Avian Influenza Adenovirus-Vectored *in Ovo* Vaccination: Combination with Marek's Disease Vaccine

by

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Abstract

Protective immunity against avian influenza (AI) can be elicited in chickens in a single-dose regimen by *in ovo* vaccination with a replication competent adenovirus (RCA)-free human adenovirus serotype 5 (Ad)-vector encoding either the AI virus H5 (AdH5) or H7 hemagglutinins (HA). *In ovo* vaccination is likely one of the most efficient mass vaccination delivery routes in commercial chickens. From an applied perspective, it is relevant to clarify whether other vaccines routinely delivered by the same route would interfere with Ad-vector vaccination when applied simultaneously. Marek's disease virus (MDV) vaccination is routinely performed *in ovo* in the U.S. poultry industry. The overall aim of this study was to evaluate the effects of combined *in ovo* vaccination with the experimental AdH5 recombinant vaccine and commercially available MDV vaccines. When the AdH5 vaccine was used in combination with MDV vaccines, chickens responding to the AdH5 vaccine had similar AI antibody levels compared to AdH5-only vaccinated birds. However, combined vaccinated groups showed reduced vaccine coverage to AI which suggests some level of interference. The combination of AdH5 with MDV Rispens/HVT affected the vaccine coverage to AI more severely. This result suggests that the replication rate of the more aggressive Rispens strain of serotype 1 may have interfered with the Ad-vectored vaccine. Increasing the Ad concentration produced similar AI antibody titers and AI vaccine coverage when applied alone or in combination with the HVT/SB1 vaccine. Adenovirus DNA was detected in hatched chickens 2 days

after hatch but was undetectable on day 9 post hatch. MDV DNA was detected in feather follicles of all vaccinated birds at 12 days of age. Thus, Ad-vector vaccination does not interfere with the efficacy of MDV vaccination using any of the commonly used vaccine strains.

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List of Abbreviations

APC	Antigen Presenting Cell
APV	Avian Pneumovirus
CAR	Coxsackievirus and adenovirus receptor
CELO	Chicken embryo lethal orphan
CTL	Cytotoxic T lymphocyte
DIVA	Differentiating infected from vaccinated animals
DPI	Days post infection
FFE	Feather follicle epithelium
FPV	Fowl pox virus
gB	Glycoprotein B
gC	Glycoprotein C
gD	Glycoprotein D
gH	GlycoproteinH
HVT	Herpes of turkeys
IBDV	Infectious bronchitis virus
IFN-ɣ	Interferon Gamma
Ig	Immunoglobulin
IL	Interleukin
MD	Marek's disease

MDV	Marek's disease virus
MDV-1	Serotype 1 Marek's disease virus
MDV-2	Serotype 2 Marek's disease virus
MDV-3	Serotype 2 Marek's disease virus
NDV	Newcastle disease virus
NK	Natural Killer Cells
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription-polymerase chain reaction
Th	T- helper lymphocyte
ThF	Follicular T-helper lymphocyte
USA	United States of America
USDA	United States Department of Agriculture
VH	Variable heavy chain
VL	Variable light chain

Introduction

Protective immunity against avian influenza (AI) can be elicited in chickens in a single-dose regimen by *in ovo* vaccination with a replication competent adenovirus (RCA)-free human adenovirus serotype 5 (Ad)-vector encoding either the AI virus H5 (AdH5) or H7 hemagglutinin (HA). *In ovo* vaccinated chickens are protected against highly pathogenic (HP) AI virus homologous challenges (174-175). Successful results have also been reported with Ad-vectored AI vaccines in mice (116). In addition to *in ovo* injection, ocular administration of Ad expressing the H5 gene has also been shown to induce specific immune responses and protection against HPAI virus challenge (173,178). *In ovo* vaccination is likely one of the most efficient mass vaccination delivery routes in commercial chickens. From an applied perspective, it is relevant to clarify whether other vaccines routinely delivered by the same route would interfere with Ad-vector vaccination when applied simultaneously as it has been previously reported for other vaccines [e.g. (8,47)]. Marek's disease virus (MDV) vaccination is routinely performed *in ovo* in the U.S. poultry industry. Theoretically, interference could occur between MDV and AdH5 as a result of at least three different mechanisms. First, different replication kinetics; a fast replicating MDV might induce innate immune responses which would prevent entry into the cell or viral gene expression by a non-replicating virus. For example, when a host cell is infected it may secrete IFN α and/or β that produces an antiviral state in surrounding cells; therefore, when the cells become

infected they die, preventing viral replication and limiting spread of the virus (138). Second, the vaccine viruses might compete for the same target cells; adenoviruses [including the chicken embryo lethal orphan virus (CELO)] use CAR (coxsackievirus and adenovirus receptor) for infection (170), which are expressed by a wide spectrum of cells including lymphocytes, which are also target cells for MDV. Third, immunodeficiency caused by MDV; MDV initially infects B-lymphocytes causing cell depletion and/or dysfunction and reduced antibody production (57,74). Even though many subsets of T-cells can be transformed by MDV, the highest proportion comprises CD4+ T-helper cells expressing a TCR2 receptor (104,142). Thus, MDV might reduce the immune response elicited by the Ad-vaccine.

The overall aim of this study was to evaluate the effects of combined *in ovo* vaccination with the experimental AdH5 recombinant vaccine and commercially available MDV vaccines.

Literature Review

Avian Influenza

History of Avian Influenza

Avian influenza (AI) was first described during 1878 in Italy by Perroncito as a serious disease of chickens. The disease has been known by different names including for example fowl plague, Geflügelpest, fowl pest, peste aviaire [rev. by (83)]. Early reports on fowl plague indicated uncertainty about the etiology of the disease and debated whether investigators were encountering a new disease or a variant of fowl cholera [rev. by (156)]. Bacteria isolated from birds that succumbed from fowl plague were unable to reproduce the disease [e.g. (12,23,54) rev. in (156)], providing evidence for fowl plague and fowl cholera to be different entities.

Classification of Avian Influenza Virus

AI virus (AIV) has been classified as a type A influenza virus based on nucleoprotein and matrix antigens. AIVs are further divided into subtypes according to the antigenic determinants on the hemagglutinin (HA) and neuraminidase (NA) surface proteins. So far 16 antigenically different HAs and 9 NAs have been identified [rev. by (36)]. Avian influenza viruses have been further classified based on pathogenicity by the World Health Organization (WHO) and the World Organization for Animal Health (OIE). Highly pathogenic AIV (HPAI) have an intravenous pathogenicity index (IVPI) in 6 week-old chickens of ≥1.2 or cause at

least 75% mortality in 4 to 8 week-old chickens. So far, only H5 and H7 subtypes have been associated with HPAI virus outbreaks, although not all viruses belonging to these subtypes cause HPAI (162). H5 or H7 viruses with IVPI indexes of ≤1.2 or causing <75% lethality undergo sequencing of the HA gene to determine the presence of multiple basic amino acids at the cleavage site of the hemagglutinin molecule. LPAI viruses containing amino acid sequences similar to HPAI isolates are classified as low pathogenicity notifiable AI viruses (LPNAI). All AIVs not meeting the IVPI or amino acid similarity criteria are classified as low pathogenic avian influenza viruses (63). According to the USDA, effective in 2006, all confirmed LPAI H5 and H7 AI subtypes must be reported to the OIE because of their potential to mutate into highly pathogenic strains (177).

Etiologic agent

Avian influenza viruses are segmented negative-sense RNA viruses belonging to the family *Orthomyxoviridae.* AIVs are pleomorphic in shape and range from spherical particles with an 80 to 120 nm diameter to filamentous forms. All influenza A viruses contain 8 different gene segments that encode at least 10 different viral proteins. The most abundant and immunologically important glycoproteins found on the viral envelope are rod-shaped trimers of hemagglutinin (HA) and mushroom-shaped tetramers of neuraminidase (NA). HA is made up of a globular domain and a stem domain (93). The HA globular head is made up solely by HA1 and contains the receptor-binding depression in addition to the majority of the antigenic sites of the molecule. The stalk consists of a complete HA2 glycoprotein as well as a section of HA1 (181). The HA glycoprotein is the most abundant surface antigen of the virion

and is responsible for the binding to host cell receptors and for fusion between the virion envelope and the host cell (181). NA is the second major surface antigen of the virion. The NA is a membrane-associated tetramer with a mushroom shaped head that is attached to a slender stalk. The head is responsible for the antigenic and enzymatic properties of the NA, containing the active site in a large cavity on its surface [rev. in (45, 61, 179)]. Unlike HA, NA molecules are not evenly distributed around the virion envelope; instead they have a tendency to collect into patches (181). NA is responsible for cleaving terminal sialic acid residues from glycoproteins or glycolipids. Therefore, it is responsible for freeing virus particles from host cell receptors and permits progeny virions to escape from the host cell, aiding in the spread of the virus (181). In addition to HA and NA proteins, a smaller amount of M2 protein is present on the surface of the envelope. The M2 protein acts as an ion channel and plays a role in triggering the viral uncoating in the endosome (93). Although antibody responses have been detected against some internal proteins (41) (151), a protective immune response is determined mainly by neutralizing antibodies produced against the two surface proteins, HA and NA (159).

Epidemiology

Avian influenza virus can infect and replicate in a large variety of host species such as chickens, turkeys, swine, horses, humans and other avian and mammalian species [rev. in (169)]. According to Stallknecht et al. (154), the concentration of virus needed to infect each species varies greatly and depends mainly on the degree of host adaptation (154). This provides some level of a species barrier (152,176). Cleavage of the precursor HA molecule (HA0) into HA1 and HA2 subunits by host

proteases is essential for infectivity and for spread of the virus. Therefore, virus activation by host proteases plays a crucial role in the spread of infection, tissue tropism and pathogenicity (93). In addition, influenza virus infectivity is primarily influenced by the type of linkage to galactose on the host cell surface (46,149). In most cases AI viruses possess HAs with a high affinity for sialic acid attached to galactose sugars with a α2, 3-linkage contributing to host specificity for only species with the preferred linkage. In comparison, human influenza viruses have a mutated HA which preferentially binds to sialic acid attached to sugars in the α2, 6-linkage. This change in the HA can result in a switch in the linkage that the virus prefers, consequently allowing the switch from one species to another (93).

Avian influenza viruses can infect a wide variety of avian species; however, orders *Anseriformes* (ducks, geese, swans) and *Charadriiformes* (gulls, terns and shore birds) are particularly recognized to be the natural reservoirs for avian influenza (86,150,153,181). A review by Alexander (3) indicates that HPAI viruses are infrequently isolated from wild birds; however high viral isolation rates have been recorded in surveillance studies of AI viruses that have a low pathogenicity for poultry: 15% of the viruses isolated from 90 species of birds were isolated from ducks and geese and around 2% from all other avian species. Undomesticated birds, specifically waterfowl, are considered the natural hosts for AI viruses and are therefore considered a constant source of viruses. These AI viruses are introduced into domestic bird populations and cause LPAI that manifests as a localized infection that results in mild disease (respiratory disease, depression and egg-production problems) (36). It is theorized that HPAI viruses emerge from H5 and H7 LPAI

viruses by mutation or recombination (58,122). According to Capua et al. (36) such mutations probably occur after the viruses have moved from their natural wild-bird host to poultry. However, the period of time needed for the AI virus to mutate to virulence is unpredictable and may occur either soon after AI introduction to poultry or after the LPAI viruses have been circulating in the flock for a longer period of time (36,106). Documented evidence supports the theory that HPAI does not constitute a separate phylogenetic lineage, instead HPAI arise from nonpathogenic strains (6,134). It is interesting that according to Webster et al. (181) wild birds are also the original source of all viral genes for both avian and mammalian lineages of influenza viruses. He found that all known HA and NA subtypes of influenza have been found in wild birds.

Avian Influenza Transmission

According to Swayne et al. (162) chickens and turkeys are not natural hosts for AIV but they can easily become infected with the virus. AI introduction into domestic poultry occurs through direct or indirect contact with infected birds shedding large quantities of the AI virus through their respiratory secretions and feces (36,164). However, other modes of dissemination to domestic poultry include exposure of poultry to infected birds in live bird markets, movement of infected poultry, introduction of the virus through untreated drinking water and exposure of susceptible turkeys to swine, allowing turkeys to become infected with the swine influenza virus (36,65,160). AI is also transmitted by contaminated personnel, supplies and equipment (165). AI virus can also be airborne transmitted and via contaminated dust/feathers (20,124). After the virus has entered the flock,

transmission of AI viruses within poultry is through respiratory secretions and contaminated feces (162).

Clinical signs and lesions

AIV can generally be divided into low or mildly pathogenic strain viruses that commonly cause mucosal infections or a respiratory/enteric tract infection and viruses that cause systemic infections [rev. in (156)]. The viruses that cause systemic infections usually cause high mortality and are referred to as HPAI. AIV causes a wide range of clinical signs. LPAI is prevalent in wild aquatic birds and causes mostly asymptomatic infections and has nominal consequences in its natural host (120). In domestic poultry clinical signs often include mild to severe respiratory signs such as coughing, sneezing, rales, rattles and excessive lacrimation. Layer and breeder hens may exhibit increased broodiness and decreased egg production. Domestic poultry may also demonstrate generalized clinical signs including huddling, ruffled feathers, depression, decreased activity, lethargy, decreased feed and water consumption and occasional diarrhea [rev. in (93)]. Infections with LPAI are usually associated with lesions of the respiratory tract (sinusitis), gross kidney lesions and pancreatitis [rev. in (2-3)].

According to Saif [rev. in (93)] HPAI viruses either do not replicate or replicate to a limited degree and produce minimal clinical signs in wild birds and domestic ducks. Chickens and turkeys with HPAI are typically found dead with few clinical signs other than depression and being in a comatose state (88,102). As the birds' age increases so does the frequency of clinical signs appearing before death. Egg production in laying hens drops to near zero within 3 to 5 days of infection. In some

cases paralysis, convulsions and rolling movements have been noted. Diarrhea and respiratory signs such as nasal discharge, rales, coughing, sneezing or respiratory distress have been infrequently reported with HPAI (7). Severe edema of the upper respiratory tract or lungs has also been reported in birds affected by HPAI [rev. in (162)]. In most cases of acute infections in which the birds die (day 1 or 2 of infection), poultry have lacked visible gross lesions (69). On the other hand, some strains have caused severe lung congestion, hemorrhagic lesions on the skin, liver, spleen, heart and kidneys, and edema of the head and face in chickens (2,157).

Control Strategies for Avian Influenza

The need for controlling LPAI was not recognized until the 1960s when there were syndromes of respiratory disease and drops in egg production observed mainly in turkeys but also seen in pheasants, quail and partridges (55). Munster et al. (106) determined that negligible genetic and antigenic differences exist between H5 and H7 LPAI viruses found in wild birds and those that caused HPAI outbreaks in domestic poultry in Europe. This observation led to the realization that it is not only important to control HPAI viruses but also LPAI viruses of the H5 or H7 subtypes because they could represent HPAI precursors (36). Therefore, it is important that the emphasis for AI control is preventing the introduction of the wild bird-origin AI viruses to domestic poultry and other domesticated birds. Because of the supporting evidence that H5 and H7 HPAI may originate from LPAI viruses, the World Organization For Animal Health (OIE) has increased the use of stamping-out programs when encountering either of these two subtypes of AI as a means to prevent emergence of HPAI viruses (90).

According to Swayne (165) there are three goals in the control of AI. These include prevention, management and eradication. The five strategies developed by the United States Department of Agriculture (USDA) to reach these goals include education, biosecurity, diagnostics and surveillance, elimination of infected poultry and decreasing host susceptibility. Control strategies may be different depending on country, subtype of virus, economic situation and risk to public health (163,165).

U.S. Strategies for HPAI outbreaks

In the U.S. HPAI control has been based on rapid eradication through depopulation of flocks and disposal of carcasses and quarantine of infected flocks. In addition, decontamination of equipment and infected areas is important in containing the outbreak. Federal laws and regulations give USDA the authority to declare an animal health emergency, to quarantine and destroy flocks, and to pay indemnities (77,107).

U.S. Strategies for H5 and H7 LPAI outbreaks

Common strategies when facing a H5 or H7 LPAI outbreak have been control or eradication. These strategies have varied with the individual situation. The control and eradication strategies encompass components of biosecurity, diagnostics and surveillance, elimination of infected birds as well as vaccination in some situations (165). Before 1995, the use of USDA-licensed AI vaccines only required approval by the poultry industry and state governments; however in 1995 the USDA implemented the requirement that federal approval must be obtained for field use of USDA-licensed H5 and H7 vaccines (107). Currently, federal regulations do not require indemnity payments for losses pertaining to H5 and H7 LPAI (165). Consequently,

in most cases, indemnities have not been paid for elimination of H5 and H7 LPAI-infected poultry.

U.S. Strategies for non-H5 and non-H7 LPAI

Similar prevention, control and eradication strategies have been employed with non-H5 and non-H7 LPAI. For example, in Minnesota since 1978, similar strategies have been utilized for the non H5/H7 LPAI as was used for the H5/H7 LPAI outbreaks. In addition, vaccination continues to be an important tool in non-H5 and non-H7 LPAI eradication strategies. For instance, it is important to vaccinate turkey breeders against H1N1 swine influenza (165).

Avian Influenza Vaccines

AI vaccines have been shown to provide protection against mortality, morbidity and declines in egg production when used properly [rev. in (168)]. When vaccination is used as part of a control program designed to eradicate a disease from a designated area, the vaccine should have three important characteristics. First, a vaccine should prevent or reduce clinical disease if the bird is infected. Second, the vaccine should prevent or significantly reduce the amount of virus that is shed into the environment, therefore limiting the source of infection for other birds or flocks. Third, a vaccine should raise the threshold of virus required to infect the birds, which may prevent infection of some exposed flocks (94). It is also important that the vaccination complies with the DIVA strategy (differentiation of infected from vaccinated animals). This aids in assuring trading partners of a safe product and may allow the

faster resumption of exportation of poultry to other countries after an AI outbreak (37).

The first AI vaccine was developed in the early 1900s based on observations that some chickens infected with fowl plague virus (e.g. H7 HPAI virus) recovered from the disease and then were resistant to re-infection by fowl plague virus (11,172). The first vaccines were produced by drying spinal cords from fowl plague infected birds or by using heat, light or chemicals to inactivate the virus in the blood or liver from birds who died from fowl plague. Problems of incomplete inactivation and insufficient quantity of inactivated virus were likely responsible for many failed vaccine attempts [rev. in (168)]. Vaccines to help control LPAI were developed in the mid-1960s. In 1978 the first licensed inactivated AI vaccine was approved by the USDA in response to severe losses in Minnesota breeder turkeys (100).

Until vaccination was used in Mexico and Pakistan in 1995, vaccination had not been used in a control and eradication program for HPAI (108,180). Vaccination was utilized in these cases because financial constraints made the stamping out method unfeasible. Currently, two types of vaccines are applied in the control and prevention of AI worldwide. These include inactivated oil emulsion vaccines and a fowlpox recombinant vaccine (94). Inactivated oil emulsion vaccines are produced by virus propagation in embryonating chicken eggs. Allantoic fluids harvested from these eggs are formalin inactivated and emulsified with an oil-based adjuvant. With proper preparation and administration these vaccines elicit high levels of neutralizing antibodies [rev. in (94)]. A whole AIV inactivated vaccine has been shown to be protective against HPAI challenge but multiple booster vaccinations were required for

clinical protection [e.g.(137)]. In addition, because the vaccines are prepared from whole virus, antibody responses are produced to all viral proteins. Therefore, birds vaccinated with a killed vaccine cannot be readily distinguished from birds that have been naturally infected (DIVA) based on standard antibody based tests (94). Also, with inactivated oil emulsion vaccines a long withdrawal time is required before the birds can be marketed. Consequently, a restriction is placed on the use of these vaccines in broilers that achieve market weight in as little as 6 weeks (94).

Differentiation of infected from vaccinated animals (DIVA), using an inactivated whole virus vaccine containing the same HA subtype as the outbreak virus, but a different NA subtype from the outbreak virus was first suggested in the late 1980's (9,155). This strategy was first used in Italy to supplement control measures for the eradication of an H7N1 LPAI virus from 2000 to 2002 (38).

Avian Influenza Recombinant Vaccines

Recombinant AI vaccines comply with the DIVA strategy as the vector exposes only an immunologically relevant antigen to the host's immune system. Fowlpox viruses (FPV) have been used successfully as viral vectors to express a variety of foreign genes for many years (10,21-22). A recombinant FPV expressing the H5 HA from an A/Turkey/Ireland/83 strain was developed in the 1980s and was shown to provide protection from lethal challenge with H5 influenza viruses (167,182). The recombinant FPV vaccine was commercialized and approved for use by several countries as one of the first viral vectored vaccines (94). In addition, Bublot et al. (24) developed a fowl pox vectored vaccine for AI consisting of a live recombinant FPV expressing the HA gene of A/Turkey/Ireland/1378/83 isolate (TROVAC-H5).

The vaccine was granted a license for emergency use in the U.S. in 1998 and full use in Mexico, Guatemala, and El Salvador. One administration of TROVAC-H5 protects chickens against AI-induced mortality and morbidity for at least 20 weeks. Protection was demonstrated against 2003 and 2004 HPAI H5N1 isolates. Anti-AI or anti-FPV maternal antibodies did not inhibit efficacy but protection against AI was significantly decreased in chickens that had been previously infected or vaccinated with FPV (24).

Additionally, a variety of other virus vectors have been utilized to produce AI vaccines. Pavlova et al. in 2009 (117) developed an attenuated infectious laryngotracheitis virus that expressed the hemagglutinin gene of HPAI virus H5N1 of A/duck/Vietnam/TG24-01/2005. They found that after a single ocular immunization all animals developed HA-specific antibodies and were protected against lethal infection by homologous isolates as well as heterologous isolates such as HPAIV A/swan/Germany/R65/2006 (H5N9) (96.1% homology) or A/chicken/Italy/8/98 (H5N2) (93.8% homology) (117).

A recombinant NDV vectored live attenuated vaccine that expresses an H5 hemagglutinin from isolate A/Bar-headed goose/Qinghai/3/2005 (H5N1) was reported to induce antibodies to both NDV and AIV in a single dose regime in chickens. This vaccine provided protection against a lethal dose of both velogenic NDV and homologous and heterologous AIV H5N1 (59).

Kalhoro et al. (84) in 2009 reported the development of a recombinant vesicular stomatitis virus expressing the HA antigen of HPAIV A/FPV/Rostock/34 (H7N1) in place of the VSV G gene. Chickens were immunized by the intramuscular route at 3

weeks of age and then boosted 3 weeks later. Two weeks after boost, birds were challenged with HPAIV A/chicken/Italy/445/99 (H7N1). Birds were shown to be protected against a lethal dose of the heterologous HPAIV strain (84).

Wu et al. (190) described an AI vaccine produced using a recombinant baculovirus encoding the HA protein of HPAI strain A/Chicken/Hubei/327/2004(HB/327). Chickens were vaccinated by intramuscular injection at day 0 and 21 days of age. On day 35 chickens were challenged with HPAI strain HB/327. It was determined that 1 x 10^8 PFU of BV-G-HA vaccine offered complete protection against challenge with HPAI HB/327.

Adenoviruses as Viral Vectors

Adenoviruses belong to the family *Adenoviridae* and are divided into two genera, *Mastadenovirus* and *Aviadenovirus*. *Aviadenovirus* is limited to viruses of birds but the genus *Mastadenovirus* includes human, simian, murine, bovine, equine, porcine, ovine, canine, and opossum viruses. Forty-nine human adenovirus serotypes have been described. Adenoviruses are non-enveloped icosahedral particles measuring 70 to 100 nm in diameter. They contain linear double-stranded DNA genomes. Adenoviruses have eight RNA polymerase II transcription units and through mRNA splicing they encode for at least 40 different proteins (56).

Adenoviruses have been used extensively as viral vectors in various applications such as gene therapy and vaccination. Adenoviruses are effective viral vectors because they can infect a variety of different cell types as well as differentiated or non-differentiated cells. They additionally allow a high expression of the

recombinant protein and accommodate an up to 8 kb transgene insert into their genome. Adenoviruses can be grown to high titers such as 10^{10}-10^{13} viral particles (vp)/ml. Another advantage of adenoviruses is that the adenovirus DNA does not integrate into the host chromosome so it does not inactivate genes or activate oncogenes (103).

Adenoviruses have successfully been utilized as vectors for gene therapy or treatment of cancer. Some examples of these applications include adenovirus-vectored recombinant viruses for the treatment of breast cancer (62,71), human laryngeal squamous cell carcinoma (171) and colorectal cancer (92).

Adenoviruses have been used as viral vectors for vaccine administration including for example HIV (99), Ebola virus (161), SARS-CoV (96), and *Pseudomonas aeruginosa* (189).

Adenovirus Vectored Avian Influenza Vaccine

In 2007 Toro et al. demonstrated that chickens can be protected against highly pathogenic avian influenza (HPAI) by *in ovo* administration of a replication competent adenovirus (RCA)-free human serotype 5 adenovirus vector encoding an avian H5 hemagglutinin (HA) from the A/turkey/Wisconsin/68 strain of AIV (AdTW68.H5). This adenovirus vectored AI vaccine does not possess a safety risk due to the deletion of the E1/E3 genes in the vector making it replication incompetent. This is also important because the vaccine cannot propagate in the field and generate revertants. It is in compliance with the DIVA strategy because the vector only encodes the viral HA. It is also important to note that the vaccine is produced in PER.C6 cells rather than embryonating eggs which would be important

in the case of an AI outbreak when embryonating chickens eggs may be in short supply. The administration of the vaccine by the *in ovo* route allows automated mass delivery, decreasing the labor cost associated with vaccination as well as allowing for uniform vaccination (174).

It was shown that the AdTW68.H5 was capable of producing an H5 HA specific antibody response when administered *in ovo* at day 18 of incubation (174-175). All *in ovo* vaccinated chickens survived a challenge with the HPAI virus A/chicken/Queretaro/14588-19/95 strain. Vaccinated birds also showed a significant reduction of detectable viral RNA in oropharyngeal swabs collected 2, 4 and 7 days after challenge indicating that *in ovo* vaccination elicited an immune response capable of controlling AI virus shedding within a week of infection. In another trial, chickens were challenged with A/swan/Mongolia/244L/2005. All control birds died from AI within 9 days post challenge. The *in ovo* immunized birds had a survival rate of 68% without clinical signs for 10 days post-challenge. It is conceivable that the survival rate against avian influenza would be improved by *in ovo* vaccination with an adenovirus vectored vaccine encoding an HA with a closer antigenic similarity to the challenge virus (174).

It was additionally demonstrated that chickens vaccinated with an RCA-free human Ad5 vector encoding a North American lineage (A/chicken/New York/13142-5/94) H7 HA (AdChNY94.H7) were protected against H7 HPAI challenge. Further reports described that when chickens were vaccinated *in ovo* with AdTW68.H5 and subsequently intramuscularly vaccinated with AdChNY94.H7 after hatch, they developed antibodies against both the H5 and H7 HA proteins. Furthermore, it was

demonstrated that the use of a synthetic AI H5 gene with codons optimized to match the chicken tRNA pool is more immunogenic than its wild type counterpart (175). When chickens were ocularly vaccinated, the Harderian glands were shown to produce H5 and Ad5 specific IgA and IgG spot-forming cells (SFCs). In addition, Ad5 and H5 specific antibodies were induced in the serum (178).

Advantages of Live Recombinant Viral Vectored Vaccines

A live virus vaccine mimics the protection produced by a natural acquired infection. This includes for example stimulation of B and T cell responses (98). In addition, it has been recognized (106, 107) that live virus vaccines produce immunity for a longer duration than a non-replicating vaccine and commonly requires less frequent booster vaccinations. The distinct advantage of vectored vaccines is the ability to introduce a specific immunogenic antigen that can facilitate a particular protective immune response (174).

Even though multiple vectors are available as vaccine carriers, recombinant avian virus vectors (*e.g.* fowlpox virus) have shown reduced protection due to preexisting immunity (maternal and active humoral immunity) associated with the birds pre-exposure to the avian virus vector (166).

Avian Influenza DNA Vaccines

Multiple DNA vaccines have also been developed to protect against avian influenza infections. Pan et al. (114) in 2010 reported development of a DNA vaccine expressing the hemagglutinin of AIV A/Goose/Jiangsu/1/2000 that was sub-cloned into pmcDNA3.1+ plasmid and transformed into attenuated *Salmonella enterica* serovar Typhimurium. Chickens were primed orally with recombinant *S.*

Typhimurium at 1 day or 2 weeks of age and this was followed by boosting with recombinant *S*. Typhimurium or killed vaccine at 4 weeks of age. Chickens were intranasally challenged with 10^5 EID_{50} of HPAIV H5N1 A/goose/Jiangsu/1/2000 at 6 weeks of age. *S*. Typhimurium was shown to be able to transfer HA DNA into chicken cells. A humoral and cell mediated response was elicited on mucosal surfaces as well as a systemic immune response against the HA antigen. Chickens vaccinated with recombinant *S*. Typhimurium followed by a killed AI vaccine boost were completely protected following viral challenge. Furthermore, they did not show signs of disease, detectable virus shedding, or death. These results indicate that chickens immunized with a DNA vaccine transported by *Salmonella* and a killed vaccine can be effectively protected from challenge with H5N1 HPAI.

In 2007 Jiang et al. (80) described an H5 subtype AI DNA vaccine that was produced using a pCAGG plasmid vector and a chicken codon biased sequence based on the HA glycoprotein of the HPAI H5N1 virus A/goose/Guangdong/1/96 (GS/GD/96). All chickens receiving this vaccine developed high levels of hemagglutination inhibition (HI) and neutralizing antibodies at 3 weeks post vaccination. These vaccinated birds were completely protected from lethal H5 virus challenge (80).

Rao et al. (128) reported a multivalent DNA vaccine for poultry protecting against multiple HPAI H5N1 strains. Plasmid expression vectors pCMV/R or pCMV/R 8kB encoding HAs from three phylogenetically diverse strains of influenza viruses were generated. Three intramuscular administrations and Agro-jet inoculation of at least 5 μg of trivalent DNA were shown to provide complete protection against HPAI

challenge. It was also shown that protective immunity was also elicited in chickens with two 5 μg of trivalent DNA vaccinations using an Agro Jet device.

Advantages and disadvantages of DNA vaccines

DNA vaccines are capable of inducing both an adaptive humoral and cellular immune response, similar to those produced by live virus infection or vaccination (94). However, the protection elicited by DNA vaccines in an avian model (e.g. chicken) have been less consistent than with inactivated whole AI virus or subunit vaccines (89,94,158). Other limitations of DNA vaccines include the requirement of large quantities of expensive (158-159) DNA needed per dose to produce a protective immune response in chickens. Multiple vaccinations are also often required to achieve protection, increasing the labor costs associated with the vaccination [rev. in (168)].

Marek's Disease Virus

Marek's disease virus (MDV) is responsible for great economic losses in the poultry industry. MDV vaccination is routinely performed *in ovo* in the United States poultry industry. Therefore, it seems appropriate to investigate if MDV vaccination would interfere with Ad-vector vaccination. Interference between avian vaccine viruses has been reported when vaccines are applied simultaneously in chickens [e.g. (8,47)]. Vaccine virus interference can occur for example as result of competition for cell receptors, different rates of replication (a fast replicating virus might induce innate immune responses that will prevent a slower replicating virus to successfully proliferate), and/or immunodeficiency caused by one of the viruses which will

prevent the development of appropriate immune responses to the second vaccine virus.

History of Marek's Disease

Marek's disease (MD) was first described by Jozsef Marek in Hungary in 1907 (60). Roosters showed paralysis caused by a mononuclear infiltration of peripheral nerves and spinal nerve roots [rev. in (17)]. In 1914 outbreaks of Marek's disease were reported in the United States, the Netherlands, Great Britain and many other countries (13). As observations were added to Marek's early description, it was recognized that lesions were not only restricted to the peripheral nerves and spinal cord. Blindness frequently accompanied paralysis and neural lesions including visceral lymphomas and infiltration of the iris and brain (187). In the early 1960's researchers successfully performed a reproducible experimental transmission of the virus (16,144) and determined the cell association of the MDV (15) allowing the causal agent to be identified. In 1967, researchers in Great Britain and the United States independently identified the agent of Marek's disease as being a herpesvirus (18,44).

The disease caused by MDV was called by many names depending on the region infected and the symptoms caused by the virus. A few examples include neuritis, neurolymphomatosis gallinarum (also had visceral, neural and ocular forms) and range paralysis (187). The term Marek's disease that is currently in use was proposed by Biggs (187) in 1961 to distinguish the condition from other lymphoproliferative diseases such as lymphoid leukosis.

Serotypes of MDV

Three serotypes of MDV have been identified. Marek's disease virus is the prototype virus of the MDV group and is designated as serotype 1. Serotype 1 MDV viruses are divided into subgroups based on their virulence, mild MDV, virulent MDV, very virulent MDV and very virulent plus MDV strains (34,188). The additional two serotypes of MDV include non-oncogenic herpesviruses isolated from turkeys (HVT) and chickens. The non-oncogenic viruses isolated from chickens are designated as serotype 2 (14,42) while the MDV viruses isolated from turkeys are designated as serotype 3 (85,186).

Etiologic agent

MDV is a highly cell associated alpha herpesvirus from the family *Herpesviridae (60).* MDV has a linear, double stranded DNA genome that is about 160-180 kb in size and the genome contains at least 90 open reading frames (76,97). MDV can be found in multiple forms such as an 85-100 nm in diameter nucleocapsid or an enveloped particle 150-160 nm in diameter. MDV is also found in feather follicle epithelium as an enveloped 273-400 nm particle (187).

All three MDV serotypes have genome structures consisting of a long unique region and a shorter unique region, each flanked by inverted repeats (40). The genomes of all three serotypes are similarly organized (73,113). However, minor but possibly important differences have been observed. For example, the size of the genome differs between serotypes. Serotype 1 has the largest genome, followed by serotype 2 then lastly serotype 3 having the smallest genome size (40,66). All three serotypes also differ in their restriction endonuclease digestion patterns [rev. in

(187)]. Nonetheless, all three serotypes share significant homology at the DNA level, specifically with certain genes such coding for gB (glycoprotein B), gC, gD and gH (48,192-193).

Specific genes have been identified among the serotype 1 MDV that are thought to be responsible for the virus' oncogenic properties. Some of these genes are Meq (81,121), vIL-8 (115), pp38 (50) and two small open reading frames encoding pp14 and p7 (68). Meq is the most consistently expressed latency gene (115). Accumulating evidence suggests that Meq may be the principle oncogene for MDV while other MDV genes serve auxiliary functions (52).

MDV Transmission

MDV spreads by direct or indirect contact and in environments that have been contaminated by infected birds. These areas can remain infectious for several months (49). According to Carrozza (39) the feather follicle epithelium is the source of infectious cell free virus and from about 13 dpi virus replication is fully productive, releasing infectious, enveloped, cell-free virus into the environment. The virus is shed with the debris of dead stratified epithelial cells which are inhaled by other chickens (39).

Infection and Replication of MDV

According to Davidson (52) MDV replicates similarly to other cell associated herpesviruses. Initial infection begins by virus binding to the cellular receptors likely by the use of glycoprotein B, C and D and fuses and penetrates the target cell. The virus goes through uncoating with the aid of cellular enzymes which releases the viral DNA to be transported to the nucleus. Messenger RNA is synthesized in the nucleus

and then transported into the cytoplasm for translation. The virus enters the cells and infects other cells by direct contact possibly through formation of intracellular bridges (82). In addition to the virus going through exocytosis in Golgi vesicles, the release of progeny viruses are accompanied by death of the target cells (52).

There are 4 basic phases of MDV pathogenesis based on the Cornell model [rev. in (35,139,143)]. These include an early cytolytic phase [2-7 days post-infection (dpi)], a latent phase (7-10 dpi), late cytolytic and immunosuppressive phase (18 dpi) and a proliferative phase (28 dpi onward). In the early cytolytic phase MDV is picked up by macrophages and ellipsoid associated reticular cells (EARCs) from the lungs and enters the blood stream; the cells then enter the secondary lymphoid tissues (e.g. spleen, gut-associated lymphoid tissue, cecal tonsil, Harderian gland) (79). Cell free MDV reaches the lymphoid organs within 24-36 hours post tracheal inoculation [rev. in (143)]. MDV enters the lymphocytes in the lymphoid organs through splenic EARC lining the blood vessels (79). The primary target cells for acute cytolytic infection are B-lymphocytes (~90%) and about 3 percent are CD4+ and CD8+ TCR$\alpha\beta^+$ T-lymphocytes increasing to ~6% by 7 dpi (5,32,148). It is generally accepted that resting T-cells are not easily infected by MDV infection but when B cells become infected, T cells are activated, allowing these T-cells to become susceptible (32). Cytolytic infection of B and T cells is semi-productive; no cell-free virus is produced. Therefore the mechanism of viral spread from cell to cell is unclear (29). External viral spread takes place by a fully productive cytolytic infection in the feather follicle epithelium that produces a large number of enveloped infectious

virions and leads to lysis of the susceptible cell and release of infectious MDV into the environment (29).

MDV goes into a state of latency at about day 6-7 dpi, when the host immune response is evident. At this stage the MDV viral genome is present but expression of viral antigens is suspended; no infectious virus is produced until after reactivation. Immunosuppression can cause a re-emergence of a cytolytic infection in the lymphoid organs and epithelia. The view that the host immune response is essential in the establishment and maintenance of latency was supported by Buscaglia et al. in *in vitro* studies on MDV latency in spleen cells grown in conditioned media harvested from immunocompetant stimulated spleen cells (27). Following the reactivation of MDV from latency the majority of the latently infected lymphocytes are T-cells with only ~ 3 percent being B cells and the infected T-cells predominantly belonging to the CD4+ TCR$\alpha\beta^+$ lymphocyte subset (33,148). In resistant chicken strains latent infection can persist at a low level in the spleen and blood lymphocytes without further effect. On the other hand, in MDV susceptible or immune suppressed birds a second pathological cycle begins 2-3 weeks post primary infection. Latently infected peripheral blood lymphocytes can spread the virus throughout the body and organs of the host [rev. in (52)].

As described by Calnek (35), during the late cytotoxic/immunosuppressive phase at 14-21 dpi, MDV undertakes a second wave of semi-productive infection and cytolysis in susceptible chickens affecting the thymus, bursa and some epithelial tissues including the FFE, kidney, adrenal gland and proventriculus. Necrosis of lymphocytes and epithelial cells is accompanied by pronounced inflammation and

infiltration of mononuclear cells and heterophils and severe atrophy of the bursa and thymus.

Three to 4 weeks post-infection the virus may enter the transformation phase. This occurs when non-productively infected lymphocytes migrate into the visceral organs or peripheral nerves and proliferate to form lymphomas. The majority of cells prepared from visceral lymphomas are T lymphocytes (75%) while only 15 % are B lymphocytes (119,136). Most of the tumor cells express MHC class II antigens and are $CD4^{+}$ $CD8^{;}$ however, CD4-CD8+ and CD4-CD8- T cells may also be transformed (111,142). According to Burgess (26) in susceptible birds there is a mass proliferation of CD4+ TCR$\alpha\beta^{+}$ cells which invade and replace the normal tissue. A mature lymphoma may be formed by 50 dpi. CD8+ cells are the predominant cells in resistant birds and lesions regress after 30 dpi because of apoptosis. These findings suggest that the CD8+ T cells are responsible for regulating the CD4+ T-cell population by cytolytic anti-tumor activity (26).

MDV Immunodeficiency

Marek's disease virus has been commonly noted to cause immunosuppression (1,64,67,74,104,141,187). According to Davison (52), the main causes of MDV-induced immunosuppression are divided into three categories; these include loss of lymphocytes as a consequence of viral replication, virus-induced changes in the regulation of immune responses and tumor cell-induced immunosuppression. Immunosuppression occurs from an acute cytolytic infection of the lymphoid tissue (e.g. bursa, thymus and spleen) at 3 dpi and reaches its peak between 5-7 days (118). B cells are the primary target of these infections but in time CD4+ and CD8+ T cells

become activated and then infected (148). The bursa and thymus undergo severe regression of the bursal lymphoid follicles and thymic cortex. MDV infected cells are destined to die (135). According to O'Brien, apoptosis is the most likely cause of MDV-induced cell death, as seen in many virus infections (53,110). In addition, Derfuss (53) stated that herpesviruses commonly trigger apoptosis during productive infections, but can also block apoptosis during latency or transformation. The virulence of the MDV strain may determine the damage caused to the lymphoid organs by a strain not establishing latency and producing a prolonged cytolytic infection (30,78).

Immunosuppression may also be caused by changes in the regulation of immune responses. Nitric oxide (NO) produced by macrophages and other cells during MDV infection, can be beneficial to the reduction of MDV replication. Therefore, it is not surprising that MDV genetically resistant chickens produce higher levels of NO than susceptible chickens (78). NO can cause apoptosis by causing mitochondrial dysfunction in T-cells inducing immunosuppression (28). NO has also been shown to suppress T-cell proliferation (19,123). This could explain the decrease in mitogen responsiveness between 5 and 8 dpi (191).

Down regulation of the MHC class I molecule was observed in MDV infected chicken embryo fibroblasts in comparison to non infected controls (72,87,95). A down regulation of CD8 antigen on CD4+ CD8+ and CD4- CD8+ lymphocytes was also reported in the thymus during cytolytic infection and in the splenic and peripheral lymphocytes at 21 dpi (105). The decreased expression of CD8 antigen may lead to a decreased CTL activity, which is critical during the secondary cytolytic

infection when MDV-specific CTLs are very important in controlling the MDV infection (141).

Immunosuppression may also be caused by MDV induced tumor cells (52). The development of tumors is often associated or preceded by the reactivation of virus replication and therefore may cause additional damage to the lymphoid tissues causing permanent immunosuppression (25,125).

MDV Vaccination

MD vaccines have been extremely effective at reducing MD losses. Losses from condemnations for MD lesions in young broiler chickens in the USA have decreased from 1.5 percent in 1970 to 0.0121 percent in 1999 by vaccination against MDV (184). Marek's disease vaccines are commonly administered by inoculation of one day old chicks (administered through the subcutaneous or intramuscular route at day 1 of age) (52)) or by inoculation of 18 day old embryos (130).

The first vaccine against Marek's disease was described by Churchill et al. in 1969 (43) and was based on the oncogenic HPRS-16 strain of serotype 1 MDV that had been attenuated by serial passages in chicken kidney cell cultures (43). This vaccine was replaced by a herpesvirus of turkeys (HVT) vaccine (FC126 Strain) (112,186) that was initially licensed in the USA in 1971 and was rapidly accepted and used by the poultry industry worldwide [rev. in (52)]. The HVT vaccine was a cell-associated vaccine but later it was also produced as a cell-free lyophilized vaccine. In 1969 Rispens et al. (131) described a low pathogenic MDV serotype 1 isolate that when inoculated into day-old MD-susceptible chicks protected them from mortality

and gross pathological lesions (131). This finding was used to produce an attenuated vaccine strain named CVI988 or Rispens and the vaccine was shown to be protective in laboratory and field trials (132-133). Rispens was not used in the USA until 1994 (52). Rispens was shown to be more protective against highly virulent challenge strains of MDV in comparison to the HVT vaccine (183). Currently most of the parental flocks (breeders) and layers are vaccinated at 1 day of age with the Rispens vaccine worldwide (52).

In 1978 another non-oncogenic strain was characterized by Schat et al. (140). The MDV strain, SB or SB-1 was a serotype 2 virus. Even though this vaccine was able to protect against pathogenic strains of MDV serotype 1 when administered alone (140) it was determined that it had a synergistic activity that provided more protection when administered in conjunction with HVT (31,185) The bivalent vaccine was introduced to the USA market in 1983 (52).

Field challenge commonly occurs when after hatching and being vaccinated the birds are placed directly in brooder houses where there is a high risk of exposure to MDV and an inadequate amount of time for chickens to produce a protective immune response against the vaccine before challenge (145). In addition, there is a large labor cost associated with manually vaccinating the quantity of chicks in the hatchery as well as there being many vaccine failures after manual vaccination (51). Sharma et al. (145-146) demonstrated that in addition to the procedure and vaccine not having appreciable adverse effects on the hatchability of the chicks, embryos could also be successfully vaccinated by the *in ovo* route against MDV at 17-18 days of incubation (145-146). This was stated as being the first successful embryonic vaccination

performed in chickens. The *in ovo* route of vaccination was determined to be more effective against early challenge then vaccination at day 1 of age. *In ovo* vaccination takes place by puncturing a small hole through the blunt end of the egg with a needle and then passing a needle into the egg to deliver a small amount of vaccine into the amniotic fluid. The amniotic fluid is taken up by the embryo prior to hatching, exposing the embryo to the vaccine (51).

According to Davison (52) the *in ovo* vaccination route is commonly used in the USA with more than 95 percent of broilers being vaccinated *in ovo*. Its use is also increasing throughout Asia, Europe, and South America.

Immunodeficiency Caused by MDV Vaccines

Friedman et al. (57) determined reduced B lymphocyte response against STM (B-lymphocyte specific mitogen) after chickens were vaccinated subcutaneously with HVT, HVT+SB-1 or Rispens in comparison to non-vaccinated controls. The antibody production in the HVT vaccinated birds was similar to non-vaccinated controls when stimulated with STM. The groups receiving HVT + SB-1 or Rispens had a significantly reduced B lymphocyte response, compared to the non-vaccinated controls. However, the Rispens vaccinated birds were able to attain normal antibody levels to STM by day 18 while the HVT + SB-1 birds did not recover throughout the experimental period. It was also shown that when MD vaccines were given at 1 day of age; there was an increase in chick mortality from a pathogenic infection such as *E. coli*. The vaccinated birds had a 156 to 218% higher mortality by 11 and 14 days post vaccination than non-vaccinated controls. Islam et al. (75) also documented that the HVT vaccine alone caused mild depletion of T and B lymphocytes but there were no

significant effects on immune organ weight or infectious bronchitis virus vaccine antibody titer.

Interference between Viral Vaccines

Although multiple vaccines have often been successfully applied together (129,147) some interference has been encountered when using multiple viruses concurrently. For example, when infectious bronchitis virus (IBV) and NDV were inoculated in parallel in chicken kidney cells (8), embryonating chicken eggs (127) or chickens (126), it appeared that IBV interfered with the replication of NDV. In chicken embryos, viral interference was determined by the rate at which the virus killed the embryo and its hemagglutinating activity. It was found that whether IBV was inoculated before, simultaneously or after NDV, NDV was unable to replicate when the titer of IBV inoculated was at a higher concentration than that of NDV. Heat inactivated IBV or anti-IBV antibody neutralized virus did not cause interference in NDV vaccination in embryonating chicken eggs (127). When IBV and NDV co-infection was investigated in chickens it was also found that IBV interfered with NDV judging by the absence of significant HI antibodies, susceptibility to ND challenge and by a prolonged incubation period for nervous symptoms in a significant percentage of the birds. Interference was present regardless of the sequence of administration of the two viruses, time intervals used and relative amounts of virus used. It was also noted that NDV did not appear to interfere with the replication of IBV as seen by the birds' resistance to IBV challenge after the co-administration of the two viruses (126).

Cook et al. (47) in 2001 also observed that when an IBV vaccine was administered one week before an avian pneumovirus (APV) vaccine that APV was detected for a shorter period of time and with reduced amounts resulting in a reduced antibody response to APV vaccination in comparison to the antibody response in birds vaccinated with APV only. No interference was detected against the IBV immune response. It was hypothesized that the interference was caused by the similarity in target cells and the higher rate of replication of IBV in comparison to APV.

Theoretical Possibilities for Interference between MDV and Ad-vectored Vaccines

1. *Competition for cell receptors.*

MDV targets lymphoid cell populations (described above). Ad5 binds to a (CAR) or an $\alpha_M\beta_2$ integrin whereas Ad2 binds to major histocompatibility complex class I (MHC-I) (194). CAR receptors are present in specialized intracellular junctions such as the cardiac intercalated disk and the tight junctions of polarized epithelial cells. CAR's tissue distribution has not been well defined but the mRNA for the receptor has been found in a number of organs indicating that there is a large distribution of CAR receptors (194). In addition, a co-receptor is needed for the virus to gain entry into the cell. Some of these include vitronectin binding α_v integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ (109).

Adenovirus type 5 was shown to be able to infect lymphocytes. It commonly infects children and persists in lymphoid tissues such as the tonsils and adenoids. Adenoviruses are capable of replicating in cultures of purified lymphocytes from human adenoids. Lymphocytes and other mononuclear leukocytes may be the cells

that maintain long-term infection with adenovirus *in vivo*, especially within lymphoid tissues (4). Lavery et al. showed that established human B and T cell lines support productive infection by adenovirus serotype 5, as indicated by viral DNA replication, viral RNA synthesis and assembly of infectious particles (91).

Another example of adenoviruses infecting lymphocytes was presented by Meeker et al. (101). They demonstrated that an adenovirus serotype 5 vector could transduce and exhibit efficient gene transfer, out of a panel of 33 lymphocyte malignancy derived cell lines that were tested; three anaplastic large cell lymphoma lines, two Hodgkin's disease cell lines, two Burkitt's lymphoma cell lines and three myeloma cell lines. Although in their study these cell lines allowed the highest rates of transduction, many of the other lymphoid cell lines also allowed gene transfer but at a lower rate. Gene transfer in some of these cell lines may have been undetectable by the assays used in the experiment.

Horvath et al. (70) presented data indicating that adenovirus infections appear to decrease the DNA synthesis of stimulated T-cells, decreasing the number of active T-cells in vitro. A cytotoxic effect was also observed in adenovirus-infected cells. These authors indicate that adenovirus infections of lymphocytes may be responsible for some immunosuppression and may lead to an increase in susceptibility to opportunistic infections.

2. MDV causing immunodeficiency

As described above, MDV wild and vaccine strains cause immunodeficiency. Thus, concurrently delivered vaccines, including Ad-vector vaccines, could be negatively affected by MDV (i.e. absence or reduced immune responses).

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Avian Influenza Adenovirus-Vectored *In Ovo* Vaccination: Target Embryo Tissues and Combination with Marek's Disease Vaccine

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*Abbreviations***:** AAALAC=Association for Assessment and Accreditation of Laboratory Animal Care; Ad=adenovirus serotype 5; AI=avian influenza; BSL=biosafety level; CAR = coxsackie-adenovirus receptor; ELISPOT=enzyme linked immunospot assay; HA=hemagglutinin; HI= hemagglutination inhibition; HP=highly pathogenic; HVT = turkey herpesvirus; IFA= immunofluorescence assay; qRT-PCR=quantitative RT-polymerase chain reaction; RCA=replication competent adenovirus; RDEII=receptor destroying enzyme; RT=reverse transcriptase; MDV= Marek's disease virus; SPF= specific pathogen free; TK = thymidine kinase.

Summary

We investigated embryo tissues targeted by replication competent adenovirus (RCA)-free recombinant adenovirus (Ad) expressing a codon-optimized avian influenza (AI) H5 gene from A/turkey/WI/68 (AdH5) when injected into 18-day embryonated eggs. We also evaluated the effects of concurrent *in ovo* vaccination with the experimental AdH5 vaccine and commercially available Marek's disease virus (MDV) vaccine combinations Rispens/HVT or HVT/SB1. Computed tomography indicates that *in ovo* injection on day 18 of incubation places the solution in the amnion and/or allantoic cavities. Ad DNA was consistently detected in the chorioallantoic membranes as well as in the embryonic bursa of Fabricius, esophagus, and thymus 3 days post-inoculation. H5 expression in these tissues was also detected by immunofluorescence assay. These results indicate possible ingesting of vaccine virus contained in the amnion. On the other hand, vaccine localization in the allantoic fluid would have allowed bursal exposure through the cloaca. When the AdH5 vaccine was used in combination with MDV, chickens responding to the AdH5 vaccine had similar AI antibody levels compared to AdH5-only vaccinated birds. However, combined vaccinated groups showed reduced vaccine coverage to AI which suggests some level of interference. The combination of AdH5 with MDV Rispens/HVT affected the vaccine coverage to AI more severely. This result suggests that the replication rate of the more aggressive Rispens strain of serotype 1 may have interfered with the Ad-vectored vaccine. Increasing the Ad concentration produced similar AI antibody titers and AI vaccine coverage when applied alone or in combination with the HVT/SB1 vaccine. Adenovirus DNA was detected in hatched chickens 2 days after hatch

but was undetectable on day 9 post hatch. MDV DNA was detected in feather follicles of all vaccinated birds at 12 days of age. Thus, Ad-vector vaccination does not interfere with the efficacy of MDV vaccination using any of the commonly used vaccine strains.

Protective immunity against avian influenza (AI) can be elicited in chickens by *in ovo* vaccination in a single-dose regimen with a replication competent adenovirus (RCA)-free human adenovirus serotype 5 (Ad)-vector encoding either the AI virus H5 (AdH5) or H7 hemagglutinin (HA). *In ovo* vaccinated chickens are protected against highly pathogenic (HP) AI virus homologous challenges (17, 18). In addition to *in ovo* injection, ocular administration of Ad expressing the H5 gene has been shown to induce specific immune responses and protection against HPAI virus challenge (16, 19). Even though *in ovo* vaccination has been routinely performed in the poultry industry for many years, little is known about virus entry sites in the embryo after the injection. This knowledge is of interest in the case of a non-replicating virus such as the RCA-free adenovirus vector. Thus, we investigated embryo tissues targeted by the AdH5-vector when injected into 18-day embryonated eggs. From an applied perspective, it is also relevant to elucidate whether other vaccines routinely delivered by the same route would interfere with Ad-vector vaccination when applied simultaneously as it has been previously reported for other vaccines [e.g. (2, 3)]. Marek's disease virus (MDV) vaccination is routinely performed *in ovo* in the U.S. poultry industry. Theoretically, MDV and Ad-vectored interference could occur as a result of at least three different mechanisms: (1) different replication kinetics (A fast replicating MDV might induce innate immune responses which would prevent infection or gene expression by a non-replicating virus.); (2) the vaccine viruses might compete for the same target cells [Adenoviruses (including the

chicken CELO) use CAR receptors for infection (15), which are expressed by a wide spectrum of cells including lymphocytes, which are also the target for MDV.]; (3) immunodeficiency caused by MDV [MDV initially infects B-lymphocytes causing cell depletion and/or dysfunction and reduced antibody production (5, 7).]. Even though many subsets of T-cells can be transformed by MDV, the highest proportion comprises CD4+ T-helper cells expressing a TCR2 receptor (11, 12). Thus, MDV might reduce the immune response elicited by the Ad-vaccine.

One aim of this study was to investigate possible target tissues for the human Ad-vaccine virus in the chicken embryos by PCR and immunofluorescence assay (IFA). We also evaluated the effects of concurrent *in ovo* vaccination with the experimental AdH5 recombinant vaccine and commercially available MDV vaccines.

Materials and Methods

Chickens

Specific pathogen free (SPF) white leghorn embryonated eggs (Sunrise Farms, Catskill, NY) were used in all experiments. Chickens were vaccinated *in ovo* on day 18 of embryonation and hatched. Hatched chickens were maintained in battery cages or in Horsfall-type isolators in biosafety level 2 (BSL-2) climate-controlled rooms and provided with water and food *ad libitum*. All experimental procedures and animal care were performed in compliance with all applicable federal and institutional animal use guidelines. Auburn University College of Veterinary Medicine is an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) -accredited institution.

RCA–free recombinant adenovirus vector expressing codon-optimized H5 HA gene

The RCA-free Ad-vectored AI vaccine encoding the AI H5 hemagglutinin ($AdTW68.H5_{ck}$) contains a synthetic AI H5 gene from the A/tk/WI/68 (H5N9) strain with the codons optimized to match the tRNA pool found in chicken cells (16). Titer [infectious units/ml (ifu)] was determined by the Adeno-X rapid titer kit (BD Clontech, Mountain View, CA).

Marek's disease virus vaccines

Available commercial MDV vaccines include the chicken-origin Rispens strain of serotype 1, the naturally non-oncogenic SB-1 of serotype 2, and the turkey herpesvirus (HVT) belonging to serotype 3. We used the commonly used commercially available MDV vaccine combinations Rispens/HVT and HVT/SB-1. These vaccines were used in accordance with the manufacturer's recommendations (kindly provided by Dr. K. Cookson, Pfizer).

Determination of Ad-vectored vaccine target tissues in embryos

We initially determined the site of injection and the location of the inoculum following inoculation of embryonated eggs. Embryonated eggs were inoculated on day 18 of incubation using 300 µl of contrast solution (iodine solution) and immediately scanned by digital radiography and computed tomography. We subsequently investigated the site of Ad-virus entry. Embryonated eggs were vaccinated on day 18 of incubation with 300 µl containing 3.9×10^{10} ifu $AdTW68.H5_{ck}$ per egg. Three days post-vaccination (21 days of incubation) both egg and embryo tissues were harvested individually for the

detection of adenovirus DNA by PCR or H5 expression by immunofluorescence (IFA) as described below.

Adenovirus DNA detection in embryo and egg tissues

The primers selected amplified a 139 base pair (bp) region of the highly conserved hexon gene as described (4) and the amplification reaction was modified by using Platinum PCR SuperMix™ (Invitrogen) with the following conditions: an initial denaturation step at 94 C for 1 min, followed by 40 cycles at 94 C for 30 sec., 55 C for 30 sec, and 72 C for 1 min. The PCR products obtained were separated by agarose gel electrophoresis and visualized by ethidium bromide or green gel staining.

Detection of H5 expression in vaccinated embryo tissues by IFA

H5 expression was determined by IFA using a biotin-avidin complex on tissue sections as previously described (19). In brief, cryo-sections (4-5 μm) were prepared from embryo tissues, fixed in cold acetone, blocked, incubated overnight (4 C) with rabbit anti-H5 IgG (eEnzyme, Montgomery Village, MD), washed, incubated overnight (4 C) with biotin(donkey)-anti rabbit IgG (Jackson ImmunoResearch, West Grove, PA), washed, and incubated for 2 h at room temperature with FITC-conjugated avidin (Southern Biotech, Birmingham, AL). After washing, samples were mounted and evaluated using a fluorescent microscope.

Effects of combined in ovo vaccination with RCA-free Ad AI vaccine and commercially available MDV vaccines.

Experiment 1

AI antibody responses in Ad-vector/MDV combined vaccinated chickens

Six experimental groups were established and inoculated *in ovo* on day 18 of

embryonation as follows: AdTW68.H5$_{ch}$ (n=18), AdTW68.H5$_{ch}$+Rispens/HVT (n=18), AdTW68.H5$_{ch}$+HVT/SB-1 (n=18), Rispens/HVT (n=16), HVT/SB-1 (n=16), and a non-vaccinated control group (n=10). AdTW68.H5$_{ch}$ was used at a dose of 2 X 10^8 ifu/200 µl and MDV vaccines were utilized at the dose recommended by the manufacturer. Individual blood samples and feather follicles were collected on days 12, 20, 32, 40, and 48 after hatch from all birds. Individual sera were inactivated in a water bath at 56 C for 30 min, treated with RDEII (receptor destroying enzyme) (9) and thus pre-diluted 1:4 before testing by hemagglutination inhibition (HI) as described (14) for antibodies against the A/turkey/WI/68 (H5N9) AI virus strain. MDV replication in the chickens was assessed by detection of MDV DNA in feather follicles by PCR. DNA extractions were performed using the Qiagen DNeasy blood and tissue kit (Qiagen, Valencia, CA) following the manufacturer's specifications. Primers used in the detection of MDV DNA extracted from chicken feather follicles were designed to amplify a 483 bp portion of the highly conserved MDV glycoprotein C gene: MDV-gC(F-1) TACATACGTGTGTGYCAACGACC and MDV-gC (R-1) GGCARAGACCTGTAACCACAG and used at a concentration of 20 pmol/µl. A platinum PCR supermix (Invitrogen, Grand Island, NY) was used as directed. The amplification reaction used the following conditions: 94 C for 1 min; 94 C for 30 sec, 61.7 C for 30 sec, 72 C for 1 min for 35 cycles, then 72 C for 10 min. The PCR products were separated by electrophoresis in 2% agarose gels and visualized by SYBR Green 1 Nucleic Acid Gel Stain (Lonza, Rockland, ME) staining.

Experiment 2

Because the AI vaccine coverage (percent chickens with detectable AI antibody titers) in chickens vaccinated with AdH5-MDV combinations was reduced compared to chickens vaccinated with the AdH5 vaccine alone in the first experiment (see Results Experiment 1), in experiment 2 we repeated the trial using only the AdTW68.$H5_{ch}$ + HVT/SB-1 combination with the following modifications: (1) the dose of AdTW68.$H5_{ch}$ vaccine was increased to 3.9 x 10^{11} ifu/300 µl; (2) viral loads of each vaccine virus were determined in the vaccinated birds by quantitative PCR (qPCR) to determine possible interference between the vaccine viruses. Thus, four groups of chickens were used: AdTW68.$H5_{ch}$+HVT/SB-1 (n=18), HVT/SB-1 (n=19), AdTW68.$H5_{ch}$ (n=17), and unvaccinated controls (n=15). The MDV HVT/SB-1 vaccine was used as recommended by the manufacturer. On days 2 and 9 after hatch five chickens from each group were euthanized by CO_2 inhalation and each whole chicken homogenized using a 700 W powered blender and different stainless steel jars for each bird. Samples of the individual chicken homogenates were tested for MDV and adenovirus DNA by qPCR as described below. On days 20, 30, and 40 post-hatch sera were collected from the remaining birds (n= 5-8) and tested for AI HI antibody as described above. Relative concentrations of viral DNA and HI titers detected in the bird groups were analyzed by ANOVA followed by a multiple comparisons posttest.

Adenovirus DNA quantitation in AdH5 + MDV vaccinated chickens

A Taqman qPCR was used for adenovirus DNA quantitation in experiment 2. DNA was extracted from an aliquot (25 mg) of whole chicken homogenates prepared days 2 and 9 after hatch. The primers used for Taqman qPCR were designed to amplify a 150 bp region of the Ad fiber gene (Ad5 fiber-F: 5'- ACG ACT CCA AAC TTA GCA TTGC -

3'; Ad5 fiber-R: 5'- AGC TAC CAG TGG CAG TAG TTA G -3'). Ad5 fiber-P: 5' [HEX]ACCCAAGGACCCCTCACAGTGTCA[BHQ1a~Q]-3' was used as probe. For normalization to copies of the chicken genome, primers and probe detecting the chicken TK gene were included in each reaction

(chick TK-F 5' TTAGTTTTAGTACGGCAGTTGCAC -3';

TK-R 5'- CCAGAGGGACCAGGTTGAGG -3'; TK-P 5'-[Cy5]-AACCTCGCCAAACCCAGCCAGCAG -[BHQ-2a]-3'). Probes and primer stock solutions at a concentration of 10 pmol/µl were added to reaction mixtures. Each 25 µl PCR reaction mixture included 50 ng template DNA extracted from homogenized tissues, 12.5 µl master mix [from QuantiTect multiplex PCR NoROX kit (Qiagen, Valencia, CA)], 1 µl each forward and reverse Ad fiber primers, 0.5 µl fiber probe, 1 µl each forward and reverse TK primers, and 0.5 µl TK probe. qPCR was performed with a Bio-Rad iCycler iQ5 real-time PCR detection system. The program used was 95 C for 15 min, then 95 C for 15 sec, 56 C for 60 sec, for 40 cycles.

MDV DNA quantitation in Ad-vector + MDV vaccinated chickens.

A SYBR Green qPCR was used to evaluate MDV DNA on days 2 and 9 after hatch (Experiment 2). DNA was extracted from an aliquot (~25 mg) of whole chicken homogenate. Primers described by others (6) for specific amplification of a 505 bp region of the ICP4 gene of MDV serotype 3 [(HVT-1 (F) ATGGAAGTAGATGTTGAGTCTTCG and HVT-2 (R) CGATATACACGCATTGCCATACAC)] at a stock concentration of 20 pmol/µl were added to reactions. Reaction mixtures contained 12.5µl RT-SYBR Green qPCR master mix (SABiosciences, Frederick, MD), 9.5 µl ddH_2O, 1µl HVT-1 (F) primer, 1µl HVT-2

(R) primer, and 1µl of template. Approximately 150 ng/µl of sample DNA was used per reaction. Steps and conditions of the qPCR were as follows: 95 C for 10 min, 95 C for 30 sec, 59.2 C for 30 sec, 72 C for 1 min for 40 cycles. MDV genome copy numbers were normalized to the chicken β-actin gene [β-actin anti-sense: ATC GTA CTC CTG CTT GCT GAT; β- actin sense: CAA CAC AGT GCT GTC TGG TGG (1)] and expressed as fold-increase of the unvaccinated control using the cycle threshold (Ct) ΔΔ Ct analysis. The relative copy numbers of MDV genomes (ICP4 genes) were determined using the $2^{-\Delta\Delta Ct}$ method (8).

Results

*Determination of Ad-vectored vaccine target tissues in embryos***.**

The site of injection and the location of the contrast solution are shown in Fig. 1. As shown in the radiograph (Fig. 1A) the needle reaches through the chorioallantoic membranes. Computed tomography (Fig. 1B) indicates that *in ovo* injection on day 18 of incubation places the contrast solution in the amnion and/or allantoic cavities. Theoretically, amnion localization of the vaccine virus allows embryo skin exposure and possible vaccine virus swallowing. On the other hand, vaccine localization in the allantoic fluid would allow bursal exposure through the cloaca.

Ad DNA was detected in different egg and embryo tissues 3 days after vaccination (day 21 of incubation). Ad PCR amplicons were confirmed by sequencing. Ad DNA was consistently detected in the egg chorioallantoic and amniotic membranes and fluids (8/8), as well as in the embryonic bursa of Fabricius (3/4), esophagus (3/4), and thymuses (5/6) (Fig. 2 A, B). The spleens and livers were consistently negative. PCR results on skin

samples were inconsistent (3/8). No Ad DNA was detected in tissues harvested from unvaccinated controls.

H5 expression was detected by IFA in esophagus, bursa of Fabricius, and in the thymus of embryos collected 3 days post-inoculation (Fig. 3). H5 detection attempts by IFA in other tissues of *in ovo* vaccinated embryos were unsuccessful.

Effects of Ad-vector/MDV combined in ovo vaccination.

Experiment 1

Individual chickens in all AdH5 vaccinated groups developed AI HI antibody levels which continued to increase through day 48 after hatch. Birds receiving AdTW68.$H5_{ch}$-only reached mean HI titers of 5 $\log_2$ at 48 days of age. At the same time, birds that developed AI antibodies in the AdH5/MDV combined vaccinated groups reached similar AI mean antibody titers without significant differences (P>0.05) between treatments. However, AdH5-only vaccinated chickens achieved a significantly higher (P<0.05) (chi square test) AI vaccine coverage (percent AI antibody positive animals) on days 32, 40, and 48 compared to chickens receiving the combined vaccines (Fig. 4). For example, on day 48 of age AI vaccine coverage in chickens vaccinated with AdH5-only was 88% while chickens vaccinated with AdH5+HVT/Rispens or AdH5+SB-1/HVT reached 39% and 56% respectively. Neither MDV only nor unvaccinated controls developed AI antibodies (not shown).

MDV DNA was detected in the feather follicles of all MDV vaccinated chickens throughout the experimental period irrespective of vaccine combination (Fig. 5 B, C). MDV DNA was neither detected in control nor in AdH5-only inoculated chickens (Fig. 5 A).

Experiment 2

As in the previous experiment, all groups vaccinated *in ovo* with AdTW68.H5$_{ch}$ showed detectable AI antibody titers without significant differences (P>0.05) between AdH5-only and AdH5/MDV combined vaccinated chickens (Fig. 6A). Unlike experiment 1 however, the percent of HI antibody positive birds did not differ significantly (P>0.05) between treatments with vaccine coverage varying between 66% and 75% on day 40 of age (Fig. 6B) in AdH5-only and AdH5/MDV vaccinated chickens.

The adenovirus DNA concentration determined by qPCR showed no significant differences (P>0.05) on day 2 post-hatch between AdH5-only and combined vaccinated chickens (Fig. 7). Unvaccinated controls were negative for Ad DNA. On day 9 after hatch adenovirus DNA was not detectable in either group suggesting that the non-replicating Ad-vaccine had been cleared from the chickens.

As seen in Fig. 8, on day 2 post-hatch the MDV DNA concentration was significantly higher (P<0.05) in MDV-only compared to dually vaccinated chickens. On day 9 after hatch the concentrations of MDV DNA were not significantly different between vaccinated groups (Fig. 8).

Discussion

Ad-vectored AI vaccination has been shown to provide protective immunity in chickens when delivered by the *in ovo* and mucosal routes (16-18). *In ovo* vaccinated birds develop specific AI antibodies and specific T cell responses (13), which explains the observation that even vaccinated birds without detectable antibodies are protected against highly pathogenic AI challenge (10). The results of egg imaging demonstrated that the vaccine is injected into the amnion/allantoic cavities. This result was

corroborated by adenovirus DNA detection in the amnion-allantoic fluids of all *in ovo* vaccinated eggs. In the current study, the bursa and esophagus samples of vaccinated embryos were consistently positive for Ad-5 DNA. The expressed AI hemagglutinin was also detected both in the bursa and in esophagus samples by IFA. These results suggest that the vaccine contained in the amniotic fluid may be swallowed by the embryo. In addition, the bursa (connected to the cloaca via the proctodeum) might be exposed to vaccine virus contained in the allantoic fluid. The fact that Ad DNA was detected in the thymus requires further investigation. The finding of Ad DNA-positive skin samples could be the result of either vaccine virus infecting the epithelial cells of the skin or detection of Ad contamination at sampling.

Ad vaccination by the *in ovo* route would impose co-administration with MDV vaccines due to cost and efficiency reasons. Based on the biology of the viruses involved it is possible that vaccination interference might occur between a replication deficient Ad virus and the highly efficient replication competent MDV. Our results showed that when the AdH5 vaccine was used in combination with MDV (Rispens/HVT or HVT/SB1) chickens responding to the AdH5 vaccine had similar AI antibody levels compared to singly (AdH5-only) vaccinated birds. However, combined vaccinated groups showed reduced vaccine coverage to AI which suggests some level of interference. We believe that this level of interference cannot be attributed to MDV causing immunodeficiency because in this theoretical scenario, we would expect detecting similar vaccine coverage but homogeneously reduced antibody levels. It was interesting that the combination of Ad with HVT and the more aggressive MDV serotype 1 Rispens strain affected more severely the vaccine coverage to AI than the combination with HVT and the avirulent

serotype 2 SB1 strain. This result suggests that the replication rate of the more aggressive Rispens strain of serotype 1 may have out-competed the Ad-vectored vaccine. This assumption is supported by the results of experiment 2 which showed that increasing the Ad concentration produced similar AI antibody titers and AI vaccine coverage when applied alone or in combination with the HVT/SB1 vaccine. From a speculative perspective it is possible that combined vaccination with MDV Rispens would require an even higher dose of the AdH5 vaccine or reducing the dose of the Rispens strain to allow effective immunization to AI.

MDV DNA was detected in feather follicles of all vaccinated birds at 12 days of age in experiment 1. Thus, Ad-vector vaccination at the dose administered in experiment 1 apparently does not interfere with the efficacy of MDV vaccination using any of the commonly used vaccine strains. In experiment 2 when we increased the concentration of the Ad-vector vaccine, a reduced MDV DNA concentration was detected in the total chicken homogenate of dually vaccinated chickens at 2 days of age. In contrast, we detected similar adenovirus DNA concentrations in combined and singly vaccinated chickens on the same day. The difference in MDV DNA levels had disappeared by day 9 of age, at a time when Ad DNA was no longer detectable. Thus, co-vaccination by the *in ovo* route with AdH5 and MDV vaccines seems to involve some level of interference, but this interference is temporary and a higher titer of AdH5 appears to compensate for the non-replicating nature of the AdH5 vaccine in its ability to compete with the MDV vaccines.

The fact that the recombinant Ad vaccine was no longer detectable by day 9 after hatch in the chickens, i.e. more than 30 days before harvest, is of distinct importance because it represents an additional safety feature of this technology.

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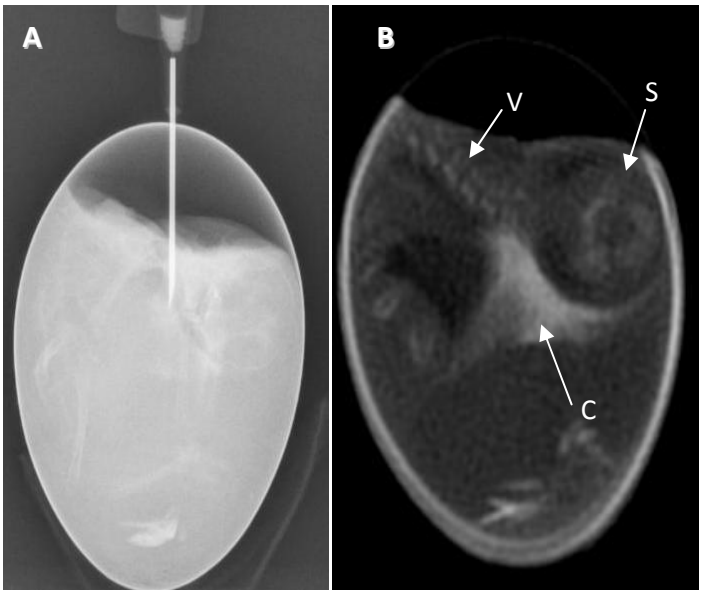

Fig. 1. The site of injection and the location of the inoculum when delivered by the *in ovo* route was evaluated by imaging techniques. A. Radiograph shows the needle reaching through the chorioallantoic membranes. B. Computed tomography indicates that *in ovo* injection on day 18 of incubation places the contrast solution in the amnion and/or allantoic cavities. V: embryo vertebra; S: embryo skull; C: contrast solution.

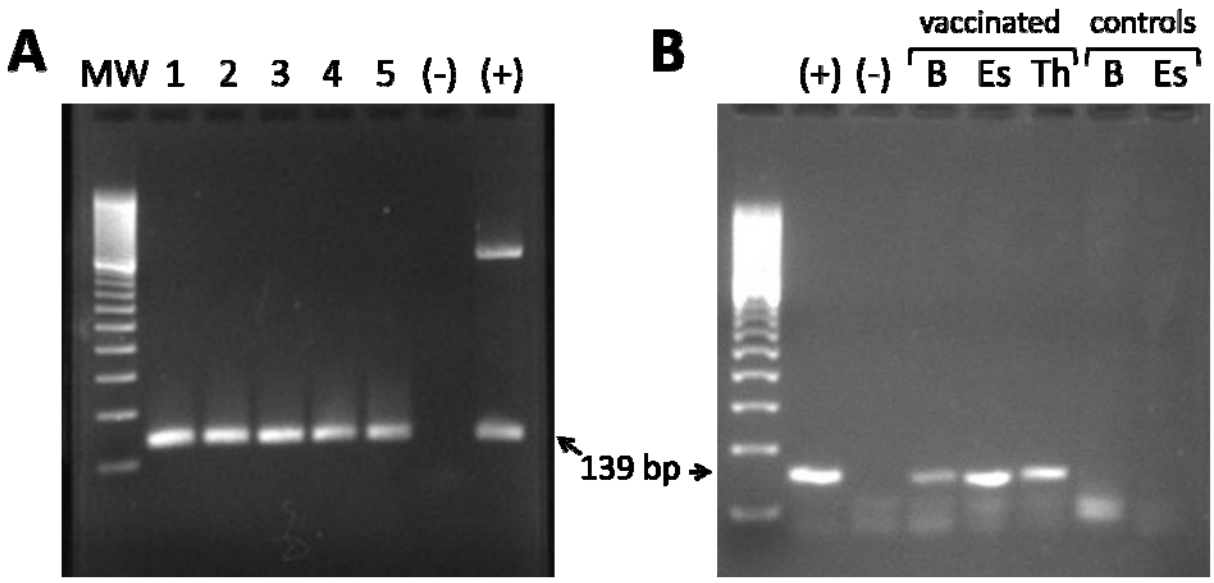

Fig 2. Embryonated eggs were injected with recombinant adenovirus (Ad) on day 18 of embryonation. Ad5 DNA (139 bp amplicons) detected in tissues 3 days after vaccination. (**A)** Ad5 DNA in chorioallantoic membrane of multiple eggs (lanes 1-5). (+): Positive control, (-): Negative control. (**B)** Ad DNA detected in b: bursa, Es: esophagus, and Th: thymus of vaccinated embryos. Ad DNA was consistently detected in chorioallantoic membranes, and in embryonic bursa of Fabricius, esophagus, and thymuses. Ad positive PCR amplicons were confirmed by sequencing. Spleen and liver were consistently negative (not shown). PCR results on skin samples were inconsistent (not shown). No Ad DNA was detected in tissues harvested from unvaccinated controls.

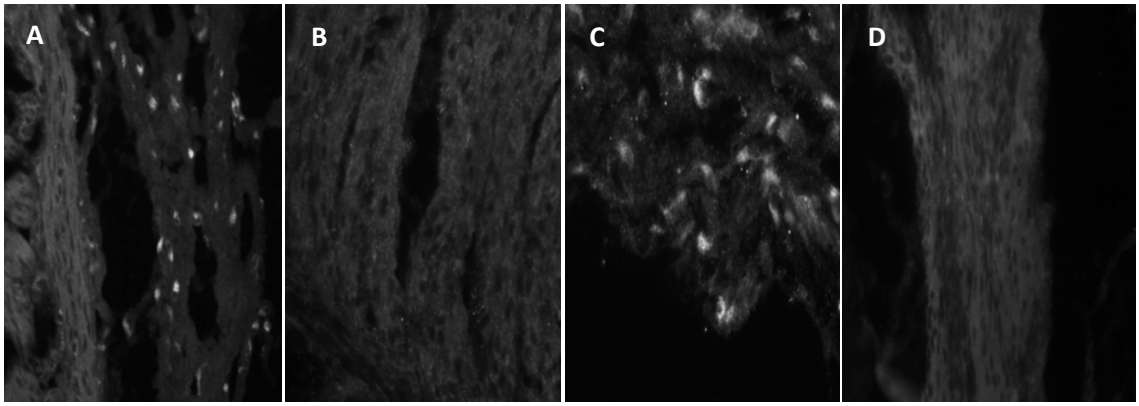

Fig. 3. Hemagglutinin (H5) detection by immunofluorescence assay (IFA) in embryo tissues collected 3 days after *in ovo* inoculation (400X). (**A)** Positively stained cells by IFA in the esophagus of vaccinated embryos. (**B)** Esophagus of unvaccinated controls. (**C)** Positively stained cells by IFA in the bursa of Fabricius in vaccinated embryos. **(D)** Bursa of unvaccinated controls.

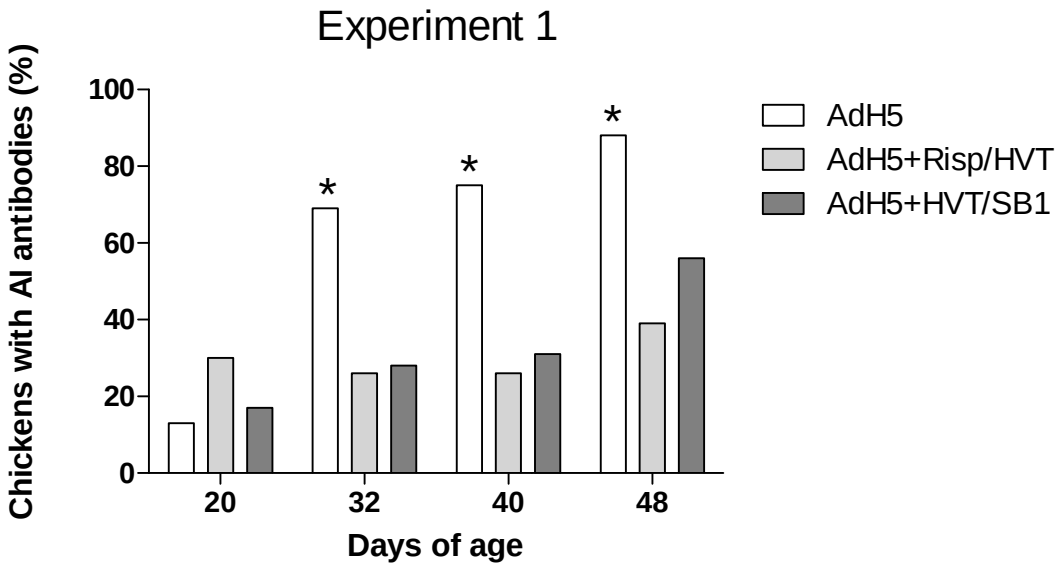

Fig. 4. Avian influenza (AI) vaccine coverage (percent chickens with detectable AI antibodies) after *in ovo* vaccination with AdH5 alone or in combination with Marek's disease virus (MDV) vaccines Rispens/HVT or HVT/SB1. AdH5-only vaccinated chickens achieved a significantly higher ($P<0.05$) AI vaccine coverage on days 32, 40, and 48 compared to chickens receiving AdH5 in combination with MDV vaccines. Birds that developed AI antibodies achieved similar mean HI titers to AI (~5 $\log_2$ at 48 days of age) without significant differences ($P>0.05$) between birds subjected to AdH5-only or AdH5/MDV combined vaccination (not shown).

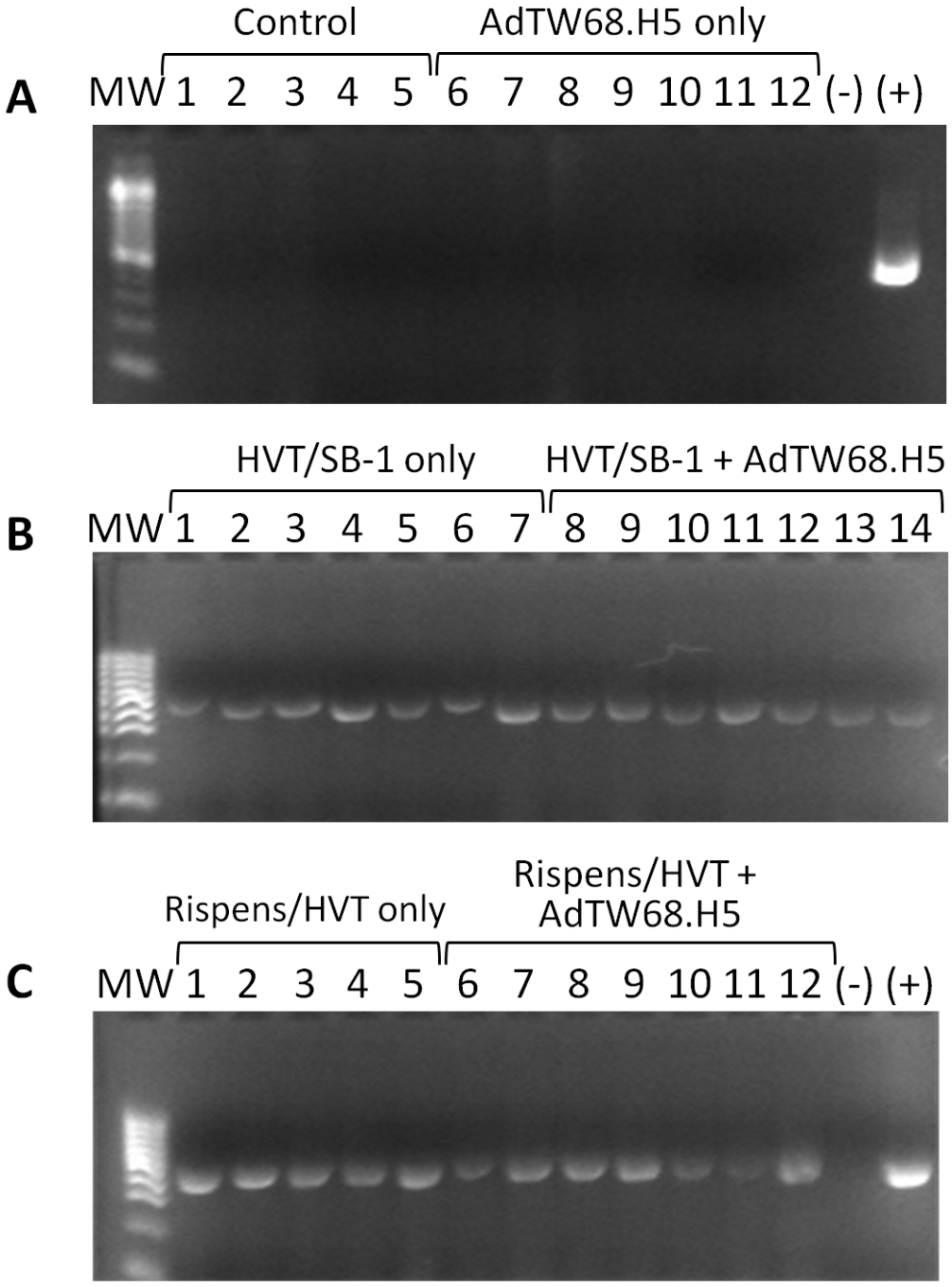

Fig. 5. Marek's disease virus (MDV) DNA detection by PCR in feather follicles of AdH5-only or AdH5/MDV vaccinated chickens. (A) Control and AdH5 alone. MDV DNA was detected neither in control nor in AdH5 vaccinated birds. (B) HVT/SB-1 alone and AdH5+HVT/SB-1 and (C) Rispens/HVT alone and AdH5+Rispens/HVT. MDV DNA was detected in all MDV vaccinated birds.

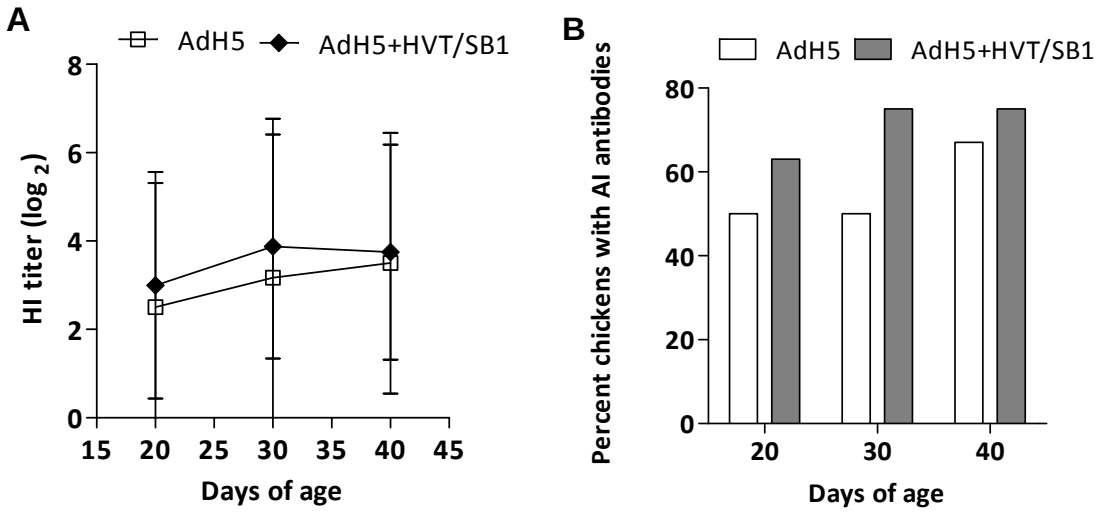

Fig. 6. Chickens were vaccinated with AdTW68.H5$_{ch}$+HVT/SB-1 (n=18) or AdTW68.H5$_{ch}$–only (n=17). The dose of AdTW68.H5$_{ch}$ vaccine was increased compared to Experiment 1. (A) Avian influenza (AI) H5 hemagglutination inhibition (HI) antibody titers detected in vaccinated chickens. No significant differences (P>0.05) in antibody titers were detected between singly or combined vaccinated groups. (B) AI vaccine coverage (percent chickens with detectable HI antibody titers) in vaccinated chickens. No significant differences (P>0.05) detected between vaccinated groups. HVT/SB-1-only (n=19) and unvaccinated controls (n=15) were negative for AI antibodies (not shown).

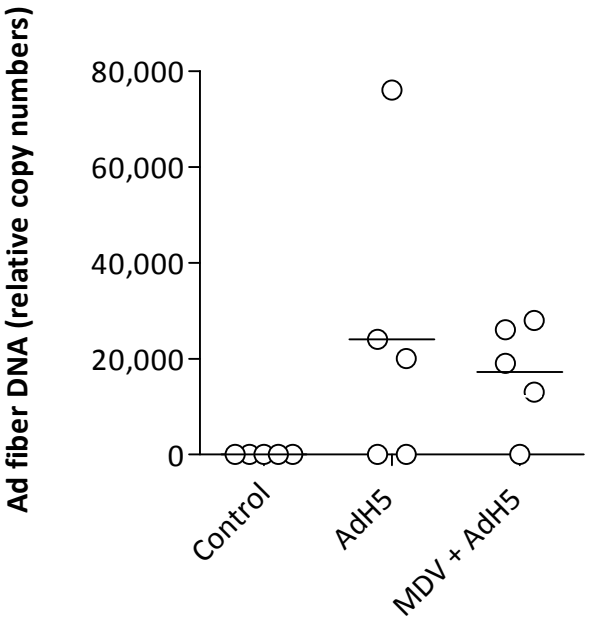

Fig. 7. Adenovirus (Ad) DNA concentration determined by qPCR. No significant differences (P>0.05) on day 2 post-hatch between AdH5-only and AdH5+MDV vaccinated chickens. Unvaccinated controls were negative for Ad DNA. On day 9 after hatch Ad DNA was not detectable in either group (not shown).

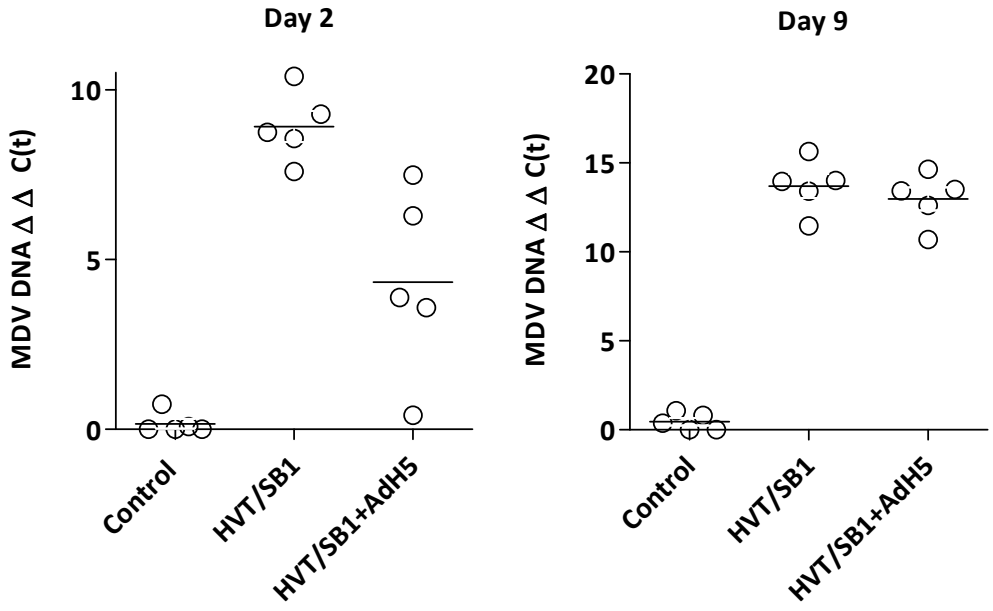

Fig. 8. Marek's disease virus (MDV) DNA concentration determined by qPCR on days 2 and 9 post-hatch. The MDV DNA concentration was significantly higher ($P<0.05$) in MDV-only compared to dually vaccinated chickens on day 2 of age. On day 9 after hatch the concentrations of MDV DNA did not vary significantly between AdH5-only and AdH5+MDV vaccinated groups. Unvaccinated controls were negative for MDV DNA.