**Cellular and Humoral Immune Responses to Avian Paramyxoviruses in Chickens**

by

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Abstract

The current dissertation (i) examined the effectiveness of a recombinant Newcastle disease virus (NDV) vaccine candidate, (ii) evaluated mucosal and systemic immune responses induced by NDV in chickens with maternally derived antibodies (MDA), and (iii) assessed wild-bird-origin avian paramyxoviruses type 1 (APMV-1) genotype X as potential vaccine candidates for poultry.

(i) We previously demonstrated that a prime-boost regime with an infectious bronchitis virus (IBV) Massachusetts (Mass)-type vaccine and recombinant NDV LaSota (rLS) co-expressing IBV Arkansas (Ark)-type trimeric spike ectodomain (Se) and granulocyte macrophage colony stimulating factor (GMCSF) enhances heterologous protection against virulent Ark-type challenge. Here we evaluated protection against Ark-type challenge conferred by administering the rLS/ArkSe.GMCSF and the attenuated Mass viruses simultaneously as a combined vaccine. Protection conferred by the combined vaccine was compared to protection induced by a commercial attenuated ArkDPI (Delmarva Poultry Industry) vaccine as well as by the attenuated Mass vaccine alone. Vaccination with the combined vaccine (rLS/ArkSe.GMCSF + Mass) as well as Mass alone provided significantly less protection against Ark challenge compared to the control using attenuated live ArkDPI vaccine. Only ArkDPI-vaccinated chickens exhibited "sterilizing immunity," i.e., no virus isolated from ≥10% of chickens after challenge. Chickens vaccinated with the combined vaccine rLS/ArkSe.GMCSF + Mass showed significantly less tracheal damage after challenge than birds vaccinated with the attenuated Mass vaccine alone. In addition, the combined vaccine also resulted in lower rate of virus isolation from the trachea. We concluded that the combined vaccine containing the recombinant virus, and the attenuated Mass

enhanced the cross-protective ability of the attenuated Mass vaccine against heterologous challenge.

(ii) The immune responses in the Harderian gland (HG) were characterized after NDV LaSota ocular vaccination in antibody naïve specific pathogen free (SPF) chickens and in chickens of commercial origin with NDV MDA. Ocular LaSota vaccination of 13-day-old white-leghorn SPF chickens elicited serum antibody levels that consistently increased after day 15 post-vaccination, while the specific IgA response in lacrimal fluids was already detectable on day 10 after vaccination. Eleven days post-vaccination, the relative abundance of B cells as well as T-helper (CD4+), and cytotoxic T cells (CD8+) in HGs was significantly increased achieving maximum frequencies 16 days post-vaccination. In a second experiment, chickens with MDA originating from NDV-vaccinated commercial white-leghorn layer breeders as well as white-leghorn SPF chickens were vaccinated with NDV LaSota. The LaSota virus successfully replicated in periocular tissues and in the trachea both in commercial and control SPF chickens after vaccination at 2 or 15 days of age (DOA). Vaccination at 2 DOA did not induce a serum NDV antibody response in chickens of commercial origin. In contrast, seroconversion was elicited in commercial chickens upon vaccination at 15 DOA, likely associated with waning of MDA. Unlike systemic IgG responses, vaccination at 2 or 15 DOA elicited strong specific IgA responses in lacrimal fluid in commercial chickens. The IgA response was highest 9 days after vaccination and showed a tendency to decline on day 15 post-vaccination. Commercial chickens vaccinated on day 2 of age showed increased B cells in HG both on days 10 and 16 post-vaccination. The expansion of B cells in the HG in these chickens is consistent with increased IgA levels detected in lacrimal fluids. In contrast, control SPF chickens showed a more limited B cell expansion in HG and lower IgA levels. Vaccination on day 15 of age also triggered a greater

increase of B cells in HGs in commercial chickens than in control SPF chickens. The B cell response was accompanied by T helper (CD4+) cell expansion occurring both in commercial and control SPF chickens. These cells expanded to a lesser extent when vaccination was performed at 2 DOA compared to vaccination at 15 DOA. Cytotoxic T cells (CD8+) showed significant expansion irrespective of vaccination day and without differences detected between control SPF chickens and chickens with MDA. We conclude that NDV LaSota elicits vigorous humoral and cell immune responses in the HG. Furthermore, unlike the interference shown by MDA on vaccine-induced serum antibody responses, MDA do not interfere with the mucosal immune response of the HG.

(iii) We examined the replication and adaptation of APMV-1 using four isolates: Mallard/US(OH)/04-411/2004, Northern pintail/US(OH)/87-486/1987, Mottled duck/US(TX)/TX01-130/2001, and Mallard/US(MN)/MN00-39/2000, and assessed their potential as vaccine candidates for chickens. The adaptability of each virus was examined by serial passages in embryonated chicken eggs (ECE) and in Vero cells. All APMVs successfully replicated in ECE. In contrast, two isolates passaged in Vero cells showed successful replication and two showed a continuous decline in viral load during passages. Whole genome sequencing analysis identified 14 genomic positions with significant variation in mean allele frequency. Changes of the predominant virus population were characterized by shifts of amino acid (aa) frequency at seven positions. Notably, four of these changes were located in the HN protein, one in matrix (M) protein, and two in the L-protein sequences. Remarkably, while the percentage of alternative amino acids in viral populations passaged in ECE showed limited variation, e.g., at aa position 127 of HN, the frequency varied from 7.4% to 19.8% and HN aa position 192 from 5.1% to 43.5%, the variation of the viral populations passaged in Vero cells was significantly

higher at the same positions (e.g. the frequency of the alternative amino acids at HN aa positions 127 and 192 changed from 20.8% to 95.2% and 7.2% to 91.2%, respectively). Isolate 2 passaged in Vero cells displayed a marked variation in alternative amino acid frequencies, specifically at positions 127 within the HN- and 100 within the M- proteins. Isolate 3, while showing no alterations at the same HN positions, showed a considerable change in alternative amino acid frequency in the L protein at position 1875, a change occurring only in the Vero cell environment. One-day-old SPF chickens inoculated with isolates passaged in ECE elicited serum antibody responses similar to those elicited by the LaSota reference strain. In contrast, APMVs passaged in Vero cells showed limited replication in chickens and reduced induction of systemic antibodies. Interestingly, one virus passaged in ECE and another in Vero cells elicited IgA levels in lacrimal fluid comparable to the LaSota strain. We concluded that the four wild-bird APMV isolates tested demonstrated successful adaptation to ECE, with one isolate eliciting overall immune responses comparable to the LaSota virus, supporting their potential as vaccine candidates.

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List of Abbreviations

| | |
|---|---|
| aa | Amino acid |
| AAvV-1 | Avian avulavirus 1 |
| AIV | Avian influenza virus |
| ANOVA | Analysis of variance |
| APHIS | Animal and Plant Health Inspection Service |
| AOAV-1 | Avian orthoavulavirus 1 |
| APMV-1 | Avian paramyxovirus 1 |
| Ark | Arkansas-type IBV |
| ArkDPI | Ark Delmarva Poultry Industry |
| ArkSe | Ark spike ectodomain |
| ATCC | American Type Culture Collection |
| CMI | Cell mediated immunity |
| DOA | Days of age |
| ECE | Embryonated chicken eggs |
| $EID_{50}$ | 50% embryo infective dose |
| ELISA | Enzyme-linked immunosorbent assay |
| GM-CSF | Granulocyte macrophage colony stimulating factor |
| HALT | Head-associated lymphoid tissue |
| HG | Harderian gland |
| HI | Hemagglutination inhibition |
| ICTV | International Committee on Taxonomy of Viruses |
| IB | Infectious bronchitis |

| | |
|---|---|
| IBV | Infectious bronchitis virus |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| ILTV | Infectious laryngotracheitis virus |
| IRF | Interferon regulatory factor |
| JAK-STAT | Janus kinase-signal transducer and activator of transcription |
| Mass | Massachusetts |
| MDA | Maternally derived antibodies |
| MHC | Major histocompatibility complex |
| MOI | Multiplicity of infection |
| ND | Newcastle disease |
| NDV | Newcastle disease virus |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NGS | Next generation sequencing |
| qRT-PCR | Quantitative reverse transcriptase polymerase chain reaction |
| rLS | Recombinant LaSota virus |
| rLS/Ark.Se | rLS-expressing IBV Ark Se |
| RIG-I | Retinoic acid-inducible gene I |
| RT-qPCR | Reverse transcription quantitative polymerase chain reaction |
| S | Spike |
| Se | Spike ectodomain |
| SISPA | Sequence-independent single-primer amplification |

| | |
|---|---|
| SPF | Specific pathogen free |
| STAT | Signal transducer and activator of transcription |
| TLR | Toll-like receptor |
| VNT | Virus neutralization test |

CHAPTER 1.

# Literature Review

## 1.1. Introduction

The global demand for animal protein continues to increase. According to projections by the Food and Agriculture Organization (FAO) in 2024, total global meat production was expected to reach approximately 371 million tons by 2025, with poultry accounting for the largest portion. Chicken meat alone was anticipated to constitute about 146 million tons, exceeding the production of all other terrestrial livestock species (52). Egg production follows a similar upward trend, with an expected output of around 1.7 trillion eggs in the same period (50). The rapid expansion of poultry production is largely attributable to efficient feed-to-protein conversion, relatively low environmental footprint compared to meat-production species, and broad consumer acceptance.

Respiratory viruses represent one of the most persistent and significant biological challenges to the continued growth of poultry production. Outbreaks caused by RNA viruses such as Newcastle disease virus (NDV), avian influenza virus (AIV), and infectious bronchitis virus (IBV), along with DNA viruses like infectious laryngotracheitis virus (ILTV), can significantly elevate mortality rates, impede weight gain, reduce egg productivity, and trigger trade restrictions with global economic repercussions (93). Among these viruses, RNA viruses are distinctively challenging to control due to their successful adaptability resulting from error-prone replication generating frequent mutations (42, 43), and genetic plasticity from recombination, or gene reassortment (in segmented viruses) (41, 42, 97). Such characteristics

enable these viruses to evade immune responses, perpetuating their global impact on poultry health and productivity. The economic impact of respiratory viruses includes increased direct costs associated with increased culling, reduced productivity (weight gain and feed conversion), and augmented mortality, as well as increased indirect costs from disrupted trade and market instability which often surpasses feed expenses within an affected production cycle. Therefore, the effective prevention and control of respiratory viral diseases, particularly those caused by RNA viruses, remain a critical priority for poultry producers, veterinarians, and policymakers worldwide.

## 1.2. Newcastle Disease Virus

Newcastle disease (ND), caused by highly virulent (velogenic) and moderately virulent (mesogenic) strains of NDV, remains one of the most contagious and economically devastating avian diseases worldwide (3, 6, 15, 19, 39). Over time, the virus has undergone multiple taxonomic revisions as virological classification systems have evolved. Historically, NDV was referred to as avian paramyxovirus type 1 (APMV-1), a designation still commonly used in both scientific literature and field practice. In 2002, it was placed within the genus *Avulavirus* in the family *Paramyxovirinae*, with members of this genus broadly labeled as "Avian paramyxoviruses" followed by numeric identifiers (e.g., APMV-1). Subsequent updates by the International Committee on Taxonomy of Viruses (ICTV) renamed the virus avian avulavirus 1 (AAvV-1) in 2016 and further reorganized it into the genus *Orthoavulavirus* in 2018, formally designating it as avian orthoavulavirus 1 (AOAV-1). In the most recent ICTV classification, the former genus *Avulavirus* has been elevated to the subfamily *Avulavirinae*,

which now includes three genera: *Metaavulavirus* (14 species), *Orthoavulavirus* (11 species), and *Paraavulavirus* (3 species), with NDV formally classified as a strain of *Orthoavulavirus javaense*, replacing the earlier AOAV-1 designation (10, 126). Historical accounts of the disease precede this nomenclature: Kraneveld first documented a lethal outbreak in Java, Indonesia, described as "pseudopeste des poules" in 1926 (84) while Doyle reported a similar epizootic near Newcastle-upon-Tyne, England, which ultimately gave rise to the common name "Newcastle disease" (46). Despite formal taxonomic changes, the revised terminology has not been widely adopted. The World Organisation for Animal Health (WOAH/OIE) continues to use "NDV" to refer specifically to virulent APMV-1 strains, and thus the traditional nomenclature remains prevalent (147).

Pathotyping studies classified NDV strains as lentogenic, mesogenic, or velogenic based on embryo mean death time and pathogenicity in chicks (5, 44). This distinction was pivotal, as the discover of naturally low-virulent strains such as B1 and LaSota enabled the development of live vaccines that have helped to reduce the disease's economic impact (64, 122).

From the 1970s onward, systematic surveillance revealed extensive genetic diversity among NDV isolates, now classified into genotypes I – XXI (38, 147). Despite substantial genetic heterogeneity, changing at approximately $10^{-4}$ substitutions per nucleotide per replication cycle due to their error-prone RNA-dependent RNA polymerase, all NDV strains belong to a single serotype (87).

Currently the control of ND relies heavily on vaccination, strict biosecurity measures, and continuous molecular monitoring. However, despite over seventy years of vaccination efforts, NDV remains endemic across many poultry-producing areas (15, 40, 126). Velogenic NDV

continues to cause significant economic losses from mortality rates exceeding 80%, increased culling, trade bans, and production drops (126, 147).

## 1.3. NDV Genome

NDV has a non-segmented, negative-sense single-stranded RNA genome that is always a multiple of six nucleotides in length, a requirement known as the “rule of six” that optimizes nucleocapsid assembly and RNA-polymerase processivity (82, 125). The genome is flanked by a 3′ leader of approximately 55 nucleotides and a 5′ trailer of approximately 114 nucleotides. These regions contain cis-acting signals that are essential for transcription, replication, and packaging (87).

NDV strains are broadly classified into two main classes based on the phylogenetic analysis of the fusion (F) gene sequence: class I and class II (33, 37). Class I strains typically have a genome size of 15,198 nucleotides and are generally associated with low virulence, often isolated from wild birds and live poultry markets (33, 79). In contrast, class II strains exhibit greater genetic diversity, with genome sizes ranging from 15,186 to 15,192 nucleotides, and include both virulent and avirulent isolates found in various avian species with a global distribution (40, 99). Importantly, class II viruses are further subdivided into multiple genotypes, reflecting their extensive evolutionary diversification and significance in both endemic circulation and epidemic outbreaks (38).

In the NDV genome, six main genes are arranged in a specific order, from 3′ to 5′, encoding six structural proteins: nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), haemagglutinin-neuraminidase (HN), and the large polymerase (L) (5, 87). Additionally, two

non-structural proteins, V and W, are generated from edited transcripts of the P gene (70, 125). The N gene encodes the N protein, which encapsidates genomic and antigenomic RNA, forming the helical ribonucleoprotein template for the polymerase complex (17). The P gene encodes the polymerase cofactor P and, through co-transcriptional RNA editing, produces the interferon-antagonist V, and a minor product W, believed to play a role in evading the host immune response (125). The M gene encodes the M protein that bridges the nucleocapsid and plasma membrane to drive virus budding (107). The F gene encodes the F glycoprotein, with a multibasic cleavage motif serving as the primary molecular marker of velogenicity (35). The F protein undergoes a significant conformational change upon cleavage, which is essential for mediating viral fusion with the host cell membrane. The HN gene encodes the multifunctional HN protein, which plays critical roles throughout the NDV infection cycle. First, its sialic acid binding domain mediates viral attachment to host cell receptors, initiating infection. Next, HN interacts with the F protein to trigger its conformational refolding, a step required for membrane fusion and entry of the viral genome into the host cell. Finally, the neuraminidase activity of HN facilitates the release of progeny virions by cleaving sialic acid residues from the host cell surface and viral glycoproteins, preventing self-aggregation and promoting efficient viral dissemination. Consequently, given the F protein critical role in membrane fusion and its conformational dynamics, alongside the HN protein role in attachment, both are major targets of neutralizing antibodies (68). Finally, the L gene encodes the 250-kDa RNA-dependent RNA polymerase that carries transcriptase, capping, methyl-transferase, and polyadenylation activities (45).

## 1.4. NDV Replication

Attachment begins when the HN glycoprotein binds α2,3-linked sialic-acid residues on respiratory and/or enteric epithelial cells. Following HN's receptor binding, the activated HN then interacts with the F protein to trigger its irreversible refolding, allowing the viral envelope to fuse with the plasma membrane and release the ribonucleoprotein complex into the cytoplasm (47, 68). It is important to note that the F protein must be proteolytically activated before the virus can become infectious. This activation involves the cleavage of the F0 precursor into F1 and F2 subunits by host proteases. This cleavage primes the F protein for its subsequent role in membrane fusion. Proteolytic cleavage of F at its multibasic site by ubiquitous furin is the key molecular switch that permits systemic spread (35).

After uncoating, the negative-sense genome, encapsidated by N and associated with the P and L proteins, serves as the template for transcription by the L-P polymerase complex. The polymerase initiates at the 3′ leader region, sequentially transcribing each gene into capped, polyadenylated monocistronic mRNAs in the order N > P > M > F > HN > L. This transcription process establishes a characteristic 3′ to 5′ gradient of mRNA abundance, typical of *Mononegavirales* (87). Once N protein accumulates sufficiently, the polymerase shifts from transcription to replication, ignoring gene-end signals to produce full-length positive-sense antigenomic RNA. These antigenomes subsequently serve as templates for new negative-sense genomic RNA, both forms obligatorily encapsidated by N and adhering to the “rule of six” for optimal encapsidation and polymerase efficiency (81, 82, 145).

Assembly begins when fresh N coats nascent genomes to form nucleocapsids, which migrate to the plasma membrane. M protein recruits nucleocapsids to membrane domains

enriched in F and HN, coordinates budding, and promotes the release of mature virions from the host cell (107).

Throughout this replication cycle, genetic and antigenic diversity arises primarily from the high mutation rate (~$10^{-4}$ substitutions per site per cycle) of the virus's error-prone RNA-dependent RNA polymerase (120). This inherent variability, further shaped by selective pressures from host immune responses and adaptation to different avian hosts, has resulted in the emergence of over 20 recognized genotypes. Nonetheless, the existence of a single serotype highlights functional constraints on surface glycoproteins, restricting their antigenic variability (38, 87).

## 1.5. NDV Pathogenesis and Clinical Presentation

The NDV pathogenesis is characterized by an unusually wide range of clinical manifestations attributable to viral genetic determinants, like the sequence at the F protein cleavage site, which dictates tissue tropism and fusogenic potential, and the efficiency of the HN attachment protein in mediating host cell binding. These viral factors interact with host factors, including the host's immune status and age-related physiological conditions. Infection is acquired by inhalation or ingestion of virus-loaded droplets or fecal dust, after which primary replication occurs in epithelial cells of the upper respiratory and intestinal tracts (7).

Isolates are empirically grouped as lentogenic, mesogenic, velogenic neurotropic, or velogenic viscerotropic according to mean-death-time in embryos and intracerebral pathogenicity in day-old chicks (5, 147). These groups differ dramatically in clinical outcome varying from mild rhinitis to fulminant visceral hemorrhage and neurological signs (5). The

molecular support of this scale lies in the F protein cleavage site. Lentogenic strains possess a monobasic motif that can only be cleaved by trypsin-like enzymes found on the mucosal surfaces, such as the respiratory and intestinal tracts. In contrast, velogenic strains have a multibasic motif that is processed by the ubiquitous intracellular furin, enabling systemic dissemination and high virulence (35, 119).

Clinical outcomes reflect the above-mentioned molecular divide. Lentogenic infections are frequently subclinical or limited to mild rhinitis and growth delay. Mesogenic strains add depression, green diarrhea, and mortality up to 25% in young birds (126). Velogenic neurotropic viruses produce severe respiratory distress followed by neurological signs – ataxia, tremors, torticollis, limb paralysis – reflecting viral replication in the brainstem and spinal cord. Velogenic viscerotropic viruses cause extensive congestion and petechial hemorrhage of the proventriculus, intestine, and cecal tonsils, abrupt egg-production drops in layers, and flock mortalities that can exceed 80% (23). Histologically, velogenic strains induce necrosis of lymphoid follicles, apoptotic depletion of the bursa and thymus, and diffuse vasculitis with hemorrhage in multiple organs, contributing to immunosuppression and secondary bacterial disease (44).

The clinical outcome is further influenced by host genetics, age, nutrition, concurrent infections, and environmental stress. While high maternal antibody titers or timely vaccination can mitigate clinical signs, they may not always prevent local replication and shedding, especially when the challenge strain differs antigenically from the vaccine strain (38, 75). Conversely, heat stress, mycotoxicosis, immunosuppressive viruses (such as infectious bursal disease virus), or high stocking density can lower the infectious dose required for manifest disease. Consequently, even in well-vaccinated poultry industry regions, the subclinical

circulation of heterologous strains can perpetuate local transmission cycles, necessitating continuous surveillance and phenotype-matched immunization strategies.

## 1.6. Host Immune Responses to NDV

Host immunity to NDV involves both innate and adaptive immune mechanisms that limit viral replication, mitigate clinical disease, and suppress transmission. Innate immunity is activated within hours of NDV exposure (94). Viral RNA in the cytoplasm is detected by endosomal Toll-like receptors 3 (TLR3) and 7 (TLR7) and the cytoplasmic RNA helicase melanoma-differentiation-associated protein 5 (MDA5), the principal RIG-I-like sensor in chickens (since chickens lack RIG-I) (153). Signaling through IRF-7 and NF-κB induces type I interferons (IFN-α/β) and proinflammatory cytokines, establishing an antiviral state and recruiting immune cells like macrophages and natural killer cells (77, 153). Velogenic NDV strains counteract host antiviral defenses via their V protein, which suppresses interferon responses by binding STAT1/STAT2 to block JAK-STAT signaling, and promoting ubiquitin-mediated degradation of phosphorylated STAT1, thereby inhibiting expression of interferon-stimulated genes (67, 104, 108).

The humoral response follows a classical chronology. Antigen-specific IgM appears in serum 4 to 6 days post-infection, switching to IgG (also known as IgY in chickens) by day 10, which persists for months and correlates with systemic protection (100). Secretory IgA from Harderian gland plasma cells reach mucosal surfaces and the upper respiratory tract, peaking about a week after ocular vaccination, and is effectively associated with reduced virus shedding (2, 116). Neutralizing antibodies target epitopes on the HN and F glycoproteins, and high-titer

sera (> 6 $log_2$ by HI test) are generally protective against death even with heterologous challenge (74, 76).

Cell-mediated immunity plays a critical role. $CD4^+$ T helper cells proliferate in lymphoid tissues during the first week after infection, secreting IL-2 and IFN-γ to support B cells and $CD8^+$ cytotoxic T lymphocytes (CTLs) (75, 88). Live vaccines are generally superior to inactivated vaccines at eliciting CTL responses because they replicate in epithelial cells and professional antigen-presenting cells, ensuring abundant MHC-I presentation (32).

Maternally derived antibodies (MDA), transferred via the yolk, offer protection during the first two to three weeks of life but can interfere with early vaccination responses by neutralizing vaccine strains (112). However, some local immune responses, such as mucosal IgA and T-cell priming, may still occur after mucosal vaccination (e.g., ocular or spray) despite systemic interference (57), a subject further expanded in the third chapter of the current dissertation.

## 1.7. NDV Vaccination

Outbreaks caused by genotypes VII, XII, and others in Asia, Africa, and the Americas illustrate the continuing need for improved vaccines. Live lentogenic vaccines such as Hitchner B1, LaSota, Clone 30, and the thermostable I2 and V4 derivatives, remain the cornerstone of ND control worldwide. They protect against clinical disease yet do not completely block replication and shedding. Cross-protection may be reduced when emerging viruses diverge antigenically from the vaccine strain, even though all belong to a single serotype (100). Applied by coarse spray, eye-drop, or via the drinking water, live vaccine viruses replicate in the mucosal

epithelium, elicit mucosal IgA, systemic neutralizing IgG, and robust CTLs responses, and can considerably decrease velogenic-virus shedding when the field challenge is antigenically similar (22, 76). However, live vaccine viruses are vulnerable to interference from high MDA titers. Chicks with HI titers $\geq 6 \log_2$ often fail to seroconvert after a single hatch-day dose, though they may still mount Harderian-gland IgA responses and T-cell priming (57, 112). Thus, optimal programs schedule an early priming spray, timed booster(s) after maternal immunity wanes, and, in high-density production systems, an additional boost in the field at around four weeks.

Inactivated oil-emulsion vaccines, delivered intramuscularly or subcutaneously, add enduring systemic antibodies and are used almost universally to protect long-lived birds such as layers and breeders. When vaccination with an inactivated preparation follows live priming, flock HI geometric-mean titers often exceed the 5 $\log_2$ threshold correlated with protection (9, 75). When applied in breeders, vaccination with inactivated vaccines also elevate maternal antibodies that protect progeny during the critical first two weeks of live. Drawbacks include higher labor costs, injection reactions, and limited induction of mucosal or cellular immunity.

Field vaccine performance is conditioned by additional variables. Coarse-spray droplets and eye drops reliably prime upper-respiratory IgA whereas water vaccination loses effectiveness if chlorine is not neutralized or drinkers are not drained (53). Heat or freeze-thaw cycles can inactivate live vaccines rapidly, and ventilation or dust can dilute spray applications (51, 103). Consequently, uneven flock titers may occur, and compartments of susceptible birds can allow silent circulation of virulent virus, even in technically “vaccinated” flocks. Genotype mismatch, leading to antigenic mismatch, is another emerging issue. For example, velogenic lineage VII and XXI viruses in Asia, Africa, and the Middle East are shed at higher titers and for longer periods in LaSota-vaccinated flocks (a genotype II vaccine strain) compared to lineage II

counterparts. This underscores the need for licensing genotype-matched live or inactivated vaccines (100, 124).

In addition to traditional live and inactivated vaccines, recombinant NDV vectors are being developed to enhance protective efficacy. Recombinant NDV allows simultaneous immunization against NDV and other pathogens and support the DIVA (Differentiating Infected from Vaccinated Animals) strategy. These topics will be explored further in the following section.

## 1.8. NDV as a Vaccine Vector

Despite the above-mentioned genetic diversity, the conserved genome architecture of NDV (3′-N-P-M-F-HN-L-5′), its strict adherence to the "rule of six", and the presence of shared cis-regulatory elements collectively form a robust genetic framework suitable for vaccine engineering. These features allow NDV to be genetically modified using reverse genetics technologies to express foreign antigens for targeted protection against additional avian pathogens.

Lentogenic NDV strains are particularly well-suited for this application due to their ability to replicate efficiently in embryonated eggs, genetic stability, and capacity to elicit strong mucosal immune responses. Since the late 1990s, reverse genetics systems based on full-length cDNA copies of lentogenic strains such as LaSota, B1, and Clone 30 have enabled the insertion of foreign genes – typically between 2 and 4.5 kb – without significantly compromising viral replication or safety (48, 85, 105). Numerous constructs have since been developed, expressing the envelope proteins of AIV, IBV, and ILTV (80, 123, 140, 154).

Three intrinsic features make NDV an attractive vaccine vector candidate. First, lentogenic NDV strains retain their attenuated phenotype even after insertion of foreign genes, as long as the F-protein cleavage site remains mono-basic, which minimizes the risk of reversion to virulence (80). Second, oculo-nasal vaccine administration stimulates potent local immune responses, including Harderian gland-derived IgA and virus-specific $CD4^+$ and $CD8^+$ T cells (49). Co-delivery of foreign antigens engages the same mucosal pathways, offering added benefits for the protection against respiratory pathogens such as IBV. Third, NDV replication naturally induces strong type-I interferon and pro-inflammatory cytokine responses (e.g., IL-1β), which provide built-in adjuvanticity (66). Furthermore, commercially available indirect ELISAs that detect NDV nucleoprotein, but not heterologous inserts allow DIVA thereby facilitating disease surveillance.

However, certain limitations remain when using NDV as a vaccine vector. Foreign gene inserts must comply with the "rule of six" and are best placed at the P–M or M–F gene junctions to maintain proper transcriptional gradients. Larger inserts can reduce viral fitness and replication efficiency, and pre-existing NDV antibodies in the host may reduce immune responses to booster doses (106, 155).

More broadly, while vectored vaccines offer advantages, their development and deployment in poultry still face challenges. Nevertheless, commercial progress is evident. For example, an NDV-based vector expressing H5 haemagglutinin has been licensed for AIV control in China (25, 101). Similarly, a fowlpox-based vaccine that delivers the NDV F gene (128) is commercially available for chickens, illustrating that vectored vaccines can be successfully developed and produced at industrial scale for poultry use.

## Statement of Research Objectives

The current dissertation consists of a three-part study with the following objectives: (i) examine the effectiveness of a recombinant NDV vaccine candidate, (ii) evaluate mucosal and systemic immune responses induced by NDV in chickens with maternally derived antibodies (MDA), and (iii) assess wild-bird-origin avian paramyxoviruses type 1 (APMV-1) genotype X as potential vaccine candidates for poultry.

CHAPTER 2.

# Cross-Protection Conferred by Combined Vaccine Containing Infectious Bronchitis Virus Attenuated Massachusetts and Recombinant LaSota Virus Expressing Arkansas Spike

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## 2.1 Summary

We previously demonstrated that a prime-boost regime with an infectious bronchitis virus (IBV) Massachusetts (Mass)-type vaccine and recombinant LaSota virus (rLS) coexpressing IBV Arkansas (Ark)-type trimeric spike ectodomain (Se) and granulocyte macrophage colony stimulating factor (GMCSF) enhances heterologous protection against virulent Ark challenge. This study evaluates protection against Ark-type challenge conferred by administering the rLS/ArkSe.GMCSF and the attenuated Mass viruses simultaneously as a combined vaccine. Chickens were vaccinated at day of hatch and challenged at 21 days of age with virulent Ark. Protection conferred by vaccination was assessed by respiratory signs, tracheal virus isolation as well as IBV RNA quantitation, and tracheal histomorphometry. Protection conferred by the combined vaccine was compared to protection induced by a commercial attenuated ArkDPI (Delmarva Poultry Industry) vaccine as well as by the attenuated Mass vaccine alone. Vaccination with the combined vaccine (rLS/ArkSe.GMCSF + Mass) as well as Mass alone provided significantly less protection against Ark challenge compared to the control using attenuated live ArkDPI vaccine. Only ArkDPI-vaccinated chickens exhibited “sterilizing immunity,” i.e., no virus isolated from ≥10% of chickens after challenge. Chickens vaccinated

with the combined vaccine rLS/ArkSe.GMCSF + Mass showed significantly less tracheal damage after challenge than birds vaccinated with the attenuated Mass vaccine alone. In addition, the combined vaccine also resulted in lower rate of virus isolation from the trachea. We concluded that the combined vaccine containing the recombinant virus and the attenuated Mass enhanced the cross-protective ability of the attenuated Mass vaccine against heterologous challenge.

## 2.2 Introduction

Novel variants of infectious bronchitis (IB) virus (IBV) continue to emerge as a result of naturally occurring recombination events between IB wild-type and vaccine viruses (24, 26, 69, 73, 83, 86, 91, 143, 148). In addition, distinct IBV vaccine subpopulations continue to be isolated from outbreaks of disease in chickens vaccinated with attenuated IBV vaccines (55, 95, 139). Accumulating evidence indicates that the long-term use of attenuated IBV vaccines has complicated the control of the disease and perpetuated IB associated economic losses to the poultry industry (130). Therefore, the use of recombinant viruses expressing IBV immunodominant epitopes instead of live vaccines of differing types appears to be an alternative to reduce emergence of novel IBV.

Expression of the IBV spike (S) from Newcastle disease virus (NDV) has shown varying degrees of protection against IBV challenge (1, 123). Expressing the S ectodomain (Se), i.e., the S protein excluding the transmembrane anchor and short cytoplasmic domain of S2, is a relevant improvement because the Se cannot be inserted into the viral envelope of the recombinant viral vector, i.e., eliminating the potential of extending the tropism of the vector.

Although initial studies using recombinant LaSota virus (rLS) expressing the IBV Se showed limited protection against IBV challenge (151), the addition of the granulocyte macrophage colony stimulating factor (GM-CSF) gene enhanced its protection significantly (78). Moreover, a prime-boost regime with an attenuated Massachusetts (Mass)-type vaccine and the rLS expressing IBV Se of an Arkansas (Ark) strain and GM-CSF (rLS/ArkSe.GMCSF) significantly enhanced protection against Ark challenge in comparison with Mass alone. We speculate that the enhancement of protection is likely due to a booster effect triggered by epitopes shared between the S of Mass and the S of Ark expressed from the recombinant construct. In this study we evaluated the option of vaccinating 1-day old chickens with a combination of the attenuated Mass and the rLS/ArkSe.GMCSF vaccines. This combined vaccine would reduce labor and comply with Animal & Plant Inspection Service (APHIS) regulations for licensing IBV vaccines.

## 2.3 Materials and Methods

**Chickens**. White leghorn chickens were hatched from specific pathogen free (SPF) embryonated eggs (Charles River, North Franklin, CT) and maintained in Horsfall-type isolators in biosafety level 2 facilities with water and food *ad libitum*. Experimental procedures and animal care were performed in compliance with applicable federal and institutional animal care and use guidelines. Auburn University College of Veterinary Medicine is an Association for Assessment and Accreditation of Laboratory Animal Care -accredited institution.

**Challenge virus**. The previously characterized virulent Ark-type IBV strain with GenBank accession no. DQ458217 (134) was used for challenge. The virus was titrated in SPF

embryonated chicken eggs as accepted (141) with minor modifications previously described (60).

**Vaccine viruses**. The previously developed rLS/ArkSe.GMCSF virus was used (78). As reported before, this recombinant LaSota vaccine effectively expresses the IBV Ark-type Se in cell culture and is stable after passages in embryonated eggs (78). The rLS/ArkSe.GMCSF virus stock was titrated by 50% embryo infective dose ($EID_{50}$) in 9-day-old SPF chicken embryos. Embryos were considered positive by detection of hemagglutinating activity in the allantoic fluid of inoculated eggs as accepted for NDV (141). In addition, commercially available live attenuated IBV vaccines belonging to the Mass and ArkDPI (Delmarva Poultry Industry) –types were used. The live attenuated IBV vaccines were titrated in SPF embryonated chicken eggs as described for the challenge virus (above). Both vaccine viruses, the recombinant NDV and the live IBV, were mixed in the same vial before administration. Due to well-known interference between IBV and NDV (110), two combined vaccines were prepared that differed in the dosage of the recombinant LaSota virus content. Specifically, we used $10^6$ $EID_{50}$ or $10^7$ $EID_{50}$ of rLS/ArkSe.GMCSF, while the dose of attenuated Mass vaccine was adjusted to $10^3$ $EID_{50}$. For simplicity reasons, these groups have been identified in the figures as rLS^6+Mass and rLS^7+Mass. All vaccinations in chickens were performed with a total volume of 100 µl (25 µl in each nostril and each eye) of appropriately diluted virus stock.

**Experimental design**. Four groups of chickens (n=21 chickens/group) were vaccinated at 1 day of age (DOA) and additional two unvaccinated groups (n=15 chickens/group) served as controls. Groups 1 and 2 were vaccinated with the combined vaccine rLS/ArkSe.GMCSF and live Mass.

Group 1 received $10^6$ $EID_{50}$ of rLS/ArkSe.GMCSF + $10^3$ $EID_{50}$ of Mass (designated rLS^6+Mass) and chickens of group 2 received $10^7$ $EID_{50}$ of rLS/ArkSe.GMCSF + $10^3$ $EID_{50}$ of Mass (designated rLS^7+Mass). Chickens of groups 3 and 4 were vaccinated with $10^3$ $EID_{50}$/per bird of the Mass and ArkDPI live vaccines, respectively. Groups 5 and 6 were age-matched nonvaccinated/challenged and nonvaccinated/non-challenged controls. Sera were collected 20 days after vaccination for IBV and NDV serum antibody determinations. NDV antibodies were determined by ELISA (IDEXX Laboratories Inc., Westbrook, ME) following the kit's manufacturer recommendations. The neutralization ability of systemic IBV antibodies elicited by the combined vaccine (rLS/ArkSe.GMCSF + Mass) versus those elicited by vaccination with Mass alone against Ark was determined by a virus neutralization test (VNT). The VNT was conducted in embryonated chicken eggs by the α method (constant-serum, diluted-virus) using pools of five sera and tenfold dilutions of the IBV Ark challenge strain. Instead of determining embryos positive for virus replication based on embryo lesions, we determined relative IBV RNA in the allantoic fluid of each egg with TaqMan© quantitative reverse transcription PCR (qRT-PCR) as described previously (21). Viral RNA data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey multiple comparisons posttest. Differences were considered significant with $P<0.05$.

At 21 DOA, all chickens in groups 1 through 5 were challenged ocularly with 100 µl containing $10^3$ $EID_{50}$/chicken of the virulent IBV Ark strain. Protection was evaluated 5 days after challenge by individual assessment of respiratory rales, tracheal IBV RNA, tracheal histomorphometry, and virus isolation in embryonated chicken eggs (8). In brief, nasal and/or tracheal rales were evaluated blindly by close listening to each bird and scored as 0 (absent), 1 (mild), 2 (moderate), or 3 (severe). Scoring data were compared by Kruskal-Wallis test followed

by the Dunn posttest. Tracheal viral load was determined by qRT-PCR. Briefly, IBV RNA was extracted from homogenized tracheal samples with TriReagent RNA/DNA/Protein Isolation Reagent (Molecular Research Center, Cincinnati, OH) following the manufacturer's recommendations. Relative viral copies were determined by qRT-PCR as described above. Viral RNA data were analyzed by one-way ANOVA followed by the Tukey posttest. Differences were considered significant at $P<0.05$.

Tracheal histomorphometry was performed as previously described (134). In brief, formalin-fixed sections of trachea were processed, embedded in paraffin, sectioned at 4–6 μm and stained with hematoxylin and eosin for histopathological examination. The tracheal mucosal thickness and the thickness of lymphocytic infiltration were measured using ImageJ (121) (https://imagej.nih.gov/ij/download.html), and the average of five measurements for each chicken calculated. Data were analyzed by ANOVA followed by the Tukey posttest.

IBV isolation from tracheal samples was performed as accepted (58). Briefly, tracheal samples were collected 5 days post challenge into tubes containing sterile tryptose broth and antibiotics, and stored frozen at -80 C. Samples were thawed and vortexed, and 0.2 ml of each supernatant inoculated into five 10-day embryonated SPF chicken eggs via the allantoic route. Eggs were incubated at 37 C for 7 days. Embryos dying prior to day 4 of incubation were eliminated. Embryo deaths occurring after day 4 of incubation were considered positive. On day 7, eggs containing a live embryo were examined for IBV typical lesions including stunting, curling, and presence of urates in the kidneys. The sample was considered positive for virus isolation if any of the five embryos showed IBV lesions. Normal embryos were scored as negative. Based on results of viral RNA quantitation and because of labor and other costs

consideration, virus isolation was restricted to chicken groups vaccinated with rLS^7+Mass, Mass, and ArkDPI.

## 2.4 Results

**NDV antibody by ELISA**. As seen in Fig. 2.1, chicken groups vaccinated with the combination of $10^6$ $EID_{50}$ of rLS/ArkSe.GM-CSF and live Mass IBV developed relatively low NDV antibody levels with sample to positive (S/P) ratios less or equal to 0.2. Both groups receiving rLS/ArkSe.GM-CSF showed NDV antibody levels higher than groups receiving only IBV, but only in group 2 (vaccinated with $10^7$ $EID_{50}$ of rLS/ArkSe.GM-CSF) did the increase in NDV antibody levels achieve statistical significance ($P$<0.05). Thus, the recombinant LaSota virus showed limited replication in the vaccinated chickens.

**IBV virus neutralization**. As indicated above, instead of determining whether embryos were positive or negative to IBV by classical lesions in each of the virus dilution mixtures with serum, we determined relative IBV RNA in the allantoic fluid of each embryonated egg. Thus, the data obtained were not transformed into a neutralization index but directly analyzed based on the detected viral loads in the eggs inoculated with increasing dilutions of the virus and constant serum mixtures. Figure 2.2 shows relative IBV RNA detected by qRT-PCR in the allantoic fluid of embryonated eggs inoculated with virus-serum mixtures at virus dilutions $10^{-5}$ and $10^{-6}$. Only at relatively high dilutions of the virus+constant serum mixtures did neutralization become evident compared to the virus alone. This result shows that limited systemic antibody levels were elicited by vaccination with either rLS/ArkSe.GM-CSF+Mass or Mass alone. In addition, no

significant differences were detected in relative amounts of IBV RNA in embryos inoculated with the mixtures of virus with serum from either vaccinated group, which indicates no difference in the quality (e.g. avidity) of the generated systemic antibodies.

**Signs and viral load**. High incidence of respiratory rales was observed only in the unvaccinated/challenged control group. The absence of respiratory signs in vaccinated chickens was not consistent with viral loads. While significantly lower levels ($P<0.05$) of IBV RNA were detected in the tracheas of all vaccinated groups in comparison with unvaccinated/challenged control chickens, only chickens vaccinated with the attenuated ArkDPI vaccine exhibited a significant ($P<0.05$) reduction in viral loads among vaccinated chickens (Fig. 2.3). The lowest IBV RNA concentration following challenge was attained by vaccination with the homologous live attenuated Ark vaccine.

**Virus isolation**. As anticipated, the attenuated Ark vaccine was most efficacious against homologous challenge. Consistent with viral RNA quantitation results, chickens vaccinated with the Ark attenuated vaccine strain showed lowest virus isolation from the trachea five days after challenge (Table 2.1). In contrast, chickens vaccinated with rLS/ArkSe.GM-CSF (rLS^7)+Mass as well as chickens vaccinated with Mass exhibited extensive viral shedding. Although challenge virus was isolated from 49.5% of rLS^7+Mass vaccinated chickens compared to 56.2% of Mass vaccinated chickens, the difference was not significant ($P<0.05$) as determined by the Fisher exact test.

**Tracheal histomorphometry**. As seen in Fig. 2.4, significantly increased tracheal mucosal thickness and lymphocyte infiltration ($P<0.05$) was detected in unvaccinated chickens challenged with virulent Ark compared to non-challenged controls. The tracheal mucosa was protected in groups vaccinated with the combination of the recombinant virus and the Mass strain and mucosal histology did not differ ($P<0.05$) from chickens vaccinated with the homologous attenuated ArkDPI vaccine. Significantly higher values for mucosal thickness and lymphocyte infiltration ($P<0.05$), i.e., increased mucosal damage, was detected in chickens vaccinated with Mass alone compared to combined vaccinated chickens.

## 2.5 Discussion

We previously showed that vaccination with rLS -expressing Ark-type Se in chickens induces a relatively low antibody NDV response with average ELISA S/P ratios barely reaching levels considered positive by the guidelines of the ELISA kit (151). In the current study, although chickens vaccinated with the Mass + rLS/ArkSe.GM-CSF combined vaccine elicited significantly higher ($P<0.05$) NDV antibody responses than controls not exposed to NDV, these antibody levels were extremely low indicating limited replication of the recombinant NDV in the chickens. An additional factor explaining the low antibody responses is that early vaccination at 1 day old has been shown to induce weak immune responses due to the immature immune system of newly hatched chickens.

The fact that IBV interferes with the replication of NDV has been known since the early 1960s (111). Thus, to avoid IBV outcompeting NDV, the dosages of each component were adjusted. However, the current results indicate that the dosage adjustment will require further

refinement. The low IBV systemic antibody response detected by virus neutralization test is consistent with this result.

The results of protection against heterologous challenge were inconsistent. On the one hand, the combined vaccine neither reduced the viral load in the trachea (based on both IBV RNA quantitation and virus isolation) nor enhanced the cross-neutralizing ability of systemic antibodies. The lack of reduction of viral shedding is most likely the result of limited replication of the recombinant LaSota virus in the vaccinees. In addition to further dosage adjustments in the combined vaccine discussed above, the virulence of the LaSota strain is significantly weakened as result of the insertion of two foreign genes. Indeed, we previously reported that the insertion of only the IBV Se gene already reduced the intracerebral pathogenicity index and mean death time of the LaSota strain to the level of the B1 strain. In addition, in this experiment we vaccinated chickens at 1 DOA, i.e., chickens with an immature immune system. Finally, in this experiment we did not use a booster vaccination.

Although the reduction of viral shedding after challenge is subpar, the results showing significant reduction of tracheal damage are promising. Indeed, the combined vaccine significantly reduced tracheal damage (as determined by histomorphometry) compared to Mass vaccination alone. Thus, the replication of the recombinant virus was sufficient to enhance the protective ability of the attenuated Mass against Ark challenge. The results showed no difference in virus neutralizing capacity of systemic antibodies in vaccinated chickens. Thus, we speculate that the protection of the tracheal mucosa observed is likely the result of mucosal immunity. Further investigation into quality and quantity of tracheal IgA elicited by vaccination with the combined vaccine is needed.

The current regulation title 9 CFR for licensing IBV vaccines (102) indicates that if less than 90 % of vaccinees are negative for virus recovery following challenge, the master seed virus efficacy is unsatisfactory. This expected level of protection can currently be achieved only by attenuated live IBV vaccines. In the current experiment, only the control using attenuated ArkDPI was able to reduce viral shedding after virulent Ark challenge at a level that meets the 9 CFR regulation. In contrast, recombinant IBV vaccines have in general failed to meet this level of protection (78, 123, 151). Use of a prime-boost regime with attenuated Mass followed by rLS/ArkSe.GM-CSF showed encouraging results as heterologous protection was enhanced; i.e., the prime and boost strategy protected against Ark challenge significantly better than Mass only (78). One option to use rLS expressing tailored IBV Se and comply with the licensing requirement would be to use it as a combined vaccine preparation with attenuated Mass. The Mass component of the combined vaccine would comply with the requirement as attenuated Mass provides excellent immunity against homologous (Mass) challenge. The addition of the tailored rLS would enhance the cross-protection provided by Mass. Finally, in the field the combined vaccine would likely be used as a booster after priming with Mass, which as we know from previous work does enhance cross-protection (78). Ultimately, this strategy should reduce the need for attenuated vaccines of multiple serotypes and therefore reduce the risk of emergence of novel recombinants.

Table 2.1. Virus isolation* from tracheal samples collected from vaccinated chickens five days after challenge with virulent Ark-type IBV.

| Vaccination | Total eggs examined | Total IBV positive | Total IBV negative | Percent IBV positive |
|---|---|---|---|---|
| rLS^7+Mass | 109 | 54 | 55 | 49.5% |
| Mass | 105 | 59 | 46 | 56.2% |
| Ark | 97 | 3 | 94 | 3.10% |

*Virus isolation conducted in chicken embryonated eggs as accepted (8)

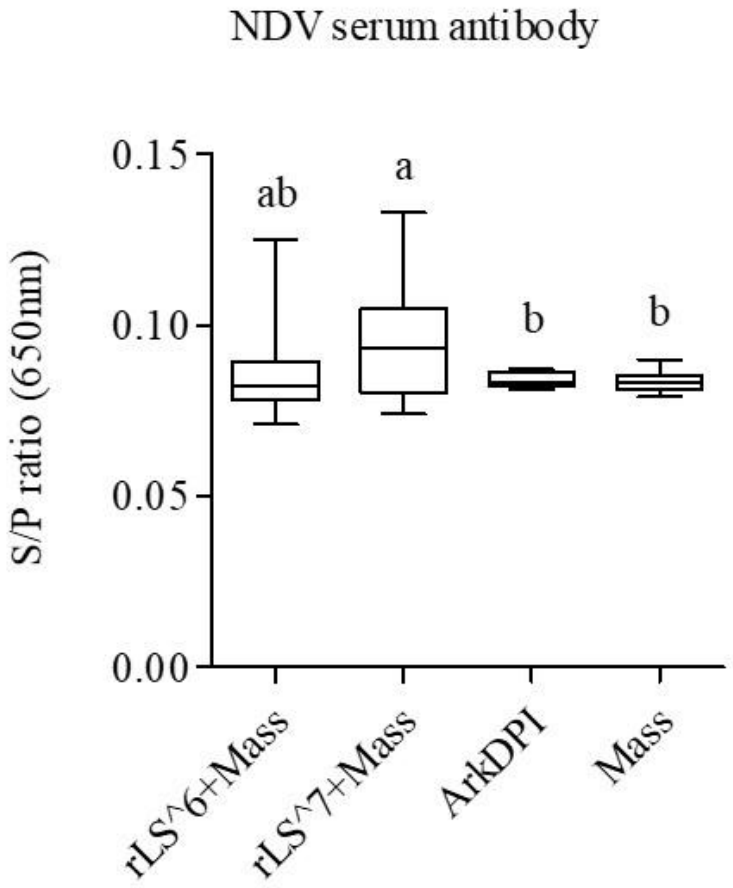


Fig 2.1. NDV antibodies determined by commercial ELISA in chickens (n=21/group) collected 20 days after single vaccination with the combined vaccine containing $10^3$ $EID_{50}$/per bird of attenuated Massachusetts (Mass) and either $10^6$ $EID_{50}$ of rLS/ArkSe.GMCSF (rLS^6+Mass) or $10^7$ $EID_{50}$ of rLS/ArkSe.GMCSF (rLS^7+Mass). Controls included chickens vaccinated with $10^3$ $EID_{50}$/per bird of attenuated Mass and Arkansas (Ark)DPI live vaccines. Data analyzed by ANOVA with the Tukey multiple comparison posttest. Different letters indicate significant differences at $P<0.05$.

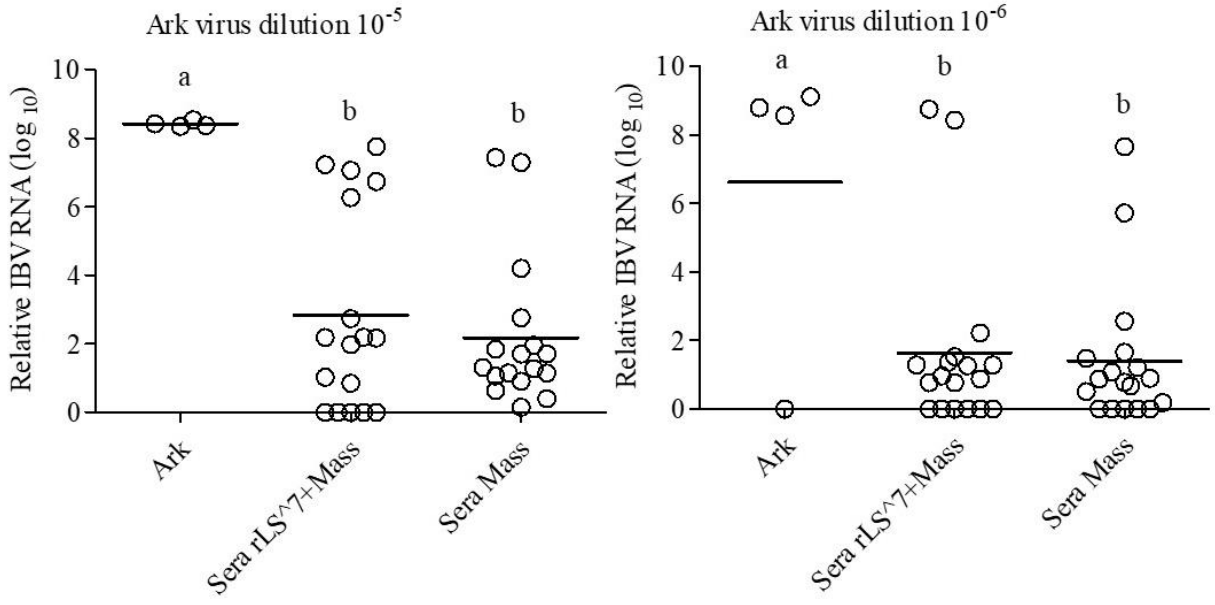


Fig 2.2. Virus neutralizing ability of sera against IBV Ark from chickens vaccinated with rLS^7+Mass or chickens vaccinated with Mass. Relative IBV RNA detected by qRT-PCR in the allantoic fluids of embryonated eggs inoculated with diluted-virus/constant-serum mixtures compared to RNA detected in allantoic fluid of embryonated eggs inoculated with Ark virus alone. For simplicity reasons, we show only Ark virus dilutions ($10^{-5}$, $10^{-6}$) at which neutralization became evident compared to the virus alone. Values analyzed by ANOVA and Tukey posttest. Different letters indicate significant differences at $P<0.05$.

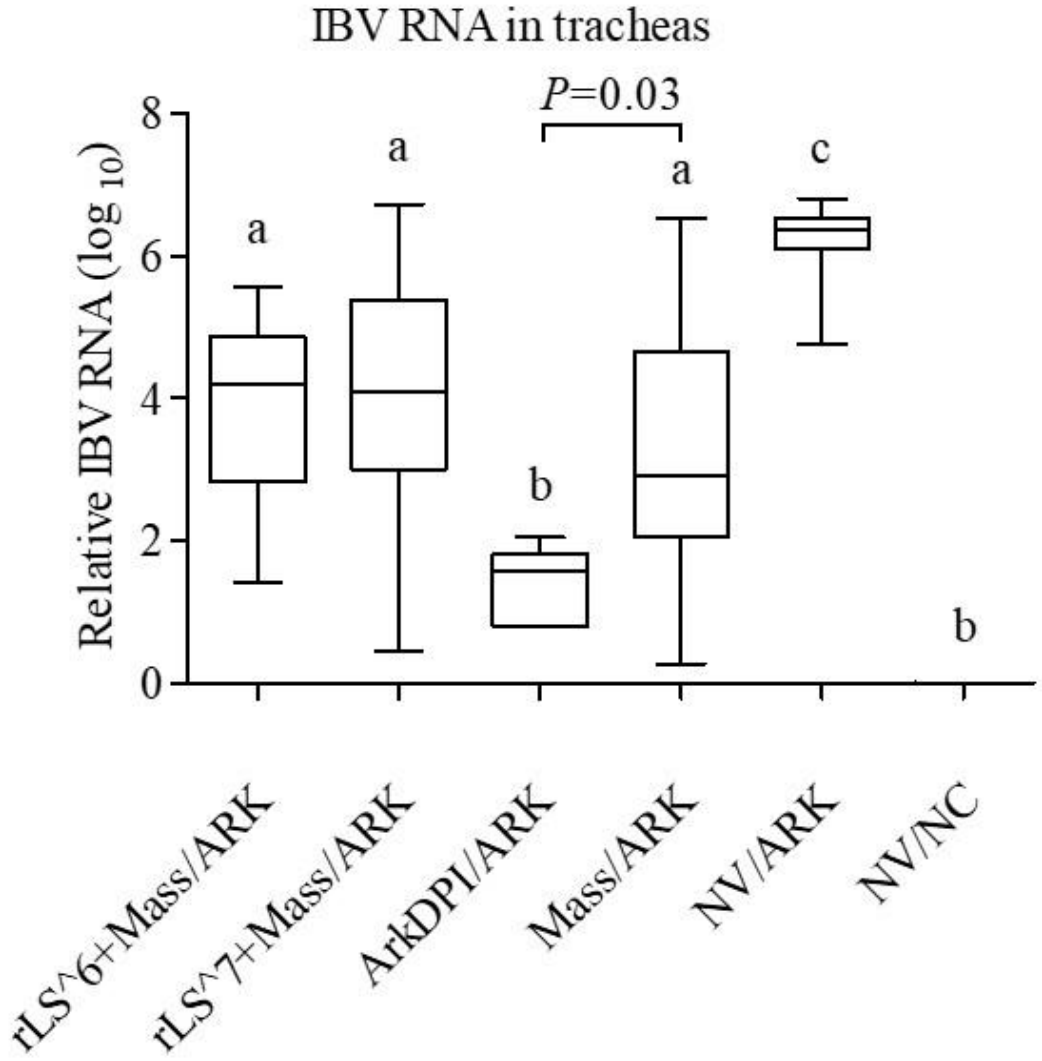


Fig. 2.3. Viral load in the tracheas of chickens (n=21/group) vaccinated as described in the legend of Fig. 2.1. Chickens were challenged with $10^3$ $EID_{50}$/bird of virulent Ark (ARK) at 21 days of age and relative IBV RNA in the tracheas determined by quantitative RT-PCR five days after challenge. NV/ARK = non vaccinated/Ark-challenged; NV/NC = nonvaccinated/nonchallenged. Data analyzed by ANOVA and Tukey posttest (boxes: 25$^{th}$ percentile, median, 75$^{th}$ percentile; whiskers: minimum and maximum). Different letters indicate significant differences at $P<0.05$. Exact $P$ values between distinct groups determined by two-tailed $t$-test.

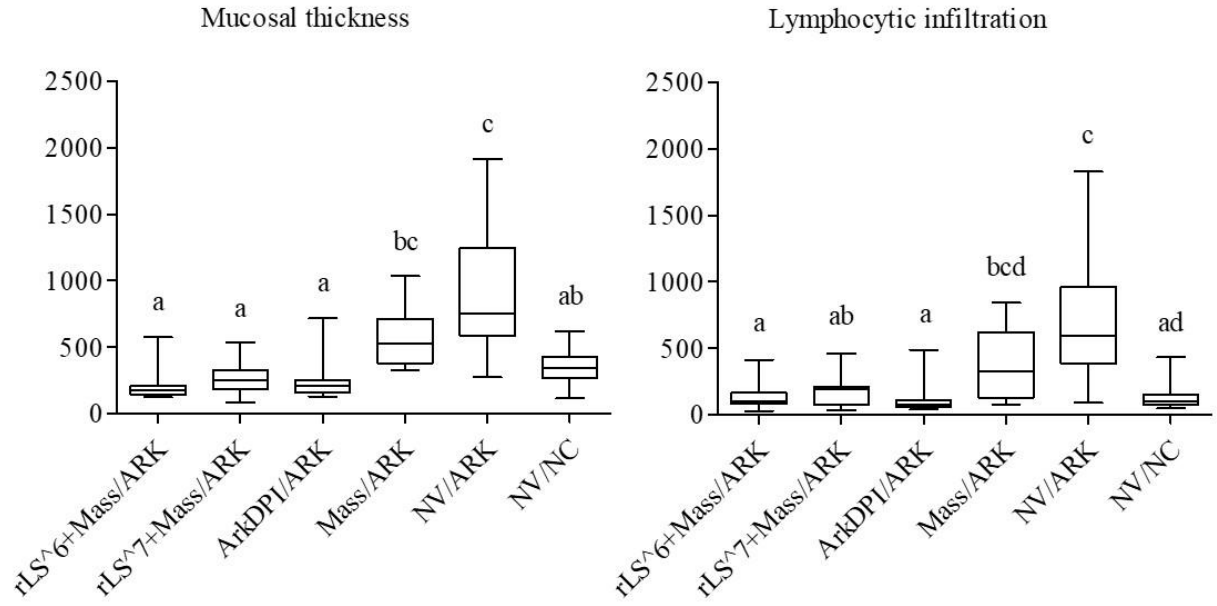


Fig 2.4. Tracheal histomorphometry of chickens vaccinated as described in the legend of Fig. 2.1 and challenged with $10^3$ $EID_{50}$/bird of virulent Ark (ARK) at 21 days of age. Histomorphometry performed 5 days after challenge. NV/NC = non-vaccinated/non-challenged and NV/ARK = non-vaccinated/Ark challenged. Tracheal histomorphometry (A) mucosal thickness and (B) lymphocytic infiltration presented in arbitrary units using ImageJ. Values analyzed by ANOVA and Tukey posttest (boxes: 25$^{th}$ percentile, median, 75$^{th}$ percentile; whiskers: minimum and maximum). Different letters indicate significant differences at $P$<0.05.

CHAPTER 3.

# Immune Responses in the Harderian Gland After Newcastle Disease Vaccination in Chickens with Maternal Antibodies

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## 3.1 Summary

The immune responses in the Harderian gland (HG) were characterized after Newcastle disease virus (NDV) LaSota ocular vaccination in antibody naïve specific pathogen free (SPF) chickens and in chickens of commercial origin with NDV maternally derived antibodies (MDA). Ocular LaSota vaccination of 13-day-old white-leghorn SPF chickens elicited serum antibody levels that consistently increased after day 15 post-vaccination, while the specific IgA response in lacrimal fluids was already detectable on day 10 after vaccination. Eleven days post-vaccination, the relative abundance of B cells as well as T-helper ($CD4^+$), and cytotoxic T cells ($CD8^+$) in HGs was significantly increased achieving maximum frequencies 16 days post-vaccination. In a second experiment, chickens with MDA originating from NDV vaccinated commercial white-leghorn layer breeders as well as white-leghorn SPF chickens were vaccinated with NDV LaSota. The LaSota virus successfully replicated in periocular tissues and in the trachea both in commercial and control SPF chickens after vaccination at 2 or 15 days of age (DOA). Vaccination at 2 DOA did not induce a serum NDV antibody response in chickens of commercial origin. In contrast, seroconversion was elicited in commercial chickens upon vaccination at 15 DOA, likely associated with waning of MDA. Unlike systemic IgG responses,

vaccination at 2 or 15 DOA elicited strong specific IgA responses in lacrimal fluid in commercial chickens. The IgA response was highest 9 days after vaccination and showed a tendency to decline on day 15 post-vaccination. Commercial chickens vaccinated on day 2 of age showed increased B cells in HG both on days 10 and 16 post-vaccination. The expansion of B cells in the HG in these chickens is consistent with increased IgA levels detected in lacrimal fluids. In contrast, control SPF chickens showed a more limited B cell expansion in HG and lower IgA levels. Vaccination on day 15 of age also triggered a greater increase of B cells in HGs in commercial chickens than in control SPF chickens. The B cell response was accompanied by T helper ($CD4^+$) cell expansion occurring both in commercial and control SPF chickens. These cells expanded to a lesser extent when vaccination was performed at 2 DOA compared to vaccination at 15 DOA. Cytotoxic T cells ($CD8^+$) showed significant expansion irrespective of vaccination day and without differences detected between control SPF chickens and chickens with MDA. We conclude that NDV LaSota elicits vigorous humoral and cell immune responses in the Harderian gland. Furthermore, unlike the interference shown by MDA on vaccine-induced serum antibody responses, MDA do not interfere with the mucosal immune response of the HG.

## 3.2 Introduction

Newcastle disease virus (NDV), an avian Orthoavulavirus 1 belonging to the *Paramyxoviridae* family, is responsible for significant disease in multiple wild bird species and enormous economic losses to the world poultry industry (126). The protection of commercial poultry has primarily relied on extensive use of live lentogenic as well as inactivated NDV

strains. The most commonly employed NDV lentogenic strains include LaSota, Hitchner B1, Clone 30 (derived from NDV LaSota), and VG/GA. Despite global availability of vaccines, NDV is highly prevalent in distinct regions of the world and causes sporadic outbreaks of disease in regions considered free of NDV. While a plethora of studies have reported that high levels of systemic antibodies are associated with effective protection against NDV, limited information is available on mucosal cell- and humoral-immune responses to NDV following vaccination (65).

Ocular and nasal mucosal surfaces are the port of entry for a variety of respiratory pathogens including NDV. The head-associated lymphoid tissue (HALT), which includes the Harderian gland (glandula lacrimalis accesoria) (HG), the conjunctiva associated lymphoid tissue, and lymphoid follicles distributed throughout the mucosal surfaces, plays a relevant role as the first line of defense against airborne pathogens. Indeed, a multiplicity of studies during the 1970s showed production of different isotypes of pathogen-specific immunoglobulins by HG lymphocytes (4, 16, 20). Subsequent studies demonstrated the relevance of mucosal immunity to respiratory RNA viruses such as infectious bronchitis virus (IBV) (131, 132). Comparatively, less information is available on mucosal IgA responses to NDV. Mucosal NDV IgA has been detected in tracheal lavages (2) as well as in the lacrimal fluid of chickens inoculated with lentogenic NDV Hitchner B1 (116) and the V4 vaccine strains (71). However, clearance of virus in lacrimal fluids was attributed to IgM rather than IgA (118). The methods used during those studies were cumbersome and likely lack sensitivity compared to current ELISA methods using commercially available anti-chicken IgA antibodies.

Unlike antibody responses, limited information is available on cell-mediated immune (CMI) responses to NDV. Initial work in the 1970s using the leukocyte migration inhibition (LMI) test showed systemic CMI responses first detected on the third day, reaching a peak

during the first or second week and gradually decreasing during the third- and fourth- week following inoculation. The authors discussed that the rapid onset of a CMI response would explain early protection (129). In contrast, a report using blood lymphocytes in a blastogenesis microtest indicated that CMI would play no role in the defense against NDV (113). Those results showed that NDV-inoculated birds were protected only when specific antibody responses were elicited; in contrast, exclusive induction of CMI was not protective. The conclusion that CMI plays no role is questionable because viruses are T-dependent immunogens, i.e. antibody responses to proteins require antigen-specific T-cell ($CD4^+$ cells) assistance for clonal expansion. Thus, it is possible that blood CMI responses do not accurately reflect their importance in protection to NDV simply due to a dilution factor, i.e. the number of NDV-activated T cells in the blood likely fell below detection levels.

Unlike systemic CMI responses, CMI responses in the HALT (both HG and conjunctiva associated lymphoid tissue) to other viruses, e.g. coronaviruses, adenoviruses, using ELISPOT and flow cytometry evaluations are readily detectable after ocular inoculation (135, 137, 138). Furthermore, a clear trend has been detected for B ($Bu\text{-}1^+$), T-helper ($CD3^+CD4^+$), and cytotoxic T ($CD3^+CD8^+$) cell frequencies in the HG after IBV vaccination on days 1 or 7 of age (152). Using immunohistochemistry, Russel et al (117) reported $Bu\text{-}1^+$ and all subpopulations of T cells ($CD4^+$, $CD8^+$, $CD3^+$, TCRγδ, TCRαβ1 TCRαβ2) in the HG to be increased at least three-fold after ocular NDV Hitchner B1 vaccination. Moreover, the authors concluded that T cells, but not B cells, were essential for virus clearance (117).

Another relevant aspect affecting the success of NDV immunization is the presence of maternally derived antibodies (MDA) in progeny chickens. The level of MDA in young chickens is mainly dependent on the intensity of the NDV immunization program used in the

breeders (61). Several studies have shown induction of low levels of specific IgG and IgA elicited by NDV vaccination in chickens with MDA compared to antibody naive chickens (57, 112, 144). Furthermore, absence of specific CMI responses in the spleen were reported in chickens with MDA that were actively vaccinated with NDV (112).

Considering the limited information available on mucosal immune responses to NDV vaccination, in the current work we aimed at further characterizing the immune responses elicited in the HG after vaccination with the lentogenic LaSota strain. In a first trial, we evaluated B and T cell responses in the HG of naïve (SPF) chickens after NDV LaSota vaccination at 13 days of age. We also determined specific IgA responses in lacrimal fluids as well as serum antibodies. In a second trial, we evaluated the effect of MDA on HG immune cell populations and antibody responses after NDV LaSota vaccination using chickens of commercial origin.

## 3.3 Materials and Methods

**Chickens**. The evaluation of immune responses in susceptible chickens was performed in white leghorn chickens free of NDV antibodies which were hatched from SPF embryonated eggs (Charles River, North Franklin, CT). The evaluation of the effect of maternally derived antibody (MDA) was performed in layer-type chickens originating from NDV-vaccinated commercial breeders. The breeders had been primed with a live B1 vaccine, boosted with LaSota, and finally revaccinated with an inactivated NDV vaccine prior to onset of lay. Fertile eggs from these breeders were hatched in our facilities and chickens designated “commercial chickens” or “chickens with MDA” throughout this manuscript. All chickens were maintained in Horsfall-

type isolators in biosafety level 2 facilities. Experimental procedures and animal care were performed in compliance with applicable federal and institutional animal care and use guidelines. Auburn University College of Veterinary Medicine is an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International)-accredited institution.

**Vaccine virus**. The NDV LaSota strain was originally obtained from the American Type Culture Collection (ATCC). The NDV LaSota virus stock was titrated by 50% embryo infective dose ($EID_{50}$) in 9-day-old SPF chicken embryos as generally accepted (141). Embryos were considered positive if hemagglutinating activity was detected in their allantoic fluid.

**Trial 1**. Adaptive immune responses of naïve (SPF) chickens after ocular NDV LaSota vaccination. Two groups of SPF chickens (n=27/group) were established. Chickens of group 1 were individually vaccinated at 13 days of age via the ocular route with 100 µl volume of virus suspension (50 µl in each eye) containing $10^4$ $EID_{50}$ of NDV LaSota. Chickens were vaccinated at this age to avoid potential weaker responses due to immune system immaturity inherent to newly hatched chickens (152). Group 2 of age-matched SPF chickens served as unvaccinated controls. Sera and lacrimal fluids were collected from all chickens on days 10, 15, 21, and 28 after vaccination for NDV antibody determinations. HGs were collected from 9 chickens of each group on days 11, 16, and 29 post-vaccination for determination of B and T lymphocyte relative frequency. The frequencies are relative to the total number of “live” and “single” events in the selected window of “lymphocytes” as shown in Supplementary Figure 3.1.

**NDV antibody in naïve (SPF) chickens after vaccination**. NDV serum antibody levels were determined using a commercial NDV ELISA kit (IDEXX Laboratories Inc., Westbrook, ME) following the manufacturer's recommendations. Lacrimal fluids (50-200/µl from each bird) were obtained as described previously (133) and NDV IgA levels determined using the commercial kit's NDV coated ELISA plates of the same source. Tear samples were diluted 1:100 and specific IgA detected using a horseradish peroxidase–conjugated polyclonal goat-anti-chicken IgA antibody (Southern Biotechnology Associates Inc., Birmingham, AL) instead of the secondary antibody provided in the commercial ELISA kit. Absorbance was measured at 650 nm. Antibody levels determined in vaccinated and unvaccinated groups were analyzed by analysis of variance (ANOVA) followed by the Tukey's multiple comparisons posttest. Differences were considered significant at $P<0.05$.

**Cell immune responses in the HG of naïve chickens**. Relative abundance of B and T lymphocytes in the HG of vaccinated and non-vaccinated chickens was performed by flow cytometry conducted essentially as previously described (149) with minor modifications. In brief, at each sampling time, the HGs were collected from 9 chickens of each group immediately after euthanasia and placed in cold Ariaans complete medium (13). In order to obtain enough cells for flow cytometry, for both vaccinated and non-vaccinated groups the HGs from three chickens were pooled, resulting in three pools for each group for each sampling day. Cells were obtained from the HG pools by mechanical disruption followed by enzyme treatment with collagenase type IA (Sigma Aldrich Corp., St. Louis, MO). Live mononuclear cells were isolated by density centrifugation over a Histopaque density gradient (1.077 g/ml) (Sigma Aldrich Corp., St. Louis, MO) as described previously (150). B- and T-cell relative frequencies were determined

by flow cytometry as previously described (152). In brief, the following commercially available antibody conjugates (Southern Biotechnology Associates Inc., Birmingham, AL) were used: phycoerythrin conjugated-mouse-anti-chicken Bu-1 monoclonal antibody; Alexa Fluor 647–conjugated anti-CD3 monoclonal antibody; fluorescein isothiocyanate–conjugated mouse-anti-chicken CD8; and Pacific Blue–conjugated mouse-anti-chicken CD4. A volume of 0.8 µl of each antibody conjugate was diluted in a volume of 50µl of buffer. One million cells were stained with 50 µl of the antibody combination. After washes and dead cell exclusion, cells were filtered and analyzed immediately in a CytoFLEX LX cytometer (Beckman Coulter, Indianapolis, IN). B (Bu-1$^{+}$), T-helper (CD3$^{+}$CD4$^{+}$), and cytotoxic T (CD3$^{+}$CD8$^{+}$) lymphocytes were counted using the CytExpert software, version 2.3.1.22. Flow cytometry data were processed using Kaluza Analysis Software, version 2.2.1. A small lymphocyte gate was defined using the single stained controls for each antibody and Ghost Dye red 780 (Tonbo Biosciences, San Diego, CA) in each sample. Cell relative abundances were compared at 11-, 16-, and 29-days post-vaccination between vaccinated chickens and non-vaccinated age-matched controls by Student's t test.

**Trial 2**. Effect of MDA on adaptive immune responses after NDV LaSota vaccination. Five groups of chickens (n=24/group) were used. Three groups included chickens with MDA from commercial origin. Serum NDV-specific MDAs in commercial chickens were determined by ELISA on day 2 post hatch using a commercial NDV ELISA kit (IDEXX Laboratories Inc., Westbrook, ME). We also included two SPF chicken groups to compare results within the same trial and to compare with results of trial 1. A group of commercial chickens and a group of SPF chickens were vaccinated at 2 days of age. A second group of commercial chickens as well as age-matched SPF chickens were vaccinated at 15 days of age. Vaccination was performed as

described in trial 1 (above) using a volume of 100µl per bird containing $10^4$ $EID_{50}$ of NDV LaSota. The remaining group of commercial chickens served as age-matched non-vaccinated controls. NDV replication after vaccination was assessed by viral RNA copy numbers in tracheal swabs and lacrimal fluids determined by qRT-PCR.

**Vaccine virus replication in chickens with MDA and SPF chickens**. Lacrimal fluids and tracheal swabs were collected from all chicken groups on days 9 and 15 after vaccination for NDV RNA determination by qRT-PCR. Briefly, lacrimal fluids were collected as described above. Tracheal swabs were placed in tubes containing 0.5 ml of PBS and maintained frozen at -80C until RNA extraction was performed. NDV RNA was extracted using a QIAamp® Viral RNA Mini Kit utilizing a QIAcube® following the manufacturer's instructions (QIAGEN, Hilden, Germany). Viral RNA was eluted in 60µl total volume. Relative viral RNA was determined by qRT-PCR using primer and probes targeting the L-gene as described by others (127). Viral RNA data were analyzed by Kruskal-Wallis test followed by a multiple comparisons posttest. Differences were considered significant at $P<0.05$.

**NDV antibody in commercial chickens after vaccination**. Sera and lacrimal fluids were collected 9 and 15 days after vaccination and tested by ELISA as describe in trial 1 (see above).

**Cell immune responses in the HG of commercial and SPF chickens**. HGs were collected from 12 chickens per group on days 10 and 16 after vaccination. The HGs of four chickens were pooled. Thus, three pools of HGs were analyzed per for each chicken group. B and T lymphocyte determinations were conducted by flow cytometry as described in trial 1 (above). Data collected

for chicken groups at different times post-vaccination were compared with age-matched non-vaccinated controls by ANOVA and Tukey's posttest.

## 3.4 Results

### Trial. 1. Adaptive immune responses of antibody naïve (SPF) chickens after ocular LaSota vaccination.

**NDV antibody responses in sera and lacrimal fluids**. As shown in Fig. 3.1, ocular NDV LaSota vaccination of 13-day-old SPF chickens elicited antibody responses in both sera (Fig. 3.1A) and lacrimal fluids (Fig. 3.1B). Serum NDV-antibody levels consistently increased between 15- and 28-days post-vaccination. Compared to unvaccinated controls, the level of NDV-antibodies in vaccinated chickens achieved significance ($P$<0.05) 21 days after vaccination. In contrast and as expected, significantly increased ($P$<0.05) NDV-specific IgA levels in lacrimal fluids of vaccinated chickens were detectable much earlier on day 10 after vaccination. Although specific IgA levels showed a slight tendency to continue to augment with time after vaccination, this slight increase did not achieve statistical significance.

**B and T cells in HG**. As shown in Fig. 3.2, ocular NDV LaSota vaccination elicited both B (Fig. 3.2A) and T cell (Figs. 3.2B and 3.2C) expansions in the HG of SPF chickens. Eleven days post-vaccination both B (Bu-1$^+$) and T cells (CD3$^+$CD4$^+$, and CD3$^+$CD8$^+$) showed significant increases ($P$<0.05) compared to age-matched non-vaccinated controls. The maximum cell abundance for all measured cell types was detected on day 16 post-vaccination. T cell

populations subsequently decreased to the levels of unvaccinated controls at 29 days post-vaccination. Similar cell counts were detected for both T-helper cells ($CD3^+CD4^+$) and cytotoxic T cells ($CD3^+CD8^+$) at all measured time points. Compared to controls, only B cells ($Bu\text{-}1^+$) maintained higher concentrations in the HG, though not achieving statistical significance, through 29 days after vaccination.

**Trial 2. Effect of MDA on immune responses after ocular NDV LaSota vaccination.**

**Serum NDV maternally derived antibody in chickens of commercial origin**. Fig. 3.3 shows NDV serum antibody levels detected at 2 days of age in chickens of commercial origin vs. SPF controls. As expected, commercial chickens showed anti-NDV serum antibodies of maternal origin, while SPF chickens were NDV antibody-negative.

**LaSota vaccine virus in the trachea and lacrimal fluids of chickens with MDA and SPF chickens**. As seen in Fig. 3.4, the LaSota virus replicated successfully both in chickens with MDA and SPF chickens irrespective of vaccination occurring on day 2 or 15 of age. For either vaccination day and both chicken types, highest viral load, as determined by NDV RNA levels, was detected in both tracheal swabs (Figs. 3.4A and 3.4B) and lacrimal fluids (Figs. 3.4C and 3.4D). While all chickens, commercial and SPF, exhibited virus RNA in lacrimal fluids on day 9 after vaccination on day 2, the incidence of viral RNA was reduced in tracheal swabs as 8/12 were positive (Table 3.1). A significantly lower relative viral RNA ($P<0.05$) was detected on day 15 after vaccination both in lacrimal fluids and tracheal swabs without significant differences with controls, except tracheal swabs of commercial chickens vaccinated at 15 days of age

showing significantly higher viral loads. It was also notable that viral replication in 15 days of age SPF birds was not significantly different from controls at 9- and 15-days post vaccination.

**Antibody responses of chickens with MDA after NDV LaSota vaccination**. NDV-specific antibody levels in sera and specific IgA in lacrimal fluids were determined 9 and 15 days after vaccination performed on days 2 (Fig. 3.5A) or 15 of age (Fig. 3.5B). Both figures show that non-vaccinated control chickens of commercial origin exhibited maternally derived NDV serum antibodies throughout the experimental period. As seen in Fig. 3.5B, antibodies were still detectable on day 30 of age. No differences in serum antibody levels were detected between commercial non-vaccinated chickens and commercial chickens vaccinated at 2 days of age at 9 and 15 days after vaccination (Fig. 3.5A); i.e. vaccination at 2 days in chickens with high levels of MDA did not induce a detectable active systemic antibody response. In SPF chickens vaccinated at 2 days of age, no detectable antibody response was evident 9 days post-vaccination compared to unvaccinated controls. However, vaccinated SPF chickens did exhibit a slight antibody increase 15 days post-vaccination, with levels not differing significantly from non-vaccinated commercial chickens with MDA. In contrast, vaccination performed in both commercial and SPF chickens at 15 days of age induced a significant increase ($P<0.05$) in NDV antibodies compared to unvaccinated controls as determined both at 9- and 15-days post-vaccination (Fig. 3.5B). Nine days after vaccination SPF chickens did not differ significantly from commercial controls. The increase was distinctively evident on day 15 post-vaccination. Following vaccination at 15 days of age, no significant differences were detected between the antibody levels achieved by vaccinated commercial origin chickens compared to SPF chickens.

Fig. 3.6 shows NDV-specific IgA levels detected in lacrimal fluids of commercial chickens vaccinated at 2 (Fig. 3.6A) or 15 (Fig. 3.6B) days of age. As expected, unvaccinated commercial chickens showed absence of specific IgA in tears. Vaccination at 2 days of age resulted in increased NDV-specific IgA in both commercial and SPF chickens as measured 15 days after vaccination with no significant difference between commercial and SPF chickens. However, no specific response was detectable in either group 9 days post-vaccination. In contrast, vaccination at 15 days of age induced a significant increase ($P<0.05$) of specific IgA as determined both at 9 and 15 days after vaccination. The IgA response was highest 9 days after vaccination and showed a tendency to decline on day 15 post-vaccination.

**B and T cell responses in the HG of chickens with MDA after NDV LaSota vaccination**. As shown in Figs. 3.7 – 3.9, ocular NDV LaSota vaccination elicited both B and T cell expansions in the HG of commercial and naïve (SPF) chickens compared to age-matched unvaccinated controls both 10 and 16 days after vaccination.

Fig. 3.7 shows B cell (Bu-$1^{+}$) abundance in the HG after NDV vaccination on days 2 (Fig. 3.7A) or 15 (Fig. 3.7B) of age. In chickens vaccinated on day 2 of age, B cells were increased on both days 10 and 16 post-vaccination in chickens with MDA compared to vaccinated SPF chickens as well as unvaccinated commercial controls. On day 16 the difference was significant ($P<0.05$). Interestingly, vaccination on day 2 of age in SPF chickens did not result in increased B cells compared to unvaccinated commercial controls on either of the days determined. In contrast, vaccination on day 15 of age induced a significant increase in B cells ($P<0.05$) on both days 10 and 16 post-vaccination in commercial chickens compared to either of the other chicken groups. Vaccination on day 15 did elicit an increase in B cells in SPF chickens

compared to unvaccinated commercial controls, as their cell count did not differ significantly from vaccinated commercial chickens. A similar result had been obtained in trial 1 where B cells were significantly increased on day 16 post-vaccination performed on day 13 of age.

Fig. 3.8 shows T-helper cell ($CD3^+CD4^+$) abundance in the HG after vaccination on days 2 (Fig. 3.8A) or 15 (Fig. 3.8B) of age. As seen in Fig. 3.8A, $CD3^+CD4^+$ cell expansion occurred both in commercial and SPF chickens as a consequence of vaccination on day 2 of age. While the increase seen in SPF chicks was significant compared to commercial unvaccinated controls ($P<0.05$), these cells also increased in commercial chickens but without achieving significance compared to either vaccinated SPF or unvaccinated commercial chicks. In contrast, vaccination on day 15 of age resulted in a considerable and significant increase of $CD3^+CD4^+$ cells in both commercial and SPF chickens 16 days post-vaccination.

Unlike T-helper cells, at 16 days post-vaccination cytotoxic T cells ($CD3^+CD8^+$) showed a significant expansion to similar levels irrespective of vaccination day i.e., vaccination on days 2 (Fig. 3.9A) or 15 (Fig. 3.9B). No significant differences were detected between MDA-negative (naïve) chickens and commercial chickens with MDA.

## 3.5 Discussion

The lentogenic Hitchner B1 and LaSota NDV strains are extensively used worldwide to protect chickens against NDV outbreaks. While these vaccines are effective at preventing severe disease and mortality in challenged chickens, they neither prevent infection nor virus shedding (22, 76), unless doses $\geq 10^6$ $EID_{50}$ are used (32). Novel NDV vaccines have been assessed by comparing viral shedding among vaccinated and challenged chickens (100) but

measurements of elicited immune responses have not been included in those evaluations. As discussed above (see Introduction section), limited information is available on cell immune responses triggered by NDV vaccination, and, to our knowledge, information on mucosal immune responses is mostly unavailable. In contrast, a considerable amount of information is available on mucosal responses generated against other RNA respiratory viruses, such as IBV. B cells, specific lacrimal IgA, and mucosal cellular responses involving $CD4^+$, $CD8^+$, and other T-cell subsets have been associated with vaccinal immunity and protection conferred against virus challenge (56). Therefore, the data collected in the current work adds to basic immunology knowledge and could be used as reference values for the evaluation of novel NDV vaccine candidates.

In trial 1, we characterized the antibody and cell immune responses of SPF chickens after vaccination. We chose vaccinating at 13 days of age to evaluate the response of a mature and fully functional immune system, which according to previous studies occurs at ~10 days post-hatch (136, 152). The results show that ocular NDV LaSota vaccination elicits serum antibody levels consistently increasing after day 15 post-vaccination. In contrast, the IgA response is already detectable on day 10 after vaccination, which is likely due to the initial replication of the virus in periocular tissues including the HG, which clearly stimulates a strong immune response. We previously reported lymphocytic infiltration of the HG 7–10 days after ocular inoculation with IBV (132). In the present study, we found that 11 days post NDV vaccination, the relative abundance of B cells as well as $CD4^+$ and $CD8^+$ T cells in HGs was significantly increased, achieving highest frequencies 16 days post-vaccination. Interestingly, T cell populations decreased to the levels of unvaccinated controls at 29 days post-vaccination, but B cells maintained higher concentrations through 29 days after vaccination.

One relevant cause for reduced vaccine efficacy in progeny chickens is neutralization of vaccine virus by MDA. In the current study, we used progeny chickens from NDV-vaccinated commercial breeders showing high levels of MDA. MDA highest levels are detected close to hatch while they wane within a few weeks after hatch. MDA waning was evident in non-vaccinated controls of commercial origin still exhibiting remnants of detectable NDV serum antibody on day 30 of age. As expected, vaccination at 2 days of age in chickens with high levels of MDA did not induce an active systemic antibody response. In contrast, SPF chickens did exhibit a slight serum antibody increase 15 days post-vaccination. Vaccination at 15 days of age, i.e. with lower MDA, induced a significant increase in NDV serum antibody in both commercial and SPF chickens compared to unvaccinated controls both at 9- and 15-days post-vaccination. The fact that no significant differences were detected between systemic antibody levels achieved by vaccinated commercial chickens and SPF chickens indicates that the lower levels of MDA at 15 days of age did not interfere with systemic responses elicited by vaccination.

Current accepted knowledge indicates that monomeric immunoglobulins, mainly IgG, are transferred from the breeders to the progeny chickens. It is also known that some systemic IgG may transudate to mucosal surfaces (133). Unlike dimeric IgA, which includes a secretory component to protect the immunoglobulin from destruction by mucosal enzymes, transudate monomeric IgG lacks protection against enzymatic activity and is therefore expected to provide limited neutralization capacity and protection at mucosal levels. The current results showed MDA was not able to impede successful replication of the LaSota virus in periocular tissues and in the trachea. Both lacrimal fluids and tracheal swabs showed highest concentrations of viral RNA 9 days post-vaccination irrespective of vaccination at 2 or 15 days of age. It was noticeable that the incidence of positive chickens differed; while in lacrimal fluids 11/12 chickens were

positive for NDV RNA, in tracheal swabs 7/12 chickens were positive for NDV RNA (Table 3.1).

Unlike systemic IgG responses, vaccination at 2 days of age elicited production of specific IgA in both commercial and SPF chickens detectable at 15 days after vaccination. A much stronger IgA response in tears was detected when vaccination was performed at 15 days of age. NDV-specific IgA responses were highest 9 days after vaccination and showed a tendency to decline on day 15 post-vaccination.

Ocular LaSota vaccination elicited both B and T cell expansions in the HG of commercial and antibody naïve chickens compared to age-matched unvaccinated controls. Commercial chickens vaccinated on day 2 of age showed increased B cells on both days 10 and 16 post-vaccination. The expansion of B cells in the HG in these chickens is consistent with the increased IgA levels detected in their lacrimal fluids. Unlike commercial chickens, SPF chickens (without MDA) showed limited B cell expansion, which was also consistent with the lower IgA levels achieved by these groups. HG follicular dendritic cells have been demonstrated to play an important role in antibody responses (36). Thus, a speculative explanation might be that even limited neutralization activity from maternal IgG transudate is able to trigger enough dendritic cell activity in the HG to increase mucosal B cell clonal expansion in chickens with MDA compared to antibody naïve chickens. An analogous mechanism has been described for the use of immune complex vaccines for infectious bursal disease, where follicular dendritic cells augment the expansion and activity of B cells (72). Although vaccination on day 15 of age also generated a stronger increase of B cells in commercial chickens than in SPF chickens, B cells in SPF chickens measured 16 days after vaccination showed a tendency to increase. A similar result had been obtained in trial 1 in which B cells were significantly increased on day 16 post-

vaccination performed on day 13 of age. As expected, the B cell expansion was accompanied by T helper ($CD4^+$) cell expansion occurring both in commercial and SPF chickens as a result of vaccination, as determined on both days 10 and 16 post vaccination. These cells expanded to a lesser extent when vaccination was performed on day 2 of age compared to vaccination on day 15 of age. The difference is likely associated with the immaturity of the immune system of younger chickens. T-helper cell activation also resulted in cytotoxic T cells ($CD8^+$) showing significant expansion irrespective of vaccination day and without differences detected between MDA-negative SPF chickens and commercial chickens with MDA. CTL activity has been correlated with reduction in viral load and protection against acute infection with other RNA viruses (31, 92, 96). We conclude that NDV LaSota elicits vigorous humoral and cell immune responses in the HG in antibody naïve chickens. Furthermore, unlike the interference shown by MDA on systemic immune responses following vaccination, MDAs do not interfere with the mucosal immune response of the HG.

Table 3.1. NDV RNA positive samples by qRT-PCR per group * 9 and 15 days after vaccination with NDV LaSota.

| Age (day) at vaccination | Sample type | No. of samples | Treatment group | | | |
|---|---|---|---|---|---|---|
| | | | Com/9 DPV** | Com/15 DPV | SPF/9 DPV | SPF/15 DPV |
| 2 | Tracheal swabs | 12 | 7 | 3 | 8 | 3 |
| 2 | Lacrimal fluid | 12 | 11 | 2 | 12 | 4 |
| 15 | Tracheal swabs | 12 | 9 | 8 | 5 | 3 |
| 15 | Lacrimal fluid | 12 | 4 | 0 | 1 | 0 |

* Com = commercial chickens with MDA; SPF = specific pathogen free.

** DPV = days post-vaccination.

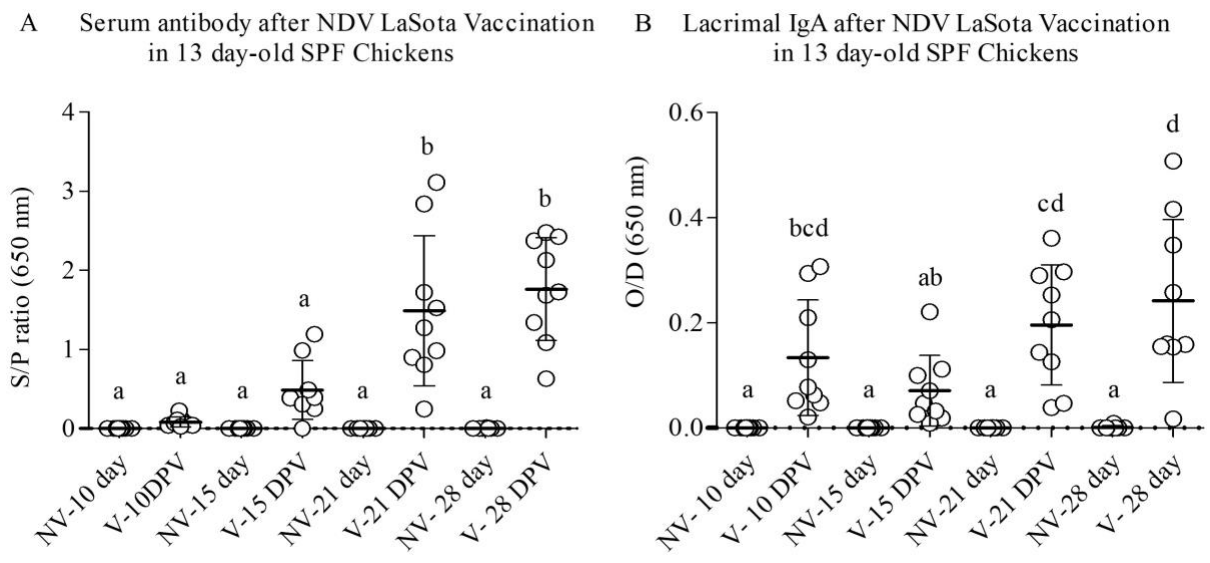


Fig. 3.1. Antibody determined by ELISA at 10, 15, 21, 28 days post vaccination (DPV) of SPF chickens vaccinated (V) on day 13 of age with NDV LaSota. (A) Serum NDV antibodies expressed as sample-to-positive (S/P) ratio. (B) NDV IgA in lacrimal fluid expressed as optical density (O/D). NV = non-vaccinated age-matched controls. Individual values, means, and standard deviations are shown. Different letters indicate significant differences determined by ANOVA and Tukey's posttest at $P<0.05$.

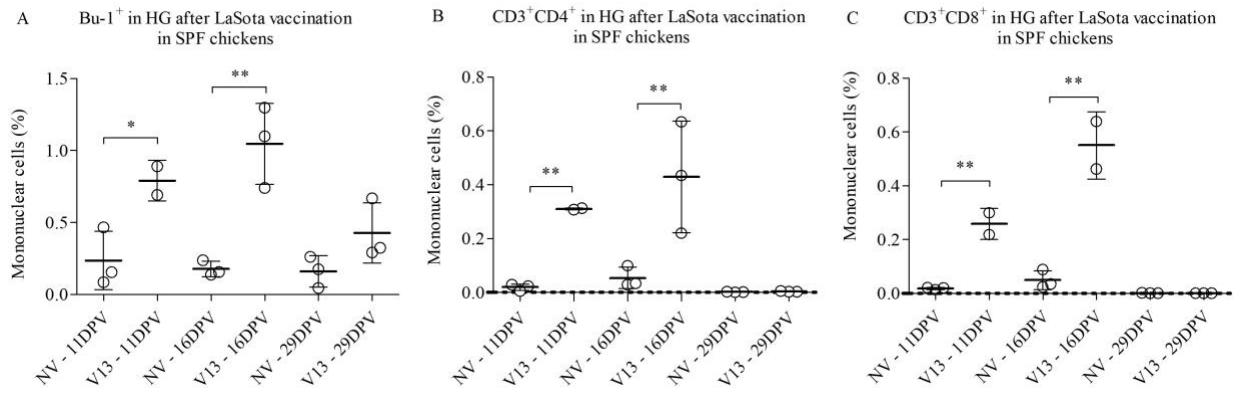


Fig. 3.2. Relative frequencies of B and T lymphocyte populations in the Harderian glands (HG) of SPF chickens vaccinated (V) on day 13 of age with NDV LaSota, determined by flow cytometry. (A) Bu-1$^+$ = B cells, (B) CD3$^+$CD4$^+$ = T-helper cells, and (C) CD3$^+$CD8$^+$ = cytotoxic T cells expressed as percentage of total mononuclear cells detected in the HG of chickens 11-, 16-, and 29-days post vaccination (DPV). NV = non-vaccinated age-matched controls. Each value represents the cells counted in pools of HGs collected from 9 individuals per time point. Means and standard deviations are shown. Significant differences between vaccinated and age matched unvaccinated controls determined by Student's *t*-test: * indicates $P$ = 0.01-0.05; ** indicates $P$ = 0.001-0.01).

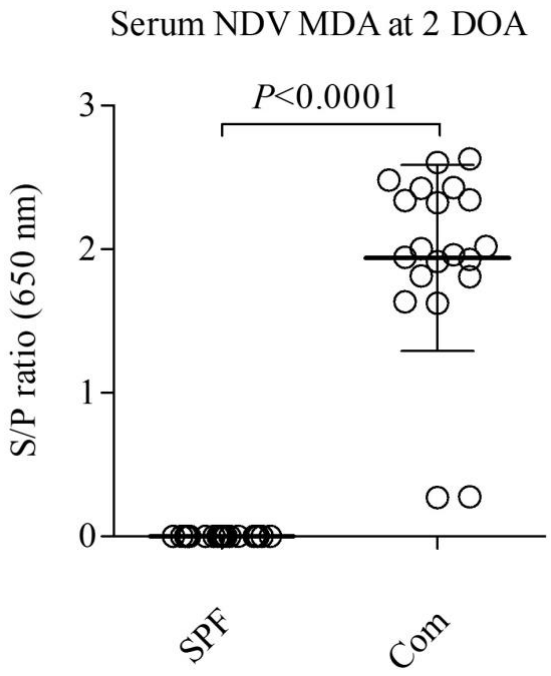


Fig. 3.3. Serum NDV maternally derived antibodies (MDA) determined by ELISA at 2 days of age (DOA). S/P = sample-to-positive ratio. Com = commercial chickens originating from NDV vaccinated breeders; SPF =specific pathogen free chickens. Individual values, mean, and standard deviations are shown. Difference evaluated by Student's *t*-test. Exact *P* value indicated.

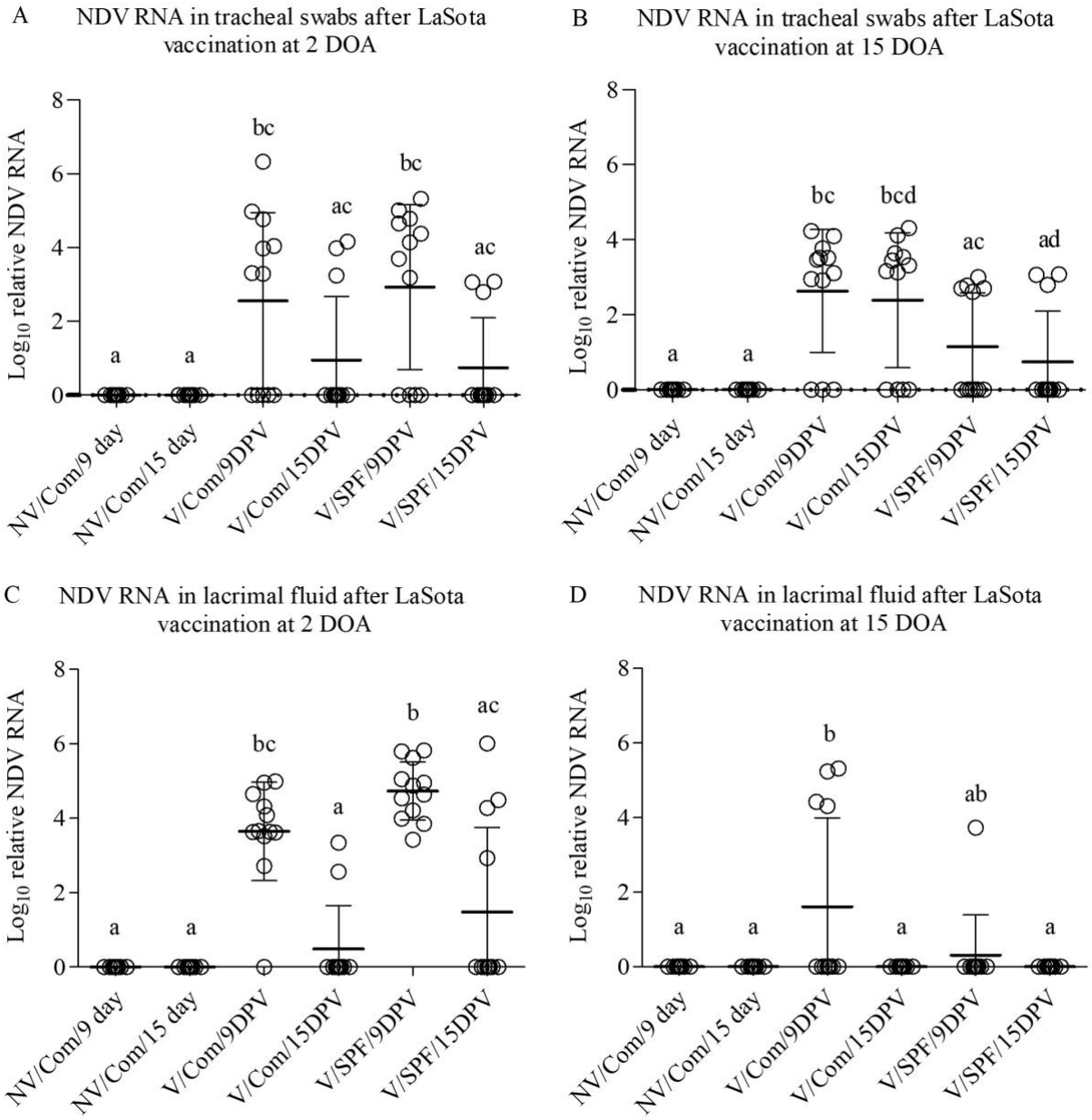


Fig. 3.4. Viral loads (NDV RNA) in tracheal swabs (A, B) and lacrimal fluids (C, D) of chickens of commercial origin (Com), i.e., with maternally derived antibodies, and SPF chickens vaccinated at 2 or 15 days of age (DOA) with NDV LaSota, determined 9 and 15 days post vaccination (DPV) by qRT-PCR. V = vaccinated. NV = non-vaccinated age matched controls. Individual values, average, and standard deviations are shown. Different letters indicate significant differences at $P<0.05$ determined by ANOVA and Tukey's posttest.

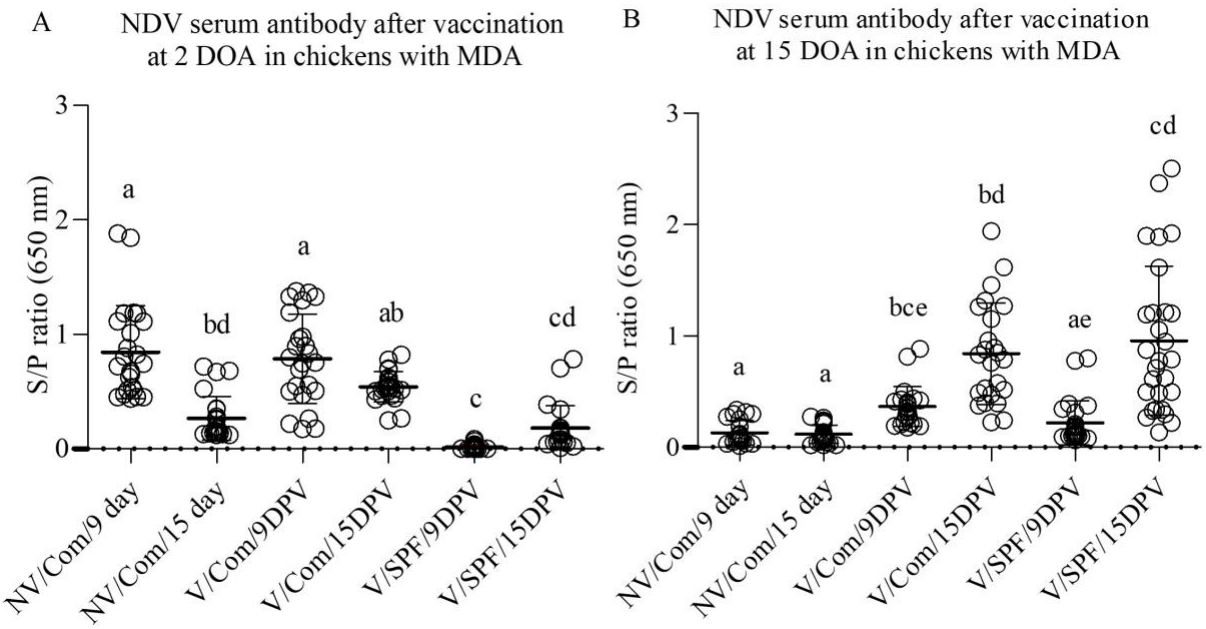


Fig. 3.5. Serum NDV-specific antibodies determined by ELISA in commercial chickens with MDA (Com) and SPF chickens vaccinated with NDV LaSota as described in Fig. 3.4. Sera collected 9 and 15 days post vaccination (DPV). S/P = sample-to-positive ratio. (A) vaccination at 2 DOA; (B) vaccination at 15 DOA. V = vaccinated; NV = nonvaccinated age-matched controls. Individual values, average, and standard deviation are shown. Different letters indicate significant differences at $P<0.05$ determined by ANOVA and Tukey's posttest.

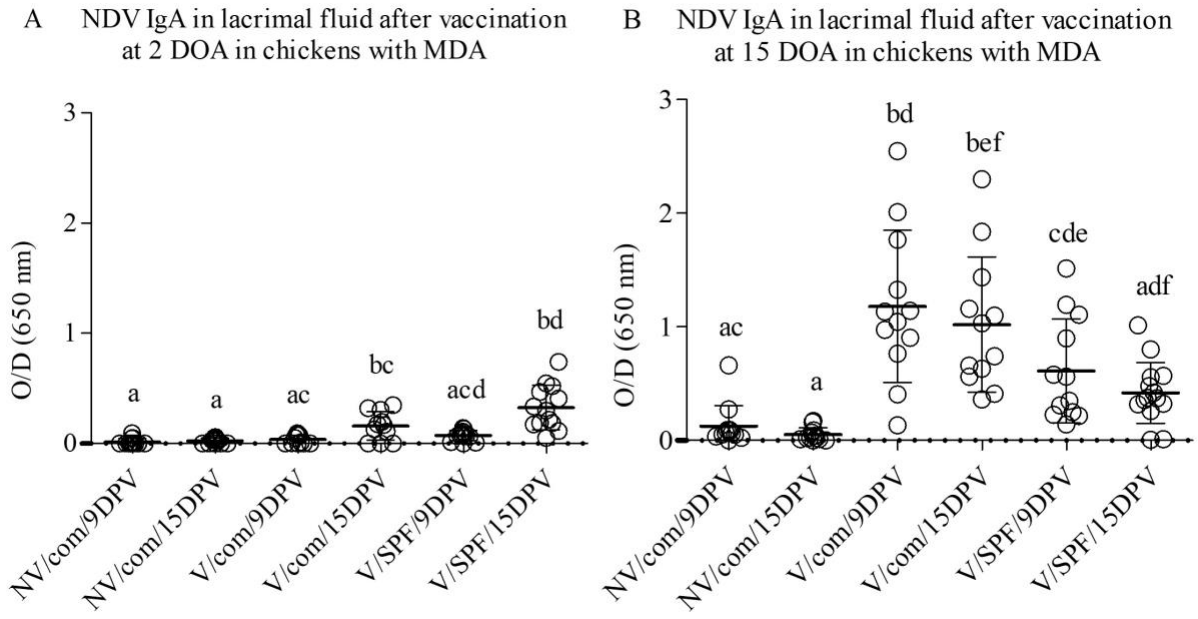


Fig. 3.6. NDV-specific IgA determined by ELISA in lacrimal fluids of commercial chickens with MDA (Com) and SPF chickens vaccinated with NDV LaSota as described in Fig. 3.4. Lacrimal fluids collected 9 and 15 days post vaccination (DPV). O/D = optical density. (A) vaccination at 2 DOA; (B) vaccination at 15 DOA. V = vaccinated; NV = nonvaccinated age-matched controls. Individual values, average, and standard deviation are shown. Different letters indicate significant differences at $P<0.05$ determined by ANOVA and Tukey's posttest.

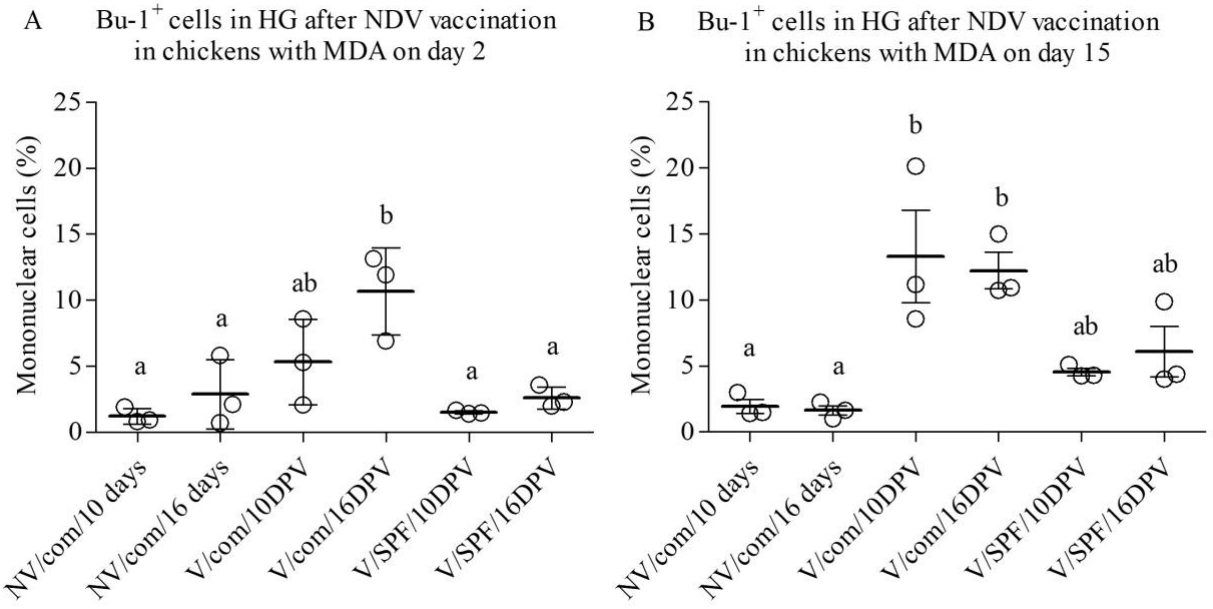


Fig. 3.7. Relative frequency of B lymphocytes (Bu-1$^{+}$) expressed as percent of total mononuclear cells in the Harderian glands (HG) of commercial chickens with MDA (Com) and SPF chickens vaccinated with NDV LaSota as described in Fig. 3.4. Harderian glands collected from each group of chickens at 10 and 16 days post vaccination (DPV). (A) vaccination at 2 days of age (DOA); (B) vaccination at 15 DOA. V = vaccinated; NV = nonvaccinated age-matched controls. Each value represents the cell counts found in pools of HG collected from four chickens, i.e. 12 individuals per time point. Averages and standard deviations are shown. Different letters indicate significant differences at $P<0.05$ determined by ANOVA and Tukey's posttest.

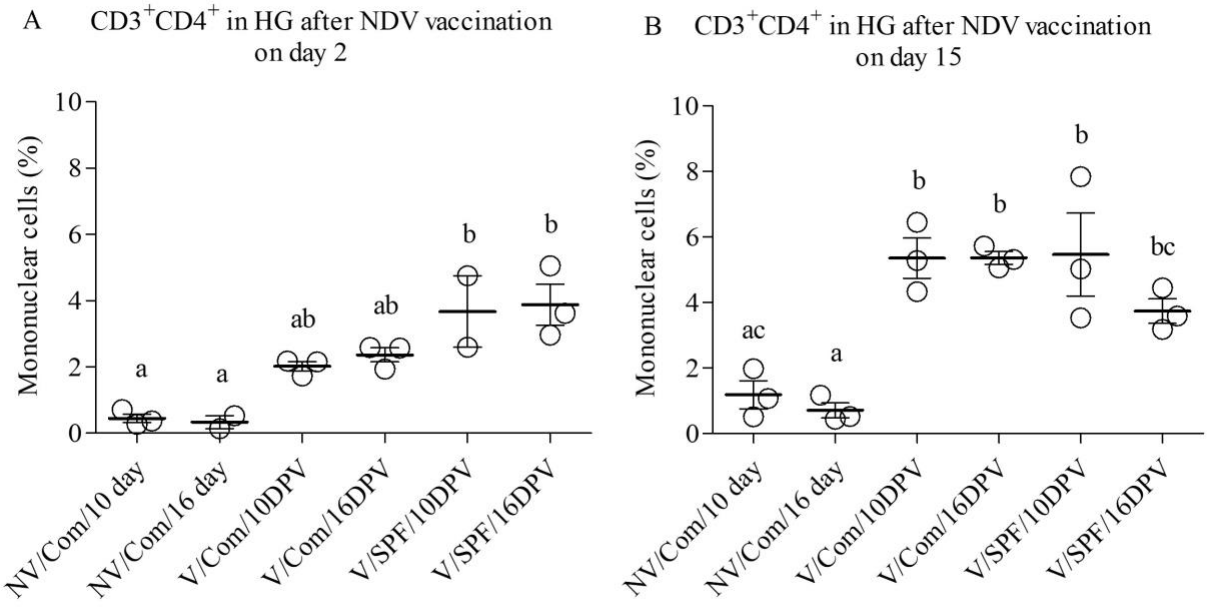


Fig. 3.8. Relative frequency of T-helper lymphocytes ($CD3^+CD4^+$) expressed as percent of total mononuclear cells in the Harderian glands (HG) of commercial chickens with MDA (Com) and SPF chickens vaccinated with NDV LaSota as described in Fig. 3.4. Harderian glands collected from each group of chickens at 10 and 16 days post vaccination (DPV). (A) vaccination at 2 days of age (DOA); (B) vaccination at 15 DOA. V = vaccinated; NV = nonvaccinated age-matched controls. Each value represents the cell counts found in pools of HG collected from four chickens, i.e. 12 individuals per time point. Averages and standard deviations are shown. Different letters indicate significant differences at $P<0.05$ determined by ANOVA and Tukey's posttest.

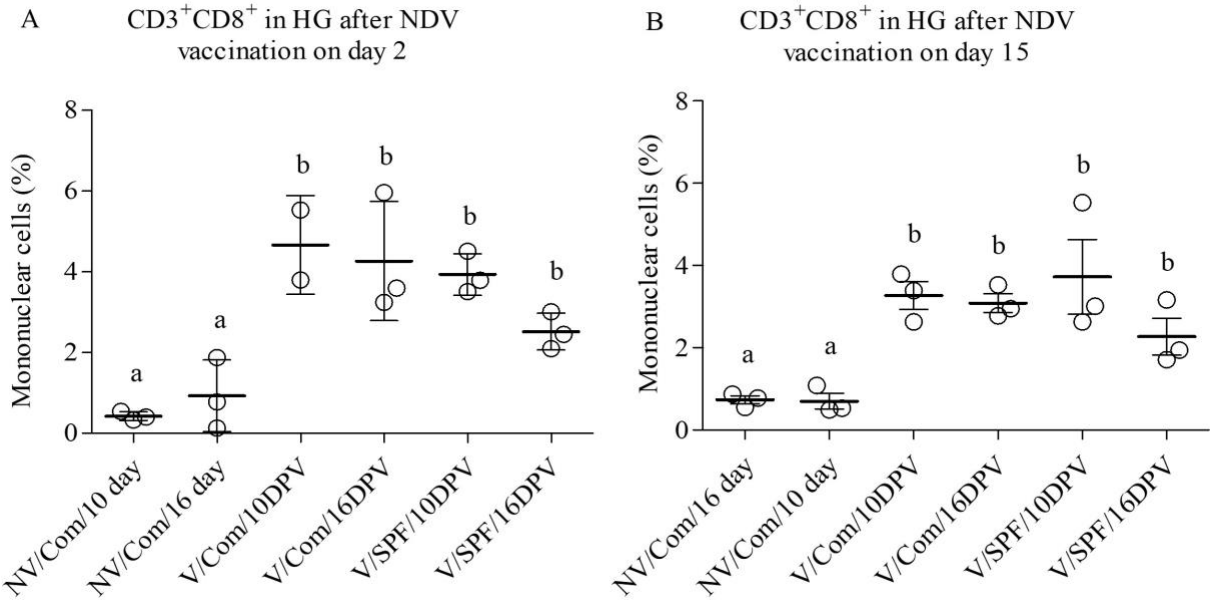


Fig. 3.9. Relative frequency of T-cytotoxic lymphocytes ($CD3^+CD8^+$) expressed as percent of total mononuclear cells in the Harderian glands (HG) of commercial chickens with MDA (Com) and SPF chickens vaccinated with NDV LaSota as described in Fig. 3.4. Harderian glands collected from each group of chickens at 10 and 16 days post vaccination (DPV). (A) vaccination at 2 days of age (DOA); (B) vaccination at 15 DOA. V = vaccinated; NV = nonvaccinated age-matched controls. Each value represents the cell counts found in pools of HG collected from four chickens, i.e. 12 individuals per time point. Averages and standard deviations are shown. Different letters indicate significant differences at $P<0.05$ determined by ANOVA and Tukey's posttest.

CHAPTER 4.

# Avian Paramyxovirus Type 1 From Wild Birds: Population Adaptations and Immunogenicity in Chickens

Avian Diseases (in press, 2025)

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## 4.1 Summary

We examined the replication and adaptation of avian paramyxoviruses serotype 1 (APMV-1) using four isolates, Mallard/US(OH)/04-411/2004, Northern pintail/US(OH)/87-486/1987, Mottled duck/US(TX)/TX01-130/2001, and Mallard/US(MN)/MN00-39/2000, and assessed their potential as vaccine candidates for chickens. The adaptability of each virus was examined by serial passages in embryonated chicken eggs (ECE) and in Vero cells. All APMVs successfully replicated in ECE. In contrast, two isolates passaged in Vero cells showed successful replication and two showed a continuous decline in viral load during passages. Whole genome sequencing analysis identified 14 genomic positions with significant variation in mean allele frequency. Changes of the predominant virus population were characterized by shifts of aa frequency at seven positions. Notably, four of these changes were located in the HN protein, one in matrix (M), and two in the L-protein sequences. Remarkably, while the percentage of alternative amino acids in viral populations passaged in ECE showed limited variation, e.g., at aa position 127 of HN, the frequency varied from 7.4% to 19.8% and HN aa position 192 from 5.1% to 43.5%, the variation of the viral populations passaged in Vero cells was significantly

higher at the same positions (e.g. the frequency of the alternative amino acids at HN aa positions 127 and 192 changed from 20.8% to 95.2% and 7.2% to 91.2%, respectively). Isolate 2 passaged in Vero cells displayed a marked variation in alternative amino acid frequencies, specifically at positions 127 within the HN- and 100 within the M- proteins. Isolate 3, while showing no alterations at the same HN positions, showed a considerable change in alternative amino acid frequency in the L protein at position 1875, a change occurring only in the Vero cell environment. One-day-old SPF chickens inoculated with isolates passaged in ECE elicited serum antibody responses similar to those elicited by the LaSota reference strain. In contrast, APMVs passaged in Vero cells showed limited replication in chickens and reduced induction of systemic antibodies. Interestingly, one virus passaged in ECE and another in Vero cells elicited IgA levels in lacrimal fluid comparable to the LaSota strain. We concluded that the four wild-bird APMV isolates tested demonstrated successful adaptation to ECE, with one isolate eliciting overall immune responses comparable to the LaSota virus, supporting their potential as vaccine candidates.

## 4.2 Introduction

During the last several decades the control of Newcastle disease virus (NDV) has been based on the use of avian paramyxovirus serotype 1 (APMV-1) vaccines belonging to genotypes I and II (64). The lentogenic B1 and LaSota strains and the avirulent variant V4 strain are commonly used globally. However, NDV strains currently circulating in poultry also include genotypes V in North America, XII in South America, and VII in Europe, Asia, and Africa (40),

which differ both genetically and antigenically from the commonly used vaccine strains (14, 62, 109, 114). These changes could lead to reduction in the effectiveness of current NDV vaccines.

The single-stranded RNA genome of NDV consists of six genes organized as 3′-NP-P-M-F-HN-L-5′, encoding six primary proteins, and the V and W proteins generated through mRNA editing of the P gene (125). Each of these proteins play a distinct role in the virus's life cycle. The fusion (F), hemagglutinin–neuraminidase (HN), and large RNA polymerase (L) proteins have been shown to contribute to the overall pathogenicity of NDV (35, 45, 68, 115). The HN and F glycoproteins facilitate attachment and membrane fusion and play a central role in eliciting virus-neutralizing antibody responses essential for effective protection against infection in chickens (18, 98). The HN protein is responsible for binding to cell receptors containing sialic acid and the F protein enables viral entry. The HN protein also has neuraminidase activity to remove sialic acid from progeny virions. The L protein, the largest protein in the virus, is involved in genome replication and in the transcription and regulation of viral replication in cells (45). As with other nonsegmented RNA viruses, the NDV's RNA large polymerase is error prone, facilitating the generation of genetic diversity (87).

APMVs of serotype 1 elicit antibodies that provide some level of protection against any NDV. However, there is considerable genetic diversity in APMV-1, with both a class differentiation and for class II viruses a genotype system. The class II APMVs have at least 20 different genotypes, with at least 10% amino acid (aa) sequence differences between genotypes (38). Vaccination studies have shown better protection when matching the genotype of the vaccine with the genotype of the virulent challenge virus, and the recommendation is to match vaccines to achieve the most effective protection. This approach is feasible for inactivated vaccines, where virulent virus can be used, but is extremely limited when using live vaccines, as

most genotypes include only virulent viruses. Naturally occurring low virulent APMV-1 viruses found in poultry and wild birds are limited to genotypes I, II and X, which do not match the most common circulating virulent strains of genotypes V, VII and XIII. Although the classic genotype II vaccine strains can be protective, a current research hypothesis is that including vaccines of different genotypes in a single vaccine or administered at different times might provide a broader immune response that would enhance vaccine protection.

In the current work, we characterized genetic and phenotype changes of four wild-bird APMV-1 isolates during passages in embryonated chicken eggs (ECE) and Vero cells. The virus populations selected in ECE and Vero cells were then evaluated for their replication ability and immunogenicity in chickens. A long-term objective for testing in future studies would include using the wild-bird APMV-1 viruses in multistrain NDV live vaccine protocols.

## 4.3 Materials and Methods

**Chickens.** White leghorn chickens were hatched from specific-pathogen-free (SPF) embryonated eggs (Charles River, North Franklin, CT) and maintained in Horsfall-type isolators in biosafety level 2 facilities. Experimental procedures and animal care were performed in compliance with applicable federal and institutional animal care and use guidelines. Auburn University College of Veterinary Medicine is an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International)–accredited institution.

**Viruses.** Four class II, genotype X APMV-1s originally isolated from wild birds were provided by the U.S. Department of Agriculture Southeast Poultry Research Laboratory. The APMV-1s

Mallard/US(OH)/04-411/2004, Northern pintail/US(OH)/87-486/1987, Mottled duck/US(TX)/TX01-130/2001, and Mallard/US(MN)/MN00-39/2000 had been previously confirmed to be low pathogenic viruses for chickens. For simplicity reasons, in this manuscript the viruses were designated APMVs 1, 2, 3, and 4, respectively. In addition, we used an NDV LaSota strain obtained from the American Type Culture Collection (ATCC) as a positive control.

**Adaptation of wild-bird APMV-1s to ECE and Vero cells.** Each virus was subjected to seven passages either in ECE or Vero cells (ATCC). The number of passages was based on previous work indicating that other RNA viruses; for example, infectious bronchitis coronavirus, required 6–7 passages for the virus population to adapt to kidney cell culture as determined by virus replication and sequencing of the spike gene (59). Similarly, in the current study viral adaptation during passages was evaluated indirectly by determining successful viral replication by quantitative reverse transcriptase PCR (RT-qPCR) and by determining genetic shift of the predominant virus population by whole-genome sequencing. Successful replication in ECE is not only relevant from a basic science perspective but also from an applied perspective, as production of live NDV vaccines in ECE is well established in the poultry pharmaceutical industry.

Each virus was passaged in 11-day-old SPF ECE. ECEs were inoculated with 0.2 ml of a 1:10 dilution from the stock solution of each virus (n = 6 eggs/virus). Allantoic fluids were harvested 72 hr postinoculation, pooled for each virus, centrifuged, and stored at –80 C until the subsequent passage. The subsequent passages were carried out in the same way, using a 1:10 dilution of the allantoic fluid from the previous passage.

Studying the adaptation of the isolates to Vero cells was performed essentially from a basic science perspective. Vero cells were cultured and maintained as described (11). For each isolate, confluent Vero cells in all six wells of a six-well plate were inoculated with 1 ml/well of a 1:10 dilution of the first ECE passage stock solution or the clarified cell lysate from the previous passage in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. After 1 hour incubation at 37 C, with gentle agitation every 10–15 minutes, 1 ml of complete medium was added to each well. After 72 hr, cells were lysed in the plate in the medium by two freeze-thaw cycles, lysates from all six wells were pooled, and cell debris was removed by low-speed centrifugation. Aliquots were removed for immediate subsequent passage. An aliquot of the remaining clarified lysate for RNA isolation and the rest of the clarified lysate were stored at –80 C until they were used for RNA isolation or inoculation of chickens.

**Viral replication quantification in ECE and Vero cells.** After each passage, RNA was extracted from allantoic fluids from ECE and clarified cell lysate from Vero cells for NDV RNA quantitation by RT-qPCR (8). In brief, RNA was extracted from 140 µl allantoic fluid or clarified cell lysate using QIAamp® Viral RNA Mini Kit using a QIAcube® following the manufacturer's instructions (QIAGEN, Hilden, Germany). Viral RNA was eluted in 60 µl total volume. Relative viral NDV RNA copy numbers were determined by RT-qPCR using primers and probes targeting the L gene as described (54).

**Next-generation sequencing of APMV-1s RNA following passages.** Total RNA extracted from 140 µl of allantoic fluids or clarified cell lysates after passage in ECE or Vero cells was

subjected to host rRNA depletion as described (63), followed by purification using RNAClean XP beads (Beckman Coulter, Brea, CA). Whole-genome sequencing was performed using sequence-independent single-primer amplification (SISPA) next-generation sequencing (NGS) method as described (28). Briefly, first-strand cDNA was synthesized from total nucleic acids using SuperScript IV Reverse Transcriptase (Invitrogen, Carlsbad, CA) and primer K-8N. The cDNA was then converted to double-stranded DNA using Klenow reaction, followed by purification using AMPure XP beads (Beckman Coulter). This purified DNA served as a template for random PCR amplification, which was conducted using primer K and Phusion High-Fidelity PCR Kit (New England Biolabs, Ipswich, MA). PCR products were purified again with AMPure XP beads, quantified using Qubit 1X dsDNA High Sensitivity assay (Invitrogen), and used to prepare multiplexed paired-end sequencing libraries using Illumina DNA Prep Kit (Illumina, San Diego, CA) according to the manufacturer's recommendations. After quantification using the Qubit 1X dsDNA High Sensitivity Assay Kit (Invitrogen) and High Sensitivity D5000 Screen Tape (Agilent Technologies, Santa Clara, CA), the libraries were pooled (4 nM, 10 µl each), spiked with a control library (5% PhiX library v3), diluted to 12 pM final concentration, and sequenced (paired-end, 2 × 300 bp) using the MiSeq Reagent Kit v3 (600-cycle; Illumina) on an Illumina MiSeq platform.

**Bioinformatics analysis.** The Illumina raw sequencing data were preprocessed within the Galaxy platform. Briefly, raw sequence reads were quality assessed using FastQC v0.63 (12); short reads were filtered and low-quality bases were trimmed using Fastp 0.23.2 (27). Host reads were removed using the Burrows-Wheeler Alignment Tool (BWA-MEM 0.7.17.2) (89), and the output was sorted using Samtools merge 1.15.1 tool (34). The remaining unmapped reads were

further used for APMV-1 genome consensus assembly by BWA-MEM mapping with reference genomes. The GenBank accession numbers for each reference virus are: virus 1 GQ288377; virus 2 GQ288378; virus 3 GQ288391; and virus 4 GQ288392. PCR duplicates were removed using RmDup 2.0.1 (90), and then final consensus sequences were generated using the bam2consensus tool (142). The consensus sequences were further analyzed with MacVector software (version 18.0). Single-nucleotide polymorphism analysis of viral genomes was also performed on the Galaxy platform. Sequencing reads were first realigned to improve alignment accuracy, followed by the insertion of indel qualities into the BAM file, both using LoFreq v2.1.5 (146). Variants were then called using LoFreq with parameters set to a minimum coverage of 10 and minimum mapping quality thresholds of 20. Postcalling, variant filtering was also performed with LoFreq, applying stringent criteria: minimum coverage of 500, a minimum allele frequency of 0.05, and filtering based on a multiple testing–corrected strand-bias *P*-value threshold of 0.001. The filtered variants were annotated using SnpEff eff v4.3 (30), and variant information was subsequently extracted from variant call format (VCF) files and converted into a tabular format using SnpSift Extract Fields v4.3 (29) for downstream analysis.

**Statistical analysis.** Variant information in tabular format was used for further in-depth evaluation carried out in the R programming environment within RStudio (version 2023.06.0+421), employing the Cochran–Armitage trend test. This statistical test identifies significant linear trends in proportions, making it suitable for the analysis of changes in the frequency of alternative alleles across successive passages. The analysis was performed separately for each genomic position, passage substrate (Vero cells or ECE), and virus isolate to capture substrate- and isolate-specific trends. The Cochran–Armitage trend test requires input

data to be structured in a specific format. Thus, because the test evaluates trends in proportions, the sequencing data, which initially represented allele frequencies as raw proportions, were reformatted to be compatible with the trend test to allow meaningful comparisons across samples. A "success" value was created for each genomic position to represent the proportion of the alternative allele, and the "total" value was set to 1 to standardize these proportions as part of a single trial. Data were grouped by genomic position, substrate, and isolate. This grouping enabled the test to evaluate trends within specific contexts, capturing substrate- and isolate-specific dynamics. Following data preparation, *P* values were calculated for each unique combination, assessing whether observed trends in allele frequency over successive passages were statistically significant. Nucleotide (nt) frequency differences at a given genomic position with $P \leq 0.05$ were considered significant, indicating a consistent increase or decrease in the frequency of the alternative allele across passages. For the positions with significant differences in nucleotide frequencies, additional metrics were calculated, including the minimum and maximum alternative allele frequencies and the range of passage numbers demonstrating these trends. To determine the significant allele frequency trends, Tables 4.1 and 4.2 include the nucleotides and encoded amino acids of the original reference sequences, which were also used to detect population shifts during passages.

**Replication and immunogenicity of wild-bird APMV-1s upon inoculation into chickens.** The seventh passage of each wild-bird APMV-1 in either ECE or Vero cells was inoculated via the ocular route into 1-day-old SPF chickens. Immunogenicity was evaluated by determining NDV serum antibodies and NDV IgA in lacrimal fluids. Virus replication in chickens was assessed by estimation of NDV RNA in the lacrimal fluids.

**Virus titration and inoculation into chickens.** The seventh passage of each virus in ECE was titrated by hemagglutinating activity (HA) as accepted (141). HA titration of isolates 2 and 3 passaged in Vero cells was not possible because HA was not detectable. Isolates 1 and 4 passaged in Vero cells were excluded from the chicken experiment because of their declining replication during passages (Fig. 4.1).

Eight groups (between 12 and 19 chickens per group) of SPF chickens were established. Chickens in all groups were inoculated ocularly with a 100 μl of virus suspension (50 μL in each eye) of one of the passaged APMVs. Each bird in Groups 1–4 was inoculated with 8 HA units of the seventh passage in ECE of one of the four viruses. Groups 5 and 6 were inoculated with 100 μl of clarified cell lysate of the seventh passage in Vero cells of isolates 2 and 3. Group 7 was included as a positive control with chickens inoculated with $10^4$ $EID_{50}$ of the LaSota strain (dose commonly employed for vaccination in the poultry industry) in a 100-μl volume. Group 8 included age-matched negative controls that were inoculated with 100 μl of buffer solution.

**Antibody responses in chickens.** Serum samples were collected 20 and 27 days postinoculation and NDV antibody levels were determined using a commercial NDV ELISA kit (IDEXX Laboratories Inc., Westbrook, ME) following the manufacturer's recommendations. Lacrimal fluids (50–200 μl from each bird) were obtained 10 days postinoculation as described previously (133), and specific NDV IgA levels were determined using the commercial kit's NDV-coated ELISA plates from the same commercial source. Tear samples were diluted 1:100 and specific IgA was detected using a horseradish peroxidase–conjugated polyclonal goat–anti-chicken IgA

antibody (Southern Biotechnology Associates Inc., Birmingham, AL) instead of the secondary antibody provided in the commercial ELISA kit. Absorbance was measured at 450 nm. Antibody levels were analyzed by ANOVA followed by Tukey's multiple comparisons *post hoc* test. Differences were considered significant at $P \leq 0.05$.

**Viral replication in chickens.** Lacrimal fluids were collected 3 and 10 days postinoculation for relative NDV RNA level determination by RT-qPCR. Briefly, RNA was extracted from 140 µl lacrimal fluids using the QIAamp® Viral RNA Mini Kit utilizing a QIAcube® following the manufacturer's recommendations (QIAGEN, Hilden, Germany). Viral RNA was eluted in 60 µl total volume. Relative viral RNA level was determined by RT-qPCR using primers and probes targeting the L gene as described by others (54). A 10-point RNA standard curve was included with every plate. Total RNA was extracted from a single 100-µl dose of commercial NDV LaSota vaccine (Boehringer Ingelheim, Ingelheim, Germany), quantified with a NanoDrop, and the copy number was estimated from the viral genome size (15.2 kb), assuming 100% viral RNA. The RNA stock was then serially diluted 10-fold in carrier RNA (salmon sperm RNA) to give $1 \times 10^{10}$ to $1 \times 10^{1}$ genome copies in the 5-µl template volume used per reaction. Cycle threshold (Ct) values were plotted against $\log_{10}$ copy number; only curves with 95–105 % efficiency (slope −3.1 to −3.4) and $R^2 > 0.99$ were accepted. Sample Ct values were converted to $\log_{10}$ genome copies per milliliter of lacrimal fluid with this curve. However, because the vaccine likely contained an unknown quantity of host RNA, viral RNA levels are expressed as relative amounts rather than absolute copy numbers. Viral loads were analyzed by ANOVA followed by Tukey's multiple comparisons *post hoc* test with differences considered significant at $P \leq 0.05$.

## 4.4 Results

**Viral replication in ECE and Vero cells.** As shown in Fig. 4.1, all APMVs (1 - 4) successfully replicated in ECE, with constant high relative NDV RNA levels maintained during successive passages. In contrast, isolates 1 and 4 were not able to adapt to Vero cells, showing a steady decline in viral RNA levels over successive passages. Isolates 2 and 3 showed adaptation to Vero cells with successful replication after the fourth passage as inferred from a pronounced increase in viral RNA levels.

**Genetic shifts in APMVs-1 during passages.** Consensus sequence analysis revealed shifts in the amino acids encoded in the genomes of APMV populations after passages both in Vero cells and ECE. In ECE, a single change was noted at genome nucleotide position 7922, codon 507 in the HN gene. The shift from aa E to K was detected in passage 5. In the genomes of viruses passaged in Vero cells, changes in amino acids encoded were observed for APMVs 2 and 3, whereas genomes of viruses 1 and 4 exhibited no changes. Specifically, virus 2 showed multiple changes from passage 5 onward including in the F gene at genome nucleotide position 4914 (encoding F aa 127) changing the aa from G to W, in the HN gene at genome nucleotide position 6819 (encoding HN aa 139) from I to N, and in the NP gene at genome position 477 (encoding NP aa 158) from G to S. APMV 3 demonstrated changes in the F gene at genome nucleotide position 4915 (F aa position 127), changing from G to V from passage 5, and at genome position 7013 ( HN aa position 204), with aa shifting from I to L starting from passage 6. Although not all aa changes involved major changes in biochemical properties, some of the changes involving

shifts from a polar hydrophilic aa to a nonpolar hydrophobic aa (G to W and G to V) and from nonpolar hydrophobic to polar hydrophilic (I to N) may be relevant for phenotypic adaptation.

The mean allele frequency trends across passages for the two biological substrates are presented in Fig. 4.2. In ECE, the mean allele frequency remained relatively stable over time, indicating minimal adaptive changes of the predominant virus population. In contrast, allele frequencies in Vero cells showed a marked increase after the third passage, reflecting a substrate-specific pattern for adaptation.

The Cochran–Armitage trend test identified genome positions with statistically significant trends of variation in allele frequencies. As shown in Table 4.1, 14 genome positions exhibited significant shifts in the alternative nucleotide over successive passages with 7 positions located in the HN gene, 6 in the L gene, and 1 in the M gene. Notably, six genome positions (6780, 6974, 7162, 7884, 8793, and 13391) demonstrated significant allele frequency trends in both biological substrates. Among those, positions 6780, 6974, 7162, and 7884 were in the HN gene. The remaining two positions (8793, and 13391) were in the L gene.

Table 4.2 shows the predicted aa changes at the positions identified with significant trends observed in allele frequencies. Among the 14 genome positions exhibiting significant trends, 6 exhibited synonymous changes, that is, not altering the encoded aa. However, eight nonsynonymous shifts led to alterations in protein sequences, suggesting functional adaptations that could impact the virus's behavior and interactions with the host cells. Notably, 5 of these nonsynonymous changes were located in the HN protein, indicating a higher propensity for structural variations in this protein during serial passages. The remaining nonsynonymous changes were found in the L- and the M- proteins.

**NDV antibody responses after inoculation of chickens.** Serum NDV antibody levels measured 20 and 27 days after inoculation of chickens on Day 1 of age are shown in Fig. 4.3. Chickens inoculated with APMVs passaged in ECE elicited antibody titers similar to those induced by the LaSota strain. Notably, these groups exhibited consistently higher serum antibody levels compared to those inoculated with virus passaged in Vero cells, indicating a possible influence of the adaptive changes on immunogenicity.

IgA-specific NDV antibodies in lacrimal fluids, measured 10 days after inoculation, are shown in Fig. 4.4. Although most groups inoculated with the APMV-1 isolates exhibited limited IgA responses, virus 4 passaged in ECE and virus 3 passaged in Vero cells elicited IgA levels similar to those elicited by the LaSota strain.

**Viral load in lacrimal fluids after inoculation in chickens.** Fig. 4.5 shows the relative viral RNA levels, as a surrogate measure for load, in lacrimal fluids for each virus measured 3 and 10 days postinoculation in 1-day-old chickens. Three days after inoculation, chickens inoculated with APMVs passaged in ECE exhibited higher viral loads in lacrimal fluids compared to those inoculated with the viruses passaged in Vero cells. Notably, viruses 2 and 3 reached viral loads similar to the LaSota strain at both time points, indicating that the passages in ECE performed on these viruses did not negatively impact their replication dynamics in chickens.

## 4.5 Discussion

Among RNA viruses, influenza viruses and coronaviruses are likely the most prone to genetic shift and drift. Other RNA viruses, including APMV-1, are considered more stable.

Indeed, the same NDV lentogenic strains of serotype 1 have been used effectively for several decades to prevent outbreaks of disease in chickens (126). This may allow speculation that the genes used as markers to genotype APMVs of serotype 1 do not play such an essential role in evolutionary adaptation. In the current work, seven passages were performed based on analogous studies showing that the predominant population of an infectious bronchitis coronavirus strain quickly shifts and stabilizes after 6–7 passages in kidney cell culture (59). In the current study, all APMVs (1 – 4) from wild birds showed effective replication during seven passages in ECE (Fig. 4.1) and the mean allele frequency remained relatively stable (Fig. 4.2), indicating minimal adaptive shifts of the predominant virus population. This natural ability of APMVs to adapt to different bird species may explain the fact that wild birds are an excellent reservoir for NDV (126). The effective replication of all viruses in ECE corroborates the robustness of egg-based systems for APMV-1s propagation and facilitates the possibility of vaccine production using APMVs from other avian species to protect poultry. In contrast, adaptation to Vero cells presented challenges to the virus population. APMVs 1 and 4 were unable to sustain replication in Vero cells as became evident from declining viral loads during successive passages (Fig. 4.1). This suggests that, in the absence of mutations, additional passages would have ended in the extermination of the virus population. Interestingly, viruses 2 and 3 showed increasing viral loads after the fourth passage, indicating partial adaptation of the virus population and potential for further optimization for growth in Vero cells.

Whole-genome sequencing revealed significant genetic changes associated with the adaptation of the APMVs-1 to different environments. As shown in Table 4.1, 14 genomic positions showing significant trends in allele frequency after passages were identified: 3576, 6780, 6974, 7074, 7162, 7883, 7884, 8242, 8793, 9485, 12395, 13391, 13908, and 13992.

Notably, six positions (6780, 6974, 7162, 7884, 8793, and 13391) demonstrated trends to variation both in ECE and Vero cells, emphasizing their potential significance in viral selection and adaptation. Four of these shared positions (6780, 6974, 7162, and 7884) were located in the HN gene. Similarly, for the infectious bronchitis coronavirus previously studied, most aa changes during adaptation of the virus population occurred in the attachment protein (spike) but changes were also observed in the replicase region (59). The remaining two shared positions (8793 and 13391) were in the L gene coding for the RNA polymerase protein essential for viral transcription and genome replication of APMVs.

Notable allele frequency shifts at specific codon positions in the HN and L genes resulted in shifts at specific aa positions (Table 4.2). Codon positions corresponding to amino acids 127 and 191 in the HN protein demonstrated substantial allele frequency shifts in the environment of Vero cells contrasting with the more stable frequencies observed during passages in ECE. This shift suggests that these positions may be important for enhanced viral fitness under the selective pressure exerted by the mammalian cell environment. Specifically, the results show successful adaptation and replication of APMVs 2 and 3 in Vero cells but steady declining replication of viruses 1 and 4. APMV 2 showed a notable alternative allele frequency shift in a specific codon in the HN gene, resulting in an aa change at position 127, perhaps indicating that this phenotype was relevant for adaptation. In addition, virus 2 showed a unique aa change in the M protein at position 100. Virus 3 did not show changes at these positions but displayed a significant shift in the L polymerase protein at aa positions 142 and 1875 that may have assisted with replication efficiency. This result aligns with results by others indicating that changes in the L gene can enhance RNA synthesis, replication efficiency, and in some cases, increased virulence (45, 115). Such evidence supports the concept that the L gene's interaction with other

genomic regions is essential in modulating viral phenotypes and underscores the importance of monitoring these adaptive changes to understand viral behavior in different host environments.

In the analysis of viral adaptation and population shifts, we acknowledge the limitations posed by the low viral loads observed in APMVs 1 and 4 after passages in Vero cells. Their reduced replication efficiency resulted in incomplete sequencing data for several genomic positions, potentially obscuring additional allele frequency trends that might be occurring in these viruses. The inability to sequence a comprehensive range of positions because of low viral RNA levels could have led to an underestimation of the genetic diversity and adaptation dynamics in these virus populations.

An interesting pattern emerged from the consensus alignment of the L, F, and HN proteins of the different APMVs after passages in both ECE and Vero cells (data not shown). Specifically, the amino acids at 12 positions in the HN protein are consistently shared by viruses 2 and 3 but differ in viruses 1 and 4. Similarly, viruses 2 and 3 share unique amino acids at four positions in the F protein and 20 in the L protein but these differed in viruses 1 and 4. Despite the amino acids at these positions being consistent in both environments, their presence in viruses 2 and 3 suggests they may confer an adaptive advantage in Vero cells, potentially explaining the different replication success observed among the viruses. In the Vero cell environment, the consensus sequence analysis revealed specific aa changes in viruses 2 and 3 that were not present in viruses 1 and 4, further supporting this hypothesis. For example, virus 2 showed a shift at nucleotide position 4875 (aa position 115) in the F gene, and 6780 (aa position 127) in the HN gene that resulted in aa changes with considerably different chemical properties (from G to W and I to N, respectively). These modifications, which change the hydrophilicity and hydrophobicity of the aa may indicate an adaptation mechanism favoring viral fitness in Vero

cells. Moreover, virus 3 demonstrated a notable aa change from I to L at position 6974 (aa position 192) in the HN gene from passage 6, a shift that seems to be beneficial for viral replication in Vero cells.

The results of ocular inoculation of the passaged isolates in chickens showed that change of the predominant population influences virus replication and immunogenicity. Chickens inoculated with ECE-passaged isolates exhibited higher viral loads in lacrimal fluid (Fig. 4.5), stronger mucosal IgA responses (Fig. 4.4), and higher serum NDV-specific antibody levels (Fig. 4.3) compared to those inoculated with Vero cell-passaged viruses, that is, the Vero adapted population was less fit in the chicken environment. From an applied perspective, these findings show that ECE-propagated isolates maintain their immunogenic properties. Indeed, viruses 2 and 3 passaged in ECE elicited responses comparable to those of the LaSota vaccine strain, supporting their potential as vaccine candidates. The reduced immunogenicity of Vero cell-passaged viruses is attributable to their lower replication efficiency.

NDV genotyping is typically based on variations in the F gene. However, the current work suggests that mutations in the HN and L genes may also be relevant for this virus's overall fitness and adaptation. Moreover, it is noteworthy that in the current work, we did not observe any changes in allele frequency trends in the F gene, although changes in the HN and L proteins may influence the functionality and efficiency of the F protein indirectly. Therefore, the inclusion of multigenic data in phylogenetic analysis and vaccine development seems very important.

Table 4.1. Genomic positions with significant allele frequency (af) trends [A] in APMVs 1 (Mallard/US(OH)/04-411/2004), 2 (Northern pintail/US(OH)/87-486/1987), 3 (Mottled duck/US(TX)/TX01-130/2001), and 4 (Mallard/US(MN)/MN00-39/2000) after seven passages in embryonated chicken eggs (ECE) and Vero cells.

| Genome position | Gene | Passage substrate | Virus | Ref. nt[B] | Alt. nt[C] | Min.[D] Pass. | Max.[D] Pass. | Min.[E] %af | Max.[E] %af |
|---|---|---|---|---|---|---|---|---|---|
| 3576 | M | Vero | 2 | G | A | 1 | 3 | 9.40 | 27.5 |
| 6780 | HN | Vero | 2 | T | A | 1 | 7 | 20.8 | 95.2 |
| 6780 | HN | ECE | 2 | T | A | 1 | 7 | 7.40 | 19.8 |
| 6974 | HN | Vero | 3 | A | C | 1 | 7 | 7.20 | 91.2 |
| 6974 | HN | ECE | 3 | A | C | 1 | 7 | 5.10 | 43.5 |
| 7074 | HN | ECE | 2 | G | A | 2 | 7 | 6.20 | 28.5 |
| 7162 | HN | Vero | 2 | C | T | 1 | 7 | 24.1 | 93.0 |
| 7162 | HN | ECE | 2 | C | T | 1 | 7 | 12.1 | 22.5 |
| 7883 | HN | ECE | 1 | G | A | 1 | 7 | 13.4 | 70.5 |
| 7883 | HN | ECE | 2 | G | A | 1 | 7 | 6.80 | 37.9 |
| 7884 | HN | Vero | 3 | A | T | 1 | 7 | 76.8 | 94.3 |
| 7884 | HN | ECE | 2 | A | T | 1 | 7 | 5.10 | 24.4 |
| 8242 | HN | ECE | 2 | T | C | 2 | 7 | 6.60 | 26.0 |
| 8793 | L | Vero | 3 | C | T | 1 | 7 | 22.5 | 29.5 |
| 8793 | L | ECE | 3 | C | T | 1 | 7 | 12.5 | 26.8 |
| 9485 | L | ECE | 2 | G | A | 1 | 7 | 43.3 | 61.0 |
| 12395 | L | Vero | 3 | C | T | 1 | 7 | 79.3 | 91.0 |
| 13391 | L | Vero | 2 | G | A | 1 | 7 | 37.5 | 93.8 |
| 13391 | L | ECE | 2 | G | A | 1 | 7 | 24.5 | 38.8 |
| 13908 | L | ECE | 2 | T | C | 1 | 7 | 30.2 | 44.4 |
| 13992 | L | Vero | 3 | G | A | 1 | 7 | 80.4 | 91.8 |

[A] Positions showing significant trends to variation determined by the Cochran–Armitage trend test ($P \leq 0.05$)

[B] Reference nucleotide: Baseline comparison for sequence analysis.

[C] Alternative nucleotide: Variant base identified post-sequencing.

[D] Passage range: Minimum and maximum passages sequenced for each position.

[E] Allele frequency range: Minimum and maximum proportions of the alternative nucleotide observed across passages.

Table 4.2. Differential alternative (alt) amino acid (aa) frequencies [A] in structural proteins of APMVs 1 (Mallard/US(OH)/04-411/2004), 2 (Northern pintail/US(OH)/87-486/1987), 3 (Mottled duck/US(TX)/TX01-130/2001), and 4 (Mallard/US(MN)/MN00-39/2000) after seven passages in embryonated chicken eggs (ECE) and Vero cells.

| Genome position | Protein | aa position | Passage substrate | Virus | Ref. nt[B] | Ref. aa[B] | Alt nt[C] | Alt aa[C] | Min.[D] %alt aa | Max.[D] %alt aa |
|---|---|---|---|---|---|---|---|---|---|---|
| 3576 | M | 100 | Vero | 2 | G | V | A | I | 9.40 | 27.5 |
| 6780 | HN | 127 | Vero | 2 | T | I | A | N | 20.80 | 95.2 |
| 6780 | HN | 127 | ECE | 2 | T | I | A | N | 7.40 | 19.8 |
| 6974 | HN | 192 | Vero | 3 | A | I | C | L | 7.20 | 91.2 |
| 6974 | HN | 192 | ECE | 3 | A | I | C | L | 5.10 | 43.5 |
| 7074 | HN | 225 | ECE | 2 | G | R | A | H | 6.20 | 28.5 |
| 7883 | HN | 495 | ECE | 1 | G | E | A | K | 13.4 | 70.5 |
| 7883 | HN | 495 | ECE | 2 | G | E | A | K | 6.80 | 37.9 |
| 7884 | HN | 495 | Vero | 3 | A | E | T | V | 76.8 | 94.3 |
| 7884 | HN | 495 | ECE | 2 | A | E | T | V | 5.10 | 24.4 |
| 8793 | L | 142 | Vero | 3 | C | P | T | S | 22.5 | 29.5 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 8793 | L | 142 | ECE | 3 | C | P | T | S | 12.5 | 26.8 |
| 13392 | L | 1875 | Vero | 3 | G | V | A | I | 80.4 | 91.8 |

[A] Only amino acids corresponding to genome positions showing a significant trend ($P \leq 0.05$), as determined by the Cochran–Armitage trend test, are included.

[B] Reference nucleotide and amino acid: Baseline comparison for sequence analysis.

[C] Alternative nucleotide and amino acid: Variant identified post-sequencing.

[D] Alternative amino acid range: Minimum and maximum proportions of the alternative amino acid observed across passages.

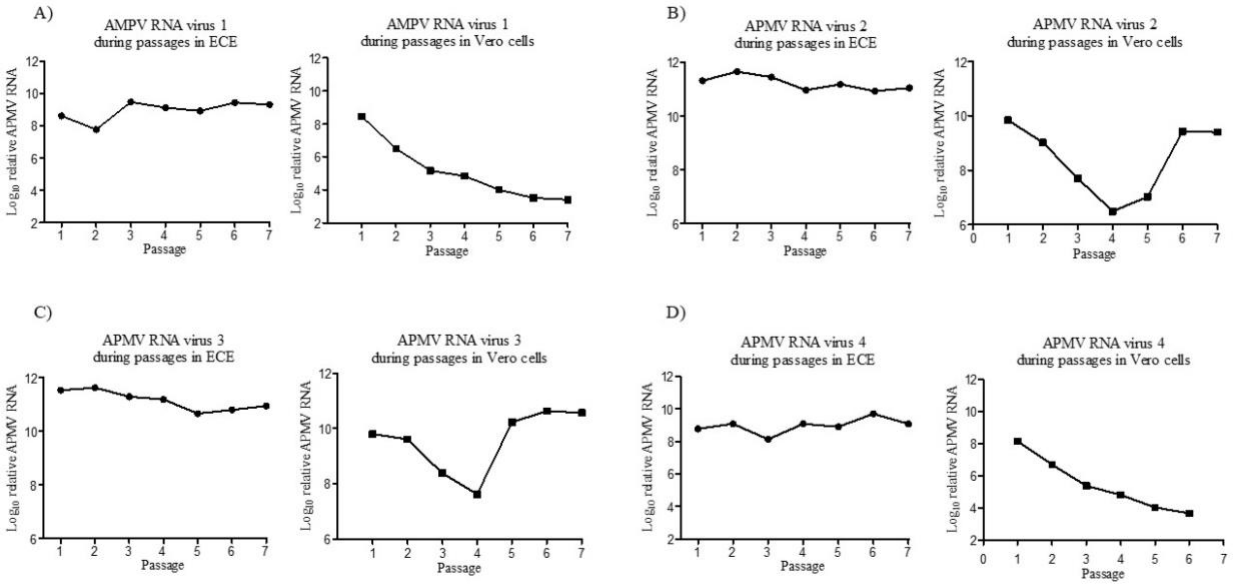


Fig. 4.1. Viral loads (log10 relative APMV RNA in allantoic fluid of embryonated chicken eggs (ECE) or Vero cell clarified lysate) by RT-qPCR during serial passages of APMV-1 viruses (A) #1 (Mallard/US(OH)/04-411/2004), (B) #2 (Northern pintail/US(OH)/87-486/1987), (C) #3 (Mottled duck/US(TX)/TX01-130/2001), and (D) #4 (Mallard/US(MN)(OH)/04-411/2004).

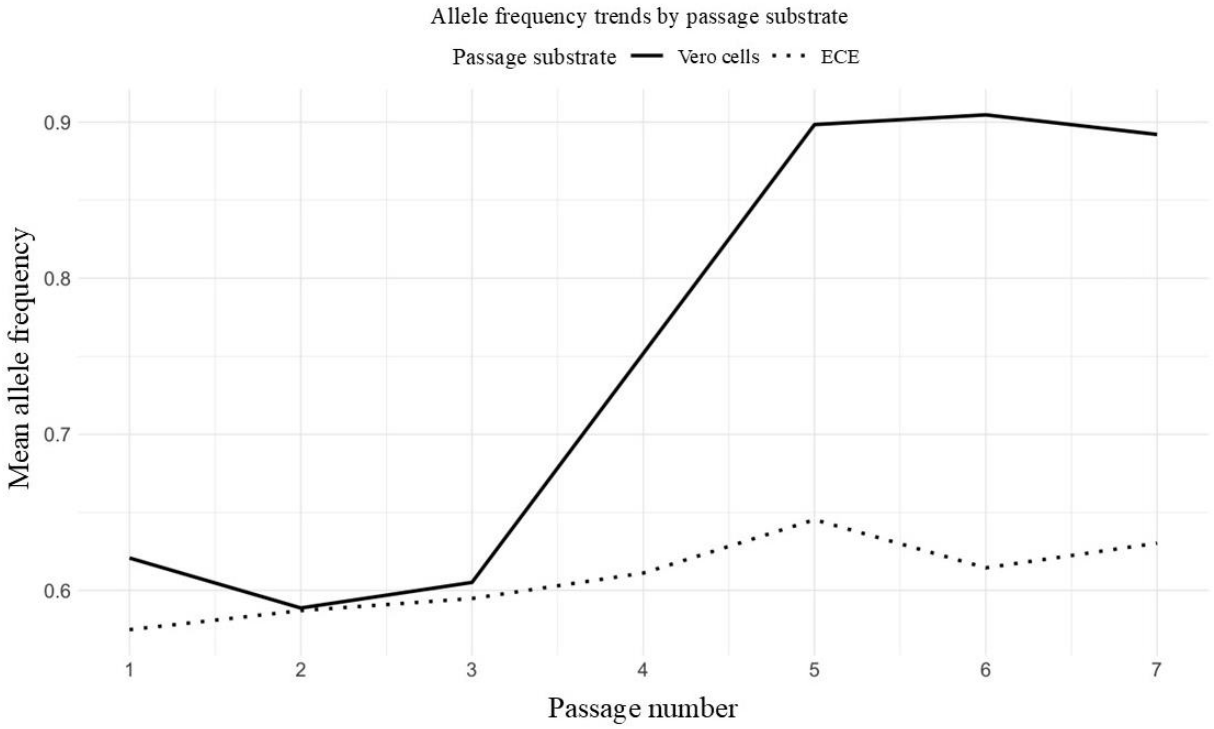


Fig. 4.2. Mean allele frequency trends of four APMV-1 viruses (Mallard/US(OH)/04-411/2004, Northern pintail/US(OH)/87-486/1987, Mottled duck/US(TX)/TX01-130/2001, and Mallard/US(MN)/MN00-39/2000) during 7 passages in Vero cells (solid line) and embryonated chicken eggs (ECE) (dotted line).

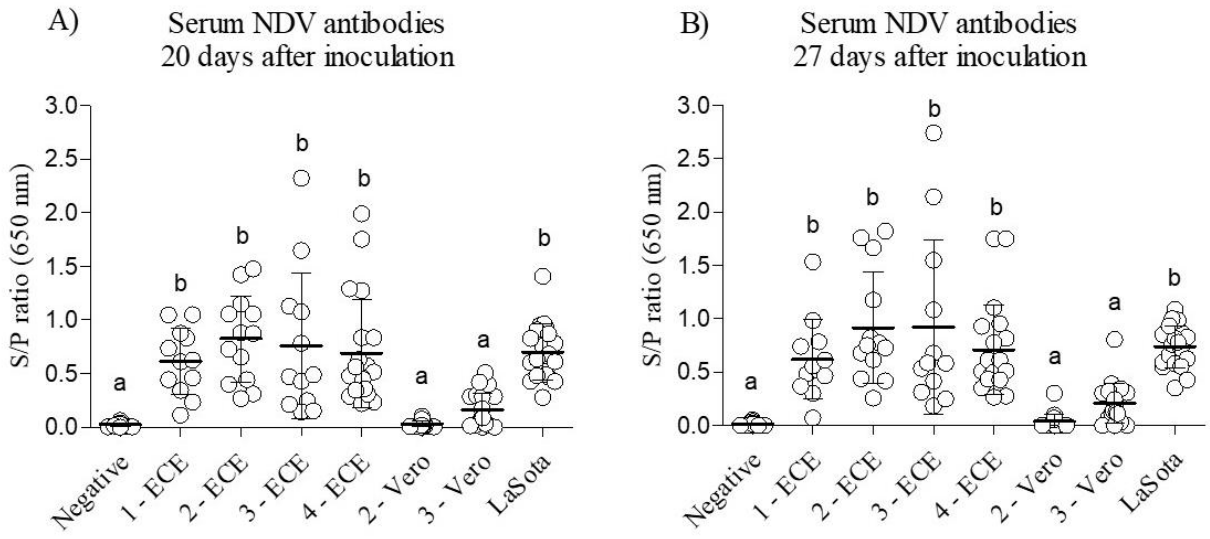


Fig. 4.3. Serum antibodies determined by NDV ELISA kit 20 and 27 days after ocular inoculation of SPF chickens at day 1 of age with APMVs 1 (Mallard/US(OH)/04-411/2004), 2 (Northern pintail/US(OH)/87-486/1987), 3 (Mottled duck/US(TX)/TX01-130/2001), and 4 (Mallard/US(MN)/MN00-39/2000) after passages in ECE or Vero cells. An additional group was inoculated with NDV LaSota. Serum NDV antibodies expressed as sample-to-positive (S/P) ratio (A) 20 days after inoculation and (B) 27 days after inoculation. Individual values, means, and standard deviations are shown. Different letters indicate significant differences $P \leq 0.05$ determined by ANOVA and Tukey's post-hoc test.

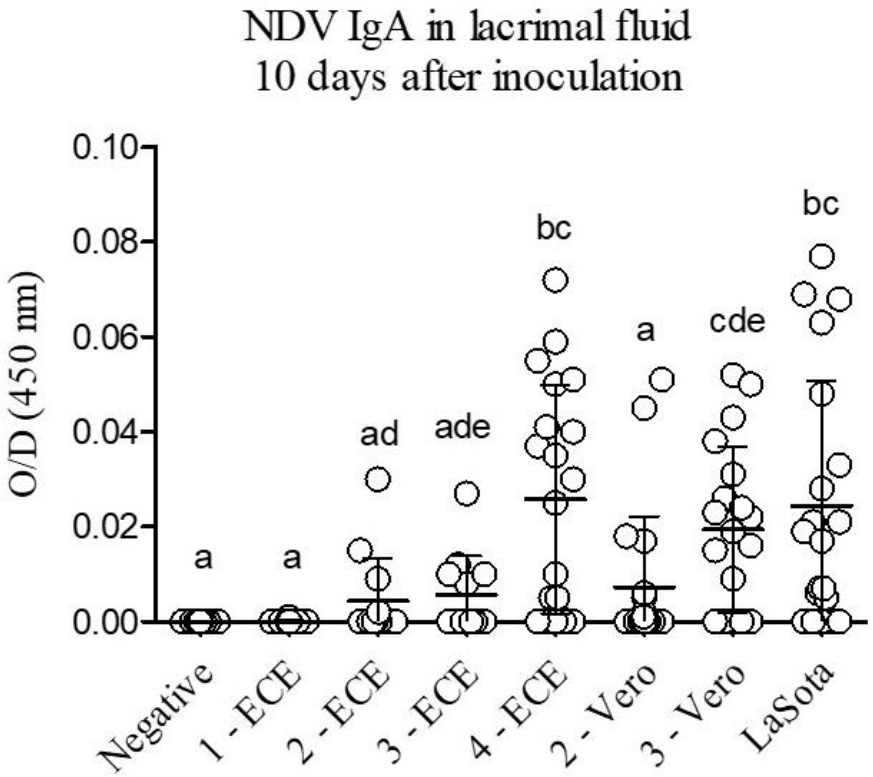


Fig. 4.4. NDV IgA in lacrimal fluid determined by ELISA 10 days after ocular inoculation in SPF chickens at day 1 of age with APMVs 1 - 4 passaged in ECE or Vero cells. An additional group was inoculated with NDV LaSota. Antibody levels are expressed as optical densities (O/D). Individual values, means, and standard deviations are shown. Different letters indicate significant differences ($P \leq 0.05$) determined by ANOVA and Tukey's post-hoc test.

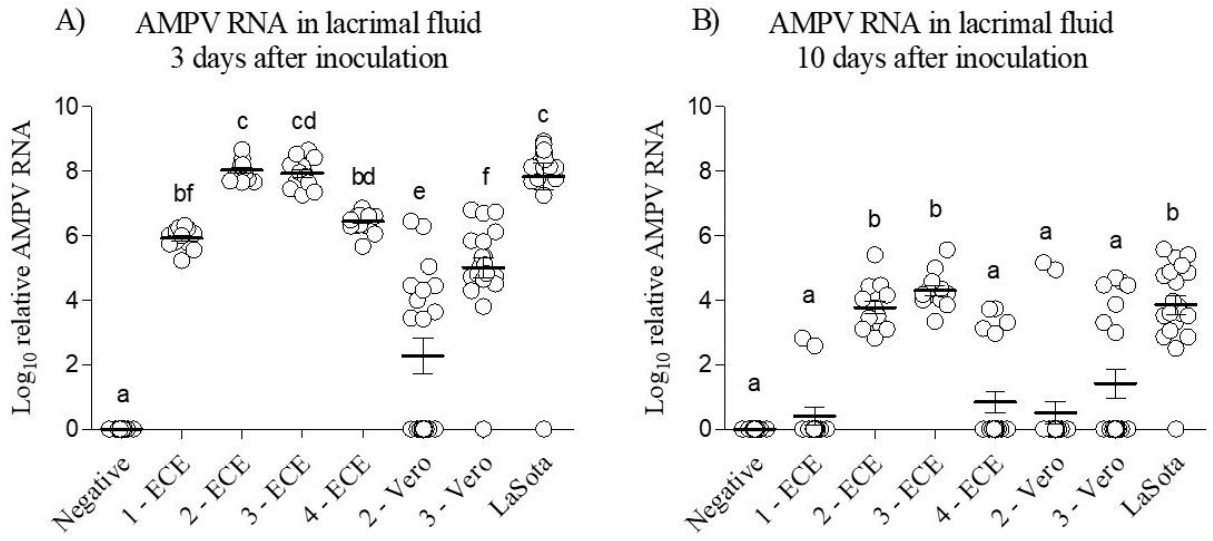


Fig. 4.5. Viral loads (log10 relative APMV RNA) in lacrimal fluid determined by RT-qPCR 3 and 10 days after ocular inoculation in SPF chickens at day 1 of age with APMVs 1 - 4 identified in Fig 4.1. Viral loads achieved by the reference LaSota strain are also shown. Individual values, average, and standard deviations are shown. Different letters indicate significant differences determined by ANOVA and Tukey's post-hoc test at $P \leq 0.05$.

CHAPTER 5.

# Conclusions

The combined vaccine (rLS/ArkSe.GMCSF and Mass) does not reduce viral shedding in vaccinated / Ark challenged chickens. The addition of the recombinant virus improves cross protection of the trachea.

Maternally derived antibodies do not seem to interfere with mucosal immune responses. The presence of maternally derived antibodies appears to favor B cell expansion in the Harderian gland.

NDV genotyping has been based solely on the F gene. Considering the variation observed in the HN gene during host adaptation, including the HN gene in genotyping efforts would be appropriate.

APMV-1s of wild bird origin elicited both systemic and mucosal immune responses in young chickens after administration via the ocular route, supporting their potential as vaccine candidates.

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