IMPROVED NEWCASTLE DISEASE VACCINE STRATEGIES TO REDUCE SHEDDING OF VIRULENT VIRUS FROM INFECTED BIRDS

by

PATTI J. MILLER

(Under the Direction of David L. Suarez)

ABSTRACT

Newcastle disease threatens the poultry industry throughout the world, adversely affecting poultry producers, backyard farmers, and the economies of entire nations. Newcastle disease virus (NDV) is also known as avian paramyxovirus serotype -1 virus (APMV-1). APMV-1 all belong to a single serotype, and by definition any Newcastle disease (ND) vaccine strain should provide protection against morbidity and mortality from any NDV challenge. However, available vaccines do not protect against infection. Vaccinated birds exposed to a virulent NDV likely will be infected and shed virus without showing symptoms of ND. Vaccination with live and killed ND vaccines is universally practiced in the United States (U.S.) and in many other countries, with some exceptions. The vaccine seed strains are viruses of low virulence that were originally isolated in the 1940s, and genetically are distant from most recent virulent viruses. Currently, when a flock has birds infected with virulent NDV the birds exposed or possibly exposed are depopulated to contain the spread of infection. The most recent

outbreaks in the U.S. are believed to have originated from viruses spread from birds that entered the U.S. from Mexico. Viruses that cause future outbreaks likely will be in the same genetic lineage as these recent outbreak viruses. The experiments described were performed to test the hypothesis that increasing the genetic relatedness of the ND vaccine virus to the likely virulent challenge virus will produce more specific neutralizing antibodies and decrease the amount of challenge virus shed from vaccinated poultry. Various inactivated and live vaccines including four recombinant viruses, some of which contained genes of the California 2002 outbreak virus were tested against virulent challenges with California/212676/2002 and chicken/U.S./(TX)GB/1948. Inactivated homologous vaccines reduced the amount of virus shed orally better than heterologous The inactivated vaccines made with recombinant viruses with varying vaccines. relatedness to the challenge virus again showed that vaccines more genetically related to the challenge virus worked best to reduce oral viral shedding. All of the live vaccines provided better protection with less virus shed even compared to the homologous inactivated vaccine. However, birds vaccinated with live vaccines more genetically related to the challenge virus often shed less virus in oropharyngeal swabs and had significantly fewer birds shedding virus compared to live vaccines less similar to the challenge virus.

INDEX WORDS: Avian paramyxovirus-1, APMV-1, Newcastle disease virus, NDV, Vaccine, Poultry

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A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2008

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DEDICATION

I would like to dedicate this dissertation to my favorite scientist and my best friend, Gary and to Preston, Anneliese, Gavin, Garrison, Aidan and Isabella, my extraordinary children who always manage to amaze and entertain me.

ACKNOWLEDGEMENTS

Many people have assisted me in my training. Most importantly, I would like to thank my advisor, Dr. David Suarez, for his guidance and leadership and Dr. Jack King for his extensive mentoring. I would like to thank my committee members, Dr. Mark Jackwood, Dr. David Stallknecht and Dr. Pedro Villegas for their helpful suggestions and continual support. I am also indebted to the members of the Southeast Poultry Research Laboratory, especially Dr. Claudio Afonso, Dr. Qing Yu and Dr. Carlos Estevez for their scientific input and advice, and to Suzanne DeBlois, Dr. Mia Kim, Aniko Zsak, Dawn Williams-Coplin, Tracy Smith-Faulkner and Tim Olivier for helping me with my animal experiments and processing of samples. A special thank you goes to Jenny Pfeiffer, my officemate and fellow student, for providing wine (and whine), wit, sustenance and sympathy during stressful times. I would also like to thank Dr. David Swayne and the Agricultural Research Service of the United States Department of Agriculture for providing the trainee position that supported my training.

Lastly, I thank my husband, Gary, and my children Preston, Anneliese, Gavin, Garrison, Aidan, and Isabella. They have been incredibly supportive and patient over the past few years and I could not have done it without them.

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CHAPTER 1

INTRODUCTION

Newcastle disease (ND) results from an infection with a virulent Newcastle disease virus (NDV) (Alexander, 2003). NDV potentially infects all species of birds, and in poultry is highly contagious, and usually fatal (Alexander, 1988). This disease is one of the most important infectious diseases of poultry because of the potential for devastating loses. It occurs on six of the seven continents of the world and is enzootic in many countries. In the United States (U.S.) virulent NDV strains are not endemic and disease caused by them is often referred to as exotic Newcastle disease (END) (USDA, 2006). The possibility of an outbreak of virulent NDV (vNDV) in the United States is a constant threat with the last major outbreak in the U.S. ending in 2003 (Nolan, 2002). In 2004 ND was named as one of the seventeen most dangerous animal diseases by the Homeland Security Presidential Directive-9 which required that a National Veterinary Stockpile of ND countermeasures be prepared due to the negative affects that an outbreak would have on agriculture and economics of the U.S. Different genetic groups or genotypes of avian paramyxovirus serotype-1 virus (APMV-1) circulate in different parts of the world. The virulent strains require monitoring and control measures even in countries where they are endemic because these occurrences impact the international trade in poultry and poultry products. Low virulence strains are also frequently endemic and while they do not require control for trade purposes, poultry producers vaccinate to prevent losses from respiratory disease caused by these viruses.

NDV is a member of the genus Avulavirus (Fauquet and Fargette, 2005; Mayo, 2002) in the Paramyxoviridae family and is a negative-sense, single stranded, nonsegmented, enveloped RNA virus, and is also known APMV-1 (Alexander, 2003). NDV is composed of six genes and their corresponding six structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and the RNA polymerase (L) (Hamaguchi et al., 1983). RNA editing of the P protein produces two additional proteins, V and W (Chambers and Samson, 1982; Collins et al., 1982). Of these only the HN and F glycoproteins, important for binding and fusion of the virus to host cells, are known to induce neutralizing antibodies after vaccination that confer protection from morbidity and mortality associated with vNDV infection (Merz et al., 1980) (Avery and Niven, 1979; Boursnell et al., 1990a; Boursnell et al., 2005; Long et al., 1986; Nakaya et al., 2001; Park et al., 2006; Taylor et al., 1990; Umino et al., 1984).

APMV-1 viruses all belong to a single serotype, and therefore by definition any NDV vaccine strain should provide protection against morbidity and mortality from any NDV challenge virus. Currently, poultry in the U.S. are vaccinated for NDV but the vaccines do not induce sterilizing immunity. In addition, the genotypes of the vaccines are different than the genotypes of the viruses that currently cause disease. Vaccinated birds may not show obvious signs of ND and the amount of virus shed will be reduced but not eliminated. If high enough levels of virus are shed, transmission to other susceptible birds is possible. For these studies the following hypothesis was tested: increasing the genetic relatedness of the NDV vaccine virus to the likely virulent challenge virus would produce more specific neutralizing antibodies which would decrease the amount of challenge virus shed from vaccinated poultry. To evaluate this hypothesis, a series of experiments were designed that focused on vaccinating specific pathogen-free (SPF) white Leghorns chickens with different NDV vaccines and then measuring how much virus was shed after challenging the birds with virulent strains.

The first experiment tested inactivated vaccines made from NDV genotypes different than that of the challenge virus along with an inactivated virus homologous to the challenge. The challenge virus is the virulent virus from the most recent outbreak in the USA, the California/212676/2002 (CA02) virus. The second experiment used both inactivated and live vaccines against the CA02 challenge virus. In addition to the commonly used B1 (chicken/U.S./B1/1948) vaccine, this experiment also utilized a recombinant virus that is in the same genotype as the CA02 virus but is not as virulent, therefore, it could be used as a live virus vaccine (Estevez et al., 2007). This recombinant virus (rA) was rescued from an infectious clone of Anhinga/U.S/(FL)/44083/1993. This backbone, rA, was manipulated using reverse genetics to include the HN gene from CA02 virus instead of the original HN of the backbone to increase the relatedness to CA02, creating a second recombinant, rA-CAHN, also to be used as a vaccine (Estevez et The third experiment tested only live vaccines and included a third al., 2007). recombinant virus as a vaccine, rA-CAFHN. This recombinant included the fusion (F) and HN of CA02 into the rA backbone. This experiment used not only CA02 but also a genotypically different challenge virus, chicken/U.S./(TX)GB/1948, to further test how the homology of vaccine to challenge virus affects the amount of challenge virus shed. The fourth experiment included a higher dose of challenge virus, a larger number of birds

per group, and a fourth recombinant virus tested as a vaccine, rA-CAHN-LSCL. This recombinant contained the HN of the CA02 virus in the rA backbone and the Anhinga fusion cleavage site was changed to that of LaSota, a commonly used vaccine virus. The fifth and last experiment tested only two live vaccines: LaSota and rA-CAFHN, the recombinant with the HN and F of the CA02, against a high challenge dose of CA02 or TXGB. A series of experiments aimed at comparing the detection of the amount of virus in oropharyngeal and cloacal swabs when using virus isolation in embryonated chicken eggs compared to using reverse transcription polymerase chain reaction-based methods were also initiated.

Currently, NDV vaccines are evaluated by regulatory agencies in the U.S. and in other countries for their ability to protect chickens from morbidity and mortality from challenge. The amount of virus that is shed from vaccinated birds after exposure to a virulent virus is not taken into account. These experiments were designed to provide a comprehensive assessment of viruses commonly used in NDV vaccines in the U.S. and recombinant NDV vaccines prepared with viruses of the same lineage as CA02 and their ability to reduce or prevent viral shed after a challenge with virulent CA02 NDV or virulent TXGB strains. While the "one serotype" hypothesis is still likely correct, it may be improved upon by increasing the relatedness of the vaccine antigen to the circulating virulent NDV strain allowing a better protective response against a virulent NDV infection of vaccinated birds. References

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CHAPTER 2

LITERATURE REVIEW

History of Newcastle disease

Newcastle disease (ND) is major disease of poultry and presents a worldwide problem for poultry producers. The disease results from infections with virulent Newcastle disease viruses, defined as strains having intracerebral pathogenicity indices of > 0.7 in day old chickens (*Gallus gallus*) and/or having multiple basic amino acids (at least three arginine (R) or lysine (K) residues) at the C-terminus of the fusion protein cleavage site along with a phenylalanine at position 117 (International Office of Epizootics. Biological Standards Commission., 2004). ND was first reported in 1926 on the island of Java, Indonesia. A year later the disease was recognized in poultry flocks in Newcastle-on-Tyne, England and found to be due to a virus (Alexander, 1980; Doyle, 1927). Mouth exudates from infected birds were still infectious after filtration with one of three different types of filters used to remove bacteria. In addition, no bacteria could be cultured aerobically or anaerobically from organs of infected birds. Doyle suggested this disease be named for this city in which it was identified. Reports from Central Europe exist suggesting earlier ND episodes, but due to the lack of definitive evidence ND is considered to have been first recognized in 1926 (Halasz, 1912). In the past ND has been referred to as avian pneumoencephalitis, Korean fowl plague, Tetelo disease, Ranikhet disease, avian distemper, avian pest, pseudo-poultry plague, atypische Geflugelpest, pseudovogel-pest, and pseudo-fowl pest (Alexander, 2003). In 1927 Doyle

reports that in Europe and Asia the birds with ND presented with respiratory and nervous signs, small hemorrhages in internal organs in 20% of the birds, along with high mortality rates (Doyle, 1927). In 1944 Beach reported that an isolate from a flock with pneumoencephalitis, a disease that had been prevalent in California poultry flocks for the previous nine years, was neutralized by Newcastle disease immune serum (Beach, 1944). The clinical signs in the California birds differed from the symptoms observed in European outbreaks in that the respiratory and nervous signs were more mild and the mortality was low (Beach, 1944). Over the following decades, the international transport of psittacine birds and poultry (initially by boat and eventually by air transport) along with the commercialization of the poultry industry assisted the spread of the virus around the world (Lancaster, 1975).

There have been four recognized panzootic episodes of ND. A panzootic is a disease that affects animals of many species over a wide area. The first one began in 1926 and took sixteen years to spread (Lancaster, 1975). The second began in the late 1960's and spread more rapidly, only taking four years to spread across the globe (Alexander, 2003). As the third panzootic spread in the early 1970's, there was an increase in the number of countries affected by ND and also a change from the virulent viruses that typically caused neurological symptoms with moderate mortality to viruses that caused intestinal lesions and higher mortality in unvaccinated birds (Lancaster, 1975). In 1970 and 1971 the United States (U.S.) was affected with outbreaks of ND in California, Florida and Texas in which these more virulent viruses were isolated (Butterfield and Graves, 1975). By the mid 1970s many birds were vaccinated for ND and, therefore, did not die upon infection. This complicated the control of ND in the U.S.

after it was determined that government sponsored vaccine teams were unintentionally spreading the disease as they vaccinated seemingly healthy, but infected flocks. The infected birds shed the virus via oral secretion and fecal matter (Alexander, 2001). Since 1973, the U.S. has restricted the importation of exotic birds by requiring that they be quarantined and tested for NDV before entering. The last panzootic mostly affected pigeons whose symptoms were more neurological without respiratory signs (Alexander, 1988a). By 1981 the virus had spread around the world and in England poultry flocks were infected by feed contaminated with pigeon feces and carcasses. The U.S. contained and eradicated the last outbreak of exotic ND (END) in 2003 and remains free from END as described by the Office of International Epizooties (OIE). However, low virulence ND viruses which circulate among waterfowl and from vaccinated poultry are commonly isolated (Zanetti et al., 2005). A continuation of strict biosecurity and vaccination of poultry remains necessary to prevent the introduction of virulent strains of NDV.

The prevalence of ND throughout the world changes rapidly. At least 150 countries have reported ND outbreaks to the OIE World Animal Health Database (www.oie.int/wahid) and virulent viruses are endemic in most of these. Fifteen countries reported active or resolved outbreaks of ND in 2006. In the last six months of 2007 the OIE reports ND outbreaks continuing in Italy, Sweden, Slovenia, Slovakia, Greece, Bulgaria, and the Republic of Macedonia. For that same time period, countries reported as having recently resolved ND outbreaks are Romania, Serbia, Estonia, Botswana, Honduras, and Chile. While commercial poultry producers have reliable programs of surveillance and reporting diseases, backyard birds and village poultry may not be as closely watched. In addition, many countries (including the U.S.) have vaccination

programs that include live vaccines. The use of live vaccines without markers and/or the ability to distinguish vaccinated from infected birds adds another complication to the ND control program.

Etiology

Newcastle disease virus is also known as avian paramyxovirus serotype-1 (APMV-1) (Alexander, 2003) and is a member of the genus Avulavirus (Fauquet and Fargette, 2005; Mayo, 2002) in the *Paramyxoviridae* family. It is a negative-sense, single stranded, non-segmented, 15.2 kb enveloped RNA virus that replicates in the cytoplasm (Alexander, 2003; Lamb, 2007). The envelope is a lipid bi-layer and is acquired from the host cell that the virus has infected (Rifkin and Quigley, 1974). As seen in Figure 2.1 (a), NDV is composed of six genes and their corresponding six structural proteins, listed from 3' to 5': nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and the large RNA polymerase (L) (Hamaguchi et al., 1983). RNA editing of the P protein produces two additional proteins, V and W (Chambers and Samson, 1982; Collins et al., 1982). When one guanine residue is added to the conserved editing site of the mRNA by the RNA dependent RNA polymerase (RNAP), the V protein is produced after transcription. The addition of two G residues results in the production of the W protein. It is believed that the RNAP adds the G residues in a similar manner that stuttering adds four to seven uracil bases. The carboxy-terminus portion of the V protein has been shown to have anti-interferon activity, which allows the virus to reduce this response of the host's innate immune

system (Park et al., 2003). The function of the W protein is unknown (Huang et al., 2003).

The HN and F are glycoproteins that allow binding and fusion to host cells (Figure 2.1 b). The HN is a homo-tetrameric, type II integral membrane attachment protein that binds to the host cell receptors and is able to agglutinate red blood cells (Villar and Barroso, 2006). Infection of birds can be confirmed by testing sampleinoculated egg fluids for hemagglutination, followed with hemagglutination-inhibition assays using serum with NDV antibodies. The neuraminidase portion of the HN prevents the progeny virions from clumping to the surface of the cell from which they are being released, and may possibly prevent the virus from being trapped in secretions of the host that contain cell receptors that the virus recognizes (Villar and Barroso, 2006). The neuraminidase activity of the HN protein allows red blood cells (RBC) to eventually elute from the virus when the host receptors are degraded by the HN protein (Alexander, 2003). It has been shown that NDV isolated from wild birds may agglutinate RBC from different species than those isolated from poultry (Ito et al., 1999). Ito and co-workers showed that, in general, isolates from wild birds were able to agglutinate horse, pig, mouse, human, cow and chicken RBC, while the chicken isolates were mostly only able to agglutinate chicken and cow RBC. This suggests that the receptors may be slightly different and that this difference may affect how easily that virus can be transmitted from a wild bird to a chicken. There is also evidence that the HN protein plays a role in cell to cell and virus to cell fusion by enhancing the activity of the F protein (Lamb, 1993). The HN protein of a few avirulent isolates such as Ulster 2C/1976, D26/1976 and V4 Queensland/1966, all class II/genotype I viruses, and Alaska 196/1998 (class I) is made



Figure 2.1. Structure of NDV genome and virion. (a) Schematic of the linear organization of the NDV genome. The virion is a negative sense virus represented by the (-), and the genome starts with the leader (l) sequence and has a trailer (t) at the 5' end. The Phosphoprotein (P) is edited to produce the W and V proteins. (b) Schematic of NDV with glycoproteins HN and F exposed on the lipid bilayer envelope. The amino (N) end of the F protein and the carboxy (C) end of the HN protein are also on the surface of the virus. The F protein is shown in the un-cleaved (F0) and cleaved (F1 and F2) form.

in a precursor format of HN0. Posttranslational cleavage is needed to remove a fortyfive-residue region of a glycosylated extension on the carboxy terminus that is thought to impair the function of the HN (Gotoh et al., 1988; Miller et al., 2007; Scanlon et al., 1999). These HN0 HN proteins are longer, having 616 amino acid compared to either 577 or 571 amino acids for the HN proteins that are active and do not need to be cleaved (Sakaguchi et al., 1989). HN0 can be cleaved by trypsin and by other proteases (chymotrypsin, thermolysin, elastase) that do not cleave the fusion protein of these low virulence viruses (Nagai and Klenk, 1977).

The trimeric F protein allows fusion of the virus to the host cell and is a type I integral membrane protein with the transmembrane domain located in the C-terminus (Lamb, 2007). The F protein is always produced as a precursor molecule, F0. The F0 of a low virulence virus containing a single basic amino acid at the fusion cleavage site is expressed on cell surface and relies on exogenous proteases to be cleaved which occurs primarily in the respiratory or gastrointestinal tract (Rott, 1979). F0 of a virulent virus having multiple basic amino acids is cleaved as it is transported through the trans Golgi at the N-terminus into F1 and F2 rendering the virus activated and infectious facilitating systemic replication (Rott, 1988). This is typically facilitated by host cell proteases, but can also be performed by bacterial proteases (Nagai et al., 1976). For cleavage of the F0 to be complete the host also needs to provide a second protease specifically a carboxypeptidase to remove basic amino acids (Lamb, 2007). The type of host proteases able to cleave the different F0 molecules determines if the virus can replicate systemically, as seen with virulent viruses, or primarily in the respiratory or gastrointestinal tract, as seen with the more mild viruses (Rott, 1979). The less virulent viruses having one basic amino acid at their fusion cleavage sites can be cleaved by only trypsin and trypsin-like proteases. The location of these proteases limits the location of where the virus can spread in the body. Collins and coworkers in 1993 compared the deduced amino acid sequence of many strains of NDV and showed that the site that F0 is cleaved contains multiple basic amino acids (Collins, 1993). Usually the sequence of the fusion cleavage site of a highly virulent virus is described as starting at position 112 as: ¹¹²R-K/R-Q-K/R-R-F¹¹⁷ (Alexander, 2003). The less virulent viruses have fewer basic amino acids in those positions and a leucine instead of a phenylalanine at position 117. The phenylalanine at position 117 is necessary for virulence, but its role in pathogenicity is unknown (Alexander, 2003). Furin, or another similar subtilisin-like (serine) endoprotease, and another protease homologous to blood clotting factor Xa that is a member of the prothrombin family have both been implicated as the protease responsible for cleaving the F0 proteins from virulent viruses with multiple basic amino acids (Gotoh et al., 1990; Gotoh et al., 1992; Klenk, 1984).

The matrix protein (M) is largely conserved and associates with the inner surface of the viral membrane (Pantua et al., 2006; Peeples and Bratt, 1984). The NP, P, and L proteins form the ribonucleoprotein complex, which is the template for RNA synthesis (Yusoff, 2001). The NP of NDV is herringbone-like structure (Alexander, 1988b). Newcastle disease virions are pleomorphic, and may be round with a diameter between 100 to 500 nm or filamentous in shape with a diameter of 100 nm (Alexander, 2003). The 3' end of the genome contains a leader sequence that is transcribed to produce the smallest and most abundant mRNA that shares a high degree of homology with the 5' end trailer sequence (Peeters et al., 2000). These sequences are involved in the regulation of replication, transcription, and encapsidation of genomic and antigenomic RNAs (Lamb, 2007). Like other RNA viruses, NDV exists as a heterogeneous population referred to as quasi-species, which provides the virus with high variability to survive under different selective pressures (Kattenbelt et al., 2006).

Virulence Phenotype

Lacking molecular techniques to easily categorize and identify endemic viruses from outbreak viruses, ND isolates historically have been divided into three groups based on their virulence in poultry: lentogen (low virulence), mesogen (moderate virulence) and velogen (high virulence) (Alexander, 2003). Standardized pathogenicity tests are used to clarify NDV pathotypes that were created based on clinical signs. The mean death time (MDT) using chicken embryos, and *in vivo* tests in poultry, such as the intracerebral pathogenicity index (ICPI) in day old chicks and the intravenous pathogenicity index (IVPI), determines the category of virulence of the isolates (Alexander, 2003; King, 1996a; King, 1996b). MDT, originally described in 1955, relies on the ability of virulent viruses to kill embryos faster than the less virulent viruses (Hanson and Brandly, 1955). The ICPI is currently used to differentiate endemic lentogenic viruses from more virulent mesogenic and velogenic viruses. The IVPI is used to distinguish mesogenic strains from the more virulent velogenic strains. The guidelines for the ICPI, IVPI, and the MDT are listed in Table 2.1.

Lentogenic vaccine-like viruses of low virulence, which are commonly isolated in the U.S., can cause respiratory disease and decreased productivity in chickens with concurrent bacterial infections, immunosuppressive viral infections, or in those flocks

Pathotype	ICPI ^a	IVPI ^b	MDT ^c
Viscerotropic velogenic	>1.5-2.0	2.0-3.0	<60 hours
Neurotropic velogenic	> 1.5-2.0	2.0-3.0	<60 hours
Mesogenic	<u>>0.7-1.5</u>	0.0-0.5	60-90 hours
Lentogenic	0.2-0.7	0.0	>90 hours
Asymptomatic	0.0-0.2	0.00	>90 hours

a-ICPI: Intracerebral pathogenicity index- Ten 24-40 hour old SPF chicks are inoculated with 0.05 ml of a 1:10 dilution of bacteria-free, isotonic, NDV containing allantoic fluid and watched daily for 8 days. Birds are rated 0 = normal 1 = sick and 2 = dead. The ICPI is the mean score per bird observation.

b-IVPI: Intravenous pathogenicity index- Ten 6 week old SPF chickens are inoculated intravenously with 0.1 ml of 1:10 dilutions of bacteria-free, isotonic, NDV containing allantoic fluid and watched for ten days. Birds are rated 0 = normal, 1 = sick, 2 = paralyzed and 3 = dead. The IVPI is the mean score per bird observation. c-MDT: Mean death time- 0.1ml of each of ten-fold dilutions are inoculated into the allantoic cavity of five 9-11 day old SPF embryonated chicken eggs and candled twice daily for seven days. Days of egg deaths are noted. The mean death time is the mean time in hours for the highest dilution that killed all of the eggs.

exposed to high ammonia levels. Lentogenic strains have been isolated from birds in live bird markets in both the U.S. and China that produce no clinical signs in their hosts (Kim et al., 2007b; Seal et al., 2005). Lentogens that replicate in the gastrointestinal tissues, such as Ulster 2C/1967 and Queensland V4/1986, are often referred to as asymptomatic enteric viruses. Mesogenic viruses are no longer found in poultry in the U.S. but are actually used in some countries that have endemic virulent strains as a booster vaccine (Kumanan et al., 2005). The correlation between the *in vivo* assay prediction of clinical disease and the presence of multiple basic amino acids in the fusion cleavage site is high with the exception being APMV-1 variant strains, referred to as PPMV-1, isolated from pigeons where the pathogenicity values and the clinical signs in chickens are mild even with the presence of multiple basic amino acids (Collins et al., 1994). Passing viruses multiple times in different cell types or different hosts may change virulence and both increases and decreases in virulence have been noted (Kommers et al., 2003; Lawton et al., 1986; Mohan et al., 2007). Virulent strains can further be divided into viscerotropic velogenic (VVND) or neurotropic (NVND) velogenic depending on the clinical signs and pathological lesions they produced (Alexander, 2003). While NVND may have a lower mortality rate, these viruses are still considered as velogens. Hemorrhagic lesions in the intestinal tract of infected birds distinguish VVND from NVND. Neurological signs may be seen with both VVND and NVND.

Definitions Set Forth by Regulatory Agencies

The United States Code of Federal Regulations, Title 9 (9CFR) defines END as a disease of birds and poultry caused by a velogenic NDV that is acute, rapidly spreading

and usually fatal (USDA, 2006). The OIE definition of ND is an infection of a bird with a virulent APMV-1 virus. Virulent is defined as having an ICPI of ≥ 0.7 or having a fusion protein cleavage site containing multiple basic amino acids. Mesogens and velogens by this definition are defined as virulent NDV (vNDV). These vNDV are not endemic to the U.S. and therefore, are defined by the U.S. as "exotic ND" (END) (USDA, 2006). A proposal was introduced in 2007 for the U.S. to amend the 9CFR to adopt the OIE definition, but at this time it has not yet been modified. One disadvantage associated with the OIE definition occurs when viruses isolated from pigeons without signs of ND have an ICPI ≥ 0.7 in day old chickens which requires reporting to the OIE even if the virus is not infecting poultry (de Oliveira Torres Carrasco et al., 2007). In contrast, there are also viruses isolated from pigeons that are shown to have multiple basic amino acids at their fusion cleavage site (virulent by OIE standards), cause disease in pigeons but not in chickens, and these also require reporting to the OIE which may disrupt trade of poultry and poultry products (Collins et al., 1994).

Strain Diversity

Although all NDV are members of APMV-1, antigenic and genetic diversity is recognized (Aldous et al., 2003; Alexander et al., 1998). For example, antigenic diversity can be seen in Table 2.2 by the different binding patterns to monoclonal antibodies produced by viruses in different genotypes: Ulster vaccine virus (class II, genotype I), B1 vaccine virus (class II, genotype II), CA02 virulent viscerotropic virus (class II genotype V), Pigeon84, a pigeon APMV-1 variant (class II genotype VIb), and AK196, an avirulent class I waterfowl virus. Table 2.2. HI test results of NDV isolates against polyclonal antiserum and NDV specific monoclonal antibodies (MAb) (Miller, unpublished).

MAbª	NDV Isolates					
	Ulster	B1	Pigeon84	AK196	CA02	
AVSf	+b	+				
10D119	c					
15C4 ^h	+	+			+	
B79 ⁱ	+	+	+	+	+	
161/617 ^j			+			
P3A11 ^k	+	+			+	
P11C9	+	+			+	
P15D7	+	+			+	
P10B8					+	
+ controld	+	+	+	+	+	
- control∘						

^amAb diluted 1:10, therefore HI negatives (---) are < 10.

^b+ = antibody-inhibited hemagglutination.

°--- = no hemagglutination inhibition.

^d = positive polyclonal antiserum from LaSota vaccinates.

• = normal serum from SPF chicken diluted 1:2.

fAVS reacts with many lentogens.

910D11 reacts with neurotropic velogens and mesogens of Class II/Genotype II.

h15C4 reacts with most NDV (APMV-1) except pigeon Paramyxovirus type-1 viruses (PPMV-1).

ⁱB79 reacts with most APMV-1 included most PPMV-1. ⁱ161/167 reacts only with PPMV-1 within the APMV-1 group.

*P3A11, P11C9, P15D7, and P10B8 identify additional antigenic epitopes among APMV-1.

Aldous and coworkers (2003) have identified at least six distinct lineages or groupings of NDV based on nucleotide sequence. A more traditional classification using full-length sequence to relate the viruses isolated over time has been reviewed by the Lomniczi laboratory (Czegledi et al., 2006) and shows two major divisions represented by class I and class II, with both classes being further divided into nine genotypes (Kim et al., 2007a). Class I strains commonly isolated from apparently healthy waterfowl, shorebirds and birds from live bird markets are genetically distinct and phylogenetically distant from the commonly isolated vaccine viruses and the more rare outbreak viruses isolated in the United States (Kim et al., 2007a). Genotyping techniques were not yet developed when B1 and LaSota vaccines were developed. The U.S. isolates of NDV identified in the 1940s and the vaccines used today to control ND are Aldous lineage 2 (class II genotype II) viruses. However, since the 1970s, Aldous lineage 3c (class II genotype V) viruses have caused the END outbreaks in Mexico, the United States and Canada.

Genotypes

APMV-1 viruses have at least three genome lengths; 15,186, 15,192 and 15,198 nucleotides (Czegledi et al., 2006). Class I viruses are avirulent in chickens (except for one known virulent virus), typically recovered from waterfowl (Family Anatidae), and recently have been divided further into nine genotypes (1-9) based on a 374 base-pair portion of the F gene (Alexander et al., 1992; Kim et al., 2007a). Class II viruses are divided into nine (I-IX) genotypes. Genotype II viruses include the avirulent vaccine viruses used in the USA such as LaSota and B1 but also the neurotropic velogenic,
virulent chicken/U.S./(TX)GB/1948 (TXGB) which was isolated in 1948 and is used in the USA as a challenge to show efficacy of ND commercial vaccines before production. Genotype III viruses were mostly isolated before 1960 in Japan, but have been isolated sporadically in Taiwan in 1969 and 1985 and Zimbabwe in 1990 (Yu et al., 2001). Genotype IV viruses were the predominant viruses isolated in Europe before 1970 (Czegledi et al., 2006). Genotypes V, VI, VII, and VIII contain only virulent viruses. Genotype V viruses emerged in South and Central American in 1970 and caused outbreaks in Europe that same year. These viruses also caused outbreaks in North America in Florida (1971, 1993) and California (1971, 2002)(Wise et al., 2004a). Genotype VI emerged in the 1960s and continued to circulate as the predominant genotype in Asia until 1985 when genotype VII became more common (Mase et al., 2002). Genotype VI is further divided into sub-lineages VIa through VIg with VIb being commonly isolated from pigeons. Genotype VII was initially divided in to two sublineages: VIIa, representing viruses that emerged in the 1990s in the Far East and spread to Europe and Asia and VIIb, representing viruses that emerged in the Far East and spread to South Africa (Aldous et al., 2003). The two sub-lineages of VII were then further divided into VIIc, d, and e and represent more isolates from China and South Africa (Wang et al., 2006). Genotype VIII viruses have been circulating in South Africa since 1960s (Abolnik et al., 2004). Genotype IX is a unique group that contains the first virulent outbreak virus from China from 1948 and members of this genotype continue to occasionally be isolated in China (Wang et al., 2006). The genotypes that are considered "early" (1930-1960) I, II, III, and IV contain 15,186 nucleotides. Viruses that emerged "late" (after 1960), V, VI, VII, VIII, and IX contain 15,192 nucleotides. Genotype IX

viruses were initially isolated in 1948, an exception to the "late" grouping. Class I viruses are the longest of the APMV-1 genomes at 15,198 nucleotides.

Potential Explanations of NDV Evolution

Before 1990 it was believed that either 1) NDV was always present in the environment and only when the poultry industry grew was the loss of income through bird deaths noticed or 2) NDV was enzootic in some unknown or wild bird species that harbored the virulent virus, passing it to poultry while itself being unaffected by the virus (Alexander, 2003). Data from several NDV outbreaks in poultry that originated in psittacines in the 1970s, pigeons in the 1980s, and by cormorants (to turkeys) in the 1990s provided evidence of the second theory seemed very likely. After isolates from an outbreak in Ireland in 1990 were analyzed, another hypothesis arose. This theory stated that low virulence viruses circulating in and among the waterfowl of Ireland, was transmitted to poultry and mutated to form quasi-species of which a virulent class I virus arose (Alexander et al., 1992; Collins, 1993). This hypothesis was strengthened when isolates from outbreaks in Australia were shown to be genetically similar to the low virulence, class II genotype I viruses that were known to be circulating in the country (Kattenbelt et al., 2006). These endemic low virulence viruses required only two point mutations to become virulent (Alexander, 2003). Recently there have been multiple accounts from China of recombination between different genotypes, one of which is usually a genotype II vaccine virus, allowing the emergence of chimeric ND viruses (Han et al., 2008; He et al., 2008; Qin et al., 2007). Three viruses isolated from Chinese outbreaks are reported to have lentogenic fusion cleavage sites, including the lack of a

phenylalanine at position 117 (¹¹²G-R-Q-G-R-L¹¹⁷), but at the same time have ICPI and IVPI values of virulent viruses (Tan et al., 2007). Further characterization of these isolates may give information on the possibility and prevalence of "natural" recombination among ND viruses. It is likely that all three theories help explain the genesis of Newcastle disease.

Hosts of NDV

NDV is reported to infect over 200 species of birds, both wild and domestic (Alexander, 2003; Kaleta, 1988). Turkeys (Meleagris gallopavo) are as susceptible to infection as chickens (Gallus gallus), but clinical signs are less severe (Alexander, 1999; Box et al., 1970; McFerran, 1988). Waterfowl and shorebirds are felt to be the most clinically resistant among the wild bird populations (Kaleta, 1988). Domesticated and feral pigeons (Columba livia) are known to carry virulent NDV and have not only infected poultry but also have been infected by chickens (Alexander et al., 1984; Pearson et al., 1987). Viruses have been isolated from cormorants and anhingas (Anhinga anhinga) and are maintained in kidney tissue for long periods of time (Kuiken, 1999). The most important outbreaks in wild birds have been in double-crested cormorants (Phalacrocorax auritus) (Blaxland, 1951; Kuiken et al., 1998). Ostriches (Struthio *camelus*) are also susceptible to NDV infection (Verwoerd et al., 1997; Verwoerd et al., 1999). Many animals including reptiles and humans are susceptible to NDV infection (Alexander, 1995). NDV will replicate in human conjunctival tissue and has been implicated in the possible death via pneumonia of an immunocompromised organ transplant recipient (Alexander, 1988c; Goebel et al., 2007; Swayne and King, 2003). In the early 1970's, imported parrots led to a ND outbreak that spread to the poultry industry in California and in 1991 an outbreak of ND in pet birds was described in six states likely due to illegal importation of pet birds (Hanson et al., 1973; Panigrahy et al., 1993). The isolation of virulent APMV-1 in 1999 from a parrot supports the continued quarantine rules and regulations imposed by the United States Department of Agriculture (Granzow et al., 1999; USDA, 2006).

Transmission, Spread and Maintenance of NDV

Newcastle disease virus is spread horizontally to susceptible birds through the inhalation or ingestion of respiratory secretions and fecal matter from infected birds (Alexander, 1988c). The infective dose of NDV per bird will depend on the virus and the susceptibility of the host. In general, the infective dose of a virulent NDV for a susceptible chicken will be in the range between 10^3 to 10^4 median embryo infectious dose 50 (EID₅₀) (Alexander, 1999; King, 1996b). Virulent NDV, being a systemic infection, can be found in the egg of infected breeder birds, but since infected birds typically have decreased egg production, it is thought that few infected eggs are laid. Because chicken embryos are highly susceptible to infection with resulting embryonic death, it is unlikely that vertical transmission of the virus occurs, although there have been reports of hatchlings born infected with vNDV (Capua et al., 1993; Chen and Wang, 2002; Roy and Venugopalan, 2005). In addition, eggs with fissures and cracks contaminated with fecal matter containing NDV may become infected. NDV in the environment from contaminated tissues, and feces can persist for days and can be spread indirectly on contaminated equipment, litter, soil, feed, vaccines and water. Vermin, wild

birds, insects, and people all need to be considered as possible routes of exposing poultry to NDV. In addition, having members of a flock come into a house together and leave at the same time (all in-all out) and excluding water sources that have exposure to wild birds are a necessary part of biosecurity. NDV has been recovered from the air of poultry houses containing infected birds (Delay, 1948), and there is evidence that the virus can be spread from an infected flock to a susceptible flock through the air (Hugh-Jones et al., 1973). Transmission of avian influenza (AI) virus has been shown to be dependent on the relative humidity and ambient temperature and it is likely the NDV stability is also affected by these factors (Lowen, 2007). Lowen and co-workers found that the transmission of AI viruses was favored by cold, dry conditions that extended the length of time that the virus was shed. Wild birds are known to have a potential role in the transmission of NDV to poultry (Aldous et al., 2007; Banerjee et al., 1994; Blaxland, 1951; Roy et al., 1998; Vickers and Hanson, 1979). Typically viruses with low virulence are isolated from wild birds in the U.S. (Kim et al., 2007a). Cormorants have been implicated in ND outbreaks in turkeys raised in open range environments with access to the same water sources as the cormorants (Heckert, 1993; Heckert et al., 1996).

Imported psittacines infected with NDV were the cause of the outbreak in California in 1971 (Utterback and Schwartz, 1973). Transmission of NDV to poultry through various insects has been investigated since the 1970s (Rogoff et al., 1975). Small amounts of CA02 NDV were isolated from flies collected from two residences that had backyard poultry infected in the CA02 outbreak (Chakrabarti et al., 2007). Laboratory infected house flies have been shown to be able to carry small amounts of NDV in their intestinal tracts for up to 96 hours (Watson et al., 2007). A novel, "upwards

vertical", route of transmission of avian polyomavirus was discovered where blowfly larvae transmit the virus to nestlings, which then pass the virus on to the parents (Potti et al., 2007). Based upon these findings, it is clear that insect control should be taken into consideration when biosecurity measures are planned for a facility. Vaccinated poultry can shed virus for at least nine days after vaccination (Kapczynski and King, 2005). Parrots have been shown to be able to shed virulent virus sporadically for years (Erickson et al., 1977). While virulent NDV has been isolated from various wild birds, the reservoir for NDV is still unknown.

Incubation, Clinical Signs and Immunity

The incubation period for NDV is usually five to six days, but can vary from two to fifteen and will depend on the species of the host, the immune status of the host, and the virulence of the virus (Alexander, 2003). Clinical signs of ND in chickens may include a marked drop in egg production followed by depression, respiratory distress, hemorrhage in multiple organs, neurological signs and acute death. In unvaccinated chickens there may be sudden death without any symptoms of illness. Vaccinated birds may show no signs of disease upon infection with NDV. However, neurological signs such as body or head tremors or torticollis may be seen ten to fourteen days after infection. The various signs and symptoms seen with ND are not pathognomonic, and therefore clinical signs alone are not specific enough for a diagnosis. Factors that determine the outcome of the disease include the host species, age, immune status, coexisting infections, and the environment of the host (Alexander, 2003; Lancaster, 1975). Feral birds such as pigeons and cormorants may not show any signs of disease upon infection with a virulent NDV. Other diseases that may resemble an infection with a virulent ND due to a high mortality rate and similar lesions are highly pathogenic avian influenza, fowl cholera, laryngotracheitis, and diptheric fowl pox (Alexander, 2003).

NDV infection induces active immunity (cell-mediated immunity (CMI), humoral, and mucosal) and allows passive immunity to be transferred to embryos (Beard and Brugh, 1975; Ewert et al., 1977; Gough and Alexander, 1973; Holmes, 1979). CMI occurs two to three days after NDV vaccination (Ewert et al., 1977) and is thought to provide protection to vaccinated poultry early in an infection when birds were found to have low antibody response (Gough and Alexander, 1973). However, it has also been shown that specific CMI induced by live or inactivated B1 NDV by itself is not protective against virulent NDV challenge using Texas-GB (Reynolds and Maraqa, 2000b). CMI induced from a live vaccine occurs earlier and stronger than that induced by inactivated vaccine (Lambrecht et al., 2004). Humoral immunity is essential for protection to ND with antibodies arising in serum six to ten days after infection, and a peak response three to four weeks later. Neutralizing antibodies primarily bind to virions preventing attachment to cells, which reduces the production of progeny and inhibits viral spread (Al-Garib, 2003). Neutralizing antibodies are measured using virus neutralization (VN) tests or hemagglutination inhibition assays that correlate well to VN (Alexander, 2003). While antibodies to the HN and F provide protection, those to the internal proteins do not (Reynolds and Maraqa, 2000a). Antibodies to HN inhibit the ability of the virus to attach to the host cell and are measured by the hemagglutination-inhibition (HI) assay. Antibodies to the F protein block the virus from entering the host cell. Local immunity ascribed to immunoglobulin A (IgA) exposed on mucosal surfaces, helps to

limit replication of the virus, but does not clear the viral infection (Al-Garib et al., 2003; Holmes, 1979; Russell and Ezeifeka, 1995). Passive immunity from maternal antibodies passed to embryos via the egg yolk may be protective depending on the amount of antibody transferred, and the dose and the virulence of the challenge virus. If present at the time of vaccination with a live vaccine these antibodies will neutralize the live vaccine NDV antigen and can lead to vaccine failure (Heller et al., 1977). Other factors that have been shown to impact clinical signs and immunity seen in poultry infected with NDV are nutrition and breed. Too little of a nutrient (Vitamin A) or too much of nutrient (Vitamin E) may lead to sub-optimal immune response (Friedman et al., 1998; Rombout et al., 1992; Sijtsma et al., 1991a; Sijtsma et al., 1991b). In the chicken, IgM, IgY (avian IgG equivalent), and IgA are produced as part of the immune response (Jeurissen et al., 2000). Experimentally, using trinitrophenyl-conjugated keyhole limpet hemocyanin as an antigen, in the Ross 508 breed of chickens, broiler-type birds, produced a high IgM response. This response in these broilers was limited to non-specific cell mediated immunity that was short lived. In contrast, white Leghorns, layer-type chickens, produced a high IgY response, with both specific and non-specific cell mediated immunity that was long lasting (Koenen et al., 2002). Interestingly, with some ND viruses, broilers (white Rocks) seem to be more resistant to morbidity than layers (white Leghorns) (King, 1996b). Variations in immune responses also have been reported with in ovo vaccines from two different lines of chickens (Dilaveris et al., 2007). Dilaveris and co-workers found that when vaccinating two SPF white Leghorn lines (line 0 and VALO light Sussex line) at eighteen days of age in ovo with live Poulvac®NDW vaccine, a strain related to Ulster 2C/1967, that 32% of the line 0 and 10% of the VALO

embryos died. Interestingly, surviving hatchlings of both groups shed virus in oral secretions and feces for eleven days and at amounts large enough to pass to sentinel birds.

Gross Pathology

Infection with NDV can lead to subtle pathological changes. Hemorrhages in the eyelids, spleen, tracheal, proventriculus, Peyer's patches, cecal tonsils, bursa, and thymus are commonly seen with ND in poultry when infected with a viscerotropic virus (Kommers et al., 2002). In addition, the spleen may be enlarged, mottled and necrotic (Wakamatsu et al., 2006a). Small hemorrhages and cyanosis may be seen in the comb and wattles. The gastrointestinal tract is often empty and the bursa small after four days post-infection. The neurotropic type of NDV does not induce gross lesions in the gastrointestinal tract but there are numerous histopathological lesions (Wakamatsu et al., 2006b). Hemorrhage of the cranial tracheal mucosa is a unique lesion found consistently with the isolates from the CA 2002 outbreak (Wakamatsu et al., 2006a). Gross lesions for lentogenic infections are typically related to thickening of the membranes of the air sacs from inflammation and pneumonia due to the virus or from a secondary bacterial infection. Birds with partial immunity are unlikely to have severe lesions.

Diagnostic Tools

For effective disease management, it is important to be able to identify birds that are infected with NDV and to distinguish vaccine viruses from virulent viruses. Fluids from eggs inoculated with specimens such as oropharyngeal and cloacal swabs from live and dead birds can be evaluated for NDV antigen using a hemagglutination assay (HA). This is a non-specific assay that tests samples from inoculated 9-11 day old specific pathogen-free (SPF) embryonated chicken eggs (ECE) and requires samples be kept refrigerated to ensure virus viability (International Office of Epizootics. Biological Standards Commission., 2004). However, multiple viruses can agglutinate chicken red blood cells (cRBC) including any of the sixteen hemagglutination subtypes of influenza A viruses, any of the other eight avian paramyxovirus serotypes, some adenoviruses, and some bacteria as well as NDV. Hemagglutination positive samples can then be used in a hemagglutination inhibition (HI) assay with NDV specific serum to positively identify the sample. If the sample has NDV antigen, the NDV antibodies will bind it and agglutination of the cRBC will be inhibited. The HI assay is used worldwide not only to test for NDV antigen, but also to show the presence of NDV antibodies (Alexander, 1998). Because the viruses must be grown in ECE this assay takes 5-7 days, which may not be optimal in an outbreak setting. HI results are also dependent on the amount of virus in the antigen, percentage of and type of red blood cells used and the temperature at which the assays are performed. Commercial ELISA kits are available to test for NDV antibodies. These assays, a convenient proxy for protective antibody, measure antibody levels of both the neutralizing antibodies to the F and HN proteins and the non-protective antibodies to internal proteins that are highly expressed (Snyder et al., 1983; Wilson et al., 1984). A variety of immunohistological assays have been developed to detect NDV antigens in tissue samples, but they cannot differentiate virulent virus strains from vaccine virus (Brown et al., 1999; Kommers et al., 2001; Wakamatsu et al., 2007). ELISA tests are convenient in that they can be automated, and they can be completed in a

few hours. Other tests that can be used to detect NDV antibodies include virus neutralization, single radial immuno-diffusion, plaque neutralization, and single radial hemolysis (Alexander, 2003; Chu, 1982; Thayer, 1998).

Molecular techniques using reverse transcription with a polymerase chain reaction (RT-PCR) are often used for rapid detection of the NDV genome in an outbreak setting (Jestin and Jestin, 1991; Wise et al., 2004b). General primers for the M gene are initially used to screen for NDV genome, and positive samples are then tested for primers made to identify a virulent cleavage site (Berinstein et al., 2001). Advantages of the RT-PCR assays are that large number of samples can be rapidly processed in an automated fashion, and they can be used to differentiate virulent from non-virulent strains (Collins, 1993; Peeters et al., 1999). However, primers and probes have to be designed to specific conserved areas of various genes, as mismatches will lead to false negative test results. Unfortunately for NDV there is no universal primer/probe set that can identify all genotypes (Kim et al., 2006). Before PCR based assays were created, monoclonal antibodies were used to glean information about the antigenicity of an isolate by comparing the binding patterns to multiple monoclonal antibodies against already characterized isolates (Alexander, 2003; Heckert et al., 1996). Some antibodies appear to only recognize virulent viruses or avirulent viruses, but with time, as more viruses are characterized, exceptions to what the monoclonal antibodies are known to bind to are found (Alamares et al., 2005). Binding patterns are often compared after viruses are passed multiple times in cell culture or in different hosts to see if the antigenicity changed (Kommers et al., 2003). Other groups have developed experimental methods using oligonucleotide microarrays to detect NDV and avian influenza (AI) simultaneously

(Wang et al., 2007). NDV pathotypes and high pathogenicity hemagglutination subtypes H5 and H7 could be discerned. However, only three of the nine genotypes for class II viruses (and no class I viruses) were tested and pigeon variant APMV-1 (PPMV-1) were not detected because their F cleavage site (¹¹²R-R-K-K-R-F¹¹⁷) differs from the other virulent viruses.

Even though molecular techniques are commonly used for the rapid diagnosis of NDV, virus isolation in SPF ECE followed with HA and HI is the gold standard for NDV identification (EFSA, 2007). As an alternative to isolating viruses in ECE, cell culture may be used. However, viruses that do not have multiple basic amino acids at their fusion cleavage site will not grow without the addition of trypsin to the media, except in chicken embryo kidney cells. Therefore, cell culture is not considered reliable for isolating new viruses, but is a convenient option for growing viruses previously characterized (EFSA, 2007; Gresland et al., 1979; Zaffuto et al., 2008).

Infectious Clones

NDV infectious clones produced through reverse genetics were first reported in 1999 (Peeters et al., 1999; Romer-Oberdorfer et al., 1999) and since then, multiple infectious clones have been reported (Estevez et al., 2007; Krishnamurthy et al., 2000; Nakaya et al., 2001; Swayne et al., 2003). Briefly, rescue of infectious clones involves the viral NP, P and L genes each encoded in an expression plasmid being transfected along with a plasmid containing the entire antigenome of NDV. The plasmids must be under the control of the phage T7 promoter and the cells either need to express the T7 polymerase or a vaccinia virus expressing the T7 polymerase can be added. Transfected cells are harvested and inoculated into SPF ECE and fluids are tested for HA activity. Sequencing should be done to ensure the correct virus is rescued. These engineered infectious clones allow the further manipulation of nucleotides and genes to create various viruses that can be tested for pathogenesis. In addition, molecular tags such as green fluorescent protein (GFP) can be added to allow visualization of infection in pathogenesis studies (Ge, 2006). Tags also allow tissue tropism to be identified (Al-Garib et al., 2003). The effects of various HN and F genes on virulence can be tested (de Leeuw et al., 2005; Estevez et al., 2007; Oldoni et al., 2005; Wakamatsu et al., 2006b). The infectious clones allow other foreign genes to be inserted creating a NDV backbone that may be used as a vaccine for not only NDV but also for additional diseases (Bukreyev et al., 2005; DiNapoli et al., 2007; Huang et al., 2001; Nakaya et al., 2001; Park et al., 2006; Steel et al., 2008; Swayne et al., 2003). Infectious clones allow the glycosylation of the HN and F proteins to be altered to see how they affect virulence and pathogenesis (Panda et al., 2004). Panda and coworkers showed the loss of Nglycosylation from the HN protein slowed the replication process, decreased the ability of the viruses to fuse, and decreased the virulence of these viruses in chickens, but did not affect the ability of the virus to recognize the receptor (Panda et al., 2004). Infectious clones created through reverse genetics allow clarification of pathogenesis and virulence factors for NDV. While the amino acid composition of the F0 protein has been proven to be a virulence factor, the role other genes have in virulence have not been specifically discerned (Peeters et al., 1999). Some infectious clones have lower virulence than the

wild type virus they were created from (Huang et al., 2004; Peeters et al., 1999; Romer-Oberdorfer et al., 1999; Wakamatsu et al., 2006b). It has been hypothesized that if a virus population is restricted, as it is when viruses are plaque purified or rescued as infectious clones, there is a decrease in the diversity found in the population and viruses not as fit are amplified (Duarte et al., 1994; Novella et al., 1999).

Surveillance

All birds imported into the U.S. must enter through one of the three Animal and Plant Health Inspection Service (APHIS) quarantine stations along with proper health certificates and permits (http://aphis.gov). Birds will be tested for NDV and other dangerous contagious bird diseases. APHIS reports select diseases, including ND, to the World Animal Health Organization (OIE). State and USDA veterinarians are trained to identify possible ND when called to look at poultry flocks with high mortality rates. The poultry industry also employs veterinarians to keep track of mortality rates for their flocks. When mortality rates spike in a flock over a threshold level, State veterinarians and APHIS officials are notified so that a diagnosis can be made rapidly and quarantine and culling protocols can be implemented quickly to keep losses at a minimum. The testing of daily mortality of a flock is an important means to monitor disease. Tissues from dead birds, not randomly selected birds in a house, give the best chance for NDV to be recovered.

Alternatively, unvaccinated SPF sentinel birds can be placed in with vaccinated flocks to monitor for ND. The National Animal Health Surveillance System integrates surveillance of state and federal agencies. Tissues and swabs from poultry that possibly could have died from NDV are submitted first to the state veterinary laboratory and then to the National Veterinary Services Laboratory (NVSL) for confirmation of the diagnosis. Investigations to find the source of the outbreak and the index case are initiated and quarantine areas are set up to determine what flocks will be depopulated and to prevent movement of birds into and out of the quarantine areas. Often NVSL will send viruses to the Southeast Poultry Research Laboratory for further evaluation of the phylogeny and to perform pathogenicity testing. Virulent ND strains isolated from wild birds are reported to OIE, but with no trade implications. Following a report of ND in poultry to OIE by the United States Department of Agriculture (USDA), trade partners often ban imports of poultry and poultry products from larger regions of the U.S. may be prohibited from import leading to staggering financial losses. In addition, states not affected by the quarantine likely will ban poultry and poultry from affected states.

Prevention of Newcastle disease

Exotic Newcastle disease is caused by a vNDV that is highly contagious, spreads rapidly, and causes high mortality and economic loss (Al-Garib, 2003). For example, it is estimated that the cost of containing the CA 1971-1973 outbreak equaled \$56 million and exceeded \$200 million for the 2002-2003 outbreak (Kapczynski and King, 2005; Kapczynski et al., 2006; Utterback and Schwartz, 1973). The primary control measure in the U.S. for an END outbreak is to quarantine and depopulate infected or likely exposed animals, with indemnification for losses. Vaccination likely reduces virus shed, but currently vaccination instead of depopulation in the face of an outbreak is not employed.

In the U.S., and in most countries worldwide, the control of ND centers on biosecurity and the vaccination of poultry with both live and inactivated vaccines by poultry producers to prevent outbreaks. Government employed vaccination teams are no longer used in the U.S. due to biosecurity concerns. Optimal vaccination occurs at a time after maternal antibody has waned allowing the vaccines to induce a good immunological response but before the birds are likely to be exposed to a virulent strain of NDV. The type of program chosen for each poultry flock will depend on several factors beginning with whether the goal is to protect the birds from infection, or from clinical disease and death. At the same time it is important not to induce iatrogenic respiratory reactions, which would lead to economic losses. When defining a vaccination program for a flock factors such as the age, the maternal antibody level, the breed, and the presence of concurrent infections are to be taken into account.

Both live and inactivated vaccines have their advantages and disadvantages, which have been reviewed previously (Bermudez, 2003; Senne et al., 2004). Live vaccines are advantageous because they are inexpensive to produce, are able to be mass applied, induce mucosal immunity, and provide rapid onset of immunity (Marangon and Busani, 2007). Live vaccines are imperfect in that they may cause disease, are inactivated by maternal antibodies, interfere with surveillance and they require proper handling as to not be inactivated before being administered (Senne et al., 2004). Inactivated vaccines are easier to store, are more able to overcome maternal antibodies,

and will not cause the disease for which they are being given to protect (Brugh and Siegel, 1978). The disadvantages of using inactivated vaccines are that they need a large amount of antigen, are expensive to produce, cannot be mass applied and have a slower onset of immunity (Marangon and Busani, 2007).

Turkeys are often vaccinated with ND vaccines with good results, but some morbidity and mortality can be associated from vaccination with live vaccines and upon challenge with a virulent NDV after vaccination (Kelleher et al., 1988; Sacco et al., 1994; Saif and Nestor, 2002). While ND vaccines are only licensed for chickens, turkeys and pigeons, ND vaccines also appear to provide good protection in partridges (Family Phasianidae) and Guinea fowl (Family Numididae) (Alexander, 2003). Ostriches respond favorably to vaccination with ND vaccines and are typically given an inactivated LaSota vaccine (Blignaut et al., 2000; Bolte et al., 1999). Protective immune responses are induced in pigeons vaccinated with PPMV-1 vaccines (Kapczynski et al., 2006; Roy et al., 2000; Stone, 1989). PPMV-1 viruses are AMPV-1 strains isolated from pigeons that are often referred to as variant due to their propensity to bind to different monoclonal antibodies.

Ideally, the goal of a vaccination program is to produce life long immunity with a single vaccine at the earliest age possible without side effects due to mortality, respiratory disease or poor growth. In addition, the vaccine should be administered by mass administration methods, be easy to store, and include markers that allow the differentiation of infected animals from vaccinated animals which could aid quarantine and eradication efforts in an outbreak setting (Veits et al., 2006). The discovery of a vaccine that satisfies all of the previously listed attributes does not appear imminent.

Initially, inactivated vaccines were used to control ND but were noted to be not as effective due to their inability to produce mucosal responses and to be mass applied (Alexander, 1988a). A transition to live vaccines was made in the 1950s when strains of low virulence were characterized and proven to be efficacious for vaccination against similar circulating viruses at the time (Beaudette, 1949).

In addition to the common live and inactivated vaccines, there have been less conventional vaccines created for NDV that include DNA vaccines, virosomes, ISCOMs, NDV as vectors for vaccines, NDV marker vaccines, pox and herpes vectors for expressing NDV proteins, baculovirus system for expressing NDV proteins and NDV proteins expressed in plant products (Boursnell et al., 1990a; Boursnell et al., 1990b; Guerrero-Andrade, 2006; Kapczynski and Tumpey, 2003; Letellier et al., 1991; Loke et al., 2005; Mebatsion et al., 2002; Meulemans, 1988; Mori et al., 1994; Morrison et al., 1990; Nagy et al., 1991; Nishino et al., 1991; Russell and Koch, 1993; Veits et al., 2006). Most of these newer vaccines, while efficacious, have not yet been used in commercial settings due to their production costs being more expensive than the current vaccines (Seal et al., 2000). All of these above-mentioned vaccines do have the potential to be used as a marker type vaccine to allow the differentiation of infected versus vaccinated animals.

Dow Agrosciences has registered but not yet made available commercially their recombinant HN NDV protein expressed from plant cells (www.dowagro.com). Innovax [™] -SB-ND, a recombinant vaccine approved for control of NDV and virulent Marek's disease in 18-day-old chicken embryos, is produced by Intervet, Inc. (www.intervetusa.com) and is currently being marketed. A few companies market a

fowlpox vectored ND vaccine. One example is Vectormune®FP-N (www.biomune.company.com) produced for NDV maternal antibody negative chickens at least one day of age and turkeys at least 4 weeks of age delivered by the subcutaneous route of delivery (Meeusen et al., 2007). Newplex[™], a vaccine made with LaSota NDV conjugated to an antibody allowing an immune response without causing mortality to the embryos for in ovo vaccination is also available (www.lahinternational.com). However, none of the new generation vaccines are widely used because of the higher cost associated with the vaccines with a perception of little advantage over more traditional vaccines.

Today the strains of NDV used to produce ND vaccines (B1/1947, LaSota/1946) being class II genotype II viruses are phylogenetically the same as the outbreak viruses isolated in the 1940's, but phylogenetically they are different from strains causing outbreaks of END in North America since the 1970's. Similar to B1 and LaSota in class and genotype, the VG/GA strain, a class II genotype II virus, isolated from a healthy turkey, marketed and used in many countries as the live ND vaccine, Avinew® by Merial, was isolated in 1989 (Seal et al., 1995). Since all of the APMV-1 ND viruses share similar antigenic epitopes they are considered to be of a single serotype, meaning the antibodies and cell mediated immunity induced by any one NDV would protect from disease and death after a challenge with any other NDV. It is widely recognized that because all NDV isolates are of one serotype, ND vaccines prepared with any NDV lineage, given correctly, can protect poultry from clinical disease and mortality from a virulent NDV challenge (Kapczynski and King, 2005). However, Kapczynski and King showed that current vaccination programs in commercial broilers in the U.S. are not

completely effective at preventing clinical disease and virus shedding after experimental challenge (Kapczynski and King, 2005). This suggests that perhaps the current vaccination programs are not optimized to prevent or at least to decrease viral shed. More recently it was shown that homology between inactivated vaccine strains and the challenge or outbreak virus was important in protecting vaccinates from infection and subsequent shed of ENDV (Miller et al., 2007).

The ability of these vaccines to protect from clinical disease and death does not mean that these same antibodies will equally protect vaccinates from infection and shedding of the virus into the environment in oral secretions and fecal excretions. Indeed, it has long been known that virulent viruses may infect, replicate and be shed by birds without clinical disease when the birds have some level of vaccinal immunity (Capua et al., 1993; Guittet, 1993; Kapczynski and King, 2005; Parede and Young, 1990). Continued vaccination with heterologous vaccines may create a situation as seen with Marek's disease where antigenic variation promotes the generation of viral strains with new virulence characteristics (Perdue, 2000). Vaccinating birds every six months with the correct dosage of NDV vaccine should protect most chickens form morbidity and mortality from a virulent infection. However, optimally vaccinated birds may still shed virulent virus, and sub-optimally vaccinated birds may shed large amounts of virus without showing obvious clinical signs of disease. The use of the hemagglutinin inhibition test has been used to evaluate the level of protection from vaccines to virulent challenge. Vaccinated birds challenged with chicken/U.K./Herts1933/56 having HI titers over sixty-four should not have mortality (FAO, 1979). Titers of 512 or greater should be protective from both morbidity and mortality from a challenge with CA02 virus

(Miller, unpublished observation). The possibility of improving protection from infection and shed induced by NDV vaccines through the manipulation of the vaccine antigen is an exciting and beneficial opportunity for the poultry industry.

Summary

ND has been documented to affect numerous avian species for nearly a century. Several approaches aimed at controlling the spread of the disease have been employed with varying degrees of success. Vaccines are able to protect vaccinates from disease, but do not prevent infection nor control shedding of the virus to other birds. There remains a need for improved strategies for disease control. Possible avenues of research include improved diagnostics, surveillance, and vaccines. While it is likely that NDV will continue to infect poultry throughout the world, conscientious practices and policies can minimize the adverse impact on the poultry industry.

Goals and Objectives

The hypothesis of the proposed research is that increasing the genetic relatedness of the NDV vaccine virus to the likely virulent challenge virus will decrease the amount of challenge virus shed from vaccinated poultry through the induction of a more specific immune response. This hypothesis is based on the following observations. First, a similar strategy for avian influenza has been shown to reduce oropharyngeal viral shed in vaccinated and infected birds (Swayne et al., 2000; Taylor et al., 1988; Webster et al., 1991; Wood et al., 1985). Second, protection from ND correlates with the HI antibody titers at the day of challenge (Kapczynski and King, 2005; Kapczynski et al., 2006) and HI titers are higher when the HN antigen used in the HI assay is homologous with the antigen used to prepare the vaccine (Stone, 1989). Third, it has been shown that circulating NDV isolates are antigenically different from vaccines strains in use (Panshin et al., 2002a; Panshin et al., 2002b; Seal et al., 2005) and it is possible that this difference in antigenicity between vaccines and potential outbreak viruses allows the "one serotype" protection of vaccinated poultry from morbidity and mortality but reduces protection from viral infection and subsequent viral shed after challenge (Kapczynski and King, 2005).

Based on these observations, the experimental focus of this proposal is to evaluate the ability of NDV vaccines manipulated to be more genetically similar to the challenge virus to induce better protection against infection in vaccinates. This will be measured by the reduction of the amount of virulent virus subsequently shed after challenge as compared to the amount of virus shed by the control sham vaccinated birds. Sterilizing immunity with absolute protection from infection is ideal; however, the more attainable goal of this proposal is to develop a vaccine that lowers the amount of virus shed into the environment, decreasing the spreading of virulent virus to susceptible birds.

Specific Aim 1. To determine if vaccines prepared from inactivated strains of different genotypes or lineages of NDV differ in their ability to protect against mortality, morbidity and shed of virus after a virulent challenge with CA02 NDV. More specifically, this aim will determine if vaccines prepared with the homologous strain protect against virus shedding better than the other vaccines.

Specific Aim 2. To determine if live and inactivated vaccines prepared with a recombinant NDV strain (Anhinga 1993) that is in the same genotype (V) as the CA 02 strain are more effective against shed of virus after a virulent challenge with CA 02 NDV. Specifically, this aim will determine if the replacement of the hemagglutinin-neuraminidase (HN) and/or the fusion (F) genes of the CA 02 virus into the recombinant Anhinga strain used for the vaccines increase protection from shed.

Specific Aim 3. To determine if commonly used vaccines such as Ulster, B1 and LaSota and the recombinants mentioned above protect as well against virulent TXGB NDV, a genotype II virus in the same genotype as LaSota and B1.

Completion of these specific aims will directly test the hypothesis that increasing the genetic relatedness of ND vaccine virus to the challenge virus will reduce virus shed in infected birds.

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CHAPTER 3

ANTIGENIC DIFFERENCES AMONG NEWCASTLE DISEASE VIRUS STRAINS OF DIFFERENT GENOTYPES USED IN VACCINE FORMULATION AFFECT VIRAL SHEDDING AFTER A VIRULENT CHALLENGE¹

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Abstract

Strains of Newcastle disease virus (NDV) can be separated into genotypes based on genome differences even though they are antigenically considered to be of a single serotype. It is widely recognized that an efficacious Newcastle disease (ND) vaccine made with any NDV does induce protection against morbidity and mortality from a virulent NDV challenge. However, those ND vaccines do not protect vaccinates from infection and viral shed from such a challenge. Vaccines prepared from ND viruses corresponding to five different genotypes were compared to determine if the phylogenetic distance between vaccine and challenge strain influences the protection induced and the amount of challenge virus shed. Six groups of four-week-old specific pathogen-free Leghorn chickens were given oil-adjuvanted vaccines prepared from one of five different inactivated ND viruses including strains B1, Ulster, CA02, Pigeon84, Alaska196, or an allantoic fluid control. Three weeks post-vaccination, serum was analyzed for antibody content using a hemagglutination inhibition assay against each of the vaccine antigens and a commercial NDV ELISA. After challenge with virulent CA02, the birds were examined daily for morbidity and mortality and were monitored at selected intervals for virus shedding. All vaccines except for the control induced greater than 90% protection to clinical disease and mortality. The vaccine homologous with the challenge virus reduced oral shedding significantly more than the heterologous vaccines. NDV vaccines formulated to be phylogenetically closer to potential outbreak viruses may provide better ND control by reducing virus transmission from infected birds.

Introduction

Newcastle disease virus (NDV), also known as avian Paramyxovirus type-1 virus, is a member of the genus *Avulavirus* (Mayo, 2002) in the *Paramyxoviridae* family. It is a single stranded, non-segmented, enveloped RNA virus with negative polarity (Alexander, 2003). NDV is composed of six genes and their corresponding six structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and the RNA polymerase (L). RNA editing of the P protein produces two additional proteins, V and W. The HN and F are glycoproteins that allow binding and fusion of the virus to the host cells to initiate a NDV infection. Antibodies to HN and F are neutralizing and represent the primary protective component induced by Newcastle Disease (ND) vaccines (Seal et al., 2000b).

Antigenic (Alexander et al., 1998) and genetic diversity (Aldous et al., 2003) are recognized within the APMV-1 serotype. At least 6 distinct lineages of NDV have been identified based on restriction enzyme analysis and nucleotide sequence of the fusion protein gene (Aldous et al., 2003; Ballagi-Pordany et al., 1996). Another classification system using full-length sequence to relate the viruses isolated over time has been reviewed by Lomniczi and coworkers (Czegledi et al., 2006) and shows two major divisions represented by Class I and Class II, with Class II being further divided into at least eight genotypes. This paper will refer to the second classification system when discussing the ND viruses used. The amino acid diversity across NDV sequences available on GenBank® for both the HN and the F genes displays on average a 10% difference between the genotypes of class II and a 15% difference between class I and class II viruses. Amino acid diversity among strains may have been the basis of the report in 1951 that certain NDV strains were antigenically superior to others when used to formulate a killed vaccine (Hanson, 1951).

Historically, NDV isolates have been divided into three groups used to describe their virulence in poultry: lentogen (low virulence), mesogen (moderate virulence) and velogen (high virulence)(Alexander, 2003). Select lentogenic strains are universally used as live vaccines in the commercial poultry industry. Experimental infections of specific pathogen-free (SPF) chickens with these lentogenic vaccine strains cause little to no clinical disease. When these viruses are used in the field they can cause decreased productivity in commercial chickens by inducing a mild respiratory disease, particularly when the birds are infected with other respiratory pathogens or in combination with Virulent NDV isolates, the cause of ND - called exotic environmental stressors. Newcastle disease (END) in the United States (U.S.), are not endemic in the U.S. and can spread rapidly leading to high mortality rates (USDA, 2006). Symptoms of a virulent NDV infection in susceptible birds may include depression, respiratory distress, hemorrhage in multiple organs, neurological signs and acute death. ND vaccines are widely administered to reduce clinical disease from endemic infections with low virulence strains and can provide protection against disease but not infection with virulent outbreak viruses. Consequently, the primary control measure in the U.S. if an ND outbreak occurs is depopulation of infected or likely exposed animals. This can create a significant financial burden, for example the estimated cost for controlling the California 2002-2003 outbreak exceeded \$200 million (Kapczynski and King, 2005).

In the U.S., and in many countries worldwide, ND prevention is focused on biosecurity and the vaccination of poultry with both live and inactivated ND vaccines. Ideally vaccines are administered after maternal antibodies have waned which allows the induction of a good immunological response before the birds are likely to be exposed to a virulent strain of NDV, but because of differences in flock immunity, vaccination is rarely ideally implemented. Both live and inactivated vaccines have their advantages and disadvantages, which have been reviewed previously (Senne et al., 2004). Today the strains of NDV used to produce ND vaccines in the U.S., such as LaSota and B1, are phylogenetically in the same genotype as viruses isolated in the 1940's, but are phylogenetically divergent from strains causing the recent outbreaks of ND in North America since the 1970's, such as Fontana/1972, Turkey North Dakota/1992, and California/2002 (see Figure 3.1). It is widely recognized that because ND isolates are of one serotype, ND vaccines prepared with any ND lineage, given correctly, can protect poultry from clinical disease and mortality from a virulent ND virus challenge (Beard et al., 1993; Benson et al., 1975; Butterfield et al., 1973). However, even as far back as 1953 the feasibility of one NDV vaccine being able to protect birds from ND without evaluating the factors for each individual outbreak has been questioned (Upton et al., 1953). In 1972, Spalatin noted that the new forms of NDV being isolated in the U.S. are able to infect vaccinated chickens and that these new viruses seem partially resistant to the antibodies induced by the current vaccines (Spalatin, 1972). More recently, Kapczynski and King showed that current vaccination programs in commercial broilers in the U.S. are not completely effective at preventing clinical disease and virus shedding after experimental challenge with a recent virulent strain (Kapczynski and King, 2005). These results along with the susceptibility of vaccinated commercial layers to virulent NDV infection in the California 2002 outbreak suggests the current vaccination programs

may not be optimized. The objective of this study was to compare the protection induced by ND vaccines prepared with viruses of five different NDV genotypes by assessing viral shed from vaccinates in addition to the standard observation of morbidity and mortality after challenge. The comparison was done with inactivated vaccines, the only feasible option to utilize the virulent CA 2002 NDV as both a vaccine antigen and a challenge virus. We found that vaccinating with a NDV homologous with the ND challenge virus induced high hemagglutination-inhibiting antibody titers and significantly reduced the amount of virus shed in oral secretions compared to the heterologous vaccines. Vaccines with the ability to reduce viral shed would enhance the role of vaccination in ND control.

Materials and Methods

Eggs and chickens

Four week old, SPF White Leghorn (WL) chickens obtained from the Southeast Poultry Research Laboratory (SEPRL) flocks were separated into six vaccination groups of 16 birds each. The chickens were wing banded and kept in Horsfall isolation units in BSL 3 Ag facilities and allowed to acclimate for 2 days prior to their being vaccinated. Additional birds from this group were bled and tested by hemagglutination inhibition (HI) assay and ELISA (IDEXX, Westbrook, ME) to confirm that the flock was negative for NDV antibodies. Birds were given food and water ad libitum throughout the experiment. The SEPRL SPF WL flock was the source of the embryonated chicken eggs (ECE) utilized for virus isolation (VI), virus titrations and for the normal allantoic fluid for preparing the control vaccine and for diluting antigens after inactivation. The SEPRL Institutional Animal Care and Use Committee approved all animal experiments.

We chose phylogenetically diverse ND viruses to use as vaccines: Ulster/1967 (Alexander, 2003), B1/1947 (Alexander, 2003), Pigeon/1984 (Pigeon84) (King, 1996a), Alaska196/1998 (Kim et al., 2007) and California/212676/2002 (CA02) (Wakamatsu et al., 2006) (see Figure 3.1 and Table 3.1). Ulster, a Class II Genotype I virus, was originally isolated in Northern Ireland and is used as a vaccine virus in that country. B1, a Class II Genotype II virus, is used worldwide as a live vaccine virus. Pigeon84, a Class II, Genotype VIb virus, is representative of the virulent pigeon paramyxoviruses and has been characterized previously as a mesogen in chickens (see Table 3.2) (King, 1996a; Kommers et al., 2001). Alaska196 is a Class I virus that was isolated in 1998 from a Northern Pintail and represents a group of viruses that are commonly found in waterfowl. Typically Class I isolates do not cause disease in poultry and genetically are highly divergent from the other isolates in the Genotypes of Class II (Aldous et al., 2003). There has been one velogenic Class I virus reported (Alexander et al., 1992). The CA02 virus, a Class II, Genotype V virus, is a velogen that is representative of the recent outbreak in the Southwestern U.S. and is used as a vaccine and challenge virus. Stocks of NDV were obtained from the SEPRL repository, and grown in 9-11 day old SPF ECE by chorioallantoic sac inoculation. Pools of infective allantoic fluid were clarified via centrifugation at 1000 x g for 15 minutes. Infectivity titers of the pools were determined by titration in ECE prior to inactivation, and hemagglutination (HA) titers were determined before and after inactivation (see Table 3.1) (Thayer, 1998). Allantoic fluid for each virus was inactivated with 0.1% beta-propiolactone (BPL) (Sigma, St. Louis, MO) (Spradbrow and Samuel, 1991) for 4 hours at room temperature and kept overnight

at 4°C for hydrolysis of the BPL. Complete virus inactivation was confirmed by failure to recover virus after embryo inoculation (Alexander, 1998). Prior to being stored at -70° C, the pH of the pools of virus antigen as allantoic fluid were adjusted to 7.0 by adding sterile sodium bicarbonate (Gibco, Invitrogen Corporation, Grand Island, NY) (Budowsky et al., 1993).

Vaccine generation

Water-in-oil emulsion vaccines were prepared with virus antigen concentration the equivalent of $10^{8.3}$ EID₅₀ (median embryo infectious dose) of virus prior to BPL inactivation. To achieve this concentration B1, Ulster, and AK196 were diluted with normal allantoic fluid. Pigeon 84, having a lower EID₅₀ titer and HA titer, was concentrated by ultra-centrifugation at 120,000 x g. CA02 was kept at the original concentration. Table 3.1 characterizes each of the viruses used for the vaccine preparation. The oil phase of the vaccine was made by adding 36 parts of Drakeol 6VR (Butler, PA), 3 parts of Span 80 (Sigma, St. Louis, MO) and 1 part of Tween 80 (Sigma, St. Louis, MO) for each vaccine to be made into a working solution. The oil phase was added to each of the virus antigens or normal allantoic fluid (the aqueous phase) to achieve a 4:1 ratio of oil to water as previously described (Stone, 1983). Vaccines were prepared by homogenization in a Waring blender (Fisher Scientific International Inc., Hampton, NH) (Stone et al., 1978) three days prior to administration and kept at 4° C prior to use.

Vaccination studies

Groups were subcutaneously vaccinated with 0.5ml of their appropriate vaccines. Twenty-one days post vaccination serum was collected and the birds were challenged with $10^{5.7}$ EID₅₀ of CA02 virus administered in 50µl into the right eye and 50µl into the choana. Oropharyngeal and cloacal swabs were collected on days 2, 4, 7 and 9 into 1.5 ml of brain heart infusion (BHI) broth (BD Biosciences, Sparks, MD) with a final concentration of gentamicin (200 µg/ml), penicillin G (2000 units/ml), and amphotericin B (4 µg/ml). Birds were monitored daily for clinical signs and death through day 14 post challenge when they were bled and euthanized. Moribund chickens were euthanized with intravenous sodium pentobarbital at a dose of 100 mg/kg and counted dead for the next day. Necropsies were completed on selected birds post-challenge to assess the presence of gross pathological lesions.

VI, HA assay, HI assay, ELISA, Monoclonal antibodies

Virus isolation (VI) and hemagglutination (HA) assays to identify virus positive fluids were conducted as described (Wakamatsu et al., 2006). VI positive samples were titrated in SPF ECE (Alexander, 1998). All virus titers were calculated using the Spearman-Kärber method. Hemagglutination-inhibition (HI) assays (micro-beta) were completed on pre-challenge sera by testing all samples against their homologous and heterologous vaccine antigens (King, 1996b). ELISA assays (IDEXX, Westbrook, ME) were also completed on the pre-challenge serum according to the manufacturer's recommendations. Geometric mean titers (GMT) of HI antibodies were determined for each vaccination group. Each of the vaccine antigens were tested against NDV specific monoclonal antibodies (MAbs) to show antigenic variation among the NDV strains as described (Kommers et al., 2003). The National Veterinary Services Laboratory provided B79, 15C4, 10D11, AVS, and 161/167. P3A11, P11C9, P15D7, and P10B8, prepared at Southeast Poultry Research Laboratory, have been previously described (Kommers et al., 2003). Four HA units of each of the viral antigens were used in completing the HI assay of MAbs, and HI titers equal or greater than 16 are considered positive (International Office of Epizootics. Biological Standards Commission., 2004; King, 1996a).

Nucleotide sequencing

All sequencing reactions were performed as previously described (Kim et al., 2006). Pigeon84 HN and F, CA02 HN and F, and Alaska196 HN were sequenced from cDNA amplified by RT-PCR from Trizol LS (Invitrogen, Carlsbad, CA) extracted RNA using gene specific primers that are available upon request. Sequences have been deposited in the GenBank® under the following accession numbers: EF520717 (CA02 HN), EF520718 (CA02 F), EF20715 (pigeon 84 HN), EF520716 (pigeon 84 F), EF520714 (AK196 HN), and EF612277 (AK196 F). Nucleotide sequences for the complete HN and F proteins for B1 (HN: AF309418 F: M24695) and Ulster (HN: M19478 F: M24694) are available from GenBank®.

Genetic Analysis

The amino acid sequences of the HN and F proteins of the vaccine viruses used in the study were compared by phylogenetic analysis and pair-wise alignment of each isolate with CA 02 with the program Megalign (DNASTAR, Madison, WI). The sequences were aligned using the Lipman–Pearson Method with a Gonnet250 Protein weight matrix and amino acid similarities are shown in Table 3.2 (Lipman and Pearson, 1985).

Phylogenetic tree assembly

Phylogenetic trees were constructed using the maximum likelihood method as implemented in the software package Phyml with the following parameters (Guindon et al., 2005): 100 boot-strapped data set, JTT model of amino acid substitution, fixed proportion of invariable sites, 4 substitution rate categories, 2 gamma distribution parameters and optimization of tree topology, branch lengths and rate parameters. Bootstrap values greater than 75 are reported.

Statistical analysis

Animal experiments were done with 16 chickens per treatment group with the exception of the B1 group in which one bird died pre-vaccination. Serology data are presented as geometric mean titers plus or minus (+/-) standard error. Group means were analyzed by ANOVA with Tukey's posthoc test when indicated. Significance is reported at the level of $P \le 0.05$.

Results

The five viruses chosen to be used as vaccines differed phylogenetically (Figure 3.1, Table 3.1) and antigenically (Table 3.2). In evaluating the deduced similarity for the

HN and F proteins between the CA02 challenge strain and the vaccine strains, Pigeon84 and Alaska196 are respectively the most and least genetically similar (Table 3.2). When using a panel of 9 different monoclonal antibodies, each virus had a different antigenic pattern of reactivity compared to the CA02 virus antigenic pattern (data not shown). The CA02 virus shared six epitopes with Ulster and B1, but only two with Pigeon84 and Alaska196.

The chickens were vaccinated at four weeks of age and the pre-challenge serum 3 weeks after vaccination was analyzed with both a cross-HI assay and a commercial NDV ELISA test (Table 3.3). Up to 5-fold titer differences were observed between Alaska196 and Pigeon84 antigens on mean HI titers when Alaska196 was the vaccine and a 3-fold difference when Pigeon84 was the vaccine. There was a 3-fold difference in mean HI titers of the B1 vaccinates when tested with the B1 and CA02 antigens. The CA02 vaccine strain produced higher serum HI titers to homologous antigen as compared to the other vaccine strains. The ELISA titers from the B1 group were higher than all the other groups, although the B1 HI titers were the lowest. Post-challenge HI and ELISA titers, measured at 14 days, revealed an anamnestic response in all

groups as expected since all vaccinates became infected with the challenge virus (data not shown).

The birds were challenged with the virulent CA02 strain and evaluated daily for morbidity and mortality. The control vaccinated birds and one bird from the Alaska196 vaccination group displayed conjunctivitis with severe depression, before dying or being euthanized between 4 to 6 days post-challenge. Necropsy of these controls and the Alaska196 bird revealed gross lesions consistent with a virulent NDV infection including petechial hemorrhages and edema in the conjunctiva of the lower eyelid, petechial hemorrhages in the thymus, multifocal hemorrhages of the proventriculus and cecal tonsils. Hemorrhage of the cranial tracheal mucosa, a unique lesion described consistently with this CA02 viral infection, was also observed (Wakamatsu et al., 2006).

Neurological signs were seen in two of the vaccinated birds: one B1 vaccinate and one CA02 vaccinate. The B1 vaccinate displayed torticollis, an inability to stand and slight body tremors. Upon necropsy at the end of the experiment, this bird was grossly normal except for petechial hemorrhages in the thymus. The CA02 vaccinate displayed a paralyzed wing, an inability to stand and to keep its head up. This bird displayed no gross lesions of a virulent NDV infection upon necropsy at the end of the experiment. Neither bird had the tracheal lesion previously described. The CA02 vaccinate with neurological lesions had pre-challenge serum HI antibodies to the CA02 antigen of 20 versus the mean titer of 1015 for the other fifteen vaccinates in this group. The B1 bird with neurological signs had a HI antibody titer of 80 to the CA02 antigen, which was similar to the mean HI antibody titer of 118 for the other vaccinates in the B1 group.

All of the oral swabs from the control and vaccinated birds were positive on days 2 and 4 post challenge with titers from all the groups peaking on day 4. By days 7 and 9 the number of vaccinated birds shedding virus was reduced. Table 3.4 demonstrates that there was no significant difference in the frequency of the number of birds shedding among the vaccination groups except for the number of positive cloacal swabs on day 2 for the Pigeon84 vaccinates. At 2 days post-challenge, vaccination with B1, Ulster, and Alaska196 had no effect on oral shedding of virus (Figure 3.2B) as measured by viral

	H	IA titer ^a		
Antigen	Pre	Post	EID ₅₀ ^b	Class/Genotype ^c
B1 Ulster Pigeon84 AK196 CA02	512 2048 32 2048 512	384 2048 1024 ^d 1024 512	$10^{9.7} \\ 10^{9.3} \\ 10^{6.9} \\ 10^{9.1} \\ 10^{8.3}$	II/II II/I II/VIb I/NA ^e II/V

Table 3.1. Characterization of the ND viral strains used for the preparation of vaccines

^aThe HA titer of each antigen is listed pre and post inactivation with BPL. ^bEmbryo Infectious Dose 50 (EID₅₀) prior to inactivation is listed per 0.1ml: All vaccines were adjusted to the equivalent EID₅₀ 10^{8.3} prior to inactivation. ^cClass and Genotype (Czegledi et al., 2006). ^dPigeon84 HA titer post-concentration.

^eNA: not applicable; no genotypes have been reported in Class I.



Figure 3.1. Phylogenetic comparison of the full-length hemagglutinin-neuraminidase (HN) and fusion (F) proteins of representatives of the NDV Classes and Genotypes [7]. Viruses utilized to prepare vaccine antigens are bolded. Bootstrap values greater than 75 are noted. Ruler distance of 0.02 represents 2 amino acid changes per 100 amino acids.



Figure 3.2. Virus isolation from oropharyngeal (oral) swabs collected on selected days after CA02 END virus challenge of all treatment groups at 21 days post-vaccination. A) Mean virus titers of oral swabs of all groups on all sample days. All control animals were dead by day 6. B) Comparison of oral virus titers at 2 day post-challenge: * indicates significant difference from Control and AK. C) Comparison of oral virus titers at 4 day post-challenge: * indicates significant difference between control and all other treatments. Data (mean \pm SE) were analyzed by ANOVA followed by Tukey's multiple comparison test. B1= B1, UL=Ulster, PG=Pigeon84, AK =AK196, CA=CA02.

Vaccine	Amino Acid similarity with challenge virus (%)			
G 4 4 2	HN	Ē		
CA02	100.0	100.0		
Pigeon84	92.3	92.9		
Ulster	90.7	89.7		
B1	89.3	88.1		
Alaska196	84.2	85.2		

Table 3.2. Deduced hemagglutinin-neuraminidase (HN) and fusion (F) protein similarity between the vaccine strain and the challenge NDV strain of CA02^a

^aAmino acid similarity analysis and pair-wise alignment of each isolate with CA 02 were performed with the program Megalign (DNASTAR, Madison, WI): The amino acid sequences were aligned using the Lipman–Pearson Method.

		ELISA				
Vaccine	B1	Ulster	Pigeon84	AK196	CA02	
B1	291 ^b	133	30	40	96	3676 ^c
Ulster	612	586	146	306	411	3045
Pigeon84	348	281	562	182	190	2816
AK196	334	174	146	829	198	3269
CA02	761	538	485	463	794	3292

Table 3.3. Pre-challenge serology completed by micro-beta HI^a and ELISA (IDEXX)

^aHI assays were completed with 4 HA units of each vaccine antigen to test pre-challenge serum of each vaccine group and group geometric mean titers are presented. ^bHomologous responses noted by bold font.

^cELISA group geometric mean titers are presented in the right column.

Vaccino	Days post challenge samples collected							
group	2		4		7		9	
	O^b	C^{c}	0	С	0	С	0	С
Control	$16/16^{a}$	16/16	16/16	13/13	NS^{f}	NS	NS	NS
B1 ^d	15/15	08/15	15/15	06/15	05/15	05/15	02/15	00/15
Ulster	16/16	10/16	16/16	07/16	02/16	00/16	00/16	03/16
Pigeon84	16/16	04/16* ^g	16/16	05/16	01/16	03/16	00/16	03/16
AK196 ^e	16/16	12/16	16/16	07/16	00/15	02/15	00/15	02/15
CA02	16/16	10/16	16/16	04/16	02/16	02/16	02/16	00/16

Table 3.4. Frequency of isolation of challenge virus in different vaccine groups

^aData are expressed as positive isolations/total number of swabs with one per bird. ^bO: oropharyngeal swabs. ^cC: cloacal swabs.

^dOne bird from the B1 group died pre-vaccination.

^eOne bird from the AK196 group died on day 5 post-challenge.

^fNS; No survivors.

^g*Denotes significance from corresponding control, p<0.05.

titers. However, both Pigeon84 and CA02 caused a significant reduction in shedding compared to the controls. On day 4 the oral virus titers of the CA02 vaccinates were significantly reduced compared to the titers of the other vaccine strains as well as the controls (Figure 3.2A,C). The heterologous NDV vaccine strains significantly reduced oral viral shed on day 4 (Figure 3.2C) compared to the control vaccine, but that reduction was not as great as in CA02 vaccinates. All of the cloacal swabs from the control group were positive on days 2 and 4. The cloacal swabs from the ND vaccinated groups contained low amounts of virus throughout the sampling period (Figure 3.3A) and the number of birds shedding virus was decreased on days 2 and 4 post-infection compared to the control birds. Unlike the oral swabs, there was no significant difference in the virus titers from the cloacal swabs of those vaccinated groups except that pigeon84 had significantly less virus isolated on day 2 compared with the amount isolated from Alaska196 (Figure 3.3B). This difference disappeared by day 4 (Figure 3.3C).

Discussion

The goal of this study was to determine if the antigenic distance of the vaccine strain, as described by phylogeny, can influence the amount of virus shed after infection with a virulent strain of NDV and thus impact decisions on vaccine formulation and challenge virus for potency testing. We identified four NDV isolates that represented four genotypes different from the CA02 outbreak strain to use in this study as vaccines that have different degrees of amino acid similarity to the CA02 HN and F proteins (Table 3.2). As shown in Figure 3.1, these isolates represent the diversity found in both the HN and F proteins. Specific antibodies to both of these glycoproteins are known to



Figure 3.3. Virus isolation from cloacal swabs collected on selected days after CA02 END virus challenge of all treatment groups at 21 days post-vaccination. A) Mean virus titers of cloacal swabs of all groups on all sample days. All control animals were dead by day 6. B) Comparison of cloacal virus titers at 2 day post challenge: * indicates significant difference from Control; ** indicates significance difference between Alaska and Pigeon C) Comparison of cloacal virus titers at 4 day post challenge: * indicates significant difference from control. Data were analyzed by ANOVA followed by Tukey's multiple comparison test. B1=B1, UL=Ulster, PG =Pigeon84, AK =AK196, CA=CA02.

be involved in the neutralization of NDV (Boursnell et al., 1990a; Boursnell et al., 1990b; Kamiya et al., 1994; Loke et al., 2005; Merz et al., 1980; Park et al., 2006; Reynolds and Maraqa, 2000; Umino et al., 1984). The majority of virulent ND strains isolated in North America since 1970 from poultry, psittacines and wild birds like cormorants and anhingas have been Class II Genotype V viruses that show nucleotide similarities to the Mexican isolates of 1996 and 1998 (Pedersen et al., 2004). If there were to be another outbreak in the U.S., the etiological agent would likely be a virulent virus similar to the Class II Genotype V viruses of the recent past and not virulent virus of the Class II Genotype II isolates like Texas GB that have not been isolated in the U. S. since the early 1970's.

In this study, the NDV vaccine homologous to the Mexican-like Class II Genotype V challenge virus (CA02) induced the highest titers of hemagglutinationinhibition antibodies using the CA02 as antigen when compared to the amounts induced by heterologous vaccines (Table 3.3). Most importantly, improved protection of vaccinated birds as measured by a significant decrease in challenge virus shedding in oropharyngeal swabs was also seen in the group vaccinated with the homologous vaccine (Figure 3.2C). The HI assay detects antibodies to the HN surface antigen, which are known to correspond to antibodies that provide protection from disease. Each vaccine group gave the highest HI titers when the antigen used in the assay was homologous to the vaccine antigen, except for B1, which has been previously shown to respond poorly in this regard (Kendal AP and Allan AW, 1970). The cross HI titers in Table 3.3 also show that the HI titers can vary greatly depending on the antigen used for testing. For example the B1 vaccinated birds had a GMT HI titer of 291 when compared with B1 antigen, but a titer of 96 when using CA02, the challenge strain as antigen. Using the vaccine antigen and not the probable challenge antigen in evaluating the GMT HI response could lead to an over estimation of the immune response and the potential level of protection they induced (Table 3.3). Testing these same vaccinates against the antigen of the likely challenge virus will give a better indication of the type of protection these birds will have. We also found that the ELISA titers (Table 3.3) for the B1 vaccinates had the highest NDV antibody response even though the B1 vaccinates had the lowest HI titers to the CA02 challenge antigen. These results suggest that either the ELISA antigen had greater homology with the B1 virus or it simply reflects the differences in levels of antibodies to conserved structural proteins other than the HN in the response measured by ELISA. The similarity of ELISA antibody titers among all vaccine groups in contrast to the variability in HI titers indicates the lesser role of the HN in the induction of the antibodies assayed by ELISA. Consequently, the ELISA response may not be as useful as the HI in predicting the level of protection induced by vaccination.

Although virus shed hasn't been widely reported as a method of monitoring protection induced by ND vaccines (Kapczynski and King, 2005), there are many reports of avian influenza (AI) vaccines being able to reduce the number of vaccinated birds shedding challenge virus (Taylor et al., 1988; Webster et al., 1996) and also of them being able to reduce the amount of challenge virus shed from the respiratory tracts (Swayne, 1999; Swayne et al., 1997; Webster et al., 1996). Notably, one report describes a similar pattern seen in these NDV experiments with significant reductions in oropharyngeal shedding with a homologous vaccine and no differences in cloacal shedding between vaccine groups (Swayne et al., 2000). While antigenic drift does

appear to be happening with NDV isolates throughout the world it is occurring at a much slower scale than that seen with AI viruses (Aldous et al., 2003; Czegledi et al., 2006; Lee et al., 2004; Seal et al., 2000a; Toyoda et al., 1989). In addition to shedding less virus into the environment, birds vaccinated for avian influenza have been shown to be more resistant to challenge by requiring a larger amount of virus to become infected (Marangon and Capua, 2006).

Control of Newcastle disease primarily consists of vaccination of flocks and culling of infected or likely infected birds. Current vaccine strategies can be effective in controlling serious illness and death in infected birds, but do not prevent infection and shedding of virus. In the U.S. where virulent ND viruses are not endemic, vaccination programs are not intensive to minimize post-vaccinal reaction (Senne et al., 2004). Transmission of virus even in a well-vaccinated flock can occur because some of the birds will have had a poor vaccine response and will be susceptible to infection. This was seen in layer flocks during the CA02 outbreak. However, in broilers because of their short life spans and the need to balance immune response with vaccine reactions, this group often has an immune response that does not provide complete clinical protection and allows high levels of virus shedding on challenge (Kapczynski and King, 2005). In countries where a virulent challenge is likely the vaccination programs may be more intensive and consequently transmission of a virulent virus may be reduced. The goal of the current study was to determine if it is possible to reduce viral shedding, and presumably, the spreading of the virus and the consequent disease, through an improved vaccine strategy. The current vaccines used to prevent ND were derived from strains isolated decades ago. In the last fifty years there has been a major shift in the types of strains of NDV that have been identified as circulating in poultry, although they still remain as a single serotype. The viral strains of greatest concern today exhibit considerable antigenic and sequence variation from the original vaccine strains (Table 3.2 and Figure 3.1). We hypothesized that if birds were vaccinated with viruses that were more antigenically similar to the challenge strain that they would shed reduced amounts of challenge virus. Indeed, the data from this study support this hypothesis.

Historically, protection induced by NDV vaccines is tested from a challenge with Texas GB/1948 in the U.S., a Class II, Genotype II virus and with Herts/1933 in Europe, a Class II, Genotype IV virus. These challenge strains do not represent the virus lineages that are currently seen in North America and around the world. Currently, protection from NDV, as evaluated for biological regulatory purposes, is defined as protection induced by vaccines against morbidity and mortality after challenge. With this definition and based on these data, the lineage of the challenge strain used to test vaccines will likely not make a difference. However in this study vaccines formulated to be similar to the challenge virus induced better protection in vaccinates as measured by the reduction in the shedding of virus after a virulent challenge. Thus, by formulating ND vaccines with a virus similar to the mostly likely outbreak virus it may be feasible to induce an immune response that not only protects against morbidity and mortality, but also against dissemination of the virulent virus.

Acknowledgments

The authors would like to thank Tim Olivier, Suzanne DeBlois, and Dawn Williams-Coplin for their excellent technical assistance, and Roger Brock for animal care assistance. We extend our appreciation to Dr. Mia Kim for her assistance with the animal

studies. USDA, ARS CRIS project 6612-32000-049, supported this research.

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CHAPTER 4

EFFICACY OF NEWCASTLE DISEASE VACCINES AS MEASURED BY VIRAL SHED AFTER VIRULENT CHALLENGE IN SPF WHITE LEGHORNS¹

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Abstract

Virulent Newcastle disease virus isolates from recent outbreaks are the same serotype but a different genotype than current vaccine strains. Prior experiments in chickens with inactivated vaccines show significantly less virus shed in birds vaccinated with a homologous vaccine (same genotype as challenge) compared to chickens vaccinated with genotypically heterologous vaccines. Subsequent experiments have compared the protection induced in chickens by live vaccines of B1 and LaSota (genotype II), Ulster (genotype I), and recombinant viruses that express the hemagglutinin-neuraminidase (HN) gene or the HN and fusion (F) genes of CA 2002 (genotype V). Vaccinates were challenged with virulent CA 2002 (genotype V) or Texas GB (TXGB, genotype II). After challenge with CA 2002 the birds vaccinated with a live recombinant genotype V virus containing the HN of CA 2002 shed significantly less virus in oropharyngeal swabs compared to B1 and had fewer birds shedding virus compared to B1, LaSota and Ulster vaccines. After challenge with CA 2002 birds vaccinated with the recombinant containing both the F and HN of CA 2002 (rA-CAFHN) shed less virus and fewer birds shed virus compared to LaSota-vaccinated birds. TXGBchallenged LaSota-vaccinated birds had less virus shed and fewer birds shedding virus compared to the TXGB-challenged rA-CAFHN-vaccinated birds. Genotypic differences between vaccine and challenge did not diminish ability of vaccines to protect against disease, but genotypic similarity did reduce virus shed and may reduce transmission. The development and use of vaccines of the same genotype as the expected field challenge may provide an additional tool for control of this important poultry pathogen.

Keywords: APMV-1 = avian Paramyxovirus serotype-1; BHI = brain heart infusion; BPL = beta-propiolactone; ECE = embryonated chicken eggs; EID₅₀= median embryo infectious dose; ELISA = enzyme-linked immunosorbent assay; F = fusion gene; HA = Hemagglutination assay; HI = Hemagglutination inhibition; HN = hemagglutininneuraminidase gene; ND = Newcastle disease; NDV = Newcastle disease virus; OE = Oil emulsion; PC= post challenge; PV = post vaccination; rA = recombinant Anhinga; SEPRL = Southeast Poultry Research Laboratory; SPF = specific pathogen-free; U.S. = United States; VI= virus isolation; vNDV = virulent NDV.

Introduction

Infection with a virulent Newcastle disease virus (NDV) leads to the disease recognized as Newcastle disease (ND) (Alexander, 2003), one of the most important infectious diseases of poultry due to the potential for devastating losses. It occurs on six of the seven continents of the world and is enzootic in many countries. The possibility of an outbreak of ND in the United States is a constant threat with the last major outbreak in the U.S. occurring in 2002-2003 (Nolan, 2002).

Newcastle disease virus, also known as avian paramyxovirus serotype-1 (APMV-1) virus, is a member of the genus Avulavirus (Fauquet and Fargette, 2005; Mayo, 2002) in the Paramyxoviridae family. It is a negative-sense, single-stranded, non-segmented, enveloped RNA virus (Alexander, 2003). The NDV genome is composed of six genes coding for non-structural and structural proteins, of which the hemagglutininneuraminidase (HN) and fusion (F) are transmembrane surface glycoproteins that allow binding and fusion of the virus to host cells. The HN and F induce specific cell-mediated immunity and neutralizing antibodies in vaccinated animals conferring protection from morbidity and mortality (Avery and Niven, 1979; Boursnell et al., 1990a; Boursnell et al., 1990b; Boursnell et al., 1990c; Edbauer et al., 1990; Jayawardane and Spradbrow, 1995a; Kamiya et al., 1994; Loke et al., 2005; Long et al., 1986; Merz et al., 1980; Nakaya et al., 2001; Park et al., 2006; Taylor et al., 1990; Umino et al., 1984).

APMV-1 viruses all belong to a single serotype, and thus by definition any ND vaccine strain should induce protection against morbidity and mortality from any NDV challenge virus. Licensed ND vaccines are currently produced for use in chickens, turkeys and pigeons to assist in the control of ND. Proper application of the available vaccines has been shown to be effective in preventing disease but not in preventing infection and virus shedding, which may result in the infection of other susceptible birds (Kapczynski and King, 2005). Currently, ND vaccines are tested for their ability to prevent morbidity and mortality, and levels of viral shedding are not taken into consideration. New outbreaks of ND, particularly in those countries that are typically free of the disease, raise the question of efficacy of current vaccines. Although the viruses isolated over the last thirty years are of the APMV-1 serotype, molecular characterization of those isolates has revealed that genotype V viruses have caused the most recent outbreaks in the U.S. and vaccines used for their control are members of genotype II (Pedersen et al., 2004). We have previously shown homology between vaccine and challenge virus to be important when comparing protection against shed induced by inactivated vaccines of different genotypes to a challenge with virulent NDV (Miller et al., 2007). In the present study, we extended the comparison to assess importance of homology of genotype in protection by using both inactivated and live vaccines expressing genotypes I, II, and V antigens and challenging separate groups of birds receiving each of the vaccines with either a genotype II virus, TX GB/1948 the standard U.S. challenge, or a genotype V virus, California/2002 isolated from the 2002-03 U.S. outbreak. The standard low virulence vaccine strains B1 (genotype II), LaSota (genotype II) and Ulster (genotype I) were utilized directly as live vaccines. Expression of live antigens of the more virulent genotype V was accomplished by utilizing an infectious clone of Anhinga/1993 virus (genotype V) and generation of additional recombinants from the Anhinga backbone which contained either the HN or the F and HN of the challenge virus, California/2002 (genotype V). In addition to observation for clinical signs and mortality, oropharyngeal and cloacal swabs were collected post-challenge from vaccinates to assess the amount of challenge virus that was shed. Antibody levels were evaluated with hemagglutination inhibition (HI) assays and with an ELISA.

We hypothesized that increasing the genetic relatedness of live ND vaccine virus to the likely virulent challenge would improve the efficacy of the vaccine by decreasing the amount of challenge virus shed from vaccinated poultry.

Materials and Methods

Eggs and Chickens. The Southeast Poultry Research Laboratory (SEPRL) specific pathogen-free (SPF) white Leghorn flock was the source of the four week-old chickens used in all of the vaccination experiments. Embryonated chicken eggs (ECE) from SEPRL were utilized for virus isolation (VI), vaccine propagation, virus titrations, and as a source of normal allantoic fluid for preparing both the inactivated sham vaccine for the control birds and for diluting antigens after inactivation for the inactivated vaccines. Additional ECE from Sunrise Farm, Inc. (Catskill, NY) were used for virus titrations.

Chickens were separated into appropriate vaccination groups for each experiment. Number of birds per group differed for each of the experiments ranging from six to twenty. The chickens were wing or leg banded and kept in negative pressure isolation units in BSL 3 Ag facilities and allowed to acclimate for two days prior to their being vaccinated. ELISA (IDEXX, Westbrook, ME) and hemagglutination inhibition (HI) assays were completed on sera from hatchmates from each experimental group to confirm the absence of NDV antibodies. Birds were given food and water *ad libitum* throughout the experiment. The SEPRL Institutional Animal Care and Use Committee approved all animal experiments.

Viruses. Working stocks of virus isolates used as vaccines were obtained from the SEPRL repository and propagated in 9-11 day old SPF ECE by chorioallantoic sac inoculation. Newcastle disease viruses representing different genotypes were selected to use as vaccines or challenge viruses: Ulster/196, B1/1947, LaSota/1946, TXGB/1948 and California/212676/2002 (CA02) (Alexander, 2003; Wakamatsu et al., 2006) (see Table 4.1). Ulster, a class II genotype I virus, is used as a live vaccine in Northern Ireland and B1 and LaSota, both class II genotype II virus, are used worldwide as live vaccines. TXGB is the virulent neurotropic challenge virus used to test efficacy of ND vaccines in the U.S. and is a class II, genotype II virus, the same genotype as B1 and LaSota. In these experiments TXGB is used as one of the challenge viruses. The CA02 viscerotropic virus, a class II, genotype V virus, is a virulent representative of the last outbreak of ND in the U.S. and was used as an inactivated vaccine and as a challenge virus. In addition, recombinant viruses created from the backbone of a genotype V NDV isolated from an Anhinga in 1993, which is in the same class and genotype as CA02,

were used as both live and inactivated vaccines (King and Seal, 1998). All but one of these recombinants are chimeras that contain genes from the CA02 virus instead of their own genes, and their creation and characterization has been previously described (Estevez et al., 2007). Recombinant Anhinga/1993 (rA), rA with the HN of CA02 replacing the HN of Anhinga (rA-CAHN), rA-CAHN with the F of CA02 replacing the Anhinga F (rA-CAFHN), and rA-CAHN with the Anhinga F cleavage site changed to that of LaSota vaccine strain (rA-CAHN-LSCL) were evaluated as vaccines. The strain of virus used for the HN and F genes is CA/212519/2002, which has the same sequence as the CA02 virus in the HN and F genes, but was isolated from a different chicken during the same outbreak. Because the sequence of the HN and F genes is the same, they both will be referred to as CA02. An additional recombinant containing the HN gene of CA02 in the Anhinga backbone along with the fusion gene cleavage site of LaSota (rA-CAHN-LSCL) was evaluated as a vaccine in experiments II and III. Pools of infective allantoic fluid were clarified via centrifugation at 1000 x g for 15 minutes. Infectivity titers of the pools were determined by titration in ECE prior to being stored at -70° C for use as live vaccine virus and prior to being inactivated for use as antigen for inactivated vaccines and for HI assays. Hemagglutination (HA) titers were determined before and after inactivation (data not shown) (Thayer, 1998). Allantoic fluid for each virus was inactivated with 0.1% beta-propiolactone (BPL) (Sigma, St. Louis, MO) (Spradbrow and Samuel, 1991) for 4 hours at room temperature and kept overnight at 4° C for hydrolysis of the BPL. Complete virus inactivation was confirmed by failure to recover virus after embryo inoculation (Alexander, 1998). Prior to being stored at -70° C, the pH of the pools of virus antigen as allantoic fluid were adjusted to 7.0 by adding sterile sodium

bicarbonate solution (Gibco, Invitrogen Corporation, Grand Island, NY) (Budowsky et al., 1993). Inactivated antigen was used for the inactivated vaccines and for the HI assays.

Vaccine generation. Water-in-oil emulsion (OE) vaccines were prepared with virus antigen concentration the equivalent of 10^{8.3} ELD₅₀ (median embryo lethal dose) of virus prior to BPL inactivation. Recombinant Anhinga (rA), having a lower ELD₅₀ titer and HA titer, was concentrated by ultra-centrifugation at 120,000 x g. The oil phase of the vaccine was made by adding 36 parts of Drakeol 6VR (Butler, PA), three parts of Span 80 (Sigma, St. Louis, MO) and one part of Tween 80 (Sigma, St. Louis, MO) for each vaccine to be made into a working solution. The oil phase was added to each of the virus antigens or normal allantoic fluid (the aqueous phase) to achieve a 4:1 ratio of oil to water as previously described (Stone, 1983). Vaccines were prepared by homogenization in a Waring blender (Fisher Scientific International Inc., Hampton, NH) (Stone et al., 1978) three days prior to administration and kept at 4° C prior to use. Infective allantoic fluid was clarified by centrifugation at 1000 x g for 15 minutes and diluted in brain heart infusion (BHI) broth (BD Biosciences, Sparks, MD) to an EID_{50} of $10^6/0.1$ ml for formulation of the live virus vaccines. All sham vaccines given to the control birds in the live vaccine experiments contained sterile BHI.

Experimental design. Viruses used in each experiment are summarized in Table 4.1. The first experiment assessed both inactivated and live vaccines containing conventional viral strains and recombinant viruses to a challenge with vNDV, CA02. Experiment II evaluated only live vaccines including an additional recombinant. A second challenge

virus, TXGB, that is genotypically different than CA02 was added to further test how homology between vaccine and challenge virus affected the amount of the virus shed post-challenge. In experiment III, larger numbers of birds were used per group, a conventional class II, genotype I vaccine (Ulster) dissimilar to both of the challenge viruses was added, a new recombinant (rA-CAFHN) was also used as a vaccine and the challenge dose per bird was increased. The last experiment assessed the commonly used LaSota vaccine and the recombinant vaccine, rA-CAFHN, against two high dose challenges with TXGB and CA02 with bird groups of twenty. In the second and third experiments, extra birds were vaccinated for each of the vaccine groups and tissues were harvested four days post-vaccination (PV) for VI to recover the vaccine viruses. Spleen and blood was harvested for experiment II and tracheas and lungs were harvested in the third experiment. For all of the experiments, oropharyngeal and cloacal were collected post-challenge on days zero, two, four, and nine into 1.5 ml of BHI broth with a final concentration of gentamicin (200 µg/ml), penicillin G (2000 units/ml), and amphotericin B (4 μ g/ml). Birds were monitored daily for clinical signs and death through day 14 PC when they were sedated, bled and euthanized. Moribund chickens were euthanized with intravenous sodium pentobarbital at a dose of 100 mg/kg and counted as dead on the next day. Necropsies were completed on selected birds post-challenge to assess the presence of gross pathological lesions.

		Cha	<u>#</u>			
<u>birds/group</u>		Vaccines	Vaccines			
Experiment I	Inactiv Live	vaccines ated Oil Emulsion A) control-OE B) rA-OE ^b C) rA-CAHN-OE ^c D) CA02-OE A) control-BHI B) B1 ^d C) rA D) rA-CAHN	CA02 (10 ^{5.7} /0.1ml) ^a	6		
Experiment II	Live	A) control-BHI B) LaSota ^f C) rA D) rA-CAHN E) rA-CAHN-LSCL ^g	CA02 (10 ^{5.9} /0.1ml) TXGB ^e (10 ^{6.1} /0.1ml)	6		
Experiment II	I Live	 A) control-BHI B) Ulster^h C) LaSota D) rA E) rA-CAHN F) rA-CAFHNⁱ G) rA-CAHN-LSCL 	CA02 (10 ^{7.1} /0.1ml) TXGB (10 ^{6.3} /0.1ml)	10		
Experiment I	V Live	A) LaSota B) rA-CAFHN	CA02 (10 ^{6.9} /0.1ml) TXGB (10 ^{6.5} /0.1ml)	20		
genotype V ^d genoty	ype II	^g genotype V				
genotype V ^e genoty	/pe II	^h genotype I				
genotype V ^f genoty	vpe II	^g genotype V				

Table 4.1. Experimental design summaries

Experiment I. Chickens were separated into eight groups of six birds, wingbanded, and allowed to acclimate for two days before vaccination. Twenty-one days PV oropharyngeal swabs, cloacal swabs and serum were collected.

Inactivated Vaccines. Groups were subcutaneously vaccinated with 0.5 ml of their appropriate inactivated vaccines. Group one was vaccinated with an OE made from normal allantoic fluid. Groups two, three, and four received recombinant Anhinga (rA) OE, recombinant Anhinga containing the HN gene of CA02 (rA-CAHN) OE and the homologous CA02 OE, respectively.

Live Vaccines. Groups were vaccinated with live B1, rA, or rA-CAHN $(10^{6}/0.1 \text{ml})$ or a sham vaccine for control birds consisting of sterile BHI.

All birds were challenged with $10^{5.7}$ median embryo infectious dose (EID₅₀) of CA02 virus administered in 50 µl into the right eye and 50 µl into the choana.

Experiment II. Chickens were separated into five groups of six birds and five groups of nine birds, leg-banded, and allowed to acclimate for two days before vaccination. There were a total of fifteen birds for each of the four vaccine groups. Live LaSota, rA, rA-CAHN, rA-CAHN with the LaSota fusion cleavage site (rA-CAHN-LSCL) ($10^{6}/0.1$ ml) or a sham vaccine was administered in 50 µl into the right eye and 50 µl into the choana. Four days PV, oropharyngeal and cloacal swabs were collected for VI of the vaccine virus. In addition, three birds from each of the isolators containing nine birds were randomly selected, sedated and euthanized for the collection of blood, and spleens for VI. Twenty-one days PV, oropharyngeal and cloacal swabs were collected for VI and sera collected for evaluation by HI and ELISA. One of the groups for each

vaccine was challenged with $10^{6.1}$ EID₅₀ of TXGB and the other was challenged with $10^{5.9}$ EID₅₀ of CA02. Challenge viruses were administered in 50 µl into the right eye and 50 µl into the choana.

Experiment III. Chickens were separated into five groups of ten birds and five groups of eleven birds, leg-banded, and allowed to acclimate for two days before vaccination. There were a total of twenty-one birds for each of the five vaccine groups. Live Ulster, LaSota, rA, rA-CAHN and rA-CAFHN ($10^{6}/0.1$ ml) or a sham vaccine was administered in 50 µl into the right eye and 50 µl into the choana. An additional group of 11 birds was vaccinated with rA-CAHN-LSCL. Because of the poor response of this vaccine from experiment II, these birds were challenged only with CA02. Four days PV, oropharyngeal and cloacal swabs were collected for VI of the vaccine virus from all birds. In addition, one bird in each of the isolators containing eleven birds was randomly selected for euthanasia for the collection of lungs and tracheas for each of the six groups for VI. Twenty-one days PV, oropharyngeal swabs, cloacal swabs and serum were collected. One of the groups for each vaccine was challenged with $10^{6.3}$ EID₅₀ of TXGB and the other was challenged with $10^{7.1}$ EID₅₀ of CA02. Both were administered in 50 µl into the choana.

Experiment IV. Four vaccine groups each with twenty birds and two control groups with six birds each were housed in the 12 isolators. Size limitations of the isolators forced uneven cage numbers for the different groups. That is; groups one (LaSota vaccine/TXGB challenge) and four (rA-CAFHN vaccine/TXGB challenge)

consisted of two isolators each with ten birds and groups two (LaSota vaccine/CA02 challenge) and three (rA-CAFHN/CA02 challenge) consisted of one isolator with ten birds and two isolators with five birds each. Two more isolators housed groups six and seven, each with six control birds vaccinated with sham vaccines. A sham vaccine, live LaSota, or rA-CAFHN at a dose of $10^{6}/0.1$ ml was administered in 50 µl into the right eye and 50 µl into the choana. Twenty-one days PV, oropharyngeal swabs, cloacal swabs and serum were collected. One of the groups for each vaccine was challenged with $10^{6.5}$ EID₅₀ of TXGB and the other was challenged with $10^{6.9}$ EID₅₀ of CA02. Both were administered in 50 µl into the right eye and 50 µl into the choana.

VI, EID₅₀, HA, HI, and ELISA. Virus isolation (VI) and hemagglutination (HA) assays to identify virus positive fluids were conducted as described (Miller et al., 2007; Wakamatsu et al., 2006). Tissues collected for VI were homogenized with BHI and VI antibiotics (described above) in a Whirlpak (NASCO, Modesto, CA) with a Stomacher 80 (Seward, West Sussex, UK) for two minutes to obtain a 10% weight: volume suspension. Serial ten-fold dilutions of VI positive swab and tissue samples were titrated in SPF ECE with eggs candled daily for seven days for mortality (Alexander, 1998). Twenty-four hour deaths were discarded and fluids from both dead and surviving eggs were tested for HA activity. Virus titers were calculated to determine the EID₅₀ using the Spearman-Kärber method (Karber, 1931). Hemagglutination-inhibition (HI) assays (micro-beta) were completed on pre-challenge sera by testing all samples against their homologous and heterologous vaccine antigens (King, 1996). ELISA assays (IDEXX, Westbrook, ME) were also completed on the pre-challenge serum according to the manufacturer's

recommendations. Arithmetic mean titers with standard errors of HI antibodies were determined for each vaccination group.

Generation of rA-CAHN with the F cleavage site of LaSota vaccine strain. Site directed mutagenesis was performed on one clone (rA-CAHN) to change the fusion cleavage site of the backbone of the Anhinga fusion gene to the less virulent cleavage site of the LaSota virus. Briefly, using PfuUltra® II Fusion HS DNA Polymerase (Stratagene, La Jolla, CA) two separate 50 µl reactions with each of the mutagenic primers (available upon request) was run for four cycles with the following parameters: 30 seconds at 92° C, 20 seconds at 58° C, 15 minutes at 68° C and held at 4° C. After which, 25 µl of each reaction product were mixed in a single tube and then run for an additional 16 cycles using the same parameters. After cycling, the reactions were digested with DpnI restriction enzyme (New England Biolabs, Ipswich, MA) for one hour and then transformed into MAX Efficiency® Stbl2[™] (Invitrogen, Carlsbad, CA) chemically competent cells and grown at 32° C for 24-48 hours. The recovered plasmids were sequenced to confirm that the mutagenesis reactions were correct. Recombinant virus was rescued as described by Estevez and co-workers (Estevez et al., 2007), and designated as rA-CAHN-LSCL.

Nucleotide Sequencing. All sequencing reactions were performed as previously described (Kim et al., 2006). Sequencing was done using the Applied Biosystems PRISM Fluorescent Big Dye Sequencing Kit and the ABI 3730 DNA Sequencer (ABI, Foster City, CA). Editing and assembly of sequence data was done using Megalign

(DNASTAR, Madison, WI). All of the recombinants were sequenced from cDNA amplified by RT-PCR from Trizol LS (Invitrogen, Carlsbad, CA) extracted RNA using gene specific primers that are available upon request. The HN and F proteins from the recombinant Anhinga (rA), the F proteins from rA-CAHN were the same sequence as the Anhinga/1993 wild-type virus that can be viewed at GenBank® under the accession number EF065682. The HN protein from the recombinant Anhinga with the CA02 HN (rA-CAHN), the recombinant Anhinga with the CA02 HN and the LaSota fusion cleavage site (rA-CAHN-LSCL), and the recombinant Anninga with the CA02 F and HN (rA-CAFHN) had HN sequences identical to the HN protein of CA/2002 wild type virus which has the following accession number; EF520717. The F protein recombinant Anhinga with the F and HN of CA02 had the same nucleotide sequence of wild type CA02: EF520718. The rA-CAHN-LSCL recombinant had the same F protein as wildtype Anhinga except the fusion cleavage site was confirmed to be that of LaSota. This F protein was not submitted to GenBank[®]. Nucleotide sequences for the complete HN for Ulster (M19478), B1 (AF309418), LaSota (AY510092), and TXGB (M21409) are available from GenBank[®]. Accession numbers for the F genes of Ulster (M24694), B1 (M24695), LaSota (DQ195265), and TXGB (M23407) are also available from GenBank[®].

Statistical analysis. HI titers are presented as arithmetic means plus or minus standard error. VI titers are presented as arithmetic mean titers plus or minus standard error. Group means were analyzed by ANOVA with Tukey's post hoc test when indicated. Fisher exact tests performed on data indicating numbers of birds shedding. Significance is reported at the level of $P \leq 0.05$.

Results

Experiment I.

Killed Vaccines. No adverse reactions were seen in the birds vaccinated with the inactivated OE vaccines. After challenge, mild conjunctivitis developed, which resolved in all of the birds except for the sham-vaccinated control group. All birds except the sham-vaccinated control group survived challenge. All of the sham-vaccinated birds were dead by day five and upon necropsy had enlarged spleens and small hemorrhages in the thymus and the mucosa of the cranial trachea. Birds vaccinated with the vaccine homologous to the challenge, CA02, shed significantly less virus in oropharyngeal swabs four days PV when compared to the other inactivated vaccines (Figure 4.1). Virus titers of cloacal swabs both two and four days post challenge (PC) show that all of the OE vaccine groups shed significantly less virus compared to the sham vaccinated control birds, but there was no significant difference among these vaccines (data not shown). Only rA-CAHN-OE had significantly fewer birds shedding challenge virus when compared to the controls two days PC in cloacal swabs, but all three of the vaccine groups shed less virus than the sham-vaccinated controls at four days after challenge (Table 4.2). The pre-challenge HI and ELISA values are presented in Table 4.3 and Figure 4.2. All vaccine groups had strongly positive ELISA and HI titers with homologous and heterologous HI antigens.

Live Vaccines. Mild conjunctivitis in the right eye of the ND vaccinated birds from application of the live vaccine resolved after two days. Mild conjunctivitis presented



Figure 4.1. Experiment I-Comparison of virus titers from oropharyngeal swabs for all vaccine groups collected on days (a) two and (b) four post-challenge with CA02 vNDV. Challenge occurred 21 days post-vaccination. * Indicates significant difference from Control-OE. ** Indicates significant difference between rA-OE and CA02-OE. P \leq 0.05

(b)

	Number of birds shedding Days Post-Challenge								
Vaccine	<u>2</u>		<u>4</u>		<u>9</u>				
	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal			
Control-BHI	6/6	6/6	6/6	6/6	NS	NS			
B1 (live)	5/6	1/6*	4/6	2/6	0/5	0/5			
rAnhinga (live)	4/6	0/6*	0/6*	0/6*	0/6	0/6			
rA-CAHN (live)	0/6*#	0/6*	0/6*	0/6*	0/6	0/6			
Control-OE	6/6	6/6	6/6	6/6	NS	NS			
rAnhinga-OE	6/6	3/6	6/6	1/6*	0/6	0/6			
rA-CAHN-OE	6/6	0/6*	6/6	1/6*	0/6	0/6			
CA02-OE	6/6	2/6	6/6	1/6*	0/6	0/6			

 Table 4.2. Experiment I-Numbers of birds shedding after challenge

NS=No Survivors

*P <0.05, vaccines compared to controls #P <0.05, rA-CAHN compared to B1

Vaccine		HI Antigens			ELISA
	B1	rA	rA-CAHN	CA02	
B1 (live)	123 +/- 24	140 +/- 41	106 +/- 25	327 +/-106	1305
rA (live)	413 +/- 178	1227 +/- 314	273 +/- 85	653 +/- 155	2438
rA-CAHN (live)	533 +/- 67	1813 +/- 347	587 +/- 53	1067 +/- 135	2337
rA (OE)	267 +/- 79	960 +/- 320	127 +/- 42	253 +/- 84	3441
rA-CAHN (OE)	320 +//- 72	360 +/- 96	213 +/- 49	693 +/- 128	1632
CA02 (OE)	293 +/- 111	813+/- 221	427 +/- 191	747 +/- 107	2045

Table 4.3. Experiment I-Pre-challenge serology completed by micro-beta HI* and ELISA**

*HI assays were completed with four HA units of each vaccine antigen to test prechallenge serum of each vaccine group. Group arithmetic means with standard errors are presented and homologous responses are noted in bold. ** ELISA group arithmetic means are presented.



Figure 4.2. Experiment I-ELISA titers from pre-challenge serum taken twenty-one days after vaccination from groups vaccinated with (a) inactivated oil-emulsion (OE) and (b) live vaccines. * Indicates significance from the sham-vaccinated controls. ** Indicates significance between vaccine groups. The 396 designates the cut off for a positive NDV antibody titer. $P \leq 0.05$.

(a)

(b)

again after challenge, but no other symptoms of ND were observed in the ND vaccine groups after challenge except for two birds. One bird from the B1 group died on day 4 PC and presented with hemorrhage of the cranial tracheal mucosa, a unique lesion found consistently in chickens infected with isolates from the CA02 outbreak (Wakamatsu et al., 2006). Another bird from the rA group had body tremors starting at day 2 postchallenge which resolved after day 9. The rA-CAHN group on day two PC had significantly fewer birds shedding compared to the control and B1 groups in oropharyngeal swabs and on day four PC the rA and rA-CAHN groups had significantly fewer birds shedding virus in oropharyngeal swabs compared to the sham-vaccinated controls (Table 4.2). All of the vaccinated birds had a significant reduction of the amount of virus shed on both two and four days PC in oropharyngeal swabs when compared to the sham-vaccinated control birds (Figure 4.3), but only the rA-CAHN group had significantly less virus shed than the B1 vaccine group on day 2PC in oropharyngeal swabs. Cloacal swabs for both days PC showed results similar to the inactivated vaccines, with a significant reduction of shedding compared only to the sham-vaccinated controls (data not shown). No significant difference was seen in the protection induced in shedding of virus in oropharyngeal swabs among the vaccinated groups at four days PC. However, the small number of birds per vaccine group may have obscured the differences between groups. Cross HI titers (Table 4.3) induced by the live vaccines in pre-challenge serum revealed B1 to have the highest antibodies to the CA02 antigen and rA and rA-CAHN to have the highest to the rA antigen. Bolded values in Table 4.3 highlight the homologous antigens to antibodies. ELISA antibodies (Figure 4.2 b) for live rA and rA-CAHN were similar in amounts and significantly higher than those



Figure 4.3. Experiment I-Comparison of virus titers from oropharyngeal swabs for all vaccine groups collected on days (a) two and (b) four post-challenge with CA02 vNDV. Challenge occurred 21 days post-vaccination. * Indicates significant difference from Controls. ** Indicates significant difference between live B1 and live rA-CAHN. P \leq 0.05.

Live Vaccine

B1

0

Control

*

rÅ

*

rA-CAHN

(b)

Day 2 Oral

induced by the B1 vaccine. Higher ELISA titers correlate with diminished virus shed in oropharyngeal swabs.

Experiment II.

No clinical signs were seen after vaccination except for a mild conjunctivitis in the right eye of some of vaccinated birds that resolved by 48 hours. To ensure infections by the vaccine viruses, the presence and amount of vaccine virus shed was assayed from oropharyngeal and cloacal swabs taken four days post vaccination (PV). Vaccine virus was recovered from birds in all of the vaccine groups except from one of the rA-CAHN-LSCL groups (Figure 4.4, Table 4.4). Figure 4.4 is pre-challenge and combines the vaccine groups. Table 4.4 divides the groups by vaccine and challenge virus. Only a few birds from the LaSota, rA, and rA-CAHN groups shed virus in cloacal swabs (data not In addition, virus was isolated and quantified from the rA and rA-CAHN shown). vaccinated extra birds from blood, and spleen tissue at four days PV, but LaSota and rA-CAHN-LSCL were not recovered from these tissues. The median embryo infectious dose for rA and rA-CAHN isolated from the spleen at four days PV was 0.5/0.1ml and in blood rA was also 0.5/0.1ml EID₅₀ and rA-CAHN was 1.1/0.1ml EID₅₀ (data not shown). Post challenge, mild conjunctivitis was seen in the right eye of most of the birds in both the TXGB and CA02 challenge groups. One LaSota vaccinated TXGB challenged bird died at day 5 PC after presenting with ataxia, depression and body and head tremors. No gross lesions were observed. All of the sham-vaccinated, CA02 challenged control birds died by day five PC. Upon necropsy they presented with petechial hemorrhages in the eyelid, occasional small hemorrhages in cecal tonsil and all had hemorrhage in the



Figure 4.4. Experiment II-Virus isolation at four days post-vaccination from live vaccine groups in oropharyngeal swabs. *Indicates significance compared to sham-vaccinated controls. Vaccine groups contain both eventual vaccine challenge groups for each vaccine. $P \leq 0.05$.

Table 4.4. Experiment II-Number of birds shedding pre and post challenge

Number of birds shedding

Days post-challenge

Vaccine	-1	7 ^a		<u>0</u>	2		4			9
	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal
Control(TX)b	N/A	N/A	0/6	0/6	6/6	6/6	6/6	6/6	0/4	1/4
Control(CA)	N/A	N/A	0/6	0/6	6/6	6/6	6/6	6/6	NS	NS
LaSota(TX)	4/6	1/6	0/6	0/6	4/6	0/6*	4/6	1/6*#	1/5°	0/5
LaSota(CA)	6/6	0/6	0/6	0/6	3/6	0/6*	3/6	1/6*#	0/6	1/6
rA(TX)	4/6	1/6	0/6	0/6	4/6	0/6*	3/6	0/6*#	0/6	0/6
rA(CA)	3/6	0/6	0/6	0/6	4/6	0/6*	6/6	0/6*#	0/6	0/6
rA-CAHN-LSCL(TX)	0/6	0/6	0/6	0/6	ND	ND	6/6	6/6	NS	NS
rA-CAHN-LSCL(CA)	3/6	0/6	0/6	0/6	ND	ND	6/6	6/6	1/1	1/1
rA-CAHN(TX)	6/6	2/6	0/6	0/6	3/6	0/6*	3/6	0/6*#	0/6	0/6
rA-CAHN(CA)	3/6	3/6	0/6	0/6	1/6*	0/6*	0/6*#	0/6 *#	0/6	0/6

a: -17 days = 4 days post-vaccination

b: Vaccine (challenge virus)

c: one LaSota vaccinate died day 5 post challenge

* < 0.05 vaccines compared to controls

< 0.05 vaccines compared to rA-CAHN-LSCL

ND: Not Done

NS: No Survivors

mucosa of the cranial trachea. None of the TXGB-challenged sham-vaccinated control birds had gross pathological lesions, except similar to the CA02 birds, the intestinal tract was empty. All but one of the rA-CAHN-LSCL-vaccinated birds died by day 6 PC, and will be discussed at the end of this section. On day two PC oropharyngeal swabs for only rA-CAHN birds challenged with CA02 show significantly fewer birds shedding virus when compared to the controls (Table 4.4). The cloacal swabs on both days two and four for LaSota, rA, and rA-CAHN challenged with both TXGB and CA02 all had significantly fewer birds shedding than the controls (Table 4.4). In day four PC oropharyngeal swabs only, rA-CAHN birds challenged with CA02 show significantly fewer birds virus positive than the controls (Table 4.4). While all vaccine groups, except rA-CAHN-LSCL, shed less virus than the controls, a trend was seen for the homologous vaccine/challenge virus combinations to shed less virus compared to the same vaccine with the genotypically heterologous challenge virus. No statistical significance was noted comparing vaccines to each other (Figures 4.5 and 4.6). LaSota vaccinated birds had the highest HI antibodies to their homologous antigen (Table 4.5). The rA and rA groups had HI antibodies highest to CA02 antigen. The rA-CAHN-LSCL group while positive (greater than 16) only had HI levels of 22 to the LaSota antigen and 24 to the CA02 antigen (Table 4.5). Highest to lowest the ELISA values for the vaccine groups were rA-CAHN, rA, LaSota and rA-CAHN-LSCL. The rA-CAHN-LSCL group was negative on ELISA with a mean antibody value of 370, less than the 396 cut off value suggested by the manufacturer (Figure 4.7). Not noted on Figure 4.7 was significance seen between 1) LaSota, rA, and rA-CAHN vaccinated birds and rA-CAHN-LSCL and 2) LaSota vaccine group and rA. Interestingly, individual rA-CAHN-LSCL-vaccinated





Day 2 Oral

(b)



Figure 4.6. Experiment II-Comparison of virus titers from cloacal swabs for all vaccine groups collected on days (a) two and (b) four post-challenge with CA02 vNDV (CA) or TXGB (TX) vNDV. Challenge occurred at 21 days post-vaccination. * Indicates significant difference from sham-vaccinated controls. $P \leq 0.05$.

(b)

Vaccine		ELISA				
-	LaSota	rA	rA-CAHN	TX	CA02	
LaSota	124+/-24	81+/-19	129+/-27	108+/-22	84+/-13	1279+/-1195
rA	421+/-73	875+/-135	560+/-109	355+/-100	896+/-215	3605+/-1941
rA-CAHN	1045+/-156	1109+/-85	1365+/- 182	877+/-135	3499+/-320	4252+/-1313
rA-CAHN-LSC	CL 22+/-11	ND	ND	ND	24+/-7	370+/- 120

Table 4.5. Experiment II- Pre-challenge serology completed by micro-beta HI* and ELISA**

*HI assays were completed with four HA units of each vaccine antigen to test prechallenge serum of each vaccine group. Group arithmetic means with standard errors are presented and homologous responses are noted in bold. ** ELISA group arithmetic means are presented. ND = Not Done.



Figure 4.7. Experiment II-ELISA titers from pre-challenge serum taken twenty-one days after vaccination from groups vaccinated with live vaccines. * Indicates significance from the sham-vaccinated controls. The 396 designates the cut off for a positive NDV antibody titer. Vaccine groups contain both eventual vaccine challenge groups for each vaccine. $P \le 0.05$.

birds that did have ELISA antibodies between 400 and 1495 died after challenge and the one surviving bird had an ELISA titer of only 311 (data not shown). The LaSota-vaccinated, TXGB-challenged bird that died had an ELISA titer of 166, however, there were other birds in the group with negative ELISA titers that lived and showed no symptoms of ND (data not shown). The back-titer of the rA-CAHN-LSCL vaccine had an EID₅₀ of $10^{5.1}$ /bird, almost a log lower than the target dose of an EID₅₀ of $10^{6.0}$ /bird. All of the rA-CAHN-LSCL birds challenged with TXGB died by day six post-challenge and five of the six CA02 challenged birds died by day six.

Experiment III.

The live vaccines caused mild conjunctivitis that resolved by day two PV as seen in prior experiments. All vaccine viruses were recovered from oropharyngeal swabs collected at four days PV, but only Ulster, LaSota, rA-CAHN, and rA-CAFHN were recovered from CL swabs (Figure 4.8). Additionally, tracheas and lungs were harvested for virus isolation to identify virus distribution to these organs post-vaccination (Table 4.6). Post-challenge mild conjunctivitis resolved by day two in the ND vaccinates. Two days PC the sham-vaccinated CA02-challenged controls had moderate conjunctivitis that worsened over time and the sham-vaccinated TXGB challenged controls had mild conjunctivitis that resolved by the next day. The sham-vaccinated CA02-challenged controls were dead by six days PC and upon necropsy had enlarged spleens, bilateral petechia of the conjunctiva, mild hemorrhage in some thymic tissues and hemorrhage of the mucosa of the cranial trachea. Sham-vaccinated TXGB-challenged controls died by day eleven PC and upon necropsy had no hemorrhage or other lesions. All ND-



Figure 4.8. Experiment III-Virus isolation from (a) oropharyngeal and (b) cloacal swabs from live vaccine groups at four days post-vaccination. * Indicates significance compared to sham-vaccinated controls. Vaccine groups contain both eventual vaccine challenge groups for each vaccine. P ≤ 0.05 .

(b)

Table 4.6. vaccination	Experiment for all vaccin	III-Virus e groups	isolation	results	of	selected	samples	four	days	post-

Sample type			VI+/- (EII	O ₅₀ /0.1ml)						
	Vaccines									
	Ulster	LaSota	rA	rA-CAHN	rA-CAFHN	rA-CAHN-LSCL				
Oral swab	+++ (1.9)	+++ (3.5)	+++ (1.1)	++ (0.7)	++ (0.9)	+++ (1.5)				
Cloacal swab	+ (0.7)									
Trachea		+++ (2.3)		+ (0.5)						
Lung	+ (0.5)		+ (0.5)		+ (0.5)					

+ = one of three eggs positive on VI ++ = two of three eggs positive on VI +++ = three of three eggs positive on VI --- = negative for VI $(\#) = EID_{50}/0.1ml$
Table 4.7. Experiment III-Number of birds shedding virus pre and post challenge

Number of birds shedding

Days post-challenge

Vaccine	-17	7a	<u>0</u>		<u>2</u>		<u>4</u>		<u>9</u>	
	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal
Control (TX) ^b	0/10	0/10	0/10	0/10	10/10	10/10	10/10	10/10	0/2°	1/2
Control (CA)	0/10	0/10	0/10	0/10	10/10	10/10	10/10	10/10	NS	NS
Ulster (TX)	10/10	8/10	0/10	1/10	10/10	0/10*	6/10	1/10*	0/10	1/10
Ulster (CA)	10/10	7/10	1/10	2/10	10/10	1/10*	9/10	6/10*	0/10	0/10
LaSota (TX)	10/10	9/10	0/10	2/10	5/10**	1/10*	1/10*	0/10*	2/10	0/10
LaSota (CA)	10/10	9/10	0/10	1/10	10/10	1/10*	2/10*	1/10*	0/10	0/10
rA (TX)	10/10	0/10	0/10	1/10	7/10	1/10*	6/10	3/10*	2/10	0/10
rA (CA)	10/10	0/10	1/10	0/10	7/10	2/10*	3/10*	0/10*	0/10	0/10
rA-CAHN (TX)	10/10	1/10	1/10	0/10	6/10	2/10*	2/10*	0/10*	0/10	1/10
rA-CAHN (CA)	10/10	2/10	0/10	0/10	3/10*#	0/10*	1/10*	2/10*	0/10	0/10
rA-CAFHN (TX)	10/10	5/10	2/10	1/10	5/10*#	0/10 *	3/10*	0/10*	0/10	0/10
rA-CAFHN (CA)	10/10	5/10	1/10	0/10	4/10*#	0/10 *	4/10*	0/10*	0/10	0/10
rA-CAHN-LSCL(C/	4)10/10	0/10	0/10	3/10	10/10	9/10	10/10	10/10	1/10	2/10

a: 17 days before challenge = 4 days after vaccination

b: Vaccine (Challenge virus) c: all Control (TX) birds expired by day 10

NS: No Survivors

* < 0.05 vaccines compared to controls and rA-CAHN-LSCL(CA)

< 0.05 from LaSota (CA)

vaccinated birds survived challenge without showing symptoms of ND except for two rA-CAHN-LSCL vaccinated birds. These two rA-CAHN-LSCL-vaccinated birds had no symptoms of ND until four days PC when bilateral conjunctivitis, diarrhea, and depression developed. At seven days PC one of these birds survived, appearing clinically normal and the other bird developed a head tilt that progressed to severe torticollis by eleven days PC. Upon euthanasia and necropsy, the bird with torticollis had bilateral petechia in conjunctival tissues but no other gross lesions.

Residual vaccine virus was isolated sporadically from all of the vaccine groups on the day of challenge (Table 4.7). The day two PC oropharyngeal swabs showed LaSotavaccinated birds challenged with TXGB, rA-CAHN-vaccinated birds challenged with CA02, and rA-CAFHN-vaccinated birds challenged with either TXGB or CA02 with significantly fewer birds shedding virus compared with the sham-vaccinated groups (Table 4.7). Day two and four PC cloacal swabs show that all vaccine groups except rA-CAHN-LSCL had significantly fewer birds shedding compared to the controls. Day four oropharyngeal swabs show LaSota, rA-CAHN, and rA-CAFHN vaccinated birds challenged with TXGB and LaSota, rA, rA-CAHN, and rA-CAFHN vaccinated birds challenged with CA02 had significantly fewer birds shedding per group compared to the controls (Table 4.7). Figure 4.9 (a) shows that all vaccine groups on average shed significantly less virus than the controls on day two PC in oropharyngeal swabs. In addition, rA-CAHN-vaccinated birds challenged with CA02 and rA-CAFHN-vaccinated birds challenged with either TXGB or CA02 shed significantly less virus compared to both Ulster vaccine groups and to LaSota vaccinated birds challenged with CA02. Other significance not noted on figure 4.9 (a) are 1) LaSota, rA, and rA-CAHN vaccinated birds



Figure 4.9. Experiment III-Comparison of virus titers from oropharyngeal swabs for all vaccine groups collected on days (a) two and (b) four post-challenge with CA02 vNDV (CA) or TXGB (TX) vNDV. Challenge occurred at 21 days post-vaccination. * Indicates significant difference from sham-vaccinated controls. ** Indicates significance between LaSota-TX and rA-TX and between rA-TX and rA-CAHN-CA. $P \leq 0.05$.

challenged with TXGB and rA vaccinated birds challenged with CA02 shed significantly less than both Ulster groups, and 2) all vaccine groups except the Ulster groups shed significantly more virus than the rA-CAHN-LSCL vaccine group. Except of rA-CAHN-LSCL, all of the vaccine groups, regardless of the challenge virus used against them, had smaller amounts of virulent challenge shed compared with the Ulster-vaccinates. Four day PC oropharyngeal swabs (Figure 4.9 b) have similar results in that all vaccine groups shed less virus than the sham-vaccinated control groups. LaSota-vaccinated birds challenged with TXGB and rA-CAHN-vaccinated birds challenged with CA02 shed significantly less than rA-vaccinated birds challenged with TXGB. Other significance not noted on figure 4.9 (b) is that all vaccine groups except Ulster shed significantly less than the rA-CAHN-LSCL group. Figure 4.10 shows that all vaccines in cloacal swabs on days two and four PC shed significantly less than the controls except for the rA-CAHN-LSCL group, which did not shed significantly less than sham-vaccinated controls challenged with TXGB. All vaccinated birds shed significantly less virus compared to rA-CAHN-LSCL, which is not noted in Figures 4.10 (a) and (b).

The three recombinants, rA, rA-CAHN and rA-CAFHN, induced strong HI antibody responses to the CA02 and the TXGB antigens (Table 4.8). The rA-CAHN-LSCL group had the highest antibodies to rA-CAFHN with a mean of 70 and a mean HI titer of 38 to the CA02 antigen. All vaccines had robust ELISA titers (Figure 4.11) except for rA-CAHN-LSCL, which had a mean value of 288, below the cut of for a positive response. Even without a positive ELISA and with low HI antibody titers all of the rA-CAHN-LSCL-vaccinate birds survived challenge. Other significance not noted on Figure 4.11 for the ELISA results: 1) LaSota is significantly lower than rA, rA-



Figure 4.10. Experiment III-Comparison of virus titers from cloacal swabs for all vaccine groups collected on days (a) two and (b) four post-challenge with CA02 vNDV (CA) or TXGB (TX) vNDV. Challenge occurred at 21 days post-vaccination. * Indicates significant difference from sham-vaccinated controls. $P \le 0.05$.

(b)

Vaccine		HI Antigens							ELISA
	Ulster	LaSota	rA	rA-CAHN	rA-CAFHN	rA-CAHN-LS	CI TX	CA02	
Ulster	94 +/-13	54 +/-9	66 +/-9	78 +/-14	195 +/-34	57 +/-35	131 +/-33	101 +/-14	1218 +/-14
LaSota	115 +/-13	162 +/-17	101 +/-14	131 +/-20	306 +/-184	68 +/-34	141 +/-31	128 +/-27	3763 +/-464
rA	922 +/-145	726 +/-146	912 +/-128	832 +/-114	1485 +/-133	1024 +/-129	1082 +/-175	1510 +/-141	5652 +/-627
rA-CAHN	1766 +/-114	1920 +/-90	1690 +/-112	1690 +/-112	1946 +/-70	1280 +/-136	1920 +/-90	1894 +/-84	7705 +/-582
rA-CAFHN	1638 +/-132	1203 +/-119	1075 +/-104	1203 +/-119	2048 +/-0	1331+/-141	1305 +/-146	1946 +/-70	6492 +/-415
rA-CAHN-LS	CL 21 +/-6	19 +/-8	24 +/-5	12 +/-3	70 +/-10	21 +/-3	46 +/-10	38 +/-11	288 +/-622

Table 4.8. Experiment III- Pre-challenge serology completed by micro-beta HI* and ELISA**

*HI assays were completed with four HA units of each vaccine antigen to test pre-challenge serum of each vaccine group. Group arithmetic means with standard errors are presented and homologous responses are noted in bold. ** ELISA group arithmetic means are presented.



Figure 4.11. Experiment III-ELISA titers from pre-challenge serum taken twenty-one days post-vaccination from groups vaccinated with live vaccines. * Indicates significance from the sham-vaccinated controls. The 396 designates the cut off for a positive NDV antibody titer. Vaccine groups contain both eventual vaccine challenge groups for each vaccine. $P \leq 0.05$.

CAHN, rA-CAFHN, and significantly higher than rA-CAHN-LSCL, 2) rA is significantly lower than rA-CAHN, rA-CAFHN, and significantly higher than rA-CAHN-LSCL, 3) Ulster is significantly lower than LaSota, rA, rA-CAHN, and rA-CAFHN, and 4) rA-CAHN-LSCL is significantly lower than rA-CAHN and rA-CAFHN.

Experiment IV.

Mild conjunctivitis resolved within two days PV in ND vaccinated birds. After challenge mild conjunctivitis in ND vaccinated birds resolved within one day. Sham-vaccinated controls challenged with TXGB also had mild conjunctivitis that resolved after one day and the group was dead by ten days PC after showing neurological signs, but with no lesions observed upon necropsy. Sham-vaccinated controls challenged with CA02 developed conjunctivitis and depression with severe respiratory symptoms leading to death by day five PC. Upon necropsy, hemorrhage in the conjunctiva and the cranial trachea mucosa was observed. Day two PC oropharyngeal swabs show both LaSotavaccinated TXGB-challenged and rA-CAFHN vaccinated CA02-challenged groups had significantly fewer birds shedding compared to controls (Table 4.9). More notable, rA-CAFHN-vaccinated birds challenged with CA02 have significantly fewer birds shedding than the rA-CAFHN-vaccinated TXGB-challenged birds. In day two PC cloacal swabs, both LaSota-vaccinated birds challenged with TXGB and rA-CAFHN-vaccinated birds challenged with CA02 have significantly fewer birds shedding than controls. Four day PC oropharyngeal swabs show LaSota-vaccinated TXGB-challenged birds and rA-CAFHN-vaccinated CA02-challenged birds had significantly fewer birds shedding compared to controls (Table 4.9). The rA-CAFHN-vaccinated CA02-challenged group

Number of birds shedding

Days post-challenge

Vaccine	<u>0</u>		<u>2</u>		<u>4</u>		<u>9</u>	
	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal
Control (TX) ^a	0/6	0/6	6/6	6/6	6/6	6/6	0/1	NS
Control (CA)	0/6	0/6	6/6	6/6	6/6	6/6	NS	NS
LaSota (TX)	1/20	1/20*	5/20*⊕	2/20*	2/20*	0/20*⊕	1/20	0/20
LaSota (CA)	0/20	7/20*	14/20	0/20*	5/20*	5/20*	0/20	1/20
rA-CAFHN (TX)	0/20	0/20*	14/20	0/20*	7/20*	3/20*	0/20	2/20
rA-CAFHN (CA)	0/20	0/20*	4/20*#	3/20*	0/20*#	1/20*	0/20	1/20

a: Vaccine (Challenge)

* P ≤ 0.05 significance from control vaccines

 \oplus P \leq 0.05 significance from LaSota (CA)

 $\# P \le 0.05$ significance from rA-CAFHN (TX)



Figure 4.12. Experiment IV- Comparison of virus titers from oropharyngeal swabs for all vaccine groups collected on days (a) two and (b) four post-challenge with CA02 vNDV (CA) or TXGB (TX) vNDV. Challenge occurred at 21 days post-vaccination. * Indicates significant difference from sham-vaccinated controls. ** Indicates significance between LaSota-TX and LaSota-CA. *** Indicates significance between rA-CAFHN-TX and rA-CAFHN-CA. $P \leq 0.05$.

(b)





(b)



Control LaSona LaSona CA INT TA CALINA CA

Vaccines

2 1 0

Control-Tt

Vaccine		ELISA			
	LaSota	rA-CAFHN	TXGB	CA02	
<u>LaSota</u> <u>rA-CAFHN</u>	140 +/- 19 621 +/-56	188 +/-26 813 +/- 51	145 +/-18 608 +/-47	133 +/-17 815 +/-60	3123+/-250 5368+/-288

Table 4.10. Experiment IV-Pre-challenge serology completed by micro-beta HI* and ELISA**

*HI assays were completed with four HA units of each vaccine antigen to test prechallenge serum of each vaccine group. Group arithmetic means with standard errors are presented and homologous responses are noted in bold.

** ELISA group arithmetic means are presented

had significantly fewer birds shedding when compared to rA-CAFHN-vaccinated TXGBchallenged birds. In four-day PC cloacal swabs all vaccine groups had significantly fewer birds shedding when compared to the control groups and LaSota-vaccinated TXGB-challenged birds had significantly fewer birds shedding when compared to the LaSota-vaccinated CA02-challenged group. Figure 4.12 (a) shows that all vaccine groups shed on average less virus when compared to sham-vaccinated controls. More importantly the LaSota-vaccinated birds challenged with TXGB shed significantly less virus compared to the LaSota-vaccinated birds challenged with CA02 and the rA-CAFHN-vaccinated birds challenged with CA02 shed significantly less virus compared to the rA-CAFHN-vaccinated birds challenged with TXGB. At four days PC oropharyngeal swabs show all vaccine groups shed significantly less virus than the control groups (Figure 4.12 b). For days two and four PC in cloacal swabs significance was seen between all vaccine groups compared to the controls, but no significance was seen between the vaccine groups compared to each other (Figure 4.13). The mean HI antibody titer of the rA-CAFHN vaccine group was six-fold higher to the CA02 antigen and four-fold higher to TXGB compared to the mean antibody titers induced by LaSota (Table 4.10). ELISA titers for both vaccine groups were robust with both significantly higher than the control groups, however, the rA-CAFHN vaccine group had a mean ELISA titer significantly higher than the LaSota ELISA titer (Figure 4.14).



Figure 4.14. Experiment IV-ELISA titers from pre-challenge serum taken twenty-one days after vaccination from groups vaccinated with live vaccines. * Indicates significance from the sham vaccinated controls and ** indicates significance of rA-CAFHN from LaSota. The 396 designates the cut off for a positive NDV antibody titer. Vaccine groups contain both eventual vaccine challenge groups for each vaccine. $P \leq 0.05$.

Discussion

Reduction of viral shed from vaccinated birds infected with NDV could potentially minimize the impact of an outbreak and help to prevent spreading of the disease. The goal of these experiments was to further explore the effect of matching genotype of ND vaccine strain to that of the virulent challenge virus. Matching AI vaccines to field strains have been shown to decrease virulent virus shed from vaccinated but infected chickens (Suarez et al., 2006). Others have also speculated that the similarity of the ND vaccine to the challenge strain may affect how much virus is shed and that testing different ND vaccines for their ability to protect against shed is warranted (van Boven et al., 2008). Previously, using viruses of different genotypes as vaccines, our laboratory has shown that homology between inactivated ND vaccine strain and virulent challenge strain does affect the amount of virulent challenge virus shed (Miller et al., 2007). In addition to investigating if this homology had the same affect in live ND vaccines, we wanted to test previously described recombinant viruses that would allow us to have viruses of the same genotype as the challenge virus, but of lower virulence to accommodate use as a live vaccine (Estevez et al., 2007). NDV strains commonly used in the preparation of commercial vaccines - Ulster (genotype I), B1 (genotype II) and LaSota (genotype II) - were compared as live vaccines for induced protection against challenge with virulent CA02 (genotype V) and TXGB (genotype II). A recombinant NDV, rAnhinga, of the same genotype V as CA02 and chimeras of rAnhinga expressing the glycoproteins HN or F and HN of CA02 were utilized as live vaccines against challenge with CA02, an isolate from the most recent U.S. ND outbreak, and TXGB, the virulent strain used in efficacy testing of ND vaccines in the U.S. The virulence of rAnhinga and chimeras are low enough to permit experimental use as a live vaccine. An additional recombinant, rA-CAHN, was modified so that the fusion cleavage site was that of LaSota for use as a vaccine of lower virulence than rAnhinga and the other chimeras. In addition to assessing protection against clinical disease and deaths as required by regulatory guidelines, vaccines were also assessed by the numbers of birds shedding virus after challenge, the amount of virus shed on average for each vaccine group and by the HI and ELISA antibodies induced after vaccination.

As anticipated, based on our prior study, the results from the inactivated vaccines of experiment I confirmed that birds vaccinated with the homologous inactivated vaccine shed significantly less virus than the other vaccine groups that were less genetically similar to the challenge (Figure 4.1 b). HI titers of the inactivated recombinants to the CA02 antigen correlated with virus shed in oropharyngeal swabs, while the ELISA values, even though robust, did not correlate with amount of virus shed, and therefore were not a good correlate of protection (Table 4.3 and Figure 4.2 a). The live vaccine group rA-CAHN, most similar to the challenge virus, in experiment I shed significantly less virus than B1 vaccinated birds with the CA02 challenge (Figure 4.3 a). Both rA and rA-CAHN had significantly fewer birds shedding compared to control birds and even though the amounts shed were negligible, small bird numbers per group may have prevented the demonstration of significant differences (Table 4.2). It is interesting to note that the CA02 OE vaccine with an HI titer 2 times higher and an ELISA titer 1.5 times higher than that of the live B1 had average oropharyngeal shedding with an EID_{50} of around $10^2/0.1$ ml that was about that same as the shedding seen with B1. This suggests a mechanism of protection from shed that is unrelated to the antibodies

measured in the ELISA and HI assays. Measuring mucosal IgA and cellular immune responses in future studies with these recombinants may provide direct evidence of this. In both parts of experiment I, increasing the genetic relatedness between the vaccine and the challenge virus improved protection from shedding overall with the live recombinant vaccines providing the best protection from shed.

Live vaccines have the advantage of being able to be mass applied, are less expensive to produce and most importantly are able to induce mucosal immunity through IgA production (Jayawardane and Spradbrow, 1995b). These attributes were the impetus to focus subsequent experiments on the protective affects of live vaccines. Throughout the first three experiments, relatively small bird numbers per treatment group demonstrated trends in improved protection but not significant differences in virus shedding. In experiment IV reduced numbers of treatment groups were utilized to allow greater experimental numbers per group. The most virulent lentogenic vaccine strain used in the U.S., LaSota, was compared against the recombinant vaccine that contained both glycoproteins of the CA02 virus and both vaccine groups were challenged with virulent viruses of a homologous and heterologous genotype. The LaSota vaccine/TXGB challenge combination had significantly fewer birds shedding and significantly smaller quantity of virus shedding than the LaSota/CA02 combination. The rA-CAFHN vaccine/CA02 challenge combination had fewer birds shedding virus compared to the rA-CAFHN/TXGB group. Because the quantity of TXGB shed in both control and vaccinated birds was lower than that seen in CA02, the differences in virus shedding was not significantly different from the amount of CA02 shed from rA-CAFHN vaccinated/CA02 challenged group (Figure 4.12 b). However, all of the oropharyngeal

swab samples from the rA-CAFHN group for day four PC were VI negative. The rA-CAFHN HI titer was six-fold higher for the CA02 antigen and ELISA titers were two-fold higher than that induced by LaSota.

Results from experiments II and III with conventional vaccine viruses and with recombinants followed the trends seen with the inactivated vaccines studies and were confirmed in experiment IV with a higher number of birds. Vaccine-challenge virus combinations with increased genetic relatedness were most efficient. Recombinants induced high HI and ELISA titers to CA02, whereas Ulster and LaSota gave lower, but protective titers. Cloacal swabs showed little variation in quantity of virus shed between the vaccines, except for rA-CAHN-LSCL. Evaluation of cloacal swabs in the initial testing of a vaccine virus is warranted, but only continued if the initial experiment shows this route of shed to be prominent. In addition, day zero, pre-challenge, and day nine PC swabs were most often negative and provided little additional information to the experiment. Spleen, blood, trachea and lung samples were harvested for VI of vaccine virus as evidence of how efficiently the virus could replicate in the host. While VI of spleen tissues reliably was positive for the mesogenic recombinants (rA, rA-CAHN, rA-CAFHN), oropharyngeal swabs at four days PV were the best sample to recover vaccine virus from all pathotypes. Our study confirms an earlier study that lentogens would not be found in the spleen or blood (Brown et al., 1999).

In experiment III, the rA-CAHN-LSCL vaccine protected birds from mortality with a 10% mortality rate, however, the 80 % morbidity rate, was not optimal. Virus isolation on cloacal swabs for this vaccine revealed shedding at levels higher than that found with the recombinant vaccines, on average an EID_{50} of $10^{2.0}/0.1$ ml. With the

higher vaccine dose most of the rA-CAHN-LSCL birds shed virus in oropharyngeal and cloacal swabs on two and four days PC. Even though the rA-CAHN-LSCL titers in experiment III successfully protected from morbidity and mortality they were not different from the HI result from experiment II where the birds died after challenge. This suggests factors other than antibodies are protecting these vaccinated birds from symptoms and death, but the low HI and ELISA titers seem related to the higher levels of virus shed and the number of birds shedding virus. Ideally, a recombinant ND vaccine would have a lentogenic fusion cleavage site, and be able to be given at a dose that is comparable and cost-effective to produce. The recombinant with the LaSota cleavage site used in this study failed to protect against morbidity at a dose that would have resulted in at least some protection from a LaSota-type of vaccine. More studies on the minimal infective dose are needed with this virus. It has been known that recombinants with homotypic HN and F genes function more efficiently than recombinants with glycoproteins from different viruses (Deng et al., 1995; Lamb et al., 2006; Melanson and Iorio, 2004). The Anhinga F and CA02 HN functioned well together until the cleavage site was changed to match the less virulent LaSota fusion cleavage site. Other NDV recombinants with lentogenic cleavage sites used as vector vaccines have demonstrated a decreased ability to spread sufficiently in host cells to achieve protective immune responses (Swayne et al., 2003). Because the MDT of this virus is > 168 hours, and because it seems to grow well in eggs (EID₅₀ $10^{7.1}$ /0.1ml) perhaps its use as an *in ovo* vaccine should be investigated if initial studies to investigate livability and hatchability were favorable. Also more information on transmission of all of the recombinants with

contact studies are needed to see how easily virus is passed among birds.

Results presented here provide insight into ND vaccine strategies in the future. Our data suggest that ND vaccines that are more related to a genotype V virus may be more effective in the U.S. In conclusion, our studies show that virus shed can be controlled by choosing vaccines that are more genetically similar to the challenge virus and suggest that minimizing virus shed may be a useful strategy to limit the spread of the disease. However, most of the recombinant vaccines, although experimentally causing only conjunctivitis similar to the lentogenic vaccines, had evidence of replication in internal organs suggesting it may cause more reaction if applied in the field. The rA-CAFHN recombinant virus experimentally provided improved protection and has higher HI titers than the commercial vaccines viruses tested, but because the virus does not grow to as high of titer as the commercial vaccines, has an increased potential for a more severe vaccine reaction, and it has a cleavage site compatible with a reportable ND virus, it is not a promising commercial vaccine candidate. However, the rA-CAFHN recombinant vaccine does provide proof of principle that antigenically matching the vaccine to the field virus can provide a significant advantage in protection as measured by the amount and number of birds shedding virulent virus. Newcastle disease virus remains endemic in many countries around the world, even though cheap, effective, and easily administered vaccines are readily available. The use of antigenically matched vaccines may provide an additional tool for control of this important poultry pathogen. In addition, when evaluating commercial viruses, it may be advisable to move away from TXGB as a challenge virus because this genotype of virulent virus does not appear to circulate in nature. It seems more reasonable to select a new challenge virus that presents an existing threat to U.S. agriculture, with the most obvious choice being a genotype V

CA02-like virus, because this genotype of virus continues to circulate in Mexico. Lastly, to optimize the use of vaccines as a tool to control the spread of virus in an outbreak, it may be useful to add protection from shed in the evaluation of the ability of a vaccine to protect birds from ND.

Acknowledgements

The authors thank Tim Olivier, Suzanne DeBlois, Aniko Zsak, and Dr. Mia Kim for their assistance with these studies. USDA CRIS 6612-32000-049 supported this research.

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CHAPTER 5

CONCLUSIONS

As noted in the Introduction, regulatory agencies across the world evaluate ND vaccines for their ability to protect chickens from morbidity and mortality after challenge. The amount of virus that is shed in exposed birds is not taken into consideration. Since virus shed from infected birds can be transmitted to other birds leading to further infections, the concept of improving vaccines to reduce virus shed warrants consideration. The completed experiments were designed to determine the ability of currently used and recombinant ND vaccines to reduce or prevent viral shed after a challenge with virulent strains of NDV. The hypothesis stated that increasing the genetic relatedness of the ND vaccine virus to the likely virulent challenge virus would decrease the amount of challenge virus shed from vaccinated poultry through the induction of a more specific immune response. The hypothesis was tested through the completion of these specific aims.

Specific Aim 1. To determine if vaccines prepared from inactivated strains of different lineages of NDV differ in their ability to protect against mortality, morbidity and shed of virus after a virulent challenge with CA02 NDV. More specifically, this aim will determine if vaccines prepared with the homologous strain protect against virus shedding better than the other vaccines. In each of the aims, the ability of the various vaccines to prevent mortality and morbidity via clinical observation was assessed. Shed of virus was

determined by measuring the amount of virus in oral and cloacal samples. The data shown in chapter 3 demonstrates that inactivated vaccines formulated from viruses homologous with the challenge virus, CA02, reduce the amount of virulent challenge virus shed when compared to inactivated vaccines formulated with viruses from genotypes different than that of CA02.

Specific Aim 2. To determine if live and inactivated vaccines prepared with a recombinant NDV strain, Anhinga/1993, that is in the same genotype (genotype V) as the CA02 strain are more effectively reduce shed of virus after a virulent challenge with CA02 NDV. Specifically, this aim will determine if the replacement of the hemagglutinin-neuraminidase (HN) or the HN and fusion (F) genes of the recombinant Anhinga (rA) virus for the genes of the CA02 virus and their use as vaccines reduces shed of the challenge CA02 virus. Since the HN and F genes are important for inducing protective immunity, evaluating how these genes impacted vaccine efficacy was critical. By utilizing recombinants that contained the HN or the HN and F from CA02, it was determined that adding these genes of the CA02 virus into the rA backbone reduce shedding of virulent challenge virus compared to rA for both live and inactivated vaccines.

Specific Aim 3. To determine if the amounts of TXGB shed are reduced after vaccination with commonly used vaccines such as Ulster, B1 and LaSota and the recombinants mentioned above. TXGB is the challenge virus utilized by vaccine companies in the U.S. to test efficacy of commercial ND vaccines. A series of

experiments were performed to address this specific aim. B1, LaSota and TXGB are all genotype II viruses. A comparison of how LaSota and B1 protect from shedding after a TXGB challenge or the less related CA02 was performed. Using recombinant vaccines with different degrees of homology to CA02 against CA02 challenge and against TXGB challenge, the role of homology on the level of virus shed was evaluated. Ulster, a genotype I virus genetically different from both challenge viruses, was also evaluated for its ability to reduce virus shedding against both challenge viruses. Similar to that seen in the above aims, increasing genetic relatedness decreased the amount of virus shed and the number of birds shedding virus.

Together, these findings provide compelling evidence that matching the genotype of the ND vaccine virus to that of the challenge virus can significantly reduce the number of birds shedding virus and the quantity of virus shed by individual birds. The strategies of matching genotypes for inactivated vaccines and of using reverse genetics, when low virulence viruses in the same genotype as the challenge virus do not exist, to engineer viruses for use as live vaccines have the potential to improve the efficacy of ND vaccines by decreasing the spread of the disease.

While recombinant vaccines for ND control have been approved for use in the U.S., they contain only the HN or HN and F genes of genotype II lentogens, inserted into other vectors such as fowl pox or turkey herpes virus. The recombinants tested here are complete viruses created from genes from multiple viruses. Essentially, using genomic information and reverse genetics, the recombinants produced, which are attenuated compared to the viruses they originated from, were created specifically to test as experimental vaccines against genotype V viruses. The presence of a virulent fusion

cleavage site, and the mesogenic backbone of the rA-CAFHN recombinant prevents its use in the U.S. as a live vaccine because it is a select agent and reportable to the World Organization for Animal Health. The rA-CAHN-LSCL recombinant did not replicate well in chickens, likely due to the mismatch of the CA02 HN gene with the Anhinga F gene. The reduction in shedding virulent virus after the mutation of the virulent fusion cleavage site of the rA-CAFHN recombinant to that of the LaSota fusion cleavage site, along with some other mutations that ensure the cleavage site cannot revert back to virulence will have to be evaluated. Additional vaccine experiments using recombinants containing the HN and F genes of the CA02 virus in the backbone of a lentogenic NDV will further elucidate the importance of the other NDV genes in reducing the shedding of virulent virus. Homology of inactivated vaccine virus to the challenge virus was a key determinant in decreasing the amount of virulent virus shed. Changing the virulent fusion cleavage site of the CA02 virus and having the virus replicate to high enough titers to be able to deliver enough antigen for a useful inactivated ND vaccine is problematic. Perhaps concentrating already approved viruses, such as LaSota using ultracentrifugation, would increase the antigen content of the inactivated vaccine, and "make up" for the mismatch in genotypes between the vaccine and the challenge virus. This would unfortunately increase the already higher expense associated with the production and administration of inactivated vaccines.

While there are limitations to the strategies proposed both with safety and regulatory issues, the research provides evidence that the type of vaccine given affects the amount of virus shed after challenge. In addition to evaluating the ability of a vaccine to induce protection from morbidity and mortality, reduced shedding of virulent virus that is similar to a likely outbreak virus should be taken into account. Decreasing the amount of virulent virus shed from vaccinated birds could improve ND containment and eradication in the event of an outbreak.

APPENDIX

QUANTIFICATION OF NEWCASTLE DISEASE VIRUS BY REAL-TIME RT-PCR 1

¹Miller, P.J., King, D.J, and Suarez, D.L. Presented at the 2005 American Veterinary Medical Association Annual meeting.

Abstract

Outbreaks of Newcastle disease (ND) cause economic losses to the poultry industry, and are controlled primarily by vaccination. For vaccination, challenge studies, morbidity, mortality, and viral shed are important measures of protection. Currently, virus shedding is measured by titrating viral samples in embryonated chicken eggs (ECE), which is time consuming and costly. Recently, a real-time RT-PCR (RRT-PCR) based assay has been developed and validated for the rapid diagnosis of NDV. The goal of this study was to determine if this RRT-PCR assay could be used to quantify viral load in clinical samples. Using a comparison of viral titrations in ECE and the RRT-PCR assay for five different NDV isolates, we demonstrated a correlation between RRT-PCR and viral titration in quantifying viral load. Using an *in vitro* transcribed RNA as reference standards, four of the isolates showed comparable sensitivity, but the fifth isolate differed by one log of virus. When clinical samples were compared, a weak correlation between virus titration and RRT-PCR was observed. This may reflect inconsistent RNA extraction from the samples coupled with low amounts of virus being shed on the days examined. Further optimization is necessary to use RRT-PCR as a way to measure virus from clinical samples.

Introduction

Newcastle disease (ND) is a highly contagious disease of many species of birds. Infections with Newcastle disease virus (NDV) produce a range of clinical forms that vary from asymptomatic to severe morbidity and mortality depending on virus virulence, host species and immune status. Typically infection results in a respiratory disease. NDV is a member of the Avian Paramyxovirus serotype 1 (APMV-1). It is non-segmented single-stranded RNA virus approximately 15 kb and encodes for 6 major proteins. Traditional detection of NDV isolates involves isolation in embryonated chicken eggs (ECE) followed by hemagglutination (HA) and hemagglutination inhibition (HI) tests for virus identification. Pathotyping involves determining intracerebral pathogenicity index (ICPI) in one day old chicks, an eight day test which requires BSL-3 Ag animal facilities for evaluation of suspected virulent viruses. Since the OIE has added the F protein cleavage site sequence as an acceptable way to pathotype NDV isolates, many molecular techniques have been developed with the goal of decreasing the time to identify an NDV isolate. The recently developed real time RT-PCR (RRT-PCR) protocols can rapidly identify NDV by combining the amplification of NDV RNA with a diagnostic probe to specifically detect and identify the amplified product. The goal of this experiment was to use a RRT-PCR protocol validated by the USDA for the matrix gene together with a standard curve to compare quantification of viral titer samples of five NDV isolates (Figure 5.1) from experimentally infected birds to virus isolation data.

Materials and Methods

Chickens. Four week old, specific pathogen-free (SPF) Leghorn chickens, both male and female, were obtained from the Southeast Poultry Research Laboratory (SEPRL) flock. Birds were moved into BSL-3Ag facilities at four weeks of age and were wing banded before being placed eight per Horsfall isolator with *ad libitum* access to water and feed. Birds were given two days to acclimate before being inoculated with NDV.

Viruses. Five strains of NDV were used in this experiment: LaSota, Ulster, Roakin, Pigeon (84), and Anhinga. All viruses were obtained from the SEPRL repository. Aliquots of each of the virus stocks were titrated in ECE and EID₅₀/0.1ml were calculated using the Spearman-Kärber method.

Experimental Design. In this experiment, four-week-old chickens were separated into six groups of 16 birds each; a control group and one group for each of the five test viruses. The chickens were wing-banded and the controls were inoculated with brain hear infusion broth (BHI). Each of the remaining groups was inoculated with 10^5 EID_{50} of one of the test viruses diluted in BHI. The inoculum was administered 50 µl into the right eye and 50 µl into the choanal slit. Oropharyngeal swabs were collected into 2 ml of BHI with a final concentration of gentamicin (200 µg/ml), penicillin G (2000 units/ml), and amphotericin B (4 µg/ml) on days two, four, and seven. Birds were euthanized on day seven. Swab samples (oral and cloacal) were tested using a real time RT-PCR protocol with a standard (in vitro transcribed RNA of B1 NDV strain) and also titrated in SPF, 9-11 day old embryonated chicken eggs (ECE) to compare the values

between the two methods. The goal was to be able to accurately predict how much virus was being shed from the birds by using real time RT-PCR, as a more expedient method of determining the levels of virus shedding in vaccine and pathogenesis experiments.

Virus isolation, HA and titration. Virus isolation and hemagglutination assays were conducted as described by Alexander (1998). Virus titers were calculated using the Spearman-Kärber method after inoculating 10-fold dilutions into 9 or 10 day old SPF ECE.

RNA extraction. RNA was extracted with the RNeasy kit (Qiagen, Valencia, CA) with a modified protocol for fluid samples recommended by the manufacturer as described (Wise et al., 2004). Briefly, 500 μ l of swab material from clinical samples was clarified by centrifugation at 12,000 x g for 2 min, or, for previously isolated viruses, 500 μ l of chorioallantoic fluid (CAF) was mixed with 500 μ l of 70% ethanol and 500 μ l of kit-supplied RLT buffer (Qiagen) and the entire sample was applied to the RNeasy spin column. Subsequently, the kit protocol for RNA isolation from the cytoplasm of cells was followed. RNA was eluted in 50 μ l of nuclease-free water, and 8 μ l per RRT-PCR was used for the template

RRT-PCR. For the initial virus dilutions the Qiagen one-step RT-PCR kit was used as previously described (Suarez, 2002). For the APMV-1 matrix 40 cycles are run as follows: 10 sec denaturation at 94° C, 30 sec annealing at 56° C, and 10 sec extension at 72° C. For the clinical samples, the same concentrations of reagents were used, however in the form of lyophilized beads (Cepheid, Inc. Sunnyvale, CA) to minimize the pipetting

steps, and reduce the risk of contamination. Each bead contained the correct concentration of NDV Matrix primers, probe, $MgCl_2$ and buffers for four reactions. Additional water (60 µl), dNTPs (3.2 µl), enzyme (4.0 µl) and RNase Inhibitor (2.0 µl) were added to each bead.

Results and Discussion

The goal of this study was to determine if this RRT-PCR assay, in combination with a single isolate of *in vitro* transcribed RNA, could be used to quantify viral load of various isolates from clinical samples. While the correlation between the RRT-PCR assay and traditional viral titration was not as strong as anticipated, the data do suggest that the RRT-PCR assay could be useful in clinical settings. For example, data in Table 5.1 indicate that the B1 in vitro transcribed RNA could be used as an internal control for four of the five isolates. When clinical samples were compared (Table 5.2), a weak correlation between virus titration and RRT-PCR was observed. This may reflect inconsistent RNA extraction from the samples coupled with low amounts of virus being shed on the days examined. In addition, the isolates that were the least sensitive for copy number (Pigeon, Ulster and Anhinga) seem to correlate with the nucleotide mismatches in the probe and primer sites used in this assay. Data from Figure 5.2 demonstrate that the slopes for each of the isolates were consistent, suggesting that the efficiency of amplification was similar among the different isolates. There are two significant modifications that could increase the utility of this approach. The first deals with the *in* vitro transcribed RNA for the standard. This may be problematic in that the RNA is not subjected to the same extraction and treatment of the samples. By using allantoic fluid
from an inoculated SPF ECE, both the standard and the test samples could be treated in an identical manner. This would minimize the differences in the RNA to be used for the standard and the samples. Secondly, when analyzing laboratory samples it may be beneficial to use the same strain for the standard as the infecting virus. While this strategy may not be useful for outbreak settings, it could help generate consistent and quantifiable data for laboratory samples. Several other minor modifications have been considered to optimize this assay, including using a wider range of dilutions when determining amplification efficiency, increasing the amount of virus inoculated to each bird, and analyzing samples on additional days post-infection. In conclusion, RRT-PCR provides a sensitive method to quantify NDV in biological samples and adoption of the aforementioned improvements could improve the sensitivity and reliability of the assay.

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Table 5.1 C	alculation c	of copy	number

Virus	Copy#/ one EID50
Lasota	1.13
Ulster	1.12
Roakin	1.30
Pigeon	12.01
Anhinga	1.10

Copy # calcuated using standard positive control values and virus titers

Viruses	Oropharyngeal swab (2 days post infection)			
	ECE	R	RT-PCR	
	EID ₅₀ /0.1ml	Copy#	Log [(Copy#/EID ₅₀)*100]	
Lasota				
Mean	3.01	1.96	1.8	
S.E.	1.09	1.11		
Ulster				
Mean	0.30	2.26	0.65	
S.E.	0.11	0.23		
Roakin				
Mean	0.42	2.57	1.8	
S.E.	0.11	2.59		
Pigeon				
Mean	0.11	1.07	0.19	
S.E.	0.16	0.36		
Anhinga				
Mean	0.19	2.00	1.68	
S.E.	0.12	0.73		

Table 5. 2. Comparison of conventional virus titration and RRT-PCR for clinical samples



Figure 5.1. Phylogenetic analysis of nucleotide sequences from NDV matrix protein genes used in this study. The phylogenetic tree was generated by the Clustal V method following alignment of sequences.



Figure 5.2. Log concentrations of virus diluted in allantoic fluid at observed cycle threshold number, which indicate PCR amplification efficiency among tested NDV isolates.