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ORIGINAL ARTICLE

Field assessment of an H5N1 inactivated vaccine in chickens and ducks in Lao PDR

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Abstract Despite the extensive use of poultry vaccines to control the spread of H5N1 influenza in poultry, H5N1 outbreaks continue to occur in domestic birds. Our objective was to determine the duration of the neutralizing antibody response under field conditions after vaccination with a laboratory-tested inactivated reverse genetics-derived H5N3 vaccine. H5N3 hemagglutination inhibition (HI) and virus neutralization (VN) antibodies were observed 40 weeks after vaccination of chickens with two doses and vaccination of ducks with one dose. Cross-clade antibodies to an H5N1 virus (A/chicken/Laos/A0464/07) antigenically distinct from the vaccine strain were detected in ducks after a single vaccination and were sustained for 28 weeks (for 40 weeks when a boost vaccination was given). Our results indicate that this inactivated H5N3 vaccine can produce long-lasting antibodies to homologous and heterologous viruses under field conditions.

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Introduction

For more than a decade, highly pathogenic H5N1 avian influenza viruses have persisted and reemerged in Asian countries, in many of which H5N1 has now become endemic. The continuing re-emergence of highly pathogenic H5N1 influenza in Asia highlights the need for H5N1 poultry vaccines that not only control disease signs but also prevent viral shedding. Highly pathogenic H5N1 viruses isolated in Asia are consistently pathogenic to chickens; however, ducks may have asymptomatic infection and shed virus, posing a silent threat to poultry and humans [9, 24]. Limited studies have examined the extent and duration of antibody responses induced by influenza vaccines in ducks. Several laboratory studies have examined whether H5 vaccines given to poultry can prevent H5N1 disease and reduce the shedding and spread of H5N1 viruses [4, 8, 11, 14, 16, 19, 22, 23, 27–31, 33]. Although antibody response and protection were correlated in these studies, they were assessed 1–3 weeks after vaccination, a time frame that is not relevant to field conditions. The duration of the antibody response induced by H5 vaccines under field conditions is not known.

Vaccination of poultry against avian influenza is a response to repeated outbreaks in recent years. Vaccination campaigns have been successful in the short term, but outbreaks have inevitably recurred. In Mexico, for example, vaccination programs against H5N2 epizootics have been under way since 1995 [32]. In the end, however, extensive vaccination caused antigenic drift from the vaccine strain, contributing to vaccination failure. In Southeast Asia, H5N1 vaccination programs have been instituted in Indonesia, Hong Kong, China, and Vietnam [5, 6, 21]. The concern in this region is inadequate vaccine coverage. In China, only 20–50% of all flocks were vaccinated, and

in Vietnam, only 40–60%. Although no outbreaks have been reported in vaccinated flocks, any H5N1 virus introduced into these flocks, e.g. virus shed by asymptomatically infected ducks, may be further disseminated by vaccinated poultry that are protected only against severe illness [7].

In Lao PDR, H5N1 vaccines are currently prohibited due to concern that vaccinated birds may shed virus without signs of infection. Control measures have included surveillance, culling or stamping out of infected flocks, disinfection, and restriction of movement within and across borders. Because of the continuing reemergence of H5N1, government authorities are re-evaluating the use of H5N1 poultry vaccines. In collaboration with the National Animal Health Centre in Lao PDR, we conducted local field-testing of a reverse genetics-derived, inactivated H5N3 vaccine shown under laboratory conditions to protect chickens and ducks from homologous and heterologous challenge and to eliminate virus shedding [33]. This study was designed to evaluate the vaccine's immunogenicity in local ducks and chickens and the duration of immunity to an H5N1 strain isolated in Lao PDR.

Materials and methods

Vaccine and viruses

The Poulvac® FluFend I AI H5N3 RG (Fort Dodge Animal Health, Fort Dodge, Iowa) inactivated vaccine was a previously reported reverse genetics-derived H5N3 (rgΔH5N3) strain containing the modified H5 from A/chicken/Vietnam/C58/04 (clade 1.0), the N3 from A/duck/Germany/1215/73, and the internal genes of A/Puerto Rico/8/34 in an oil–water emulsion [15]. The content of the hemagglutinin protein in allantoic fluid was standardized by the single radial immunodiffusion technique as described previously [34]. The clade-2.3.4 H5N1 virus used in serological analysis, A/chicken/Laos/A0464/076 (Laos/H5N1), was obtained from collaborators in Lao People's Democratic Republic, where it had recently been isolated. Stock viruses were grown in 10-day-old embryonated chicken eggs for 36–48 h at 35°C. The allantoic fluid was then harvested, and aliquots were stored at −80°C until use.

Chickens and ducks

This study was performed at the National Animal Health Center's vaccine production center in Lao PDR. Khaki Campbell layer ducks (*Anas platyrhychos*) and Vietnamese yellow chickens (*Gallus gallus domesticus*) 2–4 months of age were purchased from local commercial breeders and maintained in housing with free access to outdoor wire pens. Animal husbandry and experimental protocols were approved by the Department of Livestock and Fisheries, Lao PDR. Chickens (10 per group) and ducks (40 per group) were randomly assigned to receive no vaccine (controls), one vaccine dose (1.2 μg of HA antigen intramuscularly), or two vaccine doses 8 weeks apart. Blood was collected from the wing vein 2 weeks before and 2 weeks after primary vaccination and then approximately every 2 weeks for 40 weeks. Before vaccination, serologic testing of all birds showed HI antibody titers <10 for the H5 influenza A viruses.

Serological analyses

Hemagglutination inhibition (HI) assays were performed in microtiter plates with sera pretreated with a receptor-destroying enzyme, as described previously [18]. The antigen–antibody mixtures were tested for HA activity by addition of 0.5% chicken red blood cells (CRBCs). Virus neutralization (VN) assays were performed using Madin–Darby canine kidney (MDCK) cells in 96-well tissue culture plates, as described previously [10]. After incubation at 37°C for 72 h, supernatant from each well was tested for HA activity by adding 0.5% CRBCs. Geometric mean titers (GMT) were calculated for each group of serum samples. Neuraminidase inhibition (NI) tests were done by standard methods as described previously [1].

Results

Immunogenicity of standardized rgΔH5N3 vaccine in ducks and chickens

Serum HI antibody titers to the rgΔH5N3 (clade 1) vaccine virus and the Laos/H5N1 (clade 2.3.4) influenza virus were measured (Table 1). Only 20% of chickens administered a single vaccine dose had HI titers (mean titer 23) to the rgΔH5N3 virus; however, 1 month after a boost dose, 75% were seropositive (mean titer 66). These HI titers declined throughout the study but remained detectable at 40 weeks. A single vaccination was sufficient to induce HI titers against the rgΔH5N3 in 71% of ducks (mean titer 61). After the boost dose, the mean titer in ducks rose to 352, then declined but remained detectable in 88% of ducks at 40 weeks. In HI tests against the heterologous Laos/H5N1 influenza virus, only a single chicken had a positive titer, 1 month after boost vaccination. Ducks had low but detectable HI titers to Laos/H5N1 1 month after primary vaccination and 1 month after boost vaccination (mean titers 43 and 30, respectively). Although these cross-reactive HI titers were low, they remained detectable for up to

Table 1 Anti-HA antibodies in chickens and ducks after vaccination with clade 1 rgΔH5N3 vaccine

		Virus[a]	Mean HI titer[b] ± SD (% positive)					
			1 mpv	3 mpv (1 mpb)	5 mpv (3 mpb)	7 mpv (5 mpb)	8 mpv (7 mpb)	10 mpv (9 mpb)
Ducks	Single dose	H5N3	61 ± 25 (71%)	20 ± 13 (81%)	22 ± 13 (68%)	13 ± 1 (50%)	18 ± 6 (52%)	20 (1%)
(n = 25 per group)[c]		H5N1	43 ± 29 (19%)	10 (11%)	<	<	<	<
	Two doses	H5N3	169 ± 54 (85%)	352 ± 238 (100%)	82 ± 67 (100%)	79 ± 66 (88%)	91 ± 77 (100%)	60 ± 42 (88%)
		H5N1	25 ± 11 (32%)	30 ± 10 (42%)	16 ± 3 (40%)	24 ± 11 (30%)	18 ± 10 (30%)	17 ± 11 (27%)
Chickens	Single dose	H5N3	23 ± 15 (20%)	<	<	<	<	<
(n = 10 per group)		H5N1	<	<	<	<	<	<
	Two doses	H5N3	<	66 ± 51 (75%)	43 ± 22 (75%)	35 ± 6 (50%)	55 ± 30 (62%)	13 ± 6 (43%)
		H5N1	<	10 (11%)	10 (13%)	10 (13%)	<	<

[a] H5N3 A/chicken/Vietnam/C58/2004 (ΔH5), clade 1; H5N1 A/chicken/Laos/A0464/07, clade 2.3.4

[b] Hemagglutination inhibition (HI) titers are the reciprocal of the highest serum dilution at which agglutination was inhibited

[c] 25 of the 40 ducks in each vaccination group were randomly sampled at each collection point

mpv months post-primary vaccination; *mpb* months post-boost vaccination; < mean HI titer <10

3 months after a single vaccination and for the entire 10-month study period after a boost dose. No HI titers were observed in control birds.

NI testing of serum samples from ducks was performed to confirm that HI antibody titers to H5 viruses indicated a response to vaccination rather than H5N1 infection. The anti-neuraminidase antibodies in these samples inhibited the N3 but not the N1 reference antigens (data not shown), confirming that the antibodies were generated in response to vaccination.

Neutralizing antibodies in ducks and chickens

We next determined the vaccine's ability to induce virus-neutralizing antibodies (Fig. 1). VN titers to the rgΔH5N3 vaccine were more robust in ducks (GMT, 285) than in chickens (GMT, 25). Neutralizing titers waned after a single vaccination; titers were detectable in ducks throughout the study but were undetectable in chickens 28 weeks after vaccination. VN titers against rgΔH5N3 peaked in ducks (GMT, 753) and chickens (GMT, 71) 1 month after boost vaccination and remained detectable throughout the study. Chickens showed no VN titers against the Laos/H5N1 virus after either primary or boost vaccination. In ducks, the GMT to the H5N1 virus was 133 one month after primary vaccination, then declined rapidly but remained detectable for 28 weeks. One month after boost vaccination, this cross-reactive VN titer was 358. It then declined but remained detectable 40 weeks after primary vaccination.

Discussion

There has previously been little information about influenza vaccination of poultry under field conditions. In laboratory studies, vaccination against H5N1 influenza viruses has protected ducks [8, 11, 16, 22, 23, 29, 30] and chickens [4, 13, 14, 19, 25–27, 31] against lethal challenge, but the duration of the antibody response, which is a crucial factor in the field [3, 28], has not fully been addressed. Our results show that the vaccine responses in chickens and ducks can differ markedly under field conditions. Antibodies were observed in both species; however, the response was more robust in ducks, as were the cross-reactive HI and VN titers to an antigenically distinct, locally isolated H5N1 virus. Because ducks appear to perpetuate and spread H5N1 influenza viruses in Eurasia, vaccination of domestic ducks will help to control outbreaks.

Poultry raised for meat consumption, market, egg production, or breeding stock must be protected from infectious disease. In Lao PDR, poultry are ready for sale as meat at 2–3 months of age, but poultry for egg production

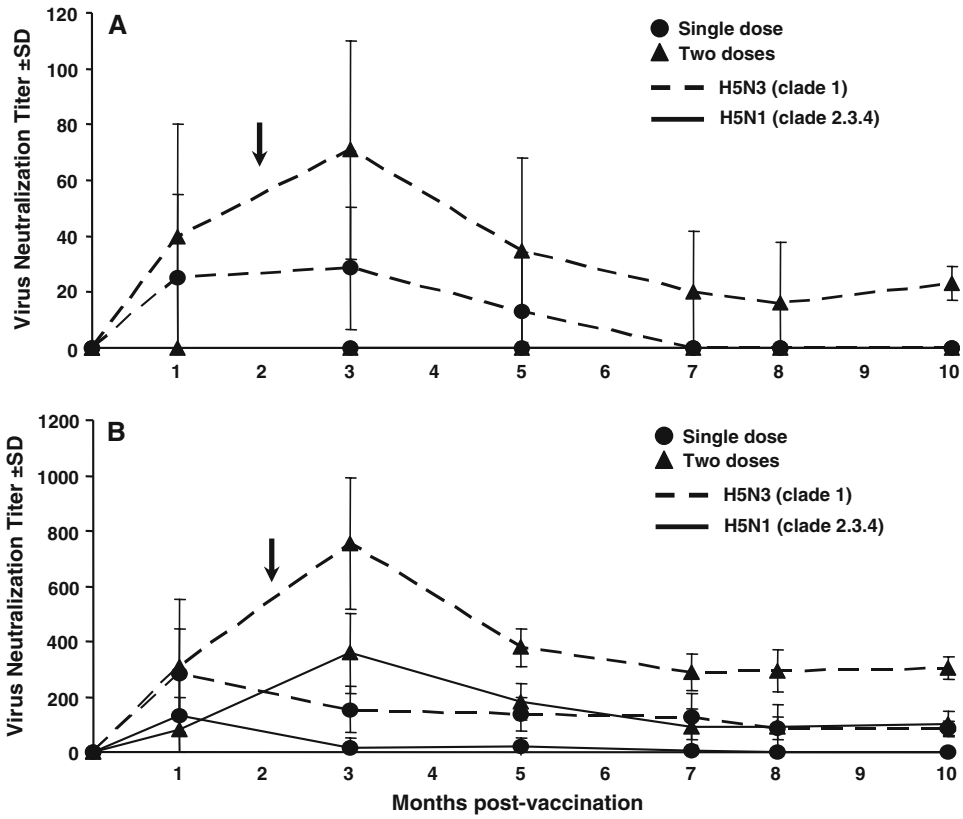

Fig. 1 Duration of neutralizing antibody response to two clades of H5 influenza virus induced by inactivated rgΔH5N3 vaccine in chickens (**a**) and ducks (**b**). *Lines* indicate mean antibody titers to the H5N3 vaccine strain (*dashed*) and the H5N1 virus (*solid*). *Bars* indicate the standard deviation. *Arrows* indicate time of boost vaccination

and breeding stock are kept for up to 3 years. We did not monitor the antibody response for more than 40 weeks. However, the robust neutralizing antibody response to the rgΔH5N3 vaccine 1 month after a single vaccination suggests that ducks raised for meat would be adequately protected for life. The maintenance of antibodies for the duration of the study suggests that with the use of a boost dose, this vaccine would also protect egg production and breeding stock.

Cross-reactive neutralizing antibodies were detected only in ducks. The failure of chicken rgΔH5N3 antibodies to cross-react with Laos/H5N1 may be attributed to differences in homology between the HA1 glycoproteins of the rgΔH5N3 and Laos/H5N1 viruses (93% homologous). Previous studies in chickens have shown a correlation between hemagglutinin sequence similarity and the ability of vaccine to reduce tracheal titers of challenge virus [13, 26]. While vaccination provided complete protection and eliminated shedding of homologous virus in chickens [12, 14, 33], it provided only partial protection [19] or did not eliminate shedding [13] of a heterologous challenge virus that shared 93.2–94.2% similarity. In ducks, antigenic disparity does not appear to affect vaccine cross-reactive efficacy even when cross-reactive HI or VN titers are low or undetectable [2, 11, 16, 30]. Understanding how species differ in their response to vaccination is crucial for future vaccine development.

Appropriate vaccination can help to control the spread of highly pathogenic H5N1 viruses in poultry. It is speculated that 90% of a flock must be vaccinated to ensure flock immunity [20]. It also requires a high-quality vaccine that elicits a lasting antibody response. In our study, chickens produced HI antibodies homologous to the inactivated vaccine strain for 28 weeks but were not challenged with homologous virus to confirm protection [8]. Tian et al. [28] reported protection from lethal challenge and reduced shedding in ducks 52 weeks after high-dose primary vaccination (4.6 and 9.2 μg of HA vs. 1.2 μg in our study). Despite the absence of challenge experiments, our results show that one vaccination stimulates a 40-week neutralizing antibody response to homologous rgΔH5N3 virus in ducks. More importantly, cross-clade-reactive neutralizing antibodies were observed in ducks for 20 weeks after a single vaccination and for 40 weeks after a boost vaccination.

In the current study, a clade 1 virus (rgΔH5N3) induced cross-reactive antibodies in ducks that neutralized a clade 2.3.4 virus (Laos/H5N1) in vitro. Induction of immunity to antigenically distinct clades is crucial in Eurasian countries where H5N1 has become endemic. Lao PDR and Vietnam experienced the introduction of H5N1 viruses from antigenically different clades due to the movement of poultry. In Vietnam, the dominant clade 1 viruses were replaced by clade 2.3.4 viruses in the north, while clade 1 remained

dominant in the south [17]. H5N1 viruses have been introduced into Lao PDR multiple times since 2003. These viruses belonged to clade 1 in 2003–2004; clade 2.3.4 viruses were then isolated in 2006–2007, and in 2008 clade 2.3.2 viruses were introduced. Vaccines are most effective against viruses that are homologous or antigenically similar to the vaccine strain; however, H5N1 vaccines that can elicit broad cross-protective immunity would be ideal. Our findings are promising for countries that experience the introduction of antigenically distinct viruses.

Our results provide information about the duration of immunity in local strains of chickens and ducks that will assist the Lao PDR authorities in evaluating the use of poultry vaccines. The availability of an H5N1 vaccine that can elicit antibodies that cross-react with antigenically different H5N1 viruses will be crucial for early control of outbreaks, in conjunction with culling and stamping out of infected flocks. Vaccination strategies implemented in China and Vietnam were initially successful at eliminating the transmission of H5N1 from poultry to humans; however, in time, outbreaks in poultry recurred. The practical difficulties of vaccination, as well as the rapid antigenic drift of H5N1 viruses, are likely to be responsible. The continued circulation of H5N1 viruses in Southeast Asia has resulted in the emergence of new strains, requiring that vaccine strains be updated each year or created for use in specific countries or regions. Short-term use of vaccination of chickens in Hong Kong in 2003 was a successful control strategy that halted transmission of H5N1 virus [6]; therefore, emergency vaccination in response to H5N1 outbreaks should be considered as an alternative to prophylactic vaccination. Although H5N1 is not yet endemic in Lao PDR, the country is subject to frequent reintroduction of H5N1 viruses from neighboring countries in which H5N1 is endemic. As outbreaks of highly pathogenic H5N1 strains continue to occur in poultry in Lao PDR, it may become necessary to re-evaluate the use of vaccines as part of the control strategy.

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