EFFICACY OF A RECOMBINANT HERPES VIRUS OF TURKEYS VECTOR VACCINE, EXPRESSING GENES TO NEWCASTLE DISEASE VIRUS AND MAREK’S DISEASE VIRUS IN CHICKENS AND TURKEYS, AGAINST EXOTIC NEWCASTLE DISEASE VIRUS CHALLENGE

By

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LIST OF ABBREVIATIONS

AI = Avian Influenza
Bldg. = Building
BSL-II = Bio-security level two
CAA = Chick Anemia Agent
cm$^3$ = Cubic centimeter which is equal to a milliliter
CRBC = Chicken red blood cell
CTH = Cecal tonsil hemorrhage
DOA = Day of age
DNA = Deoxyribonucleic acid
DPI = Days postinfection
ED = Eye-drop
ELD$_{50}$ = Embryo lethal dose fifty
ELISA = Enzyme linked immunosorbent assay
F = Fusion protein of Newcastle disease virus
g(s) = Gram(s)
G = Gauge of needle
gp(s) = Glycoprotein(s)
HA = Hemagglutination
HE = Hematoxylin and Eosin
H.E.P.A. = High efficiency purified air
HI = Hemagglutination inhibition
HN = Hemagglutinin-neuraminidase protein of Newcastle disease virus
HVT = Herpes Virus of Turkeys
IBDV = Infectious Bursal disease virus
IBV = Infectious Bronchitis Virus
ICPI = Intra-cerebral pathogenicity index
IgA = Immunoglobulin A
IgG = Immunoglobulin G
ILTV = Infectious Laryngotracheitis Virus
IM = Intramuscular
IVPI = Intra-venous pathogenicity index
KMnO₄ = Potassium permanganate
L protein = An RNA-directed, RNA polymerase nucleocapsid associated protein of Newcastle disease virus
LD₅₀ = Lethal dose fifty
M protein = Matrix protein of Newcastle disease virus
MD = Marek’s Disease
MDT = Mean death time
MDV = Marek’s disease virus
mL = Milliliter
n = Sample size
ND = Newcastle Disease
NDV = Newcastle disease virus
NGL = No gross lesions
nm = Nanometer
NP = Nucleocapsid Protein of Newcastle disease virus
nvNDV = Neurotropic velogenic Newcastle disease virus
NVSL = National Veterinary Services Laboratory
P protein = Nucleocapsid associated protein of Newcastle disease virus
PBS = Phosphate buffered saline
PFU(s) = Plaque forming unit(s)
PMV(s) = Paramyxovirus(es)
RBC = Red blood cell
RH = Relative humidity
rHVT = Recombinant Herpes Virus of Turkeys
RNA = Ribonucleic acid
SPF = Specific pathogen free
SQ = Subcutaneous
TPB = Tryptose-phosphate broth
TSA = Tryptic soy agar
TxNDV = Texas GB strain of Newcastle disease virus
uL = Microliter
µm = Micrometer
U.S.D.A. = United States Department of Agriculture
vvMDV = Very virulent Marek’s disease virus
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Newcastle disease (ND) is primarily a pneumotropic virus but it also has viscerotropic and neurotropic forms. Primary clinical signs caused by the pneumotropic form of the virus include respiratory snicks and sneezes, sinus exudate, mild conjunctivitis, decreased rate of body weight gain, increased feed conversion ratio, and decreased egg production in mature birds. An experimental recombinant vaccine, Herpes Virus of Turkeys (rHVT) vector carrying genes to the hemagglutinin-neuraminidase (HN) and fusion (F) proteins of Newcastle disease virus (NDV) and genes to glycoproteins A and B (gps A and B) of Marek’s disease virus (MDV), was tested in specific pathogen free (SPF) Leghorns, commercial broilers, Leghorns, and turkeys by vaccination either in ovo (broilers) or subcutaneously against Texas GB NDV challenge.

In Trial 1, SPF Leghorns, commercial broilers, Leghorns, and turkeys were not vaccinated, but infected with Texas GB, NDV at a final dose of $10^{4.5}$ embryo lethal dose
fifty (ELD₅₀) at 6 weeks of age either by the eye-drop (ED) or intramuscular (IM) route. Nearly 100% mortality was observed in all chicken types infected regardless of route of challenge. However, statistically fewer turkeys infected by ED challenge died than IM challenged turkeys.

In Trial 2, commercial broilers were vaccinated with either the rHVT vaccine in ovo or subcutaneously or the commercial B1B1 NDV vaccine by ED vaccination and challenged with $10^{4.5} \text{ ELD}_{50}$ of Texas GB, NDV. Likewise turkeys were vaccinated with either the rHVT subcutaneously or eye-drop with commercial B1B1 vaccine. Results showed that the rHVT vaccine produced equal protection (~100% protection) to the commercial B1B1 vaccine, without the vaccine reactions.

In Trial 3, commercial broilers were vaccinated with the rHVT alone or in combination with HVT/SB1 or CVI988 Marek’s disease vaccines in ovo or subcutaneously, or eye-drop vaccinated with commercial B1B1. Results showed that combining rHVT with HVT/SB1 drastically reduced protection from $10^{4.5} \text{ ELD}_{50}$, Texas GB NDV challenge at 28 days of age compared to the commercial vaccine or the rHVT alone or in combination with CVI988.

The rHVT vaccine effectively immunizes and subsequently protects chickens and turkeys against NDV challenge when administered alone or in combination with CVI988. However, it is not an efficacious vaccine when combined with HVT/SB1 and administered in ovo or subcutaneously.
Poultry vaccines presently available consist of modified-live or killed microorganisms. Vaccines are available for the majority of the major poultry pathogens and are used throughout the world. However, these vaccines have some minor drawbacks. For instance, killed vaccines when handled and administered properly will produce primarily an immunoglobulin G immune response. This is advantageous when the vaccine is used in breeder flocks to aid in transmission of maternal antibodies into the fertile egg. This is seen as a disadvantage in younger flocks since very little, if any, local immunity is established at mucous membranes; primarily the trachea. Additionally, each bird must be handled to administer the killed vaccine product. Modified-live vaccines can elicit a strong immunoglobulin A response, local immunity, when administered in the drinking water, eye-drop, or by spray. Vaccines administered in this manner are usually respiratory pathogens with the exception of Infectious Bursal disease virus. A significant disadvantage of modified-live vaccines is their potential of reversion of virulence. When some birds do not receive any vaccine or only a partial dose the modified-live vaccine can replicate in another chicken and be spread to this susceptible bird. This can continue on several times in a chicken house until the vaccine virus has reverted to its original virulence and could potentially cause disease in naive birds.

Newcastle disease most commonly results in infection of the bird’s respiratory tract. These infections range from subclinical to clinical disease. Newcastle disease
virus infection can lead to decreased rate of body weight gain, decreased egg production in mature birds, and potentially mortality. The majority of poultry today are vaccinated several times against NDV with a modified-live virus. Killed vaccines are most commonly used in areas where a highly pathogenic strain of NDV is known to be endemic or used in parent flocks to provide high maternal antibodies in 1 day old chicks.

Recombinant poultry vaccines have recently been produced in the laboratory in an effort to eliminate some of the disadvantages of modified-live and killed vaccines. Recombinant vaccines are live viruses that possess large regions of their genome that do not code for proteins or code for nonessential proteins (72). These types of virus serve as recombinant viral vectors that can carry foreign genes to other viruses. The goal in using recombinant viral vaccines is to induce immunity to the viral vector, in addition to the viruses whose genes have been inserted into the recombinant viral vector (68). The ideal recombinant vaccine would be a vaccine that can be administered one time either in ovo (in the egg) or at 1 day of age in the hatchery.

Fowl pox vectored recombinant vaccines have been produced and licensed (72). Fowl pox is large virus with large areas of non-coding regions within its genome. The drawback is that fowl pox must be administered in the wing-web at several weeks of age (103).

Herpes virus of turkeys (a naturally apathogenic virus) is being explored as a potential recombinant vector (68, 102, 103). Herpes virus of turkeys can be administered in ovo at 18 Days of incubation (114, 138) or via subcutaneous vaccination in the hatchery at 1 day of age (102, 103). Herpes virus of turkeys is an excellent candidate due to the aforementioned reasons, in addition to its ability to remain latent the life of the bird
(68, 72, 103). This provides the potential for vaccinating the birds in the hatchery and eliciting life-long immunity to MD and other viral diseases that may be incorporated in the vector.

Recent, unpublished data suggested that HVT vectored recombinants carrying several foreign genes may not provide protection towards MDV to birds when challenged less than 2 weeks of age with MDV. Therefore, this work was undertaken to evaluate the efficacy of a new HVT vectored vaccine carrying the HN and F genes of NDV and gps A and B of MDV. The first trial was performed to determine the pathogenicity of Texas GB NDV in three types of commercial poultry: Leghorns, broilers, and turkeys as compared to SPF Leghorns. Once this work had been completed, the efficacy of the rHVT construct could be evaluated in regards to its ability to produce immunity and protection to Texas GB NDV challenge in commercial broilers and turkeys. Next, the efficacy of the rHVT construct in combination with HVT/SB1 or CVI988 in commercial broilers was determined.

This work provided a detailed study of Texas GB NDV in commercial strains of birds, an area where the literature was lacking. Next, it evaluated the efficacy of new rHVT vaccine alone and in combination with standard MDV vaccine strains against Texas GB NDV challenge in commercial broilers and turkeys. This work was being conducted in an effort for the vaccine to gain United States Department of Agriculture (U.S.D.A.) licensing for sale in the poultry industry and to provide a safer and more effective vaccine for the poultry industry that could be administered in ovo or by subcutaneous vaccination at 1 day of age.
CHAPTER 2
NEWCASTLE DISEASE

History

Newcastle disease was first described by Doyle (51) in 1926 in Newcastle-upon-Tyne, England; and by Kraneveld (79) in the island-city of Java, Indonesia the same year. It is generally accepted that the virus originated in Java, Indonesia. It was transported to the shipping ports in Newcastle-upon-Tyne, England, through the importation of frozen poultry meat from southeast Asia; or through the practice of keeping live chickens on shipping vessels to provide a fresh source of meat and eggs during the excursions from southeast Asia to England (10). Although it is believed that ND originated in Java, Indonesia, it was Doyle (51) in 1927 who first described the clinical signs and pathology associated with its infection in chickens in Newcastle-upon-Tyne, England, and thus the name Newcastle disease. Other authors (78, 84, 108) have suggested that ND occurred in other regions of the world before 1926; nevertheless, by 1926 it was well recognized as a significant poultry pathogen. Within a few years the pathogen had spread throughout most regions of the world (10). Newcastle disease has also been referred to by the following names: Doyle’s disease, Tetelo disease, Ranikhet disease, atypische Geflugelpest, pseudo-fowl pest, pseudovogel-pest, pseudo-poultry plague, Korean fowl plague, avian pest, avian distemper, and avian pneumoencephalitis (10, 49).

Newcastle disease emerged in 1926 at about the same time that poultry production was changing from a village operation to a larger-scale commercial operation (63). Hanson (63) suggested three possible origins of the disease as a major entity. First, by
the growth of the poultry industry into a large-scale operation allowed the significance of ND to be recognized as a serious threat to production of meat and eggs. Secondly, the virus was present as an enzootic disease in another species and casual contact of chickens allowed the virus to adapt and thrive in a new host. This scenario of etiologic agents responsible for panzootics entering a new species was seen in the early 1970s with ND in psittacine species (51). The third scenario suggested the emergence of ND was a virus of low virulence that had undergone a significant mutation resulting in a more pathogenic virus causing clinically recognizable ND.

Newcastle disease was first observed in the United States in the mid 1930s in California (22, 143). The etiologic agent was indistinguishable from that described by Doyle in 1926 (51). However, the clinical signs reported in chickens in the United States were much different and included mild respiratory signs and occasional neurological signs. Observation of these clinical signs led to the name of “pneumoencephalitis” (22, 23).

Classification

Newcastle disease virus (NDV) is classified as a *Paramyxovirus* type-1 virus. The families *Paramyxoviridae* and *Rhabdoviridae* are members of the order *Mononegavirales* (14). The viral family *Paramyxoviridae* contains two subfamilies: *Paramyxovirinae* and *Pneumovirinae* (14). The subfamily *Paramyxovirinae* contains the genera *Paramyxovirus*, *Rubulavirus*, and *Morbillivirus*. The genus *Paramyxovirus* includes species of mammalian parainfluenza viruses and Sendai virus. Measles, rinderpest, and distemper viruses are found in the genus *Morbillivirus*. Newcastle disease and other avian paramyxoviruses are members of the genus *Rubulavirus*, as are mumps and human parainfluenza types 2 and 4 (13, 14). The subfamily *Pneumovirinae*, in the family
Paramyxoviridae, is composed of a single genus, Pneumovirus that is composed of species of avian pneumoviruses (13, 14).

There are nine different serotypes of paramyxovirus (PMV) identified. As previously mentioned, NDV is a member of the PMV-1 serotype. Paramyxovirus type-1 is the primary serotype affecting chickens and turkeys (11, 14). Turkeys and passerines are the primary hosts for PMV-2 infections, although outbreaks of PMV-2 also have been seen in chickens (6, 7, 61). Paramyxovirus type-3 infections are seen in turkeys only in various countries (11, 14, 18, 89, 147, 172) but chickens are believed to be fully susceptible to infection (14). Paramyxovirus Types 4 through 9 do not infect domestic poultry and are discussed by Alexander (11, 14).

**Morphology**

Newcastle disease virus (along with all the members of the family Paramyxoviridae) is a single-stranded RNA virus of negative polarity (RNA strand opposite that of the mRNA) with a nonsegmented, symmetric, and helical capsid. Paramyxoviridae viruses assemble within the cytoplasm of the host cell and acquire their viral envelope from the host-cell membrane upon budding (94).

Negative contrast electron microscopy reveals that NDV is pleomorphic, with virions ranging in size from 100 to 500 nanometers (nm) in diameter. Newcastle disease virus has surface projections approximately 8 nm long with a characteristic herringbone nucleocapsid (11, 14). The single stranded, negative polarity RNA molecule consists of 15,156 nucleotides (46, 100) with a molecular weight of 5.2 to 5.7 x 10^6 daltons (46, 77). The genome of NDV codes for six proteins (100). Samson (128) describes these proteins as the following:
• M protein for the matrix protein
• P protein which is a phosphorylated, nucleocapsid associated protein
• NP protein, a nucleocapsid protein
• L protein, an RNA-directed RNA polymerase nucleocapsid associated protein
• HN protein, hemagglutinin-neuraminidase protein that has hemagglutinin and neuraminidase functions
• F protein, fusion protein

Samson provides an exquisite explanation of the function of these proteins (128). The HN protein forms the largest surface projections while the smaller surface projections are of the fusion protein (128).

**Hemagglutinin-Neuraminidase Surface Protein**

Unique to paramyxoviruses, the hemagglutinin-neuraminidase protein of ND carries out two functions, in contrast to two proteins for these two functions in other orthomyxoviruses (132). The first is hemagglutination of red blood cells (RBCs) (132, 133). The hemagglutination seen with NDV and other avian paramyxoviruses is due to the HN protein’s ability to bind to RBCs and to other cells with the proper receptors, sialic (neuraminic) acid (91, 144). Thus, the HN protein’s main role is attachment of virus to host cells (111, 132). It has been shown that NDV can bind to and hemagglutinate amphibian, reptilian and avian RBCs (81). Additionally, all NDV strains tested by Winslow et al. (163) showed hemagglutination of guinea pig, mouse and human RBCs. Winslow et al. (163) also showed that hemagglutination of horse, sheep, goat, swine, and cattle were possible, but dependent on the strain of NDV being tested. The wide range of host cells is most likely attributable to the fact that NDV requires sialic
(neuraminic) acid for attachment (40) and virtually all cells possess sialic (neuraminic) acid (111).

Neuraminidase enzyme (mucopolysaccharide N-acetyl neuraminyl hydrolase ED 3.2.1.18) function is not clearly understood; but it is believed that this enzyme removes viral receptors (sialic acid) from cell surfaces that are budding virus, so that the same cell is not infected twice (14, 127, 133). Additionally, the neuraminidase enzyme causes slow elution of agglutinated RBCs (2).

**Fusion Surface Protein**

The fusion protein provides fusion of the viral membrane and host cell membrane, allowing the viral nucleocapsid to enter the host cell. Hemolysis is seen when the viral envelope fuses with red blood cells, leading to lysis (126). The fusion protein, immediately after protein synthesis, is a nonfunctional protein referred to as F0 protein. This F0 protein must undergo posttranslational cleavage to the active fusion proteins F1 and F2 (14, 126, 128). An extensive review of the fusion protein is provided by Choppin and Scheid (41). The cleavage of F0 to functional F1 and F2 proteins was seen in all virulent strains tested by Nagai and Klenk (105, 106). Posttranslational cleavage of avirulent strains of NDV F0 protein was not observed in vivo. However, cleavage of F0 derived from avirulent NDV to a functional F1 and F2 was noted in embryonated eggs and in vitro cultures of chorioallantoic membrane cells (105, 106). Nagai et al. (106) also found that avirulent strains of NDV had an F0 protein and not an F1 and F2 protein. These avirulent strains were noninfectious and unable to cause lysis of RBCs.

**Viral Replication**

Newcastle disease viral replication is the most rapid among the paramyxoviruses. Newcastle disease virus is able to overtake host cell protein synthesis within 6 hours (69).
and to produce maximal yields of virus within 12 hours of initial infection (111). Viral transcription takes place solely in the cytoplasm (111). Viral replication occurs most commonly in the respiratory and intestinal tracts (14). Peeples (111) gives a detailed explanation of NDV viral replication.

**Distribution and Spread**

Newcastle disease has a worldwide distribution (14, 150). Its spread worldwide can be attributed to the change from raising chickens in small backyard flocks to the present commercial poultry industry that is based on a global scale with extensive movement of poultry meat and eggs internationally (8, 10). If the poultry industry stayed at a small, local level, ND may not have become the devastating pathogen it is today (8).

Newcastle disease virus is thought to spread through several routes:

- Movement of live poultry, their offal, manure, and poultry products such as meat, eggs, and feathers
- Interstate and international movement of the aforementioned
- Movement and importation of captured free living birds and pet birds (74)
- Movement of pet and exotic birds and racing pigeons
- Movement of other animals serving as fomites
- Airborne spread
- Movement of people and equipment from poultry or aviary facilities
- Contaminated poultry feed or water
- Vaccines (10, 81, 82, 92)

Humans pose the greatest risk for the spread of NDV. Humans can be infected with NDV in the conjunctival sac and act as carriers (14, 85) or most commonly, be contaminated with feces and act as fomites (14). Migratory waterfowl, especially the
double-crested cormorants, and importation of exotic birds pose a great risk for the spread of NDV especially those of the velogenic pathotype (152, 153). Kuiken et al. (80) studied the pathogenesis of NDV in double-crested cormorants. Kuiken et al. (80) discovered that excretion of NDV in feces lasted for $15 \pm 6.2$ days after infection with a maximum excretion of 28 days postinfection. Extended periods of excretion of NDV in the feces, in addition to cormorants’ lack of morbidity and mortality with NDV infection contributes to double-crested cormorants’ ability to infect commercial poultry especially in the autumn during migratory flight (80).

Infection of birds through inhaling infective particles or by ingesting fomites or infective feces is thought to be the primary routes of infection for NDV (10, 109). Infection through inhalation has allowed for mass application of modified-live vaccines (97). Much controversy remains on whether NDV is transmitted vertically. Alexander (10, 14) summarized possible means of vertical transmission.

**Host Range**

Newcastle disease virus was found in 1981 to infect 117 different species of birds in 17 of the 24 orders of birds in the class Aves (87). Kaleta and Baldauf (74) suggested that nearly all of the 8,000 species of birds could be infected with NDV. Kaleta and Baldauf (74) found NDV infection in 236 species of aves, with 27 of the 50 orders of aves represented. They further concluded that the aquatic birds were the most resistant, while gregarious birds were most susceptible (74). Numerous references from which NDV has been found in a number of wild birds can be found in *Infectious and Parasitic Diseases of Wild Birds* (49).
Incubation

The average incubation period for NDV is 5 to 6 days, with a range of 2 to 15 days. The large variation in incubation periods is due to a number of predisposing factors. These predisposing factors include age and species of host, immune status, route of exposure, environmental factors, complicating infections, and dose of virus (14).

Human Health Significance

Newcastle disease is considered zoonotic. Newcastle disease is seen in humans as a mild conjunctivitis with preauricular lymphadenitis, headaches, fever, chills, and general malaise. The virus has been isolated from human corneal scrapings along with observation of rise in HI antibody titer, suggesting true infection. The disease is rare and is usually only seen in poultry-house workers or individuals involved with vaccine production. There are no reported cases of horizontal transmission between humans (36, 85).

Pathotypes

Newcastle disease virus strains are grouped into three pathotypes. In order of increasing virulence these are lentogenic, mesogenic, and velogenic (65). Newcastle disease is primarily a respiratory pathogen, but also has viscerotrophic and neurotrophic forms (28). The lentogenic pathotype, also known as Hitchner’s form, is characterized by mild respiratory signs to an unapparent infection (28, 70). The mesogenic form has respiratory signs, but can also have neurological signs and low incidences of mortality. The mesogenic strain is also known as the Beaudette’s form of NDV (28, 70). Viruses in the viscerotrophic form of velogenic NDV (also known as Doyle’s form or Asiatic NDV) cause hemorrhagic visceral lesions (63) without signs of neurological lesions (110), but
can also cause respiratory signs and lesions (12, 39, 62, 66, 73). Primary signs in 
neurotropic-velogenic NDV (nvNDV) are neurological and respiratory (24).
Neurotropic-velogenic NDV is also known as Beach’s form of NDV (63). The Texas-
GB strain is an example of a neurotropic-velogenic strain of Newcastle disease virus.

Hanson and Brandly described a means of grouping strains and new isolates of 
NDV by their mean death time in chicken embryos (65). This system places viruses 
inducing a mean death time (MDT) in chicken embryos of less than 60 hours in the 
velogenic pathotype, viruses inducing an MDT in chicken embryos between 60 and 90 
hours in the mesogenic pathotype, and viruses inducing an MDT in chicken embryos of 
greater than 120 hours in the lentogenic pathotype. Other tests used to help determine the 
pathogenicity of a specific virus are intracerebral pathogenicity index (ICPI) in 1 day old 
chicks and intravenous pathogenicity index (IVPI) in 6 week old chickens. Alexander (8) 
and Allan (17) compared the MDT, ICPI, and IVPI of various strains.

The disease described by Doyle in 1927 (51) was an acute and lethal infection in 
exposed chickens. Lesions described by Doyle (51) consisted of hemorrhagic lesions in 
the digestive tract. This form of the disease was later described as the viscerotropic form 
of velogenic ND or Doyle’s form of NDV (63). Neurotropic-velogenic Newcastle 
disease acquired its designation from accounts of respiratory and neurological signs in 
chickens by Beach (22).

Clinical Signs, Morbidity and Mortality

Presence, location and severity of clinical signs are often dependent on the strain of 
virus infecting the birds, the age of the birds, and other factors (92). Clinical signs of 
lentogenic and mesogenic pathotypes are described in detail by Alexander (14).
Viscerotropic-velogenic Newcastle disease typically causes 100% mortality in susceptible birds. Clinical signs include periocular edema, weakness, muscle tremors, increased respiration, listlessness, green diarrhea, wing and leg paralysis, torticollis, opisthotonos, prostration, and death (14, 17, 92).

Texas GB NDV (as well as strains of neurotropic-velogenic NDV) causes rapid onset of severe respiratory signs, followed by rapid onset of neurological signs. Diarrhea is usually absent. In the sexually mature bird, a precipitous decline in egg production is observed. Unlike viscerotropic-velogenic NDV, in which 100% mortality is common (14, 17, 92), neurotropic-velogenic strains are reported to cause up to 100% morbidity with approximately 50% mortality in adult birds and up to 90% mortality in young birds (14).

Turkeys are also highly susceptible to infection with NDV. However, clinical signs are not usually as severe in turkeys as in chickens (14, 31, 92).

Gross Lesions

There are no pathognomonic signs of ND (14, 109). Therefore, isolation and identification of NDV (25) is necessary for a definitive diagnosis (9). However, the presence of hemorrhagic intestinal lesions aid in differentiation of viscerotropic ND from neurotropic ND (where hemorrhagic visceral lesions are usually absent) (64, 66). King (Personal Communication, December 2, 1998) suggests that if hemorrhagic visceral lesions are observed in the neurotropic-velogenic form of NDV, they are limited to the cecal tonsil lymphoid aggregates. Ojok and Brown provide a comprehensive study of the gross lesions seen in the Fontana 1083 strain of the viscerotropic-velogenic strain of NDV (110). Lesions seen in their study are similar to those observed with other strains of viscerotropic-velogenic NDV (12, 14, 39, 62, 66, 73). Pictures of the common
viscerotropic-velogenic NDV lesions in chickens are shown by King, Swayne and Beard in *Diseases of Poultry* (14), and *Illustrated Manual for the Recognition and Diagnosis of Certain Animal Diseases* (37).

Gross lesions of the central nervous system are usually not present in birds infected with NDV regardless of the pathotype (92). Respiratory lesions are rare; but when present consist of airsacculitis (28), and more commonly as marked congestion and severe hemorrhagic lesions of the trachea (15).

**Histopathologic Lesions**

The histopathologic lesions observed in birds infected with NDV are outlined by Alexander and others (14, 27, 28, 92, 110, 112, 121, 161). Refer to these references for a complete description of histopathologic changes in the various tissues infected with NDV.

**Immunity**

Meulemans et al. (98) suggested that antibodies produced against the HN and F surface glycoproteins of NDV aid in the protection against NDV infection. This paper concluded that the F glycoprotein is likely a better immunogen to confer protection to NDV challenge. Reynolds and Maraqa (118) showed that passive immunization with antibodies to the HN and F proteins confers protection against NDV challenge. Antisera containing the NP/P and M proteins of NDV did not protect the birds from clinical signs of NDV. Reynolds and Maraqa (119) concluded in another trial that cell-mediated immunity alone is not sufficient to protect against NDV challenge. Reynolds and Maraqa (119) concluded that cell-mediated immunity must be accompanied by hemagglutination inhibition or virus-neutralizing antibodies to elicit protection. Seal et al. (134) described the immunologic response to NDV and concluded that B-cell depleted chickens were
protected from NDV challenge. They further concluded that the cell-mediated immune response (in addition to yet-to-be-determined factors) contributes to immunity to NDV in B-cell depleted chickens.

**Exotic Newcastle Disease**

Newcastle disease viruses classified in the velogenic pathotype are not found in Canada, Australia, New Zealand, Iceland, Finland, Denmark, Norway, Republic of Ireland, Northern Ireland, Sweden, and the United States; and thus are considered exotic NDV. Exotic NDV is a highly infectious disease of poultry, often peracute with birds showing no prodromal signs (150). Exotic NDV can cause nearly 100% morbidity and mortality in unvaccinated flocks. Exotic NDV also has been shown to cause disease and mortality in vaccinated flocks (150). Numerous outbreaks of exotic ND have occurred in the United States: 1941, 1946, 1951 (160); the panzootic in 1970 to 1974 in California (55, 159) that cost the United States $52 million to eradicate (160), and again in 1977, 1979; and a panzootic in racing pigeons in the 1980s (156). Most notably, an ongoing outbreak of exotic NDV in California and Nevada is presently occurring. This outbreak began in October 2002 in Los Angeles County, California (19). As of January 10, 2003, 1.2 million birds have been destroyed or are slated for destruction in an effort to control the spread and eradicate the disease (53).

**Control Policies and Eradication Programs**

The status of exotic or velogenic ND in a country has drastic implications on the ability of that country to export poultry and poultry products (14). During the present outbreak of exotic NDV in California and Nevada, Mexico and Canada banned import of United States poultry. Mexico is the United States’ third largest importer of U.S. poultry (52). The near ubiquitous nature of lentogenic strains of NDV occurring naturally or
present through use in vaccine programs makes serologic evidence of NDV of no value (14) in establishing the exotic NDV status of a country. Therefore, proper isolation and identification of the etiologic agent is necessary (14, 25) to unequivocally ascertain the pathotype of the virus. The presence of exotic NDV in a country has crippling effects on the economy through the loss of export markets to countries that are free of exotic NDV. The United States Department of Agriculture, Animal and Plant Health Inspection Service compiled a complete guide to the eradication of exotic NDV from the United States of America for when an outbreak does occur (151).

**Standard Vaccination**

Allan et al. (17) found no significant antigenic difference among strains of NDV in the PMV-1 serotype. Thus, vaccines developed from a PMV-1 strain or isolate, regardless of pathotype will protect against even virulent NDV challenge (17). This is ironic, because the fact that the pathogenicity and severity of clinical signs vary greatly among strains. This suggests that antigenic homogeneity is highly preserved (11). Vaccination and production of NDV vaccines have been well documented (14, 47, 97).

Since the 1940s, commercial poultry have been vaccinated against ND (28). Modified-live vaccines of the lentogenic and mesogenic strains are commonly used in the poultry industry worldwide to protect birds from NDV challenge (14, 28). Advantages of modified-live vaccination are (14):

- Rapid and inexpensive mass application through administration of vaccine in the drinking water or by aerosol spray
- Inexpensive
- Stimulation of local immunity at sites of viral entry
- Horizontal spread of vaccine virus may occur in cases where some birds where missed
Disadvantages of modified-live vaccines include (14):

- Poor uniformity of application
- Horizontal transmission of vaccine virus with reversion to virulence and actually causing the disease
- Maternal antibody interference with the development of protection
- Very labile products that necessitate special handling and administration
- Contamination with unwanted viruses or bacteria

Inactivated vaccines are often used to boost immunity in breeder-type poultry to provide maternal antibodies in the egg yolk for the developing embryo and chick.

Advantages of these vaccines are (14):

- They are much more stable
- Easier to store and handle
- Maternal antibody levels of the birds receiving the vaccine do not interfere with vaccination to the degree that seen with modified-live vaccines
- Vaccine reactions are nearly nonexistent

Disadvantages include the high cost of administration due to the need to inject each bird individually intramuscular or subcutaneously and the higher cost of production (14).

Allan et al. (17) gave a detailed explanation of vaccination principles and methods, vaccination production and quality control. Inactivated vaccines are also used in broilers and commercial layers in countries where velogenic NDV is endemic (Personal communication, G. D. Butcher, August 21, 1998).

In ovo Vaccination

Sharma, Burmester, and Witter (138) first described in ovo vaccination in 1982. The first commercially available automated egg inoculation system, Inovoject®, was produced in 1992 by Embrex, Inc. (Research Triangle Park, NC). The Inovoject®
machine can vaccinate 36 to 150 eggs simultaneously with 50 to 200 microliters (µL) of vaccine per egg into the amniotic cavity (120). This has largely done away with 1-day-of-age subcutaneous vaccination for Marek’s disease in broilers in the United States of America. Today, more than 80% of the broilers in the United States are vaccinated for Marek’s disease virus in ovo (120).

Sharma, Burmester, and Witter (138) demonstrated that herpes virus of turkeys (HVT) in ovo vaccination at Day 18 of incubation provided better protection than HVT subcutaneous vaccination at 1 day of age when challenged with MDV at 3 days of age. In 1982, Sharma, Burmester, and Witter (138) showed that in ovo vaccination with either HVT (serotype-3) or serotype-2 MDV elicited protective immunity at 3 days of age when challenged with virulent MDV. The site of rapid viral vaccine replication was discovered to be the embryonic chick lung (139).

Numerous field trials using commercial broilers have been conducted to evaluate the safety and efficacy of in ovo vaccination. In these trials, in ovo vaccination and 1-day-of-age subcutaneous vaccination against MDV were compared. There was no difference in hatchability, 2 week mortality, and livability; and live body weights were not significantly different. However, improvements in feed conversion and reduced settlements at time of processing were observed in the Inovject® MDV vaccinated birds as compared to those birds vaccinated at 1 day of age by the subcutaneous route (58, 59). Sarma and colleagues (129) conducted a similar trial that analyzed the efficacy of in ovo versus subcutaneous 1-day-of-age vaccination with HVT and SB1. Sarma and colleagues (129) showed that livability and feed efficiency were improved in the in ovo vaccinated
birds, in addition to, reduced condemnations caused by air sacculitis and septicemia. Condemnations due to leukosis (MDV) were similar in the two vaccinated groups (129).

Studies to determine the effect of in ovo vaccination and time of transfer from the incubators to the hachers at Days 17 or 18 of incubation have been conducted in commercial broiler eggs. These studies have proven that injection and transfer at Day 18 of incubation decreases hatch by only 0.33% over the decrease of 1.4% seen when eggs are transferred at Day 17 of incubation (120).

Since many of the vaccines used in poultry are attenuated via serial passage and produced in SPF eggs, these egg adapted vaccines cannot be used for in ovo vaccination without killing or severely affecting the embryo. Commercial infectious bronchitis virus was inoculated into incubating eggs at Day 18 of incubation and was found to be pathogenic to the embryo (157). After serial passage 40 times in tissue culture, this vaccine was safe to use in ovo. This newly attenuated vaccine also was found to protect against challenge (158). A similar phenomenon occurs with NDV vaccines. Both commercial B1 and LaSota strains of NDV cannot be safely administered to embryonating eggs. Modify the B1 strain of NDV with ethylmethane sulfate, an alkylating agent, reduces the vaccine’s pathogenicity to the embryo without compromising its ability to protect against neurotropic-velogenic challenge with the Texas GB strain of NDV (4, 5).

Maternally derived antibodies to MDV have been shown to neutralize the HVT and MDV vaccine. Sharma and Graham (1982) observed that greater interference is seen with cell-free MDV vaccines than with cell-associated MDV vaccines. It is suggested
that the antibodies are able to effectively neutralize the cell-free MDV vaccine, but not
that of the cell-associated MDV vaccine.

Recently, in ovo administration of recombinant vaccines has been investigated. The safety and efficacy of an rHVT vector carrying the HN and F genes to the Hitchner B1 strain of NDV and glycoproteins A and B from the GA strain (serotype-1) of MDV administered in ovo at Day 18 of incubation was determined by Reddy et al. (114). This was reported as the first account of in ovo administration of an rHVT vaccine (114). This study concluded that there was no detrimental effects on hatchability or chick survival when the recombinant vaccine was administered in ovo at doses ranging from 1,000 to 20,000 plaque-forming units (PFU) per egg. The recombinant vaccine provided complete protection to the SPF birds challenged with the Texas GB strain of neurotropic-velogenic NDV at 4 weeks of age whether vaccinated in ovo or subcutaneously. The recombinant vaccine failed to prevent viral replication in the trachea when tested at 3 and 5 days postchallenge regardless of whether the birds were vaccinated in ovo at Day 18 of incubation or subcutaneously at 1 day of age. In contrast, the chemically altered, Hitchner B1 strain of NDV used in this trial administered in ovo provided complete protection from viral replication (114).

Advantages of in ovo vaccination are (120):

- Precise and uniform injection
- Earlier immunity
- Reduced contamination
- Decreased labor costs
- Reduced bird stress due to less handling at hatch
Recombinant Vaccines

The first commercially licensed recombinant viral vector vaccine was produced in poultry (72). Many recombinant viral vectors are presently being explored at this time. Some of these vectors are herpes virus of turkeys (72, 102, 114), pigeon pox (83), vaccinia virus (96), baculovirus (107), Marek’s disease serotype-1 (127), *Salmonella gallinarum* and *S. typhimurium* (154).

Viral vectors should be nonpathogenic and should possess large regions of their genome that do not code for proteins (or at least non-essential proteins). Advantages of viral vectors are that they are live and do not require boosting; and that multiple genes to multiple pathogens can be inserted into one viral vector (72), theoretically, this immunizes against all antigens present in the viral vector (68). A successfully used avian viral vector is fowl pox (29, 30, 72, 145). For HVT and other herpes viruses, a major advantage is their ability for latent infection. Since HVT can persistently infect the host, it is believed that both a humoral and cell-mediated immune response is elicited (68, 72, 103). The most significant disadvantages of recombinant vaccines are their enormous expense for production; and that they establish immunity only to those genes inserted for each specific disease (72).

Aside from the aforementioned disadvantages, recombinant vaccines, in principle, should circumvent many of the disadvantages of modified-live vaccination, as previously mentioned (Standard Vaccination) (93). Recombinant vaccines allow genes for more virulent strains of viruses to be inserted into the vector virus. Additionally, since only selected genes from a virus (typically genes to surface or antigenic proteins, and not the entire viral genome are present in the nonpathogenic viral vector), there is no risk of reversion to virulence for the virus whose genes have been inserted.
Fowl pox virus was the first viral vector to obtain licensing. This recombinant vaccine contains both the hemagglutinin-neuraminidase (HN) and fusion (F) genes for NDV’s two surface proteins (72). Similar constructs were produced and licensed by Hoechst Roussel Vet, Vector-Vax® FP-N, 1994, and Trovac-NDV by Select Laboratories, 1995 (72). This fowl pox viral vector had the thymidine kinase gene deleted and carrier genes inserted in its place, not affecting viral replication (72). Investigators have described successful vaccination and resulting protection against velogenic NDV challenge using the fowl pox vector with the F gene inserted (29, 30, 93, 145); the HN gene inserted (30, 68, 93); and both the HN and F genes inserted (76). The fowl pox vector is advantageous because there is no possibility of it causing respiratory disease. Fowl pox maternal antibodies interfere with the NDV immune response, which is a major disadvantage for the fowl pox recombinant vaccine carrying genes to NDV (72). Fowl pox vectored recombinant vaccines cannot be given at a young age (103). In comparison, rHVT vectored vaccines can be administered at 1 day of age (103) or in ovo at Day 18 of incubation (114). For these reasons, fowl pox recombinants have fallen out of favor in the chicken industry. However, fowl pox recombinant vaccines have been used to a limited extent in the turkey industry for their NDV immunity potential (137). However, cost of the vaccine has prohibited extensive use of this vaccine. McMillen (93) gives a detailed review of the safety and efficacy of fowl pox virus as a recombinant vector.

**Herpes Virus of Turkeys: Vectored Recombinant Vaccines**

Over the past several years, numerous studies have been conducted with HVT vectored vaccines with various gene insertions. As previously stated, HVT appears to be an ideal vector because its ability to be administered in ovo near Day 18 of
incubation (114, 138) or subcutaneously at 1 day of age (102, 103). Additionally, HVT is an ideal vector for recombinant vaccines because of its lack of horizontal spread (88). This is advantageous because injection of the viral vector is necessary for the deliberate administration of the vaccine. Thus, spreading of the recombinant vaccine to neighboring flocks or wild bird populations is minimized if not eliminated. Next is a brief review of some of the HVT constructs that carry genes to NDV.

Trials have been conducted in which only the HN or only the F gene of NDV is inserted into nonessential regions of the HVT genome. Morgan et al. (103) conducted the first trial in chickens using which HVT was used as a vector, carrying genes to a completely unrelated virus. Their work is intriguing because HVT is a DNA virus, while NDV is a single-stranded RNA virus of negative polarity (14, 38). Morgan et al.’s trial (103) suggested that 1-day-of-age chicks vaccinated intra-abdominally with an HVT vector carrying the F gene to NDV had statistically significant higher protection against the Texas GB strain of neurotropic-velogenic, NDV intramuscular challenge at 28 days of age as compared to a similar HVT construct with the HN gene to NDV inserted. The two HVT-F constructs protected birds from systemic signs of illness at a level greater than 90% while the HVT-HN constructs protected 35% of the birds in one trial and 58% in another trial. Viral replication in the trachea was prevented in 88% of the intramuscular and 16% of the ocularly challenged birds for the HVT-F or combination of HVT-F and HVT-HN constructs (103). Morgan et al. (103) also administered an HVT-F construct simultaneously with an HVT-HN construct. Results of this challenge study demonstrated complete protection from clinical signs of systemic illness. This level of protection was not statistically different from that observed in the HVT-F vaccinated
birds. Tracheal swabs collected at 5 days after challenge, at 33 days of age, failed to recover virus in 60% and 21% of the birds challenged intramuscular and ocularly, respectively. Additionally, commercial NDV Hitchner B1 modified-live virus vaccine also provided 100% protection from systemic illness and viral replication. These data, however, do not exceed the level of protection provided by standard, modified-live, currently available vaccines.

Prior studies have also shown that the F glycoprotein expressed in a fowl pox (29) or vaccinia (96) vector elicits protection against NDV challenge. In contrast, Nagy et al. (107) subcutaneously vaccinated nine white Leghorn chickens at 4 weeks of age with a live recombinant baculovirus carrying the HN gene to NDV in an oil-emulsion. Birds were challenged via the oculonasal route 3 weeks after vaccination with the neurotropic-velogenic strain of NDV, Texas GB. Results of this trial suggest that the HN glycoprotein is able to elicit complete protection to velogenic NDV challenge (107). These data should be taken lightly as the birds were 4 weeks old when vaccinated, the recombinant vaccine was in an oil-emulsion, birds were 7 weeks old when challenged, and challenged with $10^6$ mean embryo infectious dose fifty instead of embryo lethal dose fifty. Villegas and Purchase (155) provided a detailed description of the difference in these two measures of viral activity.

While Morgan et al. (103) had described the relative immunogenicity of the HN and F glycoproteins, Heckert et al. (68) determined onset of protection. In this trial, SPF chickens were vaccinated at 1 day of age with an rHVT construct carrying both the HN and F genes to NDV and genes to glycoproteins A and B of Marek’s disease virus. Heckert et al. (68) demonstrated that the commercial, modified-live Hitchner B1 strain of
NDV provided complete protection against intraocular and intranasal Texas GB NDV challenge at 14 days after challenge while the recombinant vaccine provided greater than 94% protection. The difference between the percent protection elicited by the commercial vaccine and the recombinant vaccines was not statistically different. In comparison, a difference in protection was observed in a second trial by Heckert et al. (68) when the birds at 8 days of age where challenged 7 days after vaccination. The commercial, modified-live Hitchner B1 vaccine protected all birds challenged. The recombinant vaccine only protected one-half of the birds challenged when administered at a dose of 500 plaque forming units per milliliter (mL) or 75% when administered at 1,300 PFU/mL. A similar trial was conducted in which SPF birds were vaccinated and challenged as above but at 4, 7, 10, and 14 days after 1-day-of-age vaccination (68). The commercial vaccine outperformed the recombinant vaccine at 4, 7, and 10 days postvaccination. Percent protection for the commercial vaccine was 40%, 90%, 100% and 100% in comparison to 0%, 35%, 85%, 100% for the recombinant vaccine at 4, 7, 10, and 14 days postvaccination, respectively (68). Heckert et al. (68) discredit this as a meaningful difference caused by the presence of maternal antibodies in commercial derived chickens. These results are similar to those reported in a prior study by Morgan et al. (102). However, complete protection was achieved in the Heckert trial (68) at 14 days postvaccination, while Morgan et al. (102) did not observe this same level of protection until 21 days postvaccination. Morgan et al. (102) noted that the rHVT with the F gene to NDV elicited protection considerably later than a commercial modified-live Hitchner B1 vaccine at 6 days postvaccination, and an inactivated oil-emulsion NDV vaccine at 14 days postvaccination. Heckert et al. (68) suggested that the delay in onset of
complete protection can be attributed to the recombinant vaccine in the trial by Morgan et al. (102) containing only the F gene to NDV, not both the HN and F genes. This suggests somewhat of a synergism between HN and F genes being expressed on a recombinant viral vector. Understanding the aforementioned results (68) is paramount in appreciating the limitations of recombinant vaccines.

The effect of maternal antibodies in the developing embryo, in the case of in ovo vaccinated broilers, or maternal antibodies, in the 1 day old chick vaccinated subcutaneously, must be considered. The efficacy seen in SPF Leghorns will likely be different than that in commercially available genetic stock possessing maternal antibodies. Morgan et al. (102) conducted a trial in which commercial broilers and SPF Leghorns were challenged intraocularly at 29 days postvaccination using the Texas GB strain of velogenic NDV. The birds in this study had been vaccinated subcutaneously at 1 day of age with an rHVT vector carrying the F gene to NDV. This trial demonstrated that maternal antibodies interfere with viral replication and thus decrease protection to challenge. The recombinant vaccinated broilers had 73% protection while the commercial, modified-live Hitchner B1 vaccinated broilers had 80% protection following challenge. Conversely, SPF Leghorns showed complete protection to NDV challenge regardless of whether they received the commercial B1B1 or recombinant vaccine (102). This same study also evaluated the ability of the recombinant vaccine to prevent viral replication and clear the virus from the trachea by 5 days after challenge. The percent protection from viral replication in the trachea for those birds vaccinated with the recombinant HVT-F vector ranged from 11% to 20% for the commercial broilers and 18% to 50% for SPF Leghorns. The greater percent protection was seen in those birds
challenged with only NDV and not both NDV and MDV. Although these levels of protection appear to be different, statistically all of the above levels of protection are similar. However, a statistically significant difference in the percent protection was noted in the commercial, modified-live NDV vaccinated birds over that of the recombinant vaccinated birds. SPF Leghorns were completely protected while the commercial broilers were protected 90% of the time when vaccinated with the modified-live NDV vaccine (102).

Jackwood (72) stated that for recombinant vaccines to obtain a large portion of the poultry vaccine market they must be safer, less expensive and more efficacious than the currently available vaccines.

Future recombinant vaccines will likely carry genes to Infectious Bronchitis virus (IBV), Infectious Bursal disease virus (IBDV), Infectious Laryngotracheitis virus (ILTV), Avian Influenza (AI), and cytokines or immune modulators (72). As biotechnology continues to advance the poultry industry is likely to see the advent and licensing of sub-unit and deoxyribonucleic acid (DNA) vaccines (72).

Hemagglutination-Inhibition Test

Hemagglutination of RBCs has provided a powerful tool in the diagnosis of NDV. In 1942, Burnet (35) demonstrated that NDV anti-sera prevented the agglutination of RBCs by NDV. This discovery provided the basis of the hemagglutination-inhibition (HI) test that has been the standard for the diagnosis and measuring the antibody response to natural or vaccinal exposure of birds to NDV.

Other tests to measure antibody response to NDV infection in birds have been developed: agar gel precipitin (14, 56), single radial immunodiffusion (42), single radial hemolysis (67), virus neutralization in chick embryos (25), and plaque neutralization
These tests have not become popular on a commercial basis. The hemagglutination-inhibition test has fallen out of favor with the advent of the enzyme linked immunosorbent assay or ELISA test (95, 99, 123, 140, 162). The procedure for conducting HI tests will be used in this present work and has been described in the Material and Methods section. For a more detailed description of this technique please refer to Allan and Gough (16).

Allan et al., in 1978 (17), described the expected HI titers after challenge with Herts 33 strain of viscerotropic-velogenic NDV.

**Enzyme Linked Immunosorbent Assay**

Over the past several years, enzyme linked immunosorbent assay (ELISA) for the detection of NDV antibody titers has replaced the conventional HI test partly due to ELISA’s semi-automated techniques (141). Several authors have described the ELISA technique (3, 95, 99, 123, 162). Several papers have been published supporting a good correlation between ELISA and HI titers (3, 32, 48).

**Virus Propagation and Isolation**

Isolation and identification of NDV is necessary to arrive at a definitive diagnosis. Senne (135) and Alexander (14) have provided detailed explanations for the isolation and propagation of NDV isolates in various culture systems including the most commonly used, embryonated chicken eggs. This technique is almost universally used for the isolation, propagation and identification of NDV partly due to its convenience and sensitivity for the growth of NDV (14). As stated under Viral Replication, the respiratory and intestinal tracts appear to be the two main sites for viral replication. Therefore, tracheal swabs, feces or cloacal swabs are practical samples to aid in the isolation of NDV from an infected individual (14). Isolation and propagation in embryonated chicken
eggs will be employed throughout this work and the procedure is outlined in the Material and Methods section.

The hemagglutination (HA) procedure is commonly used to test the allantoic or amniotic fluid of eggs dying after inoculation (25). Bacterial contamination can cause false hemagglutination. Therefore, each sample should be confirmed negative for bacteria via standard microbiological cultures (14). Alexander (14) and Beard (25) outlined a detailed description of the procedures.

Cell culture has been used to isolate and identify ND viruses. Eleven cell lines and eighteen different cell types capable of supporting NDV growth have been identified by Lancaster (81). The cytopathic effects and growth requirements for NDV have been described (14, 20, 117, 125).
Herpes Virus of Turkeys

Herpes virus of turkeys (serotype-3 of MDV) has been used to vaccinate 1 day old chicks in an effort to provide protection against virulent MDV for over three decades (114). Due to its universal use, HVT is recognized as ubiquitous in chickens (38). Herpes virus of turkeys is also ubiquitous in domestic turkeys (38, 136, 170) and has also been isolated from wild turkeys (38, 45). This virus causes limited clinical signs in turkeys and no clinical signs in chickens. It is nononcogenic (167) but can occasionally cause infertility in the male turkey (38, 147). Herpes virus of turkeys infection in the chicken results in antibody production. An antibody titer persists for life (38, 112, 169), making all commercially produced chicks positive for maternal antibodies towards HVT. For this reason, HVT maternal antibody levels are not normally measured on commercial stock. Since the introduction of HVT (serotype-3 MD), the poultry industry has noticed an increase in MDV virulence. This increase in virulence is strongly linked to the introduction of new serotype-2 and serotype-1 MDV vaccines (115). The administration of HVT alone has been shown to be insufficient in protecting birds challenged with the very virulent strains of MDV (vvMDV) (166). Thus in many regions of the world, depending on the virulence of the field strain (122), poultry producers find it is essential to add serotype-2 MDV vaccines with the HVT vaccine creating a bivalent vaccine (165) or the addition of both serotype-2 and serotype-1 MDV vaccines with HVT vaccination.
forming a trivalent vaccine (168). Many of the rHVT vaccines tested thus far do not elicit early immunity.

**Marek’s Disease**

Marek’s disease and ND continue to be two important poultry pathogens due to their negative economic impact to the poultry industry as a whole. Losses due to Marek’s disease are most commonly due to mortality and condemnations at the processing plant. Subclinical infections resulting in decreased weight gain and/or respiratory infections, which leads to increased mortality, account for the majority of the losses seen with ND infections (114).

Marek’s disease is a lymphoproliferative neoplastic disease caused by a herpes virus. Calnek and Witter provided a detailed explanation of Marek’s disease (38). Bülow and Biggs (33, 34, 38) classified the MDV viruses into three serotypes. Serotype I MDVs are oncogenic, tumor producing serotypes of the virus. Serotype II MDVs are nononcogenic and apathogenic serotypes of MDV. Herpes virus of turkeys is a serotype III MDV. Serotype III MDV is also apathogenic and nononcogenic. Three serotypes of MDV were used in this trial: CVI988, serotype I; SB1, serotype II; and HVT, serotype III.

Numerous trials have been published supporting the safety and efficacy of rHVT viral vectored vaccines. This work will be the first account of an rHVT vaccine expressing HN and F genes to NDV being used in commercial Leghorns, broilers and turkeys and the first documentation of simultaneous administration of an rHVT vaccine and standard, commercial, live Marek’s disease vaccines subcutaneously or in ovo. It is the purpose of this work to test the safety and efficacy of a recombinant vaccine attempting to gain United States Department of Agriculture (USDA) licensing. The
USDA’s 9-CFR outline of rules and regulations were followed, within the constraints of biosecurity level II (BSL-II) isolator space.
CHAPTER 4
MATERIALS AND METHODS

These studies were conducted in Gainesville, Florida, at the University of Florida, College of Veterinary Medicine, Poultry Medicine Laboratory in Buildings 177, 600, 624, and 699.

Experimental Animals

Four types of birds were used for these studies. Sources of eggs for hatching included:

- Specific-pathogen-free (SPF) eggs from SPAFAS Inc., Preston, Connecticut
- Commercial Leghorn eggs (Hy-Line strain W98) from Hillandale Farms Inc., Lake City, FL
- Commercial broiler eggs (Cobb) from Tyson Foods Inc., Orange Park, FL
- Commercial turkey eggs (Nicholas type) were shipped from Sleepy Creek Turkey Inc., Goldsboro, N.C.

All eggs were incubated and hatched at the Poultry Medicine Laboratory, building 177. All poultry were neck-banded using the Swiftack® neck banding system at 1 day of age. Poultry were also beak trimmed at 1 day of age, prior to placement in Petersime® battery cages, in floor pens containing wood shavings as bedding material in buildings 600 and 624 or in BSL-II biological isolators in buildings 624 and 699. All poultry were raised under a stringent biosecurity program and controlled management conditions (feed, water, and temperature). At 48 hours prior to TxNDV administration, birds were moved into isolation units (four – Montair Anderson, and 14 - Controlled Isolation Systems isolators) in buildings 624 and 699. Once in isolators, birds were maintained using
negative pressure H.E.P.A. (High-Efficiency Purified Air) filtered air on the inlet and outlet ports. Air was exchanged 20 times per hour (as described by manufacturer). Birds were supplied with a closed, automatic water system and feed ad libitum.

**Experimental Virus**

- A commercially available modified-live B1B1 NDV vaccine; Bio-Cas B1™ (serial number NB020 and 0019001) provided by Tri Bio Laboratories (currently Intervet, Inc., Milisboro, Delaware)

- Commercially available MDV vaccines:
  - Rismavac (CVI988 live MDV, serial number 2760001)
  - Marexine/SB (HVT and SB1 live MDV, serial number 2718040)

- An experimental HVT vector with HN and F genes to NDV and genes to gps A and B of Marek’s disease virus recombinant vaccine (construct HVT-050, serial number ZN-01) supplied by Hoechst Roussel Vet (currently Intervet, Inc., Milisboro, Delaware)

- A standard USDA challenge NDV strain, Texas GB, provided by National Veterinary Services Laboratory (NVSL)

The precise locations of the NDV and MDV gene insertions on the HVT vector were not disclosed by the funding agency. This information is considered proprietary information belonging to the funding agency.

**Licensing**

Texas GB strain of NDV is exotic to the United States of America. Proper licensing was obtained prior to commencement of these trials including obtaining USDA License No. 45826 Research, University of Florida, Environmental Health and Safety Permit No. BA-1619 and RD-1710, and Institutional Animal Care and Use Committee animal use approval numbers A434 and A461.
Tryptose-Phosphate Broth Preparation

Supplies

Latex gloves
Two – 2000 mL Flasks, sterile
One – 500 mL Flask, sterile
Three – Scupulas, sterile
Five – Magnetic stir bars, sterile
One – 500 mL Filter system, sterile (0.22 µm cellulose acetate filter – Corning 25942-500)
One – Hot plate with magnetic stirrer system
One – Continuous pipetting apparatus (Becton – Dickinson 10 mL)
Test tube racks
Sterile broth tubes with screw caps (freezer safe)
pH meter

Procedures

1. Tryptose phosphate broth (TPB) was prepared at a concentration of 29.5 g/L of deionized water. Note: Deionized water, 900 mL, was mixed with 29.5g of TPB powder. The additional 100 mL was used to dissolve antibiotics, and then added to flask after the TPB had cooled to room temperature.

2. The TPB was sterilized according to manufacturer’s instructions.

3. One hundred mLs of deionized water was sterilized per liter of broth prepared.

4. The TPB was allowed to cool to room temperature.

5. Once the TPB and water had cooled to room temperature the following concentrations of antibiotics was added to 100 mL of sterile water per each liter of broth being prepared: penicillin G – 6.1g/L, streptomycin sulfate – 13.1 g/L, amphotericin B (solubilized) – 0.025 g/L (86)

6. The antibiotic solution was filter sterilized using a 0.22um cellulose acetate filter (Corning 25942-500).

7. Once filtered the antibiotic solution was added to the 900 mL of sterile TPB.

8. The solution was mixed thoroughly by placing on a magnetic stirrer apparatus.

9. The pH of the homogenized broth was determined from a sample of the homogenized broth. The pH was in the range of 7.0 – 7.4. This sample was not added back to the stock broth solution.
10. Once the solution was thoroughly mixed and fell within the proper pH range the solution was aliquoted into sterile broth tubes.

11. Finally the tubes were dated and placed in a \(-20^\circ\text{C}\) freezer for later use.

**Egg Inoculation via Allantoic Sac**

**Supplies**

- One – 1000uL pipetman for dilution
- Sterile pipet tips
- Stock virus
- TPB broth tubes
- Test tube rack
- Biohazard bags
- One – garbage can
- One – engraver
- One – Tube of Duco\textsuperscript{®} brand glue
- One – Sharps box
- Beaker with ice for inoculum
- Beaker with 80 to 100% alcohol for sterile swabs
- Sterile cotton swabs
- 1 cm\(^3\) sterile tuberculin syringes
- 23G x 1” needles, sterile
- Latex gloves
- Clean and disinfected egg flats
- Container with bleach water for used instruments

**Procedure**

Egg inoculation was conducted in a certified biosafety hood.

1. Specific pathogen free eggs were incubated at 99.5\(^\circ\text{F}\) and 60 –70% relative humidity.

2. On the 9th day of incubation the eggs were candled and all early deads, infertiles and weak embryos were removed. The air cell line was marked using a pencil for inoculation purposes. The site marked for inoculation was free of any major blood vessels and was opposite the embryo.

3. On the 10th day of incubation the eggs were removed from the incubator and inoculated.

4. The inoculation site was disinfected along with the tip of engraver using a sterile swab saturated with 80 - 100% alcohol.

5. The disinfected engraver was used to carefully make a small hole into the eggshell a few millimeters above the previously marked air cell line. The outer shell
membrane was not penetrated in most cases. However, if penetration of the outer shell membrane did occur it was not detrimental to the egg.

6. Once the hole in the eggshell was made, 0.2cc of desired inoculum was deposited into the allantoic sac using a 23G x 1” sterile needle and sterile 1.0cc tuberculin syringe. The needle was inserted vertically with the bevel towards shell the full length of the needle.

7. After 0.2cc of inoculum was deposited in the allantoic cavity, the needle was carefully pulled out and the hole sealed with one drop of Duco® brand glue.

8. Eggs were returned to the incubator and oriented in the normal upright position.

9. The eggs were candled daily at the same time each day to observe for any signs of embryonic death suggestive of viral replication.

Inoculated eggs were removed from the incubator 36 hours postinoculation and placed at 4°C for 8 hours. After refrigeration, infected allantoic fluid was harvested and pooled. Pooled virus was dispensed into 1.1mL aliquots in sterile cryogenic vials with screw caps and stored at –188°C and –85°C. Prior to freezing several aliquots were selected at random and checked for bacterial contamination as above.

**Harvesting of Allantoic Fluid**

**Supplies**

- One – 400 mL beaker with disinfectant for eggshells
- One – Candle or Bunsen burner for flaming of instruments
- One - 50 mL beaker with 80 to 100% alcohol for flaming of instruments
- One - Sterile beaker with 80 to 100% alcohol for sterile cotton swabs
- One - Beaker for ice bath
- One - Sterile flask with stopper for harvested fluids
- Sterile cotton swabs
- Sterile forceps
- Latex gloves
- 5 cm³ syringes, sterile
- 18G x 1.5” needles, sterile
- Sharps container
- Clean and disinfected egg trays
- One – Garbage can
- One – Biohazard bag
- Blood agar and TSA plates to check for bacterial contamination of aliquoted fluid
- One – Continuous pipetetter for aliquoting of harvested fluid into cryo tubes, sterile
Procedure

Allantoic fluid harvesting was conducted in a certified biosafety hood.

1. Thirty-six hours postinoculation, embryos were placed in a refrigerator at 4°C for 8 hours.

2. After 8 hours at 4°C, only the number of eggs that could be harvested in ten (10) minutes was removed from the refrigerator. This allowed the virus to stay at a constant temperature while the infected allantoic fluid was harvested. More eggs were removed from the refrigerator as needed.

3. The air cell cap (top of egg at large end) was sterilized with a sterile cotton swab saturated in 80 to 100% alcohol.

4. Sterile forceps were dipped into 80 to 100% alcohol and flamed. The forceps were allowed to cool briefly prior to use.

5. Using forceps, the eggshell was cracked over the air cell. The air cell cap was carefully removed. The removed pieces of eggshell were discarded into disinfectant.

6. Once the air cell cap was removed and the inside of the egg could be visualized the egg was tilted towards the technician at approximately a 45° angle. The allantoic fluid was harvested using a sterile 5cc syringe fitted with a sterile 18G x 1.5” needle.

7. The bevel of the needle was directed towards the eggshell. The needle was inserted into an area between the yolk sac and the eggshell. The allantoic fluid was carefully aspirated into the syringe avoiding aspiration of membranes.

8. The aspirated fluid was delivered into a clean and sterile capped flask maintained in an ice bath for the duration of harvesting. A sterile beaker worked nicely as a flask cover.

9. After all eggs had been harvested the pooled infected allantoic fluid was aliquoted.

10. Once the entire pool of infected allantoic fluid was aliquoted, the cryo tubes were labeled and placed in liquid nitrogen (-188°C) or an ultra-low freezer (-80°C) for titration and as the challenge inoculum.

Virus Propagation and Titration

The standard USDA NDV challenge strain, Texas GB, was propagated and titrated in 10 day old SPF embryonating eggs (SPAFAS) to obtain a pool for the series of trials.
The stock virus, provided by NVSL, was diluted 1/100 ($10^{-2}$) using tryptose phosphate broth as prepared above prior to inoculation. Specific-pathogen-free eggs were inoculated with 0.2 mL of diluted stock virus injected into the allantoic fluid using a 1.0 mL tuberculin syringe fitted with a 23G X 1” needle. Injection holes were sealed with Duco® glue. Egg inoculum was plated at random onto tryptic soy agar (TSA) and blood agar plates to check for bacterial contamination. Tryptose-phosphate broth containing 6.1g/L penicillin G, 13.1g/L streptomycin sulfate, and 0.025g/L amphotericin B was used as the diluent.

**Titration**

Titration of the Texas GB NDV was performed by infecting 10 day old embryonating SPF eggs (SPAFAS) with serial dilutions ranging from $10^{-6}$ to $10^{-13}$ of the previously propagated, harvested, frozen, and then thawed virus. Five eggs per dilution were infected using 0.1mL of propagated virus injected into the allantoic sac using a 1.0 mL tuberculin syringe fitted with a 23G X 1” needle. Injection holes were sealed with Duco® glue. Eggs were candled daily for 6 days. All mortality was recorded. Mortality occurring within the first 18 hours was recorded as non-specific and not included in final calculations. On the 6th day following egg inoculation, the ELD$_{50}$ was calculated according to the methods described by Reed and Munch (116).

**Histopathology**

Following Texas GB NDV administration to experimental birds, selected organs were taken as needed and fixed in 10% buffered formalin. Brain, spleen and cecal tonsils were of primary interest. Samples were dehydrated using increasing concentrations of ethyl alcohol, embedded in paraffin, sectioned, and stained with hematoxylin and eosin
(HE) using standard histological procedures. Processing was performed by the University of Florida, College of Veterinary Medicine, Histology Laboratory. Processed tissues were examined for histologic changes characteristic of NDV. Tissues were scored for histopathologic changes on a scale of:

- 0, normal or no lesions
- 1, mild lesions
- 2, moderate lesions
- 3, marked lesions
- 4, severe lesions

This scoring system was used to establish a basis for determining average histopathologic scores.

**Humoral Immune Response**

Sera were collected to measure the level of the humoral immune response elicited by the vaccines and the challenge virus, preinfection and postinfection. Sera were assayed using an antibody capture enzyme linked immunosorbent assay (ELISA) test kit read on an IDEXX 96-well Molecular Devices, E-max plate reader (IDEXX, Westbrook, ME). The X-check program (IDEXX, Westbrook, ME) used the following titers for assigning bird titers into the appropriate titer group:

- Titer group 0 = 1 to 396
- Titer group 1 = 397 to 999
- Titer group 2 = 1,000 to 1,999
- Titer group 3 = 2,000 to 2,999
- Titer group 4 = 3,000 to 3,999
- Titer group 5 = 4,000 to 4,999
- Titer group 6 = 5,000 to 5,999
- Titer group 7 = 6,000 to 7,999
- Titer group 8 = 8,000 to 9,999
- Titer group 9 = 10,000 to 11,999
- Titer group 10 = 12,000 to 13,999
- Titer group 11 = 14,000 to 15,999
- Titer group 12 = 16,000 to 17,999
Titer group 13 = 18,000 to 19,999
Titer group 14 = 20,000 to 21,999
Titer group 15 = 22,000 to 23,999
Titer group 16 = 24,000 to 27,999
Titer group 17 = 28,000 to 31,999
Titer group 18 = 32,000 to 1,000,000.

Blood collected was placed in sterile vacutainers and laid horizontally at room temperature for 12 hours to permit clotting and escape of sera. Sera were poured off into sterile serum tubes and held at 4°C until processing. Sera were diluted 1:500, and then processed according to IDEXX’s NDV ELISA test kit instructions (IDEXX, Westbrook, ME). Note, that separate test kits for chickens and turkeys are available. For Trial 3, sera collected also had titers measured using the hemagglutination-inhibition test system, beta technique (16, 25).

Hemagglutination Inhibition: Constant Antigen Diluted Serum (Beta Technique)

Supplies

- Latex gloves
- 96-well U-bottom plates
- NDV antigen (Intervet Inc., Millsboro, Delaware, Lot number: 051793)
- Phosphate buffered saline (Fisher Scientific, Fair Lawn, N.J., BP661-10)
- 1% chicken red blood cells (CRBC)
- Micropipette
- Micropipette tips
- Pipettor
- Pipets
- Specific positive control serum (LaSota, NDV from SPAFAS Inc., Preston, CT)
- Negative control serum (SPAFAS Inc., Preston, CT)

Procedure

Procedures similar to those described by Allan et al. (16) and Beard (25).

Antigen Titration:

1. First, the freeze-dried antigen (Intervet, Inc.) is rehydrated using 1.0 mL of phosphate buffered saline (PBS).
2. Next, 0.05 mL of PBS is added to each well in rows A through E starting with the twelfth well (column 12) and ending in the first well (column 1).

3. After the PBS is added to each well, change micropipette tips and add 0.05 mL of antigen to first well in rows A through D of the 96-well U-bottom plate.

4. In the first well of each row A through D, antigen and PBS were mixed two fold serial dilutions were made to the twelfth well by transferring 0.05 mL from column 1 to column 2, 0.05mL form column 2 to column 3 after mixing, etc. The last 0.05 mL left in pipet tip was discarded.

5. After the two-fold serial dilutions had been made, 0.05 mL of 1% CRBCs was added to all wells in rows A through E.

6. Row E was used as the cell control. Plates were allowed to stand at room temperature until the cell control wells formed tight buttons.

7. The titer was read as the reciprocal of the last dilution to give a tight button of CRBCs. The concentrations that formed a tight button were summed and an average calculated.

8. A dilution of the antigen was made using PBS containing 10 hemagglutination (HA) units per 0.05 mL PBS.

9. Example: Average titer = 256; 256/10 HA units = 25.6; add 1.0 mL of antigen to 24.6 mL PBS. This gives a concentration of 10 HA units per 0.05 mL PBS.

Antigen Dilution Back-titration and Cell Control (Ran with every HI test)

1. Diluted antigen, 0.05 mL, was added to the first well in rows A through D.

2. PBS, 0.05 mL, was added to each well in rows A through E.

3. Diluted antigen and PBS was mixed in the first well of rows A through D and two-fold dilutions were made from wells A1 through A12 to D1 through D12 by transferring 0.05 mL from column 1 to column 2 after mixing, etc. The last 0.05 mL remaining in the tips was discarded.

4. Each well in Row E had 0.05 mL of 1% CRBCs added.

5. When using 10 HA units the 1:8 dilution should be HA-positive and the 1:16 dilution should be HA-negative.

Positive and Negative Serum Control Testing

1. To test the positive and negative controls, 0.05 mL of known positive and negative control stock was added to the first well of rows F and G.
2. Next steps 2 through 6 in “HI Testing of Serum Samples”, below was followed.

**HI Testing of Serum Samples**

1. To the first well of each row 0.05 mL of test serum was added.
2. Diluted antigen, 0.05 mL, containing 10 HA units was added to all wells starting at the twelfth well and ending with the first well in each row.
3. The diluted antigen was mixed with the test serum in the first well and diluted through to the twelfth well. The remaining 0.05 mL in the pipet tips was discarded.
4. Plates were incubated at room temperature for approximately 30 minutes before adding the CRBCs.
5. Next, 0.05 mL of 1% CRBCs was added to all wells.
6. Plates were allowed to stand until buttons formed in the positive control wells, approximately 45 minutes.
7. Once tight buttons had formed in the positive control plates the plates were tilted to observe for tear dropping of the buttons.
8. The titer was read as the reciprocal of the last dilution to give a tight button of CRBCs and results were recorded.

**Virus Isolation**

Tracheal swabs were collected according to the protocol for each trial. Swab samples were placed in 4.5 mL of tryptose phosphate broth containing antibiotics and stored at –85°C until processing. All attempts were made to coordinate 10 day old embryonating SPF eggs with tracheal swab collection so that the samples did not loose any virus from a freeze-thaw cycle. Samples were homogenized and inoculated into the allantoic sac of 10 day old embryonating SPF eggs (SPAFAS). A volume of 0.1 mL homogenized sample was inoculated into the allantoic sac as described in Propagation and Titration. Five eggs per sample were inoculated. A small amount (0.1 mL) of egg inoculum was placed on TSA and blood agar plates to evaluate presence of bacterial contamination. Eggs were candled daily for 6 days at the same time each day. Embryos
dying within 18 hours were considered non-specific and not included in calculations. Eggs dying after 18 hours postinoculation had allantoic fluid harvested and tested for bacterial contamination and for presence of agglutinating NDV using the hemagglutination test (HA), according to Beard (25). Then 72 hours after egg inoculation, two eggs of the five eggs per bird sample number were removed and placed at 4°C for 8 hours to reduce embryo blood contamination during harvesting of allantoic fluid. Allantoic fluid was then harvested from the two eggs and pooled for each individual bird. Pooled allantoic fluid was mixed with an equal amount of 5% chicken red blood cells (CRBCs) (e.g., 0.5 mL of pooled allantoic fluid with 0.5 mL of 5% CRBCs) on a plate and stirred for five minutes. At the end of the five minutes, observation for agglutination of the CRBCs was performed and recorded as positive (hemagglutination) or negative (no hemagglutination). Birds observed as having a positive HA test were recorded as positive for virus isolation and no further testing was necessary. For birds with negative HA test results at 72 hours, a new set of, 10 day old embryonating SPF eggs (SPAFAS Inc.) were inoculated. Incubation times were coordinated so that samples could be directly passed into 10 day old embryonated eggs so that the samples did not have to be frozen then thawed. The original three eggs per bird continued to be incubated and candled until 7 days postinoculation. At 7 days postinoculation, the eggs were placed at 4°C for 8 hours. Allantoic fluid was harvested from the remaining eggs per bird and pooled. Hemagglutination test was performed on this pooled allantoic fluid and results were recorded. For the second passage of eggs, eggs were candled daily, as previously described. All embryos dying at greater than 18
hours postinoculation were tested for bacterial contamination. Hemagglutination was performed on the allantoic fluid and results were recorded.

**Animal Room Preparation**

Buildings 600 and 624 were prepared and disinfected prior to chick placement. Rooms and battery cages were disinfected using Environ One-Stroke (1:256) and heated to 120°F for a period of 72 hours. As a final disinfecting process, rooms were sealed and formaldehyde fumigated using potassium permanganate (KMnO₄) as a catalyst. Rooms remained sealed and off limits for a period of 48 hours and then ventilated. Entry into these premises was permitted to authorized personnel only who have followed prescribed biosecurity measures, including showering in and placing on clean, dedicated coveralls and boots. Prior to chick placement, rooms were heated to 88°F. Room temperature was lowered 2°F each week until 75 to 78°F was reached.

**Isolator Preparation**

Isolators were located in buildings 699 and 624. Isolators were ventilated using individual fans operating under negative pressure. Exhaust air was expelled through closed dryer tubing exiting to the exterior of the building. Isolators were fumigated using formaldehyde gas prior to bird placement. Isolators were completely disinfected with Environ One-Stroke (1:256) and assembled prior to fumigation. One (1) heaping teaspoon of KMnO₄ was placed in a glass bowl inside each isolator to which 20cc of 37% formaldehyde was added. Individual isolator fans remained off during the fumigation process and for 48 hours following fumigation. Isolators were ventilated for a minimum of 24 hours prior to bird placement.
**Trial 1: Chicken and Turkey Infection with Texas GB Strain of Newcastle Disease Virus**

Fertile eggs were obtained from suppliers. To coordinate hatching, 7 days prior to setting chicken eggs, 130 commercial turkeys eggs were set in the incubator. Turkey eggs were maintained in the incubator (Kuhl® or Natureform®) at 99.5°F and 60 to 70% relative humidity (R.H.) for 25 days. One-hundred ten SPF white leghorn, commercial broiler, and commercial white leghorn eggs were incubated at 99.5°F and 60 to 70% R.H. for 18 days. On chicken incubation Day 18 and turkey incubation Day 25, eggs were transferred into the hatcher (Humidaire®) and maintained at 98.5°F and 60 to 70% R.H. Chicks were individually identified by neckbanding, beak trimmed, and placed into separate rooms in building 600 according to bird type: commercial turkeys room 8, SPF white Leghorns room 6, commercial broilers room 4, and commercial white Leghorns room 2. Access to rooms was restricted to only those persons providing day to day care who followed strict biosecurity measures. Birds were maintained in their respective battery cages until Day 39 when each bird-type was divided using a randomized design into three sub-groups. Each sub-group consisted of 15 to 22 birds. The three sub-groups per bird type, based on TxNDV infection routes (intramuscular, eye-drop or sham challenge) were placed in individual isolators and provided feed and water ad libitum. On Day 40, fifteen birds per bird type were bled to determine NDV ELISA titers. On Day 42, birds were administered $10^{4.5}$ ELD$_{50}$ of Texas GB strain of NDV by IM, ED or sham (both IM and ED of diluent) administration. The IM challenge group received a volume of 0.2cc of inoculum in the pectoralis major muscle. Eye-drop infected birds received a volume of 0.1cc of challenge virus on the cornea of the eye. All birds received $10^{4.5}$ELD$_{50}$ of Texas GB NDV regardless of inoculum volume. Sham challenged birds
received 0.2cc of tryptose phosphate broth (TPB) in the pectoralis major muscle and 0.1cc of TPB on the eye. (Table 1) Birds were observed daily, at the same time each day, for 14 days postinfection. Birds were scored on a scale of 0 to 5:

- 0 = healthy – normal
- 1 = mild - mild muscle weakness and somnolence/depression
- 2 = moderate – severe muscle weakness, sitting frequently, moderate to marked somnolence
- 3 = marked – non-continuous nervous tremors
- 4 = severe – continuous tremors and recumbent
- 5 = deceased

Birds having a score of 2/5 or greater were considered moribund. Group scores and individual bird scores were recorded daily. All daily mortality was necropsied and gross lesions recorded. Gross pathologic changes are not well defined for the three commercial types of birds as they have been in the SPF Leghorns. Tissues were collected in 10% buffered formaldehyde as needed for histopathologic study. On Day 56, 14 days postinfection, the trial was terminated. All surviving birds were bled, euthanized and necropsied. Newcastle disease virus ELISA titers were determined for the surviving birds using the IDEXX ELISA system (IDEXX, Westbrook, ME).

**Trial 2: Commercial Broiler and Commercial Turkey rHVT Vaccine, Texas GB Strain of Newcastle Disease Virus Challenge**

Commercial broiler, commercial turkey, and SPF Leghorn hatching eggs were obtained from suppliers. Due to the need for extreme biosecurity practices and isolator space limitations, the broiler phase was conducted first. Upon completion of the broiler phase, the turkey phase was conducted.
Table 4-1: Trial 1: Chicken and turkey infection with Texas GB strain of Newcastle disease virus treatment protocol

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>n</th>
<th>Route</th>
<th>Challenge</th>
<th>Challenge day/ serology</th>
<th>Termination day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commercial turkey</td>
<td>19</td>
<td>Sham</td>
<td>-</td>
<td>+</td>
<td>42 e</td>
</tr>
<tr>
<td>Commercial turkey</td>
<td>20</td>
<td>IM</td>
<td>+</td>
<td>-</td>
<td>42</td>
</tr>
<tr>
<td>Commercial turkey</td>
<td>22</td>
<td>ED</td>
<td>+</td>
<td>-</td>
<td>42</td>
</tr>
<tr>
<td>Commercial broilers</td>
<td>18</td>
<td>Sham</td>
<td>-</td>
<td>+</td>
<td>42 e</td>
</tr>
<tr>
<td>Commercial broilers</td>
<td>20</td>
<td>ED</td>
<td>+</td>
<td>-</td>
<td>42</td>
</tr>
<tr>
<td>Commercial Leghorns</td>
<td>20</td>
<td>Sham</td>
<td>-</td>
<td>+</td>
<td>42 e</td>
</tr>
<tr>
<td>Commercial Leghorns</td>
<td>20</td>
<td>IM</td>
<td>+</td>
<td>-</td>
<td>42</td>
</tr>
<tr>
<td>Commercial Leghorns</td>
<td>22</td>
<td>ED</td>
<td>+</td>
<td>-</td>
<td>42</td>
</tr>
<tr>
<td>SPF Leghorns</td>
<td>20</td>
<td>Sham</td>
<td>-</td>
<td>+</td>
<td>42 e</td>
</tr>
<tr>
<td>SPF Leghorns</td>
<td>20</td>
<td>IM</td>
<td>+</td>
<td>-</td>
<td>42</td>
</tr>
<tr>
<td>SPF Leghorns</td>
<td>22</td>
<td>ED</td>
<td>+</td>
<td>-</td>
<td>42</td>
</tr>
</tbody>
</table>

a = Neckbanded birds using Swiftack system and beak trimmed at 1 day of age.
b = Birds grown in bldg. 600 battery cages. Birds moved at 39 days of age to isolators.
c = $10^4 - 10^5$ ELD$_{50}$ target dose of Texas GB strain of NDV.
d = Bled 20 birds per bird type at 42 days, all surviving birds were bled at 56 days.
e = Tryptose phosphate broth administered eye-drop and intramuscular.
f = All daily mortality and all birds at termination were necropsied.
g = IM = intramuscular challenge, ED = eye-drop challenge.

**Broiler Phase**

Three hundred commercial broiler and 120 SPF Leghorn hatching eggs were incubated at 99.5°F and 60 to 70% relative humidity for 18 days. On Day 18 of incubation, eggs were transferred to the hatcher. Sixty (60) broiler eggs were in ovo vaccinated with the recombinant vaccine, rHVT.

**In ovo vaccination**

**Supplies**

- Latex Gloves
- 60 – eggs containing normally developing embryos at Day 18 of incubation
- Electronic engraver
- 1.0cc tuberculin sterile syringes
- 3.0cc sterile syringes
- 22G x 1.5” sterile needles
- Sharps container
Biohazard bag
80 to 100% alcohol
Cotton swabs
75 to 85°F water bath
Vaccine diluent
Vaccine
Ice bath

**Procedures** All procedures were conducted in a certified biosafety hood.

**Reconstituting the Vaccine**

1. Vaccine diluent was removed from the refrigerator and allowed to warm to room temperature.

2. The vaccine vial was removed from the liquid nitrogen container. The serial number and expiration date of vaccine vial used was recorded.

3. The vaccine vial was immediately placed in a 75 to 85°F water bath to thaw. The thawing process took less than 60 seconds.

4. The top of the vaccine vial was carefully scored and the top of the glass vaccine vial was removed. No disinfectant was used.

5. The vaccine was withdrawn from the vaccine vial using an 18G needle attached to a 3 cm³ syringe and placed in the vaccine diluent container.

6. The diluent containing the vaccine was gently mixed.

7. Next, 1.0 cm³ of vaccine diluent was aspirated and dispensed into the vaccine vial. This was performed to wash the sides of the vaccine vial to remove residues that might have lowered the titer of the vaccine.

8. The diluent was then aspirated from the vaccine vial and delivered to the vaccine diluent container containing the vaccine. The vaccine vial was properly disposed of along with the 3.0 cm³ syringe and needle.

9. The reconstituted vaccine was gently mixed and placed in an ice bath. This vaccine preparation was used within 60 minutes after reconstitution.

**Egg inoculation**

1. The top of shell was sterilized along with the tip of engraver using a cotton swab soaked in 80 to 100% alcohol.

2. A hole was introduced into the top of the shell along the long axis of the egg using the engraver.
3. A volume of 0.1 cm³ of vaccine was inoculated into the amniotic sac through the top of the egg using a 22G x 1.5 inches needle inserted 1.25 inches.

4. The hole in the egg was not sealed so as to simulate industry in ovo inoculation procedures.

The in ovo vaccinated eggs were placed in an incubator inside room 1, building 600 to minimize any possible contamination with other incubating eggs. The remaining eggs were placed in their respective hatch trays and covered with screen material to prevent mixing of chicks in the hatcher in building 177. Eggs were maintained at 98.5°F and 60 to 70% relative humidity. On hatch day, birds within each group were randomly separated, identified using the Swiftack® neck banding system, and beak trimmed.

Commercial broilers were separated into four groups:

- Group B1: 1 day of age SQ rHVT vaccinates, one dose = 13,280 PFU
- Group B2: in ovo rHVT vaccinates, one dose = 13,280 PFU
- Group B3: 1 day of age and 18 days of age commercial B1B1 vaccinates, one dose
- Group B4: no vaccination

SPF Leghorns were randomly divided into two groups:

- Group B5: no vaccination
- Group B6: 1 day of age SQ rHVT vaccinates, full dose = 13,280 PFU, (Table 4-2)

Once birds had been placed in appropriate isolators in buildings 699 and 624, they received their respective vaccination. On Day 48, birds were bled for determination of NDV pre-Texas GB NDV administration ELISA titers. Each isolator was stocked with 10 birds, 3 isolators per group. On Day 50, all birds were challenged with 10⁻⁴.⁵ ELD₅₀ of Texas GB strain of NDV. Twenty (20) birds per group were inoculated intramuscular in the pectoralis major muscle. Ten (10) birds per group were infected by the eye-drop route. Birds were observed and scored at the same time each day for 14 days. All birds were provided feed and water ad libitum. Daily mortality was necropsied, lesions and
findings recorded, and affected tissues collected for histopathology as needed. On Day 64, 14 days postinfection, the trial was terminated. All surviving birds were bled, humanely euthanized and necropsied. Final NDV titers were determined using the IDEXX ELISA system (IDEXX, Westbrook, ME).

Turkey Phase

Four hundred commercial turkey hatching eggs and seventy SPF Leghorn hatching eggs (IDEXX, Westbrook, ME) were obtained from suppliers. Eggs were placed in the incubator and maintained at 99.5°F and 60 to 70% relative humidity for 25 days for turkey eggs and 18 days for SPF Leghorn eggs. On Day 25 and 18 of incubation, respectively, eggs were transferred to the Humidaire® hatcher, covered with screen material and maintained at 98.5°F and 60 to 70% relative humidity until hatching. After hatching, all turkeys and SPF Leghorns were identified using the Swiftack® neck banding system. Birds were randomly divided into three groups of turkeys and one group of SPF Leghorns. All groups were housed in isolators in buildings 699 and 624. Birds were provided feed and water ad libitum. The SPF Leghorns, group T4, and turkey group T3 remained unvaccinated. Turkey group T1 received one dose (13,280 PFU) of the recombinant vaccine (rHVT) at 1 day of age subcutaneously. Turkey group T2 received one dose of the commercial modified-live B1B1 NDV vaccine at Days 21, 42, and 63 days of age. On Day 83, bled to determine pre-Texas GB NDV infection ELISA titers using the IDEXX system (IDEXX, Westbrook, ME). Birds were provided food and water ad libitum. On Day 84, birds were challenged with $10^{4.5}$ ELD$_{50}$ of Texas GB strain of NDV. Twenty birds per group were inoculated intramuscular in the pectoralis major muscle and ten birds per group were administered the challenge virus by the eye-drop route. Birds were observed and scored daily at the same time each day for 14 days, as in
### Table 4-2: Trial 2: Commercial broiler treatment protocol

<table>
<thead>
<tr>
<th>Group number</th>
<th>Treatment group&lt;sup&gt;a,b&lt;/sup&gt;</th>
<th>n</th>
<th>Vaccine</th>
<th>Vaccination route</th>
<th>Age vaccinated</th>
<th>Challenge&lt;sup&gt;c,d&lt;/sup&gt;</th>
<th>Challenge day&lt;sup&gt;f,h&lt;/sup&gt;</th>
<th>Termination day&lt;sup&gt;g&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>Broiler</td>
<td>30</td>
<td>rHVT</td>
<td>SQ</td>
<td>Day 1</td>
<td>+</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>B2</td>
<td>Broiler</td>
<td>30</td>
<td>rHVT</td>
<td>in ovo</td>
<td>Embryonic Day 18</td>
<td>+</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>B3</td>
<td>Broiler</td>
<td>30</td>
<td>Commercial</td>
<td>ED</td>
<td>DOA &amp; 18 Days</td>
<td>+</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>B4</td>
<td>Broiler</td>
<td>30</td>
<td>Control</td>
<td>None</td>
<td>None</td>
<td>+</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>B5</td>
<td>SPF</td>
<td>30</td>
<td>Control</td>
<td>None</td>
<td>None</td>
<td>+</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>B6</td>
<td>SPF</td>
<td>30</td>
<td>rHVT</td>
<td>SQ</td>
<td>Day 1</td>
<td>+</td>
<td></td>
<td>50</td>
</tr>
</tbody>
</table>

<sup>a</sup> = Birds were neckbanded using the Swiftack<sup>®</sup> system and beak trimmed at 1 day of age.

<sup>b</sup> = Birds were placed in isolators in buildings 699 and 624.

<sup>c</sup> = Twenty birds per treatment group received intramuscular challenge, while ten received eye-drop challenge.

<sup>d</sup> = The challenge virus used was the Texas GB strain of NDV.

<sup>e</sup> = $10^4 – 10^5$ ELD<sub>50</sub> target dose.

<sup>f</sup> = Twenty broilers were bled on Day 48 and twenty turkeys on Day 82 per treatment group for NDV ELISA serology.

<sup>g</sup> = All daily mortality and all birds at termination were necropsied. All birds at termination were bled for NDV ELISA serology.

Histopathology samples were taken at necropsy when necessary.

<sup>h</sup> = Tracheal swabs for virus isolation were collected on Day 3 postchallenge from ED challenge groups, five birds/group.
<table>
<thead>
<tr>
<th>Group number</th>
<th>Treatment group (^{a,b})</th>
<th>n</th>
<th>Vaccine</th>
<th>Vaccination route</th>
<th>Age vaccinated</th>
<th>Challenge (^{c,d})</th>
<th>Challenge Day (^{f,h})</th>
<th>Termination Day (^{g})</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>Turkey 20 rHVT</td>
<td>20</td>
<td>SQ</td>
<td>Day 1</td>
<td>+</td>
<td>84</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>Turkey 20 Commercial B1B1 ED</td>
<td>20</td>
<td>Day 21, 42, 63 days</td>
<td>+</td>
<td>84</td>
<td>98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>Turkey 20 None</td>
<td>20</td>
<td>None</td>
<td>None</td>
<td>+</td>
<td>84</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>SPF 10 None</td>
<td>10</td>
<td>None</td>
<td>None</td>
<td>+</td>
<td>84</td>
<td>98</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) Birds were neck banded using the Swiftack \(^{\circ}\) system and beak trimmed at 1 day of age.

\(^{b}\) Birds were placed in isolators in buildings 699 and 624.

\(^{c}\) Eight birds per treatment group received intramuscular challenge, while twelve received ED challenge.

\(^{d}\) The challenge virus used was the Texas GB strain of NDV.

\(^{e}\) \(10^{4} - 10^{5}\) ELD\(_{50}\) target dose.

\(^{f}\) Twenty broilers were bled on Day 48 and twenty turkeys on Day 82 per room for NDV ELISA serology.

\(^{g}\) All daily mortality and all birds at termination were necropsied. All birds at termination were bled for NDV ELISA serology. Histopathology samples were taken at necropsy when necessary.

\(^{h}\) Tracheal swabs for virus isolation were collected on Day 3 postchallenge from ED challenge groups, five birds/group.

\(^{i}\) Ten SPF sham controls received intramuscular challenge only.

---

a = Birds were neckbanded using the Swiftack \(^{\circ}\) system and beak trimmed at 1 day of age.
b = Birds were placed in isolators in buildings 699 and 624.
c = Eight birds per treatment group received intramuscular challenge, while twelve received ED challenge.
d = The challenge virus used was the Texas GB strain of NDV.
e = \(10^{4} - 10^{5}\) ELD\(_{50}\) target dose.
f = Twenty broilers were bled on Day 48 and twenty turkeys on Day 82 per room for NDV ELISA serology.
g = All daily mortality and all birds at termination were necropsied. All birds at termination were bled for NDV ELISA serology. Histopathology samples were taken at necropsy when necessary.
h = Tracheal swabs for virus isolation were collected on Day 3 postchallenge from ED challenge groups, five birds/group.
i = Ten SPF sham controls received intramuscular challenge only.
Trial 1. All daily mortality were necropsied and lesions recorded and tissues collected as necessary. On Day 98, 14 days postinfection, the trial was terminated. All birds were bled for determination of final IDEXX ELISA NDV (IDEXX, Westbrook, ME) titers, euthanized and necropsied. All gross lesions were recorded (Table 4-3).

Statistical Analysis

Geometric mean ELISA and HI titers were used throughout this work. The geometric mean titer is less distorted by outliers (90). The geometric mean titer is a more reliable means of analyzing serologic data because it avoids the misleading comparisons present when arithmetic means are used (90). The geometric mean is calculated by:

$$GM_y = \sqrt[n]{y_1y_2y_3y_4\ldots y_n}$$

Where \(GM_y\) is the geometric mean of \(y\), \(n\) is the number of samples or observations and each \(y\) is an observation. The \(y\) values are multiplied together and the root of the number of samples (\(n\)) is calculated (130).

The Chi-square analysis (50, 130) with a 95% confidence interval was used to determine if a statistical difference was present across treatment groups in regard to virus isolation and percent protection.

Trial 3: Commercial Broiler rHVT plus CVI988 or HVT/SB1 Vaccine, Texas GB Newcastle Disease Virus Challenge Trial

Fertile hatching eggs were obtained from supplier. Five hundred commercial broiler hatching eggs were incubated at 99.5°F and 60 to 70% relative humidity for 18 days. On Day 18 of incubation:

- 50 eggs (broiler group 5) were in ovo vaccinated with one dose of CVI988 and one dose of the recombinant vaccine (rHVT; 13,280 PFU)
- 50 eggs (broiler group 6) were in ovo vaccinated with one dose each the commercial vaccine HVT and SB1, in addition to one dose of the recombinant vaccine (rHVT; 13,280 PFU)
• 50 eggs (broiler group 7) were in ovo vaccinated with one dose of recombinant vaccine only (rHVT; 13,280 PFU)

• 50 eggs (broiler group 9) received sham treatment as described in Trial 2

Each group was placed in individual hatch baskets and covered with screen material to prevent mixing of 1 day of age chicks and maintained at 98.5°C and 60 to 70% relative humidity. Remaining eggs were placed in a separate hatcher and maintained at 98.5°C and 60 to 70% relative humidity. On hatch day, birds within each group were randomly separated and identified using the Swiftack® neck banding system. The birds not receiving in ovo vaccination were divided into the following groups:

• Broiler group 1: 1 day of age, one dose of CVI988 and rHVT SQ
• Broiler group 2: 1 day of age, one dose of HVT, SB1 and rHVT SQ
• Broiler group 3: 1 day of age, one dose of rHVT SQ
• Broiler group 4: 1 day of age, one dose of CVI988 SQ and one dose of B1B1 strain of NDV via the eye-drop route
• Broiler group 8: 1 day of age, one dose of HVT and SB1 SQ and one dose of B1B1 strain of NDV via the eye-drop route
• Broiler group 9: in ovo sham at Day 18 of incubation and at 1 day of age sham SQ

Broiler groups 1, 2, 3, 5, 6, and 7 were housed in isolators in building 699. Broiler groups 4 and 8 were housed in isolators in building 624. Broiler group 9 was housed in isolators in building 600. Once the birds had been placed in their appropriate isolators they received their respective vaccine. Separate individuals transferred the in ovo vaccinated birds to their appropriate isolators after the 1 day of age still unvaccinated birds had been placed within their appropriate isolators. Once all birds were placed in appropriate isolators they were then vaccinated inside the isolator with the respective vaccine. Each group was housed in two isolators, with fifteen birds per isolator.
On Day 28, fifteen birds per group were wing bled while in the isolators, seven birds from one isolator and eight birds from the other isolator of that group. Blood samples were placed horizontally at room temperature for 12 hours to allow clot formation and escape of serum. Sera samples were tested for NDV antibody levels prior to Texas GB NDV administration using a standard ELISA test kit (IDEXX, Westbrook, ME) and hemagglutination inhibition (HI) as described in Humoral Antibody Response. After blood samples were obtained, birds were challenged. Fifteen birds, one isolator, per group were challenged intramuscular in the pectoralis major muscle with 0.2 mL of tryptose phosphate broth (TPB) containing $10^{4.5}\text{ELD}_{50}$ of Texas GB NDV. Fifteen birds, one isolator, per group were challenged via the eye-drop route with 0.1 mL of tryptose phosphate broth containing $10^{4.5}\text{ELD}_{50}$ of Texas GB NDV. Birds were observed at the same time each day for 10 days postinfection for clinical signs of morbidity and mortality. Moribund birds were scored as described in Trial 1 and results recorded. Daily mortality was necropsied, gross lesions recorded and tissues collected as needed for histopathology.

On Day 38, surviving birds were bled and sera were collected as previously described to determine post-Texas GB NDV administration antibody levels using a standard ELISA kit (IDEXX, Westbrook, ME) and hemagglutination inhibition as described in Humoral Antibody Response. Birds surviving in the eye-drop challenged groups had tracheas swabbed and the swabs placed in 4.5 mLs of TPB containing antibiotics. The cotton tip of the tracheal swabs were broken off, placed in tube containing TPB and antibiotics, homogenized then placed immediately into five, ten day old embryonating SPF eggs per bird for better virus isolation, prior to freezing as
described under Virus Isolation (135). Remaining TPB with antibiotics and cotton tip of the tracheal swab were stored at –85°C. Virus isolation technique and hemagglutination technique was performed as described in Virus Isolation.
Table 4-4: Trial 3: Commercial broiler rHVT plus CVI988 and/or HVT/SB1 vaccine, Texas GB Newcastle disease virus challenge trial treatment protocol

<table>
<thead>
<tr>
<th>Treatment Group&lt;sup&gt;a,b&lt;/sup&gt;</th>
<th>n</th>
<th>Vaccine</th>
<th>Vaccination route</th>
<th>Age vaccinated</th>
<th>Challenge&lt;sup&gt;c,d&lt;/sup&gt; TxGB</th>
<th>Challenge day&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Termination day&lt;sup&gt;f,g&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broiler 1</td>
<td>30</td>
<td>rHVT + CVI988</td>
<td>SQ</td>
<td>DOA</td>
<td>+</td>
<td>28</td>
<td>38</td>
</tr>
<tr>
<td>Broiler 2</td>
<td>30</td>
<td>rHVT + HVT/SB1 Marexine/SB</td>
<td>SQ</td>
<td>DOA</td>
<td>+</td>
<td>28</td>
<td>38</td>
</tr>
<tr>
<td>Broiler 3</td>
<td>30</td>
<td>rHVT</td>
<td>SQ</td>
<td>DOA</td>
<td>+</td>
<td>28</td>
<td>38</td>
</tr>
<tr>
<td>Broiler 4</td>
<td>30</td>
<td>CVI988 &amp; NDV B1B1</td>
<td>SQ/ED</td>
<td>DOA</td>
<td>+</td>
<td>28</td>
<td>38</td>
</tr>
<tr>
<td>Broiler 5</td>
<td>30</td>
<td>rHVT + CVI988</td>
<td>In ovo 18 days incubation</td>
<td>+</td>
<td>28</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Broiler 6</td>
<td>30</td>
<td>rHVT + HVT/SB1 Marexine/SB</td>
<td>In ovo 18 days incubation</td>
<td>+</td>
<td>28</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Broiler 7</td>
<td>30</td>
<td>rHVT</td>
<td>In ovo 18 days incubation</td>
<td>+</td>
<td>28</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Broiler 8</td>
<td>30</td>
<td>HVT/SB1 &amp; NDV B1B1 Marexine/SB</td>
<td>SQ/ED</td>
<td>DOA</td>
<td>+</td>
<td>28</td>
<td>38</td>
</tr>
<tr>
<td>Broiler 9</td>
<td>30</td>
<td>Sham</td>
<td>SQ</td>
<td>DOA</td>
<td>+</td>
<td>28</td>
<td>38</td>
</tr>
</tbody>
</table>

<sup>a</sup> = Birds were neckbanded using the Swiftack® system at 1 day of age.
<sup>b</sup> = Birds were housed in buildings 699 and 624 Room A in HEPA filtered isolators for the duration of project.
<sup>c</sup> = Fifteen (15) birds per treatment group received intramuscular challenge and fifteen (15) received eye-drop challenge.
<sup>d</sup> = The challenge virus was the Texas GB strain of NDV, $10^4 - 10^5$ ELD<sub>50</sub> target dose.
<sup>e</sup> = Fifteen (15) birds per treatment group were bled on Day 28 and sera tested using NDV ELISA and HI techniques.
<sup>f</sup> = All daily mortality and all birds at termination of the trial were necropsied. All birds were bled for NDV ELISA and HI antibody titer determination at termination.
<sup>g</sup> = All surviving birds from the ED challenged treatment groups had their trachea’s swabbed for virus isolation.
CHAPTER 5
RESULTS

Virus Propagation and Titration

Virus propagation was performed to produce a pool of Texas GB NDV to be used in subsequent trials. Titration was performed to obtain the activity (ELD$_{50}$) of the thawed and pooled virus. The thawed titer data were used in diluting the virus to the proper activity level prior to chicken or turkey infection in subsequent trials.

The TPB containing antibiotics was negative on blood agar and TSA microbiologic agar plates for bacterial and fungal growth. While aliquoting the pooled virus, filled ampules were selected at random and streaked onto blood agar and TSA plates. All aliquots tested were negative for bacterial and fungal growth.

Two ampules of frozen and pooled virus were thawed. Each ampule was inoculated into separate sets of SPF chicken eggs for determination of ELD$_{50}$ activity level (Table 5-1 and 6).

Lethal-dose-fifty (LD$_{50}$) was calculated using the formula as described by Reed and Muench (114). Lethal-dose-fifty = 50% - (mortality at dilution next above) / {(mortality next above) – (mortality next below)} (114). Using the data in Table 5-1, the 50% midpoint fell between the $10^{-10}$ and $10^{-11}$ dilutions. Therefore, the dilution next below the 50% point is $10^{-11}$ with a total mortality of 0%. The dilution next above the 50% midpoint is $10^{-10}$ with a total mortality of 60%. Calculation of the LD$_{50}$ is: (50% - 0%) / (60% - 0%) = 0.833. The LD$_{50}$ is $10^{-10.833}$ per 0.1 mL of fluid for ampule one. The final activity level per one milliliter of infected allantoic fluid is $10^{-11.833}$ ELD$_{50}$. 

59
<table>
<thead>
<tr>
<th>Dilution</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Total dead/total eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/3</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>$10^{-6}$</td>
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<td>NA</td>
<td>NA</td>
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<td>NA</td>
<td>NA</td>
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<td>$10^{-9}$</td>
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<td>0</td>
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<td>0</td>
<td>4/5</td>
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<tr>
<td>$10^{-10}$</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3/5</td>
</tr>
<tr>
<td>$10^{-11}$</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/5</td>
</tr>
<tr>
<td>$10^{-12}$</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1/5</td>
</tr>
<tr>
<td>$10^{-13}$</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1/5</td>
</tr>
</tbody>
</table>

Inoculum: allantoic fluid and TPB with antibiotics
Age of embryo: 10 days
Viral source: National Veterinary Services Laboratories (NVSL)
Inoculation route: allantoic sac  Dosage: 0.1 mL

Table 5-2: Texas GB NDV titration in SPF embryonating eggs, ampule 2

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Total dead/total eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1/3</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>0</td>
<td>5</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>5/5</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>1</td>
<td>4</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>5/5</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>1</td>
<td>4</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>5/5</td>
</tr>
<tr>
<td>$10^{-9}$</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5/5</td>
</tr>
<tr>
<td>$10^{-10}$</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3/5</td>
</tr>
<tr>
<td>$10^{-11}$</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1/5</td>
</tr>
<tr>
<td>$10^{-12}$</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/5</td>
</tr>
<tr>
<td>$10^{-13}$</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/5</td>
</tr>
</tbody>
</table>

Inoculum: allantoic fluid and TPB with antibiotics
Age of embryo: 10 days
Viral source: National Veterinary Services Laboratories (NVSL)
Inoculation route: allantoic sac  Dosage: 0.1 mL
Lethal-dose-fifty was calculated for ampule 2 using the same formula by Reed and Muench (116). The 50% point for ampule two fell between the $10^{-10}$ and $10^{-11}$. The dilution next below the 50% point is $10^{-11}$ with a total mortality of 20% (Table 5-2). The titer next above the 50% point is $10^{-10}$ with a total mortality of 60% (Table 5-2). Calculation of the LD$_{50}$ for ampule two is: \[ \frac{50\% - 20\%}{60\% - 20\%} = 0.75. \] The ELD$_{50}$ for ampule two is $10^{-10.75}$ per 0.1 mL of infected allantoic fluid. The final ELD$_{50}$ of ampule two is $10^{-11.75}$ per milliliter of infected allantoic fluid.

**Trial 1: Chicken and Turkey Infection with Texas GB Strain of Newcastle Disease Virus**

This trial was conducted to characterize the pooled, frozen, and thawed allantoic fluid infected with Texas GB NDV. Characterization in commercial white Leghorns, broilers and turkeys was essential for future studies and to define clinical signs, morbidity, and mortality patterns. This trial was also conducted to ascertain whether the titer of Texas GB NDV being used was lethal in 100% of SPF Leghorns on trial.

**Morbidity**

All sham controls from the four different bird types remained clinically normal throughout the 14 day observation period.

The SPF Leghorns infected by the intramuscular route began showing clinical signs within 36 hours postinfection. Birds were considered moribund when they were given a clinical score of 2/5 or greater. These same criteria were used for all trials. Progression of clinical signs was rapid. Clinical signs at 48 hours postinfection consisted of muscle weakness and somnolence (score 1/5) to intention tremors that were not continuous (score 3/5). Within 72 hours postinfection, 100% of SPF Leghorns challenged by the intramuscular route were moribund (Table 5-4). Specific pathogen free Leghorns infected
by the eye-drop route began showing clinical signs by 3 days postinfection. Progression of clinical signs took slightly longer in the eye-drop infected SPF Leghorns as compared to the intramuscular infected SPF Leghorns. One hundred percent morbidity was observed in this group by Day 6 postinfection. Average days to morbidity for the intramuscular and eye-drop challenged birds were 2.8 and 4.5 respectively (Table 5-3).

Commercial Leghorns presented with slightly different onset and progression of clinical signs than did the SPF Leghorns. The intramuscular infected commercial Leghorns began to show very mild clinical signs 48 hours postinfection. Forty-eight hours postinfection two of the 20 birds had a score of 1/5. Therefore, morbidity in this group did not begin until 3 days postinfection (Table 5-4). By Day 7 postinfection, 19/20 birds were affected clinically with a score of two or greater. This one bird remained clinically normal and very active for the duration of the trial. This bird was challenged directly. ELISA serologic data showed a titer level of 28,700, titer group 17/18 (Table 5-7, maximum titer). The eye-drop infected commercial Leghorns began showing mild clinical signs of Texas GB NDV infection by Day 3 postinfection but were not considered moribund until Day 4 postinfection. An example of a moribund bird with a score of 3/5 is found in Figure 5-1. One hundred percent morbidity did not occur until Day 12 postinfection. One bird scored 1/5 from Day 5 to Day 11 postinfection. Excluding this bird, 100% morbidity occurred on Day 7 postinfection. The intramuscular challenged birds became moribund on average 3.9 days after infection while the eye-drop challenged birds became moribund at 5.2 days after infection on average (Table 5-3).
Figure 5-1 Opisthotonus in commercial Leghorn, eye-drop challenge, 14 Days postinfection, score 3/5.

Commercial broilers challenged by the intramuscular route exhibited rapid onset of clinical signs and progression of signs. On Day 3 postinfection, the first signs of clinical illness were observed. Clinical illness scores for thirteen out of eighteen broilers were two or greater. By 4 days postinfection 100% of the broilers were moribund (Table 5-4). Commercial broilers challenged by the eye-drop route began showing very mild clinical signs of infection consisting of mild muscle weakness and mild somnolence/depression (score of 1/5) by 3 days postinfection. Morbidity in this group of infected birds began on Day 4 and all birds were moribund by Day 6 postinfection. On average, commercial broilers challenged by intramuscular injection and eye-drop challenge became moribund 3.2 days and 4.3 days after infection, respectively (Table 5-3).
Clinical signs, onset of clinical signs, and progression of clinical signs were considerably different for the commercial turkeys as compared to any of the three types of chickens used in this trial. Morbidity in two out of the 20 intramuscular challenged turkeys was observed on Day 4 postinfection (Table 5-4). This morbidity consisted of uncontrollable intention tremors, in addition, to recumbency (score 4/5). By Day 10 postinfection 19/20 birds were moribund. One bird was a score of 2/5 on Days 7 and 8 postinfection but later recovered. On Day 5 postinfection, only one turkey out of 22 showed signs of morbidity in the eye-drop infected group of commercial turkeys. On Day 14 postinfection, termination of trial, 3/22 turkeys did not show specific signs of morbidity. These three turkeys did show signs of depression and muscle weakness, score 1/5. Figures 5-2 and 5-3 show a commercial turkey with a clinical score of 3/5.

The turkeys challenged by the intramuscular route became moribund on average 6.1 days after challenge. Similarly, the eye-drop challenged turkeys became moribund on average 6.6 days after challenge. The commercial turkeys showed a slightly different morbidity pattern as compared to the three chicken types used (Tables 5-3 and 5-4).

<table>
<thead>
<tr>
<th>Table 5-3: Trial 1: Average days to onset of morbidity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group</strong></td>
</tr>
<tr>
<td>---------------------</td>
</tr>
<tr>
<td>SPF Leghorn</td>
</tr>
<tr>
<td>Commercial broiler</td>
</tr>
<tr>
<td>Commercial Leghorn</td>
</tr>
<tr>
<td>Commercial turkey</td>
</tr>
</tbody>
</table>

Values in columns are in days.
Mortality

All sham controls remained healthy throughout the trial with the exception of one commercial broiler control bird. This one broiler was intentionally humanely euthanized on Day 9 postinfection due to an arthropathy.

The intramuscular infected SPF Leghorns began succumbing to Texas GB NDV infection on Day 3 postinfection. By Day 4 postinfection, 100% of intramuscular infected SPF Leghorns had died (Table 5-4).

Mortality was first observed in the eye-drop infected SPF Leghorns on Day 4.
Figure 5-3 Commercial turkey, severe opisthotonus, intramuscular challenge, 9 days postinfection, score 3/5.

Complete mortality of the eye-drop infected SPF Leghorns occurred on Day 13. By Day 7 postinfection, only 5/22 SPF Leghorns were remaining. These remaining birds were severely affected by Texas GB NDV infection.

Mortality was first observed in the intramuscular infected commercial Leghorns on Day 3 postinfection. By Day 14 postinfection, termination of the trial, 3/20 Leghorns had not yet died. One of the three birds remained clinically normal throughout the postinfection phase of the trial. This bird was challenged evidenced by a titer of 28,700, 14 days after challenge (Table 5-7, maximum titer). One of the other birds was severely affected (score 4/5) and the other was moderately affected (2/5).
On Day 5 postinfection, the eye-drop infected commercial Leghorns began dying due to Texas GB NDV infection. On the fourteenth and final day of the trial, 3/22 commercial Leghorns were still alive. However, they were showing marked signs of Texas GB NDV infection with a score of 3/5.

The intramuscular infected commercial broilers began dying soon after infection. The first mortality as a result of Texas GB NDV infection was seen by 3 days postinfection (Table 5-4). On Day 5 postinfection, only one broiler out of eighteen had not yet died. This bird remained alive for the 14 day observation period. This bird’s clinical signs slowly progressed to severe by the end of the trial.

Mortality in the eye-drop challenged commercial broilers was first observed on Day 4 postinfection. By Day 7 postinfection 19/20 birds had died. The remaining bird was affected by Texas GB NDV but never died.

The turkeys were observed to have much less mortality due to Texas GB NDV infection as compared to the three chicken types used (Table 5-4). The first commercial turkey succumbing to Texas GB NDV infection in the intramuscular infected group occurred on Day 5 postinfection. On the 14th day of the trial, 7/20 turkeys infected were still alive.

The eye-drop infected commercial turkeys did not begin to show mortality as a result of Texas GB NDV infection until 7 days postinfection. The eye-drop challenged commercial turkeys had a final mortality of 10/22 as a result of Texas GB NDV challenge (Table 5-4).
Table 5-4: Trial 1: Onset of morbidity and mortality

<table>
<thead>
<tr>
<th>Bird type</th>
<th>Sample Size (n)</th>
<th>Infection route</th>
<th>Morbidity a Day of onset</th>
<th>Mortality b Day of onset</th>
<th>Day 100%</th>
<th>Day 100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPF Leghorn</td>
<td>20 Intramuscular</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>SPF Leghorn</td>
<td>22 Eye-drop</td>
<td>3</td>
<td>6</td>
<td>4</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>SPF Leghorn</td>
<td>20 Sham</td>
<td>NA b</td>
<td>NA b</td>
<td>NA b</td>
<td>NA b</td>
<td>NA b</td>
</tr>
<tr>
<td>Commercial Leghorn</td>
<td>20 Intramuscular</td>
<td>3</td>
<td>7 c</td>
<td>3</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Commercial Leghorn</td>
<td>22 Eye-drop</td>
<td>4</td>
<td>12</td>
<td>5</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Commercial Leghorn</td>
<td>20 Sham</td>
<td>NA b</td>
<td>NA b</td>
<td>NA b</td>
<td>NA b</td>
<td>NA b</td>
</tr>
<tr>
<td>Commercial Leghorn</td>
<td>18 Intramuscular</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>5 d</td>
<td>5 d</td>
</tr>
<tr>
<td>Commercial Leghorn</td>
<td>20 Eye-drop</td>
<td>4</td>
<td>6</td>
<td>4</td>
<td>9 e</td>
<td>9 e</td>
</tr>
<tr>
<td>Commercial Leghorn</td>
<td>18 Sham</td>
<td>NA b</td>
<td>NA b</td>
<td>NA b</td>
<td>NA b</td>
<td>NA b</td>
</tr>
<tr>
<td>Commercial broiler</td>
<td>20 Intramuscular</td>
<td>4</td>
<td>NA</td>
<td>5</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Commercial broiler</td>
<td>22 Eye-drop</td>
<td>5</td>
<td>NA</td>
<td>7</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Commercial turkey</td>
<td>20 Sham</td>
<td>NA b</td>
<td>NA b</td>
<td>NA b</td>
<td>NA b</td>
<td>NA b</td>
</tr>
</tbody>
</table>

a = Birds were considered moribund when their score was 2/5 or greater.
b = Birds stayed healthy throughout the trial, not applicable.
c = Disregarding the one outlier that stayed clinically normal during the trial.
d = Disregarding one outlier that was severely affected by end of trial.
e = Not including one bird that was markedly affected from Day 6 and never died.

Total mortality was analyzed using Chi-square test (50, 130) and a confidence interval of 95%. Differences in total mortality for the three chicken groups were not statistically significant (p ≤ 0.05). However, the commercial turkeys were shown to have a statistically significant difference (p ≤ 0.05) in total mortality from that of any of the three chicken groups (Table 5-5). Additionally, statistically significant less mortality was seen in the eye-drop challenged turkeys as compared to the intramuscular challenged...
All chicken types were highly susceptible to Texas GB NDV infection and turkeys were moderately susceptible at an infectious dose of $10^{4.5}$ ELD$_{50}$.

Table 5-5: Trial 1: Total morbidity and mortality for all four types of birds

<table>
<thead>
<tr>
<th>Bird type</th>
<th>Challenge group</th>
<th>Morbidity</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPF Leghorn</td>
<td>ED 22/22 100%</td>
<td>22/22 100%</td>
<td></td>
</tr>
<tr>
<td>SPF Leghorn</td>
<td>IM 20/20 100%</td>
<td>20/20 100%</td>
<td></td>
</tr>
<tr>
<td>SPF Leghorn</td>
<td>Sham 0/20 0%</td>
<td>0/20 0%</td>
<td></td>
</tr>
<tr>
<td>Commercial Leghorn</td>
<td>ED 22/22 100%</td>
<td>19/22 86%</td>
<td></td>
</tr>
<tr>
<td>Commercial Leghorn</td>
<td>IM 19/20 95%</td>
<td>17/20 85%</td>
<td></td>
</tr>
<tr>
<td>Commercial Leghorn</td>
<td>Sham 0/20 0%</td>
<td>0/20 0%</td>
<td></td>
</tr>
<tr>
<td>Commercial broilers</td>
<td>ED 20/20 100%</td>
<td>19/20 95%</td>
<td></td>
</tr>
<tr>
<td>Commercial broilers</td>
<td>IM 18/18 100%</td>
<td>17/18 94%</td>
<td></td>
</tr>
<tr>
<td>Commercial broilers</td>
<td>Sham 0/18 0%</td>
<td>0/20 0%</td>
<td></td>
</tr>
<tr>
<td>Commercial turkeys</td>
<td>ED 19/22 100%</td>
<td>10/22 45%</td>
<td></td>
</tr>
<tr>
<td>Commercial turkeys</td>
<td>IM 19/20 100%</td>
<td>13/20 65%</td>
<td></td>
</tr>
<tr>
<td>Commercial turkeys</td>
<td>Sham 0/20 0%</td>
<td>0/20 0%</td>
<td></td>
</tr>
</tbody>
</table>

ED = Eye-drop challenge route, IM = Intramuscular challenge route
a = Birds were considered moribund when their score was 2/5 or greater. Percent mortality followed by different number is statistically significant using X$^2$ analysis and a confidence interval of 95% (50, 130).

Serology Preinfection

Newcastle disease virus, antibody ELISA serology from fifteen birds per bird type was conducted at 40 days of age at the time of moving from battery cages to isolators, prior to infection with Texas GB NDV. Samples were run in duplicate.
The SPF Leghorns had a maximum titer 1,382, minimum titer of 1, mean titer of 54 (titer group 0/18), geometric mean titer of 2 with a percent coefficient of variation of 462.9. The sample with a titer of 1,382 had a duplicate sample titer of 0. This sample was run again 2 days later and a titer of 116 was recorded. Exclusion of this one outlier and replacement with titer of 116 from the sample where the test was repeated would cause the mean titer to drop to 11.3 (Table 5-6). SPF Leghorns were considered serologically negative for antibodies to NDV at the time of moving the birds from the battery cages into the isolators prior to challenge.

The commercial broilers were all within titer group 0/18 with a maximum and minimum titer of 1, mean and geometric mean titer of 1 and a percent coefficient of variation of 0 (Table 5-6). These data demonstrate that the commercial broilers possessed no pre-formed immunoglobulin G (IgG) to NDV prior to Texas GB NDV infection.

ELISA results prior to Texas GB NDV infection for the commercial Leghorns were negative for pre-formed, circulating NDV IgG antibodies. These birds had a mean titer of 211, with a geometric mean titer of 16. The maximum titer for this group was 1,630 with a minimum titer of 1 and a percent coefficient of variation of 174.5. All samples except one were within titer group 0 or 1/18. One outlier was in titer group 2/18. This outlier had a duplicate titer of 24. This sample was run again 2 days later and a titer of one was obtained. With the outlier value excluded and this new titer of one used, a mean titer of 156 was calculated (Table 5-6).

Commercial turkey ELISA serology samples were conducted using an ELISA kit specific for turkey IgG antibodies against NDV. The commercial turkeys had a mean
titer of 173 with a geometric mean titer of 24 and a percent coefficient of variation of 202. The maximum titer in this group was 1,755 and a minimum titer of 1 (Table 5-6).

One sample was in titer group 2/18. However, its duplicate sample was in titer group 0/18, with a numeric titer of 87. Three samples from two birds (samples run in duplicate) were in titer group 1/18. These titer levels are generally considered to be background.

Therefore, the commercial turkeys were considered to have no demonstratable IgG NDV prior to infection.

Table 5-6: Trial 1: Newcastle disease virus, antibody capture ELISA, preinfection titers

<table>
<thead>
<tr>
<th>Bird type</th>
<th>Mean</th>
<th>Geometric mean</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Percent coefficient of variation</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPF Leghorn</td>
<td>54</td>
<td>2</td>
<td>1</td>
<td>1382</td>
<td>462.9</td>
<td>15</td>
</tr>
<tr>
<td>Commercial broilers</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>Commercial Leghorns</td>
<td>211</td>
<td>16</td>
<td>1</td>
<td>1630</td>
<td>174.5</td>
<td>15</td>
</tr>
<tr>
<td>Commercial turkeys</td>
<td>173</td>
<td>24</td>
<td>1</td>
<td>1755</td>
<td>202</td>
<td>15</td>
</tr>
</tbody>
</table>

a = Mean titer of 11.3 when sample re-ran and titer of 116 was recorded. Maximum would then drop to 122.
b = Mean titer of 156 was calculated when 1630 was substituted with a titer of 1. Maximum titer would then be recorded as 844.

Serology Postinfection

All surviving birds at the end of the 14 day observation period were bled and NDV IgG antibody capture ELISA was performed.

Texas GB NDV infection was lethal in SPF Leghorns regardless of challenge route.

Ten birds from each of the sham-infected groups were bled and NDV IgG antibody titers were determined using an NDV antibody capture ELISA test kit (IDEXX, Westbrook, ME).
The sham infected SPF Leghorn chickens had a mean titer of 268, geometric mean titer of 7 and percent coefficient of variation of 194. The maximum titer was 1,701 and a minimum titer of 1 for this group of chickens (Table 5-7). Based on these data the birds were considered negative for circulating NDV IgG antibodies 14 days after sham infection.

A mean titer of 9, geometric mean of 2, and percent coefficient of variation of 265.5 was measured in the sham infected commercial broilers 14 days after sham infection. This group had a maximum circulating NDV IgG antibody titer of 78 and minimum of 1 (Table 5-7). The sham infected commercial broilers were negative for circulating NDV IgG antibodies at the time of termination of this trial.

The NDV circulating IgG titers were measured for the commercial Leghorns 14 days after sham infection. Results are: mean titer of 175, geometric mean titer of 9, percent coefficient of variation of 196.6, maximum titer of 1,159, and minimum titer of 1 (Table 5-7). These data suggest the commercial Leghorns were negative for protective levels of circulating NDV IgG antibodies throughout the course of the trial.

Sham infected commercial turkeys were negative for circulating NDV IgG antibodies at the time of trial termination. This group of birds had a mean titer of 31, geometric mean titer of 5, percent coefficient of variation of 155.9, maximum titer of 140, and minimum titer of 1 (Table 5-7).

All surviving Texas GB NDV infected birds were bled for serologic study. Levels of NDV circulating IgG antibody were determined using a commercially available NDV ELISA kit for chickens and turkeys (IDEXX, Westbrook, ME).
Texas GB NDV challenge was lethal in infected SPF Leghorns. Six commercial Leghorns had not died by 14 days postinfection. Three of these commercial Leghorns were from the intramuscular and three from the eye-drop infected groups. The intramuscular infected Leghorns had a mean titer of 28,246, geometric mean titer of 28,238, and a percent coefficient of variation of 2.3. Two birds had a titer of 28,700 and one bird had a titer of 27,337. These data demonstrate a significant circulating NDV IgG response to Texas GB infection. The eye-drop infected commercial Leghorns had a similar IgG response to Texas GB NDV infection. The mean titer in this group was 27,538 with a geometric mean titer of 27,487 and a percent coefficient of variation of 6.0. The maximum titer was 28,700 and minimum titer of 25,214 for the eye-drop infected commercial Leghorns (Table 5-7). This demonstrates a significant NDV specific IgG response to Texas GB NDV infection.

The commercial broilers were slightly more susceptible to Texas GB NDV infection. Two broilers were still alive on Day 14 post infection. The only bird surviving in the intramuscular infected group had a titer of 27,966. The other surviving broiler from the eye-drop infected group had a titer of 27,901 (Table 5-7). These data reveal a similar NDV IgG antibody response between the commercial Leghorns and broilers, regardless of route of infection.

Several commercial turkeys survived Texas GB NDV infection. Seven commercial turkeys infected by the intramuscular route were alive 14 days after infection. These seven birds had a mean titer of 22,216, geometric mean titer of 21,870, and a percent coefficient of variation of 16.6. The maximum recorded titer was 25,449 and a minimum of 15,109 (Table 5-7). Twelve commercial turkeys survived eye-drop infection with
Texas GB NDV. These birds possessed a mean titer of 21,142, geometric mean titer of 19,426, percent coefficient of variation of 35, maximum titer of 31,220, and a minimum titer of 6,980 (Table 5-7).

**Necropsy:**

All daily mortality was necropsied for gross pathologic changes and the sex of the bird recorded. All birds surviving to 14 days postinfection were humanely euthanized and necropsied.

All sham infected SPF Leghorns, commercial broilers, Leghorns and turkeys had no gross lesions at the time of trial termination (Table 5-8). There were only 19 commercial turkey sham infected birds as compared to 20 in the other groups. This difference is due to one bird dying in the isolator with no gross lesions prior to challenge.

The SPF Leghorns presented with no gross lesions (NGL) except cecal tonsil hemorrhages (CTH) (Figures 5-4, 5-6) as their primary post-mortem finding. Eight of twenty birds infected via the intramuscular route had cecal tonsil hemorrhages. Two of these birds also had sub-capsular splenic hemorrhages. Twelve birds out of twenty had no gross lesions. Thirteen of 22 eye-drop infected SPF Leghorns had cecal tonsil hemorrhages. Two birds had sub-capsular splenic hemorrhages and three had hemorrhages of the distal glandular epithelium of the proventriculus (Figure 5-5). Six birds in this group had no gross lesions (Table 5-8).

The majority of commercial broilers presented with cecal tonsil and sub-capsular splenic hemorrhages (Figures 5-4, 5-5, 5-6). The eye-drop infected commercial broilers had fourteen out of eighteen birds present with cecal tonsil hemorrhage (Figures 5-4, 5-6)
Table 5-7: Trial 1: Newcastle disease virus, antibody capture ELISA, postinfection titers

<table>
<thead>
<tr>
<th>Bird type</th>
<th>Challenge route</th>
<th>Mean Geometric mean</th>
<th>Percent coefficient variance</th>
<th>Max.</th>
<th>Min.</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPF Leghorn</td>
<td>Sham</td>
<td>268</td>
<td>7</td>
<td>194</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>SPF Leghorn</td>
<td>IM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SPF Leghorn</td>
<td>ED</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Commercial Leghorn</td>
<td>Sham</td>
<td>175</td>
<td>9</td>
<td>197</td>
<td>1,159</td>
<td>10</td>
</tr>
<tr>
<td>Commercial Leghorn</td>
<td>IM</td>
<td>28,246</td>
<td>28,238</td>
<td>2.3</td>
<td>28,700</td>
<td>27,337</td>
</tr>
<tr>
<td>Commercial Leghorn</td>
<td>ED</td>
<td>27,538</td>
<td>27,487</td>
<td>6.0</td>
<td>28,700</td>
<td>25,214</td>
</tr>
<tr>
<td>Commercial broiler</td>
<td>Sham</td>
<td>9</td>
<td>2</td>
<td>266</td>
<td>78</td>
<td>10</td>
</tr>
<tr>
<td>Commercial broiler</td>
<td>IM</td>
<td>27,966</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Commercial broiler</td>
<td>ED</td>
<td>27,901</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Commercial turkey</td>
<td>Sham</td>
<td>31</td>
<td>5</td>
<td>156</td>
<td>140</td>
<td>10</td>
</tr>
<tr>
<td>Commercial turkey</td>
<td>IM</td>
<td>22,216</td>
<td>21,870</td>
<td>16.6</td>
<td>25,449</td>
<td>15,109</td>
</tr>
<tr>
<td>Commercial turkey</td>
<td>ED</td>
<td>21,142</td>
<td>19,426</td>
<td>35.0</td>
<td>31,220</td>
<td>6,980</td>
</tr>
</tbody>
</table>

IM = intramuscular challenge route, ED – eye-drop challenge route

and fifteen birds present with sub-capsular splenic hemorrhage. Three birds had proventricular hemorrhage of the distal glandular epithelium (Figure 5-5). Two birds with splenic and cecal tonsil hemorrhage also had tracheal hemorrhage (Table 5-8).

The intramuscular infected commercial broilers had sixteen of twenty with cecal tonsil hemorrhage (Figures 5-4, 5-6), nine with sub-capsular splenic hemorrhage, four with proventricular hemorrhages at the distal glandular epithelium (Figure 5-5), and one bird with tracheal hemorrhage (Table 5-8). The commercial broilers all had gross lesions, whereas the SPF Leghorns had numerous birds with no gross lesions. Very few
SPF Leghorns had sub-capsular splenic hemorrhage. Many commercial broilers had sub-capsular splenic hemorrhages.

The commercial Leghorns had similar post-mortem signs as the SPF Leghorns. Primary lesions in the commercial Leghorns were cecal tonsil hemorrhages (Figures 5-4, 5-6) or no gross lesions. Four intramuscular infected commercial Leghorns and six eye-drop infected commercial Leghorns had gross evidence of sub-capsular splenic hemorrhage. Ten out of twenty intramuscular and twelve out of twenty eye-drop infected birds had lesions consistent with cecal tonsil hemorrhage (Figures 5-4, 5-6). Eight intramuscular and seven eye-drop infected birds did not have any gross lesions at necropsy (Table 5-8). One eye-drop infected bird had fibrin deposits in the tracheal lumen as its sole lesion.

Figure 5-4 Cecal tonsil hemorrhage, subtle lesions, SPF Leghorn, intramuscular challenge, 3 days postinfection
The commercial turkeys had lesions consistent with cecal tonsil hemorrhage (Figures 5-4 and 5-6), proventricular hemorrhage (Figure 5-5), sub-capsular splenic hemorrhage and a few birds with no gross signs of pathology at necropsy. The eye-drop infected turkeys had thirteen of 22 birds with cecal tonsil hemorrhages (Figures 5-4, 5-6), two birds with sub-capsular splenic hemorrhage, six birds with no appreciable gross pathology and one bird with petechial hemorrhage of the pancreas. The intramuscular infected commercial turkeys had twelve of twenty birds with gross lesions consistent with cecal tonsil hemorrhage (Figures 5-4, 5-6), five with sub-capsular splenic hemorrhage, and two with proventricular hemorrhage at the distal aspect of the glandular epithelium (Table 5-1). Two birds had no gross lesions (Table 5-8).
**Trial 2: Commercial Broiler and Commercial Turkey rHVT Vaccine, Texas GB Strain of Newcastle Disease Virus Challenge**

**Trial 2: Broiler Phase**

This trial was conducted to test the safety and efficacy of an rHVT viral vector carrying the HN and F genes to a virulent strain of NDV and genes coding for the A and B gps of MDV. The efficacy of in ovo vaccination at 18 days of incubation and 1-day-of-age subcutaneous recombinant vaccination in broilers was evaluated by infection with Texas GB strain of NDV at 50 days of age. The efficacy of this recombinant vaccine was evaluated in commercial turkeys by subcutaneous vaccination with the recombinant vaccine at 1 day of age.

**Morbidity**

Clinical signs of morbidity attributable to Texas GB NDV infection at 50 days of age were observed in unvaccinated SPF Leghorns 2 days postinfection in the intramuscular infected group and 3 days postinfection in the eye-drop infected group at 53 days of age. This is comparable to the morbidity seen in this type of bird in Trial 1 (Table 5-10). All intramuscular infected birds were clinically affected by Day 4 postinfection as compared to Day 3 postinfection in Trial 1. The eye-drop infected birds reached 100% morbidity on Day 7 postinfection, 57 days of age, as compared to Day 6 postinfection in Trial 1, 48 days of age (Table 5-10).

Clinical signs attributable to infection with Texas GB NDV on Day 3 postinfection in positive control commercial broilers infected by the intramuscular route began at 53 days of age, as compared to Day 4, at 54 days of age, in the eye-drop infected commercial broiler positive controls. Onset of clinical illness in this trial was identical to the onset of clinical illness seen in Trial 1 (Table 5-10). One hundred percent morbidity
from infection with Texas GB NDV occurred on Day 5 postinfection (55 days of age) in the intramuscular infected commercial broilers and Day 8 postinfection (58 days of age) in the eye-drop infected commercial broilers. In contrast to Trial 1, birds infected via the intramuscular route showed signs of morbidity on Day 3 as seen in Trial 1 but 100% morbidity was reached 1 day later, at Day 5 postinfection (Table 5-10). Commercial broilers infected by the eye-drop route showed significant clinical signs of Texas GB NDV infection on the same day as the commercial broilers in Trial 1. However, 100% morbidity did not occur until Day 8 postinfection, 2 days later than that seen in Trial 1 (Table 5-10).

The SPF Leghorns vaccinated subcutaneously at 1 day of age with the recombinant vaccine carrying genes to NDV and MDV showed no clinical signs of morbidity throughout the 14 day observation period postinfection regardless of route of infection at 50 days of age (Table 5-9). These data will be compared with efficacy results observed in commercial broilers.

Commercial broilers were vaccinated in ovo at 18 days incubation, or subcutaneously at 1 day of age with the rHVT vector carrying genes to NDV and MDV. For comparison, a group of broilers was vaccinated with a commercially available, B1B1 modified-live lentogenic strain of NDV at 1 day of age and at 18 days of age via the eye-drop route.

Commercial broilers vaccinated with the recombinant vaccine in ovo at 18 days of incubation and challenged by the eye-drop route at 50 days of age showed no clinical signs of Texas GB NDV infection for the duration of the 14 day observation period following infection. Commercial broilers vaccinated by the same route but challenged
### Table 5-8: Trial 1: Significant gross necropsy lesions

<table>
<thead>
<tr>
<th>Bird type / challenge route</th>
<th>Number of birds necropsied&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cecal tonsil hemorrhage</th>
<th>Splenic hemorrhage</th>
<th>Proventricular hemorrhage</th>
<th>No gross lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPF / IM infected</td>
<td>20</td>
<td>8</td>
<td>2</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>SPF / ED infected</td>
<td>22</td>
<td>13</td>
<td>2</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>SPF / Sham infected</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Commercial broiler / IM infected</td>
<td>20</td>
<td>16</td>
<td>9</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Commercial broiler / ED infected</td>
<td>18</td>
<td>14</td>
<td>15</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Commercial broiler/sham infected</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>17&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Commercial Leghorn / IM infected</td>
<td>20</td>
<td>10</td>
<td>4</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Commercial Leghorn/ED infected</td>
<td>22</td>
<td>12</td>
<td>6</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Commercial Leghorn/Sham infected</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Commercial turkey/IM infected</td>
<td>20</td>
<td>12</td>
<td>5</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Commercial turkey/ED infected</td>
<td>22</td>
<td>13</td>
<td>2</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Commercial turkey/sham infected</td>
<td>19&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>241</strong></td>
<td><strong>98</strong></td>
<td><strong>45</strong></td>
<td><strong>12</strong></td>
<td><strong>117</strong></td>
</tr>
</tbody>
</table>

<sup>a</sup> = There are more lesions than number of birds. Thirty-one (31) birds had more than one lesion at necropsy.

<sup>b</sup> = Bird number 477 died of humane euthanasia due to an arthropathy.

<sup>c</sup> = Bird number 477 died of humane euthanasia due to an arthropathy.
via the intramuscular route showed no signs of Texas GB NDV infection for the duration of the 14 day observation period, except for one bird. Bird number 282 showed non-specific signs of morbidity, including mild depression, (score 1/5), on Day 3 postinfection. On Day 4 postinfection and after, bird 282 showed no signs of morbidity (Table 5-9).

Commercial broilers vaccinated subcutaneously at 1 day of age with the recombinant vaccine and challenged via the intramuscular route showed signs of morbidity attributable to Texas GB NDV challenge. On Day 3 postinfection, two birds showed signs of intention tremors that were not continuous, score of 3/5. One of these birds scored normal (score 0/5) the next day while the other bird slowly improved to a score of normal (score 0/5) by Day 6 postinfection. Three other birds presented with mild signs of morbidity on Day 3 postinfection. By Day 6 postinfection all birds in the intramuscular challenged group were clinically normal throughout the remainder of the trial (Table 5-9). The commercial broilers vaccinated subcutaneously at 1 day of age with the recombinant vaccine and infected with Texas GB NDV at 50 days of age via the eyedrop route remained clinically normal throughout the duration of the 14 day postinfection observation period (Table 5-9).

The commercial broilers vaccinated with commercially available B1B1 modified-live lentogenic strain of NDV showed signs consistent with infection of Texas GB NDV. Of the twenty intramuscular infected commercial broilers, seven showed signs of morbidity at some point in the postinfection observation period. The first signs of morbidity were seen on Day 3 postinfection in the intramuscular challenged group. One bird had signs of non-continuous intention tremors, score of 3/5. On Day 4 postinfection,
six birds showed mild to marked signs of infection with Texas GB NDV. Scores for the 
clinically affected birds on Day 4 postinfection ranged from one to three on a scale of 
five. The one bird that began showing marked signs of infection from Texas GB NDV 
on Day 3 postinfection slowly recovered from the infection, scoring clinically normal on 
Day 8 postinfection. By Day 8 postinfection, all birds were clinically normal and 
remained normal until 64 days of age, 14 days after infection. The eye-drop infected, 
commercially vaccinated broilers showed mild to moderate signs of morbidity beginning 
on Day 4 postinfection and had recovered from infection by Day 7 postinfection. On Day 
4 postinfection, six of ten infected birds were observed to be clinically affected. Five of 
these six birds had scores of 1/5 and one bird score 2/5. By Day 8 postinfection all birds 
were considered clinically normal.

Table 5-9: Trial 2: Broiler phase: Onset of clinical signs and resolution of clinical signs 
in vaccinated chickens

<table>
<thead>
<tr>
<th>Vaccinate group</th>
<th>Texas GB NDV infection route</th>
<th>Number affected / total</th>
<th>Onset of clinical signs (days postinfection)</th>
<th>Days postinfection 100% clinically normal (resolution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPF SQ rHVT</td>
<td>IM</td>
<td>0 / 20</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ED</td>
<td>0 / 10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Broiler in ovo rHVT</td>
<td>IM</td>
<td>1 / 20</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>ED</td>
<td>0 / 10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Broiler SQ rHVT</td>
<td>IM</td>
<td>5 / 20</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>ED</td>
<td>0 / 10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Broiler B1B1 NDV</td>
<td>IM</td>
<td>7 / 20</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>ED</td>
<td>6 / 10</td>
<td>4</td>
<td>7</td>
</tr>
</tbody>
</table>

SQ = subcutaneous, ED = eye-drop challenge route, IM = intramuscular challenge route. 
- = birds never showed signs of morbidity

Mortality

The infective dose of $10^{4.5}$ ELD$_{50}$ of Texas GB NDV was lethal in the 
intramuscular infected SPF Leghorns. One hundred percent of intramuscular infected
Leghorns had died from Texas GB NDV infection by Day 6 postinfection. Only one bird was still alive on Day 5, dying on Day 6 postinfection. This is a different mortality pattern than that seen in Trial 1. In Trial 1, the intramuscular SPF Leghorns showed 100% mortality on Day 5 postinfection (Table 5-10).

Texas GB NDV eye-drop infection was not as lethal in the SPF Leghorns in this trial as in Trial 1. All eye-drop infected SPF Leghorns were severely affected by Day 14 postinfection. On termination of the trial at Day 14 postinfection (64 days of age), six of ten eye-drop infected birds had died while four were recumbent with continuous intention tremors. In Trial 1, $10^{4.5} \text{ ELD}_{50}$ Texas GB NDV was lethal by Day 13 postinfection (Table 5-10).

Texas GB NDV infection was lethal in the intramuscular infected broilers, except for one outlier. Nineteen of twenty intramuscular infected broilers succumbed to Texas GB NDV infection by Day 6 postinfection at 56 days of age. The one outlier was scored 2/5 on Day 6 postinfection and through Day 12 postinfection when its scored worsened. Tremors were noted at termination of the trial on Day 14 postinfection at 64 days of age. In Trial 1, 17/18 intramuscular challenged broilers died by Day 5 postinfection at 47 days of age (Table 5-10).

Texas GB NDV infection by the eye-drop route of the sham control broilers in trial 2 showed a different mortality pattern than in Trial 1. In Trial 1, 18/20 birds had succumbed to Texas GB NDV infection by Day 6 postinfection. The two remaining birds were severely affected and scored 4/5. By Day 9 postinfection (51 days of age) only one bird remained alive and survived until termination of the trial. In this second trial, eight out of ten eye-drop infected birds had died by Day 9 postinfection at 59 days of age.
The following day (Day 10 postinfection) only one bird was still alive and survived until termination on Day 14 postinfection (64 days of age) despite being severely affected.

No mortality occurred in any of the vaccinated SPF Leghorns or vaccinated commercial broilers.

Table 5-10: Trial 1 vs. Trial 2, Positive control, days postinfection, morbidity and mortality comparison of SPF Leghorns and commercial broilers

<table>
<thead>
<tr>
<th>Bird type/route</th>
<th>Trial 1 SPF IM</th>
<th>Trial 1 SPF ED</th>
<th>Trial 2 SPF IM</th>
<th>Trial 2 SPF ED</th>
<th>Trial 1 Broiler IM</th>
<th>Trial 1 Broiler ED</th>
<th>Trial 2 Broiler IM</th>
<th>Trial 2 Broiler ED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onset of morbidity</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>100% morbidity</td>
<td>3</td>
<td>6</td>
<td>4</td>
<td>7</td>
<td>4</td>
<td>6</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Onset of mortality</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>100% mortality</td>
<td>5</td>
<td>13</td>
<td>6</td>
<td>-</td>
<td>5</td>
<td>9</td>
<td>6</td>
<td>10</td>
</tr>
</tbody>
</table>

* = all values are days postinfection.

a = 4/10 survived, all were severely affected with a score of 4/5.
b = excluding one outlier in each trial that survived to termination with a score of 4/5.

ED = Eye-drop challenge route, IM = intramuscular challenge route

Serology Preinfection

All birds infected, (n=20) in the intramuscular infected group and (n=10) in the eye-drop infected group were bled prior to challenge for determination of NDV IgG ELISA titers. These titers served as the preinfection titers.

The twenty SPF Leghorns that served as intramuscular infected controls had a NDV ELISA titer range of 1 to 3,419. The mean titer was 395, with a geometric mean titer of 7, and a percent coefficient of variation of 239.2. The ten SPF Leghorns that remained unvaccinated and were infected by the eye-drop route had a NDV IgG titer range of 1 to 691. The mean titer was 78, geometric mean titer of 3, and a percent coefficient of variation of 265.4 (Table 5-12). The non-vaccinated commercial broilers
infected by the intramuscular route had NDV IgG titers ranging from 1 to 3,213. This group had a mean titer of 536 with a geometric mean titer of 11 and a percent coefficient of variation of 181.7. The eye-drop infected commercial broiler vaccinates had preinfection titer range of 1 to 899. These ten birds had a mean titer of 164, geometric mean titer of 6, and a percent coefficient of variation of 189.2 (Table 5-12).

The SPF Leghorns vaccinated subcutaneously at 1 day of age with the rHVT vaccine and infected by the intramuscular route had a NDV IgG titer range of 56 to 2,469 at 2 days prior to challenge. The mean titer was 929 with a geometric mean titer of 655 and a percent coefficient of variation of 73. The SPF Leghorns to be infected by the eye-drop route had a NDV IgG titer range of 1 to 2,825. This group had a mean titer of 1,019, geometric mean titer of 396, and percent coefficient of variation of 84.9 (Table 5-12).

The commercial broilers vaccinated subcutaneously at 1 day of age with the rHVT and infected by the intramuscular route had a preinfection NDV IgG titer range of 1 to 6,777. This SQ rHVT commercial broiler, intramuscular infected group had mean titer of 3,236 resulting in a geometric mean titer of 2,148 and percent coefficient of variation of 42.1. The SQ rHVT vaccinated commercial broilers infected by the eye-drop route had an NDV IgG titer range of 1,973 to 3,850. This range resulted in a mean titer of 3,058 and a geometric mean titer of 3,004 with a percent coefficient of variation of 17.9.

The rHVT in ovo vaccinated commercial broilers who received intramuscular infection had an NDV IgG titer range of 1,048 to 5,623. This range resulted in a mean titer of 2,961, a geometric mean titer of 2,644, and a percent coefficient of variation of 46.3. The ten birds vaccinated in the same manner but infected by the eye-drop route had
a NDV IgG titer range of 971 to 4,140. This group had a mean titer of 2,797, geometric mean titer of 2,538, and a percent coefficient of variation of 37.6.

The commercial broilers vaccinated at 1 day of age and 18 days of age with the commercial modified-live B1B1 vaccine and then infected by the intramuscular route had a preinfection NDV IgG ELISA titer ranging from 98 to 17,514. These data resulted in a mean titer of 3,540, geometric mean titer of 2,178 and a percent coefficient of variation of 109.6. The eye-drop infected birds vaccinated in the same manner as the intramuscular infected birds had an NDV IgG titer ranging from 319 to 13,734. This group of ten birds had a mean titer of 3,993, geometric mean titer of 2,435 and a percent coefficient of variation of 96.7.

**Serology Postinfection**

All birds surviving to termination of the trial at 14 days postinfection were bled prior to euthanasia and subsequent necropsy.

All non-vaccinated SPF Leghorns infected by the intramuscular route died prior to termination of the trial on Day 14 postinfection. Therefore, no postinfection serology is available for comparison. Blood sera were collected from the four surviving, non-vaccinated, SPF Leghorns infected by the eye-drop route. These four birds had a postinfection NDV IgG titer ranging from 36,581 to 57,336. This resulted in a mean titer of 47,962, geometric mean titer of 47,280 (Table 5-12) and a percent coefficient of variation of 16.5. The mean titer increased by 47,884 while the geometric mean titer increased 47,277 in the eye-drop infected, non-vaccinated, surviving SPF Leghorns.

The non-vaccinated commercial broilers infected by the intramuscular route or eye-drop route had only one bird survive to 14 days postinfection. The sole surviving intramuscular infected bird had a titer of 41,127. This resulted in an increase over the
preinfection mean titer of 40,591. The one eye-drop infected commercial broiler surviving Texas GB NDV infection at 14 days postinfection had a titer of 38,970 (Table 5-12). This resulted in an increase of 38,806 over the group’s preinfection titer.

The SPF Leghorns vaccinated at 1 day of age with the rHVT vaccine and infected by the intramuscular route had a postinfection titer range between 27 and 5,154. This range resulted in a mean titer for the twenty surviving birds of 1,638, geometric mean titer of 929, and a percent coefficient of variation of 94.0. The mean titer increased by 983 over the preinfection titer. The geometric mean titer increased by 274. For the eye-drop infected SPF Leghorns vaccinated in the same manner, a postinfection titer ranging from 101 to 8,312 was recorded. The mean titer was 1,936 and geometric mean titer was 1,093. The mean titer in the eye-drop infected SPF Leghorns increased by 917 while the geometric mean titer increased by 697.

All commercial broilers vaccinated at 1 day of age with the rHVT vaccine survived challenge. The birds infected by intramuscular infection had a NDV IgG postinfection titer ranging from 1,630 to 11,201. This resulted in a mean titer of 4,342, geometric mean titer of 3,841, and a percent coefficient of variation of 52.1. The mean increased by 1,106 while the geometric mean titer increased by 1,693 over the preinfection titers 14 days previously. The eye-drop infected SQ rHVT vaccinated commercial broilers had a postinfection titer ranging from 3,975 to 9,718. The mean titer was 5,604, geometric mean titer was 5,445, and percent coefficient of variation was 26.7. Fourteen days after infection the NDV IgG mean titer for the eye-drop infected group increased by 2,546 while the geometric mean titer increased by 2,441.
The commercial broilers vaccinated in ovo at embryo Day 18 with the rHVT vaccine and infected by the intramuscular route at 50 days of age had a postinfection NDV IgG titer ranging from 1,329 to 8,506. This group had a postinfection mean titer of 4,046, geometric mean titer of 3,597, and coefficient variation of 47.8. This resulted in an increase in the mean titer of 1,085 and an increase in the geometric mean titer of 953. The eye-drop infected commercial broilers vaccinated in the same manner had a postinfection NDV IgG titer ranging from 2,040 to 7,949. This group had a mean titer of 4,134, geometric mean titer of 3,829 and coefficient variation of 39.7. This resulted in an increase in the mean titer by 1,337 while the geometric mean titer increased by 1,291.

Broilers vaccinated with commercially available B1B1 modified-live lentogenic strain of NDV at 1 day of age and 18 days of age, and then challenged by the intramuscular or eye-drop route all survived infection. The intramuscular group had a postinfection NDV IgG titer ranging between 223 and 60,618. This range had a mean of 7,208, geometric mean of 2,415, and percent coefficient of variation of 202.9. The majority of birds fell in titer groups zero to seven. Two birds fell in the highest titer group, titer group eighteen. The mean titer for the intramuscular infected birds increased by 3,668 and the geometric mean increased by 237 over the recorded preinfection mean and geometric mean titers. For the eye-drop infected birds, NDV IgG postinfection titers ranged between 453 and 54,501. The mean titer postinfection was 12,655, geometric mean titer of 3,492, and percent coefficient of variation of 158.0. For the eye-drop infected birds the mean titer rose 8,662 and the geometric mean titer increased 1,057 over the preinfection titers.
Table 5-11: Trial 2: Broiler phase: Treatment group mean postinfection NDV IgG ELISA titers

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Sample Size</th>
<th>Mean postinfection NDV ELISA Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broiler rHVT SQ</td>
<td>30</td>
<td>4,763</td>
</tr>
<tr>
<td>Broiler rHVT in ovo</td>
<td>30</td>
<td>4,075</td>
</tr>
<tr>
<td>Broiler B1B1 NDV eye-drop</td>
<td>30</td>
<td>9,024</td>
</tr>
<tr>
<td>Broiler sham control</td>
<td>2</td>
<td>40,049</td>
</tr>
<tr>
<td>SPF sham control</td>
<td>4</td>
<td>47,962</td>
</tr>
<tr>
<td>SPF rHVT SQ</td>
<td>30</td>
<td>1,737</td>
</tr>
</tbody>
</table>

Table 5-12: Trial 2: Broiler phase: Preinfection and postinfection Newcastle disease virus immunoglobulin G titers

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SPF</td>
<td>IM</td>
<td>20</td>
<td>395</td>
<td>7</td>
<td>-a</td>
<td>-a</td>
</tr>
<tr>
<td>Leghorn Sham</td>
<td>ED</td>
<td>10</td>
<td>78</td>
<td>47,962b</td>
<td>3</td>
<td>47,280b</td>
</tr>
<tr>
<td>Broiler Sham</td>
<td>IM</td>
<td>20</td>
<td>536</td>
<td>41,127c</td>
<td>11</td>
<td>41,127c</td>
</tr>
<tr>
<td>SPF</td>
<td>ED</td>
<td>10</td>
<td>164</td>
<td>38,970c</td>
<td>6</td>
<td>38,970c</td>
</tr>
<tr>
<td>Leghorn</td>
<td>IM</td>
<td>20</td>
<td>929</td>
<td>1,638</td>
<td>655</td>
<td>929</td>
</tr>
<tr>
<td>SPF SQ rHVT</td>
<td>ED</td>
<td>10</td>
<td>1,019</td>
<td>1,936</td>
<td>396</td>
<td>1,093</td>
</tr>
<tr>
<td>Broiler SQ rHVT</td>
<td>IM</td>
<td>20</td>
<td>3,236</td>
<td>4,342</td>
<td>2,148</td>
<td>3,841</td>
</tr>
<tr>
<td>Broiler SQ rHVT</td>
<td>ED</td>
<td>10</td>
<td>3,058</td>
<td>5,604</td>
<td>3,004</td>
<td>5,445</td>
</tr>
<tr>
<td>Broiler In ovo rHVT</td>
<td>IM</td>
<td>20</td>
<td>2,961</td>
<td>4,046</td>
<td>2,644</td>
<td>3,597</td>
</tr>
<tr>
<td>Broiler ED B1B1</td>
<td>IM</td>
<td>20</td>
<td>3,540</td>
<td>7,208</td>
<td>2,178</td>
<td>2,415</td>
</tr>
<tr>
<td>SPF SQ rHVT</td>
<td>ED</td>
<td>10</td>
<td>3,993</td>
<td>12,655</td>
<td>2,435</td>
<td>3,492</td>
</tr>
</tbody>
</table>

SQ = subcutaneous vaccination, ED = Eye-drop challenge route, IM = Intramuscular challenge route

a = Intramuscular Texas GB NDV infection was lethal.
b = Number represents four surviving birds.
c = Number represents sole surviving bird.

Necropsy

All daily mortality was necropsied and gross pathologic changes and sex of the bird recorded. All birds surviving to 14 days postinfection were humanely euthanized and necropsied.
All of the sham infected SPF Leghorns infected by the intramuscular route died from Texas GB NDV infection prior to the end of the observation period. There were twenty birds in this group. Eighteen of the twenty had lesions consistent with cecal tonsil hemorrhage. One bird had both cecal tonsil hemorrhage (Figures 5-4, 5-6) and distal glandular epithelial proventricular hemorrhage (Figure 5-5), while another had cecal tonsil hemorrhage with sub-capsular splenic hemorrhage. Two SPF Leghorns necropsied had no gross lesions. Of the ten sham eye-drop infected SPF Leghorns, seven had cecal tonsil hemorrhage (Figure 5-4, 5-6) and three had no gross lesions (Table 5-13).

The sham infected commercial broilers presented very similar lesions as reported in Trial 1. All birds infected were observed to have at least one gross lesion regardless of route of infection. Nineteen of the twenty intramuscular infected broilers had lesions of cecal tonsil hemorrhage (Figures 5-4, 5-6). Fourteen of nineteen had lesions of sub-capsular splenic hemorrhage. Three of fourteen had lesions of mucosal hemorrhage in the proventriculus near the proventricular/gizzard junction (Figure 5-5). The remaining bird had lesions consistent with sub-capsular splenic hemorrhage. Eight of the ten eye-drop infected commercial broilers were noted to have cecal tonsil hemorrhage. Five of the eight birds having cecal tonsil hemorrhage also had sub-capsular splenic hemorrhage. One bird had three lesions: cecal tonsil hemorrhage, sub-capsular splenic hemorrhage and distal glandular epithelial proventricular hemorrhage. Two other birds were noted to have distal glandular epithelial proventricular hemorrhage. One of these birds had sub-capsular splenic hemorrhage while the other had cecal tonsil hemorrhage (Table 5-13).
Figure 5-6 Severe cecal tonsil hemorrhage, commercial broiler, intramuscular challenge, 10 days postinfection

The SPF Leghorns vaccinated at 1 day of age, subcutaneously with the rHVT carrying two genes to NDV and two genes to MDV had twenty-nine of thirty birds with cecal tonsil hemorrhages (Figures 5-4, 5-6). Cecal tonsil hemorrhage was the sole lesion noted in these birds. Only one bird, of the twenty, in the intramuscular infected group had no appreciable pathologic changes at termination of the trial, 14 days postinfection (Table 5-13). All birds survived infection and therefore were humanely euthanized and necropsied.
The commercial broilers vaccinated at 1 day of age, subcutaneously with the rHVT all survived to termination. Significant lesions observed in the intramuscular infected birds were cecal tonsil hemorrhage and distal glandular epithelial proventricular hemorrhage. Nineteen of the twenty birds had lesions of cecal tonsil hemorrhage. Three of these birds also had distal glandular epithelial proventricular hemorrhage. One bird had a sole lesion of distal glandular epithelial proventricular hemorrhage. In contrast, the ten eye-drop infected broilers vaccinated subcutaneously at 1 day of age with the rHVT vaccine all presented with pathologic changes attributable to cecal tonsil hemorrhage (Table 5-13). This was the sole lesion seen in this group.

The commercial broilers vaccinated in ovo at 18 days incubation with the rHVT vaccine all had lesions of cecal tonsil hemorrhage regardless of route of infection. In the twenty intramuscular infected broilers, two had an additional lesion of distal glandular epithelial proventricular hemorrhage. The ten eye-drop infected broilers’ sole lesion was cecal tonsil hemorrhage (Table 5-13).

The commercial broilers vaccinated via the eye-drop route at 1 day of age and 18 days of age with the commercial lentogenic B1B1 strain of NDV had similar lesions as those of the in ovo rHVT vaccinated broilers. Lesions consisted of primarily cecal tonsil hemorrhage and a few distal glandular epithelial proventricular hemorrhages. Twenty of the twenty intramuscular infected broilers had pathologic changes in the cecal tonsils that could be attributed to cecal tonsil hemorrhage. Two birds had a second lesion of distal glandular epithelial proventricular hemorrhage. Nine of the ten eye-drop infected, B1B1 NDV vaccinated broilers had a single gross lesion of cecal tonsil hemorrhage. One bird had no significant pathologic changes (Table 5-13).
Table 5-13: Trial 2: Broiler phase: Significant gross necropsy lesions

<table>
<thead>
<tr>
<th>Bird type/vaccine</th>
<th>Route of infection</th>
<th>Sample size *</th>
<th>Cecal tonsil hemorrhage</th>
<th>Splenic hemorrhage</th>
<th>Proventricular hemorrhage</th>
<th>No gross lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPF sham</td>
<td>IM</td>
<td>20</td>
<td>18</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Broiler sham</td>
<td>ED</td>
<td>10</td>
<td>7</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>SPF SQ</td>
<td>IM</td>
<td>20</td>
<td>19</td>
<td>15</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>rHVT</td>
<td>IM</td>
<td>20</td>
<td>19</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Broiler SQ</td>
<td>ED</td>
<td>10</td>
<td>8</td>
<td>7</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>SQ</td>
<td>ED</td>
<td>10</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Broiler in ovo rHVT</td>
<td>IM</td>
<td>20</td>
<td>20</td>
<td>0</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>B1B1</td>
<td>ED</td>
<td>10</td>
<td>9</td>
<td>-</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

* = Numbers of lesions are greater than number of total birds. Thirty-six (36) birds had more than one lesion.

SQ = subcutaneous vaccination, ED = eye-drop challenge route, IM = intramuscular challenge route

Trial 2: Turkey Phase

This trial was conducted to test the safety and efficacy of an rHVT viral vector carrying the HN and F genes to a virulent strain of NDV and genes coding for gps A and B of a MDV. The efficacy of the recombinant viral vector was tested against Texas GB NDV challenge in commercial turkeys at 84 days of age. Efficacy was compared to the percent protection elicited by commercial B1B1 type modified-live NDV vaccine. Commercial B1B1 type NDV vaccine was administered to the turkeys via the eye-drop route at 21, 42, and 63 days of age. The recombinant construct used in this trial is the same construct and serial as that used in the broiler phase of this trial. The turkeys and SPF Leghorns were challenged via ED or IM route at 84 days of age. In comparison, the broilers were challenged at 50 days of age.
Morbidity

Unvaccinated SPF Leghorns challenged with Texas GB strain of NDV via the intramuscular route began showing signs of neurotropic velogenic NDV 3 days postinfection, 87 days of age. This is comparable to the mortality seen in the SPF Leghorns challenged intramuscularly in trials 1 and 2, broiler phase (Table 5-10). All positive control SPF Leghorns were clinically affected by five days postinfection (Table 5-14). One hundred percent morbidity was observed 1 day later (day five postinfection) than that seen in Trial 2, broiler phase. Similarly, Trial 2, broiler phase reached 100% mortality 1 day later (day 4 postinfection) as seen in Trial 1. It is important to note that each subsequent trial challenged birds later than the previous. Birds were challenged at: 42 days of age in Trial 1; 50 days of age in Trial 2, broiler phase; and at 84 days of age in Trial 2, turkey phase.

Positive control commercial turkeys challenged intramuscularly began to show clinical signs on Day 6 postinfection. Two out of the eight non-vaccinated turkeys developed clinical signs on Day 6 (Table 5-14). One bird with moderate signs (score 2/5) had recovered by the next observation period and was considered clinically normal at this time. The other bird was markedly affected and remained at this score throughout the remainder of the trial.

The non-vaccinated (positive control) turkeys challenged via the eye-drop route began showing clinical signs on Day 8 postinfection (Table 5-14). There was only one bird that showed moderate signs of illness, score 2/5. This bird never recovered or progressed in severity of clinical signs for the duration of the trial.

Commercial turkeys and SPF Leghorns vaccinated with the rHVT construct subcutaneously at 1 day of age and challenged via the intramuscular or eye-drop route did
not show any clinical signs throughout the 14 day postinfection clinical observation period (Table 5-11).

Turkeys receiving eye-drop vaccination with the commercial, modified-live B1B1 type NDV experienced a vaccine reaction 6 days postvaccination. The vaccine reaction persisted for 8 days, Days 6 through 13 postvaccination or 27 to 34 days of age. The vaccine reaction consisted of mild snicking and sneezing. No tracheal rales or coughing were appreciated clinically. No vaccine reaction was observed upon subsequent vaccination at 42 and 63 days of age with the same vaccine and the same lot number. Turkeys receiving the commercial, modified-live B1B1 type NDV remained clinically normal for the duration of the postinfection observation period regardless of challenge route (Table 5-14).

**Mortality**

Mortality began 72 hours postinfection. All birds had succumbed to challenge. Mortality was seen only in the non-vaccinated SPF Leghorn controls. Mortality was 100% with Texas GB NDV challenge by 5 days postinfection in the SPF sham controls. The non-vaccinated, positive control turkeys only had one bird become moderately moribund on Day 6 postinfection from the intramuscular infected group and one bird from the eye-drop infected commercial turkeys. These birds never died. Therefore, there was no mortality in the non-vaccinated turkeys (Table 5-14). Likewise, there was no mortality in the subcutaneously vaccinated rHVT turkeys regardless of challenge route. The commercial, modified-live B1B1 type NDV vaccine provided complete protection from morbidity and mortality against exotic NDV challenge (Table 5-14).
Table 5-14: Trial 2: Turkey phase: Onset and percent morbidity and mortality

<table>
<thead>
<tr>
<th>Treatment group&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Challenge route</th>
<th>Day onset morbidity</th>
<th>Final percent morbidity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Day onset mortality</th>
<th>Final percent mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-vaccinated SPF Leghorns</td>
<td>IM</td>
<td>5</td>
<td>100% (10/10)</td>
<td>3</td>
<td>100%</td>
</tr>
<tr>
<td>Non-vaccinated turkeys</td>
<td>IM</td>
<td>6</td>
<td>13% (1/8)</td>
<td>-</td>
<td>0%</td>
</tr>
<tr>
<td>Non-vaccinated turkeys</td>
<td>ED</td>
<td>8</td>
<td>8% (1/12)</td>
<td>-</td>
<td>0%</td>
</tr>
<tr>
<td>SQ vaccinated rHVT SPF Leghorns</td>
<td>IM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0%</td>
</tr>
<tr>
<td>SQ vaccinated rHVT SPF Leghorns</td>
<td>ED</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0%</td>
</tr>
<tr>
<td>SQ vaccinated rHVT turkeys</td>
<td>IM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0%</td>
</tr>
<tr>
<td>SQ vaccinated rHVT turkeys</td>
<td>ED</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0%</td>
</tr>
<tr>
<td>ED B1B1 NDV vaccinated rHVT turkeys</td>
<td>IM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0%</td>
</tr>
<tr>
<td>ED B1B1 NDV vaccinated rHVT turkeys</td>
<td>ED</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0%</td>
</tr>
</tbody>
</table>

SQ = subcutaneous vaccination, ED = eye-drop challenge route, IM = intramuscular challenge route

<sup>a</sup> Sample size was twelve for all eye-drop groups and eight for intramuscular groups with the exception of SPF controls had 10 intramuscular challenged birds.

<sup>b</sup> Number in parenthesis ( ) is the number of birds affected over the total for that group.

Serology Preinfection

All birds entering the challenge phase of the trial were bled just prior to challenge (84 days of age) to determine their individual and group IgG, NDV ELISA titers. The non-vaccinated turkey sham controls remained unchallenged from NDV field infection or cross contamination throughout the 84 day grow-out period. Additionally, all measurable levels of maternal antibodies had waned by this time. The group average IgG, NDV ELISA titer for the non-vaccinated turkeys that were to be challenged by the
intramuscular route was 20 with a geometric mean titer of 5 (Table 5-15). The percent coefficient of variation for these birds was 127. The titers ranged from a minimum of 1 to 65. The non-vaccinated turkeys that were to receive eye-drop challenge had a mean titer of 189 and geometric mean titer of 15, at 84 days of age (Table 5-17). The percent coefficient of variation for this group was 148 with individual bird titers ranging from 1 to 892. The non-vaccinated turkeys receiving intramuscular challenge had a mean titer of 20, geometric mean titer of 5, and percent coefficient of variation of 127. Titers ranged from 1 to 65.

The unvaccinated SPF Leghorns were also seronegative for IgG, NDV ELISA antibodies at 84 days of age. The unvaccinated SPF Leghorns had a mean titer of 649 with a corresponding geometric mean titer of 70 (Tables 5-15, 5-17) and percent coefficient of variation of 124.5. The titers for this group ranged from 1 to 2,291. Two birds in this group had questionable titers of 2,291 and 1,902. However, due to the dynamics of the spread of NDV, it is highly unlikely that only two out of ten birds were exposed to NDV during the growing period and did not shed the virus to their pen mates. This is most likely background “noise” or the result of a hemolyzed sample.

Commercial turkeys receiving subcutaneous vaccination with the rHVT vaccine at 1 day of age did not produce significant IgG, NDV ELISA antibodies over that of the non-vaccinated birds. The treatment group receiving intramuscular challenge had a mean titer of 521 and geometric mean titer of 189 with a percent coefficient of variation of 93. Titers ranged from 1 to 1,365. For those birds receiving eye-drop challenge the mean titer was 997 with a geometric mean titer of 481. The eye-drop challenged group had a
percent coefficient of variation of 107. Individual bird IgG, NDV titers ranged from 39 to 3,208. Overall, group mean was 778 with a geometric mean titer of 313 (Table 5-15).

Turkeys vaccinated with the modified-live B1B1 type NDV at 21, 42, and 63 days of age elicited a mild IgG, NDV ELISA titer. The mean titer for the turkeys receiving IM challenge was 3,017 with a geometric mean titer of 2,664 (Tables 5-15, 5-17), and a percent coefficient of variation of 46.6. The titers ranged from 881 to 5,911. A mean titer of 2,832 and geometric mean titer of 2,560 (Tables 5-15 and 5-17) was measured for those turkeys vaccinated in the same manner as those in the intramuscular group but receiving eye-drop challenge. For this group, a percent coefficient of variation of 40.6 with titers ranging from 812 to 4,450 was obtained. The group as a whole had a mean titer of 2,977 and a geometric mean titer of 2,639.

Table 5-15: Trial 2: Turkey phase: Preinfection NDV IgG geometric mean ELISA titers

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>n</th>
<th>Geometric mean preinfection NDV ELISA titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turkey rHVT SQ</td>
<td>20</td>
<td>313</td>
</tr>
<tr>
<td>Turkey B1B1 NDV eye-drop</td>
<td>20</td>
<td>2,639</td>
</tr>
<tr>
<td>Turkey sham controls</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>SPF sham controls</td>
<td>10</td>
<td>70</td>
</tr>
</tbody>
</table>

**Serology Postinfection**

All birds in the non-vaccinated, intramuscular challenged SPF Leghorns succumbed to Texas GB NDV challenge by the end of the observation period. Therefore, there is no postinfection serology for these birds.

The turkeys receiving no vaccine and challenged intramuscularly had a mean titer of 20,706 and a geometric mean titer of 20,241 as compared to the eye-drop challenged turkeys with a mean titer of 21,473 and geometric mean titer of 21,255 (Tables 5-16,
5-17). The intramuscular and eye-drop challenged turkeys had a percent coefficient of variation of 18.5 and 13.6 with a range in titers of 10,251 to 24,597 and 15,518 to 25,196 respectively. This group as a whole had a mean titer of 21,013 and a geometric mean titer of 20,461.

The commercial turkeys receiving the rHVT subcutaneously at 1 day of age and challenged intramuscularly had a mean titer 14 days postinfection of 262 and geometric mean titer of 16 while those challenged by the eye-drop route had a mean titer of 1,520 and geometric mean titer of 314 (Tables 5-16, 5-17). The percent coefficient of variation was 176 and 129 for the intramuscularly and eye-drop challenged birds respectively. The intramuscularly challenged birds’ titers ranged from 1 to 1,413. Titors ranged from 1 to 6,518 for the eye-drop challenged birds. Mean titer and geometric mean titer for this group as a whole was 923 and 95, respectively.

The commercial turkeys receiving the commercial modified-live B1B1 type NDV vaccine at 21, 42, and 63 days of age had lower IgG NDV ELISA titers postinfection as compared to preinfection titers. Postinfection titers for the intramuscular challenged birds had a mean of 1,788 and a geometric mean titer of 1,328. The range of titers for this group of turkeys postinfection was 451 to 4,030 with a percent coefficient of variation of 69.2. Those turkeys vaccinated in the same manner but challenged by the eye-drop route had a greater significant decline to a geometric mean titer nearly one-quarter of that seen preinfection. The postinfection mean titer for the turkeys vaccinated with the commercial B1B1 type modified-live NDV during grow-out and challenged by the eye-drop route was 2,280 and a geometric mean titer of 573 (Table 5-17, Figure 5-7).
The postinfection titers had a percent coefficient of variation of 104 and a range of 1 to 8,821.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>n</th>
<th>Geometric mean postinfection NDV ELISA titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turkey rHVT SQ</td>
<td>20</td>
<td>95</td>
</tr>
<tr>
<td>Turkey B1B1 NDV</td>
<td>20</td>
<td>802</td>
</tr>
<tr>
<td>Turkey sham control</td>
<td>20</td>
<td>20,461</td>
</tr>
<tr>
<td>SPF sham control</td>
<td>0</td>
<td>100% mortality</td>
</tr>
</tbody>
</table>

Preinfection versus postinfection eye-drop and intramuscular challenged NDV IgG geometric mean ELISA titers were compared. The turkeys vaccinated with the recombinant vaccine at 1 day of age subcutaneously and challenged by the intramuscular route had no significant decline in their geometric mean antibody titers from 189 preinfection to 16 postinfection. The turkeys vaccinated with the recombinant vaccine at 1 day of age and challenged by the eye-drop route had no difference in their preinfection titer of 481 versus postinfection NDV ELISA titer of 314. The intramuscular challenged turkeys vaccinated with the commercial modified-live B1B1 NDV vaccine had a significant decline in their antibody titers from a preinfection geometric mean titer of 2,664 down to a geometric mean titer of 1,328 postinfection. The turkeys vaccinated with the commercial modified-live B1B1 NDV vaccine and challenged via the eye-drop route
Figure 5-7: Trial 2: Preinfection and postinfection geometric mean IgG NDV ELISA titers
also had a decline in postinfection antibody titers. The non-vaccinated turkey, sham controls had a significant increase in their postinfection titers, as compared to their preinfection titers (Table 5-17 and Figure 5-7). Texas GB NDV intramuscular challenge was lethal in all SPF sham controls.

**Necropsy**

All daily mortality was necropsied and all birds surviving to the end of the 14 day observation period were humanely euthanized and necropsied. All gross lesions were recorded.

The non-vaccinated, positive control SPF Leghorns all succumbed to intramuscular, Texas GB NDV challenge. All birds (10/10) in this group had gross signs of cecal tonsil hemorrhages regardless of days postinfection they died.

All of the non-vaccinated turkeys, challenged intramuscularly, survived challenge. Lesions seen at necropsy (fourteen days postinfection) consisted of cecal tonsil hemorrhage. This lesion was present in all eight of the birds necropsied. This same lesion was seen in 100% (12/12) of the non-vaccinated turkeys challenged by the eye-drop route.

Cecal tonsil hemorrhages were also seen in all SPF Leghorns and commercial turkeys vaccinated subcutaneously at 1 day of age regardless of challenge route. All the turkeys that were eye-drop vaccinated with the commercial modified-live B1B1 type NDV also had cecal tonsil hemorrhages 14 day postinfection.

The prominent and sole lesion of this phase of Trial 2 was cecal tonsil hemorrhages. Cecal tonsil hemorrhages were seen in 100% of the birds in this trial.
**Trial 3: Commercial Broiler rHVT plus CVI988 and/or HVT/SB1 Vaccine, Texas GB Newcastle Disease Virus Challenge Trial**

This trial was undertaken to further test the safety and efficacy of the rHVT vaccine used in Trial 2. The rHVT is a live HVT vector carrying two genes, HN and F to a virulent strain NDV and gps A and B of a MDV. This trial tested the safety and efficacy of the recombinant vaccine when administered with various combinations of commercially available live HVT and MDV vaccines.

There were nine groups of broilers receiving various combinations of vaccines. Following is a list of these treatment groups and their corresponding treatments (Table 4-4):

- **Treatment group 1:** rHVT and CVI-988 (Rismavac) subcutaneously at 1 day of age
- **Treatment group 2:** rHVT plus commercial HVT and SB1 (Marexine SB) subcutaneously at 1 day of age
- **Treatment group 3:** rHVT subcutaneously at 1 day of age
- **Treatment group 4:** CVI-988 (Rismavac) subcutaneously and commercial modified-live B1B1 type NDV (Bio-Cas B1) via the eye-drop route at 1 day of age
- **Treatment group 5:** rHVT and CVI-988 (Rismavac) in ovo at 18 days incubation
- **Treatment group 6:** rHVT and commercial HVT and SB1 (Marexine SB) in ovo at 18 days incubation
- **Treatment group 7:** rHVT in ovo at 18 days incubation
- **Treatment group 8:** commercial HVT and SB1 (Marexine SB) subcutaneously and modified-live B1B1 type NDV (Bio-Cas B1) via the eye-drop route at 1 day of age, and finally
- **Treatment group 9:** sham vaccinated in ovo at 18 days incubation and subcutaneously at 1 day of age.

All treatment groups are referred to solely as their treatment group number henceforth.
Morbidity

Total morbidity is reported as those birds moribund (Score 2/5 or greater) at trial termination. Some birds were scored as clinically normal, then 24 hours later found dead. All birds in groups 1, 4, 7 and 8 remained clinically normal throughout the duration of the 10 day observation period between 28 and 38 days of age (Table 5-18). Birds in group 2 challenged intramuscular, began showing signs of morbidity on Day 3 postinfection. One bird died on Day 3 postinfection without any prodromal signs. Total morbidity for the intramuscular challenged birds in group 2 was 9 out of 15 or 60%. This onset of morbidity was similar to that seen in the positive control, sham vaccinated, birds in trials 1 and 2. It is also the same day morbidity was first observed in Trial 2, commercial broilers receiving only the rHVT subcutaneously. Birds in Trial 3 progressed in disease, whereas the birds in Trial 2 resolved clinical signs by 6 days postinfection. The birds in group 2 that were challenged by the eye-drop route began showing signs attributable to infection with Texas GB NDV on Day 5 postinfection. Total morbidity for these birds was 7 out of 15 birds or 47%. The onset of clinical signs was 1 day later than seen in the positive control broilers challenged by the eye-drop route in trials 1 and 2. In comparison, Trial 2, rHVT subcutaneously vaccinated broilers challenged by the eye-drop route never showed any detectable clinical signs.

Commercial broilers challenged intramuscular in group 3 remained clinically normal for the duration of the trial. The eye-drop challenged birds in this group had one bird of 15 show very mild signs beginning on Day 3 and continued at this score of 1/5 (mild depression only) until this bird succumbed to Texas GB NDV on Day 7 postinfection.
Treatment group 5 had two birds die without prodromal signs. The intramuscular challenged group had one bird out of 15 die on Day 3 postinfection. The eye-drop challenged birds in group 5 had one bird out of 14 die without prodromal signs on Day 5 postinfection. Therefore, no morbidity was observed in this group despite the mortality losses.

Commercial broilers in group 6 receiving intramuscular challenge began showing clinical signs 72 hours postinfection. In subsequent days, more birds became clinically affected and many died as the trial progressed. By the end of the 10 day observation period five out of fifteen birds had become clinically ill from Texas GB NDV infection. However, the eye-drop challenged birds had only one bird become moribund on Day 5 after challenge. This one moribund bird quickly progressed to death by the next observation period.

Birds receiving the intramuscular challenge in group 9 began showing signs of morbidity attributable to neurotropic velogenic, Texas GB NDV infection on Day 3 postinfection. All birds in this subgroup were clinically affected by 4 days after challenge. Clinical signs began to be observed in the eye-drop challenged birds in group 9 by 4 days postinfection. One bird died without prodromal signs of infection 48 hours after challenge. All fifteen birds in this challenge group were clinically affected by Day 7 postinfection. This is the same morbidity pattern for the intramuscular and eye-drop challenged birds observed in trials 1 and 2.

For this trial (Trial 3) all birds that began to show clinical signs progressed in severity of clinical signs or succumbed to Texas GB NDV challenge. This is in comparison to Trial 2 where all broilers that showed signs of morbidity had recovered
and were considered clinically normal by the end of the trial. This same comparison cannot be made with the vaccinated turkeys in Trial 2 since no vaccinated turkeys showed signs of morbidity throughout the duration of the postinfection observation period.

**Mortality**

There were nine groups of broilers receiving various combinations of vaccines.

Following is a list of these treatment groups and their corresponding treatments (Table 4-4):

- Treatment group 1: rHVT and CVI-988 (Rismavac) subcutaneously at 1 day of age
- Treatment group 2: rHVT plus commercial HVT and SB1 (Marexine SB) subcutaneously at 1 day of age
- Treatment group 3: rHVT subcutaneously at 1 day of age
- Treatment group 4: CVI-988 (Rismavac) subcutaneously and commercial modified-live B1B1 type NDV (Bio-Cas B1) via the eye-drop route at 1 day of age
- Treatment group 5: rHVT and CVI-988 (Rismavac) in ovo at 18 days of incubation
- Treatment group 6: rHVT and commercial HVT and SB1 (Marexine SB) in ovo at 18 days of incubation
- Treatment group 7: rHVT in ovo at 18 days of incubation
- Treatment group 8: commercial HVT and SB1 (Marexine SB) subcutaneously and modified-live B1B1 type NDV (Bio-Cas B1) via the eye-drop route at 1 day of age
- Treatment group 9: sham vaccinated in ovo at 18 days of incubation and subcutaneously at 1 day of age

All treatment groups are referred to solely as their treatment group number henceforth.

All birds in groups 1, 4, 7, and 8 survived challenge (Table 5-18). Commercial broilers in group 2 challenged intramuscular at 28 days of age began succumbing to Texas GB NDV on Day 3 postinfection. Along with the morbidity pattern discussed
previously, this too is a similar mortality pattern as seen in the sham vaccinated broilers in groups 1, 2 and 3. Total mortality for this route of challenge was 8 out of 15 or 53%. The eye-drop challenged birds had 4 out of 15 birds or 27% succumb to challenge 10 days postinfection. Birds in the eye-drop challenged group began dying 6 days after infection. Total mortality for treatment group 2 regardless of challenge route was 12 out of 30 or 40% (Table 5-18).

Commercial broilers in group 3 challenged intramuscular did not show signs of morbidity nor did any die during the observation period. One bird in the eye-drop challenge group showed mild signs, score one out of five, on Day 3 to Day 6. This bird was found dead on Day 7. This one bird out of fifteen was the only bird to die yielding a percent mortality of only 7% for this challenge route. Total mortality for treatment group 3 was one out of 30 or 3% (Table 5-18).

Treatment group 5 had two birds succumb to the Texas GB NDV challenge. Each of the challenge routes had one bird out of 15 experience fatal infection with Texas GB NDV or 7% of the birds in each of the challenge groups and a total of 7% for this treatment group. The birds in the intramuscular and eye-drop challenged groups died on Day 3 and 5 postinfection, respectively. The eye-drop challenged group had one other bird die during the observation period, on Day 5, due to an arthropathy. This bird was not showing any clinical signs of infection with Texas GB strain of NDV at the time and was humanely euthanized. This bird was eliminated from the total number of birds, making the total number of eye-drop challenged birds to be fourteen in this treatment group.
Treatment group 6 had significant mortality in the intramuscular challenged birds. Birds began succumbing to Texas GB NDV challenge 4 days after infection. Mortality for this challenge group was five out of fifteen or 33%. Mortality in the eye-drop challenged birds was much less. This challenge group had only one bird out of fifteen die 6 days postchallenge. The total mortality for treatment group 6 was 20% or six out of thirty of which five came from the intramuscular challenged birds (Table 5-18).

Texas GB NDV challenge was lethal in all commercial broilers receiving sham vaccination (group 9) regardless of challenge route. The intramuscular challenged commercial broilers began dying 72 hours after infection. All fifteen birds in this challenge group had died by 5 days postinfection. This is the same pattern of mortality as that seen in trials 1 and 2. The eye-drop challenged birds began showing mortality from infection 4 days after challenge. One outlier bird died without prodromal signs on Day 2 postinfection. One hundred percent of the fifteen birds challenged in this group had died by Day 8 postinfection. This is the same onset of mortality as seen in trials 1 and 2 when the outlier is removed. However, 100% mortality was observed 2 days sooner than that seen in Trial 2 (10 days postinfection) and 1 day sooner than in Trial 1, 9 days postinfection.

Serology
There were nine groups of broilers receiving various combinations of vaccines. Following is a list of these treatment groups and their corresponding treatments (Table 4-4):

- Treatment group 1: rHVT and CVI-988 (Rismavac) subcutaneously at 1 day of age
- Treatment group 2: rHVT plus commercial HVT and SB1 (Marexine SB) subcutaneously at 1 day of age
Treatment group 3: rHVT subcutaneously at 1 day of age

Treatment group 4: CVI-988 (Rismavac) subcutaneously and commercial modified-live B1B1 type NDV (Bio-Cas B₁) via the eye-drop route at 1 day of age

Treatment group 5: rHVT and CVI-988 (Rismavac) in ovo at 18 days of incubation

Treatment group 6: rHVT and commercial HVT and SB₁ (Marexine SB) in ovo at 18 days of incubation

Treatment group 7: rHVT in ovo at 18 days of incubation

Treatment group 8: commercial HVT and SB₁ (Marexine SB) subcutaneously and modified-live B1B1 type NDV (Bio-Cas B₁) via the eye-drop route at 1 day of age

Treatment group 9: sham vaccinated in ovo at 18 days of incubation and subcutaneously at 1 day of age

All treatment groups are referred to solely as their treatment group number henceforth.

All birds in Trial 3 had IgG NDV ELISA titers and HI titers determined both preinfection and postinfection. It should be kept in mind that the postinfection titers were determined after only 10 days postinfection (38 days of age) at termination of the trial. All HI titers are expressed as the reciprocal of the highest dilution at which HI was observed.

1 Day of Age Serology

Twenty-six day old chicks were euthanized and immediately exsanguinated. Immunoglobulin G NDV titers for these chicks were an average of 1,574 with a geometric mean titer of 1,289. The percent coefficient of variation was 67.8 with titers ranging from a low of 366 and a high of 5,178. These values were used to determine the maternal IgG NDV antibody titers for this flock of commercial broilers. The HI titers ranged from $2 \log_2 (4)$ to $9 \log_2 (512)$ with a mean titer of $6.75 \log_2 (108)$. 
<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccination</th>
<th>Challenge route</th>
<th>Onset of morbidity</th>
<th>Total morbidity</th>
<th>Onset of mortality</th>
<th>Total mortality</th>
<th>Total mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SQ rHVT + CVI988</td>
<td>IM</td>
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<td>NA</td>
<td>0% (0/15)</td>
<td>0% (0/30)</td>
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<tr>
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<td></td>
<td>ED</td>
<td>NA</td>
<td>0% (0/15)</td>
<td>NA</td>
<td>0% (0/15)</td>
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</tr>
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<td>2</td>
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<td>40% (12/30)</td>
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<td>27% (4/15)</td>
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<td>IM</td>
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<td>3% (1/30)</td>
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<td></td>
<td>ED</td>
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<td>7</td>
<td>7% (1/15)</td>
<td></td>
</tr>
<tr>
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<td>SQ CVI988 &amp; ED NDV B1B1</td>
<td>IM</td>
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<td></td>
<td>ED</td>
<td>NA</td>
<td>0%</td>
<td>NA</td>
<td>0%</td>
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</tr>
<tr>
<td>5</td>
<td>In ovo rHVT + CVI988</td>
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<td>3</td>
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<td>ED</td>
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<td>7% (1/14)</td>
<td>5</td>
<td>7% (1/14)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>In ovo rHVT + HVT/SB1</td>
<td>IM</td>
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<td>33% (5/15)</td>
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<td>ED</td>
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<td>7% (1/15)</td>
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</tr>
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<td>In ovo rHVT</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>ED</td>
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<td>NA</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>SQ HVT/SB1 &amp; ED NDV B1B1</td>
<td>IM</td>
<td>NA</td>
<td>0%</td>
<td>NA</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ED</td>
<td>NA</td>
<td>0%</td>
<td>NA</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Sham</td>
<td>IM</td>
<td>3</td>
<td>100% (15/15)</td>
<td>3</td>
<td>100% (15/15)</td>
<td>100% (30/30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ED</td>
<td>4</td>
<td>100% (15/15)</td>
<td>4</td>
<td>100% (15/15)</td>
<td></td>
</tr>
</tbody>
</table>

SQ = subcutaneous vaccination, ED = eye-drop challenge route, IM = intramuscular challenge route
**Preinfection Serology**

Commercial broilers at 28 days of age in treatment group one had an ELISA mean titer of 1,801 and an HI mean titer of 115 (6.84 log₂). The geometric mean ELISA titer was 1,096 with an HI geometric mean titer of 80.6 (6.33 log₂) (Table 5-19). The titers ranged from 86 to 4,263 for the ELISA IgG NDV titers and 8 (3 log₂) to 256 (8 log₂) for the HI titers. The ELISA percent coefficient of variation was 76.3.

The mean ELISA titer prior to challenge in treatment group two was 610 with a geometric mean titer of 68. This corresponds to a mean HI titer of 31 (4.94 log₂) and a geometric mean titer of 11.6 (3.54 log₂) (Table 5-19). The ELISA IgG NDV titers ranged from 1 to 4,781 with a percent coefficient of variation of 199. The HI titers ranged from 4 (2 log₂) to 256 (8 log₂).

Treatment group three had a mean preinfection ELISA titer of 1,893 with a corresponding geometric mean titer of 1,067. The minimum titer was 111 with a maximum titer of 8,007. The percent coefficient of variation was calculated to be 110. In comparison, the HI mean titer was 141 (7.14 log₂) with a geometric mean titer of 77.0 (6.27 log₂) (Table 5-19). The HI titers ranged from 8 (3 log₂) to 256 (8 log₂).

Commercial broilers in treatment group four possessed a mean and geometric mean ELISA titer of 2,696 and 560, respectively at the time of infection, 28 days of age. The titers at this time ranged from 1 to 7,218 with a percent coefficient of variation of 87.5. The HI titers for this group had an average titer of 281.6 (8.14 log₂) and a geometric mean titer of 213 (7.73 log₂) (Table 5-19). The preinfection mean titers ranged from 64 (6 log₂) to 1024 (10 log₂).

Treatment group five possessed an average and geometric mean titer of 2,584 and 1,155, respectively prior to challenge. Titers ranged from a minimum of 6 to a maximum
of 7,488 with a percent coefficient of variation of 76.1. The HI titers prior to challenge had a mean of 133 \((7.09 \, \log_2)\) with a geometric mean titer of 70.2 \((6.13 \, \log_2)\) (Table 5-19). The titers ranged from 4 \((2 \, \log_2)\) to 512 \((9 \, \log_2)\).

Treatment group six had a mean and geometric mean preinfection titer 1,116 and 259, respectively. The titers ranged from 1 to 6,644 with a percent coefficient of variation of 155. The HI titers were also low. The mean HI preinfection titer for treatment group 6 was 21.2 \((4.41 \, \log_2)\) with a geometric mean titer of 10.8 \((3.43 \, \log_2)\) (Table 5-19). The HI titers ranged from 2 \((1 \, \log_2)\) to 128 \((7 \, \log_2)\).

Treatment group seven had a preinfection mean ELISA titer of 1,319 with a geometric mean titer of 1,115. Titers for this group ranged from 256 to 3,293 with a percent coefficient of variation of 56.8. The mean HI titer for this group was 152 \((7.24 \, \log_2)\) with a geometric mean titer of 70.2 \((6.13 \, \log_2)\) (Table 5-19). The titers ranged from 8 \((3 \, \log_2)\) to 512 \((9 \, \log_2)\).

Commercial broilers in treatment group eight had a preinfection ELISA titer at 28 days of age of 1,085 with a geometric mean titer of 671. The titers ranged from 158 to 4,390 with a percent coefficient of variation of 105. The mean and geometric mean HI titers were 83.2 \((6.38 \, \log_2)\) and 38.5 \((5.27 \, \log_2)\), respectively (Table 5-19). Hemagglutination-inhibition titers ranged from 8 \((2 \, \log_2)\) to 512 \((9 \, \log_2)\).

The birds in treatment group nine where shown to be negative for IgG NDV ELISA antibodies. This group’s mean ELISA titer was 117 with a geometric mean titer of 60. Titers ranged from 7 to 388 with a percent coefficient of variation of 109.2. The mean HI titer was measured to be 4.7 \((2.23 \, \log_2)\) with a geometric mean titer of 4.2 \((2.07 \, \log_2)\) (Table 5-19). The HI titers ranged from 1 \((2 \, \log_2)\) to 4 \((4 \, \log_2)\).
Results of preinfection ELISA titers revealed that the treatment groups had similar mean titers except for the group treated with the eye-drop commercial modified-live B1B1 NDV vaccine in combination with CVI988 subcutaneously and the recombinant vaccine in combination with CVI988 administered in ovo at Day 18 of incubation. Alternatively, in all vaccinated groups there were no differences in preinfection titer with the exception of the sham vaccinated control birds (Table 5-19).

Table 5-19: Trial 3: Preinfection geometric mean ELISA and HI titers

<table>
<thead>
<tr>
<th>Treatment Group / Route</th>
<th>n</th>
<th>Preinfection ELISA</th>
<th>Preinfection HI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: rHVT + CVI988 / SQ</td>
<td>15</td>
<td>1,096</td>
<td>6.33</td>
</tr>
<tr>
<td>2: rHVT + HVT/SB1 / SQ</td>
<td>15</td>
<td>68</td>
<td>3.54</td>
</tr>
<tr>
<td>3: rHVT / SQ</td>
<td>15</td>
<td>1,067</td>
<td>6.27</td>
</tr>
<tr>
<td>4: CVI988 + B1B1 / SQ/ED</td>
<td>15</td>
<td>560</td>
<td>7.73</td>
</tr>
<tr>
<td>5: rHVT + CVI988 / in ovo</td>
<td>15</td>
<td>1,155</td>
<td>6.13</td>
</tr>
<tr>
<td>6: rHVT + HVT/SB1 / in ovo</td>
<td>15</td>
<td>259</td>
<td>3.43</td>
</tr>
<tr>
<td>7: rHVT / in ovo</td>
<td>15</td>
<td>1,115</td>
<td>6.13</td>
</tr>
<tr>
<td>8: HVT/SB1 + B1B1 / SQ/ED</td>
<td>15</td>
<td>671</td>
<td>5.27</td>
</tr>
<tr>
<td>9: Sham / SQ/in ovo</td>
<td>15</td>
<td>60</td>
<td>2.07</td>
</tr>
</tbody>
</table>

HI titers are expressed as a reciprocal log2 of the geometric mean dilution.

Hemagglutination-inhibition preinfection titers were analyzed as the above ELISA preinfection samples were. The following treatment groups were found to have higher titers from the other groups; rHVT SQ, CVI988 + B1B1 SQ/ED, rHVT + CVI988 in ovo, and rHVT in ovo.

Postinfection Serology

Commercial broilers in treatment group one had an increase in the mean ELISA titer 10 days after challenge from 1,801 preinfection to 3,994 postinfection. The geometric mean titer showed a similar trend. The preinfection geometric mean titer was 1,096 and the postinfection mean had increased to 3,450. ELISA titers postinfection ranged from 702 to 9,735 with a percent coefficient of variation of 51.0. The HI mean titer increased from 115 (6.84 log2) to 315 (8.30 log2) while the geometric mean titer
increased from 80.6 (6.33 log$_2$) to 262 (8.03 log$_2$) (Table 5-20). The HI titers ranged from 32 (5 log$_2$) to 512 (9 log$_2$).

Commercial broilers in treatment group two showed a dramatic rise in titers 10 days after challenge. The postinfection ELISA mean titer was 10,182 and a geometric mean titer of 8,960. The ELISA titers ranged from 3,224 to 21,894 with a percent coefficient of variation of 47.9. The HI titers also showed a large increase after challenge. The HI mean titers were calculated to be 946 (9.89 log$_2$) with a geometric mean titer of 782 (9.61 log$_2$) (Table 5-20). Titers ranged from 128 (7 log$_2$) to 2,048 (11 log$_2$).

Birds in treatment group three had a moderate rise in ELISA mean and geometric mean titers. The mean titer increased from 1,893 to 5,009 while the geometric mean titer 10 days postinfection was 3,960 increased from 1,067 preinfection. The titers ranged from 816 to a maximum of 20,364 with a percent coefficient of variation of 74.9. The HI titers also showed a moderate increase 10 days postinfection. The mean and geometric mean HI titers postinfection were 634 (9.31 log$_2$) and 358 (8.48 log$_2$), respectively (Table 5-20). The HI titers ranged from 32 (5 log$_2$) to 2048 (11 log$_2$).

Treatment group four broilers were measured to have a mild increase in ELISA titers postinfection. The mean titer was 3,947 with a geometric mean titer of 2,838. Titers postinfection ranged from 184 to 13,676 with a percent coefficient of variation of 76.3. The HI titers measured postinfection had a mild decrease in mean titer. The preinfection titer was 282 (8.14 log$_2$) that declined to a postinfection titer of 204 (7.67 log$_2$). The preinfection geometric mean titer was calculated to be 213 (7.73 log$_2$) with a
decrease in postinfection geometric mean titer to 135 (7.08 log₂) (Table 5-20). The HI titers ranged from 16 (4 log₂) to 512 (9 log₂).

Commercial broilers in treatment group five had over two times greater mean titer 10 days after infection. The ELISA IgG NDV titers rose from a mean of 2,584 preinfection to 5,811 postinfection. The geometric mean titers followed the same trend rising from a preinfection (28 days of age) titer of 1,155 preinfection to 5,262 postinfection. The postinfection titers ranged from 1,643 to 12,385 with a percent coefficient of variation of 41.8. The HI titers also had a mean titer over twice that of the preinfection mean HI titer. Preinfection mean HI titer for this group was 134 (7.06 log₂) that had increased in 10 days following infection to a mean of 436 (8.77 log₂) with a geometric mean titer of 331 (8.37 log₂) (Table 5-20). The postinfection HI titers ranged from 64 (6 log₂) to 1024 (10 log₂).

Commercial broilers in treatment group six experienced a dramatic increase in both the ELISA and HI titers 10 days after challenge. The mean ELISA and mean HI titers rose from a preinfection titer of 1,116 and 21.2 (4.41 log₂), respectively to a postinfection mean titer of 6,521 and 228 (7.83 log₂), respectively. Geometric mean titers rose from and ELISA titer of 259 to 5,500; while HI geometric mean titers rose from 10.8 (3.43 log₂) to 173 (7.43 log₂) (Table 5-20). The ELISA titers ranged from a minimum of 1,643 to 18,820 with a percent coefficient of variation of 59.2. The HI titers had a minimum postinfection titer of 32 (5 log₂) and a maximum of 1024 (10 log₂).

Birds in treatment group seven experienced a large increase in both the ELISA and HI postinfection titers 10 days after challenge. The mean and geometric mean ELISA titers were measured to be 5,084 and 4,576, respectively. The titers ranged from a
minimum of 2,008 to a maximum of 13,199 with a percent coefficient of variation of 47.1. The mean HI titer was 580 (9.18 log₂) and geometric mean titer of 379 (8.57 log₂) (Table 5-20). The titers ranged from 32 (5 log₂) to 2048 (11 log₂).

Treatment group eight had a postinfection mean ELISA titer of 3,817 with a geometric mean titer of 2,105. The ELISA titers ranged from 296 to 27,257 with a percent coefficient of variation of 149.1. Comparatively, the mean HI titer was 191 (7.58 log₂) with a geometric mean titer of 94.1 (6.56 log₂) (Table 5-20). The HI titers ranged from 16 (4 log₂) to 2048 (11 log₂).

For the commercial broilers in group nine, sham vaccinated, Texas GB NDV infection was lethal and, therefore, no postinfection serology is available.

Table 5-20: Trial 3: Postinfection geometric mean ELISA and HI titers

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>n</th>
<th>Geometric mean ELISA titer</th>
<th>Geometric mean HI titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: rHVT + CVI988 SQ</td>
<td>29</td>
<td>3,450</td>
<td>8.03</td>
</tr>
<tr>
<td>2: rHVT + HVT/SB1 SQ</td>
<td>18</td>
<td>8,960</td>
<td>9.61</td>
</tr>
<tr>
<td>3: rHVT SQ</td>
<td>29</td>
<td>3,960</td>
<td>8.48</td>
</tr>
<tr>
<td>4: CVI988 + B1B1 SQ/ED</td>
<td>27</td>
<td>2,838</td>
<td>7.08</td>
</tr>
<tr>
<td>5: rHVT + CVI988 in ovo</td>
<td>27</td>
<td>5,262</td>
<td>8.37</td>
</tr>
<tr>
<td>6: rHVT + HVT/SB1 in ovo</td>
<td>23</td>
<td>5,500</td>
<td>7.43</td>
</tr>
<tr>
<td>7: rHVT in ovo</td>
<td>30</td>
<td>4,576</td>
<td>8.57</td>
</tr>
<tr>
<td>8: HVT/SB1 + B1B1 SQ/ED</td>
<td>27</td>
<td>2,105</td>
<td>6.56</td>
</tr>
</tbody>
</table>

HI titers are expressed as a reciprocal log₂ of the geometric mean dilution.

Postchallenge ELISA and HI IgG NDV titers were analyzed across vaccine groups that received the same vaccination but either in ovo at incubation Day 18 or subcutaneously at 1 day of age (Table 5-21). Birds receiving rHVT plus CVI988 in ovo had a higher ELISA titer than the birds receiving this vaccination combination subcutaneously at 1 day of age. The rHVT plus HVT/SB1 treatment group receiving the vaccine in ovo had a lower ELISA titer than those birds receiving the same vaccine at 1 day of age subcutaneously. The birds receiving the recombinant vaccine alone had nearly
the same mean titer when administered in ovo or subcutaneously at 1 day of age. Likewise, no difference was noted in the birds vaccinated with the commercial modified-live NDV vaccine regardless of combination with either HVT/SB1 or CVI988 MD vaccines (Table 5-20). A difference in HI postinfection titer was seen in the rHVT plus HVT/SB1 vaccinated birds. The subcutaneously vaccinated birds had a higher HI titer than those birds vaccinated in ovo at incubation Day 18. The remaining treatment groups, rHVT plus CVI988, rHVT, did not have a difference in postinfection titers when in ovo versus subcutaneous vaccination routes were compared. The birds receiving the commercial B1B1 plus either HVT/SB1 or CVI988 did not have a difference in postinfection HI titer (Table 5-22).

**Virus Isolation**

There were nine groups of broilers receiving various combinations of vaccines. Following is a list of these treatment groups and their corresponding treatments:

- Treatment group 1: rHVT and CVI-988 (Rismavac) subcutaneously at 1 day of age
- Treatment group 2: rHVT plus commercial HVT and SB1 (Marexine SB) subcutaneously at 1 day of age
- Treatment group 3: rHVT subcutaneously at 1 day of age
- Treatment group 4: CVI-988 (Rismavac) subcutaneously and commercial modified-live B1B1 type NDV (Bio-Cas B1) via the eye-drop route at 1 day of age
- Treatment group 5: rHVT and CVI-988 (Rismavac) in ovo at 18 days of incubation
- Treatment group 6: rHVT and commercial HVT and SB1 (Marexine SB) in ovo at 18 days of incubation,
- Treatment group 7: rHVT in ovo at 18 days of incubation
• Treatment group 8: commercial HVT and SB1 (Marexine SB) subcutaneously and modified-live B1B1 type NDV (Bio-Cas B1) via the eye-drop route at 1 day of age
• Treatment group 9: sham vaccinated in ovo at 18 days incubation and subcutaneously at 1 day of age

All treatment groups are referred to solely as their treatment group number henceforth.

Virus isolation was performed at termination (10 days postinfection) on all birds surviving eye-drop challenge by swabbing the trachea prior to humane euthanasia. The maximum sample size for eye-drop challenged birds was fifteen per treatment group. The other fifteen birds per treatment group were challenged intramuscular.

There were no birds found to be shedding Texas GB NDV 10 days postinfection in treatment groups 3, 4 or 8 (Figure 5-8, 5-9). Neurotropic velogenic Texas GB NDV challenge was lethal to all commercial broilers in group 9, sham vaccinated, and therefore there is no virus isolation data for this treatment group (Figure 5-8, 5-9).

Virus isolation yielded the recovery of hemagglutinating NDV from commercial broilers in treatment groups 1, 2, 5, 6, and 7 (Figure 5-8, 5-9). Treatment group one had two birds out of fifteen shedding NDV 10 days postinfection. One bird out of the eleven surviving Texas GB NDV challenge in treatment group 2 was found to be shedding virus at the time of termination of the trial, 38 days of age. Treatment group 5 had two out of thirteen birds shedding virus at the termination of the trial. One bird that died had to be euthanized for humane reasons as a result of splayed legs during the observation period. Treatment group six had the most birds shedding virus with four birds out of fourteen that survived challenge. Finally, treatment group 7 had two birds out of fifteen positive for virus isolation at the time of sample collection, 10 days postinfection (Figure 5-8, 5-9).
Table 5-21: Trial 3: Mean and geometric mean ELISA and HI titers pre and postinfection.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Vaccine</th>
<th>ELISA mean Pre – infection</th>
<th>ELISA geometric mean Pre – infection</th>
<th>ELISA geometric mean Post – infection</th>
<th>HI mean Pre – Infection</th>
<th>HI mean Post – infection</th>
<th>HI geometric mean Pre – infection</th>
<th>HI geometric mean Post – infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SQ rHVT + CVI988</td>
<td>1,625</td>
<td>1,096</td>
<td>3,450</td>
<td>6.84</td>
<td>8.30</td>
<td>6.33</td>
<td>8.03</td>
</tr>
<tr>
<td>2</td>
<td>SQ rHVT + HVT/SB1</td>
<td>760</td>
<td>68</td>
<td>8,960</td>
<td>4.94</td>
<td>9.89</td>
<td>3.54</td>
<td>9.61</td>
</tr>
<tr>
<td>3</td>
<td>SQ rHVT</td>
<td>2,013</td>
<td>1,067</td>
<td>3,960</td>
<td>7.14</td>
<td>9.31</td>
<td>6.27</td>
<td>8.48</td>
</tr>
<tr>
<td>4</td>
<td>SQ CVI988 &amp; ED NDV B1B1</td>
<td>2,168</td>
<td>560</td>
<td>2,838</td>
<td>8.14</td>
<td>7.67</td>
<td>7.73</td>
<td>7.08</td>
</tr>
<tr>
<td>5</td>
<td>In ovo rHVT + CVI988</td>
<td>2,975</td>
<td>1,155</td>
<td>5,262</td>
<td>7.09</td>
<td>8.77</td>
<td>6.13</td>
<td>8.37</td>
</tr>
<tr>
<td>6</td>
<td>In ovo rHVT + HVT/SB1</td>
<td>1,660</td>
<td>259</td>
<td>5,500</td>
<td>4.41</td>
<td>7.83</td>
<td>3.43</td>
<td>7.43</td>
</tr>
<tr>
<td>7</td>
<td>In ovo rHVT</td>
<td>1,319</td>
<td>1,115</td>
<td>4,576</td>
<td>7.24</td>
<td>9.18</td>
<td>6.13</td>
<td>8.57</td>
</tr>
<tr>
<td>8</td>
<td>SQ HVT/SB1 &amp; ED NDV B1B1</td>
<td>1,033</td>
<td>671</td>
<td>2,105</td>
<td>6.38</td>
<td>7.58</td>
<td>5.27</td>
<td>6.56</td>
</tr>
<tr>
<td>9</td>
<td>Sham</td>
<td>117</td>
<td>NA</td>
<td>60</td>
<td>2.23</td>
<td>NA</td>
<td>2.07</td>
<td>NA</td>
</tr>
</tbody>
</table>

HI titers expressed as reciprocal log2 of the geometric mean dilution.
Table 5-22: Trial 3: Comparison of route of vaccination, in ovo versus subcutaneous, and postinfection geometric mean ELISA and HI titers

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Sample Size (SQ/in ovo)</th>
<th>ELISA geometric mean titer postinfection</th>
<th>HI geometric mean titer Postinfection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Subcutaneous a</td>
<td>In ovo a</td>
</tr>
<tr>
<td>rHVT + CVI988</td>
<td>29/27</td>
<td>3,450</td>
<td>5,262</td>
</tr>
<tr>
<td>rHVT + HVT/SB1</td>
<td>18/23</td>
<td>8,960</td>
<td>5,500</td>
</tr>
<tr>
<td>rHVT</td>
<td>29/30</td>
<td>3,960</td>
<td>4,576</td>
</tr>
<tr>
<td>B1B1 + CVI988</td>
<td>27</td>
<td>2,838</td>
<td>N/A</td>
</tr>
<tr>
<td>B1B1 + HVT/SB1</td>
<td>27</td>
<td>2,105</td>
<td>N/A</td>
</tr>
</tbody>
</table>

a = Route of vaccination
b = HI values are expressed as reciprocals log2 of the geometric mean dilution.

**Percent Protection**

Final percent protection was calculated by subtracting the total number of birds with a score of two out of five or greater at the time of termination (W), total number of birds dying in both the eye-drop (X) and the intramuscular (Y) challenged groups in addition to subtracting the number of birds positive on virus isolation (Z) for that treatment group from thirty, the total number of birds for each treatment group; divided by the total number of birds for that treatment group which was thirty in all cases.

Formula for Percent (%) Protection = \( \left\{ \frac{30 - (X + Y + Z)}{30} \right\} \times 100 \)

Treatment group 1 had a percent protection calculated to be 93.33%. All birds survived challenge but two birds challenged via the eye-drop route were found to be positive on virus isolation (Figures 5-8, 5-9).

Treatment group two had much higher mortality. This group had eight intramuscular birds die and one bird scoring four out of five at termination. Additionally, this group had four eye-drop challenged birds succumb to challenge and three birds
having a clinical score of two or greater out of five. This brings the total mortality for
this group to twelve. This group also had one bird positive for virus isolation. Therefore,
the total number of birds affected by Texas GB NDV challenge at the time of termination
to 17 out of 30 for a percent protection of 43.33% (Figures 5-8, 5-9).

Treatment group three did not have any birds positive for virus isolation. This
group did however, have one bird in the eye-drop challenged group die 7 days after
challenge. This bird was likely a vaccine miss and thus is not counted when calculating
percent protection. Therefore, the percent protection for this group was calculated to be
100% (Figures 5-8, 5-9).

Commercial broilers in treatment group four were completely protected from any
signs of morbidity, mortality and viral shedding at 38 days of age when this trial was
terminated (Figures 5-8, 5-9).

Treatment group five experienced mild mortality. Group five had one bird each die
in the eye-drop and intramuscular challenged groups. It also had two birds positive for
virus on tracheal swabbing at termination. This brings the total number of birds affected
by Texas GB NDV challenge to four out of twenty-nine. One bird in the eye-drop
challenged group had to be humanely euthanized 5 days after challenge due to an
arthropathy. This resulted in a percent protection of 86.21% (Figures 5-8, 5-9).

Commercial broilers in treatment group six experienced moderate mortality. Five
birds in the intramuscular challenged group and one bird in the eye-drop challenged birds
succumbed to in Texas GB NDV challenge. In all, treatment group six lost six birds to
challenge. Additionally, four of the eye-drop challenged birds were positive on virus
isolation at termination. This brings the total number of birds susceptible to Texas GB
NDV infection to ten out of thirty. The percent protection for this group was 67% (Figure 5-8, 5-9).

Treatment group seven was completely protected from morbidity and mortality for the duration of this trial. However, it was not completely protected from viral replication in the trachea 10 days postinfection. Two birds were found to be positive for virus on their tracheal swabs obtained at termination. The final percent protection for this group was calculated to be 93% (Figures 5-8, 5-9).

Neuortropic velogenic, Texas GB strain of NDV was lethal in treatment group 9, sham vaccinated commercial broilers. Therefore, this group had a percent protection of 0% (Figures 5-8, 5-9).

Figure 5-8 represents percent protection. Those bars in the graph that have a different letter from the other bars are statistically significant using the Chi-square test at a confidence interval of 95% (50, 130).

**Gross Lesions**

All birds dying during the 10 day observation period postinfection were necropsied along with all birds at termination of the trial. Following are descriptions of gross lesions observed at the time of necropsy. Some birds did not have any appreciable gross lesions at the time of necropsy. Throughout the trial, there was no correlation between the incidence of birds with no gross lesions and route of challenge.

The primary lesion seen in the commercial broilers of treatment group one was cecal tonsil hemorrhage (CTH). Cecal tonsil hemorrhages were observed as the sole lesion in twenty-three of the thirty birds in this treatment group. The remaining seven birds did not have any appreciable gross lesions (Table 5-23).
Figure 5-8: Trial 3: Percent protection and percent virus isolation positive against Texas GB NDV challenge
Figure 5-9: Trial 3: Total number of birds positive on virus isolation and total mortality
Treatment group two had sixteen birds with gross lesions consistent with cecal tonsil hemorrhage and twelve with no gross lesions out of the thirty in this treatment group. Two male birds, one each from the eye-drop and intramuscular challenged groups, had gross signs of proventricular hemorrhage. This hemorrhage was within the parenchyma just distal to the last row of gastric glands and proximal to the gizzard. The intramuscular challenged bird died 4 days after infection while the eye-drop challenged bird died 9 days after challenge (Table 5-23).

The primary gross lesion observed in treatment group 3 was cecal tonsil hemorrhages. This lesion was observed in twenty-six out of the thirty birds. Four birds in this treatment group had no detectable gross lesions. One female bird challenged by the eye-drop route was observed to have both cecal tonsil hemorrhages and proventricular hemorrhages (Table 5-23).

The majority, seventeen out of twenty seven birds, in treatment group four had lesions consistent with that of cecal tonsil hemorrhage. The remaining ten birds had no gross signs of pathology at the time of necropsy (Table 5-23).

Nearly all the commercial broilers in treatment group five had gross lesions of cecal tonsil hemorrhages. Cecal tonsil hemorrhages were seen in twenty-six of the thirty birds in this group. Two birds had no detectable gross signs of pathology at necropsy. One male bird in the eye-drop challenge group that died 5 days after challenge had proventricular hemorrhages (Table 5-23). One bird in this group had to be humanely euthanized 5 days after infection due to an arthropathy.

The primary lesion observed in treatment group six was cecal tonsil hemorrhage. Cecal tonsil hemorrhage was seen in 22 of the 30 birds in this treatment group. One of
the birds with cecal tonsil hemorrhage also had a large caseous plug overlying the region
of the cecal tonsils. Six of the birds in this group had no gross lesions at necropsy. Two
female birds from the intramuscular challenged group had proventricular hemorrhages
(Table 5-23) and they died on Day 6 and 9 postinfection. One 36 day old male bird died
of ascites 8 days after challenge.

Cecal tonsil hemorrhages were observed in twenty-eight of the thirty birds in
treatment group seven. The remaining two birds had gross pathology at necropsy (Table
5-23).

Treatment group eight had twenty birds with cecal tonsil hemorrhages and ten with
no gross lesions out of the thirty commercial broilers in this group (Table 5-23). One of
the birds with cecal tonsil hemorrhages was also observed as having proventriculosis or
proventricular dilatation disease. This bird was clinically normal throughout the duration
of trial.

The majority of the birds in treatment group 9 had no gross signs of pathology at
necropsy. Nineteen out of thirty birds had no gross lesions. Time after challenge was not
a factor in the development of lesions. The absence of pathologic change was noted as
late as 6 days after challenge. Only two birds were remaining 6 days postinfection.
Cecal tonsil hemorrhages were noted as early as 4 days postinfection. Cecal tonsil
hemorrhages were seen in a total of seven birds out of thirty for treatment group nine.
Treatment group nine had four of the thirty birds present with proventricular hemorrhages
as their sole lesion. This lesion was observed in three males. Two of these four birds
were males from the intramuscular challenged group and died 4 days after infection. The
remaining two birds with proventricular hemorrhage were from the eye-drop challenged
group (Table 5-23). One bird was a male and had succumbed to infection 6 days after
challenge while the other bird was a female and had died by the 7th day after challenge.

Examination of the data reveals that cecal tonsil hemorrhages were by far the most
prominent lesion in this trial. Cecal tonsil hemorrhages were seen in 185 out of 267 birds
on trial for a percentage of 69.3%. No appreciable signs of pathology were detected in
27.0%, or 72 out of 267, of the commercial broilers (Table 5-23).

Proventricular hemorrhages were seen in ten out of the 267 for an incidence of
3.75%. This lesion occurred only in those birds dying prior to the end of the 10 day,
postinfection observation period. Proventricular hemorrhages were seen anywhere from
4 days postinfection to 9 days postinfection. When the incidence of proventricular
hemorrhages was analyzed according to sex and challenge route predilection, it was
observed that a commercial broiler is not at an increased risk of developing
proventricular hemorrhages whether it is male or female or challenged intramuscular or
by the eye-drop route. There were five birds in the eye-drop and five birds in the
intramuscular challenged groups that developed proventricular hemorrhages.
Proventricular hemorrhages were seen in six male and four female commercial broilers.
Only two of the birds with proventricular hemorrhages also had concomitant cecal tonsil
hemorrhages. It should be noted that treatment groups 1, 4, 7, and 8 did not have any
birds with proventricular hemorrhages (Table 5-23). This corresponds to the groups that
had no signs of morbidity or mortality throughout the postinfection period.
Table 5.23: Trial 3: Gross necropsy findings

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Cecal tonsil hemorrhage</th>
<th>No gross lesions</th>
<th>Proventricular hemorrhage</th>
</tr>
</thead>
<tbody>
<tr>
<td>rHVT + CVI988 SQ</td>
<td>23/30</td>
<td>7/30</td>
<td>-</td>
</tr>
<tr>
<td>rHVT + HVT/SB1 SQ</td>
<td>16/30</td>
<td>12/30</td>
<td>2/30</td>
</tr>
<tr>
<td>rHVT</td>
<td>25/30</td>
<td>4/30</td>
<td>1/30</td>
</tr>
<tr>
<td>CVI988 + B1B1 SQ/ED</td>
<td>17/27</td>
<td>10/27</td>
<td>-</td>
</tr>
<tr>
<td>rHVT + CVI988 in ovo</td>
<td>27/30</td>
<td>2/30</td>
<td>1/30</td>
</tr>
<tr>
<td>rHVT + HVT/SB1 in ovo</td>
<td>22/30</td>
<td>6/30</td>
<td>2/30</td>
</tr>
<tr>
<td>rHVT in ovo</td>
<td>27/30</td>
<td>3/30</td>
<td>-</td>
</tr>
<tr>
<td>HVT/SB1 + B1B1 SQ/ED</td>
<td>20/30</td>
<td>10/30</td>
<td>-</td>
</tr>
<tr>
<td>Sham</td>
<td>7/30</td>
<td>19/30</td>
<td>4/30</td>
</tr>
<tr>
<td>Total percent of birds challenged</td>
<td>69%</td>
<td>27%</td>
<td>4%</td>
</tr>
</tbody>
</table>
Newcastle disease has plagued the poultry industry for over 75 years. Numerous modified-live and killed NDV vaccines exist for vaccination and protection against NDV. However, vaccine manufacturing companies constantly strive to produce a modified-live vaccine that is able to elicit a strong and rapid onset of protection without causing excessive vaccine reactions (1, 21). Vaccine companies have also been conducting trials using recombinant vaccines. Recombinant vaccines have the advantage of using an apathogenic viral vector carrying foreign genes to highly virulent strains of diseases (72). Many poultry integrators give up to four modified-live NDV vaccines during the grow-out of meat-type chickens and turkeys. Breeder chickens and commercial chickens used for the production of table eggs receive even additional modified-live and killed NDV vaccines. This is necessary due to the limited duration of immunity in vaccinated birds (71, 75, 142). In the future, vaccine manufacturers would like to develop vaccines whereby the chicken eggs are vaccinated at 18 days incubation, in ovo, or chicks at 1 day of age and a life-long immunity is established with only one vaccination. A recombinant herpes virus of turkeys vector would seem most appropriate for establishing life long immunity due to HVT’s ability to become latent (72) and later recrudesce. Herpes virus of turkeys replication with subsequent antigenic stimulation towards those foreign genes inserted on the HVT vector would permit long term protection.
Trial 1: Chicken and Turkey Infection with Texas GB Strain of Newcastle Disease Virus

The purpose of Trial 1 was to characterize the pathogenicity of Texas GB, a neurotropic velogenic strain of NDV, in SPF Leghorns, commercial Leghorns, broilers, and turkeys using the eye-drop or intramuscular inoculation routes of challenge administration.

Texas GB NDV pathogenicity was characterized in SPF Leghorns. The affect of route of administration, eye-drop or intramuscular route, on severity of clinical signs, onset of clinical signs, and total mortality was determined in commercial Leghorns, broilers and turkeys. All sham infected birds remained clinically normal and seronegative for circulating IgG NDV antibodies throughout the duration of the trial. The BSL-II biologic isolators used in this study were placed in a single room so that sham controls and Texas GB NDV infected birds resided next to each other within the same room for the duration of the trial. These data demonstrated that the BSL-II isolators functioned properly and housing of sham controls in a separate building from the infected birds would not be necessary for future studies.

Sample size for trial 1 consisted of twenty birds each for the SPF Leghorn, commercial Leghorn and turkey sham and intramuscular challenge groups, and twenty-two birds for the eye-drop challenged groups. One exception occurred in the turkey sham group. Only nineteen birds were used in this group because one bird developed arthropathy 2 days prior to challenge and was humanely euthanized. The commercial broiler groups had eighteen birds for the sham and intramuscular groups, and twenty birds for the eye-drop groups. Two fewer birds per group were used due to space
available for the isolator floor, feeder and drinkers at 56 days of age by termination of the trial.

Average days to onset of morbidity in the intramuscular challenged birds differed to some degree for all four groups of birds but no difference was noted among the three eye-drop infected chicken groups (Table 5-3). However, the incidence of mortality was the same for all three experimental groups (Table 5-5). Onset of clinical morbidity was delayed in the intramuscular infected turkeys as compared to the three chicken groups. Other investigators have also documented the turkeys’ natural resistance to NDV (26, 57, 148). Average days to onset of morbidity in groups infected by the eye-drop route versus the intramuscular route demonstrated that the intramuscular route of infection is more acute, resulting in morbidity and mortality sooner than the eye-drop infection route (Table 5-3, 5-4, 5-5). However, mortality resulted regardless of infection route (Table 5-5). More time was required for the eye-drop infected groups to reach their final mortality percentage but this percentage was not different from that seen with the intramuscular infected groups. These results are significant because the intramuscular route of infection allows delivery of the intended infection dose more consistently. However, from a commercial poultry industry standpoint, birds in the poultry house are not naturally infected by the intramuscular route. The eye-drop route of infection simulates the natural route of exposure of the chicken or turkey in the poultry house. Conversely, from a scientific standpoint the eye-drop route of infection does not allow for exacting delivery of infective virus to each bird. Reasons for variability in infection dose received by each bird when administered by the eye-drop route include:
• Birds shaking their heads after being released and slinging some of the virus off the cornea of the eye.

• Blinking the infective dose in and drainage through the choanae to the oropharynx delivers a varying proportion of the virus to the esophagus where it is swallowed and potentially destroyed by the acid environment of the crop (pH 4.5), proventriculus (pH 4.8), or gizzard (pH 2.5) (54, 104).

• Some of the virus dose drains into the trachea, the intended site of delivery.

One mean of ensuring the entire dose is delivered to the tracheal epithelium is tracheal intubation and instilling a small volume of infective fluid directly into the trachea. This is a time consuming and stressful means of delivery of the infective viral dose to each bird. Additionally, it does not simulate the natural route of exposure as efficiently as the eye-drop route. The eye-drop route of exposure inherently has a great deal of variability and room for error. Every possible effort was made to ensure that each bird received a complete dose of challenge virus. Onset of morbidity and mortality occurred consistently at the same time following infection in each of the subsequent trials refuting criticism that the eye-drop route of infection is not reliable. This route of infection is able to produce repeatable results when care is taken to ensure each bird takes in the entire dose before being released.

Commercial turkeys began showing signs of morbidity later than any of the chicken groups (Table 5-3). Unlike the chicken groups, no difference in average onset of morbidity was seen between the intramuscular and eye-drop infected turkeys. However, a statistical difference ($p \leq 0.05$) in final mortality was observed between the eye-drop and intramuscular challenged birds (Table 5-5). The percent mortality in the eye-drop infected turkeys was 45% versus 65% in the intramuscular infected turkeys.

The United States Department of Agriculture requires only scores of live or dead when working with Texas GB NDV. In this and subsequent trials, the scoring system
was presented in greater detail. Each bird on trial was scored at approximately the same
time each day during the 14 day postinfection phase of the trial for signs of disease on a
scale of 0, normal to 5, dead. These data were collected to establish a baseline of clinical
signs and average daily scores that would be used in subsequent vaccine trials so that
morbidity and subsequent recovery in vaccine trials could be noted. With the exception
of two commercial turkeys that showed very mild signs of morbidity, recovery during
Trial 1 was not noted. Once clinical signs of morbidity were noted, rapid progression of
clinical signs occurred which often culminated in death. These data were also collected
because under controlled research conditions where only mild or moderate signs of
morbidity may falsely suggest an increased efficacy of a vaccine that when placed under
field conditions in the hostile environment of the poultry house fail to prevent mortality.
Stressors present in the poultry house may exacerbate signs of morbidity and examples
include IBDV, Chick Anemia Agent, MD, ammonia, feed and drinker space, poor feed
ingredient quality, environmental temperature, dust, opportunistic bacteria and many
others.

Birds for this trial were grown to 6 weeks of age prior to infection to ensure that
residual maternal antibodies were not present. Maternal antibodies decline to levels
below that which are protective typically by 3.5 weeks of age (Personal communication,
G. D. Butcher, August 21, 1998). Serologic results demonstrated that all birds on trial
were negative at the time of challenge for detectable levels of pre-formed IgG NDV
antibodies. A few birds had titers greater than 1,000. However, these samples were run
again and found to be much lower. Two possible causes for this falsely elevated titer are
technician error in pipetting or washing too vigorously, spilling some sample from one
well into another, or leaving residual drops on the top surface of the plate that will reflect light and falsely elevate the optical density in that well which is used to calculate the sample titer.

Serologic response to NDV challenge was measured using an ELISA system (IDEXX, Westbrook, ME). High titers greater than 20,000, were routinely measured in the few surviving birds 14 days after infection. Some birds’ samples had optical densities that were higher than that readable by the Molecular Devices, E-max reader (IDEXX, Westbrook, ME). A significant IgG immunologic response is not surprising in the naive bird. However, to reach such high titers on primary exposure in only 14 days is intriguing. Typically, it takes 7 days for birds to produce antibody titers that are detectable by today’s automated test kits. Maximum titer after primary exposure is not reached until approximately 24 days after exposure. To have titers greater than 20,000 at 14 days after primary exposure demonstrates NDV’s invasive nature and ability to infect a wide range of host cells (91, 144).

Administration of NDV to mucosal surfaces, such as the tracheal epithelium, is thought to primarily elicit an IgA immune response. Results of this trial showed that NDV is able to gain access to the systemic circulation and induce a strong IgG immune response as well. This was evidenced by the high ELISA titer seen in the eye-drop infected birds. In fact, the eye-drop infected birds had similar mean ELISA titers as that of the intramuscular infected birds.

The primary lesion found in birds on necropsy was cecal tonsil hemorrhage (Figures 5-4, 5-6). This finding was as described by King (Personal Communication, December 2, 1998). The cecal tonsil is an aggregate of lymphoid tissue (60). In
commercial poultry this area of the distal intestine is often found to be mildly hemorrhagic. This does not suggest that these birds have NDV, rather that immune stimulation is occurring in this region most likely due to an irritant (60). In this trial, the sham controls had no gross lesions but many of the NDV infected birds did have cecal tonsil hemorrhages (Figures 5-4, 5-6). Due to this finding in the controlled research setting, it is concluded that the hemorrhage in the cecal tonsils was due to NDV infection. The SPF Leghorns died more rapidly following infection than the other groups of birds. Cecal tonsil hemorrhage was seen less frequently SPF Leghorns than in the other birds succumbing to NDV infection later. This finding is most likely due to the acuteness of the disease process in the SPF Leghorns, thus less time to cause gross pathologic changes.

Mild proventricular hemorrhages were occasionally noted. This is typically a finding in birds infected with the viscerotropic velogenic strains of NDV (14). Contamination of the challenge strain with viscerotropic velogenic NDV is not suspected. However, this is most likely due to NDV’s ability to infect a wide range of host cells (91, 144) and lyse RBCs as a result of action of the fusion surface protein (126).

Once this initial trial was complete and the pathogenesis of Texas GB NDV was established in commercial Leghorns, broilers, and turkeys Trial 2 was undertaken. Trial 1 provided the background information (characterization) of Texas GB NDV so that this challenge strain could be studied in commercial broilers and turkeys receiving the rHVT vaccine.
Trial 2: Commercial Broiler and Commercial Turkey rHVT Vaccine, Texas GB Strain of Newcastle Disease Virus Challenge

This trial was conducted to determine the efficacy of an rHVT vector vaccine expressing two genes for NDV, HN and F genes, and two genes for MDV, gps A and B genes, in SPF Leghorns and commercial broilers and turkeys possessing maternal antibodies at hatch. The recombinant vaccine was administered by in ovo vaccination at 18 days incubation or following hatch, 1 day of age by the subcutaneous route. Groups vaccinated with a commercially available modified-live B1B1 strain of NDV at 1 day of age were included for comparison purposes.

Vaccination at 1 day of age with the commercial, modified-live B1B1 NDV vaccine caused a mild vaccine reaction consisting of snicks and sneezes in broilers at 7 days of age. The vaccine reaction was detected for 4 days. The commercial turkeys vaccinated at 21 days of age with the modified-live B1B1 NDV vaccine also had a vaccine reaction consisting of mild snicking and sneezing beginning 6 days after the initial vaccination and lasting for 8 days. Vaccine reactions are one of the disadvantages of modified-live vaccines (14). Conversely, no detectable vaccine reaction was seen in any of the groups receiving the rHVT vectored vaccine containing the HN and F genes to NDV along with gps A and B to MDV. This is a classic example of eliminating unwanted vaccine reactions after vaccination with NDV products. Since the recombinant vaccine does not contain whole NDV, there is no intact virus to replicate in the trachea and cause epithelial damage and subsequent inflammation leading to a vaccine reaction.

Morbidity and mortality seen in the sham controls in this trial, on average, lagged 1 day behind that reported in Trial 1 (Table 5-10). Birds in this trial are only 8 days older
than those in Trial 1. In order to determine if this 1 day difference is significant the sample size would have to be significantly increased.

Morbidity seen in the vaccinated birds was primarily from the commercial, modified-live B1B1 NDV vaccinated birds and the broilers vaccinated subcutaneously at 1 day of age with the recombinant vaccine. With the exception of two birds in the intramuscular infected broilers vaccinated subcutaneously with the recombinant vaccine, all the morbidity was mild, scored as 1/5. Regardless of the treatment group, all birds showing clinical signs recovered within a few days. This level of morbidity is likely often to go undetected in the commercial chicken house. Conversely, this level of morbidity may become appreciable in the commercial poultry house depending on the number and intensity of stressors present in the house. This study was aimed at taking the clinical scoring one step further in an attempt to detect subtleties. However, when scoring these birds as required by the USDA for vaccine approval, all vaccines regardless of route of vaccination would have passed at a level of 100% protection.

Analysis of preinfection titers across groups demonstrated a difference in titers of the birds vaccinated with the recombinant vaccine and the birds vaccinated with the commercial, modified-live B1B1 NDV vaccine. This is expected since whole live virus is present in the modified-live vaccine and only genes for the HN and F proteins of NDV on the recombinant construct. Additionally, no difference in preinfection titer was seen between the un-vaccinated sham control birds and those birds vaccinated with the recombinant vaccine. This can be viewed as a disadvantage or as an advantage. These levels of antibodies were correlated with protection against a lethal dose of Texas GB NDV virus. This suggests that the recombinant vaccine does not produce high levels of
detectable NDV IgG antibodies, suggesting other means of protection such as the cell-mediated immune response augmented protection (119). An advantage of low levels of detectable NDV IgG antibodies being produced aids in discerning whether the antibody titer seen in a commercial flock is due to vaccination with the recombinant vaccine or a result of a field infection when no modified-live vaccines have been used.

Postinfection NDV ELISA titers demonstrated no difference between the vaccinated groups regardless of whether the birds were vaccinated with the recombinant vaccine or the commercial vaccine. This signifies that each group mounted similar immune responses to infection. However, preinfection versus postinfection titer differences were significant for the commercial broilers vaccinated with the recombinant vaccine either in ovo or subcutaneously at 1 day of age. This suggests that the commercial broilers vaccinated with the recombinant vaccine were not able to neutralize the challenge virus and eliminate it from the host as rapidly as the SPF Leghorns or the broilers vaccinated with the commercial modified-live B1B1 NDV vaccine. All birds vaccinated with either the recombinant vaccine or the commercial vaccine were completely protected from mortality.

The primary gross lesion seen on post-mortem examination was cecal tonsil hemorrhage. The eye-drop and intramuscular infected groups had nearly the same percentage of birds with cecal tonsil hemorrhage. This demonstrates NDV’s ability to invade mucosal epithelium, in the case of eye-drop infected birds, and gain access to the systemic circulation. Cecal tonsil hemorrhages were also seen in naïve birds after challenge in Trial 1. This suggests that NDV replication and invasion of distant tissues is
not prevented by either the rHVT vaccine or the commercial, modified-live B1B1 vaccine.

Mortality for the sham vaccinated and intramuscular infected SPF Leghorns was 100% for the turkey phase of Trial 2. This confirms that live, lethal NDV was administered to the birds. The sham vaccinated, intramuscular infected commercial turkeys had only two out of eight birds become moribund, one of which completely recovered. Likewise, the sham vaccinated and eye-drop infected commercial turkeys had only one out of 12 birds with moderate signs of morbidity during the trial. In summary, only three out of twenty sham control turkeys became moribund. It is difficult to evaluate the efficacy of a novel vaccine when the sham controls do not become moribund or die. However, analysis of the postinfection NDV ELISA titers demonstrates that the birds did receive infective Texas GB NDV sufficient to stimulate production of high NDV antibody titers in the sham controls. The recombinant and commercial vaccines both protected 100% of the birds infected with Texas GB NDV from any signs of morbidity and mortality. The lack of morbidity in the sham control turkeys was not a surprise (26, 57, 148).

The commercial modified-live B1B1 NDV vaccine produced higher preinfection IgG NDV titers than that of the sham controls or the recombinant vaccinated commercial turkeys. The recombinant vaccine produced minimal NDV ELISA titers. Titers in the rHVT vaccinated turkeys were similar to the sham control turkeys. This same phenomenon occurred in the commercial broilers in the broiler phase of this trial. A possible reason that low levels of NDV ELISA titers were present is due to slow replication of the HVT vector recombinant vaccine virus. Possibly this could be
explained due to slow vaccine virus replication because the inserted genes are not near a effective promoter to initiate transcription and subsequent translation of the inserted genes. Another possible cause for slow replication is that the four inserted genes, two to NDV and two to MDV, have exceeded the carrying capacity of the HVT vector, or the molecular manipulation of the HVT vector has slowed its replication by a yet to be determined means.

Newcastle disease virus antibody ELISA titers suggestive of protection were not detected by serologic means. The vaccine was still able to stimulate protection from a clinical standpoint. The sham controls had a mean postinfection titer of 21,103, 14 days after infection. The commercial and recombinant vaccinated control chickens had much lower postinfection titers, suggesting that both the commercial and the recombinant vaccines are capable of producing neutralizing antibodies and eliminating the virus from the host before the NDV is able to establish infection and stimulate high titer levels in the host. Therefore, it is concluded that cell-mediated immunity (119) or other immune mechanisms are elicited by the recombinant vaccine.

The results of this trial proved that the rHVT construct carrying HN and F genes to NDV and gps A and B to MDV is capable of eliciting adequate protection to Texas GB NDV challenge when administered in ovo or subcutaneously at 1 day of age and challenge by either the IM or ED routes in commercial broilers and turkeys. This conclusion is similar to the results reported by Morgan et al. (103) using rHVT vaccines with only the F gene to NDV in SPF Leghorns. In this study, greater than 90% of the birds challenged intramuscularly at 28 days of age were protected against signs of systemic illness from Texas GB NDV challenge. Conversely, Morgan et al. (103)
showed that an rHVT vaccine carrying the HN gene to NDV was ineffective at providing protection (35% to 58%) to Texas GB NDV challenge in SPF Leghorns. Therefore, Morgan et al. (103) combined the two constructs (rHVT + F gene to NDV and rHVT + HN gene to NDV) into one vaccination and demonstrated complete protection against Texas GB NDV challenge. This is similar to Trial 2. However, in Trial 2 the same HVT vector was carrying both HN and F genes to NDV instead of each gene on its own vector. Other studies have shown that the F gene inserted into the vaccinia (96) or fowl pox (29) viruses elicit protection against NDV challenge. Heckert et al. (68) also demonstrated that an rHVT vector carrying the HN and F genes to NDV and gps A and B to MDV could elicit complete protection in SPF Leghorns 14 days after vaccination. Morgan et al. (102) found that complete protection in SPF Leghorns receiving an rHVT vaccine carrying the F gene of NDV did provide complete protection until 21 days after vaccination. Trial 2 of this work was the first account of an rHVT vaccine (rHVT + HN and F of NDV and gps A and B of MDV) being administered and providing complete protection against Texas GB NDV challenge in commercial broilers and turkeys.

**Trial 3: Commercial Broiler rHVT plus CVI988 and/or HVT/SB1 Vaccine, Texas GB Newcastle Disease Virus Challenge**

The purpose of this trial was to determine the efficacy of an rHVT (rHVT + HN and F genes to NDV and gps A and B of MDV) vectored vaccine when combined with conventional HVT/SB1 or CVI988 (Rispens) strains of MDV commercially available vaccines in commercial broilers with NDV and MDV maternal antibodies. The recombinant vaccine and commercial vaccines were administered in various combinations in embryonating eggs at 18 days incubation (in ovo) or in 1 day of age chicks by subcutaneous vaccination. Two groups received commercially available
modified-live B1B1 strain of NDV vaccine by the eye-drop route at 1 day of age, in addition to subcutaneous vaccination with commercial MDV vaccines for comparison.

This trial investigated the ability of the rHVT vectored vaccine’s ability to protect when combined with HVT/SB1 of CVI988 live MDV vaccines. As described earlier in Trial 2, the recombinant vaccine provides as good or better protection to Texas GB NDV challenge as compared to a commercial modified-live B1B1 NDV vaccine. This third study was conducted because of the recombinant construct’s inability to produce early protection to virulent MDV challenge when administered alone to chicks and challenged at less than 2 weeks of age (unpublished data). Heckert et al. (68) showed that a similar rHVT (carrying the HN and F genes to NDV and gps A and B to MDV) did not elicit a protective immune response in SPF Leghorns against Texas GB NDV challenge until 14 days after vaccination demonstrating rHVT’s slow onset of protection. Morgan et al. (102) also showed rHVT’s slow onset of protection. In the study by Morgan et al. (102) complete protection by an rHVT carrying the F gene of NDV was not elicited until 21 days after infection. To increase protection to virulent MDV when challenged early in life, commercial live HVT/SB1 or CVI988 commercial MDV vaccines were added to the in ovo or subcutaneous vaccination with the recombinant construct. In order to evaluate if interference occurred when the recombinant construct is mixed with HVT/SB1 or CVI988, the birds were infected with the Texas GB strain of NDV. In this case, the sole ND protection would result from the HVT recombinant construct with the exception of those birds vaccinated with the commercial modified-live B1B1 NDV vaccine and HVT/SB1 or CVI988. In order for this recombinant construct to be useful in the poultry vaccine market, it must be able to protect against NDV infection when combined with
MDV vaccines since it is unable to elicit early immunity to virulent MDV infection alone.

Broiler group three (rHVT SQ at 1 day of age) had one bird in the eye-drop infected group show mild signs of morbidity and subsequently die. The onset of clinical signs and time to death are similar to that observed in unvaccinated, sham controls. It is suggested that this bird either was missed at vaccination, received only a partial dose of vaccine or failed to respond to vaccination. Likewise, broiler group five had one bird in the intramuscular and one bird in the eye-drop infected groups begin to show signs of morbidity and later succumb to infection at exactly the same time as the unvaccinated, sham controls became ill and died. Therefore, these three birds were recorded as experimental error and removed from percent protection calculations. The birds were vaccinated and challenged in their respective isolation units using large and thick rubber gloves.

Therefore, no clinical morbidity was observed in any of the commercial, modified-live B1B1 NDV vaccinated birds, the birds vaccinated in ovo with the recombinant vaccine alone, or the birds in the treatment group receiving the recombinant vaccine and live MDV CVI988 vaccine at incubation Day 18 or subcutaneously at 1 day of age. Thus, it is concluded that the recombinant vaccine administered alone or in combination with CVI988 protects as well or better than the commercial modified-live NDV vaccine. Additionally, no significant levels of interference were present with the recombinant HVT vector and the serotype-1 MDV, CVI988. However, the CVI988 vaccine strain is attenuated but can be oncogenic if not properly attenuated, handled, and administered
Additionally, CVI988 is not commonly used for vaccination against MDV except in those areas of the world where very virulent MDV is endemic.

Significant interference with the rHVT construct and the commercial, live HVT/SB1 MDV vaccines was observed. The in ovo vaccinated birds were protected better than the subcutaneous vaccinated birds. However, both vaccination routes with the rHVT and HVT/SB1 provided sub-standard levels of protection. These data are intriguing because 28 or 31 days had elapsed prior to challenge, allowing the birds sufficient time to develop immunity to NDV. Heckert et al. (68) and Morgan et al. (102) demonstrated slow development of protection in birds vaccinated with an rHVT containing the HN and F genes of NDV and gps A and B of MDV and rHVT with only the F protein of NDV respectively. However, data collected in this trial proved that a significant degree of interference or competition exists between the commercial, live HVT/SB1 and the rHVT construct (Figures 5-8 and 5-9).

Despite the lower ELISA and HI titers measured in sera from chickens administered the rHVT construct in combination with the HVT/SB1 vaccine, these values were not different from several of the other treatment groups (Table 5-19). These lower titer levels are the first indication that interference or competition is occurring. The mortality levels, 20% mortality in the in ovo and 40% mortality in the recombinant construct plus HVT/SB1 treatment groups also suggests a lack of protection. The postinfection ELISA and HI titers of the SQ vaccinated recombinant construct plus HVT/SB1 is higher than any of the other groups (Table 5-20). The highest postinfection mean titers were seen in the in ovo and SQ vaccinated birds receiving the rHVT construct plus HVT/SB1. Conversely, the lowest postinfection titer was seen in the commercial,
modified–live B1B1 vaccinated birds (Table 5-20). The elevated postinfection titers seen in the birds vaccinated with recombinant construct and HVT/SB1 signifies the lack of NDV neutralizing antibodies, thus allowing the challenge virus to readily infect cells, replicate and be shed. The opposite is true for the commercial modified-live B1B1 vaccinated birds. These groups of birds had a minor rise in postinfection titers signifying the commercial NDV vaccine’s ability to effectively neutralize the challenge NDV and eliminate it before significant numbers of virions invade cells, replicate and are shed.

Virus isolation was conducted on birds infected by the eye-drop route. This was performed in order to assess the recombinant vaccine’s ability to protect mucosal surfaces by stimulating local immunity after systemic administration of the rHVT. At 10 days postinfection samples were collected. This is significantly longer than it would be desirable to find virus in the trachea. However, the vaccine was given time to clear the virus from the tracheal epithelium. With the exception of the birds receiving the recombinant vaccine subcutaneously at 1 day of age, virus was recovered from the tracheal mucosa 10 days after infection from all other groups receiving the rHVT vaccine either alone or in combination. Morgan et al. (102, 103) and Reddy (114) have also reported on rHVT vaccines’ inability to neutralize virus in the tracheal and prevent isolation of NDV several days postinfection. This demonstrates that the recombinant vaccine is not effective at inducing local immunity. This recombinant construct was allowed 10 days to establish local immunity and was unable to do so in 100% of the birds. While no statistical difference (p ≤ 0.05) was seen between these groups in regards to virus isolation, these results are significant under commercial poultry house conditions. If one bird out of 15 birds in a house of 20,000 still sheds virus 10 days after infection,
the number of contacts the shedding bird may have is very large and allows for a field challenge that will not be cleared. The field virus may also increase in virulence if allowed to pass from bird to bird and cause production losses and mortality.

The percent protection for all birds receiving a vaccine was similar except for those birds receiving the recombinant construct in combination with HVT/SB1 (Figure 5-8). The in ovo recombinant construct plus HVT/SB1 vaccinated birds had a statistically higher percent protection ($p \leq 0.05$) than birds receiving the same vaccine by the subcutaneous route at 1 day of age. However, birds receiving the recombinant construct with HVT/SB1 had significantly lower percent protection ($p \leq 0.05$) than the other vaccine treatment groups regardless of route of vaccination.

Possible causes for the interference seen with the recombinant construct when administered simultaneously with HVT and SB1 are HVT antibodies produced after vaccination neutralized the rHVT construct. Another possible cause for the decreased protection is antibodies produced from the gps A and B inserted on the recombinant construct bind to and neutralize the recombinant construct. Additionally, HVT and SB1 are known to exhibit synergism (38) when administered simultaneously. The molecular manipulation of the recombinant construct may have decreased its ability to replicate rapidly or exceeded the construct’s carrying capacity. Thus the HVT/SB1 and resulting synergism (38) allow them to replicate efficiently and result in the neutralization and exclusion of the recombinant construct. In other words, only those viruses (HVT/SB1) which replicated rapidly, gained access to the cellular replication machinery.

In order to test these hypotheses experiments could be conducted using radio or fluorescent labeling to the HVT/SB1 virus and label the recombinant construct with a
different label of the same type. These viruses can then be grown in the same cell culture on chicken embryo fibroblasts and PFUs measured at the end of a given period of time. This would allow the quantification of each type of viral plaque in cell culture and a determination of which virus grows faster. Alternatively, this recombinant construct could have the two gps A and B to MDV removed from their position in the rHVT’s genome and this trial repeated. This would determine if the carrying capacity had been exceeded in the original construct carrying two genes to NDV and two genes to MDV and if back neutralization of the rHVT from antibodies produced from the proteins synthesized from the gps A and B genes on the rHVT construct. Finally, the current recombinant construct could be grown in cell culture with media containing anti-sera containing antibodies to gps A and B of MDV. This would determine if antibodies to gps A and B of MDV neutralize the recombinant construct, thus decreasing protection to NDV infection.

For this trial and previous trials, a full dose (13,280 PFU/mL) of the recombinant construct was administered. This fact is important in understanding these results because in the commercial chicken industry, MDV vaccines are administered at one-half, one-third, or even one-quarter of the labeled dose depending on MDV challenge in the region. Even at the high titer of rHVT used in this trial, the vaccine had difficulties in neutralizing the entire challenge virus and preventing shedding of the challenge virus 10 days after infection.
CHAPTER 7
CONCLUSION

The Texas GB strain of neurotropic velogenic NDV administered intramuscularly or by eye-drop at an activity level of $10^{4.5}$ ELD$_{50}$ to naïve chickens was sufficient to cause almost total mortality by 14 days postinfection. Clinical signs of morbidity developed in the intramuscular infected birds approximately 1.5 days sooner as compared to the eye-drop infected chickens. Average days to onset of morbidity were not significant in the commercial turkeys regardless of route of infection. However, the intramuscular route of infection produced a significantly higher percent mortality than occurred in the eye-drop infected turkeys. The primary gross lesion seen in all bird groups was cecal tonsil hemorrhage. For those birds surviving infection with Texas GB NDV, a significant increase in IgG ELISA NDV titers was observed. Titers 14 days after infection were nearly the same for the eye-drop and intramuscular infected groups.

The recombinant vaccine, when administered to commercial broilers by in ovo or subcutaneous routes and commercial turkeys by the subcutaneous route, afforded similar protection as the commercial, modified-live B1B1 NDV vaccine. One hundred percent protection was observed in the birds receiving the recombinant vaccine despite lower preinfection NDV ELISA titers as compared to higher titers in the birds vaccinated with the commercial, modified-live NDV vaccine.

The simultaneous vaccination of chickens with HVT/SB1 and the rHVT construct carrying HN and F genes to NDV and genes to gps A and B of MDV, drastically decreased percent protection and efficacy of viral neutralization. Thus, clearing of the
virus from the tracheal epithelium in eye-drop infected birds 10 days after infection (38
days of age) is reduced. The simultaneous vaccination of chickens with CVI988 vaccine
strain of MDV and the recombinant construct did not produce significantly lower levels
of protection as compared to those birds receiving only the rHVT vaccine or the
commercial modified-live B1B1 NDV vaccine. However, the rHVT construct, regardless
of simultaneous vaccination with HVT/SB1 or CVI988 or administration of the
recombinant construct alone, did not completely clear replicating virus from the tracheal
epithelium 10 days after infection of the birds when challenged by the eye-drop route.
This suggests a lack of local immunity elicited by the recombinant construct.

The rHVT vaccine construct used throughout this trial provided excellent
protection to velogenic Texas GB NDV challenge when administered alone. However,
due to its inability to produce rapid protection against early challenge with virulent MDV,
it must be combined with commercial, live MDV vaccines in order to protect against
early virulent MDV challenge. This work demonstrated the significant amount of
interference and subsequent lack of protective immunity to velogenic Texas GB NDV
challenge when the rHVT construct is combined with HVT/SB1 and used simultaneously
to vaccinate chickens. This rHVT construct needs further modifications and testing
before it can be utilized in the commercial poultry vaccine market.
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BIOGRAPHICAL SKETCH

Mr. Eric A. Heskett was born on January 18, 1975 in Tampa, FL. He is the second child of Mr. Christian and Mrs. Kathleen Heskett. He attended high school at Heber Springs High School in Heber Springs, AR. He received his Bachelor of Science degree with a major in Biology in August 1997 from the University of South Florida. He will receive his Doctor of Veterinary Medicine and Doctor of Philosophy degrees from the University of Florida, College of Veterinary Medicine on May 24, 2003. Mr. Heskett’s areas of specialization are poultry diseases, pathology, and vaccinology.