ROLE OF INDIVIDUAL GENES, GENOTYPES AND CYTOKINES IN THE PATHOGENESIS AND VIRULENCE OF NEWCASTLE DISEASE

by

LEONARDO SUSTA

(Under the Direction of CORRIE C. BROWN and CLAUDIO L. AFONSO)

ABSTRACT

Newcastle disease (ND) is a limiting disease of poultry worldwide caused by virulent strains of Newcastle disease virus (NDV), and often responsible for major panzootics and extensive poultry mortality. ND requires notification to the Office of International Epizootics (OIE). Although all isolates of NDV belong to the same serotype, different isolates can produce very different clinico-pathological disease in susceptible hosts. Such diversity has been imputed to the contribution of the different genes that compose the NDV genome, and to the intrinsic innate immune response of the host. In addition, recent evidence shows that numerous new genotypes of NDV are emerging, possibly further increasing the variability of ND presentation and NDV host range. Therefore, a better understanding of NDV pathogenesis should be pursued through the characterization of isolates that belong to new genotypes, and through the evaluation of the molecular pathogenesis of the disease, as determined by the viral genes and the cytokine response during viral infection.

In a first set of experiments, NDV infectious clones with substitutions of the HN (hemagglutinin-neuraminidase) and F (fusion) genes or the insertion of chicken

Interferon Gamma (IFN-γ) were rescued by reverse genetic techniques, and were tested in 4-week-old chickens. Results showed that mere substitution of HN and F belonging to virulent viruses did not significantly increase virulence when inserted into a low virulence backbone; whereas insertion of IFN-γ into a virulent backbone markedly decreased its virulence. Furthermore, results showed that addition of extra genes within the viral backbone decreased the overall virulence, but enhanced the neurotropic behavior of NDV.

In another set of experiments, three wild type strains from recent outbreaks and belonging to newly described genotypes were characterized in pathogenesis experiments to assess possible changes in pathogenicity. Results showed that tested strains acted similarly to what was previously observed, with a marked tropism for lymphoid tissues. Strain belonging to genotype I, class II (Australia strain) had attenuated pathogenicity, which was unexpected based on the results of standard pathogenicity indices. This underscores the importance of animal experimentation to completely characterize newly isolated NDV genotypes.

INDEX WORDS: Avian Paramyxovirus-1, Avian Virology, Chickens, Immunohistochemistry, Infectious Clones, *In Situ* Hybridization, Interferon Gamma, Newcastle disease, Newcastle Disease Virus, Pathogenesis, Pathogenicty, Reverse Genetics, Veterinary Pathology, Virulence

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DEDICATION

This dissertation is dedicated to my mother, Cristina. I know that without her continuous and relentless support, I could have never accomplished this.

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CHAPTER 1

INTRODUCTION

Newcastle disease (ND) is a reportable disease caused by virulent strains of Newcastle disease virus (NDV), a single stranded, non-segmented, negative-sense RNA virus, which belongs to the Mononegavirales order, Paramyxoviridae family, Avulavirus genus.[1] All NDVs are encompassed in a single serotype, avian paramyxovirus-1 (APMV-1).[1] The RNA genome consist of approximately 15,000 nucleotides that encode for 6 structural proteins, in order from 3' to 5': nucleoprotein (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and large protein (L).[1,4] The non-structural V protein is encoded by alternative mRNA editing of the P gene.[6]

Newcastle disease has a global distribution, affects numerous types of birds and is a constant threat to the poultry industry worldwide. In countries with an intensive poultry industry, ND causes severe economic losses due to the cost of control efforts and from trade restrictions. In developing countries, where chickens are reared in "backyard" systems, ND can markedly decrease the availability of dietary protein, and severely damages the microeconomy through loss of extra eggs or chickens for sale.[4]

One of the main characteristics of ND is its protean presentation, and the numerous degrees of virulence that different NDV isolates often produce in animals. On a clinico-pathological point of view, NDV isolates have been divided, from the most to the least virulent, into velogenic, mesogenic and lentogenic. Velogenic viruses can be further

divided into viscerotropic, if they cause widespread intestinal hemorrhages, or neurotropic, if they induce marked neurological diasease.[1-4] This variability, together with the increased number of new genotypes arising around the globe,[10] and the vast host range of this virus, makes it challenging to pinpoint ND as a single clinico-pathological entity, and hard to diagnose.

The determinants of NDV pathogenicity are not completely understood. Viral genes definitely play an important role, especially the F,[11] HN, [8] L[13] and the non-structural V protein.[6,7] However, the chicken immune system has also been implicated in the pathogenesis of the disease.[5,12] The adverse role of the immune system has been already established for other infectious diseases, and it has been established for highly pathogenic avian influenza, which elicits widespread, systemic activation/release of cytokines (cytokine storm) that contribute to multiorgan failure and death.[9,14]

The aim of this study is to contribute to the understanding of NDV pathogenesis through: 1) the use of recombinant viruses, produced by reverse genetic techniques, to investigate the molecular pathogenesis of NDV; and 2) to characterize new strains of NDV isolated during recent outbreaks, to assess possible changes in viral pathogenesis.

In the first set of experiments, we evaluated: 1) the effect of replacement HN and F genes from a velogenic strain on the virulence of a mesogenic backbone, 2) the effect of inserting an extra gene without known immunological activity (the green fluorescent protein, GFP) when inserted into a velogenic NDV backbone, and, 3) the effect of inserting chicken IFN-γ gene within a velogenic NDV backbone, to gain insight on how the innate host immunity can contribute to NDV pathogenesis.

In the second set of experiments, strains of NDV isolated from recent outbreaks were characterized to evaluate the possibility of changes in pathogenicity.

In all of the experiments, viruses were characterized by standard pathogenicity indices: ICPI (intracerebral pathogenicity index) and/or MDT (mean death time in eggs), and by full pathogenesis assessment in 4-week-old chickens to evaluate the extent of tissue damage. Viral distribution in tissues was assessed by the use of immunohistochemistry and *in situ* hybridization.

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CHAPTER 2

LITERATURE REVIEW*

*Cattoli G, Susta L, Terregino C, Brown CC. 2011. *Journal of Veterinary Diagnostic Investigations* 23: 637-656.Partially reprinted in section "PATHOLOGY AND PATHOGENESIS" with permission of the publisher.

DEFINITION

Newcastle disease (ND) is a limiting disease of poultry worldwide caused by virulent strains of Newcastle disease virus (NDV), and is often responsible for major panzootics and extensive poultry mortality.[7] ND is a disease that requires notification to the World Animal Health Organization, once known as Office of International Epizootics (OIE),[108] has the potential for rapid spread, serious economic losses and can markedly impair trade due to international embargoes.[108]

Given its enormous global impact, ND definition is strictly categorized and defined by international standards. According to the World Animal Health Organization (OIE) "Newcastle disease is defined as an infection of birds caused by a virus of *avian paramyxovirus serotype 1* (APMV-1) that meets one of the following criteria for virulence:

a) the virus has an intracerebral pathogenicity index (ICPI) in day-old chicks (Gallus gallus) of 0.7 or greater;

or

b) multiple basic amino acids have been demonstrated in the virus (either directly or by deduction) at the C-terminus of the F2 protein and phenylalanine at residue 117, which is the N-terminus of the F1 protein. The term 'multiple basic amino acids' refers to at least three arginine or lysine residues between residues 113 and 116. Failure to demonstrate the characteristic pattern of amino acid residues as described above would require characterization of the isolated virus by an ICPI test."

The amino acid residues are numbered from the N-terminus of the amino acid sequence deduced from the nucleotide sequence of the F0 gene. Residues 113-116 correspond to positions -4 to -1 from the cleavage site of F0.[108] This definition entails that not all the

strains or isolates of APMV-1 are the causative agent of ND, therefore strictly speaking, "Newcastle disease" should be applied only to infections with the isolates that meet the afore-mentioned criteria.

HISTORY

In the mid-eighteenth century, there were reports of poultry outbreaks with typical clinical characteristics of Newcastle disease in Europe and Eastern Asia. However these reports might have also described outbreaks of Avian Influenza (Fowl Plague) to which, clinically, virulent pathotypes of NDV are very similar.[83]

The first official records of NDV outbreaks were reported starting from 1926, when the disease arose as a new entity at the same time in two different parts of the world: in the state of Java, now part of Indonesia, and Newcastle-upon-Tyne, England.[6,7,37] Kraneveld (1926) described the event in this manner ''In March 1926, the Veterinary Medical Laboratory received a number of diseased chickens from Batavia (now Djakarta) on the island of Java, Indonesia, which had fallen victim to a disease appearing in that city for the first time. It is now known across the entire island of Java and in some parts of the outer regions". Kraneveld (1926) also noted that among kampong (village) chickens, "the disease often rages uncontrolled".[83] By 1928, the disease had spread throughout all the provinces of the British India.[41] By strange coincidence or, more likely, by some fact of which we have no knowledge, the disease occurred at Newcastle-on-Tyne in England in the spring of 1926,[37] this being exactly the period and year when the disease was reported by Kraneveld (1926) in Indonesia.[83] By 1927, Doyle recognized that the disease "is caused by a filter-passing virus and is characterized by a difficulty in respiration and a high mortality" and that "fowls immune

to Newcastle disease are susceptible to artificial and natural infection with plague" (Avian Influenza). Therefore, the important distinction with Avian Influenza was already made.

Two very striking facts about the first reports of Newcastle disease are its very abrupt insurgence, and that it presented almost simultaneously in two very distant areas of the world. To explain these peculiarities, three main hypotheses have been proposed:

a) The virus had always been present as an unrecognized disease of chickens.

b) The virus, essentially in its virulent form, was enzootic in some other species in which it produced no disease or unrecognized disease.

c) The virus originated at some point in time and place as the result of a major genomic mutation.[6,8]

HISTORY IN THE US

Currently (2011), the United States (US) is considered free of Newcastle disease (ND), meaning that the strains of NDV that circulate in poultry in the US are considered non-reportable by the OIE definition, having mild virulence and cause minimal clinical signs. Virulent NDV cases in the US are thought to be introduced from abroad and are considered to be exotic, hence the name Exotic Newcastle Disease. Therefore, according to the United States Department of Agriculture (USDA), Exotic Newcastle Disease (END) and Velogenic Newcastle Disease (VND) are synonymous.[1,48] However, this distinction is mainly applied to poultry, since virulent strains are known to circulate among wild pigeon and cormorant populations in the USA.[14,80]

In the first part of the 20th century, in the US, ND was known as avian pneumo-encephalitis, as reported in an article by J.R Beach in 1942.[18] In 1944, Beach proved that

the disease was caused by the virus of Newcastle disease, and this can be considered the first identification of a ND virus isolate in the US.[19] Since then the US experienced several outbreaks of ND. Of these, two have been very extensive, both in terms of animals destroyed and money spent for eradication.[48]

The first occurred in southern California in 1971, when commercial poultry flocks experienced a major outbreak. The disease threatened the California poultry industry which now ranks first in the nation for table egg production and fourth for turkey production. Spread of the disease beyond California's borders could have impacted the entire country's egg and poultry supply. The outbreak in California was finally eradicated in 1974, but the toll was high with nearly 12 million birds destroyed on 1,341 infected premises. Eradication efforts cost taxpayers $56 million, and severely disrupted the operations of many producers. Consumers paid the price, too, as prices of poultry and poultry products increased.[1,26,48]

The second outbreak occurred in October 2002, and was first diagnosed in backyard flocks (non-commercial). The disease was confirmed in California and later confirmed in Nevada (January 2003), Arizona (February 2003) and Texas (April 2003). By May 1, 2003 there were approximately 17,000 premises quarantined in the four states with over 3.5 million birds depopulated in over 2,500 different premises.[1,48,143]

After the 2002-2003 outbreaks, the USDA Animal and the Plant Health Inspection Services (APHIS), started an Exotic Newcastle disease (END) surveillance. This program aims to facilitate early detection of END in commercial and non-commercial populations within the United States and to identify at-risk populations to enhance targeted surveillance efforts during an outbreak.[1]

THE VIRUS

Newcastle disease virus (NDV) is classified in the the order Mononegavirales, family Paramyxoviridae, subfamily Paramyxovirinae, genus *Avulavirus*, and all strains, regardless of virulence, comprise avian paramyxovirus type 1 (APMV-1) within that genus. Newcastle disease virus is a non-segmented, single-stranded, negative sense RNA virus. On electron microscopy, the viral particles are enveloped, pleomorphic, and measure between 150 to 300 nm in diameter.[6-8]

The RNA genome is composed of approximately 15,000 nucleotides that encode for 6 structural proteins, in order from 3' to 5': nucleoprotein (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and large protein (L).[6,155] Three non-structural proteins are encoded by alternative mRNA editing of the P gene: the V protein, and the putative W^{155} and X^{91} proteins. However the X protein has been recently demonstrated not to be produced by NDV.[118] The length of the genome may vary, and three lengths have been observed: 15189, 15198 and 15192.[33] Regardless of its actual length, the number of nucleotides in the genomes is usually a multiple of six ("rule of six"). The proposed reason for this numerical ration is that stochiometrically, a monomer of the nucleocapsid (N) is best suited to hinge with 6 nucleotides.[117]

The N protein has the function to tightly bind genomic RNA, and on electron microscopy this protein shows a characteristic "Herring-Bone" pattern, with a diameter varying between 13-18 nm.[70,120,155] The L protein is the RNA-dependent RNA polymerase. The P protein is phosphorylated and, together with the L protein, is associated with the nucleocapsid. The RNA, together with the N, P, and L proteins forms the Ribonucleoprotein complex (RNP) that has the main functions of regulating and directing:

1) RNA *transcription* into messenger RNAs; and

2) the *replication* of the whole length genomic RNA for the new viral particles.[6]

The HN protein has both hemagglutinin and neuraminidase activities, and has the ability to agglutinate avian, reptile, human and guinea pig red blood cells.[6] The F protein is important in the fusion of the viral envelope with the cellular cytoplasmic membrane, and is the cause of syncytia formation as a cytopathic effect in cell culture.[6] The HN and F protein on electron microscopy are identified as 8-12 nm spikes that project from the viral surface. The M protein has a major role in the budding of virions from the host cells and interacts with the RNP.[6]

STRATEGIES OF REPLICATION OF NDV

NDV replicative activity, as with other negative strand non-segmented (NNS) RNA viruses, has two primary roles: 1) to produce messenger RNAs (with a 5' cap and a 3' poly-A tail) that can be translated into structural proteins; and 2) to produce faithful copies of the genomic RNA for the future viral particles. Transcription and replication of the RNA take place when the ribonucleoprotein (RNP) is intact, suggesting that the N and P proteins regulate the activity of the L protein, which has catalytic activity as a polymerase.[137]

As soon as the virus enters the cell, the polymerase produces a small, uncapped, RNA leader (Le+), and five to ten capped and polyadenylated mRNAs that encode for viral structural proteins. At this stage, viral replication can begin. The polymerase produces an antigenomic (sense) RNA intermediate that is encapsidated by the N protein. This antigenome serves as the template for the full length progeny genome (antisense).

Replication is asymmetric, meaning that usually there is 5-10 times molar excess of genomic RNA (negative sense {3'→5'}) versus antigenomic (positive sense {5'→3'}) RNA in the cell. This is explained by the fact that the RNA polymerase (L protein) has higher affinity for the promoter of the antigenome rather than the one in the genome.[31]

It is not clear what signal triggers replication versus transcription of the RNA. High concentrations of N protein have been associated with replication, while low concentrations with transcription; thus, it has been proposed that the availability of nucleoprotein in the cytoplasm may be the trigger to start viral replication.[21,22]

The transcriptional activity of NDV, as for other NNS RNA viruses, is sequential and polar.[15,29,49] Elegant experiments with UV light demonstrated that the polymerase engages the RNA at the 3' end of the genomic RNA and produces, as it proceeds in its reading, as many mRNAs (with poly-A tails and caps) as there are open reading frames (ORFs) in the NDV genome.[29] However, this activity has a gradient, meaning that the genes that are present at the 3' end are more frequently transcribed than those at more downstream (5') positions.[29] This may be the reason why the length of experimentally inserted foreign genes within the backbone of NDV and other similar viruses is proportional to the decrease in viral replication.[79,125]

PATHOLOGY AND PATHOGENESIS

Classification of viral pathogenicity

Newcastle Disease (ND) affects a wide range of domestic and wild avian species, however the severity of the disease varies greatly, spanning from peracute disease with almost 100% mortality, to subclinical disease with no lesions. This variability makes it difficult to pinpoint ND as a single clinicopathologic entity. Based on the severity of

clinical disease, the strains of NDV were originally classified into four pathotypes, known as Doyle, Beach, Beaudette and Hitchner forms.[6] Nowadays, pathotypes are more commonly referred, from least to most pathogenic as: "asymptomatic enteric", "lentogen" (former Hitchner form), "mesogen" (former Beaudette form) and "velogen". The velogens have been further divided into "viscerotropic" (former Doyle form; velogenic viscerotropic NDV; VVNDV) or "neurotropic" (former Beach form; velogenic neurotropic NDV; VNNDV) according to their ability to cause primarily visceral or nervous signs.[6] Additionally, some laboratory testing in embryos or chickens using standard pathogenicity parameters can be done, including MDT (mean death time), IVPI (intravenous pathogenicity index) and ICPI (intracerebral pathogenicity index). All involve the use of numeric criteria. The MDT is the time to death, measured in hours, after inoculation of embryonated eggs – if the embryos die in less than 60 hours, it is classified as a velogen; if the embryos survive for more than 90 hours, it is classified as a lentogen; anything in between is a mesogen.[6,108] The IVPI test involves scoring chickens' illness (0=normal; 1=sick; 2=paralyzed or nervous signs; 3=death) after intravenous inoculation of 6-week-old chickens. IVPI scores are computed in similar way to ICPI (see below) and range from 0 to 3. According to some authors,[7] velogenic NDV have IVPI scores between 2 and 3, mesogenic between 0.0 and 0.5, while lentogens have 0; however, to the author's knowledge, there are no IVPI cut-off values to define notifiability to the international community. IVPI test is not in widespread use today.

At present, according to international standards,[108] the definitive *in vivo* assessment of virus virulence is based on the ICPI test, which is regarded as the most sensitive and widely used test for measuring virulence. The ICPI is a calculation done by scoring sick

or dead birds (0=normal; 1=sick; 2=dead) every day for eight days after inoculation of virus intracerebrally into ten 1-day-old chicks.[108,139] Scores range from 0-2, and any strain with an ICPI≥0.7 is considered virulent or "notifiable" to the OIE.[108] The correspondence between these standard tests and pathotypes are reported in tables 2.1.[7] Additionally, the OIE recognizes specific sequences of the F protein as a qualifier for virulence: "notifiable" are those strains that have, with respect to the amino acid sequence of the fusion (F) protein, one pair of basic amino acids at residues 116 and 115 plus a phenylalanine at residue 117 and a basic amino acid (R) at residue 113.[108]

Some drawbacks to these tests and difficulties exist in the interpretation of pathotype results. For example, Pearson *et al.* [115] reported 10 NDV isolates from pigeons to have ICPI values between 1.2 and 1.45 and a range of IVPI values from 0 to 1.3, suggesting the viruses were virulent; however the lowest MDT recorded was 98 hours, a characteristic of lentogenic viruses. In fact, not all virulent strains have an MDT<60h (see table 2.2). The *in vivo* tests on strains isolated from species other than chickens can present some problems and may not produce accurate readings until passaged in chickens or embryonated chicken eggs.[12] Personal experiences show that a more accurate indication of the real 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}) administrated via natural routes (e.g. oro-nasal route).

Bird Studies

Although the standard pathogenicity indices can often offer a good idea of the virulence, they do not always correlate exactly with what is observed in animal experiments, especially when the virus is administered to adult birds via a possible natural route of inoculation.[135,145] In other words, the MDT and ICPI do not always correlate with the clinicopathologic syndrome, and the "disease inducing ability" of each strain. Furthermore, it should be noted that the status of "notifiability", as indicated by the OIE (ICPI≥0.7 and/or virulent fusion protein cleavage sequence) covers a broad span of pathogenic potential, encompassing viruses that are capable of causing very severe lesions or none (OIE).[108] Therefore, animal experiments done in conditions similar to those in the field (for example 4-week-old chickens inoculated via eyedrop instillation), are useful as they help to completely characterize newly isolated strains. Such information can augment our understanding of the strains and can improve diagnostic ability by describing more accurately the clinical and pathologic presentations associated with a particular isolate.

In the next sections, the clinical signs and pathologic findings will be presented based upon species infected. Data and findings are drawn from numerous sources, but mainly from original research papers, case reports, and textbook chapters. In addition, much is based on observations in one of our laboratories (SEPRL-ARS-USDA and UGA), where a large number of strains have been studied in an identical and systematic way. In this system, a standard target dose (10^5 EID_{50}) is given via a natural route (eyedrop inoculation) to uniform age (4-week-old) and source (SEPRL source flock) birds, with consistent observation, collection, and examination methods.

Clinical signs and pathologic findings with NDV infection

Chickens

The vast majority of references on NDV in poultry are related to chickens, as this species is the most seriously impacted by NDV.[6] There are such widely varying disease forms that for this species clinical findings are further divided according to pathotypes. However, the severity of clinical signs does not vary only accordingly to the inherent virulence of the virus, but also according to some host-related factors. These are mainly age, route of infection, immune status and concomitant environmental stress. For example, younger animals tend to have more severe and acute disease than older animals, intravenous inoculation is more likely to elicit neurologic signs, and aerosolization of high viral doses tends to impact the upper respiratory tract preferentially.[2,6,7,9,78]

Velogenic Viscerotropic ND (VVND)

With VVND, mortality easily can reach 100%, and in experimental conditions the course of disease is rapid, usually 2 to 4 days. Clinical signs are first recognizable starting at 2 days postinfection (dpi).[26,74,77,135,143] The main signs are conjunctival swelling and reddening centered over the lymphoid patch located in the lower eyelid, anorexia, ruffled plumage, prostration, weakness, tremors and diarrhea; labored breathing is variably reported.[6,26,74,77,135,143] In numerous animal experiments conducted with the same technique in our laboratory (infection via eye drop instillation in 4-week-old chickens) respiratory signs were observed very rarely, and were limited to open-mouth breathing in a few animals.[135] In the absence of respiratory lesions (as reported in the same studies), the open-mouth breathing was interpreted as polypnea and a consequence of a generalized febrile state.

The presence of multifocal hemorrhages seen through the serosal surface of the intestines, multifocal areas of necrosis/ulceration of the gut-associated lymphoid tissue, and disseminated foci of necrosis in the spleen are highly suggestive of VVNDV infection.[6-8,26,74,77,135,143] The cecal tonsils, which are especially prominent gut lymphoid aggregates located in the proximal portion of the ceca, are often regarded as the "old faithful" lesion for VVND, as they most consistently display hemorrhage and necrosis grossly. Other common intestinal lesions are multifocal hemorrhages and ulceration in the junction between proventriculus and gizzard, which is a site of lymphoid aggregate development. Spleens are enlarged and severely mottled, in the most severe cases showing multiple foci of white to yellow discoloration (necrosis).[26,74,77,135,143] Perithymic hemorrhages are occasionally observed,[26] and as the disease progresses, there is severe atrophy of thymus and bursa.[77,135] Tracheal hemorrhages have been rarely described, but were a notable features in many chickens infected with the CA02 isolate, a VVNDV isolated from the 2002 outbreak in California,[143] especially in the cranial portion of the trachea, and were the consequence of necrosis in the laryngeal tonsils.[143] Comb/wattle edema are variably present.[94] Eyelid edema and hemorrhage are consistent findings in animals inoculated via the conjunctival route.[26,103]

The most unifying histologic feature is severe necrosis of the lymphoid tissues scattered throughout the body, most especially prominent in spleen and gut-associated lymphoid tissue, which corresponds to the foci hemorrhage and ulceration noted grossly.[6,26,74,77,135,143] In the less severe, or initial stages, there is lymphoid depletion, and hyperplasia of macrophages with large vacuolated cytoplasm (commonly is referred as "starry-sky" effect). In later stages, there is accumulation of cellular and karyorrhectic

debris, pyknosis and numerous macrophages with vacuolated cytoplasm that contain nuclear debris (tingible body macrophages).[26,74,77,135,143] In the thymus very early in the infection there is almost complete necrosis of the cortex. The medulla usually has less severe lymphoid depletion. In the bursa of Fabricius, there is severe loss of lymphocytes both in the cortex and medulla of numerous follicles. The intrafollicular epithelial cells become prominent and there is accumulation of numerous macrophages. Occasionally there is formation of epithelium-lined cysts within the lymphoid-depleted lobules. Although numerous follicles are affected at the same times, it is not unusual to observe severely affected lobules adjacent to less affected or normal ones.

Microscopic changes in the brain are minimal with VVND, even in birds dying with neurologic signs. Perivascular cuffing is occasionally described.[6,26,74,77,135,143] One report of a field outbreak caused by a VVNDV strain, describes multifocal necrotizing encephalitis characterized by multifocal extensive areas of malacia. However, when the same isolate was inoculated into SPF chickens, no encephalitic lesions were observed, suggesting a co-participation of other factors in the pathogenesis of the field lesions, rather than just pathogenic potential of the virus alone.[104]

Occasionally, vascular changes such as hydropic degeneration of the tunica media, hyalinization and development of hyaline thrombosis are reported in VVNDV infections.[6,103] However, in numerous experiments involving SPF chickens infected via eyedrop instillation,[25,26,74,75,77,103,135,143,146] vasculitis has never been a prominent feature. Most likely, it is possible that vessels adjacent to intense inflammatory foci might undergo nonspecific inflammatory changes that alter the vessel's morphologic appearance. In the experience of the authors, vessels adjacent to primary areas of

infection can show some degree of hyalinosis, most likely caused by intense exudation of proteins.

With respect to other body organs, VVND infection has been reported to cause multifocal areas of necrosis in the pancreas, liver and gall bladder.[6,74,77,135,143] A recent NDV outbreak in a poultry facility Japan[104] was characterized, among other lesions, by hepatic necrosis. When the same strain was inoculated in SPF chickens, fibrin thrombi occurred in the liver.

In general, there is a paucity of evidence that VVND affects the lung. In multiple experiments using various VVND strains in our laboratory, there has been no indication of pulmonary parenchymal involvement.[26,74,77,135,143] One report of a VVNDV field outbreak in Japan describes a pneumonia characterized by histiocytic proliferation and tracheitis.[104] However when the same isolate was inoculated into SPF chickens, no pulmonary lesions were observed.

Velogenic Neurotropic ND (VNND)

Morbidity with VNND often reaches 100%, and mortality is usually 50% (but can rise to 100% in young chickens). The most prominent clinical signs are neurologic and consist of head twitch, tremors, opisthotonus and paralysis.[6,26,139] Despite the fact that the neurologic involvement can be dramatic, the animals are characteristically bright and alert and if able to reach the food, will eat. The course of the disease is longer than with VVND, and the neurological signs are most prominent between 5 and 10 days post-infection (dpi), which is beyond the point of survival with most VVND strains, where animals often die at 4 or 5 dpi.[26]

While according to some key reviews,[6,139] respiratory signs are considered a prominent feature of infection with velogenic neurotropic strains, there is an absence of original reports (at least in the recent literature) that describe respiratory clinical signs or respiratory lesions in animals experimentally infected with VNNDV. When 4-week-old chickens were infected via eyedrop instillation with four neurotropic strains (Turkey ND, Texas GB, Cor-MI, Cor-MN) respiratory distress was not observed, and the neurologic signs predominated.[26,73] To the authors' knowledge, only one original report, published in 1976, of experimental infection with VNNDV induced severe respiratory signs, and these were described as mouth breathing and gasping by 4 dpi, followed by nervous signs at days 11-12 dpi.[133]

Gross lesions are often absent and the involvement of the visceral organs appears to be minimal, although animals euthanized in the early stages of disease may have splenic or proventricular congestion.[26] Despite the neurotropism of these strains, gross lesions in the central nervous tissue are not present.[6,26] In comparison to VVND, there are no characteristic gross lesions for VNND. In fact, in most cases, gross lesions are completely absent.

Histopathologic changes in chickens infected with VNND strains are largely restricted to the central nervous system. There is multifocal mononuclear perivascular cuffing, associated with hypertrophy/hyperplasia of vascular endothelium, moderate gliosis and multifocal necrosis of the Purkinje cells.[26,151] The lesions are more prominent in the cerebellum, especially within the molecular layer, where they first appear around 5 dpi.[151] Nervous lesions are most prominent between 5 and 10 dpi. Other reported

histologic lesions with VNNDV are lymphoid depletion and myocarditis.[26] No reports of documented pneumonia with VNNDV were found in the literature.

Mesogenic ND

As reported by Alexander,[6] mesogenic viruses in field conditions cause mild clinical signs, mainly respiratory. Field outbreaks with mesogenic strains also have been associated with a drop in egg production and misshapen eggs.[6] Concurrent viral and secondary bacterial infections are thought to be common complications of mesogenic NDV that result in more severe morbidity.[6,20,42,105,139]

In contrast to what is observed in the field with mesogenic strains, experimental inoculation of SPF chickens with most mesogenic strains causes very minimal clinical signs (mostly slight depression), but not any signs specifically related to the respiratory system. In numerous animal experiments conducted with similar methodology, mesogenic strain infection will in rare cases result in neurologic signs, similar to those observed with VNND, but much milder, and with lower mortality rates.[74,135,136]

Gross lesions with mesogenic strains are minimal. As shown by Brown,[26] SPF chickens infected with mesogenic strains had mild splenomegaly and some degree of conjunctivitis when inoculated via eyedrop instillation. In the field, infection with mesogenic strains is often associated with secondary bacterial infections, which have their own set of morphologic correlates.[6,8,9]

Histologically, there is a range of changes seen with mesogenic strains. The more virulent strains, those that cause a notable degree of clinical disease, consist mainly of nonsuppurative encephalitis that has many similarities to the cases caused by the VNND strains, i.e., perivascular cuffing and gliosis. Some birds may also have myocarditis,

especially between 5 and 10 dpi.[26,74,135] In addition, splenic and pancreatic necrosis can be observed.[156]

When pigeon-isolated strains, which were considered to be mesogenic after passage in chickens,[76] were inoculated into 4-week-old chickens via eyedrop instillation, nervous lesions were the most severe, and were characterized by perivascular cuffing, gliosis, chromatolysis and neuronal necrosis, all of which were most prominent in the cerebellum and medulla oblongata between 5 and 10 dpi.[75,77] In the same studies, multifocal myocardial necrosis and mild splenic necrosis were observed.

Lentogenic ND

It is generally accepted that lentogenic viruses do not cause disease in adult chickens. Although some textbooks refer to the LaSota strain as causing severe respiratory disease in very young animals, no peer-reviewed references could be found in the scientific literature.[6] When the lentogens B1 and QV4 were experimentally inoculated into 4-week-old chickens,[26] or when QV4 was inoculated into 7-week-old chickens,[53] in both cases via eyedrop instillation, no clinical signs were observed.

Some lentogenic isolates in Australia have been associated with respiratory disease in commercial broilers in the field ("late respiratory syndrome"), with very low mortality, detectable gross lesions (reddening of the trachea) and chronic non-suppurative tracheitis histologically.[57] However, *E. coli* was consistently isolated from the tracheas of the diseased birds, indicating that the clinical disease may well have been multifactorial in nature.[56]

In another report, there were mild clinical signs consisting of rales, coughing, anorexia, and depression observed between 2 and 12 dpi when a lentogenic strain (Ishii)

was aerosolized at high concentrations into 40-day-old SPF chickens.[78] However, the high dose delivered directly to the respiratory system may have affected the clinicopathologic syndrome.

Lentogenic strains produce minimal, if any, gross lesions.[6] In one report, mild pulmonary hemorrhages and splenomegaly were described with QV4 strain, when inoculated via eyedrop instillation.[53] In another experiment conducted by Brown, inoculation of B1 and QV4 via eye-drop (using our laboratory's standard methodology and dosing) caused no gross lesions.[26]

In a report by Hooper, in which fields outbreaks are described in farmed broilers in Australia, lentogenic strains of NDV had been isolated together with *E. coli*, and gross lesions consisted mainly of tracheal hemorrhages;[57] when the same NDV isolate was experimentally inoculated into SPF chickens, no gross lesions were detected.[56]

Histologic changes seen in lentogen-infected chickens are minimal. When the lentogenic strains B1 and QV4 are inoculated via eyedrop into 4-week-old chickens, hyperplasia of the lymphoid follicles in the spleen and air sacs were present.[26] In a similar experiment with QV4, but with slightly older birds (7 weeks), there was lymphoid follicle proliferation mainly in the lamina propria of the trachea.[53] Some lentogenic isolates in Australia caused non-suppurative tracheitis in association with *E. coli* in field outbreaks, or, when experimentally inoculated into SPF chickens, induced mild changes, including lymphocytic infiltration, loss of cilia, and squamous metaplasia in the proximal trachea.[56,57] Aerosol delivery of lentogenic virus in an experimental setting commonly results in tracheal changes - deciliation, congestion, goblet cell hyperplasia, edema, and multifocal submucosal infiltration of scattered heterophils, lymphocytes and plasma

cells.[78,93] In one report of B1 infection via aerosol and air sac instillation, the main lesion consisted of lymphoid follicle proliferation in the lung and in the air sacs.[52]

NDV in other avian species:

Turkeys

Turkeys are susceptible to NDV and clinical signs are similar to those present in chickens but are less severe.[11,24] As shown by Wakamatsu, 3-week-old SPF and 6-week-old commercial turkeys infected with CA02, a VVNDV isolated from the 2002 outbreak in California,[143] all became sick, with first clinical signs appearing around 2 dpi and all were dead or humanely euthanized by 5 dpi. Clinical signs consisted mainly of depression, nasal discharge, blood-tinged diarrhea and incoordination. In the same study, commercial turkeys appeared more resistant than SPF turkeys. In another experiment conducted by Piacenti *et al.* ,[119] SPF turkeys infected with the velogenic viscerotropic strain CA1083 had body tremors, dyspnea, incoordination and died by 7 dpi; those infected with TurkeyND and Iowa 1519 (velogenic neurotropic) were recumbent and uncoordinated, with leg and wing paralysis, and head twitching by 5 dpi, with all animals infected with Iowa 1519 strains dying by 7 dpi. In the same experiments, 4-week-old commercial turkeys had less severe clinical signs, and longer disease progression.

Turkeys infected with VVND strains developed splenic necrosis and/or splenomegaly, conjunctivitis, multifocal areas of hemorrhage and ulceration mainly in the small and, to a lesser extent, large intestine, multifocal hemorrhages in the upper third of the trachea that are associated with necrosis of the laryngeal tonsils (just caudal to the epiglottis), cloudy air sacs, and multifocal pancreatic necrosis. Histologically the most

prominent lesion was lymphoid depletion and necrosis of the organs and ulceration of the intestine overlying the affected lymphoid patches.[119,143] Turkeys infected with VNNDV (Turkey ND and Iowa 1519) had multifocal gliosis and perivascular cuffing in the brain, necrosis of Purkinje cells, multifocal necrotizing myocarditis and mild lymphocytic infiltration within the airsacs.[119] Turkeys infected via eyedrop with Roakin strain had conjunctivitis, splenomegaly, multifocal myocardial necrosis, lymphocytic infiltration in tracheal mucosa and airsacs and by 10 dpi, multifocal areas of pancreatic necrosis. Infection with LaSota (a lentogen) via eyedrop did not cause any lesions.[119] In turkeys aerosolized a very high dose of lentogenic NDV (LaSota, B1, ET, 2024), the most prominent lesion was mild to moderate fibrino-necrotizing tracheitis.

Ducks

Ducks can become infected and spread the virus, however no clinical signs are usually reported when animals are experimentally infected, even with velogenic strains.[107,110] Only one paper describes neurological signs in ducklings that were experimentally infected with a mesogenic strain that was responsible for a previous field outbreak in ducks.[55]

Geese

Geese are considered susceptible to infection, but the development of clinical disease is variable. Recently there have been numerous reports[59,67,87,148,158] of clinical disease in geese caused by NDV strains in China. As reported by Hongquan *et al*,[148] the same field isolates were able to experimentally reproduce similar clinical disease in SPF geese. The involved strains belonged to genotype VIId (isolated more frequently), VI and IX. Clinical signs started to appear at 3 dpi and were characterized by moderate to severe

depression, anorexia, diarrhea, ocular and nasal discharges, and swelling of the eyelids. Deaths occurred between 3 and 12 dpi.[148]

Both natural and experimental infections of geese with some novel strains circulating in China[148] were characterized by multifocal areas of ulceration and hemorrhages in the esophagus, gizzard and multifocal necrosis of the intestinal mucosa. Histologically, there was ulceration and fibrin deposition in the intestinal mucosa and over the cecal tonsils, severe atrophy of lymphoid organs and lymphoid depletion in some animals, multifocal areas of necrosis in the pancreas and, less frequently, in the liver. In a few cases, the brain was affected, with neuronal degeneration present.

Pigeons

Birds in the *Columbiformes* order, which includes pigeons and doves, can be infected with NDV.[44,143] Most Newcastle disease in pigeons, however, is due to pigeon-specific viruses, which are known as pigeon paramyxovirus-1 (PPMV-1) to distinguish them from the rest of the APMV-1 viruses. The first PPMV-1 outbreak was first reported in the Middle East during the 1970s, then spread to Europe during the 1980s[86] and nowadays is considered to be endemic worldwide.[142] Clinical signs in pigeons vary mainly according to age. In young animals mortality can reach 100%, whereas in adults mortality is minimal, and morbidity around 10%. Incubation period is 10-14 days and viral shedding can be observed beginning at 2 dpi[13] Clinical signs consist mainly of nervous signs (most prominent in young birds) and diarrhea.[6,142]

Unlike PPMV-1, NDV strains isolated from chickens, even when highly virulent, cause minimal or no clinical disease in pigeons. In a study conducted by Wakamatsu *et al.*,[143] eye drop instillation of CA02, a velogenic viscerotropic strain, caused observable

clinical disease (mild tremors) in only one of ten inoculated birds. In other studies when pigeons were inoculated via eyedrop with CA1083 (also a VVND), clinical disease was observed in 9/21 juvenile birds and 5/10 adult birds. Clinical signs consisted of head tremors, wry neck, opisthotonus, wing droop, and leg paralysis. In the juvenile pigeons, 7 or 21 died, and in the adults, only 1 in 10 died.[44]

Lesions associated with NDV in pigeons vary according to the virulence of the strains. In general, isolates from chickens cause minimal pathological changes, whereas the pigeon variant of NDV (PPMV-1) can cause a series of lesions that vary based upon age and inoculation route.[72] Gross lesions in pigeons infected with PPMV-1 from natural outbreaks consist of pancreatic necrosis, enteritis and proventricular hemorrhages. Histologically, lesions consist of non-suppurative encephalitis, multifocal necrosis in the spleen, bursa, liver, larynx and pancreas and multifocal accumulation of lymphocytes in several organs.[92,115,156] Grossly, pigeons infected with viscerotropic velogenic CA02 showed only moderate splenic enlargement at gross inspection, while histological lesions consisted mainly of perivascular cuffing and gliosis in the cerebellum and brainstem by 14 dpi.[143]

Upland gamebirds

Partridges and pheasants are considered to be extremely susceptible to NDV.[4,5] Clinical signs have been considered similar to those observed in chickens, and can span from acute onset with rapid death, severe nervous signs, to inapparent infection.[6,32,71] Also, lesions are similar to those observed in poultry.[6]

Cormorants

There are several reports describing NDV outbreaks in cormorant populations. Reports from the field describe mainly neurologic signs,[14] however, experimental infection of cormorants infected with strains isolated from recent outbreaks did not cause mortality in 16-week-old cormorants. Clinical signs, including tremors and ataxia, were recorded in a small number of inoculated birds.[80]

In field reports[14,16] the most prominent gross lesions were enlarged and mottled spleen associated with bursal atrophy and multifocal hemorrhagic foci in the meninges. Histologically, lesions consisted of multifocal non-suppurative encephalitis with areas of gliosis, which appeared more prominent in the cerebellar white matter, interstitial nephritis and multifocal myocarditis. In experimentally infected 16-week-old cormorants, no lesions were observed at necropsy.[80]

Pet birds

In psittacine birds clinical signs vary from inapparent to severe neurologic disease. The incubation period is usually short (2-3 days), but can be up to 14 days.[139] In a survey including seven species of wild birds in a zoological collection, three species of parrots (macaw parrot, white cockatiel, red breasted parakeet) shed VVND without showing clinical signs.[123] In 1991, Panigrahy[112] reported cases of ND in psittacine birds from six states of the USA, in four of which the disease assumed outbreak proportions. Clinical signs included tremors, lateral recumbency, respiratory distress, greenish diarrhea, ruffled plumage, head drawn back between the shoulders and often death. The isolated viruses were categorized as VVNDV. Birds affected were yellow-headed Amazon parrots (*Amazona ochrocephala oratrix*), yellow-naped Amazon parrots

(*Amazona ochrocephala auropalliata*), cockatiels (*Nymphicus hollandicus*), and conures (*Aratinga* spp).

In another study,[45] aerosol exposure of budgerigars (*Melopsittacus undulates*), Amazon parrots, and conures with a VVNDV isolated from an Amazon parrot, caused mainly neurological signs consisting of tremors, ataxia, wing droop, and uni- or bilateral leg paralysis that culminated about 2 weeks post infection. Animals with bilateral leg paralysis died, while those with single leg paralysis adapted to the condition or recovered. Budgerigars showed the most severe signs, followed by Amazon parrots and conures. In the same study, budgerigars, Amazon parrots and conures were able to spread the virus and to infect cage-mates.

Psittacine birds also have been implicated in the maintenance of NDV infections and in transmission of the disease to poultry species. The California outbreak of 1971 has been traced back to a psittacine isolate.[141] This appears even more important since excretion of VVNDV has been shown to last for more than one year after exposure in Amazon parrots and for more than 80 days in budgerigars.[45]

Gross and histological lesions in psittacines after NDV exposure are not well documented. In one report,[44] following aerosol exposure to VVNDV, conures, Amazon parrots and budgerigars had hemorrhages and necrosis of the intestinal mucosa, hemorrhages on the skull cap and around the orbit, fibrinous peritonitis, hepato-splenomegaly, focal hepatic necrosis, airsacculitis and hemorrhagic tracheitis.

Canaries (*Serinus canarius*) show variable clinical disease. In one study,[45] aerosolization of a VVNDV strain into canaries caused viral shedding and cumulative mortality of 25%, however, no characteristic clinical findings were observed before

death. In another study, canaries showed very low mortality upon infection with a VVNDV strain and, when present, clinical signs included severe depression prior to death and neurological deficits.[140]

Immunohistochemistry and *in situ* hybridization as tools for better understanding the pathogenesis of NDV

Immunohistochemistry and *in situ* hybridization (ISH) have been used extensively to study the distribution of NDV protein in the tissues of infected birds[26,58,74-77,90,109,143-146,151] These studies have allowed a more thorough understanding of the tropism and distribution of the virus. As such, they help to supply information about pathogenesis and so can clarify for the diagnostician the pattern of lesions seen grossly and histologically. They are not in routine use as diagnostic assays but the information from the experimental studies can supply a roadmap in understanding the clinicopathologic picture presented with the various pathotypes. The pathotypes - VVNDV, VNNDV, mesogens, and lentogens - all appear to have different tropism and viral distribution in body tissues.

Velogenic Viscerotropic ND (VVND)

Animal experiments in chickens conducted with similar methodologies[74-77,143] showed that the VVNDV strains, in comparison with the other pathotypes, have the most intense and widespread distribution of virus in various tissues. Virus was constantly found as early as 2-3 dpi in the lymphoid tissues throughout the body, including the thymus and bursa (primary lymphoid organs), spleen and cecal tonsils. Within the spleen, both by IHC and ISH, signal was mainly observed in the macrophages surrounding the penicillary arteries, whereas in the other lymphoid organs virus was detected in the center

of the lymphoid follicles, in cells morphologically compatible with macrophages and lymphocytes. In the cecal tonsil, NDV first appears in cells morphologically consistent with macrophages within the lamina propria, and this is followed rapidly by depletion and ulceration. Other sites where NDV immunohistochemical signal has been observed are the conjunctiva, nasal turbinates, multifocally and rarely in the esophageal mucosa, crop, bone marrow (in the lymphoid dependent areas and within the osteoclasts), in the epithelium of the comb, interstitium and proximal tubules of the kidney, in Kupffer cells in the liver, in cardiac myocytes, pancreas and in epicardial and pericardial lining cells. VVND viruses have been detected by both IHC and ISH within scattered neurons in the cerebrum and cerebellum, and in the submucosal and myenteric plexuses within the intestine.[104,135] In the respiratory tract, moderate signal has been detected in laryngeal tonsils by IHC, alveolar septa by ISH, and occasionally in the epithelial cells lining both air capillaries and atria and within the epithelium of the air sacs, by IHC.[26,135,143] So it appears that after initial replication in lymphoid tissue, the VVNDV extensively disseminate to multiple body systems.

Turkeys had viral distribution very similar to that observed in chickens, with the main target of viral replication being the lymphoid organs.[119,143] Pigeons infected with CA02, had minimal positive signal only by ISH in bursa, spleen and thymus.[143]

Velogenic Neurotropic ND (VNND)

In chickens the VNNDV strains Texas GB and Turkey ND were detected only in the brain (few scattered neurons), myocardium and the air sacs by ISH or IHC.[26] In general, the detection signals increase with time, starting at 5 dpi and increasing until the animals become severely clinically ill.[26,77] In turkeys infected with VNNDV, distribution

of immunohistochemical labeling was similar, and mainly detected in the cerebellum, pancreas and heart.[119]

Mesogenic ND

Numerous IHC and ISH studies performed on tissues of chickens infected with mesogenic strains demonstrate that localization of the virus is mainly limited to the site of inoculum (mainly conjunctiva), heart and, in those strains that cause neurologic disease, the brain.[74,75,77,135] In these neurologic cases, immunolabeling or hybridization demonstrate that the virus is preferentially located in the clustered neurons within the cortex, medulla oblongata, scattered Purkinje cells and within multifocal areas of the molecular layer in the cerebellum. With Roakin, Anhinga, Pigeon TX, Pigeon GA, Pigeon 84 and 84-44407 strain (all mesogens), viral mRNA or nucleoprotein was constantly detected in clusters of cardiac myocytes (usually associated with areas of inflammation) by 5 dpi.[26,74,75,77] With ISH technique, Roakin and Anhinga strains were also detected in the air sac epithelium.[26] Rare positive cells by ISH were observed in the spleen of animals infected with Anhinga.[26] Turkeys infected with Roakin strain, showed minimal immunolabeling by IHC, only in few cells in the heart and crop.[119]

Lentogenic ND

Systemic detection of lentogens is challenging. In an experiment conducted with 4-week-old chickens inoculated via eyedrop instillation, hybridization with B1 strain occurred only in very small amounts in the air sac epithelium and in the myocardium; QV4 was detected only in the heart.[26] Immunolabeling for NP protein was detected only minimally in few epithelial cells of the trachea in commercial turkeys infected with LaSota strain. No systemic spread was observed.[119]

Newcastle disease infection and respiratory pathology

Newcastle disease virus is often grouped with the respiratory pathogens and there seems a common assumption that NDV is primarily a respiratory disease. Based on a review of the literature as well as our own experimental findings, it seems advisable to caution against labeling NDV as such.

The velogens have specific tropisms for lymphoid tissue (VVND) or central nervous system (VNND). Although respiratory tissues may show evidence of infection, as detected by IHC or ISH, the involvement of this system seems relatively minor compared to the massive damage to other body systems. The mesogenic strains have a particularly wide array of clinicopathologic presentations, with experimental infection resulting in anything from mild depression to severe neurologic impairment. Field outbreaks associated with mesogenic strains are often reported to be respiratory in nature, although these most commonly have additional secondary bacterial pathogens. In experimental studies with mesogens, there is a lack of respiratory involvement.

Although some lentogenic NDV strains have been shown experimentally to cause moderate lesions in the respiratory system, these changes were obtained only through aerosolization or use of very high viral titers,[2,53,78] direct air sac instillation of the virus[52] or when very young chickens (one-day-old) were used.[93] In our ISH and IHC studies, we have identified viral RNA or protein within cells throughout the respiratory tract, in varying degrees, especially with the VVNDVs. However, the progression to lesion development is not remarkable.

It seems that NDV is truly a kaleidoscope of disease presentations, with the most virulent viruses targeting lymphoid tissues and CNS, and minor presence of virus with or

without pathologic changes in multiple other body systems. In the field, where secondary respiratory pathogens are always lurking, minor pathologic changes in airways induced by the less virulent NDV strains might allow for these pathogens to become established and create a respiratory syndrome.

MOLECULAR DETERMINANTS OF VIRULENCE

To date, many studies have focused on the specific molecular determinants of NDV virulence associated with the F, HN, P and L proteins.

The fusion protein cleavage site has received the most attention. The presence of cellular protease is required for the activation of the viral fusion protein precursors. The inactive precursor F0 is synthesized as a single chain molecule. In order for the viral particles to become infectious, F0 has to undergo post-translational endoproteolysis by a host cell protease.[99] The virulent (velogenic and mesogenic) strains have a polybasic fusion protein cleavage motif, $_{112}$R/K-R-Q-R/K-R-F$_{117}$, which is cleaved by furin (or paired basic amino acid-cleaving enzyme; furin/PACE), a subtilisin-like endoprotease that is a ubiquitous protease present in almost every tissue. Therefore the infection with virulent NDV can occur in most body tissues.

In contrast, the lentogenic strains have a monobasic cleavage motif, $_{112}$G/E-K/R-Q-G/E-R-L$_{117}$ (E = glutamate; G = glycine), which can be cleaved only by trypsin, which naturally exists in only a few tissues, specifically respiratory and gastrointestinal, thus rendering the infection limited to these mucosal surfaces.[7,8,50,99-101] Studies from Wakamatsu *et al.* showed that site-directed mutagenesis of the LaSota F gene (very low virulence) resulting in a virulent amino acid sequence, dramatically increased the overall virulence of the virus (as demonstrated by ICPI and MDT), severity of lesions and tissue

distribution of the virus.[145] On the other hand, when the virulent Fusion gene from virulent strains, such as Turkey North Dakota (Turkey/US(ND) 43084/92) and California (Game fowl/US(CA)/212510/02) were inserted into a mesogenic backbone (Anhinga), the expected increase in virulence was not observed.[46]

The fact that some virulent viruses have been traced back to previously circulating non-pathogenic strains that acquired virulence by mutation of the F protein cleavage site, further substantiates the importance of the F cleavage site as a virulence factor. This has been demonstrated with the 1998-2000 NDV outbreaks in Australia, and in Ireland in 1991.[10,51]

The HN protein is responsible for the attachment of virus particles to sialic acid-containing receptors of the host cells.[98,111,126] It can be translated in three different sizes (571, 577, or 616 amino acids) depending on the position of stop codons in the gene.[124] The longest form ($HN0_{616}$) requires proteolytic cleavage to become catalytically active.[100] The other two forms (HN_{557} and HN_{571}) appear to be constitutively active without a proteolytic step. Very low virulence strains, such as Ulster 2C, D26, and QV4, produce a HN0.[28] The protein is normally glycosylated and it is known that loss of HN glycosylation will alter NDV pathogenicity, rendering those strains with non-glycosylated HN less virulent.[98] However, experiments conducted by Wakamatsu showed that insertion of the HN proteins from the virulent strain Beaudette C into a LaSota Backbone (low virulence) did not increase the virulence of the virus in 4-week-old chickens.[146]

The P protein is essential for viral replication as it stabilizes the L protein in a P:L complex that acts as a viral RNA-dependent RNA polymerase. It is also of great

importance since its post-transcriptional editing can potentially produce at least 2 different non-structural proteins.[131] The V protein is produced by post transcriptional editing of the P gene mRNA at the site (UUCUUUCC), where addition of a G is produced by the RNA polymerase (+1). Production of putative W protein occurs when 2Gs are inserted (+2).

Among the non-structural proteins of the Mononegavirales, the V proteins are the most widespread, being produced by all five genera in the Subfamily Paramyxovirinae.[47] In NDV, as in other Mononegavirales, the amino-terminal of the V protein is the same as the one of the P protein, but the carboxy-terminal differs, in that it is composed of multiple cysteine residues and zinc-binding activity. This portion is considered to be biologically relevant.[47]

The activity of the V protein as a class I interferon antagonist has been demonstrated for NDV,[62] and these data are in accordance with the activity of other V proteins produced by viruses of the same genus and family.[102] The carboxy terminal region of the V protein is able to inhibit the interferon response through the degradation of Signal Transducer and Activators of Transcription 1 (STAT1) via the proteasome.[113] Several experiments *in vivo* demonstrated that viruses defective in V protein had decreased growth, while viruses with increased production of V protein had higher growth titers.[65,95,146] In one study, the importance of the V protein for viral replication was underscored by the fact that viruses defective for the V protein reacquired the ability to express the V protein after 7 serial passages in chicken embryos.[95] The V protein has been also implicated in prevention of apoptosis. Park and colleagues showed that a V protein-defective NDV induced severe cytopathic effects and augmented levels of

apoptosis *in vitro*, while the same V protein-competent NDV triggered apoptosis to a lesser extent.[113] It is not clear whether the V protein of NDV is able to inhibit apoptosis *per se*, or if this effect is linked to its anti-interferon activity. In addition, it should be considered that the V protein has a role in the formation of the nucelocapsid, potentially operating as a real structural protein.[82,134] Thus, hampered viral replication when V protein expression is lost, may reflect both lack of interferon antagonism and also a possible alteration of viral assembly.

The role of the W protein in NDV pathogenesis has been minimally investigated, and there are few reports about the actual production of W protein by NDV viruses and its role in NDV pathogenesis.[91] Reports about other viruses in the *Mononegavirales* order, especially Nipah virus, describe the W protein as another class I interferon inhibitor, with a STAT-1 binding domain similar to that observed for the V proteins.[114,129] Another function associated with this protein, is its ability to inhibit the Toll-like Receptor 3 (TLR-3) pathway, thus down-regulating the innate immune response.[128] In Nipah virus-infected cells, the W protein has a nuclear localization, whereas the V protein has a cytoplasmic localization.[128]

The L protein is the largest viral protein and is an RNA-dependent RNA polymerase.[82] Its contribution to the virulence of NDV is not clear. Insertion of an L protein from a lentogenic (LaSota) strain increased virulence when inserted in a mesogenic (Baudette C) backbone.[122] However, LaSota itself displays a minimal pathogenic potential, therefore it seems that for the L protein to increase virulence, a well-defined set of other genes has to be present in the virus. Probably, the ability of the L protein to affect the virulence resides in its polymerase processivity.[122]

Another recent work suggested that the NP, P and L proteins complex takes part in the virulence of NDV by contributing to viral replication, thus linking the replicative activity of NDV to its virulence.[38]

REVERSE GENETICS AND PRODUCTION OF RECOMBINANT VIRUSES

Reverse genetics is the most frequently used technique for exploring the molecular basis of NDV pathogenesis and the reasons for the different virulence of NDV strains.[31,150] Because "naked" RNA from NDV, as from other viruses of the Mononegavirales order, is not infectious by itself, the rescue of virus through the use of reverse genetics requires three conditions – 1) transfection of competent cells with a plasmid having the full length genome of the virus; 2) cotransfection of the same cells with helper plasmids encodings for N (nucleoprotein), M (matrix) and P (phosphoprotein) protein; and 3) the presence of a cellular environment supporting the constitution of full ribonucleoproteins (RNPs).[150]

Once the RNPs are formed, the RNA-dependent RNA polymerase can transcribe all the other mRNAs that will translate into the proteins necessary for the formation of a complete virion, together with the full length negative sense (genomic) RNA.[150]

This technique makes it possible to engineer negative-strand RNA viruses with insertion of heterologous genes or to generate mutations that can be examined for their contribution to pathogenicity.

For NDV, reverse genetics has been used in various ways:

1- addition or mutations of genes from other strains of NDV with different virulence and mutation of the intergenic sequences.[27,46,63,66,79,89,109,116,145,146,152]

2- insertion of proteins from different pathogens (such as infectious bursal disease virus) in order to create multivalent vaccines.[27,35,36,60,61,97,106]

3- insertions of cytokines into an oncolytic NDV backbone in order to increase the oncolytic activity and to elicit anti-neoplastic immunity, using the cytokines as molecular adjuvant.[66,130]

Moreover the insertion of proteins that allow better localization of the virus in cells, such as fluorophores, has been also attempted with numerous viruses, such as NDV, vesicular stomatitis virus, rabies virus, Sendai virus, Respiratory syncytial virus, SV5 virus and rinderpest.[3,43,54,68,88,132,147,157] So far, only two low pathogenicity NDV strains, LaSota and B1, have been engineered to produce GFP.[43]

Several NDV viruses, along with numerous non-segmented negative strand RNA viruses, have been rescued with the use of reverse genetics.[27,30,60,61,63,69,79,84,109,127,145,149,153] However, especially for the viruses carrying heterologous genes, a substantial drop in the pathogenicity has often been observed.[46,79,120,145,146] This has also been observed, even though more rarely, in wild-type viruses inserted into a plasmid vector, and then rescued. This is peculiar, since these viruses have very similar genomic sequences of the parental strains.[46]

Some reasons have been proposed for inability of infectious clones to maintain their virulence. These include:

1- "genomic bottlenecking",[39,40] which consists of voluntary/involuntary selection of a particular viral clone during the purification procedures. If the overall fitness of a heterogeneous viral population is the sum of the contribution of each single clone (with all its mutations), then narrowing the population (bottle-necking) to a

single or few clones during purification, causes a decrease of fitness in the viral population;

2- variation of the intergenic sequences during the cloning process;[153]

3- the fact that certain genes work better in the context of a few selected other genes from the same strain, i.e. homotypic interaction;[34,85,96,138]

4- the length of the inserted gene and the position of the foreign genes in the genome. The replication machinery of NDV, as of that of other viruses in the same order, is polar and obligatorily sequential, as demonstrated with UV light experiments.[150] This means that genes at the 3' of the genomic viral RNA are more abundantly transcribed than those at the 5', following a gradient. Therefore, if foreign genes are inserted in the viral backbone, a decrease in the transcription of the downstream genes will result, causing unfavorable ratio between the viral structural proteins that might limit viral replication and fitness. This decrease in fitness has been demonstrated to be proportional to the length of the insert.[125]

HOST RESTRICTIONS

The determinants of host range in NDV are not completely understood. The F and HN genes must play a role, since they allow the attachment to and the penetration of the virus in the cells, however, there are no reports in the literature regarding the role that HN and F proteins play in NDV host range restriction.

In other members of the Paramyxoviridae family, the non structural proteins with class I interferon inhibitory activity play a critical role in host range restriction.[47] For example, NS1 and NS2 proteins from human respiratory syncytial virus (RSV) better

inhibit the interferon pathway in human cell culture, rather than in bovine cells.[23] In another report, when the NS1 gene from a mouse-adapted avian influenza virus was exchanged with the NS1 from the 1918 influenza virus pandemic (highly adapted to humans), the virus was extremely attenuated in mice.[17]

Specifically in NDV, the V protein is considered to function as a type I interferon antagonist, and acts via degradation of the STATI and STATII complexes via the proteasome.[62,113,114] Chimeric NDV viruses expressing the NS1 protein from avian influenza virus were able to grow better and induce less apoptosis in human cell lines, compared to NDV expressing only the wild type V protein.[113] The rationale between the relatedness of V protein and host range, is that the NDV V protein should inhibit the interferon pathway only of those species that are permissive to NDV replication. Furthermore, different V proteins belonging to NDV strains may contribute to the variability of virulence.[47] For example, the V protein from a virulent NDV is better able to block the IFN- pathway (*in vitro*), when compared with the V protein of a lower-virulence NDV.[64]

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Table 2.1. Pathotype designation based on standard pathogenicity tests

Pathotype	**MDT**[a]	**ICPI**[b]
Velogenic	<60	>1.5
Mesogenic	60-90	0.7-1.5
Lentogenic	>90	<0.7

[a] MDT = Mean Death Time, measured in hours to death

[b] ICPI = Intracerebral Pathogenicity Index, based on an averaged score of clinical signs over time (min. 0.0 – max 2.0).

Table 2.2. Biological proprieties of selected well-characterized strains of Newcastle disease virus

Biological propriety	**VIRUS**						
	Lentogenic			**Mesogenic**		**Velogenic**	
	Hitchner B1	La Sota	Ulster 2C	Komarov	Roakin	Texas GB	Herts '33
Plaque presence in CEF (size in mm)	No*	No*	No*	Yes (1-2)	Yes (1)	Yes (0.5-4)	Yes (0.5-4)
MDT	120	103	>150 (140)^	69	68	55	49
ICPI	≤0.2	≤0.5	≤0.1	1.4	1.45	1.75	1.9
IVPI	0.0	0.0	0.0	0.0	0.0	2.7	2.7
Lethality in 8 weeks old chicks	0	0	0	0	0	≥70%	≥95%
Main tissue tropism	r	r	r/d	r	r	r/n	r/n/d
Elution rate at 4°C from chicken erythrocytes	R	S	S	S	S	S	S
Thermostability of hemagglutinin (minutes at 56°C)	5	5	120	5	5	60	50

only with trypsin or DEAE; ^ after adaptation in eggs;

CEF=chicken embryo fibroblast;

MDT= mean dead time in hours of chicken embryos infected with one minimum lethal dose;

ICPI = intracerebral pathogenicity index;

IVPI= intravenous pathogenicity index; r = respiratory system; n = nervous system; d = digestive system;

R = rapid (< 1 hour) ; S = slow (1 to 24 hours)

CHAPTER 3

CLINICOPATHOLOGICAL CHARACTERIZATION IN POULTRY OF THREE STRAINS OF NEWCASTLE DISEASE VIRUS ISOLATED FROM RECENT OUTBREAKS*

*Susta L, Miller PJ, Afonso CL, Brown CC. 2011. *Veterinary Pathology*. 38: 349-360. Reprinted with permission of the publisher.

ABSTRACT

Newcastle disease is a severe threat to the poultry industry and is caused by Newcastle disease virus (NDV), a member of the genus *Avulavirus*, Family Paramyxoviridae. The virus is rapidly evolving, with several new genotypes having been discovered in the last few years. Characterization of these strains is important to evaluate field changes, to anticipate new outbreaks and to develop adequate control measures. In this paper three Newcastle disease isolates (APMV-1/duck/ Vietnam, Long Bien /78/2002 [Long Bien], APMV-1/chicken/Australia/9809-19-1107/1998 [Australia], APMV-1/double crested cormorant/ U.S., Nevada/ 19529-04/ 2005 [Nevada Cormorant]) from recent outbreaks were investigated via clinicopathological assessment, immunohistochemistry (IHC), in situ hybridization (ISH), virus isolation, and serology in experimentally infected 4-week-old chickens. Phylogenetic studies showed that Australia isolate belongs to Class II genotype I, Long Bien to Class II genotype VIId and Nevada Cormorant to Class II genotype V. Even though all three viruses had a virulent fusion protein cleavage site and ICPI values greater than 1.5, they all differed in their ability to cause clinical signs, lesions and in their viral distribution in body tissues. The Long Bien isolate showed the most severe clinicopathological picture, and the most widespread viral distribution. The Australia and Nevada Cormorant isolates had a milder pathological phenotype, with viral replication restricted to only a few organs. The variability in clinicopathological characteristics despite the similarity in ICPI, suggests that full clinicopathological assessment is necessary to fully characterize new isolates, and that there are differences in pathogenesis among viruses of different genotypes.

Keywords: Avian paramyxovirus 1; Newcastle disease virus; chickens; pathogenesis; immunohistochemistry; in situ hybridization

INTRODUCTION

Newcastle disease virus (NDV), synonymous with Avian Paramyxovirus-1 (APMV-1), is the causative agent of Newcastle disease (ND), an infection which threatens poultry worldwide.[1-3] The poultry industry loses millions of dollars annually from morbidity, mortality, and trade losses due to ND.[3] NDV belongs to the Mononegavirales order, Paramyxoviridae family, Paramyxovirinae subfamily and *Avulavirus* genus. The genome is negative sense RNA of approximately 15,200 bases and it contains six genes that encode an equal number of structural proteins, which are from 3' to 5': nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and polymerase (L). Although all ND viruses belong to a single serotype, APMV-1 serotype, isolates differ in virulence in poultry, which translates into a wide range of clinical signs, often making it challenging to recognize NDV as the cause of the clinical problem in the field.[1] Historically strains were classified according to the time to death after inoculation of embryonating eggs, with those strains that caused death the fastest (and therefore more virulent) termed "velogens" and those in which the embryos survived for much longer periods of time termed "lentogens." Those strains falling in between were "mesogens".[1, 31] Today, classification of the different strains is carried out by two internationally recognized methods. The intracerebral pathogenicity index (ICPI) involves inoculating one-day-old chicks intracerebrally and scoring birds as normal, sick or dead over a period of 8 days, to result in a score from 0.0 to 2.0. Those strains scoring > 0.7 are considered "notifiable" to the international community through the World Organization for Animal Health and any strain with a score >1.5 is considered a velogen.[2, 31] The second internationally recognized test is to sequence the fusion (F)

protein cleavage site to evaluate the presence of multiple basic amino acids and phenylalanine, which is considered an indication of virulence and therefore notifiable.[11,29]

Outbreaks of ND are reported frequently from many parts of the world, with the most severe disease being caused by virulent strains. The USA is considered free of virulent NDV strains, but occasionally pathogenic strains enter through wild birds or informal (illegal) trade. The last outbreak of Newcastle disease occurred in California in 2002/2003 and resulted in depopulation of more than 3 million birds and containment costs exceeding US$160 million.[37]

There is some correlation between viral virulence and phylogenetic analysis of virus evolution. According to phylogenetic distances, often carried out on the sequence of the F gene, NDV isolates are divided in 2 classes (I and II), and class II is further divided into 9 genotypes.[26,27] Comparison of phylogenetic analysis with biological characteristics of the NDV isolates demonstrates that viruses of class I are mainly composed of avirulent strains, usually circulating among wild birds, while viruses of class II are more frequently virulent[8,26] and mainly isolated from poultry. Within Class II, worldwide, most outbreaks are caused by viruses of genotypes V, VI and VII, even if occasional outbreaks caused by viruses from other genotypes do occur. Particularly important are viruses of genotype VIId, which lately have caused several outbreaks in China, Vietnam, Indonesia, Pakistan and Africa.[17,27,28]

NDV is a continuously evolving virus with evidence of accelerated evolution of the virulent strains,[26] and there are reports of increased pathogenicity, outbreaks in vaccinated animals and increased host range.[15,25,26,28,30,38] Therefore, it is important to conduct clinicopathological characterization of new strains that are isolated during the

course of outbreaks worldwide, to determine how the viruses are changing. Full characterization of the clinical disease and pathogenesis in poultry could also impact the diagnostic aspects of the disease and implementation of control measures. In addition, characterization of the genomic sequences, genotyping of the new isolates and identification of their nearest ancestors, could help discern the source of new outbreaks.

In this study, three different strains of NDV from recent outbreaks were characterized through sequencing, phylogenetic analysis, ICPI, and clinicopathological assessment. The latter was performed by inoculating 4-week-old chickens via a natural route (eyedrop instillation). The resulting disease was characterized through virus isolation (VI), serology and clinicopathological observations, including histology, immunohistochemistry and in situ hybridization in multiple body tissues.

The first virus, APMV-1/duck/ Vietnam, Long Bien /78/2002, and in this paper referred as "Long Bien", was initially isolated from ducks in a Vietnam market during 2002. The second virus, APMV-1/chicken/Australia/9809-19-1107/1998, here referred to as "Australia" was isolated during a large outbreak in Australia that occurred from 1998 to 2000. This virus and other isolates from this same outbreak were unusual in that they are the only known virulent representatives of class II, genotype I. There is evidence [12,17,39] that the origin of these viruses can be traced back to low virulence NDV circulating in waterfowl just prior to the outbreak. The third virus, APMV-1/double crested cormorant/U.S., Nevada/ 19529-04/ 2005, and here referred as "Nevada Cormorant" is an isolate responsible for significant morbidity and mortality in cormorants in the state of Nevada in 2005 and in other U.S. states in 2008.

MATERIAL AND METHODS

Viruses

APMV-1/duck/ Vietnam, Long Bien /78/2002 (Long Bien), APMV-1/chicken/Australia/9809-19-1107/1998 (Australia) and APMV-1/double crested cormorant/ U.S., Nevada/ 19529-04/ 2005 (Nevada Cormorant) were obtained from the Southeast Poultry Research Laboratory (SEPRL) NDV repository. Each virus was propagated by one passage in 9 to10-day-old embryonated chicken eggs inoculated by the chorioallantoic route.

Eggs and chickens

The source of embryonating chicken eggs and chickens was the SEPRL SPF White Leghorn flock. Birds were housed in negative pressure isolators under biosafety level (BSL)-3 enhanced (E) conditions at SEPRL and provided food and water *ad libitum*.

Intracerebral Pathogenicity Index Test

To characterize the viruses, ICPI was performed using standard protocols.[2] Briefly, chickens were inoculated at one-day of age with 0.1 ml of a 1:10 dilution of infective allantoic fluid. Chicks were monitored daily and scored as normal, sick or paralyzed and dead to compile a score for the 8-day observation period.[2]

Clinicopathological assessment in chickens

Three groups of ten 4-week-old SPF White Leghorn chickens were inoculated bilaterally in the conjunctival sac with 0.1 ml of viral inoculum. Phosphate buffered

saline (PBS) was used for the non-infected control birds. The target dose of inoculum was $10^{5.0}$ 50% embryo infectious doses (EID_{50}). The actual infectious doses as determined by back titration in embryonating chicken eggs were: $10^{5.5}$ EID_{50} for Long Bien; $10^{4.5}$ EID_{50} for Australia and $10^{5.3}$ EID_{50} for Nevada Cormorant. The birds were monitored clinically every day, and two birds of each group were euthanized after taking oropharyngeal and cloacal swabs at 2, 5, 10 and 14 days post inoculation (dpi). Birds whose condition became critical were euthanized regardless of the scheduled sampling day.

Blood samples for serology were collected at 10 and 14 dpi. Tissues (eyelid, spleen, bursa of Fabricius, thymus, Harderian gland, proventriculus, small intestine, cecal tonsils, large intestine, air sac, trachea, lung, heart, esophagus, pharynx, crop, brain, liver, pancreas, kidney, comb, head of left femur including bone marrow, and nasal turbinate) were collected, and fixed by immersion in 10% neutral buffered formalin for approximately 52 h. The sections of femur and turbinate were decalcified in 5% formic acid for 3-4 h. All sampled tissues were routinely processed into paraffin, and 3µm sections were cut for hematoxylin and eosin staining (HE), immunohistochemistry (IHC) and in situ hybridization (ISH).

Immunohistochemistry

All sampled tissues were examined by immunohistochemistry (IHC) to detect viral nucleoprotein. Briefly, after deparaffinization, tissue sections were subjected to antigen retrieval by microwaving for 20 min at minimum power in Vector antigen unmasking solution (Vector Laboratories, Burlingame, CA) followed by application of

universal blocking reagent (Biogenex, San Ramon, CA) as recommended by the manufacturer. The primary antibody, made in rabbit, was raised against a synthetic nucleoprotein peptide (TAYETADESETRRIC) and used at 1:8000 dilution. The detection system was an alkaline phospatase-labeled polymer specific for the Fc portion of rabbit immunoglobulin (LabVision, Fremont, CA). Chromogen was a naphthol-based dye (Fast Red, Dako, Carpinteria, CA). Sections were counterstained lightly with hematoxylin and coverslipped with Permount for a permanent record.

In Situ Hybridization

Selected tissue sections from the NDV infected birds were probed with a negative-sense digoxigenin-labeled 850 base riboprobe representing the 5' end of the matrix gene of the NDV Fontana (CA1083) as previously described.[7] The matrix gene from the Fontana strain was cloned into pCRII transcription vector (Invitrogen, Carlsbad, CA) and anti-sense riboprobes were generated using RNA polymerase in the presence of labeled nucleotides. This probe was used for hybridization with all the three strains in this study, because sequence information indicated a very high grade of identity (Long Bien 92%; Nevada Cormorant 89%; Australia 88%) with the probed strains (NCBI accession number: AY562988).

For hybridization, sections from selected blocks were deparaffinized, rehydrated, digested with 100 µg/ ml Proteinase K for 15 min, and hybridized overnight at 42 °C with approximately 20 ng of probe in standard sodium citrate (SSC), 50% formamide, 5% blocking reagent (Boehringer Mannheim, Indianapolis, IN), 1% *N*-lauroylsarcosine, and 0.02% sodium dodecyl sulfate. Following stringent washes, bound probes were

visualized by the addition of anti-digoxigenin alkaline phosphatase and the chromogen/substrate nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP).

Virus isolation and titration of swabs

For those birds used in the clinicopathologic assessment experiment, oral and cloacal swabs were obtained from each bird immediately prior to euthanasia and placed in separate tubes containing 1.5 ml of brain-heart infusion broth (BHI) with antibiotics (2000 U penicillin G/ ml, 200 µg gentamicin sulfate/ ml, and 4 µg amphotericin B/ ml; Sigma Chemical Co., St. Louis, MO). Swab sample tubes were centrifuged at 1000×g for 20 min, and the supernatant removed for virus isolation and titration. Virus infectivity titers were calculated from the result of inoculation of 9- to 10-day old SPF embryonating chicken eggs with serial 10-fold dilutions in BHI containing antibiotics (100 U penicillin G/ ml and 50 µg gentamicin sulfate/ ml). NDV in infected dead or surviving embryos was identified by hemagglutination (HA) activity in amnioallantoic fluid harvested from chilled eggs. NDV was confirmed in HA positive samples by hemagglutination inhibition (HI) test with NDV-specific antiserum.

Serology

The HA and HI tests were conducted by conventional microtiter methods with serum separated from the blood samples taken at 10 and 14 dpi. Four HA units per 25 µl of betapropiolactone-inactivated NDV LaSota was used as test antigen in completing the HI test.

RNA isolation, PCR amplification and sequencing

RNA was extracted from allantoic fluids using Trizol LS (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Briefly, 750 µl of Trizol LS reagent was added to 250 µl of allantoic fluid, vortexed, and incubated at room temperature for 7 min. The RNA was separated into the aqueous phase with the addition of 200 µl of chloroform, precipitated with isopropanol, and then centrifuged to pellet the RNA. After one wash with 70% ethanol, the RNA was dried and resuspended in RNAse-free water. PCR amplification of the RNA was performed using the Qiagen One Step reverse transcription PCR (RT-PCR) kit (Qiagen, Valencia, CA). Amplified products were separated on a 1% agarose gel, the bands excised and eluted using the QIAquick Gel extraction kit (Qiagen), and the samples quantified using a standard spectrophotometer. All sequencing reactions were performed with fluorescent dideoxynucleotide terminators in an automated sequencer (ABI 3700 automated sequencer; Applied Biosystems Inc., Foster City, CA). Nucleotide sequence editing and analyses were conducted with the LaserGene sequence analysis software package (LaserGene, version 5.07; DNAStar, Inc., Madison, WI) using the full-length genome positions from the NDV LaSota vaccine strain complete genome. The regions sequenced were a 374 bp partial F gene (positions 4554 to 4917), and the complete coding region for the F gene (positions 4544 to 6205), as previously described.[18] All RNA and DNA procedures were performed in a biological safety cabinets and use of RNase-free reagents and standard anti-contamination protocols were followed at all times to minimize contamination.

Sequence alignment and tree construction

The coding sequence of 51 NDV full Fusion genes were aligned using ClustalW as implemented in the BioEdit Sequence Alignment Editor (http://www.mbio.ncsu.edu/BioEdit/bioedit.ht ml) and edited manually based on the amino acid sequence. Maximum likelihood (ML) phylogenetic analysis with bootstrap values for n = 100 replicates was performed using PHY ML V 2.4.4 software and employing the general time reversible 7 (GTR) model of nucleotide substitutions, ML estimates of base frequencies, estimated transition/transversion ratio, proportions of invariable sites with 4 categories of substitution rates estimated Gamma shape parameter 1.136, and estimated proportion of invariant sites 0.292.[13]

RESULTS

Pathogenicity index-ICPI

The ICPI values were 1.88 for Long Bien, 1.88 for Australia, and 1.53 for Nevada Cormorant.Therefore, all the viruses could be considered velogens according to the OIE standards and notifiable to the international community.[2,31]

Clinical disease, virus isolation, and serology

Results for observed clinical signs are presented in table 3.1. Results for virus isolation are presented in table 3.2. For all three strains, all infected birds with the three strains developed clinical disease, and virus was successfully isolated from at least one oral and cloacal sample from all three groups of animals. Birds inoculated with the Long Bien isolate had severe illness, characterized by marked depression, reluctance to move and high mortality, with all of the birds dying or being euthanized by 3 dpi. Two birds

inoculated with the Australia isolate showed depression and open-mouthed breathing between day 3 and 5. Also, mild neurological signs, such as head tremors and twitch were noted in this group between 4 and 9 dpi, but all became clinically normal by 10 dpi. Birds inoculated with the Nevada Cormorant isolate had marked neurological signs by 4 dpi consisting of head tremors, twitching, recumbency and paralysis; by 10 dpi all these animals were euthanized with severe neurologic signs.

NDV was isolated from all the birds inoculated with Long Bien and Australia isolates starting from 2 dpi. In the group inoculated with Nevada Cormorant virus, at 2 dpi NDV was isolated only from one bird, while all the sampled birds were positive by 5 dpi. By day 10, virus isolation was positive (Australia and Nevada Cormorant isolates only as all Long Bien birds were dead by this time point), but at very low titers and only for the cloacal swabs. Seroconversion (≥:128) occurred in birds infected with Australia and Nevada Cormorant isolates at 10 dpi (data not shown). Serology was not performed on animals infected with Long Bien because they were all euthanized by day 3.

Pathology

Gross findings are presented in table 3.1. The most striking findings were in birds inoculated with the Long Bien isolate. By 2 dpi the birds had severe edema and petechial hemorrhages of the eyelid and mottled spleens. On day 3, the spleens were unifor mly enlarged and had multifocal to coalescing white stippling (necrosis), both on the surface and in cut section (fig. 3.1). Other lesions were thymic hemorrhages, proventricular hemorrhages, multiple foci of necrosis in the small intestine (fig. 3.2), and edema, necrosis and hemorrhage of the cecal tonsils.

The birds infected with the Australia isolate had minimal to moderate petechiation of eyelids and moderately enlarged spleens by days 2 and 3. At days 5 and 6, the spleens were enlarged and proventricular hemorrhages were present in two birds.

The birds infected with the Nevada Cormorant isolate had minimal gross lesions. There was mild conjunctivitis by 2 dpi, moderately congested and mottled spleen at 5 dpi, and at day 9 there was moderate thymic atrophy in one bird. There were no abnormal gross findings in the noninfected control birds.

Histology

Histological as well as IHC findings are presented in table 3.3. The most severe lesions were observed in birds infected with the Long Bien isolate, starting at day 2 and culminating at day 3. These were mainly confined to the site of inoculation (eyelid), the lymphoid organs (spleen, thymus, bursa) or the lymphoid aggregates of the intestines (cecal tonsils and other gut-associated lymphoid tissue). In the eyelid the lesions were characterized by severe edema, hemorrhage, multifocal areas of necrosis associated with fibrin exudation, and pleomorphic inflammatory infiltrate, mainly composed of heterophils and macrophages. In the lymphoid organs (thymus, bursa, spleen, and intestinal lymphoid patches, especially cecal tonsils) lesions consisted of severe necrosis, marked lymphocyte depletion, infiltration of numerous macrophages, and moderate heterophilic infiltrate. In the intestines, the severe necrosis of the lymphoid-dependent areas was associated with focal to locally extensive ulceration of the epithelium and accumulation of necrotic material within the intestinal lumen. Multifocal areas of necrosis were observed within the pancreas. By 3 dpi, at the level of the physis and

metaphysis, especially in the areas beneath the articular cartilage, there were multifocal areas of bone marrow necrosis.

In birds infected with the Australia strain, similar lesions were observed within the eyelid and lymphoid organs, but these were much less intense than those with Long Bien, and reached their peak at 4 dpi. Overall, the most severe lesions were in the bursa and cecal tonsils, and consisted mainly of necrosis, lymphocyte depletion and accumulation of macrophages. The heart, at 5 and 6 dpi, had multifocal myonecrosis with accumulation of macrophages. At day 10, there was moderate non-suppurative encephalitis, consisting of multifocal areas of lymphoplasmacytic perivascular cuffing.

Birds infected with the Nevada Cormorant isolate had minimal lesions within the lymphoid tissues, but showed severe non-suppurative encephalitis, and moderate myocarditis. The encephalitis was confined between day 5 and 10 and was characterized by perivascular accumulations of lymphocytes, macrophages, rare plasma cells and hypertrophy of the endothelial cells (fig. 3.3). The myocarditis was characterized by multifocal necrosis associated with moderate macrophage infiltration. One bird infected with Nevada Cormorant at day 5 had severe laryngitis, with necrosis of the epithelial layer (ulceration), accumulation of fibrin and infiltration with heterophils and macrophages.

Immunohistochemistry

In the infected cells, the immunolabeling for NDV was intracytoplasmic and finely to coarsely granular. Long Bien-infected birds had the broadest viral distribution (23/25 different tissues were positive), and the most intense signal, starting from day 2

and culminating at day 3. The organs with the strongest signal were the eyelids, the lymphoid organs and the mucosa-associated-lymphoid aggregates (MALT) in multiple organs (fig. 3.4). In these tissues, the positive cells consisted mainly of lymphocytes and macrophages. In the spleen the immunoreactivity was confined to the fixed-macrophage dependent areas around the penicillary arteries, while the lymphocyte-dependent areas were devoid of signal. In the respiratory system the positive signal was confined to the nasal and tracheal mucosa, in scattered lymphoid aggregates closely associated with the secondary and tertiary bronchi (BALT), and to the squamous epithelium of the atria, air capillaries and air sacs. In the digestive tract, intense positivity for NDV was observed only within the submucosal lymphoid aggregates, and no staining was observed associated with the epithelial lining. Scattered Kupffer cells in the liver and several epithelial cells in the distal tubules of the kidneys had positive signal for NDV. In the bone marrow, numerous osteoclasts and mononuclear cells just beneath the growth plate or surrounding areas of necrosis showed intense immunolabeling (fig. 3.5). In the heart, the staining was limited to the epicardium. Birds infected with the Australia isolate had a more limited viral distribution (15/25positive tissues) and lesser amounts of signal even in the positive tissues when compared to Long Bien, but the pattern of staining and the type of infected cells was the same as described above. The viral distribution was mainly limited to the lymphoid organs and lymphoid aggregates, and reached its peak at 5 dpi. With these animals, in the intestines, immunolabeling was also present within the submucosal and myenteric plexuses in cells (fig. 3.6) consistent with neurons and glial cells. Tissues from birds infected with the Nevada Cormorant isolate had minimal signal for NDV, and it was primarily confined to the portal of entry (eyelid), with the exception

of moderate immunolabeling of scattered neurons within the brains of birds at day 6 and 10dpi (fig. 3.7).

In situ Hybridization

In selected sections the negative sense riboprobe for NDV matrix gene labeled the same structures and cells immunolabeled with immunohistochemistry. The type of staining was cytoplasmic and evenly distributed (figs. 3.8, 3.9).

Sequencing and phylogeny

Results of sequencing and phylogenetic trees are presented in fig.10 (detailed correspondence between names used in the tree and NCBI accession numbers are available in on line supplement).

The Genome sequences of the viruses were submitted to Gene Bank under the following names:

APMV-1/duck/ Vietnam, Long Bien /78/2002: **GU332646**

APMV-1/chicken/Australia/9809-19-1107/1998: **GU332645**

APMV-1/double crested cormorant/ U.S., Nevada/ 19529-04/ 2005: **253317813**

Phylogenetic analysis with full fusion sequences for the three selected strains revealed that the Nevada Cormorant isolate belongs to a new branch of genotype V that is clearly separated from other viruses that caused outbreaks in Cormorant in 2008.[35] The Australia isolate belongs to genotype I viruses and groups with other contemporary Australian viruses. The Long Bien isolate falls within genotype VIId, and is most closely related to isolates from China in 2000 from chickens and geese (fig. 10). All three viruses,

Australia, Long Bien and Nevada Cormorant have a virulent fusion cleavage site motif. [31] The -R R K K R F- motif was present in Long Bien, -R R Q R R F- in the Australia isolate, and -R R Q K R F- in the Nevada Cormorant virus.

DISCUSSION

Although all three viruses were considered virulent, both by ICPI and fusion protein amino acid sequence, these strains showed differing clinicopathological pictures, both in severity and in distribution of the virus through various tissues of the body.

The Long Bien isolate belongs to genotype VIId, and to the authors' knowledge this is the first full clinicopathological characterization of an isolate belonging to this genotype. Clinicopathologically, the phenotype of Long Bien was similar to other previously described virulent NDV strains belonging to genotype II, V, VI and VIIa.[7,19-21,37] Long Bien strain caused severe disease, with birds succumbing rapidly after infection. By IHC, the viral tropism was mainly for lymphoid tissues, but viral protein was also very evident in the respiratory epithelium of trachea, lung, air sacs and also in the renal epithelium. Within the lymphoid tissues, and particularly in the spleen, the majority of the immunolabeled cells were morphologically compatible with macrophages, specifically those surrounding the penicillary arteries in the spleen. This tropism for cells belonging to the monocyte-macrophage series was supported by visualization of positive osteoclasts within the bone marrow, as these cells are derived from a subset of circulating monocytes.[6,40]

The Australia isolate, another velogen, had a less severe clinicopathological presentation. Lesions were milder and viral distribution, as detected by IHC, was more limited. Lymphoid tissue appeared to be the primary target, but damage in these areas and

associated IHC signal was much less than what was seen with Long Bien. An unexpected finding was the presence of abundant viral nucleoprotein, and ISH staining within the myenteric plexuses of the intestines, which was most notable at 5dpi, and that was not associated with any inflammatory changes. The positive ISH signal using a negative riboprobe indicates that the virus is actively replicating in these cells, as the probe will bind only to positive sense RNA, which would be the replicative intermediate of the virus (which is negative sense) or the viral mRNA. Although some NDV strains are known for their ability to invade nervous tissue, it is usually the CNS that is affected, making this tropism for the peripheral nervous system unusual. Only one report in the literature, describing an outbreak in Japan in vaccinated flocks, describes immunohistochemical staining of the intestinal nerves, but the genotype of the investigated virus was type VII, whereas the Australia virus is genotype I.[30]

The marked differences in the clinicopathological assessment between the two velogenic strains with the highest ICPI, Australia and Long Bien, is also mirrored by the phylogenetic distances. Genotype VIId (Long Bien) encompasses numerous markedly virulent strains that are currently circulating in Southeast Asia, Africa, Middle East, and have also been reported to have increased host range.[15,16,26,27,38] On the other hand, the Australia isolate belongs to genotype I, a clade that contains mainly non-virulent strains that often circulate in wild birds and waterfowl. It has been known that on rare occasions these strains can mutate and become virulent. This has been demonstrated for the 1998-2000 NDV outbreaks in Australia (of which our strain is an isolate), and the 1990 outbreaks in Ireland. In these cases, the virulent isolate viruses have been traced back to previously circulating non-pathogenic strains that acquired virulence by mutation of the F

protein cleavage site.[4,8,12] Even if both Australia and Long Bien strains had the same ICPI value (1.88) and a virulent F gene cleavage site, they displayed strikingly different clinicopathologic syndromes when inoculated into 4-week-old birds. Long Bien behaved as a full-fledged VVND, acting very similarly to other virulent strains even if genetically distant, while Australia had a much milder pathotype. The reason for this is unknown, but it might be linked to the fact that Class I viruses are usually adapted to waterfowl, and not usually to chickens.[12] In addition, it should be considered that the F gene is not the only determinant of virulence, but that other genes may also be involved in the disease inducing ability of NDV strains. For example, the HN protein has been shown to influence tissue tropism and to trigger apoptosis *in vitro*;[14, 33] the P gene has been demonstrated to inhibit the interferon pathway through the production of the V protein,[32] and the L gene has been associated with virulence through RNA processing ability.[34] The work presented here confirms that neither ICPI nor fusion protein cleavage are sufficient to fully predict the outcome of virus infection in poultry.[9]

Phylogenetic analysis revealed that our third strain (Nevada Cormorant) belongs to a distinct subgroup of genotype V recently isolated from Cormorants.[35] Although the ICPI of this virus was 1.53, which makes this virus technically a velogen (>1.5)[2] and its fusion protein has a virulent cleavage sequence, Nevada Cormorant isolate behaved similarly to many previously described mesogens of genotype V,[7] although the degree of neurologic involvement was more marked than what had been described.[7] Mesogens do not usually produce severe clinical signs in SPF chickens, but can occasionally cause neurological signs.[1] The virus was detected by immunohistochemistry only at the inoculation site (eyelid) and seldom (4 birds) in the brain. The presence of viral

nucleoprotein and RNA, as revealed by immunohistochemistry and in situ hybridization, was mainly in neurons of the cerebral cortex, the nuclei of the brainstem and the cerebellum, thus accounting for the neurological signs. The involvement of the respiratory system was minimal. As described for the cormorants affected with NDV,[5, 10, 22-24] the clinicopathological findings in the chickens involved mainly the nervous system, suggesting that the tropism and the behavior of the virus was similar in different hosts. The development of clinical disease in chickens inoculated with a strain rescued from wild birds (cormorant) underlines the important role that wild birds could have in the natural or accidental transmission of a virulent strain to commercial flocks.

In conclusion, this paper is the first to fully characterize through sequencing and clinicopathological observation the recently isolated strains Australia, Nevada Cormorant and Long Bien. The constant characterization of newly isolated strains that circulate within and outside the United States will allow for assessment of eventual variation in their pathogenicity for poultry species. Such information will be helpful in devising appropriate prevention and control measures. Also, this paper reinforces the concept that standard indices and sequence analysis of NDV isolates may not fully predict their pathogenicity in susceptible animals, therefore, making animal experiments important tools for a complete characterization of new isolates.

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Table 3.1. Clinical (Clin) and pathological (Path) findings of chickens infected with NDV strains.

	2 dpi†		3 dpi		4 dpi		5 dpi		6 dpi		10 dpi	
Strain inoculated	**Clin**	**Path**	**Clin**	**Path**	**Clin**	**Path**	**Clin**	**Pathol**	**Clin**	**Path**	**Clin**	**Pathol**
PBS	Normal.	Normal.	Normal.	Normal.	Normal.	Normal.	Normal.	Normal.	Normal.	Normal.	Normal.	Normal.
Long Bien	Depression. Severe conjunctivitis.	Eyelids-Ed. (2/2)*. Spleen-Nec. (2/2). Cecal tonsils-Ed. (1/2).	Severe depression, conjunctivitis. One spontaneously dead bird.	Eyelid-Ed.+Hem. Spleen-Nec. Proventriculus-Hem. Intestine-Hem+Nec. (4/4).	All birds dead.	-	-	--	-	-	-	-
Australia	Normal.	Conjunctivitis (2/2).	Slight depression. One bird open mouth breathing.	Eyelid-Hem. (1/2). Spleen-Mot. (1/2). Cecal Tonsils-Hem. (1/2).	Depression. One bird very sick.	Eyelid-Hem. Intestine-Hem. Kidney-Hem. (1/1)	One Bird very sick with open mouth breathing. Another bird slightly depressed.	Proventriculus and Intestine-Hem. (1/2). Enlarged spleen (1/2).	One bird severe depression. Two slight depression.	Proventriculus and Intestine-Hem. (1/1).	Recovered.	No gross lesions.

Nevada Cormorant	Normal.	Thymus-Hem. (2/2). Spleen-Mot. (1/2).	Depression, Slight conjunctivitis.	Slight Conjunctivitis.	Depression. Nervous signs. Comb hemorrhages.	No necropsy at this time.	Depression. Nervous signs.	Spleen-Mot. (4/4). Eyelid-Hem. (1/4).	Two depressed. Two paralyzed.	Cecal tonsils-Ed. (1/2). Spleen-Mot. (1/2).	Paresis, recumbency and depression. One head twitch.	Thin and easily pliable femurs. (2/2). Thymic atrophy (1/2).

Ed.= edema.

Hem.= hemorrhage.

Mot.= mottled.

Nec.= necrosis.

*numbers of birds showing lesions / on the total number of birds per group necropsied on that day.

†Days Post Infection.

Table 3.2. Virus isolation and titration of oral (O) and cloacal (C) swab samples.

	PBS		Nevada Cormorant(5.3)*		Australia(4.5)		Long Bien(5.5)	
Dpi†	***O***	***C***	***O***	***C***	***O***	***C***	***O***	***C***
2	-§	-¦	$<10^{0.5}$(1/2) #	- (0/2)	$10^{0.7}$(1/2)	$10^{0.7}$(1/2)	$>10^{3.5}$(2/2)	$>10^{3.5}$(1/2) $10^{3.1}$(1/2)
3	nc¶	nc	nc	nc	$>10^{3.5}$(2/4)	$10^{0.9}$(1/2) $10^{1.7}$(1/2)	$>10^{3.5}$(4/4)	$>10^{3.5}$(4/4)
4	nc	nc	nc	nc	$>10^{3.5}$(1/1)	$>10^{3.5}$(1/1)	nc	nc
5	-	-	$10^{3.3}$(1/4) ‡ $10^{3.5}$(3/4)	$10^{1.9}$(1/4) $10^{3.1}$(1/4) $10^{1.5}$(1/4) $>10^{3.5}$(1/4)	$>10^{3.5}$(2/2)	$10^{2.9}$(1/2) $10^{2.7}$(1/2)	nc	nc
6	nc	nc	$10^{2.9}$(1/2) $10^{3.5}$(1/2)	$10^{1.5}$(1/2) $10^{0.5}$(1/2)	$10^{2.5}$(1/1)	$10^{1.7}$(1/1)	nc	nc
10	-	-	- (0/2)	$<10^{0.5}$(1/4)[f]	-(0/2)	$10^{1.5}$(1/2)	nc	nc

* Number in parentheses is the viral titer of inoculum per 0.1 ml.

†Days postinoculation.

‡Number of birds displaying that value / total number of animals sampled.

§ No virus recovered from undiluted swab fluids inoculated into three eggs, 0.2 ml per egg.

¦ Viral titer of swab sample per 0.1 ml.

#low titer but positive.

¶ (nc) non collected.

Table 3.3. Distribution and intensity of the lesions* and immunohistochemical† staining for NDV nucleoprotein.

		Vietnam		**Nevada Cormorant**			**Australia**		
Organs	DPI[#]	2	3	2	5-6[ǀ]	9	2-3	4-6	10
Eyelid	HE[‡]	++	+++	+	+	+	+	++	-
	IHC[§]	+++	+++++	-	+	-	+	++	-
Spleen	HE	++	++++	+	+	+	+	+	+
	IHC	+++	++++	-	-	-	+/-	++	-
Thymus	HE	++	+++	-	-	-	-	-	-
	IHC	+++	+++++	-	-	-	+	++	-
Bursa	HE	++	+++	-	-	-	-	++	-
	IHC	++	++++	-	-	-	-	+	-
Cecal Tonsils	HE	++	+++	-	+	+	-	++	-
	IHC	++++	++++	-	-	-	+	++	-
Pancreas	HE	-	++	-	-	-	-	-	-
	IHC	-	++	-	-	-	-	-	-
Lung	HE	-	-	-	-	-	-	-	-
	IHC	+	++	-	-	-	-	+/-	-
Heart	HE	-	-	-	+	+	+	++	-
	IHC	-	++	-	-	-	-	+/-	-
Brain	HE	-	-	-	+++	+++	-	-	++
	IHC	-	-	-	+	+	-	-	-
Bone marrow	HE	-	+++	-	-	-	-	-	-
	IHC	+	+++	-	+/-	-	-	+	-

Histological grading:

* Spleen: +Moderate hyperplasia of lymphocytes; ++ mild lymphocytic depletion ; +++moderate (<50%) lymphocyte depletion, histiocytic accumulation and multifocal necrosis; ++++ (>50%) severe lymphocytic depletion, histiocytosis and necrosis.

Thymus, Cecal tonsil, GALT, Bursa and Thymus: +mild lymphocytic depletion, ++ (<50%) moderate lymphocytic depletion with necrosis and histiocytosis, +++ (>50%) severe lymphocytic depletion, necrosis and histiocytosis.

Bone marrow: mild + (<20%) bone marrow necrosis; ++ mild (20-50%) bone marrow necrosis; +++ severe (>50%) bone marrow necrosis.

Pancreas: + mild (<3 areas) vacuolation and degeneration; ++ moderate (>3 areas) vacuolation and degeneration.

Brain: + vascular reactivity; ++ vascular reactivity and perivascular cuffing; +++vascular reactivity, perivascular cuffing, gliosis.

Immunohistochemical grading:

† - = no IHC signal present.

+ = rare cells in the section are positive on IHC.

++ = positive cells seen, <50% of all high power fields (HPF).

+++ = positive signal seen in 50 to 75% of HPF.

++++ = abundant positive signal in more than 75% of HPF.

#(DPI)Day Post Infection.

‡(HE)Hematoxylin and Eosin.

§(IHC)Immunohistochemistry.

¦ results shown from days on which tissues had most intense staining.

Figure 3.1. Spleen; chicken infected with Long Bien strain, 2 dpi. Enlarged and diffusely mottled spleen indicating necrosis.

Figure 3.2. Small intestine; chicken infected with Long Bien strain, 3 dpi. Areas of hemorrhages and necrosis in the small intestine.

Figure 3.3. Brain; chicken infected with Nevada Cormorant strain, 5 dpi. Perivascular cuffs in the white matter, and focal area of gliosis. HE.

Figure 3.4. Spleen; chicken infected with Long Bien strain, 3 dpi. Extensive presence of viral antigen (red) within the histiocytes surrounding the penicillary arteries. Immunohistochemistry for NDV antigen, alkaline phosphatase method, hematoxylin counterstain.

Figure 3.5. Femur, physis; chicken infected with Long Bien strain, 3 dpi. Numerous osteoclasts and fewer mononuclear cells are strongly positive for NDV nucleoprotein. Immunohistochemistry for NDV, alkaline phosphatase method, hematoxylin counterstain.

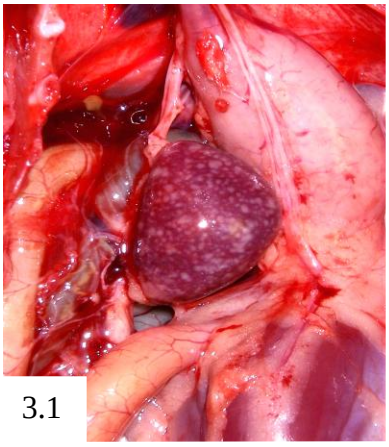
Figure 3.6. Intestine; chicken infected with Australia strain, 4 dpi. IHC (red) demonstrates viral protein within neurons in submucosal and myenteric plexuses. Immunohistochemistry for NDV, alkaline phosphatase method, hematoxylin counterstain.

Figure 3.7. Cerebellum; chicken infected with Nevada Cormorant strain, 6 dpi. NDV nucleoprotein (red) is present within the axons and the glial cells of the molecular layer, and in few Purkinje cells. Immunohistochemistry, alkaline phosphatase method, hematoxylin counterstain.

Figure 3.8. Intestine; chicken infected with Australia strain, 4 dpi. Riboprobe ISH (brown) shows that the viral mRNA is present in the submucosal and myenteric (inset) plexuses. NBT-BCIP, hematoxylin counterstain.

Figure 3.9. Intestine; chicken infected with Australia strain, 5 dpi. ISH (brown) demonstrates intracytoplasmic presence of viral mRNA in macrophages of the cecal tonsils. NBT-BCIP, hematoxylin counterstain.

Figure 3.10. Evolutionary relationships of three selected NDV isolates in comparison to reference genotypes. The evolutionary history was inferred using the Maximum Likelihood method. The optimal tree with the sum of branch length is shown and bootstrap values greater than 70 are included in the nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Correspondence between tree names and GenBank accession numbers are presented in table 4 as online supplement. The name of the viruses described in the paper are followed by an asterisk and underlined. Roman numbers within circles indicate the genotype.



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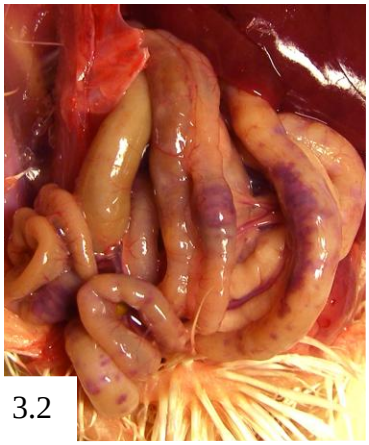

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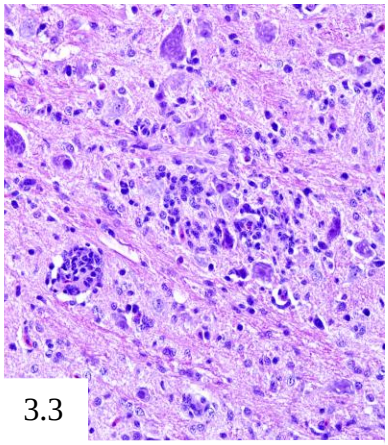

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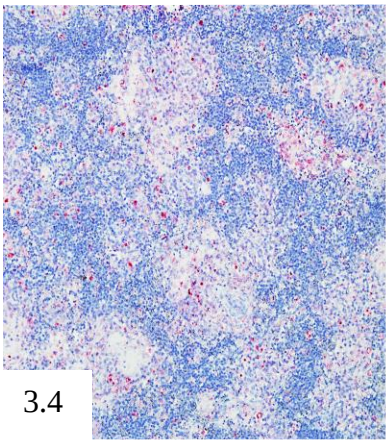
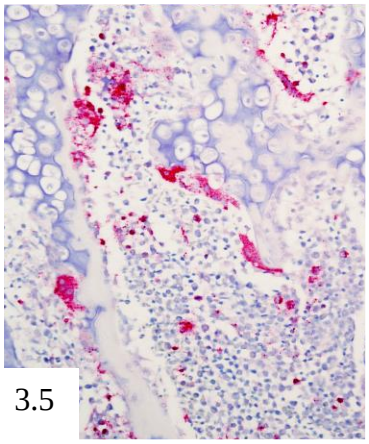


3.4



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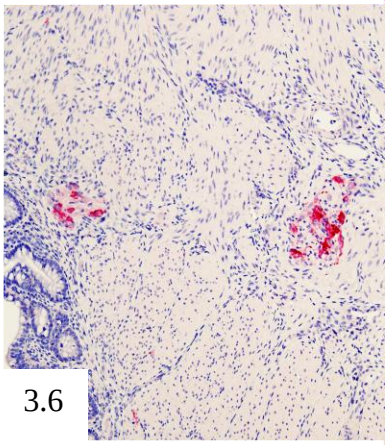

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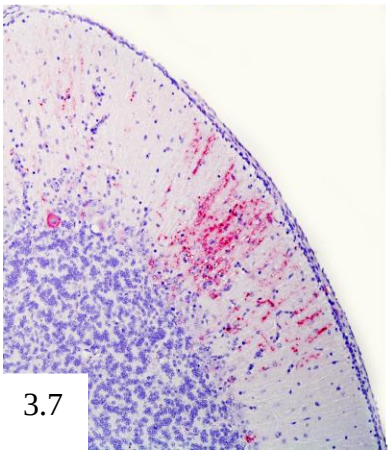

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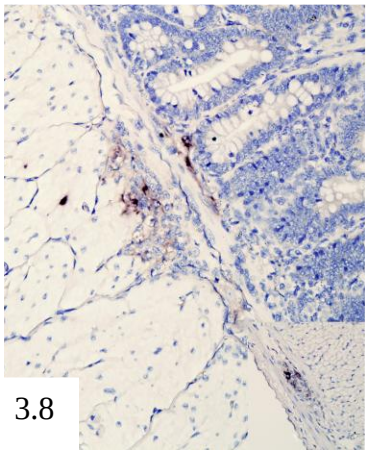

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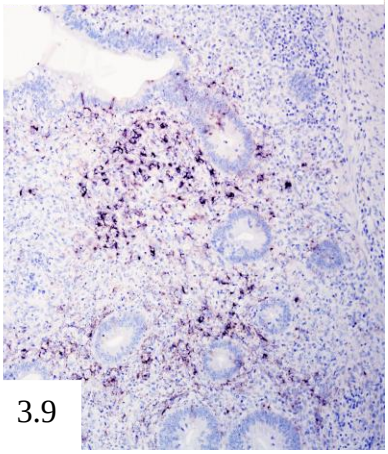

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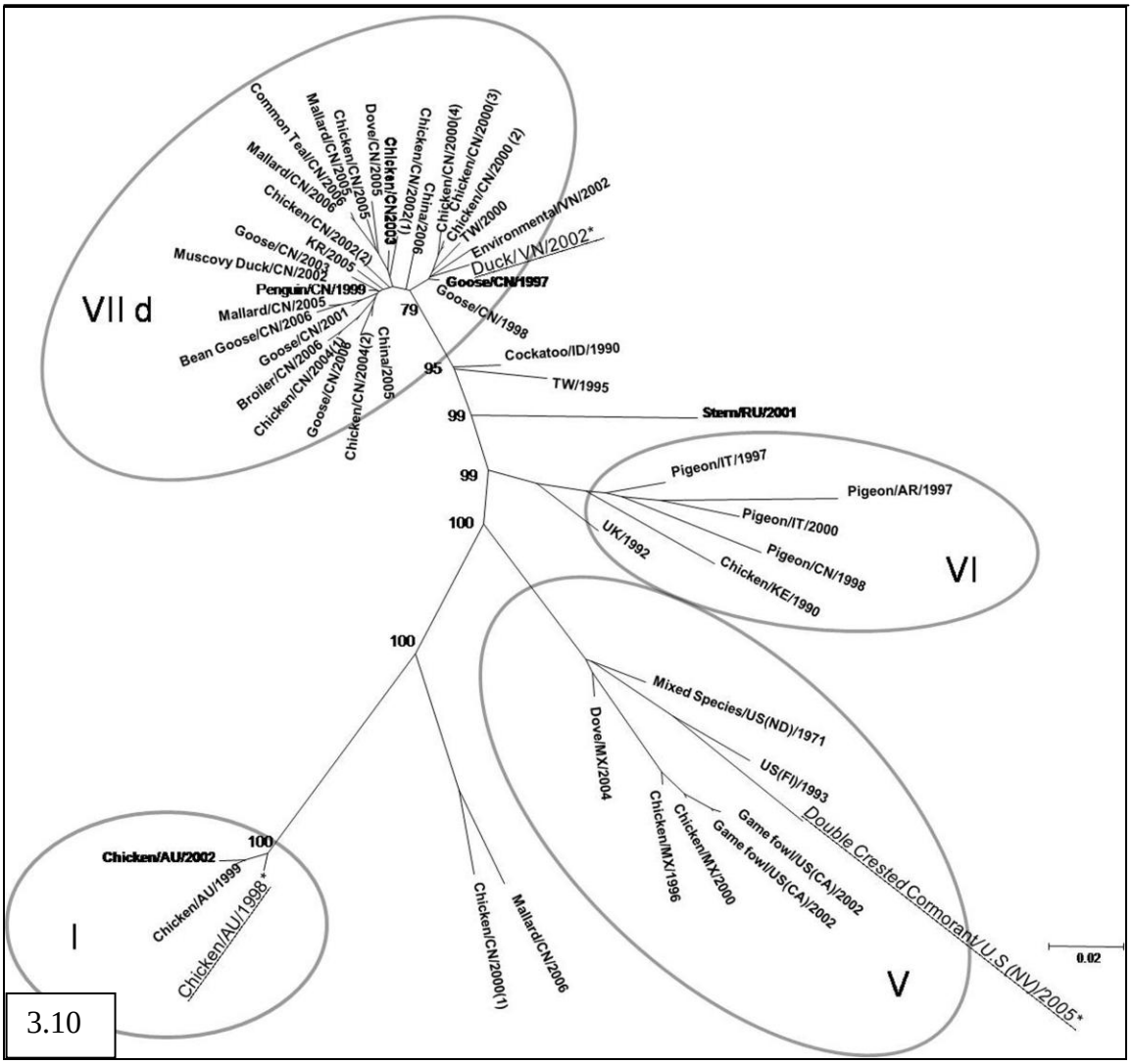
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CHAPTER 4

PATHOGENICITY EVALUATION OF DIFFERENT NEWCASTLE DISEASE VIRUS CHIMERAS IN 4-WEEK-OLD CHICKENS*

*Susta L, Miller PJ, Afonso CL, Estevez C, Yu Q, Zhang J, Brown CC. 2010. *Tropical Animal Health and Production* 42: 1785-1795. Reprinted with permission of the publisher.

ABSTRACT

The aim of this study was to evaluate the disease-inducing ability of four chimeric Newcastle disease viruses (NDV) by clinicopathological assessment. The infectious clones were previously generated by replacement of hemagglutinin-neuraminidase (HN) and/or fusion (F) genes from virulent strains (Turkey North Dakota and California 02) into a mesogenic strain (Anhinga) backbone. Groups of 4-week-old chickens were inoculated via eye drop instillation, clinical signs were monitored daily, and necropsies with collection of tissues were performed at 2, 5, 10 and 14 days post infection (dpi). Tissue sections were evaluated for histopathology and immunohistochemistry for NDV nucleoprotein. All viruses replicated successfully in the natural host, although viral recovery, seroconversion and extent of immunohistochemical staining were greatest from birds infected with those viruses containing both F and HN genes from the same virulent virus. There was minimal to no increase in clinicopathologic disease due to infection with the chimeras compared to the recombinant backbone. However, all birds developed histological evidence of encephalitis. The results suggest that the inherent virulence of Turkey North Dakota and California 2002 strains is due to more than the simple presence of their F and HN genes.

Key words Avian paramyxovirus-1; fusion protein; hemagglutinin-neuraminidase; immunohistochemistry; infectious clones; Newcastle disease; pathogenesis; reverse genetics; chimeras.

Abbreviations APMV-1 = avian paramyxovirus-1BHI = brain-heart infusion broth; dpi = days postinoculation; EID_{50} = 50% embryo infectious dose; HA = hemagglutination;

HI = hemagglutination inhibition; HN = hemagglutinin-neuraminidase; F = fusion protein; ICPI = intracerebral pathogenicity index; IHC = immunohistochemistry; MDT = mean death time; ND = Newcastle disease; NDV = Newcastle disease virus; OIE = Office International des Epizooties; PBS = phosphate-buffered saline; RT-PCR = reverse transcription–polymerase chain reaction; SEPRL = Southeast Poultry Research Laboratory; SPF = specific pathogen free

INTRODUCTION

Newcastle disease (ND), caused by virulent strains of ND virus (NDV) is a widespread and economically significant disease of poultry (Alexander 2003; Alexander 2001). The virus is classified in the order Mononegavirales, family Paramyxoviridae, subfamily Paramyxovirinae, genus *Avulavirus* and all strains regardless of virulence comprise avian paramyxovirus-1 (APMV-1) within that genus. The NDV is a non-segmented, single-stranded, negative sense RNA virus. The RNA genome is approximately 15,000 bases and it is composed of 6 proteins, in order from 3' to 5': nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and large protein (L) (Alexander 2003). The virulence of the different NDV strains varies greatly according to the host susceptibility, and the intrinsic virulence of the viral strain (Alexander 1995; Brown *et al.* 1999; Kommers *et al.* 2002). Because there is great variability in the type and severity of the disease it produces, there are often difficulties in recognizing the clinical problem as Newcastle disease (ND) (Alexander 1998, 2003; Alexander 1995, 2001).

There are several virulence classification schemes for NDV. Historically, virus strains have been classified into highly virulent (velogenic) NDV, moderately virulent (mesogenic) NDV, and low virulent (lentogenic) NDV by inoculation into embryonating eggs and recording time to death (Mean Death Time or MDT). Velogenic strains are further subdivided into viscerotropic and neurotropic, based upon their ability to cause visceral hemorrhagic lesions or nervous system lesions respectively (Alexander 1998, 2003). An intracerebral pathogenicity index (ICPI) in 1-day-old chicks is the internationally recognized standard and consists of inoculating 1-day-old chicks intracerebrally and scoring birds as normal, sick, or dead over a period of 8 days, to result

in a score from 0.0 to 2.0. Those strains scoring $\geq$0.7 are considered "notifiable" to the international community through the World Organization for Animal Health (formerly known as the Office International des Epizooties and still recognized by the designated abbreviation, OIE) (OIE 2008). The amino acid sequence at the fusion protein cleavage site has been postulated to be a primary determinant of NDV virulence (Glickman *et al.* 1988; Nagai *et al.* 1976) and the presence of multiple basic amino acids and phenylalanine at that site in an isolate is another criterion recognized by the OIE as determining a notifiable agent. Usually, both ICPI and fusion protein sequence are seen as equally important in determining if a strain should be classified as notifiable (OIE 2008).

To date, many studies have focused on the specific molecular determinants of virulence associated with the F and HN proteins. The fusion cleavage protein has received the most attention. The presence of cellular protease is required for the activation of the viral fusion protein precursors. The virulent (velogenic and mesogenic) strains have the fusion protein cleavage motif $_{112}$R/K-R-Q-R/K-R-F$_{117}$, which is cleaved by ubiquitous proteases present in almost every tissue, allowing for successful infection of most body tissues. In contrast, the lentogenic strains have a fusion protein sequence which can be cleaved only by trypsin, which naturally exists in only a few tissues, specifically respiratory and gastrointestinal, thus rendering the infection limited to these mucosal surfaces (Alexander 1998; Gotoh *et al.* 1992; Nagai 1995; Nagai and Klenk 1977). The HN protein is responsible for the attachment of virus particles to sialic acid containing receptors of the host cells (Morrison *et al.* 1990; Panda *et al.* 2004; Scheid

and Choppin 1973), and modification of its glycosylation pattern has been shown to modify NDV virulence (Panda *et al.* 2004).

Studies with recombinant viruses have proved very useful in determining the role of various genes in pathogenesis and virulence. However, some studies have shown that international standard virulence parameters, i.e. ICPI, do not always correlate with behavior of the virus in adult chickens. For example, some recombinant viruses with identical ICPIs displayed different lesions and viral distribution when inoculated via a natural route into susceptible chickens (Wakamatsu *et al.* 2006c). Conversely, viruses with different ICPIs showed very similar clinicopathologic manifestations when inoculated via a natural route into susceptible chickens (Wakamatsu *et al.* 2006b). Studies in our laboratory have focused on assessing clinicopathological characteristics of NDV, through inoculation of 4-week-old chickens and systematic examination of multiple tissues collected at defined time points, by histopathology for lesion development, immunohistochemistry for detection of viral proteins, and in-situ hybridization for demonstration of viral replication. More than 50 strains have been assessed in this manner (Brown *et al.* 1999; Kommers *et al.* 2001, 2003a, b; Kommers *et al.* 2002; Oldoni *et al.* 2005; Wakamatsu *et al.* 2006a, 2006b, 2006c). Recently, Estevez *et al.* (2007) created chimeric viruses on a mesogenic backbone. A rescued mesogenic virus (Anhinga) was developed, and then the HN gene alone or both F and HN genes from two virulent viruses (Turkey North Dakota and California 2002) were inserted. The ICPI of viruses with both genes inserted had the highest increase in ICPI among the other recombinant viruses (Estevez *et al.* 2007). The aim of this study was to assess the clinicopathological features of these chimeric viruses using our

laboratory procedure to assess how these viruses behaved when inoculated via a natural route into adult chickens.

MATERIAL AND METHODS

Viruses

All viruses and their clones were obtained from the Southeast Poultry Research Laboratory (SEPRL). The infectious clones have been previously described (Estevez *et al.* 2007). Briefly, all were produced on a common Anhinga backbone in which cloned hemagglutinin-neuraminidase (HN) and fusion protein (F) genes from two different virulent viruses were inserted. Anhinga virus (Anhinga/U.S. (FL)/44083; ICPI = 1.45) was originally isolated from a captive bird in a zoological park in Florida. It is classified among the mesogenic viruses. The virulent HN and F genes were taken from Turkey North Dakota (TkND) and California 2002 (CA02) strains. Turkey North Dakota, a neurotropic velogenic NDV (Turkey/U.S. (ND)/43084/92; ICPI = 1.63), was isolated from turkeys believed to be infected through contact with feral birds, probably cormorants (Estevez *et al.* 2007; Seal *et al.* 1995). The viscerotropic velogenic California virus CA02 (fowl/U.S. (CA)/212519/02; ICPI = 1.85) was isolated from backyard flocks and is related to NDV strains that were circulating in Mexico at the time of its isolation and is the virus responsible for the extensive US outbreak in 2002-2003 (Pedersen *et al.* 2004). Full clinicopathological characterization, including immunohistochemical examination, of Anhinga (Anhinga/U.S (FL)/44083), Turkey North Dakota (Turkey/U.S. (ND)/43084/92) and California 02 (fowl/U.S.(CA)/212519/02) strains has been described previously in our laboratory using

identical systems to what is described in this report (Brown *et al.* 1999; Wakamatsu *et al.* 2006a).

The designation for the chimeric viruses is indicated below, along with their ICPI as previously determined (Estevez *et al.* 2007) and they are graphically represented in Figure 4.1.

1) rAnh: recombinant Anhinga as rescued from a plasmid, with no foreign gene insertion; ICPI = 0.89
2) rAnhTkHN, recombinant genes: HN from TkND strain; ICPI = 1.00
3) rAnhTkFHN, recombinant genes: HN and F from TkND; ICPI = 1.16
4) rAnhCAHN, recombinant gene: HN from CA02; ICPI = 0.86
5) rAnhCAFHN, recombinant genes: HN and F from CA02; ICPI = 1.14

Viruses were propagated in embryonating eggs at the Southeast Poultry Research Laboratory (SEPRL), and the amnioallantoic fluid harvested from those infected eggs was used as inoculum after appropriate dilution.

Eggs and chickens

The source of embryonating chicken eggs and chickens was the SEPRL SPF White Leghorn flock. Birds were housed in negative pressure isolators under biosafety level (BSL)-3 enhanced (E) conditions at SEPRL and provided food and water ad libitum. Embryonating eggs were inoculated for viral propagation, isolation, and titration (Alexander 1998). Chickens were inoculated for the pathogenesis (clinicopathologic assessment) study.

Pathogenesis assessment in chickens

Six groups of ten 4-week-old SPF White Leghorn chickens were inoculated bilaterally in the conjunctival sac with 0.1 ml of viral inoculum. Phosphate buffered saline (PBS) was used for the noninfected control birds. The target dose of inoculum was $10^{5.0}$ 50% embryo infectious doses (EID_{50}). The birds were monitored clinically every day, and two birds of each group were euthanized after taking oropharyngeal and cloacal swabs at 2, 5, 10 and 14 days post inoculation (dpi). Blood samples for serology were also collected at 10 and 14 dpi. Tissues (eyelid, spleen, bursa, thymus, Harderian gland, proventriculus, small intestine, cecal tonsils, large intestine, air sac, trachea, lung, heart, esophagus, pharynx, crop, brain, liver, pancreas, kidney, comb, head of femur including bone marrow, and turbinate) were collected, and all sample tissues were fixed by immersion in 10% neutral buffered formalin for approximately 52h. The sections of femur and turbinate were decalcified in 5% formic acid for 3-4h. All sampled tissues were routinely processed into paraffin, and 3μm sections were cut for hematoxylin and eosin staining and immunohistochemistry (IHC).

Immunohistochemistry

All sampled tissues were examined by immunohistochemistry (IHC) with the following protocol to detect viral nucleoprotein. After deparaffinization, tissue sections were subjected to antigen retrieval by microwaving for 20 min at minimum power in Vector antigen unmasking solution (Vector Laboratories, Burlingame, CA) followed by application of universal blocking reagent (Biogenex, San Ramon, CA) as recommended by the manufacturer. The primary antibody, made in rabbit, was anti-peptide

(nucleoprotein), used at 1:8000 dilution, and the detection system was an alkaline phosphatase-labeled polymer specific against the Fc portion of rabbit immunoglobulin (LabVision, Fremont, CA). Chromogen was a naphthol-based dye (Fast Red, Dako, Carpinteria, CA). Sections were counterstained lightly with hematoxylin and coverslipped with Permount for a permanent record.

Virus Isolation and Titration of swabs

Immediately prior to euthanasia, oral and cloacal swabs were obtained from each bird and placed in separate tubes containing 1.5 ml of brain-heart infusion broth (BHI) with antibiotics (2000U penicillin G/ ml, 200 µg gentamicin sulfate/ ml, and 4 µg amphotericin B/ ml). Swab sample tubes were centrifuged at 1000 × g for 20 min, and the supernatant removed for virus isolation and titration. Virus infectivity titers were calculated from the result of inoculation of 9- to 10-day-old SPF embryonating chicken eggs with serial 10-fold dilutions in BHI containing antibiotics (100U penicillin G/ ml and 50µg gentamicin sulfate/ ml). NDV in infected dead or surviving embryos was identified by hemagglutination (HA) activity in amnioallantoic fluid harvested from chilled eggs. NDV was confirmed in HA positive samples by hemagglutination inhibition (HI) test with NDV-specific antiserum.

Serology

The HA and HI tests were conducted by conventional microtiter methods with serum separated from the blood samples taken at 10 and 14 dpi. Four HA units per 25µl

of betaproprionolactone-inactivated NDV LaSota was used as test antigen in completing the HI test.

RNA extraction and RT-PCR

To better determine if the histopathological lesions that were observed in the brain were associated with NDV infection, PCR was performed in the paraffin embedded brains of all birds sampled at 14 dpi. The RNA extraction protocol was as described previously (Wakamatsu *et al.* 2007). Briefly, each formalin-fixed tissue embedded in paraffin blocks was trimmed with a razor blade to obtain 35 mg of tissue, which was then placed into a microcentrifuge tube. These tissue pieces were deparaffinized with 1 ml xylene, mixed on a vortex mixer, and centrifuged at 10,000xg at room temperature for 10 min. The supernatant was discarded, and the deparaffinization step was repeated. The resultant tissue pellet was washed with 1 ml of cold 100% ethanol and dried for 15 min in a vacuum centrifuge. For digestion of proteins and nucleases, the dried sample was incubated at 50°C for 4 h with 600 µl of digestion buffer (20 mM Tris-HCl pH7.6, 20 mM ethylenediaminetetraacetic acid, and 1% sodium dodecyl sulfate with 300 µg of proteinase K). To extract RNA, 600 µl of phenol:chloroform 5:1 was added to each tube, and the tubes were centrifuged at 10,000 x *g* at 4°C for 3 min. This step was repeated with the top layer. Then, 600 µl of chloroform:isoamyl alcohol 24:1 was added to the top layers collected, and the resulting mixture was centrifuged at 10,000 x *g* at 4°C for 3 min. For precipitation of purified nucleic acid, the top sample layer was mixed with 50 µl of sodium acetate (pH7.0) and 1 ml of cold 100% ethanol and placed at –80°C overnight. The precipitated nucleic acid was centrifuged at 14,000 x *g* for 30 min at 4°C, and the

pellet was washed with cold 70% ethanol and dried in a vacuum centrifuge for 15 min. The pellet was resuspended in 30 µl of nuclease-free water and stored at –80°C. All buffers and reagents were prepared with ribonuclease-free water. Total nucleic acid yield was calculated using a spectrophotometer at 260 nm absorbance. For each RT-PCR, 1 µg total RNA was used.

First strand cDNA was synthesized from 1 µg RNA with random primers, 0.25 mM dNTPs and Superscript reverse transcriptaseII (Invitrogen), according to the manufacturer. The pairs of NDV matrix protein specific primers were designed according to GenBank for specific NDV nucleotide sequences to identified desired fragments.

One pair of primers was synthesized as following: NDV-Anhinga Sense (S): 5'---GAG TTA CTT TCT TCT GCA ATG CTC TGC CTA GG—3'and NDV-Anhinga Anti Sense (AS): 5'---GAA AAA GGT GCC AGC TTG GTC CGT GCA---3'for primary PCR (639 bp).

Another pairs of primers was NDV-Anhinga Sense (S): 5'--- GAG TTA CTT TCT TCT GCA ATG CTC TGC CTA GG—3' and NDV –Anhinga Nested Anti Sense (NAS): 5'---GTT CCG CTC CCG GGG ATC TTC TCT---3' as nested primers (251bp).

As positive controls, 1 µl of total RNA extracted from amnioallantoic fluid of SPF embryonating chicken egg infected with NDV Anhinga strain was used. Paraffin embedded tissues from eyelids positive by IHC were used as extraction positive control. For a negative control, RNA extract from non-infected tissues was used.

The primary PCR was performed in a 27 µl volume containing 3 µl of sample cDNA, 12.5 µl of TaqPCR Master Mix, 1 µl of each primer (NDV-S and NDV-AS), and 9.5 µl of nuclease-free water. The reaction was then incubated in a thermocycler under

the following conditions: 94°C for 5 min; 35 cycles of 94°C for 30 sec (denaturation), 55ºC for 30 sec (annealing), and 72°C for 1 min (extension). The samples were then chilled at 4°C. The nested PCR was performed in a 20 µl volume containing 1 µl of the primary PCR product, 10 µl TaqPCR Master Mix, 7 µl of nuclease free water, and 1 µl of each primer. The product was amplified by using the same reaction conditions used for the first PCR, with the primers NDV- Anhinga S and NDV- Anhinga NAS.

A total of 20 µl of each nested PCR product was subjected to electrophoresis on a 2% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM ethylenediaminetetraacetic acid [EDTA]) for 45 min at 90 V. For staining, 1.25 µl of ethidium bromide per 30 ml was added to the gel. The gel was photographed with UV illumination.

The PCR products obtained were confirmed by comparing their size with a product obtained with the same primers applied to positive control DNA. The positive control was a TA-Vector plasmid with insert of specific NDV-Anhinga matrix protein gene (sequence verified by the Molecular Genetics Instrumentation Facility, University of Georgia).

RESULTS

Pathogenesis assessment in chickens - clinical disease, gross pathology, histopathology, IHC

Appearance of clinical signs and the main pathologic findings are presented in Table 4.1. Only three birds displayed clinical signs - two infected with rAnhCAHN and one with rAnhTKFHN. The signs were not dramatic and occurred late in infection. Two birds infected with rAnhCAHN began showing slight depression at 9 dpi, which lasted through day 14. One bird infected with rAnhTKFHN had head twitching at 10 dpi.

Gross lesions were minimal to absent. Three animals infected with rAnh, rAnhCAHN and rAnhTKHN had eyelid petechiation at 5 and 10 dpi. Histologic changes, with the exception of brain, were mild. At 10 and 14 dpi, the eyelids of all the infected birds showed moderate hyperplasia of the lymphoid tissue. The hearts of two birds infected with rAnhTKFHN and rAnhCAFHN at 14 dpi had moderate lymphocytic infiltration dissecting between cardiac myofibers. However, distinct and consistent lesions were seen only in brain. Severity and distribution of brain lesions are summarized in table 4.2. The lesions were observed both in the neuraxis and in the cerebellum, and were characterized by multifocal perivascular cuffing, up to 4-5 rows of cells, consisting of lymphocytes, plasma cells and lesser numbers of macrophages; moderate lymphocytic infiltration of the adjacent parenchyma; multifocal or coalescing glial nodules, consisting of microglia and astrocytes; and moderate perivascular and intracellular (neuropil rarefaction) edema (fig. 4.2). In the cerebellum, the perivascular lesions were mostly limited to the cerebellar peduncles (figs. 4.3 and 4.4) and molecular layer (fig. 4.5), with multifocal necrosis and neuronophagia of the Purkinje cells adjacent to the inflammatory foci. The most severe changes were observed in the birds infected with rAnhCAHN, at 10 dpi, and consisted of multifocal to coalescing gliosis, involving the whole neuraxis, with severe lymphoplasmacytic and histiocytic perivascular cuffing and perivascular edema.

Using immunohistochemistry there was positive labeling for NDV nucleoprotein in only three birds and only in non-nervous tissue. In two birds infected with rAhnhCAFHN and sampled at 5 dpi, there was scattered positive signal in the lymphoid tissue of the eyelid, pharynx and crop (fig. 4.6). Additionally, infrequent cells were positive for NDV nucleoprotein in the eyelid of a bird infected with rAnhTKFHN at 5 dpi.

Virus isolation and titration of swabs

Results for virus isolation from oral and cloacal swabs are presented in Table 4.3. Briefly, virus was isolated from oral swabs from birds infected with rAnh at 2, 5 and 10 dpi, from birds infected with rAnhTKHN at10 dpi, from birds infected with rAnhTKFHN at 5 and 10 dpi, from birds infected with rAnhCAHN at 2 and 5 dpi, and from birds infected with rAnhCAFHN at 2 and 5 dpi. The highest titers were seen with rAnhCAFHN at 5 dpi, with both birds having $10^{2.5}$ EID_{50} or greater in the oral swabs. There was no virus isolated from oral swabs from any birds at 14dpi. From cloacal swabs, virus was isolated at 5 and 10 dpi only. No virus was isolated from cloacal swabs from birds infected with rAnhTKHN. Highest titers from cloacal swabs were obtained from birds infected with rAnhCAFHN.

Serological Examination

Results of serologic testing are presented in Table 4.4. All serum samples from infected birds, except two birds infected with rAnhCAHN at 14 dpi, had evidence of antibody response to NDV. Antibody titers from rAnhTKFHN had the highest (1:128) value at day 10. Serum from birds infected with rAnhCAFHN at 14dpi had the highest titers compared to birds infected with the other viruses at the same time points.

PCR

Results of semi-nested RT-PCR amplification of matrix gene from formalin-fixed paraffin-embedded brain samples of infected birds euthanized at 14 dpi are presented (fig. 4.6). A specific NDV product, a part of matrix gene (251 bp), was successfully

amplified from nine out of 10 infected birds. The only negative sample from the infected birds was the brain of a bird infected with rAnhCaHN at 10 dpi. The non-infected control birds were negative.

DISCUSSION

This study investigated the effect of NDV gene substitution involving the F and HN genes from virulent NDV strains within a mesogenic backbone, in 4-week-old chickens inoculated via a natural route of infection. The clones were previously characterized (Estevez *et al.* 2007) by the standard virulence test, ICPI. In the present study, potential for disease causation was assessed through inoculation into fully susceptible 4-week-old chickens, as already done in our laboratory for characterization of numerous NDV strains.

All of the recombinant strains proved to be capable of infecting adult birds using a natural (eyedrop) route of infection. The virus could be retrieved from cloacal and/or oral swabs of all the inoculated groups, even if not at all time periods, confirming that all the viruses replicated in the host organism. Levels of recovered virus were low, but this is consistent with the natural behavior of parental Anhinga, as previous infection studies have shown that there is relatively limited replication *in vivo* (Brown *et al.* 1999). Serologic titers demonstrated a humoral response to the inoculated viruses for all groups, a further indication of successful replication. The groups inoculated with the chimeric viruses containing both the F and HN genes from virulent viruses had the strongest serologic response as compared to the others, which is most likely an indication of more extensive replication.

In terms of clinical assessment, none of the birds became very ill. A head twitch was seen in one bird (rAnhTKFHN) at 14 dpi and slight depression, beginning at 9 dpi in some others (rAnhCAFHN). Visceral gross lesions were not evident in any birds. This is similar to what observed with experimental infection with wild type Anhinga, which caused neither clinical disease nor gross lesions in chickens over a period of 10 dpi (Brown *et al.* 1999). Using immunohistochemistry the recombinants that had the two genes inserted, rAnhCAFHN and rAnhTKFHN, had more immunohistochemical evidence of antigen. There was overall minimal viral antigen in all tissues which could be explained by a low multiplication rate of the viruses in the tissues, resulting in a viral burden near the sensitivity of the immunohistochemical technique, thus allowing antigen detection only in or near the inoculation site. This viral distribution is slightly lower than reported with experimental infection with wild type Anhinga, using a system identical to our experimental design, which described minimal reactivity by immunohistochemistry and *in situ* hybridization in scattered cells in eyelid, spleen and heart (Brown *et al.* 1999). For rAnhTKFHN virus was present at the site of inoculation (eyelid) and for rAnhCAFHN, virus was found at the eyelid as well as in very small amounts in the submucosa of pharynx and crop. It is probable that these clones might have a better replication activity than the others. These are also the two viruses with the highest ICPIs among the recombinants.

The wider distribution of the immunohistochemical staining, and the higher viral loads of the chimeric viruses bearing both virulent genes (HN and F) mirror the pathogenicity index data, in that the recombinant viruses with virulent HN and F substitutions had higher ICPIs (Estevez *et al.* 2007). This could be a reflection of the fact

that HN and F genes work optimally when derived from the same strain. There are several reports suggesting that the HN and F may play a combined role in the pathogenesis of NDV (Deng *et al.* 1995; Huang *et al.* 2004; Lamb *et al.* 2006). However, even if the strains with virulent substitution (F+HN) caused the most clinicopathological changes, these were still considerably less severe than those that usually occur with the parental strains (TkND, CA02) that contributed the inserted genes (Brown *et al.* 1999, Wakamatsu *et al.* 2006a). In addition, none of the chimeras displayed increased disease-inducing ability when compared to the Anhinga wild type (Brown *et al.* 1999).

It is possible that the substitution of a few genes is not enough to successfully modify the pathogenicity, and that the new genes have to "fit" within the general backbone, and that some chimeras display a better fitness than others just because of the way the different parts interact with each other. Support for this comes from an experiment conducted by Wakamatsu et al (2006c), in which the F gene of LaSota (a lentogen) was mutated by insertional mutagenesis to display a virulent motif similar to that of Beaudette C strain. In this case, only minimal genomic mutations were produced (few bases), and no genes from other strains were inserted, but there was dramatic increase in ICPI, severity of lesions and spread of the virus, comparable to those produced by velogenic viruses.

None of the rescued clones, including rAnhinga which had the same genetic makeup as the wild type strain, had ICPIs approaching those of the parental backbone (wild-type Anhinga). This loss of virulence is often observed with numerous viruses rescued by reverse genetics, and a possible explanation includes genomic bottlenecking during the

cloning processes that induces loss of genomic variability and viral population fitness (Duarte *et al.* 1994a, b).

Histologically, the most dramatic finding in this study was the presence in all the inoculated groups of at least one bird with lymphoplasmacytic encephalitis at 10 and 14 dpi. As mentioned previously, there was no immunohistochemical signal at any site except in some birds and only at or near the site of inoculation. There was never any IHC signal associated with the brain lesions. To better determine if NDV was associated with the encephalitic lesions, RT-PCR for a portion of the matrix gene from the brains of birds was done. Amplification was successful in nine of ten, strongly suggesting that the virus itself may have been causing the active lesion, and supporting the hypothesis that the viruses had a systemic spread and successfully replicated in brain. The only brain to have no amplified segment was the one showing the most severe inflammatory infiltrate. This may be explained by the fact that the more vigorous inflammatory response had served to eliminate the virus from this tissue. Negative immunohistochemical staining in the brain lesions might be explained by the fact that the virus replicated at levels below the sensitivity threshold of the IHC technique, but still enough to yield PCR positivity. In a previous clinico-pathological assessment of wild-type Anhinga, encephalitis was not described (Brown *et al.* 1999); however, this study ended at 10 dpi rather than at 14 dpi, as in our experimental protocol. Furthermore, neurological signs are occasionally reported with mesogenic strains (Alexander 2003), and encephalitis can be observed (Bhaiyat *et al.* 1994). Because neurological lesions were seen in all birds, including the rAnh, it is unlikely that the inserted genes might have accounted for an increased neurotropism.

Some degree of lymphocytic infiltrate in the myocardium was observed with two strains (rAnhTKFHN and rAnhCAFHN). This involvement of the heart, even if more severe, has been described also with experimental infection of chickens with wild-type Anhinga (Brown *et al.* 1999).

In summary this study demonstrates that the tested chimeric viruses were able to successfully infect and replicate when introduced via a natural route of infection. All the viruses had a slightly attenuated ability to induce disease when compared to wild-type Anhinga, as observed by severity of lesions and extent of immunohistochemical staining. Comparing the chimeric viruses, those with substitution of both virulent HN and F genes showed a slightly increased ability to replicate, as the viral titers were higher and the immunohistochemical staining was more intense. However, the clinical disease was far less severe than those caused by the parent strains that contributed the virulent genes, indicating that the overall virulence is not determined only by few genes, but rather may be the result of complex interaction between all the elements of the viral genome.

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Table 4.1 Clinical and pathologic assessment of inoculated chickens

	2 dpi		5 dpi		10 dpi		14 dpi	
Strain inoculated	**Clin**	**Pathol**	**Clin**	**Pathol**	**Clin**	**Pathol**	**Clin**	**Pathol**
PBS								
rAnh				Eyelid petechiae (1/2)[a]		Encephalitis (1/2) Eyelid petechiae (2/2)		Encephalitis (2/2)
rAnhTKHN				Eyelid petechiae (1/2)				Encephalitis (1/2)
rAnhTKFHN					Head twitch (1/6)[b]	Encephalitis (2/2)		Encephalitis (2/2)
rAnhCAHN				Eyelid petechiae (1/2)	Depressed (1/6)	Encephalitis (2/2)	Depressed (2/2)	Encephalitis (2/2)
rAnhCAFHN						Encephalitis (1/2)		

[a]Number of birds with lesions over the number of animals necropsied for each group at each time point

[b]Number of birds showing clinical signs over the total number of the animals still in the cage

Table 4.2 Distribution and score of the inflammatory changes in the brain of the chickens infected with infectious clones at day 10 and 14.

Strain	PBS				rAhn[f]				rAnhTKHN				rAnhTKFHN				rAnhCAHN				rAnhCAFHN			
	10dpi		14dpi		10dpi		14dpi		10dpi		14dpi		10dpi		14dpi		10dpi		14dpi		10dpi		14dpi	
Cerebellum	-[a]	-[b]	-[c]	-	-	+	-	-	+[d]	-	+	+	+	+	-	+	+	+	+	-	-	+	-	-
						+											+[e]	+	+			+		
Brainstem	-[a]	-[b]	-	-	-	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+	-	-
																+	+	+	+	+				

[a] First animal of two in the same post infection time group.

[b] Second animal of two in the same post infection time group.

[c] (-)Negative: no inflammatory foci observed.

[d] (+)Moderate: inflammatory infiltrates seen in one or two foci of the neuraxis or cerebellum.

[e] (++)Severe: evidence of more than 2 inflammatory foci in the neuraxis or cerebellum.

[f] Type of strain instillated via eyedrop.

Table 4.3 Virus isolation and titration of oral (o) and cloacal (c) swab samples from 4-wk-old chickens infected 2, 5, 10, and 14 days previously with recombinant and chimeric viruses

		PBS		rAnh		rAnhTKHN		rAnhTKFHN		rAnhCAHN		rAnhCAFHN	
Dpi[a]	ID[b]	*O*	*C*	*O*	*C*	*O*	*C*	*O*	*C*	*O*	*C*	*O*	*C*
2	A	-[c]	-		-	$10^{0.9}$[d]	-	-	-	-	-	$<10^{0.5}$[e]	-
	B	-	-		-	$10^{1.7}$	-	-	-	$10^{0.9}$	-	$10^{1.5}$	-
5	C	-	-	$10^{1.3}$	-	$<10^{0.5}$	$<10^{0.5}$	$10^{0.9}$	-	$10^{1.7}$	$<10^{0.5}$	$10^{2.5}$	$10^{1.5}$
	D	-	-	$10^{0.7}$	-	$10^{1.5}$	-	-	-	$10^{0.9}$	-	$10^{2.9}$	$10^{2.3}$
10	E	-	-	$<10^{0.5}$	$<10^{0.5}$	$10^{0.7}$	$10^{0.7}$	-	$10^{0.9}$	-	$10^{0.7}$	-	-
	F	-	-	$<10^{0.5}$	$10^{0.5}$	-	$<10^{0.5}$	$<10^{0.5}$	-	-	$10^{0.7}$	-	$<10^{0.5}$
14	G	-	-	-	-	-	-	-	-	-	-	-	-
	H	-	-	-	-	-	-	-	-	-	-	-	-

[a] Days post-inoculation.

[b] Bird identification.

[c] No virus recovered from undiluted swab fluids inoculated into three eggs, 0.2 ml per egg.

[d] Viral titer of swab sample per 0.1 ml.

[e] low titer but positive.

Table 4.4 Newcastle disease virus hemagglutinin-inhibition (HI) titers of serum collected from 4-wk-old chickens infected 10 and 14 days previously with the recombinant and chimeric viruses.

Dpi[a]	ID[b]	PBS	rAnh	rAnhTKHN	rAnhTKFHN	rAnhCAHN	rAnhCAFHN
10	1	<2	32 [c]	4	128	32	32
	2	<2	64	16	128	64	128
14	1	<2	64	128	128	16	1024
	2	<2	32	128	128	64	128

[a] Days postinoculation.

[b] Bird identification.

[c] HI titer.

Figure 4.1. Schematic representation of the recombinant viruses, with inserted genes

Figure 4.2. Chicken infected with rAnhCAHN at 10 dpi. Brainstem. HE. There are scattered perivascular accumulations of lymphocytes and extensive gliosis. Bar = 100µm.

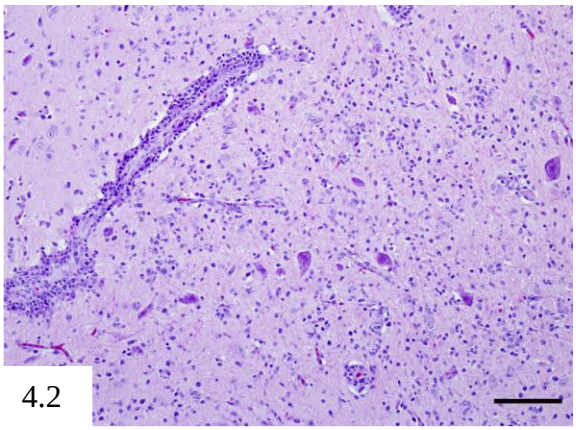
Figure 4.3.Chicken infected with rAnh at 10 dpi. Cerebellum. HE. Perivascular accumulations of lymphocytes and prominent small vessels are present in the molecular layer. Bar = 200µm.

Figure 4.4. Chicken infected with rAnhCAFHN at 10 dpi. Cerebellum. HE. Perivascular accumulation of lymphocyte, degenerating Purkinje cells, and prominent small vessels are present. Bar = 200µm.

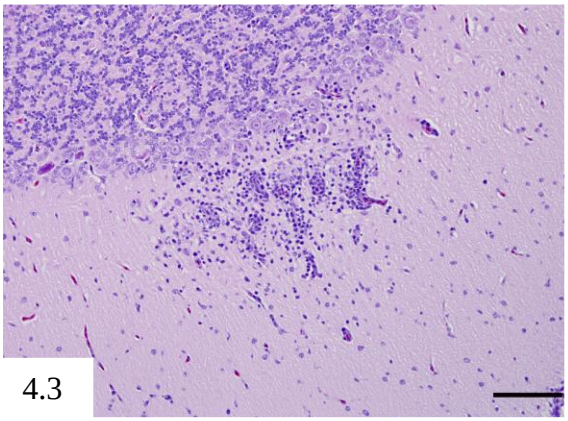
Figure 4.5. Chicken infected with rAnhCAFHN at 5 dpi. Eyelid. IHC. Scattered cells are immunolabeled for NDV nucleoprotein (red). Hematoxylin counterstain. Bar=100µm.

Figure 4.6. Seminested RT-PCR products to detect NDV matrix gene, RNA extracted from brain. Lane 1, 2 : negative controls; lane 3,4: rAnhCAFHN; lane 5,6: rAnh; lane 7,8: rAnhCAHN; lane 9,10: rAnhTKFHN; lane 11,12: rAnhTKHN. lane 13,14: positive controls.

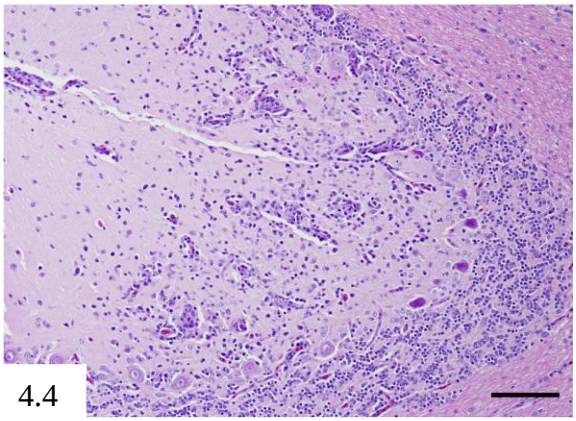
4.1



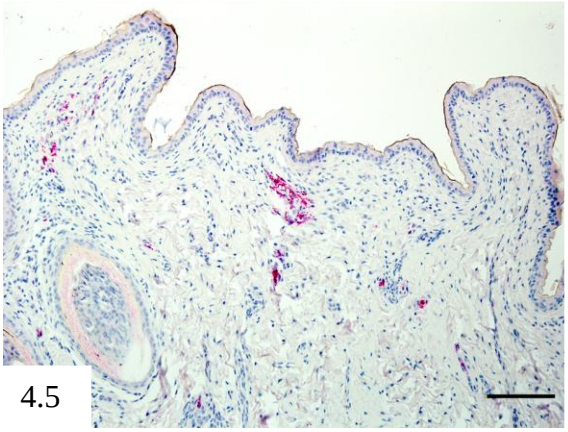
4.2



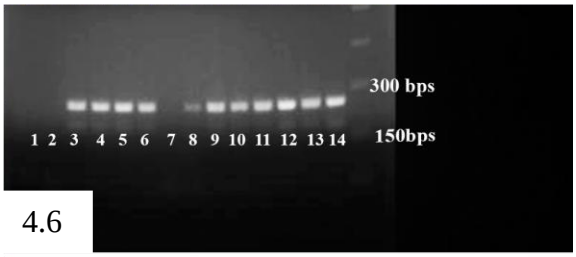
4.3



4.4



4.5



4.6

CHAPTER 5

INSERTION OF THE GREEN-FLUORESCENT PROTEIN (GFP) INTO THE BACKBONE OF A VELOGENIC VISCEROTROPIC NDV ALLOWS MANIFESTATION OF NEUROLOGICAL DISEASE*

*Susta L, Miller PJ, Hu S, Liu X, Rue CA, Afonso CL, Brown CC. 2011. To be submitted to *Avian Pathology*.

ABSTRACT

Reverse genetics was used to create two recombinant Newcastle disease viruses derived from a velogenic viscerotropic strain from China (wt-ZJ1). One of the recombinant viruses (rZJ1) was constructed to be identical to the wild type and the other had the gene for the green fluorescent protein (GFP) inserted between the M and P genes (rZJ1-GFP). Growth characteristics were assessed *in vitro* on chicken fibroblast cell line (DF-1) by multicycle growth curves and the ability to cause clinical disease was investigated *in vivo* by Mean Death Time (MDT), intracerebral pathogenicity index in one-day-old chickens (ICPI) and by detailed pathogenesis studies in 4-week-old naïve Leghorn chickens. The insertion of GFP into the rZJ1 genome caused a delay in viral replication *in vitro*, and slightly decreased pathogenicity, as revealed by MDT (69.6 hrs rZJ1 vs 72 hrs for rZJ1-GFP) and ICPI scores (1.89 rZJ1vs 1.62 rZJ1-GFP). In 4-week-old chickens, rZJ1 behaved as a typical velogenic viscerotropic strain; however, animals infected with rZJ1-GFP survived slightly longer and had severe neurological disease, with all infected animals having paresis and paralysis. The neurological signs observed in rZJ1-GFP were identical to those observed in typical velogenic neurotropic pathotypes. This report suggests that the capacity to cause neurological damage was present in the velogenic viscerotropic rZJ1, and that a typical neurotropic phenotype can be manifested upon attenuation.

Keywords: GFP; IHC; ISH; NDV; neuropathogenesis; recombinant viruses

INTRODUCTION

Newcastle disease (ND) is a disease of poultry worldwide caused by selected strains of Newcastle disease virus (NDV), and it is responsible for major panzootics, extensive poultry mortality, and economic losses due to international embargoes for animals and animal products (Office International des Epizootes, 2008). Newcastle Disease requires notification to the Office of International Epizooties (Office International des Epizootes, 2008).

Newcastle disease virus belongs to the Mononegavirales Order, Paramyxoviridae family, Paramyxovirinae subfamily and *Avulavirus* Genus. All the strains in this genus belong to a single serotype, avian paramyxovirus-1 (APMV-1).

NDV is a non-segmented, single-stranded, negative-sense RNA virus. The RNA genome is approximately 15,000 bases and is composed of 6 genes, in order from 3' to 5': nucleoprotein (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and large protein (L) (Alexander & Senne, 2008). The transcriptional activity of the RNA-dependent RNA polymerase of NDV (as of other viruses belonging to the same family) is polar and sequential, with the genes that are closer to the 3' being transcribed in larger amount than those close to the 5', following a gradient (Collins, *et al.* 1980).

The different isolates of Newcastle disease display a wide variety of virulence potential. Several methods have been adopted to classify the virulence of NDV strains, such as Mean Death Time (MDT) of embryonated eggs and Intracerebral Pathogenicity Index (ICPI), each of which have numerical scoring divisions for classification (Office International des Epizootes, 2008). Intracerebral pathogenicity index and the putative amino acid sequence of the fusion protein cleavage site have been adopted by the

international community as methods of classification (Office International des Epizootes, 2008). According to the OIE, notifiable strains are those viruses which have an ICPI equal or greater than 0.7 or those whose F cleavage site has at least 3 arginine or lysine residues between positions 113 and 116 and a phenylalanine residue at position 117 (Office International des Epizootes, 2008).

Based upon the clinicopathological syndrome elicited, NDV has been classified into 3 different pathogenicity classes, from the most to the least virulent, respectively: velogenic, mesogenic, and lentogenic. Lentogenic NDVcauses inapparent infection or minimal disease in young animals; mesogenic NDV has an intermediate virulence with neurological and respiratory signs; velogenic viruses cause high mortality and morbidity and have been further divided into viscerotropic (VVNDV) and neurotropic (VNNDV), according to whether they cause primarily visceral or primarily neurological disease (Alexander & Senne, 2008; Cattoli *et al.* , 2011)

The clinical characteristics of the neurological signs elicited by NDV and the respective lesions have been extensively characterized (Ecco *et al.* , 2010; Wilczynski *et al.* , 1977), however, little is known about the mechanism of neurovirulence in ND.

From the data in the literature, it appears that the NDV strains that are able to elicit neurological signs have an attenuated phenotype compared to velogenic viscerotropic strains. In other words, most of the neurotropic velogenic strains have ICPI values consistently lower than the viscerotropic strains (Alexander, 1998; Alexander & Senne, 2008; Brown *et al.* 1999; Kommers *et al.* , 2002; Kommers *et al.* , 2003a; Susta *et al.* , 2010; Susta *et al.* , 2011). In addition, neurological signs are a relatively late event in the progression of the disease, becoming evident 5 to 10 days post infection (d.p.i), in

contrast to the velogenic viscerotropic strains which, when inoculated in the same manner, usually cause death of the birds by 5 d.p.i, with severe systemic illness and marked depression (Brown, *et al.* , 1999; Kommers *et al.* , 2003a; Kommers *et al.* , 2003b; Susta *et al.* , 2010; Susta *et al.* , 2011).

Using immunohistochemistry (IHC) or *in situ* hybridization (ISH), in VVNDV, there is minimal presence of virus in the brain of birds at 4 d.p.i, suggesting that these strains can indeed invade the nervous tissue (Ecco *et al.* , 2010). Therefore, the ability to invade the central nervous system (CNS) seems to be an intrinsic characteristic of VVNDV, but CNS signs are not usually observed (other than depression) because the animals die with generalized systemic illness, most likely before neurological signs are fully developed.

In order to gain more insight into the neurovirulence of Newcastle disease, we conducted clinicopathological assessment in 4-week-old chickens inoculated with rZJ1 (a plasmid-rescued VVNDV) and rZJ1-GFP, the same virus as rZJ1 with the open reading frame (ORF) of the green fluorescent protein (GFP) inserted between the P and M genes (Liu *et al.* , 2007). Because the transcriptional activity of the RNA-dependent RNA polymerase of NDV is polar and sequential (genes that are closer to the 3' end being transcribed in larger amounts than those close to the 5' end) (Collins *et al.* , 1980), we expected the insertion of the GFP gene to cause viral attenuation by reducing the transcription of the downstream genes. In fact, decrease in replication efficiency has been observed in the Mononegavirales order, and also in NDV, when foreign genes have been inserted into the viral backbone (Bukreyev *et al.* , 1996; Krishnamurthy *et al.* , 2000). We hypothesized that this decrease in virulence might increase the ability of rZJ1-GFP to cause neurological signs, while losing some of its viscerotropic characteristics. The rZJ1

and rZJ1-GFP viruses are identical, with the exception of the insertion of the GFP ORF, therefore any alteration in virulence pathogenicity will be likely caused by the gene-insertion-derived attenuation, rather than by acquisition of a new function (Liu *et al.* , 2007). In fact, the green fluorescent protein has no known intrinsic influence on the virulence or pathogenesis, and has been widely used as a tool for tagging numerous viruses in both *in vivo* and *in vitro* systems (Agungpriyono *et al.* , 2000; Engel-Herbert *et al.* , 2003; He *et al.* , 1997; Kaku *et al.* , 2009; Liu *et al.* , 2008; Song *et al.* , 2009; Walsh *et al.* , 2000; Zhang *et al.* , 2005). Insertion of GFP ORF has been extensively used with lentogenic and mesogenic NDV backbones, and no major alterations in viral characteristics were reported (Engel-Herbert *et al.* , 2003; Kim & Samal, 2010; Romer-Oberdorfer *et al.* , 1999; Wong *et al.* , 2010), however, the effects induced by insertion of the GFP gene in a viscerotropic velogenic backbone has never been reported (Liu *et al.* , 2007).

In this paper, rZJ1 and rZJ1-GFP were characterized *in vivo* by means of MDT, ICPI and detailed pathogenesis study in 4-week-old chickens, as well as *in vitro* characterization through multicycle growth curves.

MATERIALS AND METHODS

Cells

DF-1 cells (Chicken embryo fibroblast cell line; ATCC CRL 12203) and HEp-2 (American Type Culture Collection, ATCC, Manassas, VA, CCL-23) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS).

Plasmids

The two plasmids containing the full-length clone (FLC) of rZJ1 and rZJ1-GFP were previously generated (Liu *et al.* , 2007):

rZJ1: contains the complete genome for ZJ1 (wt-ZJ1) circulating in China.

rZJ1-GFP: the same virus as rZJ1, with insertion of the GFP gene in the intergenic region between the P and M genes.

The inserted GFP gene is not continuous with the ORF of any flanking genes and therefore is produced independently of the other viral structural proteins.

Viruses and viral rescue

The modified Vaccinia virus Ankara recombinant expressing the T7 RNA polymerase (a generous gift of Bernard Moss, National Institute of Health) was grown in primary chicken embryo fibroblast cells and used to rescue the virus.

The chimeric viruses were recovered from the full-length plasmids as described previously (Estevez *et al.* , 2007). Briefly, in a six well plate, HEp-2 cells at 80-90% confluence were infected with MVA-T7 at a multiplicity of infection (MOI) of 3. After 1h of incubation at 37C, the *Vaccinia* inoculum was removed, and cells were transfected with 1µg of FLC, 0.75 µg of pNP (expression plasmid for nucleoprotein), 0.5 µg of pP (expression plasmid for Phosphoprotein), 0.1 µg of pL (expression plasmid for Polymerase).

LipofectamineTM (Invitrogen) was used for transfection according to the manufacturer's protocol. After 5 h, the supernatant was discarded and fresh DMEM containing 5% FBS was added. Supernatant and cells were collected after 72 h, frozen and thawed twice, and

gently centrifuged (1500g X 10 min) to separate cellular debris. The supernatant was inoculated into 9-day-old embryonating specific pathogen free chicken eggs. Eggs were chilled either upon death of the embryo or after 3 days; and allantoic fluid from eggs that had a positive HA titer, was re-inoculated into eggs to propagate the virus. Allantoic fluid from chilled eggs was harvested, centrifuged, and the supernatant used as virus stocks and stored at -80°C for future use. In order to confirm viral sequence and identity, RNA was extracted from the allantoic fluid of eggs during second passage and used for RT-PCR and sequencing, as previously described (Kim *et al.*, 2007).

Growth curve

The growth kinetics of rZJ1 and rZJ1-GFP were determined using a multicycle growth curves in DF-1 cell line. Cells were infected at a MOI of 0.01 and each time point was done in triplicate. Whole-cell lysates were collected at 6, 12, 24, 36 and 60 hours. The virus titers in the samples were quantified by plaque assay in DF-1 cells. Briefly, cleared whole-cell lysates collected from virus-infected DF-1 cell samples were serially diluted and each dilution was inoculated into 12-well plate DF-1 cells. After 1 h of virus adsorption, supernatants were removed from wells; wells were washed with PBS three times, and then overlaid with M199 supplemented with 0.9% bacto-agar. After 72 h, cells were fixed with 10% formalin and stained with crystal violet for enumeration of plaques.

Eggs and chickens

The source of embryonating chicken eggs and chickens was the SEPRL SPF White Leghorn flock. Birds were housed in negative pressure isolators under biosafety level (BSL)-3 enhanced conditions at SEPRL and provided food and water *ad libitum*.

Specific pathogen free embryonating eggs were inoculated for viral propagation, isolation, and titration (Alexander & Senne, 2008; Office International des Epizootes, 2008).

Pathogenicity index test (MDT and ICPI)

The MDT was performed by inoculating serial 10-fold dilutions of the rescued viruses stocks into 9-day-old embryonated chicken eggs, as previously reported (Alexander & Senne, 2008; Office International des Epizootes, 2008). The ICPI test was performed according to standard protocol (Alexander & Senne, 2008; Office International des Epizootes, 2008). Chickens were inoculated intracerebrally at one-day of age with 0.1 ml of a 1/10 dilution of infective allantoic fluid. Chicks were monitored daily and scored as normal, sick or paralyzed and dead to compile a score for an 8-days observation period. Calculation of the ICPI and MDT were performed as described elsewhere (Alexander & Senne, 2008; Office International des Epizootes, 2008).

Pathogenesis assessment in chickens

Two groups of ten 4-week-old SPF White Leghorn chickens were inoculated in the left conjunctival sac and the choanal slit with 0.1 ml of viral inoculum. Phosphate buffered saline (PBS) was used for the non-infected control birds. The target dose of inoculum was $10^{5.5}$ 50% embryo infectious doses (EID_{50}). The actual infectious dose as determined by back titration in embryonating chicken eggs were: $10^{5.7}$ EID_{50} for rZJ1 and $10^{5.5}$ EID_{50} for rZJ1-GFP. The birds were monitored clinically every day, and two birds of each group were euthanized after taking oropharyngeal and cloacal swabs at 2, 5 and

10 d.p.i. Birds whose condition became critical were swabbed and euthanized regardless of the scheduled sampling day.

Tissues (eyelid, spleen, bursa, thymus, Harderian gland, proventriculus, small intestine, cecal tonsils, large intestine, air sac, trachea, lung, heart, esophagus, pharynx, crop, brain, liver, pancreas, kidney, comb, head of left femur including bone marrow, and turbinate) were collected, and all tissue samples were fixed by immersion in 10% neutral buffered formalin for 52 h. The sections of femur and turbinate were decalcified in 5% formic acid for 3-4 h. All sampled tissues were routinely processed into paraffin, and 3 μm sections were cut for hematoxylin and eosin staining (HE), immunohistochemistry (IHC) and *in situ* hybridization (ISH).

Immunohistochemistry

All sampled tissues were examined by IHC to detect viral nucleoprotein. In addition, on consecutive selected sections from tissues of animals infected with rZJ1-GFP, IHC was performed for Green Fluorescent Protein (GFP). Briefly, after deparaffinization, tissue sections were subjected to antigen retrieval by microwaving for 20 min at minimum power in Vector antigen unmasking solution (Vector Laboratories, Burlingame, CA), followed by application of a universal blocking reagent (Biogenex, San Ramon, CA) as recommended by the manufacturer. The primary antibodies, both made in rabbit, were anti-peptide (nucleoprotein), used at 1:8000 dilution, and anti-GFP (Abcam antibody), used at 1:1000 dilution. The detection system was an alkaline phosphatase-labeled polymer (LabVision, Fremont, CA) or an avidin–biotin–alkaline phosphatase system (Vector Laboratories, Burlingame, CA). Chromogen was a naphthol-based dye

(Fast Red, Dako, Carpinteria, CA). Sections were counterstained lightly with hematoxylin and coverslipped with Permount for a permanent record.

In Situ Hybridization

Selected tissue sections (spleen, bursa, thymus, cecal tonsils and brain, of birds at 2, 3, 4 and 5 d.p.i from the NDV-infected birds were probed with a negative-sense digoxigenin-labeled 850 base riboprobe representing the 5' end of the M gene of NDV Fontana (CA1083), as previously described (Brown *et al.* , 1999). The M gene from the Fontana strain was cloned into pCRII transcription vector (Invitrogen, Carlsbad, CA) and anti-sense riboprobes were generated using RNA polymerase in the presence of labeled nucleotides. This probe was used to hybridize both the viruses in this study, since sequence information indicated a very high (92%) grade of identity with the probed strains (www.pubmed.org). Positive hybridization with antisense-riboprobe confirmed mRNA production and viral replication in tissues.

For hybridization, sections from selected cassettes were deparaffinized, rehydrated, digested with 100 µg/ ml Proteinase K for 15 min, and hybridized overnight at 42°C with approximately 20 ng of probe in 5X standard sodium citrate, 50% formamide, 5% blocking reagent (Boehringer Mannheim, Indianapolis, IN), 1% *N*-lauroylsarcosine, and 0.02% sodium dodecyl sulfate. Following stringent washes, bound probes were visualized by the addition of anti-digoxigenin alkaline phosphatase and the chromogen/substrate nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate.

Virus Isolation and Titration of swabs

For those birds used in the pathogenesis assessment experiment, immediately prior to euthanasia, oral and cloacal swabs were obtained from each bird and placed in separate tubes containing 1.5 ml of brain-heart infusion broth (BHI) with antibiotics (2000 U penicillin G/ ml, 200 µg gentamicin sulfate/ ml, 4 µg amphotericin B/ ml; Sigma Chemical Co., St. Louis, MO). Swab sample tubes were centrifuged at 1000 × g for 20 min, and the supernatant removed for virus isolation and titration. Virus infectivity titers were calculated from the result of inoculation of 9 to 10 day-old SPF embryonating chicken eggs with serial 10-fold dilutions in BHI containing antibiotics (100 U / ml penicillin G and 50 µg / ml gentamicin sulfate, 4 µg amphotericin B/ ml; Sigma Chemical Co., St. Louis, MO). NDV in infected dead or surviving embryos were identified by hemagglutination (HA) activity in amnioallantoic fluid harvested from chilled eggs. NDV was confirmed in HA positive samples by hemagglutination inhibition (HI) test with NDV-specific antiserum.

RESULTS

Virus rescue

Virus was rescued from the recombinant plasmid and propagated by two passages into eggs. Identity of the two viruses was confirmed by sequence of the RT-PCR of the positions 2858-3676 of the ZJ1 genome. Allantoic fluid from eggs infected with both viruses was HA positive.

Growth curve

The growth characteristics of rZJ1 and rZJ1-GFP viruses were assessed by multicycle growth curves in DF-1 cells (figure 5.1). rZJ1 grew at 1-log higher titers than ZJ1-GFP, starting at 36h. A delay in growth of rZJ1-GFP was observed early during infection, and was maintained for up to 60 hours (end of experiment), with final titers of 3.67×10^7 PFU / ml (ZJ1-GFP) and 2.67×10^8 PFU / ml (ZJ1), indicating that, overall, the ZJ1-GFP has a reduced capacity to replicate in DF-1 cells.

Pathogenicity index test (MDT and ICPI)

The MDT for rZJ1 and rZJ1-GFP were 69.6 h and 72 h, respectively.

The ICPI for rZJ1 and rZJ1-GFP were 1.87 and 1.64, respectively.

Pathogenicity assessment in chickens

Results for observed clinical signs and gross lesions are presented in table 5.1. All of the birds infected with rZJ1 and rZJ1-GFP strains developed clinical disease. Birds inoculated with rZJ1 had severe acute illness, characterized by marked depression and open-mouthed breathing by 2 d.p.i. At 3 d.p.i, there was severe depression and reluctance to move. All animals died or were euthanized *in extremis* by 4 d.p.i.

Birds inoculated with rZJ1-GFP showed conjunctivitis and depression starting at 2 d.p.i. Depression and severe neurological signs (head twitch) were observed in three birds starting at 4 d.p.i. By 5 d.p.i all the remaining birds had very severe neurological signs, consisting of head twitch, paralysis, wing drop and ataxia (figure 5.2). All animals were euthanized at 5 d.p.i.

Gross Findings

Gross findings are presented in table 5.1. By 2 d.p.i, the birds infected with rZJ1 had severe edema and petechial hemorrhages of the eyelid, mottled spleen and edema of the pancreas. On day 3, the spleens were uniformly enlarged and had multifocal to coalescing white stippling (necrosis), apparent on both capsular and cut surfaces, thymic hemorrhage, proventricular hemorrhages, necrotic plugs in the ceca, and edema, necrosis and hemorrhage of the cecal tonsils (figure 5.3). The birds infected with rZJ1-GFP, showed conjunctivitis starting at 2 d.p.i, intestinal hemorrhages, thymic atrophy and enlarged and mottled spleen by 5 d.p.i.

Histopathology

Histological findings are presented in table 5.2. The most severe lesions were observed with rZJ1, starting by day 2 and increasing in severity through day 4. These were mainly confined to the site of inoculation (eyelid), the lymphoid organs (spleen, thymus, bursa of Fabricius) or the lymphoid aggregates of the intestines. In the eyelid, the lesions were characterized by severe edema, hemorrhages, multifocal areas of necrosis associated with fibrin exudation and pleomorphic inflammatory infiltrate, mainly composed of heterophils and macrophages. In the lymphoid organs (thymus, bursa, spleen, and intestinal lymphoid patches, especially cecal tonsils), the lesions consisted of severe necrosis, marked lymphocyte depletion, infiltration of numerous macrophages, and moderate heterophilic infiltrate. In the intestines, the severe necrosis of the lymphoid-dependent areas was associated with focal to locally extensive ulceration of the epithelium and accumulation of necrotic material within the intestinal lumen. Multifocal areas of necrosis were observed within the exocrine pancreas. In the bone marrow, at 3

d.p.i there were multifocal areas of necrosis, especially in the areas beneath the articular cartilage.

Birds infected with rZJ1-GFP had lesions comparable to those observed with rZJ1. Lymphoid organs were the most affected and the severity of lesions was similar to those induced by rZJ1, but appeared slightly later in the time-course of the disease. Lesions were most severe at 5 d.p.i. In the brain, birds at 5 d.p.i showed few vessels with plumped endothelium, perivascular edema and accumulation of scattered lymphocytes in the Virchow-Robins spaces (figure 5.4). In one bird, there was multifocal accumulation of lymphocytes within the myocardium. Necrotizing pancreatitis and submucosal proventricular hemorrhages were a common histological finding in both strains.

Immunohistochemistry (IHC*)*

Immunohistochemical findings are presented in table 5.2. Using immunohistochemistry for NDV nucleoprotein, birds infected with rZJ1 had the broadest viral distribution (24/24 positive tissues), associated with the most intense reactivity for NDV, between day 3 and 4, reaching a peak of intensity at 4 d.p.i. The tissues with the strongest signal were the eyelids (site of inoculation), the lymphoid organs (thymus, spleen, bursa) (figure 5.5) and the mucosa-associated lymphoid aggregates in the intestine and lungs. Immunolabeled cells consisted mainly of lymphocytes and macrophages. In the spleen, the immunoreactivity was mainly confined to those areas surrounding the penicillary arteries and in cells consistent with macrophages, while the lymphocyte-dependent areas were negative. In the respiratory system, the signal was minimal and confined to the nasal and tracheal mucosa, in scattered lymphoid aggregates

closely associated with the secondary and tertiary bronchi, and to the squamous epithelium of the air capillaries and air sacs. In the digestive tract, intense positivity for NDV was observed only within the lymphoid aggregates of the lamina propria, and no signal was observed associated with the epithelial lining. Scattered Kupffer cells in the liver and several epithelial cells in the distal tubules of the kidneys were immunoreactive for NDV. In the bone marrow, numerous osteoclasts, mononuclear cells just beneath the growth plate or surrounding areas of necrosis showed intense immunolabeling. In the heart, the signal was observed within scattered cardiac myocytes.

In comparison to rZJ1, birds infected with rZJ1-GFP isolate had a diminished viral distribution (18/24 positive organs), but the type of immunolabeled tissues and cells was very similar to that of rZJ1. In addition, in the lymphoid organs, and especially within the spleen, the signal was less intense when compared to rZJ1, whereas in some other organs, such as the trachea, air sacs and kidney the signal was more intense at 5 d.p.i. In animals infected with rZJ1-GFP at 5 d.p.i, but not with rZJ1, immunolabeling was also extensively present within the submucosal and myenteric plexuses of the intestine and esophagus, in cells consistent with neurons and glial cells (figure 5.6), and in scattered, extra intestinal nerves.

To confirm that GFP was produced by the inoculated rZJ1-GFP construct, consecutive sections of tissues that were positive for NDV protein were subjected to IHC for GFP. Immunoreactivity for GFP was finely granular and labeling was in the same areas that were positive for NDV nucleoprotein IHC (figures 5.8 and 5.9).

In situ Hybridization

In selected sections (spleen, bursa, thymus, cecal tonsils and brain, of birds at 2, 3, 4 and 5 d.p.i) the negative sense riboprobe for NDV matrix gene labeled the same structures and cells immunolabeled with IHC. The signal was cytoplasmic and evenly diffuse. With ISH, the submucosal and myenteric plexuses were positive as well (figure 5.7).

Virus isolation and titration of swabs

Results of titration of the swabs are presented in table 5.3. Virus was isolated from all the birds inoculated with rZJ1 and rZJ1-GFP starting from 2 d.p.i. At this time point, the birds infected with both rZJ1 and rZJ1-GFP had positive oral and cloacal swabs. By day 3, for rZJ1 infected birds and by day 5 for rZJ1-GFP infected birds, high titers of virus could be isolated from all of the swabs.

DISCUSSION

Birds inoculated with rZJ1 displayed severe clinicopathological disease, with all birds dying by day 4. Based on numerous pathogenesis studies done in our laboratory in a consistent manner with multiple strains, death of all birds by 4 d.p.i indicates a strain of considerable virulence, and is comparable to many other velogenic viscerotropic NDV isolates (Brown *et al.* , 1999; Kommers *et al.* , 2003b; Kommers *et al.* , 2002; Wakamatsu *et al.* , 2006). It is reasonable to believe that rZJ1 is very similar in pathogenesis to the parent ZJ1, and that the rescue system probably did not affect the overall fitness of the virus. Histologically and by IHC, rZJ1 behaved as a typical highly virulent strain (Brown *et al.* , 1999; Kommers *et al.* , 2001; Kommers *et al.* , 2003a, 2003b; Wakamatsu *et al.* ,

2006), with extensive necrosis of lymphoid tissues, especially those of the spleen and intestine, and widespread viral replication. Overall, the rZJ1 virus was positive in 24 of 24 tissues examined by IHC, indicating a marked systemic pantropism, as is typical of the viruses in this highly virulent group.

The infectious clone rZJ1-GFP was also pathogenic in chickens and displayed a velogenic phenotype with wide viral distribution, as detected by IHC (18/24 tissues). However, in comparison to rZJ1, rZJ1-GFP showed moderate attenuation in 4-week-old chickens, in that animals survived slightly longer (one day). Besides, chickens infected with rZJ1-GFP developed severe neurological signs that were not observed with the rZJ1 lacking the GFP insert; and that are similar to what could be expected with velogenic neurotropic viruses (Brown *et al.* , 1999; Ecco *et al.* , 2010). Nervous signs consisted of paralysis, paresis, head-twitching and tremors, indicating involvement of the brain and possibly of the spinal cord. In the animals infected with rZJ1-GFP, neurological dysfunction started at 4 d.p.i and peaked at 5 d.p.i, day of euthanasia, and this timeline is in partial agreement with what previously described for other VNNDVs (Brown *et al.* , 1999; Ecco *et al.* , 2010). However, even when neurological signs are present as early as 5 d.p.i, animals inoculated with typical neurotropic strains of NDV usually die or are euthanized *in extremis* at 9 or 10 d.p.i, which are later time points than what was observed for rZJ1-GFP (5 d.p.i) (Brown *et al.* , 1999; Kommers *et al.* , 2002; Susta *et al.* , 2010). This suggests that ZJ1-GFP maintained some characteristics of the VVNDVs, which usually kill the animals within four to five days post inoculation (Brown *et al.* , 1999; Kommers *et al.* , 2003a; Susta *et al.* , 2010).

Mild lesions were observed in the brain of birds infected with rZJ1-GFP and no immunohistochemical staining was observed. A recent retrospective study of NDV-induced encephalitidies demonstrated that lesions have their highest intensity between 5 and 10 d.p.i, with lesions caused by mesogenic strains shifted toward 9 and 10 d.p.i. The fact that all the animals infected with rZJ1-GFP were euthanized at 5 d.p.i (the lower limit of the usual time frame) might have limited the possibility to observe more severe lesions and/or IHC staining in the brain. Nevertheless, there was evidence that rZJ1-GFP was infective for neurologic tissue as demonstrated by the intense NDV immunohistochemical staining in the intestinal plexuses of all animals, at 5 d.p.i. NDV in the intestinal plexuses has been reported very rarely (Nakamura *et al.* , 2008; Susta *et al.* , 2010).

Lack of immunohistochemical staining in the brain and the mild encephalitis, in the face of such overt neurological signs, could be explained by the fact that a minimal viral load could cause neurological dysfunction, without eliciting severe encephalitis. Presence of encephalitis without immunohistochemical staining in the brain of NDV-infected animals has been previously described (Susta *et al*, 2010; Susta *et al.* , 2011), and even when histopathological lesions were severe, immunohistochemical staining was often patchy and not necessarily associated with the areas of encephalitis (Ecco *et al.* , 2010).

In addition, paralysis and paresis could have been caused by lesions in the spinal cord (upper and lower motor neuron disease), but unfortunately sampling of the spinal cord was not included in our experimental protocol and so cannot be ruled out. In this context, it could be possible that the virus might have travelled in a centripetal fashion from the

intestinal ganglia, where IHC staining was observed at highest intensity, to the peripheral nerves, spinal cord and finally the brain. This explanation would consider involvement of the brain a later event, thus accounting for the negative IHC results on the brains of rZJ1-GFP animals, all of which were euthanized *in extremis* by 5 d.p.i (early time point). Despite its neurological properties, rZJ1-GFP maintained the ability to cause severe lesions comparable to rZJ1 or other velogenic viscerotropic NDV, and definitely more severe than what could be expected with other velogenic neurotropic or mesogenic NDVs (all able to cause neurological signs) (Brown *et al.* , 1999; Ecco *et al.* , 2010; Kommers *et al.* , 2003a; Susta *et al.* , 2010; Wakamatsu *et al.* , 2006).

The differences between the abilities of rZJ1 and rZJ1-GFP to induce disease are also reflected by the differences in ICPI and MDT scores (1.64 vs. 1.87; 69.6 hrs and 72 hrs) and most likely are caused by the insertion of the GFP gene. This has been also confirmed by the growth curve kinetics, which confirm that rZJ1-GFP has a lower rate of growth compared to rZJ1. It has been shown by other researchers that insertion of a foreign gene into the backbone of mononegavirales generally leads to some attenuation (Bukreyev *et al.* , 1996; Hasan *et al.* , 1997; Sakai *et al.* , 1999). In this case, proposed reasons for the attenuation could be the length of the inserted gene and the position of foreign gene in the genome. The replication machinery of NDV, as of that of other viruses in the same order, is polar and obligatorily sequential (Whelan *et al.* , 2004). This means that genes at the 3’ end of the genomic viral RNA are more transcribed than those at the 5’ end, following a gradient. The GFP was located between the phosphoprotein and matrix genes, meaning that it was inserted between genes number 2 and 3 in order of transcription. This would certainly further decrease transcription of the downstream

genes, causing a potentially compromised ratio between the viral structural proteins which would in turn limit viral replication and fitness. At least for Sendai virus, this decrease in fitness has been demonstrated to be proportional to the length of the insert (Sakai *et al.* , 1999). Evidently, also the position of the gene counts, since depending on the insertion site, only some genes have an impaired transcription. However, regardless of the attenuation, there was still abundant evidence of replication in multiple tissues, with positive IHC and ISH signal, similar to those observed with highly virulent ND (Brown *et al.* , 1999; Kommers *et al.* , 2003a, 2003b; Kommers *et al.* , 2002; Wakamatsu *et al.* , 2006). Moreover, positive immunohistochemistry for GFP revealed that this gene was successfully expressed by the virus.

These results seem to agree with the fact that strains able to elicit neurological disease (velogenic neurotropic and mesogenic) usually have lower ICPI and virulence than the velogenic viscerotropic strains: in the present work, a neurotropic behavior has been elicited when a decrease in viral replication and ICPI score was induced by insertion of the GFP gene.

It seems that somehow the ability to cause neurological disease is already potentially present within the VVNDVs, but can be observed only when there is some attenuation. Why a decrease in virulence determines a more intense neurological syndrome is open to debate, however, a longer course of the disease, as observed with velogenic neurotropic and mesogenic, could enable the neurotropic symptoms to express completely and cause disease. In fact, the strains that induce neurological symptoms often show encephalitic lesions at later time points during the course of the disease, 5 to 10 d.p.i, while velogenic viscerotropic cause death of infected animals within 4 d.p.i (Brown

et al. , 1999; Ecco *et al.* , 2010; Kommers *et al.* , 2003a; Kommers *et al.* , 2002; Susta *et al.* , 2010).

In summary, this paper describes for the first time the change of velogenic viscerotropic NDV phenotype to a velogenic neurotropic. Furthermore the change was caused by the insertion of a gene that is expected not to have any other effect in pathogenicity other than decreased levels of gene expression in gene located downstream. Reduced virus replication observed in vitro and in vivo in the GFP viruses, and full manifestation of the neurogenic pathotype supports the idea that expression of the neurogenic phenotype is likely and intrinsic property of the velogenic viruses that was manifested only with a reduced capacity of the virus to replicate.

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Table 5.1. Clinical (clin) and pathological (path) findings of infected animals.

	2 d.p.i		3 d.p.i		4 d.p.i		5 d.p.i	
Strain inoculated	**Clin**	**Path**	**Clin**	**Path**	**Clin**	**Path**	**Clin**	**Path**
PBS	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
rZJ1 (5.7)[a]	Depression.	Severe conjunctivitis (2/2).[b] Spleen-Mot (1/2). Pancreas, cecal tonsils, thymus-Ed (1/2).	Severe depression. Two animals with open mouth breathing (2/4).	Eyelid-Ed+Hem (1/4). Spleen-Nec(3/4). Proventriculus-Hem (4/4). Intestine-Hem+Nec (2/4).	1 animal died spontaneously, 3 very depressed and non-responsive.	Eyelid-hem(1/3). Proventriculus-Hem(1/3). Intestinal hem (3/3). Spleen-Nec (2/3).	No survivors at this time point.	No sample collected.
rZJ1-GFP (5.5)	Normal.	Conjunctivitis.	Ruffled plumage.	Conjunctivitis.	Depression. 3 animals with head twitch	No animals sampled for necropsy	Depression. 5 animals head twitch. 5 animals paralysis.	Thymic atrophy (2/3). Spleen-Nec (1/3). Intestinal-hem (1/3).

d.p.i: days post inoculation

a: in parenthesis is the viral titer per 0.1 ml of the inoculum (back-titration)

b: in parenthesis is the number of chickens with the described lesions over the total of sampled animals for that time point

Ed: edema

Hem: hemorrhage

Mot: mottled

Nec: necrosis

Table 5.2. Distribution and intensity of the lesions and NDV immunolabelling

		ZJ1			ZJ1-GFP	
Organs	D.P.I[†]	2	3	4	2	5
Eyelid	HE[a]	++	+++	+++	++	+++
	IHC[b]	++	++++	++++	-	++
Spleen	HE	+	+++	+++	+	+++
	IHC	+	++	+++	-	++
Thymus	HE	+	++	+++	-	+++
	IHC	++	++++	++++	-	+++
Bursa	HE	-	+	++	-	+++
	IHC	-	++	+++	-	++
Harderian G	HE	-	-	-	-	-
	IHC	-	+	++	-	-
Proventriculus	HE	-	++	+++	-	+++
	IHC	+	++	++	-	+
Small Intestine	HE	-	-	+++	-	++
	IHC	+	-	++	-	+/-
Cecal Tonsils	HE	+/-	++	+++	+	+++
	IHC	+	+++	++++	-	++
Large Intestine	HE	-	-	-	-	+++
	IHC	-	++	+++	-	+
Pancreas	HE	-	+	+	-	++
	IHC	-	+	++	-	+
Air sacs	HE	-	-	-	-	-
	IHC	-	-	++	-	+
Trachea	HE	-	-	-	-	-
	IHC	+/-	-	+	-	+
Lung	HE	-	-	-	-	-
	IHC	-	-	+	-	+
Heart	HE	-	-	-	-	+
	IHC	-	++	++	-	-
Esophagus	HE	-	-	-	-	-
	IHC	-	-	+	-	++
Tongue	HE	-	-	-	-	-
	IHC	+	-	+	-	-
Pharynx	HE	-	++	-	-	-
	IHC	++	++	++	-	++
Crop	HE	-	-	-	-	-
	IHC	-	-	-	-	+
Brain	HE	-	-	-	-	++
	IHC	-	-	+	-	-
Liver	HE	-	-	-	-	-
	IHC	+	+	+	-	-
Kidney	HE	-	-	-	-	-
	IHC	-	-	+	-	++
Comb	HE	-	-	-	-	-
	IHC	-	+	-	-	-
Femur	HE	-	+	++	-	+
	IHC	+	+++	++	-	+
Turbinates	HE	-	-	-	-	-
	IHC	++	+++	++	-	+/-

Histological grading:

Spleen: +Moderate hyperplasia; ++lymphocytic depletion; +++moderate (<50%) lymphocyte depletion, histiocytic accumulation and multifocal necrosis; ++++ (>50%) severe lymphocytic depletion, histiocytosis and necrosis.

Thymus, Cecal tonsil, GALT, Bursa and Thymus: +mild lymphocytic depletion, ++ (<50%) moderate lymphocytic depletion with necrosis and histiocytosis, +++ (>50%) severe lymphocytic depletion, necrosis and histiocytosis

Bone marrow: mild + (<20%) bone marrow necrosis; ++ mild (20-50%) bone marrow necrosis; +++ severe (>50%) bone marrow necrosis.

Pancreas: + mild (<3 areas) vacuolation and degeneration; ++ moderate (>3 areas) vacuolation and degeneration.

Brain: + vascular reactivity; ++ vascular reactivity and perivascular cuffing; +++vascular reactivity, perivascular cuffing, gliosis.

Immunohistochemical grading:

- = no IHC signal present

+/-=very rare positive cells are observed

+ = rare cells in the section are positive on IHC

++ = positive cells seen, <50% of all high power fields (HPF)

+++ = positive signal seen in 50 to 75% of HPF

++++ = abundant positive signal in more than 75% of HPF

[†]Day Post Infection

[a]Hematoxylin and Eosin

[b]Immunohistochemistry

Table 5.3. Virus isolation and viral titer (EID_{50}) of oral (o) and cloacal (c) swabs per 0.1 ml.

	PBS		ZJ1 (5.7)[a]		ZJ1-GFP (5.5)	
D.p.i[b]	***O***	***C***	***O***	***C***	***O***	***C***
2	-[d]	-[e]	$>10^{3.5}$(2/2)[c]	$10^{1.7}$(1/2)	$10^{3.3}$(1/2)	$10^{0.9}$(1/2)
				$10^{2.9}$(1/2)	$10^{1.9}$(1/2)	$10^{0.7}$(1/2)
3	nc[e]	nc	$>10^{3.5}$(4/4)	$>10^{3.5}$(4/4)	nc	nc
4	nc	nc	$>10^{3.5}$(3/3)	$>10^{3.5}$(3/3)	nc	nc
5	-	-	nc	nc	$10^{5.5}$(1/3)	$10^{4.5}$(1/3)
					$10^{5.7}$(1/3)	$10^{5.3}$(1/3)
					$10^{4.5}$(1/3)	$10^{4.5}$(1/3)

[a] Number in parentheses is the viral titer (EID_{50}) of inoculum per 0.1 ml.

[b] (d.p.i): Days post inoculation.

[c] Number of birds displaying that value over the total swabbed animals.

[d] No virus recovered from undiluted swab fluids inoculated into three eggs, 0.2 ml per egg.

[e] Not collected

EID_{50}: mean embryo infectious dose

Figure 5.1. Multicycle growth curve of rZJ1 and rZJ1-GFP in DF-1 cells. MOI= 0.01. Error bars indicate standard error.

Figure 5.2. 4-week-old chicken, experimentally infected with rZJ1-GFP strain. 5 d.p.i. Neurological signs consists of wing droop and inability to stand.

Figure 5.3. Intestine; 4-week-old chicken, experimentally infected with rZJ1 strain. 4 d.p.i. Multifocal hemorrhages within the small intestine and necrotic foci in the cecal tonsils.

Figure 5.4. Bursa; 4-week-old chicken, experimentally infected with rZJ1 strain. 3 d.p.i. NDV nucleoprotein is present in scattered follicles. IHC, alkaline phosphatase method, hematoxylin counterstain.

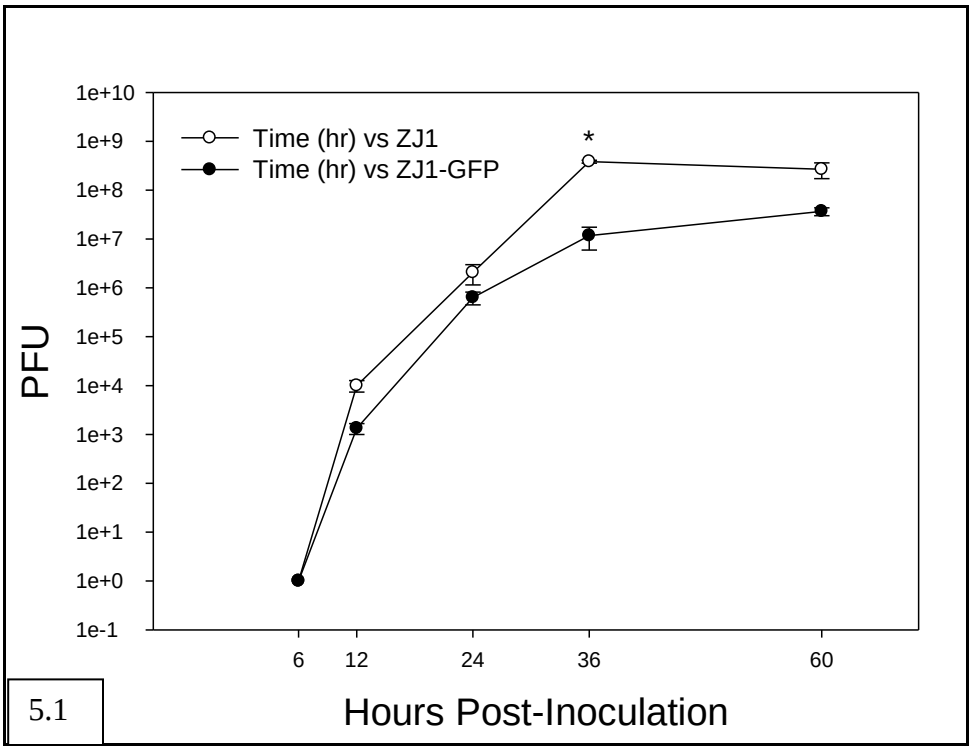
Figure 5.5. Cerebellum; 4-week-old-chicken, experimentally infected with rZJ1-GFP. 5 d.p.i. Scattered vessels have plumped endothelium and perivascular lymphohistiocytic cuffings. HE.

Figure 5.6. Intestine; 4-week-old chicken, experimentally infected with rZJ1-GFP strain. 5 d.p.i. NDV nucleoprotein is present within neurons and glial cell in submucosal plexuses. IHC, alkaline phosphatase method, hematoxylin counterstain.

Figure 5.7. Intestine; 4-week-old chicken, experimentally infected with rZJ1-GFP strain. 5 d.p.i. Consecutive section of figure 6 is represented. Riboprobe for NDV nucleoprotein labels the same structures (neurons and glial cells of submucosal plexuses) highlighted by IHC in figure 5.6. ISH, alkaline phosphatase method, hematoxylin counterstain.

Figure 5.8. Cecal Tonsil; 4-week-old chicken, experimentally infected with rZJ1-GFP strain. 5 d.p.i. NDV nucleoprotein labels numerous areas within the cecal tonsils surrounding the secondary follicles. IHC, alkaline phosphatase method, hematoxylin counterstain.

Figure 5.9. Cecal tonsils; 4-week-old chicken, experimentally infected with rZJ1-GFP strain. 5 d.p.i. IHC for GFP labels areas similar to those observed in figure 5.8. IHC, alkaline phosphatase method, hematoxylin counterstain.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1e+6
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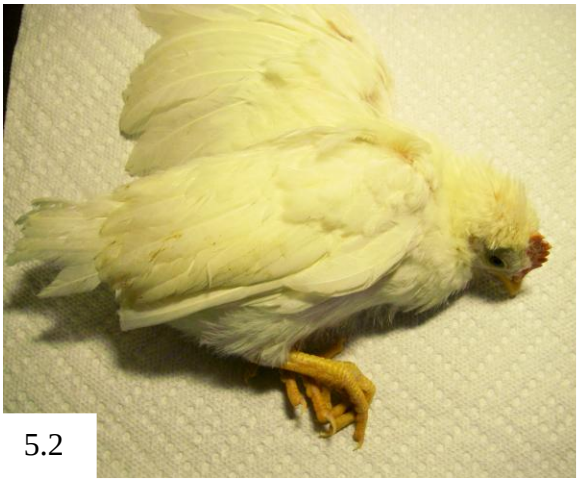
1e+4
1e+3
1e+2
1e+1
1e+0
1e-1
PFU
Time (hr) vs ZJ1
Time (hr) vs ZJ1-GFP
*
6
12
24
36
60
Hours Post-Inoculation

5.1



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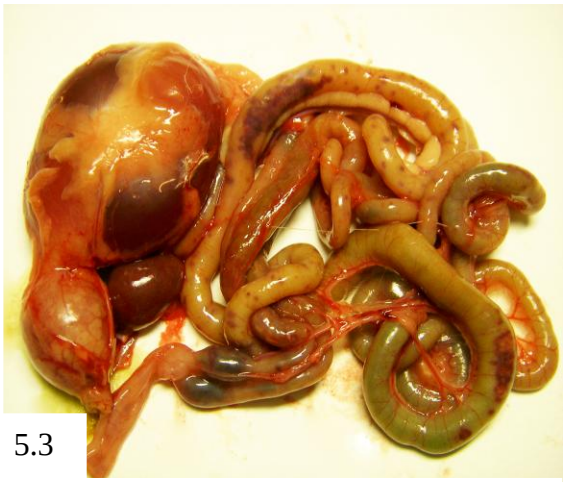

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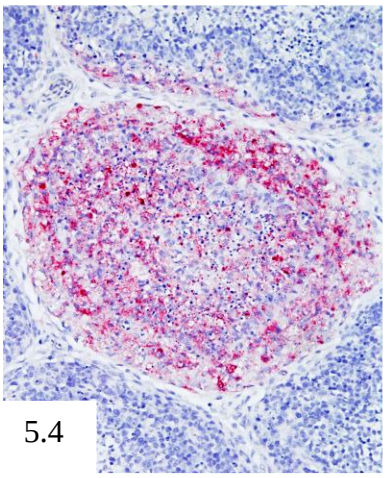

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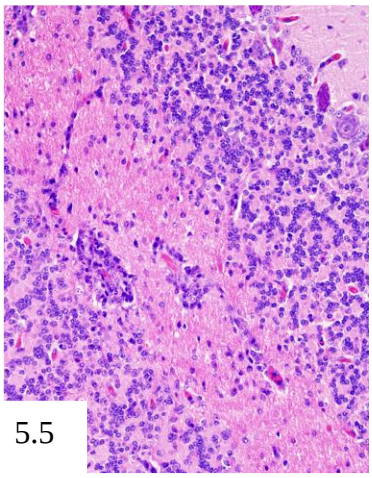

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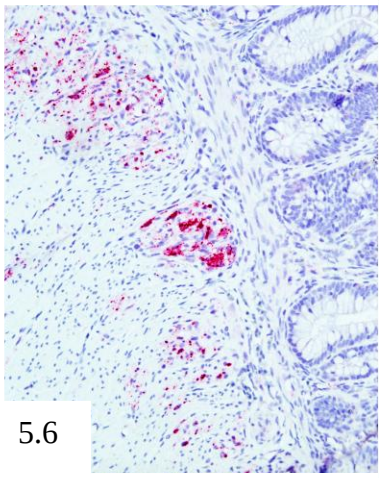

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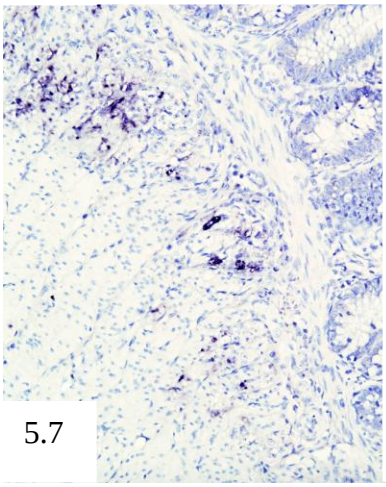

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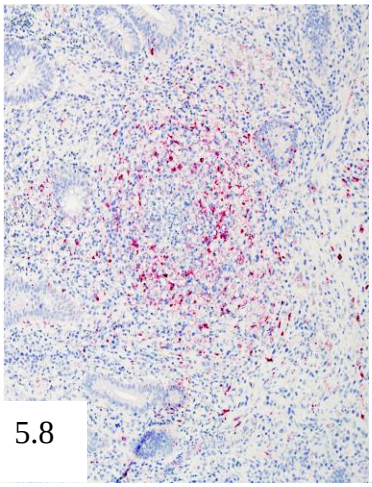
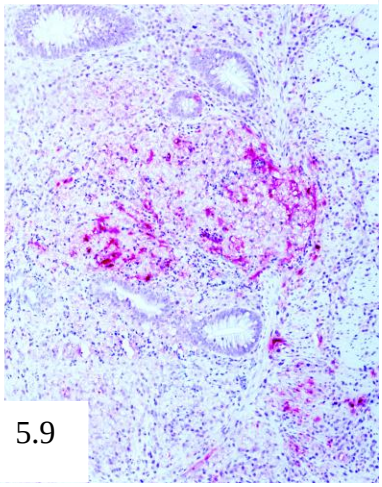


5.8



5.9

CHAPTER 6

CONSTRUCTION OF RECOMBINANT NEWCASTLE DISEASE VIRUS EXPRESSING CHICKEN IFN-γ: EFFECT OF ELEVATED LEVELS OF EXPRESSION ON THE TRANSCRIPTIONAL HOST RESPONSE, VIRAL REPLICATION AND PATHOGENESIS*

*Susta L, Cornax I, Cardenas S, Miller PJ, Brown CC, Afonso CL. 2011. To be submitted to *Journal of Virology*.

ABSTRACT

Newcastle disease (ND) is a severe disease of poultry and other avian species, characterized by high morbidity and mortality. It is caused by virulent strains of Newcastle disease virus (NDV), part of the Mononegavirales class, Paramyxoviride family, Avulavirus genus. Interferon-gamma (IFN-γ) is a cytokine that has pleiotropic biological effects including intrinsic antiviral activity, induction of innate immunity, as well as stimulation and regulation of immune responses. Here we studied the effect of IFN-γ expression on NDV pathogenesis by inserting the open reading frame (ORF) of chicken IFN-γ within the backbone of a virulent strain of NDV (ZJ1). The resulting recombinant virus (rZJ1-IFNγ) was characterized by single step growth curve, ICPI and inoculation into 4-week-old chickens and was compared with the same virus with inserted the green fluorescent protein (GFP) gene at the same position of IFNγ (rZJ1-GFP), and its revertant, rZJ1-Rev (the same virus with elimination of the IFN-γ ORF). rZJ1-IFNγ was able to express biologically active IFN-γ, and the insert did not alter its growth kinetics in chicken fibroblast cell line. rZJ1-IFNγ had markedly decreased virulence, as shown by ICPI value, compared to rZJ1-GFP and rZJ1-Rev. In 4-week-old chickens infected with rZJ1-IFNγ, there was dramatic increase in survivability (compared to the two controls), but the ability to cause lesions was partially maintained, especially in the central nervous system. The data suggest that IFN-γ expressed during viral replication can markedly decrease the severity of the damage caused by virulent NDV, and that NDV can be used as a system to deliver cytokines in the avian system.

INTRODUCTION

Newcastle disease (ND), caused by virulent strains of ND virus (NDV) is a widespread and economically significant disease of poultry (2, 4). The virus is classified in the Order Mononegavirales, family Paramyxoviridae, subfamily Paramyxovirinae, genus Avulavirus and within that genus, all strains regardless of virulence comprise avian paramyxovirus type 1 (APMV-1). NDV is a non-segmented, single-stranded, negative sense RNA virus. The RNA genome is approximately 15,000 bases and it is composed of six genes encoding for six structural proteins, in order from 3' to 5': nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and large protein (L) (2).

The severity of clinical and pathological signs elicited by different NDV strains varies with host susceptibility, and the intrinsic virulence of the each viral strain (5, 7, 19). Clinically, virus strains have been classified into highly virulent (velogenic) NDV, moderately virulent (mesogenic) NDV, and low virulent (lentogenic) NDV (2). The intracerebral pathogenicity index (ICPI) in 1-day-old chicks is the internationally recognized standard system for pathotyping and consists of inoculating one-day-old chicks intracerebrally and scoring birds as normal, sick, or dead over a period of 8 days, to result in a score from 0.0 to 2.0. Those strains scoring $\geq$ 0.7 are considered "notifiable" to the international community through the World Organization for Animal Health (formerly known as the Office International des Epizooties and still recognized by the designated abbreviation, OIE) (27). Presence of a notifiable strain within a country has serious negative implications for trade and therefore is damaging to the economic viability of the poultry industry (27).

The amino acid sequence at the fusion protein (F) cleavage site has been regarded to be a primary determinant of NDV virulence (14, 24) and the presence of multiple basic amino acids and phenylalanine at that site is another of the criteria recognized by the OIE to classify an NDV isolate as a notifiable agent. However, many strains (especially those defined as mesogens) with a virulent fusion cleavage site have variable disease-inducing ability when characterized in animal experiments conducted with specific pathogen free (SPF) chickens (7, 18, 35); thus indicating that the virulent phenotype is the complex result derived from the contribution of several viral genes at the same time. Also the V protein has been considered as an important factor for NDV pathogenesis: it is a non-structural protein, produced by post-transcriptional mRNA editing of the P gene, and it has IFN-α antagonistic activity through degradation of the STAT1 via the proteasome pathway (25). When the coding region of the P gene was modified to inhibit the production of the V protein (an IFN-α antagonist), the recombinant NDV had marked attenuated virulence both in vivo and in vitro (38).

Few data are available on the innate immune response and early pathogenesis of NDV. Peripheral blood monocytes from chickens selected for type IV hypersensistivity showed marked upregulation of IFN-γ mRNA and increased nitric oxide (NO) production when infected with velogenic NDV at 0.1 multiplicity of infection (MOI). However, in this work no pathogenesis experiments were attempted (1). As shown by Rue *et al.* (31) with qRT-PCR, IFN-γ, IFN-α/β and IL-6 mRNA were upregulated in chicken splenocytes infected with velogenic NDV California (CA) strain at 2 days post infection (dpi), whereas the same genes were not upregulated when splenocytes were infected with lentogenic LaSota strain. In the same study, microarrays carried out on whole RNA from

the spleen of chickens infected with CA strain at 2 dpi, showed that there was upregulation of many IFN-responsive genes, IL-6 and IFN-γ RNA. In the same report, NO was markedly increased in the serum of animals infected with CA strains at 2 and 3 dpi; and at the same time there was increased immunohistochemical staining for inducible nitric oxide synthetase (iNOS) in the spleen of chickens at 3dpi (31). In the avian system, as well as in mammals, IFN-γ has been demonstrated to markedly induce expression of iNOS (22).

Ecco *et al.*, using formalin-fixed, paraffin-embedded spleens of animals infected with different velogenic NDV strains, have shown marked upregulation of IFN-γ RNA when compared to spleens from birds inoculated with mesogenic strains and PBS (controls). In this same work, Ecco and coauthors speculated that increased production of IFN-γ could contribute to tissue damage by inducing release of lysosomal enzymes and reactive oxygen species by activated macrophages (one of the main cellular targets of IFN-γ) (9, 21).

Based on these reports, it seems that the IFN-γ may play an important role in the pathogenesis of the NDV as a key mediator of tissue damage or as a possible mediator of the "cytokine storm", which has been often implicated in the pathogenesis of multi-organ failure during systemic viral diseases (15, 40). To test this hypothesis, and to better understand the role of IFN-γ in the pathogenesis of ND, we constructed a velogenic NDV with the chicken IFN-γ ORF inserted between the P and M genes. Because the virus itself produces IFN-γ, i.e., simultaneously with the viral proteins and at the same localized position of viral replication, this system allows for evaluation of the effect of IFN-γ at the very site of early replication.

MATERIALS AND METHODS

Cells and Viruses

DF-1 cells (Chicken embryo fibroblast cell line; ATCC CRL 12203) and HEp-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS). The modified *Vaccinia* virus Ankara recombinant expressing the T7 RNA polymerase (a generous gift of Bernard Moss, National Institute of Health) was grown in primary chicken embryo fibroblast cells.

Cloning and construction of the viral DNA full-length clones

Primers used in the cloning strategies are listed in table 6.1.

IFN-γ cloning

Total RNA was extracted from chicken spleen using Trizol according to manufacturer's instructions, with slight modifications, as previously described (31). IFN-γ cDNA was produced from total RNA using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions and standard protocols, with the following primers: forward (IFN-γ1f), 5'→3'; reverse (IFN-γ1r), 5'→3'. The forward primers contained an NcoI restriction site, which was not used in this study and was eliminated in the following PCR reactions to insert the flanking regions around the IFN-γ amplicon. Bands of correct size were excised from the gel, DNA was extracted and amplicons were cloned into a pCR2.1 vector according to manufacturer's instructions. The correct sequence was confirmed by sequencing of the plasmid-clones.

The IFN-γ was additionally subcloned into pCR2.1 with primers (IFN-γ1f: 5'ct**gggccc**tc*ttagaaaaaat*a*cgggtagaagt*accATGACTTGCCAGACTTACA 3' and IFN-γ2r: 5' ggccggtt**gggccc**tc***tta***ttaGCAATTGCATCTCCTCTGAGACT 3') in order to add ApaI restriction sites and "gene start" (GS) and "gene end" (GE) sequences flanking the IFN-γ open reading frame cDNA. In the primer sequences, the bold sequence corresponds to the ApaI site, the italicized sequence to the GS and the italicized and underlined sequence to the GE. In order to maintain the nucleotide base number from the two excision sites of ApaI as a multiple of six, an additional stop codon was inserted at the end of the IFN-γ ORF (bold and italicized in the primer sequence). PCR was performed using a high fidelity PCR kit (Promega, Madison WI), according to manufacturer instructions. Correct sequence was confirmed by sequencing using the same primers used in the cloning strategy. The resulting clone was named pCRIFNγ.

Construction of pNDV/ZJ1-IFN-γ and pNDV/ZJ1-Rev.

The whole genomic cDNA of the wild type ZJ1 NDV strain had been previously cloned into the expression vector TVT7 (named TVT-FLNDV-ZJ1) (23). The ORF of IFN-γ was inserted within the untraslated regions (UTRs) of the P gene, between the P and M genes, of the ZJ1 cDNA. The strategy adopted was similar to what was previously described for insertion of the green fluorescent protein (GFP) gene into TVT-FLNDV-ZJ1 (named pNDV/ZJ1GFP) (23). Both Plasmids TVT-FLNDV-ZJ1 and pNDV/ZJ1GFP were provided by Dr. X.F. Liu.

The fragment between positions 2857–5637 of the ZJ1 genome was amplified using primers Z1 and Z8 (table 6.1), and cloned into pCR2.1. This fragment contains both AgeI (position 2880 of ZJ1 genome) and PsiI (position 5266 of ZJ1 genome) restriction

sites, which are unique in the ZJ1 genome, and an ApaI restriction site (position 3144). From the pCR2.1, the 2857-5637 fragment was subcloned into the plasmid pUC19 using HindIII and XbaI restriction enzymes, resulting in plasmid pUCZJ1. Plasmid pUC19 was chosen because its polylinker does not contain ApaI restriction sites, thus making the APAI site at position 3134 of the ZJ1 genome unique in the pUCZJ1 vector. The IFN-γ gene (pCRIFNγ) was inserted through the ApaI site, with creation of pUCZJ1-IFN-γ intermediate plasmid. The correct sequence of pUC19ZJ1-IFN-γ was confirmed by sequencing using the primers Z1 through Z9 (see table 6.1). The AgeI/PsiI fragment of TVT-FLNDV-ZJ1 was substituted by the AgeI/PsiI fragment of pUCZJ1-IFN-γ. The new full-length clone plasmid was designated pNDV/ZJ1-IFN-γ.

To construct the full-length cDNA clone of the revertant virus, the intermediate plasmid pUCZJ1-IFN-γ was cut with ApaI and re-ligated to eliminate the insertion at position 3134 of the ZJ1 genome. The intermediate plasmid pUCZJ1-Rev was sequenced as described above and the sequence confirmed. The resultant plasmid was then re-inserted within the TVT-FLNDV-ZJ1 as described. The resulting full-length clone plasmid was designated pNDV/ZJ1-Rev.

Rescue of the viruses

The viruses were recovered from the full-length clone (FLC) plasmids (pNDV/ZJ1-IFN-γ, pNDV/ZJ1-Rev and pNDV/ZJ1-GFP) as described previously (12). In order to confirm viral sequence and identity, the 2857-3676 (primers Z6, in table 6.1) region of the ZJ1 genome was amplified from allantoic fluid derived total RNA using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase kit

(Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Recovered viruses were named rZJ1-IFNγ, rZJ1-Rev and rZJ1-GFP.

Growth curve

The growth kinetics of rZJ1-IFNγ, rZJ1-GFP and rZJ1-Rev were determined by single-step growth curve in DF-1 cells in 6-well plates. Cells were infected at a multiplicity of infection (MOI) of 10 and each time point was done in triplicate. Supernatant (100 µl) was collected at 1, 4, 8, 12, 16, 20, 24, 28 and 32 hours post infection, and replaced with the same amount of fresh medium. The virus titers in the samples were quantified by plaque assay in DF-1 cells. Briefly, samples were serially diluted and each dilution was inoculated into 12-well plate DF-1 cells. After 1 h of virus adsorption, supernatants were removed from wells; wells were washed with PBS three times, and then overlaid with DMEM supplemented with 0.9% methylcellulose. After 72 h, cells were fixed with 10% formalin and stained with crystal violet for enumeration of plaques.

Eggs and chickens

The source of embryonating chicken eggs and chickens was the SEPRL specific pathogen free (SPF) White Leghorn flock. Birds were housed in negative pressure isolators under biosafety level (BSL)-3 enhanced (E) conditions at SEPRL and provided food and water *ad libitum*. SPF embryonating eggs were inoculated for viral propagation, isolation, and titration (3).

IFN-γ Cytokine Expression

SPF eggs at 10 days of embryonation were injected with 100 µl of PBS-control, rZJ1-GFP or rZJ1-IFNγ at $10^{2.5}$ 50% embryo infectious doses (EID_{50}) per 0.1 ml. Sixteen eggs were injected per treatment. At 30, 50, 70 and 80 hours post-injection, 4 eggs from each group were candled to determine mortality and chilled at 4°C overnight. After eggs were chilled allantoic fluid was collected and stored at -80°C. Chicken interferon-gamma was assayed by ELISA method in allantoic fluid in triplicate according to the manufacturer's instructions for measurement of IFN-γ in serum (Chicken IFNγ Antibody Pair Kit, Invitrogen, Carlsbad, CA).

Chicken macrophage-like HD11 cells that produce high levels of nitric oxide in response to IFNγ (22), were plated in DMEM growth media at 10^5 cells per well in a 96-well culture plate and allowed to incubate overnight at 39°C at 5% CO2. Allantoic fluid samples were diluted in DMEM growth media and added to HD11 cell culture wells. After 48 hours incubation at 39°C, nitrite concentration in the growth media was measured by Griess assay (Promega, Madison, WI) according to manufacturer's protocols.

Intracerebral Pathogenicity index test (ICPI)

The ICPI test was performed according to standard protocol (3). Chickens were inoculated intracerebrally at one day of age with 0.1 ml of a 1:10 dilution of filtered, infective allantoic fluid with one of the following: PBS (control), rZJ1-Rev, rZJ1-IFNγ and rZJ1-GFP. Chicks were monitored daily and scored as normal, sick or paralyzed, and

dead to compile a score for an 8-day observation period. Calculation of the ICPI was performed as described elsewhere (3).

Pathogenesis assessment in chickens

Three groups of ten 4-week-old SPF White Leghorn chickens were inoculated bilaterally in the left conjunctival sac and the choanal slit with 0.1 ml of viral inoculum of rZJ1-IFNγ, rZJ1-GFP or rZJ-1-Rev. Phosphate buffered saline (PBS) was used for the non-infected control birds. The target dose of inoculum was $10^{5.5}$ EID_{50}. The actual infectious doses as determined by back-titration in embryonating chicken eggs were: $10^{6.1}EID_{50}$ for rZJ1-IFNγ, $10^{5.5}$ EID_{50} for rZJ1-GFP and $10^{4.6}EID_{50}$ for rZJ1-Rev. The birds were monitored clinically every day, and two birds of each group were euthanized after sampling oropharyngeal and cloacal swabs at 2, 5, 10 and 14 days post inoculation (dpi). Birds, whose condition became critical, were swabbed and euthanized regardless of the scheduled sampling day.

Tissues (eyelid, spleen, bursa, thymus, Harderian gland, proventriculus, small intestine, cecal tonsils, large intestine, air sac, trachea, lung, heart, esophagus, pharynx, crop, brain, liver, pancreas, kidney, comb, head of left femur including bone marrow, and turbinate) were collected, and all tissue samples were fixed by immersion in 10% neutral buffered formalin for 52 h. The sections of femur and turbinate were decalcified in 5% formic acid for 3-4 h. All sampled tissues were routinely processed into paraffin, and 3 μm sections were cut for hematoxylin and eosin staining (HE) and immunohistochemistry (IHC).

Immunohistochemistry

Tissue samples from animals infected with rZJ1-IFNγ and rZJ1-GFP were examined by IHC to detect viral nucleoprotein. Briefly, after deparaffinization, tissue sections were subjected to antigen retrieval by microwaving for 20 min at minimum power in Vector antigen unmasking solution (Vector Laboratories, Burlingame, CA) to expose antigenic sites, followed by application of a universal blocking reagent (Biogenex, San Ramon, CA) as recommended by the manufacturer. The primary antibody, made in rabbit, was an anti-nucleoprotein) antibody, used at 1:8000 dilution. The detection system was an avidin–biotin–alkaline phosphatase (Vector Laboratories, Burlingame, CA). Chromogen was a naphthol-based dye (Vector Red, Dako, Carpinteria, CA). Sections were counterstained lightly with hematoxylin and coverslipped with Permount for a permanent record.

Analysis of cytokine RNA levels

RNA levels of IFN-γ were measured at 2 dpi in the lung, spleen, thymus and bursa of animals infected with rZJ1-IFNγ, rZJ1-GFP and in PBS-inoculated control animals. RNA levels of IFN-γ, IL-2, Il-6 and IFN-β were assessed in the lung and spleen of animals infected with rZJ1-IFNγ, rZJ1-GFP and PBS-inoculated control at 2 dpi. Additionally, serum amyloid protein A (SAA) RNA was assayed in the liver of rZJ1-IFNγ, rZJ1-GFP and PBS-control infected animals at 2 dpi. For each treatment (infection) group, qRT-PCR was performed in four animals.

RNA Isolation

Lung, spleen, bursa, thymus and liver cell suspensions were generated by pushing freshly isolated tissue through a 70 µm pore size mesh cup, immediately into Trizol (Invitrogen, Carlsbad, CA) and stored at -80°C after collection of all samples for that day. Samples were later thawed at room temperature and chloroform was added according to the manufacturer's protocol. Samples were incubated for 10 min on ice. Following centrifugation at 2400 x g for 15 min, the aqueous phase was removed, mixed with an equal volume of 70% ethanol, and applied to Qiagen RNeasy midiprep columns (Qiagen, Valencia, CA). RNA quantity was assessed using a Nanodrop spectrophotometer on the RNA-40 setting and quality was assessed using a nanochip on a Bioanalyzer (Agilent Technologies, Santa Clara, CA). All RNA samples had a RNA integrity number greater than 8.6 with an average of 9.7.

Quantitative RT-PCR

Sequences of primers for IFN-γ, IL-6, IFN-β, IL-2, β-catenin (9) and serum amyloid protein A (SAA) (13) have been previously published.

One-step quantitative RT-PCR amplification and detection were performed using a 7500 FAST Real-time PCR System (Applied Biosystems, Foster City, CA) with SYBR green fluorescence detection of PCR product on 20 µl reaction mixtures containing: 10 µl of Power SYBR® Green RNA-to-CT™ 1-Step Kit (Applied Biosystems, Foster, CA), 500 nmol / L each of forward and reverse specific primer and 60 ng of RNA. Thermocycler conditions were as follows: one cycle at 48°C for 30 min, one cycle at 95°C for 15 min, 40 cycles at 95°C for 15 sec and at 58°C at 32 sec. One cycle for dissociation curve for all reactions was added and the melting curve analyzed.

Relative changes in gene expression were calculated according to the Pflaffl method (28) using LinRegPCR version 12 (29) to calculate individual specific primer efficiency by linear regression. All cycle threshold fluorescence (Ct) values were corrected to the average Ct of the PBS, mock-infected control for each gene of interest, and β-actin was used as the endogenous control.

Virus Isolation and Titration of swabs

For those birds used in the pathogenesis assessment experiment, immediately prior to euthanasia, oral and cloacal swabs were obtained from each bird and placed in separate tubes containing 1.5 ml of brain-heart infusion broth (BHI) with antibiotics (2000 U / ml penicillin G, 200 µg / ml gentamicin sulfate, and 4 µg / ml amphotericin B; Sigma Chemical Co., St. Louis, MO). Swab sample tubes were centrifuged at 1000 × g for 20 min, and the supernatant removed for virus isolation and titration. Virus infectivity titers were calculated from the result of inoculation of 9 to 10 day-old SPF embryonating chicken eggs with serial 10-fold dilutions in BHI containing antibiotics (100 U / ml penicillin G and 50 µg / ml gentamicin sulfate). NDV in infected dead or surviving embryos were identified by hemagglutination (HA) activity in amnioallantoic fluid harvested from chilled eggs. NDV was confirmed in HA positive samples by hemagglutination inhibition (HI) test with NDV-specific antiserum.

Data Analysis

Significant differences between treatment groups (rZJ1-GFP, rZJ1-reverant, rZJ1-IFNγ and PBS-control) in cytokine expression were determined by one-way ANOVA and

student t-means comparisons using JMP version 8.0 software (SAS Institute, Raleigh, NC).

RESULTS

Virus rescue

Virus was rescued from the recombinant plasmid and propagated by two passages into eggs. Identity of the three viruses was confirmed by sequencing the 2857-3676 region of the ZJ1 virus genome, which was amplified by RT-PCR on total RNA from the allantoic fluid of infected eggs.

Growth curve

The growth characteristics of rZJ1-IFNγ, rZJ1-GFP and rZJ1-Rev viruses were assessed by single-step growth curves in DF-1 cells (Fig. 6.1). No significant differences in growth kinetics or viral yields were observed between ZJ1-IFN-γ, ZJ1-GFP and ZJ1-Rev. All viruses reached maximum concentration at 20 h and remained constant through the end of the experiment (32 h). Highest titers (+/- standard error) were 1.43 (+/-1.73) $X10^7$ PFU / ml for rZJ1-IFNγ, 3.7(+/- 1.88) $X10^7$ PFU / ml for rZJ1-GFP and 3.33(+/-3.34) $X10^7$ PFU / ml for rZJ1-Rev.

Expression of functional IFN-γ

Allantoic fluids from eggs inoculated with rZJ1-IFNγ had significantly higher levels of functional IFN-γ protein than PBS-control and rZJ1-GFP as measured by commercial IFN-γ ELISA and IFN-γ bioassay in HD11 cells (Fig 6.2, panels a and b).

The highest concentration of IFN-γ occurred at 50 hours post-inoculation with rZJ1-IFNγ and slowly reduced over the next 30 h. The rZJ1-GFP virus induced a minor IFN-γ response at 70 h post-injection just prior to causing embryonic death.

Intracerebral pathogenicity index

The ICPI for rZJ1-IFNγ was 1.56, for rZJ1-GFP was 1.64 and for rZJ1-Rev 1.85

Pathogenesis assessment in chickens

Results for observed clinical signs, gross lesions and virus isolation are presented in tables 6.2, 6.3 and 6.4, respectively. All birds infected with rZJ1-Rev and rZJ1-GFP had the most severe clinical disease. In the rZJ1-Rev-inoculated birds, marked depression was evident as early as 2 dpi. By 4 dpi, these birds were recumbent and unable to stand. At 4 dpi, one chicken (1/10) infected with rZJ1-Rev showed neurological signs characterized by paralysis and head twitch, another birds showed intentional tremors. All of them died spontaneously or were euthanized *in extremis* by 5 dpi.

In birds inoculated with rZJ1-GFP, conjunctivitis and depression were evident at at 2 dpi. By 3 dpi, three (out of 10) animals had head twitch. At 5 dpi, 10/10 animals had neurological signs consisting of head twitch (5/10) or paralysis (5/10). At 5 dpi, all animals were euthanized due to marked neurological signs.

In contrast to the other two infected groups (described above), birds inoculated with rZJ1-IFNγ showed much less severe clinical disease. Birds were bright and alert through 5 dpi. From 5 to 7 dpi, all (8/8) birds were moderately depressed and lethargic. At 7 dpi, two (2/8) birds had neurological signs, consisting of hemiparesis of the

hindlimbs. However, even these two birds were still able to move to reach food and water and continued to eat and drink. At this time point one chicken with neurological signs and one without (for comparison) were euthanized. At day 10, the second bird with neurological signs had not recovered and was euthanized. No other clinical signs were noted until the end of the experiment (14 dpi).

Gross Findings

Gross findings are summarized in table 6.1. By 2 dpi, one bird (1/2 birds sampled) infected with rZJ1-Rev had conjunctivitis, thymic atrophy, hemorrhages of the Harderian gland, multifocal necrosis of the large intestine and enlarged spleen. On day 4, birds showed multifocal necrosis of the cecal tonsils (2/2) and proventricular and thymic hemorrhages (1/2). At 5dpi, animals had cyanotic combs (2/2), marked dehydration (2/2), conjunctival hemorrhages (2/2), hemorrhage and necrosis of the proventriculus (2/2), multifocal hemorrhages in the small intestine (2/2), necrosis of the cecal tonsils (2/2), and severe bursal and thymic atrophy with multifocal petechiation (1/2).

The birds infected with rZJ1-GFP, showed conjunctivitis starting at 2 dpi (1/3), thymic atrophy (2/2), intestinal hemorrhages, and enlarged and mottled spleen (1/3) (Fig. 6.3) by 5 dpi.

In birds infected with rZJ1-IFNγ, no lesions were observed at 2 dpi; at 5 dpi, birds had enlarged spleen (1/2), petechial hemorrhages in small intestine (Fig. 6.4) and proventriculus (Fig. 6.5) (1/2) and thymic atrophy (2/2). At 6 dpi, animals had prominent air sacs (2/2) and moderate thymic atrophy (1/2). At 7 dpi there was moderate thymic atrophy (2/2).

Histopathology

Histological and IHC findings are presented in table 6.2.

The most severe lesions were observed with rZJ1-Rev and rZJ1-GFP, and in both groups lesions were similar in severity and timing, starting at 2 dpi and increasing in severity through 5 dpi. Lesions invariably involved the site of inoculation, the lymphoid organs (spleen, thymus, bursa of Fabricius) and the lymphoid aggregates of the intestines. In the eyelid, lesions were often bilateral (not only limited to the site of inoculation) and were characterized by an acute inflammatory reaction, with severe edema, hemorrhages, multifocal areas of necrosis especially in the mucosa-associated lymphoid tissue (MALT), and ulceration of the mucosal layer, with fibrin exudation and inflammatory infiltrate, composed of heterophils and macrophages. In the lymphoid organs (thymus, bursa, spleen, and intestinal lymphoid patches, especially the cecal tonsils), lesions consisted of multifocal to coalescing severe necrosis, severe lymphocyte depletion, infiltration of numerous macrophages, and moderate heterophilic infiltrate (Fig. 6.5). In the intestines, there was multifocal ulceration and accumulation of necrotic debris involving the mucosal layer beneath the areas of the severe necrosis of the lymphoid-dependent areas. Scattered multifocal areas of necrosis were observed within the exocrine pancreas. Meningeal vessels have hypertrophic endothelial cells and scattered lymphocytes were observed within the Virchow Robin spaces in the brain in animals infected with both rZJ1-Rev and rZJ1-GFP.

Lesions caused by rZJ1-IFNγ were in general markedly less severe than those caused by rZJ1-GFP and rZJ1-Rev. Birds infected with rZJ1-IFNγ had most prominent lesions at 5 dpi, in the eyelid (bilateral) and lymphoid organs (spleen, thymus, bursa),

mainly characterized by lymphoid depletion, necrosis, and prominent macrophages (Fig. 6.6 and 6.7). By 7 dpi, lesions markedly decreased in severity and lymphoid-depleted areas were gradually repopulated by lymphocytes, mainly evident in both spleen and liver as hyperplasia of secondary lymphoid follicles. In the liver, these were mainly in the portal spaces, and more rarely scattered in the parenchyma, without specific orientation. Starting at 5 dpi, and reaching the peak of severity by 10 dpi, rZJ1-IFNγ-infected birds had prominent encephalitis, characterized by plumped endothelial cells, and accumulation within the Virchow-Robin spaces of numerous lymphocytes and fewer macrophages (Fig. 6.8). At 10 dpi, rare lymphocytes were observed infiltrating the myocardium.

Immunohistochemistry

Distribution and intensity of immunohistochemical staining are presented in table 6.2. Overall staining appeared to mirror the distribution and severity of the lesions. Animals infected with rZJ1-IFNγ showed immunoreactivity in 8/24 total organs. Reactivity was mainly restricted to the lymphoid organs at 5 dpi (Fig. 6.9 and 6.10) and in the eyelid at 2 dpi. At 5 dpi, one out of two sampled birds had multifocal immunohistochemical staining in scattered intestinal nervous plexuses; this kind of labeling was restricted only to this animal and was not observed in any other rZJ1-IFNγ-infected chicken at any other time point.

Animals infected with rZJ1-GFP showed marked immunohistochemical staining in numerous organs, with a total of 18/24 positive tissues, the lymphoid tissues showing the most reactivity. A unique feature of rZJ1-GFP-infected animals was marked IHC

staining in neurons and glial cells of numerous submucosal intestinal plexuses, mainly at 5 dpi.

Analysis of cytokine RNA levels

RNA levels of IFN-γ were assessed in spleen, bursa, thymus and lungs (Fig. 6.11). Changes in IL-2, IL-6 and IFN-β RNA levels were measured in the lung as an indicator of local immune regulation, and in the spleen (Fig. 6.12) as an indicator of systemic changes in immune regulation. RNA levels of IFN-γ were significantly higher in the spleen, lung, bursa and thymus of rZJ1-IFNγ-infected chickens when compared to PBS-control ($p<0.01$, $p=0.02$, $p=0.01$ and $p=0.05$, respectively). RNA levels of IFN-γ were significantly higher in the spleen and bursa ($p<0.05$) (but not in the thymus and lung) of rZJ1-IFNγ infected chickens when compared to rZJ1-GFP infected chickens (Fig. 6.11). rZJ1-GFP and rZJ1-IFNγ both had significantly reduced IFN-β RNA levels in the lung (3.75 and 2.5 folds respectively) compared to PBS-control ($p=0.028$ and $p=0.021$, respectively). There were no significant differences between groups in IL-2 and IL-6 RNA levels in the lung (lowest p-value=0.09). In the spleen of rZJ1-GFP infected chickens, there was a significant decrease (five-fold) of IL-2 RNA expression, compared to PBS-control ($p=0.027$). In the spleen of rZJ1-IFNγ infected animals, IL-2 RNA expression was unaffected compared to PBS-control ($p=0.69$). IFN-β RNA levels were decreased in the spleen of rZJ1-GFP infected animals compared to PBS-control, however the difference was not statistically significant ($p=0.08$). There were no significant differences between groups in IL-6 RNA in the spleen (lowest p-value=0.33).

RNA levels of SAA in liver of birds infected at 2 dpi, showed that rZJ1-GFP induced a significant increase in transcription of SAA when compared to rZJ1-IFNγ ($p<0.05$) (Fig. 6.13).

Virus isolation and titration of swabs

Results of titration are summarized in table 3. Results showed that all three viruses were able to replicate in the chickens and shed the virus through the oropharynx and the cloaca. rZJ1-Rev and rZJ1-GFP had the highest titers. rZJ1-IFNγ had lower titers throughout the experiment; titers peaked at 5 dpi, decreased at 7 dpi and were negative from 10 dpi onwards.

DISCUSSION

Through reverse genetics, we successfully rescued a recombinant NDV harboring the chicken IFN-γ open reading frame (ORF) between the P and M genes. A revertant virus (same as rZJ1-IFNγ, with elimination of IFN-γ ORF) was also rescued and characterized similarly to prove that the phenotype induced by IFN-γ could be reverted by its exclusion. rZJ1-GFP was used as an additional control to exclude that the changes in the phenotype could have been caused simply by the insertion of an extra gene, since GFP has no known intrinsic biological or immune activity. In fact, insertion of a foreign gene within the viral backbone of viruses belonging to the Paramyxoviridae family has been shown to cause a decrease in the viral replication, most likely as the consequence of the replication mechanism, which is necessarily polar and sequential, causing the genes at the 3’ end to be transcribed at higher amounts than those at the 5’ end (8, 11, 32, 39). The

rZJ1-IFNγ, rZJ1-GFP and rZJ1-Rev viruses were evaluated *in vitro,* in order to assess viral kinetics and production of IFN-γ, and *in vivo,* to determine pathogenesis in 4-week-old chickens.

Reverse transcriptase PCR of the 2857-3676 region from the allantoic fluid of rZJ1-IFNγ-infected eggs, showed that the IFN-γ ORF was complete, and was maintained during two consecutive passages on eggs. This is in accordance with what was previously described for negative sense, non-segmented (NNS) RNA viruses in general, and NDV in particular, for which foreign genes inserted into a viral backbone have been shown to be extremely stable, even after several passages *in vitro* (39). In addition, studies on NDV have demonstrated that insertion of extra genes within the untranslated, non coding regions (UTRs) at the end of the P gene (between P and M; where both IFN-γ and GFP were inserted in our constructs), does not cause sensible alteration of viral replication (17, 23).

Production of IFN-γ by rZJ1-IFNγ was demonstrated directly with ELISA on allantoic fluid of infected eggs, and was greater than IFN-γ produced when eggs were infected with rZJ1-GFP. The produced IFN-γ was also shown to have maintained biological activity, as shown by Griess assay in HD11 cells. Production of IFN-γ was also indirectly assessed by qRT-PCR, which showed that lung, thymus, bursa and lung of rZJ1-IFNγ-infected chickens at 2 dpi had significantly greater amount of IFN-γ RNA in bursa and spleen when compared to ZJ1-GFP and PBS controls. This increase, however, is not specific for the inserted viral IFN-γ, and could also reflect upregulation of genomic RNA.

Single-step growth curves with a MOI of 10, revealed that ZJ1-IFN-γ, ZJ1-Rev and ZJ1-GFP have similar replication kinetics. We opted to assay the growth curve with a high MOI, so that the produced cytokine could not interfere with viral replication by activating the IFN-γ pathway in cell culture. The results confirm that manipulation of the ZJ1 backbone and insertion of IFN-γ did not intrinsically alter the ability of rZJ1-IFNγ to replicate in DF-1 cells, and that there are no alteration of the backbone. This is further confirmed by the revertant virus (rZJ1-Rev), where the ICPI increased to a level similar to the wild-type (1.89 (23)) when the IFN-γ ORF was removed.

In vivo characterization by ICPI and clinicopathological assessment showed that expression of IFN-γ during viral replication is sufficient to markedly attenuate a velogenic viscerotropic NDV to levels similar to a mesogenic strain; in addition, this attenuated phenotype was successfully reverted by elimination the IFN-γ ORF insert, as shown by rZJ1-Rev.

In animal pathogenesis experiments, rZJ1-IFNγ was compared with the revertant virus (same backbone, without cytokine) and rZJ1-GFP, a virus that has the green fluorescent protein inserted in the same position as the IFN-γ ORF in rZJ1-IFNγ. In the present study, ICPI and clinicopathological assessment demonstrated that rZJ1-IFNγ had marked attenuation, when compared to both rZJ1-Rev and rZJ1-GFP. This indicates that the decrease in virulence induced by rZJ1-IFNγ could be reversed by elimination of the IFN-γ ORF and that this effect is not caused (or only minimally caused) merely by "hindrance" of another gene within the genome, since rZJ1-GFP was still markedly virulent. The ICPI value of rZJ1-IFNγ (1.56) was dramatically lower than that of rZJ1-Rev (1.85) and 0.8 points lower than rZJ1-GFP. These differences in ICPI are mirrored

by the clinicopathological assessment. Animals infected with rZJ1-Rev and rZJ1-GFP had the most severe disease and lesions, which were almost comparable in intensity and distribution, mainly affecting the lymphoid organs and the intestinal tract. We expected rZJ1-GFP to be slightly attenuated compared to rZJ1-Rev, however, this did not seem to be the case in the animal experiment. Since the ICPI of rZJ1-Rev was higher than rZJ1-GFP, a possible explanation could the lower inoculation titer of rZJ1-Rev, as revealed by the back-titration for the animal experiment.

Similarly to what was observed with rZJ1 and rZJ1-GFP, lesions in rZJ1-IFNγ-infected animals were mainly restricted to the lymphoid organs, but these were much milder and transient, being observed only at one time point: 5 dpi. By 7 dpi (next time point), the lymphoid organs were repopulated with lymphocytes, with formation of secondary lymphoid follicles. Animals infected with rZJ1-IFNγ had encephalitis, which was most severe at 10 dpi. At this time point, four animals were sampled and all showed moderate to severe encephalitis. Encephalitis was also observed with animals infected with rZJ1-GFP and 5 dpi.

Overall, for rZJ1-GFP and rZJ1-IFNγ viruses, the immunohistochemical staining for NDV nucleoprotein paralleled the distribution and severity of the lesions and is similar to what has previously been reported for other NDV strains (7, 18, 19, 36, 37). rZJ1-IFNγ showed immunoreactivity only at 5 dpi and only in the lymphoid organs, and in areas with lesions. One animal had scattered staining in the intestinal plexuses, which has been previously described in few other cases of NDV infection (26, 36), and that in our study were observed in a more consistent way with the animals infected with the rZJ1-GFP control. Lesions in the brain were not associated with immunohistochemical

staining. Discrepancies between the presence of lesions and IHC staining for the virus in the central nervous system have been reported with NDV infection, and even with the neurotropic strains of NDV, immunohistochemical staining is often scattered and limited (7, 10).

Taken together, the pathology data show that rZJ1-IFNγ behaved similarly to a mesogenic strain (7, 18, 19), while rZJ1-GFP and rZJ1-Rev behaved as velogenic, and in a similar way to the rZJ1 rescued from the TVT-FLNDV-ZJ1 plasmid (Susta L, Miller PJ, Hu S, Liu X, Rue CA, Afonso CL, Brown CC. Clinicopathological Characterization of Two Recombinant Newcastle Disease Viruses Derived From a Virulent Chinese Strain. Annual Meeting of the American Association of Avian Pathologists, Atlanta, July 2010).

The data in this study suggest that production of IFN-γ simultaneous to the production of viral particles (transcription in NDV is sequential) induces enough cellular antiviral status to enhance elimination of the virus. IFN-γ has been previously shown to have anti-viral/parasitic activity in the avian system (other than the mammalian), as seen with Duck Hepatitis B Virus *in vitro*, and *in vivo* by enhancing resistance to Coccidia (16, 22, 34). However, even if IFN-γ has been shown to potentiate the immune response against microbes, recent publications about NDV pathogenesis (9, 31), have shown that IFN-γ is upregulated in chicken tissues during infection with velogenic viscerotropic viruses, suggesting that IFN-γ might have acted as one of the possible mediators of tissue damage (9, 21). Nonetheless, the data of the work presented here suggest that IFN-γ is most likely protective. The upregulation of IFN-γ observed in the tissue of VVNDV-infected animals might be a late and ineffective response to NDV replication, however,

when IFN-γ is produced in association within the initial stages of NDV replication, as in the case of rZJ1-IFNγ, its activity seems to be protective and able to restrain viral production and spread. Nonetheless, the exact mechanism of IFN-γ protection is not completely elucidated in our experiment, and this remains to be investigated.

In mammals, in which IFN-γ has been most extensively studied, the main antiviral effects of IFN-γ are mediated by upregulation of: 1) protein kinase R (PKR, induces phosphorylation of eIF2α causing blockade of the cell cycle), 2) adenosine deaminase acting on RNA (ADAR, which edits viral RNA by inducing punctiform mismatches) and 3) inducible nitric oxide synthase (iNOS, which may have an antiviral effect due to the production of NO) (33). If this is the case also for the IFN-γ-mediated attenuation of rZJ1-IFNγ, then a decrease in NDV virulence may be driven by the IFN-γ-mediated innate immune response. Based on the data from IHC and titration of swabs in this study, it seems that the attenuation is most likely the consequence of a decreased ability of viral replication and an overall decrease in viral load *in vivo*. In fact, the immunohistochemical signal for rZJ1-IFNγ was much lower than that for rZJ1-GFP; and titration of the swabs showed lower titers at 2 dpi when compared to rZJ1-GFP and rZJ1-Rev. It is possible to speculate that the anti-viral mechanisms triggered by IFN-γ might have directly decreased part of the viral progeny, thus lowering the amount of viable virus for replication.

Another possible explanation is that the virally encoded IFN-γ might act and modulate the production of other cytokines, somehow counteracting the effect of a life-threatening cytokine storm and restraining an unchecked inflammatory response. Data from qRT-PCR partially support this hypothesis; in fact, at 2 dpi, in the liver of animals infected with rZJ1-IFNγ, there is decreased production of SAA RNA, in comparison to

rZJ1-GFP-infected animals. SAA is an acute phase protein, produced by the liver under the effect of pro-inflammatory cytokines such as TNFα, IL-1 and IL-6 (21). A decreased expression in rZJ1-IFNγ-infected chickens may indicate that IFN-γ down-regulates the systemic inflammatory response in response to NDV infection, thus resulting in decreased morbidity and mortality. However, it is possible that a decreased expression of SAA production may simply be a reflection of a decreased viral burden (still caused by IFN-γ), as discussed above. In addition, rZJ1-GFP induced marked downregulation of IFN-β in the spleen, however, this decrease was not significantly different from the rZJ1-IFNγ-infected and PBS-control animals. To better assess the overall effect of IFN-γ on the production of other cytokines, other time points and a larger variety of cytokines should be studied.

Lastly, the rZJ1-IFNγ virus is the first virulent NDV recombinant to harbor a cytokine and to be used in the field of avian medicine (Lentogens are already used in human oncolytic therapy (30)). The construct proved able to produce the inserted cytokine and to deliver IFN-γ in the animal system. Therefore this work can possibly offer preliminary data for the production of better NDV vaccines that use inserted cytokines genes as true molecular adjuvants to amplify the immune response (6).

CONCLUSIONS

In conclusion, the present work demonstrates that simultaneous expression of IFN-γ with viral replication in the animal system can markedly decrease the virulence of a velogenic NDV, and that NDV can be successfully used to deliver cytokines whose ORFs are present in the viral genome. In addition, this is the first time that IFN-γ-bearing

NDV recombinant viruses have been used to study the role of a specific cytokine during viral infection in birds. This rZJ1-IFNγ virus could be a useful tool in the further investigation of NDV pathogenesis. The description and characterization of rZJ1-IFNγ could also be the first step in the formulation of safe and more immunogenic NDV vaccines that aim to use the cytokines as true molecular adjuvants.

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Table 6.1. List of Primers used; F=forward, R=reverse.

Name	Position in ZJ1 genome	Sequence
Z1F	2-25	CCAAACAGAGAATCGGTGAGTTAC
Z1R	974-994	GATATCACCTGTGAGGCTGCT
Z2F	670-689	TGAGACAGCAGATGAGTCAG
Z2R	1587-1606	GCTGGGTGTTGTCGATCAGT
Z3F	1351-1371	TCAACAGGCGGGAGTCCTCAC
Z3R	2184-2200	TCGCTTGCGGCCTGAGT
Z4F	1895-1915	GGCCACTTTTACAGATGCGGA
Z4R	2505-2530	GGTACAGGAGTATTGTCTTGGCTCTG
Z5F	2222-2242	GACTGGAGCAAGCAACTCCCT
Z5R	3054-3073	TTCAGCGCAAGGCGTTTGAT
Z6F	2857-2876	TGACGCTCAATAAGCTCTCG
Z6R	3676-3697	TGCCTGCACTATCGAGAAGACT
Z7F	3389-3414	GACGGGAAGAAGCAAATCACCCCACA
Z7R	4223-4248	TGGCTCCAGAGTATCTTGGCAACCTG
Z8F	4008-4028	ATATCGGGCTTATGTCCACTG
Z8R	4994-5014	CTTAAGCCGGAGGATGTTGGC
Z9F	4715-4732	TCTCAGACAGGGTCAATC
Z9R	5637-5657	AAGCTGACGTATTGCCGCTCA
IFN-γF		AAAACCATGGCTTGCCAGACTTACAACTTG
IFN-γR		GCAATTGCATCTCCTCTGAGACTGGCT
IFN-γ2F	IFN-γ primers with flanking sites, forward	5'CT**GGGCCC**TC*TTAGAAAAAAT*A*CGGGTAGAAGT*ACCATGACTTGCCAGACTTACA3'

IFN-γ2R	IFN-γ primers with flanking sites, reverse	5’ GGCCGGTTGGGCCCTCTTATTAGCAATTGCATCTCCTCTGAGACT 3’

Table 6.2. Summary of clinical signs and lesions in 4-week-old chickens infected with rZJ1-IFNγ, rZJ1-GFP and rZJ1-Rev.

Strain	Clinical signs	Lesions
rZJ1-IFNγ	Transient depression at 5 dpi. Paralysis starting at 7 dpi in two birds. One bird left alive maintained paralysis until 10 dpi, when was euthanized.	Thymic atrophy, proventricular and intestinal hemorrhages at 5 dpi. No other lesions noted at other time points.
rZJ1-Rev	Depression, prostration, severe conjunctivitis. Two birds at 5 dpi had neurological sign. All animal died or euthanized by 5 dpi	Conjunctivitis and thymic atrophy by 2 dpi. Necrosis and hemorrhages of proventriculus, small intestine and cecal tonsils at later time points. Mottled spleen. Atrophy of bursa at 5 dpi.
rZJ1-GFP	Depression, conjunctivitis, prostration. Neurological signs by 4 dpi. At 5 dpi, all 10 remaining animals had neurological signs. All animals were euthanized by 5 dpi.	Conjunctivitis, necrosis of the lymphoid organs (thymus, spleen, bursa and cecal tonsils) starting at 4 dpi and culminating at 5 dpi.
PBS	None recorded	None recorded

Table 6.3. Distribution and intensity of the lesions (HE) and immunohistochemical staining (IHC) for NDV nucleoprotein.

		rZJ1-IFNγ			rZJ1-Rev			ZJ1-GFP	
Organs	DPI[†]	2	5	10	2	4	5	2	5
Eyelid	HE	++	++	-	+++	+++	+++	++	+++
	IHC	+	++					-	++
Spleen	HE	+	++	+	++	+++	++++	+	+++
	IHC	-	+					-	++
Thymus	HE	-	+	+/-	-	++	+++	-	+++
	IHC	-	++					-	+++
Bursa	HE	-	++	-	-	+	+++	-	+++
	IHC	-	++					-	++
Cecal Tonsils	HE	+/-	+	-	+	+++	+++	+	+++
	IHC	-	+					-	++
Pancreas	HE	-	-	-	-	+	+	-	++
	IHC	-	-					-	+
Lung	HE	-	-	+/-	-	-	-	-	-
	IHC	-	-					-	+
Heart	HE	-	-	-	-	-	-	-	+
	IHC	-	-					-	-
Brain	HE	-	-	++	-	+/-	+/-	-	++
	IHC	-	-					-	-
Bone marrow	HE	-	-	-	-	-	-	-	+
	IHC	-	-					-	+

Histological grading:

Spleen: +Moderate hyperplasia; ++lymphocytic depletion; +++moderate (<50%) lymphocyte depletion, histiocytic accumulation and multifocal necrosis; ++++ (>50%) severe lymphocytic depletion, histiocytosis and necrosis.

Thymus, Cecal tonsil, GALT, Bursa and Thymus: +mild lymphocytic depletion, ++ (<50%) moderate lymphocytic depletion with necrosis and histiocytosis, +++ (>50%) severe lymphocytic depletion, necrosis and histiocytosis

Bone marrow: mild + (<20%) bone marrow necrosis; ++ mild (20-50%) bone marrow necrosis; +++ severe (>50%) bone marrow necrosis.

Pancreas: + mild (<3 areas) vacuolation and degeneration; ++ moderate (>3 areas) vacuolation and degeneration.

Brain: + vascular reactivity; ++ vascular reactivity and perivascular cuffing; +++vascular reactivity, perivascular cuffing, gliosis.

Immunohistochemical grading:

- = no IHC signal present

+/-=very rare positive cells are observed

+ = rare cells in the section are positive on IHC

++=positive cells seen, <50% of all high power fields (HPF)

+++ = positive signal seen in 50 to 75% of HPF

++++ = abundant positive signal in more than 75% of HPF

[†]Day Post Infection

Table 6.4. Virus isolation and titration of oral (O) and cloacal (C) swab samples per 0.1 ml.

	PBS		rZJ1-IFNγ(6.1)*		rZJ1-GFP(5.5)		rZJ1-Rev(4.6)	
Dpi †	***O***	***C***	***O***	***C***	***O***	***C***	***O***	***C***
2	-§	-	$<10^{0.5}$ (1/2)# $10^{2.1}$ (1/2) ‡	$<10^{0.5}$ (1/2) - (1/2)	$10^{3.3}$ (1/2) $10^{1.9}$ (1/2)	$10^{0.9}$(1/2) $10^{0.7}$ (1/2)	$10^{3.1}$(1/2) $10^{2.3}$ (1/2)	$<10^{0.5}$(1/2) $10^{1.5}$ (1/2)
4	nc	nc	nc	nc	nc	nc	$>10^{4.5}$(1/2) $10^{4.3}$(1/2)	$>10^{4.5}$ (1/2) $10^{4.1}$ (1/2)
5	-	-	$10^{4.3}$ (1/2) $10^{5.5}$ (1/2)	$10^{2.7}$ (1/2) $10^{3.9}$ (1/2)	$10^{5.5}$ (1/3) $10^{5.7}$ (1/3) $10^{4.5}$ (1/3)	$10^{4.5}$ (1/3) $10^{5.3}$ (1/3) $10^{4.5}$ (1/3)	$>10^{4.5}$(2/2)	$>10^{4.5}$(2/2)
7	nc	nc	$10^{1.3}$ (1/2) $10^{0.7}$ (1/2)	$<10^{0.5}$ (2/2)	nc	nc	nc	nc
10	-	-	- (2/2)	- (2/2)	nc	nc	nc	nc
14			- (2/2)	- (2/2)	nc	nc	nc	nc

* Number in parentheses is the viral titer of inoculum per 0.1 ml (backtitration).

†Days postinoculation.

‡Number of birds displaying that value / total number of animals sampled.

§ No virus recovered from undiluted swab fluids inoculated into three eggs, 0.2 ml per egg.

#low titer but positive.

nc: non collected.

Figure 6.1. Single step growth curve for rZJ1-IFNγ, rZJ1-GFP and rZJ1-Rev. Results show that there is no significant difference in the growth of the three viruses.

Figure 6.2. IFN-γ concentration and biological function in allantoic fluids of eggs injected with ZJ1-IFNγ, ZJ1-GFP or PBS control as measured by ELISA (**A**), and induction of nitric oxide production by chicken macrophage-like HD11 cells (**B**). *-indicates significant difference versus ZJ1-GFP and PBS-control groups within a time point ($p<0.05$). Bars represent standard error of the mean (SEM), n=4.

Figure 6.3. 4-week-old chicken, experimentally infected with rZJ1-GFP strain, 5 dpi. The spleen is enlarged, mottled and has multifocal specks of necrosis.

Figure 6.4. 4-week-old chicken, experimentally infected with rZJ1-IFNγ strain, 5 dpi. There are multifocal areas of hemorrhages through the serosa of an intestinal loop.

Figure 6.5. Spleen; 4-week-old chicken experimentally infected with rZJ1-GFP strain. 4 dpi. There are multifocal areas of necrosis and accumulation of fibrin. HE.

Figure 6.6. Spleen; 4-week-old-chicken, experimentally infected with rZJ1-IFNγ. 5 dpi. There is diffuse lymphoid depletion in the white matter and accumulation of macrophages within the splenic ellipsioid. HE.

Figure 6.7. Bursa; 4-week-old chicken, experimentally infected with rZJ1-IFNγ. 5 d.p.i. Multifocal bursal follicles show lymphoid depletion and accumulation of prominent macrophages. HE.

Figure 6.8. Cerebellum, cerebellar nuclei; 4-week-old chicken, experimentally infected with rZJ1-IFNγ. 7 dpi. There is a focally extensive area of neuropil and white matter vacuolation, infiltration with lymphocytes and macrophages. Scattered neurons are necrotic. HE.

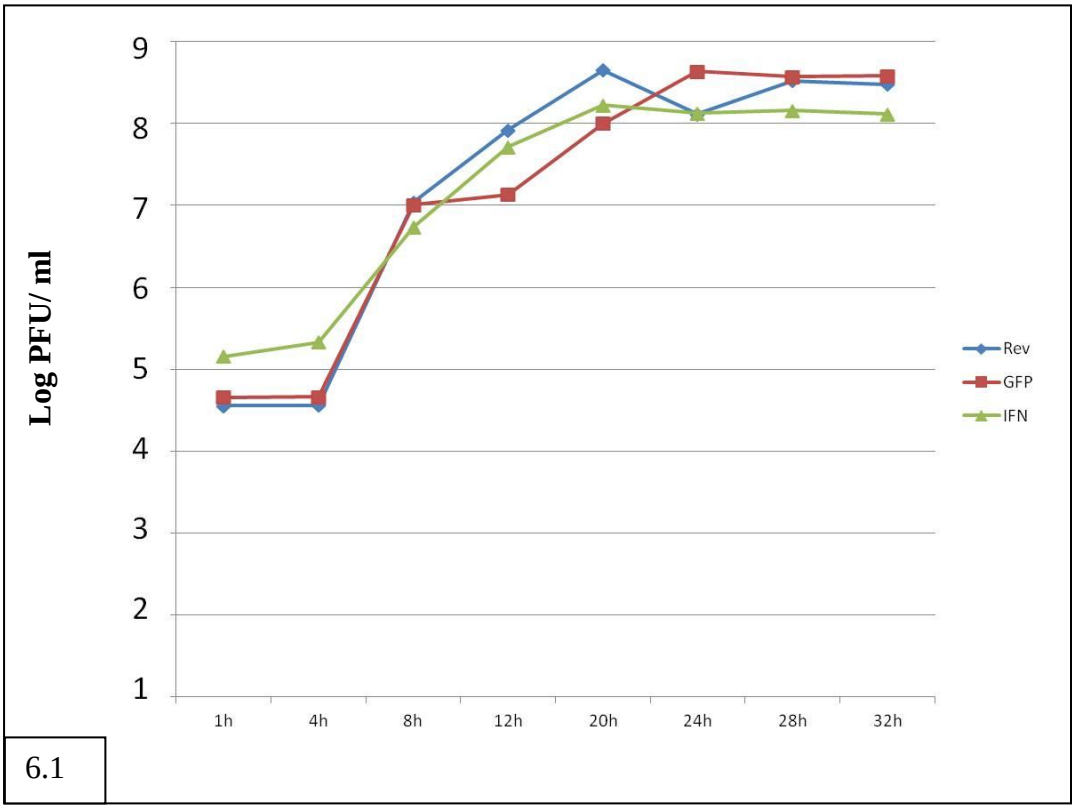
Figure 6.9. Spleen; 4-week-old chicken, experimentally infected with rZJ1-IFNγ. 5 dpi. Scattered macrophages in the splenic ellipsoid are immunolabelled for NDV. IHC, alkaline phosphatase method, hematoxylin counterstain.

Figure 6.10. Bursa; 4-week-old chicken, experimentally infected with rZJ1-IFNγ. 5 dpi. NDV nucleoproten is detected within macrophages within multifocally affected bursal follicles. IHC, alkaline phosphatase method, hematoxylin counterstain.

Figure 6.11. IFN-γ RNA expression in bursa, lung, spleen and thymus 48 h post-inoculation with rZj1-IFNγ, rZJ1-GFP and PBS. *-indicates significant difference versus ZJ1-GFP and PBS-control groups within a tissue ($p<0.05$). Bars represent standard error of the mean (SEM), n=4.

Figure 6.12. IFN-β, IL-2, IL-6, IFN-γ RNA expression in the spleen of 4-week-old chicken infected with rZJ1-IFNγ, rZJ1-GFP and PBS 48 hours previously.

Figure 6.13. Relative fold change of SAA RNA expression in the liver of 4-week-old chicken infected with rZJ1-IFNγ, rZJ1-GFP and PBS 48 h previously.
*-indicates significant difference versus ZJ1-GFP and PBS groups ($p<0.05$). Bars represent standard error of the mean (SEM), n=4.



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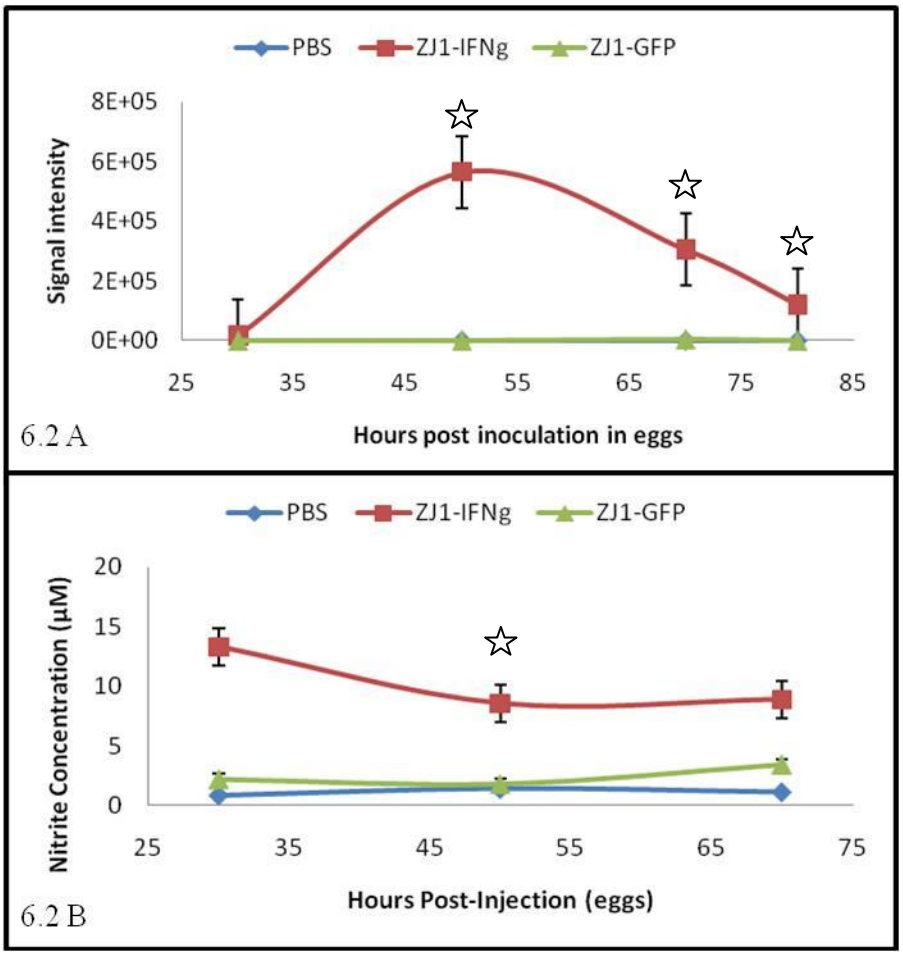
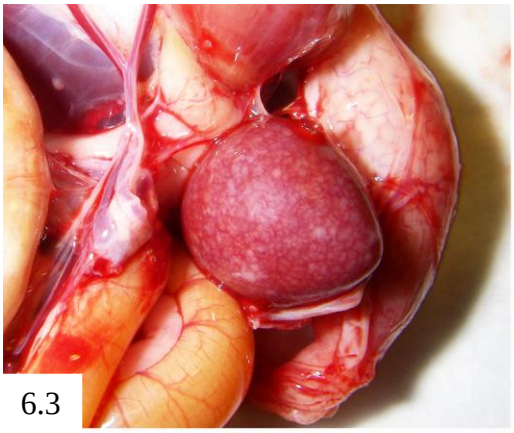

PBS
ZJ1-IFNg
ZJ1-GFP
Signal intensity
8E+05
6E+05
4E+05
2E+05
0E+00
25
35
45
55
65
75
85
Hours post inoculation in eggs
6.2 A
PBS
ZJ1-IFNg
ZJ1-GFP
Nitrite Concentration (µM)
20
15
10
5
0
25
35
45
55
65
75
Hours Post-Injection (eggs)
6.2 B



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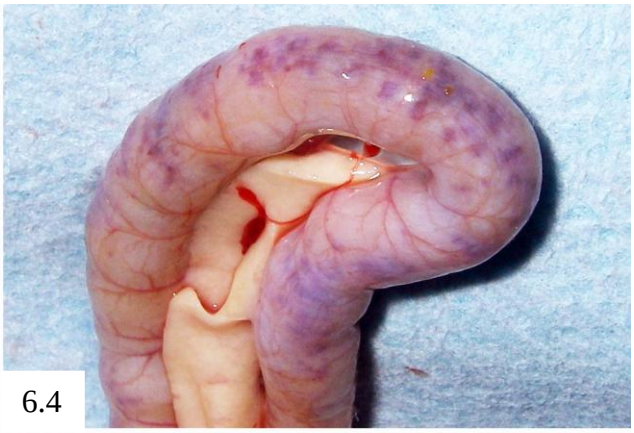

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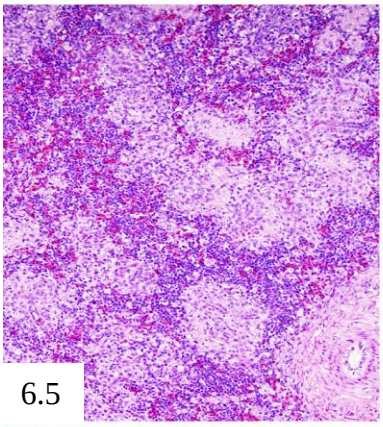

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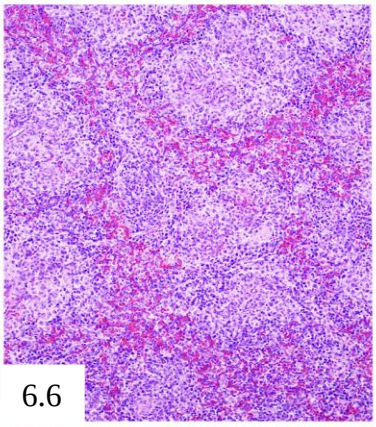

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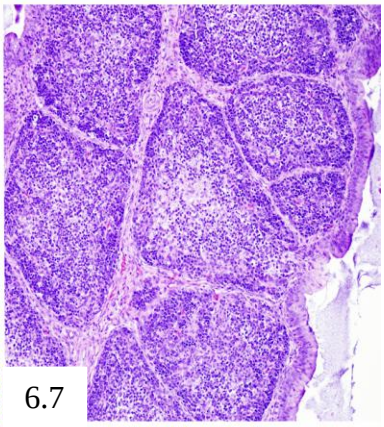

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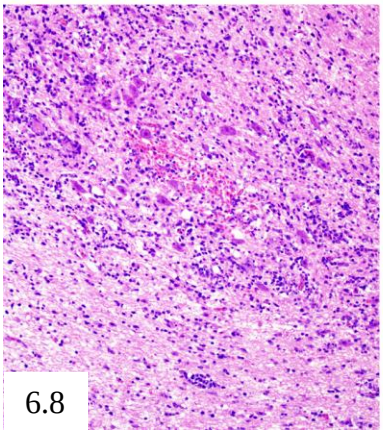

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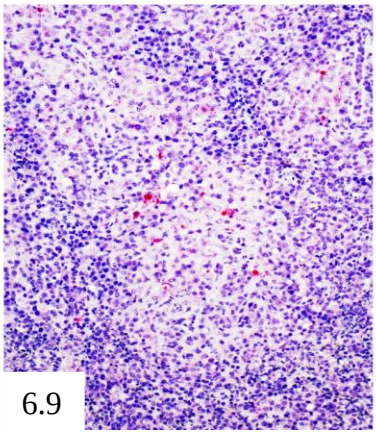

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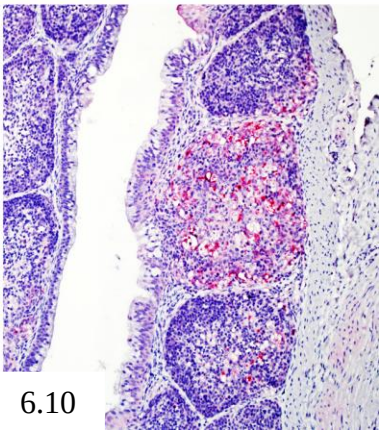

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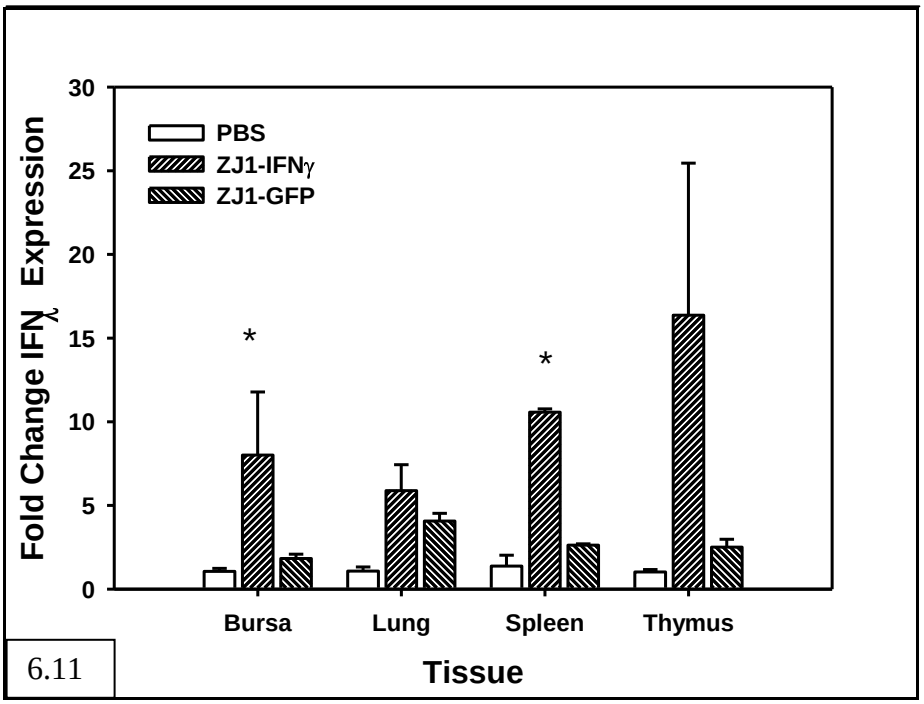
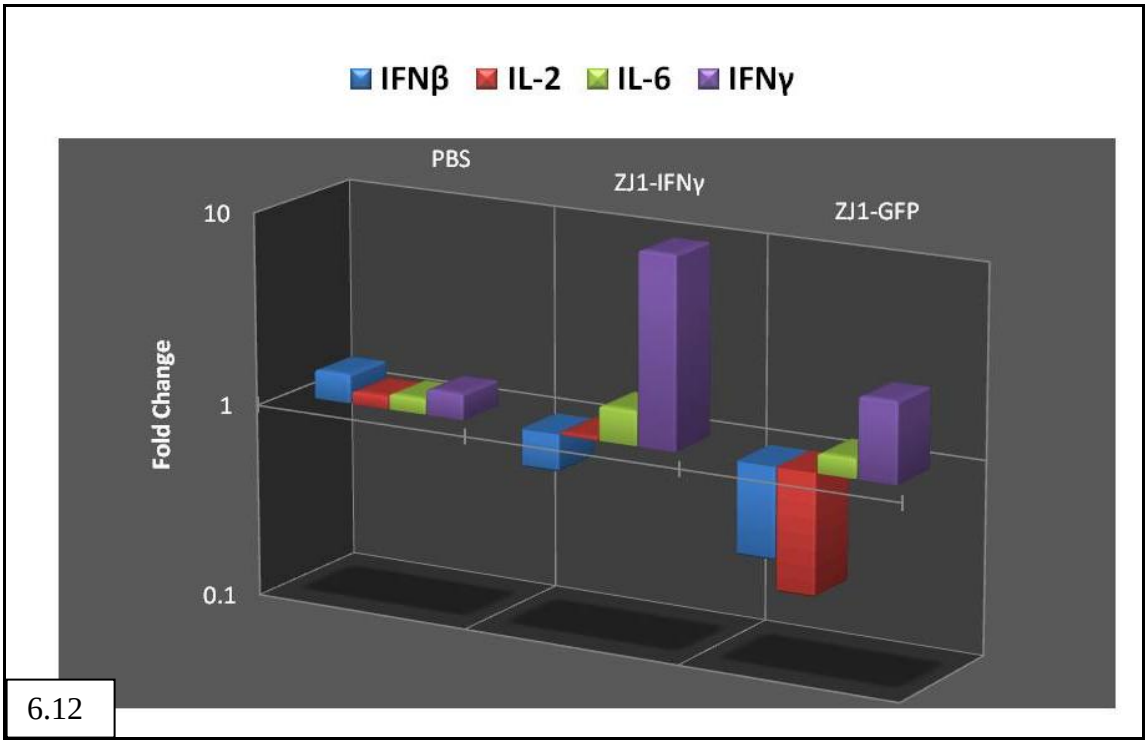

30
25
20
15
10
5
0
Fold Change IFNγ Expression
PBS
ZJ1-IFNγ
ZJ1-GFP
*
*
Bursa
Lung
Spleen
Thymus
Tissue

6.11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IL-6
IFNγ
PBS
ZJ1-IFNγ
ZJ1-GFP
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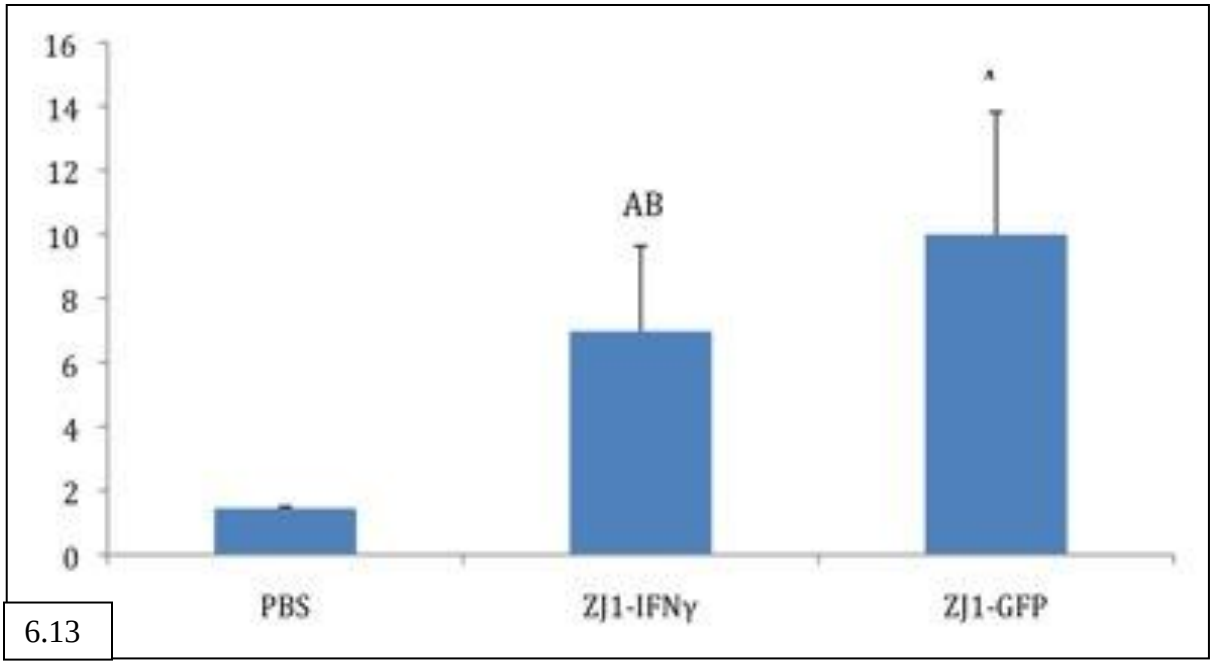
1
0.1
Fold Change

6.12



6.13

CHAPTER 7

CONCLUSIONS

Understanding the mechanism of the extreme variability in NDV virulence and, therefore, dissecting its pathogenesis is one of the most challenging aspect in NDV research but also one of the most rewarding in terms of achieving satisfactory measures for its control. One approach to the study of NDV pathogenesis is the construction of recombinant viruses through the use of reverse genetics. This technique has been used for NDV in various ways, including addition or mutations of genes from strains of differing virulence, mutation of the intergenic sequences and insertion of extra foreigner genes. Another, more conventional approach for the study of NDV pathogenesis is full characterization of newly isolated strains to detect possible variation of virulence associated with the raise of new genotypes.

In the present study we rescued a virulent NDV (rZJ1) with the insertion of the chicken IFN-γ gene (rZJ1-IFNγ). IFN-γ dramatically decreased rZJ1 virulence, as shown by ICPI and animal experiment in 4-week-old chickens, and decreased viral load, as shown by IHC and low viral shedding in oral and cloacal swabs. This study shows that IFN-γ is indeed efficacious in decreasing the virulence of NDV and is not a mediator of tissue damage during NDV infection. This is partially surprising, since IFN-γ has been shown to be upregulated in the tissues of animals infected with velogenic NDV. It is possible that IFN-γ upregulation in these cases might be a late response in tissues that are

already overwhelmed by viral replication. The reasons why IFN-γ might have decreased rZJ1 virulence are not completely understood. Mechanism might entail the intrinsic antiviral activity of IFN-γ; or, on the other hand, IFN-γ may modulate the immune response, leading to a decreased systemic inflammatory response (as shown by the data of SAA transcription in the liver).

In order to create a suitable control for insertion of IFN-γ, we also rescued a velogenic virus with inserted a gene with unknown immunological activity, the green fluorescent protein (rZJ1-GFP). Although GFP has not effect on the viral pathogenesis *per se*, we have shown that insertion of GFP decreased viral replication (as shown by multicycle growth curve), and ICPI when compared with the same backbone without any inserted genes (rZJ1). In pathogenesis experiments in 4-week-old chickens, rZJ1-GFP caused marked neurological signs, practically behaving as a velogenic neurotropic NDV, characteristic that was not shared with rZJ1. Decreased viral replication has been observed also with other viruses of the paramyxoviride family with inserted extra gene, however, the mechanism by which a decrease in replicative ativity of NDV leads to a more neurovirulent behaviour, remains to be investigated. A timing factor seems to be likely, since also with wild-type velogenic neurotropic viruses, neurological signs usually appear at late time points during infection.

In another set of experiments, we have shown that HN and F genes from virulent NDV strains, when inserted within the context of a mesogenic backbone, were not able to sensibily change virulence of the newly produced recombinant viruses. These results suggest that only few genes deriving form virulent NDV are not sufficient to reproduce the virulent behaviour of their parental strain, when inserted in a less virulent backbone.

Lastly, NDV pathogenesis was investigated through the characterization of viruses islated form recent ND outbreaks in poultry (Australia and Vietnam) and in wild birds (Cormorant, Nevada). All viruses had marked tropism for lymphoid tissues and macrophages in numerous organs, while no or minimal lesions were observed in the respiratory system. This striking lack of respiratory signs confirms the observation of previously published report on NDV experimental pathogenesis. Therefore, caution is warranted in labelling NDV as a primary respiratory pathogen.

The Australia strain behaved unexpectedly, since, in spite of an high ICPI score (typical of a velogenic viscerotropic), animals lived longer, developed neurological signs and had moderate lesions. These data empirically confirm that neurotropism of NDV might be an intrinsic activity of viscerotropic strains that become attenuated. In addition, the discrepancy between the ICPI values and the results of the clinco-pathologial assessment underscores the importance of animal experiment to completely characterize NDV strains.