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Correction Details: In the originally published article, the ethics section incorrectly stated ***"Ethical Considerations: None."*** The study was part of a ***postgraduate student's dissertation*** and had received ***local ethical approval (EE/1400.2.24.36770/scu.ac.ir)*** through the institutional procedures in place at the time. Specifically, the research proposal was reviewed and approved by the Faculty Educational Committee and subsequently endorsed by the University Educational Council. Although the university did not have a centralized Institutional Ethics Committee at that time, this process served as the official ethical oversight mechanism.

This correction does not affect the scientific validity, methodology, or conclusions of the original article.

The article has been updated accordingly.

The editorial office and the authors apologize for any inconvenience caused.

Editor-in-Chief
Dr. Jamshid Razmyar
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Evaluation of Serological Response of Low and Highly Pathogenic Influenza Vaccines in Japanese Quails

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ABSTRACT

Avian influenza A virus (AIV) causes one of the most transmissible diseases. This virus can infect the quails and be spread to other animal species. Vaccination in chickens and ducks has shown that highly pathogenic avian influenza viruses (HPAI) can be controlled. This study evaluated the serological response of low and highly pathogenic influenza vaccines in quails. One hundred forty-day-old quails were divided into seven groups. Before vaccination, 20 blood samples were randomly collected from the quail wing vein. At 21 days of age, Group 2 was vaccinated with the H9N2 vaccine. Quails in Group 4 were vaccinated with the H5N1 influenza vaccine (Harbin). Quails in Group 6 received the H5N1 vaccine (Livaning). At 42 days of age, Groups 3, 5, and 7 were re-vaccinated with the same vaccines as in the previous stage. Blood samples were collected from each group from 20 quails at 20, 42, and 56 days to determine AIV antibodies by the HI test. Three weeks after the second vaccination (H9N2), the antibody titer was higher than in the group that received the vaccine once, but the difference was insignificant. The antibody titer after the second Harbin vaccine (H5N1) was higher than in the group receiving only one dose, but the difference was negligible. The antibody titer at 63 days was higher in the group that received one dose of the Livaning (H5N1) vaccine, and this difference was significant. After the second vaccination, there was a significant difference in the titers between the two doses of H9N2 and H5N1 for the Livaning and Harbin vaccines. The average increase in antibody production following the two doses of H9N2 and Harbin vaccines showed similar trends. However, the Livaning vaccine produced a significantly higher antibody response than the other two (p<0.05).

Keywords: *Serology, Influenza, Quail, Vaccination, detection.*

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1 Introduction

Influenza A viruses of the H9N2 subtype usually cause low to moderate disease but can lead to severe disease and mortality in birds when combined with other infections (1). Avian influenza virus (AIV) is the agent of one of the most transmissible diseases caused by type A viruses belonging to the *Orthomyxoviridae* family (2). It is associated with economic damage and health threats to animals and humans. The avian influenza virus causes various illness signs, from subclinical infections to very severe diseases, with up to 100% mortality in birds (3). Varying in only one amino acid cleavage site of hemagglutinin protein can modify the pathogenicity of the virus (2). The H9N2 serotype usually causes low to moderate disease but can result in acute disease and high mortality in birds when combined with other infections (1).

Avian influenza (AI) in Japanese quail (Coturnix japonica) was first reported in Italy (1966-1968), after which different strains of influenza virus from quail were seen as sporadic outbreaks in North America, Europe, and Asia. (4). The H9N2 virus widespread in Asian poultry in 1999 was similar to the H9N2 virus isolated from quail in Hong Kong in late 1997. The highly pathogenic avian influenza H5N1 (HP) was identified in China in 1996 with reports of death (5). Recently, a report on the pathogenicity and transmissibility of the H5N1 influenza virus in cattle shows the importance of controlling this disease (6). Different experimental infections in Japanese quail have different sensitivity levels to the H5 virus (HPAIV) compared to chickens (higher to similar or lower sensitivity). Recently, H5N1 and H9N2 related to human viruses have been identified in quail (7, 8). Because quails have both types of receptors for influenza viruses, sialic acid α2,3-galactose (SAα2,3-gal) and α2,6-galactose (SAα2,6-gal) act as a type of mediator. It is a place for the emergence and transmission of new viruses that can cross the interspecies barrier between domestic poultry and humans. Also, adaptation of wild bird influenza viruses can occur in quail. Therefore, quail vaccination seems necessary as part of the flu prevention program (9).

The correct use of avian influenza virus vaccines increases resistance to infection and reduces disease severity and death, virus replication, shedding, and transmission. The protection of these vaccines against H5 HPAI was seen in chickens, geese, and ducks. The Asian H5N1 HPAI virus did not cause illness or death in ducks, but the vaccine reduced virus replication in their respiratory and intestinal tracts.

This study compares the serological response to low and highly pathogenic vaccines in quail under similar conditions.

2 Materials and methods

2.1 Experiment Design

One hundred and forty-one Japanese quails were purchased, and blood samples were randomly taken from the wing veins of 20 quails at 20 days. The quails were then randomly divided into seven experimental groups (Table 1), housed in separate rooms, and given free access to water and litter. Group 1 did not receive a vaccine and served as a negative control. Group 2 quails were vaccinated at 21 days, while Group 3 quails were vaccinated at 21 and 42 days with the H9N2 bird flu vaccine (Razi Vaccine and Serum Institute). quails in Group 4 were vaccinated at 21 days, and quails in Group 5 were vaccinated at 21 and 42 days with the Harbin Avian Influenza Inactivated Vaccine (H5) (China). quails in Group 6 received one dose at 21 days, and quails in Group 7 received two doses (at 21 and 42 days of the H5N1 influenza vaccine (Livaning, H5, China). All birds were vaccinated subcutaneously in the back of the neck (Table 1).

Table 1. Vaccination of studied quails

Groups	1st round of H9N2 vaccine	2sd round of H9N2 vaccine	1st round of H5N1 vaccine (Harbin)	2nd round of H5N1 vaccine (Harbin)	1st round of H5N1 vaccine (Livaning,)	2nd round of H5N1 vaccine (Livaning)
1	-	-	-	-	-	-
2	+	-	-	-	-	-
3	+	+	-	-	-	-
4	-	-	+	-	-	-
5	-	-	+	+	-	-
6	-	-	-	-	+	-
7	-	-	-	-	+	+

2.2 Sampling

2.2.1 Blood collection

The blood samples were collected via wing veins at 20, 42, and 63 days of age via wing veins of all quail groups. The blood sera were collected, transferred to microtubes, and stored in the freezer until testing.

2.2.2 Hemagglutination Inhibition (HI) Test

The HI test of avian influenza was performed using the beta method with 4 HA antigens, H9 and H5 (specific to Harbin and Livaning). Results were recorded as log2 X values of the highest dilution that showed complete hemagglutination inhibition.

2.3 Statistical Method

For the statistical analysis of the results, SPSS software version 26 (IBM, USA) was used, employing One-way ANOVA and Univariate Analysis of Variance methods.

3 Results

The results of the hemagglutination inhibition (HI) test of quail blood serum are listed in Table 2. Two groups of quail vaccinated once and twice with the low-intensity vaccine (H9N2) were statistically compared using a two-way ANOVA test for the equality of the mean titer. In the group that received the second round of vaccine three weeks after the second vaccination, the antibody titer was higher than in the group that received the vaccine once. However, the difference was not statistically significant (P>0.05). In the next stage, two groups of quail vaccinated once and twice with the high-virulence Harbin vaccine (H5N1) at 42 and 63 days were statistically compared. The antibody titer after the second vaccination was higher in the twice-vaccinated group than in the group that received only one dose of the Harbin (H5N1) vaccine, but the difference was not statistically significant (P>0.05).

The antibody titers after the first and second rounds of vaccination at the ages of 42 and 63 days in two groups vaccinated with the high-intensity Livaning vaccine were compared using a two-way ANOVA test for equality of mean titers. After the second vaccination with the Livaning (H5N1) vaccine, the average antibody titer at the age of 63 days was higher than in the group that received one dose of the vaccine, and this difference was significant ($P \leq 0.05$).

Table 2. HI titers (Mean±SD) of avian influenza virus blood serum titer based on logarithm 2 in experiment groups

		Before vaccination (20 days old)	Before the second vaccination (42 days old)	63 days old
	Non-Vaccine control	0^{c}	0^{c}	0^{c}
vaccine H9N2	One time vaccine	0^{c}	2.95^{a} ±0.14	2.89^{a} ±0.51
	Two times vaccines	0^{c}	2.95^{a} ±0.14	3.58^{a} ±0.56
vaccine H5N1 (Harbin)	One time vaccine	0^{c}	3.13^{a} ±0.34	3.00^{a} ±0.33
	Two times vaccines	0^{c}	3.13^{a} ±0.34	4.0^{a} ±0.71
vaccine H5N1(Livaning)	One time vaccine	0^{c}	3.38^{a} ±0.32	3.33^{a} ±0.33
	Two times vaccines	0^{c}	3.38^{a} ±0.32	5.89^{b} ±0.51

Different subscribe letters in each column indicate a significant difference (p<0.05).

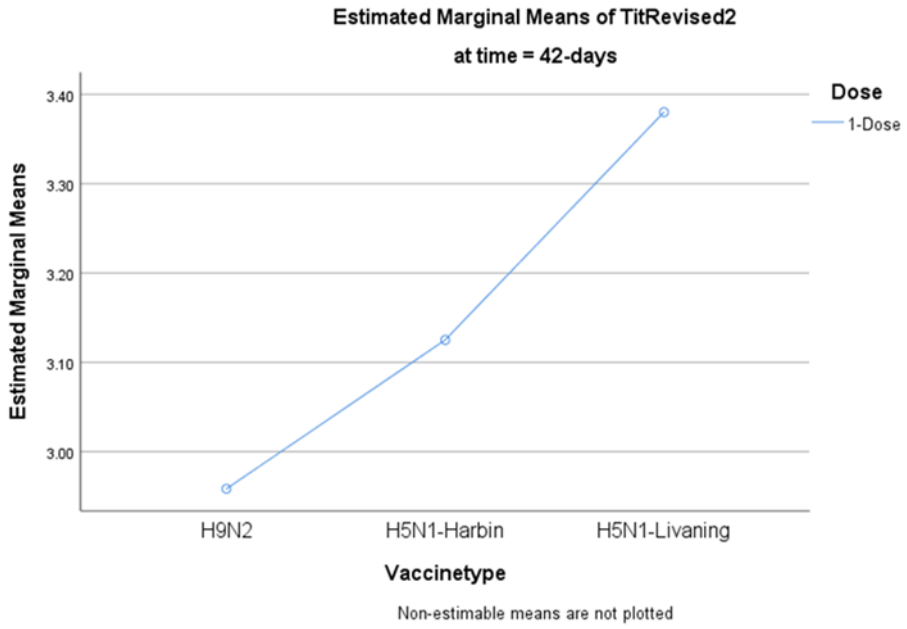

Figure 1. Blood serum antibody titer against influenza virus at the age of 42 days

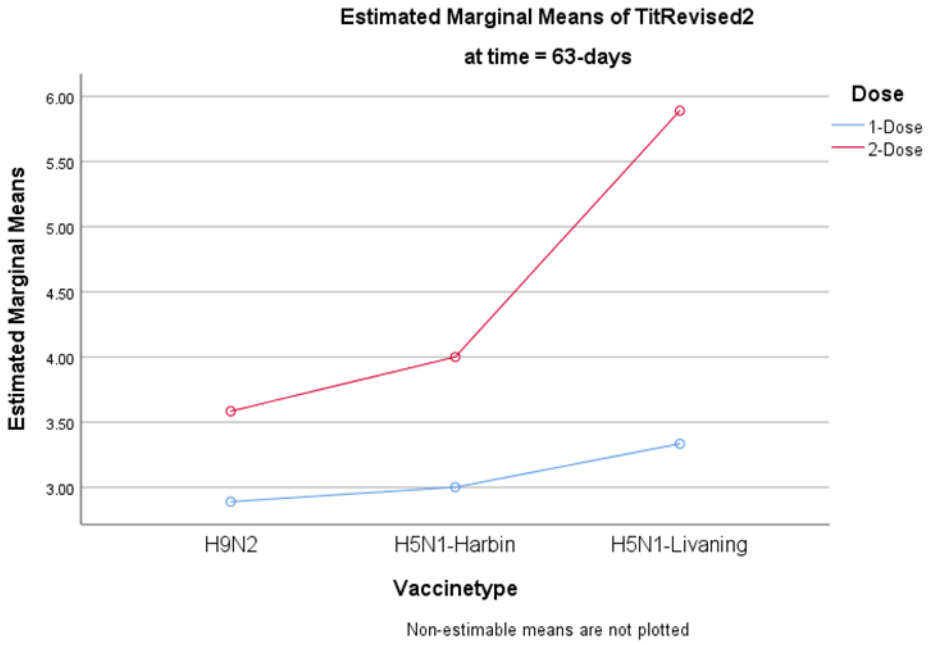

Figure 2. Blood serum antibody titer against influenza virus in 63 days

After the second vaccination, there was a significant difference in the average antibody titers between the two doses of H9N2 and H5N1 vaccinations for the Livaning and Harbin vaccines. The average increase in antibody production following the two doses of H9N2 and Harbin vaccines showed similar trends. However, the Livaning vaccine produced a significantly higher antibody response than the other two ($P \leq 0.05$).

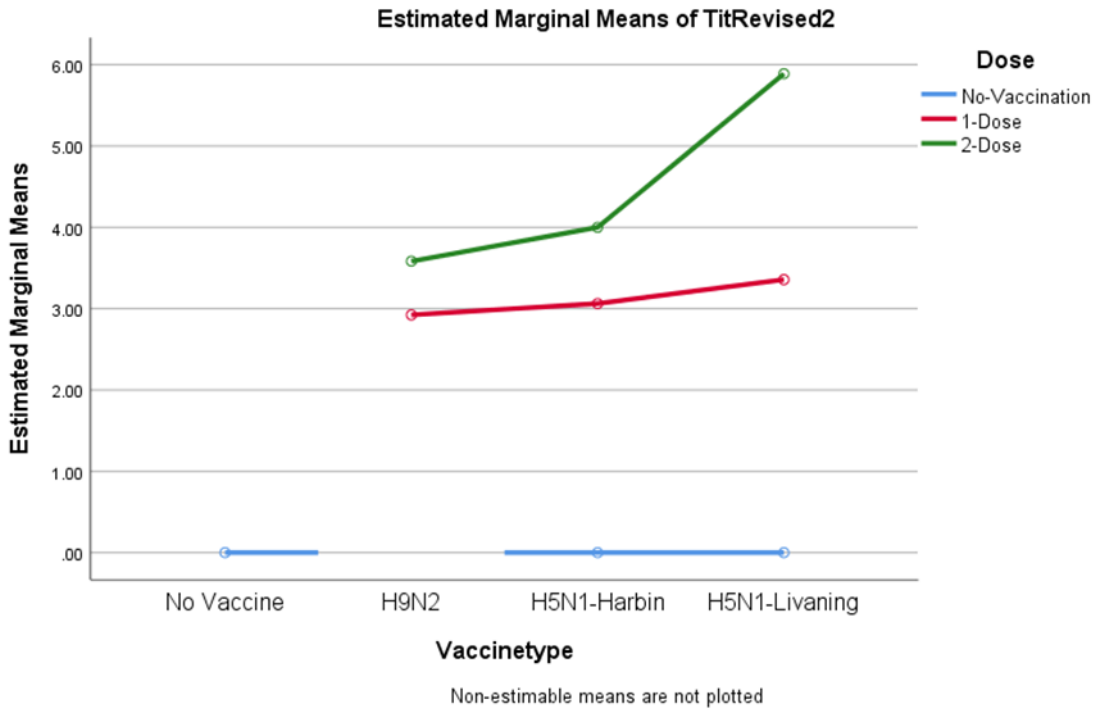

Figure 3. Comparison of blood serum antibody titers against influenza virus of different doses of vaccines

4 Discussion

Avian influenza is the most contagious, deadly, and damaging poultry disease, posing significant health threats to animals and humans. According to research findings, the cleavage of the hemagglutinin surface protein (HA) into HA1 and HA2 by intracellular or extracellular proteases is essential for creating infectious virus particles and the multiplication cycles of the influenza virus (10).

Examining changes in the hemagglutinin cleavage site of influenza isolates shows that the transformation of LPAI (Low Pathogenic Avian Influenza) viruses into HPAI (Highly Pathogenic Avian Influenza) has increased significantly over the last 30 years. LPAI viruses in the H9N2 subtype usually cause low to moderate disease, often with respiratory, gastrointestinal, renal, and genital symptoms. They do not cause significant losses in infected herds unless concurrent with other viral and microbial infections, leading to acute respiratory complexes, where the mortality rate can reach up to 97% (11).

Highly contagious H5N1 avian influenza (HP) was first identified in China in 1996 with a fatality (12). H5 and H7 subtype viruses have been reported in 2,634 cases of humans and animals worldwide, of which more than a thousand deaths have been seen. Multiple outbreaks of these viruses in wild and domestic birds have resulted in the deaths of at least 422 million domestic birds since 2005. The third wave continues from 2020 until now. In Europe and North America, highly pathogenic influenza is often controlled by culling suspected contaminated birds (elimination strategy), while some countries (China) control the disease by vaccination (13).

During three global outbreaks of H5 avian influenza, China, the world's largest poultry producer, suffered relatively low poultry losses and nearly eliminated the widespread H7N9 virus that emerged in 2013 with vaccination. H7N9 viruses have lost their affinity for human cell receptors, which is necessary for human-to-human transmission (14).

In this study, the serum titer of all quails before vaccination at 20 days old was negative for H9N2 and H5N1 avian influenza virus, indicating the absence of infection and lack of protective antibodies against the influenza virus. This study compared the response to low-intensity and high-intensity vaccines and the effect of multiple vaccinations in quails. After the first vaccination, the blood serum antibody levels in quails increased, which correlated with the severity of the vaccine virus. Influenza vaccines with high pathogen influenza virus stimulated the bird's immune system more than vaccines with low pathogen and produced more

antibodies. With the second vaccination, the blood serum antibody levels in quails increased, and this increase was significantly higher in the group receiving the Livaning vaccine compared to quails receiving a second dose of low vaccines and the Harbin vaccine (p<0.05). This highlights the importance of vaccine type in stimulating the bird's immune system, a factor that must be considered when choosing a vaccine. Despite low HI titers, most birds did not show clinical signs, but virus shedding was still significant. If this occurs in the field, it may lead to the gradual extent of wild viruses in vaccinated farms, and new virus types may emerge, posing risks not only to poultry flocks but also to public health (15). It may then become impossible to eradicate the virus by vaccination alone (16). Recent discoveries of new variants in Indonesia suggest this may already happen (17). Based on the results of Sarkadi et al. (18), vaccination of quails with the H5N1 vaccine provides adequate immunity against challenges with HPAI strains in quails. Our study also observed an increase in titer after vaccination with the H5N1 vaccine. The high susceptibility of quails to H5N1 raises concerns about their role in the persistence of HPAI viruses, warranting further monitoring and research.

Poetri et al. (5) showed in their research that vaccination with an inactivated vaccine containing an acute influenza virus strain (H5N1 A/chicken/Legok/2003) in most birds caused hemagglutination inhibition (HI) titers below 4 (log2). Challenged vaccinated birds with the H5N1 virus showed no clinical signs, and virus shedding was limited; however, almost all vaccinated birds exhibited a fourfold or greater increase in HI titer after challenge, indicating infection. This suggests that there is a possibility of virus transmission. Their study demonstrated that single-dose vaccination under field conditions can prevent clinical signs but is insufficient to prevent virus transmission, potentially allowing gradual virus shedding in vaccinated commercial flocks.

Indriani et al. (19) vaccinated thirty quail flocks with an inactivated bivalent H5N1 AI vaccine. Quails were vacFcinated intramuscularly with two doses on days 23 and 45. After the first dose, the antibody titer was not optimal, but after the second dose, it was approximately 4 (log2) on average, and up to 70% protection was observed in quails challenged with the H5N1 influenza virus. However, virus shedding was detected in these birds seven days after the challenge.

Abotaleb et al. (20) evaluated the effectiveness of two commercial inactivated H5 AI vaccines administered weekly in quails. Due to the presence of two types of avian influenza receptors, these birds allow the recombining of different types of mutated AIV viruses, which may threaten human health and the poultry industry. Susceptible quails received two doses of the H5N1 vaccines studied at three-week intervals. Blood samples were collected weekly, and AI antibodies were measured from sera using the HI test with homologous H5N1 and heterologous H5N8 antigens containing 4 HA units. Vaccinated quails were intranasally challenged with 100 LD50 of HPAI subtype H5N8 four weeks after receiving a booster dose. Vaccination of quails with one or two doses of imported H5N1 vaccine induced a stronger immune response than the local commercial vaccine against the homologous H5N1 antigen.

Elsayed et al. (21) studied genetic mutations in the NA and HA genes of H9N2 influenza strains isolated from quails compared to original viruses isolated from quails in Egypt. The transmissibility of the virus to humans and its virulence in poultry may be affected by mutations in the NA protein. Mutations in the HA gene of the H9N2 virus may reduce the effectiveness of H9N2 vaccine strains and increase the likelihood of infection with a common strain between humans and animals.

Gol et al. (22) evaluated the effect of the killed H9N2 avian influenza (AIV) vaccine on tissue distribution and virus shedding in quails. They did not observe any clinical signs or necropsy lesions in quails. On the first, third, and sixth days after the challenge, the virus was detected in different tissues of the non-vaccinated challenged groups. These researchers demonstrated that quail vaccination against AIV H9 is necessary to prevent clinical signs and virus replication in the respiratory and intestinal tracts. Two doses of vaccination, compared to one, significantly protected the respiratory tract and intestines (P≤0.05). Their study emphasized that Japanese quail vaccination against both low-pathogenicity and high-pathogenicity avian influenza viruses is crucial to reducing virus shedding in the environment. Double vaccination showed better performance than single vaccination, and the vaccine quality significantly influenced antibody titers and the success of vaccination.

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Conflict of Interest

The authors declared no conflicts of interest.

Author Contributions

Authors contributed equally to this article.

Data Availability Statement

Data are available from the first author upon reasonable request.

Ethical Considerations

None.

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