Pathological Study on the Pathogenesis of the Pancreatic and Renal Lesions in Chickens after Experimental Infection with the 9a5b Newcastle Disease Virus Mutant Isolate

高病原性変異ニューカッスル病ウイルス（9a5b）の実験感染による膵臓病変および腎臓病変の病理発生に関する病理学的研究

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Thesis submitted to the United Graduate School of Veterinary Science at Yamaguchi University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Contents

Abstract

Newcastle disease virus (NDV) infection in bird usually causes systemic infection and widespread pathological lesions in most of body organs. Pancreatic and renal pathologic lesions are usually reported during NDV infection in several avian species for example chicken, turkey, pigeon, goose and double-crested cormorants. Until now, little is known about pancreatic and renal lesions in NDV infections and the pathogenesis of NDV in the pancreas and kidney of infected chickens remains unclear. Previously, a comparative study between chickens and ducks after experimental infection with 9a5b mutant NDV was performed by our laboratory and the pancreatic and renal lesions were more prominent in chickens than ducks; therefore, we aimed in this study to investigate the pathogenesis of pancreatic and renal lesions in chickens using the same isolate in detail from an early infection phase 6 hours postinoculation (6 hpi) till the late phase of infection 10 days postinoculation (10 dpi). Also, to investigate the pathogenesis of NDV strains that may spontaneously mutate from avirulent to virulent ones. The virulent 9a5b mutant NDV isolate was inoculated intranasally in 32-day-old specific pathogen-free white Leghorn chickens. The pancreas and kidney was grossly examined and both fixed in formalin for histopathological and immunohistochemical investigations. Pancreas was fixed in gluteraldehyde for electron microscopic examinations. The kidney was further examined by RT-PCR and virus titration. In the chicken pancreas, inflammatory changes were observed in the peripancreatic tissue at the early stage of infection (12 hpi) and became more prevalent towards the end of the experiment. Multifocal areas of necrotizing inflammation were detected in the exocrine portion of the pancreas by 5 dpi and became more severe at 10 dpi. The endocrine islets were generally

preserved, but slight degenerative changes were observed at 10 dpi. Immunohistochemistry (IHC), NDV-nucleoprotein (NP) positive signals were detected in the peripancreatic soft tissues (macrophages and other lymphoid cells) by 1 dpi. In the exocrine portion of the pancreas, NDV-NP positive signals were detected at 5 dpi and increased in intensity and distribution by 10 dpi. NDV particles were confirmed in the cytoplasm of exocrine acinar cells by transmission electron microscopy. CD3-postive cells were observed preferentially in the peripancreatic soft tissues earlier than in the pancreatic tissue. Moreover, in comparing with control chicken, insulin immunopostivity was unchanged, except on the last day of experiment, when insulin IHC was slightly reduced. In the kidney study, histopathologically, tubulointerstitial nephritis was detected in both renal cortex and medulla but it was more abundant in the medulla. Nephrotropism of the 9a5b NDV isolate in the chicken kidneys was confirmed by IHC, RT-PCR, and virus isolation from an early time after infection (1 dpi). Virus detection was consistent among these three methods and started at 1 dpi, peaked at 5 dpi, and diminished at 10 dpi. Massive degenerative changes and infiltration of CD3 immunopositive cells accompanied replication of the 9a5b NDV isolate in chicken kidneys from 1 dpi.

In conclusion, the 9a5b NDV infection in the chicken pancreas initially induced inflammatory reaction and viral replication in the peripancreatic soft, and spread to the pancreatic parenchymal tissue. Furthermore, necrosis affected mainly the exocrine portion of the pancreas while the endocrine portion was generally unaffected by the NDV infection. In the chicken kidney, the 9a5b NDV mutant isolate had showed a nephrotropism to the chicken kidney from an early time after infection (1 dpi). Tubulointerstitial nephritis was more severe in the renal medulla than the cortex. Local inflammatory response in

kidney tissues may play an important role in suppressing viral replication. Pathological changes that were caused by NDV in the chicken kidneys were similar to those caused by avian influenza virus, infectious bronchitis virus, and avian nephritis virus and this highlights the importance of including ND in the differential diagnosis of kidney disease in chickens.

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications:

El-Bahrawy, A., Zaid, A., Sunden, Y., Sakurai, M., Ito, H., Ito, T., Morita, T. Pathogenesis of Pancreatitis in Chickens after Experimental Infection with 9a5b Newcastle Disease Virus Mutant Isolate. *Journal of Comparative Pathology*, 2015;153(4):315-23.

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LIST OF ABBREVIATIONS

APMV	:Avian Paramyxoviruses
CD3	:Cluster Differentiation 3
cDNA	:Complementary DNA
dpi	:Days postinoculation
EID_{50}	:50% Egg Infectious Dose
F	:Fusion Protein
H_2O_2	:Hydrogen Peroxide
HE	:Hematoxylin and Eosin Stain
HN	:Hemagglutinin-Neuraminidase Protein
hpi	:Hours postinoculation
Hrs	:Hours
ICPI	:Intracerebral Pathogenicity Index
IHC	:Immunohistochemistry
IVPI	:Intravenous Pathogenicity Index
L	:RNA Polymerase Protein
M	:Matrix Protein
MDT	:Mean Death Time
mm	:Millimeter
ND	:Newcastle Disease
NDV	:Newcastle Disease Virus
NDV-NP	:Newcastle Disease Virus Nucleoprotein
ng	:Nanogram
nm	:Nanometer
NP	:Nucleoprotein

OIE	:Office International des Epizootics
P	:Phosphoprotein
RNP	:Ribonucleoprotein Complex
RT-PCR	:Reverese Transcriptase Polymerase Chain Reaction
SPF	:Specific Pathogen Free
TEM	:Transmission Electron Microscopy
Vol	Volume
wt	Weight

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INTRODUCTION

Newcastle disease virus (NDV) is the causative agent of Newcastle disease (ND). NDV is a single-stranded, nonsegmented, negative-sense enveloped RNA virus (de Leeuw and Peeters, 1999; Dortmans *et al*., 2011). The genome of NDV encodes six structural proteins: nucleoprotein (NP), fusion protein (F), RNA polymerase protein (L), matrix protein (M), hemagglutinin-neuraminidase protein (HN), and phosphoprotein (P) (de Leeuw and Peeters, 1999; Dortmans *et al*., 2011). Cleavability of F protein gene is an important factor that influences tissue tropism and viral spread. Based on F protein gene cleavability by cellular proteases, NDV strains differ in their virulence (Gotoh *et al.,* 1992; Murphy *et al.,* 1999; Nagai, 1995). Also, the HN gene (De Leeuw et *al*., 2005; Dortmans *et al*., 2011; Huang *et al.,* 2004; Wakamatsu *et al.,* 2006b) and the P gene (Wakamatsu *et al.,* 2006b) have been identified as key players in NDV virulence. According to the World Organization for Animal Health (Known as Office International des Epizootics; OIE), NDVs can be categorized into strains of high virulence (velogenic), moderate virulence (mesogenic), and low virulence (lentogenic). In chickens, the velogenic strains have viscerotropic form (characterized by marked lesions in the gastrointestinal tract and high mortality) and neurotropic form (characterized by respiratory and neurologic signs with high mortality). Mesogenic strains cause acute

respiratory disease but with low mortality. Lentogenic strains induce mild respiratory infections (OIE, 2012).

The pathogenicity of NDV isolate can be determined using the intracerebral pathogenicity index (ICPI) in 1-day-old chicks. The virulent viruses will give scores close to 2.0, whereas lentogenic strains will give scores close to 0.0 (OIE, 2012). The clinicopathological characters of NDV infections are varied and depend on many factors, including the method of infection, host, and virus strain (Alexander, 2003).

The F protein cleavage site sequence of avirulent strains is ^{112}(G/E)(K/R)Q(G/E) RL^{117} which is only susceptible to trypsin-like enzymes found in the respiratory and digestive tissues, causing localized infection. While, the F protein cleavage site sequence of virulent strains is ^{112}(R/K)-R-Q-(R/K) RF^{117} which is susceptible to ubiquitous intracellular host proteases, enabling them to induce systemic infection (de Leeuw *et al*., 2005; Glickman *et al*., 1988; Miller and Koch, 2013; Toyoda *et al*., 1987). Avirulent wild waterfowl viruses, having the typical avirulent fusion protein cleavage site sequence, have the potential to become velogenic after passage in chickens (Shengqing *et al*., 2002; Tsunekuni *et al*., 2010). The virulent 9a5b NDV mutant isolate was generated from the lentogenic Goose/Alaska/415/91 strain by nine consecutive passages in chicken air sacs, followed by five passages in

chicken brain (Shengqing *et al*., 2002). The original strain has an ICPI of zero and avirulent F protein cleavage site compared with the 9a5b NDV mutant isolate that has an ICPI equal to 1.88 and a virulent F protein cleavage site (Shengqing *et al*., 2002). Furthermore, a genetic comparison between the original lentogenic Goose/Alaska/415/91 and the 9a5b NDV mutant isolate demonstrated that 3 amino acid substitutions in the HN proteins, causing change in the inactive HN_0 precursor (found in avirulent viruses) to biologically active HN protein (found in virulent viruses and are responsible for the virus attachment to the host cell and promotes F protein fusion activity) (Huang *et al*., 2004; Lamb and Kolakofsky, 2001; Robert, 1993; Takimoto *et al*., 2002; Tsunekun *et al*., 2010).

Pancreatic and renal pathologic lesions are usually reported during NDV infection in several avian species for example chicken, turkey, pigeon, goose and double-crested cormorants. However, little is known about pancreatic and renal lesions in NDV infections and the pathogenesis of NDV in the pancreas and kidney of infected chickens remains unclear. Previously, a comparative study between chickens and ducks after experimental infection with 9a5b mutant NDV was performed and the pancreatic and renal lesions were more prominent in chickens than ducks (Anis *et al*., 2013a); therefore, we aimed in this study to investigate the pathogenesis of pancreatic and renal lesions in

chickens using the same isolate in detail from an early infection phase (6 hpi) till the late phase of infection (10 dpi). Also, to investigate the pathogenesis of NDV strains that may spontaneously mutate from avirulent to virulent ones.

Review Of Literature

2.1. Newcastle disease history

In 1926, the first official outbreak of ND was reported in Java, Indonesia (Kraneveld, 1926). In 1927, the disease was reported in Newcastle-upon-Tyne, England (Doyle, 1927), from where the name "Newcastle disease" was originated (Doyle, 1933). Many authors believed that ND was presented before its official documentation for example, outbreaks in Central Europe (Halasz, 1912), Korea in 1924 (Levine, 1964), Ranikhet, India (Edwards, 1927) and in the Western Isles of Scotland in 1896 (Macpherson, 1956). ND has spread throughout the world within a few years (Senne *et al*., 1983; Spradbrow, 1988) and continues to emerge in both endemic and epidemic forms (Brown *et al*., 1999).

Since the first recognition of the disease, four panzootics of NDV had occurred (Alexander, 2001). Southeast Asia was the location of the first panzootic and this panzootic extended slowly through Asia to England and Europe in 1926 and then worldwide spread has occurred by the early1960s. By the late 1960s, the second panzootic started in the Middle East and most countries infected by 1973 (Within 4 Years) (Hanson, 1972). The increase in the international trade of poultry and caged psittacine species and poultry industry development are among the reasons which have facilitated the spread of the second panzootic (Francis, 1973; Walker *et al*., 1973). This panzootic has entered California, USA through caged birds (Francis,

1973; Hanson, 1972). The third panzootic has occurred in the late 1970s and was recognized from the antigenic and genetic analysis of NDV, which indicated that probably there is a spread of a third virulent virus different from the other 2 panzootic. In comparing with the previous two panzootic, the start and spread of this panzootic were unclear probably due to the universal vaccination against ND (Herczeg *et al*., 2001; Lomniczi *et al*., 1998). The fourth panzootic of ND was occurred in the Middle East in the late 1970s and originated from the pigeons and doves (Kaleta *et al*., 1985). This panzootic was characterized by nervous signs similar to the neurotropic form of ND in chickens but without respiratory signs. By 1981, ND had reached Europe (Biancifiori and Fioroni, 1983) and became a worldwide pathogen.

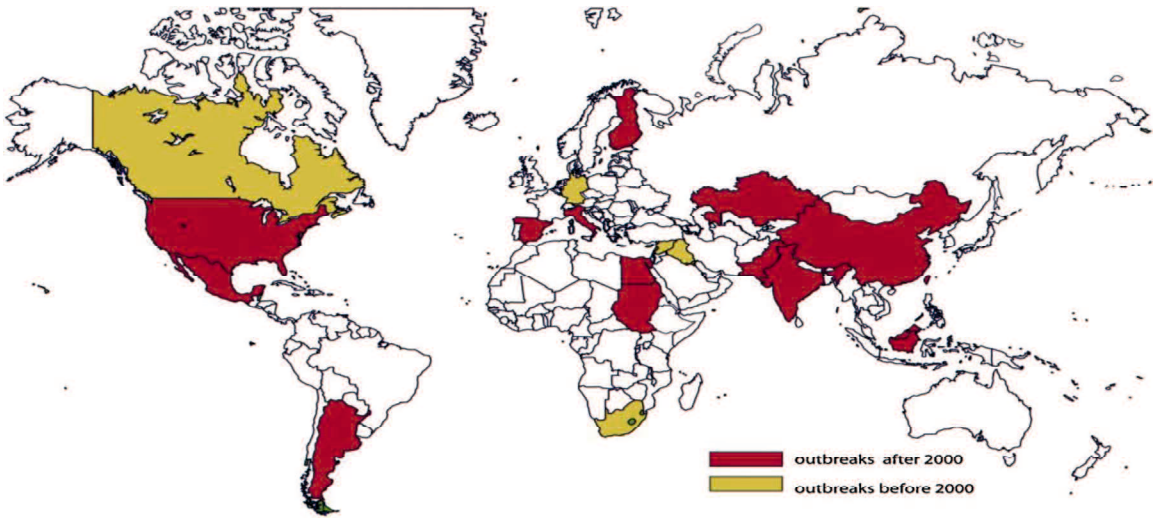


Fig. 1: The global scenario of NDV outbreaks in the world (Ganar *et al*., 2014).

2.2. Etiology of Newcastle Disease

Newcastle disease virus (NDV) is the causative agent of Newcastle Disease (ND) (Brown *et al*., 1999). NDV is a member of the order Mononegavirales in the family Paramyxoviridae, genus Avulvirus (Wise *et al*., 2004). NDV is a nonsegmented, single-stranded, negative-sense enveloped RNA virus (de Leeuw and Peeters, 1999; Dortmans *et al*., 2011). Newcastle disease virions or Avian paramyxoviruses virions are pleomorphic, filamentous and/or round in shape (Alexander *et al*., 1983a; Briand *et al*., 2012; Yamamoto *et al*., 2015; Yusoff and Tan, 2001) with a diameter ranged from 100 to 500 nm (Miller and Koch, 2013; Yusoff and Tan, 2001). The viral genome encodes six structural proteins: nucleoprotein (NP), fusion protein (F), RNA polymerase protein (L), matrix protein (M), hemagglutinin-neuraminidase protein (HN), and phosphoprotein (P) (Fig. 2).

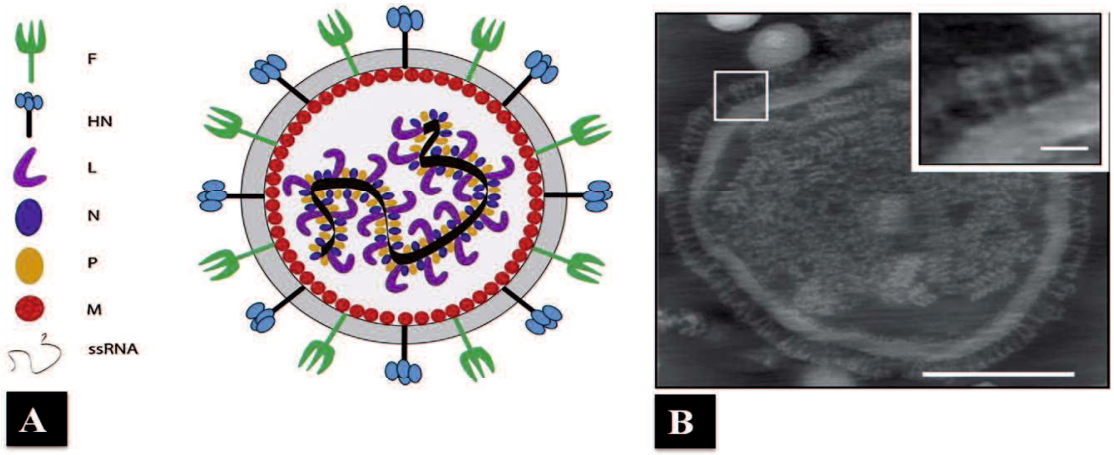


Fig. 2: NDV structure: A. Schematic diagram of Newcastle disease virus structure (Ganar *et al*., 2014); B. Negative staining and electron tomographic analysis of Newcastle disease virus. 44-nm thick digital section taken at the level of the envelope. Bar = 100 nm. Inset; Glycoprotein protruding from the envelope. Bar = 10 nm (Miller and Koch, 2013).

The viral nucleocapsid has a diameter of about 18 nm and 1 mm in length (Alexander *et al*., 1983a; Briand *et al*., 2012; Yusoff and Tan, 2001) and is formed by three proteins; NP to which the P and L proteins connected forming the herringbone-like ribonucleoprotein complex (RNP) (Lamb and Parks, 2007; Yusoff and Tan, 2001). The L protein is the largest structural protein of NDV (Yusoff *et al*., 1987) and its precise functions is unknown (Yusoff and Tan, 2001) but it can contribute with the P protein in synthesis of the virus RNA (Hamaguchi *et al*., 1983). The P gene encodes two further proteins V and W by the RNA editing phenomenon (Fig. 3) which means to add one or two G at the RNA editing site (Catolli *et al*., 2011; Lamb and Parks, 2007; Steward *et al*., 1993). V and W proteins are present only in the virus infected cells (Ganar *et al*., 2014). The virus attachment and fusion to the host cell membrane is a responsibility of the two surface glycoproteins (F and HN). Also, the F and HN proteins have the ability to induce protective immunity (Meulemans *et al*., 1986; Nagy *et al*., 1991). The F protein has trimer spikes whereas the HN protein has tetramer spikes (Ganar *et al*., 2014) and the spikes length is varied from 8 nm to 10 nm (Lamb and Parks, 2007; Yamamoto *et al*., 2015). The M protein is a hydrophobic non-glycosylated matrix protein (Yusoff and Tan, 2001) located under the virus membrane and is responsible for virus assembly and budding (Pantua *et al*., 2006).

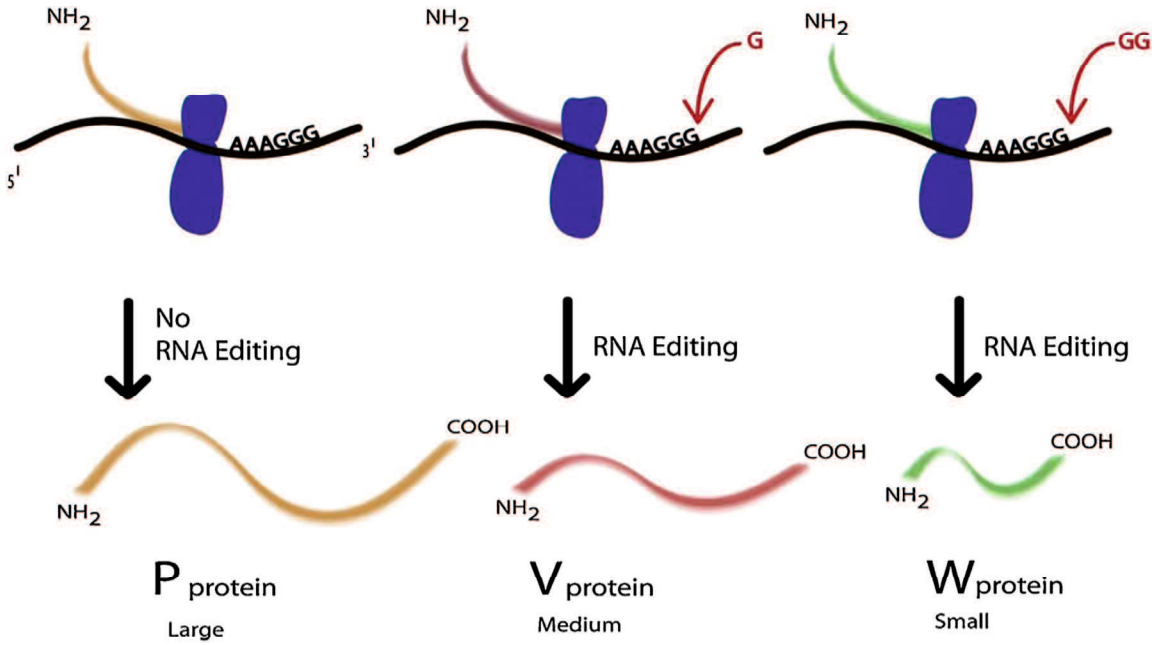


Fig. 3: Newcastle disease virus RNA editing Phenomenon; incorporation of single G or two G residues at the RNA editing site could give rise to V or W, respectively (Ganar *et al*., 2014).

NDV strains are classified into two classes, class I and class II. Class I includes mildly virulent viruses circulating among wild birds in the US live-markets (Seal *et al*., 2005), while class II includes 16 genotypes with varying degree of virulence (Kapczynski *et al*., 2013). The viral genome (Fig. 4) of class I and class II viruses consists of 15,198 and 15,186 or 15,192 nucleotides, respectively (Czeglédi *et al*., 2006).

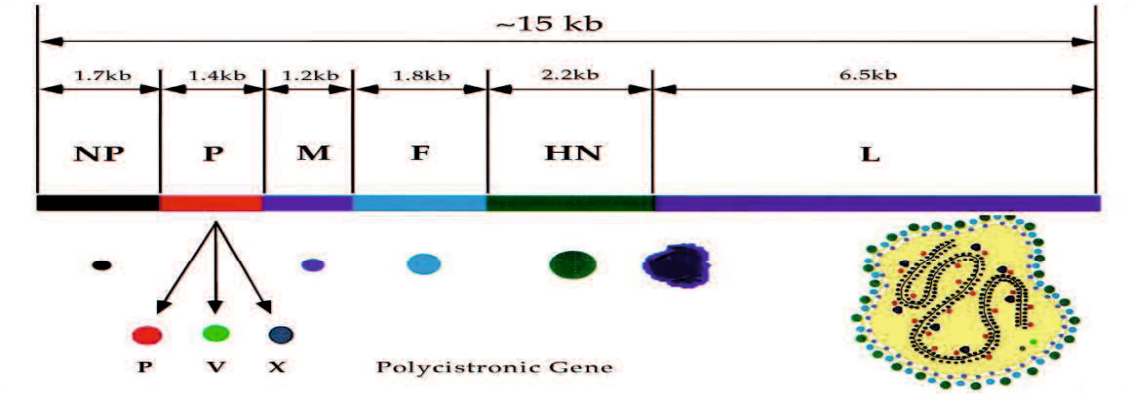


Fig. 4: Newcastle disease virus genomic organization (Seal *et al*., 2000).

The "rule of six" (Fig. 5) is a phenomena needed by all NDV isolates for efficient viral replication (Calain and Roux, 1993) where the genomes are in multiple of six nucleotides in the vast majority, if not all NDV isolates (Peeters *et al*., 2000).

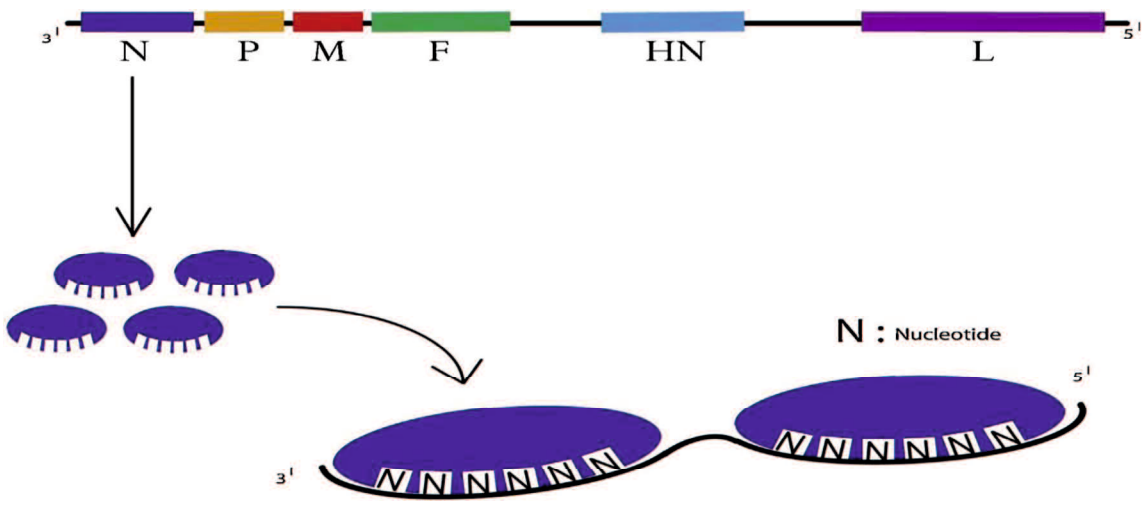


Fig. 5: Schematic representation of Newcastle disease virus genome highlighting the rule of six essentially means that a nucleocapsid protein can bind effectively to six nucleotides. The area between the genes shown in black represents Inter Genomic Sequences that vary in size from 1 to 47 nucleotides (Ganar *et al*., 2013).

2.3. Paramyxovirus serotypes

The paramyxoviruses isolated from avian species are divided into nine serotypes (APMV 1~9) based on haemagglutination inhibition and neuraminidase inhibition assays (Ganar *et al*., 2014). Recently, new serotypes have been identified and paramyxoviruses extended to include 13 known serotypes (Briand *et al*., 2012; Fornells *et al*., 2012; Goraichuk *et al*., 2016; Terregino *et al*., 2013; Yamamoto *et al*., 2015). Summary of APMV serotypes (1-13) is present in Table 3.

NDV is known as avian paramyxovirus serotype-1 (APMV-1) to be distinguished from other serotypes of avian paramyxoviruses (Tumova *et al*., 1979a). NDV is the only well-characterized serotype due to its economic importance and infection to most of avian species (Nayak *et al*., 2012); in contrast to serotype-1, little is known about the other APMV serotypes and their biological characteristics and pathogenicity still remains unclear (Nayak *et al*., 2012). APMV (2~9) usually isolated from wild birds (Mundt, 2013). Complete genome sequences of APMV (2-9) were reported (Kumar *et al*., 2008; Nayak et al., 2008; Paldurai *et al*., 2009; Samuel *et al*., 2009, 2010; Subbiah *et al*., 2008; Xiao *et al*., 2009, 2010). APMV serotypes were classified into specific lineages or clades based on serological (monoclonal antibodies) and phylogenetic studies (Alexander, 2000).

2.4. Newcastle disease virus (APMV-1) Pathotypes

Based on the severity of clinical signs seen in infected chickens (Alexander and Senne, 2008), NDV strains can be divided into five pathotypes (viscerotropic and

neurotropic velogenic, mesogenic, lentogenic and asymptomatic) (OIE, 2012). The velogenic strains are divided into viscerotropic and neurotropic strains; Viscerotropic strains characterized by marked lesions in the gastrointestinal tract and high mortality while, the neurotropic strains characterized by respiratory and neurologic signs with high mortality. Mesogenic strains cause acute respiratory disease but with low mortality. Lentogenic strains induce mild respiratory infections. Asymptomatic usually considered a subclinical enteric infection (OIE, 2012).

These pathotypes are formerly named Hitchner form for Asymptomatic enteric and lentogen, Beaudette form for mesogen, Doyle form for velogenic viscerotropic and Beach form for velogenic neurotropic (Beard and Hanson, 1984). Pathotype classification is not clear-cut because considerable change and overlapping of the clinical signs sometimes observed (Alexander and Allan, 1974).

2.5. Pathogenicity tests

Standard pathogenicity parameters is performed to classify NDV pathotypes and this includes mean death time (MDT), intravenous pathogenicity index (IVPI), and intracerebral pathogenicity index (ICPI).

2.5.1. Mean death time (MDT): In this assay, the time of chick embryo death is recorded after inoculation of the allantoic sac. The higher NDV virulence is the shorter the MDT. Lentogenic viruses have an MDT greater than 90 hours (hrs), mesogenic strains have an MDT of 60-89 hrs, whereas the velogenic isolates have an MDT of less than 60 hrs (Hanson and Brandly, 1955).

2.5.2. Intravenous pathogenicity index (IVPI): This test is performed by intravenous inoculation of 6-week-old chickens with infective allantoic fluid (0.2 ml/ bird) and observing birds daily for 10 days. After inoculation, numbers of healthy, sick, paralyzed and dead birds were used to calculate the IVPI of an isolate. The IVPI values ranged from 0 to 3.0; velogenic strains have IVPI approach 3.0, while lentogenic strains and some mesogenic strains have IVPI values of zero.

2.5.3. Intracerebral pathogenicity index (ICPI): This test is performed in chicks over 24-hrs and under 40-hrs old at the time of inoculation. 0.05 ml of the fresh infective allantoic fluid with a haemagglutinin titer greater than 16 and diluted in 1/10 sterile isotonic saline are injected intracerebrally in each of 10 chicks. The birds are examined daily for 8 days and scored: 0 if normal, 1 if sick, and 2 if dead. The ICPI is the mean score per bird per observation over the 8-day period. The virulent viruses will give scores close to 2.0, whereas lentogenic strains will give scores close to 0.0 (OIE, 2012).

In the past, intravenous pathogenicity test and mean death time were used to characterize NDV virulence (Alexander and Senne, 2008), but now no longer used and the ICPI is the official recognized test by the World Organization for Animal Health (OIE). Newcastle disease has to be reported if the virus has an ICPI greater than 0.7 or have multiple basic amino acids at the fusion cleavage site (molecular basis for NDV isolate pathogenicity) (OIE, 2012).

2.6. Molecular basis for NDV isolate pathogenicity

Genome sequence of NDVs is now the most convenient step performed by many laboratories to characterize NDV strains (Miller and Koch, 2013).

Envelope of NDV has a surface glycoprotein called the F protein which is a key player in NDV virulence, enabling the virus to fuse with the host cell membrane (De Leeuw *et al*., 2005; Panda *et al*., 2004; Romer-Oberdorfer *et al*., 2003). The F protein is present as inactive precursor (F0) and must be cleaved by the host proteases to active forms (F1 and F2) for the virus particles to be infectious (Ganar *et al*., 2014; Scheid and Choppin, 1974). The F protein cleavage and activation does not require acidic pH (Ganar *et al*., 2014).

The cleavage specificity is determined by the amino acid sequence present at the cleavage site and varies with the type of the strain (Glickman *et al*., 1988; Toyoda *et al*., 1987). The F protein cleavage site sequence of avirulent strains has a monobasic amino acid sequence motif at the C-terminus of the F2 protein and a

leucine at the N-terminus of the F1 protein ^{112}(G/E)(K/R)Q(G/E) RL^{117} which is cleaved by trypsin-like enzymes found in certain tissues such as the respiratory and digestive tissues, causing localized infection. While, the F protein cleavage site sequence of virulent strains has a multibasic amino acid sequence motif at the C-terminus of the F2 protein and a phenylalanine at the N-terminus of the F1 protein ^{112}(R/K)-R-Q-(R/K) RF^{117} and is cleaved by ubiquitous intracellular host proteases, enabling them to induce systemic and fatal infection (de Leeuw *et al*., 2005; Glickman *et al*., 1988; Miller and Koch, 2013; Toyoda *et al*., 1987).

Pathogenicity indices have some drawbacks and can be criticized (Cattoli *et al*., 2011; Dortmans *et al*., 2011). For example, strains that have been classified as virulent strains based on their pathogenicity indices and F protein cleavage site (Susta *et al*., 2011; Wakamatsu *et al*., 2006a) but does not produce severe clinical disease (Anis *et al*., 2013a; Dortmans *et al*., 2011, Susta *et al*., 2011; Wakamatsu *et al*., 2006a). These drawbacks usually rose with strains isolated from species other than chickens (Anis *et al*., 2013a; Pearson *et al*., 1987). Also, intracerebral inoculation, not being the natural way of infection, may lead to a difference in ICPI value when compared with the natural route of infection (Dortmans *et al*., 2011) and NDV virulence is multigenic and the F protein gene is not the only key player in NDV virulence (De Leeuw et *al*., 2005; Dortmans *et al*., 2011; Huang *et al.,* 2004; Wakamatsu *et al.,* 2006b). (Cattoli *et al*., 2011) reported that “A more accurate

indication of the true pathogenicity of ND viruses for a susceptible species could come from experimental infection of a statistically significant number (≥10) of young and adult birds with a viral standard dose (e.g. 10^5 EID_{50}) administered via natural routes (e.g. oro-nasal route)".

2.7. NDV pathogenesis and replication

NDV pathogenesis is multigenic and several viral genes have been identified as key players in the pathogenesis such as F, HN, P, NP, L and V proteins (Brown *et al*., 1999; De Leeuw *et al*., 2005; Dortmans *et al*., 2010, 2011; Huang *et al*., 2003; Huang *et al*., 2004; Romer-Oberdorfer *et al*., 2003; Wakamatsu *et al*., 2006b). NDV is a negative sense RNA virus and has the same replication strategy followed by the vast majority of negative sense RNA viruses (Lamb *et al*., 2005). NDV replication cycle is the fastest among parmyxoviruses with viral yields peaked within 12 hpi (Hightower and Bratt, 1974).

Replication process started with the virus attachment to the host cell (Ganar *et al*., 2014; Hines and Miller, 2012; Yusoff and Tan, 2001). All viral replication events occur within the host cell cytoplasm (Hines and Miller, 2012). NDV infection occurs primarily via pH-independent pathway and sometimes by receptor mediated endocytosis or caveolae-dependent endocytosis (Ganar *et al*., 2014).

NDV attaches through its surface protein HN to the cell receptors such as sialic acid residues (gangliosides and N-glycoproteins receptors) (Ganar *et al*., 2014;

Hines and Miller, 2012; Yusoff and Tan, 2001). Actual viral receptors on the host cells are still unknown (Yusoff and Tan, 2001). Due to the attachment of the HN protein to the host membrane some conformational changes may occur, allowing the activation and exposure of the F protein to the host membrane and then successful virus cell fusion (Hines and Miller, 2012; Yusoff and Tan, 2001).

After fusion, the M proteins detached from the nucleocapsid by unknown mechanism to free the viral nucleocapsid into the cell cytoplasm and begin the replication and transcription process (Yusoff and Tan, 2001). NDV genome is negative-sense RNA, so the L proteins alone (Hines and Miller, 2012) or in combination with the P protein act as the viral RNA polymerase (Yusoff and Tan, 2001), helping the negative genomic RNA to transcribe to the positive sense mRNA which then translates into viral proteins (Ganar *et al*., 2014). The positive sense RNA is then used as a template for the synthesis of negative sense genomic RNA (Ganar *et al*., 2014). The HN0 and F0 glycoproteins, are synthesized in the rough endoplasmic reticulum, whereas the viral structural proteins (NP, P, L and M) and the non-structural proteins V and W are produced in the cytoplasm (Yusoff and Tan, 2001). The glycoproteins undergo some post-translational modifications and then transported across the endoplasmic reticulum and Golgi apparatus (Yusoff and Tan, 2001). N, P and L proteins are essential for nucleocapsid assembly (Ganar *et al*., 2014). The nucleocapsid proteins align within the new membrane to form the RNP

complex (Hines and Miller, 2012) and then transported to the plasma membrane (Yusoff and Tan, 2001). The M protein is essential for mature NDV assembly and budding from the host cell membrane (Ganar *et al*., 2014; Yusoff and Tan, 2001). The replication process is summarized in Fig 6.

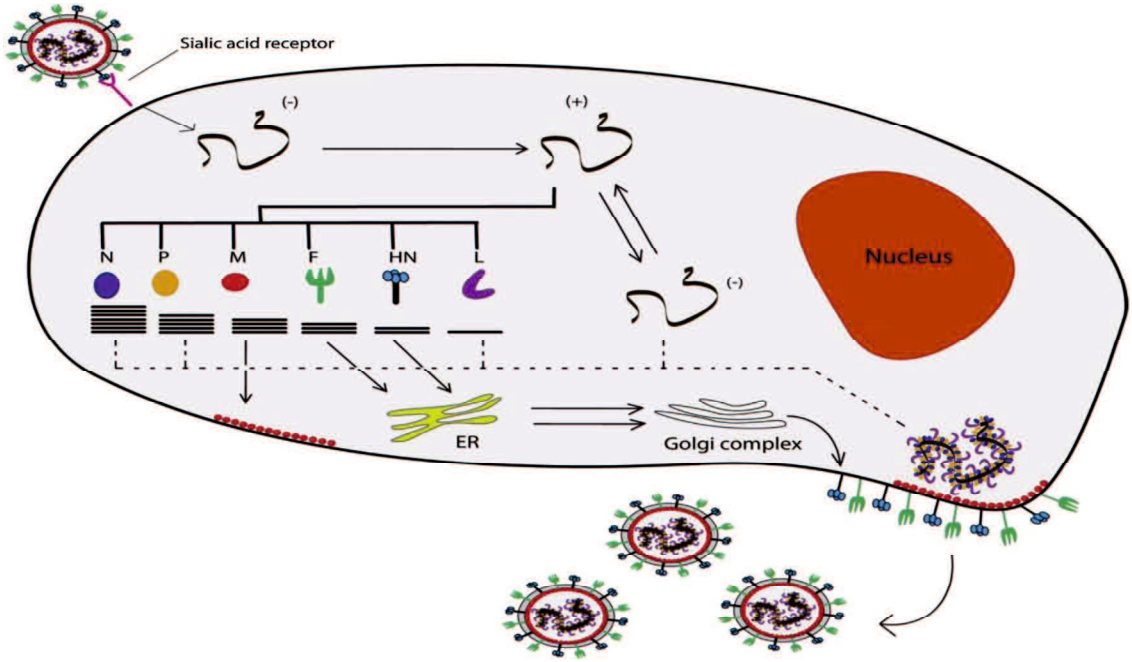


Fig. 6: Schematic representation of Newcastle disease virus replication. HN protein attaches to sialic acid receptors on cell surface, resulting in fusion. The negative sense viral RNA is transcribed to produce the structural mRNAs, with the help of RNA dependent RNA polymerase. The negative sense RNA is converted to positive sense template for the synthesis of new negative sense RNA genome. This newly formed genomic RNA is then wrapped in N, P and L proteins to form the nucleocapsid that is assembled with matrix and surface glycoproteins and released from the host cell (Ganar *et al*., 2014).

2.8. NDV transmission

2.8.1. Sources of NDV particles

Birds infected with NDV usually shed the virus particles in their feces, oropharyngeal secretions (Lee *et al*., 2016; Samuel *et al*., 2012; Susta *et al*., 2014) and skin feathers (Lee *et al*., 2016).

2.8.2. Type of NDV transmission

Horizontal and vertical transmission of NDV has been reported. However, horizontal transmission is more documented and reported in literatures as it is easy for birds to be infected through ingestion, drinking and/or inhalation of materials contaminated with NDV particles (Miller and Koch, 2013). Examples of horizontal transmission were reported such as eating of contaminated food by diseased pigeons (Alexander *et al*., 1984) or oral application of live freeze-dried vaccine (Thekisoe *et al*., 2004) and finally air borne transmission occurs through inhalation of virus aerosols (Li *et al*., 2009) or live virus vaccine (Mazija *et al*., 2010).

Vertical transmission or transmission of NDV from parents to their offspring is rarely reported. This way of transmission is still questionable due to the possibility of infection by contaminated feces through broken or cracked eggshell and/or exposure newborn chick to a virus contaminated materials (Miller and Koch, 2013). Another possibility is with high virus titer in embyonated eggs, a prompt embryo death and no vertical transmission will occur (Chen and Wang, 2002). However,

vertical transmission and NDV tropism for the reproductive tract have been reported (Biswal and Morril, 1954; Bwala *et al*., 2011; Silva *et al*., 2016). Isolation of NDV have been documented in eggs (Lancaster, 1963), embryonated eggs after vaccination (Bivins *et al*., 1950; Capua *et al*., 1993; Prier *et al*., 1950) and from breeder hen (Roy and Venugopalan, 2005). Also, vaccine strain has been detected in the reproductive tract (Raszewska, 1964; Silva et al., 2016). Moreover, vaccination of chicken has decreased internal egg contamination with NDV (Silva *et al*., 2016).

2.8.3. Factors contribute to the widespread of NDV

There are some factors help the massive spread of NDV between flocks such as movement of live birds including exotic, feral, game and racing pigeons, movement of farm equipments, poultry products, and mechanical transmission of the virus through contact with farm workers and vaccines (Burridge *et al*., 1975; Alexander, 2000). With open rearing systems, the chance of infection is more through contact with feral birds, while in closed rearing systems the chance of contact with feral birds and infection is less (Lancaster and Alexander, 1975).

2.9. Virus hosts

It is generally accepted that all birds are susceptible to NDV based on a report that NDV has been documented in at least 241 species of birds (Kaleta and Baldauf, 1988). Chickens are by far the most susceptible among birds to infection, while other

birds are resistant and does not produce much clinical signs such as duck (Higgins, 1971).

2.10. Incubation period

The clinicopathological characters of NDV infections are varied and depend on many factors, including the host species, age, host immunity, virus strain, dose and method of infection (Alexander, 2003; Cattoli et al., 2011). Natural infections have average incubation period of 6 days but in other cases incubation period can be extend to 15 days. With more virulent strains, infection with viscertropic velogenic NDV has a short incubation period of 1-4 days (Miller and Koch, 2013).

2.11. Clinical signs

The severity of clinical signs widely differs and depends on the virulence of the virus and the host immune status, route of infection and age. Younger birds usually manifest more severe and acute disease than older one (Cattoli *et al*., 2011). NDV strains can be divided into five pathotypes (viscerotropic and neurotropic velogenic, mesogenic, lentogenic and asymptomatic) based on the severity of clinical signs seen in infected chickens (OIE, 2012). Neither the clinical signs nor the gross lesions are pathognomonic for ND (Miller and Koch, 2013).

With highly virulent viruses, the disease may appear suddenly with high mortality and absence of the clinical signs (Miller and Koch, 2013). Other studies described the clinical signs with experimental infection of viscerotropic velogenic strains as

started 2 dpi and include the following; mortality can reach to 100%, anorexia, weakness, green diarrhea, conjunctival swelling, ruffled feathers, tremors, prostration (Brown *et al*., 1999; Kommers *et al*., 2002, 2003), respiratory signs were observed very rarely (reviewed in Cattoli *et al*., 2011).

For velogenic neurotropic form of ND, it is mainly reported in the United States (Alexander and Senne, 2008) and has longer disease course than with velogenic viscerotropic form (Cattoli *et al*., 2011). The disease has 100% morbidity and up to 50% mortality and started with sudden onset of severe respiratory disease followed by the neurological signs (Alexander and Senne, 2008). The clinical signs are most prominent between 5 and 10 dpi and include the following; tremors, opisthotonus, head twitch, and paralysis (Brown *et al*., 1999; reviewed in Cattoli *et al*., 2011) and diarrhea is not always observed (Alexander and Senne, 2008).

The clinical signs of mesogenic strains of NDV in field infection are respiratory signs and drop in egg production (Alexander and Senne, 2008). Mortality rate is low except in very young and compromised birds when secondary infections are common complications of mesogenic NDV infections and result in more severe morbidity (Bhaiyat *et al*., 1994; El Tayeb and Hanson, 2002; Nakamura *et al*., 1994).

The clinical signs are not observed in previous experimental infection with lentogenic viruses (Brown *et al*., 1999; Hamid *et al*., 1990) except in cases with very young birds (Cattoli *et al*., 2011). On the other hand, in one report mild clinical signs

were observed in 40-day-old specific pathogen-free (SPF) chickens when a lentogenic strain was aerosolized with high concentration (Kotani *et al*., 1987).

2.12. Gross pathology of NDV

The presence of hemorrhages and necrosis in the intestinal wall and/or necrosis and hemorrhage in the gut-associated lymphoid tissue are pathogonomic for velogenic visceotropic NDV strains (Brown *et al*., 1999; Hanson *et al*., 1973; Kommers *et al*., 2002, 2003; Susta *et al*., 2011). Cecal tonsils are one of the prominent lesions in visceotropic velogenic NDV strains and usually have hemorrhage and necrosis (Cattoli *et al*., 2011). Enlarged and mottled spleens, severe atrophy of thymus and bursa, perithymic hemorrhages and multifocal hemorrhages and ulceration in the junction between proventriculus and gizzard are usually observed with viscerotropic strains (reviewed by Cattoli *et al*., 2011).

For velogenic neurotropic NDV strains, gross lesions are absent in the central nervous system and no characteristic gross lesions have been described (Brown *et al*., 1999).

Mesogenic strains have minimal lesions such as mild splenomegaly and some degree of conjunctivitis (Brown *et al*., 1999).

Lentogenic strains produce minimal or no gross lesions. In one report mild pulmonary hemorrhages and splenomegaly have been observed with QV4 strain

(Hamid *et al*., 1990), on the other hand another experiment with the QV4 does not induce any gross lesions (Brown *et al*., 1999).

2.13. Histopathology of NDV

The lymphoid organs (spleen, thymus, bursa and cecal tonsils) are the target organs for NDV pathology. Several studies have addressed these pathologic changes in the lymphoid organs and varied between lymphoid depletion, necrosis, sometimes apoptosis and fibrin deposition (Anis *et al*., 2013b; Brown *et al*., 1999; Harrison *et al*., 2011; Susta *et al*., 2011).

2.13.1. Other organs

The brain is mainly involved in infection with velogenic neurotropic NDV strains and has multifocal perivascular cuffing, neuronal degeneration, moderate gliosis and hypertrophy of the endothelial cells (Brown *et al*., 1999; Kommers *et al*., 2003; Susta *et al*., 2011; Wilczynski *et al*., 1977). With velogenic viscerotropic NDV, perivascular cuffing has been reported (Brown *et al*., 1999; Kommers *et al*., 2002, 2003).

The heart has myocarditis (Susta *et al*., 2011) associated with myofibers disruption and infiltration of macrophages and lymphocytes (Brown *et al*., 1999; Kommers *et al*., 2003). Vascular changes have been reported with velogenic neurotropic NDV and include hyalinization, hyaline thrombosis in small vessels and hydropic degeneration of the blood vessels media (Nakamura *et al*., 2004). These vascular

changes are seen in vessels near to the primary site of infection and are caused by intense exudation of proteins (Cattoli *et al*., 2011).

The eye usually has prominent edema, hemorrhage, and necrosis associated with fibrin exudation especially at the site of inoculation (Brown *et al*., 1999; Nakamura *et al*., 2004; Susta *et al*., 2011).

The gastrointestinal tract usually has ulceration and necrosis mainly in the area of gut lymphoid tissues such as the cecal tonsils (Brown *et al*., 1999; Susta *et al*., 2011). Evidence of accumulated necrotic materials in intestinal lumens has been reported (Susta *et al*., 2011). Small focal degeneration and necrosis are observed in the liver, gall bladder, gizzard and proventriculus (Alexander and Senne, 2008)

Several experimental studies with velogenic strains (viscerotropic and neurotropic) concluded that the lung is not involved during infection or has little pathological changes (reviewed by Cattoli *et al*., 2011). Only one report from Japan has described pneumonia in an outbreak with velogenic neurotropic NDV (Nakamura *et al*., 2008).

The reproductive tract of chicken has mild interstitial edema, sporadic glandular ectasia, accumulations of lymphocytes and plasma cells and mild interstitial fibrosis. These changes were more observed in the uterus than in the magnum and the isthmus (Bwala *et al*., 2011).

The pancreas usually has multifocal necrotic areas mainly in the exocrine epithelium in chicken (Alexander, 2003; Anis *et al*., 2013a; Kommers *et al*., 2002, 2003; Susta *et al*., 2011; Wakamatsu *et al*., 2006a;), turkey (Piacenti *et al*., 2006), pigeon (Barton *et al*., 1992; Zanetti *et al*., 2001), and geese (Wan *et al*., 2004).

The kidney usually has multifocal necrosis of the tubular epithelium and mononuclear cells infiltration in chicken (Anis *et al*., 2013a; Courtney *et al*., 2013; Kommers *et al*., 2001), duck (Anis *et al*., 2013a; Njagi *et al*., 2012; Zhang *et al*., 2011), turkey (Piacenti *et al*., 2006), and double-crested cormorants (Kuiken *et al*., 1999).

2.14. Ultrastructural changes in NDV infections

Several authors have performed transmission (TEM) and scanning electron microscopy to describe the ultrastructural changes in organs after infection with NDV. The trachea has been reported to have hypertrophy of goblet cells, excessive globular mucous secretion, increase in the proportion of nonciliated to ciliated epithelium, deciliation and erosions of the mucosal epithelial tracheal surface (Anis *et al*., 2013a; Mast *et al*., 2005). In the lung, after aerosol vaccination, there is decrease or absence of lung surfactant and their producer cells (pneumocytes) lost their cell junction and had apoptotic changes, serofibrinous exudate was observed in most of the air capillaries between the 24 and 72 hrs. Later most of these changes were reversed and lung restored to almost a normal state (Kazachka, 2008). In the

esophagus, intracytoplasmic inclusion body was occasionally observed in the endothelial cells of esophageal blood vessels (Crespo *et al*., 1999). In the heart, shortening of sarcomeres, damaged mitochondrial (ghost-like), hemorrhage with no or little infiltration of inflammatory cells, edema and apoptosis were observed (Lam, 1996b). Blood cells (heterophils, lymphocyte and macrophages) have condensed chromatin, apoptosis (Lam, 1996a,c). In addition, macrophages have the viral particles and lost their phagocytic activity (Lam, 1996c).

AIM OF THE STUDY

NDV infection in bird usually causes systemic infection and widespread pathological lesions in most of body organs. The pancreas is frequently involved during NDV infection and the pathologic lesions in the pancreas have been reported in chickens (Alexander, 2003; Anis *et al.,* 2013a; Kommers *et al.,* 2002; Kommers *et al.,* 2003; Susta *et al.,* 2011; Wakamatsu *et al.,* 2006a), turkey (Piacenti *et al.,* 2006), pigeon (Barton *et al.,* 1992; Zanetti *et al.,* 2001), and geese (Wan *et al.,* 2004). Also, the renal lesions induced by NDV were previously reported in chickens (Anis *et al*., 2013a; Courtney *et al*., 2013; Kommers *et al.,* 2001), duck (Anis *et al*., 2013a; Njagi *et al*. 2012; Zhang *et al*., 2011), turkey (Piacenti *et al.,* 2006), and double-crested cormorants (Kuiken *et al*., 1999). Kidney might be implicated in the virus excretion and possible transmission to other birds (Kuiken *et al*., 1999). Until now, little is known regarding pancreatic and renal lesions in NDV infections and the pathogenesis of pancreatic and kidney damage during NDV infection in birds is still unclear. An experimental infection has been performed by our laboratory to compare the pathogenesis of 9a5b NDV mutant isolate in chickens and ducks and the pancreatic and renal lesions were reported to be more prominent in chickens than ducks (Anis *et al*., 2013a); therefore, we aimed in this study to investigate the pathogenesis of pancreatic and renal lesions in chickens in detail

using the same NDV isolate from an early infection phase (6 hpi) till the late phase of infection (10 dpi). Also, to investigate the pathogenesis of NDV strains that may spontaneously mutate from avirulent to virulent ones.

Table 1: Study design

Twenty-nine (Pancreas study) or twenty- six (Kidney study) 32-day-old male white leghorn SPF chickens were acclimatized for one week and then intranasally inoculated with the 9a5b NDV mutant isolate, and sampled as shown in this table

Inoculation	Chickens*		
	Infected		Control
	Pancreas	Kidney	
6 hpi*	4*	3	
12 hpi	4	3	
1 dpi***	4	3	
2 dpi	3	3	
3 dpi	3	3	
5 dpi	3	3	
10 dpi	3	3	5
Total	24	21	5

*; number of chickens sampled at each time point
**; hour postinoculation
***; days postinoculation

Table 2: Collected tissues samples and their methods of investigations.

Analysis	Tissue samples	
	Pancreas	Kidney
HE*	+	+
NDV-NP IHC**	+	+
CD3 IHC	+	+
Insulin IHC	+	–
TEM***	+	–
Virus titration	–	+
PCR	–	+

+: done; –: not done
*; Hematoxylin and eosin staining
**; Immunohistochemistry

***; Transmission electron microscopy

MATERIALS AND METHODS

1. Virus

The 9a5b NDV mutant isolate used in this study was provided by the department of Veterinary Public Health, Tottori University, Japan. The virus was propagated in SPF eggs and 100 µl of the virus suspension, containing $10^{8.75}$ 50% egg infective dose (EID_{50}) was inoculated by intranasal route in chickens (Shengqing *et al*., 2002).

2. Chickens

Twenty-nine 32-day-old male white leghorn SPF chickens, purchased from Nippon Institute for Biological Science, were divided into 2 groups, control group (5 chickens) and infected group (24 chickens for pancreas study) or (21 chickens for kidney study). Chickens were reared in negative-pressure isolators under biosafety level 2, and were supplied with water and food ad libitum (Fig. A). The chickens were monitored clinically two times every day throughout the experiment. After one week of acclimatization, the 9a5b mutant isolate was inoculated in chickens by intranasal route (Fig B). Four chickens from the infected group were sacrificed at each time point 6 hpi, 12 hpi, and 1 dpi, and three chickens at each time point 2 dpi, 3 dpi, 5 dpi, and 10 dpi. The control-group chickens were sacrificed on the last day of the experiment (10 dpi). The birds were sacrificed under Halothane inhalation anesthesia. All experimental procedures were performed according to Tottori University guidelines for

animal welfare, and the ethics committee of the Tottori University approved the study (http://www.tottori-u.ac.jp/kouhou/kisokusyuu/reiki_honbun/u0950581001.html).

3. Necropsy and sampling

Immediately after sacrificing the birds, necropsy examination was performed and the pancreatic and renal tissues were examined for any gross abnormalities.

3.1. Tissue preservation

Collected tissues were preserved in the proper way that will be used in the analysis.

a. Neutral Buffered Formalin (NBF) fixation

Both of pancreatic and kidney tissues were fixed in 10% NBF for light microscopy and immunohistochemistry (IHC).

b. Gluteraldhyde fixation

Pancreatic tissues were fixed in 2.5% gluteraldehyde for transmission electron microscopy (TEM) investigation.

c. Freezing

Kidney tissues were preserved at -80ºC for RNA isolation and virus titration.

3.2. Histopathology and IHC

After 60 hrs of fixation in NBF, fixed tissues from pancreas and kidney were

processed and embedded into paraffin blocks. Tissue sections of 4-µm thickness were cut for haematoxylin and eosin (HE) and IHC staining.

3.2.1. IHC staining of 9a5b Newcastle Disease Virus-nucleoprotein (NDV-NP).

a. Preparation of NDV-NP primary antibody

The primary NDV-NP antibody was prepared in rabbits according to the method described by Kommers *et al*. (2001). Briefly, a peptide antigen was prepared with the sequence TAYETADESETRRIC, representing residues 181-194 of the NP protein with a Cystein (C) addition for coupling. The peptide was conjugated to keyhole limpet haemocyanin and this conjugate was used to immunize a rabbit. Serum was collected from the immunized rabbit and the immunoglobulin G fraction was purified by affinity chromatography (Hokudo Bioscience, Tokyo, Japan) (Anis *et al*., 2013 a, b).

b. Protocol for NDV-NP IHC in chicken pancreas and kidney

For NDV-NP IHC, 4-µm thickness paraffin tissue sections from pancreas and kidney were dewaxed, rehydrated, treated with 3% H_2O_2 in distilled water for 15 minute (min) at room temperature (RT) to block the endogenous peroxidase activity, and then subjected to antigen retrieval in citrate buffer (pH 5.4) by microwaving at full power for 10 min. Sections were blocked with 10% normal goat serum for 5 min by microwaving at low power without heating and then

incubated with the primary antibody (1:8,000 dilution) overnight at 4ºC (Anis *et al*., 2013 a, b). After stringent washing with phosphate-buffered saline (PBS), sections were incubated with a labeled polymer (ChemMate^{TM} DAKO EnVision^{TM}/HRP [DAP]; Dako, Carpinteria, CA) for 30 min at RT and then subjected to stringent washing. The sections were detected with 3, 3′-diaminobenzidine tetrahydrochloride (Dako), counterstained with haematoxylin and covered with DPX mounting medium (Sigma Life Science, Steinheim, Germany).

3.2.2. Cluster differentiation 3 (CD3) IHC staining in chicken pancreas and kidney

The protocol used for CD3 IHC was similar to the aforementioned protocol, except that the time for antigen retrieval was 20 min and the primary antibody used was polyclonal rabbit anti-human CD3 (Dako) at 1:300 dilution.

3.2.3. Insulin IHC staining in chicken pancreas

For detection of insulin, the time for antigen retrieval was 20 min, the sections were incubated with the primary antibody (polyclonal guinea pig anti-swine insulin; Dako) at 1 : 100 dilution for 10 min with microwaving and biotinylated guinea pig immunoglobulinG (H+L) (Vector laboratories, Burlingame, California, USA) was used as the secondary antibody at 1 in 400 dilution for 5 min with microwaving.

3.3. Transmission Electron Microscopy (TEM) of chicken pancreas

Sampling, trimming and labeling of chicken pancreas; Small parts from the pancreas were cut by a new razor blade (0.25 cm^2) on a colorless plastic plat and add a few drops of 2.5 % glutraldehyde on the sample and fix overnight at 4ºC. Labeling; the samples were added in small well-caped labeled bottle.

The samples were washed in 0.1M PBS 3 times for 5 min each at RT; Post fixed in 1% Osmium Tetraoxide for 1 hr; then washed again in 0.1M PBS (until clear fluid) and dehydrated in ascending grades of alcohol for 20 min each (50%, 70%, 80%, 90%, 95% and 99%) and in absolute alcohol for 1 hr each (I and II). Followed by

1. QY-I (n- Butyl glycidyl ether) 30 min/ on vibrator.
2. QY-II (Methyl glycidyl ether) / 40 min/ on vibrator.
3. QY-I: Epon (2: 1) / 30 min/ on vibrator.
4. QY-I: Epon (1: 2)/ 30 min/ on vibrator.
5. Epon (Quelol- 812)/ 60 min/ on vibrator.
6. Epon/ 60 min or overnight/ on vibrator.
7. Embedding in Epon. →

Preparation of Epon

Epon 812----- Quetol 812
DDSA---------- Dodecenyl Succinic Anhydride
MNA---------- Methyl Nadic Anhydryde
DMP-30-------- dimethylaminomethyl phenol
Mix the first 3 components together in paper cub and mix on magnetic stirrer for 5 min then add the fourth component (DM-30) and continue mixing on the stirrer for about 15 min and the color will changed from dark yellow to reddish.

8. Put the embedded samples at 60ºC for 3 days.
9. Store the hard embedded samples in closed tube with silica gel to absorb the moisture until semi-thin and ultrathin sections preparation and examination.

10. Semi-thin sections (1-μm-thick) were obtained and stained with 1% toluidine blue for light microscopy; representative fields were selected to make ultrathin sections (70 nm).

11. After staining with uranyl acetate and lead citrate, ultrastructural investigation was performed using a transmission electron microscope (TEM-100CX; Japan Electron Optical Laboratory, Tokyo, Japan).

3.4. Virus titration of chicken kidney

Virus titers were determined using 10-day-old embryonated SPF eggs. Briefly, kidney tissues were homogenized in PBS [1:10 (wt:vol)], and then a 10 fold serial dilution of clear homogenate (100 μl) was inoculated into the allantoic cavity of the eggs. After inoculation, the eggs were incubated at 37ºC for 3 days, followed by 1 day at 4ºC. Heamagglutination test was performed and the virus titer was expressed as EID_{50} per gram (Reed and Muench, 1938).

3.5. RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR) from chicken kidney

Total RNA was extracted from kidney tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The amount of RNA was measured by Nanodrop spectrophotometer (ND-1000, Thermo

Scientific, USA). Extracted RNA (500 ng) was reverse transcribed to complementary DNA using PrimeScript™ II first strand cDNA synthesis kit (Takara, Japan). For PCR, a primer set which amplify 167 base pair (pb) from the F gene was used. Primer sequence was as the following (Forward) 5′- CGC AGA TCA CAG CGG CTT CTG - 3′ and (Reverse) 5′- GGT CGT TTA CAA ACT GCT GC - 3′ (Tsunekuni *et al*., 2010). PCR condition was 40 cycles consisted of : 94ºC for 2 min; 94ºC for 30 sec for denaturation, 56ºC for 30 sec for annealing, 72ºC for 20 sec for primer extension and final extension at 72ºC for 5 min. 10 µl of PCR product was then analyzed using 2 % agarose gel electrophoresis. The gel was precasted with gel red nucleic acid stain (Biotium).

Fig. A: Chickens were reared in negative-pressure isolators under biosafety level 2 and were supplied with water and food *ad libitum.*

Fig. B: Specific pathogen free (SPF) chickens were inoculated intranasally with 100 µl ($10^{8.75}$ 50% EID_{50}) of 9a5b NDV mutant isolate.

3.1. Pathogenesis of the 9a5b NDV mutant isolate in the chicken pancreas

Clinical signs

Clinical signs of NDV were transient and started from the third dpi in the form of inappetence, sleepiness, and diarrhoea and peaked at 4 and 5 dpi, with symptoms such as depression, lethargy, open-mouthed breathing with oral discharges, and closed eyes (Fig. 1). All symptoms began to decrease from 6 dpi, and the chickens resumed appetite to food. Only mild depression persisted until the end of the experiment (Fig. 2). All the inoculated chickens completed the experiment without any mortality. No clinical signs were observed in control chickens.

Gross pancreatic pathology

Grossly, the pancreas had slight congestion, oedema, and mottled appearance from 1 dpi to 3 dpi (Fig. 3). At 5 dpi, pale-whitish foci were observed on the surface of the pancreas which increased at the last day of the experiment (Fig. 4). No gross lesions were found in control chickens.

Histopathology of chicken pancreas

The histopathological findings in the chicken pancreas were summarised in Table 4. Lymphoid-like nodules were observed in peripancreatic soft tissue. At 12 hpi, slight infiltration of lymphocytes and macrophages in the peripancreatic soft tissue was observed (Fig. 5). The inflammatory reaction increased in

intensity toward the end of the experiment (Fig. 6). The inflammation in the peripancreatic tissues occurred earlier than that in the pancreatic tissue (Table. 4). In the pancreas, mild infiltration of heterophils and mononuclear cells (lymphocytes and macrophages) in the interstitial tissue of the exocrine tissue was seen at 1 and 2 dpi. At 3 dpi, slight vacuolation and necrosis of pancreatic exocrine cells were observed, along with increased number of heterophils and mononuclear cells. By 5 dpi, some inflammatory nodules, mononuclear cells and heterophilic infiltration, mild to moderate necrosis and vacuolation of the exocrine cells were observed (Fig. 7). At 10 dpi, this necrotizing pancreatitis became more severe and involved all the exocrine portion of the pancreas. Necrotizing pancreatitis was characterized by destruction of the exocrine gland, severe vacuolation and infiltration of mononuclear cells (Fig. 8) and heterophils with marked lymphoid nodules proliferation. Mononuclear cells infiltration was prominent around the pancreatic duct, along with mild vacuolation of the duct epithelium. The main pancreatic ducts and their branches did not exhibit any obstructive lesions. The endocrine portion of the pancreas was not affected throughout the experiment and remained intact, except at 10 dpi, when the islets showed slight atrophy. No histological lesions were detected in control chickens.

NDV-nucleoprotein IHC in chicken pancreas

The immunohistochemical findings in the peripancreatic and pancreatic tissue were summarised in Table 5. Strong positive signals were observed in the macrophages and lymphocytes infiltrating the peripancreatic soft tissue from 1 dpi (Fig. 9) and monocytes within the blood vessels (Fig. 10). In the pancreatic tissue, NDV-positive signals were detected in the cytoplasm of the degenerated exocrine cells, within vacuolated lesions, and in macrophages at 5 dpi (Fig. 11). On the last day of the experiment, NDV-NP positive signals were more abundant in the necrotic pancreatic acinar cells (Fig. 12) and macrophages (Fig. 12 inset). The peripancreatic and the pancreatic tissues from the control group tested negative for NDV.

TEM

In the infected group, virus particles were seen in the cytoplasm of the exocrine cells at 10 dpi. The virus was enveloped and round in shape with a diameter of 200 nm ~ 500 nm (Fig. 13).

CD3 IHC in chicken pancreas

By 12 hpi, CD3-immunopositive cells were observed in the peripancreatic soft tissues and these cells started to infiltrate the adjacent pancreatic tissue (Fig. 14). CD3-positive cells infiltration in the pancreatic tissue increased with time (Fig. 15) and eventually diffused throughout the whole pancreas especially

between the exocrine tissue (Fig. 16) and around pancreatic duct branches (Fig. 17), the pancreatic endocrine islets, and blood vessels by the last day of the experiment.

Insulin IHC in chicken pancreas

The immunohistochemical findings of insulin immunopostivity were summarised in Table 6. Compared with the control group (Fig. 18), insulin immunoreactivity in the endocrine islets was not affected throughout the infection, except on the last day of the experiment, when slight reduction of insulin immunopositive signals were seen in the pancreatic islets and most of the islets were surrounded by severe inflammatory reaction (Fig. 19).


1


2

3.2. Pathogenesis of the 9a5b NDV mutant isolate in the chicken kidney

Gross renal pathology

The findings are summarized in Table 7 and 8. Grossly, the kidneys were pale with mild lesions. Multifocal small whitish areas were observed in cross sections of kidneys in 2 and 3 chickens at 5 and10 dpi, respectively.

Histopathology of chicken kidney

Histologically, about 1~2 small lymphoid foci were observed in the interstitium of the kidney in 2 of the 5 control chickens and in the ureteral mucosa of all examined birds. Areas of hematopoiesis composed of red blood cells and granulocytes were observed in both control and infected birds. No histopathological changes were observed in infected kidneys at 6 and 12 hpi. At 1 dpi, 2 chickens had congestion, hemorrhage and marked mononuclear cell infiltration. At 2 and 3 dpi, 2 chickens in each had mild multifocal lymphohistiocytic tubulointerstitial nephritis within the cortex and lymphoid nodules were observed and gradually increased. The medullary cones were mildly dilated and infiltrated with heterophils and other mononuclear cells (Fig. 20). At 5 dpi, 3 chickens had moderate multifocal lesions and occasionally these lesions coalesced to form diffuse cortical tubulointerstitial nephritis (Fig. 21). Marked degeneration, necrosis, and apoptosis of the renal tubular epithelium in the cortical region were identified (Fig. 22). The medullary cone showed moderate dilatation and degeneration of its components (collecting tubules and ducts,

medullary loops, and ureteral branches) associated with calcification and inflammatory reaction. At 10 dpi, 3 chickens had severe cortical tubulointerstitial nephritis. The medullary cones were markedly dilated and more severely damaged than the cortex. Mild to moderate interstitial fibrosis and protein casts were observed in the renal tubular lumen. Severe dystrophic calcification and inflammatory response were observed (Fig. 23). Clinical signs, gross and histopathologic lesions were absent in control chickens.

NDV-NP IHC in chicken kidney

Immunohistochemically, NDV-NP was detected in infected renal tissues at 1 dpi, followed by an increase in intensity and distribution at 2–5 dpi and then decreased or became absent by the final experimental day (Table 8). Most immunostaining for NDV-NP was distributed in a multifocal pattern in the intact (Fig. 24) and vacuolated epithelium of proximal and distal tubules (Fig. 22 *inset*), in infiltrating mononuclear cells, and occasionally in some cells of reptilian-type glomeruli in the cortex (Fig. 25). In the renal medulla, NDV-NP immunostaining was mainly present in the tubular epithelium and the infiltrating mononuclear cells. Kidney of control chickens was negative to NDV-NP IHC.

CD3 IHC in chicken kidney

CD3 positive cells were observed rarely in the interstitium of the kidneys in 2 chickens of the control group and in the ureteral mucosa of all control chickens. In

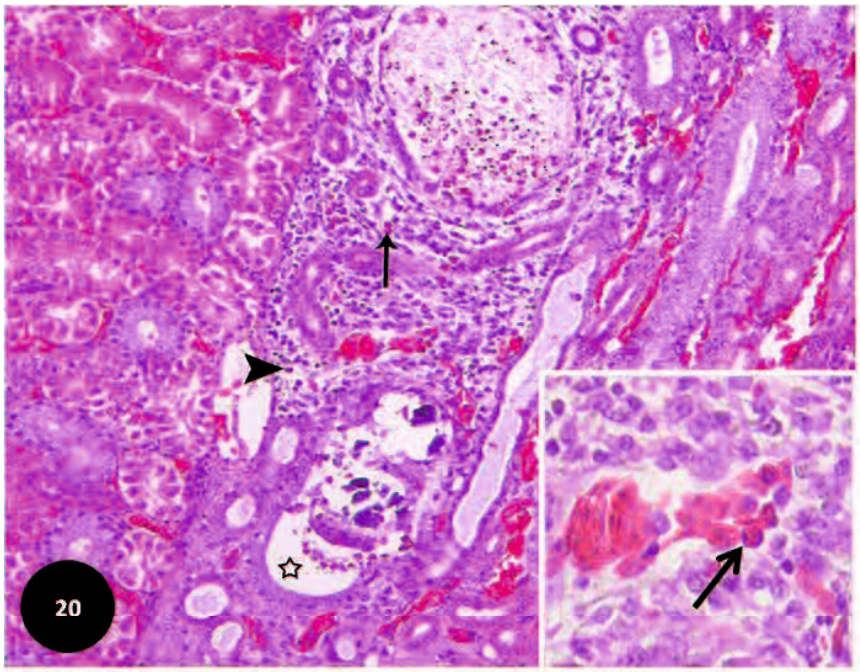
the infected renal tissues, infiltration of CD3 immunopositive cells commenced at 1 dpi (Fig. 26) and became marked at 10 dpi (Fig. 27). CD3 positive cells had focal and diffuse infiltration throughout the entire kidney (Fig. 28) and in areas with immunostaining signals of 9a5b NDV mutant isolate (Fig. 29).

Virus titration of chicken kidney

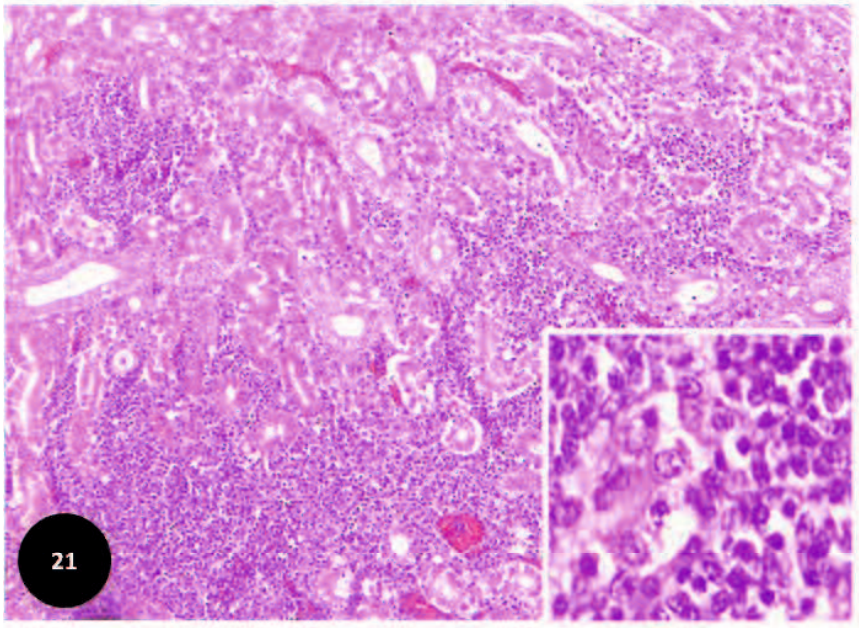
Virus titration results are summarized in Fig. 30. At 6 and 12 hpi, 9a5b NDV mutant isolate was not detected in kidneys. Virus was detected at 1 dpi, in one chicken at 1 dpi, in 2 chickens at 2 dpi, in all examined chickens at 3 and 5 dpi with a replication peak at 5 dpi, and in only one chicken at 10 dpi. No virus was detected in control chickens.

RT-PCR

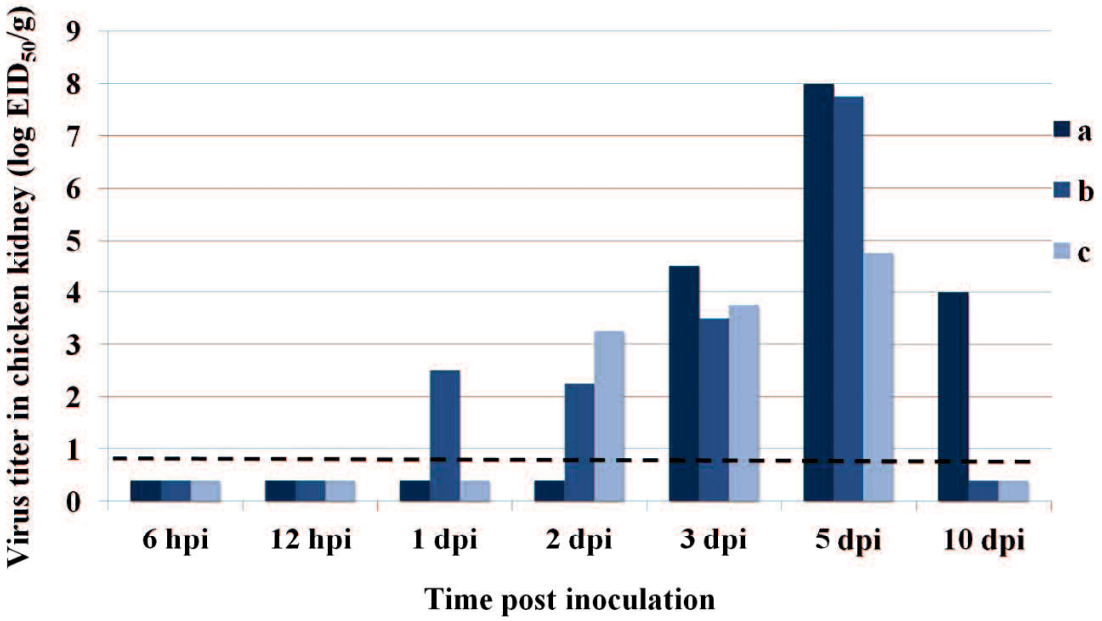
RT-PCR was performed to detect the genome of NDV. The results in infected chicken kidneys coincided with IHC and virus titration results (Fig. 31). Kidneys of control chickens were negative by RT-PCR.



20



21


Virus titer in chicken kidney (log EID50/g)
9
8
7
6
5
4
3
2
1
0
6 hpi
12 hpi
1 dpi
2 dpi
3 dpi
5 dpi
10 dpi
Time post inoculation
a
b
c

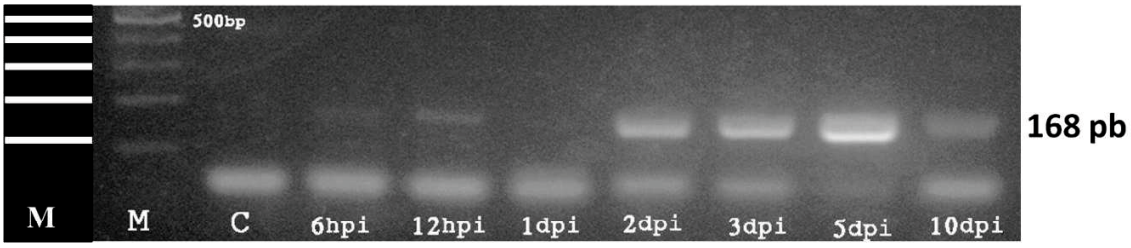


Fig. 31: RT-PCR of individual chicken kidneys after experimental infection with the 9a5b NDV mutant isoalte showed postive band at 168 pb.

Discussion

This study was conducted to investigate the pathological effect of the 9a5b NDV mutant isolate on chicken pancreas. The clinical signs were mild and were compatible with our previous report (Anis *et al*., 2013a). Also, similar findings were observed for virulent NDV strains that does not produce much clinical disease (Dortmans *et al*., 2011; Ecco *et al*., 2011a; Susta *et al*., 2011) although, those previously studied viruses were classified as virulent strains based on their ICPI and the sequence of F protein cleavage site (Susta *et al*., 2011). Intracerebral inoculation, not being the natural way of infection, may lead to a difference in ICPI value when compared with the natural route of infection (Cattoli *et al*., 2011; Dortmans *et al*., 2011). In addition, NDV virulence is multigenic and the F gene is not the only key player in NDV virulence (De Leeuw *et al*., 2005; Dortmans *et al*., 2011; Huang *et al*., 2004; Wakamatsu *et al*., 2006b).

Viral replication and an associated inflammatory reaction occurred in the peripancreatic tissues and then in the pancreas. The virus most likely reached the peripancreatic tissues and pancreas at the same time by viraemia. Infiltrating CD3-positive cells were detected in the peripancreatic tissues as early as 12 hpi and these cells started to infiltrate the adjacent pancreatic tissue from this time. The infiltration extended within the pancreas, eventually

invading the entire exocrine pancreas. Moreover, NDV-NP signals were detected in macrophages and other lymphoid cells at 1 dpi in the peripancreatic tissue were then observed in the pancreas by 5 dpi. Additionally, in the present study and in a previous study on the same strain of NDV (Anis *et al*., 2013a), the gross and histological pancreatic lesions were mild until 5 or 6 dpi and then increased in severity and distribution, causing multifocal necrotizing pancreatitis (Kommers *et al*., 2003; Piacenti *et al*., 2006; Wakamatsu *et al*., 2006a). The same pattern of spread of inflammation in peripancreatic tissue has been reported previously for other viruses such as Rift valley fever virus (Gommet *et al*., 2011). The infected chickens had lymphoid-like nodules in their peripancreatic tissues, which were also infiltrated by macrophages and other lymphoid cells at an early stage after infection. In-vivo studies support the role of macrophages and other lymphoid cells in NDV replication and further dissemination to other tissues (Brown *et al*., 1999; Kommers *et al*., 2003; Wakamatsu *et al*., 2006a; Susta *et al*., 2011; Anis *et al*., 2013b; Lu *et al*., 2014). In addition, an in-vitro study confirmed that NDV could replicate within macrophages (Lam, 1996). Replication of NDV in macrophages disrupts their function and phagocytic ability (Lam, 1996; Qureshi, 1998; Qureshi *et al*., 2000). We speculate that the abundance of macrophages and other lymphoid

elements in the peripancreatic tissues at an early stage after NDV infection provides a suitable environment for viral replication.

In this study, the exocrine portion of the pancreas is mainly affected by the 9a5b NDV mutant isolate infection while the endocrine islets were slightly affected at the last day of the experiment. It is possible that that the damage in the exocrine gland after NDV infection may be attributed to the proteases content in the exocrine cell which may enhance the viral replication. This hypothesis agrees with the results of previous reports (Gotoh *et al*., 1992; Murphy *et al*., 1999; Nagai, 1995), which suggested that NDV exhibits differences in tropism and virulence depending on the activation of a viral fusion protein precursor by cellular proteases.

The slight degenerative changes in the endocrine islets at the last day of the experiments (10 dpi) may explain the weaker and fainter insulin IHC-positive signals in the infected group than that of the control group. The endocrine islets appear to be resistant to NDV infection. The resistance of endocrine islets may be explained by the following: firstly, avian pancreas contains a high level of glutathione which is 10 times more than the level found in mammalian pancreas (Hazelwood, 2000). Glutathione was proposed to have a protective action against cytotoxic studies on avian endocrine beta cells (Hazelwood, 2000); therefore, may be a similar protective effect for glutathione in the endocrine

pancreas against viral infections is present. Secondly, differences in the tissue distribution of the cellular proteases like furin and proprotein convertase (PC2 and PC3) were previously mentioned. Furin is located on the Golgi membrane and ubiquitously present in a variety of tissues; while, PC2 and PC3 are only expressed in the endocrine tissues. Furin is completely capable of F protein gene cleavability of virulent viruses; on the other hand, PC2 and PC3 are fully incapable or partially capable of F protein gene cleavability. Cleavage by furin can increase the virus infectivity (Gotoh *et al*., 1992; Nagai, 1995). However, the real cause for endocrine resistance is still unknown which may need further studies. In the present study, changes in the endocrine islets may be secondary to the massive necrosis of the exocrine glands or may be due to indirect effect of the virus because NDV cannot be detected by IHC or by TEM in the endocrine cells. Late virus dissemination from the peripancreatic soft tissues into the pancreatic tissues is possible and this may explain the late dramatic massive extension of pancreatic necrosis from 5 dpi.

In conclusion, the 9a5b NDV mutant isolate infection in chicken induced inflammatory reaction and viral replication in the peripancreatic soft tissues earlier than in the pancreatic tissue; therefore, the peripancreatic soft tissues may play a preparatory role in late widespread necrotizing pancreatitis induced by NDV in chickens. Furthermore, necrosis affected mainly the exocrine

portion of the pancreas while the endocrine portion was generally unaffected by the NDV infection.

The second part of this study was performed to investigate the pathogenesis of NDV in the kidney of chickens after experimental infection with 9a5b NDV mutant isolate. To the best of our knowledge, there have been no previous studies that have addressed the relationship between histopathological alterations in chicken kidney tissues and NDV replication.

Nephrotropism of the 9a5b NDV mutant isolate in the chicken kidneys was confirmed by IHC, RT-PCR, and virus isolation from an early time after infection (1 dpi). Virus detection was consistent among these three methods and started at 1 dpi, peaked at 5 dpi, and diminished at 10 dpi. The histopathological changes in the kidneys of infected chickens were consistent with the viral replication. The 9a5b NDV mutant isolate caused severe degeneration and necrosis in the renal epithelium with positive viral immunostaining in both tubular epithelium and mononuclear cells as previously reported for other birds (Anis *et al*., 2013a; Courtney *et al*., 2012; Kuiken *et al*., 1999; Piacenti *et al*., 2006; Susta *et al*., 2011). The virus distribution was in the cortex and medulla of kidney as previously described (Kuiken *et al*., 1999) Moreover, in this study positive viral immunostaining were observed occasionally in some glomerular cells.

In this study, NDV tubulointerstitial nephritis can be classified into cortical tubulointerstitial nephritis and intratubular medullary cone nephritis as previously described in the case of AIV infection in chickens (Swayne and Slemons, 1990). The 9a5b NDV mutant isolate caused more severe tubulointerstitial nephritis in the renal medulla than in the cortex similar to findings with IBV infection in chickens (Albassam *et al*., 1986). In humans, the medulla is more susceptible to infection than the cortex due to the low pH, high osmolality, and high concentration of ammonia in the medulla (Schaeffer, 2001). In birds, the different susceptibility of the renal cortex and the medulla to infection is unknown as the pH is variable (Echols, 2005). Tubular injury in the medulla more likely results from plugging of the lumina by cell debris or calcified materials or both, subsequently increasing inflammation as proposed by others in the case of AIV infection in chickens (Swayne and Slemons, 1990). Loss of renal function combined with water deprivation during the peak of clinical signs may enhance the effect.

CD3-positive cells were the main inflammatory component in chicken kidneys as previously reported in chicken brain during NDV infection (Ecco *et al*., 2011b). Infiltration of CD3-positive cells in the infected kidneys were associated with a decrease or complete absence of viral replication at 10 dpi.

In conclusion, the 9a5b NDV mutant isolate showed a nephrotropism to chicken kidneys early after infection (1 dpi). Tubulointerstitial nephritis was more severe in the renal medulla than in the cortex and the local inflammatory response may play a role in suppressing viral replication. Lesions that were caused by NDV in the kidneys of chickens were similar to those caused by AIV, IB, and ANV and this highlights the importance to include NDV in the differential diagnosis of kidney disease in chickens.

Summary and conclusion

This study aimed to investigate the pathology of NDV in two of the organs (pancreas and kidney) those are usually involved during NDV infection in several avian species. Moreover, scarce data are present regarding the pathogenesis of pancreatitis and nephritis in birds infected with NDV.

Usually with velogenic strains studies birds succumb to infection and die within 3-5 dpi. In the present study, the clinical signs were mild with no mortality in chickens after the infection with the velogenic 9a5b NDV mutant isolate. The 9a5b NDV mutant isolate has ICPI of 1.88 and the F protein cleavage site of virulent strains. This result can confirm that NDV virulence is multigenic and the F protein gene is not the only key player in virulence. In addition, intracerebral inoculation, not being the natural way of infection, may lead to a difference in ICPI value compared with the natural route of infection.

In the chicken pancreas, the 9a5b NDV infection induced inflammatory reaction and viral replication in the peripancreatic soft tissues earlier than in the pancreatic tissue; therefore, the peripancreatic soft tissues may play a supporting role in late widespread necrotizing pancreatitis induced by NDV. Furthermore, the exocrine portion of the pancreas was mainly affected while the endocrine portion was slightly resistant to the infection. Further studies are warranted to investigate the different

susceptibility between the exocrine and the endocrine portion of the pancreas to NDV infections.

In the chicken kidney, the 9a5b NDV mutant isolate had a marked nephrotropism to chicken kidneys from an early time after infection (1 dpi). Kidneys might be implicated in the virus excretion and further transmission to other birds. Tubulointerstitial nephritis was more severe in the renal medulla than in the cortex. The local inflammatory response may play a role in suppressing viral replication. Lesions that were caused by NDV in the kidneys of chickens were similar to those caused by AIV, IB, and ANV and this highlights the importance to include NDV in the differential diagnosis of kidney disease in chickens.

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