



Published in final edited form as:

*Influenza Other Respir Viruses*. 2013 November ; 7(6): 1194–1201. doi:10.1111/irv.12140.

## Effect of Passive Immunization on Immunogenicity and Protective Efficacy of Vaccination against a Mexican Low Pathogenic Avian H5N2 Influenza Virus

Heather L. Forrest<sup>a,\*</sup>, Alejandro Garcia<sup>b,\*</sup>, Angela Danner<sup>a</sup>, Jon P. Seiler<sup>a</sup>, Kimberly Friedman<sup>a</sup>, Robert G. Webster<sup>a</sup>, and Jeremy C. Jones<sup>a,#</sup>

<sup>a</sup>Division of Virology, Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, Tennessee, USA

<sup>b</sup>Laboratorios Avilab, Loma Morad 7652 Col. Lomas del Manantial, Tonalá, Jalisco, Mexico

### Abstract

**Background**—Despite the use of vaccines, low pathogenic (LP) H5N2 influenza viruses have continued to circulate and evolve in chickens in Mexico since 1993, giving rise to multiple genetic variants. Antigenic drift is partially responsible for the failure to control H5N2 influenza by vaccination; the contribution of maternal antibodies to this problem has received less attention.

**Methods**—We investigated the effect of different antisera on the efficacy of vaccination and whether booster doses of vaccine can impact immune suppression.

**Results**—While single doses of inactivated, oil emulsion vaccine to currently circulating H5N2 influenza viruses provide partial protection from homologous challenge, chickens that receive high-titer homologous antisera intraperitoneally before vaccination showed effects ranging from added protection to immunosuppression. Post-infection antisera were less immunosuppressive than antisera obtained from field-vaccinated chickens. Homologous, post-infection chicken antisera provided initial protection from virus challenge but reduced the induction of detectable antibody responses. Homologous antisera from field-vaccinated chickens were markedly immunosuppressive, annulling the efficacy of the vaccine and leaving the chickens as susceptible to infection as non-vaccinated birds. Booster doses of vaccine reduced the immunosuppressive effects of the administered sera.

**Conclusion**—Vaccine efficacy against LP H5N2 in Mexico can be severely reduced by maternal antibodies. Source dependent antisera effects offer the possibility of further elucidation of the immunosuppressive components involved.

### Keywords

avian influenza; H5N2; immunosuppression; maternal immunity; Mexico; vaccine

---

#Corresponding Author: jeremy.jones@stjude.org.

\*Designates equal principal authors

## INTRODUCTION

Of the 17 subtypes of influenza A (H1–H17), H5 and H7 are of the most concern because low-pathogenicity strains of these two subtypes have the potential to mutate to the highly pathogenic form of avian influenza (AI) during circulation in poultry. Highly pathogenic avian influenza (HPAI) can cause high mortality in chickens and can have a significant impact in the areas of both agricultural and public health (1).

Many countries have discouraged AI vaccination, fearing it would hinder rapid diagnosis (2) and negatively affect serologic surveillance in poultry (3). Since the late 1990s, the severity of AI outbreaks has increased worldwide, leading to a re-evaluation of vaccination (1,2). Mexico began to vaccinate poultry against AI shortly after a 1993 confirmed case of H5N2 low pathogenic avian influenza (LPAI)(4-6). In 1994, the LPAI H5N2 virus mutated into HPAI (5,7). An AI vaccination program was established to control and eradication (8) with a commercial vaccine using the virus strain A/CK/Mexico/CPA-232/1994 (H5N2)(4). The last HPAI H5N2 virus was isolated in 1995 (8), and Mexico decided to continue vaccination to protect commercial flocks from LPAI (4). From 1995 to 2001, more than 1 billion doses of AI vaccine were administered. However, the LPAI H5N2 outbreaks in Mexico persist, and related viruses have spread to neighboring countries (8,9).

One explanation for persistence of LPAI H5N2 in Mexico is that antigenic differences developed between circulating viruses and vaccine seeds. Significant antigenic drift has been observed between 1994 vaccine seed A/CK/Mexico/CPA-232/1994 (H5N2) and field isolates (4,5,10,11). Another possible explanation is the interference of passive, or maternal immunity. In birds, maternal antibodies are transferred into the yolk, and the chick draws from this source of passively acquired immunity during the first weeks of its life (12). Although these antibodies can protect the chicks against viral disease (13), they also can hinder the immune response to vaccination as seen with infectious bursal disease (14), Newcastle disease virus (NDV)(15), and AI vaccines (12, 13, 16). The majority of studies on the influence of maternal immunity have focused on Egypt, where the circulation of H5N2 HPAI viruses in poultry continues despite vigorous control efforts. Results indicate that when chicks received maternal antibody through passive transfer (12) or passive immunization with anti-H5 chicken serum (16), the antibodies interfered with vaccine efficacy. We hypothesized that a similar situation may be occurring in Mexico and contributing to the persistence of LPAI H5N2 despite nearly two decades of vaccination, surveillance, and biosecurity measures. Therefore, we examined the immunogenicity and protective efficacy of an inactivated influenza vaccine made with H5N2 LPAI isolate A/Chicken/Mexico/IA05/09 (H5N2) in chicks that were passively immunized with antisera to homologous virus.

## MATERIALS & METHODS

### Viruses

LPAI H5N2 virus, A/Chicken/Mexico/IA05/09 (H5N2), was grown in 10-day-old embryonated chicken eggs for 48 hours at 37°C and stored at –80°C. The 50% chicken infectious dose (CID<sub>50</sub>) was determined in white leghorn chickens as described (16).

## Chickens

Specific pathogen-free (SPF) white leghorn chickens were purchased from Charles River Laboratories (North Franklin, CT) and used for all experiments. Animal experiments were approved by the Animal Care and Use Committee of St. Jude Children's Research Hospital and performed in compliance with National Institutes of Health and the Animal Welfare Act. Challenge experiments were performed in biosafety level 2 containment with restricted access to the animal facilities and as outlined in the Biosafety and Microbiological and Biomedical Laboratories (BMBL) guidelines (17) with the following enhancements: 1) experimental groups of infected birds were housed in individual cubicles under negative pressure and HEPA filtration, 2) researchers were required to wear personal protective equipment including N-95s, disposable tyvek suits, head, face, hands and shoes covers and all PPE was discarded prior to leaving the biosafety suite. Birds were observed for clinical signs of influenza for 14 days and assayed for virus as described (16).

## Preparation of antisera and passive immunization

Vaccinated chicken serum was obtained from a commercial farm in Mexico from layer chickens vaccinated at 3-weeks- of-age against AI and NDV; at 8 weeks of age with monovalent vaccine containing AI; and at 19 weeks of age with a bivalent vaccine containing AI and NDV. All three vaccines were adjuvanted and inactivated, and contained the A/Chicken/Mexico/IA05/09 (H5N2) strain. These poultry were administered vaccinations for other common viral and bacterial pathogens. Serum was collected from chickens at 32 weeks of age and HI titered with a resulting value of 1280 HI units/50 $\mu$ l.

To prepare serum from infected chickens, 8-week-old SPF chickens were inoculated with 1.0 ml of 1:10 dilution of A/Chicken/Mexico/IA05/09 (H5N2) via the natural route (oculo-nasal administration). Chickens were boosted at 22 days post-infection with undiluted virus stock (1.0 ml IV + 1.0 ml IP or 2.0 ml IV), serum was collected 1 week later, and it was titered in the HI assay with a value of 2560 HI units/50 $\mu$ l.

For passive immunization, 3-week-old chickens in groups of 11 received 1.0 ml of antisera intraperitoneally (IP) either neat or 1:2 dilution from the stock sera (described above) to simulate maternal immunity (Table 1). Prior to administration, sera was tested for bacterial contamination and for the presence of immunosuppressive chicken anemia virus and found to be negative in both cases (data not shown).

## Vaccines

The vaccine used for immunization was a whole-virion, inactivated, mineral-oil emulsion preparation containing the strain A/Chicken/Mexico/AvilabLA10509/09 (H5N2, AVILAB Laboratories, Jalisco, Mexico). One day after passive immunization, all birds (excluding naïve group which received PBS) were administered 0.5 ml of vaccine subcutaneously in the lower neck. Nine days later, groups 5 and 8 received a vaccine boost (Table 1).

## Challenge

Twenty-five days after boost, all animals were challenged with  $10^{4.2}$  EID<sub>50</sub> (equivalent to 100 CID<sub>50</sub> units) intranasally (0.25 ml), intratracheally (0.25 ml), and intraocularly (1 drop per eye) of A/Chicken/Mexico/IA05/09 (H5N2) in PBS.

## Swab collection

Oropharyngeal and cloacal swabs were collected from all birds before challenge and on days 3, 5, 7, 9, 11 and 13 after challenge and assayed for infectivity by titration in embryonated chicken eggs as described (16). The lower limit of detection was 0.75 log<sub>10</sub>EID<sub>50</sub>/ml.

## Blood sampling and serology

Blood was collected from 2–5 birds from each group prior to passive immunization and tested to determine whether antibodies to influenza A were present. Additionally, blood was collected 10, 21, 35 and 50 days after passive immunization and subjected to the HI assay as described. Absolute values from the assay were converted to Log<sub>2</sub> for statistical analysis. The lower limit of detection was 1:10 serum dilution ( $\approx 3$  Log<sub>2</sub>).

## Statistical analyses

The arithmetic mean values  $\pm$  standard deviations and statistical significance of infectious titers, serum antibody titers and duration of shedding average values was determined by the student's t-test using Excel (Microsoft, Redmond, WA) or GraphPad Prism v5 (GraphPad Software, La Jolla, CA) software. A Fisher's exact test was used to compare total number of birds shedding throughout the experiment using GraphPad Prism v5 software.

# RESULTS

## Virus shedding after challenge

Chickens were vaccinated with a recent LPAI H5N2 isolate (A/Ck/Mexico/IA05/09) homologous to the challenge virus to minimize the influence of antigenic drift on the vaccine's efficacy. After the challenge, all chickens remained free from clinical signs of infection.

Shedding was more frequent and occurred at higher titers via the oropharyngeal route (Table 2) than the cloacal route (Supplementary Table 1) and oropharyngeal shedding was present in all groups by 3 days post-challenge (dpc). As expected, all naïve animals (group 1) had positive oropharyngeal swabs (Table 2). One dose of vaccine was sufficient to protect against shedding in 5 of 9 chickens in the vaccine control group (group 2) and reducing mean oropharyngeal titers as compared to naïve birds (3,5 dpc,  $p < 0.05$ ). Surprisingly, neat laboratory derived post-infection antisera (group 3) appeared to provide additional protection, decreasing both the number of birds shedding and duration of shedding ( $p < 0.05$ ); only 2 of 10 birds (one bird at 3 and another at 5 dpc) had detectable virus in oropharyngeal swabs. Diluting the laboratory post-infection antisera 1:2 (group 4) appeared to qualitatively reduce the protective effect. Four of 11 birds were shedding via the oropharyngeal route 9 days post challenge, but despite this, no difference was detected by our statistical methods between group 4 and groups 3 or 5. The booster vaccine administered

to group 5 did not reduce the number of birds with oropharyngeal shedding; 6 of 11 birds were shedding 7 dpc.

Oropharyngeal shedding in groups 6–8, which consisted of chickens passively immunized with field-derived, post-vaccination antisera, provided a different picture of vaccine efficacy and antibody interference. In group 6 (neat serum) and 7 (serum diluted 1:2), all birds shed virus at levels statistically insignificantly different to those of the naïve group, and the number of birds shedding was higher than the vaccine control group ( $p = 0.01$ ). Additionally, group 6 birds shed at higher titers than the vaccine control group at multiple time points (3,5 dpc,  $p < 0.05$ ). These data combined provide clear evidence of the interference of antibodies on the efficacy of the vaccine. Conceivably, boosting may have provided some benefit because the number of birds shedding at any given time was lower in group 8 than in group 7 ( $p = 0.05$ ); however, approximately half the birds in group 8 had late onset shedding (9 or 11 dpc), so virus was detected as late as 13 dpc.

Limited cloacal shedding was seen in all groups (Supplementary Table 1). Group 2 (vaccination alone) had fewer birds shedding from the cloaca than did group 1, with 1 of 9 and 8 of 9 birds shedding respectively ( $p = 0.01$ ).

### Passive antibody transfer

Approximately half of the animals from each experimental group had detectable antibody titers 1 day after passive immunization with sera against A/Ck/Mexico/IA05/09 (H5N2) (Table 3). Average circulating antibody titers on day 1 were statistically higher in groups receiving post-infection antisera than those receiving post-vaccination antisera ( $p = 0.05$ ). Titers from passive immunization quickly waned and were no longer detectable on day 10 (Table 3).

### Serological response to vaccination after passive immunization

A single dose of vaccine induced high HI titers in all 9 vaccine control birds, with titers ranging from 8.9–9.4  $\text{Log}_2$  (Table 3). These birds' immunity was marginally boosted after challenge, with HI titers increasing to an average 10.3  $\text{Log}_2$ . Chickens in group 3, administered neat laboratory derived sera, had a delayed antibody response. A 21 days post-vaccination, antibody titers were detectable in only 5 of 10 birds and significantly lower than titers from the vaccine control group ( $p < 0.005$ ). Titers improved by 35 days post-vaccination, with all 10 birds responding. Although this improvement continued after challenge, HI titers of group 3 were still lower than those of vaccine controls (group 2) at both 35 and 50 days post vaccination ( $p < 0.005$  and  $0.05$  respectively). Antibody titers in group 4 displayed a trend similar to group 3. Chickens in group 4 and 5 had similar antibody titers until day 50 post vaccination when group 5 titers were higher than group 4 ( $p < 0.005$ ), an indication that boosting increased seroconversion in the face of passively administered antibody.

In group 6, administered neat, field-derived serum, only 3 of 10 birds had detectable HI titers (mean, 5.3  $\text{Log}_2$ ) 21 days after vaccination. An additional 5 birds had detectable titers by day 35, but mean titers remained lower than those of the vaccine control group at this timepoint and for the remainder of the study ( $p < 0.01$ ). In group 7, which received diluted

field antisera, 7 of 10 birds responded to vaccination by day 21, and by day 35, all birds had detectable HI tiers. At both timepoints, titers were higher than in birds receiving undiluted sera (group 6,  $p < 0.05$ ). Similar to the laboratory sera group that received a second administration of vaccine, boosting improved the number of group 8 birds with detectable antibody titers on day 21 (10 of 11) and those titers were higher than group 6 and 7 at day 35 and beyond ( $p < 0.05$ ). By day 35, the average HI titer in group 8 was statistically insignificant to those of the vaccine control group and titers remained as high as those of the vaccine controls after challenge.

### Seroelevation after challenge

In addition to virus shedding, a 4-fold increase in HI titer 14 dpc was also suggestive of infection. All birds in the naïve group met these criteria, and 2 birds from the vaccine control group increased their titers after challenge (Figure 1). Of the experimental groups, group 6 (neat, field-derived post-vaccination antiserum) had the most animals that showed increased titers ( $n=7$ ), and group 3 (neat, laboratory-derived post-infection antiserum) had the fewest animals with increases ( $n=3$ ). Some of the animals in the vaccine control group and groups 3–5 showed increased HI titers but did not shed after challenge. Although vaccination may not have been effective in preventing infection in these specific instances, it did eliminate viral shedding. If both seroelevation and virus shedding are taken as evidence of infection, then all birds passively immunized with field-derived post vaccination antisera had evidence of infection despite vaccination with or without boost.

### Duration of virus shedding

To estimate duration of shedding in each group, we averaged the number of swab time points (6 total) at which group members had a positive oropharyngeal or cloacal swab (only animals that shed were included in this calculation). The naïve birds had the highest average (4.2) and group 3 (neat, laboratory-derived post-infection antiserum) had the lowest average (2.0). Notably, group 3 also had the lowest number of animals shedding and the earliest cessation of shedding (day 7). Groups 4 and 5 had shedding averages higher than that of the vaccine control group (2.70 and 3.50 respectively) suggesting a longer shedding duration, though these were not statistically significant. Groups receiving field-derived post-vaccination antiserum also had higher averages (group 6 – 3.50 and group 7 – 3.70) and in contrast to laboratory sera, group 7 birds statistically shed longer ( $p < 0.05$ ) than the vaccine control group suggesting the influence of maternal antibodies on duration of shedding.

## DISCUSSION

The continuing difficulty in controlling low pathogenic H5N2 influenza in chickens in Mexico with vaccination may in part be due to the influence of maternal antibody on vaccine efficacy. In this study, we mimic the influence of maternal antibody by passively administering antiserum and show that sera from H5N2 vaccinated chickens in the field in Mexico is immunosuppressive whereas undiluted, laboratory prepared post-infection H5N2 antiserum provides added protection from infection. The difference between the sera provides us with the unique opportunity to investigate the mechanism responsible for this

phenomenon in the future. Additionally, we demonstrate that multiple doses of vaccine can partially alleviate the suppressive effect mediated by this passively acquired immunity.

Birds from the vaccine control group shed virus following challenge, despite having high HI titers. These findings were consistent with a recent reviews of avian influenza vaccination practices worldwide, in which single vaccinations failed to provide a level of immunity capable of preventing virus shedding (18). The failure was especially evident when maternally derived antibodies were present (18,19). Although our finding that vaccinated birds shed virus could be attributed to the lack of a boost, maternal antibodies were not a factor because the birds were SPF and HI titers averaged 9.4 Log<sub>2</sub> on the day of challenge, which should have provided adequate protection. The establishment of a single minimal HI titer required for immune protection in poultry is not possible because it is dependent on a variety of factors including species of bird and type of vaccine. Notably, HI titers required for protection tend to be higher in SPF laying chickens than in commercial broilers and breeders (20). Although Kumar et al. reported an association between HI titer (>1:40 serum dilution) and clinical protection in a study of chickens vaccinated with inactivated H5N2 prior to H5N1 challenge (21), another study showed that an HI titer of 1:120 was required for clinical protection against highly pathogenic H5N2 (20).

Both humoral and adaptive immune responses have been described for poultry (22). Whereas the systemic component of the poultry humoral antibody response is well described and similar to other species, the mucosal component and cell-mediated responses are not well characterized (23, 24). Secretory antibodies of the poultry mucosal immune response have long been believed to have a functional analogy with those of the mammalian secretory immune system (i.e. secretory IgA)(22,25). One potential explanation for the varying degree of vaccine interference seen with the two sources of passively immunized antisera used in this study could be the amount of IgA in the antisera (26-28). Further studies are warranted to determine the differences between the antisera and the component(s) responsible for interference and protection.

Our data presented here and data from others demonstrate maternal antibodies inhibit the humoral immunity, but additional studies suggest the T cells responses remain largely unaffected or even enhanced (29). The degree of change in both breadth and quality of cell-mediated immunity in the face of influence of maternal immunity remains an important subject for analysis. Additionally, it will be beneficial to explore other laboratory methods of simulating maternal immunity in poultry that more closely resembles the levels and decline rate of naturally acquired immunity, such as inoculating yolk antibody preparations in the yolk sac of day old chickens (30).

We observed that the passively administered field-derived antiserum antibodies interfere with the efficacy of influenza vaccination despite a lack of prolonged circulation of these antibodies. We found that antibodies from sera passively administered via the IP route were undetectable 10 days after injection – sooner than one would expect if the maternal immunity was acquired naturally. This is perhaps not surprising, as studies have demonstrated that the absence of detectable maternal antibodies does not necessarily mean that antibodies are not influencing the immune response. Chicks in which maternal

antibodies are undetectable can still be protected against disease (31,32) and undetectable maternal antibodies have been shown to interfere with efficacy of vaccination against AI (13). In general, maternally derived antibodies in poultry decline linearly and reach peak titers in circulation approximately 2–3 days post hatch (10) and recent data indicates vaccination of maternal antibody-positive chicks 10 days post-hatch or later may be the best approach, as maternal antibodies can potentially interfere with vaccination as late as 3 weeks post-hatch (33).

It is also important to continue to explore methods to circumvent maternal antibody interference through better vaccination strategies. The H5 fowlpox-vectored vaccine currently used in Mexico offers some promise because it is not inhibited by maternal antibodies and does not interfere with routine serological surveillance; however, its efficacy appears to be compromised by active immunity (9, 34). A recent publication by Kim et al. offers insight into the mechanism controlling the inhibition of vaccine-induced seroconversion by maternal antibodies in the rate model of measles virus vaccine and a way to overcome this inhibition. They showed that maternal antibodies inhibit B cell responses by interacting with the inhibitory Fc $\gamma$ RIIB receptor for IgG and that this inhibition can be partially overcome by injecting rats with virus-specific monoclonal IgM antibody, which stimulates the B cell directly (35). This finding is not directly transferable to poultry because only a small amount of information exists about Fc receptors in non-mammalian vertebrates. Still, research has described the chicken Fc $\gamma$  receptor CHIR-AB1 that is expressed on B cells, binds IgY (the functional equivalent of mammalian IgG) with high affinity, and has both activating and inhibitory motifs (36, 37). Therefore, the role of IgM in overcoming the inhibitory effect of maternally derived antibodies should be investigated.

## CONCLUSIONS

Our findings illustrate and support the paradigm that immunogenicity and protective efficacy of inactivated avian influenza vaccination is susceptible to passively (e.g. maternally) derived antibodies and that this likely contributes to the failure of AI vaccination programs in Mexico. Furthermore, the suppressive effects of the antisera on the response to vaccination differed with the source of the serum (i.e. from field vaccinated birds or laboratory challenged birds). Antibody characterization of the two types of antisera is needed to determine what factors may contribute to these variations. Although no vaccination strategy has successfully escaped the influence of maternal antibodies, recent studies appear to be progressing towards identifying the mechanism behind the suppression. Uncovering this mechanism will guide us in developing new vaccination approaches that can evade maternal antibody influence.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.



## Acknowledgments

This work was supported by Contract No. HHSN266200700005C from the National Institute of Allergy and Infectious Disease, National Institutes of Health, Department of Health and Human Services, and by the American Lebanese Syrian Associated Charities (ALSAC).

We thank Chandra Savage, Ginger Redd, Ghazi Kayali, Sook-San Wong, Christine Oshansky-Weilnau for technical assistance, Cherise Guess for editorial assistance, and James Knowles for administrative assistance.

## References

1. Maas R, Tacken M, van Zoelen D, Oei H. Dose response effects of avian influenza (H7N7) vaccination of chickens: serology, clinical protection and reduction of virus excretion. *Vaccine*. 2009; 27:3592–7. [PubMed: 19464539]
2. Capua I, Alexander DJ. Avian influenza vaccines and vaccination in birds. *Vaccine*. 2008; 26(Suppl 4):D70–3. [PubMed: 19230164]
3. van den Berg T, Lambrecht B, Marche S, Steensels M, Van Borm S, Bublot M. Influenza vaccines and vaccination strategies in birds. *Comp Immunol Microbiol Infect Dis*. 2008; 31:121–65. [PubMed: 17889937]
4. Escorcía M, Vazquez L, Mendez ST, Rodríguez-Ropon A, Lucio E, Nava GM. Avian influenza: genetic evolution under vaccination pressure. *Virology*. 2008; 378:15. [PubMed: 18218105]
5. Lee CW, Senne DA, Suarez DL. Effect of vaccine use in the evolution of Mexican lineage H5N2 avian influenza virus. *J Virol*. 2004; 78:8372–81. [PubMed: 15254209]
6. Horimoto T, Rivera E, Pearson J, Senne D, Krauss S, Kawaoka Y, et al. Origin and molecular changes associated with emergence of a highly pathogenic H5N2 influenza virus in Mexico. *Virology*. 1995; 213:223–30. [PubMed: 7483266]
7. Eggert D, Thomas C, Spackman E, Pritchard N, Rojo F, Bublot M, et al. Characterization and efficacy determination of commercially available Central American H5N2 avian influenza vaccines for poultry. *Vaccine*. 2010; 28:4609–15. [PubMed: 20470798]
8. Villarreal-Chavez C, Rivera-Cruz E. An update on avian influenza in Mexico. *Avian Dis*. 2003; 47:1002–5. [PubMed: 14575101]
9. Bublot M, Pritchard N, Swayne DE, Selleck P, Karaca K, Suarez DL, et al. Development and use of fowlpox vectored vaccines for avian influenza. *Ann N Y Acad Sci*. 2006; 1081:193–201. [PubMed: 17135511]
10. JMS. Host factors for disease resistance. In: Saif, YMFA.; Glisson, JR.; McDougald, LR.; Nolan, LK.; Swayne, DE., editors. *Diseases of Poultry*. 12. Hoboken, New Jersey: Wiley-Blackwell; 2008.
11. Escorcía M, Carrillo-Sanchez K, March-Mifsut S, Chapa J, Lucio E, Nava GM. Impact of antigenic and genetic drift on the serologic surveillance of H5N2 avian influenza viruses. *BMC Vet Res*. 2010; 6:57. [PubMed: 21172021]
12. Abdelwhab EM, Grund C, Aly MM, Beer M, Harder TC, Hafez HM. Influence of maternal immunity on vaccine efficacy and susceptibility of one day old chicks against Egyptian highly pathogenic avian influenza H5N1. *Vet Microbiol*. 2012; 155:13–20. [PubMed: 21920677]
13. Maas R, Rosema S, van Zoelen D, Venema S. Maternal immunity against avian influenza H5N1 in chickens: limited protection and interference with vaccine efficacy. *Avian Pathol*. 2011; 40:87–92. [PubMed: 21331952]
14. Naqi SA, Marquez B, Sahin N. Maternal antibody and its effect on infectious bursal disease immunization. *Avian Dis*. 1983; 27:623–31. [PubMed: 6314972]
15. van Eck JH, van Wiltenburg N, Jaspers D. An Ulster 2C strain-derived Newcastle disease vaccine: efficacy and excretion in maternally immune chickens. *Avian Pathol*. 1991; 20:481–95. [PubMed: 18680044]
16. Kim JK, Kayali G, Walker D, Forrest HL, Ellebedy AH, Griffin YS, et al. Puzzling inefficiency of H5N1 influenza vaccines in Egyptian poultry. *Proc Natl Acad Sci U S A*. 2010; 107:11044–9. [PubMed: 20534457]

17. Centers for Disease Control and Prevention. Biosafety in Microbiological and Biomedical Laboratories (BMBL). St. Louis, MO: U.S. Government Printing Office; 2009.
18. Swayne DE, Pavade G, Hamilton K, Vallat B, Miyagishima K. Assessment of national strategies for control of high-pathogenicity avian influenza and low-pathogenicity notifiable avian influenza in poultry, with emphasis on vaccines and vaccination. *Rev Sci Tech.* 2011; 30:839–70. [PubMed: 22435196]
19. Schijns, VEJCSJ.; Tarpey, I. Practical aspects of poultry vaccination. In: Davison, TFKB.; Schat, KA., editors. *Avian Immunology.* 1. London: Academic Press; 2008.
20. Swayne, DEKD. Vaccines, vaccination, and immunology for avian influenza viruses in poultry. In: Swayne, D., editor. *Avian Influenza.* 1. Hoboken: Wiley-Blackwell; 2008.
21. Kumar M, Chu HJ, Rodenberg J, Krauss S, Webster RG. Association of serologic and protective responses of avian influenza vaccines in chickens. *Avian Dis.* 2007; 51:481–3. [PubMed: 17494614]
22. Swayne DE, Kapczynski D. Strategies and challenges for eliciting immunity against avian influenza virus in birds. *Immunol Rev.* 2008; 225:314–31. [PubMed: 18837791]
23. Swayne, DEHD. Influenza. In: Saif, YMFA.; Glisson, JR.; McDougald, LR.; Nolan, LK.; Swayne, DE., editors. *Diseases of Poultry.* 12. Hoboken: 2008.
24. Suarez DL, Schultz-Cherry S. Immunology of avian influenza virus: a review. *Dev Comp Immunol.* 2000; 24:269–83. [PubMed: 10717293]
25. Porter P, Parry SH. Further characterization of IgA in chicken serum and secretions with evidence of a possible analogue of mammalian secretory component. *Immunology.* 1976; 31:407–15. [PubMed: 1027723]
26. Gelb J Jr, Nix WA, Gellman SD. Infectious bronchitis virus antibodies in tears and their relationship to immunity. *Avian Dis.* 1998; 42:364–74. [PubMed: 9645328]
27. Takada A, Kida H. Protective immune response of chickens against Newcastle disease, induced by the intranasal vaccination with inactivated virus. *Vet Microbiol.* 1996; 50:17–25. [PubMed: 8810004]
28. van Ginkel FW, Tang DC, Gulley SL, Toro H. Induction of mucosal immunity in the avian Harderian gland with a replication-deficient Ad5 vector expressing avian influenza H5 hemagglutinin. *Dev Comp Immunol.* 2009; 33:28–34. [PubMed: 18773917]
29. Siegrist, C. Vaccine immunology. In: Plotkin, SAOW.; Offit, PA., editors. *Vaccines.* 5. China: Saunders-Elsevier; 2008.
30. Stone HD, Brugh M, Xie Z. Simulation of maternal immunity by inoculation of immune yolk preparations into the yolk sac of 1-day-old chickens. *Avian Dis.* 1992; 36:1048–51. [PubMed: 1485856]
31. Nemeth NM, Bowen RA. Dynamics of passive immunity to West Nile virus in domestic chickens (*Gallus gallus domesticus*). *Am J Trop Med Hyg.* 2007; 76:310–7. [PubMed: 17297041]
32. Heller ED, Leitner H, Drabkin N, Melamed D. Passive immunisation of chicks against *Escherichia coli*. *Avian Pathol.* 1990; 19:345–54. [PubMed: 18679943]
33. De Vriese J, Steensels M, Palya V, Gardin Y, Dorsey KM, Lambrecht B, et al. Passive protection afforded by maternally-derived antibodies in chickens and the antibodies' interference with the protection elicited by avian influenza-inactivated vaccines in progeny. *Avian Dis.* 2010; 54:246–52. [PubMed: 20521640]
34. Swayne DE, Beck JR, Mickle TR. Efficacy of recombinant fowl poxvirus vaccine in protecting chickens against a highly pathogenic Mexican-origin H5N2 avian influenza virus. *Avian Dis.* 1997; 41:910–22. [PubMed: 9454926]
35. Kim D, Huey D, Oglesbee M, Niewiesk S. Insights into the regulatory mechanism controlling the inhibition of vaccine-induced seroconversion by maternal antibodies. *Blood.* 2011; 117:6143–51. [PubMed: 21357766]
36. Viertlboeck BC, Schweinsberg S, Hanczaruk MA, Schmitt R, Du Pasquier L, Herberg FW, et al. The chicken leukocyte receptor complex encodes a primordial, activating, high-affinity IgY Fc receptor. *Proc Natl Acad Sci U S A.* 2007; 104:11718–23. [PubMed: 17606923]

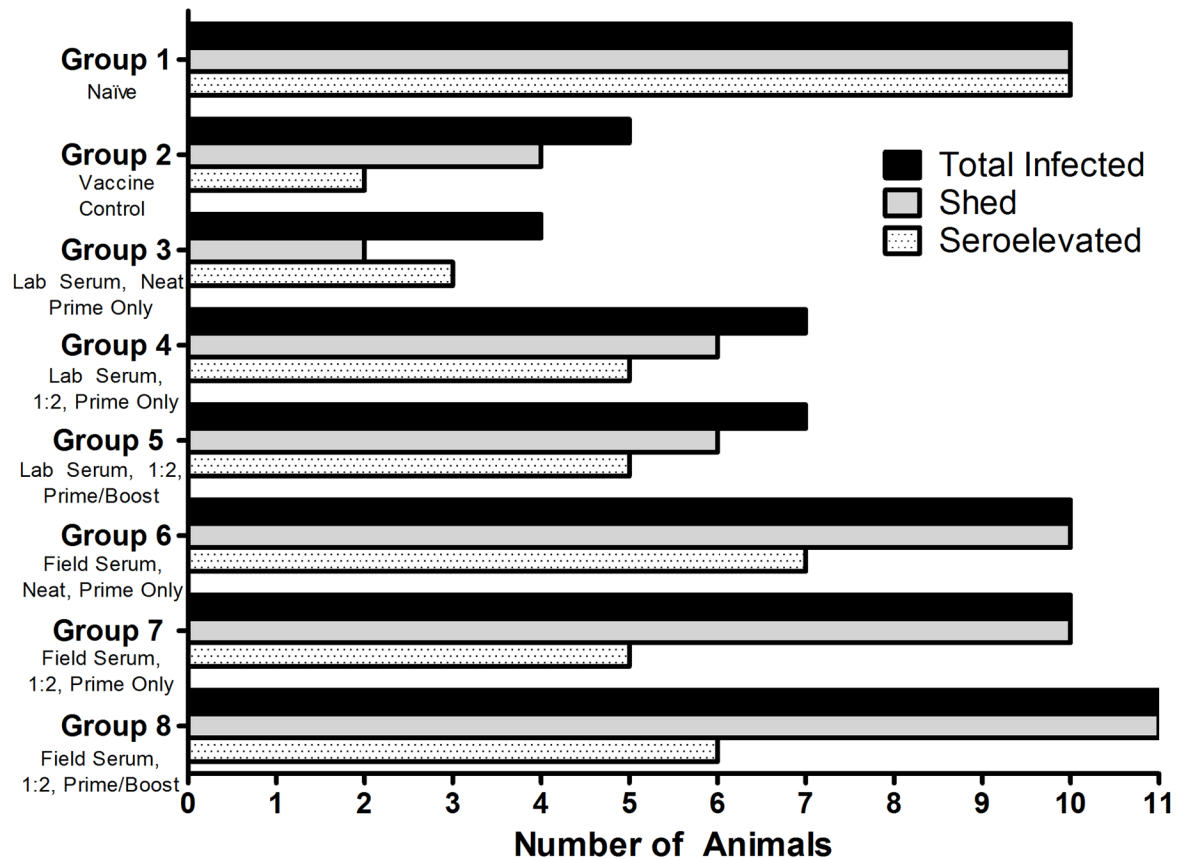
37. Viertlboeck BC, Schweinsberg S, Schmitt R, Herberg FW, Gobel TW. The chicken leukocyte receptor complex encodes a family of different affinity FcY receptors. *J Immunol.* 2009; 182:6985–92. [PubMed: 19454695]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript



**Figure 1.**

Total number of animals per group that were infected post-challenge as determined by virus shedding and seroelevation. A chicken was considered to be shedding if virus was isolated from oropharyngeal and/or cloacal swabs on at least one occurrence. A four-fold increase in HI titer 14 days post-challenge (compared to pre-challenge titer) was considered to be seroelevation suggestive of infection. The total number of animals infected per group is the combined total of animals that were shedding and/or seroelevated after challenge. Groups are as described in Table 1. Group 1, n=9; group 2, n=9; group 3, n=10; group 4, n=11; group 5, n=11; group 6, n=10; group 7, n=10; group 8, n=11.

Table 1

## Experimental Groups

| Group | Passive Immunization                | Days from Start of Experiment |          |                    |
|-------|-------------------------------------|-------------------------------|----------|--------------------|
|       |                                     | 1 Prime                       | 10 Boost | 35 Challenge       |
| 1     | PBS <sup>a</sup>                    | PBS                           | No       | Virus <sup>e</sup> |
| 2     | PBS                                 | 0.5 ml <sup>d</sup>           | No       | Virus              |
| 3     | Lab Antiserum <sup>b</sup> , Neat   | 0.5 ml                        | No       | Virus              |
| 4     | Lab Antiserum, 1:2 dilution         | 0.5 ml                        | No       | Virus              |
| 5     | Lab Antiserum, 1:2 dilution         | 0.5 ml                        | Yes      | Virus              |
| 6     | Field Antiserum <sup>c</sup> , Neat | 0.5 ml                        | No       | Virus              |
| 7     | Field Antiserum, 1:2                | 0.5 ml                        | No       | Virus              |
| 8     | Field Antiserum, 1:2                | 0.5 ml                        | Yes      | Virus              |

<sup>a</sup> Phosphate-buffered saline, negative control for immunization, vaccination and/or challenge

<sup>b</sup> Obtained from birds infected with A/Chicken/Mexico/Avilab/A0509/09 (H5N2)

<sup>c</sup> Obtained from birds vaccinated in the field with A/Chicken/Mexico/Avilab/A0509/09 (H5N2)

<sup>d</sup> Inactivated vaccine prepared against A/Chicken/Mexico/232/94 (H5N2)

<sup>e</sup> Challenged with A/Chicken/Mexico/Avilab/A0509/09 (H5N2)

**Table 2**  
Virus Shedding from Oropharynx in Chickens Challenged with Mexican Lineage H5N2 LPAI

| Group | Condition/Immunization        | Virus Titers <sup>a</sup> |                   |                   |                  |                  |                  |
|-------|-------------------------------|---------------------------|-------------------|-------------------|------------------|------------------|------------------|
|       |                               | 3 dpc <sup>b</sup>        | 5 dpc             | 7 dpc             | 9 dpc            | 11 dpc           | 13 dpc           |
| 1     | Naïve                         | 5.8 ± 1.7 (9/9)           | 6.4 ± 0.6 (9/9)   | 4.4 ± 1.4 (8/9)   | 2.0 ± 1.0 (8/9)  | 1.3 ± 0.7 (2/9)  | < <sup>c</sup>   |
| 2     | Vaccine Control               | 2.5 ± 4.2 (2/9)           | 4.4 ± 2.5 (4/9)   | 5.4 ± 1.3 (3/9)   | 6.5 ± 1.4 (2/9)  | <                | <                |
| 3     | Lab Antiserum, Neat           | 2.5 ± 0.0 (1/10)          | 3.5 ± 0.0 (1/10)  | 0.8 ± 0.0 (1/10)  | <                | <                | <                |
| 4     | Lab Antiserum, 1:2 dilution   | 3.9 ± 0.9 (2/11)          | 4.9 ± 0.5 (2/11)  | 6.8 ± 0.4 (2/11)  | 4.0 ± 1.8 (4/11) | 5.8 ± 1.2 (3/11) | 6.9 ± 0.5 (2/11) |
| 5     | Lab Antiserum, 1:2 dilution   | 6.1 ± 0.7 (3/11)          | 6.0 ± 0.9 (6/11)  | 4.1 ± 2.3 (6/11)  | 3.8 ± 2.5 (4/11) | 0.8 ± 0.0 (1/11) | <                |
| 6     | Field Antiserum, Neat         | 6.3 ± 1.8 (8/10)          | 6.5 ± 0.4 (10/10) | 4.3 ± 1.6 (10/10) | 3.8 ± 0.6 (5/10) | 2.3 ± 0.0 (1/10) | <                |
| 7     | Field Antiserum, 1:2 dilution | 4.2 ± 1.9 (4/10)          | 6.7 ± 1.0 (10/10) | 6.2 ± 0.4 (10/10) | 4.1 ± 2.5 (9/10) | 1.0 ± 0.4 (3/10) | <                |
| 8     | Field Antiserum, 1:2 dilution | 5.5 ± 0.0 (1/11)          | 5.8 ± 2.4 (6/11)  | 6.0 ± 0.9 (4/11)  | 5.4 ± 1.4 (4/11) | 4.3 ± 2.9 (6/11) | 7.0 ± 0.8 (6/11) |

<sup>a</sup>Expressed as the arithmetic mean (± sd), log<sub>10</sub> EID<sub>50</sub>/ml from 5 positive samples. Values below the limit of detection (0.75 log<sub>10</sub>EID<sub>50</sub>/ml) were excluded from calculations. Values in parenthesis are the number of birds shedding/total. For timepoints having fewer than 5 birds shedding, all positive samples were used to determine mean titer.

<sup>b</sup> days post-challenge

<sup>c</sup> < indicates all chickens at this time point had titers below the cutoff value.

HI Titers Induced by Passive Immunization, Vaccination & Challenge with Mexican Lineage H5N2 LPAI

Table 3

| Group | Condition/Immunization        | Mean Serum HI Antibody Titer ( $\text{Log}_2$ ) <sup>a</sup> |            |             |                |              |
|-------|-------------------------------|--|------------|-------------|----------------|--------------|
|       |                               | 1 (Prime)  | 10 (Boost) | 21          | 35 (Challenge) | 50           |
| 1     | Naïve                         | nd <sup>b</sup>  | nd         | nd          | < <sup>e</sup> | 5.8 (10/10)  |
| 2     | Vaccine Control               | nd   | nd         | 8.9 (9/9)   | 9.4 (9/9)      | 10.3 (9/9)   |
| 3     | Lab Antiserum, Neat           | 5.0 (6/9) <sup>c</sup>                                       | nd         | 5.3 (5/10)  | 7.1 (10/10)    | 9.1 (10/10)  |
| 4     | Lab Antiserum, 1:2 dilution   | 4.2 (7/11)   | <          | 6.0 (6/11)  | 7.5 (10/11)    | 8.5 (11/11)  |
| 5     | Lab Antiserum, 1:2 dilution   | 3.9 (7/11)   | <          | 5.9 (7/11)  | 8.4 (11/11)    | 10.0 (11/11) |
| 6     | Field Antiserum, Neat         | 3.6 (7/10)   | nd         | 5.3 (3/10)  | 6.8 (8/10)     | 8.4 (10/10)  |
| 7     | Field Antiserum, 1:2 dilution | 2.8 (5/10) <sup>d</sup>                                      | <          | 4.9 (7/10)  | 7.7 (10/10)    | 9.6 (10/10)  |
| 8     | Field Antiserum, 1:2 dilution | 3.3 (6/11)   | <          | 5.4 (10/11) | 8.7 (11/11)    | 10.1 (11/11) |

<sup>a</sup>Expressed as the  $\text{Log}_2$  of the reciprocal of the highest dilution that inhibited 4 hemagglutinating units of homologous virus. Results are presented at the arithmetic mean of positive sera (HI titer 1:10 dilution). The number of positive sera/total is indicated in parenthesis.

<sup>b</sup>Not determined

<sup>c</sup>1 bird found dead from natural causes prior to study commencement

<sup>d</sup>1 bird was removed from the study after being transferred to the wrong area of biocontainment

<sup>e</sup>< indicates all chickens at this time point had HI titers below the cutoff value