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Avian influenza H5N1 vaccination efficacy in Egyptian backyard poultry

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Abstract

Raising backyard poultry under low biosecurity conditions is a common practice in Egypt. While vaccination is routinely applied in Egypt in commercial settings to curb the spread of avian influenza viruses, it remains less commonly used in backyard settings. We assessed the immunogenicity and protective efficacy of a H5N1 vaccine based on a contemporary Egyptian clade 2.2.1.2 virus among turkeys, ducks, geese, and chickens raised together in a backyard setting. Results showed that this vaccine elicits an immune response in all tested species reaching up to a hemagglutination inhibition titer of 10 log₂ after a booster dose. However, this response varied between species. When challenged, vaccinated birds survived and shed less virus in comparison with unvaccinated birds. However, unvaccinated ducks showed no symptoms of infection and survived the duration of the experiment. Moreover, vaccinated ducks shed more virus as compared to vaccinated birds of other species. Hence, we recommend avoiding mixing various species in the backyards of Egypt. Our data indicates that vaccination can be effective in the backyard setting in Egypt, although planning should consider the species covered.

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Conflict of interest

The authors declare no conflict of interest.

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Keywords

Avian influenza virus; H5N1; Vaccine; Egypt; Backyard

Introduction

Poultry production in Egypt is classified into four sectors. Sector 1 includes industrial integrated farms raising more than 20,000 birds per cycle. Sectors 2 and 3 comprise commercial production systems raising 5,000–20,000 and 500–5,000 birds per cycle, respectively. Sector 4 refers to backyard poultry rearing [1]. In Egypt, backyard poultry production is in close geographical contact with Sector 2 and 3 farms that are commonly found in rural areas. Raising backyard poultry is considered as a main source of protein and sustenance for the clear majority of Egyptian farmers. Backyard poultry are generally raised without control conditions or biosecurity measures making them susceptible to acquiring and transmitting infectious diseases [2].

Avian influenza (AI) is a highly contagious viral disease that can affect several domestic bird species[3]. During the first wave of AI H5N1 outbreaks in Egypt in 2006, massive die-off of domestic poultry was recorded [4]. Therefore, the national veterinary authorities devised a comprehensive action plan to control the spread of the virus in Egypt. This included increasing public awareness through media campaigns, culling infected poultry, poultry movement restrictions and emergency vaccination [5, 6]. Although the number of recorded H5N1 outbreaks decreased as a result of the implemented activities [6, 7], the control strategy plan failed to restrain the spread of H5N1 virus in Egypt eventuating an enzootic situation [8]. Accordingly, two subclades of H5N1 viruses, 2.2.1 and 2.2.1.1, co-occurred in poultry from late 2009 through 2011 [9]. Subclade 2.2.1.1 viruses are thought to have emerged as vaccine escape mutants as a consequence of vaccine application [10]. Subclade 2.2.1 viruses evolved into a new phylogenetic cluster recently classified as clade 2.2.1.2 [11]. Those subclades (2.2.1, 2.2.1.1 and 2.2.1.2) were antigenically distinct [6]. Recently, Kayali *et al.* investigated the efficacy of 24 commercial inactivated avian influenza H5 vaccines that were authorized to use in Egyptian poultry [6]. Different influenza A/H5 viruses were thus used as vaccine seed strains, including classical low pathogenic H5Nx viruses or H5N1 reassortants with surface glycoprotein genes (HA and NA) of H5N1 viruses in the genetic background of A/Puerto Rico/8/1934 (H1N1) strain. Abdelwhab *et al.*, reviewed vaccine efficacy studies against different Egyptian H5N1 viruses under experimental conditions [12]. Interestingly, some of the tested vaccines were immunogenic and protected chickens when challenged by infection with Egyptian highly pathogenic H5N1 viruses under laboratory conditions [13–15]. However, the H5N1 and H5N2 experimentally prepared vaccines fared poorly when tested in the field in Egypt [16]. Previous studies on the cross-reactivity of commercial H5 poultry vaccines against H5N1 Egyptian isolates in the field setting in Egypt showed that only one vaccine based on an Egyptian H5N1 virus induced high cross-reactive antibody titers [17]. This discrepancy between the laboratory and field settings, regarding the protective response of H5-based vaccines, must be assessed knowing that backyard-bird rearing is wide spread in Egypt hence representing a major obstacle to the eradication of HPAI H5N1 virus from Egypt.

However, the efficacy and the feasibility of AI vaccination in different poultry hosts in backyards is yet unevaluated. Here, we assessed the immunogenicity and efficacy of a reassortant/inactivated H5N1 vaccine based on a currently circulating Egyptian H5N1 clade 2.2.1.2 virus in different avian hosts which are commonly reared in Egyptian backyards.

Materials and methods

Virus

H5N1 isolate, A/chicken/Egypt/D10552B/2015 (clade 2.2.1.2, D10552B), was used for preparation of an experimental vaccine and laboratory challenge experiments. The pathogenicity of HA of D10552B virus (accession no. AMN14801) was attenuated by changing the multibasic amino acids at the cleavage site (KRRKKR) to be monobasic amino acid (R). For vaccine strains preparation, plasmid-based reverse genetics system was applied using six internal genes of A/Puerto Rico/8/34 (H1N1) and two surface LP-HA and NA genes of D10552B [18]. The generated vaccine strain, rgD10552B, was propagated in 10-day-old specific pathogen free-embryonated chicken eggs (SPF-ECE) (Koum Oshiem SPF Chicken Farm, Fayoum, Egypt). The mutation at the cleavage site of rescued rgD10552B virus was confirmed by sequencing. The rgD10552B virus was propagated in SPF-ECE for 3 passages and did not result in embryo death.

LPAI G1-like A/chicken/Egypt/S10489C/2015 (H9N2) isolate was used as antigen for HI assays against H9N2 viruses.

Vaccine

To make the experimental vaccines, the rgD10552B was propagated in SPF-ECE for three successive passages to a titer of $10^{7.5}$ EID₅₀/0.1ml. Allantoic fluids were harvested, tested for sterility, and inactivated by addition of 0.1% formalin and mixed with Montanide ISA 70 VG (Seppic, France) in the ratio recommended by the manufacturer (30 antigen/70 adjuvant). The inactivated virus fluid was then examined for residual infectivity in SPF-ECE and tested for sterility and safety [19].

Vaccine Efficacy in SPF chickens

SPF chicken eggs were hatched at the Center of Scientific Excellence for Influenza Viruses, National Research Centre, Cairo, Egypt. Sera collected from 10 randomly selected chicks were tested for H5 and H9 antibodies resulting from maternal passively transferred immunity at weeks one and two of age. Using hemagglutination inhibition assay (HI) with Chicken red blood cells [20], the antibody titer against H5N1 and H9N2 viruses were monitored and corresponding Log₂ titer was calculated. Ten chickens received 0.5 ml of the vaccine by intramuscular injection through the thigh at 14 days old. One group was used as control and received 0.5 ml PBS. Weekly and up to the fourth week post vaccination (wpv), blood samples were withdrawn from chickens in each group to evaluate antibody titer against rgD10552B by HI.

At 4 wpv, five animals of each group were infected with 0.5 ml of challenge virus at a dose of $10^{6.5}$ EID₅₀/ml of HP D10552B virus via the natural route (i.e., intranasal, intraocular,

and intratracheal) Birds were then monitored daily for morbidity and mortality. Cloacal and oral swabs were collected from each bird at days 2, 3, 5, and 7 post infection for virus titration in eggs. All animal experiments were approved by the Ethics Committee of the National Research Centre, Egypt.

Backyard trial

The trial was conducted during March and April 2016 concurrently at three different backyard sites in Kafr Elshekh, Sharkia and Gharbia governorates in the Nile Delta region of Egypt. Breeding conditions typically applied in Egyptian backyard settings were implemented including allowing free range, supplementing the diet with crop waste or food leftovers, and using household water source for drinking. Forty eight of each: chickens, Pekin ducks, domestic geese (White Embden geese), and turkeys raised at specific nursery farms were purchased at about 14–21 days of age as is the practice in Egypt [21]. To enable monitoring maternal immunity antibody (MIB) levels, 8 serum samples were collected per bird type and tested for H5 and H9 antibodies using HI. Sixteen of each bird type were distributed over the three backyard sites and housed at the same location. A week after placement for adaptation, 8 birds of each type per site were vaccinated with 0.5 ml of the experimental vaccine by intramuscular injection and 8 birds of each type served as unvaccinated controls. On vaccination day and on a weekly basis thereafter, blood was collected from 5 birds per type from each group to test for vaccine immunogenicity. In the 5th week post vaccination, a booster dose of 0.5 ml of experimental vaccine was administered. The birds remained on site until the 7th week post-first vaccination dose.

Before starting the experiment and weekly thereafter, we collected oropharyngeal swabs, cloacal swabs, surface swabs from each breeding site, water sample and air sample to monitor the presence of influenza A viruses (IAV) by PCR according to previously published methods [9, 22] to ensure that the birds remained influenza-free during the study. Air sampling was done using a Coriolis cyclone sampler (Bertin Technologies, Montigny-le-Bretonneux, France). Ten milliliters of water with 0.01% Tween 20 was used in the sampling cones of the Coriolis sampler, which was run for a period of 10 min at 300 liters/min.

Challenge of vaccinated backyard poultry with HPAI H5N1

Three animals from each group were randomly removed from all sites, placed in BSL-3 certified isolators and infected with 0.5 ml of challenge viruses at a dose $10^{6.2}$ EID₅₀/bird of HP D10552B virus via the natural route at 7 wpv. Birds were then monitored daily for morbidity and mortality. Cloacal and oral swabs were obtained from each bird at days 1, 3, and 5 post infections for virus titration in eggs.

Statistical analyses

Statistical analyses were done using GraphPad Prism V5 (GraphPad Inc., CA, USA). ANOVA with Tukey post hoc test was used to compare antibody titers. Differences were considered statistically significant at $p < 0.05$.

Results

Immunogenicity and protective efficacy of inactivated vaccine in SPF chickens

Immunized SPF chickens developed a significant immune response at 3 wpv ($P < 0.001$) that increased significantly by the fourth week when compared to the unvaccinated chickens (Figure 1A). Vaccinated chickens had 100% survival without any observed symptoms of AI infection. All chickens in the non-vaccinated control group died after two days post infection with typical severe symptoms of H5N1 infection including cyanotic combs and wattles, edema of the head, and leg shank hemorrhage. Besides, no AI virus was recovered from the cloaca or oral swabs of all vaccinated SPF chicken groups at days 2, 3, 5 and 7 post infection. Virus shedding was detected in unvaccinated chickens at day 2 in oral and cloacal swabs with $\text{Log}_{10} \text{EID}_{50}$ 6.5 ± 0.5 and 2.6 ± 0.45 , respectively (Table 1).

Inactivated vaccine immunogenicity and efficacy in backyard poultry

Four backyard poultry species were immunized using the inactivated rgD10552B and kept at three different sites of Egyptian delta region for 5 weeks, followed by booster vaccination and housing for 2 more weeks. Before and after starting the experiment, AI virus was not detected in any weekly randomly collected swabs from the different poultry species.

a) Duck—The HI antibody profile in the vaccinated vs unvaccinated ducks in backyards is shown in Figure 2A. Vaccinated ducks developed a significant HI antibody titer ($P = 0.001$) after 2 wpv (week 6 of age) compared to unvaccinated ducks. There were no significant changes in HI titers of vaccinated ducks from the 2nd week to the 5th wpv ($P > 0.05$). The antibody titer of vaccinated ducks increased sharply and reached the peak ($9.4 \log_2 \text{HI}$) at the first week post booster vaccination dose then slightly decreased ($8.8 \log_2 \text{HI}$) at the following week. There were no significant differences in the level of HI titer ($P > 0.05$) among the three districts in seropositive vaccinated ducks (Figure S1A).

In the challenge experiment, all vaccinated and non-vaccinated ducks did not show any observed symptoms of AI infection. At 1 dpi, no virus was recovered from the cloaca of all vaccinated ducks but two ducks of control were shedding virus with $\text{Log}_{10} \text{EID}_{50} = 2.25 \pm 0.35$ in cloacal swabs. Only one duck was shedding virus in oral swab with $\text{Log}_{10} \text{EID}_{50} = 2 \pm 0.0$ in vaccinated and control group at day 1 (Table 2). At day 3, AIV was isolated from 3 surviving ducks in oral and cloacal swabs of the control group ($\text{Log}_{10} \text{EID}_{50} = 3.8$). One of the 3 vaccinated ducks was shedding virus in oral swab ($\text{Log}_{10} \text{EID}_{50} = 2 \pm 0.0$). By day 5, virus was detected in the 3 survived ducks in the control group with 2.5 and 2.6 $\text{Log}_{10} \text{EID}_{50}$ in oral and cloacal swab, respectively. No virus was detected in the cloacal swabs of the vaccinated ducks at day 5 while all ducks in the control group was shedding virus with 2.5 $\text{Log}_{10} \text{EID}_{50}$ (Table 2).

b) Turkey—The HI antibody profile in the vaccinated vs unvaccinated turkeys in the field is shown in Figure 2B. Compared to unvaccinated, the vaccinated turkeys developed significant HI antibody titers after 2 weeks of the first shot of the vaccine ($P = 0.001$), and the titers reached 8.2, 6.8 and 5.2 $\log_2$ at Sharkia, Gharbia and Kafr Elshekh, respectively (Figure S1A). By week 3 post vaccination, the HI titer increased in vaccinated turkeys at all

sites. No increase was observed in the vaccinated turkeys at week 4 in Sharkia and Gharbia while the titer decreased two fold in the vaccinated turkeys of Kafr Elshekh. The antibody increased at the first week after booster immunization and reached its peak at the following week. AI virus was not detected in any randomly collected swabs from turkeys every week.

Following challenge infection, all vaccinated turkeys survived without any observed symptoms of influenza infection. All unvaccinated turkeys died after 8 dpi (at days 5, 6 and 8) with remarkable clinical signs of AI infection. At 1 dpi, no AIV was recovered from the cloacal and oral swabs from vaccinated or control turkeys (Table 2). At day 3, AIV was isolated from all surviving turkeys in the control group with virus titer 2.5 and 3.2 $\text{Log}_{10}\text{EID}_{50}$ in oral and cloacal swabs. Two of the 3 vaccinated turkeys were shedding virus in oral ($\text{Log}_{10}\text{EID}_{50}$ 2.25±0.35) and cloacal swabs ($\text{Log}_{10}\text{EID}_{50}$ =2.75±0.35). By day 5, AIV was detected in the 2 surviving turkeys of the control group with 2.75 and 2.5 $\text{Log}_{10}\text{EID}_{50}$ in oral and cloacal swab respectively. No virus was detected in the collected swabs of the vaccinated turkeys at day 5 (Table 2).

c) Goose—The HI antibody profile in the vaccinated vs unvaccinated geese in the field is shown in Figure 2C. No significant differences were observed between vaccinated and unvaccinated geese until week 2 post vaccination ($P > 0.05$). Vaccinated geese developed a significant HI antibody titer after 3 weeks of the first dose of the vaccine ($P = 0.001$). After the booster dose, the antibody titer increased gradually and peaked at the second week (week 11 of age).

All vaccinated geese showed 100% survival without any observed symptoms of infection after challenging with influenza A/chicken/Egypt/D10552B/2015 (H5N1). One of the unvaccinated geese died at 6 dpi. At 1 dpi, no virus was recovered from the cloacal and oral swabs from vaccinated geese. Two geese in the control group shed virus at the first day post infection in cloacal swabs with virus titer $\text{Log}_{10}\text{EID}_{50}$ 2.25±0.35 (Table 2). At day 3, AIV was isolated from all geese in the control group with virus titer 2.3±0.5 and 2.6±0.5 $\text{Log}_{10}\text{EID}_{50}$ in oral and cloacal swabs respectively. One of the 3 vaccinated geese was shedding virus in oral ($\text{Log}_{10}\text{EID}_{50}$ 1.5) and cloacal swabs ($\text{Log}_{10}\text{EID}_{50}$ 2.5). By day 5, virus was detected in the 3 surviving geese of the control group with 3.3 and 2.8 $\text{Log}_{10}\text{EID}_{50}$ in oral and cloacal swabs, respectively. No AIV was detected in the cloacal swabs of the surviving vaccinated geese at day 5 but only one goose was shedding virus in the oral swab with viral titer 2.5 $\text{Log}_{10}\text{EID}_{50}$ (Table 2).

d) Chicken—The HI antibody profile in the vaccinated vs unvaccinated chickens in the field is shown in Figure 2D. No significant differences were observed among the compared vaccinated chicken group at 1 wpv ($P > 0.05$). Chicken developed significant HI antibody titer after 2 weeks of the first vaccine shot ($P = 0.001$) in the vaccinated group that increased with time till week 5 of the first dose. The HI titer slightly decreased in the vaccinated chickens after one week of the second dose that reached the peak at the second week of the booster shot.

Vaccinated and non-vaccinated chickens were inoculated with homologues HPAI H5N1 viruses via natural routes of infection with 10^6 $\text{EID}_{50}/\text{ml}$ of virus in a total volume of 0.5 ml.

Vaccinated chickens showed 100% survival without any observed symptoms of AI infection. All unvaccinated chickens died after 6 dpi (at day 3, 4 and 5) with typical clinical signs of HPAI infection.

No AIV was recovered from the oral and cloacal swabs from vaccinated chicken group at day 3 and 5 post infection. At day 3, AIV was isolated from 2 of 3 survived chickens of the control group with virus titer $3.8 \text{ Log}_{10} \text{EID}_{50}$ in oral and cloacal swabs. By day 5, virus was detected in the only survived chicken of the control group with $3 \text{ Log}_{10} \text{EID}_{50}$ of oral swab (Table 2).

Intraspecies immune response variability

The comparison of the immune response of inactivated vaccine between different vaccinated hosts showed that the vaccinated geese produced significantly the lowest antibody titers in response to AI vaccination as measured by HI ($P = 0.001$) at the first three weeks post vaccination (Figure 2E) which were moderately recovered ($P = 0.01$) at week 4 post vaccination. However, the vaccinated chicken showed HI titers at 3 weeks post vaccination contrary to ducks and turkeys (Figure 2E). At week 4, the vaccinated turkeys revealed significant HI titers ($P = 0.01$) compared to vaccinated geese. The vaccinated turkeys showed significantly higher HI titers ($P = 0.05$) than all other vaccinated hosts at 3 weeks post vaccination and the mean HI titer was $7.9 \log_2$ (Figure 2E). At week 4, the vaccinated turkeys had significant higher HI titers ($P = 0.01$) than vaccinated geese.

Discussion

Backyard poultry rearing is a common practice across rural areas of Egypt and other countries. This sector is of high importance in sustaining AI transmission due to the lack of biosecurity measures and lack of vaccination. Here we sought to investigate the immunogenicity and efficacy of a reassortant/inactivated H5N1 vaccine based on a currently circulating Egyptian H5N1 clade 2.2.1.2 virus in different avian hosts which are commonly reared in backyards at three different locations of the Egyptian Delta region with high intensity of backyard poultry rearing. Normal breeding conditions were applied without any restrictions and biosecurity measures.

Despite the extensive use of poultry vaccines to control the spread of H5N1 influenza in poultry, few studies have been conducted evaluating the efficacy of vaccination in the field especially under backyard conditions [17, 23]. Several laboratory studies showed that the vaccination against H5N1 influenza viruses has protected ducks and chickens against lethal challenge. These experimental studies cannot completely simulate field conditions where the birds can be immunosuppressed by other infectious agents and/or environmental stress. In this study, the efficacy of inactivated vaccine was first evaluated under laboratory conditions. The results showed that the tested vaccine was immunogenic in SPF chickens under laboratory conditions. The high titers of antibodies induced in chickens after a single dose of vaccine suggest that a single immunization dose would provide protective immunity. By challenge infection, vaccinated chickens survived without any observed symptoms of AI infection and without virus shedding either in cloacal or oral swabs.

Previous observations in the field and from experimental studies detected MIB titers that might interfere with the vaccine [14, 17, 24, 25]. We detected MIB titers against H5N1 clade 2.2.1.2 virus ranging from 3.5 to 4.5 Log₂ HI titer at 3 weeks of age in all tested poultry species. Contrary to the previous study, the MIB titers slightly increased at age of 4 weeks (day of vaccination) despite the absence of the influenza virus infection as previously observed [17]. Our results indicate that the currently developed vaccine is immunogenic with homologue Egyptian H5N1 virus in different poultry species when used in the backyard even after MIB levels increase. Ducks and geese produced significantly lower antibody titers in response to AI vaccination than chickens and turkeys after the first shot suggesting that the vaccination program for different avian species should be carefully planned for application under field conditions. The level of the antibody titer increased markedly after 2 weeks of a booster dose in all poultry types. Similarly to our results, a previous study showed that chickens and ducks developed HI antibody titers very quickly compared to geese that needed a booster dose to increase titers [26]. Also, a previous study indicated that a single dose of inactivated vaccine did not provide adequate immunity for ducks and geese against HPAI H5N1 virus [27].

Previous studies showed a correlation between serologic response and protection against mortality and viral shedding [28, 29]. Also, the OIE terrestrial manual suggests that the protection from mortality and protection from virus shedding depend on serological potency [30]. Kumer *et al.* showed that low antibody titers of 10 to 40 prevent mortality but not viral shedding while titers greater than 40 prevent mortality and reduce shedding [28]. Previous evidence showed that serological titers are associated with protection when the challenge and vaccine viruses are genetically and antigenically closely related. The presence of HI antibodies predicted protection in the field as well [31].

In the challenge experiment, all vaccinated birds regardless of species survived while unvaccinated chickens, turkeys, and geese encountered morbidity and mortality. Interestingly, non-vaccinated ducks did not show any observed signs of AI infection and survived the challenge. Survival of infected ducks with HPAI H5N1 virus indicates a low sensitivity of Pekin ducks to this clade 2.2.1.2 H5N1 challenge virus. This finding is consistent with a previous study [32]. Furthermore, vaccinated ducks continued to shed virus at higher titers than vaccinated birds of the other species. This indicates that ducks may be playing a significant role in transmission of HPAI H5N1 in Egypt as they show no symptoms after infection and shed virus. It is important to note that the group sizes in the challenge experiment allowed us to quantify differences in mortality and shedding.

However, it was not sufficient to enable proper statistical comparisons.

Consistent with our results, many studies showed that inactivated oil adjuvanted vaccines were effective in protecting domestic poultry against HPAI H5N1 viruses [33–35]. Nevertheless, a successful vaccination campaign should consider not only the poultry production sector, but also the species of the birds and the appropriate age for vaccination. In Egypt, particular attention should target backyard setting with work focusing on increasing awareness, enhancing biosecurity, separating species and vaccinating under emergency situations. It is common for backyard poultry growers in Egypt to purchase different types of

birds at 3 to 4 weeks of age from specific nursery farms [21]. Hence, vaccination at nursery farms may be a feasible vaccination strategy. Authorities should also consider the logistics of such a campaign and who would carry the cost without adding a financial burden on the relatively poor backyard growers. Similar strategies can be implemented in countries where avian influenza viruses are enzootic especially in the backyard sector.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Immunogenicity and protective efficacy of inactivated vaccine in SPF chickens. **A)** Weekly antibody titers of vaccinated SPF chickens against H5N1 using HI assay. Arrow indicated time of vaccination. **B)** Survival curves of immunized and unimmunized SPF chickens (n = 5 per group) after challenge with an HPAI H5N1 virus.

Figure 2.

Immunogenicity of inactivated vaccine in backyard poultry by weekly antibody titers measure of vaccinated poultry against H5N1 using HI assay. Arrow indicated time of vaccination **A)** ducks, **B)** turkeys, **C)** geese, **D)** chickens, **E)** comparing different vaccinated hosts.

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Figure 3.
Survival curves of immunized and unimmunized poultry after challenge with an HPAI H5N1 virus.

Table 1

Titers of A/chicken/Egypt/D10552B/2015(H5N1) virus in cloacal and oral samples obtained from immunized and unimmunized SPF chicken groups^a.

Day post challenge	Control		Vaccinated	
	Oral swab	Cloacal swab	Oral swab	Cloacal swab
2	6.5±0.5 (5/5)	2.6±0.45 (5/5)	<1 ^b	<1
3	ND	ND	<1	<1
5	ND	ND	<1	<1
7	ND	ND	<1	<1

^aData are the mean Log₁₀ EID₅₀/ml ± SD for positive samples. Values in parentheses are number of positive chickens out of each group.

^bThe titer was below the limit of detection (<1 Log₁₀ EID₅₀/ml)

ND: Not determined because of death.

Table 2

Titers of A/chicken/Egypt/D10552B/2015(H5N1) virus in cloacal and oral samples obtained from immunized and unimmunized poultry^a.

	Day post challenge	Control		Vaccinated	
		Oral swab	Cloacal swab	Oral swab	Cloacal swab
Chicken	1	2±0(2/3)	<1 ^b	<1	2±0(1/3)
Duck		2±0(1/3)	2.25±0.35(2/3)	2±0(1/3)	<1
Goose		2±0(1/3)	2.25±0.35(2/3)	<1	<1
Turkey		<1	<1	<1	<1
Chicken	3	3.8±2.3(3/3)	3.8±1.8(3/3)	<1	<1
Duck		3.1±1.25(3/3)	2.3±0.5(3/3)	2±0(1/3)	2±0(2/3)
Goose		2.3±0.5(3/3)	2.6±0.5(3/3)	1.5(3/3)	2.5±0(1/3)
Turkey		2.5±0(3/3)	3.2±1(3/3)	2.25±0.35(2/3)	2.75±0.35(2/3)
Chicken	5	3±0(1/1)	>1	<1	<1
Duck		2.5±0(3/3)	2.6±0.28(3/3)	2.5±0(1/3)	<1
Goose		3.3±0.28(3/3)	2.8±0.5(3/3)	2.5±0(1/3)	<1
Turkey		2.75±0.35(2/2)	2.5±0(2/2)	<1	<1

^aData are the mean Log₁₀ EID₅₀/ml ± SD for positive samples. Values in parentheses are number of positive animals out of each group.

^bThe titer was below the limit of detection (<1 Log₁₀ EID₅₀/ml).