

ABSTRACT

Title of Document: Determination of genetic factors involved in the pathogenesis of Newcastle disease virus

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Newcastle disease is economically the most important disease of poultry. The causative agent Newcastle disease virus (NDV) is a large, enveloped virus containing single stranded non-segmented negative-sense RNA genome. The genome of NDV contains six genes in the order of 3'Leader-N-P-M-F-HN-L-5'Trailer. NDV has at least three different genome size categories: 15,186, 15,192 and 15,198 nucleotides (nt) in length. The virulence of NDV is considered to be contributed by multiple genes. The importance of genome lengths and the roles of individual genes in virulence of NDV in its natural host, chickens, have not been determined. In this study, the effects of naturally occurring nucleotide insertions in NDV genome and roles of individual genes in the virulence of NDV in chickens were determined. To achieve this goal, reverse genetic systems for two strains of NDV were established for a highly virulent strain Texas GB (GBT) and a moderately virulent strain Beaudette C (BC). Both GBT and BC are isolated from chickens and belong to genotype II of class II NDV strains and have the genome length of 15,186 nt. The 6- and 12-nt insertions in the backbones of rBC and rGBT showed little

attenuation in virus replication and in pathogenicity of the parental recombinant viruses. The reciprocal swap between NDV strains BC and GBT for the genes, nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN) and large polymerase (L) protein genes, showed that F protein gene is most important for NDV virulence, followed by the L protein gene. M, HN, N and P genes appeared not to affect the pathotypes of their parental recombinant viruses in chickens. The observations of the present study paves the way for future directions: to use the naturally occurring insertion site in the coding region of the phosphoprotein gene for insertion of potential marker sequences; to determine the amino acid residues important in fusion protein and polymerase protein for replication and pathogenesis of NDV.

DETERMINATION OF GENETIC FACTORS INVOLVED IN THE
VIRULENCE OF NEWCASTLE DISEASE VIRUS

By

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Dedication

To Amma, Appa, Nandhu, Kalai and my beloved advisor Prof. Samal

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List of Abbreviations

aa	amino acid
APMV	avian paramyxovirus
bp	base pair
BC	Beaudette C
BSL-3+	enhanced biosafety level 3
cDNA	complementary DNA
CEF	primary chicken embryo fibroblast cells
CPE	cytopathic effect
Da	Daltons
DF-1	Douglas Foster 1
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DPI	days post infection
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme linked immunosorbent assay
EMEM	essential modified Eagle's medium
F	Fusion protein
FBS	fetal bovine serum
GE	gene-end
GS	gene-start
HA	hemagglutination
HDV-Rz	hepatitis delta virus ribozyme
HI	hemagglutination inhibition
HMPV	human metapneumovirus
HN	hemagglutinin-neuraminidase protein
HPIV-2	human parainfluenza type 2
HPIV-3	human parainfluenza type 3
HRSV	human respiratory syncytial virus
IACUC	institutional animal care and use committee
ICPI	intracerebral pathogenicity index
IGS	intergenic sequence
IVPI	intravenous pathogenicity index
kDa	kilo Daltons
L	large polymerase protein
M	matrix protein
mRNA	messenger RNA
MDT	mean death time
MOI	multiplicity of infection
MV	measles virus
MVA-T7	recombinant modified vaccinia strain Ankara expressing T7 polymerase
N	nucleocapsid protein
NA	neuraminidase

ND	Newcastle disease
NDV	Newcastle disease virus
nm	nanometer
NCR	non coding region
nt	nucleotide
NV	Nipah virus
ORF	open reading frame
P	phosphoprotein
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFU	plaque forming units
PI	post infection
rBC	recombinant NDV strain Beaudette C
RBC	red blood cell
rGBT	recombinant NDV strain Texas GB
RdRp	RNA dependent RNA polymerase
RNA	ribonucleic acid
RNP	ribonucleocapsid
RSV	respiratory syncytial virus
RT	reverse transcriptase
SeV	Sendai virus
SDS-PAGE	sodium dodecyl sulfate-poly acrylamide gel electrophoresis
SPF	specific pathogen free
SV5	simian virus 5
TCID ₅₀	50% (or median) tissue culture infective dose
UTR	untranslated region
VSV	vesicular stomatitis virus

Chapter 1:

1.1 Title

General introduction

1.2 Introduction

Newcastle disease virus (NDV) causes a highly contagious respiratory, enteric and neurological disease in poultry worldwide and accounts for major economic losses in the poultry industry (Miller *et al.*, 2009; Alexander, 2000; Samal, 2011). Newcastle disease (ND) varies in degree of severity, ranging from mild infection with no apparent clinical signs to a severe form causing 100% mortality. Based on the severity of disease, NDV strains are categorized into three main pathotypes: lentogenic, mesogenic, and velogenic. Lentogenic strains cause inapparent or mild diseases and are considered to be avirulent. Mesogenic strains are of intermediate virulence causing respiratory infection with moderate mortality, while velogenic strains are highly virulent with 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 prominently produce neurological and respiratory disorders (Alexander, 1998).

Prophylactic vaccination with live 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, has 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 protect effectively against highly virulent NDV strains. Therefore, better control and prevention of ND is necessary, which can be achieved by identifying the viral genes involved in pathogenesis. Identification of the viral genes involved in pathogenesis will enable us to develop better and safer vaccines for the poultry industry.

NDV is a member of the genus *Avulavirus* under the subfamily *Paramyxovirinae* of the family *Paramyxoviridae*. The virus is enveloped and contains a single linear strand of negative-sense RNA molecule of 15,186 nucleotides (nt) in length (Krishnamurthy and Samal, 1998; Romer Oberdorfer *et al.*, 1999). There are two other genome size classes reported among strains of NDV, which are 15,192 nt and 15,198 nt. The longer genome sequences are due to the presence of 6 and 12 additional nt in the 5' noncoding region (downstream) of N gene and in the open reading frame (ORF) of P gene, respectively. The genome contains six genes which encode at least seven proteins (Peeples, 1998). The genes are arranged in tandem in the order of 3'Leader-N-P-M-F-HN-L-5'Trailer, which encode for nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN) and large polymerase protein (L), respectively. Each gene is flanked by conserved sequences at both ends called gene start (GS) and gene end (GE), which are recognized as transcription initiation and termination signals by viral RNA polymerase. Between each gene, noncoding sequences of variable

length called intergenic sequences are present, whose functions are not known. The 3' end of the NDV genome contains a 55 nt-long viral promoter and at the 5' end is a 114 nt-long viral antigenome promoter. These are called leader and trailer, respectively (Krishnamurthy and Samal, 1998). The leader and trailer serve as cis-acting elements in replication and packaging of viral RNA (Lamb and Kolakofsky, 1996). The N, P and L together constitutes a transcriptase complex that is tightly bound to the viral genome. NDV follows the general mechanism of transcription and replication of other non-segmented, negative-strand RNA viruses (Lamb and Kolakofsky, 1996). The polymerase complex starts transcription from the 3' end (leader) and produces mRNAs by a sequential start-stop mechanism at every gene start and gene end, respectively. Replication occurs when the polymerase complex switches from transcription where transcription signals are ignored (Lamb and Parks, 2007).

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 viral genome into the host cell cytoplasm. The HN glycoprotein helps in attachment of virion to host cells, fusion promotion, and removal of sialic acids from progeny virion particles, thus facilitating budding of new viral particles from the plasma membrane. The M protein forms the inner layer of viral envelope and is responsible for maintaining viral structural integrity. The N protein is the major constituent of NDV nucleocapsid and is tightly associated with the viral genome. This encapsidation protects viral genomic RNA against RNase digestion. In paramyxoviruses, the amino terminal region of the N protein involves 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 N protein also plays an important role in switching from transcription to replication (Blumberg *et al.*, 1981).

The P protein plays major roles in genome replication and transcription (Hamaguchi *et al.*, 1983; Horikami *et al.*, 1992). In paramyxoviruses, the P protein complexes with N:RNA and L protein to form the minimal transcription unit that is capable of transcription and replication *in vivo* as well as *in vitro* (Curran *et al.*, 1993). The P protein also inhibits formation of N self-assembly thus preventing uncontrolled encapsidation of non-viral RNA (Errington and Emmerson, 1997). In common with several other paramyxoviruses, an additional V protein is produced by NDV (Steward *et al.*, 1993). V protein acts as a type I-interferon antagonist and affects virulence and pathogenesis (Huang *et al.*, 2003). V proteins of other paramyxoviruses have also shown to play important roles in viral pathogenesis and virulence.

The L gene, which constitutes approximately half of the NDV genome, is the last gene to be transcribed and its product (RNA dependent RNA polymerase (RdRp) or L protein) is the least abundant viral protein (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 polymerase gene of paramyxovirus plays a major role in transcription and replication.

Previous studies from our laboratory and from other groups have shown that envelope glycoproteins, F and HN, of NDV play important roles in pathogenesis (Huang *et al.*, 2003; Panda *et al.*, 2004). The multibasic amino acids at the F protein cleavage site of virulent NDV strains play an important role in pathogenicity. However, velogenic and mesogenic strains have the exact same F protein cleavage site but they vary in virulence, indicating that other sequences of the F gene or other viral genes contribute to virulence. Recent report of association of L protein in the virulence of NDV accounts for the fact that viral polymerase complex proteins in the viral pathogenesis and virulence (Rout and Samal, 2008; Dortmans *et al.*, 2010). To date, the contribution of paramyxovirus envelope proteins and internal proteins to pathogenesis and virulence has not been categorically examined. Therefore, the goal of our study is to examine the role of envelope proteins M, F and HN versus internal proteins N, P and L in viral pathogenesis and virulence. Our approach is to rescue a highly virulent NDV strain using reverse genetics and reciprocally swap the virion envelope protein and the polymerase complex genes with a phylogenetically close mesogenic NDV strain. We have chosen a highly virulent neurotropic NDV strain Texas GB as the velogenic virus (Paldurai *et al.*, 2010) and a moderately virulent BC as the mesogenic virus, for this study. Moreover, the importance of presence of three genome lengths (15,186, 15192 and 15198 nt) in NDV strains is not known. Therefore in the proposed study we will examine the significance of different genome lengths in NDV and to determine significance of the membrane

associated protein genes and the polymerase complex associated protein genes in NDV virulence and pathogenesis.

1.3 Objectives

1. To determine the complete genome sequence of highly neurotropic velogenic NDV strain Texas GB (GBT) and to compare it with mesogenic strain Beaudette C (BC) and other NDV strains
2. To establish reverse genetics systems for NDV strains Texas GB and Beaudette C
3. To determine the effects of naturally occurring 6- and 12-nt insertions in the NDV replication and pathogenesis
4. To determine the role of membrane associated protein genes, M, F and HN in NDV pathogenesis and virulence
5. To determine the role of polymerase complex associated protein genes N, P and L in NDV pathogenesis and virulence

Chapter 2:

2.1 Title

Review of Literature

2.2 Classification

2.2.1 General classification

Newcastle disease virus (NDV) is a member of the genus *Avulavirus* under the subfamily *Paramyxovirinae*, family *Paramyxoviridae* and the order *Mononegavirales* (Mayo, 2002, Murphy *et al.*, 1995). NDV is a well-studied member of avian paramyxoviruses (APMV) and is designated APMV serotype 1. Genus *Avulavirus* also includes APMV serotypes 2 to 10 (Samal, 2011), and recently there was a proposal for inclusion of APMV-11, isolated from common snipes (Briand *et al.*, 2012). The family paramyxoviruses also include important members such as, measles virus, mumps virus, parainfluenza virus type 2 (PIV2), simian virus 5 (SV5), Nipah virus and Hendra virus.

2.2.2 Classes and Genotypes

There are numerous NDV strains isolated worldwide showing distinct genetic differences. NDV isolates are classified into various genotypes based on the phylogenetic analysis of partial sequence of about 375 nt of fusion protein gene including the cleavage site sequence (Aldous *et al.*, 2003; Miller *et al.*, 2009). Major clusters of genotypes were called lineages and classes that contain overlapping genotypes. Class I and Class II system of classification is more recent and it is widely accepted. Class I viruses includes

genotypes 1 through 9 and all these viruses are avirulent in nature and have the genome length of 15,198 nt (Czegledi *et al.*, 2006). Class II viruses include genotypes I through XI and contains avirulent viruses and virulent viruses that are commonly in circulation around the world.

The following information on NDV genotype classification is adapted from the article by Miller *et al.* (2010), with little modification:

Class I viruses are avirulent in chickens (except for one known virulent virus, that is a passage variant of Alaska 415) and historically have been recovered from waterfowl (Family *Anatidae*) and shorebirds (Alexander *et al.*, 1992; Kim *et al.*, 2007a). Class I viruses have the longest of the APMV-1 genomes at 15,198 nucleotides (Czegledi *et al.*, 2006). These viruses comprise at least nine (1–9) genotypes that may be distributed worldwide in wild birds (Kim *et al.*, 2007a) and are frequently isolated in live bird market samples (Kim *et al.*, 2007a,b).

Class II viruses have been studied in more detail and comprise eleven (I–XI) genotypes. The genotypes that are considered “early” (1930– 1960) I, II, III, IV and IX contain 15,186 nucleotides (Czegledi *et al.*, 2006). Viruses that emerged “late” (after 1960), V, VI, VII, VIII, X, and XI contain 15,192 nucleotides. Except for recent vNDV responsible for the 1998–2000 Australian outbreak, all other known class II, genotype I viruses are of low virulence and some are often used as live vaccines (chicken/Australia/QV4/1966 and chicken/N. Ireland/Ulster/1967). Class II, genotype II includes viruses of low virulence that are used as vaccine viruses worldwide, such as LaSota, B1 and VG/GA. It also includes the neurotropic virulent chicken/U.S. (TX)

GB/1948 (Texas GB) isolate, which was isolated in 1948 and is used in the USA as a challenge to show efficacy of ND commercial vaccines before production. In the U.S., in the 1940s and 1950s the abovementioned vaccines were highly effective against the circulating neurotropic vNDV, such as TX/GB and Kansas-Manhattan strains. In fact, virulent neurotropic viruses of genotype II no longer circulate in the U.S. Genotype III viruses were mostly isolated before 1960 in Japan, but have been isolated sporadically in Taiwan in 1969 and 1985 and in Zimbabwe in 1990 (Yu *et al.*, 2001). Genotype IV viruses were the predominant viruses isolated in Europe before 1970 (Czegledi *et al.*, 2006). Genotypes V, VI, VII, and VIII are the predominant genotypes circulating worldwide and contain only virulent viruses. Genotype viruses emerged in South and Central America in 1970 and caused outbreaks in Europe that same year (Ballagi-Pordany *et al.*, 1996). These viruses also caused outbreaks in North America in Florida (1971, 1993) and California (1971, 2002) (Wise *et al.*, 2004a) and are still circulating in Mexico (Perozo *et al.*, 2008). Genotype VI emerged in the 1960s and continued to circulate as the predominant genotype in Asia until 1985 when genotype VII became more common (Mase *et al.*, 2002). Genotype VI is further divided into sub-genotypes VIa through VIg with VIb being commonly isolated from pigeons. Genotype VII was initially divided into two sub-genotypes: VIIa, representing viruses that emerged in the 1990s in the Far East and spread to Europe and Asia and VIIb, representing viruses that emerged in the Far East and spread to South Africa (Aldous *et al.*, 2003). The two sub-genotypes of VII are further divided into VIIc, d, and e, which represent isolates from China, Kazakhstan and South Africa (Bogoyavlenskiy *et al.*, 2009; Wang *et al.*, 2006), and VIIf, g, and h, which represent African isolates (Snoeck *et al.*, 2009). Genotype VIII

viruses have been circulating in South Africa since 1960s (Abolnik *et al.*, 2004a) and continue to circulate in Southeast Asia. Genotype IX is a unique group that includes the first virulent outbreak virus from China from 1948 and members of this genotype continue to occasionally be isolated in China (Wang *et al.*, 2006). Genotype X viruses have been isolated exclusively from Taiwan in 1969 and 1981 (Tsai *et al.*, 2004). The isolation reports of NDV strains indicates that multiple genotypes are circulating simultaneously worldwide and reveal an increase in the number of (reported) genotypes circulating at the present time. With the exception of genotype IV, which was believed to have not been reported to GenBank since 1989, but recently, the NDV isolates from the southern Indian state of Tamilnadu has reported the existence of genotype IV (Tirumurugaan *et al.*, 2011), viruses from most genotypes still continue to circulate at the present time. Genotype XI viruses are recently reported from Madagascar, the genome sequence indicates that these genotype could have risen from the from the IV ancestor (Maminaiaina *et al.*, 2010).

2.3 Genome organization

The genome of NDV is a single non-segmented negative-sense RNA molecule that consist of 6 genes arranged in tandem in order of 3'Leader-N-P-M-F-HN-L-Trailer5' encoding at least seven proteins (Peeples, 1988; Steward *et al.*, 1993). The genome at its 3' end contains a 55 nt 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) (Fig 2.1). The length of the IGS varies from 1-47 nt (Chambers *et al.*, 1986b; Krishnamurthy and Samal, 1998;), recently it was identified in our laboratory that the IGS between HN/L is 6 nt longer in an African NDV strain AKO18 that extends the HN/L IGS length from 47 to 53 nt (Kim *et al.*, 2012).

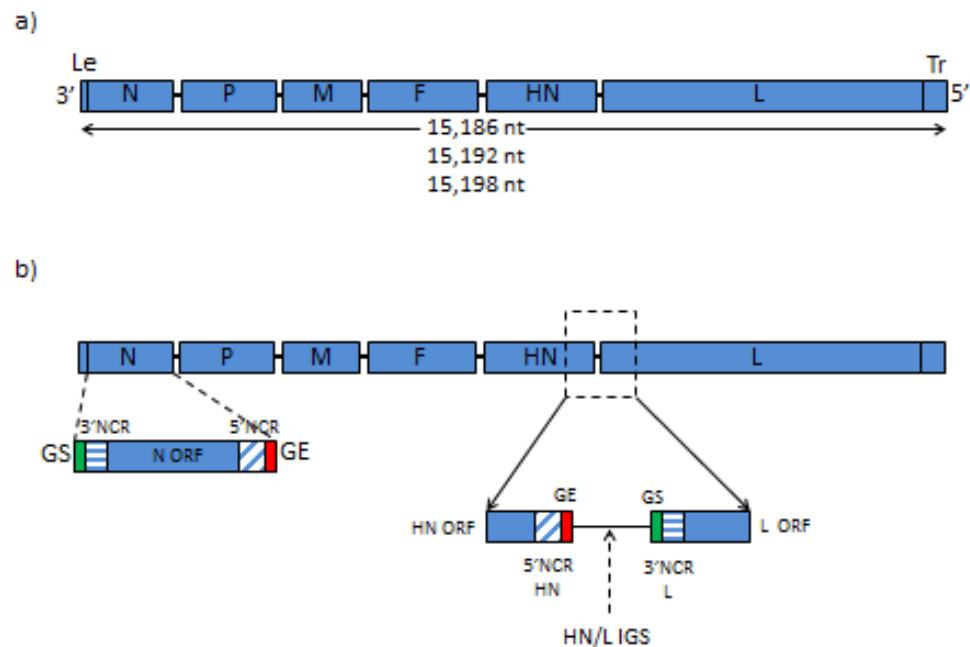


Fig 2.1. Genome map of Newcastle disease virus (a) and genetic structure of nucleocapsid protein (N) gene (b) and the gene junction of HN and L genes (dotted box). The Arabic numerals inside the arrow indicate the three genome categories observed in NDV. Gaps between the boxes indicate intergenic sequences. NCR-Non coding region, GS-Gene start, GE-Gene end, ORF-open reading frame, the coding region, IGS-Inter genic sequence, HN-Hemagglutinin-Neuraminidase protein gene, L-large polymerase protein gene, nt-Nucleotides.

2.4 Genome lengths

The genome of NDV is a single molecule of negative-sense RNA. There are three genome lengths recorded to date, namely, 15,186, 15,192 and 15,198 nt (Samal, 2011; Krishnamurthy and Samal, 1998; Czegledi *et al.*, 2006) (Fig 2.2). The longer genome sequences are due to the presence of 6 and 12 additional nt in the 5' noncoding region (downstream) of N gene (or 3' untranslated region in mRNA sense) and in the open reading frame (ORF) of P gene, respectively. Based on the phylogenetic analysis, NDV strains are classified into two divisions, class I and class II. Previously, the viruses with genome lengths of 15,198 nt were classified in class I and these viruses were isolated from birds of live bird markets and are avirulent in nature. The genome lengths, 15,186 nt and 15,192 nt were classified in division class II and this included viruses of avirulent and virulent pathotypes. Recently, in our laboratory, a genome length of 15,198 nt was recorded in a west African NDV strain AKO18 that belonged to division class II (Kim *et al.*, 2012) and which features a 6-nt insert in the 5' noncoding region (downstream) of N gene and an additional 6-nt insert in the IGS between HN/L genes.

2.5 History

Newcastle disease was first recorded on Java, Indonesia by Kraneveld in 1926. Similar disease was reported in the same year in Newcastle-upon-Tyne in England by Doyle (1927). Doyle named it Newcastle disease to distinguish it from fowl plaque, then highly pathogenic avian influenza (Doyle, 1935). ND was also reported in Korea in 1926 (Kanno *et al.*, 1929), in Ranikhet, India, in 1927 (Edwards, 1928); in Ceylon (Srilanka)

and in Manila in 1927 (Crawford, 1930; Rodier, 1928). And the enzootic nature of ND was reported from many countries worldwide, since then (Samal, 2011).

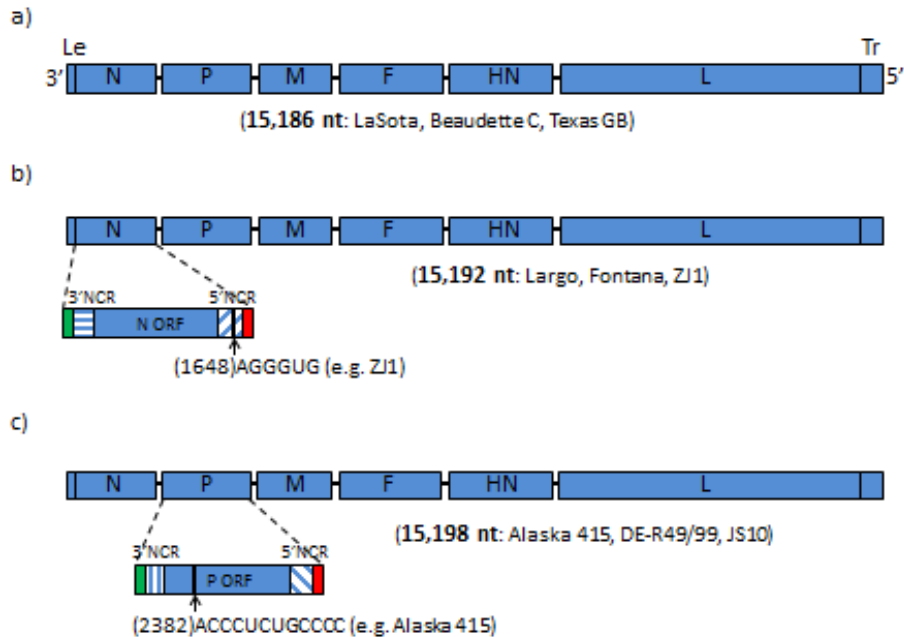


Fig 2.2 Gene map of three genome lengths observed in Newcastle disease virus. Genome lengths 15,186 nt (a), 15,192 nt (b) and 15,198 nt (c) are depicted with examples of representative NDV strains in each genome length. The longer genome lengths (b, c) were provided with extra nucleotide insertion site and sequence (with specific example). The numbers left of insert sequence denotes genome nt position.

2.6 Virion

The NDV virions are large, enveloped, pleomorphic particles ranging from 150-300 nm in size (Fig 2.3). The viral envelope is derived from the host cell membrane. The virus surface is studded with two glycoproteins, namely, fusion (F) protein, and hemagglutinin-neuraminidase (HN) protein and they protrude out from the viral membrane about 8-12 nm. 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 functional template for all RNA synthesis. The core structure is formed by nucleocapsid (N) 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). The inner layer of the viral membrane is lined by the matrix (M) protein. M protein plays a key role in morphogenesis of the virion (Peebles, 1991).

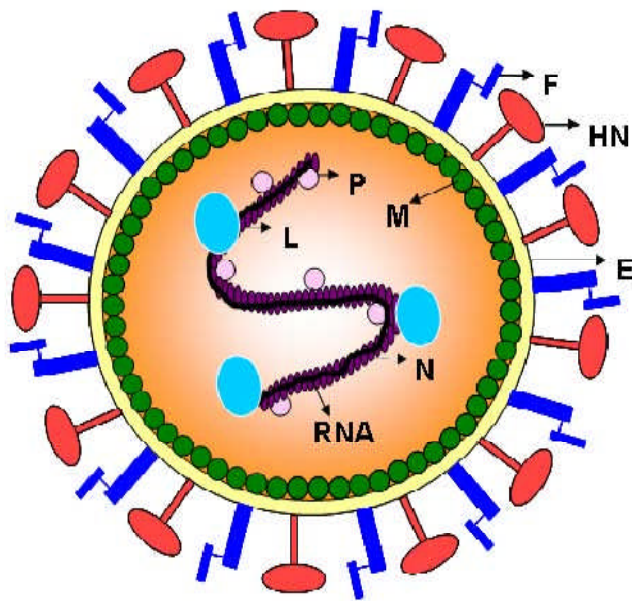


Fig 2.3 Schematic representation of Newcastle disease virion (Not drawn to scale). N-nucleocapsid protein, P-phosphoprotein, M-matrix protein, F-fusion protein, HN-Hemagglutinin-Neuraminidase protein, L-large polymerase protein. Yellow concentric circle denotes viral membrane in which surface glycoproteins F and HN. RNA genome encased in N protein is herring bone shaped. (F is a trimer and HN is a tetramer and interactions of M protein with surface and internal proteins are not shown).

2.7 Viral genes and proteins

The genome of the NDV encodes for at least seven proteins: N, P, M, F, HN, L, and V. There could be an eighth protein W, which is not found in all NDV strains and is not well characterized. V and W proteins are formed by co-transcriptional insertion of non-templated G residue(s) into P mRNA by viral RNA polymerase by a process called RNA editing (Steward *et al.*, 1993). Among NDV proteins, M, F and HN are the membrane associated proteins and N, P and L are the polymerase complex associated proteins. 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).

2.7.1 Membrane associated proteins

The envelope of NDV is associated with three proteins, namely, the morphogenic matrix protein and two integral membrane glycoproteins, 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.

Matrix protein: 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 kDa (Chambers *et al.*, 1986b). M protein is the most abundant protein inside the virion particle. 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).

Fusion protein: The F gene is 1792 nt long encoding for a 553 aa 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 (F₀) that requires host-cell proteolytic enzyme(s) for its cleavage. This cleavage of F₀ yields two subunits F₁ and F₂ connected to each other by disulfide link which is biologically active protein (Scheid and Choppin, 1974). F₀ has a predicted molecular weight of around 66 kDa whereas F₁ and F₂ are approximately, 55 kDa and 12.5 kDa, 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.

Hemagglutinin-Neuraminidase 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 kDa (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).

2.7.2 Polymerase complex associated proteins

The nucleocapsid protein (N), 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 transcription-replication complex, which is the active polymerase complex and the minimum infectious unit of NDV.

N protein: The N gene of NDV is 1,747 nt long encoding for a 489 amino acid residues long N protein. The molecular weight of NP is predicted to be 54 kDa (Krishnamurthy and Samal, 1998). The functions of N protein include, encapsidation of viral genomic RNA, thus making the nucleocapsid RNase resistant; association of N protein with P and L proteins during transcription and replication; interactions of N protein with M protein during virus assembly. The intracellular concentration of unassembled N 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 products of P gene editing: 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 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 N and L forms viral polymerase complex thus acting as a transcriptive and replicative factor. It also forms P-N0

complexes with the unassembled N (N0) thus preventing nonspecific assembly or self-aggregation of N0 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 kDa (Yusoff *et al.*, 1987). The L protein also possesses 5' capping and 3' poly (A) polymerase activities on the nascent viral mRNAs.

2.8 Virus replication cycle

First step is the attachment of the virus onto the receptors containing sialic acids mediated by the HN protein (Huang *et al.*, 1980). Upon attachment HN protein undergoes a conformational change that facilitates interaction with the fusion protein to initiate the process of membrane fusion or virus penetration. This process facilitated by the HN is also called fusion promotion. Once the F protein is activated, it fuses the virus membrane with the cell membrane thereby enabling the release of the viral nucleocapsid inside the cytoplasm of the cell (Fig 2.4). This fusion process is pH independent and occurs at neutral pH. Immediately after the fusion process the matrix protein falls off to release the viral ribonucleocapsid in the cytoplasm.

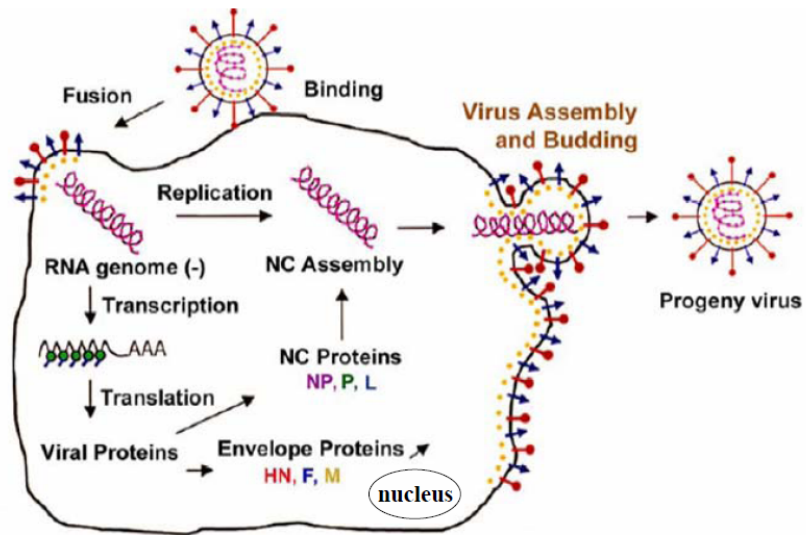


Fig 2.4 Virus replication cycle. Process of virus attachment, virus ribonucleocapsid release, transcription, genome replication, assembly, budding and release of mature virion are schematically described. (source: [http://www.urmc.rochester.edu/SMD/mbi/education/courses/MBI456 files/ParamyxovirusII](http://www.urmc.rochester.edu/SMD/mbi/education/courses/MBI456_files/ParamyxovirusII)).

Immediately after entry due to the availability of genome encased in the N protein, P and L proteins, the transcription begins. 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 N gene mRNA synthesis from N 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 in mRNA synthesis in which genes closer to the 3' end of the genome have higher concentration than those of downstream genes (Cattaneo

et al., 1987) (Fig 2.5). The mRNA produced are capped and polyadenylated in nature. The intergenic regions located between each gene are not transcribed.

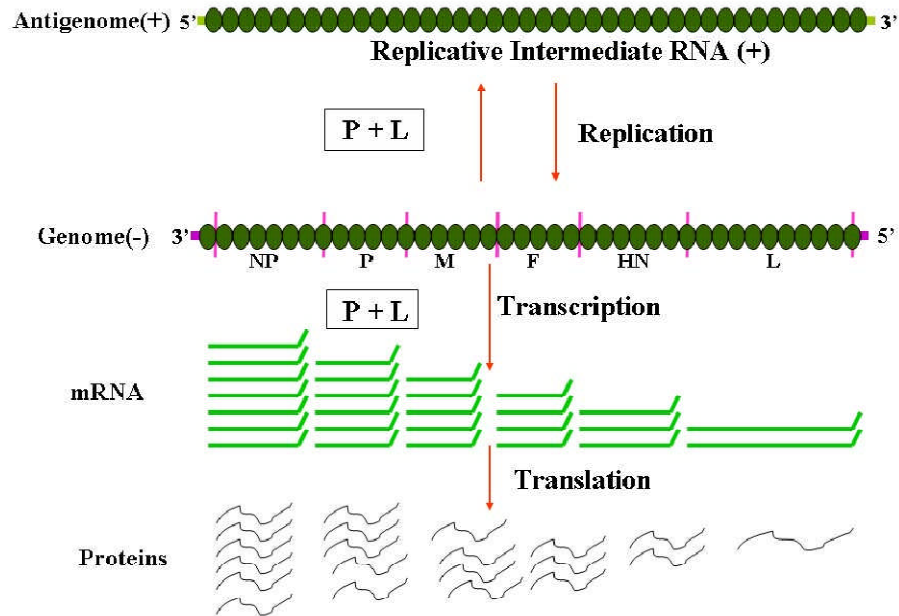


Fig 2.5 Schematic representation explaining the modular nature of transcription and translation in paramyxoviruses. The stop-start mechanism of transcription is depicted schematically. Note the fact that more number of mRNA is produced towards the 3' end of the genome and there is a gradual decrease noticed for the genes towards the 5' end. The polarity in the translated proteins is provided at the bottom.

Once the primary transcription produces sufficient mRNAs for translation of viral proteins, there is an increase in the concentration of viral proteins, especially N proteins that induce replication of viral genome by RdRp. The sufficient amount of unassembled N 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. Since the promoter to produce the genome (- sense) is stronger, more genome sense RNA is produced from the antisense RNA template.

The nucleocapsid core gets assembled inside the host cell cytoplasm. The nucleocapsids are thought to be assembled in two steps: first, free N 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 the 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 the ER to the 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 in providing the 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.

2.9 Reverse genetics

Reverse genetics is a method that allows the generation of infectious virus from the cloned cDNA of the viral genome. In non-segmented 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 N, P and L along with full length antigenome plasmid have made it possible to recover genetically engineered virus.

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.

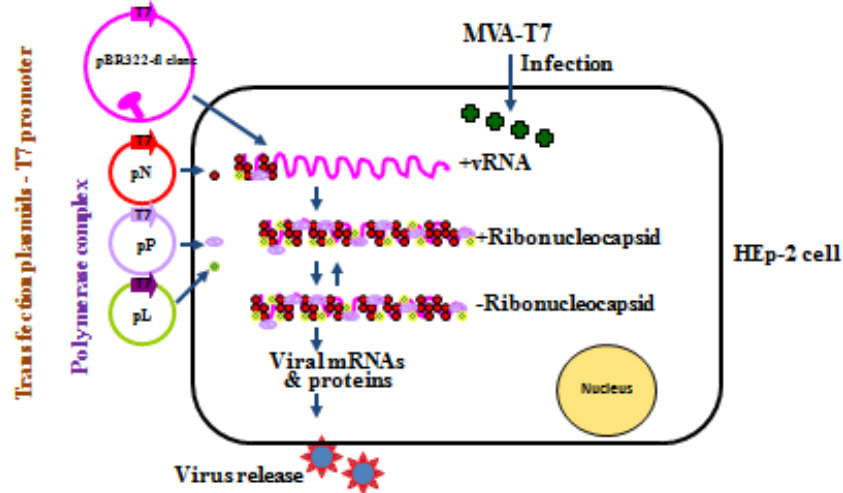


Fig 2.6 Reverse genetics: schematic representation of MVA-T7 based Newcastle disease virus rescue system. Transfection of full length cDNA clone of NDV in pBR322-HDrz, with NDV support plasmids N, P and L was performed onto the monolayer of HEP-2 cells. Simultaneously, the transfected cells are infected by modified vaccinia strain Ankara carrying T7 polymerase (MVA-T7). The T7 pol synthesizes the (+) sense transcript of NDV fl clone and the products of support plasmids, N, P and L initiates the first round of replication. Once the negative sense genome is synthesized, the virus begins the self-RdRp dependent transcription and virus replication. Virus release occurs in the supernatant. After 48 h of tranfection, the cell culture supernatant is collected and inoculated in to allantoic cavity of nine-day-old embryonated chicken eggs. After 48 h, allantoic fluid is harvested and presence of virus is confirmed by HA assay using 1% chicken RBC. Rescued virus is confirmed by the RT-PCR and sequencing.

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). Please refer to the fig 2.6 for the NDV rescue system used in our laboratory. 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.

Chapter 3:

3.1 Title

Complete genome sequence of highly virulent neurotropic Newcastle disease virus (NDV) strain Texas GB and comparison with other NDV strains

3.2 Abstract

Newcastle disease virus (NDV) strain Texas GB is a highly virulent neurotropic virus that is used as a standard vaccine challenge virus in the U.S. In this study, the complete genome sequence of strain Texas GB was determined and compared with the complete genome sequences of other NDV strains. The genome is 15,186 nucleotides (nt) long and consists of six genes in the order of 3'leader-N-P-M-F-HN-L-5'trailer. The genome contains a 55-nt leader region at the 3' end and a 114-nt trailer region at the 5' end. The intergenic regions are 2, 1, 1, 31 and 47 nt between N/P, P/M, M/F, F/HN and HN/L genes, respectively. The putative cleavage site of fusion protein showed amino acid sequence of R-R-Q-K-R↓F in position 112 to 117, which corresponds to those of virulent NDV strains. The phylogenetic analysis showed that strain GB is closely related to the neurovirulent mesogenic strain Beaudette C (BC) and to NDV viruses isolated in China and Egypt than to other strains of NDV.

3.3 Introduction

Newcastle disease (ND) is a highly contagious and fatal disease of chickens leading to huge economic losses in the poultry industry worldwide (Alexander, 2000; Samal, 2011). The causative agent Newcastle disease virus (NDV), is a member of the

genus *Avulavirus* in the family *Paramyxoviridae* (Mayo, 2002; Lamb and Parks, 2007). NDV isolates display a spectrum of virulence in chickens, ranging from inapparent infection to 100% mortality. Based on their pathogenicity in chickens, NDV isolates are categorized into three main pathotypes, lentogenic (low virulence), mesogenic (intermediate virulence) and velogenic (high virulence) (Alexander, 2000). The velogenic strains cause severe outbreaks in poultry. Based on the intracloacal inoculation test, velogenic strains are further classified into viscerotropic, which induce mortality with hemorrhagic lesions in the intestinal tract and neurotropic, when nervous signs predominate without hemorrhagic lesions in the intestine (Alexander, 2000; Sinha *et al.*, 1952; Schloer and Hanson, 1968).

NDV strain Texas GB is considered as the prototype neurotropic velogenic strain and is widely used as the standard challenge virus in vaccine efficacy studies in the U.S. Although, strain Texas GB is biologically one of the well characterized NDV strains to date (Sinha *et al.*, 1952; Banerjee *et al.*, 1994), the complete genome sequence of this strain has not been reported. Only partial or full sequences of few genes dating from 1988 to 2004 are available (Schaper *et al.*, 1988; Palmieri *et al.*, 1991; Ward *et al.*, 2000; Locke *et al.*, 2000; Seal, 1996, 2004; Wise *et al.*, 2004). The complete sequences of phosphoprotein and large polymerase genes, the intergenic sequences between N/P, P/M, HN/L genes and the trailer sequence are not available. Since the current sequences of strain Texas GB were determined by different groups at different times, it is necessary to determine the complete genome sequence of strain Texas GB to understand its pathogenicity and neurovirulence. In this study, we report the complete genome sequence of the NDV strain Texas GB (APMV-1/chicken/U.S.(TX)/GB/1948).

3.4 Materials and Methods

3.4.1 Virus

The NDV strain Texas GB was isolated from a fatal ND outbreak in chickens with severe neurological involvement, in Texas, U.S.A., in 1948 (Sinha *et al.*, 1952; Schloer and Hanson, 1968). We received this strain from National Veterinary Services Laboratory, Ames, Iowa, U.S.A. We passaged the virus three times in 9-day-old specific pathogen free (SPF) embryonated chicken eggs and made stocks in -70°C for future use. The work was performed in our USDA approved enhanced biosafety level 3 (BSL3+) facility.

3.4.2 Mean Death Time (MDT) and Intracerebral pathogenicity index (ICPI)

The pathogenicity of NDV strain Texas GB was determined by mean death time (MDT) in 9-day-old embryonated specific pathogen free (SPF) chicken eggs and by intracerebral pathogenicity index (ICPI) in one-day-old SPF chicks. Briefly, for MDT, a series of 10-fold (10^{-6} to 10^{-9}) dilutions of fresh infective allantoic fluid was made with sterile phosphate-buffered saline, and 0.1 ml of each diluent was inoculated into the allantoic cavities of five 9-day-old SPF embryonated chicken eggs. Each egg was examined three times daily and times of embryo death were recorded. The minimum lethal dose is the highest virus dilution that causes all embryos inoculated with the dilution to die. The MDT is the mean time in hours for the minimum lethal dose to kill all inoculated embryos. The MDT has been used to classify NDV strains into the following groups: velogenic strains (taking less than 60 h to kill), mesogenic strains (taking 60 to 90 h to kill), and lentogenic strains (taking more than 90 h to kill).

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

3.4.2 Viral RNA isolation and RT-PCR

The virus was grown in nine-day-old embryonated SPF chicken eggs. After 48 h of inoculation, infective allantoic fluid was harvested and used for genomic RNA extraction using RNeasy mini kit protocol (Invitrogen). The genomic RNA was tested virus free by inoculation into eggs and allantoic fluid was harvested after 48 h and assayed for HA activity using 1% chicken RBCs. RNA was removed from the BSL-3+ facility after it was shown free of viruses by the HA assay. The genomic RNA was subjected to reverse transcription using random hexamer primers and Superscript II (Invitrogen) reverse transcriptase, using the manufacturer's protocol. The cDNA served as a template for PCR using specific primer sets designed from the available sequences of strain Texas GB (accession numbers: AF144730, AF138899, AY505057 and U22293) and from the complete genome sequence of BC (Krishnamurthy and Samal, 1998). The

primers used for the sequencing of the complete genome of NDV strain Texas GB is given in table 3.1.

All PCR reactions were performed in volumes of 50 µl using Platinum *Pfx* polymerase (Invitrogen). The general conditions used for PCR were 95°C for 5 min, 30 cycles of 95°C for 30 s (denaturation), 52°C for 30 s (annealing) and 68°C for 45 s (extension) for 1 Kb size band, followed by 68°C for 10 min (final extension). PCR amplicons were sequenced directly after gel-purification.

Table 3.1. Primers used for complete genome sequencing of strain Texas GB

Primer name	Primer sequence
GBT-44fwd	5'-AATTGAAGTTGCACGGGTAGAA
GBT-797fwd	5'-GCAGTCCGCATCTTTTTGGTTA
GBT-1650fwd	5'-GACCACACCCTCAAACAAACATC
GBT-958rev	5'-AGGCTACTAAGTGCAAGGGCTGAT
GBT-2700fwd	5'-CTTGACACAGACATCCTCCATCC
GBT-1900rev	5'-GCTCAGCGCCTTGGTTTTG
GBT-2800rev	5'-GGCGGGTTTACTCAAGTCAGATGG
GBT-3900fwd	5'-CGTAGATAGGAGGGGGAAGAAAGT
GBT-4100rev	5'-CTGAGGAGAGGGCGTTTGCTAT
GBT-5000fwd	5'-TGCAGGGATCGTGGTAACAG
GBT-6000fwd	5'-ACTGTATAGGGACCGACTTGGATT
GBT-5200rev	5'-GCACCTATAAAGCGTCTCTGTCTC
GBT-6300rev	5'-CAGGCTAAGTACACCAAAAACAAG
GBT-7000fwd	5'-AGATTAATGGAGCTGCGAACAA
GBT-8000fwd	5'-GCAACAAAACAGCCACTCTTCATA
GBT-7200rev	5'-TGAGTGTAGCAGTAATGGGTAGCA
GBT-8200rev	5'-CTGCTTTGGTGCTGCTTGAACTC
GBT-9000fwd	5'-GAGTCACACCTGTCTTCACCATTG
GBT-1000 fwd	5'-CAGTCAGGAGCCAAATGTGC
GBT-11000fwd	5'-GTGACCCTACTGACTGTGACCTCT
GBT-12000fwd	5'-ATGCAATGCTGTTTAGAGACGAT
GBT-13000fwd	5'-GGAGACTTTGCGAGACTTGACTTA
GBT-14000fwd	5'-CATTTTTGCAAGAGTTAGAT
GBT-14600fwd	5'-TCTCTACTGACGGGAAAAG
GBT-9200rev	5'-CAGTTCTCCATATCTCGTGTT
GBT-10200rev	5'-GTGTGCGATTGCCTTGTCTTTTAG
GBT-11200rev	5'-TTTAGATGAATTTTTGAGGACTTG
GBT-12200rev	5'-CATCTTGTGCACCCTCCGCTTAC
GBT-13200rev	5'-TCAGATAATAGAGTTGGTAAGAA
GBT-14200rev	5'-TCATTTGAAAAGAGCGTATT

GBT-15100rev	5'-CTGCATTGCCTATAGTTTTTCAT
GBT-N307rev	5'-CCTGAGTGGTTTGTGGCATCT
GBT-N246rev	5'-GGAGGCAGAATACCGCAAAGTT
GBT-450fwd	5'-CAGTTCAACAATAGGAGTGGAGTG
GBT-14400fwd	5'-GTGACAGACATCCTATCCAGT

3.4.3 Rapid Amplification of cDNA ends (RACE)

The leader sequence of the strain GBT was determined using 3'-rapid amplification of cDNA ends (3'RACE) (Li *et al.*, 2005; Troutt *et al.*, 1992). Briefly, the genomic RNA was ligated to a 3'-blocked RNA oligo-(5'-GGTTTTGCGGTAAAGGTG GAAGAGAAG-3'-blocked) using T4 RNA ligase according to manufacturer's protocol (Promega). RT-PCR was performed using a DNA complementary oligo-(5'-CCAAAAC GCCATTTCCACCTTCTCTTC-3') with sequence specific Nrev primer 5'-GGAGGCA GAATACCGCAAAGTT. The resultant PCR product was cloned in TOPO TA vector and sequenced. The sequence of the trailer region was determined using 5'RACE technique, in which Lfwd1 primer 5'-GTGACAGACATCCTATCCAGT was used to reverse-transcribe the genomic RNA sequence into cDNA, and the cDNA was poly dATP-tailed using T4 terminal deoxynucleotidyl transferase according to the manufacturer's procedure (Invitrogen). PCR was performed using Lfwd2 primer 5'-TCTCTACTGACG GGAAAAAG and oligo-(dT) reverse primer (5'-ACCACGCGTATCGATGTGCGACTTT TTTTTTTTTTTTTV-3') and polyadenylated cDNA as a template. The obtained amplicon was cloned and sequenced.

3.4.4 Sequencing method and phylogenetic analysis

Sequencing was done using the BigDye terminator v 3.1 cycle sequencing kit (Applied Biosystems) that is based on Sanger's Dideoxy method and a 3130xl genetic analyzer, using the manufacturer's instructions. Sequences were aligned and analyzed using SeqMan and EditSeq programs, and additional primers were designed for genome walking using the PrimerSelect program of DNASTAR Lasergene 8 software package. Fourfold coverage was achieved for each nucleotide of strain GBT by sequencing four times independently from the PCR bands using a different lot of viral RNA. Phylogenetic tree was constructed using MEGA 4 software (Kumar *et al.*, 2008; Tamura *et al.*, 2007).

3.5 Results

3.5.1 Mean death time (MDT) and intracerebral pathogenicity index (ICPI)

The Mean death time value of strain Texas GB in embryonated chicken eggs was 50 h which was consistent with the velogenic NDV strains. The intracerebral pathogenicity index value was 1.925 out of the maximum value 2.0, which also was consistent with the virulence profile of velogenic NDV strains.

3.5.2 Complete genome sequence

The genome of NDV strain Texas GB is 15,186 nt (GenBank accession number GU978777), a length that is present in most NDV strains. Similar to other NDV strains, the genome of strain Texas GB consists of six genes encoding six different proteins in the order of a nucleocapsid protein (N), a phosphoprotein (P), a matrix protein (M), a fusion protein (F), an attachment protein called the hemagglutinin-neuraminidase (HN), and a

large polymerase protein (L) (3'leader-N-P-M-F-HN-L-5'trailer). The length, position and characteristics of the six genes and their intergenic sequences (IGS) are summarized in Table 3.2, and the comparison of its proteins with other NDV strains in Table 3.3.

Table 3.2. Genomic features and protein characteristics of NDV strain Texas GB.

Genes	*Hexamer phasing position at gene-start	Gene characteristics (nt)				Intergenic sequence (nt)	Deduced protein characteristics		
		Total length	5'UTR	ORF	3'UTR		Size (aa)	MW (kDa)	pI
		N	2	1746	66		1470	210	2
P	4	1451	83	1188	180	1	395	42.2	6.341
P/V	4	1452	83	720	649	-	239	25.4	6.200
P/W	4	1453	83	666	704	-	221	24.1	9.533
M	4	1241	34	1095	112	1	364	39.6	9.494
F	4	1792	46	1662	84	31	553	58.9	8.453
HN	3	2002	91	1734	177	47	577	63.3	6.750
L	6	6703	11	6615	77	-	2204	248.6	6.861

Note: Table 3.2 shows hexamer phasing positions along with individual genes, coding and non-coding, and intergenic sequences of strain Texas GB with their protein profiles. * refers to the nt position of first nt of the gene-start sequence of each gene calculated based on the six nt repeats from the 3'-end of the viral genome.

The 3' leader sequence of strain Texas GB consists of 55 nt, a length present in all NDV strains (Alexander, 1998). The leader sequence of strain Texas GB showed 100% identity with the leader sequence of strain BC, but there are 2, 3, 7, 8, 7 and 6 nt differences between the leader sequence of strain Texas GB and leader sequences of strains LaSota, B1, Anhinga, Fontana, Largo and Alaska 415, respectively. Comparison

of the sequences of NDV strains Texas GB with those of NDV strains BC and LaSota showed differences in the stretch of 6 or 7 Uracil residues in the gene-ends of N and L genes and that alters the putative IGS between N/P genes to 1 or 2 residues and the putative trailer sequence to 113 or 114 nt, respectively.

Table 3.3. Percent nucleotide and amino acid identities of Texas GB with other strains of Newcastle disease virus

NDV strains	Lentogens		Mesogens		Velogens	
	LaSota	B1	BC	Anhinga	Fontana	Largo
Complete genome [*]	96.9	97.0	99.1	83.6	85.5	85.4
Viral proteins [†]						
N	99.2	98.8	99.8	92.2	93.0	93.0
P	97.5	93.7	98.7	79.0	84.3	82.8
P/V	95.0	91.2	98.3	69.5	78.7	74.9
P/W	93.9	93.9	97.7	60.9	75.1	69.8
M	97.3	97.0	98.9	89.3	90.4	90.4
F	96.6	96.4	98.6	88.6	90.1	87.9
HN	95.5	96.4	98.1	87.6	89.1	89.3
L	97.0	97.6	99.4	93.5	94.4	94.5

Note: Table 3.3 shows nt sequence identity of the complete genome sequence (^{*}) and percent amino acid sequence identity ([†]) of different proteins encoded by NDV strain Texas GB with other strains of NDV.

This leads to the putative length of N gene to 1,746 or 1,747 nt (in case of genomes with 15,186 nt) or 1,753 nt (in case of genomes with 15,192 nt) and the putative length of L gene to 6,703 or 6,704 nt (Fig 1.a, b). The putative length of P gene consists 1,451 (in

genome lengths 15,186 and 15,192 nt) or 1,463 nt (in genome length of 15,198 nt). The P gene of strain Texas GB contains a putative editing site ²²⁸⁰UUUUUCCC²²⁸⁷ (genome sense) that is identical in position to other NDV strains (Lamb and Parks, 2007). The 5' trailer sequence of the strain Texas GB is 114 nt long and showed a 5 nt differences from the trailer sequence of strain BC.

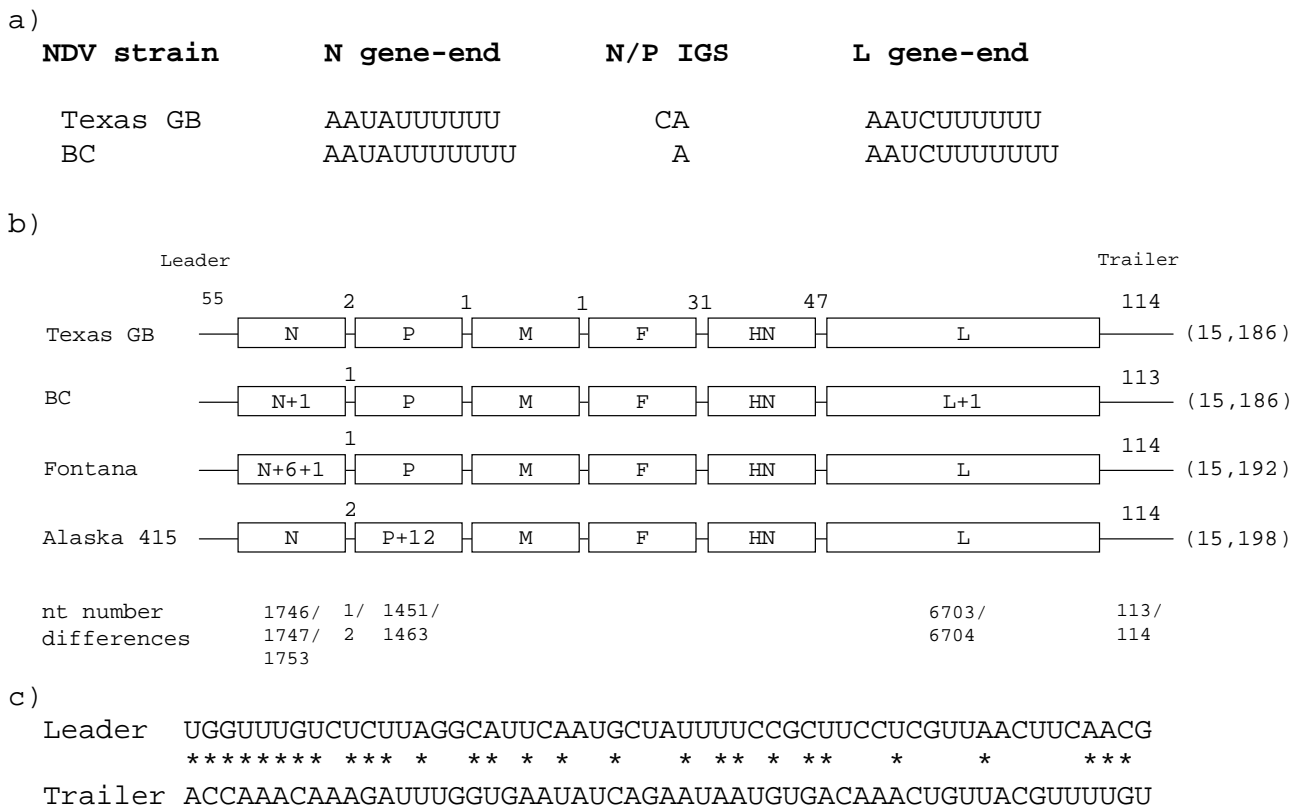


Fig 3.1. Comparison of nucleotides (nt) sequences of gene-end of N and L genes and IGS between N/P genes (given in genome sense) (a), gene map of NDV strains Texas GB, BC, Fontana and Alaska 415 with their genome length given in the parenthesis (not drawn to scale) (Arabic numerals indicate number of nt) (b), nt sequence complementarity between 3'leader and 5'trailer (last 55 nt) sequences of strain Texas GB (stars indicate base pairs) (c).

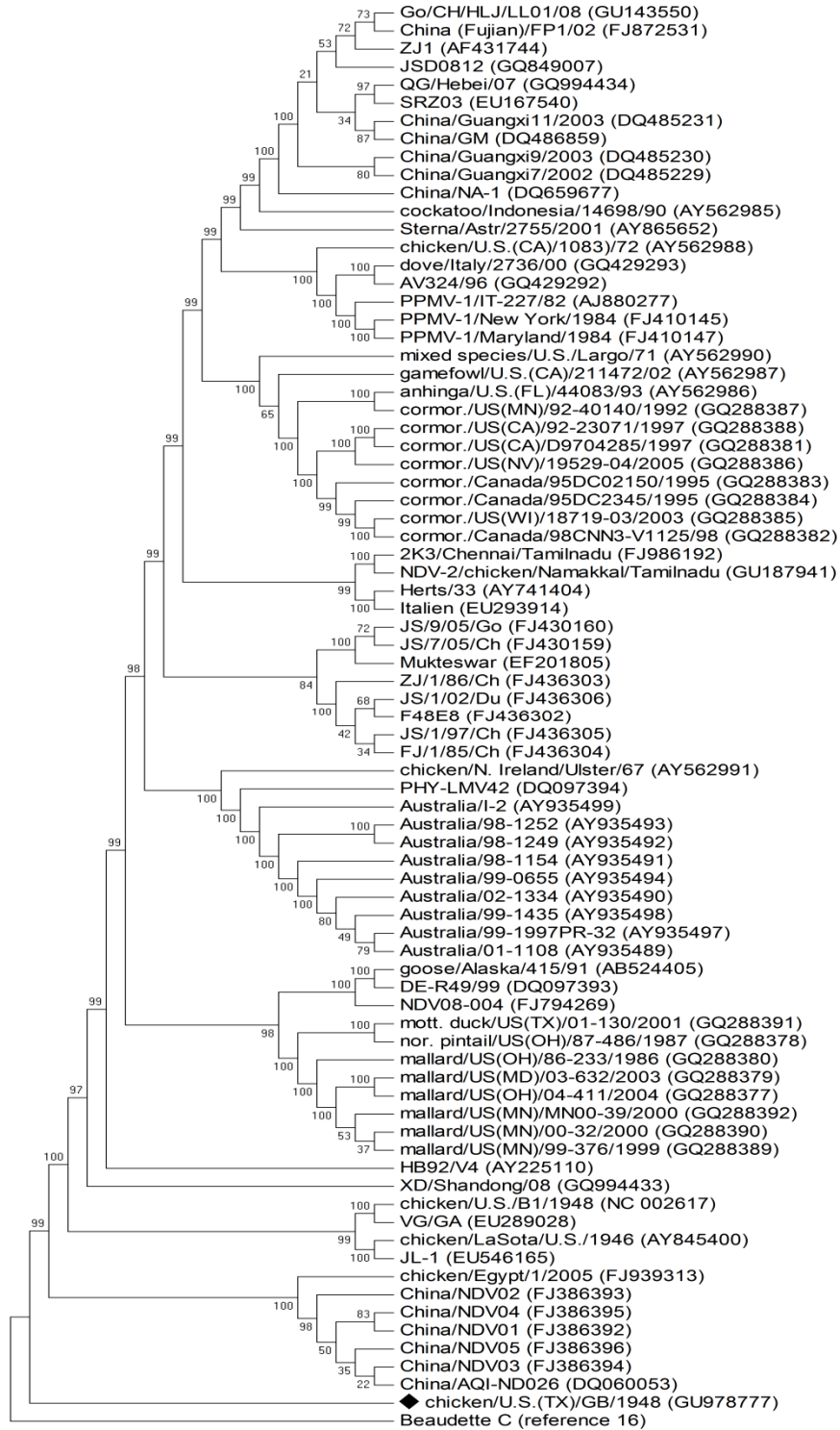


Fig 3.2. Phylogenetic tree of strain Texas GB with available genome sequences of NDV strains was constructed using maximum parsimony method using MEGA4 program with bootstrap values calculated for 1000 replicates (strain Texas GB is indicated by a solid diamond)

3.5.3 Amino acid differences between strains GBT and BC

All the six major proteins of strain Texas GB invariably showed higher amino acid sequence identity with the cognate proteins of other NDV strains that are well characterized (Table 3). The comparison of complete genome sequences showed that strain Texas GB is more related to strain BC (99.1% nt identity) than to other NDV strains (Table 3.3 and Fig 3.2). Comparison of the amino acid (aa) sequences between strains Texas GB and BC showed differences in all six proteins; one aa in N (K421E), four aa in P (P30S, K36R, A98T, V104A and D336E), four aa in M (M132V, D152N, P216S and A336T), eight aa in F (S10P, A11V, S265G, E304G, T457I, T510I, A520V and A550T), 11 aa in HN (Q7R, V9A, A34V, M35V, I191V, N228S, V271A, S310G, E332G, L454P and A571V) and 14 aa in L (T75A, I89V, N265D, C698R, K889N, F1379Y, D1531N, N1643S, R1668K, F1706L, L1734P, S1758A, M1785I and I2067V). There was no aa difference between the unique C terminal regions of V and W proteins of strains Texas GB and BC.

3.6 Discussion

The genome of NDV strain Texas GB is 15,186 nt (GenBank accession number GU978777), a length that is present in most NDV strains. Similar to other NDV strains, the genome of strain Texas GB consists of six genes encoding six different proteins in the order of a nucleocapsid protein (N), a phosphoprotein (P), a matrix protein (M), a fusion protein (F), an attachment protein called the hemagglutinin-neuraminidase (HN), and a large polymerase protein (L) (3'leader-N-P-M-F-HN-L-5'trailer). The length, position

and characteristics of the six genes and their intergenic sequences (IGS) are summarized in Table 3.2, and the comparison of its proteins with other NDV strains in Table 3.3.

The 3' leader sequence of strain Texas GB consists of 55 nt, a length present in all NDV strains (Alexander, 1998). The leader sequence of strain Texas GB showed 100% identity with the leader sequence of strain BC, but there are 2, 3, 7, 8, 7 and 6 nt differences between the leader sequence of strain Texas GB and leader sequences of strains LaSota, B1, Anhinga, Fontana, Largo and Alaska 415, respectively. Comparison of the sequences of NDV strains Texas GB with those of NDV strains BC and LaSota showed differences in the stretch of 6 or 7 Uracil residues in the gene-ends of N and L genes and that alters the putative IGS between N/P genes to 1 or 2 residues and the putative trailer sequence to 113 or 114 nt, respectively. This leads to the putative length of N gene to 1,746 or 1,747 nt (in case of genomes with 15,186 nt) or 1,753 nt (in case of genomes with 15,192 nt) and the putative length of L gene to 6,703 or 6,704 nt (Fig 1 a, b). The putative length of P gene consists 1,451 (in genome lengths 15,186 and 15, 192 nt) or 1,463 nt (in genome length of 15,198 nt). The P gene of strain Texas GB contains a putative editing site ²²⁸⁰UUUUUCCC²²⁸⁷ (genome sense) that is identical in position to other NDV strains (Lamb and Parks, 2007). The 5' trailer sequence of the strain Texas GB is 114 nt long and showed a 5 nt differences from the trailer sequence of strain BC.

The F protein cleavage site is a well-characterized determinant of NDV pathogenicity in chickens (Panda *et al.*, 2004; de Leeuw *et al.*, 2005). Virulent NDV strains typically contain a polybasic cleavage site (R-X-K/R-R↓F), which is recognized by intracellular proteases of most cells. The cleavage site of strain Texas GB is ¹¹²R-R-Q-

K-R↓F¹¹⁷, which contained four basic amino acids at positions 112-116, corresponding to those of virulent NDV strains (Alexander, 2000; Lamb and Parks, 2007).

Phylogenetic tree analysis of complete genome sequence of strain Texas GB with 78 full length genomes (or genome sequences >15 Kb) of other NDV strains was performed by maximum parsimony method using MEGA 4 software (Molecular Evolutionary Genetics Analysis) (Kumar *et al.*, 2008; Tamura *et al.*, 2007). It was observed that strain Texas GB is more closely related to strain BC and viruses isolated in China (Chen *et al.*, 2008) and Egypt (Mahamed *et al.*, 2009) than to other NDV strains (Fig 2). The recently-sequenced virulent and avirulent NDV isolates from cormorants in the U.S. (Miller *et al.*, 2009) did not show phylogenetic relatedness to strain Texas GB at the genome level (Fig 3.2). The high degree of nt identity and phylogenetic relatedness of velogenic strain Texas GB with a mesogenic strain BC suggest that subtle differences at the genome level may have a profound effect on the pathogenicity of a NDV strain. Availability of the complete genome sequence of strain Texas GB will be useful in further understanding of the NDV pathogenesis and neurovirulence.

Since this study is the first report of the complete genome sequence of the velogenic neurotropic NDV strain Texas GB which is used as standard challenge virus for vaccine testing, this would be valuable for future studies to understand the molecular determinants of NDV virulence and neurotropism in chickens.

Chapter 4:

4.1 Title

Effects of naturally occurring six- and twelve-nucleotide inserts on Newcastle disease virus replication and pathogenesis

4.2 Abstract

Newcastle disease virus (NDV) isolates contain genomes of 15,186, 15,192, or 15,198 nt. The length differences reflect a 6 nt insert in the 5' (downstream) noncoding region of the N gene (15,192-nt genome) or a 12 nt insert in the ORF encoding the P and V proteins (15,198-nt genome). We evaluated the effects of these inserts on replication and pathogenicity by adding them to two NDV strains that have natural genome lengths of 15,186 nt and represent two different pathotypes. Our results showed both the 6 nt and 12 nt inserts did not affect replication but slightly attenuates the virulence of NDV.

4.3 Introduction

Newcastle disease virus (NDV) causes a highly contagious infection in chickens that, depending on the virulence of the virus strain, can range in severity from inapparent to highly lethal (Aldous *et al.*, 2003; Alexander, 2003; Alexander, 1989). NDV is a member of the genus *Avulavirus* in the family *Paramyxoviridae* (Lamb and Parks, 2007; Mayo, 2002). The genome of NDV is a single strand of negative-sense RNA. The genome contains six genes encoding the nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and

large polymerase protein (L). In addition, the P gene encodes an additional protein called V that is expressed by RNA editing.

The NDV strains that initially were analyzed by sequencing were found to have a genome length of 15,186 nucleotides (nt) (Samal, 2011; de Leeuw and Peeters, 1999; Lamb and Parks, 2007; Romer-Oberdorfer *et al.*, 1999; Krishnamurthy and Samal, 1998). Subsequently, NDV strains were found whose genomes contained 15,192 nt (Huang *et al.*, 2004; Miller *et al.*, 2010; Yan and Samal, 2008) or 15,198 nt (Czegledi *et al.*, 2006; Kim *et al.*, 2007a, b; Liu *et al.*, 2010; Takeda *et al.*, 2005). Remarkably, sequence analysis showed that the 15,192 nt genome length viruses all contain a 6 nt insert (e.g., AGGGUG in strain ZJ1) in the 5' (downstream) noncoding region of the N gene after genome position 1647 (Huang *et al.*, 2004; Ujvari, 2006) (Fig 1a), and the 15,198 nt genome length viruses all contain a 12 nt insert (e.g., ACCCUCUGCCCC in strain Alaska 415) in the ORF encoding the P and V proteins after genome position 2381 (Czegledi *et al.*, 2006; Liu *et al.*, 2010; Seal *et al.*, 2005) (Fig. 1b). The 12 nt insertion increases the lengths of the P and V proteins by four amino acids without affecting their reading frames.

The presence of these inserts in NDV strains varies depending on their natural history: the 15,186 nt genome length viruses circulated between 1930 and 1960, and the 15,192 and 15,198 nt genome length viruses emerged after 1960 (Czegledi *et al.*, 2006; Huang *et al.*, 2004; Liu *et al.*, 2010; Seal *et al.*, 2005; Ujvari, 2006). The evolutionary significance of these inserts is not known. In this study, the effects of the 6- and 12-nt insertions in the N and P genes of NDV, respectively, is evaluated in two viruses, namely, the mesogenic Beaudette C (BC) and the velogenic Texas GB (GBT).

4.4. Materials and methods

4.4.1 Cells and viruses:

A chicken embryo fibroblast cell line (DF-1), African green monkey kidney cell line (Vero) and a human epidermoid carcinoma cell line (HEp-2) were grown in Dulbecco's minimal essential medium (DMEM) with 10% fetal bovine serum (FBS) and maintained in DMEM with 2% FBS. The modified vaccinia virus strain Ankara (MVA) expressing T7 RNA polymerase was kindly provided by Dr. Bernard Moss (NIH, Bethesda, MD) and propagated in primary chicken embryo fibroblast cells in DMEM with 2% FBS. The moderately pathogenic (mesogenic) NDV strain BC and highly pathogenic (velogenic) NDV strain GBT and its recombinant derivatives were grown in 9-day-old embryonated specific-pathogen-free (SPF) chicken eggs in an enhanced biosafety level 3 (BSL-3+) containment facility certified by the USDA following the guidelines of the IACUC, University of Maryland.

4.4.2 Construction of full length antigenome cDNA clone of NDV strains BC and GBT:

The construction of the full-length antigenome cDNA of NDV strains BC and GBT was done in a low copy number vector containing hepatitis delta ribozyme sequence, pBR322-HDrz. Identical restriction enzyme sites were introduced in the cDNA sequences for the plasmid of full length BC (pBC) and GBT (pGBT). This was achieved by analyzing the RE site profile of both the virus antigenome and the vector pBR322-HDrz. The RE sites which were not available in the antigenome and the vector were chosen for designing a linker sequence which would have the nine RE sites in the order

AscI, PacI, PmeI, AsiSI, AgeI, SnaBI, BstBI, MluI and RsrII. The linker sequence is 112 nt long with the sequence of 5'-ACTTggcgcgccAATACCTtaattaaGGAGCTTTgtttaaacGGCAgcgatcgcGTTAACaccggtCGGCtacgtaCCAAttcgaGGCGacgcgtCGATTcggaccgAATT. The cloning of the linker sequence into pBR322-HDrz using RE sites AscI and RsrII to generate pBR322-HDrz-Linker that enabled the sequential cloning of antigenome of strains BC and GBT. The primer sets used for the sequential cloning of N, P, M, F and HN as segments 1, 2, 3, 4 and 5. The L gene was cloned in three fragments, namely, 6, 7 and 8. Primers used for the gene-wise cloning of the antigenome of strain GBT is given in Table 4.1.

Table 4.1. Primers used for the construction of full length cDNA clone of NDV strain Texas GB (GBT)

Fragment	Primer Name	Primer sequence
1	rGBT-N (AscI)	5'-AGTTGGCGCGCCTAATACGACTCACTATAGGGACCAAAC
	Fwd	AGAGAATCCGTAAGTTACGATAAAAAG
	rGBT-N (PacI)	5'-TCTGCCTTAATTAATAGTGAGCCGCATTGTGCCTGTGG
	Rev	
2	rGBT-P (PacI)	5'-TCAGCCTTAATTAACAGAGCCGAGGAAATTAGAAAAA
	Fwd	AGTACG
	rGBT-P (PmeI)	5'-CGCGCCGTTTAAACACGGTTGCGCGATCATTTAGTGGG
	Rev	
3	rGBT-M (PmeI)	5'-CGCGCCGTTTAAACTAGCTACATTAAGGATTAAGAA
	Fwd	AAAATACG
	rGBT-M (AsiSI)	5'-ACGATTGCGATCGCGACAGATTAGTTTTTGGTGTTCATG
	Rev	
4	rGBT-F (AsiSI)	5'-ACGATTGCGATCGCTTACAGTTAGTTTACCTGTCTATC
	Fwd	
	rGBT-F (AgeI)	5'-TCTACAACCGGTAGTTTTTTCTAAACTC
	Rev	
5	rGBT-HN (AgeI)	5'-AAAACACTACCGGTTGTAGATGACCAAAG
	Fwd	
	rGBT-HN (SnaBI) Rev	5'-ATCAGGTACGTACATTTTTTCTTAATCGAGGGACTATTG
6	rGBT-L1 (SnaBI)	5'-ATCAGGTACGTAGCAATGAGATACAAGGCAAAACAGC
	Fwd	
	rGBT-L1 (BstBI)	5'-AATCCATTCTGAAGAAGTGAGGTAGGCCCATCAAC
	Rev	
7	rGBT-L2 (BstBI)	5'-CACTTCTTCTGAATGGATTCACCTAAGACTGATGGACAC
	Fwd	TACAATG
	rGBT-L2 (MluI)	5'-GTGTGAACCGGTCATACCTTGCTGGAGCACTCAAATCT

8	Rev	AACTCTTG
	rGBT-L3 (MluI)	5'-GGTATG <i>ACGCGT</i> TCACACTAAGTCAGATTCATC
	Fwd	
	rGBT-L3 (RsrII)	5'-GGTCC <i>GGACCG</i> CGAGGAGGTGGAGATGCCATGCCGACCC
	Rev	ACCA ACAAAGATTTGGTGAATAACAAGAC

Note: The restriction enzyme sites are given in italics and bold.

The platinum *Pfx* PCR was used to amplify the segments and the gel purified fragments were digested and sequentially cloned into the pBR322-HDrz-Linker to generate the full length antigenome of strains BC and GBT, pBC and pGBT, respectively. Upon cloning each fragment, the sequence of the fragment was confirmed by completely sequencing the fragment and then next fragment cloning was started. In the P gene a naturally occurring RsrII site was modified using an overlap PCR to enable the reciprocal swap of each gene separately between the pBC and pGBT. In total 16 nt changes were introduced in the antigenome of strains BC and GBT to introduce RE sites and to modify the RsrII site in the P gene. Nt changes in the L coding regions were introduced such a way that the amino acid sequence was not altered. The details of 16 nt changes of strains BC and GBT are given in table 4.2.

Table 4.2. The position and identical nucleotide changes introduced into the cDNA clones of pBC and pGBT

Location	Nucleotide changes	RE sites	Amino acids unchanged
N gene 5'NCR	A(1768)T, C(1769)T, C(1773)T	PacI	-
P gene 5'NCR	A(3221)G, A(3222)T, T(3227)A	PmeI	-
M gene 5'NCR	T(4452)G, T(4453)C, T(4457)C,	AsiSI	-

	A(4458)G, T(4459)C		
HN-L IGS	A(8327)C, G(8330)A	SnaBI	-
L ORF	G(10396)A, A(13537)G	BstBI, MluI	Glu (GAG to GAA), Ala (GCA to GCG)
P ORF	A(13537)G	RsrII	Arg (CGG to CGA)

4.4.3 Support plasmids construction:

The open reading frame (ORF) of the nucleocapsid protein, phosphoprotein and large polymerase protein genes of NDV strain GBT were cloned in expression plasmid pGEM7Z(+) to generate support plasmids pN, pP and pL respectively. The primer sets used for the construction of support plasmids is given in table 4.3.

Table 4.3. Primer sequences used for the construction of NDV strain Texas GB support plasmids.

Primer Name	Primer sequence
N orf F(EcoRI)	5'-CATAGAATTCATGTCTTCCGTATTTGA CGAGTACGAACAGCTCC
N orf R(HindIII)	5'-GGTCAAGCTTTCAATACCCCGAGTCG GTGTCGTTATCTTGGGATG
P orf F(EcoRI)	5'-CATAGAATTCATGGCCACCTTTACAGATG CGGAGATCGACGAGC
P orf R(HindIII)	5'-GGTCAAGCTTTTAGCCATTTAGTGCAA GGCGCTTGATTTTCCTG

L1 orf F(ApaI)	5'-CATAGGGCCCATGGCGAGCTCCGGTC CTGAAAGGGCAGAGCATCAG
L1 orf R(AatII)	5'-CAACTCGACGTCGGTTCTTGCTCTTCGG GTCATGATTGCGGTTTGAAG
L2 orf F(AatII)	5'-GAACCGACGTCGAGTTGCAACCTTCATAAC AACTGACCTGCAAAAAG
L2 orf R(KpnI)	5'-GAAAGGTACCGCAACAGGTGCTTCCCTGA TACAGCAACTAAATTTAC
L3 orf F(KpnI)	5'-GTTGCGGTACCTTTTCGAGCTACTTGGGGT GGCACCGGAGCTAAGG
L3 orf R(ClaI)	5'-GGTCATCGATTTAAGAGTCACAGTTACTGT AATATCCTTTGACTGC

4.4.4 Virus rescue system:

A recombinant vaccinia virus-based transfection system was used to rescue infectious recombinant BC and GBT from their respective cDNA clones. Briefly, HEp-2 cells were transfected using Lipofectamin 2000 with 5 µg cDNA clone along with support plasmids, 3 µg of pN, 2 µg of pP and 1 µg of pL. Along with the transfection, the infection of modified recombinant vaccinia virus (MVA/T7) capable of synthesizing T7 RNA polymerase was also done. In a parallel transfection, plasmid pL was excluded in the experiment to serve as a negative control. Two days after transfection, the supernatant was injected into the allantoic cavities of 9-day-old embryonated eggs. The allantoic fluid of the eggs injected with the transfectant gave a positive hemagglutination (HA) titer of

2^6 to 2^8 HA units. The viruses were passaged three times in 9-day-old embryonated eggs and genomic RNA was extracted and the presence of the virus is confirmed by RT-PCR and sequencing using virus specific primers.

4.4.5 Construction of rBC and rGBT with 6- and 12-nt inserts:

In the present study, we investigated the functional significance of these inserts by introducing them separately by reverse genetics into the genomes of two 15,186 nt genome length viruses representing two different NDV pathotypes, namely the highly virulent strain Texas GB (Paldurai *et al.*, 2010) and the moderately virulent strain Beaudette C (Krishnamurthy and Samal, 1998; Krishnamurthy *et al.*, 2000) (Fig 1b). Consensus sequences for the 6 and 12 nt inserts were determined by aligning the sequences of the N and P genes of representative 15,192 and 15,198 nt genome length NDV strains, using Clustal W alignment analysis of Mega 4.0 software (Kumar *et al.*, 2008). This showed that the 6 nt insert had conserved residues at positions 2, 3 and 4 (-GGG--) (Fig 4.1), while the 12 nt insert was conserved except at positions 1, 6 and 8 (-CCCU-U-CCCC) (Fig 4.2). A 6-nt (AGGGUG) or 12-nt (ACCCUCUGCCCC) insert conforming to the respective consensus sequence was introduced separately into the N (after nt position 1647) or P (after nt position 2381) genes of strains Texas GB and Beaudette C.

Genotype/strain (NDV classII)		Nucleocapsid protein gene 5' non coding region	
I	Ulster 67	(1620)	UAGUAGGGUUAGGGGGCGAGCGUGGGG-----UGGGAGGUCAGGCGUCAG
II	LaSota	(1620)	UUGUAGGGUACGGGAGUGGGCAUCAGC-----UGGGGAGCUAAAACGCCGA
	Beaudette C	(1620)	UAGUAGGGUUAUGGGAGUGGGCAUCAGC-----UGGGGAGCUAAAACGCCGG
	Texas GB	(1620)	AAGUAGGGUUAUGGGAGUGGGCAUCAGC-----UGGGGAGCUAAAACGCCGG
	Mukteswar	(1620)	UAGUAGAGCUAAGAAGACGGACAUUGGA-----UUGGGGACCAAGGUGUCAG
IV	Herts 33	(1620)	UGGUGGGGUUGGGGAGACGGCCGUGGGG-----UGGGGGACUAAGCCGUCGG
V	Largo 71	(1620)	CAGCGGGGUUUAAGGAGCGGGCUUGGGGAGGGAGUGAGGGGCUUGGGUGUCGG
VI	Fontana	(1620)	CAGUAGGGUUUGGGAGGCGGCCUAGAGAAGGDUUGAGGGGCUAAGGUGUCGA
VII	ZJ1	(1620)	UGGUGUGUUUGGGGGCGGGUUUUGAGAAGGUGUGAGGGGCUUGGGUGUUGG
	SFO2	(1620)	UAGUGUGUUUGGGAGGCGGGUUUUGGGAGGGUGUGAGGGGCUUGGGUGUUGG
	GUANGXI9	(1620)	UAGUACA GUUUAGAGAGCGGGUUUUGGGAGGGUGUGGGGGACUGGGUGUUGG
VIII	QH1	(1620)	CGAUAGGGUUUAGGAGGCAGACUGGGGGGGGGGUGGGGGACUGGGUGUCGG
IX	F48E8	(1620)	UGGUAGGGUUUGGGGAGCGUGCGUGGGGGGGGGGUGGGGGACUAAGGUGUCGG
XI	MG 725 08	(1620)	UGCGGGGUUGAGGGGGUGGGUUUGGGGGGGGUGGGGGGUGUGCGUCGG

Fig 4.1. Nucleotide sequence alignment of 5'non coding region (downstream) of NDV nucleocapsid protein gene. Gaps indicate absence of the insert sequence. Roman letters denotes the genotypes of featured class II NDV strains. Numbers in the parentheses indicate the nt position of NDV genome.

Genotype/Strain		Phosphoprotein gene coding region	
NDV Class II	I Ulster 67	(2360)	UUUGUCAGUUCUCUCUGGCGUC-----UUUGUCCAGUUCGCGGCGGGGACCUUCG
	II LaSota	(2360)	UUUGUCAGUCCUUUCUGGCGUC-----UUUGUUCAGUUCGCGGCGGGGACCUUUG
	Beaudette C	(2360)	UUUGUCGGUCCUUUCUGGCGUC-----UUUGUUCAGUUCGCGGCGGGGACCUUUG
	Texas GB	(2360)	UUUGUCGGUCCUUUCUGGCGUC-----UUUGUUCAGUUCGCGGCGGGGACCUUUG
	III Mukteswar	(2360)	UUUGUCAGUCCUCUCUAGCGU-----UUUGUCCAGUUCGCGGCGAGACCUUCG
	IV Herts 33	(2360)	CUUGUUGUCCUUUCUGGCGU-----UUUGUCCGGUUCGCGGCGGGGACCUUCG
	V Largo 71	(2366)	CUUGUUGUCCUUUUAAGGUC-----GUUGUCCGGUUCGCGGCGGGGACCUUCG
	VI Fontana	(2366)	UUUGUUGUCCUUUCUGGCGUC-----GUUGUCCGGUUUAGGCGGGGACCUUCG
	VII ZJ1	(2366)	UUCGUUGUCCUUUCUGGCGUC-----GUUGUCCGGUUCGCGGAGGGGACCUUUG
	VIII QH1	(2366)	UUCGUUGUCCUUUCUAGCGUC-----UUUGUUCGGUUCGCGGCGAGGGGACCUUCG
	IX F48E8	(2366)	UUUGUCAGUCCUCUCUGGCGUC-----UUUGUCCAGUUCGCGGCGGGGACCUUCG
NDV Class I	XI MG 725 08	(2366)	CUUGUUGUCCUCUCCAGCGCC-----UGGUGUCCGGCCUCGAGUUGGACCUUCG
	DE-R49/99	(2360)	CCGGUUGUCCUCCUCCCGGGUCACCCUCUGCCCGGUUGUCCAGCGGGUUCGGGACCUUGG
	Alaska 415	(2360)	CCGGUUGUCCUCCUCCCGGGUCACCCUCUGCCCGGUUGUCCAGCGGGUUCGGGACCUUGG
	JS10	(2360)	CCGGUUGUCCUCCUCCCGGGUCACCCUCUGCCCGGUUGUCCAGCGGGUUCGGGACCUUGG
	NDV08-046	(2360)	CCGGUUGUCCUCCUCCCGGGUCACCCUCUGCCCGGUUGUCCAGCGGGUUCGGGACCUUGG
	JX07	(2360)	CCGGUUGUCCUCCUCCCGGGUCACCCUCUGCCCGGUUGUCCAGCGGGUUCGGGACCUUGG
	ZJ 10-03	(2360)	CCGGUUGUCCUCCUCCCGGGUCACCCUCUGCCCGGUUGUCCAGCGGGUUCGGGACCUUGG
D-AH 10-02	(2360)	UCAGUUGUCCUCCUCCCGAGUUACCCUCUGCCCGGUUGUCCAGCGGGUUCGGGACCUUGG	

Fig 4.2. Nucleotide sequence alignment of coding region of NDV phosphoprotein gene. Gaps indicate absence of the 12-nt insert sequence. Roman letters denotes the genotypes of featured class II NDV strains. Numbers in the parentheses indicate the nt position of NDV genome.

4.4.6 Construction of BC minigenome system:

BC minigenome CAT reporter assay was developed to determine the effect of 12 nt (4 aa) insert sequence in the P protein. The reporter chloramphenicol acetyl transferase (CAT) open reading frame was combined with the 5' and 3' end sequences of BC genome using overlap PCR. BC 5'-genome end of 265 nt, that included 114 nt trailer and 151 nt of terminal part of L gene containing 5'UTR and last 42 nt of coding region. BC 3'-genome end of 221 nt, that included leader and complete N gene 3'UTR. Briefly, three PCR products were generated using the primer sets given in table 4. Each PCR product had 15 nt overlap sequence with the other product and all three fragments were joined using two rounds of overlap PCR reaction. The orientation of the trailer, CAT ORF and the leader was maintained in reverse.

Table 4.4. Primers used for BC minigenome construction

Primer Name	Primers for BC minigenome
BC-MG trailer F	5'-AGTTGGCGCGCCTAATACGACTCACTATAGGG ACCAAACAAAGATTTGGTGAATAACAA
BC-MG trailer R	5'-GGCAATGCAGTCAAAGGATAT
BC-MG CAT gene F	5'-TTTGACTGCATTGCCTTACGCCCCGCCCTGCCACTCATCG 5'-AAGCCTTCTGCCAACATGGAGAAAAAAT
BC-MG CAT gene R	CACTGGATATA
BC-MG leader F	5'-GTTGGCAGAAGGCTTTCTCGA 5'-GGTCCGGACCGCGAGGAGGTGGAGATGCCATGCCG
BC-MG leader R	ACCCACCAAACAGAGAATCCGTAA

Primer sequences of BC support plasmids for minigenome	
BC-NP HindIII F	5'-GCTGACAAGCTTATGTCTTCCGTATTTGACGAG
BC-NP EcoRI R	5'-GACGTTGAAATTCTCAATACCCCCAGTCGGTGTC
BC-P HindIII F	5'-GCTGACAAGCTTATGGCCACCTTTACAGATGCG
BC-P EcoRI R	5'-GACGTTGAAATTCTTAGCCATTTAGTGCAAGGCG
BC-L1 NheI F	5'-ATCTTAGCTAGCATGGCGAGCTCCGGTCCTGAA
BC-L1 NheI R	5'-CTCATCGCTAGCATACTCTTGGTCAACGATATG
BC-L2 NheI F	5'-GAGTATGCTAGCGATGAGTCAACTGTCTTTTAA
BC-L2 NotI R	5'-ATCTTAGCGGCCGCTTAAGAGTCACAGTTACTGTAATATCC

4.4.7 Virus growth kinetics and plaque morphology:

The growth kinetics of rBC and rGBT and their mutant viruses were determined under multiple-cycle growth conditions in DF-1 cells. The virus was inoculated at a multiplicity of infection (MOI) of 0.01 into DF-1 cells grown in DMEM with 10% FBS at 37°C. The supernatant was collected at 8-h intervals until 64 h postinfection (p.i.). The virus content in the samples was quantitated by Tissue culture infective dose 50 (TCID₅₀) in DF-1 cells using the method of Reed and Muench (1938). Plaque assay was performed to determine the plaque morphology. Briefly, supernatants collected from virus-inoculated samples earlier, were serially diluted, and 100 µl of each serial dilution was added per well of confluent DF-1 cells in 12-well plates. After 60 min of adsorption, cells were overlaid with DMEM (containing 2% FBS and 0.81% methylcellulose) and then incubated at 37°C for 4 days. The cells were then fixed with absolute methanol and stained with 1% crystal violet for plaques.

4.4.8 Pathogenicity studies in chickens:

To test the pathogenicity of the recovered viruses *in vivo*, an intracerebral pathogenicity index (ICPI) test was performed according to standard procedures (Alexander, 1989). For ICPI, 10^{-1} dilution of 2^9 HA units of each virus/chicken was inoculated intracerebrally into groups of 10 one-day-old SPF chicks. Inoculation was performed with a 27-gauge needle attached to a 1-ml stepper syringe dispenser that was set to dispense 0.05 ml of inoculum per inoculation. The birds were inoculated by inserting the needle up to the hub into the right or left rear quadrant of the cranium. The birds were observed for clinical signs and mortality once every 12 h for 8 days. ICPI values were calculated as described by Alexander, 1989. Briefly, the birds were scored daily: 0 if normal, 1 if sick, and 2 if dead. The ICPI value was the mean score per bird per observation. Highly virulent (velogenic) viruses give values approaching 2; avirulent (lentogenic) viruses give values close to 0.

4.4.9 Pathogenicity in 2-week-old chickens, virus titration:

Groups of 8 two-week-old SPF chickens were inoculated with 0.2 ml of 10^6 PFU of each virus by the oculonasal route. The birds were observed daily and scored for clinical signs for 14 DPI. Three birds from each group were euthanized at 3 DPI and lung, trachea, spleen, brain and intestine were collected. For virus titration, the tissue samples were homogenized, and the supernatant was serially diluted and used to infect DF-1 cells, with duplicate wells per dilution. Infected wells were identified by HA assay of the supernatant, and the $TCID_{50}/g$ of tissue was calculated using the method of Reed and Muench (1938).

4.4.10 Scoring analysis of clinical signs and mortality:

The remaining 10 chickens in each virus group were observed for clinical signs of disease until 14 days post infection (DPI). The chickens in each virus group was clinically scored; 0 for normal, 1 for sick, 2 for paralysis/twitching/wing drop, 3 for prostration, and 4 for death, with scores taken daily until 14 DPI. A mean score per virus group per day was generated for comparison. The mortality pattern was observed daily for the entire 14 days to generate the survival pattern of chickens among the virus groups.

4.5 Results

4.5.1 Virus construction and recovery:

The recombinant viruses were rescued using recombinant MVA-T7 rescue procedure as described in materials and methods section 4.4.4 and designated rGBT and rBC for the parental viruses, rGBT-N6 and rBC-N6 for the 15,192 nt genome length mutant viruses, and rGBT-P12 and rBC-P12 for the 15,198 nt genome length mutant viruses (Fig 4.3). The parental recombinant and genome length mutant viruses were serially passaged 5 times in 9-day-old embryonated chicken eggs and 5 times in chicken embryo fibroblast DF-1 cells and the stability of the created restriction enzyme sites and insert sequences were confirmed by sequence analysis. All infectious virus research was performed in a USDA approved enhanced biosafety level 3 (BSL-3+) containment facility. It should be also noted that this study reports the first rescue of a highly virulent NDV strain of U.S. origin (strain Texas GB).

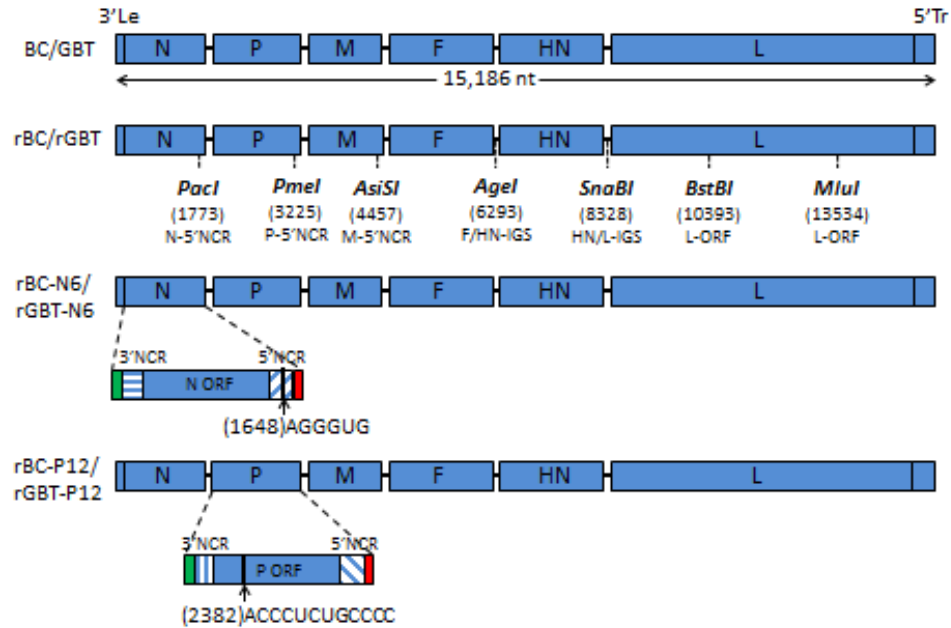


Fig 4.3. Gene map and cloning strategy for full-length cDNAs of NDV strains GBT (rGBT) and BC (rBC). The top diagram shows the gene map. Le and Tr are the leader and trailer sequences, respectively. The second diagram shows the sequence positions of restriction enzyme (RE) site used in the cloning (except *AgeI* site, which occurred naturally in both strains, all other RE sites were introduced by making identical nt changes in both strains). The last two diagrams show the sequences and positions of the 6 nt and 12 nt inserts in rGBT and rBC to generate genome lengths of 15,192 nt (rGBT-N6 and rBC-N6) and 15,198 nt (rGBT-P12 and rBC-P12).

4.5.2 Multicycle growth kinetics and plaque morphology:

The multicycle growth kinetics and plaque morphology of the genome length mutant viruses were evaluated *in vitro* in DF-1 cells using standard procedures (Yan and Samal, 2008). The biological and recombinant versions of the parental GBT strain (wtGBT and rGBT, respectively) had similar growth patterns and plaque morphologies (data not shown). Compared with the parental viruses, rGBT-N6 and rGBT-P12 showed similar titers in DF-1 cells throughout the course of 64 h post infection (Fig 4.4). Similarly, rBC group mutant viruses also showed similar growth pattern compared to that of the parental recombinant. But the growth kinetics of these viruses was much lower

than those of rGBT viruses. The peak virus titers of rBC viruses were around 32 h but in rGBT viruses it was around 24 h (Fig 4.4).

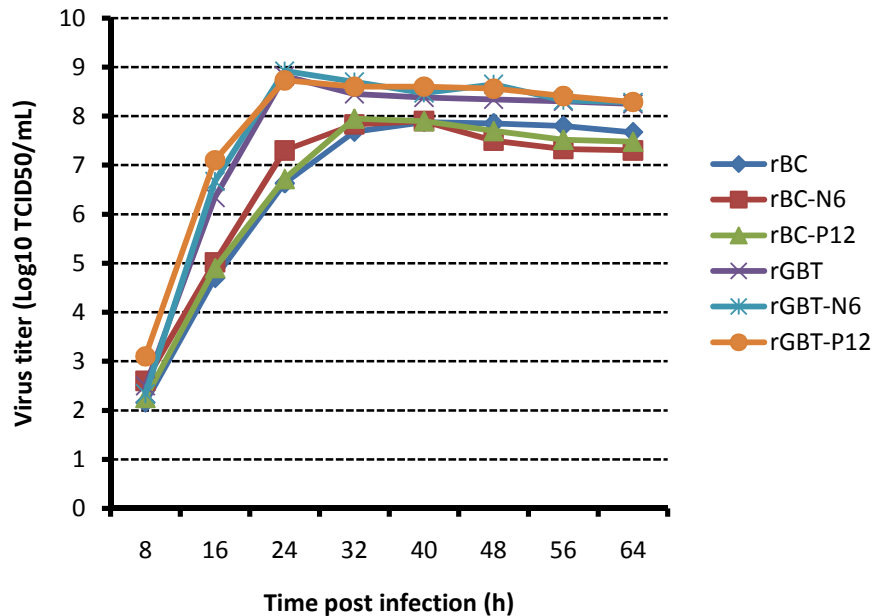


Fig 4.4. Multicycle growth kinetics in DF-1 cells of biological and recombinant GBT and BC in parallel with their genome length mutants. Cells were infected at a multiplicity of infection of 0.01, supernatant samples were collected at 8 h intervals until 64 h post infection, and virus titers were determined by TCID50 calculations as following the method of Reed and Muench (1938).

The plaque size of the viruses was compared by measuring the diameters of 20 randomly picked plaques (Fig 4.5). The mean plaque diameters (in mm) of rBC group of viruses were smaller compared to the rGBT viruses: 1.83 ± 0.12 , 1.76 ± 0.1 and 1.87 ± 0.15 for rBC, rBC-N6 and rBC-P12, respectively; 2.82 ± 0.16 , 2.91 ± 0.19 and 2.87 ± 0.17 for rGBT, rGBT-N6, and rGBT-P12, respectively (Fig 4.5). The plaque sizes indicates no significant differences among the mutant viruses and their respective parental recombinant viruses.

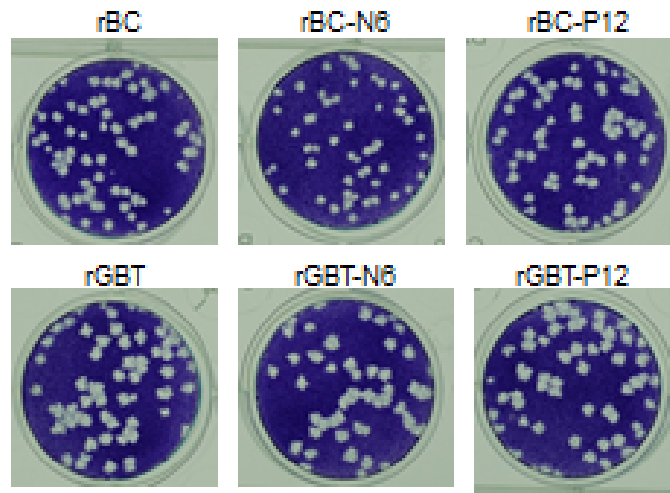


Fig 4.5. Plaque morphology of rBC parental and mutant viruses of strain GBT and BC. Note the larger plaque size of GBT viruses compared to BC viruses with their mutants viruses also similar to the recombinant parental virus plaques.

4.5.3 Nucleocapsid protein expression and virus spread:

To determine the influence of 6 nt expression in N protein expression and virus growth we performed western blot analysis for N protein expression and flowcytometry analysis to determine percentage infected cells 24 h post infection of 0.01 moi of rBC group viruses. N protein expression as the function of band intensity ratio recorded for rBC, rBC-N6 and rBC-P12 were 1.0, 0.9881 and 0.9699, respectively (Fig 4.6a) from DF-1 cell lysates, 24 h post infection of 0.01 moi of viruses. N protein peptide antibody was used to detect the N protein band in the western blot. Protein concentration in the DF-1 cell lysate was determined using Coomassie Plus (Bradford) protein assay reagent kit (Thermo Scientific) and 10 μ g of protein content of the lysate was loaded in each well for running the SDS-PAGE. In flowcytometry analysis, 41.3%, 40.6% and 42.4% of infected cells were recorded for rBC, rBC-N6 and rBC-P12 virus groups when the DF-1 cells were processed after 24 h post infection. The multiplicity of 0.01 moi of viruses was

used for infection. HN monoclonal antibody was used as the primary antibody to detect the virus infected cells in flowcytometry (Fig 4.6b). All the three groups of viruses showed a 40-42% range of infection, indicating that the foreign nt insertion did not affect the virus replication.

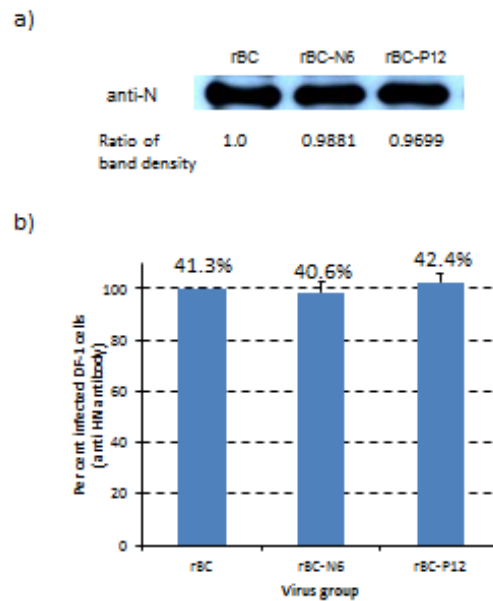


Fig 4.6. Nucleocapsid protein expression and its quantitative ratio measured in ImageJ program. 10 μ g of protein each lane, measured using Commassie Plus (Bradford) Protein assay (upper panel, a). (Lower panel, b) Flowcytometry analysis of virus growth using NDV HN monoclonal antibody to quantitate infected DF-1 cells after 24 h of 0.01 moi virus infection. Percentage of cells of rBC groups is scored 100 and rBC-N6 and rBC-P12 are calculated with respect to rBC group.

4.5.4 BC minigenome CAT reporter assay:

BC minigenome assay was used to determine the effect of 12 nt (4 aa) insert sequence in the P protein. The reporter chloramphenicol acetyl transferase (CAT) ORF was combined with the 5' and 3' end sequences of BC genome using overlap PCR. BC 5'-genome end of 265 nt, included 114 nt trailer and 151 nt of terminal part of L gene containing 5'UTR and last 42 nt of coding region. BC 3'-genome end of 221 nt, included

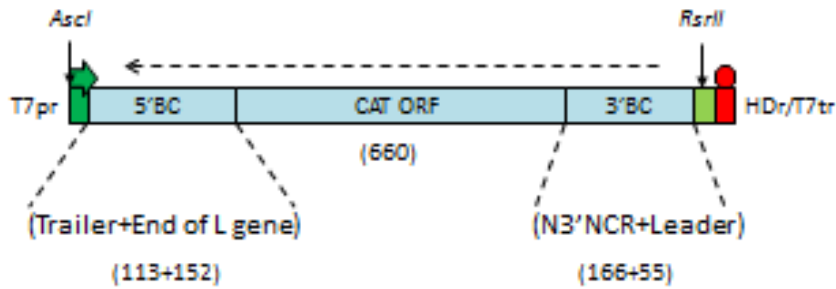
leader and complete N gene 3'UTR. The minigenome amplicon was cloned in between AscI and RsrII sites in the pBR322-HDrz vector under the promoter of T7 (Fig 7a). Expression plasmids of BC N, P, L and P with 12 nt also were cloned separately in pCDNA3.1(+) expression vector. 0.8 µg of minigenome plasmid and 0.8 µg of N, 0.6 µg of P and 0.5 µg of L plasmids were transfected using Lipofectamine 2000 reagent on to a 80% monolayer of 293T cells in 12 well cell culture plate, media was changed after 3 h, and incubated for 48 h and samples for CAT-ELISA were processed following the manufacturer's protocol. The minigenome with N, P and L recorded a value of 2.412 and with N, P4 (P12 in gene) and L recorded an OD value of 2.462 at 405 nm (Fig 4.7.b). The BC minigenome with N and P and with N and P4 (P4 denotes P protein with 4 aa insert) were kept as controls which recorded 0.186 and 0.158, respectively. This suggests that there is no significant difference in the expression of reporter CAT enzyme between wild type P protein and P protein with 4 aa insert using the minigenome system.

4.5.5. Mean death time (MDT) and Intracerebral inoculation index (ICPI):

To determine the effect of the 6 nt and 12 nt inserts on the virulence of NDV, the parental and mutant viruses were evaluated for mean death time (MDT) in 9-day-old embryonated chicken eggs and for intracerebral pathogenicity index (ICPI) in 1-day-old chicks (Alexander, 1989). The MDT and ICPI values of wild type and recombinant viruses of strains Texas GB and Beaudette C were similar, indicating that the presence of the insert sequences in the recombinant viruses did not detectably alter their pathogenicity. All four genome length mutant viruses (rGBT-N6, rGBT-P12, rBC-N6,

and rBC-P12) had MDT values similar to those of their respective parental biological and recombinant viruses (Table 4.5).

a)



b)

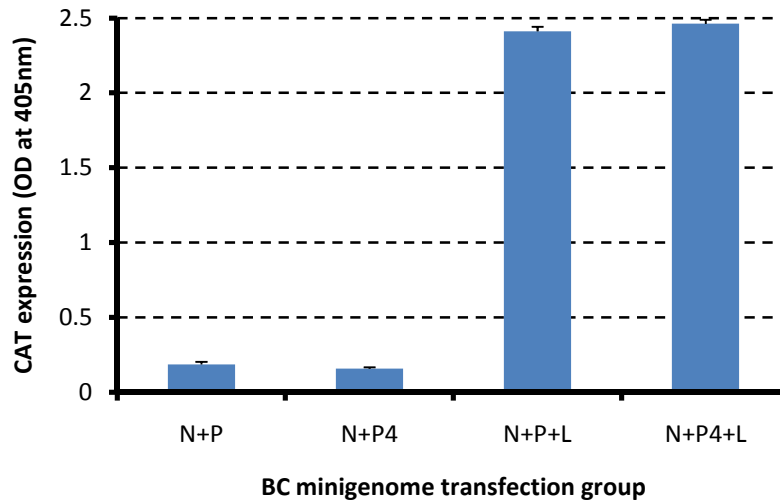


Fig 4.7. BC minigenome CAT reporter assay. a) Minigenome map of NDV strain Beaudette C (a). Arabic numerals indicate number of nucleotide residues and their sum is 1146 nt, which is multiple of six. Reverse dotted-arrow represents the orientation of the cloned 5'BC, chloramphenicol acetyl transferase (CAT) open reading frame and 3'BC sequences. HDr refers to hepatitis delta ribozyme sequence. b) BC minigenome expressing CAT enzyme quantitated by CAT-ELISA. Support plasmid L free controls (N+P and N+P4) and treatment groups (N+P+L and N+P4+L).

Table 4.5. Pathogenicity^a of genome length mutant NDV strains Texas GB and Beaudette C

Virus	MDT (h) ^b	ICPI score ^c
wtGBT	48	1.90
rGBT	47	1.88
rGBT-N6	46	1.90
rGBT-P12	47	1.86
wtBC	60	1.60
rBC	59	1.58
rBC-N6	58	1.54
rBC-P12	61	1.52

^aThe virulence of the parental and mutant Texas GB (GBT) and Beaudette C (BC) viruses was evaluated by mean embryo death time (MDT) in 9-day-old chicken embryos and intracerebral pathogenicity index (ICPI) in 1-day-old chicks. The values are the means of three independent experiments.

^bPathotype definitions by MDT: >90 h, lentogenic; 60 to 90 h, mesogenic; <60 h, velogenic.

^cPathotype definitions by ICPI: velogenic strains approach the maximum score of 2.00, whereas lentogenic strains give values close to 0.

In the ICPI test, highly virulent strains characteristically have values approaching 2.0. The ICPI values of wtGBT, rGBT, rGBT-N6 and rGBT-P12 were 1.9, 1.88, 1.90 and 1.86, respectively (Table 4.5). Similarly, the ICPI values of wtBC, rBC, rBC-N6 and rBC-P12 were 1.60, 1.58, 1.54 and 1.52 respectively (Table 4.5). These results indicate that the 6 and 12 nt insert mutant behaved in a similar way or slightly attenuated compared to the parental recombinant viruses.

4.5.6 Virus replication and pathogenesis in two-week-old chickens:

To study the pathogenesis of these viruses under conditions modeling natural infection, groups of eight two-week-old chickens were infected by the oculonasal route with 200 µL of PBS containing 1.0×10^6 PFU of each recombinant virus per bird. Three

days later, 3 birds from each group were sacrificed and virus titers in the trachea, lungs, spleen, intestine and brain were determined (Fig 4.8).

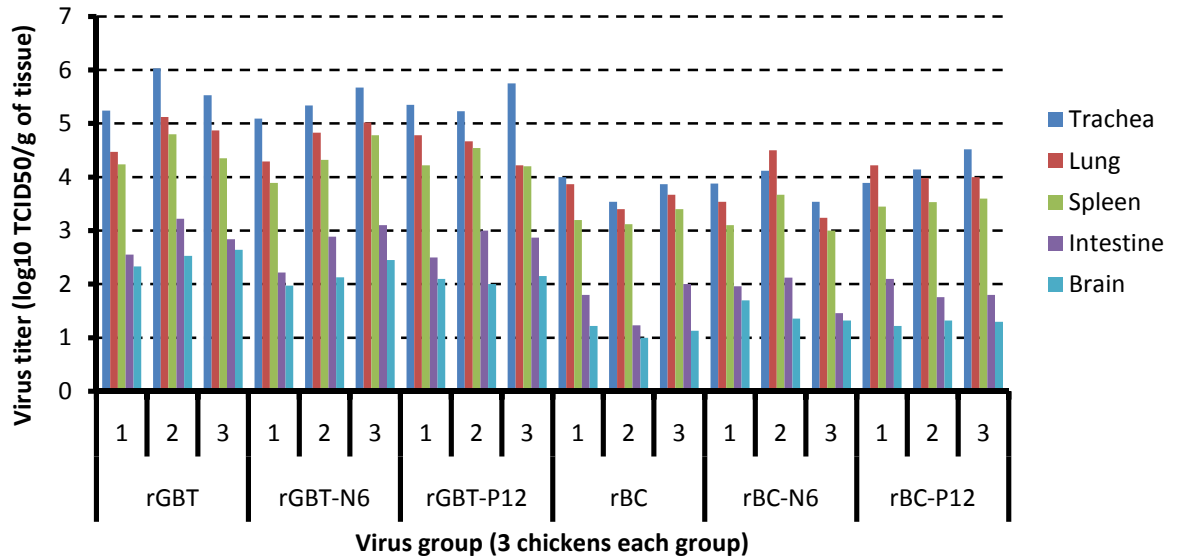


Fig 4.8. Replication and pathogenesis of genome length mutants of NDV strains GBT and BC in 2-week-old chickens following oculonasal inoculation. Virus titers in the trachea, lungs, spleen, intestine and brain of 3 chickens from each indicated virus group on day 3 post infection. The chickens in each group are numbered 1-3.

Chickens infected with rGBT, rGBT-N6, or rGBT-P12 had high virus titers in all five organs compared to the rBC, rBC-N6 and rBC-P12 (Fig 4.8).

4.5.7 Clinical disease scoring and mortality pattern in two-week-old chickens:

The remaining 5 chickens in each virus group were observed for clinical signs of disease until 14 days post infection (DPI). In the rGBT, rGBT-N6 and rGBT-P12 groups, mild clinical signs were first observed on 3 DPI and the chickens were severely paralytic on 4 DPI. All five chickens died on day 5 in rGBT, rGBT-N6 and rGBT-P12 groups (Fig 4.9).

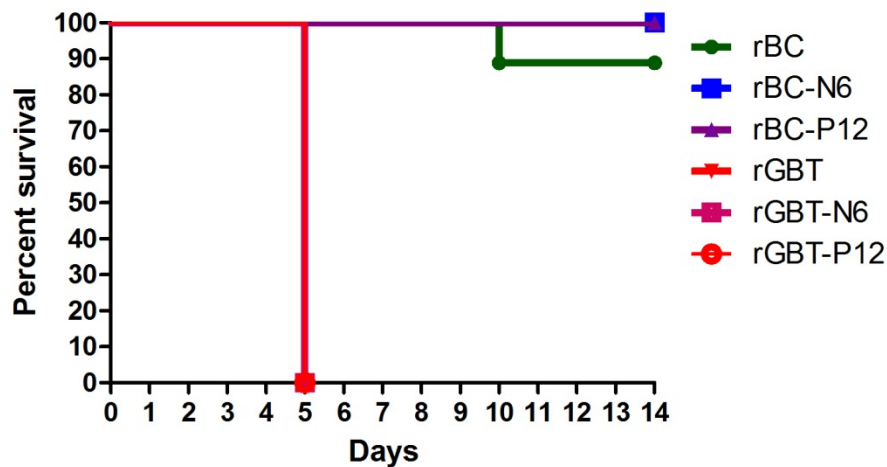


Fig 4.9. Percent survival curve based on 5 chickens in each virus group. Note that all 5 chickens in the rGBT, rGBT-N6 and rGBT-P12 groups died on day 5. In contrast, all 5 chickens in the rBC, rBC-N6 and rBC-P12 groups survived till day 14, while 1 chicken died on day 10 in rBC group.

In the case of the BC virus groups, all of the chickens survived the 14 day experiment except for 1 chicken in the rBC group that died on day 10. The chickens in the BC virus groups were clinically scored 0 for normal, 1 for sick, 2 for paralysis/twitching/wing drop, 3 for prostration, and 4 for death, with scores taken daily until 14 DPI. A mean score per virus group per day was generated for comparison (Fig 4.10).

Chickens in the rBC group first showed clinical signs on 6 DPI, which increased in severity till 11 DPI and then decreased during recovery. In the rBC group, chickens were severely sick, with scores substantially exceeding those of the rBC-N6 and rBC-P12. One chicken died on 10 DPI, as noted, while the others began to recover 12 DPI.

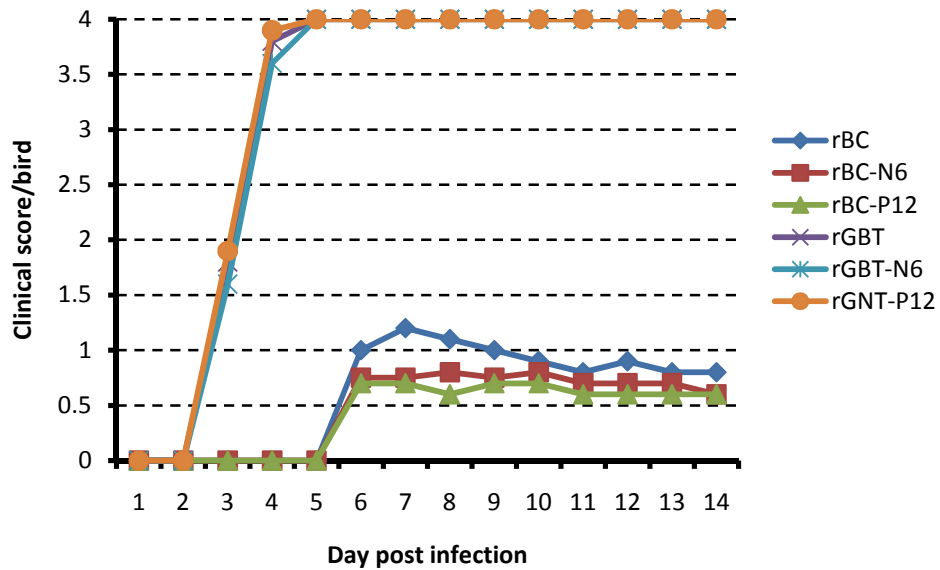


Fig 4.10. Clinical scoring of chickens from day 6 through day 14 after oculonasal infection with rBC and rGBT with their N6 and P12 viruses. Chickens (5 birds per group) were scored as 0, normal; 1, sick; 2, paralysis/twitching/wing drop; 3, prostration; and 4, death. The mean scores per group per day are shown.

4.6 Discussion

Newcastle disease virus (NDV) is an important disease in poultry. The presence of numerous genotypes with varying virulence phenotypes raises the question of the importance of the naturally occurring nt insertions in the genome. NDV has three genome categories, namely, 15,186, 15,192 and 15,198 nt. The increase in the nt lengths were due to the 6-nt and 12-nt inserts that occur naturally in, respectively, the 5' (downstream) non-coding region of the N gene and in the coding sequence for the P and V proteins. The 6 nt insert in the 5' noncoding region of the N gene of strains GBT and BC resulted in a similar growth characteristics and viral titers in DF-1 cells, as well as similar plaque

sizes. *In vivo*, the 6 nt insert in the N gene and the 12 nt insert in P gene were associated with slightly decreased virulence. In 1-day old chicks, both the rGBT and rBC viruses showed increased virulence compared to their mutant viruses.

The origin of these inserts in nature is unknown, although they appear to have been introduced after 1960 and then maintained with some sequence variation (Czegledi *et al.*, 2006; Huang *et al.*, 2004; Liu *et al.*, 2010; Seal *et al.*, 2005; Ujvari, 2006). Possible mechanisms of introduction include polymerase stuttering and RNA recombination. Each insert conforms to the “rule of six” (Calain and Roux, 1993; Kolakofsky *et al.*, 1998).

In the case of the 12 nt insert in coding sequence for the P and V proteins, the addition of 4 amino acids in the middle of the P and V proteins had no substantial effect on CAT expression indicating that the 4 aa insert “WETG” did not affect the function of the P protein. The maintenance of these inserts in nature suggests that they have adaptive value. One more possibility is that, the reduced replication and virulence of these insert mutants in DF-1 cells and in natural host chickens, implies that these viral inserts are foreign and is not related to replication and virulence of rBC and rGBT.

The virus strains which contain these inserts differ greatly in nt identities of viruses with the genome size of 15,186 nt. BC and GBT share 99.1% at the genome level. The nt identities of BC and GBT with other NDV strains in class II genotypes are I-Ulster (90.4, 90.7), II (BC and GBT are this genotype)-LaSota (97.0, 97.5), III-Mukteswar (87.9, 88.1) and IV-Herts 33(88.0, 88.3). BC and GBT share nt % identities with NDV class II strains that rose lately and possess the additional 6 nt, are genotype V-Largo (85.4, 85.6), VI-Fontana (85.5, 85.7), VII-ZJ1 (83.4, 83.6), VIII-QH1 (84.5, 84.6), IX-F48E8 (87.9, 88.1), XI-MG 725 08 (82.6, 82.8). And BC and GBT share nt % identities

of class I viruses, Alaska 415 (72.6, 72.6) and JS10 (72.5, 72.5). These genome nt identities indicate that NDV class II, genotype II viruses like BC and GBT are very distantly related with the class I viruses with 72 % nt. Compared within class II viruses, BC and GBT are closely related to genotypes I, III, IV to 88-97%, these strains are of genome length 15,186 nt, like that of BC and GBT. The Class II NDV genotypes V, VI, VII, VIII and XI except for genotype IX (88%), share a very low range of 82-85 % nt identity and also the fact that these strains are isolated from hosts like, chickens, geese, parrots, pigeons and/or mixed species, indicate the possibility that the viruses with these inserts could have evolved independently. In future, deletion studies involving the 6 and 12 nt inserts from their native viruses of longer genome lengths would shed more light on their importance.

Chapter 5

5.1 Title

The role of envelope associated protein genes in Newcastle disease virus virulence and pathogenesis

5.2 Abstract

Newcastle disease virus (NDV) is the most important disease of poultry worldwide. To study the virulence of NDV envelope associated protein genes were reciprocally exchanged between a highly virulent strain Texas GB (GBT) and a moderately virulent strain Beaudette C (BC), both strains have identical F protein cleavage site sequence $^{112}\text{RRQKR}\downarrow\text{F}^{117}$ and are phylogenetically closely related. The F gene of GBT increased replication, plaque size and fusogenicity of the recombinant BC (rBC) virus *in vitro*. It increased the virulence of rBC from 1.61 to 1.88 in intracerebral pathogenicity index in one-day-old chicks and resulted in the mortality of two-week-old chickens from 0% to 100% indicating that the fusion protein gene is the major determinant of virulence in NDV.

5.3 Introduction

Newcastle disease (ND) is the most important poultry disease in the world and accounts for huge economic losses (Alexander, 2000; Samal, 2011). The causative agent Newcastle disease virus (NDV) can infect a majority of the avian species in the class *Aves* resulting in isolation of numerous virus strains across domestic, wild birds and

waterfowl worldwide. NDV strains show a spectrum of virulence. Historically, NDV is considered as primarily a disease of chickens and based on the virulence in chickens, it has been grouped into lentogenic, mesogenic and velogenic strains representing, low-, moderate- and high-virulence strains, respectively (Alexander, 1989, 2000). Velogenic viruses establish a systemic infection leading to a respiratory and enteric disease (viscerotropism) or neurological disease (neurotropism).

NDV is a large pleomorphic enveloped virus containing single-stranded negative-sense RNA genome. NDV belongs to the genus *Avulavirus* under the subfamily *Paramyxovirinae* in the family *Paramyxoviridae* (Mayo, 2002; Lamb and Parks, 2007). There are at least three genome size categories identified that have nucleotide (nt) lengths of 15,186, 15,192 and 15,198 nt (Krishnamurthy and Samal, 1998; Romer oberdorfer, 1999; Huang *et al.*, 2004; Miller *et al.*, 2010; Czegledi *et al.*, 2006;Ujvari, 2006). The genome contains six genes flanked by extra-genic sequences required for replication in the order of 3'leader-N-P-M-F-HN-L-5'trailer. The genes code for Nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN) and large polymerase protein (L). P gene can also code for an additional protein called V protein using a co-transcriptional mechanism known as RNA editing (Lie *et al.*, 2010; Seal *et al.*, 2005; Czegledi *et al.*, 2006).

Determination of the virulence factors for NDV is very important since there are numerous viruses isolated around the world that vary greatly in virulence. NDV virulence determinants are multigenic but multibasic amino acids in the fusion protein cleavage site is considered as the most important factor for virulence (Huang *et al.*, 2004). The presence of a multibasic furin protease cleavage site helps the virus to spread

systemically and cause disease. The viruses that have monobasic cleavage site get restricted to upper respiratory tract and intestine where they can be cleavage activated by exogenous trypsin-like proteases. But this is true only with viruses with a same natural host. Modification of cleavage site sequences from other avian paramyxoviruses does not increase their virulence in chickens. HN protein is responsible for the attachment to host cells and that determines tropism of the virus. V protein is known to be involved in antagonizing antiviral defense by binding to and targeting STAT1 to proteasomal degradation. Large polymerase (L) protein is the driving force behind transcription and replication that determines virus growth and spread (Rout and Samal, 2008; Dortmans *et al.*, 2010).

In this study, we determine the role of membrane associated protein genes, M, F and HN of NDV in pathogenesis and virulence. Two chicken origin viruses with identical F protein cleavage site sequences, but differing in pathotypes, have been generated using reverse genetics; NDV strains Beaudette C (BC), a moderately virulent virus and Texas GB (GBT), a highly virulent virus with pronounced neurotic clinical signs. BC is associated with clinical illness in young chickens and not usually fatal but GBT is associated with 100% mortality and is used as a standard challenge virus in the US. Both these viruses belong to the genotype II of class II NDV strains that have identical genome length of 15,186 nt. The reciprocal exchange of membrane associated protein genes between strains BC and GBT and natural infection in their natural host chickens would give a better understanding of the virulence determinant of NDV.

5.4. Materials and methods

5.4.1 Cells and viruses.

A chicken embryo fibroblast cell line (DF1), African green monkey kidney cell line (Vero) and a human epidermoid carcinoma cell line (HEp-2) were grown in Dulbecco's minimal essential medium (DMEM) with 10% fetal bovine serum (FBS) and maintained in DMEM with 2% FBS. The modified vaccinia virus strain Ankara (MVA) expressing T7 RNA polymerase was kindly provided by Dr. Bernard Moss (NIH, Bethesda, MD) and propagated in primary chicken embryo fibroblast cells in DMEM with 2% FBS. The moderately pathogenic (mesogenic) NDV strain BC and highly pathogenic (velogenic) NDV strain GBT and its recombinant derivatives were grown in 9-day-old embryonated specific-pathogen-free (SPF) chicken eggs in an enhanced biosafety level 3 (BSL-3+) containment facility certified by the USDA following the guidelines of the IACUC, University of Maryland.

5.4.2 Plasmid construction, transfection, virus rescue and sequence analysis:

The construction of plasmid pBC and pGBT, carrying the full-length antigenome cDNA of NDV strains BC and GBT using identical restriction enzymes has been described in material and methods section of chapter 4. In the present study, M, F and HN genes were reciprocally swapped between the pBC and pGBT using RE sites, PmeI and AsisI, AsiSI and AgeI and AgeI and SnaBI, respectively. PCR products of M, F and HN genes were generated using *Pfx* polymerase and used for cloning into the full length cDNA. Lipofectamine 2000 reagent was used for transfecting HEp-2 cells with the respective full length cDNA clone with three support plasmids made of N, P and L

proteins of NDV strain GBT in pGEM-7Z(+). MVA-T7 used as the source of T7 polymerase. The plasmid constructs were screened for the appropriate gene-swaps by sequencing using gene specific primers. The recovered gene-swap mutant viruses were subjected to five passages in 9-day-old SPF chicken embryos. From the fifth passage, total RNA was isolated using RNeasy RNA purification kit (Invitrogen). Gene specific primers were used to confirm the entire sequence of the gene and the flanking regions of the backbone to confirm the gene swap virus.

5.4.3 Virus growth kinetics and plaque morphology:

The growth kinetics of rBC and rGBT and their mutant viruses were determined under multiple-cycle growth conditions in DF-1 cells. The virus was inoculated at a multiplicity of infection (MOI) of 0.01 into DF-1 cells grown in DMEM with 10% FBS at 37°C. The supernatant was collected at 8-h intervals until 64 h postinfection (p.i.). The virus content in the samples was quantitated by Tissue culture infective dose 50 (TCID₅₀) in DF-1 cells using the method of Reed and Muench (1938). Plaque assay was performed to determine the plaque morphology, briefly, supernatants, collected from virus-inoculated samples earlier, were serially diluted, and 100 µl of each serial dilution was added per well of confluent DF-1 cells in 12-well plates. After 60 min of adsorption, cells were overlaid with DMEM (containing 2% FBS and 0.81% methylcellulose) and then incubated at 37°C for 4 days. The cells were then fixed with absolute methanol and stained with 1% crystal violet for plaques.

5.4.5 Fusion index assay:

The fusogenic abilities of the recombinant viruses were examined as described by Kohn. Virus was inoculated into confluent (10^6 cells/ml) Vero cells in 6-well plates at an MOI of 0.1. Cells were maintained in 2% DMEM at 37°C under 5% CO₂. At 36 h post infection, the medium was removed and cells were fixed with methanol for 20 min at room temperature. Cells were stained with hematoxylin-eosin (Hema 3). Fusion was quantitated by expressing the fusion index as the ratio of the total number of nuclei to the number of cells in which these nuclei were observed (i.e., the mean number of nuclei per cell). The fusion index values of all viruses were expressed as percentages of these values for the parental recombinant viruses rBC and rGBT, which were considered to be 100%.

5.4.6 Pathogenicity studies in chickens:

To test the pathogenicity of the recovered viruses *in vivo*, an intracerebral pathogenicity index (ICPI) test was performed according to standard procedures (Alexander, 1989). For ICPI, 10^{-1} dilution of 2^9 HA units of each virus/chicken was inoculated intracerebrally into groups of 10 one-day-old SPF chicks. Inoculation was performed with a 27-gauge needle attached to a 1-ml stepper syringe dispenser that was set to dispense 0.05 ml of inoculum per inoculation. The birds were inoculated by inserting the needle up to the hub into the right or left rear quadrant of the cranium. The birds were observed for clinical signs and mortality once every 12 h for 8 days. ICPI values were calculated as described by Alexander (1989). Briefly, the birds were scored daily: 0 if normal, 1 if sick, and 2 if dead. The ICPI value was the mean score per bird per

observation. Highly virulent (velogenic) viruses give values approaching 2; avirulent (lentogenic) viruses give values close to 0.

5.4.7 Pathogenicity in 2-week-old chickens, virus titration, histopathology:

Groups of 12 two-week-old SPF chickens were inoculated with 0.2 ml of 10^6 PFU of each virus by the oculonasal route. The birds were observed daily and scored for clinical signs for 14 DPI. Two birds from each group were euthanized 3 DPI and lung, trachea, spleen, brain and intestine were collected in two parts. One part was used virus titration and other part was fixed in 10% buffered formalin for histopathology. For virus titration, the tissue samples were homogenized, and the supernatant was serially diluted and used to infect DF-1 cells, with duplicate wells per dilution. Infected wells were identified by HA assay of the supernatant, and the TCID₅₀/g of tissue was calculated using the method of Reed and Muench (1938). For histopathology, tissue samples collected on 3 DPI were fixed in 10% neutral buffered formalin. The fixed tissues were sectioned and stained with Hematoxylin and Eosin (H&E) (Histoserv Inc, Germantown, MD, US). The histopathology of the tissue sections were evaluated by a certified veterinary pathologist, Dr. Heather Shive PhD, DACVP, for inflammation scores and photomicrographs of specific lesions.

5.4.8 Scoring analysis of clinical signs and mortality:

The remaining 10 chickens in each virus group were observed for clinical signs of disease until 14 days post infection (DPI). The chickens in each virus group was clinically scored; 0 for normal, 1 for sick, 2 for paralysis/twitching/wing drop, 3 for

prostration, and 4 for death, with scores taken daily until 14 DPI. A mean score per virus group per day was generated for comparison. The mortality pattern was observed daily for the entire 14 days to generate the survival pattern of chickens among the virus groups.

5.5 Results

5.5.1 Recovery of infectious M, F, HN gene-swap viruses of rBC and rGBT:

Forty eight hours after the transfection, cell culture supernatant was collected to inoculate 9-day-old embryonated chicken eggs. After 2 days, the virus replication was detected by HA assay. The recombinant viruses yielded HA titers between 2^6 and 2^9 . The support plasmids, pN, pP and pL from GBT were used for the rescue of all viruses. Subsequent passages in eggs, the virus yield was about 2^9 HA units consistently and plaque assay was performed. The infective allantoic fluids with 2^8 - 2^9 PFU/mL were used as a preliminary viral stock. Part of this material was used to isolate viral genomic RNA, which was subjected to RT-PCR and complete gene sequencing. In the F swap mutant viruses, both F and HN gene sequences were confirmed, showing that the reciprocal gene-swap viruses were stable without any adventitious mutations (Fig 5.1).

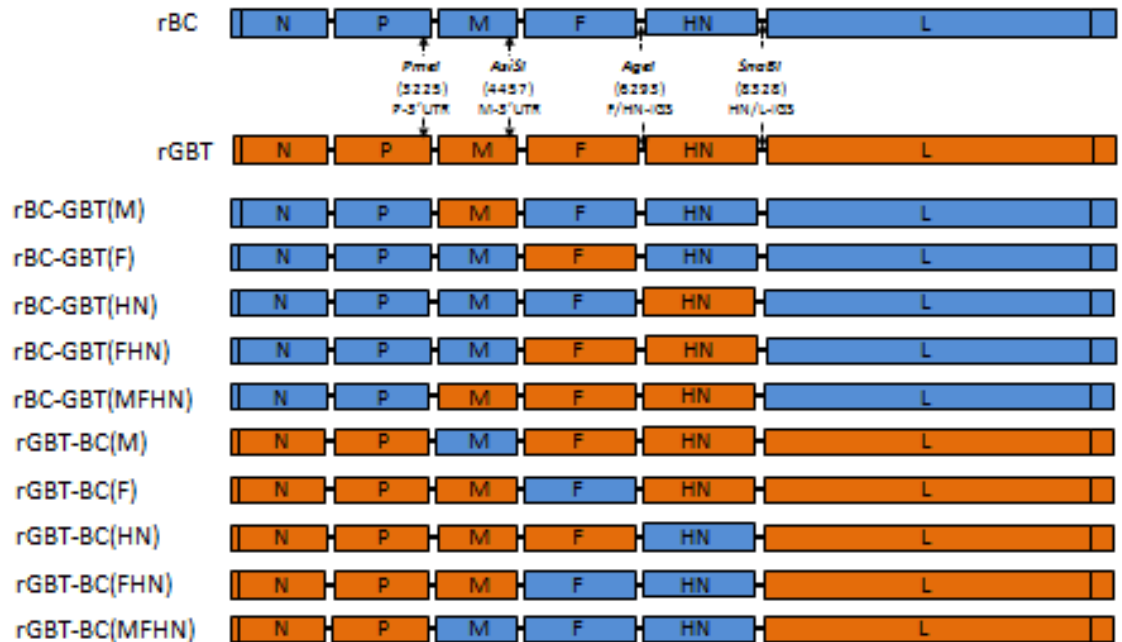


Fig 5.1. Gene map and cloning strategy for M, F and HN genes between NDV strains BC and GBT. The top diagram shows the gene map of rBC and rGBT and the restriction enzyme sites available for cloning of M (Pme I and AsiSI), F(AsiSI and AgeI) and HN (AgeI and SnaBI) genes. Last 10 gene maps show reciprocal exchange of M, F, HN, FHN and MFHN between the rBC and rGBT

5.5.2 Growth kinetics and plaque morphology of recombinant viruses:

The growth pattern of rBC and rGBT and their mutant viruses were determined in DF-1 cells at a moi of 0.01. Between the parental viruses, rGBT showed early initiation of cytopathic effects (cpe) and higher growth than rBC. rBC-GBT(FHN) showed increased growth compared to, rBC-GBT(F). At 24 h post infection rBC-GBT(F) and rBC-GBT(FHN) recorded 7.8×10^7 and 8.56×10^8 TCID₅₀/mL, whereas rBC recorded below 6.7×10^6 TCID₅₀/mL and rBC-GBT(MFHN) showed reduced growth kinetics compared to rBC-GBT(F) and rBC-GBT(FHN) throughout 64 h post infection. rGBT-BC(F), rGBT-BC(FHN), rGBT-(MFHN) showed lower titers compared to rGBT (Fig 5.2, 5.3).

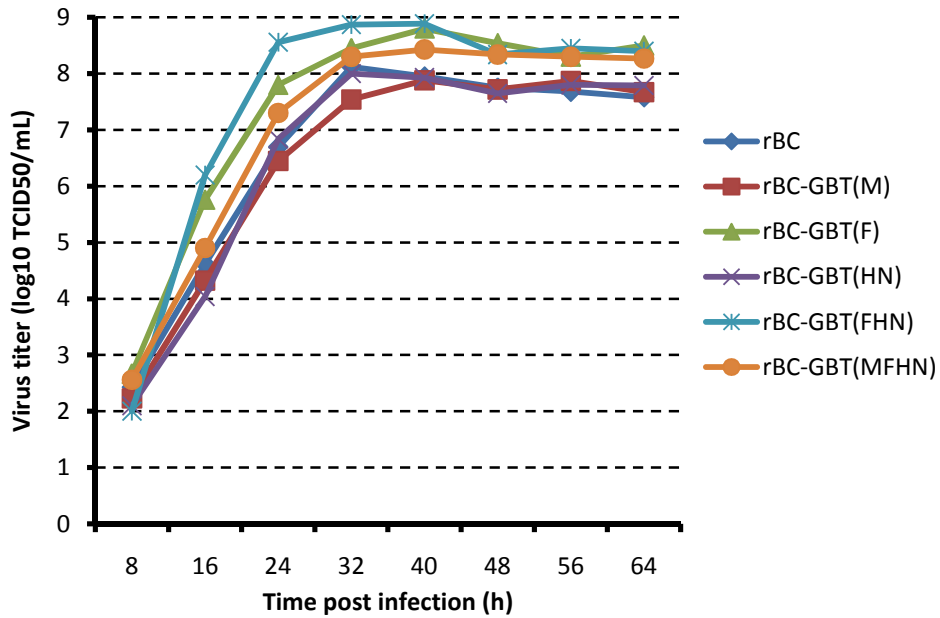


Fig 5.2. Multicycle growth kinetics in DF-1 cells of recombinant BC with the GBT envelope protein gene-swap mutants. Cells were infected at a multiplicity of infection of 0.01, supernatant samples were collected at 8 h intervals until 64 h post infection, and virus titers were determined by TCID₅₀ calculation using the method of Reed and Muench (1938).

Plaque sizes of rBC group viruses were 1.55 ± 0.06 , 1.54 ± 0.12 , 2.08 ± 0.18 , 1.71 ± 0.13 , 1.85 ± 0.13 and 2.32 ± 0.32 mm in diameter for rBC, rBC-GBT(M), rBC-GBT(F), rBC-GBT(HN), rBC-GBT(FHN) and rBC-GBT(MFHN). rGBT group of viruses recorded 2.06 ± 0.14 , 1.73 ± 0.07 , 1.95 ± 0.08 , 2.28 ± 0.25 , 1.67 ± 0.05 and 1.77 ± 0.07 mm in diameter for rGBT, rGBT-BC(M), rGBT-BC(F), rGBT-BC(HN), rGBT-BC(FHN) and rGBT-BC(MFHN), respectively, indicating that the presence of GBT-F in the BC backbone increased the plaque size and also showed increase in the virus growth kinetics in DF-1 cells (Fig 5.4).

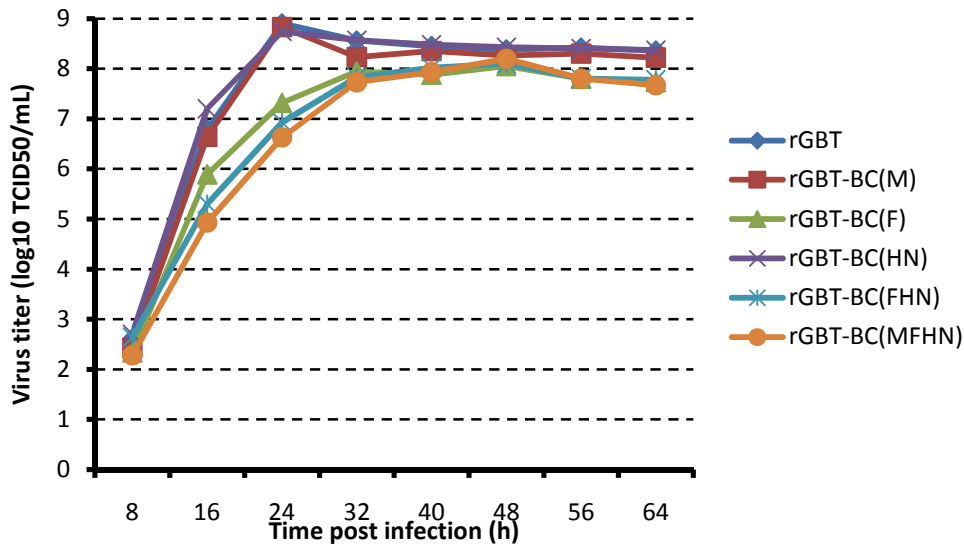


Fig 5.3. Multicycle growth kinetics in DF-1 cells of recombinant GBT with the BC envelope protein gene-swap mutants. Cells were infected at a multiplicity of infection of 0.01, supernatant samples were collected at 8 h intervals until 64 h post infection, and virus titers were determined by TCID50 calculation using the method of Reed and Muench (1938).

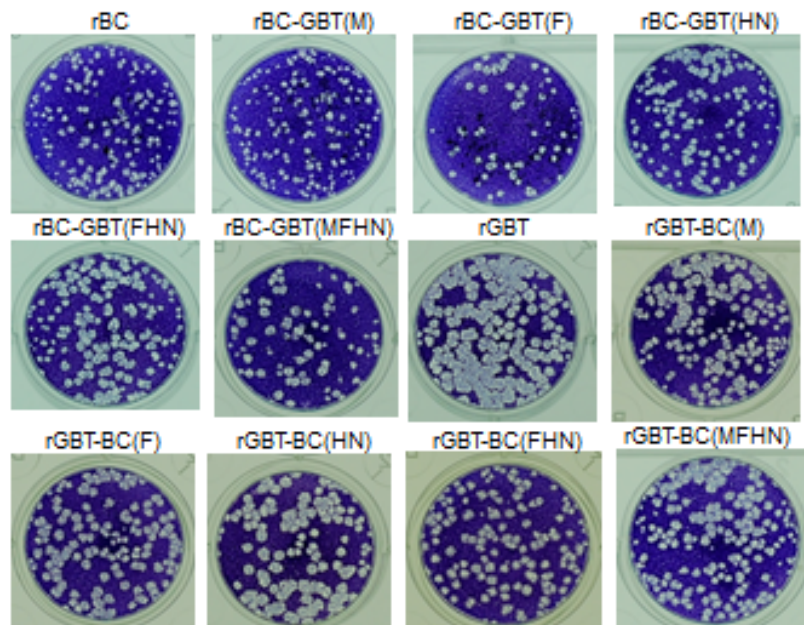


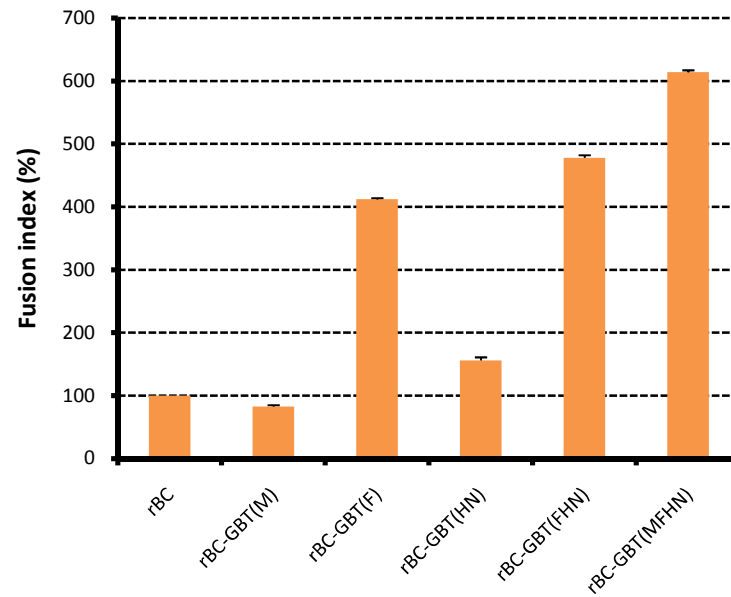
Fig 5.4. Plaque morphology of parental and mutant viruses of strain GBT and BC. Note the larger plaque size of rGBT compared to rBC. The rBC-GBT(F), rBC-GBT(FHN), rBC-GBT(MFHN) were larger than rBC. rGBT viruses with BC-F showed reduction in size compared to the parental recombinant virus plaques.

5.5.4 The fusion index:

Cell to cell fusion activity of the recombinant viruses were tested on the monolayer of verocells with 0.1 moi of virus infection. The fusion index is calculated by scoring the percentages of nuclei in syncytia to the total nuclei counted. When the fusion index of rBC was scored 100%, the rBC-GBT(M), rBC-GBT(F) and rBC-GBT(HN), rBC-GBT(FHN) and rBC-GBT(MFHN) were recorded 83%, 412%, 156%, 478% and 614%, respectively (Fig 5.5a). In the rGBT group of viruses, when rGBT was scored 100%, the rGBT-BC(M), rGBT-BC(F), rGBT-BC(HN), rGBT-BC(FHN), rGBT-BC(MFHN) were 98%, 17%, 95%, 13% and 10%, respectively (Fig 5.5b). The rBC-GBT(F) showed increased fusion index compared to rBC and rGBT-BC(F) showed reduced fusion index compared to rGBT.

While rGBT showed higher fusion index and was similar with rGBT-BC(M) and rGBT-BC(HN) but differed greatly with rGBT combinations with F gene of BC. This indicates that GBT-F is more fusogenic and retained its fusogenicity when swapped with rBC. The rBC-GBT(F) showed increased fusion index compared to rBC and rGBT-BC(F) showed reduced fusion index compared to rGBT. While rGBT showed higher fusion index and was similar with rGBT-BC(M) and rGBT-BC(HN) but differed greatly with rGBT combinations with F gene of BC. This indicates that GBT-F is more fusogenic and retained its fusogenicity when swapped with rBC.

a)



b)

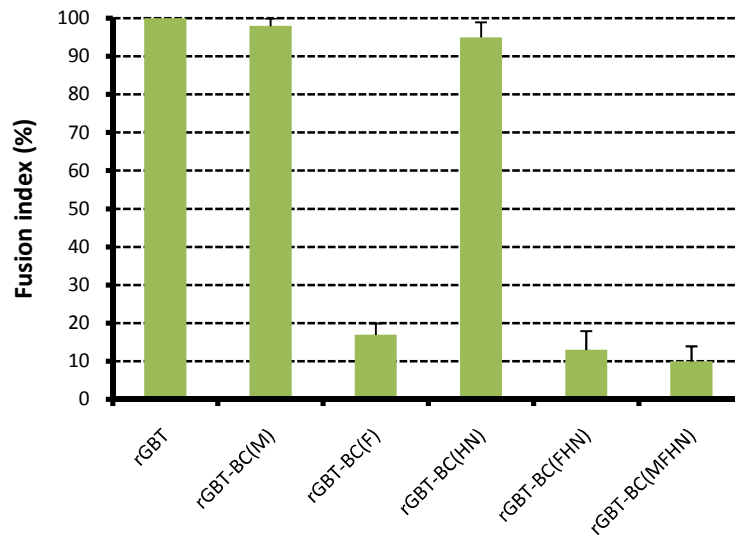


Fig 5.5. Comparison of the fusogenicities of rBC with envelope protein genes of GBT (a) and rGBT with the envelope protein genes of BC (b). Vero cells were infected with the indicated viruses at an MOI of 0.1, fixed at 36 h p.i., and stained with Hematoxylin-Eosin. The fusion index was calculated as the ratio of the total number of nuclei in multinuclear cells to the total number of nuclei in the field. Data represent the means of the results from three independent experiments.

5.5.5 Intracerebral pathogenicity index (ICPI):

Intracerebral inoculation of the rBC and rGBT with their respective mutant viruses were performed on one-day-old SPF chicks to assess the virulence. After eight days of observation, the ICPI score was determined. ICPI values of 1.61, 1.58, 1.88, 1.71, 1.8 and 1.82 were scored for rBC, rBC-GBT(M), rBC-GBT(F) and rBC-GBT(HN), rBC-GBT(FHN) and rBC-GBT(MFHN), respectively (Table 1). The ICPI values of 1.91, 1.85, 1.62, 1.88, 1.78 and 1.75 were recorded for rGBT, rGBT-BC(M), rGBT-BC(F), rGBT-BC(HN), rGBT-BC(FHN) and rGBT-BC(MFHN), respectively (Table 5.1). This indicates that rBC with GBT-F increased the virulence of the virus from 1.61 to 1.88. Similarly, rGBT with BC-F reduced its ICPI score from 1.91 to 1.62. Whereas, M and HN genes did not alter the pathotype of the virus mutants.

Table 5.1. ICPI values of recombinant viruses

Viruses	ICPI value ^a
rBC	1.61
rBC-GBT(M)	1.58
rBC-GBT(F)	1.88
rBC-GBT(HN)	1.71
rBC-GBT(FHN)	1.80
rBC-GBT(MFHN)	1.82
rGBT	1.91
rGBT-BC(M)	1.85
rGBT-BC(F)	1.62
rGBT-BC(HN)	1.88
rGBT-BC(FHN)	1.78
rGBT-BC(MFHN)	1.75

^aPathotype definitions by ICPI: velogenic strains approach the maximum score of 2.00, whereas lentogenic strains give values close to 0.

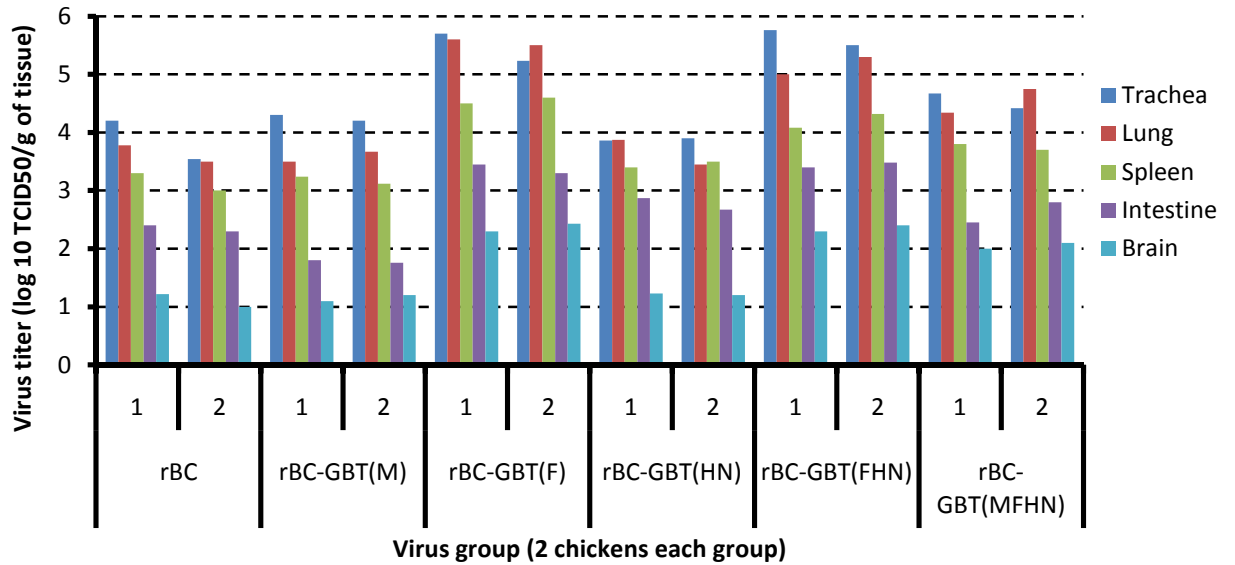
5.5.6 Virus replication and pathogenicity in two-week-old chickens:

To study the pathogenesis of rBC and rGBT with their mutant viruses, 12 groups of 12 two-week-old chickens were infected with each virus via oculonasal route. The 12 virus groups were six rBC group viruses (rBC, rBC-GBT(M), rBC-GBT(F), rBC-GBT(HN), rBC-GBT(FHN) and rBC-GBT(MFHN) and another six rGBT group viruses (rGBT, rGBT-BC(M), rGBT-BC(F), rGBT-BC(HN), rGBT-BC(FHN) and rGBT-BC(MFHN). After 3 days post infection, two chickens were sacrificed from each group and tissue samples were collected from trachea, lung, spleen, intestine, and brain. The tissue samples were divided into two parts: 1) for virus titration and 2) for histopathology. In rBC group, the tissue titers were higher in rBC-GBT(F) and rBC-GBT(FHN) compared to other virus mutants (Fig 6a). In rGBT group, the virus titers were lower in rGBT-BC(F), rGBT-BC(FHN) and rGBT-BC(MFHN) compared to other mutant viruses (Fig 5.6b).

The clinical signs were recorded for rBC and rGBT and their mutant viruses (Fig 5.7a). The rBC group showed that rBC-GBT(FHN) was most virulent, reaching the maximum score of 4 in 6 days followed by rBC-GBT(F) which showed that all birds died on day 9. Interestingly, rBC-GBT(MFHN) showed reduced clinical score and mortality compared to the above two viruses. rBC showed moderate clinical score whereas rBC-GBT(M) and rBC-GBT(HN) chickens did not show any clinical signs after day 6. The same pattern was reflected in the mortality pattern of these chickens (Fig 5.7b).

In rGBT virus group, rGBT-BC(F) and rGBT-BC(FHN) recorded low clinical score. Interestingly, in rGBT-BC(MFHN) group, all chickens died on day 6. In GBT-BC(F) all chickens survived until 14 days. However, the chickens were very sick. rGBT-

a)



b)

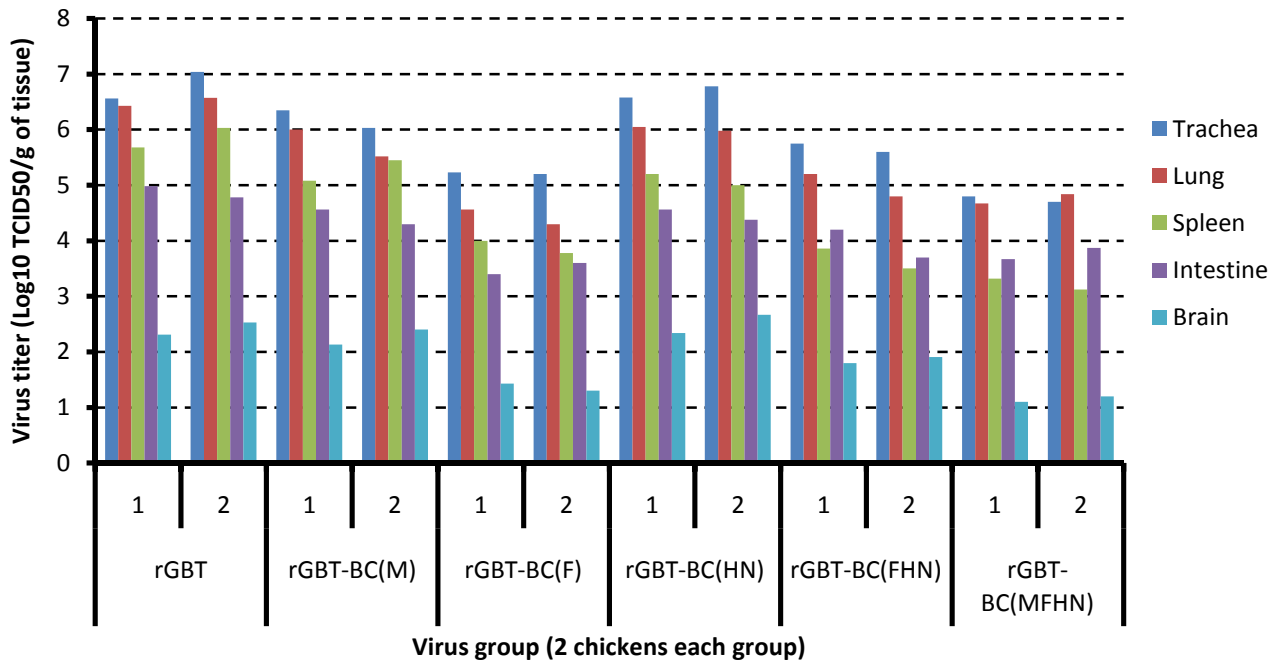
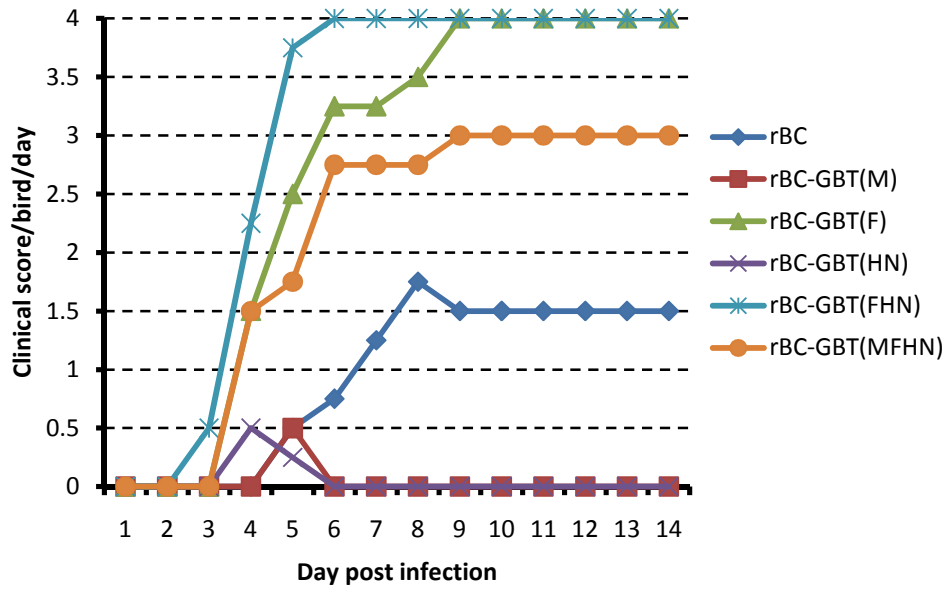


Fig 5.6. Virus titers in organs of 2-week-old chickens infected with rBC group (a) and rGBT group (b) viruses following oculonasal inoculation. Virus titers in the trachea, lungs, spleen, intestine and brain of 2 chickens from each indicated virus group on day 3 post infection. The chickens in each group are numbered 1-2.

a)



b)

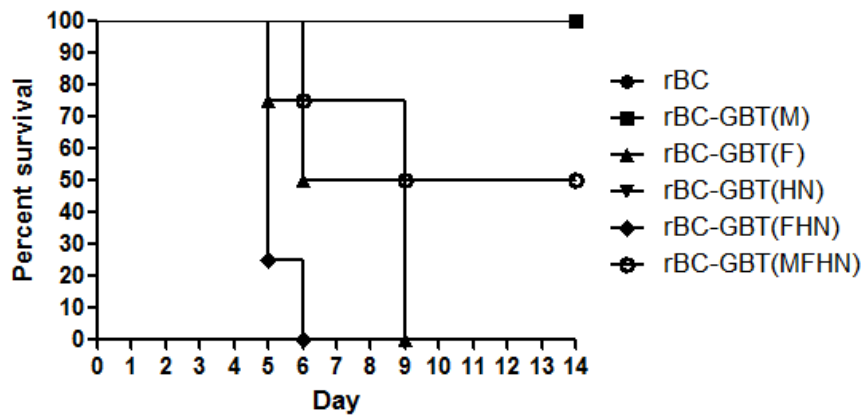
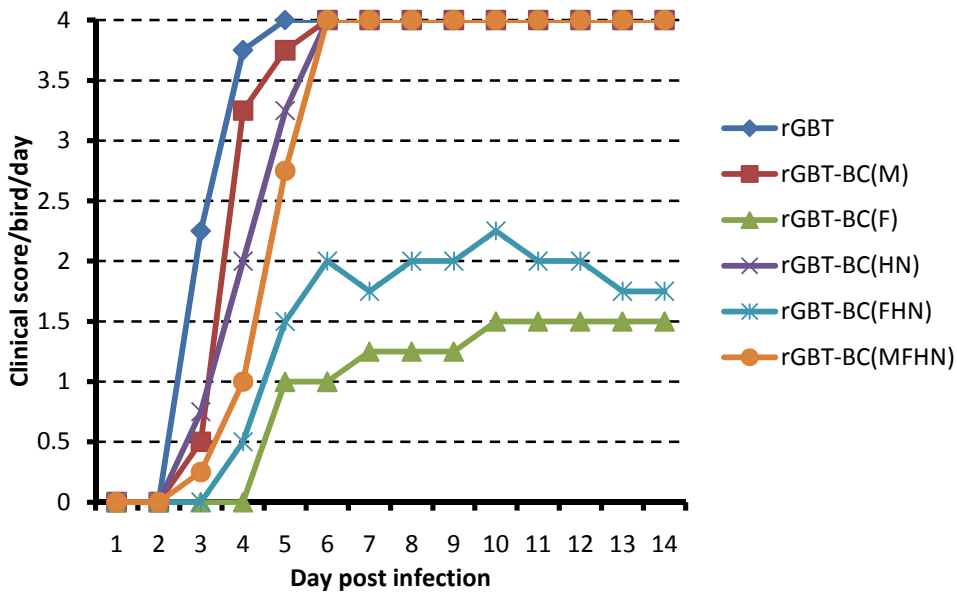


Fig 5.7. Clinical scoring (a) and survival curve (b) of chickens infected with rBC mutant viruses. Note that clinical scores of rBC-GBT(M) and rBC-GBT(HN) were lower than that of rBC; rBC-GBT(FHN) and rBC-GBT(F) showed greater scores than rBC-GBT(MFHN). Scoring of chickens (10 birds per group) was based on the clinical signs that were scored as 0, normal; 1, sick; 2, paralysis/twitching/wing drop; 3, prostration; and 4, death. The mean scores per group per day are shown.

a)



b)

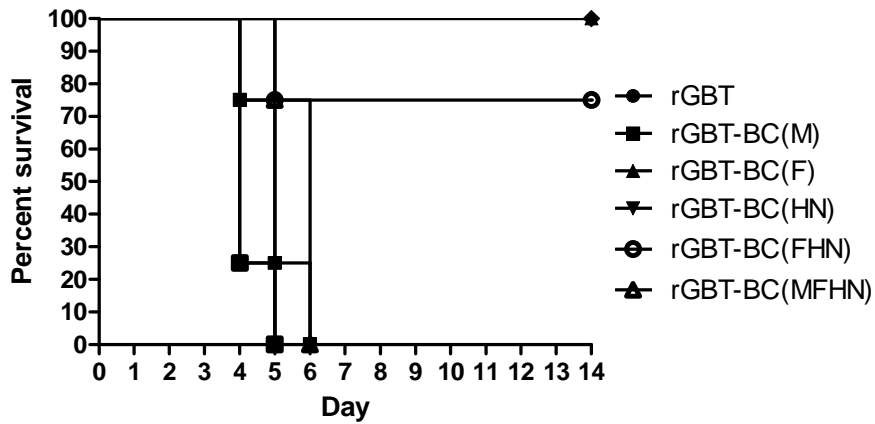


Fig 5.8. Clinical scoring (a) and survival curve (b) of chickens infected with rGBT mutant viruses. Note that rGBT-BC(F) showed the lowest scores followed rGBT-BC(FHN). Scoring of chickens (10 birds per group) was based on the clinical signs that were scored as 0, normal; 1, sick; 2, paralysis/twitching/wing drop; 3, prostration; and 4, death. The mean scores per group per day are shown.

BC(FHN) had higher scores compared to rGBT. The rGBT with BC (M, HN and MFHN) showed similar scores compared to rGBT. The mortality pattern is also reflected the clinical score pattern (Fig 5.8).

5.5.6 Histopathology descriptions:

Overall, inflammatory lesions in the tissue sections of samples collected from chickens were subacute in nature. Lesions in the lung and trachea primarily included lymphocytic inflammation with variable epithelial hyperplasia in the parabronchial epithelium and attenuation of the tracheal mucosa. Trachea cross section of rBC and rBC-GBT(F) is given in fig 5.9.

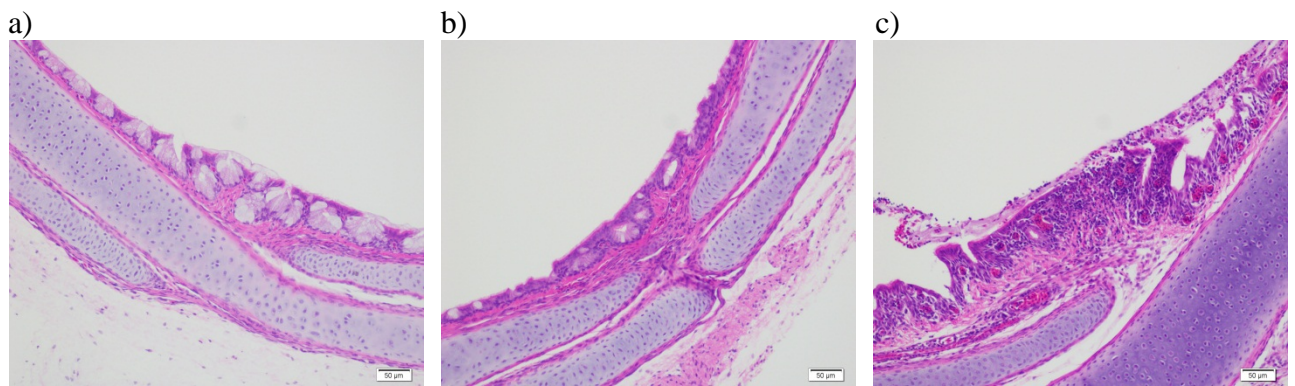


Fig 5.9. Histopathology of 2-week-old chicken trachea. a) control trachea, b) rBC infected and c) rBC-GBT(F) infected. Note mild inflammation in rBC infected trachea (b) and compare with the moderately inflamed rBC-GBT(F) trachea (c). The tissue samples were collected at day 3 post infection.

Intestinal specimens generally exhibited proliferative and lymphohistiocytic enteritis, although the severity and distribution of these lesions were quite variable. In general, inflammatory and hyperplastic lesions in the intestines were more pronounced in specimens from the rBC group. All specimens exhibited significant reactive lymphoid hyperplasia in the spleen in comparison to the control specimen. Lymphoid hyperplasia was more pronounced in specimens from the rGBT group, potentially reflecting relatively greater systemic inflammatory response. Inflammatory lesions in the brain (meningitis and encephalitis) were most observed in of the specimens from the rGBT group and several specimens from the rBC group. See table 5.2 for complete summary of histopathological descriptions, provided by Dr. Heather Shive PhD, an ACVP certified pathologist.

Table 5.2: Summary of lesions in lung, trachea, spleen, intestine, and brain in control, rBC, and rGBT groups

	control		rBC		rBC-M		rBC-F		rBC-HN		rBC-FHN		rBC-MFHN		rGBT		rGBT-M		rGBT-F		rGBT-HN		rGBT-FHN		rGBT-MFHN			
	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D		
Lung																												
Bronchial lymphoid aggregates	1	1	0	0	1	1	1	1	0	0	1	1	3	1	1	1	1	2	1	1	1	1	1	2	1	2	1	2
Parabronchitis	0	0	2	2	1/2	2	1/2	2	1/2	2	1/2	2	1/2	2	1/2	2	1/2	2	1	2	1	2	1/2	2	1/2	2	1/2	2
Epithelial hyperplasia	0	0	2	2	2	2	2	2	2/3	2	2/3	2	2/3	2	1/2*	2*	1	2	1	2	0	0	1/2	2	2*	2*	2*	
Trachea																												
Tracheitis	0	0	0	0	1	2	1 - 3	2	1	2	1 - 3	2	1	2	1	2	1	2	1	2	1/2	2	3*	2*	1	2	2	
Mucosal attenuation	0	0	2	2	2	2	2*	2*	2/3	2	0	0	2	2	3*	2*	2*	2*	1	2	0	0	2/3	2	1 - 3	2	2	
Mucosal hyperplasia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	2	2	3*	2*	0	0	0	
Necrotic/apoptotic mucosal cells	-		-		-		-		-		-		-		+		+		-		+		+		+		+	
Spleen																												
Lymphoid hyperplasia	0	0	3	5	3	5	2/3	5	3/4	5	3	5	3/4	5	4	5	3/4	5	3/4	5	3	5	3/4	5	3/4	5	3/4	5
Intestine																												
Lymphohistiocytic enteritis	0	0	2 - 4	1 - 3	3	2	1 - 4	2 - 4	2/3	2	3*	2*	0	0	0	0	2*	1*	2*	2*	3*	1*	1/2*	2*	1/2*	1/2*	1/2*	
Mucosal hyperplasia	0	0	2/3	2	3/4	2	2/3	2	2/3	2	2/3	2	2	2	1/2	2	1/2	2	1/2	2	1/2	2	0	0	2	2	2	
Brain																												
Meningitis	0	0	1*	1*	1/2*	2*	1	1	0	0	0	0	0	0	0	0	2	2	1*	2*	1*	2*	0	0	1/2	2	3*	2*
Encephalitis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2*	1*	3*	2*
Severity (S)																												
absent	0		Distribution (D)																									
minimal	1		absent																									
mild	2		focal																									
moderate	3		multifocal																									
marked	4		locally extensive																									
severe	5		multifocal and coalescing																									
			diffuse																									
+, present; -, absent																												
* indicates that lesion is not present in all specimens (see histologic descriptions)																												

5.6 Discussion

The products of Newcastle disease virus envelope associated protein genes; matrix (M), fusion (F) and hemagglutinin-neuraminidase (HN) play important roles in the virus replication cycle. The M protein orchestrates morphogenesis and the surface glycoproteins F and HN are responsible for membrane penetration and cell attachment, respectively. During viral infection of the hosts, antibodies against the surface glycoproteins confer protection; hence F and HN play a key role in establishing infection. NDV virulence and pathogenesis in natural host chickens has been previously studied with the strains isolated from birds like cockatoo, pigeon and Anhinga etc. (Dortmans *et al.*, 2009, 2010; Estevez *et al.*, 2007). This study presents a suitable model for the comparative study of the role of individual envelope associated protein genes, since two virus strains Texas GB and BC are genetically closely related and their natural host is chickens. They belong to genotype II of NDV class II viruses with nucleotide identity of 99.1% at genome level but differ in their virulence with BC causing 0% mortality to GBT causing 100% mortality. Both these viruses possess identical F protein cleavage site but still differ in virulence. Also, these strains have identical genome length of 15,186 nt. These attributes would enable us to discern the roles of individual genetic elements of NDV systematically.

The recombinant viruses of rBC group showed that rBC-GBT(F), rBC-GBT(FHN) and rBC-GBT(MFHN) showed increased growth compared to rBC, rBC-GBT(M) and rBC-GBT(HN). The plaque morphology of rBC-GBT(F), rBC-GBT(FHN) and rBC-GBT(MFHN) were larger than those of rBC, rBC-GBT(M) and rBC-GBT(HN), indicating that GBT(F) played a major role in the increased replication and larger plaque

size. The attenuation of growth and reduction of the plaque size were recorded in the rGBT with BC-F gene swap constructs which is in line with the fact that major differences existed in the F gene when compared with M and HN genes. Fusion index also served as the indicator that the GBT-F is more fusogenic and the rBC group having GBT-F increased the fusogenicity of the mutant viruses.

The pathogenicity of the rBC and rGBT group mutant viruses in one-day-old chicks revealed that the ICPI value of rBC increased from 1.61 to 1.88 by the incorporation of GBT-F and similarly, the reverse was also true when the BC-F was introduced to rGBT showed reduction of rGBT value from 1.91 to 1.62. And similar patterns were noticed with constructs having the F genes. M and HN genes did not alter the pathotypes of the mutant viruses.

In two-week-old chickens, rBC group of viruses had higher clinical scores when it had the GBT-F gene and the presence of M and HN genes of GBT reduced the clinical score with those of rBC indicating their role in the envelope proteins interactions. NDV strains GBT and BC have amino acid assignment differences of four aa in M protein: M132V, D152N, P16S and A336T; eight aa in F protein: S10P, A11V, S265G, G304E, T457I, T510I, A520V and A550T; 11 aa in HN protein: Q7R, V9A, A34V, M35V, I191V, N228S, V271A, S310G, E332G, L454P and A571V. It would be interesting to know the importance of these amino acid assignment differences in NDV virulence. rBC-GBT(MFHN) showed comparatively reduced virulence than that of rBC-GBT(F) and rBC-GBT(FHN), which might be due to the reduced compatibility of the polymerase complex proteins interaction with the M proteins. In contrary, rGBT-BC(MFHN) was highly virulent and it caused 100% mortality that could be partly due to some

compatibility of the polymerase complex proteins of GBT with BC M protein and also the part of virulence associated with the GBT polymerase complex genes might also play a key role. In the pathological sections of tissues, it was observed that the lesions were subacute in nature and the rGBT group had greater inflammation scores compared to the rBC group. rGBT showed increased proliferation of lymphocytes indicating a more systemic infection than that of rBC. rBC showed increased inflammation in the intestine compared to the rGBT viruses. The pathological scores of mild to moderate inflammation were recorded throughout but in the rBC group, increase in the infiltration of cells and tissue damage was noticed frequently in the rBC group with the GBT-F gene.

In summary, the present study found that the F gene of the highly virulent NDV strain GBT caused gain in virulence of the moderately virulent NDV strain BC in chickens. The replication, plaque morphology and the fusogenicity index were also indicative of the greater role of the NDV F gene in virulence. When introduced in rBC, GBT-F gene increased virulence of rBC to the original ICPI score of GBT in one-day-old chicks. It also increased the virulence of BC to 100% mortality in two-week-old chickens. Conversely, replacement of the F gene of GBT with that of BC decreased the virulence of GBT. These results suggest that the F gene is a major determinant of NDV virulence.

Chapter 6:

6.1 Title

The role of polymerase complex protein genes and trailer sequence in Newcastle disease virus virulence and pathogenesis

6.2 Abstract

Newcastle disease is a very important disease in poultry and causes severe economic losses worldwide. The causative agent Newcastle disease virus (NDV) has a spectrum of virulence, causing mild inapparent infection to 100% mortality. The virulence determinants are multigenic. We reciprocally exchanged the polymerase complex associated genes between a highly virulent strain Texas GB (GBT) and a moderately virulent strain Beaudette C (BC). The recombinant viruses showed that L gene of strain GBT increased the replication and plaque morphology of the BC *in vitro*. The L protein gene of GBT showed increased virulence over the N, P and L protein genes in combination. The trailer sequence did not alter the phenotypes of the recombinant viruses.

6.3 Introduction

Newcastle disease virus is a very important poultry disease that causes huge economic loss worldwide. There are numerous strains of NDV available throughout the world and their virulence ranges from mild inapparent infection to 100% mortality (Alexander, 2003; Samal, 2011). The virulence is considered to be multigenic and the

fusion protein gene is the major virulence determinant of NDV. The roles of polymerase complex protein genes N, P and L and the trailer sequence were not been determined in virulence and pathogenesis of NDV strains that have chickens as natural hosts.

NDV belongs to the genus *Avulavirus* under the subfamily *Paramyxovirinae* in the family *Paramyxoviridae* (Mayo, 2012; Lamb and Parks, 2007). There are at least three genome size categories identified that have nucleotide (nt) lengths of 15,186, 15,192 and 15,198 nt (Krishnamurthy and Samal, 1998; Czegledi *et al.*, 2006; Ujvari *et al.*, 2005). The genome contains six genes flanked by extra-genic sequences required for replication in the order of 3'leader-N-P-M-F-HN-L-5'trailer. The genes code for nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and large polymerase protein (L). P gene can also code for an additional protein called V protein using a co-transcriptional mechanism known as RNA editing.

Determination of the virulence factors for NDV is very important since there are numerous viruses isolated around the world that vary greatly in virulence. NDV virulence determinants are multigenic but multibasic amino acids in the fusion protein cleavage site is considered as the most important factor for virulence. The presence of a multibasic furin protease cleavage site helps the virus to spread systemically and cause disease. The viruses that have monobasic cleavage site get restricted to the upper respiratory tract and intestine where they can be cleavage activated by exogenous trypsin-like proteases. However, this is true only with viruses with the same natural host. Modification of cleavage site sequences from other avian paramyxoviruses does not increase their virulence in chickens. HN protein is responsible for the attachment to host cells and that

determines tropism of the virus. V protein is known to be involved in antagonizing antiviral defense by binding to and targeting STAT1 to proteasomal degradation. Large polymerase (L) protein is the driving force behind transcription and replication that determines virus growth and spread (Rout and Samal, 2008; Dortmans *et al.*, 2010).

In this study, we determine the role of polymerase complex protein genes, N, P and L, and trailer sequence of NDV in pathogenesis and virulence. Two chicken origin viruses with identical cleavage site sequences, but differing in pathotypes, have been generated using reverse genetics; NDV strains Beaudette C (BC), a moderately virulent virus and Texas GB (GBT), a highly virulent virus with pronounced neurological clinical signs. BC is associated with clinical illness and not usually fatal but GBT is associated with 100% mortality and is used as a standard challenge virus in the US. Both these viruses belong to the genotype II of class II NDV strains that have identical genome length of 15,186 nt. The reciprocal exchange of polymerase complex associated protein genes and trailer sequence between strains BC and GBT and their natural infection in their natural host chickens would give a common understanding of the virulence determinant of NDV. The reciprocal exchange of leader sequence is not done as a part of this work because the NDV strains BC and GBT have identical 55-nt leader sequence at the 3' end of their genomes.

6.4 Materials and Methods

6.4.1 Cells and viruses:

A chicken embryo fibroblast cell line (DF1), African green monkey kidney cell line (Vero), and a human epidermoid carcinoma cell line (HEp-2) were grown in

Dulbecco's minimal essential medium (DMEM) with 10% fetal bovine serum (FBS) and maintained in DMEM with 2% FBS. The modified vaccinia virus strain Ankara (MVA) expressing T7 RNA polymerase was kindly provided by Dr. Bernard Moss (NIH, Bethesda, MD) and propagated in primary chicken embryo fibroblast cells in DMEM with 2% FBS. The moderately pathogenic (mesogenic) NDV strain Beaudette C (BC) and highly pathogenic (velogenic) NDV strain Texas GB (GBT) and its recombinant derivatives were grown in 9-day-old embryonated specific-pathogen-free (SPF) chicken eggs in an enhanced biosafety level 3 (BSL-3+) containment facility certified by the USDA following the guidelines of the IACUC, University of Maryland.

6.4.2 Plasmid construction, transfection, virus-rescue and sequence analysis:

The construction of full length antigenome cDNA clone of NDV strains BC and GBT using identical restriction enzymes has been described in the Materials and Methods section of Chapter 4. In the present study, N, P, L genes and trailer were reciprocally swapped between the pBC and pGBT RE sites *AscI* and *PacI*, *PacI* and *PmeI*, *SnaBI* and *RsrII*, respectively. PCR products of N, P and L genes generated using *Pfx* polymerase was used for cloning. The trailer region was cloned into pBC and pGBT using overlap PCR designed in the L3 fragment of L gene and cloned using RE sites *MluI* and *RsrII*. Lipofectamine 2000 reagent was used for transfecting HEp-2 cells with the respective full length cDNA clone with three support plasmids made of N, P and L proteins of strain GBT in pGEM-7Z(+). MVA-T7 used as the source of T7 polymerase. The plasmid constructs were screened for the appropriate gene-swaps by sequencing using gene specific primers. The recovered gene-swap mutant viruses of BC and GBT backbones

were subjected to five passages in 9-day-old SPF chicken embryos. From the fifth passage, total RNA was isolated using RNeasy RNA purification kit (Invitrogen). Gene-specific primers were used to confirm the entire sequence of the gene and the flanking regions of the backbone to confirm the gene swap virus.

6.4.4 Virus growth kinetics and plaque morphology:

The growth kinetics of rBC and rGBT and their mutant viruses were determined under multiple-cycle growth conditions in DF-1 cells. The virus was inoculated at a multiplicity of infection (MOI) of 0.01 into DF-1 cells grown in DMEM with 10% FBS at 37°C. The supernatant was collected at 8-h intervals until 64 h post infection (p.i.). The virus content in the samples was quantitated by tissue culture infective dose 50 (TCID₅₀) in DF-1 cells using the method of Reed and Muench (1938). Plaque assay was performed to determine the plaque morphology, briefly, supernatants, collected from virus-inoculated samples earlier, were serially diluted, and 100 µl of each serial dilution was added per well of confluent DF-1 cells in 12-well plates. After 1 h of adsorption, cells were overlaid with DMEM (containing 2% FBS and 0.81% methylcellulose) and then incubated at 37°C for 4 days. The cells were then fixed with absolute methanol and stained with 1% crystal violet for plaques.

6.4.5 Pathogenicity studies in chickens.

To test the pathogenicity of the recovered viruses *in vivo*, an intracerebral pathogenicity index (ICPI) test was performed according to standard procedures (Alexander, 1989). For ICPI, 10⁻¹ dilution of 2⁹ HA units of each virus/chicken was

inoculated intracerebrally into groups of 10 one-day-old SPF chicks. Inoculation was performed with a 27-gauge needle attached to a 1-ml stepper syringe dispenser that was set to dispense 0.05 ml of inoculum per inoculation. The birds were inoculated by inserting the needle up to the hub into the right or left rear quadrant of the cranium. The birds were observed for clinical signs and mortality once every 12 h for 8 days. ICPI values were calculated as described by Alexander (1989). Briefly, the birds were scored daily: 0 if normal, 1 if sick, and 2 if dead. The ICPI value was the mean score per bird per observation. Highly virulent (velogenic) viruses give values approaching 2; avirulent (lentogenic) viruses give values close to 0.

6.4.6 Pathogenicity in 2-week-old chickens, virus titration:

Groups of 12 two-week-old SPF chickens were inoculated with 2×10^6 PFU in 0.2 mL of each virus by the oculonasal route. The birds were observed daily and scored for clinical signs for 14 days. Two birds from each group were euthanized 3 DPI and lung, trachea, spleen, brain and intestine samples were collected. For virus titration, the tissue samples were homogenized, and the supernatant was serially diluted and used to infect DF-1 cells, with duplicate wells per dilution. Infected wells were identified by HA assay of the supernatant, and the $TCID_{50}/g$ was calculated using the method of Reed and Muench (1938).

6.5. Results

6.5.1. Recovery of infectious N, P, L and Trailer swap-viruses of rBC and rGBT:

Forty eight hours after the transfection, cell culture supernatant was collected to inoculate in 9-day-old embryonated chicken eggs. After 2 days, the virus growth was detected by HA assay. The recombinant viruses yielded a HA titers between 2^6 and 2^9 . The support plasmids, pN, pP and pL from GBT were used for the rescue of all viruses. Upon subsequent passages in eggs the virus yield was about 2^9 HA units consistently and plaque assay was done. The infective allantoic fluids with 2^8 - 2^9 PFU/mL were used as a preliminary viral stock. Part of this material was used to isolate viral genomic RNA, which was subjected to RT-PCR and complete gene sequencing. The exchanged segment was completely sequenced to confirm that particular gene-swap construct (Fig 6.1).

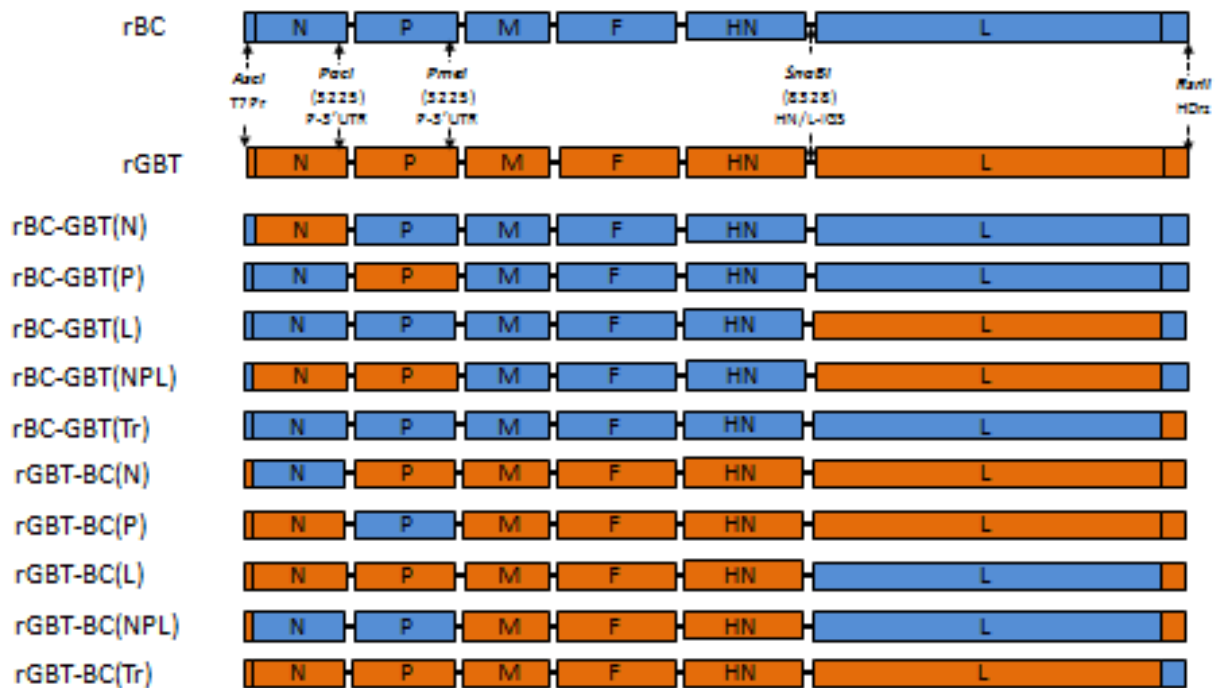


Fig 6.1. Gene map and cloning strategy for N, P, L genes and Trailer sequence between NDV strains Beaudette C (BC) and Texas GB (GBT). The top diagram shows the gene map of rBC and rGBT and the restriction enzyme sites available for cloning of N(AscI and PacI), P(PacI and PmeI) and L(SnaBI and RsrII) genes. Last 10 gene maps show reciprocal exchange of N, P, L, NPL and Trailer sequence between the rBC and rGBT

6.5.2 Virus growth kinetics and plaque morphology:

The growth pattern of rBC and rGBT group viruses were determined in DF-1 cells at a moi of 0.01. Between the parental recombinant viruses, rGBT showed early initiation of cpe higher growth compared to rBC. rBC-GBT(L), rBC-and GBT(NPL) have showed higher titers than that of rBC, rBC-GBT(N), rBC-GBT(P) and rBC-GBT(Tr). rGBT-BC(L) and rGBT-BC(NPL) showed lower titers compared to rGBT (Fig 6.2, Fig 6.3).

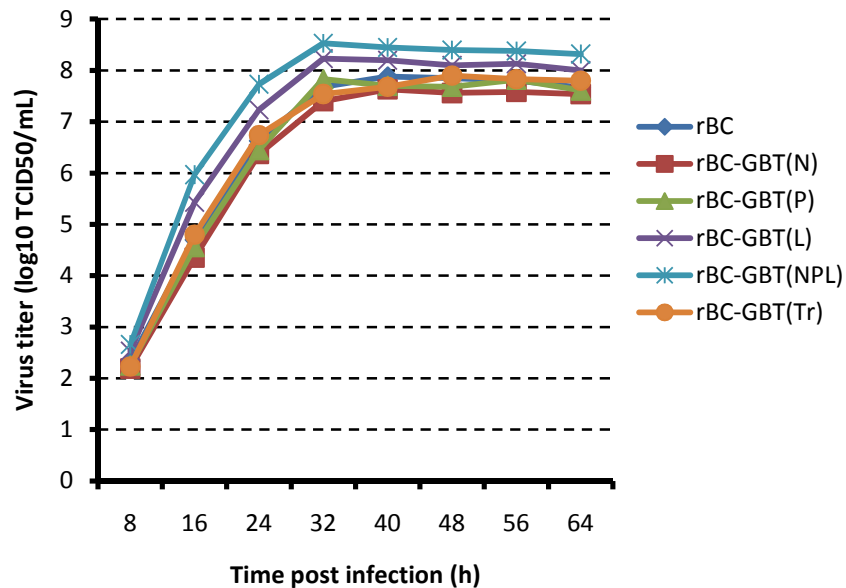


Fig 6.2. Multicycle growth kinetics in DF-1 cells of recombinant Beaudette C (rBC) with the Texas GB (GBT) polymerase complex protein genes and trailer sequence mutants. Cells were infected at a multiplicity of infection of 0.01, supernatant samples were collected at 8 h intervals until 64 h post infection, and virus titers were determined by TCID50 calculation using the method of Reed and Muench (1938).

The plaque sizes of rBC group viruses, rBC, rBC-GBT(N), rBC-GBT(P), rBC-GBT(L), rBC-GBT(NPL) and rBC-GBT(Tr) are 1.73 ± 0.17 , 1.44 ± 0.09 , 1.4 ± 0.07 , 1.43 ± 0.13 , 2.01 ± 0.11 and 1.4 ± 0.18 mm in diameter, respectively. The plaque sizes of rGBT group

viruses, rGBT, rGBT-BC(N), rGBT-BC(P), rGBT-BC(L), rGBT-BC(NPL) and rGBT-BC(Tr) are 2.19 ± 0.1 , 1.99 ± 0.23 , 2.2 ± 0.12 , 1.58 ± 0.16 , 1.34 ± 0.08 and 2.12 ± 0.09 mm in diameter, respectively. rBC with N, P and L genes of GBT showed decrease in the plaque size compared to GBT L alone, which indicates that optimum interaction is necessary for the increased size of the plaque (Fig 6.4).

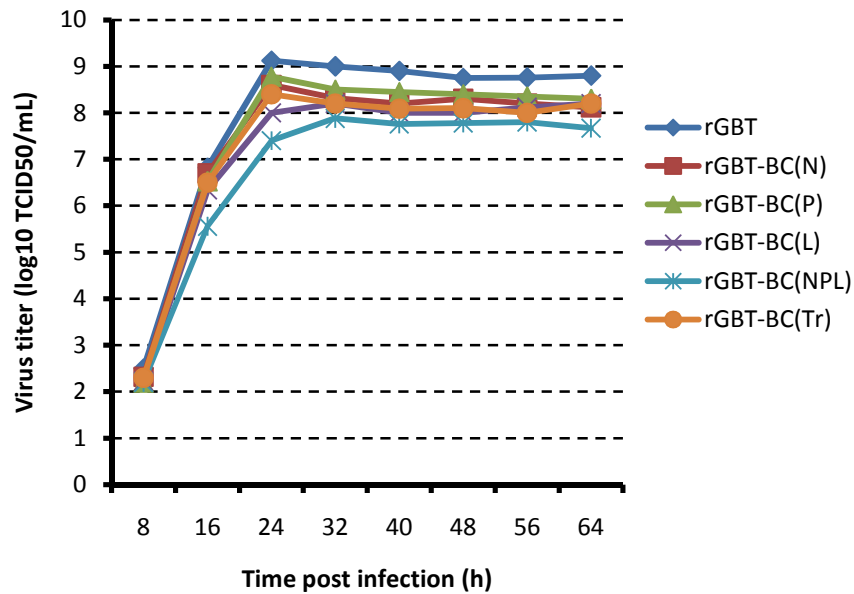


Fig 6.3. Multicycle growth kinetics in DF-1 cells of recombinant Texas GB (GBT) with the Beaudette C (BC) polymerase complex protein genes and trailer sequence mutants. Cells were infected at a multiplicity of infection of 0.01, supernatant samples were collected at 8 h intervals until 64 h post infection, and virus titers were determined by TCID50 calculation using the method of Reed and Muench (1938).

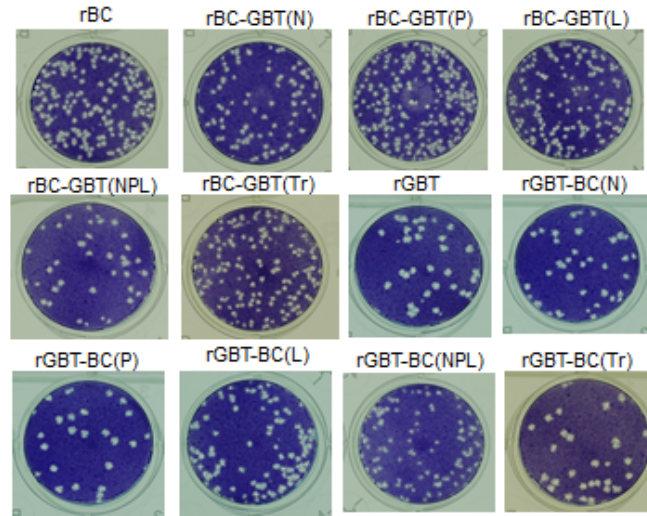


Fig 6.4. Plaque morphology of parental and mutant viruses of NDV strains Beaudette C (BC) and Texas GB (GBT). Note the larger plaque size of rGBT compared to rBC. The plaque size of rBC-GBT(NPL) is greater than rBC, rBC. rGBT viruses with BC-L showed reduction in size compared to the parental recombinant virus plaques.

6.5.3 Intracerebral pathogenicity index (ICPI):

Intracerebral inoculation of the rBC and rGBT with their respective mutant viruses were performed on one-day-old SPF chicks to assess their virulence. After eight days of observation the ICPI score was determined. ICPI values of 1.58, 1.62, 1.60, 1.71, 1.75 and 1.61 were scored for rBC, rBC-GBT(N), rBC-GBT(P) and rBC-GBT(L), rBC-GBT(NPL) and rBC-GBT(Tr), respectively (Table 6.1). The ICPI values of 1.91, 1.88, 1.90, 1.83, 1.78 and 1.90 were recorded for rGBT, rGBT-BC(N), rGBT-BC(P), rGBT-BC(L), rGBT-BC(NPL) and rGBT-BC(Tr), respectively (Table 6.1). This indicates that rBC with GBT-L increased the virulence of the virus from 1.61 to 1.71 but when GBT-N, P and L combination was swapped it increased the ICPI value to 1.75. Conversely, rGBT with BC-L reduced its ICPI score from 1.91 to 1.83 and when BC-N, P and L were swapped, the ICPI score was decreased to 1.78 signifying the combination of all three polymerase complex genes. The Trailer sequence swaps of both rBC and rGBT did not

affect the ICPI scores, suggesting that the Tr sequence played a minimal role between rBC and rGBT virulence in the one-day-old chicks (Table 6.1).

Table 6.1. ICPI values of recombinant viruses of N, P, L and Trailer swap constructs

Viruses	ICPI value ^a
rBC	1.58
rBC-GBT(N)	1.62
rBC-GBT(P)	1.60
rBC-GBT(L)	1.71
rBC-GBT(NPL)	1.75
rBC-GBT(Tr)	1.61
rGBT	1.91
rGBT-BC(N)	1.88
rGBT-BC(P)	1.90
rGBT-BC(L)	1.83
rGBT-BC(NPL)	1.78
rGBT-BC(Tr)	1.90

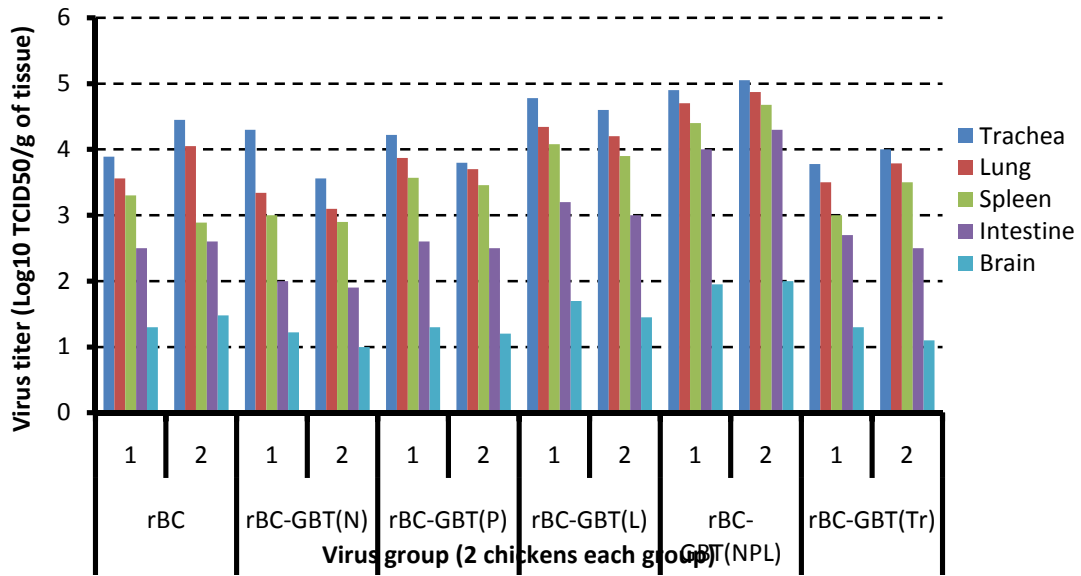
^aPathotype definitions by ICPI: velogenic strains approach the maximum score of 2.00, whereas lentogenic strains give values close to 0.

6.5.4 Virus replication and pathogenicity in two-week-old chickens:

To study the pathogenesis of rBC and rGBT with their mutant viruses, 12 groups of 12 two-week-old chickens were infected with each virus via oculonasal route. The 12 virus groups were six rBC group viruses (rBC, rBC-GBT(N), rBC-GBT(P), rBC-GBT(L), rBC-GBT(NPL) and rBC-GBT(Tr) and another six rGBT group viruses (rGBT, rGBT-BC(N), rGBT-BC(P), rGBT-BC(L), rGBT-BC(NPL) and rGBT-BC(Tr). After 3 days post infection, two chickens were sacrificed from each group and tissue samples were collected from trachea, lung, spleen, intestine and brain for virus titration. In rBC group, the tissue titers were higher in rBC-GBT(L) and rBC-GBT(NPL) compared to

other virus mutants (Fig 6.5a). In rGBT group, the virus titers were lower in rGBT-BC(L) and rGBT-BC(NPL) compared to other mutant viruses (Fig 6.5b).

a)



b)

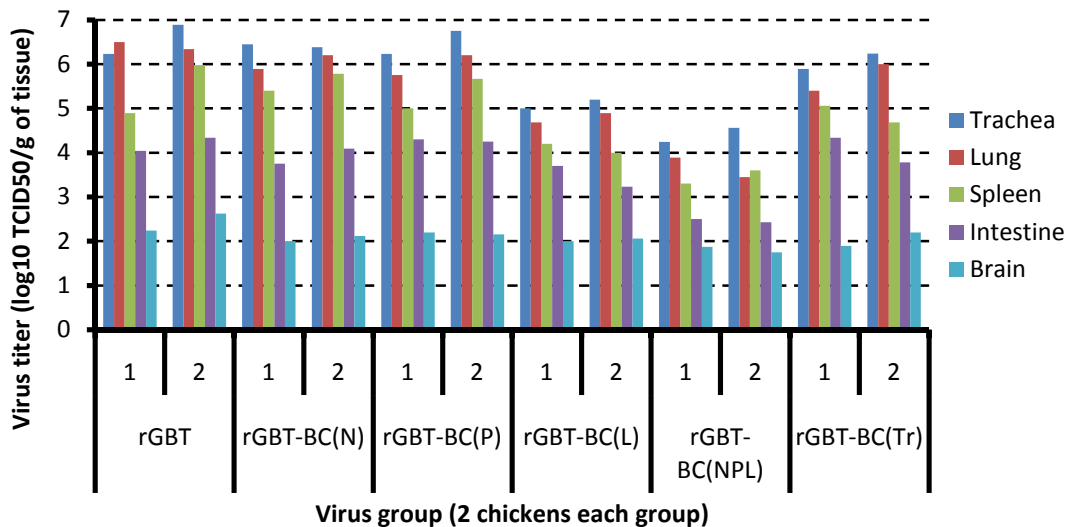
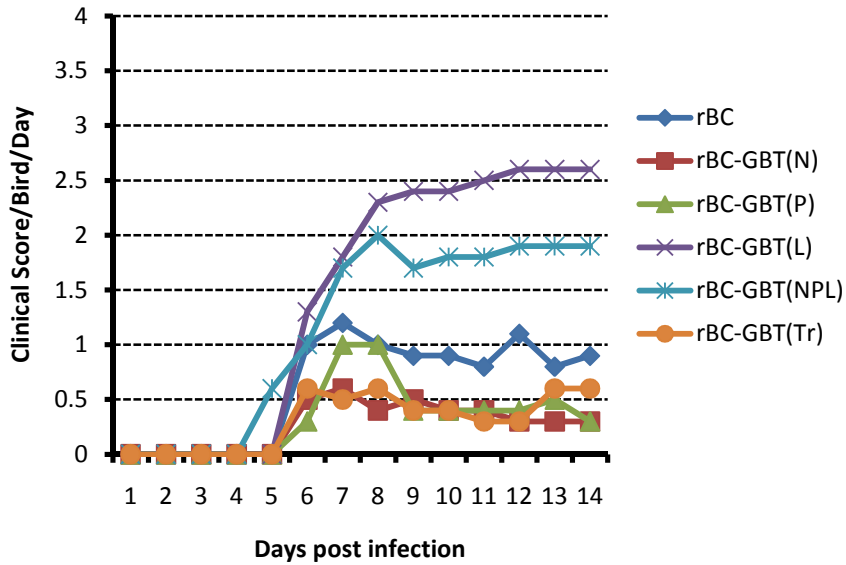


Fig 6.5. Virus titers in organs of 2-week-old chickens infected with rBC group (a) and rGBT group (b) viruses following oculonasal inoculation. Virus titers in the trachea, lungs, spleen, intestine and brain of 2 chickens from each indicated virus group on day 3 post infection. The chickens in each group are numbered 1-2.

The clinical signs were recorded for rBC and rGBT and their mutant viruses (Fig 6.6a). In the rBC group, it was observed that rBC-GBT(L) was the most virulent, reaching the maximum score of 2.9 in 12 days, whereas rBC-GBT(NPL) showed a score of 1.9 on the same day. The reduction in the clinical score in rBC with GBT-N, P and L genes combination suggests that this could be due to interaction of the GBT-N, P and L proteins with that of BC-M, F and HN proteins or the interference of Trailer sequence of the rBC or both. The survival curve also reflected the same trend (Fig 6.6b).

In rGBT group, rGBT, rGBT-BC(N) and rGBT-BC(Tr) reached the maximum score of 4.0 in day 5; rGBT-BC(P) reached the maximum score in day 6, whereas the rGBT-BC(L) reached the maximum score on day 8 but rGBT-BC(NPL) did not reach the maximum clinical score till 14 days (Fig 6.7a). This finding shows that the BC-L protein interacted better with the GBT-N and P proteins compared to BC-N, P and L proteins in the backbone of rGBT which also might suggest the interactions with the envelope associated proteins. The survival curve also reflected the same trend (Fig 6.7b).

a)



b)

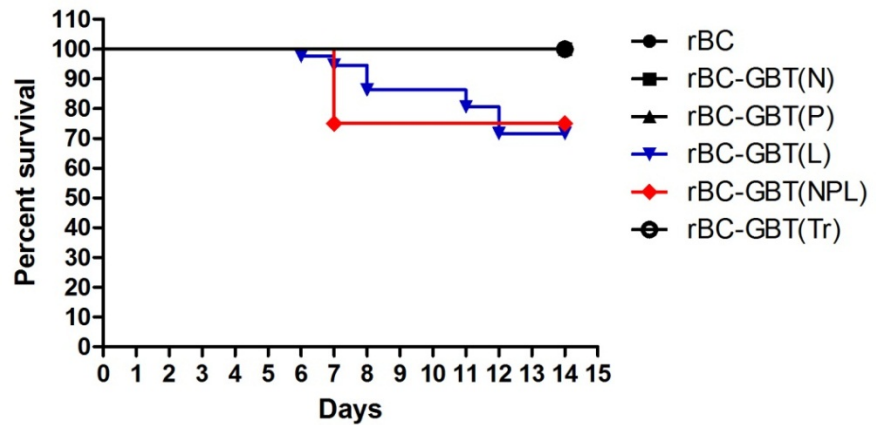
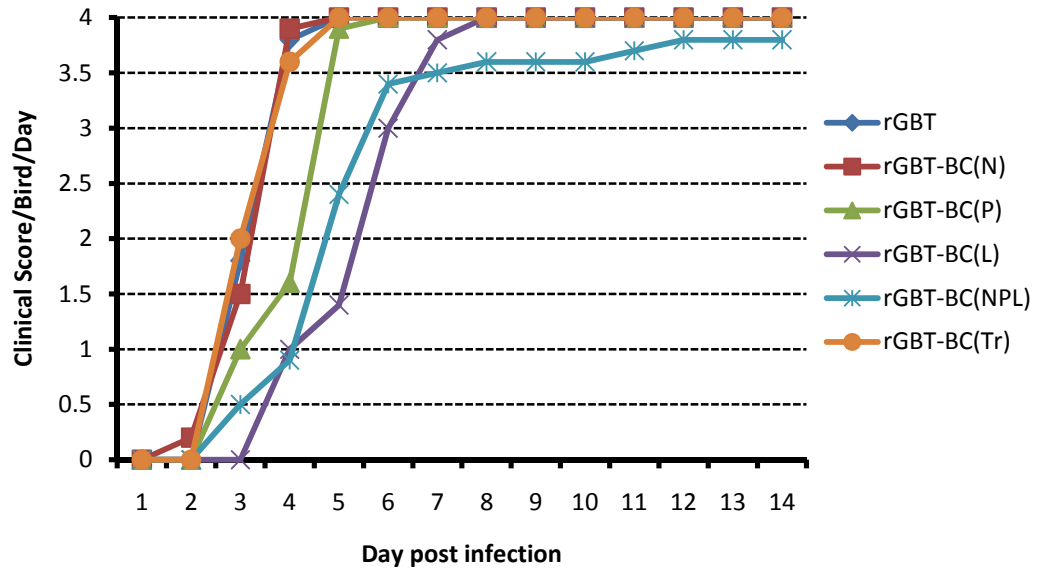


Fig 6.6. Clinical scoring (a) and survival curve (b) of chickens infected with recombinant Beaudette C (BC) mutant viruses. Note that clinical score of rBC-GBT(L) was greater than rBC-GBT(NPL) and was reflected in the survival curve. Scoring of chickens (10 birds per group) was based on the clinical signs that were scored as 0, normal; 1, sick; 2, paralysis/twitching/wing drop; 3, prostration; and 4, death. The mean scores per group per day are shown.

a)



b)

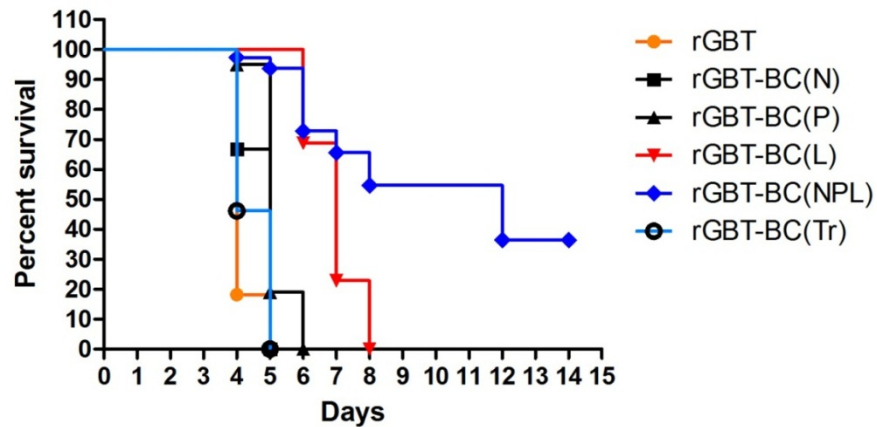


Fig 6.7. Clinical scoring (a) and survival curve (b) and of chickens infected with recombinant Beaudette C (BC) mutant viruses. Note that clinical score of rGBT-BC(NPL) was lower than rGBT-BC(L) and the same observation reflected in the survival curve. Scoring of chickens (10 birds per group) was based on the clinical signs that were scored as 0, normal; 1, sick; 2, paralysis/twitching/wing drop; 3, prostration; and 4, death. The mean scores per group per day are shown.

6.6 Discussion

The replication and gene expression in the paramyxoviruses depend on the polymerase complex associated proteins nucleocapsid (N), phosphoprotein (P) and large polymerase protein (L) which are coded by their respective genes N, P and L (Lamb and Parks, 2007). The N protein binds entirely onto the negative-sense RNA genome and imparts the status of the genome to functional template for replication and transcription (Blumberg and Kolakofsky, 1981; Blumberg *et al.*, 1981). N protein also confers nuclease resistance to the RNA. The P protein, with no known catalytic function associates with the N and L proteins and functions as the scaffold protein in virus replication and gene expression. The L protein as RNA dependent RNA polymerase plays a key role in replication and transcription (Kolakovsky and Blumberg, 1982; Banerjee, 1987; Tordo *et al.*, 1988). In this study, the roles of N, P, L and the trailer sequences were examined for their role in virus growth, pathogenesis and virulence. Two phylogenetically closely related recombinant NDV strains Beaudette C (rBC) and Texas GB(rGBT) were used for the reciprocal exchange of polymerase complex associated genes and trailer sequence between them. The two recombinant viruses were created to have identical restriction enzyme site at identical positions in the genome. The two NDV strains differ greatly in virulence; BC is a moderately virulent virus and GBT is highly virulent virus that causes 100% mortality.

The growth kinetics of rBC and rGBT differed greatly in DF-1 cells, in which rGBT replicated to high titer at 24 h post infection whereas rBC reached the maximum titer at 32 h and the titers were significantly lower than the rGBT throughout 64 h. In rBC group of viruses, rBC-GBT(L) and rBC-GBT(NPL) replicated to maximum titer

compared to rBC, rBC-GBT(N), rBC-GBT(P), rBC-GBT(L) and rBC-GBT(Tr). Conversely, in rGBT group of viruses, rGBT-BC(L) and rGBT-BC(NPL) showed lowest titers compared to other viruses in the group. The plaque size of rBC-GBT(NPL) was larger than rBC and other viruses. rGBT-BC(L) and rGBT-BC(NPL) showed smaller size plaques compared to rGBT while latter was the smallest. This indicates that the L gene of GBT increased the replication of the rBC and the converse was observed in rGBT with L gene of BC. There was no effect of trailer sequence observed in virus replication in DF-1 cells.

In one-day-old chicks, the intracerebral pathogenicity values increased when the L gene of GBT was introduced into rBC by increasing the ICPI from 1.58 to 1.71 and when N, P and L genes in combination showed increase to 1.75; N and P genes showed 1.62 and 1.6, respectively, suggesting that the L gene of GBT alone was responsible for the increase in virulence of rBC. In the rGBT, the introduction of L gene of BC resulted in lowering of ICPI value from 1.91 to 1.83; N, P and L genes in combination reduced the ICPI to 1.78, suggesting that L gene of BC played a important role in the lowering of virulence in rGBT. The N, and P genes and the trailer sequence showed the ICPI values 1.88, 1.90 and 1.90, respectively suggesting that N, P and trailer sequences did not affect the virulence of rGBT. Though the L gene played a role in altering the virulence profile of the parental recombinant viruses, it was not sufficient to change the virulence of rBC and rGBT to their original pathotypes in ICPI scores, suggesting that L gene plays a minor part in the overall virulence of NDV but it is the important gene among the polymerase associated genes in virulence determination.

In two-week-old chickens, the highest clinical scores were recorded for the rGBT but the rBC group showed sick chickens gradually recovering during the course of the experiment. The rBC group where L gene of GBT was present showed significant increase in clinical signs and mortality, increasing the clinical scores over the parental recombinant viruses. The reverse was true in rGBT but the clinical signs were far more severe than the rBC groups. In rBC, when N, P and L genes of GBT were introduced in combination, it was observed that the clinical signs and mortality pattern was less severe than of L gene. This could be due to the interactions of GBT-N, P and L proteins with those of BC-M, F and HN proteins; or L protein of GBT might had little advantage of interacting with N and P proteins of BC in which N protein potentially interacts with the envelope associated proteins through M protein. Conversely, in rGBT, the group with the combination of BC N, P and L genes showed little decrease in virulence compared to the L gene alone group, with the latter showing increased mortality gradually in the course of the experiment, suggesting that similar protein interactions are possible. However, the N, and P genes and the trailer sequence of BC did not alter the pathotype of the rGBT viruses, suggesting that they might have a minimal role in the virulence.

In summary, among the polymerase complex-associated genes and the trailer sequence, the L gene of GBT increased the virulence of the rBC indicating its role in replication, pathogenesis, and virulence in chickens. The N and P genes and trailer sequences did not alter the pathotypes of the recombinant BC and GBT viruses, indicating their minimal role in the replication, pathogenesis, and virulence of NDV. In the future, the amino acids responsible for the virulence in L protein could be characterized.

Chapter 7:

7.1 Title

Conclusions and Prospects

7.2 Conclusions and Prospects

Newcastle disease virus (NDV) is a very important disease of poultry which causes significant economic losses worldwide. There are numerous strains being isolated all over the world across many bird species. NDV, also called as avian paramyxovirus 1 (APMV-1), is a member of genus *Avulavirus* in the subfamily *Paramyxovirinae* under family *Paramyxoviridae*. NDV is a large, enveloped virus with a single molecule of negative stranded RNA as genome. The genome has six genes in the order, 3'Leader-N-P-M-F-HN-L-5'Trailer. There are at least three genome size categories present namely, 15,186, 15,192 and 15,198 nucleotides (nt) in length. The larger sizes of the genome are due to the presence of nt insertions of 6-nt and 12-nt in 5' non coding region (downstream) of N gene and ORF of P gene, respectively.

NDV strains display a spectrum of virulent phenotypes starting from viruses that cause mild and inapparent infection to 100% mortality. Virulence of NDV is a multigenic phenomenon and fusion protein cleavage is one of the major determinants of virulence. In this study, the effects of naturally occurring insertions in NDV genome size and the roles of membrane associated (M, F and HN) and the polymerase complex associated (N, P and L) protein genes were evaluated. We found that the naturally occurring insert sequences on the backbones of rBC and rGBT, attenuated the parental viruses in both

cases in replication and pathogenesis. Regarding the NDV genes, F gene is the major determinant of virulence followed by the L gene compared to other genes. Based on the outcome of this study, three major directions that can be pursued in the future, are given below:

1. **Candidate marker insertion site in the P gene of NDV:** It was observed that the 6- and 12-nt insert sequences were successfully retained in the genomes of rBC and rGBT. This provides information that these insertion sites can be potential insert sites for foreign sequences. The 12-nt insert site in the P gene ORF can be used as a site for diagnostic markers. This could be a potential site for the insertion of markers that could be useful in differentiating a vaccine virus strain with that of a field strain.
2. **BC minigenome:** We established BC minigenome to test the significance and functionality of the P protein with the four amino acids insertion. It was observed that the P with 4 aa insert functioned quantitatively similar to the parental P protein in the BC minigenome CAT reporter assay. This minigenome system can be of use in the future to determine the minimum regulatory sequences required for replication and transcription in NDV; the amino acids important for the functions of N, P and L proteins, in minigenome replication. The determination of the amino acid residues that is responsible for the increased minigenome replication would be useful to improve the efficiency of viral polymerase of potential vaccine virus vector candidates of closely related avian paramyxoviruses.

3. **F protein as the virulence determinant in NDV:** The reciprocal swapping of genes between rBC and rGBT revealed that the F protein played a major role in the virulence of NDV. In the future, studies can be targeted to examine the individual domains of the F protein and the amino acid residues that differ between the strains BC and GBT to figure out the molecular determinants of F protein in NDV virulence.

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