ABSTRACT

LAURA HARRISON

OCCURRENCE OF APOPTOSIS IN LYMPHOID TISSUES FROM CHICKENS ACUTELY INFECTED WITH STRAINS OF NEWCASTLE DISEASE OF VARYING VIRULENCE
(Under the Direction of DR. CORRIE BROWN)

Newcastle Disease Virus (NDV) poses many threats to the poultry industry. Virulence of different strains varies widely and creates a wide range of disease manifestations. Low virulence strains often circulate with minimal disease, while high virulence strains can rapidly sicken and kill an entire house of chickens. Presence of highly virulent viruses within our country will seriously damage our abilities to export chicken meat or live birds. Because apoptosis, or programmed cell death, serves as a key defense mechanism during viral infections, increased knowledge concerning apoptosis is essential in understanding host-pathogen interactions. Additionally, comparing apoptosis among strains of varying virulence generates better understanding of clinical consequences from infection with different viral strains. Immunohistochemistry (IHC) for active caspase-3, a key enzyme in apoptosis, was done on formalin-fixed, paraffin embedded sections from chickens infected with various strains of NDV. Apoptotic activity was examined in the spleen, thymus, bursa, and intestine. All tissues were harvested at two days post-infection. To confirm virus presence, IHC was done for NDV nucleoprotein. Active caspase-3 expression in lymphoid tissue infected with virulent strains was significantly increased when compared to tissue infected with milder strains. Heightened apoptosis in the virulent strains may be a key component of the severe disease manifestations seen. Because severe disease manifestation results in losses to the poultry industry, generating greater understanding of apoptosis in response to NDV infection exists as an important research initiative.

INDEX WORDS: Apoptosis, Immunohistochemistry, Caspase
OCCURRENCE OF APOPTOSIS IN LYMPHOID TISSUES FROM CHICKENS ACUTELY INFECTED WITH STRAINS OF NEWCASTLE DISEASE OF VARYING VIRULENCE

by

LAURA HARRISON

A Thesis Submitted to the Honors Council of the University of Georgia in Partial Fulfillment of the Requirements for the Degree

BACHELOR OF SCIENCE

in MICROBIOLOGY

with HIGH HONORS

and CURO SCHOLAR DISTINCTION

Athens, Georgia

2009
OCCURRENCE OF APOPTOSIS IN LYMPHOID TISSUES FROM CHICKENS ACUTELY INFECTED WITH STRAINS OF NEWCASTLE DISEASE OF VARYING VIRULENCE

by

LAURA HARRISON

Approved:

Dr. Corrie Brown 04/30/2009
Dr. Corrie Brown Date
Faculty Research Mentor

Approved:

Dr. Leonardo Susta 04/30/2009
Dr. Leonardo Susta Date
Reader

Approved:

Dr. David S. Williams 05/08/09
Dr. David S. Williams Date
Director, Honors Program, Foundation Fellows and Center for Undergraduate Research Opportunities

Approved:

Dr. Pamela B. Kleiber 05/08/09
Dr. Pamela B. Kleiber Date
Associate Director, Honors Program and Center for Undergraduate Research Opportunities
ACKNOWLEDGEMENTS

Many people have played essential roles during my research and thesis experience. Both in and out of the lab, I have been fortunate to have strong support. Whether it was advice directly related to research, a reminder to relax, a good joke, an encouraging phone call, good food, or a variety of other things, I am owe thanks to many people. The people who have been most essential to this process constantly reminded me of a very important lesson I learned to embrace while in the lab: LAB RULE #1- Ask lots of questions. COMMUNICATE. Mistakes are okay. The only unacceptable mistakes are those we don’t recognize and learn from.

Although I could probably never list everyone to which I owe thanks, here are some people who must be thanked:

The Lab- Dr. Corrie Brown, Jian Zhang, Leonardo Susta, Janildo Reis, Caroline Colden, Raquel Rech, Lindsay Stein, and Dr. Fernando Torres-Velez.

Other faculty- Dr. Karl Espelie, Dr. Pam Kleiber, Jessica Hunt, and the rest of the CURO staff

Everyone else- My family, Margaret Faenza, Alyssa Anderson, and Dorothy Futch. Also, I have to thank anyone else that sends me inspiring articles on infectious diseases.
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CHAPTER 1
INTRODUCTION

Newcastle disease virus (NDV) is a concern of the poultry industry worldwide as it causes significant morbidity and mortality as well as economically damaging trade restrictions. The NDV, also known as avian paramyxovirus-1 (APMV-1), belongs to the family Paramyxoviridae, subfamily Paramyxoviridae, and genus Avulavirus. It is an enveloped virus and the genome is non-segmented, single-stranded, negative-sense RNA. Virulence varies among NDV strains, and historically the viruses have been classified into three pathotype groups: low (lentogen), moderate (mesogen), or high virulence (velogen). Within the velogenic group, a subdivision between viscerotrophic velogenic and neurotrophic velogenic viruses exists. Neurotrophic velogenic normally causes nervous signs, while viscerotrophic velogenic causes hemorrhagic lesions in the intestine. The most widely internationally accepted classification of virulence for NDV is the intracerebral pathogenicity index (ICPI) test, in which day-old chicks are inoculated with virus intracerebrally and illness and death scored, to result in a numeric value. Those strains with ICPI >0.7 are considered to be of greatest concern and require reporting to international authorities.

Better understanding of strain differences requires increased elucidation of NDV pathogenesis. Host response affects pathogenic mechanisms; thus, the consideration of host activities such as apoptosis provides insight into viral mechanisms. Apoptosis, or programmed cell death, is an active and tightly regulated cascading process that is critical for many body processes, including innate immunity. It represents one of the body’s key responses to viral invasion, as activation of apoptosis in infected and neighboring cells limits viral replication and
dissemination respectively. The main actors of the apoptotic process are a group of proteolytic enzymes that have the ability to cleave the peptide chain between a cysteine and an aspartic acid residue (thus the name CAspases)\(^9\). Numerous caspases have been identified, and they can be divided in two groups: initiator and effector caspases. The initiator caspases (mainly caspases 8 and 9) are activated by a wide variety of stimuli and signals that can set a cell into a cascade which leads to apoptosis. Basically caspase 8 is activated by events external to the cell, mainly involving the Fas-Fas ligand, which is also known as death receptor pathway. Tumor Necrosis Factor -alpha (TNF-\(\alpha\)) and Type-1 interferons (IFNs) promote the Fas-Fas ligand pathway of apoptosis activation. Caspase 9 becomes active due to intracellular events, specifically, damage to the mitochondria. All pathways converge on the effector caspases, of which caspase 3 is the primary actor. Caspase 3, when activated, results in a downstream chain of proteolytic reactions committing a cell to specific DNA fragmentation, coagulation, and shrinkage of the cytoplasm. At this point, from the apoptotic cell there is budding of membrane coated portion of cytoplasm and nuclear material, called apoptosomes. Eventually, these are phagocytized by macrophages. Consequently, visualization of active-caspase 3 allows a snapshot of imminent apoptosis, whether the trigger was intracellular or extracellular. Previously, Kommers et al (2003) briefly investigated apoptosis \textit{in vivo} during velogenic NDV infection through TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling). There was increased apoptosis in the spleen, thymus, and bursa\(^7\). To better characterize apoptosis during NDV infection, we used immunohistochemistry (IHC) for active-caspase 3 in lymphoid organs taken from birds infected with strains of varying virulence at 2 days post-infection (dpi).
Tissues

Tissues for this study included archived formalin-fixed, paraffin-embedded eyelid, spleen, thymus, bursa, and cecal tonsils (intestinal lymphoid aggregate) that had been harvested in identical manners from several different pathogenesis experiments. In all cases, tissues were taken from birds at 2 days post-infection after administration of approximately $10^5$ TCID$_{50}$ units of virus via eyedrop instillation. In those studies, birds were humanely euthanized with intravenous injection of pentobarbital and tissues collected into 10% neutral buffered formalin immediately postmortem. After 52h in formalin, tissues were processed for histology. Archived paraffin blocks were stored at room temperature. All studies involved three-week-old White Leghorn chickens from the same source flock kept at the Southeast Poultry Research Laboratory (SEPRL).

Viruses and Pathogenicity Indices

Nine strains of NDV were examined in this study. Table 1 lists the viruses, their origin, the pathotype, and the intracerebral pathogenicity index (ICPI). Briefly, four velogenic viscerotropic strains were used: 93-28710, isolated from a Chinese parakeet at a US quarantine station; Chicken CA/S0212676, from a 2002 outbreak in California; ZJ1, a 2000 isolate from an outbreak in geese in China; Long Bien 78, a 2002 isolate from a duck at the Long Bien market in Vietnam. Three mesogenic strains were used: Roakin, a classic mesogenic strain initially isolated in birds in New Jersey in 1946; Anhinga, originally isolated from an anhinga in a captive...
population in a water park in Florida in 1993; NV Cormorant, a 2005 isolate from a double crested cormorant in Nevada, USA. Two lentogenic strains were used: B1, a low virulence
strains used in many vaccines and first isolated in New Jersey in 1947; QV4, isolated in 1966 in Australia. In addition, there were noninfected control birds (inoculated with PBS). Intracerebral pathogenicity index values were determined at the time of the original bird experiment using the same standard technique and using chickens from the same source flock at SEPRL.

**Immunohistochemistry**

Formalin-fixed paraffin-embedded tissues from chickens infected with 9 different strains as well as PBS were used. Histologic sections were cut at 4μm onto positively charged slides, and deparaffinized immediately prior to the procedures.

- **Active Caspase-3**

  Sections were unmasked through steaming for 15 minutes in citrate buffer (Biogenex Antigen Retrieval Citra). Following antigen retrieval, blocking was done with universal blocking reagent (Biogenex, San Ramon, CA). Primary antibody made in rabbit for active-caspase 3 (Anti-ACTIVE® Caspase-3 pAb, Promega) was diluted at 1:2000 in antibody diluent (DAKO buffer). Sections were incubated with primary antibody for one hour at room temperature. Negative controls for the techniques were performed using normal rabbit serum as primary antibody.

- **NDV Nucleoprotein**

  Sections used for detection of NDV nucleoprotein were unmasked using a citrate buffer solution (Vector Antigen Unmasking Solution) and microwaved at lowest power for fifteen minutes. The primary antibody was a polyclonal anti-peptide made in rabbit to the viral nucleoprotein. Primary antibody was applied to the sections at 1:8000 in antibody diluent (Zymed® Laboratories Incorporated) at 37°C for 1 hour or at 4°C overnight.
After incubation with primary antibodies, the slides were washed and incubated with an alkaline phosphatase linked polymer system (Labvision Corp) directed against the Fc fragment of the rabbit antibodies. The reaction was revealed through using a naphthol based Vector Red (Vector Labs), or tetrazolium based (NBT-BCIP) chromogens. Slides were lightly counterstained with Mayer’s hematoxylin and coverslipped using Permount for a permanent record. Serial slides immunostained for active caspase-3 and NDV nucleoprotein were compared to assess immunodetection for the two different antigens in the same areas.

**Statistical Analysis**

Cells positive for Active Caspase 3 staining were counted at 40X magnification in at least ten randomly chosen fields within each organ. The results were expressed as the arithmetic means ± S.D of a representative experiment p. The means were compared using ANOVA and Dunnett multiple comparison test for matched groups. A p<0.05 was considered statistically significant.
CHAPTER 3
RESULTS

NDV Nucleoprotein (NP)

IHC results for NDV nucleoprotein are presented in Table 2. Positive signal was detected only in tissues harvested from birds infected with the viscerotropic velogenic strains and was confined mainly to spleen and cecal tonsils. In spleen the viral nucleoprotein was detected mainly in macrophages/histiocytes surrounding the penicilliary arterioles. In intestinal lymphoid tissue (cecal tonsil), it appeared to be within the macrophages and dendritic cells. Noticeably numerous large macrophages with vacuolated cytoplasm showed the highest reactivity. In viscerotropic velogenic strains there was rare staining in cortical and medullary regions of the bursa and no staining in the thymus. In all cases where there was positive signal for NDV, it was in the cytoplasm and present as a fine to coarse granularity. There was no staining in any organs with mesogenic or lentogenic strains. Similarly, tissues from non-infected control birds were all negative for the presence of NDV nucleoprotein.

Active Caspase-3

IHC results for active caspase 3, along with the statistical analyses, are presented in Table 3. Scores were highest with the velogenic viscerotropic strains, with statistical significance at p<0.05 that apoptosis was greater in all lymphoid organs than with any other strain categories, and also negative controls. In the mesogenic strains, apoptosis levels in bursa and intestinal tissue were significantly higher than for those organs from birds infected with lentogens or negative controls. Thymic rates of apoptosis in the mesogenic strains were significantly higher than the controls, but not from the lentogens. Although the numeric values for lentogens and the
control birds were different, there was not a statistically significant difference between these two groups.

Most intense signal for active caspase 3 was seen with the viscerotropic velogenic strains. Signal within the spleen was strongest at the periarteriolar lymphoid sheaths and based on morphology of positive cells, i.e., large amount of pale cytoplasm and a vesicular often bean-shaped nucleus, cells were presumed to be macrophages (Figure 1A and 1B). In contrast, in both lentogens and negative control birds, apoptosis was minimal (Figure 2A and 2B). In both the thymus and bursa, staining occurred in both cortical and medullary regions (Figure 3). In the bursa of the birds infected with a mesogenic strain, there was some apoptosis, less than in the velogenic strains, but more than in the lentogens or control birds (Figure 4). Areas of positive active-caspase 3 staining were also found in intestinal lymphoid tissue. Staining within the bursa and thymus appeared to be occurring predominantly in lymphocytes, while in the cecal tonsils, the immunoreactivity was present in both macrophage-like cells and lymphocytes. The pattern of staining was cytoplasmic and diffuse, with a fine dust-like appearance. Sequential tissue sections show that in the event of NDV nucleoprotein detection, the immunodetection of active-caspase 3 was present in both large viral load areas and in areas where the viral nucleoprotein was minimal or absent (Figure 5).
CHAPTER 4
DISCUSSION

In this study we examined the presence of Newcastle disease virus and apoptosis in lymphoid organs of birds acutely infected with strains of varying virulence, using immunohistochemistry to detect both viral protein and active caspase 3, an indicator of imminent apoptosis. At least some apoptosis was seen in all organs of all birds, although there were notable differences according to virulence of strains. Apoptosis in those tissues from birds infected with viscerotropic velogenic strains was statistically greater than the amount of apoptosis seen in tissues from birds infected with mesogenic or lentogenic strains. The average number of apoptotic cells in spleen, thymus, and intestinal lymphoid tissue was three to four times greater than the average number of apoptotic cells in the same organs affected by the mesogenic strains and an even greater difference was seen when velogens were compared with lentogens. Very low levels of apoptotic staining occurred in lymphoid tissues of even the mock-inoculated birds, as apoptosis is an expected phenomenon due to normal clonal selection, expansion, and turnover of cells in the immune system. It was only the velogenic strains that had evidence of virus in lymphoid organs at 2dpi, as detected by IHC for viral nucleoprotein. This suggests that greater virus replication is important for induction of apoptosis. Also our results suggest that indirect effects of viral infection are playing a role in the increased apoptosis within the lymphoid tissues, as there was often apoptosis visible in tissues in which there was no evidence of viral infection.

Apoptosis due to NDV has been investigated by several researchers, but predominantly in *in vitro* systems and mostly in the context of anti-tumor therapy. NDV has been used in
experimental studies to attack tumor cells and reduce tumor burden. It has been shown to have inherent oncolytic activity and replicates selectively in tumor cells, leading to death of the neoplastic cell. Elankumaran et al. (2006) investigated the cytotoxicity of rNDV against tumor cells. The recombinant virus was a combination of low and moderately virulent NDV strains. It was found that infected cells did not depend on TNF-related apoptosis-inducing ligand (TRAIL) for induction of apoptosis, so the intrinsic pathway was assumed to be of greater importance. Furthermore, they illustrated the importance of intrinsic activation of the caspase pathway by localizing cytochrome C in the cytosol of NDV-infected cells. It is believed that cytoplasmic dsRNA intermediates and endoplasmic reticulum stress cause mitochondrial membrane destabilization as they cause the BH3 proteins to bind pro-apoptotic Bak and Bax proteins. Another study by Ravindra et al. (2009) showed that NDV infection upregulated Bak and Bax, while it downregulated Bcl-2, an anti-apoptotic protein. Bak and Bax undergo conformational changes and then increase the permeability of the mitochondrial membrane permeability, which allows the release of cytochrome C, which then binds apoptosis activating factor-1 (Apaf-1), which creates a complex known as the apoptosome. The apoptosome can activate procaspase-9, which in turn activates caspase 3. So it appears that in tumor therapy, the predominant mechanism is intrinsic activation, most likely due to NDV infection of the tumor cells.

In an in vivo system, Kommers et al. (2003) used a TUNEL assay, which detects the end-state of apoptosis, in experimentally infected chickens. Examining only virulent strains, there was increased TUNEL labeling in all lymphoid tissues at 2-4dpi. Our study also utilized the natural host and examined strains of varying virulence early in infection. At only 2dpi, there was extensive positive signal for active caspase 3 in multiple lymphoid organs, well beyond the amount of virus visualized, and even in organs where no virus was visualized. In acute NDV
with virulent strains, chickens are clinically ill, so it is presumed that acute phase cytokines are circulating, especially TNF-α and Type I IFNs, known initiators of apoptosis. Although intracellular virus replication may lead to apoptosis (intrinsic pathway), it is also known that the viscerotropic velogenic viruses infect macrophages, causing them to be upregulated, which would lead to enhanced secretion of cytokines⁵. Increased levels of acute phase response cytokines, such as TNF-α and IL-1, is the major cause of severe malaise, and termed “cytokine storm” effect. These two mediators are also known inducers of the extrinsic pathway of apoptosis, so it would be expected that in natural infection with NDV, apoptosis would be increased because of high levels of these cytokines.

In studies with other paramyxoviruses, specifically canine distemper and measles, the lymphoid tissues are recognized as the site of early viral replication and lymphopenia is a prominent characteristic of infection³,¹². For both these viruses, increased apoptosis rates are reported without direct co-infection of the cells undergoing apoptosis. Such indirect effects could be the consequence of cytokine storm, impairment of dendritic cells’ survival and function or alteration of antigen processing³,¹². There is also severe immunodepression associated with these two infections, starting at around 3dpi. In comparison, with NDV, studies have demonstrated that by 2 days after eyelid inoculation, viscerotropic velogenic strains are replicating in lymphoid tissue and by 5 dpi birds have become severely ill and have either died or are moribund⁴. Histopathologic examination of birds infected with virulent NDV strains reveals extensive lymphoid depletion and necrosis at 5 dpi⁷,¹⁰. It is likely that in NDV, the observed increased apoptosis within the lymphoid tissues could result in systemic immunodepression, rendering the chickens more susceptible to opportunistic infections. For example, although previous in vivo experiments with SPF chickens using mesogenic strains showed minimal to
moderate disease, natural outbreaks of mesogenic NDV are known to have high morbidity and some mortality, whereas mortality with mesogenic strains under laboratory conditions is infrequent\textsuperscript{7,8,10,14}. Therefore, it is probable that a transient immunodepressed status is responsible for more severe disease in non-SPF chickens, which are constantly challenged by minor pathogens and have different husbandry conditions than the experimental ones.

In summary, this study demonstrates that NDV virus induces apoptosis in the lymphoid tissues of infected chickens and that the increased apoptotic rate is statistically correlated with the virulence of the viral strains. Nonetheless, apoptosis does not seem to be only an immediate result of the viral replication within the cell and it is most likely the consequence of both direct viral replication and indirect effects.
Table 1. Virus source, amount inoculated, and intracerebral pathogenicity index (ICPI)

<table>
<thead>
<tr>
<th>Virus</th>
<th>Source</th>
<th>Amount of virus inoculated</th>
<th>ICPI *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long Bien 78</td>
<td>2002 isolate from a duck at the Long Bien market in Vietnam</td>
<td>$10^{5.5}$</td>
<td>1.88</td>
</tr>
<tr>
<td>Chicken CA/S0212676</td>
<td>plaque purified; isolated from an outbreak in 2002 in small flocks of backyard birds, including some used for illegal cockfighting (Wakamatsu et al. 2006, 933)</td>
<td>$10^{6.1}$</td>
<td>1.85</td>
</tr>
<tr>
<td>ZJ1</td>
<td>isolated from a 2000 outbreak in geese in China</td>
<td>$10^{5.7}$</td>
<td>1.86</td>
</tr>
<tr>
<td>93-28710</td>
<td>isolated from a Chinese parakeet at a US quarantine station</td>
<td>$10^{5.1}$</td>
<td>1.88</td>
</tr>
<tr>
<td>Nevada Cormorant</td>
<td>collected in 2005 from a double crested cormorant in Nevada, USA</td>
<td>$10^{5.3}$</td>
<td>1.53</td>
</tr>
<tr>
<td>Roakin</td>
<td>classic mesogenic strain originally isolated from birds in New Jersey in 1946</td>
<td>$10^{5.45}$</td>
<td>1.60</td>
</tr>
<tr>
<td>Anhinga</td>
<td>originally isolated from an anhinga in a captive population in a water park in Florida in 1993</td>
<td>$10^{5.0}$</td>
<td>1.31</td>
</tr>
<tr>
<td>B1</td>
<td>low virulence strain used in many vaccines and first isolated in New Jersey in 1947</td>
<td>$10^{5.02}$</td>
<td>0.13</td>
</tr>
<tr>
<td>QV4</td>
<td>isolated from chickens in 1966 in Australia</td>
<td>$10^{5.1}$</td>
<td>0.39</td>
</tr>
</tbody>
</table>
Table 2. Presence of NDV as detected by immunohistchemistry for nucleoprotein

<table>
<thead>
<tr>
<th>Strain</th>
<th>Organ</th>
<th>Spleen</th>
<th>Thymus</th>
<th>Bursa</th>
<th>Intestinal lymphoid tissue</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Velogenic Viscerotropic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Long Bien 78</td>
<td>+++</td>
<td>0</td>
<td>+</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Chicken CA/S0212676</td>
<td>++</td>
<td>0</td>
<td>++</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>ZJ1</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>93-28710</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td><strong>Mesogenic</strong></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Nevada Cormorant</td>
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<td>0</td>
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</tr>
<tr>
<td>Roakin</td>
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</tr>
<tr>
<td>Anhinga</td>
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<td></td>
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<tr>
<td><strong>Lentogenic</strong></td>
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</tr>
<tr>
<td>B1</td>
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<td>0</td>
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<td>QV4</td>
<td>0</td>
<td>0</td>
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<tr>
<td><strong>Negative Control</strong></td>
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<td>0</td>
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</tbody>
</table>

Key: 0 = no positive signal in any part of the organ  
+ = rare scattered in few high power fields  
++ = staining in several high power fields  
+++ = staining in every high power field
Table 3. Labeling for active caspase 3 and statistical significance

<table>
<thead>
<tr>
<th>Sample size (n)</th>
<th>Organs</th>
<th>Mean±SD</th>
<th>S.S**</th>
<th>Mean±SD</th>
<th>S.S</th>
<th>Mean±SD</th>
<th>S.S</th>
<th>Mean±SD</th>
<th>S.S</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Spleen</td>
<td>Thymus</td>
<td>Bursa</td>
<td>Gut</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>106</td>
<td>VEL-OGENS</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>74</td>
<td>MES-OGENS</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>52</td>
<td>LENT-OGENS</td>
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<tr>
<td>50</td>
<td>Negative</td>
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</tr>
</tbody>
</table>

- *Mean±SD positive cell /40X field
- ** S.S( Yes= statistically significant higher than other NDV strains at p value <0.05 for same organ; No = not statistically significant higher than other NDV strains at p value <0.05 for same organ)
Figure 1. Splenic staining induced by velogenic strains. (A) Apoptosis as seen by IHC for active-caspase 3. Apparent cell morphology suggests that cells are macrophages. 100X. (B) Virus presence as seen by IHC for NDV nucleoprotein. 40X.

Figure 2. Staining induced by lentogenic strains. (A) Cecal tonsils showing low levels of active caspase 3 staining. 40X. (B) Splenic tissue showing low levels of active-caspase 3. 20X.
Figure 3. Medullary and cortical active-caspase 3 staining in the bursa induced by velogenic strain. 40X.

Figure 4. Active-caspase 3 staining in the bursa caused by infection with mesogenic strain. 40X.

Figure 5. Lack of staining for viral nucleoprotein in birds infected with mesogenic strain despite high levels of active caspase 3 staining (Fig. 4). 40X.
WORKS CITED


