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***Original Article***

# Avian Influenza-Killed Vaccine on Tissue Distribution and Shedding of Avian Influenza Virus H9N2 in Ducklings

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**Abstract**

Ducks play an important role in the transmission of avian influenza to poultry farms. Because of the importance of vaccination in reducing virus shedding, this study evaluated avian influenza-killed vaccine H9N2 on tissue distribution and shedding of avian influenza virus H9N2 in ducklings. One hundred-day-old ducklings were purchased and, after bleeding from 20 birds, were kept in four separate rooms under standard conditions. Groups 1 and 2 were vaccinated at 9 days, and groups 2 and 3 were challenged with 0.1 ml of allantoic fluid containing $10^5$ $EID_{50}$ (A/chicken/Iran/Aid/2013(H9)) virus intranasally at 30 days. Group 4 chicks were kept as the control group. Chicks were observed two times daily. On days 1, 3, 5, and 8 after inoculation, 3 chicks were randomly selected from each group and cloaca and trachea swabs samples were collected from each bird. Then the ducklings were euthanized and trachea, lung, spleen, intestine, liver, and brain tissue samples were collected for molecular detection. The virus was detected in the tissues and tracheal and cloacal swabs by polymerase chain reaction (PCR), and anti-AIV titres were measured by HI test. The results showed no clinical signs in the challenged groups. In the vaccinated challenged group, virus was detected only in cloacal swabs, but in the unvaccinated challenged group, virus was detected more in tracheal swabs than in cloacal swabs. In challenged-unvaccinated chicks, virus was detected in the trachea and lungs, and in challenged-vaccinated birds, virus was detected in the intestines. In conclusion, vaccinating ducks against the AI H9N2 virus reduced shedding and tissue distribution of AI viruses in challenged ducks.

**Keywords:** avian influenza virus H9N2, replication, shedding, vaccination, ducklings

**Vaccin tué de la Grippe Aviaire sur la Distribution Tissulaire et l'excrétion du Virus de la Grippe Aviaire H9N2 Chez les Canetons**

**Résumé:** Les canards jouent un rôle important dans la transmission de la grippe aviaire aux élevages avicoles. En raison de l'importance de la vaccination dans la réduction de l'excrétion du virus, cette étude a évalué le vaccin tué de la grippe aviaire H9N2 sur la distribution tissulaire et l'excrétion du virus de la grippe aviaire H9N2 chez les canetons. Des canetons de cent jours ont été achetés et, après saignée de 20 oiseaux, ont été gardés dans quatre pièces séparées dans des conditions normales. Les groupes 1 et 2 ont été vaccinés à 9 jours, et les groupes 2 et 3 ont reçu 0.1 ml de liquide allantoïdien contenant $10^5$ virus $EID_{50}$ (A/chicken/Iran/Aid/2013(H9)) par voie intranasale à 30 jours. Les poussins du groupe 4 ont été gardés comme groupe témoin. Les poussins ont été observés deux fois par jour. Aux jours 1, 3, 5 et 8 après l'inoculation, 3 poussins ont été choisis au hasard dans chaque groupe et des échantillons de cloaque et de trachée ont été prélevés sur chaque oiseau. Ensuite, les canetons ont été euthanasiés et des échantillons de tissus de trachée, de poumon, de rate, d'intestin, de foie et de cerveau ont été prélevés pour une détection moléculaire. Le virus a été détecté dans les tissus et les écouvillonnages trachéaux et cloacaux par réaction de polymérisation en chaîne

(RPC), et les titres anti-AIV ont été mesurés par test HI. Les résultats n'ont montré aucun signe clinique dans les groupes testés. Dans le groupe vacciné provoqué, le virus a été détecté uniquement dans les écouvillonnages cloacaux, mais dans le groupe non vacciné provoqué, le virus a été détecté davantage dans les écouvillonnages trachéaux que dans les écouvillonnages cloacaux. Chez les poussins non vaccinés provoqués, le virus a été détecté dans la trachée et les poumons, et chez les oiseaux vaccinés provoqués, le virus a été détecté dans les intestins. En conclusion, la vaccination des canards contre le virus de l'IA H9N2 a réduit l'excrétion et la distribution tissulaire des virus de l'IA chez les canards infectés.
**Mots-clés:** virus de la grippe aviaire H9N2, réplication, excrétion, vaccination, canetons

## 1. Introduction

Avian influenza (AI) is caused by type A viruses belonging to the *Orthomixoviridae* family (1). Avian influenza viruses have a fragmented genome; rearrangement is an important mechanism in genetic variation. One important feature of influenza viruses is frequent alteration by the antigenic mechanisms of drift and shift to produce antigenic variants (2). Avian influenza is a highly contagious disease that is linked to economic damage and threatens human and animal health. It causes symptoms ranging from subclinical infection to very acute illness with 100% mortality in birds. The difference between low pathogen viruses (LPAI) and high pathogen (HPAI) viruses can be as much as a change in the amino acid at the hemagglutinin protein cleavage site. Ducks belong to the *Anatidae* family, which are the most abundant species of *Anseriformes* birds (3). Wild birds, especially aquatic birds from the order *Anseriformes*, are known as natural reservoirs for most low pathogenic avian influenza types (4). Transmission of LPAI generally occurs through the fecal-oral route in polluted aquatic habitats without any apparent symptoms of disease or mortality (4). Wild aquatic birds are the primary natural reservoirs for type A influenza viruses, which play a major role in the global spread of the virus and the emergence of new type A influenza viruses that threaten human and animal health (2). Various duck species are naturally resistant to HPAI viruses, yet ducks can spread the virus through the digestive and respiratory tract with or without clinical signs of the disease, while HPAI viruses can cause up to 100% mortality in broilers and other *Gallinaceous* birds. Accordingly, ducks have been identified as leading agents for the HPAI virus (3). The avian influenza virus enters the body of the bird through contaminated water or food (3). The spread of the virus, especially in the H4N7, H11N9, H7N3 subtypes in ducks that have been experimentally infected, is greater through the feces than through the trachea and respiratory tract (3). Avian influenza viruses also have high persistence in water and are isolated from the surface of wetlands and lakes where large numbers of ducks reside (5). Although aerosol transmission should not be overlooked, the large number of positive specimens of cloaca and tracheal swabs, high fecal virus titers, and water persistence of the virus indicate that subacute influenza viruses (LPAIs) have high survival rates in duck populations. This mechanism could be the cause of more infections in surface water-feeding ducks than those feeding from deep water (3). A review of available published articles showed that few studies on hybrid duck vaccination against avian influenza virus H9N2 are available in Iran. Therefore, the present study was conducted to evaluate the avian influenza-killed vaccine H9N2 on tissue distribution and shedding of the virus in ducklings.

## 2. Material and Methods

### 2.1. Virus

Avian influenza virus H9N2 isolated from poultry A/chicken/Iran/Aid/2013 (H9) with accession number

(KP455991.1) was used. The AIV was propagated two times in 9- to 11-day-old embryonated chicken eggs. The 50% embryo infective dose ($EID_{50}$) was calculated for the second passage according to the method of Reed and Muench (1).

**2.2. Experiment Design**

The experiment was designed according to ethical permission EE/98.24.3.38674/scu.ac.ir. One hundred-day-old ducklings (hybrid strain) were purchased and, after bleeding randomly from the saphenous vein, divided into four equal groups (20 birds in each group). Birds were reared in separate rooms in the Poultry Research Unit of the Faculty of Veterinary Medicine in Ahvaz and received feed and water *ad libitum* during the experimental period. Groups 1 and 2 were vaccinated against avian influenza virus H9N2 subcutaneously at the back of the neck at 9 days old, and chicks in groups 2 and 3 were challenged with 0.1 ml allantoic liquid containing $10^5EID_{50}$ )A/chicken/Iran/Aid/2013(H9) virus intranasally at 30 days. Group 4 chicks were kept as the uninfected unvaccinated control group. The ducklings were observed twice daily. The AI virus used in this study was isolated from broiler flocks in Ahvaz city by Boroomand, Jafari (6).

**2.3. Sampling**

**2.3.1. Serology**

Blood samples were collected from 20 hybrid ducklings one day old and at days 31 and 41 from 10 ducklings of each group via the saphenous vein to determine AIV antibodies using the HI test (1).

**2.4. Molecular Detection**

Three ducks from each experimental group were randomly selected at 1, 3, 5, and 8 days post-AIV challenge, and tracheal and cloacal swabs were collected and kept in tubes containing normal saline solution. Then the ducks were euthanized by intravenous injection of sodium pentobarbital (50.00 mg/kg), and trachea, lung, bursa, spleen, intestine, liver, and brain samples were collected and stored at -70 °C until molecular detection.

**2.5. Virus Detection**

To detect the influenza virus, the RT-PCR test was performed on tissue samples and trachea and cloaca swabs after challenge.

**2.6. RNA Extraction**

To extract RNA virus, 50-100 mg of homogenous tissue was removed separately using RNX_ plus Solution extract (Manufactured by CinnaGen Co., Iran) according to the manufacturer's instructions.

**2.7. Synthesis of cDNA**

For c-DNA synthesis, a random primer and cDNA Synthesis Kit (Yekta Tajhiz Azma, Iran) were used.

**2.8. cDNA Amplification Using PCR**

A pair of H9 influenza virus gene primers (Lee et al., 2001), F (5'- CAC CTY ACA GAR CAC GG AAT -3) and R (5'- GTC ACA CTT GTT Azam GTR TC -3') were used. The reaction factors included Mastermix 2 X (1.5 mM MgCl 2) (Amplicon, Canada) 10 µL, F primer (10 picomol per microliter), R primer (10 picomol per microliter), DNA template 3 microliter, and 6 microliter water. The final volume of 20 µl was processed with a thermocycler gradient apparatus as follows: 35 cycles 95, 53, and 72 each for 1 min followed by 72 for 10 min.

**2.9. PCR Product Evaluation**

PCR products were electrophoresed in 1% agarose gel at 100V and, after safe-staining, were visualized under UV light. 100bp DNA marker (CinnaGen, Iran) was used.

**2.10. Hemagglutination Inhibition Test (HI)**

Blood serum was separated and the HI (beta) test was performed (1).

**2.11. Statistical Method**

A 2x2 ANOVA was run to compare Group and Time, and a significant interaction between them was observed ($p<0.001$). One-way ANOVAs were run at each point to determine how groups differed.

## 3. Results

**3.1. Clinical Signs and Autopsy**

Birds in all groups showed no clinical signs, and gross lesions in post mortems of euthanized ducklings were not observed.

### 3.2. PCR Test Results

No virus excretion was observed on the first and eight days after the challenge (Table 1). In the unvaccinated challenged group, the virus was detectable from day 1 to day 5 after challenge and from day 3 to day 5 in the vaccinated-challenged group. In the challenged group, positive tracheal swabs were more than cloaca swabs, but in the vaccine-challenged group only cloaca swabs were positive. The virus was detected in the respiratory and gastrointestinal tissues of the ducklings in the challenged group but only in the intestines of the vaccine-challenged groups. The highest frequencies of positive cases in different tissues were observed on days 3 and 5 (Figure 1). Lymphoid tissue samples, spleen and bursa as well as brain and kidney tissues were negative in all groups (Table 1).

**Table 1.** Detection of influenza virus by PCR in different tissues of experimental groups

| | **Day 1 after challenge** | | | | **Day 3 after challenge** | | | | **Day 5 after challenge** | | | | **Day 8 after challenge** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tissue | Ch | V-Ch | V | C | Ch | V-Ch | V | C | Ch | V-Ch | V | C | Ch | V-Ch | V | C |
| Trachea | $\frac{1}{3}$ | - | - | - | $\frac{3}{3}$ | - | - | - | $\frac{3}{3}$ | - | - | - | - | - | - | - |
| Lung | - | - | - | - | $\frac{2}{3}$ | - | - | - | $\frac{2}{3}$ | - | - | - | - | - | - | - |
| Intestine (Pieces of duodenum, jejunum, ileum) | - | - | - | - | $\frac{1}{3}$ | $\frac{2}{3}$ | - | - | $\frac{1}{3}$ | $\frac{2}{3}$ | - | - | - | - | - | - |
| Spleen | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Brain | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Bursa | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Kidney | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

V: vaccine Ch: challenge V-Ch: vaccine-challenge C: control

$$Fraction = \frac{Positive\ samples}{Total\ samples}$$

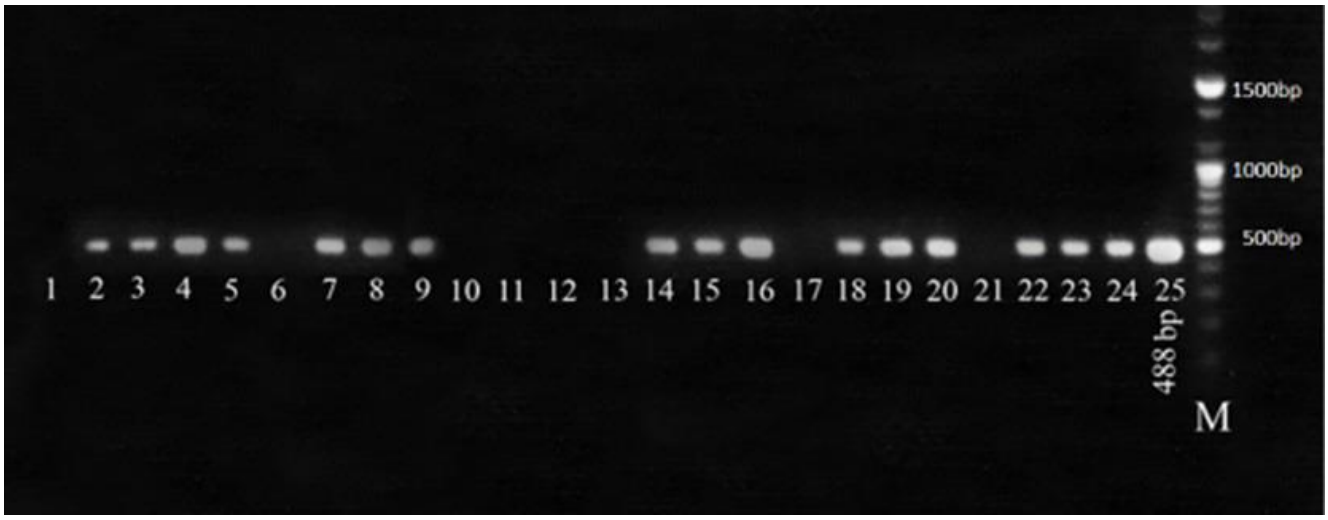


**Figure 1.** Electrophoresis of PCR product for detection of the H9 gene of avian influenza virus in 1% agarose gel with a band size of 488 bp with 100 bpDNA marker. M; marker, no. 1: negative control, no. 2: positive control, no. 3-25: positive and negative samples.

### 3.3. Haemagglutination Inhibition (HI) Test Results

Days 31 and 41 had group differences ($p$<0.001). Post hoc comparisons with a Tukey adjustment were run to determine how groups differed. On day 31, the vaccine-challenged and vaccine-control ducks differed from both non-vaccine groups ($p$<0.001) but not from each other ($p$=1.000). The non-vaccine groups did not differ from each other ($p$=1.000). On day 41, the vaccine-challenged and vaccine-control ducks differed from both non-vaccine groups ($p$<0.001) but not from each other ($p$=0.152). The two non-vaccine groups differed ($p$<0.001) with the challenged ones being higher than the control. The HI test results are shown in Table 2. The control group had a decrease in HI antibody titer. In the vaccine group, the titer increased after vaccination, and in the vaccine-challenged group, the titer of hemagglutination inhibition was higher than it was in the other groups. In the challenged group, the rise in the titer showed that the virus, which originated in chickens, was able to stimulate the immune response in ducks, and although the level of antibodies in the challenged group increased, virus excretion was also detected in them. Therefore, the immunity created by the vaccine was able to reduce the excretion of the virus.

On the day before vaccination, the mean geometric mean of the antibody against the influenza virus was 4.4, and the range of the antibody was $2^3$ to $2^5$ (with a frequency of 50%, 40%, and 10%, respectively). The geometric mean of antibody against the influenza virus in the vaccine group was 5.7, and the antibody grade on this day for the non-vaccine group was 1.1. The increase in antibody titers in the vaccine group suggests that the influenza vaccine, which was injected intramuscularly in the ducklings of the vaccine group 14 days prior, was able to provide protection against the influenza virus in ducks. The titer from the non-vaccine group indicated that the birds of these two groups had not been infected in these 14 days.

**Table 2.** HI titers, (Mean±std) of avian influenza virus based on logarithm 2

| Days | Day 8 (Before vaccination and challenge) | Day 31 (2 days before challenge and 21 days after (vaccination) | Day 41 (8 days after challenge and 31 days after vaccination) |
|---|---|---|---|
| **Groups** | Mean±std | Mean±std | Mean±std |
| Vaccine Challenged | 4.4±0.70 | 5.7±0.95$^{bd}$ | 7.1±.88$^{bd}$ |
| Non-Vaccine Challenged | 4.4±0.70 | 1.1±.32$^{ac}$ | 3.5±1.43$^{ad}$ |
| Vaccine control | 4.4±0.70 | 5.7±.95$^{bd}$ | 6.1±.99$^{bd}$ |
| Non-Vaccine control | 4.4±0.70 | 1.1±.32$^{ac}$ | 1±.67$^{abc}$ |

Different subscribe letters in each column indicate a significant ($p$<0.001) difference.

## 4. Discussion

The immune response depended on the type and age of the duck, the origin of the influenza virus, the tissues selected for sampling, and the method of inoculation (2). Ducks exhibit a weaker immune response to the H9N2 virus than mammals and poultry (2). There is limited information on H9N2 infection in ducks (2). All experiments with the LPAI virus have been performed in the laboratory and mostly on domesticated Mallard ducks. Almost all studies have shown no signs of disease or pathological lesions, even when the virus was spread through feces. In only one case, Cooley and Van Campen (7) reported pulmonary injuries in ducks infected with LPAI viruses while they were healthy and observed macrophages in the bird's lungs two days after challenge. We found that the Avian influenza virus H9N2 from a chicken origin could not cause clinical or autopsy symptoms in hybrid ducks, and this finding correlated with other research; Kida and Yanagawa (8) reported duck influenza lacking evidence of disease signs and immune response; Munster and Baas (9) reported that following intratracheal and intrapharyngeal inoculation of the LPAI virus, the virus did not cause clinical symptoms, but a marked reduction in weight gain was observed; Wang and Wang (10) inoculated H9N2 virus into ducks through the intranasal route and did not observe any specific clinical signs. In challenged unvaccinated hybrid ducks, virus was detected in the lungs as well as the intestinal tract; this finding correlated with some research reports. Kida and Yanagawa (8) inoculated Pekin ducks with LPAI virus isolated from the respiratory tract of love birds and observed that the virus was replicated in the lower end of the duck gastrointestinal tract; Munster and Baas (9) studied spatial, temporal, and species variations in the prevalence of influenza A viruses in wild migratory birds and concluded that influenza A viruses could be isolated from respiratory and cloacal swab samples. Thayer and Beard (2) reported that the main sites for virus replication in ducks and chickens are the gastrointestinal and respiratory tracts, respectively. Daoust and Kibenge (11) studied the replication of low pathogenic avian influenza virus in naturally infected mallard ducks (Anas platyrhynchos) and concluded that low pathogenic avian influenza A viruses can be isolated from oropharyngeal and cloacal swabs. Parmley and Soos (5) compared two sampling

methods, cloacal swabs alone and combined oropharyngeal and cloacal samples, to detect low pathogenic avian influenza viruses in wild ducks in Canada; they reported that combined samples improved virus detection. Daoust and van de Bildt (12) studied the replication of 2 subtypes of low-pathogenicity avian influenza virus of duck and gull origins in experimentally infected Mallard ducks and concluded there is a possible clinical significance of LPAI virus-associated pulmonary lesions and intestinal tract infection. Wang and Li (13) reported that the virus was detectable in cloacal swabs up to day 14. The present study showed that in challenged vaccinated hybrid ducks, virus was detected only in the intestinal tract, and vaccination prevented proliferation of low pathogenic influenza virus H9N2 in the respiratory tract of hybrid ducks. No report in this regard could be found; thus, no comparisons with other research findings could be made. In the present work, viral excretion was observed up to day 5 after inoculation. LPAI viruses can pass through the anterior gastrointestinal tract of ducks and propagate to the distal parts of the digestive tract without clinical symptoms. The main site of LPAI virus replication is the Lieberkuhn gland's crypts in the colon. Another target organ for LPAI viruses in ducks is the respiratory tract (4). Some researchers have shown that HPAI viruses multiply in the duck's respiratory tract, while LPAI viruses multiply in the gastrointestinal tract (12). Wang and Wang (10) reported that virus excretion was higher in the cloacal swabs than in the tracheal swabs. In the present study, increases in antibody titers in the vaccine group suggest that the influenza vaccine, which was injected intramuscularly into ducklings of the vaccine group, was able to provide protection against the influenza virus in ducks. The challenged group that did not receive the vaccine had a higher tracheal than cloacal virus shedding. Parmley and Soos (5) showed by fluorescent antibody that the virus replicates more in gastrointestinal and respiratory epithelial cells than in lymphocyte cells. In the present study, lymphoid tissue samples including spleen and bursa were also negative, and the H9N2 virus of chicken origin was detected in both intestinal and respiratory tracts, indicating the ability of the virus to replicate in intestinal and respiratory tracts, and killed vaccine prevents virus replication in the respiratory tract. Kim and Negovetich (4) inoculated LPAI virus in Mallard and Muscovy duck egg embryos and observed fewer deaths in Mallard embryos than Muscovy embryos. Viral antigen was detected in the internal organs of Mallard embryos including the nasal sinus, pharynx, trachea, bronchus, lung, and air sac, but in Muscovy embryos, virus antigen was not detected. The reason for this paradox was unclear. Mallard ducks are a natural reservoir for LPAI viruses and the virus has adapted to them. Information on duck immune responses to influenza viruses is limited. By intranasal inoculation of H9N2 in Pekin, Mallard, and Muscovy ducks, Wang and Wang (10) showed that Muscovy ducks are susceptible to the H9N2 virus, but Pekin and Mallard ducks are resistant. In the present study, the chicken-origin virus was able to stimulate the duck's immune response. The level of antibodies in the challenged group increased. Therefore, the vaccine immunity response was able to reduce virus excretion. Kida and Yanagawa (8) isolated the influenza virus in pin teal wild ducks, but no immune response was observed in their blood, and their findings were not correlated with those of the present study. They reported that in the first phase of virus inoculation, no antibody response was observed.

The protective immune mechanism against AI virus infection in avian species has not yet been identified (8). Kim and Negovetich (4) reported that White Pekin ducks infected with the H7N2 virus, despite shedding the virus through feces 7 days after inoculation, produced poor hemagglutination level responses. The ducks were re-inoculated with the same virus 46 days later and the antibody titers were measured, but the virus was not isolated in any of the organs.

In conclusion, the results of this study indicate that the vaccination of ducks against AI H9N2 virus reduces

shedding and tissue distribution of AI viruses in challenged ducks.

## Authors' Contribution

Study concept and design: M. M.
Acquisition of data: M. M.
Analysis and interpretation of data: M. M.
Drafting of the manuscript: M. M.
Critical revision of the manuscript for important intellectual content: M. M.
Statistical analysis: M. M.
Administrative, technical, and material support: M. M.

## Ethics

All procedures performed in studies involving animals were in accordance with with accession number (KP455991.1)

## Conflict of Interest

The authors declare that they have no conflict of interest.

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