tightly interact with each other during the replication of the viral genome (Curran *et al*., 1995; Nishio *et al*., 1996; Kho *et al*., 2004). Although there was no visible difference in the growth pattern of those

chimeric viruses in DF-1 cells as well as in chicken embryo, it is possible that interaction of NP and P proteins originated from heterologous strains affected NDV pathogenesis of chimeric virus in 1-day-old chicks. However, there was no difference in ICPI value between chimeric rLaSota virus bearing P gene of BC (rLaSoBCP), chimeric rLaSota virus bearing NP gene of BC (rLaSoBCN) and chimeric rLaSota virus bearing both NP and P gene of BC (rLaSoBCP+N).

The P protein of NDV encodes for a V protein through co-transcriptional insertion of G nucleotide to P gene ORF by viral polymerase (Steward *et al*., 1993). Several studies have demonstrated that the V protein of paramyxovirus plays an important role in antiviral activities by blocking interferon in host. In NDV, the mutant virus with deleted V protein has shown to affect the virus pathogenesis in its natural hosts as well as in vitro by antagonizing alpha interferon activity (Huang *et al*., 2003; Park *et al*., 2003a). The anti-interferon activity of V protein is also found to be species specific (Park *et al*., 2003b). The NDV mutant with altered V protein has shown to be highly attenuated in chicken embryo (Mebatsion *et al*., 2001). Despite the expression of V protein in all NDV strains, some velogenic strains are highly virulent whereas some lentogenic strains are completely avirulent. This indicates that the V protein is not a major factor in affecting the pathogenesis. In this study, when we compared the pathogenicity of rBCLaSoP and rBCLaSoN and between rLaSoBCP with rLaSoBCN by ICPI, there was no change in pathogenicity indicating that, although V protein is required for the pathogenesis in NDV, probably it is not responsible for the spectrum of virulence exhibited by different NDV strains.

In summary, by exchanging NP or P gene individually between virulent NDV strain BC and avirulent NDV strain LaSota, several chimeric recombinant viruses were recovered, which indicated that the heterologous NP and P proteins are functional. The pathogenicity of recombinant chimeric viruses showed that there was a decrease in pathogenicity in case of virulent NDV strain with avirulent NP or P protein. But when both the NP and P proteins of avirulent strain were exchanged on virulent NDV backbone, the virulence of the recombinant chimeric virus was closer to its parental value, indicating that probably efficient NP and P interaction between homologous proteins is necessary for optimum pathogenesis of the virus. With the availability of an established reverse genetics system for a virulent and an avirulent NDV strain, it would be interesting to explore the effect of NP and P proteins of other velogenic and lentogenic strains in NDV pathogenesis.

Chapter 5 :

5.1. Title

Development of temperature sensitive mutant of NDV by importation of single amino acid mutations from heterologous *paramyxovirus* polymerase protein

5.2 Abstract

Newcastle disease (ND) is a major disease of poultry caused by Newcastle disease virus (NDV). Being a major threat to poultry industry, there is a need to develop an efficient and suitable vaccine against highly virulent NDV strains. In this study, using a moderately virulent NDV strain rBeaudette C (rBC), we intended to develop a temperature sensitive (ts) mutant of NDV by heterologous importation of a mutation responsible for temperature sensitive phenotypes from other paramyxovirus. The phenylalanine of the L gene of rBC at position 452 was mutated to leucine or isoleucine. Transfection of these two full length cDNA clones in HEp-2 and then successive passages in HEp-2 or DF-1 cells at different temperatures did not yield any viable virus. For detailed examination, the green fluorescent protein (GFP) gene was inserted into these full length cDNA clones carrying ts mutations. Transfection of these GFP inserted clones to HEp-2 cells showed fluorescence in the transfected cells. However, the fluorescence was gradually diminished as the supernatant was passaged indicating the following mutation is lethal in NDV. To confirm this study, phenylalanine at the homologous position on support plasmid (pL) was mutated to

either leucine or isoleucine and then subjected to transfection with full length rBC expressing GFP. However, no observable fluorescence was detected in transfected cells, indicating that the mutation from phenylalanine at position 452 to leucine or isoleucine on NDV L protein could be lethal and cannot be applied for the development of the ts phenotype of NDV.

5.3 Introduction

Newcastle disease virus (NDV) is one of the most important viral agents of poultry that causes respiratory disease called Newcastle disease (ND) worldwide. NDV has several strains with a wide range of virulent spectrum from completely nonpathogenic to highly virulent form. Any outbreak of highly virulent NDV infections in poultry must be reported to the Office of International Epizootics. Currently, the US poultry industry controls NDV through vaccination using low virulent lentogenic strain live virus and inactivated virus vaccines. The available vaccines are targeted towards endemic low virulent field strains. However, the available vaccine strains grow slowly and the immunogenic surface proteins do not provide adequate protection against highly virulent NDV strains. The main goal for producing a better NDV vaccine is to induce good protective immunity and at the same time with minimal vaccine reaction in chickens. In commercially bred chickens, the vaccine reaction causes air sac diseases and stunts growth, thus increasing economic losses to poultry industry. Although efficacy of currently available NDV vaccines is widely accepted, several recent outbreaks of velogenic exotic NDV in California and western US has raised the need to develop a better vaccine that can

provide adequate protection against a wide variety of NDV strains. This can be achieved by making a virus, which will have immunogenic surface proteins of virulent origin but have limited replication ability without spreading extensively into internal organs. It is widely known that temperature sensitive recombinant virus would be able to replicate only in the respiratory tract stimulating anti-viral immunity without spreading to the visceral organs.

In other paramyxoviruses, several strategies have been adopted to develop temperature sensitive mutant viruses. One approach is to propagate the wild type virus for several passages at progressively lower temperature to a final temperature where the virus is attenuated with limited replication ability towards certain organs. Using this approach, temperature sensitive attenuated vaccine candidate, PIV3 cp5, was developed from wild type JS strain, PIV3 (Belshe and Hissom, 1982). The other approach is to identify the key amino acids on any viral proteins responsible for temperature sensitivity and then introduce this identified amino acid mutation to the virus of interest.

In paramyxovirus, the recombinant virus with desired mutations can be generated using reverse genetics method. The recovery of infectious NDV from full length cDNA has been helpful for adopting strategies to express foreign proteins (Haung *et al*., 2001; Bukreyev *et al*., 2005; DiNapoli *et al*., 2007) and study pathogenesis of the virus (Huang *et al*., 2004; Mebatsion *et al*., 2001; Park *et al*., 2003a,b). Using this technique, the genetic make up of the virus can be altered by

introducing desired mutation for the development of suitable vaccines. Mutations or substitutions in the L gene of several paramyxoviruses have resulted in development of temperature sensitive (ts) phenotypes that are attenuated *in vivo* (Haller *et al*., 2001; Tang *et al*., 2002).

The L gene is the largest gene of the NDV genome and encodes for the L protein or viral RNA dependent RNA polymerase (RdRP). The L protein is a 2204 amino acid long polypeptide having several functions, such as initiation of viral mRNA synthesis, polyadenylation, capping of viral mRNA and replication of the viral genome. Sequence comparison of NDV L protein with several other paramyxovirus L proteins shows the presence of six highly conserved domains on L protein with different predicted catalytic functions (Poch *et al*., 1989, 1990; Sidhu *et al*., 1993). Similar to other negative-strand RNA viruses, the genome of NDV is fully encapsidated by NP protein to form ribonucleoprotein complex which is used by L protein as template for transcription and replication of the viral genome.

In this study, we aligned the L protein of moderately virulent NDV strain Beaudette C(BC) and avirulent NDV strain LaSota with several other paramyxovirus L proteins to identify conserved amino acid responsible for ts phenotypes and transferred that mutation to the full length cDNA of mesogenic strain rBC. The mutated full length cDNA was transfected several times for recovery at different temperatures in two different cell lines, HEp-2 and DF-1, as well as passaging in 9-day-old embryonated eggs. We also inserted an enhanced GFP gene to these full

length cDNA containing ts mutation to track the outcome of transfection. Our results indicated that the ts mutations imported from heterologous paramyxovirus RSV L gene to the NDV L protein abrogated the function of the L protein and failed in producing the viable virus.

5.4 Materials and Methods

5.4.1 Sequence comparison among L genes of paramyxoviruses

The L genes of NDV strains rBC and rLaSota were aligned with those of L gene of the other nine paramyxoviruses using Clustral W alignment with Lasergene software (DNASTAR, Madison). The amino acid sequences of the L gene of BC (Krishnamurthy *et al*., 2000) and LaSota (GenBank, AAC28375) were compared to those of other 9 paramyxoviruses L genes. This sequence analysis showed the presence of phenyl alanine at position 452 in the L protein of BC, LaSota and most of other paramyxoviruses (Fig 18). Specific mutations were introduced at the nucleotide level of the L gene of full length cDNA of rBC. The phenyl alanine at position 452 of the L gene was either mutated to leucine or isoleucine.

Fig. 18. Alignment of L proteins of several paramyxoviruses.

Alignment results revealed that the position 521 of the respiratory syncytial virus
which is homologous to 451 position of the NDV strain BC and LaSota, contained
conserved amino acid residue phenylalanine.

5.4.2 Insertion of ts mutations in the rNDV full length antigenomic cDNA and

support plasmid pL

The plasmid pNDV-fl expressing the full-length antigenome of NDV

Beaudette C has been described previously (Krishnamurthy *et al*., 2000) and was used

to construct the temperature sensitive mutant clones by importation of the amino acid

mutation from heterologous paramyxovirus RSV. The *Age*I-*Afl*II fragment from

pNDV-fl was subcloned into pGEM-7Z (+) (Promega, Madison, WI) between *Aat*II

and *Nsi*I by using a specific primer pair with *Aat*II and *Nsi*I sites. To introduce

desired temperature sensitive (ts) mutation at the position 452 of the L protein, amino

acid phenylalanine was either changed to leucine or isoleucine. To introduce leucine

in place of phenylalanine, overlapping PCR was performed using primer Aat/AgeF

(5'-TCAGGACGTCACCGGTAAATAGTACGGGTAGGACATGGCG-3') and

primer F452L/R (5'-TTCTATACATGGCTCGAGTTCAAGTGCAGATAA

ACTCTTATA-3') to obtain PCR product A. Primers F452L/F (5'-GCACTTGAAC

TCGAGCCATGTATAGAATACGACCCTGTC-3') and NsiAfl/R (5'-

CCATGCATCTTAAGAACAATGTTTGGGCTTGCAACAG-3') were used to

obtain PCR product B. Then AgeI-AflII fragment was amplified using primer pair

Aat/AgeF, Nsi Afl/R and PCR product A and B. These PCR products A and B were

used as templates to PCR amplify the AgeI-AflII fragment containing the desired

mutation which was sub-cloned into *Aat*II and *Nsi*I sites of pGEM-7Z (+). The

resulting clone was sequenced to confirm the presence of mutation. In addition, to

introduce isoleucine in place of phenylalanine, overlapping PCR was performed using

primers Aat/AgeF (5'-TCAGGACGTCACCGGTAAATAGTAC

GGGTAGGACATGGCG-3') and F452I/R (5'-TTCTATACATGGCTCGATTTCA

AGTGCAGATAAACTCTTATA-3') to get the PCR product, A1. Primers F452I/F

(5'-GCACTTGAAATCGAGCCATGTATAGAATACGACCCTGTC-3') and

NsiAfl/R (5'-CCATGCATCTTAAGAACAATGTTTGGGCTTGCAACAG-3') were

used to obtain PCR product B1. Subsequently these two PCR products were used to

amplify the AgeI-AflII fragment using primer pair Aat/AgeF, Nsi Afl/R. This AgeI-

AflII fragment containing desired mutation was cloned into *Aat*II and *Nsi*I sites of

pGEM-7Z (+) and subsequently, sequenced to confirm the presence of desired amino

acid change. Then the mutated AgeI-AflII fragment was excised and used to replace

the corresponding fragment in pNDV-fl. The resulting clones were designated as

pNDV-fl (F452L) and pNDV-fl (F452I) respectively. The same mutations were also

introduced into the support plasmid pL (BC) by PCR mutagenesis method using one

of the primer containing desired mutation and the resulting plasmids were named as

pL (F452L) and pL (F452I) respectively. To insert GFP gene into the full length

cDNA of BC, we used pNDV-fl (*Pme* I) described in Chapter 4. Briefly, GFP gene

was PCR amplified from pGFP1 plasmid (Clonetech) with *Pme*I site using primers

GFP (Pme)-F (5'GATCGTTTAAACTTAGAAAAAATACGGGTAGAACATGGTG

AGCAAGGGCGAGGAGC-3') and GFP (Pme)/R (5'-TAGCGTTTAAACTTAT

GATCTAGAGTCGCGGCCGC-3'). The following PCR product was cloned into

*Pme*I site of the full length plasmid pNDV-fl (Pme) to obtain pNDV-fl (GFP/PmeI).

Subsequently, pNDV-fl (GFP/PmeI) was sequenced to confirm the presence of proper

orientation of the ts mutations, F452L and F452I, were excised out from pGEM 7Z

(+) and cloned into pNDV-fl (GFP/Pme) by replacing the normal fragment. These

clones were called as pBC-F452L-GFP and pBC-F452I-GFP, respectively. The

plasmids were sequenced to confirm the presence of mutation and absence of any

undesired mutations.

5.4.3 Transfection and recovery of the full length NDV plasmids

The mutant plasmids were transfected along with support plasmids pN, pP and

pL to obtain infectious virus as described previously (Krishnamurthy *et al*., 2000).

Briefly, in a six-well plate, HEp-2 cells at 80-90% confluence were infected with

MVA-T7 at 1 focus forming unit per cell. The cells were then transfected with 2.5 µg

of pN, 1.5 µg of pP, 1.0 µg of pL and 5.0 µg of full length plasmid. Two days post-

transfection, the supernatants were harvested, clarified and then used to infect fresh

HEp-2 cells or DF-1 cells at two different set of temperatures 34 °C and 37 °C. After

48 h of infection, the supernatants were collected, clarified and injected to three 9-

day-old embryonated eggs. The clarified supernatants (50 µl) were also subjected to

hemagglutination assay to detect the presence of any virus particles.
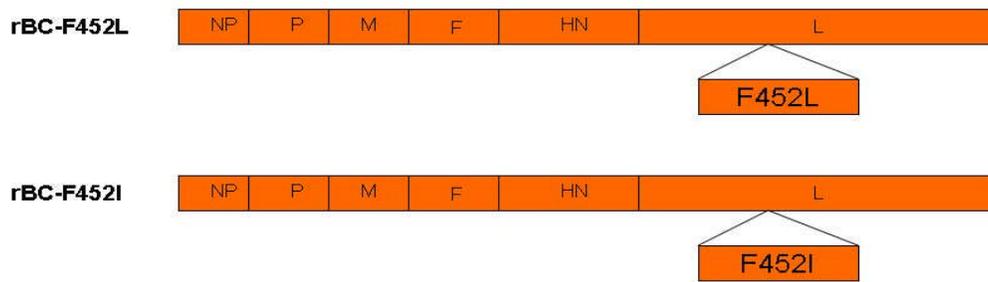
Fig. 19(a)
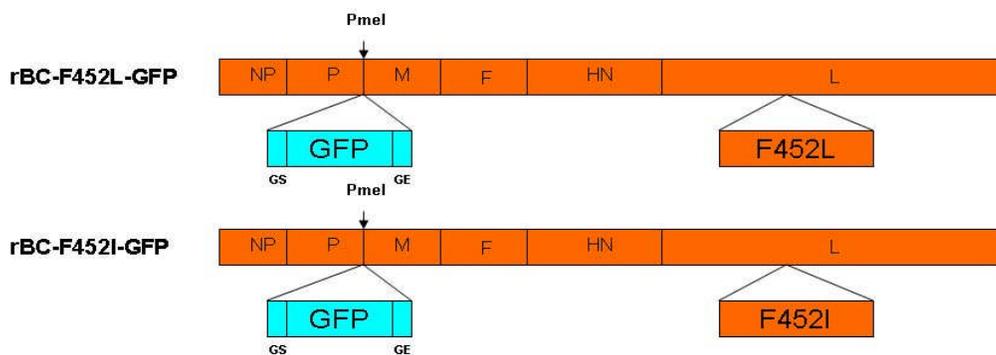
Fig. 19(b)

Fig. 19. Construction of NDV cDNA full length clones containing ts mutations.

Fig. 19(a) Construction of rNDV clones containing the ts mutations. The amino acid

at position 452 on the L protein phenyl alanine was converted to leucine. In another

clone it was converted into isoleucine. Fig. 19(b) The GFP gene was inserted between

P and M gene using unique restriction site *Pme*I. The GFP gene was modified

optimally to contain the gene start and gene end of NDV genes.

5.5 Results

5.5.1 Sequence comparison among paramyxovirus L genes

Sequence comparison of the L proteins of 10 different paramyxovirus strains along with NDV strains BC and LaSota revealed that phenylalanine at position 452 of BC and LaSota is conserved (Fig 18). This suggests a possible structural and functional role in L protein functions. The difference in position number for this residue in RSV (521) when compared with other paramyxoviruses is due to the presence of an amino-terminal extension of 70 amino acids (Stec *et al*., 1991). At the amino acid position 452, the phenylalanine was mutated to either leucine or isoleucine to find out whether the change will confer temperature sensitivity phenotypes as shown in case of RSV cpts530 (Juhasz *et al*., 1997) and HPIV 3 cp45 (Skiadopoulos *et al*., 1999). The codon from phenylalanine to leucine or isoleucine was replaced by changing two nucleotides in the codon to prevent the reversion of the mutants to wild type. All the full length clones were sequenced to verify the presence of mutations.

5.5.2 Transfection and recovery of recombinant NDV containing ts mutations within L gene

The full length cDNA clones pBC-F452L and pBC-F452I along with support plasmids pN, pP and pL were transfected into 6 well plate confluent monolayer HEp-2 cells at two different temperatures, 34 °C and 37 °C. Simultaneously, the plates were infected with MVA-T7 at 1 focus forming unit per cell. The supernatants were collected 48 h post infection, clarified, and then passaged to fresh batch of HEp-2 as

well as DF-1 cells. The hemagglutination (HA) test with 50 µl samples from each

plate showed negative in detecting any virus. After 48 h post infection, the

supernatants were again passaged to DF-1 as well as HEp-2 cells at two different

temperatures, 37 °C and 34 °C. The supernatant samples collected from DF-1 and

HEp-2 cells at both temperatures showed negative for HA test, indicating absences of

any mutant virus. To amplify if any virus recovered, 100 µl of supernatant samples

were injected to 9-day-old embryonated eggs, allantoic fluids were harvested 48 h

post injection and subjected to HA test. The samples were negative for HA test,

indicating absence of any virus. Transfection of rBC as a positive control was also

carried out along with the above clones. The above procedures were repeated for

three successive transfections and each time we were able to recover rBC virus as a

positive control, indicating that the mutation might be lethal for the recovery of virus.

In order to understand the reason for not recovering the infectious NDV containing

the mutation, we inserted GFP gene into the mutant clones, rBC-F452L and rBC-

F452I, and then transfected these clones as described above. After 48 h post

transfection, we observed enough fluorescence scattered throughout the HEp-2 cell

monolayer for both mutants rNDV-fl (F452L) and rNDV-fl (F452I) at both 37 °C and

34 °C (Fig 20). However, the intensity of fluorescence rapidly decreased with

passage, and after second passages the fluorescence was completely diminished. To

confirm our findings, the ts mutations F452L and F452I, were incorporated into the

support plasmid pL and transfected along with full length cDNA of rBC expressing

GFP. Three successive transfections did not show any visible fluorescence, indicating

that the mutation at position 452 affected the functional activity of L protein of NDV.
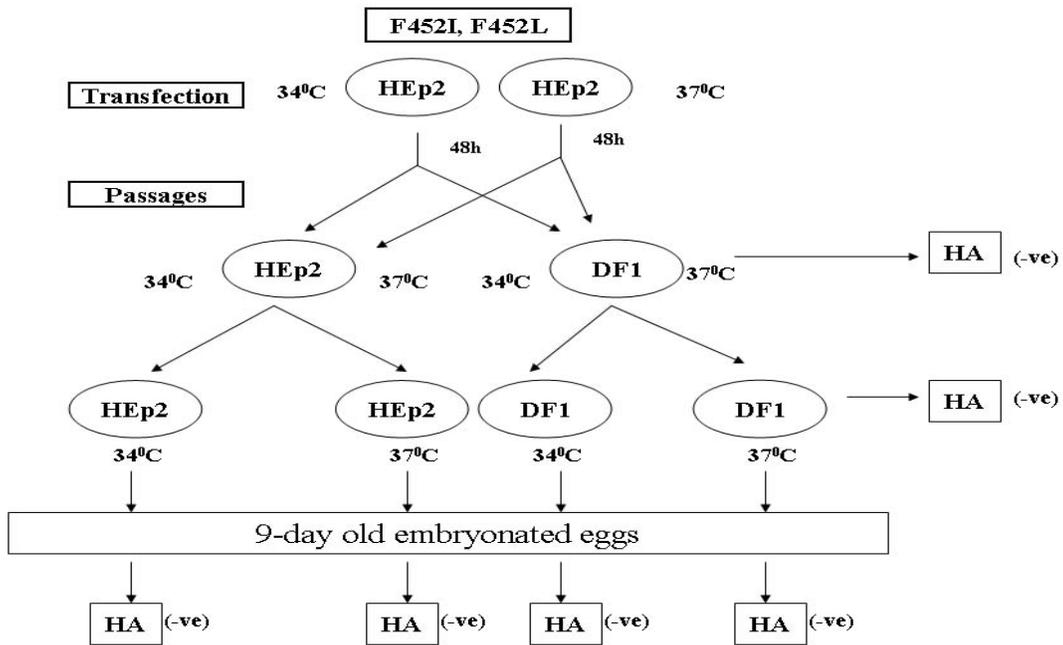
Fig. 20. Transfections of NDV ts mutant clones. The clones rBC-F452L and rBC-F452I were transfected into HEp-2 cells. After 48 h of transfection, supernatant samples were clarified and further passaged into two different cell lines HEp-2 and DF-1 cells. Further passages were performed at two different temperatures, 34 °C and 37 °C. Then, 100 μl of supernatants were inoculated into 9-day-old embryonated eggs for amplification of the recovered viruses. 48 h post inoculations, embryos were chilled and allantoic fluid samples were harvested and examined for presence of recovered viruses. All the samples obtained from each passage were checked by HA for the presence of mutant viruses.
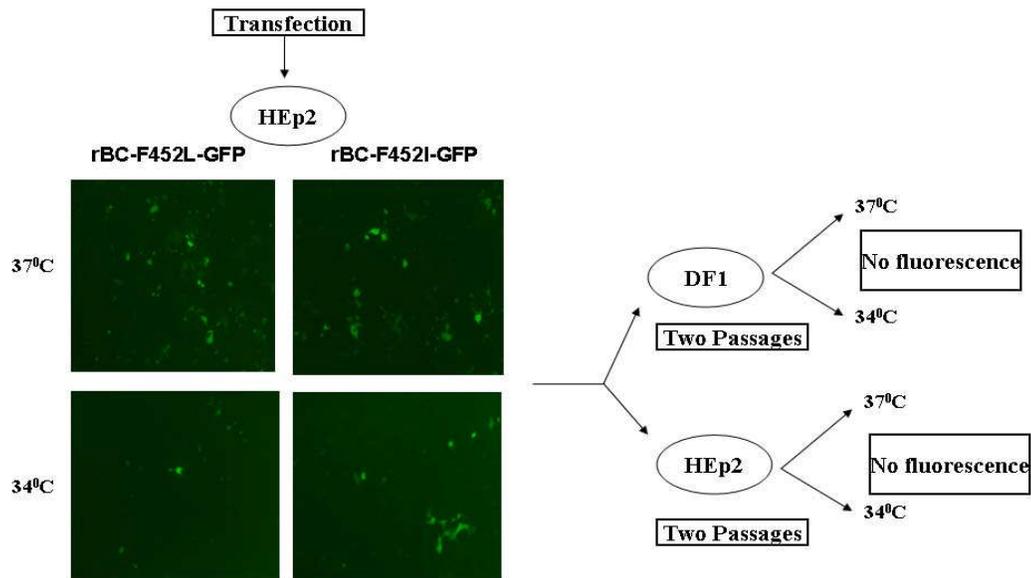
Fig. 21. Transfections of NDV ts mutant clones cotaining GFP at two different temperatures. The clones rBC-F452L-GFP and rBC-F452I-GFP were transfected in HEp-2 cells and then passaged in two different temperatures, 34 °C and 37 °C. After 48 h of transfection, supernatant samples were clarified and further passaged into two different cell lines HEp-2 and DF-1 at two different temperatures. The results showed there was a rapid decrease in fluorescence with passage. Also the samples from each passage were subjected to HA test which yielded no viable virus particles.

5.6 Discussion

Using reverse genetics, one can manipulate the viral genome in a predetermined way by introducing mutations for the development of stable, efficient and improved live attenuated viral vaccines. One way to develop a better and efficient NDV vaccine is to create a temperature sensitive mutant virus that is capable of replicating in the respiratory tract, stimulating anti-viral immunity while limiting infection to upper and lower respiratory tract without spreading to the visceral organs, thus reducing side effects to the vaccine. Several studies have shown that the mutation in the conserved L protein of paramyxoviruses can give rise to a temperature sensitive (ts) mutant with increased attenuation that can be used as a vaccine candidate (Stokes *et al*., 1993; Ray *et al*., 1996; Whitehead *et al*., 1999). Subsequently, other researchers have shown that the change in amino acid conferring to the temperature sensitivity can be imported from the heterologous paramyxovirus to the homologous region of the L gene to induce ts phenotypes (Juhasz *et al*., 1997, 1999; Skiadopoulos *et al*., 1999). Especially, in this study, we investigated whether any of the mutation can be transferred to NDV L protein for ts induction using reverse genetics. For this purpose we targeted our focus on L gene of NDV because this gene is highly conserved among NDV strains (>99.00%) and this protein plays an important role in transcription and replication of virus. We selected the position 452 of L protein of NDV as previous studies had shown that substitution of phenylalanine to leucine at position 521 in the L protein of RSVcpts530 yielded a ts mutant (Juhasz

et al., 1997) and the same mutation was imported to the homologous position of PIV3 yielding a ts mutant (Skiadopoulos *et al*., 1998). When L proteins of 10 paramyxovirus strains were aligned, the 452 positions amino acid phenylalanine was found to be conserved among several paramyxoviruses (Fig. 18). The presence of phenylalanine at position 452 in L gene is also conserved among several virulent as well as avirulent NDV strains. This suggests this amino acid position may play a critical role in L protein structure and function in NDV. So phenylalanine was mutated to either leucine or isoleucine on the L gene of full length cDNA. Several transfections of pBC-F452L and pBC-F452I in HEp-2 cells and successive passage in both HEp-2 cells and DF-1 cells at two different temperatures 34 °C and 37 °C did not yield any viable virus. Therefore, in order to explore the transfection in detail, we introduced eGFP into the full length cDNA of mutant clones and transfected as described before. Transfection of these GFP mutants showed fluorescence in the the transfected cells but the fluorescence successively diminished as the supernatants were passaged. This fluorescence in the first transfection may be due to the first round of transcription and replication of the viral genome by wild type support plasmid pL. To confirm this study, support plasmid expressing wild type polymerase gene (pL) was mutated to have the same mutations F452L and F452I and then transfected with full length rBC expressing GFP. Several transfections did not show any observable fluorescence, whereas transfection of BC-GFP with wild type support plasmid pL showed extensive fluorescence in trasfected HEp-2 cells, indicating that the mutation from phenylalanine at position 452 to leucine or isoleucine abrogated the function of the L protein. Hence our study showed that transfer of ts mutation at

position 452 from other paramyxovirus to the homologous position on L protein of NDV did not yield any viable virus. It will be worth exploring other key amino acids on L proteins of NDV for development of ts mutants.

Chapter 6

6.1 Title

Conclusions and prospects

6.2 Conclusions and prospects

Newcastle disease (ND) is a worldwide, highly contagious and fatal viral disease caused by Newcastle disease virus (NDV). The disease can cause huge economic loss to the poultry industry (Alexander, 1997). Since its first appearance in chickens in 1926, the disease continues to re-emerge throughout the world in both epidemic and endemic form causing mass mortality and destruction of poultry and disruption of international trade.

NDV is a member of genus *Avulavirus*, family *paramyxoviridae* under order *Mononegavirales* (Mayo, 2002). The paramyxovirus family also contains several other important viruses such as the sendai virus, mumps virus, simian virus 5, human parainfluenza virus and nipah and hendra viruses. Nipah and hendra viruses are highly pathogenic and fatal viruses that can possibly be used as weapons of bioterrorism. Indeed infection by highly virulent NDV strains can wipe out entire flock of poultry within a very short span of time. Thus, highly virulent NDV strains can be used as potential agent for agro-bioterrorism. Current vaccination programs for NDV involves using live attenuated lentogenic strains Hitchner B1 (Hitchner and

Johnson, 1948) and LaSota (Goldhaft, 1980). Although currently available vaccines are capable of providing a certain level of protection against NDV by routine vaccination, occasional outbreaks of virulent NDV strains in the US have been reported. Moreover, NDV vaccines cause disease signs depending on the types of strains used for vaccination, hosts, environmental condition and immune status of the bird. Therefore, it is necessary to develop a highly stable and efficient NDV vaccine. To achieve this goal, it is necessary to identify the role of each viral protein in pathogenesis and virulence.

The development of reverse genetics techniques has made possible to investigate host interaction and pathogenicity of negative strand RNA virus at a molecular level in greater detail. Particularly genetic manipulation of the viral RNA genome can be performed at the cDNA level and infectious virus can be recovered and studied in greater detail for their virulence and pathogenesis.

NDV is an ideal agent to study the underlying molecular mechanisms for pathogenesis of paramyxovirus because there are several NDV strains with a wide spectrum of virulence in chickens from completely non pathogenic to highly pathogenic. The tissue tropism of some strains is neurotropic whereas some strains is viscerotropic. With the help of a reverse genetics system, we have investigated the role of the L protein in NDV pathogenesis. Since the L protein of NDV is a multifunctional protein which is involved in viral transcription and replication, studying the role of L gene in virus pathogenesis is important to understand the

molecular basis of the viral pathogenesis. Using the reverse genetic system of a moderately virulent NDV strain rBC and an avirulent NDV strain LaSota, we exchanged the L gene between BC and LaSota and then studied those chimeric recombinant viruses for their replication and pathoegenesis. Our studies showed that the L protein of NDV plays an important role in pathogenesis. Additional studies using L gene of other velogenic and lentogenic strains will also provide more insight towards the critical role of polymerase gene in NDV pathogenesis. This information can be applied to attenuate the virulence of a virulent virus to a less pathogenic form without altering its immunogenicity that can be used as a novel efficient vaccine against ND.

Using reverse genetics system as described above, we have also examined the role of NP and P proteins in NDV virulence. We exchanged the NP gene and P gene individually or in combination between rBC and LaSota. All the chimeric recombinant viruses were recovered indicating that NP and P genes under heterologous background are functional. These chimeric viruses were tested for their replication in DF-1 cells and pathogenesis in 9-day-old as well as 1-day-old chickens indicating that the replication of virus was not affected. However, the pathogenesis study revealed that, in case of the chimeric NDV rBC with avirulent NP or P protein, the pathogenicity was slightly decreased. Interestingly when both the NP and P proteins of avirulent virus were replaced to the rBC backbone, the virulence of the chimeric recombinant viruses approached its wild type, indicating that homotypic interaction between NP and P genes is necessary for optimal pathogeneis of NDV.

Further studies using NP and P genes of other lentogenic and velogenic strains using different backbone of NDV will provide detailed insight on the involvement of these internal proteins in NDV pathogenesis. Our findings indicated that the pathogenesis of NDV is multigenic in nature.

Our studies will also help in the development of NDV as anticancer agents. Several clinical trials using NDV as antineoplastic agent on different type of cancer have shown successful results (Csatary et al., 1999; Nelson 1999; Schirrmacher et al., 1998). Several characteristics of NDV make it an excellent anticancer agent. It infects all kind of cells including non-dividing cells, such as neurons. It preferentially binds replicates and kills human tumor cells (Reichard et al., 1992). As NDV can express foreign gene efficiently and stably, pro-apoptotic and oncolytic proteins can be expressed and targeted towards specific cancer types with enhanced oncolytic activity. NDV can also be exploited to treat cancer by induction of immune responses to tumor specific antigens or by temporal expression of anti-angiogenic factors. Recent studies on different proteins of NDV have shown that the HN protein of NDV is responsible for induction of interferon and tumor necrosis factor related apopotosis in human blood mononuclear cells (Zeng et al., 2002). Recombinant NDV strain expressing chicken anemia virus apoptin or death effector domain associated factor has shown to increase oncolysis in cancer cells (unpublished data). Therefore, a more detailed study of NDV internal proteins will provide us clues to increase oncolytic activity which may be helpful in cancer treatment and gene therapy in future.

The use of reverse genetics method in molecular biology of paramyxoviruses has explored several helpful insights for designing and developing better vaccines. For example, the virulent cleavage site of F protein of NDV could be mutated to produce a better and stable vaccine without altering immunogenicity of surface glycoproteins thus making vaccine more effective. Another way was by developing an NDV strain lacking V protein for *in vivo* vaccination with increased attenuation and unaltered immunogenicity in chicken embryo (Mebatsion *et al*., 2001). Several lentogenic strains of NDV with rearranged gene order can be developed with more attenuation and increased immunogenicity for the development of a suitable vaccine as shown in the case of vesicular stomatitis virus (Wertz *et al*., 1998). Another strategy to develop a better vaccine is to create a temperature sensitive (ts) mutant phenotype of NDV having immunogenic surface proteins of virulent origin but at the same time with limited replication ability without spreading extensively into internal organs. For other paramyxoviruses, several researchers have developed ts mutant phenotypes with increased attenuation and high immunogenicity by identifying and introducing desired mutations into the L gene (Feller *et al*., 2000; Haller *et al*., 2001; Ray *et al*., 1996; Tang *et al*., 2002; whitehead *et al*., 1999). Other researchers have successfully developed ts mutant by importation of identified mutation from heterologous paramyxoviruses (Newman *et al*., 2004; Skiadopoulos *et al*., 1999). Using this strategy, we incorporated the identified mutation of L gene responsible for ts phenotype from heterologous paramyxovirus to a virulent NDV backbone and then tried to recover the virus. Our results showed that the mutation was lethal and the mutation sought can not be imported from other paramyxovirus. However, careful

sequence analysis of other temperature sensitive mutations in other paramyxovirus strains may provide a valuable insight for the development of a suitable ts phenotype strain for vaccine purpose.

A deeper understanding of the molecular basis of NDV pathogenicity will help us to efficiently control the outbreak of Newcastle disease. NDV can also be used as a model agent for studying the paramyxovirus pathogenesis in its natural host. Identification of genes responsible for NDV pathogenesis will enhance our understanding in studying the pathogenicity of other negative strand RNA viruses.

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