PATHOGENESIS OF NEW SUB-GENOTYPES OF NEWCASTLE DISEASES VIRUS STRAINS FROM ISRAEL AND PAKISTAN

By

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(Under the Direction of Corrie C. Brown)

ABSTRACT

In the past few years, NDV strains belonging to sub-genotypes VIIi and XIIIb have emerged in the Middle East and Asia, where they co-circulate with pre-existing genotypes. Strains of sub-genotype VIIi have epizootic characteristics, spreading rapidly in different regions with high mortality. In this study two NDV strains, one representative of sub-genotype VIIi isolated in Israel (APMV1/Broiler-Breeders/Israel (Kvuzat-Yavne)/NDV/826/2013 [*Kvuzat/13*]) and one representative of sub-genotype XIIIb isolated in Pakistan (APMV1/chicken/SPVC/Karachi/NDV/33/2007 [*Karachi/07*]), were characterized by intra-cerebral pathogenicity index (ICPI) and detailed clinico-pathological assessment. The ICPI values for *Kvuzat/13* and *Karachi/ 07* were 1.89 and 1.85, respectively, classifying these strains as virulent NDV by international standards. Groups of 4-week-old, White Leghorn chickens were inoculated in the right conjunctival sac and choanal slit with $10^{5.5}$ Embryo Infectious Dose 50% (EID_{50}) units of virus. Viruses caused 100% mortality within 4 days (*Kvuzat/13*) and 5 days (*Karachi/07*) post infection. Gross and histologic lesions in all infected birds included: severe hemorrhagic conjunctivitis, extensive necrosis of lymphoid tissues, segmental necrosis, and

hemorrhage in the small and large intestine. As shown by immunohistochemistry for NDV nucleoprotein, intense positive signal was observed in the lymphoid organs (spleen, bursa, thymus), the mucosa-associated lymphoid tissue in the intestine (cecal tonsils) and the respiratory tract (larynx), trachea, and Harderian glands. In lymphoid organs, immunohisotchemical signal was observed mainly in macrophages, while in the trachea and Harderian gland was observed also in epithelial cells. Results of the animal experiment confirm that both *Kvuzat/13* and *Karachi/07* are highly virulent and behave as velogenic viscerotropic NDV strains.

INDEX WORDS: Newcastle disease, Newcastle disease virus, Velogenic viscerotropic NDV, Pathogenesis, Clinico-pathological assessment, Sub-genotype VIIi, Sub-genotype XIIIb, Fifth NDV panzootic

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DEDICATION

This thesis and degree are dedicated to my parents and my siblings. To my father, Bapa Pandarangga: I hope you can see me from above and I am so grateful that you taught me to become a tough girl to face all of the obstacles in my life. I miss you so much, Bapa! To my mother, Rambu Lika: You show me how to treat disadvantaged people with compassion and it makes a big impact in my life when I interact with people around me.

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CHAPTER 1

INTRODUCTION

Newcastle disease is caused by virulent strains of Avian Paramyxovirus serotype 1 (APMV-1), members of the Paramyxoviridae family in the new genus Avulavirus[12]. The terms Newcastle Disease Virus or NDV and APMV-1 are synonymous. The genome of NDV is a non-segmented, negative-sense RNA virus that has six genes encoding for the six structural proteins: nucleo-protein (NP), phosphoprotein (P), matrix (M), fusion (F), haemagglutinin-neuraminidase (HN), and large (L) polymerase protein[11]. Newcastle disease has a worldwide distribution that negatively impacts both commercial poultry flocks and backyard chicken production[2]. NDV has the potential to cause illness in many species of birds all over the world, which increases the possibility of a panzootic. Since it was first reported in 1926, NDV has re-emerged repeatedly and been responsible for four documented panzootics[2, 13]. A proposed, rapidly spreading, 5th panzootic has been recently identified in the Middle East and Indian Subcontinent[1, 5, 13].

In developing countries in Asia, Africa, Central America, and some parts of South America, the village chicken is an extremely important resource that provides cash for smallholders, and also represents a significant source of dietary protein in the form of eggs and meat[2]. As an essential resource for many communities, poultry stocks must be maintained in good health. To ensure healthy animals, producers must implement disease control by using preventative methods, such as vaccination. In order to fully understand emerging NDV strains, the genetic diversity of all pathogenic strains of the virus needs to

be evaluated, along with the subsequent clinicopathological changes in the animals affected by disease. The data from these analyses will provide the scientific communities with the information needed to implement surveillance protocols in efforts to identify new strains, and in the future, anticipate or even prevent panzootics. This data will also aid in vaccine development and vaccine efficacy in relation to emerging NDV strains.

NDV is phylogenetically classified by the nucleotides that compose the aforementioned, six, structural proteins. At present, there are two distinct classes, class I and class II[14]. The class I viruses only have a single genotype, while the class II viruses contain eighteen genotypes, designated I-XVIII[4, 19]. All class I NDV strains are classified as low virulence, except for one isolate collected from Ireland in 1990[3]. For class II viruses, genotypes I and II contain viruses of low virulence. Genotypes V, VI, and VII emerged in the 1960s, are virulent (vNDV), and are the predominant genotypes currently observed circulating worldwide[6, 15]. Pakistan is an area with endemic vNDV and is located between Central Asia, the greater Middle East, and the region of South Asia. Due to its proximity to many countries, it is very important to correctly classify the characteristics of the viruses circulating in this country and their potential movement to neighboring countries[16]. The majority of the historically documented disease in Pakistan was caused by genotype VII. This genotype VII was further sub-classified as sub-genotype VIIi, which includes the strain chicken/Pakistan/Karachi/SPVC/33/2007 or 2007/PK/33[9, 14, 18]. Over time, the mean evolutionary distance of the chicken/Pakistan/Karachi/SPVC/33/2007 has changed more than 10% of the mean evolutionary distance from the previous sub-genotypes, and thus, has been re-classified as XIIIb, a new genotype of vNDV[7].

Israel is another country of importance when evaluating vNDV. Prior to 1979, Israel was free of vNDV; however, since 1997, there have been increasing reports of vNDV isolated from poultry and wild birds[10]. The most common genotype observed over the past 18 years has been genotype VII[8]. Genotype VII is subdivided into sub-genotypes, and in the past, sub-genotypes VIIb, d, e, f, and g have been the most prevalent identified strains[7, 14]. However a new sub-genotype, VIIi, which includes strain chicken/Israel/Kvuzat-Yvne/50-826/2013, has been identified as the most prevalent sub-genotype observed in the Israeli vNDV-infected population.

The objective of the present study was to characterize 2 different strains: chicken/Pakistan/Karachi/SPVC/33/2007 and chicken/Israel/Kvuzat-Yavne/50-826/2013 of velogenic NDV from Pakistan and Israel through observation of clinical signs and histologic assessment of tissues from 4-week-old chickens inoculated with vNDV via a natural route of infection through the eyelid and the choanal slit. Immunohistochemistry (IHC) of multiple tissues, including thymus, spleen, bursa of fabricius, cecal tonsil, heart, kidney, and intestines was performed to determine the presence and amount of virus. Characterization of the clinicopathologic effects of vNDV strains from Pakistan and Israel will not only help determine how the viruses are changing but also provide the diagnostic parameters to properly diagnose the disease and enhance surveillance efforts for this disease.[17].

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CHAPTER 2

LITERATURE REVIEW

History and Epidemiology

Newcastle Disease (ND) is caused by Avian Paramyxovirus serotype 1 (APMV-1) and has a long history with reports of disease dating back almost 100 years. Since the first reported cases on Java Island, Indonesia, and Newcastle, England in 1926, there have been 4 confirmed panzootics and a potential 5th panzootic has been suggested[2, 7]. Phylogenetically, Newcastle Disease Virus is classified into two classes: class I (1 genotype) and class II (18 genotypes); however, only a few genotypes can cause panzootics such as genotype II-VII and XIII [1, 2, 16, 20, 27, 37, 55]. Based on the OIE (World Organization for Animal Health) description, NDV has the ability to infect over 200 species of birds worldwide and can spread to cause serious socioeconomic disruption, and so it is considered a transboundary animal disease[1]. Many modes of disease transmission have been associated with NDV outbreaks, including common poultry industry practices, such as feeding carcasses or commercialized poultry feed containing offal in hatcheries and movement of imported caged birds around the world[34, 38, 42].

The first NDV panzootic was identified in Europe and persisted from the mid-1920s to the 1960s. Origins of this panzootic were recognized in Southeast Asia with subsequent spread to England the same year[7]. Although the disease was first identified in the 1920s, extensive spread occurred 15 years later, believed to be associated with the

Second World War. [7]. Evaluation of the pathogenic strains from this panzootic revealed that Class II genotypes II, III, and IV were responsible for this outbreak[2, 63].

The second panzootic was first recognized in Europe during the late 1960s and had disseminated worldwide within 4 years[7, 25]. Imported caged birds were identified as the infective source in California, USA during 1970 and 1972[25, 42]. The dominant genotype responsible for the second panzootic was Class II genotype V[38].

A third panzootic emerged from the Middle East in the late 1980s and was different from the first two panzootics in that the virus was found in pigeons, and testing demonstrated some differences from APMV-1, so it was named Pigeon Paramyxovirus-1 (PPMV-1)[42]. To distinguish the PPMV-1 from APMV-1, haemagglutination inhibition tests using mouse monoclonal antibodies were developed and validated to demonstrate the unique epitopes of PPMV-1[10]. This virus was classified as sub-genotype VIb[27].

In 1985, a fourth panzootic was first recognized in Southeast Asia with rapid spread to Africa, Western Europe, and South America[49, 65]. Unvaccinated racing pigeons from the Middle East that were susceptible to NDV infection significantly contributed to the spread in this panzootic[7]. Virus from genotype VII was responsible for this panzootic[2].

At present, a new, recently described sub-genotype VIIi has been identified from Indonesia, Israel, and Pakistan. Sub-genotypes VIIi and VIIh do not appear to have originated directly from other genotype VII strains, but they are related to the present and the past Indonesia NDV virus isolates from wild birds since the 1980s[2]. Sub-genotype XIIIa is a predominant sub-genotype causing outbreaks in Pakistan since 2012, and this is a similar pattern to sub-genotype VIIi, which is dominant in Israel. Sub-genotype VIIi,

along with sub-genotypes VIIh, XIIIa and XIIIb are potentially responsible for a proposed fifth panzootic[2].

Economic Impact

In developing countries around the world, the village chicken is an extremely important source of protein in the form of eggs and meat[7]. Outbreaks of ND can cause tremendous economic impact not only on village/backyard chicken production but also in commercial poultry production. Presence of the virus circulating within a country can decrease possibilities for trade, and although commercial birds are often vaccinated, the immunity is not sufficient to totally protect them from disease[64]. Data collected in 2009 and 2010 from Indonesia revealed that outbreaks of NDV in commercial poultry caused mortality up to 70%-80%[62]. Panzootics cause high economic losses, through loss of animal stocks and the efforts to control the spread of disease. For instance, the cost for depopulation of more than 3 million birds was approximately US$160 million for the 2002-2003 outbreak in California[57, 60]. In Southern Africa in 1996, it cost AUS $ 9.6 million (US$ 12.7 million) to control this disease[51].

Taxonomy

The International Committee on Taxonomy of Viruses places Avian Paramyxoviruses (APMVs) in the order Mononegavirales, family Paramyxoviridae, subfamily Paramyxovirinae, and genus Avulavirus[40]. In addition to the family Paramyxoviridae, the order Mononegavirales has 3 other families: Bornaviridae (1 genera), Filoviridae (2 genera), and Rhabdoviridae (6 genera). The family Paramyxoviridae is divided into 2 subfamilies: Paramyxovirinae (5 genera) and Pneumovirinae (2 genera). Viruses of the Subfamily Paramyxovirinae are responsible for

human and animal diseases, including some in genera Henipavirus, Morbillivirus, and Respirovirus. The Avulavirus genus causes disease only in avian species[40]. Avulavirus is a relatively new genus for the subfamily Paramyxovirinae, and there is only one virus, avian paramyxovirus, with 12 serotypes: APMPV 1-12, with APMV 10-12 being the most recently described[41, 42, 58]. Based on phylogenetic evaluation and calculation of evolutionary distance, the APMV-10 serotype was isolated from rock-hopper penguins from the Falkland Islands[43]. APMV-10 also has been reported in Magellanic penguins on the Brazilian tropical coast[22]. The APMV-11 was described from common snipes in France, and has the largest P genome of all serotypes of APMV and unusual P gene mRNA editing[11]. APMV-12, as the newest APMV, was isolated from wild birds in the Rovigo province of Nothern Italy[58]. APMV-2, APMV-3, and APMV-6 can use wild birds as a reservoir and cause disease in poultry[42]; however, APMV-1, also known as Newcastle Disease Virus, continues to be the most important serotype due its ability to cause disease in poultry worldwide [42].

Morphology

NDV is a non-segmented, negative sense virus that has a single stranded RNA genome, 3'-NP-P-M-F-HN-L-5'[32]. This genome encodes for a Nucleocapsid protein (NP), Phosphoprotein (P), Matrix protein (M), Fusion protein (F), Haemagglutinin-neuraminidase (HN), and Large (L) RNA-dependent RNA polymerase. The structure of the virion consists of a lipid envelope containing two glycoproteins, F and HN, and a core virion that contains ribonucleoproteins (RNP), L, NP and P [32]. The matrix protein separates the envelope and core virion of NDV. NDV is a pleomorphic virus that is roughly spherical with a diameter of 100-150 nm surrounded by an envelope covered by

spikes[5, 13]. The thickness of the envelope measures 44 nm in width and 17 nm in length and has HN protein and F proteins on the surface[39]. The glycoprotein-like spikes have heads and stalks with diameters of approximately 6 nm and 2 nm, respectively[39]. The P protein and L protein are associated with NP protein and characterized by a herringbone pattern[39, 42].

Chemical and Physical Properties

The NDV genome contains 15,186, 15,192, or 15,198 nucleotides that comprise the RNA genome. The ends of the genome have a 3' extracistronic region as the leader that contains 50 nucleotides, and a 5' extracistronic region as the trailer that contains 50 to 160 nucleotides[18, 32]. The virion is composed of 20%-25% weight of weight (w/w) of lipid and 6% w/w of carbohydrate[42].

The NP, P, and L proteins come together to form a nucleocapsid as the center of the virion. The nucleocapsid protein (NP), with molecular weight 53-57 kDa, has two main structures: N core N-terminal domain and N tail C-terminal domain[32]. The phosphoprotein contains 400-600 amino acids with heavy phosphorylation at serine and threonine residues predominantly in the N-terminal region[32]. The NP protein and P protein are connected at the C-terminal N tail. The 250 kDa L protein has six domains and also binds with the P protein. The virion of NDV has three layers that comprise the envelope. Two layers F and HN protein contain glycoprotein, and one layer contains the unglycosylated membrane M protein [31, 42]. The V protein contains a cysteine and zinc-binding domain that plays a role in blocking the interferon pathway in host cells[26, 42].

Biological Properties and Molecular Pathogenesis

The virulence of NDV is controlled by the combination of F protein and HN proteins[19, 46, 52, 60]. In addition, the V protein is a viral accessory gene that contributes to viral virulence by blocking the IFN-alpha pathway through degradation of transcription factors, STAT1 and STAT2, in host cells[26, 32]. The other proteins composing the NDV genome, such as NP, M, P, and L proteins, do not contribute to the virus' virulence [19]. However, the M protein plays an important role by creating the specific morphology of the virion, which promotes release from the core during virus entry[32].

There are three functions of the HN protein: (1) attaching to the surface of host cells through sialic acid, (2) promoting the fusion activity of F protein to enable transport through the cell surface[19], and (3) contributing to the removal of sialic acid by neuraminidase to inhibit viral self-agglutination[31]. An additional function of the HN protein is to neutralize host antibodies[17]. The size of the HN protein precursor is 616 aa in avirulent strains, 577 aa in virulent strains, and 571 aa in highly virulent strains; however, the HN protein promotes virulence only when in combination with a virulent F protein[52].

The F protein contains a specialized cleavage site specific to virulent strains of NDV, which can be cleaved by host proteases found throughout the body of the host[53]. Proteases that can cleave the F protein are contained in membrane-bound cellular organelles in almost all host cells, and the trans Golgi membranes seem to have the highest content. These proteases will become activated at pH ranges from 6-8. On the other hand, the F protein associated with NDV strains of low virulence is cleaved by a trypsin-like enzyme, which is only found in cells of the respiratory and gastrointestinal

tracts. When the F protein undergoes cleavage it is divided into two pieces with a phenylalanine at the amino terminus of F_1 and basic amino acids at the carboxy terminus of F_2[52]. The F protein within the envelope of the virion will subsequently fuse with the plasma membrane after the NDV attaches to the host cells through HN binding to sialic acid [19, 32]. After release into the cytoplasm of host cell, the negative sense RNA is synthesized strictly to make another negative sense RNA strand. This process is called primary transcription. The negative-sense RNA strand is used in the next step as a template to transcribe positive sense strands. At the 3' end of the anti-genome, there is a promoter that enables the virus to produce more negative-sense genomes [32, 42]. To make a new virion, also known as the budding process, the virus must make the nucleocapsid and the N subunits of this nucleocapsid associate with the template RNA to form the helical RNP. Next, the helical RNP associates with P-L protein complex creating the envelope. M protein plays an important role in the envelope[32].

Replication

After the negative sense nucleocapsid is released into the cytoplasm of the host cell, the genome will produce more progeny virions. For virulent NDV, this process takes approximately 10 hours [32]. At the beginning of infection under the cis-acting of RNA sequences, the genome strictly synthesizes negative-sense RNA, 3'-5', and this is called the primary transcription phase[32]. After translation, the negative-sense genome will be transcribed into a positive-sense genome or anti-genome, 5'capped and 3' polyadenylated messenger RNAs (mRNA)[32, 42]. The 3' end of the anti-genome is a promoter that can produce negative sense genomes, which enables the template to make more anti-genomes. This phase is called secondary transcription[32].

Virulence Determinants

Since NDV was first described in 1926, there have been various systems used to classify NDV strains according to virulence. The first system was devised based on mean death time of embryos. After inoculation, embryonating eggs were examined to the time of death. If death occurred in less than 60 hours, the strains were classified as "velogenic". If embryos were still alive at 90 hours, the strains were classified as "lentogenic." Any strains causing death between these two time periods were referred to as "mesogenic." Then, a system to distinguish the velogens was developed as well. Strains were inoculated into the cloaca of young birds, and if the birds developed lesions within the viscera (spleen and intestines), they were referred to as Velogenic Viscerotropic Newcastle Disease (VVND). If the process of disease was predominantly neurological, they were referred to as Velogenic Neurotropic Newcastle Disease (VNND). This system created a way of referring to the viruses according to virulence.

Then, more sophisticated and accurate systems were developed, and these are the systems that are predominantly in use today, although the terms "lentogen", "mesogen", and "velogen" are still in popular usage. These newer systems are the ones currently utilized worldwide to classify NDV strains[(44)].

The first system was introduced by Aldous and focused on a portion of the fusion gene containing the region encoding for the cleavage activation site and the signal peptide of the fusion protein gene[(4)]. This system divided the NDV strains into six distinct groups referred to as lineages: lineage 1 to 6, with lineages 3 and 4 further divided into four sub-lineages (a to d) and lineages 5 into five sub-lineages (a to e)[(8)].

The second system of NDV classification divides APMV-1 into two classes, I and II. Class I strains have a 15,198 bp genome, and class II have a 15,186 bp genome. In the 20th century, a second division occurred within the genome size classification that has 15,192 bp[18, 43, 65]. The genotype classification describes a stronger correlation between the phylogenetic topology and the evolutionary distances, and it reflects the diversity of APMV-1[20].

Recent evaluation of the mean evolutionary distance reveals greater than 10% difference between the viruses in each class. Class I consists of only a single genotype, and class II is comprised of 18 genotypes[16, 20, 55]. The 18 genetic groups or genotypes of class II include 10 previously described genotypes: I-IX and XI, and 8 new genotypes: X, XII, XIII, XIV, XV, XVI, XVII, and XVIII.

Class I NDV has been isolated from numerous live bird markets in the northeastern US[29]. The class I NDV was previously divided into 9 genotypes based on comparison of partial nucleotide sequences of the F gene[44]. However, the new classification criteria derived from the current classification system used for highly pathogenic avian influenza viruses describes the class I NDV as only having a single genotype[20]. The phylogenetic topology indicates that genotype I of class I has three sub-genotypes: Ia, Ib, and Ic[20].

The classification of class II NDV genotypes is also supported by phylogenetic topology and evolutionary distance between groups through evaluation of the complete F gene sequence. Class II NDV is separated into 18 genotypes: I-XVIII where genotype X, XII, XII, XIV, XV, XVI, XVII and XVIII, are the novel genotypes[20, 28, 36, 59, 61, 65]. Genotype I is composed of two sub-genotypes, Ia and Ib, and is primarily identified as

having low virulence; thus, it is used in live vaccines, such as chicken/Australia/QV4/1966[29, 44]. However, genotype I has one known virulent strain that was isolated as the etiological agent of the 1998 ND outbreak in Australia[23].

Genotype II was originally divided into sub-genotypes II and IIa and was used worldwide for vaccines, such as LaSota, B1, and VG/GA[29, 44]. However, after further analysis, sub-genotype IIa was found to be greater than 10% different than the other viruses in genotype II and was thus reassigned into genotype X[21]. Genotype III was isolated in Zimbabwe in 1990 and in China from 2002 to 2004[61, 65]. Genotype IV of NDV class II was the predominant isolate in Europe before 1970[18]. Genotype V has a diverse group of viruses and is divided into two sub-genotypes: Va and Vb[20]. Genotype VI is composed of 8 sub-genotypes (VIa-VIh). VIb and VIh were isolated from Taiwan from 1984 to 1985; VIf and VIg were isolated from China during 1985 to 2001[20, 36, 59, 61, 65]. Genotype VII has 8 sub-genotypes: VIIa - VIIh. Sub-genotype VIIa was isolated in Taiwan between 1969 and 1996, and sub-genotypes VIIc, VIId, VIIe were isolated from the China Taiwan during 1996 to 2001[59, 61]. Genotype IX was also isolated in China from 2002 to 2004 as well as genotype X in 1984[59, 61]. Genotype XII was a novel genotype that was isolated from chickens in South America and geese in China[20, 21]. Genotype XIII, previously known as genotype VII, was isolated in Rusia, Iran, and Pakistan from 1995 to 2008[20, 28]. Genotype XV, which had been previously classified as sub-genotype VIId and VIIe, was isolated from chickens and geese in China[20, 36]. Genotypes XIV, XVII and XVIII were found in Central and West Africa (Nigeria, Central African Republic, and Cote d'Ivoire) between 2006 and 2011[20, 55].

Pathogenicity and Virulence Tests for NDV

Based on international agreement, the World Organization for Animal Health (OIE) uses Intracerebral Pathogenicity Index as the standard method to measure the virulence of NDV[1]. Today, the OIE classifies "reportable" vs. "non-reportable" NDV according to ICPI[6].

Several pathogenicity tests have been developed to differentiate between NDV isolates of high and low virulence with some level of standardization[5]. To monitor the variation in virulence of different NDV isolates, OIE requires isolates to be evaluated by either the ICPI or the fusion cleavage site sequence. The ICPI is an in vivo test to assess the virulence of the isolate of Newcastle disease virus using one-day-old, specific pathogen-free (SPF) chickens[1]. ICPI should only be used where there is strong justification based on the epidemiological circumstances. For example, the first isolate from an outbreak should be evaluated by ICPI. This test is not for routine surveillance of healthy birds.

Proper sample collection is an important part of identifying strains of NDV. For all avian paramyxoviruses, the samples can be isolated by taking swabs of feces or the cloaca, as well as taking swabs from the trachea/oropharyngeal area[5]. The swabs should be placed in media containing an antibiotic with phosphate-buffered saline at pH 7.0-7.4. Protein media, such as brain heart infusion (BHI) or Tris-buffered tryptose broth (TBTB), enhances stability of virus especially for shipping. Penicillin, streptomycin, gentamycin, and nystatin are all common antibiotics used for control of bacterial growth with a suggested level of 10,000 IU/ml, 10 mg/ml, 0.25 mg/ml, and 5,000 IU/ml, respectively. Samples should be held at 4^0 C to prevent the decay of virus.

To isolate the viral particles, the supernatant, after washing the swab, tissue, or organ, is inoculated into the allantoic cavity of four or five, SPF, 9 to 11 day-old, embryonating chicken eggs. The allantoic fluid is harvested after 5-7 days, then tested for hemagglutinating activity. Allantoic fluid positive for NDV is confirmed to be APMV-1 with the hemagglutination inhibition (HI) assay, and then diluted in isotonic saline and filtered to perform the ICPI.

To perform ICPI, ten, 20 to 40 hour-old, SPF chicks are injected intra-cerebrally with 0.05 ml of 1:10 dilution of bacteria-free, isotonic saline containing NDV[3]. The birds are observed every 24 hours for 8 days. The scoring is 0 for a normal bird, 1 for a sick bird unable to eat or drink, and 2 for a dead bird. Any sick birds that cannot eat or drink should be euthanized humanely and scored as dead at the next observation. The ICPI is calculated as the mean score per bird observation for each day over the eight-day observation period. ICPI results range from 0.0 to 2.0 and strains having ICPI $\geq$ 0.7 are considered virulent or "reportable".

Clinicopathologic features of NDV

Chickens infected with lentogenic strains have neither clinical signs nor mortality[12, 30, 57]. However, grossly, the birds may have some eyelid petechiation, expanded lymphoid follicles, or mild tracheitis at 5 days post infection (dpi).

Birds inoculated with mesogenic NDV strains may show no clinical signs of disease, or they may have mild respiratory illness[12, 57]. Gross lesions will be present starting at 5 days post infection, characterized by reddening of the pharyngeal area, with perhaps some minor exudate in the air sacs. Mesogenic NDV is thought to predispose to other respiratory pathogens, so when present in the field, there may be secondary

infections. These secondary infections are not seen in experimentally infected SPF chickens.

For the velogens, both VVNDV and VNNDV cause high morbidity and mortality. VVNDV will cause death within 4-6 days, often with severe hemorrhage and necrosis in multiple lymphoid tissues and the gut. The clinical signs of VVNDV are depression, reluctance to move, open mouth breathing, bilateral conjunctivitis, diarrhea, and high mortality. In contrast, VNNDV does not cause the very severe early depression, with the virus largely sparing the lymphoid tissue and visceral tissue adjacent to lymphoid tissues (i.e., gut), but will move on to the brain, causing severe depression, lateral recumbency, and unilateral leg paresis starting at 5 dpi[12, 30, 57]. Gross examination of birds infected with VNNDV will have minimal gross lesions, with perhaps only a modestly enlarged spleen[12, 57]. Histopathologically, VVNDV produces severe damage in the lymphoid organs (cecal tonsil, spleen, bursa,thymus, bone marrow) characterized by accumulation of fibrin, hemorrhage, and karyorrhectic cellular debris with lymphoid depletion in the bursa and in the spleen at 4 to 5 dpi[12, 56, 57]. Birds infected with VNNDV present with neural degeneration and perivascular cuffing throughout the brain and neuronal degeneration.

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CHAPTER 3

PATHOGENESIS OF NEW SUB-GENOTYPES OF NEWCASTLE DISEASES VIRUS STRAINS FROM ISRAEL AND PAKISTAN

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Abstract

In the past few years, NDV strains belonging to sub-genotypes VIIi and XIIIb emerged in the Middle East and Asia, where they co-circulate with pre-existing genotypes. Strains of sub-genotype VIIi have epizootic characteristics, spreading rapidly in different regions with high mortality. In this study, two NDV strains, one representative of sub-genotype VIIi isolated in Israel (APMV1/Broiler-Breeders/Israel (Kvuzat-Yavne)/NDV/826/2013 [*Kvuzat/13*]) and one representative of sub-genotype XIIIb isolated in Pakistan (APMV1/chicken/SPVC/Karachi/NDV/33/2007 [*Karachi/07*]), were characterized by intra-cerebral pathogenicity index (ICPI) and detailed clinico-pathological assessment. The ICPI values for *Kvuzat/13* and *Karachi/ 07* were 1.89 and 1.85, respectively, classifying these strains as virulent NDV by international standards. Groups of 4-week-old, White Leghorn chickens were inoculated in the right conjunctival sac and choanal slit with $10^{5.5}$ Embryo Infectious Dose 50% (EID_{50}) units of virus. Viruses caused 100% mortality within 4 days (*Kvuzat/13*) and 5 days (*Karachi/07*) post-infection. Gross and histologic lesions in all infected birds included severe hemorrhagic conjunctivitis, extensive necrosis of lymphoid tissues, segmental necrosis, and hemorrhage in the small and large intestine. As shown by immunohistochemistry for NDV nucleoprotein, intense positive signal was present in the lymphoid organs (spleen, bursa, thymus), the mucosa-associated lymphoid tissue in the intestine (cecal tonsils), and the respiratory tract (larynx), trachea, and Harderian glands. In lymphoid organs, the immunohisotchemical signal was observed mainly in macrophages, while in the trachea and Harderian gland, signals was observed also in epithelial cells. Results of the animal

experiment confirm that both *Kvuzat/13* and *Karachi/07* are highly virulent and behaved as velogenic viscerotropic NDV strains.

Introduction

Newcastle disease (ND) is an important disease of poultry, caused by virulent strains of Newcastle disease virus (NDV)[3]. ND is a global concern, causing severe economic losses due to high mortality of infected birds and trade restrictions imposed on countries that declare outbreaks[3]. In developing countries, ND is particularly prevalent in small backyard flocks and poses a crippling economic problem for small and large poultry growers due to loss of productivity affecting the availability of protein for dietary consumption or sale[3,13,28].

Newcastle disease virus, known as Avian Paramyxovirus serotype 1 (APMV-1), is a member of the *Paramyxoviridae* family and *Avulavirus* genus[16]. The genome of NDV is composed of a single stranded, non-segmented, negative-sense RNA molecule, with six genes that encode for six structural proteins, from 3' to 5': nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and large protein/polymerase (L)[3,17,31].

Based on their virulence, NDV isolates are divided into asymptomatic enteric, lentogenic (minimal respiratory lesions in young birds), mesogenic (intermediate virulence) and velogenic (high morbidity and mortality). Velogenic strains can be divided into neurotropic, if they cause severe neurological disease, or viscerotropic, if they cause intestinal and visceral hemorrhages[3,8]. The intracerebral pathogenicity index (ICPI) is the internationally recognized method to categorize the virulence of NDV strains, and entails ten inoculating intracranially, one-day-old, specific pathogen-free (SPF) chickens with filtered and diluted NDV stock. The ICPI score is the mean score for each bird over an eight-day period, and ranges between 0 and 2[3,8,23]. According to

the World Organization for Animal Health (former OIE, Office International des Epizooties)[23], NDV strains with ICPI ≥ 0.7 are considered virulent and notifiable to the international community[23]. Virulence of NDV strains can also be indirectly determined by the deduced amino acid sequence of the F protein cleavage site: NDV strains with a polybasic amino and configuration between residues 113 and 116, plus phenylalanine at residue 117, cause systemic infection in susceptible birds, and are considered to be virulent, and therefore, notifiable[3,5,23].

Phylogenetically, NDV strains are divided into two distinct classes: class I and class II[19]. Class I encompasses a single genotype and class II is divided into 18 different genotypes[3,6,11,12,19,27]. Based on the evolutionary mean genetic distance, genotypes can be divided into sub-genotypes (reviewed in[10]). Class I NDV strains are commonly isolated from waterfowl and are of low virulence, with the exception for one Irish isolate that caused a chicken outbreak in 1990[6]. Within class II, genotype I encompasses mostly low virulence NDV strains (except for Australian virulent strains[12]), and genotype II includes both virulent and low-virulence NDV strains, some of which are used as vaccines[3,19]. All other recorded class II genotypes (III-XVIII) include only virulent strains. Genotypes V, VI, and VII, which emerged in the 1960s, constitute the predominant genotypes currently circulating worldwide[3,19]. NDV strains of genotype VII have worldwide distribution, and are considered to be responsible for the fourth NDV panzootic that began around 1985 in Asia and is still continuing today[2,3,19,22,24]. Genotype VII has been previously divided into five sub-genotypes (VIIb, d, e, f, g)[10,19], of which strains of sub-genotype VIIb and d are responsible for most outbreaks across the Middle East[1,25], Asia[7,19,32,33], South Africa[2] and South America[24]. In January of 2015

it was reported that NDV strains of novel sub-genotype VIIi have been detected spreading rapidly through the Middle East (Israel) and Asia (Pakistan, Indonesia)[4]. Sub-genotype VIIi has shown high infectivity and mortality rates, and it is considered to be the cause of a possible fifth ND panzootic[4]. Between 2009 and 2013, in Israel, a NDV isolate of sub-genotype VIIi became more prevalent than previously circulating sub-genotypes VIIb and VIId[4,10,22]. Similarly, in Pakistan, viruses of sub-genotype VIIi have caused several outbreaks, and at the same time, strains of sub-genotypes XIIIb have become more prevalent than previously circulating strains of genotype XIIIa[4,10]. Phylogenetic analysis suggests that both sub-genotypes VIIi and XIIIb derive from ancestral NDV strains isolated in the 1980s, rather than directly evolving from sub-genotypes of VII co-circulating in these regions[4]. The marked genetic heterogeneity of NDV strains, and the constant evolution of new genotypes poses a threat for NDV surveillance and control. For instance, new genotypes could have increased virulence or wider host-range[9]; commonly deployed vaccines could prove less effective against genetically distant new genotypes[20,21], and/or molecular-based tests could become less sensitive to detect new NDV strains[14,26]. Therefore, it is paramount to biologically and genetically characterize new NDV strains in order to implement control measures and to increase preparedness levels.

In this experiment, we describe the clinico-pathological characteristics of one representative strain of sub-genotype VIIi (*chicken/Israel/Kvuzat-Yavne/50-826/2013*, referred here as *Kvuzat/13* strain) and another representative of sub-genotype XIIIb (*chicken/Pakistan/Karachi/SPVC/33/2007*, referred here as to *Karachi/07*)[4]. NDV strain *Kvuzat/13* was isolated in 2013 from an outbreak in a broiler breeder farm in the Israel

coastal plain region; NDV strain *Karachi/07* was isolated in 2007 from an outbreak in poultry from the Pakistani region of Karachi[4]. This is the first report of detailed clinico-pathological assessment of these two newly described sub-genotype in chickens.

Materials and Methods

Viruses

NDV strains *Chicken/Israel/Kvuzat-Yvne/50-826/2013* (*Kvuzat/13*) and *Chicken/Pakistan/Karachi/SPVC/33/2007* (*Karachi/07*) were obtained from the Southeast Poultry Research Laboratory (SEPRL) repository. Each virus was propagated in 9- to 10-day-old, specific pathogen-free (SPF), embryonating chicken eggs through chorioallantoic inoculation[5,18]. As previously described, NDV strains *Kvuzat/13* and *Karachi/07* are classified within sub-genotypes VIIi and XIIb, respectively[4].

Chickens

Eggs and chickens were obtained from the SEPRL specific pathogen free (SPF), White Leghorn flock. Chickens were housed in negative pressure isolators at the facilities of the SEPRL (ARS, USDA) under Biosafety Level-3 (BSL-3) enhanced (E) agriculture conditions. Food and water were provided *ad libitum*.

ICPI

The ICPI test was performed for NDV strains *Kvuzat/13* and *Karachi/07*, as prescribed by the OIE.[23] Briefly, ten, one-day-old, SPF chickens were inoculated intracranially with 0.05 ml of a 1:10 dilution in phosphate-buffered saline (PBS) of infective allantoic fluid or PBS only (control group). Birds were observed daily and clinical signs scored (0-

normal, 1-sick or paralyzed, 2-dead) every day for eight days. The ICPI score was calculated as the mean score for each bird over the eight-day period.[23]

Clinicopathological Assessment in Chickens

Thirty, 4-week-old, White Leghorn chickens were divided into three groups of ten birds each, consisting of *Kvuzat/13*-, *Karachi/07*-infected birds, and a control group. Target dose of inoculum per each bird was $10^{5.5}$ Embryo Infectious Dose 50% (EID_{50}) diluted in 0.1ml of Brain Hearth Infusion (BHI) broth. Half of the inoculum (0.05 ml) was dispensed in the right conjunctival sac and the other half in the choanal slit. The control group received BHI only. After inoculation, birds were monitored daily and clinical signs recorded. At days 2, 5, 10, and 14 post infection (pi), two birds per each group were swabbed (oral and cloacal swabs) and euthanized (sodium pentobarbital, 100 mg/kg intravenous). Birds that were terminally ill were swabbed and then euthanized, regardless of the sampling schedule. Immediately after euthanasia, tissues (n = 26, eyelid, spleen, thymus, bursa of Fabricius, Harderian gland, crop, esophagus, proventriculus, duodenum and pancreas, jejunum, ileum, Meckel's diverticulum, large intestine, cecal tonsils, larynx, trachea, air sac, lung, heart, kidney, liver, brain, bone marrow, nasal turbinates, and comb) were collected and fixed by immersion in 10% neutral buffered formalin for 52 h. After fixation, the head of the femur and the beak sections containing the nasal turbinates were decalcified in 5% formic acid for 3-4 hours. All the tissues were processed into paraffin, and 3 μm section were cut for hematoxylin and eosin staining (HE) and immunohistochemistry (IHC).

Immunohistochemistry (IHC)

NDV nucleoprotein (NP) was detected in sampled tissues using the Novolink Polymer Kit (Leica Biosystems, Newcastle Ltd, UK). Briefly, after deparaffinization, tissues were subjected to antigen retrieval by microwaving for 10 minutes in Vector antigen unmasking solution (Vector Laboratories, Burlingame, CA) at maximum power. After cooling, quenching of endogenous peroxidase and protein block steps were carried out at room temperature according to the kit instructions. The primary antibody, raised in rabbit, was a polyclonal antibody directed against the NDV nucleoprotein synthetic peptide (TAYETADESETRRIC)[15]. The primary antibody was diluted 1:8000 in PBS with 0.02% Tween 20 and was applied to the slides for 1 h at 37^0C. After washes, tissues were incubated with the post primary block solution, followed by application of the Novolink polymer for 30 minutes at room temperature. Chromogen, 3-3'-diaminobenzidine (DAB), was applied for 5 minutes. Sections were counterstained lightly with hematoxylin and cover-slipped with Permount for a permanent record. Grading was carried out as specified in Table 1. When organs from birds at the same sampling interval had different scoring, the value was averaged and, if needed, rounded to the next integer.

Virus Isolation and Titration of swabs

Oral and cloacal swabs from each bird were placed into separate tubes containing 1.5 ml of BHI containing antibiotics (2000 U penicillin G/ml, 200 µg gentamicin sulfate / ml, 4 µg amphotericin B/ml; Sigma Chemical Co., St. Louis, MO). Swab samples were cleared by centrifugation at 1000 × g for 20 minute and the supernatant removed for virus isolation and titration. Virus isolation and titration of positive samples were conducted

according to standard procedures in of 9- to 10-day-old, SPF, embryonated chicken eggs[5,18]. Titers were expressed as embryo infectious dose 50% (EID_{50}) / 0.1 ml.

Results

Pathogenicity Index – ICPI

The ICPI values for *Kvuzat/13* and *Karachi/ 07* were 1.89 and 1.85, respectively. These results are in agreement with the sequences of the fusion cleavage site reported for these strains[4], confirming that these viruses are virulent by international standards.

Clinical disease

Detailed results of mortality and gross lesions for both strains are presented in Table 1. All *Kvuzat/13*- and *Karachi/07*-inoculated birds developed clinical signs of disease and were euthanized by days 4 and 5 post infection (pi), respectively. Overall clinical signs were similar between the two isolates, and consisted of conjunctivitis, ruffled feathers, and diarrhea starting at day 2 pi, progressing to severe depression and inability to stand at day 4 and 5 pi. The only surviving bird at day 5 pi in the *Karachi/07*-infected group showed severe depression and mild tremors. No abnormal clinical signs were observed in the control groups.

Gross Pathology

Gross findings are presented in Table 1. At day 2 pi, *Kvuzat/13*-infected birds showed moderate unilateral conjunctivitis, characterized by slight petechial hemorrhages and edema, enlarged mottled spleens, and petechial hemorrhages in the cecal tonsils. At day 4 pi, birds presented with marked splenomegaly associated with multifocal to coalescing foci of necrosis throughout the parenchyma (Fig. 1), severe diffuse thymic atrophy, diffuse bursal atrophy, bilateral severe conjunctivitis, and distended gall bladders.

Karachi/07-infected birds showed moderate conjunctivitis and splenomegaly with multifocal, white stippling throughout the parenchyma at days 2 and 3 pi. By day 4 pi, birds showed severe bilateral conjunctivitis, severe diffuse thymus atrophy with petechial hemorrhages, mottled spleens, bursal atrophy, and hemorrhages throughout the intestinal tract (Fig. 2), including the proventriculus, small intestine, and cecal tonsils. At day 5 pi, birds had small and mottled spleens, thymic atrophy with hemorrhage, and multifocal hemorrhage in the pharynx, duodenum, jejunum, and proventriculus. A urate deposit pattern was observed in the kidney of *Karachi/07*-inoculated birds at day 5 pi. Notably, birds infected with *Karachi/07* NDV strain had a very severe form of thymic serous atrophy at day 5, characterized by almost complete loss of thymic structure and accumulation of large amount of gelatinous material lodged in the jugular groove (Fig. 3).

Histopathology

Intensity and distribution of microscopic lesions are detailed in Table 2. Overall, both strains caused similar lesions, which started at day 2 pi and peaked at day 4 or 5 pi, respectively, for *Kvuzat/13* and *Karachi/07* NDV strains. Lesions for the two strains are described together, unless otherwise noted. Conjunctivitis was characterized by accumulation of edema fluid, heterophils, and macrophages at day 2 pi, which progressed to multifocal fibrin deposition, necrosis, and extensive hemorrhages by days 4 and 5 pi. In the spleen, at day 2 pi, there was multifocal, mild accumulation of karyorrhectic debris in the white pulp, and the splenic ellipsoids were expanded prominently by macrophages and appeared confluent. By day 4 pi, the splenic tissues were characterized by multifocal to coalescing areas of necrosis, associated with

accumulation of fibrin and large numbers of heterophils and macrophages (Fig. 4 & Fig. 5). In the thymus, multifocal necrosis was observed at day 2 pi, which became more severe at days 4 and 5 pi (marked lymphocyte depletion, loss of demarcation between cortex and medulla, fibrin deposition). The gut-associated lymphoid tissue (GALT) (Fig. 6), including the cecal tonsils, showed mild to moderate lymphocyte depletion and multifocal necrosis by day 2 pi, which progressed to severe coalescing necrosis at days 4 and 5 pi. In the intestines, the necrosis of the lymphoid-dependent areas was occasionally associated with focal to locally extensive ulceration of the epithelium and accumulation of necrotic material within the intestinal lumen. In the bursa, mild lymphocyte depletion was observed at day 2 pi, and by days 4 and 5 pi, lesions consisted of multifocal loss of cells in follicles and cortico-medullary junctions (punched-out appearance), which left prominent follicular epithelium and occasional formation of cystic structures. Multifocal necrosis in the pancreas developed for both strains at day 4 pi. In the respiratory system, the most remarkable finding was multifocal to coalescing, severe necrosis of the laryngeal tonsils, which was evident with both strains at day 2 pi, becoming more extensive at days 4 and 5 pi (Fig. 7). Other lesions consisted of multifocal necrosis of the tracheal epithelium at days 4 and 5 pi (Fig. 8), and multifocal necrosis and lymphoid depletion of the bronchial-associated lymphoid tissue (BALT) surrounding the tertiary bronchi. In the Harderian glands, lesions consisted of multifocal coagulative necrosis, glandular degeneration, and extensive heterophilic infiltrate. In the brain, there was multifocal, moderate infiltration of lymphocytes and plasma cells in the choroid plexuses at days 4 and 5 pi, which appeared more severe in *Karachi/07*-infected birds at day 5 pi. In the *Karachi/07*-infected bird, there was multifocal, necrotizing

myocarditis characterized by loss of myocardiocytes and accumulation of macrophages (Fig. 10). Multifocally, there was necrosis in the reticular stroma of the bone marrow at days 4 and 5 pi. Both strains caused multifocal tubular necrosis in the kidney starting at day 4 pi. By day 2 pi, there was multifocal to coalescing necrosis in the superficial and deep dermis of the comb. Lesions in the comb included accumulation in the dermis of necrotic debris, heterophils, macrophages, and edema fluid, and this change was associated with multifocal, epidermal necrosis and ballooning degeneration of keratinocytes and multifocal, serocellular crusts. There were multifocal areas of necrosis associated with lymphoid aggregates in the liver (at the level of portal triad) of *Kvuzat/13*- and *Karachi/07*-infected birds at days 4 and 5 pi, respectively.

IHC

Detailed results for IHC are presented in Table 2. Staining for NDV NP was mainly cytoplasmic and finely to coarsely granular, although extracellular staining was observed in larger areas of necrosis. Tissues from control birds were all consistently negative. Distribution and intensity of IHC labeling for the two strains were similar, and are described together unless otherwise noted. Birds infected with *Kvuzat/13* and *Karachi/07* strains had 24/26 and 26/26 positive tissues for NDV NP at least at one time point, respectively (Table 2). The most extensive and intense signal was observed in the eyelids, lymphoid organs (spleen, bursa, thymus), GALT (especially cecal tonsil), and BALT (especially larynx). In the spleen, immunolabelling was first detected at day 2 pi in macrophages surrounding the penicillary arteries, and subsequentially spread in association with the necrotic lesions at days 4 and 5 pi (Fig. 5). In the thymus, staining was present in cells compatible with both macrophages and lymphocytes, starting

multifocally at day 2 pi and progressively becoming diffuse at days 4 and 5 pi. In the other lymphoid tissues, including the GALT (Fig. 6), staining was initially present at day 2 pi in scattered cells morphologically compatible with macrophages, and at later time points spread through the whole tissue as lymphoid depletion progressed and macrophages became more prominent. In the respiratory system, prominent immunolabelling was detected in the epithelium of the trachea and larynx (Fig. 7 inset, 9), and in cells within the lymphoid aggregates of the BALT. In the Harderian glands, IHC signal was observed in scattered epithelial cells and in macrophages within the glandular stroma. Immunolabeling was also observed in the cytoplasm of scattered neurons in the brainstem and rare Purkinje cells in the cerebellum at days 4 and 5 pi. In the femur, multifocal signal was detected in areas of necrosis of the marrow stroma, mainly beneath the articular and growth cartilages, in large round cells with prominent cytoplasm consistent with macrophages. In *Karachi/07*-infected birds at day 5 pi, immunolabelling was observed also in the heart. Staining was multifocally present in macrophages that accumulated between myocardiocytes (Fig. 10, inset).

Virus isolation and titration of swabs

Results for virus isolation from oral and cloacal swabs are presented in Table 3. Both *Kvuzat/13*- and *Karachi/07*-infected birds shed virus in large amounts at days 2, 4 and 5 pi. The highest titers were obtained in swabs from oral secretions, compared to cloacal swabs. The magnitude of virus in clocal swabs increased at days 4 and 5 pi.

Discussion

NDV strains of sub-genotypes VIIi and XIIIb have been recently emerging in the Middle East and Pakistan. In particular, strains of sub-genotype VIIi have been

considered the cause of a possible fifth panzootic of NDV[4]. In the present study, one representative of each of the sub-genotypes VIIi and XIIIb were characterized by detailed clinico-pathological assessment. Results showed that these strains are markedly virulent, have broad tissue tropism, and are shed in large amounts from oral and cloacal secretions.

ICPI scores were 1.89 and 1.85 for *Kvuzat/13* and *Karachi/07* NDV strains, confirming that they are both virulent. These values are comparable to what is observed with other virulent NDV strains, such as representatives of genotypes VII (*LongBien/02*, 1.88; *SouthAfrica/08*, 1.91), XIV (*Niger/06*, 1.84), and XVII (*Nigeria/06*, 1.90)[29,30]. Pathogenesis experiments showed that both strains are highly pathogenic for chickens, with a rapid course of disease and death in all birds by day 4-5 pi. The time frame of clinical signs and the type of gross lesions observed in this study are consistent with what has been observed in previous pathogenesis experiments conducted with other virulent NDV strains with similar methodology[8,11,29,30].

Histopathology and immunohistochemistry revealed that both tested strains could cause lesions and replicate in multiple organs, suggesting a broad tissue tropism that was not only limited to the lymphoid organs (typically the primary target of very virulent NDV strains[8]). Especially severe lesions were observed in the laryngeal tonsils, and multifocal areas of erosion/necrosis were observed in the tracheal epithelium. In the lung, lesions consisted of lymphoid depletion and necrosis of the lymphoid patches associated with the tertiary bronchi. These lesions in the respiratory tract are considerably more severe than previously reported in similar experiments – especially in the tracheal epithelium[8]. The extensive immunolabeling in the laryngeal tonsils, trachea and, to a lesser extent, harderian glands and nasal turbinates, reflects the

high magnitude of virus shedding from oral secretions. Extensive lesions and virus replication were also observed in the intestine (small and large), which accounts for the high level of virus shedding in cloacal secretions. In the kidneys, lesions consisted of multifocal tubular necrosis and were associated with virus replication, supporting the notion of virus shedding not only through the feces, but also urine. In the brain, signal was observed, with both strains starting at day 4 pi in scattered neurons and occasionally in glial cells within glial nodules. Although replication was present in the brain, perivascular lymphocytic cuffing was not observed, most likely due to the acute course of the disease. These observations above confirm the broad tissue tropism of these strains and their replicative fitness *in vivo*.

Based on these results, strain representatives of sub-genotypes VIIi and XIIIb appeared to be markedly virulent, and showed broad tissue tropism and high pathogenicity. The very high virulence of *Kvuzat/13* NDV might partially account for field reports regarding sub-genotype VIIi outbreaks, i.e., high morbidity and mortality rates in wild birds (usually known to be non-clinically susceptible), and mortality rates between 40% and 60% in broiler production facilities where intensive vaccination practices are in place[4]. Overall, the results of the pathogenesis experiments carried out with the *Kvuzat/13* strain showed that this strain is highly virulent for poultry and has a marked capacity to replicate in oral, respiratory, and intestinal mucosae, thus supporting the hypothesis that NDV strains of sub-genotype VIIi might be shed extensively from infected birds and have the potential for efficient transmission and quick spread. This supports the idea that strains of sub-genoype VIIi may indeed be the cause of a fifth NDV panzootic quickly spreading throughout the Middle East[4]. The representative of

sub-genotype XIIIb, strain *Karachi/07*, appeared to have similar virulence to that of *Kvuzat/13*, causing comparable lesions and having comparable tissue tropism. Although strains of sub-genotype XIIIb seem to have limited geographic mobility compared to those within sub-genotype VIIi[(4)], scrupulous surveillance is warranted given their highly pathogenic potential.

In conclusion, the two NDV strains representing of sub-genotype VIIi and XIIIb were highly virulent for chickens and displayed a strikingly broad tissue tropism, suggesting that new NDV strains with panzootic features currently circulating in Middle East and Asia have a very high pathogenic potential. These findings underscore the importance of ongoing surveillance for newly-emerging NDV strains throughout the world, and the need for experimental infection studies to fully assess potential pathogenicity in poultry and other species.

Table 1. Summary of mortality and macroscopic lesions in 4-week-old chickens infected with *Kvuzat/13, Karachi/07* and BHI control.

NDV Strain	**Birds sacrificed at each day**	**Gross lesions at day 2 pi**	**Gross lesions at day 4 pi**	**Gross lesions at day 5 pi**
Kvuzat/13	Day 2 pi: 2 birds sacrificed for necropsy. Day 4 pi: 7 birds died spontaneously, one bird was euthanized *in extremis* (end of experiment). Collected samples from 3 birds.	Enlarged, mottled spleen (2/2)*; Petechial hemorrhages in cecal tonsils (1/2); Slight hemorrhage and edema or the left eyelid (2/2).	Hemorrhages in eyelid (2/3); Mottled and necrotic spleen (2/3); Thymic atrophy with serous atrophy (3/3); Bursal atrophy (1/3); Distended gall bladder (1/3).	All birds were dead

Karachi/07	Day 2 pi: 2 birds sacrificed for necropsy. Day 4 pi: 2 birds were euthanized. Day 5 pi: 5 birds died spontaneously, one bird was euthanized in extremis. Collected samples from 3 birds.	Enlarged, mottled spleen (2/2); moderate hemorrhage and edema on the left eyelid (2/2)	Hemorrhages in eyelid (1/2), proventriculus (2/2), duodenum (1/2), jejunum (1/2), cecal tonsils (1/2); Spleen necrosis (2/2); Thymic atrophy with serous atrophy (2/2); Dehydration (1/2).	Hemorrhage in eyelids (3/3), proventriculus (3/3), thymus (3/3), brain (1/3), duodenum (2/3), jejunum (2/3); Mottled and atrophic of spleen (3/3); Severe edema in the neck (2/3); Urate pattern in kidneys (1/3)
BHI (mock control)	2 birds euthanized for sampling at days 2, 4 and 5 pi.	No clinical signs observed.	No lesions observed.	No lesion observed

*Number of birds showing the lesion / the total number of birds necropsied that day

Table 2. Severity of microscopic lesions (HE) and distribution of NDV NP (IHC) in chickens infected with *Kvuzat/13* and *Karachi/07* NDV strains.

	Kvuzat/13		**Karachi/07**		
Tissues	Day 2 pi	Day 4 pi	Day 2 pi	Day 4 pi	Day 5 pi
	HE[a] / IHC[b]	HE / IHC	HE /IHC	HE / IHC	HE/IHC
Eyelid	++/+	+++/+++	+++/+++	+++/+++	+++/+++
Spleen	++/++	+++/+++	++/++	+++/+++	+++/+++
Bursa	+/+	+++/+++	+/+	+++/+++	+++/+++
Thymus	+/+	+++/+++ +	+/++	+++/+++ +	+++/+++ +
Harderian Gland	+/+	+/+	-/-	++/+	+/+
Esophagus	-/+	+/+	-/-	+/+	+/+
Crop	+/+	-/+	-/+	-/+	+/+
Cecal Tonsils	+/+	++/++	++/++	+++/+++	+++/+++
Proventriculus	+/+	-/-	++/+	+++/++	+++/++
Duodenum	+/+	++/++	-/+	+/++	+++/+++

Jejenum	-/-	+/+	NA/NA	NA/NA	++/++
Ileum	-/-	+/++	++/+	++/++	+++/+++
Meckel's Diverticulum	-/-	++/NA	++/++	++/++	++/++
Large Intestine	-/+	++/+++	++/++	++/+++	++/+++
Pancreas	-/-	+/+	-/+	+/+	+/++
Larynx	+/+	+++/+++ +	+/++	++/+++	+++/+++
Trachea	-/+	+/+++	-/-	+/+++	+/+++
Lung	+/+	-/++	+/+	+/++	+/++
Air Sac	-/+	+/+	+/NA	+/++	-/+
Heart	-/-	-/-	-/-	+/+	+/+
Kidney	-/-	+/+	-/+	+/+	+/++
Liver	-/+	+/-	-/+	-/-	+/++
Brain	-/-	+/+	-/+	+/+	++/++
Bone Marrow	-/+	+/++	-/++	+/++	++/+++

Comb	+/++	++/++	NA	++/+++	+/-
Nasal Turbinate	-/+	+/++	+/++	-/++	-/++

a: **Lymphoid organs**: (spleen, thymus, bursa, cecal tonsil) + = mild lymphocyte depletion / apoptosis; ++ = moderate (<50%) lymphocyte depletion, histiocyte accumulation, and multifocal necrosis; +++ = severe lymphocyte depletion, histiocyte accumulation, and necrosis (> 50% of sections). **Other organs**: + = mild / focal necrosis, inflammation (or gliosis in brain), and / or acute hemorrhage; ++ = moderate necrosis or inflammation; +++ severe necrosis, inflammation, or hemorrhage affecting > 50% of examined sections.

b: - = no IHC signal present; + = rare cells positive on IHC, ++ = positive cells seen, <50% of all 40 x HPF, +++ = positive signal seen in 50 to 75% of HPF, ++++ = abundant positive signal in more than 75% of the HPF.

N/A: not examined

Table 3. Virus isolation and titration of oral (O) and cloacal (C) swab samples and titers are expressed as EID_{50} / 0.1 ml.

	BHI		*Kvuzat/13*		*Karachi/07*	
Dpi†	***O***	***C***	***O***	***C***	***O***	***C***
2	ne	ne	$10^{3.9}$ (3/3)*	$10^{2.9}$ (3/3)	$10^{3.5}$ (3/3)	$10^{1.9}$ (3/3)
	ne	ne	$10^{3.7}$ (3/3)	$10^{3.1}$ (3/3)	$10^{2.7}$ (3/3)	$10^{1.7}$ (2/2)
4	-	-	$10^{6.3}$ (3/3)	$10^{6.5}$ (3/3)	$10^{6.3}$ (3/3)	$10^{5.5}$ (2/2)
			$10^{6.1}$(3/3)	$10^{5.9}$(3/3)	$10^{6.3}$ (3/3)	$10^{6.1}$ (2/2)
			$10^{6.3}$ (3/3)	$10^{5.5}$(3/3)	-	-
5	ne	ne	-	-	$10^{6.7}$(3/3)	$10^{6.3}$ (3/3)
			-	-	-	-
10	ne	ne	-	-	-	-
	ne	ne	-	-	-	-

* Number in parentheses indicates the number of eggs positive by virus isolation.

†Days postinoculation.

ne = negative swabs

- = non collected, either because was not sampling time, or because nobirds were left.

Figures: Lesions of Kvuzat/13- and Karachi/07 strains-infected birds

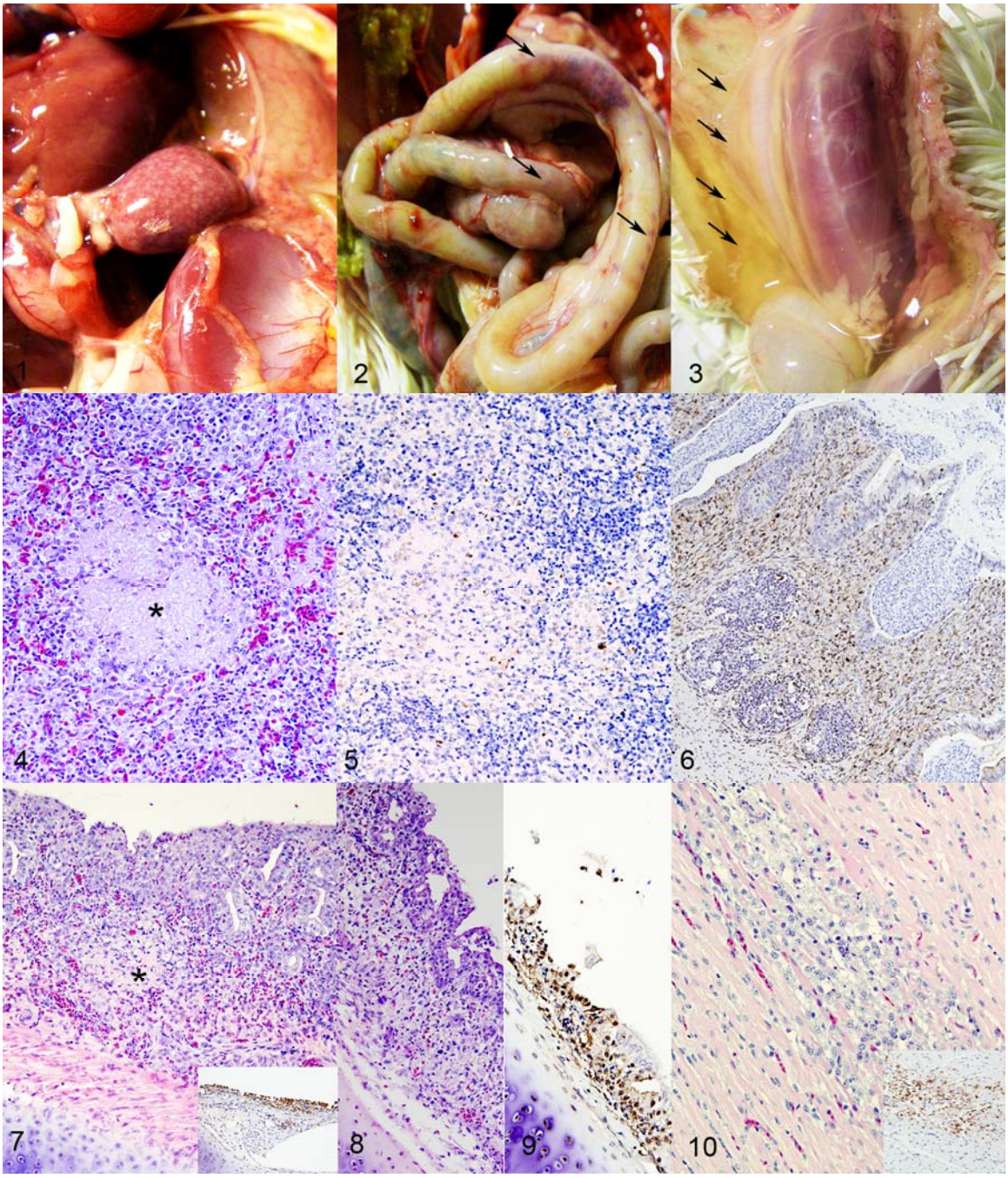

Figure Legends

Fig. 1. Spleen; 4-week-old, White Leghorn chicken infected with *Kvuzat/13* strain, day 4 pi. Multifocal specs of necrosis are present throughout the spleen.

Fig. 2. Small intestine; 4-week-old, White Leghorn chicken infected with *Karachi/07* strain, day 4 pi. Multifocal areas of hemorrhage and necrosis are observed though the serosa of the small intestine (arrows).

Fig. 3. Thymus; 4-week-old, White Leghorn chicken infected with *Karachi/07* strain, day 5 pi. There is diffuse, severe, serous atrophy of the organ and accumulation of gelatinous material in the jugular groove (arrows).

Fig. 4. Spleen; 4-week-old, White Leghorn chicken infected with *Kvuzat/13* strain, day 4 pi. Area necrosis characterized by loss of tissue architecture and accumulation fibrin (asterisk). HE.

Fig. 5. Spleen; 4-week-old, White Leghorn chicken infected with *Kvuzat/13* strain, day 4 pi. Positive signal (brown) is present interspersed within areas of necrosis. IHC for NDV nucleoprotein antigen, DAB method, hematoxylin counterstain.

Fig. 6. Cecal tonsil; 4-week-old, White Leghorn chicken infected with *Karachi/07* strain, day 4 pi. Diffuse positive signal for NDV is observed in large cells consistent with macrophages within the lamina propria. Lymphoid nodules show large macrophages that have engulfed cells debris. IHC for NDV nucleoprotein antigen, DAB method, hematoxylin counterstain.

Fig. 7. Larynx; 4-week-old, White Leghorn chicken infected with *Kvuzat/13* strain, day 4 pi. The epithelial cells show diffuse degeneration, loss of cilia, and attenuation. The lamina propria is edematous and shows diffuse accumulation of heterophils (some of

which undergo exocytosis through the epithelial layer) and multifocal areas of necrosis (asterisk). HE. Inset: immunolabelling is present within the cytoplasm of epithelial cells. IHC for NDV nucleoprotein antigen, DAB method, hematoxylin counterstain.

Fig. 8. Trachea; 4-week-old, White Leghorn chicken infected with *Karachi/07* strain, day 4 pi. The tracheal epithelium is attenuated and epithelial cells show degeneration and deciliation; inflammatory cells are undergoing exocytosis through the epithelium. The lamina propria is edematous and expanded by edema, necrotic debris, and heterophils.

Fig. 9. Trachea; 4-week-old, White Leghorn chicken infected with *Karachi/07* strain, day 4 pi. There is immunolabelling in multifocal areas of the tracheal epithelium, often associated with areas of ulceration or erosion. IHC for NDV nucleoprotein antigen, DAB method, hematoxylin counterstain.

Fig. 10. Heart; 4-week-old, White Leghorn chicken infected with *Karachi/07* strain, day 5 pi. Multifocally, there is a loss of myocardiocytes and accumulation of large cells with vacuoated cytoplasm consistent with macrophages. HE. Inset: degenerated (vacuolated) myocardial fibers and macrophages show intracytoplasmic immunolabelling. IHC for NDV nucleoprotein antigen, DAB method, hematoxylin counterstain.

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CHAPTER 4

CONCLUSION

In this study, two NDV strains, one representative of sub-genotype VIIi from Israel (APMV1/Broiler-Breeders/Israel (Kvuzat-Yavne)/NDV/826/2013 [*Kvuzat/13*]) and another representative of sub-genotype XIIIb from Pakistan (APMV1/chicken/SPVC/Karachi/NDV/33/2007 [*Karachi/07*]) were characterized by intra-cerebral pathogenicity index (ICPI) and detailed clinico-pathological assessment. Viruses caused 100% mortality of 4-week-old chickens within 4 (*Kvuzat/13*) and 5 days (*Karachi/07*) post infection. Gross and histologic lesions in all infected birds included: severe hemorrhagic conjunctivitis, extensive necrosis of lymphoid tissues, and segmental necrosis and hemorrhage in the small and large intestines. The ICPI scores were 1.89 and 1.85, respectively. Clinical signs and the patterns of lesions are similar with the results of previous pathogenesis experiments in birds infected with other virulent NDV strains.

Results of this animal experiment confirm that both *Kvuzat/13* and *Karachi/07* behaved similarly to other strains of velogenic viscerotropic NDV strains, causing death of all birds by 4-5 days post infection. There was a broad tissue tropism. Histopathologically, the lesions are most intense in lymphoid tissues, characterized by necrosis, fibrin deposition, hemorrhage, large numbers of heterophils and macrophages, and lymphoid depletion and all of these signs were present since 2 days pi. Both *Kvuzat/13* and *Karachi/07* caused necrosis in organs, such as conjunctiva, pancreas, and

brain, especially in the choroid plexus, bone marrow, and comb. Only *Karachi/07* caused multifocal necrosis of the myocardium.

As shown by immunohistochemistry for NDV nucleoprotein, both strains had systemic distribution in multiple organs, with the most intense and widespread signal in lymphoid organs. In general, the amount, timing, and location of virus signal in multiple organs is similar to other virulent ND strains, but both viruses caused more intense signals in the tracheal, larynx, and bronchial lymphoid patches compared to previously examined strains.

Overall, the results of this pathogenesis experiment carried out with *Kvuzat/13* strain showed that this strain is highly virulent for poultry and has a marked capacity to replicate in oral, respiratory, and intestinal mucosae, thus supporting the hypothesis that NDV strains of sub-genotype VIIi might be shed extensively from infected birds and have the potential for efficient transmission and quick spread. All the results showed that these strains have similarities with previous experiments with NDV velogenic strains. The results of virus titration demonstrated that the highest titers were obtained from oral secretions compared to cloacal swabs. Based on these characteristics and circulation of these viruses in the Middle East and Asia, both viruses have considered as participating in the new panzootic.