EFFECTS OF LOW PATHOGENICITY AVIAN INFLUENZA ON TURKEY BREEDING FLOCKS AND IMMUNE EVALUATION OF AN H1N2 DNA VACCINE

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ABSTRACT

Low pathogenicity avian influenza (LPAI) viruses can have substantial negative effects on egg production in turkey breeding flocks. Despite vaccination, turkey breeding hens continue to face disease associated with LPAI. Additionally, detecting a measurable immune response post-vaccination using traditional IAV killed vaccines has been challenging. In the present study, a plasmid-based DNA vaccine expressing the HA gene of an H1N2 LPAI virus in addition with immunostimulatory genes was developed and evaluated in turkeys. Turkeys were challenged with an H1N2 virus to determine the protective efficacy of the vaccine. Virus was neither detected in the birds post challenge by RT-PCR of oropharyngeal swabs nor were antibodies detected by ELISA from their sera. In conclusion, our study suggests DNA vaccine technology may be a good alternative to the currently administered autogenous vaccine, but due to the lack of a challenge model we were unable to properly evaluate immune efficacy of the vaccine.

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NOMENCLATURE and ABBREVIATIONS

IAV	Influenza A virus
HA	Hemagglutinin
NA	Neuraminidase
SA-gal	Sialic acid-galactose
HPAI	Highly pathogenic avian influenza
WOAH	World Organization for Animal Health
LPAI	Low pathogenicity avian influenza
ELISA	Enzyme-linked immunosorbent assay
NP	Nucleoprotein
NDV	Newcastle disease virus
PAMPs	Pathogen-associated molecular patterns
PRRs	Pattern recognition receptors
TLR	Toll-like receptors
RIG-1	Retinoic acid-inducible gene 1
RLR	Retinoic-like receptors
NOD	Nucleotide oligomerization domain

IFN	Interferons
MHC	Major histocompatibility complex
FPV	Fowlpox virus
HVT	Herpesvirus of turkeys
vRNP	Viral ribonucleoprotein
RdRp	RNA-dependent RNA polymerase
PB1	Polymerase basic 1
PA	Polymerase acidic
mRNA	Messenger RNA
FBS	Fetal bovine serum
NS1	Non-structural protein 1
PB2	Polymerase basic 2
IL-15	Interleukin 15
NK	Natural killer
IRF-1	Interferon regulatory factor 1
GM-CSF	Granulocyte macrophage colony stimulating factor
DC	Dendritic cells

RT-PCR	Real time polymerase chain reaction
MDCK	Madin-Darby canine kidney
HEK293	Human embryonic kidney 293 cells
AP	Alkaline phosphatase
CMV	Cytomegalovirus
Co	Codon Optimized
IM	Intramuscular
HI	Hemagglutination Inhibition

CHAPTER 1

INTRODUCTION

1.1 Avian Influenza Virology and Host Range

Avian influenza is a disease caused by influenza A viruses (IAV) (Swayne and Sims, 2021; Swayne et al., 2020). All IAV's are classified into subtypes according to the combinations of the virus surface proteins, hemagglutinin (HA) and neuraminidase (NA). There are 18 recognized HA subtypes and 11 different NA subtypes (CDC, 2023). Wild aquatic birds are the natural reservoirs of IAV and sources of viral genetic diversity (Webster et al., 1992). The prevalence of IAV in wild birds varies by species, age, season, and geographic location (Stallknecht and Shane, 1988). Poultry may be accidental or primary hosts of IAV depending on the poultry sector (Artois et al., 2018), viral lineage (Cheung et al., 2007; Guo et al., 2000; Huang et al., 2010; Yoon et al., 2014), and viral status in a region (Swayne, 2012). In some mammalian species including swine, horses, dogs, and bats, IAVs are endemic (Choi et al., 2004). Individual IAV strains are generally host-specific; although, endemic strains can be transmitted to other species and cause disease and/or lead to additional viral diversity as the virus adapts to a new host (Choi et al., 2004). One of the major selective pressures for influenza viral infections is the availability of receptors on host cell surfaces (Matrosovich et al., 1997; Rogers and Paulson, 1983). Mammalian influenza viruses preferentially bind to α2,6 sialic acid (SA)-galactose (gal) terminated sialyloligosaccharides, whereas avian viruses prefer α2,3SA-gal terminated residues (Matrosovich et. al., 2003). Pigs express both α2,3SA-gal and α2,6SA-gal receptors on their tracheal epithelium and are implicated as potential mixing vessels of avian and mammalian influenza viruses (Scholtissek et al., 1998; Ito et al., 1998). Poultry, however, are susceptible to a wide variety of IAV subtypes. The term poultry encompasses multiple commercial avian species; this research will focus on turkeys (*Meleagris gallopavo*). Turkeys are most commonly infected by influenza viruses of wild bird and mammalian (human and pig) origin, and although not widely recognized as IAV mixing vessels, they possess the host characteristics to be so.

Turkeys may become infected with IAV through direct contact with infected species or through contact with IAV contaminated surfaces or environments. As they become infected with IAVs from other species, these viruses potentially evolve to become novel reassortant or recombinant strains that may be more adaptable in other species such as humans (Zhang et al., 2020). Turkey breeding hens have been infected with IAV from humans during the process of artificial insemination (Evans et al., 2014). Mammalian-origin influenza viruses have greater affinity for the turkey hen reproductive tract (Pantin-Jackwood et al., 2010) and cause frequent infections that impact egg production. Viruses that may cause little clinical disease in swine or commercial poultry when present can have devastating effects on turkey breeding stock.

1.2 Avian Influenza Pathogenicity

The diversity of IAV genetics results in viruses that can cause a broad range of clinical signs in poultry (Swayne, 2012). The most severe clinical presentations in susceptible poultry are classified as highly pathogenic avian influenza (HPAI). The World Organization for Animal Health (WOAH) Terrestrial Animal Health Code (Terrestrial Code) defines HPAI as an infection with a highly pathogenic avian influenza (HPAI) virus. In contrast, most IAV infections are of low pathogenicity (low pathogenicity avian influenza (LPAI)) and although clinical signs may range from moderate to severe based on the viral strain, co-infections, and host species, most LPAI virus strains cause few or no discernable signs (Swayne et al., 2020). However, LPAI viruses of subtypes H5 and H7 can evolve into HPAI variants, which cause significant disease. Two main phylogenetic lineages further divide LPAI viruses: Eurasian and American lineages, which are based on the long-term geographic separation of IAV (Chen and Holmes, 2006; Webster et al., 1992; Olsen et al., 2006). The segmented genome of influenza viruses enables evolution by a process known as genetic reassortment, the mixing of genomic segments from two (or more) viruses (Webster et al., 1992). Reassortment is one of the driving forces of the genetic variation of LPAI and HPAI viruses and contributes greatly to their phenotypic variation. LPAI viruses may also be classified by species origin. Hereafter, we will separate LPAI viruses based on three main reservoirs of origin: mammalian, wild bird, and domestic poultry. LPAI viruses of H1 and H3 subtypes are most commonly of mammalian origin, while H1-H13 are isolated from wild birds (Joseph et al., 2017). Poultry are susceptible to most subtypes from the wild bird reservoir, however HA subtypes H1, H3, H5, H6, H7, and H9 comprise most infections of domestic poultry (Joseph et al., 2017). Turkeys, in particular, are susceptible to H7 and H10 subtypes (Joseph et al., 2017) while apparently are less susceptible to H3 of avian origin (Guo et al., 2015).

In turkeys, low pathogenicity IAVs replicate in epithelial cells of the gastrointestinal and respiratory tracts, producing a variety of conditions including asymptomatic and subclinical infections, or, occasionally, respiratory disease (Pantin-Jackwood and Swayne, 2009). In reproductively mature hens, replication can also occur in the oviduct leading to a decrease in egg production (Pillai et al., 2009). Many LPAIV infections present clinically in turkeys as mild-to-severe respiratory disease with signs of coughing, sneezing, rales, rattles, and excessive ocular discharge (Pantin-Jackwood and Swayne, 2009). Breeding hens may express increased broodiness and transient decrease in egg production. In general, birds may have decreased activity, mild weight loss and may huddle into groups. Individual infected birds may be lethargic and have ruffled feathers, show decreased feed and water consumption leading to mild weight loss and, occasionally, diarrhea (Pantin-Jackwood and Swayne, 2009). Gross lesions typically vary depending on the virus strain and time to death. In breeding hens, ovaries in infected hens may regress and mature ova rupture producing egg yolk peritonitis. The oviduct may be swollen with luminal exudates. Inflammation in the shell gland results in reduced eggshell matrix deposition, depigmentation, and misshapen eggshells. Eggshell abnormalities are commonly the only sign of infection and may lead to an increase in non-viable hatching eggs. Egg production decreases range from a mild to severe drop with recovery time anywhere from 2 weeks to never fully recovering to baseline production depending on viral strain. Figure 1 shows a comparison of egg production from two different breeder hen flocks affected with two different strains of LPAIV.

1.3 LPAI Vaccination

Biosecurity measures and vaccination are key components implemented in control programs against LPAI in turkey breeders. Vaccination to control LPAI has been attempted since the discovery of the disease (Alexander, 2003). The purpose of vaccination is largely to reduce the production impacts of the disease such as decreased egg production in laying poultry or decreased weight gain in meat type birds. Immunization not only minimizes clinical signs of disease but also decreases viral shedding, reducing the amount of virus in the environment, thus limiting viral spread. Vaccination against LPAI is not aimed at prevention of an index case, but rather is used to respond to ongoing outbreaks in avian or mammalian hosts. Given the diversity of IAV that might be introduced from the wild bird reservoir, new challenges are impossible to predict. Due to the need for specific protection and low demand for vaccines for a non-endemic disease, autogenous vaccines are the logical solution. Autogenous vaccines are inactivated and delivered in an oil adjuvant, requiring handling and injection of every bird, thus their use is time consuming and costly but effective in creating uniform flock-level immunity (Palomino-Tapia, 2023).

Vaccination of turkey breeders for swine influenza, which is a term used to refer to LPAI viruses of mammalian origin, is common practice in many turkey breeding systems. Autogenous vaccines commonly of the H1 and H3 subtypes are used to protect breeders from the clinical impacts of IAVs that are endemic in both swine and human populations (Corzo et al., 2012). Seed viruses that are representative of strains circulating in swine or turkey flocks near to breeding stock are used for autogenous inactivated vaccines. New strains are selected regularly to ensure vaccines are a good match to the challenge strains that continuously emerge in swine and human populations. Extensive training is required for bird handlers vaccinating hens to ensure minimal tissue damage and stress. Vaccination in turkey breeders is aimed at protection against disease in the production period from onset of lay at 31-weeks of age, until the end of lay, at 58-weeks of age. Birds are typically immunized before the onset of egg production (pre-production) during the growing phase between 22- and 26-weeks of age. Most vaccination strategies require two injections to boost the bird’s immune response. Vaccine efficacy is evaluated using a commercially available enzyme-linked immunosorbent assay (ELISA). The assay is a blocking ELISA that detects serum antibodies against the nucleoprotein (NP), which is highly conserved across IAV strains (Spackman and Killian, 2020). The assay does not differentiate between immunity formed in response to vaccination or infection, although in clinical practice, baseline serology taken before vaccination is used to measure infection status.

Despite the importance of turkey breeders in the ecology of IAV and the role of vaccination in their protection, there have been limited studies on the immune response to viral vaccines in turkey breeder candidates. Studies on the distribution and size of lymphocyte populations in the oviduct indicate that the immune response is different throughout the life-stages of the hen (Kowalczyk et al., 2020). This is an important determinant for the timing of vaccination and persistence of protection. Poor immune responses are often related to stress factors (Kaboudi, 2020), which breeder candidates frequently encounter when they undergo activities such as vaccination, light stimulation, artificial insemination, and movements. The turkey breeder hen is an immunologically complex animal whose immune capacity changes over her lifetime because of physiology and stressors and, thus, the response to influenza vaccines and her protection should be well-monitored. It has also been observed that turkey breeding hens have a predominance of α2,3SA-gal receptors in the oviduct which some subtypes of IAVs utilize for infection (Pillai et al., 2009). The type and number of receptors in the oviduct is responsible for the localization of clinical signs observed in turkey breeders and also helps explain why commercial turkeys may be infected with the same IAV subtype but may not show clinical signs.

1.4 Avian Influenza History in Minnesota

Minnesota, known as the land of 10,000 lakes, has the optimal ecosystem intertwined with multiple commercial and wildlife species for IAV to persist. Figure 2 shows the history of IAV detections and outbreaks in commercial poultry in Minnesota over the last 57 years. This figure depicts the shift in IAV HA subtypes throughout the years as the industry evolved and overcame the challenges associated with avian strains of IAV. Prior to 1985, IAV from wild birds were most commonly detected. In 1984, the Minnesota AI control plan (personal communication, Dave Halvorson, 2022) was implemented, and there was shift towards a mix of HA subtypes of wild bird, mammalian, and avian origins. The practice of rearing turkeys on range, or outdoors, ended in 1997 by industry agreement which led to a decrease in the incidence of IAV from wild bird sources in commercial turkeys. Further, the mammalian origin H1 and H3 viruses have dominated turkey cases since 2000, which arose as swine and poultry operations increased in size and decreased proximity to each other. These shifts in the influenza ecosystem as observed in Minnesota are a result of various management strategies employed throughout the years by producers and regulatory agencies (personal communication, Carol Cardona, 2022). Previous outbreaks have also demonstrated a difference in the way that commercial operations need to prevent, control, and respond to wild bird, avian and mammalian IAVs. Biosecurity, vaccination, controlled movements, active surveillance testing and stamping out are all strategies that have been utilized for various IAV LPAI strains and have had varying degrees of success depending on viral characteristics, sector of the industry involved, and the mode of viral transmission.

1.5 LPAI Cases in Minnesota Turkey Breeder Hens

During the Spring of 2020, H6N1 LPAI was perpetuated via movements through commercial turkey flocks in Minnesota and eventually the virus infected turkey breeder flocks. Infected breeder flocks had decreased water consumption, substantial decreases in egg production, followed by increased eggshell abnormalities, and mortality. Unlike other LPAI viruses in turkey breeder hens, mortality was one of the first signs of H6N1 infection. Although there was proactive surveillance for IAV at the time, including environmental sampling with drinker swabs for IAV PCR (Munoz-Aguayo et al., 2019), infections with the H6N1 IAV were rarely detected prior to the onset of mortality. The increased mortality triggered diagnostics, and after diagnosis by tracheal swab testing, flocks were voluntarily quarantined by producers to contain the virus. Despite these efforts, the H6N1 LPAIV spread throughout the region including the infection of multiple turkey breeder flocks. Eventually the disease outbreak ended due to the rapid elimination of infected flocks and the resultant decrease in available susceptible hosts (Personal observation, Marissa Studniski, 2021).

In December of 2020 in Minnesota, active surveillance continued as a result of the H6N1 outbreak earlier in the year. A turkey breeding company had a positive IAV detection from environmental drinker swabs from one barn on a lay farm. Successive tracheal swabs confirmed the diagnosis of H1N2 LPAIV in turkey breeding hens. One week after detection, the flock experienced a decrease in egg production followed by eggshell changes (soft and white eggshells) two days later. The virus spread through all flocks housed on the farm within three weeks. Weekly tracheal swabs were collected to monitor viral shedding, and all flocks were negative by 7-weeks post-detection. However, the egg production on the farm never fully recovered to baseline throughout the life of flock. Unlike the H6N1 outbreak, this case was contained on one farm in one breeder company and was not transmitted to other poultry flocks in the system or region. Genomic sequencing of the HA1 segment of the isolated virus indicated the virus was a delta 2 swine lineage H1N2 virus of clade 1B.2.1. This virus was concurrently circulating in IAV-infected swine herds in the US (Vincent et al., 2009 and personal communication, Marie Culhane, 2020). Swine were housed on a farm less than 2 miles from the infected commercial turkey breeder operation and were the likely sources of the virus.

1.6 Rationale and Significance

In Minnesota, turkey breeder hens have repeatedly been the gateway cases to LPAI and HPAI outbreaks as a result of their susceptibility to infection. Despite autogenous vaccination strategies and enhanced biosecurity measures, annual detections of IAV in Minnesota have led to substantial economic loss. Serological data are routinely collected to monitor turkey breeder disease protection as a result of vaccination. Routine monitoring of flocks occurs at approximately 32- and 51-weeks of age (point of lay and mid lay, respectively), depending on the breeding company's vaccination program. Historical data shows that turkey breeders mount a consistent antibody response to Newcastle disease virus (NDV), where an injectable killed vaccine is preceded by live vaccinations (Figure 3). In contrast, breeder hen flocks frequently fail to mount a detectable immune response post influenza vaccination using two doses of a killed autogenous vaccine (Figure 4) or mount a poor immune response which is evident with low antibody titers. Similar results have been reported by Kapczynski et al., 2009. Figures 3 and 4 show post- vaccination titers from two representative turkey breeder hen flocks on the same farm at the onset of egg production. Data collected over the last decade from multiple breeder flocks within various companies across different regions of the US repeatedly show similar results upon evaluation of titers to IAV, regardless of vaccine manufacturer (unpublished data). However, when turkey breeder flocks are infected with a live, field strain of low pathogenicity IAV of any subtype or origin, a measurable antibody response using the same ELISA test kits used to evaluate the vaccination program is detected (Figure 5). These data demonstrate that turkey breeder hens mount antibody responses to both NDV and IAV as measured with a standard ELISA test, but the response is not detected when hens are given a killed IAV vaccine. Due to poor immunological response post-vaccination, some turkey breeder companies in the US no longer use vaccination as a preventative against LPAI (personal communication, Carrie Cremers, 2021). This indicates the need for development of updated vaccination platforms that can be safely used on turkey hens, create a detectable immune response, and protect them from influenza clinical signs.

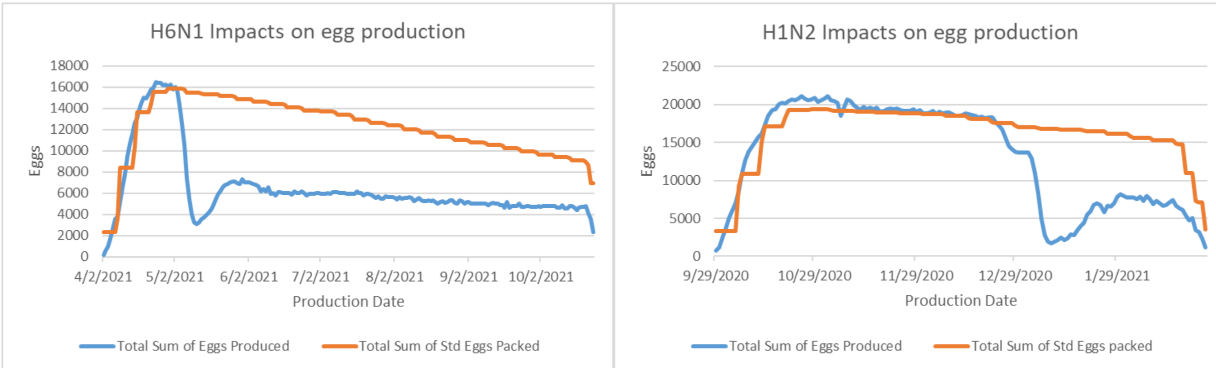

Fig. 1. Impact on egg production from two different strains of LPAIV; H6N1 (left) and H1N2 (right). Standard egg production by day for the life of flock (orange) graphed in comparison of actual eggs produced throughout the life of flock (blue) for two different past outbreaks of LPAI in turkey breeder hens.

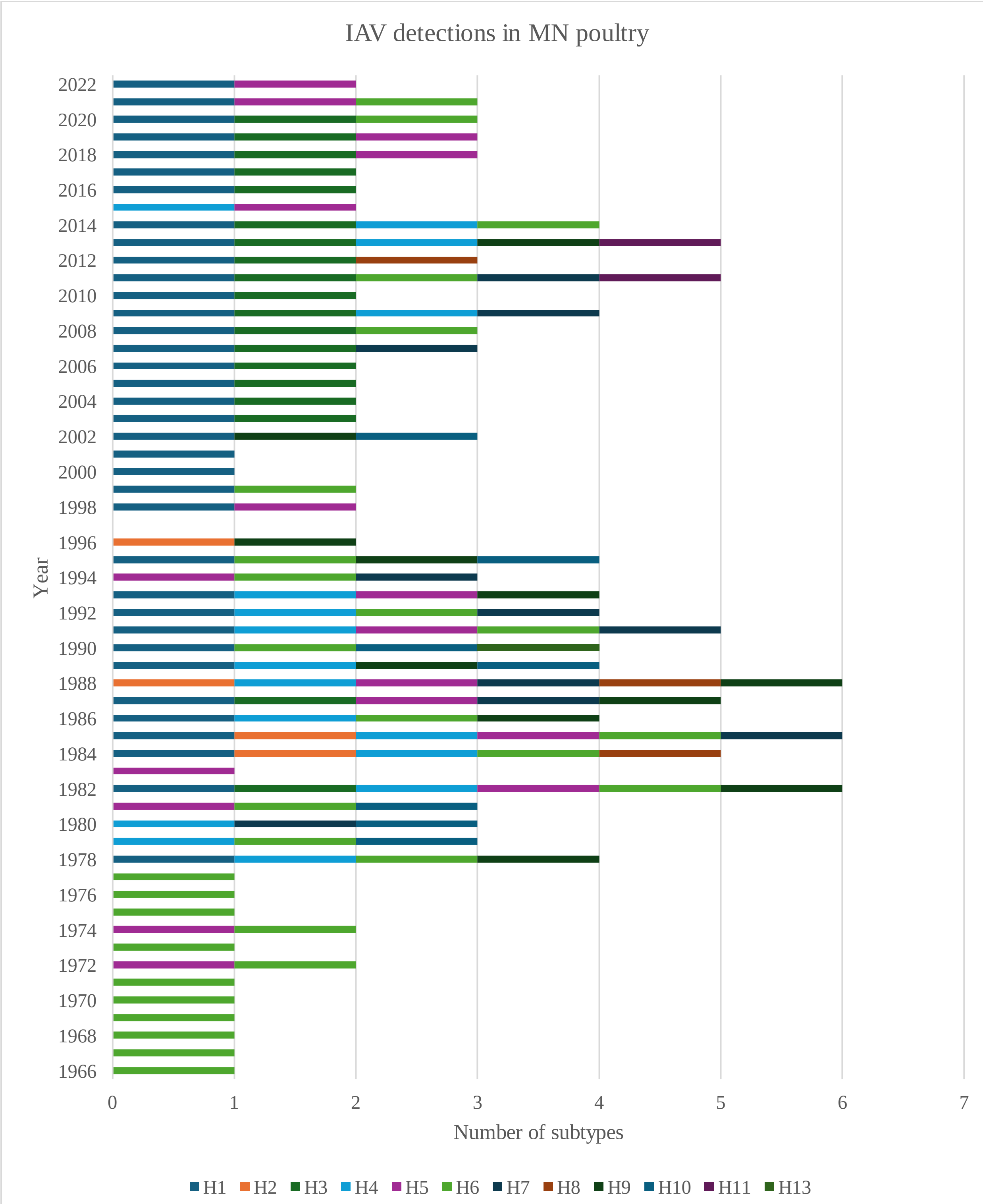
IAV detections in MN poultry
Year
2022
2020
2018
2016
2014
2012
2010
2008
2006
2004
2002
2000
1998
1996
1994
1992
1990
1988
1986
1984
1982
1980
1978
1976
1974
1972
1970
1968
1966
0
1
2
3
4
5
6
7
Number of subtypes
H1 H2 H3 H4 H5 H6 H7 H8 H9 H10 H11 H13

Fig. 2. 57-year history of IAV hemagglutinin subtypes diagnosed in commercial poultry by year in Minnesota by the state laboratories. The colors indicate hemagglutinin subtype and the total bar length by year indicates how many cases were diagnosed.

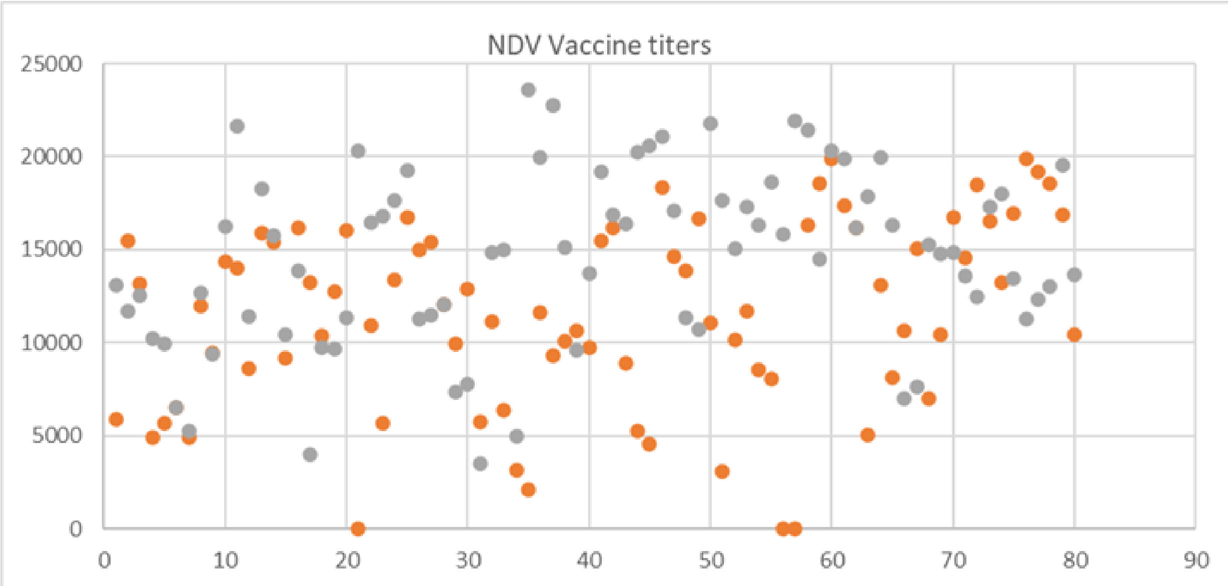

Fig. 3. Individual bird Newcastle vaccine (AviPro 233 ND-PMV3, type 3 killed virus) titers from two flocks on the same farm at 32 weeks of age. 77/80 samples from flock 1 (indicated by orange dots) are seropositive and 80/80 samples from flock 2 (indicated by grey dots) are seropositive. Titers were measured using the Synbiotics ELISA commercial test kit.

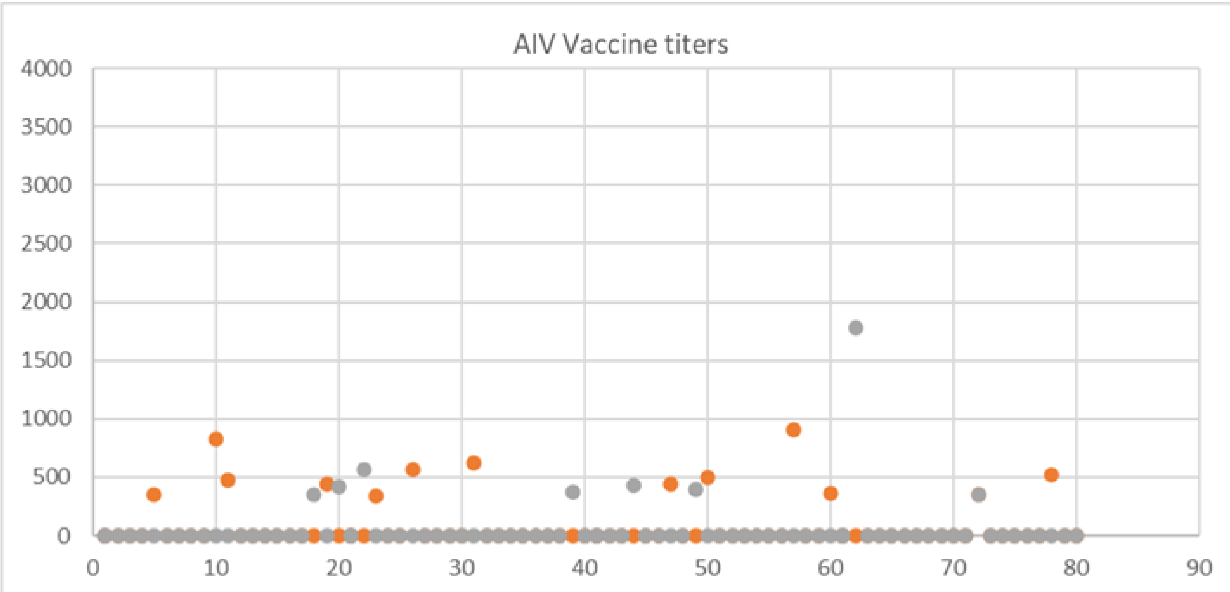

Fig. 4. Antibody titers of individual birds vaccinated with H1/H3 killed autogenous vaccine (Lohmann Animal Health) from the two flocks on the same farm at 32 weeks of age. 13/80 samples from flock 1 (indicated by orange dots) are seropositive and 8/80 samples from flock 2 (indicated by grey dots) are seropositive. Titers were measured using the Synbiotics ELISA commercial test kit.

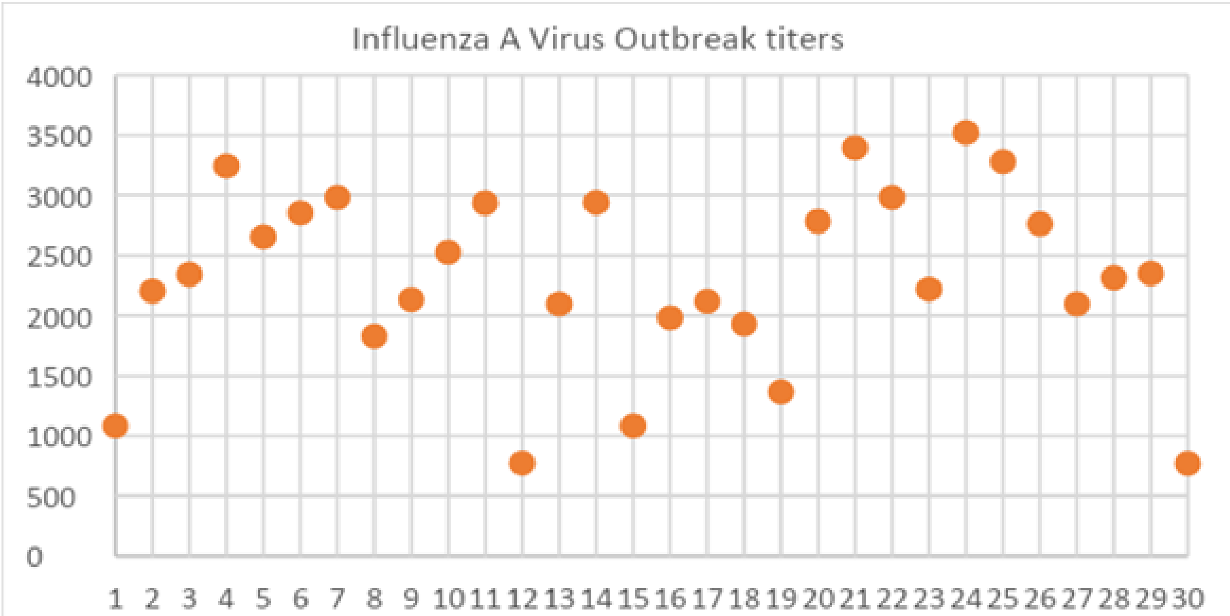

Fig. 5. Individual bird IAV titers 6 weeks post H1N2 infection. 30/30 samples are seropositive. Titers were measured using the Synbiotics ELISA commercial test kit.

Chapter 2

LITERATURE REVIEW

2.1 Avian Immune Response to IAV

The IAV genome is made up of eight gene segments of single-stranded, negative-sense RNA (Figure 1A; Bouvier and Palese, 2008). Each gene segment has a viral ribonucleoprotein (vRNP) comprising an RNA-dependent RNA polymerase (RdRp) complex formed by the association of three polymerase proteins: polymerase basic 2 (PB2), polymerase basic 1 (PB1), and polymerase acidic (PA), with viral genomic RNA integrated to the nucleoprotein (NP) molecules (Figure 1B; Eisfeld et al., 2015; Weis and Aartjan, 2021). The IAV virions have spike-like projections at the outer surface, which are composed of two surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA) (Byrd et al., 2017). The existing high antigenic variability within surface glycoproteins classifies the members of IAV into 18 HA and 11 NA subtypes (Jang and Bae, 2018). The HA protein on the IAV surface recognizes and binds to sialic acid receptors on host cells. After virus entry into a host cell, all eight viral genome segments are released into the nucleus to initiate virus transcription and replication. Protein synthesis of IAV is entirely dependent on the translation machinery of the host cell. The neuraminidase (NA) protein facilitates the release of new virions from the host cell membrane, which are then disseminated throughout the host. Figure 2 summarizes the replication cycle of IAV in host cells (Nuwarda et al., 2021). Since HA is a surface glycoprotein on the virus particle, it is readily recognized by host antibodies when an IAV infects a host (Luo, 2012). However, IAV strains are quite capable of escaping natural- or vaccine-induced host defense mechanisms by accumulating antigenic changes in the HA surface protein-producing viral variants, which immunized hosts may not recognize (Luo, 2012; Park et al., 2009).

The HA protein plays a central role in virus entry and induces an immune system response upon exposure via infection or when delivered in a vaccine. Traditional vaccines depend on the HA as their primary antigen but also include additional proteins (Suarez and Schultz-Cherry, 2000). The protection afforded by vaccines is a result of the stimulation of cellular and/or humoral immunity (Kapczynski et al., 2017). However, the level of protection produced by vaccination is directly correlated with amount of similarity between the HA of the vaccine and the challenge virus (Kapczynski et al., 2013; Swayne et al., 2000), especially for inactivated vaccines (Kapczynski et al., 2017). Though protection from IAV infection or disease has been reported between seemingly diverse strains, i.e., strains of virus that are heterosubtypic or heterologous, successful protection is most likely to occur when the vaccine and challenge viruses or components are highly similar or homologous (Kapczynski et al., 2013).

The innate immune response is the first line of defense against pathogens and ultimately regulates the adaptive immune response. In IAV infection, the viral pathogen-associated molecular patterns (PAMPs) are recognized as foreign by host pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), retinoic acid-inducible gene 1 (RIG-1)- like receptors (RLRs), and the nucleotide oligomerization domain (NOD)- like receptor family, which induce the expression of type 1 interferons (IFNs), pro-inflammatory cytokines and chemokines (Iwasaki and Pillai, 2014). TLRs are intracellular receptors that recognize influenza viral RNA and activate signaling, resulting in the activation of transcription factors which induce the expression of type 1 IFNs and pro-inflammatory cytokines. These trigger the antiviral response and lead to the recruitment of heterophils, macrophages, and dendritic cells to the site of infection (Mishra et al., 2017). Upon infection with IAV, the virus evades the host's immune system by disruption of IFN induction so it can replicate in the host's tissues (Mishra et al., 2017). In addition to the rapid response of the hosts' innate immune system to IAV infection, the host also has an adaptive or acquired immune system comprised of B and T lymphocytes that responds later during an infection. Unlike the PRRs on innate immune cells, lymphocytes use RAG recombinase to generate a diverse number of antigen receptors (Perera et al., 2012). Humoral immunity provided by B lymphocytes and their secreted antibodies protect against extracellular pathogens (Perera et al., 2012). In contrast, cellular immunity mediated by T lymphocytes defends against intracellular pathogens by killing infected cells (Perera et al., 2012). Memory T and B lymphocytes and their products provide a more rapid and robust immune response upon re-exposure of the same pathogen which helps prevent re-infection or significantly reduces the severity of disease.

2.2 Vaccination Approaches Against IAV in Poultry

Vaccines are broadly categorized into live and inactivated vaccines. Live vaccines for IAV are not used in poultry due to the potential to spread to new flocks. Live-attenuated or adapted live vaccines could be used in poultry but the research and development has not been invested due to the limited market. Multiple vaccine platforms have been studied in poultry against IAV with various degrees of success. Currently, there are no licensed vaccines for use in poultry against LPAI. The main vaccination approaches in poultry will be reviewed.

2.2.1 Inactivated vaccines

The most widely used IAV vaccines are inactivated viruses (Karunakaran et al., 1987) delivered with an oil adjuvant. The efficacy of these traditional vaccines is dependent on the seed virus used, it's relatedness of the HA protein to the challenge strain as well as the ability to deliver the vaccine to a host. Inactivated vaccines generally require two doses given via intramuscular injection and separated by a minimum interval of two weeks. The delivery of inactivated vaccines requires a skilled and available workforce increasing the cost of vaccination. Although skilled handling improves immunization uniformity, since most poultry are raised in large populations the expense of extra labor is usually considered a drawback (Beard, 2003). Whole-inactivated virus vaccines should induce anti-matrix (M) protein or anti-nucleoprotein (NP) antibodies and serological assays such as agar gel immunodiffusion (AGID) tests or enzyme-linked immunosorbent assays (ELISA) can be used for antibody detection as an indicator of vaccination. Protective antibodies can be measured with hemagglutination inhibition (HI) or serum neutralization (SN) assays (Suarez and Schultz-Cherry, 2000). The majority of laboratory and field IAV vaccine trials in turkeys have been carried out utilizing whole inactivated vaccines. Results from these studies demonstrate protective seroconversion titers, decreased clinical signs and significant reduction in viral shedding when challenged with a homologous IAV (Elfeil et al., 2022; Kapcynski et al., 2009; Abraham et al., 1988; Karunakaran et al., 1987). However, inactivated vaccines have not shown successful protection against disease when challenged with heterologous IAV strains (Naguib et al., 2017).

2.2.2 Recombinant vaccines

Recombinant vaccines have advantages over inactivated vaccines because they can deliver foreign antigens (IAV HA, for example) alone or in combination with other proteins.

Recombinant vaccines may reduce the need for adjuvants as the antigen itself or delivery platform represents a potent target for the immune system. Additionally, recombinant vaccines can be targeted to induce both cellular and humoral immune responses associated with major histocompatibility complex (MHC) class I and class II molecules, respectively (Peyre et al., 2009). Vectored vaccines against IAV can be used for emergency vaccination because the production platform is already in place making it much easier to change antigenic targets (Gerlach et al., 2019; Rahn et al., 2015). Finally, some vaccine platforms can be delivered *in ovo*, which is a tremendous advantage when large populations need to be vaccinated (Williams, 2007).

2.2.3 Vectored vaccines

Vectored vaccines provide protection against a pathogen by using a non-pathogenic agent as a vector to deliver selected immunogens into the host (McCann et al., 2022). The most common vectored IAV vaccines in poultry utilize viruses as vectors, namely Newcastle disease virus (NDV), fowlpox virus (FPV), and herpesvirus of turkeys (HVT) (Hein et al., 2021), and all three have been used in IAV vaccination campaigns.

Fowlpox virus-vectored H5 vaccine (FPV-H5), as well as inactivated H5N2 vaccine, were used as part of the program to eradicate H5N2 HPAI from Mexico (1994-5) and continue to be used in the control of LPAI in Mexico, El Salvador, and Guatemala (Senne, 2007). Although the original vaccine is less protective today against drifted field strains, FPV vaccines with updated HA inserts have demonstrated efficacy (Bertran et al., 2020). The FPV-H5 is given as a single dose in the hatchery and its protective efficacy is monitored with the HI assay (Bublot et al., 2006). Because this vaccine lacks the NP and matrix proteins of IAV, immunized birds that have not been challenged with a field strain will be negative for IAV antibodies on AGID and most commercially available ELISA diagnostic tests.

Mexico deployed an FPV-H7 vaccine in response to an HPAI H7N3 outbreak in central Mexico in 2012 (Bertran et al., 2020; Kapczynski et al., 2013). Unlike the 1994 outbreaks, the 2012 outbreak strain was not eradicated. This virus has continued to circulate (Navarro-Lopez et al., 2022; Youk et al., 2019) and Mexican authorities have updated the HA portion of the FPV-H7 vaccine and the killed strains at least five times to keep up with emerging strains (Navarro-Lopez et al., 2022).

HVT-H5 vaccines are delivered at one day of age in the hatchery and have been widely used in commercial poultry as a control tool for HPAI (Kapczynski et al., 2016) as well as for H9N2 LPAI (Chen et al., 2023). The HVT vectored vaccines stimulate humoral immunity, which is HA specific, in addition to cell-mediated immunity, which stimulates cross protective (heterosubtypic) immunity (Kapczynski et al., 2015).

The past couple of decades have shown significant progress in vaccine generation based on optimization of the NDV vector (Hu et al., 2020). NDV differs from both FPV and HVT vectors in that it can be mass applied via drinking water or spray (Ma et al., 2017) which can be an advantage for the delivery of vaccines to birds that have already been housed. Several countries have used NDV-vectored influenza vaccines including Mexico and China (Kim et al., 2019).

2.2.4 DNA and RNA vaccines

DNA and RNA vaccines contain genetic information that instructs cells in the host to produce a protein with a structure similar to that of the virus. Unlike traditional vaccines, neither

RNA nor DNA vaccines contain live virus. For this reason, nucleic acid vaccines can be targeted to specific challenges and produced more quickly than traditional vaccines. RNA and DNA vaccines induce similar immune responses, but DNA vaccines may require an electrical pulse to reach the cell.

DNA vaccines deliver a foreign antigen through inoculation of the host with plasmid DNA encoding the IAV antigen, which will then be produced in the host (Meunier et al., 2016). Both DNA and RNA vaccines are relatively easy to produce, but the manufacturing process differs slightly between them. Once DNA encoding the antigen has been chemically synthesized, it is inserted into a bacterial plasmid. Multiple copies of the plasmid are then produced within giant vats of rapidly dividing bacteria, before being isolated and purified. The first DNA vaccine studied in poultry in 1993 was directed against IAV (Deviatkin et al., 2022; Robinson et al., 1993) and the strategy continues to be widely studied in chickens (Meunier et al., 2016). Despite early promise, DNA vaccines induced limited immune responses and/or protection, and none have become commercially available. Many strategies have been evaluated to increase the protective immune responses of poultry to DNA vaccines. These include optimization of the route of inoculation, the plasmid DNA dose, booster injections, and the age of the birds inoculated, promotion of uptake of plasmids by the host cells, the addition of immune modulators, the optimization of the plasmid backbone and host codon usage, association of vaccine antigens, and the use of a heterologous prime-boost regimen (Meunier et al., 2016).

RNA vaccines encode the antigen of interest in messenger RNA (mRNA). RNA vaccines are easier to synthesize because this can be done chemically, from a template in the lab, without the need for any bacteria or cells. Unlike DNA vaccines, mRNA vaccines are one step ahead and do not rely on the host cell nucleus, as transcription takes place inside the cell cytoplasm to produce proteins. Therefore, mRNA vaccines tend to produce a greater immune response in comparison to DNA vaccines which must penetrate the cell nucleus to be effective (Shukla, 2021). However, a single plasmid DNA can produce numerous copies of mRNA and once plasmid DNA enters the nucleus, it can produce more viral protein than a single molecule of an mRNA vaccine. RNA vaccines are not as stable as DNA vaccines and require controlled temperatures until administration (Shukla, 2021). The possibility of using mRNA vaccines against IAV is already the subject of active study; several prototypes are being tested in humans (Deviatkin et al., 2022) and may also be developed for poultry.

2.2.5 Reassortant vaccines

Reassortant IAV vaccines have advantages because they present more viral epitopes to the host than vectored vaccines, thus improving the host's response to vaccination as well as increasing cross protective potential over inactivated vaccines. In a reassortant vaccine, reverse genetics is used to create clones of a target virus. The IAV is then attenuated through the introduction of mutations in the hemagglutinin, non-structural protein (NS1), and/or polymerase basic 2 (PB2) coding regions. Reassortant vaccines have shown characteristics desirable for live attenuated vaccines and show potential as vaccine candidates in poultry (Steel et al., 2009). In addition, using this approach, the viral NA can be changed from the original to create a vaccine that can be differentiated from the circulating IAV strain (Capua et al., 2003; Suarez, 2012). Reassortment can also be used to develop seed viruses with ideal characteristics to use as inactivated vaccines (Suarez, 2012).

2.2.6 Other vaccine platforms

Numerous other vaccine platforms against IAV have been tried in both human and animal medicine. Some have shown promising results including temperature sensitive mutant live vaccines (Calzas and Chevalier, 2019), protein nanoparticle vaccines (Deng et al., 2018), baculovirus expression of HA (Olsen et al., 1997;), and Pichinde virus-vectored vaccines expressing multiple IAV gene segments (Dhanwani et al., 2017). There are other delivery platforms, which are used in poultry and could be used to deliver IAV antigen. Live recombinant attenuated *Salmonella*-vectored vaccines also show promising results by achieving long-lasting mucosal, humoral, and cellular immunity against a variety of non-*Salmonella* pathogens at a low cost. The recombinant *Salmonella* strain expressing the heterologous gene can be orally administered to elicit an immune response against the pathogen from which the heterologous gene was derived (Roland and Brenneman, 2013).

2.3 Role of Cytokines in Immune Response and Vaccination

Several strategies have been employed to develop vaccines with improved effectiveness against viral pathogens, especially those that display extensive genetic variability such as IAV. One such strategy that is being explored is the use of immune enhancing cytokines as adjuvants (Tovey and Lallemand, 2010). Interleukin-15 (IL-15) is a cytokine that plays a major role in the development of inflammatory and protective immune responses to pathogens by modulating immune cells of both the innate and adaptive immune systems (Perera et al., 2012). IL-15 plays an essential role in the development, differentiation, and survival of natural killer cells (NK cells). In macrophages, IL-15 also functions as a potent regulator of proinflammatory cytokine production by these cells. Numerous studies have indicated the effectiveness of IL-15 as a vaccine adjuvant in increasing cellular and/or humoral immune responses against infectious pathogens employing multiple techniques to target the pathogen including DNA vaccines, virus vector-based vaccines, and using different methods to deliver IL-15 (Tovey and Lallemand, 2010, Perera et al., 2012). One study demonstrated a combined DNA-IL-15 vaccine against *Brucella abortus* in mice induced a robust humoral response and a protective $CD8^+$ T cell response, although $CD4^+$ T cells also played a contributory role in the protection against infection (Hu et al., 2010).

Interferon regulatory factor 1 (IRF-1) is a key factor that binds and activates the promoters of type 1 IFNs. IRF-1 has been shown to suppress the replication of RNA viruses, and it plays a key role in host antiviral defense (Feng et al., 2021). The role of IRF-1 during IAV infection in turkeys has not been studied. However, inhibition studies in mammals of IRF-1 have shown increased susceptibility to disease (Antonczyk et al., 2019), which may suggest IRF-1 plays a key role in immune response during IAV infection. IRF-1 also regulates the early apoptotic response during influenza infection (Campbell and Magor, 2020). In a study by Castaldello et al., 2010, *in vivo* administration of plasmid DNA encoding *tat* of HIV-1 and IRF-1, or a mutated version of IRF-1 deleted of the DNA-binding domain, enhanced *tat*-specific immune responses in mice and shifted them towards a predominant T helper 1-type immune response with increased IFN-γ production and cytotoxic T lymphocytes responses. These findings define IRF-1 as efficacious T helper 1-inducing adjuvants in the context of *tat*-based vaccination and providing a new promising candidate to enhance immune response to vaccines.

Granulocyte macrophage colony stimulating factor (GM-CSF) is a glycoprotein and pro-inflammatory cytokine. GM-CSF regulates proliferation and differentiation of hematopoietic progenitors (Tan et al., 2009). It has a potent effect on the differentiation and maturation of dendritic cells (DCs) as well as the expression of MHC and co-stimulatory molecules (Shi et al., 2006). The DC system is the initiator and modulator of the immune response (Banchereau and Steinman, 1998). Co-inoculation with plasmid DNA vaccines expressing GM-CSF has been reported to increase protective immunity against herpes simplex virus (Parker et al., 2006), classical swine fever virus (Andrew et al., 2006), foot-and-mouth disease virus (Du et al., 2007), infectious bronchitis virus (Tan et al., 2009), and human immunodeficiency virus (Qiu et al., 2007), as a result of enhancement of antigen-specific humoral and cellular immune responses.

The majority of IAV vaccination trials have been carried out in chickens and mammals leaving turkeys understudied and veterinarians left with assumptions regarding vaccine efficacy and protection against disease in turkeys relative to other species. Though multiple vaccine technologies have been developed for IAV in poultry and humans, mainly inactivated vaccines have been studied and used in turkeys, leaving a gap in potential vaccine platforms for turkeys. Studies on the impact of LPAI in turkey breeders are even more limited, despite the fact that outbreaks can severely impact their economic viability. Vaccine trials are typically carried out in chicks or poults due to the cost of rearing birds in laboratory settings or the cost of adult birds in production. However, as discussed, turkeys undergo age-dependent changes in immune function and response, therefore limiting the knowledge of vaccine efficacy in adult hens. In conclusion, there is a large research gap in IAV vaccine technologies and immune response in turkeys demonstrating the need for future research specifically in turkey breeder hens.

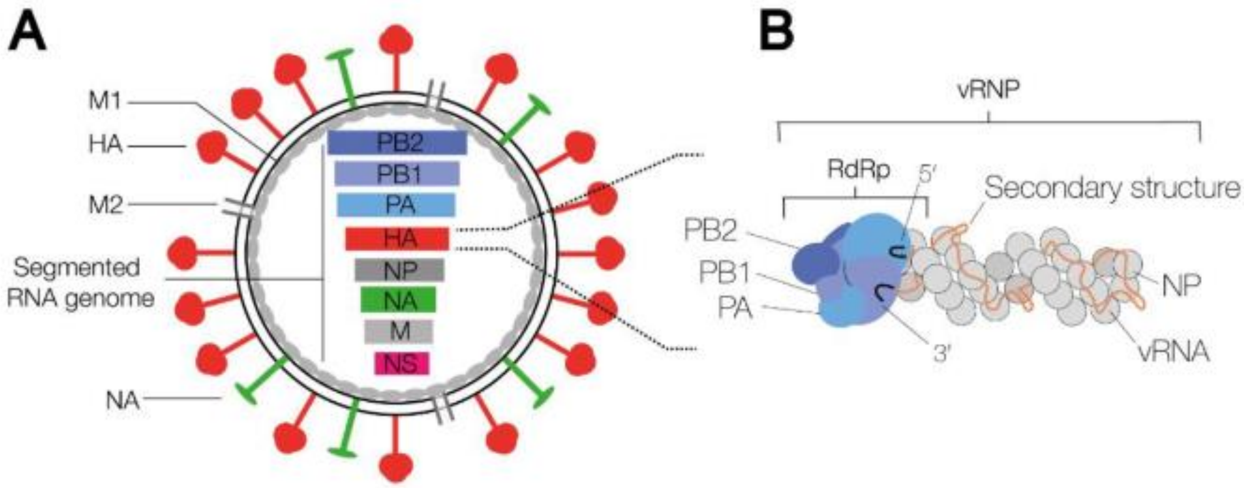

Fig. 1. Schematics of the IAV virion and the viral ribonucleoprotein complex. (**A**) Schematic of the IAV virion. The virion consists of a host cell-derived membrane, the surface glycoproteins HA and NA, the M2 ion channel, the M1 protein capsid, and the viral ribonucleoprotein (vRNP) complexes. The vRNPs each contain a segment of single-stranded, negative-sense viral RNA (vRNA). Eight of these vRNA segments make up the viral genome. (**B**) Schematic representation of a vRNP. The 5′ and 3′ termini of the vRNA (black) are bound by the heterotrimeric RdRp, which consists of the proteins polymerase basic 1 (PB1) (bright blue), PB2 (dark blue) and polymerase acidic (PA) (grey blue). The rest of the vRNA is associated with nucleoprotein (NP) monomers, forming an antiparallel double helix with a closing NP loop (Weis and Aartjan, 2021).

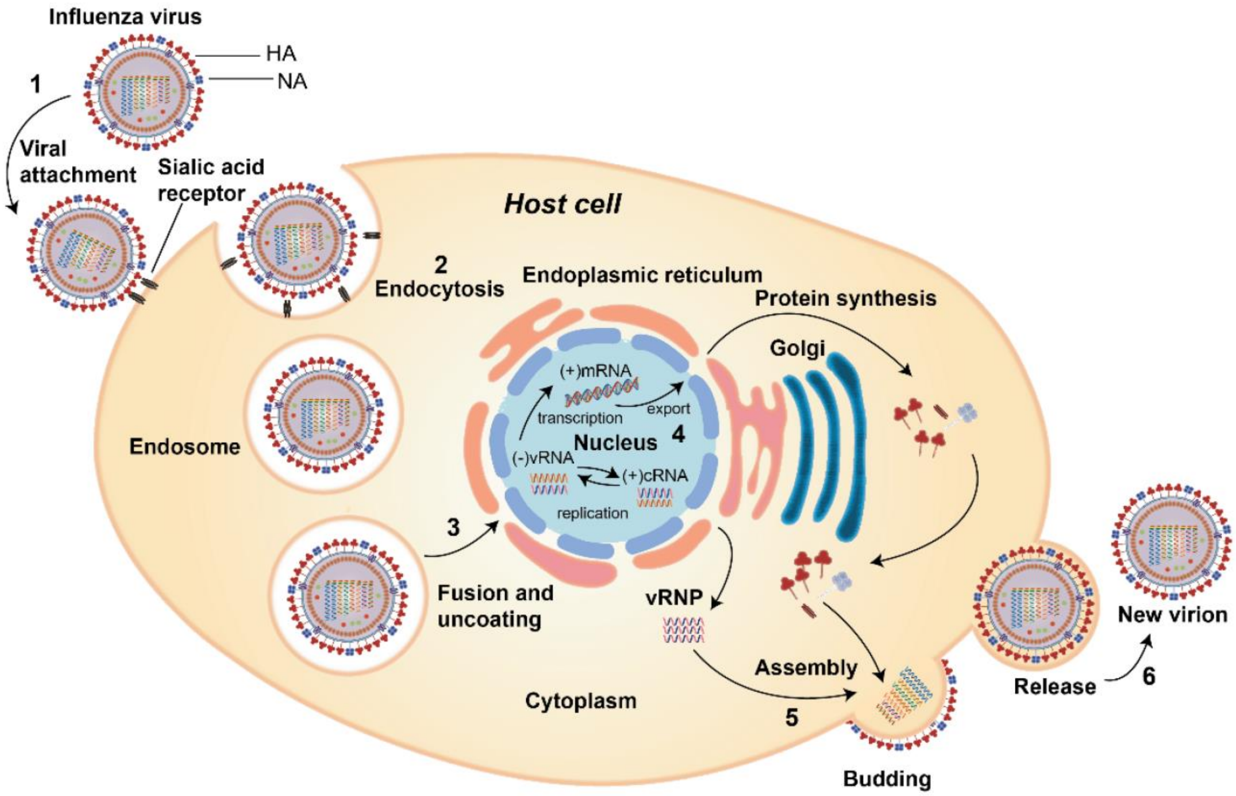

Fig. 2. The life cycle of influenza A virus: (1) virus attachment to sialic acid receptor via HA; (2) entry of the virus into the host cell via endocytosis; (3) fusion and uncoating of virus particle; (4) vRNPs entry into the nucleus followed by transcription and replication of the viral RNA genome and then export of vRNPs from the nucleus; (5) assembly of viral components and budding at the host cell membrane; (6) new virion release from the host cell (Nuwarda et al., 2021).

CHAPTER 3

RESEARCH OBJECTIVES AND JUSTIFICATION

3.1 Objective 1

The focus of this research stems from the need for additional studies specifically related to turkey breeder IAV vaccination and the continual exposure to IAV variants in this poultry sector leading to significant economic impacts on the industry. The first objective of this research is to generate a platform to produce a DNA vaccine expressing viral hemagglutinin (HA1). We also intend to booster the immunogenicity of the DNA vaccine with co-expression of poultry immunostimulatory factors, which has not been previously evaluated. A DNA vaccine is the target for this research due to the feasibility of production and its numerous advantages over inactivated vaccines. Namely, DNA vaccines have been shown to stimulate robust cell-mediated and humoral immunity. These vaccines express only the viral antigen needed for inducing neutralizing antibodies; unlike inactivated vaccines in which all viral proteins are included which increase risks for an antibody-enhanced pathogenicity effect. DNA vaccines also have advantages over mRNA vaccines. Ease of manufacture, enhanced stability, simpler storage and transportation conditions, and lowered costs of production are a few key advantages (Khan, 2013, Lee et al., 2018). In comparison to the concerns for their use in human medicine, risks for genomic integration of exogenous DNA can be minimized when used in veterinary settings due to the shorter lifespan of poultry. A turkey (or chicken) immunostimulatory factor, such as a cytokine or interferon response factor-1 (IRF1), may boost the immune response to HA vaccination in turkey hens to overcome previous research challenges involved with poor immune protective efficacy of DNA vaccination. A key advantage with this type of platform for LPAI vaccination is that once the vaccine platform is designed, the HA subtype can be substituted to match the current circulating IAV field strains to provide homologous protection in a timely manner.

3.2 Objective 2

The second objective is to further demonstrate humoral and cell-mediated immunity and protection in vaccinated turkey poults against an H1N2 viral challenge. This objective aims to both ensure birds mount a measurable immune response when properly vaccinated and that the vaccine provides sufficient immunity to minimize clinical disease impacts. It is critical that veterinarians have the tools to measure vaccination in turkeys to ensure the success of their programs. During a live homologous virus challenge, clinical signs will be compared across poults that have been vaccinated with the DNA vaccine vs. those given the currently used inactivated vaccine. Challenge studies are essential to determine the protective efficacy of one vaccine compared to another and any discernable differences. Turkey hen poults will be used in this study in place of mature breeder hens due to the cost and time of maturation of the adult hens which is limited in the time frame of this research project. In future studies, our work should be repeated in field trials with adult turkey breeder hens to replicate the results.

CHAPTER 4

IMMUNIZATION OF TURKEYS WITH A DNA VACCINE EXPRESSING THE HAEMAGGLUTININ GENE OF AN H1N2 SWINE LINEAGE INFLUENZA A VIRUS

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4.1 Abstract

Low pathogenicity avian influenza viruses (LPAIV) can have substantial negative effects on egg production in turkey breeding flocks. Vaccination can be an effective tool to reduce the impacts of disease. Despite vaccination against LPAI, turkey breeding flocks continue to face disease associated with LPAI leading to decreased egg production and viability of progeny. In the present study, a plasmid-based DNA vaccine expressing the HA gene of an H1N2 virus was developed and evaluated in turkeys. The cDNAs of immunostimulatory factors, including interleukin-15 (IL-15), interferon regulatory factor-1 (IRF-1) and granulocyte-macrophage colony-stimulating factor (GM-CSF) of turkey or chicken origin, were cloned into the plasmid vector to determine if the addition would enhance immunity of the DNA vaccine in poultry. Vaccine humoral immunity was evaluated by hemagglutination inhibition (HI) antibody titers in turkey poults administered two consecutive intramuscular injections. DNA vaccine combinations had greater HI titers in vaccinated birds two weeks post booster administration compared with

the unvaccinated and autogenous vaccine groups. Turkeys were challenged with H1N2 virus to determine the protective efficacy of the vaccine. The challenge virus was neither detected in the birds post challenge by RT-PCR of oropharyngeal swabs nor were antibodies detected by ELISA from the sera. In conclusion, our study suggests DNA vaccine technology may be a good alternative to the currently used killed autogenous vaccine, but due to the lack of a challenge model we were unable to properly evaluate the protective efficacy of the developed vaccine.

4.2 Introduction

Low pathogenicity avian influenza (LPAI) viruses can have substantial clinical and economic impacts on turkey breeder hens including decreases in egg production and the subsequent loss of viable hatching eggs. Turkey breeder hens are routinely vaccinated using inactivated vaccines of H1 and H3 subtypes, commonly of swine lineage. Despite vaccination, turkey breeding hens continue to face disease challenges associated with mammalian subtypes, typically of H1 and H3 LPAI viruses. Mammalian-origin influenza viruses have greater affinity for the turkey hen reproductive tract than the respiratory tract (Pantin-Jackwood et al., 2010) and cause frequent infections that negatively impact egg production. Infection with viruses that may cause little clinical disease in swine or commercial poultry can have devastating effects on turkey breeding stock.

Vaccinated flocks are routinely monitored for influenza A virus (IAV) antibodies using a commercially available enzyme-linked immunosorbent assay (ELISA), which detects the antibody induced by viral nucleoprotein (NP). Observations from the turkey breeding industry have noted that turkey breeding hens typically mount poor antibody responses after vaccination using inactivated autogenous vaccines of the H1 and H3 subtypes. However, when faced with a field challenge of H1 or H3 LPAI, hens mount measurable immune responses (unpublished data, Marissa Studniski).

Alternatives such as new vaccination techniques, or vaccines that are not traditional autogenous types are needed in the turkey industry to help alleviate the clinical impact of LPAI viruses. Very few studies have evaluated alternative methods of vaccination against mammalian subtype LPAI viruses in turkeys. DNA immunization is a potential alternative to conventional LPAI vaccination. This approach consists of inoculating plasmid DNA encoding the vaccine antigen into the birds so that it is produced by the bird's own protein expression system. The expressed antigen is presented to the immune system inducing both humoral and cellular immune responses (Meunier et al., 2016). The first DNA vaccine studied in poultry in 1993 was directed against IAV (Robinson et al., 1993) and the strategy continues to be widely studied in chickens (Meunier et al., 2016). Despite early promise, DNA vaccines induced limited immune responses and/or protection, and none have become commercially available. Many strategies have been evaluated to increase the protective immune responses of poultry to DNA vaccines. One such strategy that is being explored is the use of immune enhancing cytokines as adjuvants (Tovey and Lallemand, 2010).

The present investigation aimed to develop a DNA vaccine and evaluate its efficacy against LPAI H1N2 virus in turkeys. Three immunostimulatory factors including turkey IL-15 (tkIL-15) and IRF1 (tkIRF-1), and chicken GM-CSF (ckGM-CSF) were also expressed in the vaccine construct in order to improve the immunogenicity of the vaccine.

4.3 Materials and Methods

Virus and Animals

An H1N2 avian influenza virus, A/turkey/Minnesota/M20-043874/2020 (H1N2), was used in this study. It was isolated from a tracheal swab obtained from a clinically affected turkey breeder hen in Minnesota during an outbreak in 2020 and submitted to the University of Minnesota Veterinary Medicine Diagnostic Laboratory (Saint Paul, MN). According to sequencing data, this virus originated from swine. To prepare the viral inocula, the virus was inoculated into the allantoic sac of 9-day-old turkey embryonated eggs and was incubated at 39^{o}C for 48 hrs before allantoic fluid was harvested. Hemagglutination (HA) titers were determined for the allantoic fluid using 0.5% turkey red blood cells. Infectious viral titers were determined with a standard plaque assay (Baer and Kehn-Hall, 2014) before the allantoic fluid was aliquoted and stored at -80^{o}C.

Vaccine trials were conducted in a private research facility managed by a large turkey breeder company. Research protocols were reviewed for ethical considerations by the company veterinarian who also oversaw daily monitoring of the birds. Nicholas Select turkey hen poults were hatched and housed in a BSL-2 biosafety room under standard commercial housing conditions in accordance with American Veterinary Medical Association (AVMA) guidelines and the breeder company welfare standards. The birds were bled at two-weeks of age for avian influenza antibody detection using a commercial ELISA test (Zoetis; Parsippany, NJ) to test antibodies for viral NP before their use in the immunization trials.

Cell Lines, Plasmid and Reagents

Madin–Darby canine kidney (MDCK) cells and human embryonic kidney cells (HEK293) were purchased from the ATCC (Manassas, VA) and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Turkey or chicken whole blood was purchased from Colorado Serum Co (Denver, CO). Monoclonal anti-Flag antibody (M2) and alkaline phosphatase (AP)-conjugated rabbit anti-mouse IgG were purchased from Sigma-Aldrich (St Louis, MO) and monoclonal anti-actin antibody was a product of Santa Cruz Biotechnology (Santa Cruz, CA). A plasmid, pVAX-1 (Thermo Fisher Scientific; Waltham, MA) of 3.0 kb in length, was used as an expression vector for cloning and expressing viral and poultry cDNA both *in vitro* and in turkeys. pVAX-1 is specifically designed for use in the development of DNA vaccines, which contains the human cytomegalovirus immediate-early (CMV) promoter for high-level expression in a wide range of eukaryotic cells.

Viral RNA Preparation and cDNA Synthesis

A Viral RNA extraction kit (Qiagen; Hilden, Germany) was used to prepare total RNA from the allantoic fluid that was harvested from the H1N2 virus-inoculated turkey embryonating eggs. The final RNA was eluted from the column with ddH_2O and the quantity and integrity of the resultant RNA were examined using an RNA Nano (Thermo Fisher Scientific). The RNA preparations were used for cDNA synthesis and polymerase chain reaction (PCR). For cDNA synthesis, 4 μl of RNA was mixed with 0.5 μl of Uni12 primer (1.0 μl/μl) in a volume of 10 μl reaction at 70^{o}C for 5 mins, followed by the addition of 4 μl each of 2.5mM dNTPs and 5x Reverse Transcription Buffer, and 1 μl each of RNasin (40 U/ml, Promega; Madison, WI) and Reverse Transcriptase AMV (Invitrogen; Waltham, MA). The reverse transcription reaction was carried out at 42^{o}C for 1 hr before it was discontinued at 70^{o}C for 10 mins. The cDNA was then ready for viral genomic segment amplification.

Synthesis of the Viral Hemagglutinin Gene by Polymerase Chain Reaction

One μl of cDNA was used for Polymerase Chain Reaction (PCR) in a reaction which contained 5 μl each of Ex Taq Buffer and 10mM dNTP Mix, 1 ml of Ex Taq Polymerase from Takara (Japan) in a total volume of 50 μl. Primers for the H1N2 viral HA cDNA (5'-CGGGATCCGCCACCATGGGAATGAAAGTAAAACTAATGGT and 3'-CCGCTCGAGTTAGATGCATATTCTACACTG) were synthesized by Integrated DNA Synthesis (IDT, Coralville, IA). Two restriction endonuclease sites, *BamHI* and *XhoI* were included in the 5' and 3' primers, respectively, and the 5' primer also contained the Kozak sequence, GCCACC, in front of the ATG start codon to ensure efficient eukaryotic protein translation. After 30 cycles in a thermocycler with each cycle consisting of 98^{o}C for 2 minutes pre-incubation, 94^{o}C for 30 seconds for denaturing, 58^{o}C for 30 seconds for annealing and 72^{o}C for 2 minutes for elongation, and lastly 72^{o}C for 7 minutes for final synthesis, PCR product was electrophoresed in a 1% agarose gel. The gel was stained with ethidium bromide and the DNA band at the expected size was excised and purified with a Gel Purification Kit (Qiagen; Hilden, Germany). The purified DNA was cleaved with restriction endonucleases and cloned in the expression vector pVAX-1 (Thermo Fisher Scientific) using *BamHI* and *XhoI* sites to generate the DNA construct, pVAX-HA. For amplification of the plasmid construct, pVAX-HA was transformed into DH5α competent *E.coli* cells and the positive clones were screened before they were sent out for Sanger DNA Sequencing (ACGT Inc; Wheeling, IL) for confirmation.

Chemical Synthesis of Viral HA cDNA

To enhance the peptide expression efficiency and immunity, we optimized the nucleotide composition of the full-length HA cDNA sequence for avian expression and obtained a chemically synthesized HA cDNA with the addition of a Flag tag sequence at the 3' terminus. The synthesis was conducted by Synbio Technologies (Monmouth Junction, NJ). The optimized HA cDNA had the same translated amino acid sequence as the authentic version (Appendix 1) and the nucleotide sequence had a homology of 77% compared to the original and was subsequently cloned into pVAX-1 for expression (Appendix 2).

Identification and Cloning of Poultry Immunostimulatory Genes

Poultry immunostimulatory gene cDNAs were identified in NCBI Database (Sayers, et al., 2022) with homologous searching (BLAST and TBLASTN), of which three were identified and selected for use in this study. They are tkIRF-1, tkIL-15 and ckGM-CSF. Full length cDNA of tkIRF-1 (939 bp), tkIL-15 (429 bp), and ckGM-CSF (432 bp) clones encode peptides with amino acids of 313, 143, and 144 in length, respectively, were chemically synthesized, with the addition of a Flag tag sequence to the 3' terminus, and subsequently cloned into pVAX-1.

Plasmid Transfection

HEK293 cells were prepared in 6-cm plates in DMEM supplemented with 10% FCS at 37^{o}C for 16 hrs before transfection took place. One μg of plasmid and 6 μl of TransIT-LT1 (Mirus) were added in 200 μl of Opti-MEM (Invitrogen) and incubated for 20 mins before an additional 800 μl of Opti-MEM was added into the mixture. After mixing, the total 1 ml of plasmid-TransIT-LT1 transfectant was added to HEK293 cells. 24 hrs later the transfected cells were ready for cell lysate preparation and analysis.

Western Blot Analysis

Protein lysates were prepared from the plasmid-transfected HEK293 cells in a hypotonic buffer as described previously (Xing et al., 2004). Total cell lysates were prepared and separated by using 12-15% SDS-PAGE (Bio-Rad; Hercules, CA). Proteins were transferred to an Immun-Blot PVDF membrane (Bio-Rad) and Western blot analysis was performed according to a standard protocol (Mohmood and Yang, 2012) and using recommended dilutions of a rabbit polyclonal anti-HA1 (1:200) (Invitrogen; Cat# PA5-81678) or mouse anti-Flag antibody (1:1000) (Sigma-Aldrich; Cat# F1804), followed by an AP-conjugated goat anti-rabbit (Sigma-Aldrich; Cat# A3812) or rabbit anti-mouse IgG antibody (Sigma-Aldrich; Cat# A4312) both diluted at 1:10,000. BCIP/NBT reagents (Sigma-Aldrich) in pH 9.5 were used to develop colorimetric signals on the PVDF membrane. The membrane was also blotted with a monoclonal anti-actin antibody (Santa Cruz Biotechnology; Dallas, TX) to show the levels of cellular actin as an input control.

Immunization of Turkeys with DNA Vaccines

Two experiments were conducted to examine the immunogenicity of the constructed DNA vaccines with and without avian immunostimulatory factors in turkeys. The DNA vaccines were pVAX-HA or pVAX-HAco (codon optimized). Three avian immunostimulatory gene cDNAs were also cloned into pVAX-1 to become pVAX-tkIRF-1, pVAX-tkIL-15, and pVAX-ckGM-CSF. All plasmids were confirmed with Sanger sequencing and expanded in DH5α *E.*

coli, then purified with HiPure Maxiprep columns following the manufacturer's protocol (Invitrogen) and quantitated with NanoDrop 2000c (Thermo Scientific). Plasmid DNA was mixed with an adjuvant (ISA 70 VG water-in-oil emulsion) in a ratio of 3:7 (volume:volume) right before inoculation of the turkeys.

The first trial was a pilot study to determine which clones would be incorporated into the final vaccine combinations. In trial one, 17 two-week-old turkey hen poults (IAV-negative by ELISA) were divided into nine groups for inoculation of the DNA vaccine intramuscularly (IM) at 50 μg/per poult with or without the immunostimulatory genes at 25 μg/per poult. The initial trial aimed to compare groups between the codon optimized DNA vaccine (HAco1 and HAco2) and the original HA vaccine (HA1), and two clones of the constructs were included (1 and 2) for additional comparison. Blank plasmid (pVAX-1) was inoculated as a negative control. Fourteen days after the primary injection, the birds were bled for serum preparation and inoculated IM with a booster injection using the same amount of DNA as the primary injection. After another fourteen days, all birds were bled for serum preparation and serological tests before they were humanely euthanized by cervical dislocation. The experimental timeline is depicted in Figure 1A. Sera were collected for detection of IAV antibodies by the hemagglutination inhibition (HI) antibody test. The HI test, which detects antibodies to the HA antigen (Comin et al., 2013), was used in this study to detect immune responses specifically to our DNA vaccine containing the HA gene of H1N2 IAV.

In a second trial, the scope of the turkey immunogenicity experiment was expanded to 48 poults, which were assigned to six groups for the inoculation of only pVAX-HAco with or without immunostimulatory genes along with autogenous H1N2 field inactivated vaccine (Hygieia Biological Laboratories, Davis, CA), produced with the same H1N2 strain used to create the DNA vaccine. The empty plasmid, pVAX-1, was used as a control. Two weeks after the primary inoculation, a booster was administered IM and blood samples were collected. After another two weeks, blood samples were collected, and the poults were challenged with the H1N2 virus by oropharyngeal and intraocular routes at a dose of 10^5 PFU/per poult. Oropharyngeal swabs were taken on 2, 4, and ,7 days post-challenge for RNA extraction and all birds were bled and humanely euthanized on day 14 post-challenge. The duration of the second trial was six weeks after initiation with the primary DNA vaccine inoculation. Figure 1B shows the timeline of the second trial. Sera samples were tested for IAV antibodies by ELISA (IDEXX Westbrook, ME) at the onset and end of the trial and HI at 4, 6 and 8 weeks of age.

Statistics

HI serum titer results from the second trial were statistically analyzed using a one-way ANOVA at each blood collection time point looking for differences in the log-transformed IAV titer between vaccination groups ($p<0.05$ was considered significant). Data were log-transformed based on a prior understanding of the nature of the distribution of titer values. After collection, data were visually inspected to confirm that the log-transformed data more closely matched assumptions of normality than did non-transformed data. Pairwise comparisons within timepoints with a significant ANOVA result were conducted using Tukey's Honest Significant Difference test. All statistical analysis was completed in R Version 4.3.2 (R Core Team, 2023).

4.4 Results

Molecular cloning and in vitro expression

The HA1 gene (1.6 Kb) from the influenza A virus strain A/turkey/Minnesota/20-036975-001/2020 (H1N2) was amplified by PCR and the identity of the cDNA was confirmed by gel agarose electrophoresis and Sanger DNA sequencing. The HA1 cDNA of H1N2 as well as the cDNAs of tkIRF-1, tkIL-15 and ckGM-CSF were successfully cloned into pVAX-1 for DNA vaccine preparation (Figure 2). Protein expression of HA1 and avian immunostimulatory genes in transfected HEK293T cells was confirmed using Western blot analyses. A band between 50-60 KDa was detected on the membrane that was for HA, 14 KDa for ckGM-CSF, 17 KDa for tkIL-15, and 45-50 KDa for tkIRF-1, respectively, when specific anti-HA1 antibody (for HA1) and anti-flag antibody were used for staining after the HEK293 cell lysates were electrophoresed in SDS-PAGE and the proteins were transferred to PVDF membranes (Figure 3).

Evaluating vaccine combinations

Experiment one

Inoculation with the codon optimized DNA vaccines (HAco1 and HAco2) resulted in numerical increases in serum HI antibody titers by 4 weeks post vaccination compared to the HA1 only DNA vaccine (Table 1). Results also indicated that addition of an immunostimulatory gene (either tkIL-15 or ckGM-CSF) resulted in higher antibody titers from 2 to 4 weeks post vaccination as compared to HAco alone. As expected, there were no differences between clones HAco1 and 2.

Experiment two

By 2 weeks post-booster vaccination, all birds given pVAX-1 combinations or the autogenous vaccine developed significantly ($p<0.05$) detectable HI antibody titers as compared to the control group given only pVAX-1 (Figure 4). Among the groups at 2 weeks post-vaccination, only pVAX1-HAco + tkIL-15 and pVAX1-HAco + ckGM-CSF vaccination groups had significantly higher antibody titers than the autogenous vaccine group ($p<0.05$).

After challenge with the swine origin H1N2 virus, birds showed no signs of morbidity or mortality. Virus was not detected in the oropharynx or trachea of birds by qRT-PCR at 2, 4, or 7-days post-challenge. However, by 2 weeks post-challenge all birds had detectable HI antibody titers including the sham vaccinated control group with pVAX-1 and the average mean HI titer increased from the previous sample collection at the time of challenge (Figure 5). Seroconversion was detected by ELISA two weeks post challenge in 2/8 samples in the pVAX1-coHA1 + tkIRF-1 vaccination group, 1/8 samples in the pVAX1-coHA1 + ckGM-CSF group, 2/8 positive in the pVAX-1 negative control, and 5/8 positive from the H1N2 autogenous vaccine group. All other sera were negative for IAV antibodies as measured by ELISA, which is for the detection of antibodies to viral NP.

4.5 Discussion

DNA vaccines against influenza viruses have been tested in several animal models including poultry with a more recent emphasis on strategies to improve their effectiveness. In this study, codon optimization was used to enhance the cloning efficiency of the HA gene. Trial one suggests that codon-optimization of the HA antigen may enhance the immune response in vaccinated turkeys compared to the wildtype plasmid. Similarly, previous DNA immunization studies have demonstrated that codon optimization of the antigen encoding expression plasmids enhanced the immunogenicity of the vaccine, primarily through increased antigen expression (Tenbusch et al., 2010; Shehata et al., 2020). The sample size was small and few birds were evaluated in trial one, and therefore conclusions are preliminary. Trial one was aimed at establishing the methods used to measure a post-inoculation immune response.

In trial two, all birds inoculated with the pVAX1-HAco combinations in comparison to the autogenous inactivated H1N2 vaccine developed detectable HI antibodies by two weeks post booster vaccination. Higher HI titers were observed in turkeys vaccinated with pVAX1-HAco alone or in combination with an immunostimulatory factor compared to the autogenous vaccine, although the differences in HI titers were not statistically significant. Upon vaccination with the DNA vaccine, the antigen is produced by the cells of the host, which leads to activation of both cellular and humoral immune responses due to antigen presentation by MHCI and MHCII molecules (Grunwald and Ulbert, 2015). This provides an advantage over inactivated viral vaccines widely used in the commercial industry, which predominantly induce only a humoral immune response (Meunier et al., 2016). This may account for the numerical differences observed between the DNA vaccine combinations and the autogenous vaccine in this study.

Vaccine adjuvants have previously been shown to enhance the immune response of DNA vaccines (Grunwald and Ulbert, 2015) but few studies have investigated the addition of poultry immunostimulatory factors to further enhance the immune response to IAV in turkeys. Our results suggest that the addition of some immunostimulatory factors, such as tkIL-15 and ckGM-

CSF genes may contribute to an additional immune response over pVAX-HAco alone. However, additional studies need to be undertaken to properly assess the effects on the immune response of addition of these genes to DNA vaccine candidates with larger samples of birds in the study. Future studies also need to evaluate cell-mediated immunity as our test only measured humoral immunity due to the time stringency of the study.

Our challenge experiment did not provide expected results, and in fact, the data appear to be inconclusive and confusing, which probably stemmed from the turkey poult challenge model used in this study. All vaccinated and control groups had increased HI titers 2 weeks-post H1N2 challenge compared to the previous serology monitoring at 2 weeks post-booster, despite virus not being detected by RT-PCR. Few birds had detectable ELISA antibody titers 2-weeks post challenge. Previous clinical experiences have shown that when turkey breeders are infected with wild type LPAI viruses in the field, birds typically take 4-6 weeks to fully seroconvert (personal observation, Marissa Studniski, 2024). Therefore, 2 weeks may not have been sufficient time to fully evaluate seroconversion post-viral challenge.

In this study, we detected neither viral RNA (with RT-PCR) nor antibodies (with ELISA for viral NP) in any birds challenged with live H1N2 virus. This prevented the evaluation of the protective efficacy of the DNA vaccine. The absence of clinical signs post-challenge is not surprising as LPAI infections are often asymptomatic in commercial poultry. The challenge dose and route of inoculation were based on transmission routes and previous studies (Mady et al., 2017; Shehata et al., 2020), however, it is possible the dose was insufficient to cause disease for this strain of IAV. The absence of viral detection may be attributed to differences in the pathobiology and viral antigen distribution in the host considering this is a swine origin H1N2 virus. Previous LPAI challenge studies have demonstrated that there are age-related differences in susceptibility to infection which is dependent on particular HA subtypes (Bergervoet et al., 2019). There has been limited research conducted on swine origin IAV infection in turkeys, therefore additional repetitions of vaccination trials are required to fully assess the immunogenic effectiveness of the DNA vaccine in turkeys. Another limitation to this study is the age of the birds vaccinated and challenged. The birds used in these trials were not reproductively mature which may affect the distribution of sialic acid receptors adding to the difficulty of successful viral challenge and successive immune response. Challenge studies are difficult in turkey breeders due to the lack of a published challenge model. Turkey breeder hens are a minor species in the poultry market, with only two companies providing most of the genetic breeding stock to the world. This production niche makes it difficult to provide a profitable supply market for vaccine manufacturers, thus limiting the available options for vaccines. In addition, research trials utilizing mature breeding hens can be costly, as each hen averages $74 USD in 2024 at the onset of production at 29 weeks of age (economic trends 2024, Select Genetics, USA). Delivered, day-of-age turkey breeder hen poult cost on average in 2024 is $20 USD compared to $1.41 USD for a commercial hen poult (economic trends 2024, Select Genetics, USA). The cost of breeder hens is often the primary reason why commercial turkeys are not used in research trials and was the justification for why young commercial turkey poults were used in this study. However, it is not known if immature commercial poults suffice as a substitute for accurate evaluation of the immune system in mature hens. The cost, size of the bird, and turkey breeder production systems limit the feasibility of large-scale field trials, limiting the reliability of successful tools used on farm. These challenges often need to be overcome by companies invested in research priorities. However, challenges are also numerous to the companies including limited facilities, expertise, and funds just to name a few, thus why research is limited in turkey breeders.

In conclusion, our study suggests that pVAX-HAco alone or in combination with poultry immunostimulatory factors may be a promising alternative to conventional autogenous vaccinations against IAV.

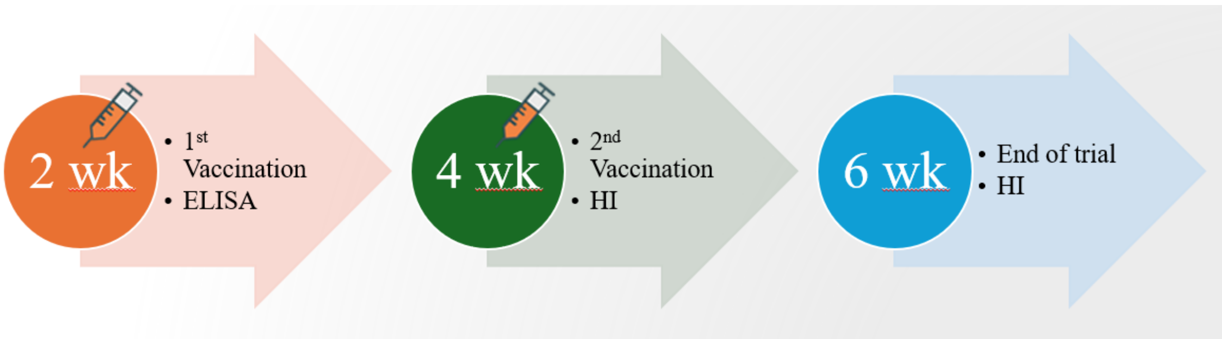

Fig 1**A**. Experimental timeline for trial 1 immunogenicity study. Bird age by week and trial activity indicated.

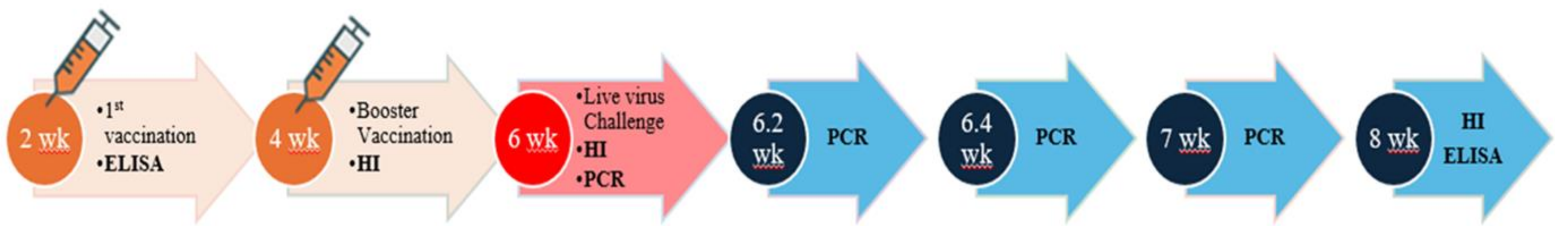

Fig. 1**B**. Experimental timeline for trial 2 immunogenicity study. Bird age in weeks and trial activity indicated.

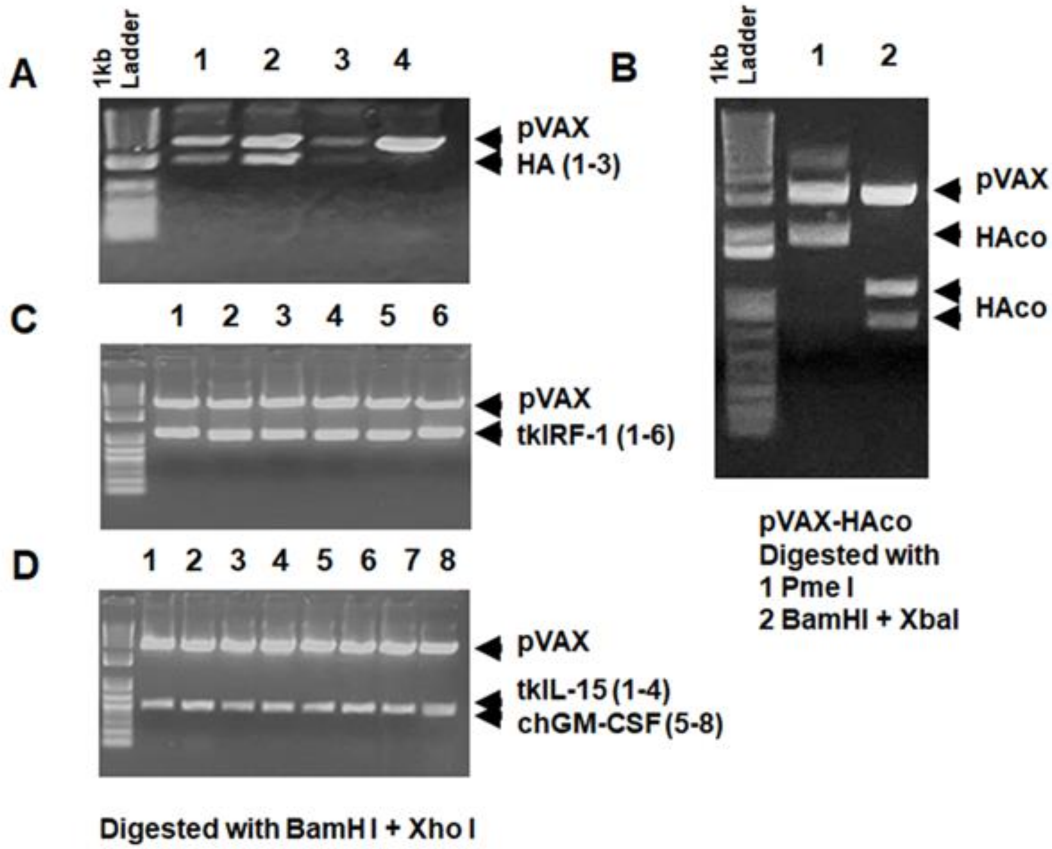

Fig 2: Restriction enzyme digestion analysis using gel agarose electrophoresis. A 1kb ladder was used for reference (**A**) three clones of the original HA gene digested with *BamHI* and *Xho I*, with expected bands at 1.6 kb for HA and 3.0 kb for pVAX 1 plasmid vector. (**B**) Codon optimized HA gene digested with *Pme I* in lane 1 and *BamHI* and *XbaI* in lane 2. HAco was identified in pVAX-HAco by the digestion with *BamHI* and *XbaI* to show that it has an internal *XbaI* site at 1.2 Kb, which is not present in the original HA cDNA. (**C**) Six clones of tkIRF-1 digested with BamHI and XhoI with expected bands at 939 bp for tk-IRF and 3.0 kb for pVAX 1. (**D**) Lanes 1-4 are clones of pVAX-tkIL-15 and lanes 5-8 are clones of pVAX-ckGM-CSF digested with BamHI and XhoI with expected bands at 429 bp for tkIL-15, 432 bp for ckGM-CSF and 3.0 kb for pVAX 1.

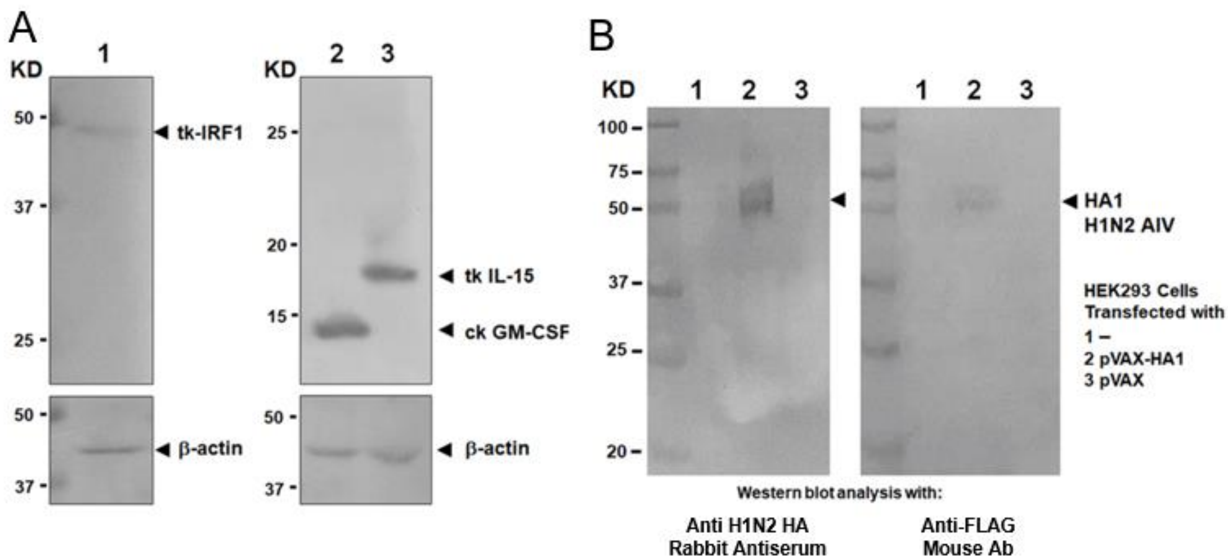

Fig 3: (**A**) Western blot analysis of immunostimulatory genes expressed in HEK293 cells. Lane 1 shows the expressed protein of tkIRF with a band at approximately 48 KDa. Lane 2, protein expression of ckGM-CSF with a band at approximately 14 KDa. Lane 3, protein expression of tkIL-15 with a band at approximately 17 KDa. β-actin was used as an input control and is expressed at 42 KDa. (**B**) Western blot analysis of H1N2 HA gene using a rabbit polyclonal anti-HA5 or mouse anti-Flag antibody. Lane 1 is a negative control and lane 3 is empty plasmid vector pVAX. Lane 2 is a band between 50-60 kDa representing the HA protein of H1N2 IAV.

Table 1: Hemagglutination inhibition titers post-vaccination by birds within each vaccination group in trial 1.

Bird	Vaccination	HI titer post-inoculation	
		2 weeks	4 weeks
1	pVAX-1	0	0
2	pVAX-1	0	0
3	HAco1	0	1:32
4	HAco1	0	1:16
5	HAco2	1:4	1:16
6	HAco2	1:4	1:16
7	HA1	0	0
8	HA1	0	0
9	HAco1 + tkIL-15	1:4	1:16
10	HAco1 + tkIL-15	1:2	1:128
11	HAco2 + tkIL-15	0	1:16
12	HAco2 + tkIL-15	0	1:128
13	HA1 + tkIL-15	0	1:8
14	HA1 + tkIL-15	0	1:256
15	HAco1 + ckGM-CSF	0	1:256
16	HAco1 + ckGM-CSF	0	1:16
17	HAco2 + ckGM-CSF	0	1:256

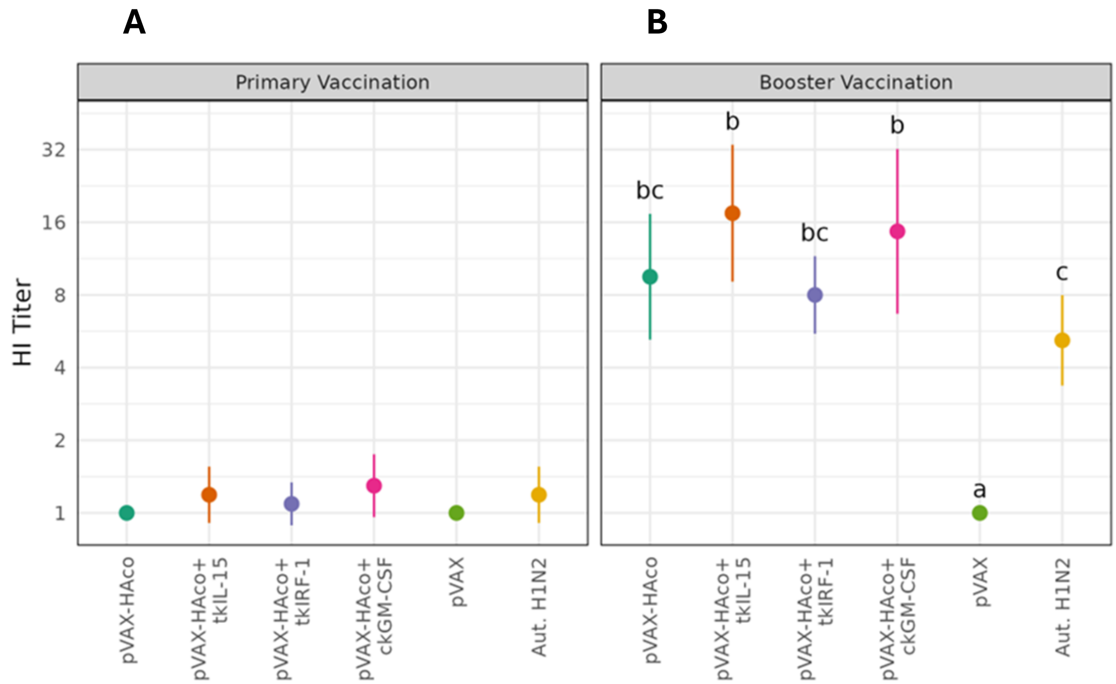

Fig 4. Mean hemagglutination inhibition titers with 95% confidence intervals for turkeys immunized in each vaccination group for trial two at 2 weeks after the primary vaccination (**A**) and 2 weeks after booster vaccination (**B**). There was a significant effect of group two weeks after booster vaccination (ANOVA; p>0.05), but not two weeks after primary vaccination. The pairwise results of Tukey's HSD test are denoted by lowercase letters. Groups that do not share a letter differ significantly (p > 0.05). All vaccine groups were statistically significantly higher than the control group pVAX, and pVAX1-coHA1 + IL-15 & pVAX1-coHA1 + GM-CSF were significantly higher than the autogenous H1N2 vaccine group.

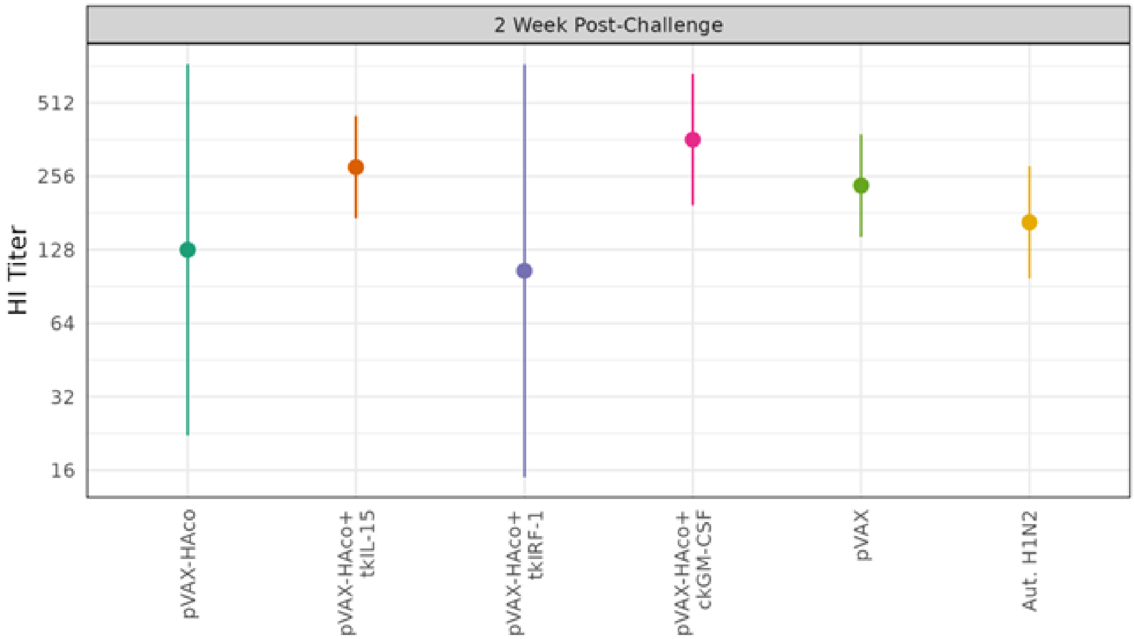

Fig. 5: Mean hemagglutination inhibition antibody titers with 95% confidence intervals for each vaccination group for trial two at 2 weeks after challenge. There was not a significant effect of group at this time point (ANOVA, $p > 0.05$).

CHAPTER 5

CONCLUSIONS

5.1 Importance of the Research

New methods and technologies for vaccination are important to stay abreast of the transient changes in the ecosystem of disease transmission. The longer lifespan of turkey breeder hens and the rearing locations of these birds among other livestock species and wildlife, enables the turkey breeder hen to act as both a gateway and a prime mixing vessel for IAV. Breeder hens are highly susceptible to disease with the amount of stress placed on the birds in order to maintain their reproductive longevity. The main defense against all infections is focused upon prevention, primarily enhanced biosecurity measures and vaccination. Vaccination is a key tool aimed to reduce the production impacts of IAV. Prevention of disease is vital as LPAI infections in turkey breeder flocks are economically damaging to flock performance. A typical breeder hen produces 116 eggs over a lay cycle of 28 weeks, and which ultimately results in an average of 98 poults in her lifetime (Aviagen Turkeys, USA, Breeder performance 2024). Depending on when a flock is affected with LPAI, these numbers can be drastically reduced. Added long-term effects such as decreased hatchability can last the lifetime of the breeder hen making these flocks economically challenging to maintain. Additionally, the performance effects of an outbreak extend further than the breeder farm operation, as the poult customer often faces decreased poult availability and may also see small, weak poults when the breeder hens are at peak infection (Personal communication, Michelle Behl, 2023).

5.2 Challenges

Vaccine availability and licensing can be costly, especially when the market is small among the US poultry sector. The result of this leaves autogenous vaccines as the only available option for turkey breeder hens against IAV. Autogenous vaccines are killed viruses, requiring individual injection of birds, limiting their use to turkey breeders. As previously emphasized, killed autogenous vaccines have not provided adequate immune protection for turkey breeders based on historical serological data and continuation of disease outbreaks. Not all turkey breeders are vaccinated against LPAI viruses in the US due to the ineffectiveness of the vaccine (personal communication, Minnesota turkey veterinarians, 2022). This leaves a major opportunity for the development of new vaccines to prevent LPAI infections, thus the primary reason for this research initiative.

Due to the cost of turkey breeder hens and the age at reproductive productivity, there has been limited research on vaccination against LPAI in turkey breeders. Thus, there is a high demand for this research for this niche market- the turkey breeder industry. Vaccine manufacturers are not actively seeking opportunities to create LPAI vaccines. The effect is that only a few private parties are working to develop LPAI vaccine research initiatives. Additionally, a second difficulty is the lack of adequate challenge models. A challenge model is needed to determine immunogenicity of a vaccine as well as its protective efficacy. We were not able to detect viral RNA by RT-PCR after challenge with the LPAI H1N2 virus of a swine origin. Many LPAI viruses are not highly adapted to turkeys, and therefore may not replicate well leading to a reduced antibody response (personal communication, David Suarez, 2024). There are a lot of unknowns that need to be further investigated to determine a successful study model. Key factors to consider are route(s) of infection, age of infection, receptor site locations, viral titer/dose, viral subtype, and viral shed duration upon others.

Cellular immune response plays a critical role in the protection against viral diseases; however, we often fail to evaluate it due to the lack of readily available assays compared to measuring the humoral immune response. Previous methods that have been used to study cell-mediated immunity include flow cytometry, intracellular cytokine staining, proliferation assay and enzyme-linked immunospot assay (Hao et al., 2021). However, these assays were developed and applied in chickens for the studies of T cell immunity to avian viral diseases, they are suboptimal and not standardized compared to similar approaches used in humans and mice (Hao et al., 2021). One of the goals of this research was to produce a measurable immune response with our vaccine platform. However, the assays that have been used in previous studies are difficult to use in high throughput settings and thus are not offered at diagnostic labs and can be time -consuming and costly to conduct in a research lab which is a limiting factor for using new vaccines. While the ability to measure cell-mediated immunity (CMI) has steadily improved, there is much to learn regarding their contribution to the protection of birds, specifically turkeys, against diseases induced by IAV (Kapczynski and Segovia, 2020). Future studies must evaluate cellular immune responses to understand the role and regulation of lymphocytes following viral challenge and defining a host response to vaccination.

5.3 Applications

This research is most valuable as the framework for future studies evaluating vaccine efficacy against LPAI and other viral diseases in turkey breeder hens. Two key shortcomings were identified in these trials; 1. Lack of a good challenge model, 2. Need to evaluate CMI, and 3. The ease of monitoring overall immune response. Studies focusing on those individual aspects will aid future vaccine development trials in turkeys.

The DNA vaccine trialed in this study may be used in commercial turkey breeder flocks as an alternative to traditional killed autogenous vaccines. A side-by side field application may be an alternative analysis of efficacy in the midst of an IAV challenge.

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APPENDIX 1. Amino acid sequence similarity comparing the original (ori) and codon optimized (opt) HA1 of H1N2.

```
                                10        20        30        40        50        60
Consensus          MKVKLMVLLCTFTATYADTICVGYHANNSTDTVDTVLEKNVTVTHSVNLLEDNHNGKLCL   60
H1N2 HA Opt Protein ............................................................   60
H1N2 HA Ori Protein ............................................................   60

                                70        80        90       100       110       120
Consensus          LKGIAPLQLGSCSVAGWILGNPECELLTSKESWSYIVETSNPENGTCYPGYFEDYEELRE  120
H1N2 HA Opt Protein ............................................................  120
H1N2 HA Ori Protein ............................................................  120

                               130       140       150       160       170       180
Consensus          QLSSVSSFKKFEIFPKKSSWPNHTVTGVSSSCSHNGNSSFYKNLLWLTVKNNLYPNLSKS  180
H1N2 HA Opt Protein ............................................................  180
H1N2 HA Ori Protein ............................................................  180

                               190       200       210       220       230       240
Consensus          YTNKEEKEILVLWGVHHPSNMEDQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRNQE  240
H1N2 HA Opt Protein ............................................................  240
H1N2 HA Ori Protein ............................................................  240

                               250       260       270       280       290       300
Consensus          GRINYYWTLLEPGDTIIFEASGNLIAPRYAFELSKGFGSGIITSNAPMGECNTKCQTPQG  300
H1N2 HA Opt Protein ............................................................  300
H1N2 HA Ori Protein ............................................................  300

                               310       320       330       340       350       360
Consensus          AINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNTPSIQSRGLFGAIAGFIEGGWTGM  360
H1N2 HA Opt Protein ............................................................  360
H1N2 HA Ori Protein ............................................................  360

                               370       380       390       400       410       420
Consensus          VDGWYGYHHQNEQGSGYAADQQSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRM  420
H1N2 HA Opt Protein ............................................................  420
H1N2 HA Ori Protein ............................................................  420

                               430       440       450       460       470       480
Consensus          ENLNKKVDDGFLDIWTYNAELLVLLENERTLDFHDSNVKNLYEKVKSQLKNNAKEIGNGC  480
H1N2 HA Opt Protein ............................................................  480
H1N2 HA Ori Protein ............................................................  480

                               490       500       510       520       530       540
Consensus          FEFYHKCDDECMDSVKNGTYDYPKYSEESKLNREKIDGVKLESMGVYNILAIYSTIASSL  540
H1N2 HA Opt Protein ............................................................  540
H1N2 HA Ori Protein ............................................................  540

                               550       560
Consensus            VLLVSLGAICFWMCSNGSLQCRICI                                     565
H1N2 HA Opt Protein  .........................                                     565
H1N2 HA Ori Protein  .........................                                     565
```

APPENDIX 2. Nucleotide sequence similarity comparing the original (ori) and codon optimized (opt) HA1 of H1N2.

```
>Nucleotide alignment 3 Alignment of 2 sequences: H1N2 HA Opt, H1N2 HA Ori

Score = 5095.0, Identities = 1323/1699 (77%),
Positives = 1323/1699 (77%), Gaps = 2/1699 (0%)

H1N2 HA Opt     1 ATGAAAGTGAAGCTGATGGTACTGCTTTGCACGTTTACTGCAACCTATGCTGATACAATT   60
                  ATGAAAGT AA CT ATGGT CTG T TG AC TTTAC GC AC TATGC GA ACAAT
H1N2 HA Ori     1 ATGAAAGTAAAACTAATGGTTCTGTTATGTACATTTACAGCTACATATGCAGACACAATA   60

H1N2 HA Opt    61 TGTGTGGGCTACCATGCCAACAACAGCACAGACACTGTGGACACCGTTCTGGAAAAGAAT  120
                  TG GTGGGCTACCATGCCAACAAC   AC GACACTGT GACAC GT CT GA AAGAAT
H1N2 HA Ori    61 TGCGTGGGCTACCATGCCAACAACTCAACTGACACTGTTGACACAGTACTTGAGAAGAAT  120

H1N2 HA Opt   121 GTCACCGTGACCCACAGTGTCAACCTTCTCGAGGACAATCACAACGGGAAGCTGTGCCTG  180
                  GT AC GTGAC CAC  TGTCAACCT CT GA GACAATCACAA GGGAA CT TG CT
H1N2 HA Ori   121 GTGACAGTGACACACTCTGTCAACCTACTTGAAGACAATCACAATGGGAAACTATGTCTA  180

H1N2 HA Opt   181 TTAAAAGGAATCGCTCCACTGCAACTTGGGAGCTGTAGTGTGGCTGGTTGGATCTTGGGG  240
                   TAAAAGG AT GCTCCACT CAA T GG AG TG AG GT GC GG TGGATCTT GGG
H1N2 HA Ori   181 CTAAAAGGGATAGCTCCACTACAATTGGGTAGTTGCAGCGTTGCCGGGTGGATCTTAGGG  240

H1N2 HA Opt   241 AACCCTGAGTGTGAGCTGCTGACCTCAAAAGAATCATGGAGCTACATTGTGGAGACTTCC  300
                  AACCC GAGTG GA  TGCTGAC TC AA GAATCATGG  CTACATTGT GAGAC TC
H1N2 HA Ori   241 AACCCAGAGTGCGAATTGCTGACTTCCAAGGAATCATGGTCCTACATTGTAGAGACATCA  300

H1N2 HA Opt   301 AATCCAGAAAATGGAACATGTTATCCTGGTTACTTTGAAGATTATGAAGAGCTTCGGGAA  360
                  AATCC GA AATGGAACATGTTA CC GG TA TT GAAGA TATGAAGA CT  GGGAA
H1N2 HA Ori   301 AATCCTGAGAATGGAACATGTTACCCAGGATATTTCGAAGACTATGAAGAACTGAGGGAA  360

H1N2 HA Opt   361 CAGCTGAGCTCTGTATCAAGTTTCAAAAAGTTCGAAATATTCCCCAAGAAGTCCTCTTGG  420
                  CA  TGAG TC GTATC    TT AA AAGTTCGAAATATTCCCCAA AAG  CTC TGG
H1N2 HA Ori   361 CAATTGAGTTCAGTATCTTCATTTAAGAAGTTCGAAATATTCCCCAAAAAGAGCTCATGG  420

H1N2 HA Opt   421 CCAAACCATACGGTGACAGGTGTGTCAAGTAGCTGCTCACATAATGGGAATAGCTCTTTC  480
                  CC AA CA AC GT AC GG GT TCA      TGCTC CATAA GGGAA AGC   TTC
H1N2 HA Ori   421 CCCAATCACACCGTAACCGGAGTTTCATCATCATGCTCCCATAACGGGAACAGCAGCTTC  480

H1N2 HA Opt   481 TACAAGAATCTGCTGTGGCTGACAGTGAAGAACAACCTTTACCCAAATCTCAGCAAATCA  540
                  TACAA AAT TGCT TGGCTGACAGT AA AACAA  T TACCCAAA CT AGCAA TC
H1N2 HA Ori   481 TACAAAAATTTGCTATGGCTGACAGTAAAAAACAATTTGTACCCAAACCTGAGCAAGTCC  540

H1N2 HA Opt   541 TATACTAATAAAGAGGAGAAGGAGATACTCGTATTATGGGGCGTCCATCACCCTTCTAAC  600
                  TA AC AA AAAGAGGAGAA GA AT CT GTATT TGGGG GT CATCACCC TCTAAC
H1N2 HA Ori   541 TACACAAACAAAGAGGAGAAAGAAATCCTTGTATTGTGGGGTGTTCATCACCCATCTAAC  600

H1N2 HA Opt   601 ATGGAGGATCAAAGGGCGCTGTATCACACAGAAAATGCCTATGTTTCTGTGGTGTCTTCT  660
                  ATGGAGGA CAAAGGGC CT TATCA ACAGAAAATGC TATGT TCTGT GTGTCTTC
H1N2 HA Ori   601 ATGGAGGACCAAAGGGCCCTCTATCATACAGAAAATGCTTATGTCTCTGTAGTGTCTTCA  660

H1N2 HA Opt   661 CACTACTCGC-GCCGATTCACTCCTGAAATTGCGAAGAGGCCGAAGGTTCGTAACCAGGA  719
                  CA TA T GC G  GATTCAC CC GAAAT GC AAGAG CC AAGGT CG AA CAGGA
H1N2 HA Ori   661 CATTA-TAGCAGAAGATTCACCCCAGAAATAGCCAAGAGACCCAAGGTGCGAAATCAGGA  719

H1N2 HA Opt   720 GGGGCGCATTAACTACTACTGGACCCTGTTGGAGCCCGGAGATACCATCATATTTGAGGC  779
                   GG  G AT AA TA TA TGGAC CTG T GA CCCGG GATAC AT ATATTTGAGGC
H1N2 HA Ori   720 AGGAAGAATAAATTATTATTGGACTCTGCTAGAACCCGGGGATACAATAATATTTGAGGC  779

H1N2 HA Opt   780 GAGTGGCAACCTGATTGCACCGAGATACGCCTTCGAGCTATCCAAGGGGTTTGGTAGTGG  839
                   AGTGG AA  T AT GCACC AG TA GCCTTCGA  T    AAGGG TTTGG    GG
H1N2 HA Ori   780 AAGTGGAAATTTAATAGCACCAAGGTATGCCTTCGAATTGAGTAAGGGTTTTGGATCAGG  839

H1N2 HA Opt   840 GATTATAACATCTAATGCTCCAATGGGAGAGTGTAATACAAAGTGCCAGACGCCCCAAGG  899
                   AT AT ACATC AATGC CCAATGGG GA TG AATACAAA TG CA AC CC CA GG
H1N2 HA Ori   840 AATCATCACATCAAATGCACCAATGGGTGAATGCAATACAAAATGTCAAACACCTCAGGG  899

H1N2 HA Opt   900 TGCCATAAACTCCAGCCTCCCGTTTCAAAATGTTCATCCCGTTACAATCGGAGAATGTCC  959
                   GC ATAAAC  CAGCCT CC TT CA AATGT CA CC GT ACAAT GGAGA TGTCC
H1N2 HA Ori   900 GGCTATAAACAGCAGCCTTCCCTTCCAGAATGTACACCCAGTAACAATAGGAGAGTGTCC  959

H1N2 HA Opt   960 AAAATATGTCAGATCCGCTAAGTTACGCATGGTGACTGGACTGCGGAACACACCTTCCAT 1019
                  AAA TATGTCAG    GC AA TTA G ATGGT AC GGACT  GGAACAC CC TCCAT
H1N2 HA Ori   960 AAAGTATGTCAGGAGTGCAAAATTAAGGATGGTTACAGGACTAAGGAACACTCCATCCAT 1019

H1N2 HA Opt  1020 TCAGTCTAGAGGCCTCTTTGGAGCCATTGCTGGCTTCATTGAAGGGGGCTGGACAGGAAT 1079
                   CA TC AGAGG  T TTTGGAGC AT GC GG TT ATTGAAGG GG TGGAC GGAAT
H1N2 HA Ori  1020 CCAATCCAGAGGTTTGTTTGGAGCTATCGCCGGTTTTATTGAAGGAGGATGGACTGGAAT 1079
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