In Ovo-Vaccination with a Non-Replicating Adenovirus-Vectored Avian Influenza: Maternal Immunity and Effects on Vaccination

by

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Abstract

Protective immunity to avian influenza (AI) virus can be elicited in chickens by *in ovo* or intramuscular (IM) vaccination with RCA-free adenovirus vector encoding AI virus H5 (AdH5). We evaluated persistence of specific antibody after *in ovo* vaccination, transfer of antibody to progeny chickens, and possible interference of maternal antibody with *in ovo* and mucosal immunization. One flock of breeder birds was vaccinated *in ovo*-only, another flock was vaccinated *in ovo* and boosted IM on week 16 of age, and a third group was the unvaccinated control. AdH5 *in ovo* vaccination elicited anti-H5 hemagglutination inhibition (HI) antibodies in breeder hens for up to 34 weeks of age. Intramuscular revaccination of *in ovo* vaccinated hens increased HI antibodies significantly. Breeder hens vaccinated *in ovo* with AdH5 effectively transferred anti-H5 maternal antibodies to progeny chickens. Maternally derived anti-H5 antibodies (MDA) from breeders vaccinated *in ovo* and boosted with AdH5 interfered with active immunization in progeny chickens.

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Introduction I.

Highly pathogenic (HP) avian influenza (AI) virus (HPAIV) threatens the world poultry industry and constitutes a risk for the humans. Vaccination of poultry has been recommended as a cost effective tool to control HPAIV by the World Health Organization (40). A replication competent adenovirus (RCA)-free adenovirus vector encoding the AI virus H5 (AdTW68.$H5_{ch}$) has been shown to elicit antibody responses and protection against HP AI challenge (42). Resistance against AI virus infection is associated with the presence of anti-HA specific antibodies (10). Initial studies on antibody persistence after *in ovo* immunization with AdTW68.H5ch demonstrated that anti-H5 antibody titers lasted at least until 52 days of age (43). Thus, in the current study we expanded the period for antibody monitoring of chickens vaccinated via the *in ovo* route to 34 weeks.

Maternally derived antibodies (MDA) represent an integral part in disease prevention in young chickens. To increase antibody levels in breeders prior to lay onset, we evaluated booster vaccination in breeder hens that were vaccinated *in ovo*. In the current study, we evaluated transfer of maternal antibodies from breeder hens, which were either singly immunized *in ovo* with AdTW68.$H5_{ch}$ or received an intramuscular booster vaccination at 16 weeks of age. However, MDA have been shown to interfere with active vaccination (4). Thus, we evaluated active *in ovo* immunization in progeny chickens with maternal immunity originating from AdTW68.$H5_{ch}$ vaccinated breeders.

Literature Review II.

Avian Influenza

Avian influenza (AI) virus (AIV) is a member of the *Orthomyxoviridae* family. The AIV genome is characterized by a single stranded RNA comprised of 8 gene segments. AIV is further subdivided into subtypes based on antigenic differences of the surface glycoproteins; hemagglutinin (HA) and neuraminidase (NA) of which there are 16 HA and 9 NA currently recognized. Only viruses of the influenza virus A genus are known to infect birds. All influenza A viruses have been isolated from avian species. Currently, only viruses belonging to the H5 and H7 subtypes have been shown to cause highly pathogenic (HP) outbreaks of disease, however not all H5 and H7 viruses are HP (26). Because of such variability in viral virulence the World Health Organization (OIE) has adopted the term notifiable avian influenza (NAI) to include both HPAI and low pathogenic (LP) AI when caused by H5 and H7 strains. HPNAI have an intravenous pathogenicity index in 6 week-old chickens > 1.2 or cause at least 75 % mortality in 4-8 week-old chickens inoculated intravenously. LPNAI include all influenza A viruses belonging to the H5 and H7 subtype that are not HPNAI viruses (40). HPAI can cause mortality in poultry approaching 100 percent. H5 and H7 HPAI viruses have been shown to arise from low pathogenic avian influenza of the same subtypes by antigenic drift and shift(26). Of the several changes that occur in the viral genome, which mediates viral virulence, most notable are the basic amino acid residues in the region connecting HA_1

and HA_2 in the HA_0 precursor. In LPAI viruses, cleavage of this precursor protein is performed by extracellular, trypsin-like proteases found primarily in the respiratory and gastrointestinal tract. HPAI viral strains possess multi-basic amino acid residues that allow cleavage by multiple intracellular proteases, such as furin, found in all organs resulting in viral dissemination and dead of the host (26).

Vaccination

By increasing the resistance of poultry populations to AIV the risk for human infections is reduced. Control of avian influenza involves a set of strategies including education, diagnostics and surveillance, elimination of infected poultry and decrease host susceptibility. The latter can be achieved by increasing resistance through active vaccination or passive immunity against the AI viral hemagglutinin or neuraminidase proteins (38). Commercially available AI vaccines include inactivated whole influenza viruses and recombinant vaccines. Numerous other experimental vaccines have been developed including DNA vaccines and recombinant protein vaccines. International organizations such as the OIE do not recommend the use of attenuated AI vaccines in poultry, especially strains belonging to the H5 and H7 subtypes. Adaptation to chickens of such influenza subtypes and/or reassortment with field influenza virus may potentially generate HPAI mutants which could be positively selected. In addition, differentiation between infected and vaccinated animals (DIVA) becomes unfeasible (44). AI viruses isolated from outbreaks in poultry or from surveillance in wild or domesticated birds constitute the primary viral strains employed for the development of inactivated AI vaccines. These viruses are grown in 9-11-day-old embryonated chicken eggs, the

infected allantoic fluid harvested, the virus inactivated by the use of chemicals (formalin and β-propiolactone), and the crude preparation or purified hemagglutinin emulsified in oils and surfactants (36). AI vaccines using the oil adjuvant system have the advantage of producing high antibody titers in poultry and effective protection against homologous field strains over long periods of time (37). However, AI inactivated vaccine use is limited because of high labor cost due to the requirement of parenteral administration of the vaccines and difficulty in identifying infected birds by routine serological tests in the vaccinated population i.e. differentiating infected from vaccinated animals (DIVA). In addition, the protective responses are dependent on antigen quantity in each dose and adjuvant system used (36).

In chickens, there are currently two types of AI H5 vectored vaccines licensed in several countries. One type is based on the fowlpox virus (FPV) vector, and the other is based on a Newcastle disease virus (NDV) vector. These vectors induce humoral, cellular, and mucosal immune responses. NDV vectors do not express the NP and M proteins. Thus commercial anti-NP-based ELISA tests or agar gel precipitation (AGP) can be used as DIVA test to detect infection. Vectors of avian origin also induce homologous immunity and constitute therefore bivalent vaccines (44). The recombinant FPV vector has some limitations including that it can only be used in chickens without preimmunity to the vector and that it requires parenteral administration.

Replication competent adenovirus (RCA)-free Ad recombinant vaccines

RCA-free Ad5 has been used experimentally as a vaccine vector in several animal studies. Researchers were able to demonstrate that a recombinant replication-defective adenovirus type 5 expressing the feline immunodeficiency virus (FIV) ENV gene was able to effectively transduce feline cells and elicit an antibody response (13). However, most studies on the use of replication-defective Ad5 as transgene carriers have focused on its use in mammalian species, while few have focused on its potential use for the development of recombinant vaccines in poultry. Eloit et.al (7) were able to demonstrate that chicken embryo fibroblasts (CEF) were effectively transduced by the a replication defective Ad5. This showed that CEF have receptor(s) for binding and penetration of the Ad5. In the same study, researchers showed that a single intramuscular vaccination with a replication defective-defective Ad5 vectoring the gD gene of pseudorabies virus induced an effective and specific vaccine response with a robust antibody titer in adult and 1 day old chickens (7).

Toro et al. (43) developed a replication competent adenovirus (RCA)-free human adenovirus encoding either the AI H7 (AdCHNY94.H7) or the H5 hemagglutinins (Ad H5). Chickens vaccinated *in ovo* with the RCA-free Ad encoding the AI H5 and boosted intramuscularly with the AdCHNY94.H7 developed high antibody titers against both the H5 and H7 AI glycoproteins. Subsequent studies with the same vaccine constructs showed that immunization either *in ovo,* via parenteral routes, and

via mucosal routes effectively protects chickens against homologous challenge (42,43). This type of vaccine has the following advantages: reduction of labor vaccination costs, less stress for the birds, precise and uniform dosage of vaccine delivery to each egg, and earlier protective immunity than post hatch vaccination (4). In contrast to other vectored vaccines like the FPV-H5 in which preexisting immunity to the virus hinders an effective immune response, successive immunizations with the replication free Ad virus induces a strong humoral response and a booster effect. The latter has been demonstrated by Toro et al. (43) in which chickens vaccinated *in ovo* with an Ad vector encoding an AI H5 (AdH5) and subsequently vaccinated IM with AdCHNY98.H7 responded against both glycoproteins with robust antibody titers. In another study an adenovirus based H5N1 highly pathogenic avian influenza (HPAI) was efficacious in eliciting high levels of anti-H5 antibodies, when administered subcutaneously (SQ). A dose as low as 10^7 viral particles (vp) of the adenovirus-based H5N1 vaccine per chicken was sufficient to generate a strong humoral immune response (35).

Immune responses elicited by RCA-free AdH5 avian influenza vaccine

Ocular delivery of AdH5 and subsequent H5 transgene expression in the Harderian gland induces specific IgA and IgG secreting cells (45). Intramuscular immunization with AdH5 has been shown to induce MHC-I restricted AIV specific $CD8^+$ T lymphocyte responses. Effector memory cells were elicited between 3 to 8 weeks post immunization (34). In addition Ad and H5 specific antibodies were induced in the serum. Intranasal immunization with replication defective (Ad-AI) in mice promoted a more potent $CD8^+$ T cell response when compared to other routes of immunization (e.g. IM), and resulted in better protection against viral challenge. This superior protection was due to influx of antigen-specific cytotoxic lymphocytes, which caused a destruction of infected cells and resulted in a better clearance of the virus. In addition mucosal IgA and IgG specific antibody responses were significantly higher when compared to parenteral routes of administration (9). Recent reports indicated that humoral and cellular immune responses induced by adenoviral vector-based vaccines can be enhanced in chickens by CD154 glycoprotein (28). In this study, fusion of the H5 avian influenza viral hemagglutinin with CD154 expressed in adenovirus vector resulted in significantly better HI antibody titers at different viral doses. The effects were attributed to a better exposure of the H5 glycoprotein to professional antigen presenting cells (APCs) via CD40 which would result in increased activation, resulting in an enhanced HA-specific T and B lymphocyte response. In addition, it caused a substantial

production of interferon γ, which have been implicated in protection (28) .

Vaccination *in ovo*

Sharma et al. (33) showed that chicken embryos could be successfully vaccinated against Marek's disease virus (MDV) at 17–18 days of incubation. *In ovo* vaccination in the poultry industry is routinely performed at 18 days of embryonation when eggs are transferred from the incubators to the hatchers. Vaccination *in ovo* is currently performed with automated in ovo injectors, which allow mass application of vaccines to large numbers of eggs (30,000-50,000 eggs per hour) (11). Using this method of mass immunization almost all broilers in the USA (approximately 7 billion per year) are vaccinated against Marek's disease virus. *In ovo* vaccination has several advantages over traditional vaccination methods including a marked reduction of labor vaccination costs, less stress for the birds, precise and uniform dosage of vaccine delivery to each egg, and earlier protective immunity than post hatch vaccination (4).

Passive immunity and interference of maternal immunity

The development of the adaptive immune system in chickens begins when embryonic stem cells migrate from the yolk sac at 5 to 7 days of embryonation to seed the embryo's bursa and thymus. At 16-18 days of incubation the chicken embryo can develop antibodies to specific antigens. The first immunoglobulin isotype to be expressed on the surface is IgM at around 14 days of embryonation (8) while IgY surface expression appear by the time of hatching (21 days) and IgA secreting cells appear on mucosal surfaces up to one week post hatch (8) . Even though embryos can develop immune responses to different antigens, which constitute the basis of *in ovo* vaccination, the response is sub-optimal in comparison to a fully matured immune system, with peak immunological maturity occurring several weeks after hatching (4). Passive immunologic protection through the transfer of maternal immunoglobulins (Ig) during the first stages of neonatal development is required for commonly encountered pathogens. This becomes especially important in natural and industry settings. In mammals the uptake of IgG has been shown to involve a MHC I like receptor (FcRn) (20). The delivery of IgG varies across mammalian species and it can involve the transfer of immunoglobulins through the placenta prenatally or through the ingestion of immunoglobulin's rich colostrum after birth (20). In birds this process involves the uptake of primarily IgY, the mammalian equivalent of IgG, from the blood and into the egg yolk, while IgA and IgM are incorporated into the albumin from

oviduct secretions and diffused into the amniotic fluid where they are ingested by the chick. At the time of hatching the chick is protected locally by IgA coating of the intestinal mucosa and systemically by circulating IgY (31). The first step in this multistep process involves the transport of maternal IgY from the hen plasma across the oolemma into the maturing oocyte in the ovarian follicle and finally uptake of IgY across the yolk sac and into the fetal circulation. The concentration of IgY in the yolk is similar albeit less than the concentration in the hen's serum. The uptake of IgY from the yolk into neonatal circulation is maximal at about 18 days when it reaches transport as high as 600 µg/day (3,21). An 8-day old embryo only possesses a single receptor class with a K_D 3.4 $X10^{-7}$ M, while an 18-day old embryo exhibits an additional receptor with a lower affinity. In addition to possessing an additional high affinity receptor the 18-day old embryo also contains an increase of low affinity receptors when compared to the 8-day old embryo. This relative increase in low affinity receptors along with the appearance of the high affinity receptors may in part explain the high uptake of IgY observed during the last three days of embryonation (31). The FcRY receptor responsible for the uptake of IgY has been recently isolated and characterized and shown to be a homolog of the mammalian phospholipase A_2 receptor (PLA_2R), a member of the mannose receptor family (47). Similar to the mammalian FcRn receptor it exhibits pH depending binding of IgY in the acid environment of endosomes and ligand release at a slightly basic pH such as blood (39). Several aspects have been shown to influence the receptor-mediated transfer of immunoglobulins from the hen serum into the egg yolk: Immunoglobulin form (monomeric or pentameric), immunoglobulin class, Fc fragment and C3H and C4H domains(23). Chickens IgY is transported into the egg yolk up to 8 times faster than

human IgA, and chicken polymeric IgM and dimeric forms of IgA are either totally absent or poorly incorporated into the egg yolk, respectively. In addition, the receptor mediated transport of Ig requires an intact Fc fragment as well as C3H and C4H domains, as it has been shown that truncated forms of IgY such as ducks are poorly incorporated into the egg yolk (23).

Persistence of maternal derived antibodies (MDA) in chickens depends on the age of the hen as well as the laying cycle and antibody titers in the mothers serum, the type of test employed for antibody detection and the growth rate of the chicken (41). MDA decline to marginal levels by 2 to 3 weeks of age (41). Although most studies on transfer of MDA have been performed in domestic poultry, many others avian species have been shown to protect their progeny through antigen-specific neutralizing antibodies such as flamingos and eastern screeched owls (1,15). In addition, maternal antibodies has been shown to decline between 3 to 4 weeks in other species such as sparrows in a fashion similar to that in chickens (22).

Because of the importance passive immunity has in protecting chickens in a field situation, vaccination of breeder hens is commonly practiced in the poultry industry (2,5,25). Certain pathogens such as infectious bursal disease virus and chicken anemia virus are highly stable in the environment and can persist in poultry houses despite cleaning and sanitation, thus vaccination of parent birds is routinely performed as a method of disease prevention (25,44).

Transfer of maternal antibodies confers protection to chickens against viral, bacterial and protozoal infections (4). The following examples illustrate the importance passive antibodies have in affording protection to neonates: broilers chicks after receiving an intramuscular injection with egg-yolk IgY from breeder hens hyper immunized with avian pathogenic *E. coli* (APEC) specific antigens were protected against development of systemic and respiratory disease after homologous challenge (90-100%) (19). Immunosuppressed broilers (treated with cyclophosphamide) are protected against *Ornithobacterium rhinotracheale* infection after passive transfer of sera from immunocompetent birds previously challenged against the bacterium, underscoring the importance of specific humoral response in conferring protection against this economically important disease (32). Breeder hens immunized against *Salmonella gallinarum* porins effectively transferred antibodies to the progeny with 50 to 70% surviving a lethal challenge with a virulent strain of the bacterium (12) while progeny from broilers breeders exposed to *Salmonella* Enteritidis bacterin had reduced bacterial shedding and fewer positive samples when compared to progeny from unvaccinated controls (18). Layer hens exposed to West Nile virus transferred antibodies that protected chickens against viremia (24). Pullets vaccinated intramuscularly with a live Mukteswar strain of Newcastle protected their offspring against a homologous lethal challenge with mortalities ranging from 18 to 90 % at 1 day and 25 days of age, respectively. As expected a higher mortality rate corresponded to a decline in maternal antibodies (29). An early study done to elucidate the role of IgY antibodies induced by maternal vaccination of laying hens with live protozoa (*Eimeria*

sp.) was able to deduced that transfer of pathogen specific IgY in yolk afforded 100 % protection in the offspring after homologous challenge (30).

Despite high titers of MDA, full protection of the offspring is not always possible since protection of antibodies is often subtype specific and subtle antigenic differences among pathogenic viruses and bacteria often limits its protective role. In addition, the presence of strong maternal immunity may protect the offspring against clinical disease but not against infection, and in some cases such as infectious bursal disease moderate to severe damage to the bursa can still be elicited (17).

Hemodilution affects maternal antibody titers which decline differently among chicken breeds. For example, due to the difference of total blood volumes between light breeds such as leghorns and heavy breeds such as broilers, maternal antibodies decline faster in broilers than in layer chicks, due to a faster rate of development and body mass (3).

Passive immunity in chicks can also be affected by components in the parent's diet, as it has been shown that the ratio of linoleic acid to α-linolenic acid can reduced the total IgY concentration in the chick serum (46). Adequate nutrition is important for the development of a healthy immune system in the embryo, and the seeding of lymphoid organs during the post-hatching period. Antigenic exposure of the neonate results in partition of nutrients normally used for growth and maintenance of basal metabolism instead of the development of an immune response, inflammation and restitution of normalcy (17). Maternal antibodies ameliorate the growth reducing effects of immune challenge after vaccination by suppressing the innate response associated with antigenic stimulation. In a recent experiment Japanese quail (*Coturnix japonica*)

were immunized with specific antigens (LPS, killed avian reovirus, or PBS), and chicks from each group of breeder hens were challenged with either the same antigens their mothers were exposed to or a novel one. At the end of the experimental period chicks exposed to the same antigens as their mothers obtain antigen specific passive antibodies which spare them from the growth retardation effects of immunization while those chicks exposed to a novel antigen (from which they lacked maternal antibodies) had reduced growth rates (14).

Despite the role maternal antibodies in protection to progeny chickens during the first few days of life, MDA can interfere with the ability of the offspring to develop active immune responses. The basic mechanism behind this interference seems to involve neutralization of pathogens (natural challenge or live vaccines) before they can replicate (4). Another possibility is direct competition of the antigen with cellular receptors that would cause blockade of priming, induction and maintenance of suppressor T cells, which function as inhibitors of memory T helper cells involved in IgY production (16). IgG specific antibodies are thought to inhibit antigen specific immune responses, possibly by attaching to antigen specific epitopes effectively shielding them from recognition by plasma cells thus eliminating the stimulus required for their proliferation . Once MDA decline after 3 or 4 weeks of age chickens become susceptible to the pathogens to which they were passively protected against (4). In a recent study chickens obtained from breeders immunized with inactivated vaccines against H5N2 AI showed that MDA^+ chickens had reduced antibody titers upon vaccination and higher rates of virus excretion after viral challenge, when compared to control chicks (MDA). In addition, it was shown that interference could be overcome and clinical protection could

still be elicited when vaccination was performed at 10 days regardless of the presence of MDA (5), thus underscoring the importance of timing of vaccination and MDA levels to determine when to best immunize chicks from MDA positive flocks. In another study commercial broiler chickens with MDA to Newcastle disease virus failed to respond to a DNA vaccine encoding the fusion and HN (Hemagglutinin-Neuraminidase) genes of NDV. Challenge with a pathogenic Newcastle strain resulted in no protection against clinical signs and mortality. The authors concluded that MDA neutralized the immunogenic effects of the administered DNA vaccine (27). Chickens obtained from layers, which were immunized against dinitrophenyl-keyhole limpet hemocyanin (DNP-KLH), and had received high levels of anti-keyhole limpet hemocyanin maternal antibodies [measured via enzyme-linked immunosorbent assay (ELISA)] showed that after active immunization antigen-specific immune responses were suppressed. In contrast progeny chickens from control breeder hens (lacking anti-DNP-KLH specific antibodies) immunized with DNP-KLH and progeny chickens obtained from breeder hens immunized with rabbit serum albumin were able to develop an active vaccinal response against DNP-KLH. In the same experiment immunization of maternally derived DNP-KLH positive chickens were able to overcome the immunosuppressive effects of maternal antibodies when given an optimal dose of the DNP-KLH antigen, which was able to break through maternal immunity (6). These results bring forth two important concepts: first the protective and immunosuppressive role of maternal antibodies is antigen specific, and second the immunosuppressive effects of maternal antibodies can be overcome if a sufficient concentration of antigen is administered.

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Research article III.

Avian Influenza *In Ovo* Vaccination with Replication Defective Recombinant Adenovirus in Chickens: Vaccine Potency, Antibody Persistence, and Maternal Antibody Transfer

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Abbreviations. Ad=adenovirus serotype 5; AI=avian influenza; BSL=biosafety level; HA=hemagglutinin; HI= hemagglutination inhibition; HP=highly pathogenic; ifu=infectious units; IM= intramuscularly; MtAb =maternal antibody; PD_{50} =protective dose 50%; qRT-PCR=quantitative reverse transcriptase- polymerase chain reaction; RCA=replication competent adenovirus; RDEII=receptor destroying enzyme; SEPRL=Southeast Poultry Research Laboratory; SPF= Specific pathogen free; VACC =vaccine accordance or intra-potency test repeatability.

Summary

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 the AI virus (AIV) hemagglutinin. We evaluated vaccine potency, antibody persistence, transfer of maternal antibodies (MDA), and interference between MDA and active *in ovo* or mucosal immunization with RCA-free recombinant Ad expressing a codon-optimized AIV H5 hemagglutinin gene from A/turkey/WI/68 (AdTW68.$H5_{ck}$). Vaccine coverage and intra-potency test repeatability were based on anti-H5 HI antibody levels detected in *in ovo* vaccinated chickens. Even though egg inoculation of each replicate was performed by individuals with varying expertise and with different vaccine batches, the average vaccine coverage of 3 replicates was 85%. The intra-potency test repeatability, which considers both positive as well as negative values, varied between 0.69 and 0.71 indicating effective vaccination. Highly pathogenic (HP) AIV challenge of chicken groups vaccinated with increasing vaccine doses showed ~90% protection in chickens receiving $\geq 10^8$ifu/bird. The protective dose 50% (PD_{50}) was determined to be $10^{6.5}$ ifu. Even vaccinated chickens which did not develop detectable antibody levels were effectively protected against HPAI virus challenge. This result is consistent with previous findings of Ad-vector eliciting T lymphocyte responses. Higher vaccine doses significantly reduced viral shedding as determined by AI virus RNA concentration in

oropharyngeal swabs. Assessment of antibody persistence showed that antibody levels of *in ovo* immunized chickens continued to increase until 12 weeks and started to decline after 18 weeks of age. Intramuscular (IM) booster vaccination with the same vaccine at 16 weeks of age significantly increased the antibody responses in breeder hens and these responses were maintained at high levels throughout the experimental period (34 weeks of age). AdTW68.$H5_{ch}$ immunized breeder hens effectively transferred MtAb to progeny chickens. The level of MtAb in the progenies was consistent with the levels detected in the breeders; i.e. IM boosted breeders transferred higher concentrations of antibodies to the offspring. Maternal antibodies declined with time in the progenies and achieved marginal levels by 34 days of age. Chickens with high MtAb levels which were vaccinated either *in ovo* or via mucosal routes (ocular or spray) did not seroconvert. In contrast, chickens without MtAb successfully developed specific antibody after either *in ovo* or mucosal vaccination. These results indicate that high levels of MtAb interfered with active Ad-vectored vaccination.

Introduction

Highly pathogenic (HP) avian influenza (AI) viruses belonging to the H5 or H7 subtypes threaten the world poultry industry and are zoonotic agents with pandemic potential for humans (17). We previously reported that protective immunity against 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 (Ad5)-vector encoding either the AI virus H5 (AdTW68.H5) or H7 (AdCN94.H7) hemagglutinins (HA). Vaccinated chickens were protected against HPAI homologous virus challenges (15, 16). We have shown that chickens vaccinated *in ovo* with AdTW68.H5 and subsequently vaccinated intramuscularly with AdCN94.H7 after hatch develop antibodies against both the H5 and H7 HA proteins. This result suggests that pre-existing Ad5 immunity in chickens does not significantly interfere with the potency of Ad5-vectored vaccines. Similarly, simultaneous *in ovo* vaccination with AdTW68.H5 and AdCN94.H7 also elicits robust hemagglutination inhibition (HI) antibody levels to both H5 and H7 AI strains (13) allowing the adoption of an immunization strategy with a broad antigen repertoire. Others evaluated a replication defective Ad-vector encoding the M2eX-HA-hCD40L or M1-M2 fusion from a human HPAI (H5N2) isolate administered via the IM and intranasal route in mice (4). Their vaccine delivery regimen resulted in both potent mucosal immunity as well as strong systemic humoral and T cell responses. A natural follow up of these studies was to evaluate vaccine potency,

antibody persistence, and transfer of maternal antibodies (MtAb) to progeny chickens. Evaluation of vaccine potency includes determination of the 50% protective dose (PD_{50}) as well as demonstration of repeatability. In this study PD_{50} was determined by HPAI challenge. Because serum HI antibody titers in poultry are strongly correlated with protection against HPAI (9) repeatability of *in ovo* immunization was determined from antibody levels achieved by the vaccinated chickens as described (2). Initial studies on antibody persistence showed that *in ovo* immunization with AdTW68.H5 induced anti-H5 antibody titers lasting at least until 52 days of age (16). Thus, in the current study we extended the time span for antibody monitoring in chickens vaccinated via the *in ovo* route to 34 weeks of age. Passively transferred MtAb are relevant in disease prevention in the poultry industry. However, maternally derived antibodies have been shown to interfere with active vaccination (reviewed in (6) and references therein). In the current study, we evaluated transfer of MtAb from breeder hens which were either singly immunized *in ovo* with AdTW68.$H5_{ch}$ or in addition received an intramuscular booster vaccination at 16 weeks of age. We also evaluated active *in ovo* or mucosal (ocular, spray) vaccination in progenies originating from AdTW68.$H5_{ch}$ vaccinated breeders; i.e. with maternal immunity.

Materials and Methods

Chickens. Specific pathogen free (SPF) (Sunrise Farms, Catskill, NY) white leghorn chickens or their progenies were used in the experiments described below. Experimental procedures and animal care were performed in compliance with all applicable federal and institutional animal use guidelines both at Auburn University College of Veterinary Medicine and at the USDA Southeast Poultry Research Laboratory (SEPRL).

A) To determine PD_{50} and repeatability, SPF chickens were hatched and maintained in Horsfall-type isolators in biosafety level (BSL) 2 facilities. Challenge with HPAI was performed in BSL 3+ facilities at SEPRL. Feed and water were provided *ad libitum*.

B) To determine antibody persistence, effect of booster vaccination, and transfer of maternal immunity, a vaccinated and an unvaccinated breeder flock were established. The vaccinated flock consisted of 70 females and 8 males. This flock was divided into two flocks (with or without booster vaccination) of 35 females and 4 males at 16 weeks of age (see experimental design below). The unvaccinated flock consisted of the same number of birds. Each breeder flock was maintained in environmentally controlled BSL-2 floor pen facilities. Feed, temperature, and light were adjusted to meet the breeder's physiological requirements during the rearing and egg production periods.

RCA–free recombinant adenovirus vector expressing codon-optimized H5 HA gene. The RCA-free Ad-vectored AI vaccine encoding the AI H5 HA was developed essentially as previously described using a synthetic AI H5 HA gene from the A/tk/WI/68 (H5N9) strain with the codon optimized to match the tRNA pool found in chicken cells (15, 16). Modifications included the use of AdHigh technology which allows the homologous recombination between the Ad-backbone and a shuttle vector to occur in *E. coli* (12). In brief, the fragment containing the full-length synthetic H5 HA gene was inserted into the *Hind*III-*Bam*HI site of the newly developed shuttle plasmid pAdHigh (12) to generate the plasmid pAdHigh-TW68.H5 with the H5 HA gene under transcriptional control of the human cytomegalovirus immediate-early promoter. An RCA-free, E1/E3-defective Ad vector encoding the codon-optimized A/tk/WI/68 HA gene (AdTW68.$H5_{ck}$) was subsequently generated in PER.C6 cells using the AdHigh system as described (12). The AdTW68.$H5_{ck}$ recombinant virus was validated by DNA sequencing. Titer [infectious units/ml (ifu)] was determined by the Adeno-X rapid titre kit (BD Clontech, Mountain View, CA).

Determination of repeatability. Determination of repeatability was performed by *in ovo* injection with 0.2 ml of vaccine suspension containing an escalating dose (10^5, 10^6, 10^7, 10^8, 10^9 ifu) of AdTW68.$H5_{ck}$. Each virus dose was applied to 3 replicates, each replicate consisting of 20 eggs. In order to stress the model, each replicate was inoculated with a different freshly thawed Ad batch and by a different person. Among the three persons, one was an expert (had performed *in ovo* vaccination for several years), another was a PhD student with moderate experience, and the 3rd person was a new graduate student without prior experience (this person was trained *in ovo* injection

just prior to performing an independent experiment). Total number of hatched chickens for each vaccine dose was as follows: 10^5 ifu, n=54; 10^6 ifu, n=52; 10^7 ifu, n=57, 10^8 ifu, n=55, 10^9 ifu, n=56. Each group was subsequently divided into 3 subgroups (17-19/group) and placed in different Horsfall-type isolators. Thus, a total of 15 groups were established. An additional group without vaccination was included as the unvaccinated control (n=19). Blood samples were obtained from all chicken groups by wing vein puncture on days 7, 18, 25, 32, and 39 after hatch. Individual serum samples were inactivated in a water bath at 56°C for 30 min, treated with RDEII (receptor destroying enzyme) (3) and thus pre-diluted 1:4 before testing by haemagglutination inhibition (HI) as described (11) for antibodies against the A/turkey/WI/68 (H5N9) AI strain. Vaccine coverage was determined and it was based on the number of antibody positive chickens in each group. The vaccine accordance (VACC) or intra-potency test repeatability was assessed based on antibody titers due to the congruence of HI antibody titer and disease protection (9). Repeatability analysis within vaccine dose (VACC) utilizes the frequency of animals testing antibody positive (p^2), the frequency of animals testing negative (q^2), as well as the total number of replicates per vaccine dose group (N) according to the formula described by Goris et al (2). According to Goris et al (2) it is clear from the formula that high repeatability is achieved when vaccination is either highly effective or ineffective because VACC is lowest (0.50) at $p = q = 0.5$.

Determination of protective dose 50% (PD_{50}). Fertile chicken eggs were injected *in ovo* with an increasing dose (10^5, 10^6, 10^7, 10^8, 10^9 ifu) of AdTW68.$H5_{ck}$ as described above. Each dose was injected into 20 fertile eggs (0.2 ml/egg) on day 18 of embryonation. Hatched chickens were placed in separate Horsfall-type isolation units.

The total number of hatched chickens in each group was as follows: 10^5 ifu, n=15; 10^6 ifu, n=17; 10^7 ifu, n=14, 10^8 ifu, n=18, 10^9 ifu, n=15. An additional group without vaccination was included as the unvaccinated control (n=17). Challenge was performed at 42 days of age mainly as previously described (15) in a BSL-3+ facility by oropharyngeal instillation of $10^{6.5}$ embryo infective doses (EID_{50}) /bird of the HPAI virus strain A/chicken/Queretaro/14588-19/95 (H5N2) (1). The H5 HA of this challenge strain has 94% deduced amino acid sequence similarity with the H5 HA of the A/tk/WI/68 (H5N9) strain expressed from the Ad5 vector (GenBank accessions U79448 & U79456). Challenged birds were observed daily for mortality throughout 8 days. Mortality data were analyzed by logistic regression using SAS® PROC NLMIXED. Degrees of freedom for the calculation of confidence intervals were adjusted to number of treatment groups minus number of fitted parameters as suggested by Schabenberger and Pearce (5).

Oropharyngeal swabs from individual birds were obtained for quantitation of AI RNA by quantitative reverse transcriptase-PCR (qRT-PCR) on days 3 and 5 after challenge, suspended in 1 ml of brain heart infusion medium (Difco, Kansas City, MO), and stored at -70ºC. RNA was extracted using the RNeasy mini kit (Qiagen Inc., Valencia, CA). qRT-PCR was performed with primers specific for type A influenza virus matrix RNA as described (8). Copy number of viral RNA was interpolated from the cycle thresholds using standard curves generated from known amounts of control A/chicken/Queretaro/95 RNA ($10^{1.0}$ to $10^{6.0}$ EID_{50}/ml).

Antibody persistence, effect of booster vaccination, and transfer of MtAb. Breeders of the vaccinated flocks were vaccinated *in ovo* at 18 days of embryonation with 300 µl of AdTW68.H5$_{ck}$ containing 1.5 x10^{9} ifu as previously reported (15, 16). The vaccinated breeder flock was further divided into two flocks at 16 weeks of age. One group received a booster vaccination (10^{8} ifu/300 µl) intramuscularly (IM) at 16 weeks of age and the 2nd group was not given the booster application. An unvaccinated control breeder flock was the control. Blood samples were collected from all breeders starting at 55 days of age and at monthly intervals until 34 weeks of age. Sera were tested for AI antibodies as described above. Fertile eggs were collected from all breeder flocks at 28 weeks of age, incubated and hatched. Progeny chickens (n=15) from breeders vaccinated *in*-ovo only, from breeders vaccinated *in ovo* + IM boost (n=18), as well as progenies (n=12) from unvaccinated breeders were maintained in BSL-2 facilities and blood samples were obtained at 3, 11, 20, 28, and 34 days of age. Sera were tested for anti-H5 HA [maternally derived] antibodies by HI as described above. HI titers detected in the groups were compared by ANOVA followed by a multiple comparisons posttest.

In ovo* vaccination in progeny chickens with maternal immunity.** To assess possible interference between maternal immunity and active *in ovo* vaccination, chicken groups (30/group) were vaccinated *in ovo* with 4.33 x 10^{11} ifu/***300 µl of AdTW68.H5$_{ck}$. Chicken groups were the progeny of breeders at 41 weeks of age which had been either vaccinated *in-ovo*, vaccinated *in ovo* + IM boost or maintained as unvaccinated controls (described above). Blood was collected from the progeny chickens at 11, 20, 28, 34 days of age and sera tested by HI for the presence of anti-H5 antibodies. HI titers

detected in the chicken groups were compared by ANOVA followed by a multiple comparisons posttest.

Mucosal vaccination in progeny chickens with maternal immunity. To assess possible interference between maternal immunity and active mucosal vaccination, chickens (18/group) were vaccinated either ocularly or by spray at 3 days-old and boosted at 18 days-old. These chickens originated either from unvaccinated breeders or from breeders that had been vaccinated *in ovo* and boosted intramuscularly with the Ad-vectored AI vaccine (described above). Ocular vaccination was performed with 100 µl $AdTW68.H5_{ck}$ per eye of vaccine suspension containing 1.3 x 10^{10} ifu. Spray vaccination was performed in a Spra-Vac® vaccination cabinet (Merial Select, Inc.) with a vaccine volume of 60mls at the same concentration as used in the ocular vaccination. Additional chickens from each breeder group were maintained as untreated controls. Blood was collected from the progeny chickens at 17, 32, 42, and 59 days of age and sera tested by HI for the presence of anti-H5 antibodies. HI titers detected in the groups were compared by ANOVA followed by a multiple comparisons posttest.

Results

Vaccine coverage. HI antibodies were detected in chickens vaccinated with 10^8 and 10^9 ifu on days 25, 32, and 39 of age. Only a few chickens inoculated with 10^7 (5/54) showed detectable antibodies on day 39 of age. Vaccine dosages $<10^7$ ifu did not elicit detectable antibody responses throughout the experimental period. Unvaccinated control birds maintained an antibody negative status. A dose response kinetic was observed with highest antibody levels achieved by chickens inoculated with the highest vaccine dose (10^9 ifu). Vaccine coverage (number of antibody positive birds) did not vary significantly ($P>0.05$) between days 25, 32 and 39 of age. For example, groups vaccinated with 10^9 ifu showed percentages of antibody positive birds on day 25 of age (3 replicates) of 69% (11/16), 87% (13/15), and 100% (16/16) with an average of 85%. The variation in coverage between the groups is likely associated with the level of expertise of each of the individuals participating in the vaccination process (described above).

Intra-potency test repeatability. The values for VACC or intra-potency test repeatability are shown in table 1. As seen in this table, highest VACC were obtained in the dose group 10^9 ifu with values varying between 0.69 and 0.71 indicating effective vaccination. Values for 10^8 ifu varied between 0.51 and 0.64. Values of 1, as seen at lower vaccine dosages, are based on the fact that the negative results (absence of a response) also had a high repeatability.

Protective dose 50% (PD_{50}). Antibody titers detected in 29 day-old SPF chickens vaccinated *in ovo* with increasing doses 10^5, 10^6, 10^7, 10^8, 10^9 ifu of AdTW68.$H5_{ck}$ are shown in Fig. 1. The values obtained showed the same tendency as the results shown above; i.e. highest vaccine dosages (10^8 and 10^9 ifu) elicited the highest antibody levels and frequency of antibody positive birds. However, the percent of AI antibody positive birds was lower: 61% (11/18) in the 10^8 and 50% (8/16) in the 10^9 groups.

The survival rate of chickens vaccinated with increasing vaccine dosage is shown in Fig. 2. Unvaccinated chickens showed 0% survival; all birds (17/17) had died by day 5 after challenge. In contrast, all vaccinated groups, even with the lowest vaccine dosage (10^5 ifu) showed some level of protection against HPAI challenge. Highest protection was observed in the groups receiving 10^8 or 10^9 ifu with 90% (16/18) and 88% (13/15) survival, respectively. Dose 10^7 ifu resulted in 74% (10/16) of birds surviving the challenge followed by 41% (7/17) survival of dose 10^6, and 7% (1/15) survival of dose 10^5. The PD_{50} was calculated by logistic regression based on mortality which declined with increasing vaccine dose. The PD_{50} (Fig. 3) was estimated to be $10^{6.55}$. The 95% confidence interval ranged from $10^{5.83}$ to $10^{7.27}$.

AI viral RNA of HPAI A/chicken/Queretaro/19/95 (H5N2) in challenged chickens determined by qRT-PCR in oropharyngeal swabs collected 3 and 5 days after challenge are shown in Fig. 4. AI virus genomes were detected in all bird groups at 3 and 5 days after challenge without significant differences between days within each group. Birds vaccinated with 10^7 and 10^8 ifu showed significantly lower ($P<0.05$) AI RNA

concentrations in oropharyngeal swabs than birds vaccinated with lower vaccine doses or unvaccinated controls on day 3 after challenge (Fig. 4 A). On day 5 after challenge there was a tendency showing that higher vaccine dosage may reduce viral shedding (Fig. 4B). However, the differences did not reach statistical significance which can be attributed to the reduced number of survivors both in unvaccinated controls and in chickens vaccinated with the lowest vaccine dose.

Antibody persistence in AdTW68.H5$_{ck}$ *in ovo* vaccinated breeder hens. Anti-H5 antibody titers in breeders peaked at 12 weeks of age (mean 4.6 $\log_2$), the plateau was maintained through 18 weeks, and slowly declined overtime averaging 2.8 $\log_2$ at 34 weeks of age (Fig. 5A). Chickens vaccinated *in ovo* + IM *b*oost at 16 weeks of age showed a significant increase (P<0.05) of anti-H5 antibody titers at 21 weeks of age (mean 8.4 $\log_2$) compared to *in ovo* -only or unvaccinated controls. High antibody titers in *in ovo* + IM boost chickens were maintained through 34 weeks of age (Fig. 5B). Unvaccinated breeder hens maintained an AI antibody negative status throughout the experimental period (not shown).

Anti-H5 maternal antibodies in progeny chickens. Anti-H5 antibodies were detected in progeny chickens from AdTW68.H5$_{ck}$ vaccinated breeders (Fig. 6). Highest anti-H5 maternally derived antibodies (mean 7.2 $\log_2$) were detected in progenies from breeders that received an in *ovo* + IM boost vaccination. Mean antibody titers declined with time and achieved marginal levels at 34 days of age. Thirty three percent (5/15) of chickens from breeders vaccinated *in ovo*- only showed antibody levels varying between 2 and 4 $\log_2$ at 3 days of age while the rest remained antibody negative. With

the exception of 1 bird, no antibodies were detected in this group (Fig. 6) on or after day 11 after hatch. Progeny chickens from unvaccinated controls maintained an AI antibody negative status throughout the experimental period (not shown).

Anti-H5 antibody in progeny chickens actively immunized *in ovo.* Progeny chickens from breeders vaccinated with AdTW68.H5$_{ck}$ *in ovo* + IM boost showed homogeneous anti-H5 antibody levels averaging 6.4 $\log_2$ on day 11 after hatch. As seen in Fig. 7, these antibody levels steadily declined through day 34 of age. In contrast progenies from both breeders vaccinated *in ovo*-only and unvaccinated controls increased their antibody levels significantly ($P<0.05$) after day 20 of age. Thus, high levels of MtAb seemed to interfere with AdTW68.H5$_{ck}$ *in ovo* vaccination.

Anti-H5 antibody in progeny chickens actively immunized by mucosal routes. As seen in Fig. 8A both ocular and spray vaccinated chickens originating from unvaccinated breeders develop antibodies against AI [levels increased significantly ($P<0.05$) compared to unvaccinated controls]. In ocularly and spray vaccinated groups as well as unvaccinated chickens the maternal antibodies declined steadily and similarly (without significant differences between groups) throughout the experimental period and never increased antibody levels (Fig. 8B). Thus, high levels of MtAb seemed to interfere with AdTW68.H5$_{ck}$ mucosal vaccination.

Discussion

Serum HI antibody titers in poultry have been reported to be strongly correlated with protection against HPAI (9). Thus, in the current study vaccine coverage and intra-potency test repeatability were based on anti-H5 HI antibody levels detected in the chickens. We observed variation between replicates in vaccine coverage which was consistent with the expertise of the individuals inoculating the eggs. Still the average in vaccine coverage of 3 replicates was 85%. Such variation would likely be reduced by using automated *in ovo* injectors as routinely used by the industry. The intra-potency test repeatability or VACC, which considers both positive as well as negative values, varied between 0.69 and 0.71. Even though these values indicate effective vaccination, they were rather low which may also be explained by the differences in the expertise of the vaccinators.

The challenge study aimed at determining the PD_{50} was performed using $10^{6.5}$ EID_{50} of HPAI A/chicken/Queretaro/19/95 (H5N2) per bird. This dose was rather high as the lethal dose 50% of this strain has been determined to be 10^3 EID_{50}. Indeed all unvaccinated birds had died by day 5 after inoculation. Based on dose and mortality the PD_{50} was determined to be $10^{6.6}$ ifu. This dosage seems rather high when compared to replicating live vaccines used routinely by the poultry industry against other viral pathogens. However, Ad-vector vaccine production in the PER C6 cell line can potentially produce $5x10^{15}$ifu (several million doses over the PD_{50}) in a few days (12)

and the safety of a non-replicating virus provides remarkable advantages over replicating viruses.

The results of the challenge study aimed at determining the PD_{50} clearly indicate that the antibody response induced by the recombinant vaccine is not exclusively responsible for protection against AI. Recent results have shown that RCA-free Ad-vectored vaccines encoding the AI HA hemagglutinin gene elicit effector, memory, and effector memory CD8+ T lymphocyte responses in chickens (7). In the current study several chickens which did not develop detectable antibody levels (Fig. 1) were effectively protected against HPAI challenge (Fig. 2). These results emphasize the importance of T cell responses after Ad-mediated vaccination (7) as the protection achieved by these antibody negative animals was likely associated with T lymphocyte responses. At the same time, they indicate that measurement of the potency of Ad-vectored vaccines should not only be based on antibody responses.

In the present study only higher doses (10^7, 10^8, 10^9 ifu) of the Ad-vectored vaccine reduced but not eliminated viral shedding as determined by AI RNA concentration in oropharyngeal swabs. This finding was rather disappointing because the risk of virus spread within or between flocks would not be completely eliminated using these vaccine dosages. In a previous study we vaccinated chickens intramuscularly with 1.1×10^{11} ifu at day 28 of age with an Ad vector expressing the H7 HA gene of the AI strain A/chicken/NY/13142-5/94. AI RNA of the challenge AI strain [A/chicken/Chile/4957/02 (H7N3)] was detectable in oropharyngeal swabs of only 1 of 11 immunized chickens but in almost all (8/11) non-immunized control chickens at 2

days after challenge (16).Therefore, dosage higher than 10^9 ifu would be necessary to considerably reduce or even eliminate viral shedding.

In initial studies we followed antibody persistence in chickens immunized *in ovo* with AdTW68.H5 through 40 days of age (16). The current results indicate that antibody levels continue to increase achieving a plateau at 12 weeks of age. Antibodies declined after 18 weeks of age but antibody persisted in some birds through 34 weeks of age. This long lasting antibody persistence is an excellent result which we cannot explain at this time point. We know that the vaccine virus is replication deficient and that naive birds do not seroconvert when raised in the same cage with vaccinated birds. Thus, virus is not spreading between birds and does not assist explaining the long lasting antibody responses. We have recently attempted to detect the Ad-vector DNA by qPCR in newly hatched chickens vaccinated *in ovo*. We were able to detect the virus at 2 days after hatch but all birds were negative by 9 days after hatch (14). Thus the virus seems to be cleared from the host rather quickly. The enduring antibody response may be attributed to a robust activation of the immune system which carries on after the vaccine disappears.

It is very interesting that intramuscular booster vaccination with the same vaccine construct increases the antibody responses significantly. We had previously shown that successive vaccination with Ad-vectors expressing the H5 and the H7 genes successfully induces strong antibody responses against both AI proteins (16). Thus, in contrast to fowlpox vectored vaccines (10), pre-existing immunity to the vector does not seem to affect booster vaccination with Ad-vectored vaccines. In the current study IM boost elicited a significant increase of specific antibody levels which were maintained

throughout the experimental period (34 weeks of age). From an applied perspective, these results indicate that IM boosting of hens (layers or breeders) which have been primed by the *in ovo* route with Ad-vectored vaccines would provide protection throughout most of the production period.

Maternally derived antibodies represent an integral part in disease prevention in young chickens. In the current study, we evaluated transfer of MtAb from breeder hens which were either singly immunized *in ovo* with AdTW68.H5$_{ck}$ or received an intramuscular booster vaccination at 16 weeks of age. As expected, specific H5 antibodies were effectively transferred to progeny chickens of Ad-vectored vaccinated breeders. The level of MtAb in the progenies was consistent with the levels detected in the breeders; i.e. breeders that had been boosted transferred higher concentrations of antibodies to their offspring. Maternal antibodies declined with time and achieved marginal levels by 34 days of age, similar to MtAb resulting from other vaccines routinely used in the poultry industry. Chickens with maternal immunity were not challenged in the current study but because specific antibodies are associated with protection (9), we would anticipate that they should be protected against homologous challenge at least during the first 20 days of life.

Maternally derived antibodies have been shown to interfere with active vaccination [reviewed by (6)]. The current results showed that progeny chickens with high maternal immunity (originating from breeders primed and boosted with AdTW68.H5$_{ck}$) never increased their antibody levels after *in ovo* or mucosal (ocular or spray) vaccination. Instead, they steadily declined in the same pace as progeny

chickens that were not immunized (described above). In contrast, both negative control progeny chickens (from naïve breeders) as well as the offspring from breeders that were vaccinated only once (*in ovo*) increased their antibody levels significantly following *in ovo* vaccination. A similar result was obtained when vaccinating MtAb positive chickens via mucosal routes. These results indicate that high levels of maternally derived antibodies actively interfered with Ad- vaccination.

It is interesting to notice that *in ovo*-vaccinated breeders responded to the IM booster vaccination in spite of the presence of specific antibodies. In contrast, progenies with high levels of specific antibodies did not respond to *in ovo* vaccination. These apparently contradictory results may be explained by the different antibody levels (~8 $\log_2$ in the progenies vs. ~4 $\log_2$ in the hens at the time of booster vaccination), by the route of vaccination (IM used to booster adult hens vs. *in ovo* or ocular used in progeny chickens), and by the maturity of the immune system (adult vs. 18-day old embryo or 3 day-old chicken).

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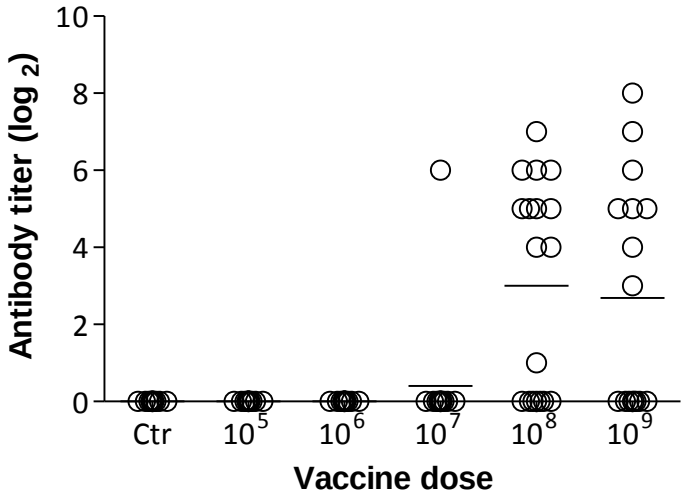

Fig.1. Individual HI anti-H5 antibody titers (log_2) detected in 29-day-old SPF chickens (14-18/group) vaccinated *in ovo* with increasing doses (10^5, 10^6, 10^7, 10^8, 10^9 ifu) of AdTW68.$H5_{ck}$ prior to HPAI challenge.

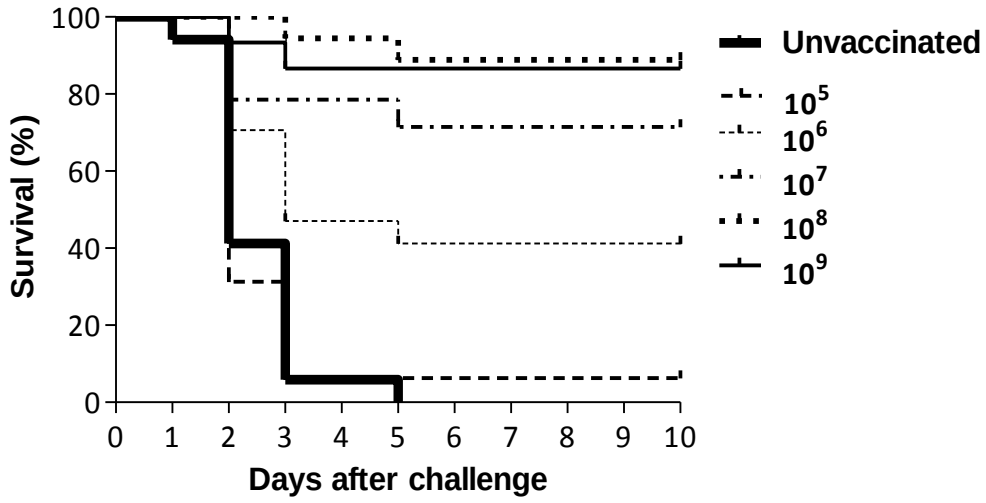

Fig.2. Survival rate of SPF chickens (14-18 /group) vaccinated *in ovo* with increasing dose of AdTW68.H5$_{ck}$ and challenged with $10^{6.5}$ EID$_{50}$/bird of HPAI virus A/chicken/Queretaro/14588-19/95 (H5N2) at 42 days of age. Highest protection was observed in the groups receiving 10^8 or 10^9 ifu with 90% (16/18) and 88% (13/15) survival, respectively.

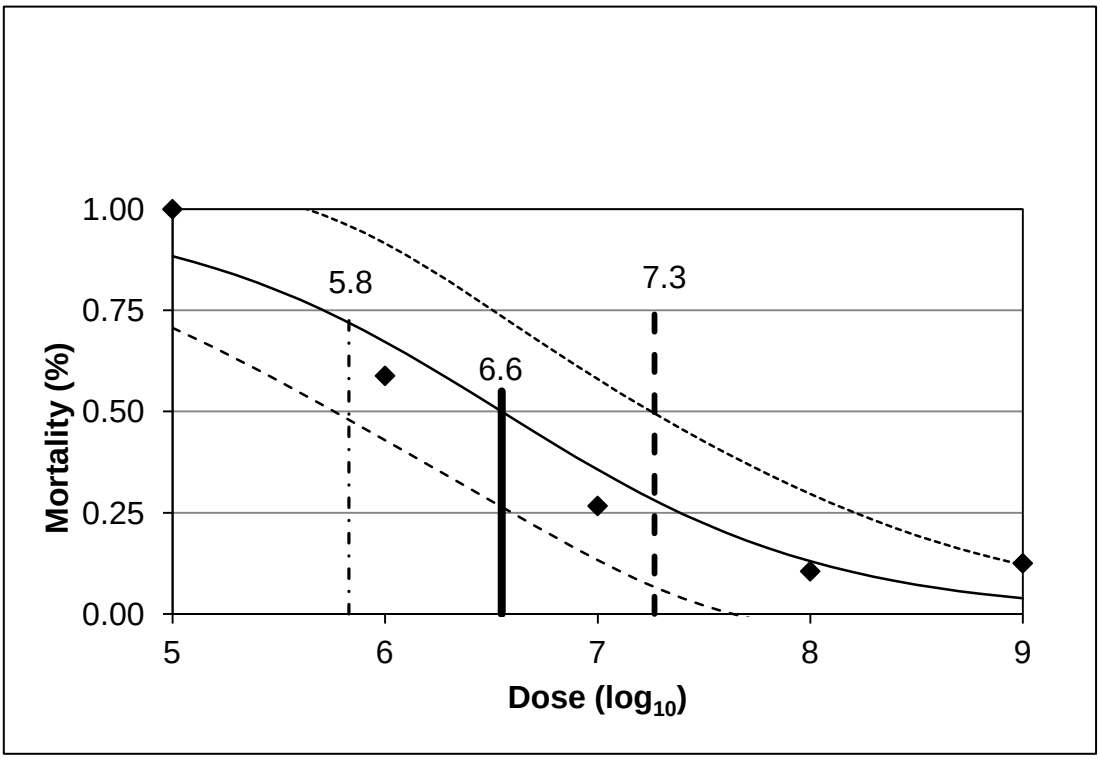

Fig.3. Protective dose 50% (PD_{50}) based on mortality vs. dose. Logistic regression adjusted to n of treatment groups minus n of fitted parameters as suggested by Schabenberger & Pearce (2003). CL=confidence limit. The PD_{50} was estimated to be $10^{6.55}$. The 95% confidence interval ranged from $10^{5.83}$ to $10^{7.27}$.

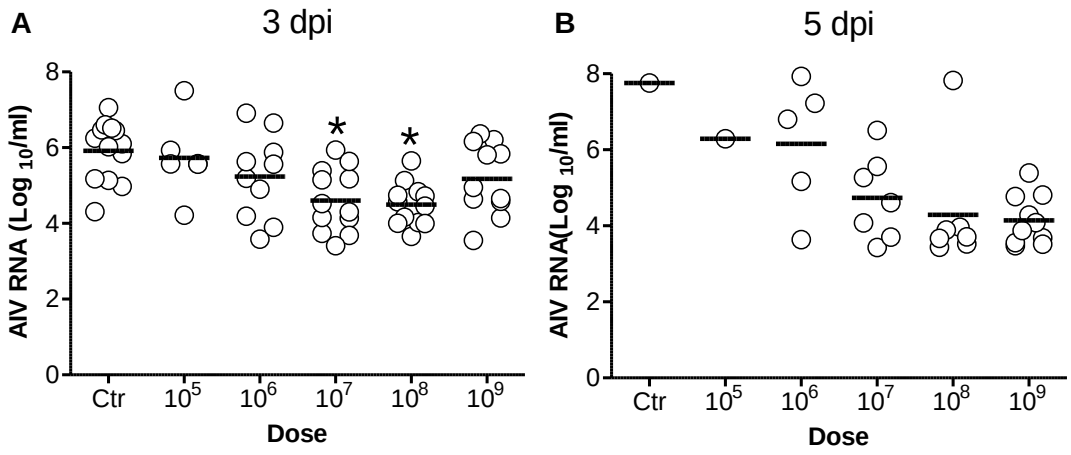

Fig.4. Viral shedding determined in SPF chickens (16/group) which were vaccinated *in ovo* with 10^5, 10^6, 10^7, 10^8, or 10^9 ifu of AdTW68.H5$_{ck}$ and challenged with $10^{6.5}$ EID_{50}/bird of HPAI virus A/chicken/Queretaro/14588-19/95 (H5N2) at 42 days of age. Viral shedding determined by qRT-PCR in oropharyngeal swabs at (A) 3 or (B) 5 days post inoculation (dpi). (*) indicates significant difference (P<0.05) vs. unvaccinated control (Ctr) [Kruskal-Wallis & Dunn's tests]. No significant differences were achieved at 5 days after challenge due to the reduced number of unvaccinated survivals on this day.

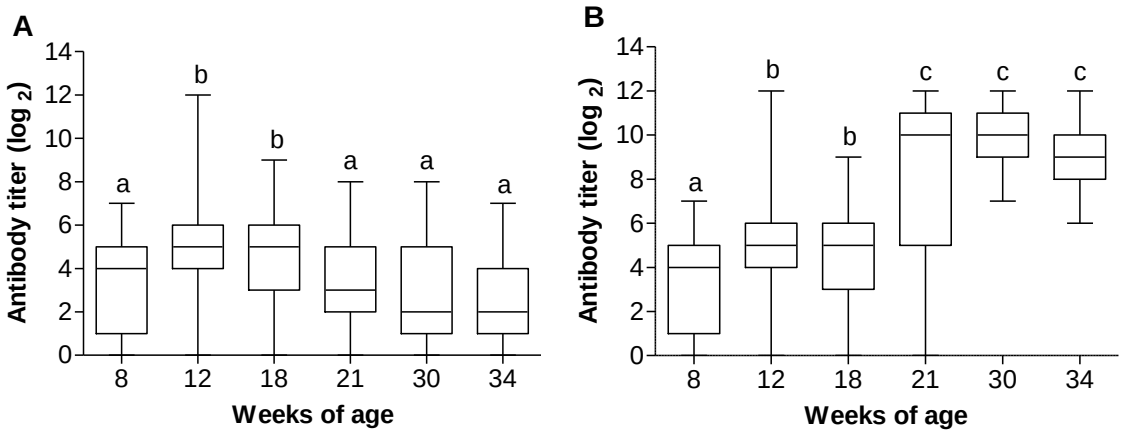

Fig.5. Anti-H5 antibody titers detected by HI in SPF white leghorn hens vaccinated either (A) *in ovo*-only (n=35), or (B) *in ovo*+IM boost (n=35) at 16 wk of age with 10^8 ifu/300 µl of AdTW68.$H5_{ck}$. Boxes: 25th percentile, median, 75th percentile; Whiskers: Min & Max. Control hens (n=10) maintained a negative AI antibody status (not shown). Different letters indicate significant differences (P<0.05).

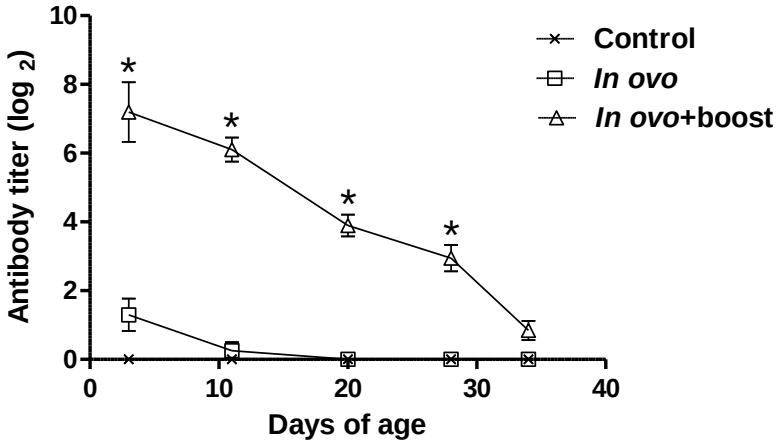

Fig.6. Anti-H5 HI antibodies in progeny chickens from breeders vaccinated with AdTW68.H5$_{ck}$ *in ovo* (n=15), vaccinated *in ovo*+IM boost (n=15), or unvaccinated controls (n=10). Sera collected at 3, 11, 20, 28, 34 days of age and anti-H5 antibodies measured by hemagglutination inhibition test. Bars: SEM. (*) Significant difference (P<0.001) vs. controls (two-way ANOVA followed by Bonferroni posttest).

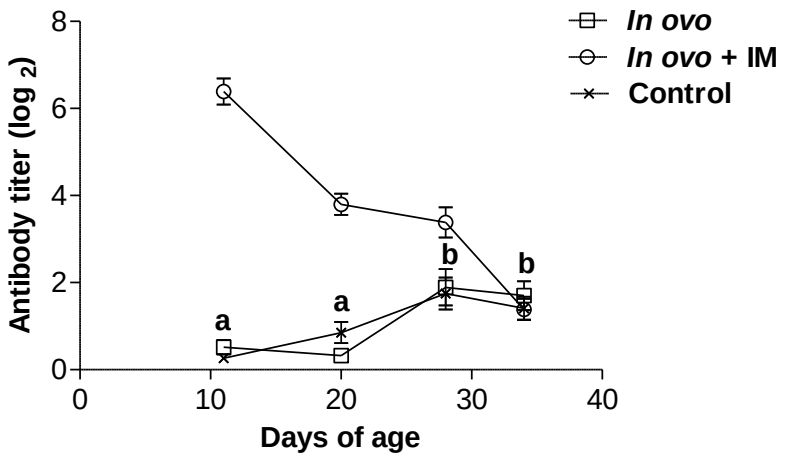

Fig.7. Anti-H5 antibodies in progeny chickens from breeders vaccinated with AdTW68.H5$_{ck}$ *in ovo* or *in ovo*+boost or unvaccinated breeders. Progeny chickens were vaccinated *in ovo* with 4.3 x 10^{11} ifu/300 µl of AdTW68.H5$_{ck}$. Bars: SEM. A significant increase (P<0.05) in anti-H5 antibodies was detected in chickens originating both from breeders vaccinated *in ovo*-only (different letters) and unvaccinated controls (not indicated). In contrast, no increase in antibody levels was detected in progeny chickens from breeders receiving a booster vaccination, i.e., their levels steadily declined throughout the experimental period.

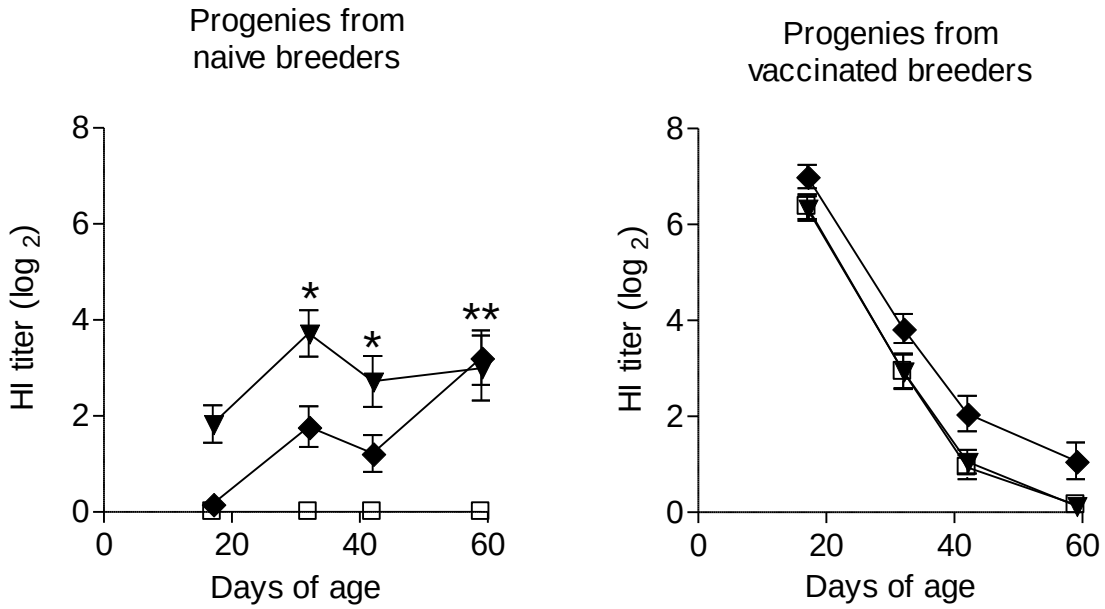

Fig. 8. HI antibodies detected in chickens (18/group) vaccinated with AdTW68.H5$_{ck}$ at 3 days-old and boosted at 18 days-old either ocularly or by spray. These chickens originated either from (A) unvaccinated breeders or from (B) breeders subjected to *in ovo* and intramuscular boost with AdTW68.H5$_{ck}$. (A) Both ocular and spray vaccinated progeny chickens from unvaccinated breeders develop antibodies against AI [levels increased significantly (P<0.05) compared to unvaccinated controls]. (*) indicates significant difference compared to control (two-way ANOVA followed by Bonferroni posttest).(B) Active antibody response were neither detected in vaccinated nor in unvaccinated progenies from vaccinated breeders; instead maternal antibodies declined steadily in all groups throughout the experimental period without significant differences between the groups.

Table 1. Repeatability analysis within vaccine dose (VACC*) using the frequency of animals testing antibody positive as well as the frequency of animals testing negative.

	Day 0		**Day 25**		**Day 32**		**Day 39**	
log_{10}	Freq(+)	VACC	Freq (+)	VACC	Freq (+)	VACC	Freq (+)	VACC
5	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00
6	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00
7	0.00	1.00	0.00	1.00	0.00	1.00	0.25	0.84
8	0.00	1.00	0.69	0.64	0.44	0.54	0.44	0.51
9	0.00	1.00	**0.69**	**0.69**	**0.63**	**0.71**	**0.75**	**0.69**

* VACC calculated from three replicates (cages; each n=15) per dose x day combination using the formula by Goris et al. (2007). Any antibody level (log_2) >0 was considered a positive reaction