Original Article

Effect of Vaccination on Distribution and Immune Response of Avian Influenza Virus H9N2 in Coturnix coturnix

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ABSTRACT

Influenza viruses can multiply in quails and be transmitted to other animal species. As vaccination reduces virus shedding in chickens, the effect of the killed H9N2 avian influenza virus (AIV) on tissue distribution and virus shedding was evaluated in quails. One hundred 20-day-old quails were divided into six equal groups, kept in separate pens, and fed ad libitum. Before vaccination, blood samples were randomly collected from the wing veins. Four groups were vaccinated with the inactivated H9N2 Razi Institute vaccine at 21 days subcutaneously at the back of neck. Three weeks later, two groups were re-vaccinated. Two weeks later, at the age of 56 days, three groups were challenged with 100 µL of allantoic fluid containing 10^5 EID_{50} H9N2 through the oculonasal route. Blood samples were collected from quails at 42, 56, 63, and 70 days from each group to determine AIV antibodies by the hemagglutination inhibition test. Three quails were randomly selected and euthanized from each group on days 1, 3, and 6 post-inoculation (PI). Tissue samples were collected, and the RT-PCR test was performed. No clinical signs or gross lesions existed in any of the groups during the experiment. However, the virus was detected in different tissues on the first, third, and sixth days after the challenge in unvaccinated challenged birds. Virus detection was significantly more frequent in the quails vaccinated once and challenged than in the twice-vaccinated challenged group ($P \leq 0.05$). On the third day of PI, the virus was detected in some organs of the challenged groups. On the sixth day of PI, the virus was detected only in the lungs of two unvaccinated and once-vaccinated challenged birds. It was concluded that the vaccination of quails against AIV H9 is necessary to protect them from clinical signs, as well as respiratory tract and intestine replication. Two-time vaccination significantly protects the respiratory and intestine tracts, compared to one-time vaccination ($P \leq 0.05$).

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Keywords: Antibody titer, Clinical signs, Detection, Gross lesion

1. Introduction

Avian influenza is a contagious disease with many economic losses in the poultry industry and a threat to human health. It occurs with various symptoms, from a subclinical infection to a very acute disease in poultry. Low-pathogenicity (LPAI) and high-pathogenicity (HPAI) viruses are related to the amino acid sequences in the hemagglutinin protein cleavage site. The H9N2 virus has caused severe damage in places where the principles of biological security are not appropriately applied and where there is dense poultry farming. In such situations, simultaneous infection with other pathogenic agents leads to severe complications and death (1, 2). The H9N2 infection was first reported in the Middle East in 1998, and large outbreaks occurred in commercial chickens in Iran (2) and Pakistan (3), often with serious complications. In 1999, human infection with H9N2 was reported with mild respiratory symptoms (4).

The human-isolated H9N2 was similar to an earlier H9N2 isolated from a quail in Hong Kong in 1997. Considering the reports of the transmission of H5N1, H7N7, and H9N2 viruses from chickens and quails to humans, avian influenza viruses (AIVs) might infect humans without an intermediate host (5, 6). This direct virus transmission can lead to an epidemic and endanger public health (4, 7). Yamada et al. (2012) pointed out the role of quails in genetic changes and the emergence of new influenza A virus varieties. Duck flu viruses can adapt to the quail body, multiply, and create new varieties that can infect other bird species. Chickens and quails act as intermediate hosts, and the virus in their bodies can transform into a new variety and be transmitted to other animal species. Quail infection with the H9N2 virus was first reported in Italy and was associated with respiratory symptoms and mortality in younger birds. It has been reported that the highly pathogenic H5N3 virus in turkeys has caused mild clinical symptoms in quails and that they can transmit this deadly virus to other birds (7). In addition to showing the diversity of hosts and the ability of quails to transmit AIVs to different species of birds, these studies also point to the importance of quails in the genetic recombination of these viruses. Considering the increasing trend of quail breeding in the world in the last two decades and the high speed of virus propagation in quails compared to other species, special attention should be paid to these birds in controlling the spread of the virus (6, 9, 10).

The present study aimed to investigate the effect of the inactivated influenza H9N2 vaccine on the immune response and tissue distribution of the H9N2 virus in quails.

2. Materials and Methods

2.1. Virus

To create an experimental infection, the A/chicken/Iran/Aid/2013 (H9) virus isolate with accession number (KP455991.1) was used with a 50% embryo infectious dose (10^5 EID_{50}) (14).

2.2. Experiment Design

One hundred 20-day-old Japanese quails were divided into six equal experimental groups. The birds were raised in separate pens in the Faculty of Veterinary Medicine' Poultry Diseases section and fed freely during the study. Before vaccination, 10 blood samples were randomly taken from the wing veins of quails. Groups 3, 4, 5, and 6 were vaccinated at 21 days with 0.2 mL of inactivated avian influenza H9N2 vaccine (Razi Vaccine and Serum Research Institute, Karaj, Ian), subcutaneously at the back of the neck. Three weeks later, groups 5 and 6 were re-vaccinated after bleeding. Two weeks later, at the age of 56 days, groups 2, 4, and 6 were challenged with 100 µL of allantoic fluid containing 10^5 EID_{50} H9N2 (A/chicken/Iran/Aid/2013) through the oculonasal route. Group 1 was kept as the control group, and group 2 was the vaccinated control group. All groups were observed for clinical signs twice daily.

2.3. Sampling

2.3.1. Serology

In order to check the level of antivirus antibodies by the hemagglutinin inhibition (HI) method, blood samples were taken from 10 quails in each group

through the wing veins on days 20, 42, 56, 63, and 70 of the experiment. Serum samples were separated and stored until the test was performed using four HA avian influenza H9N2 antigens. Prior to the HI test, serum samples received heat treatment at 56°C for 30 min, and chickens' red blood was utilized (1,14).

2.4. Molecular Analysis

In order to detect the virus in different tissues of the birds' bodies by RT-PCR, three quails from each group were euthanized on the first, third, and sixth days post-inoculation (PI). Samples were taken from the trachea, lung, spleen, liver, intestine, and brain tissues.

2.5. RNA Extraction

One mL of RNX solution was added to 50 to 100 mg of homogenous tissue samples separately, and the extraction steps were performed according to the manufacturer's instructions (Cinna Gen, Iran).

2.6. Synthesis of cDNA

In this step, a cDNA synthesis kit (Yekta Tajhiz Azma, Iran) and a general primer (random hexamer) were used.

2.7. Amplification and Gel Electrophoresis

The factors included in the PCR reaction included 10 µL of Mastermix 2X (1.5 mM MgCl 2) (Amplicon, Canada), 10 pmol per µL of each primer, F (5'- CAC CTY ACA GAR CAC GG AAT -3), R (5'- GTC ACA CTT GTT Azam GTR TC -3') (Lee et al., 2001), a 3 µL DNA template, and 6 µL distilled water. The temperature program was as follows: 5 min at 94°C, 35 cycles of 94°C (30 sec), 50°C (30 sec), 72°C (1 min), and 72°C (4 min). In order to evaluate the PCR product, electrophoresis was performed on a 1% agarose gel at a voltage of 100 V. Safe dye and a 100-bp DNA marker were also used (CinnaGen, Iran).

2.8. Statistical Analysis

A two-way analysis of variance (ANOVA) was used in data analysis. In addition, to compare groups and time, a one-way ANOVA was used at each point to show the difference between groups. The level of significance was set at ($P \leq 0.05$).

3. Results

3.1. Clinical Signs and Necropsy

There were no clinical signs or gross lesions in any of the birds in the experimental groups.

3.2. PCR Test Results

The virus was detected in the trachea, lungs, liver, and intestine on the first, third, and sixth days after the challenge in unvaccinated birds (Figure 1). The virus detection frequency in the tissues of birds vaccinated once and challenged was significantly higher than that in those vaccinated twice and challenged ($P \leq 0.05$), as shown in Table 1. On the third day after the challenge,

Table 1. The results of virus detection in different tissues of experimental groups

	Tissues																	
	Trachea			Lung			Liver			Spleen			Intestine			Brain		
	Days after Challenge																	
	1	3	6	1	3	6	1	3	6	1	3	6	1	3	6	1	3	6
Non-Vaccinated control(a)	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Non-Vaccinated Challenge(b)	3/3	3/3	0/3	2/3	2/3	2/3	3/3	2/3	0/3	0/3	0/3	0/3	3/3	3/3	3/3	0/3	0/3	0/3
One time Vaccination + Challenge(cd)	2/3	2/3	0/3	2/3	2/3	1/3	2/3	2/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Two times Vaccination + Challenge(dc)	1/3	2/3	0/3	1/3	1/3	0/3	1/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

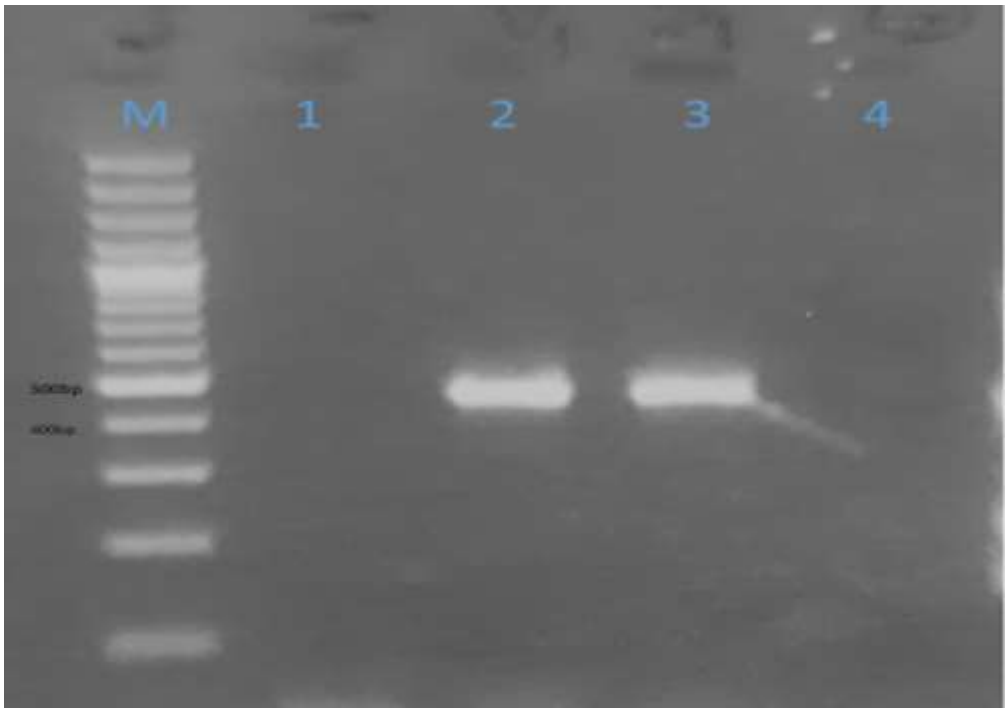

Figure 1. M: marker 100 base pare 1: negative control 2: positive control 3: positive sample 4: negative sample

Table 2. HI titers, (Mean±std) 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)	Before the challenge (56 days)	Days after the challenge	
				7 63 days old	14
	Mean titer	Mean titer	Mean titer	Mean titer	
Non-Vaccinated control(a)	-	-	-	-	-
Non-Vaccinated Challenge(b)	-	-	-	6.4±0.095	4.3±0.69
One time vaccinated Control(cd)	-	2.414±0.41	3.2±0.394	3±0.51	
One time vaccinated + Challenge(cd)	-	2.414±0.41	3.2±0.394	5.909±0.99	6.091±0.503
Two times vaccinaed Control(ebf)	-	2.414±0.41	4.278±0.357	2.5±0.99	
Two time vaccinated Challenge(fbe)	-	2.414±0.41	4.278±0.357	6.364±0.52	6.182±0.228

Different subscribe letters in each column indicate a significant difference ($P \leq 0.05$).

the virus was detected in all organs of the challenged groups except the spleen and brain. On the sixth day after the challenge, the virus was detected only in the lungs of two birds (Table 1).

3.3. Hemagglutination Inhibition Test

The serum titer against AIV H9N2 in the unvaccinated control group was not detected at the end of the experiment, indicating that the control group was not infected with the virus (Table 2). Two weeks after the second vaccination (at 56 days of age), serum antibody titer changes in the two-time vaccination group were significantly higher than those in the one-time vaccination group ($P \leq 0.05$). One week after the challenge with the influenza virus, the H9N2 serum titer increased in the challenged group, but the difference was not significant ($P \geq 0.05$).

4. Discussion

Although the H9N2 serotype is in the category of viruses with low pathogenicity, this serotype caused high mortality in an outbreak in Pakistan in 1998 (15). In some regions of Iran, this virus has caused high mortality in disease outbreaks (2, 16). However, there is limited research on quails' contamination and susceptibility to H9N2. Therefore, in this study, experimental infection with one of the isolates of H9N2, A/chicken/Iran/Aid/2013, was performed in vaccinated and non-vaccinated Japanese quails (*Coturnix coturnix japonica*) to investigate the clinical symptoms, the tissue distribution of the virus, and the effect of vaccination on antibody production and virus shedding. The fact that quails are more resistant than chickens and turkeys to the A/Ontario/7732/66 (H5N2) infection was also suggested by Slemons and Easterday (17). These researchers observed high antibody production and low mortality in quails infected with the virus. According to these findings, it can be concluded that wild galliforms, such as quails and pheasants, can transmit pathogenic AIVs to chickens and turkeys. In their research, Nili and Asasi (16), pointed out that while experimental infections with MPAI viruses have low mortality, natural infections in poultry farms mainly show respiratory diseases with a high mortality rate. In the present study, the experimental infection of quails with A/chicken/Iran/Aid/2013 (H9N2) isolated from chickens with clinical symptoms and mortality did not show clinical signs or deaths in quails. The highest virus detection was from the lungs, trachea, liver, and intestine; therefore, according to the present study, the best places for virus detection are the lungs and trachea. The influenza virus is first located in the respiratory system and then multiplies and spreads to other tissues. In the study of Marangon and Bortolotti (9), influenza viruses isolated from waterfowl were inoculated on quails through natural routes (oral, nasal, and ocular inoculation) and multiplied in their bodies. These researchers stated that the site of influenza virus replication in quails (the respiratory tract) differs from that observed in natural hosts (intestinal epithelial cells). Keshtkari and Nili (18) experimentally infected Japanese quails (*Coturnix Coturnix Japonica)* with H9N2 and observed clinical symptoms such as sneezing, panting, and depression, followed by reduced egg production. The virus did not affect food and water consumption or quail growth. Ebrahimi and Ziapour (7), in a study of experimental infection of vaccinated and non-vaccinated Japanese quails with the Iranian H9N2 virus, showed that all non-vaccinated birds were infected and showed clinical symptoms. However, the infection and clinical symptoms were lower in the vaccinated ones. The antibody titer was higher in the vaccinated group, but food and water consumption decreased in this group. These results indicate that the inactivated vaccine does not entirely prevent infection but can reduce clinical symptoms and the viral titer in the lung. Bertrand and Doles (19) showed no clinical or pathological symptoms in experimentally infected quails with LPAIVs. They concluded that quails may play an essential role in the epidemiology of these viruses. In examining the shedding pattern of the H9N2 virus in different wild birds, Umar and Asif (20) observed virus shedding in all infected birds with a different pattern. Virus shedding in Japanese quails was lower than in other birds.

Interestingly, virus shedding in sparrows and minnows was observed mainly through the digestive tract, and in guinea fowl and Japanese quails, through the respiratory tract. Germeraad and Sanders (21) stated that factors such as species, virus origin, age, and route of inoculation affect the AIV shedding pattern. Islam and Amin (22), in their investigation of the infection rates of AIV (H5) and (H9) in pigeons and quails in poultry markets in Bangladesh, found that AIV is more common in quails than in pigeons. In the study of the evolution of the H9N2 virus inside the body of quails by Ferreri and Geiger (23), it was shown that quails were very effective in the evolution of this virus.

The high shedding of the H9N2 virus through the respiratory tract without developing clinical signs following infection in quails is consistent with the results of other researchers (6, 24). Usually, quails are infected with a lower dose of the virus than chickens and turkeys as the virus multiplies in their bodies (24, 25), and virus excretion through their respiratory system is greater than that of chickens (21). Subclinical infections with high excretion of the virus cause long-term viral circulation. Because both types of viral sialic acid receptors (bird and mammalian receptors) are present in quails, they can cause the recombinant emergence of viruses (13, 24, 26).

Our study showed the absence of serum antibody titers against influenza virus H9N2 in the control groups up to the end of the experiment. It indicated that this group was not infected with influenza. Furthermore, the second vaccination increased blood serum antibodies, and the changes were significantly greater than the first vaccination ($P \leq 0.05$). The existence of a difference between the titers of the vaccinated and non-vaccinated groups two weeks after the challenge shows that the vaccination creates a protective titer in the quails and could decrease quail virus shedding. These results indicated that the inactivated vaccine could protect birds from clinical signs and significantly reduce viral shedding in the respiratory and intestinal tracts. Moreover, two-time vaccination significantly protects the respiratory and intestine tracts, compared to one-time vaccination ($P \leq 0.05$).

It was concluded that quail vaccination against AIV H9 is necessary to protect birds from clinical signs and respiratory tract and intestine replication. Two-time vaccination significantly protects the respiratory and intestine tracts, compared to one-time vaccination ($P \leq 0.05$).

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Ethics

The current study was approved by and carried out under the license (EE/1400.2.24.36770/scu.ac.ir) of the Scientific Committee of the Veterinary Medicine of Shahid Chamran University of Ahvaz . Ahvaz-Iran.

Conflict of Interest

The authors declare that they have no conflict of interest.

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