Evaluation of avian influenza killed vaccine on tissue distribution and shedding of avian influenza virus H9N2 in ducklings

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Abstract

Ducks have an outstanding role in the transmission of avian influenza to poultry farms and due to importance of vaccination in reducing shedding the virus, this study was performed to evaluate 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 condition. 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 intra-nasally at 30 days. Group 4 chicks were kept as control group. The chicks were observed two times daily. On days 1, 3, 5 and 8 after inoculation, 3 chicks were randomly selected from each groups and cloaca and trachea swabs samples were collected from each bird. Then the ducklings were euthanized, and trachea, lungs, spleen, intestine, liver and brain tissues 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 vaccinated challenged group, virus was
detected only in cloacal swabs but in unvaccinated challenged group, detection of virus in tracheal swabs was more than cloacal swabs. In challenged-unvaccinated chicks, virus was detected in trachea and lungs and in challenged-vaccinated birds, virus was detected in intestine. In conclusion, vaccination of ducks against AI H9N2 virus reduce shedding and tissue distribution of AI viruses in challenged ducks.

**Key words:** avian influenza virus H9N2, replication, shedding, vaccination, ducklings

**Introduction**

Avian influenza (AI) is caused by type A viruses belonging to the *Orthomixoviridae* family (Villegas et al., 2008). Avian influenza viruses have a fragmented genome; rearrangement is an important mechanism in genetic variation. One of the important features of influenza viruses is the frequent alteration by the antigenic mechanisms of drift and shift to produce antigenic variants (Thayer et al., 2008). Avian influenza is a highly contagious disease that is linked to economic damage and threatens human and animal health. The avian influenza virus 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 in the *Anseriformes* birds (Zsuzsanna et al., 2017). Wild birds, especially aquatic birds from the order *Anseriformes*, are known as natural reservoirs for most low pathogenic avian influenza types (Kim et al., 2009). Transmission of LPAI generally occurs through the fecal-oral route in polluted aquatic habitats without any apparent symptoms of disease or mortality (Kim et al., 2009). 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 (Thayer et al., 2008). Various duck species are naturally resistant to HPAI viruses, 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 (Zsuzsanna et al., 2017). The avian influenza virus enters the body of the bird through contaminated water and food (Zsuzsanna et al., 2017). The spread of the virus, especially in the H4N7, H11N9, H7N3 subtypes in ducks that have been experimentally infected through the feces, is greater than the trachea and respiratory tract (Zsuzsanna et al., 2017). Avian influenza viruses have also high persistence in water and are isolated from the surface of wetlands and lakes where large numbers of ducks reside (Parmley et al., 2011). 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 (LPAI) have high survival in the duck population. This mechanism could be the cause of more infections in surface water-feeding ducks than feeding ducks from deep water (Zsuzsanna et al., 2017). Review of available published articles showed that few articles regarding hybrid duck vaccination against avian influenza virus H9N2 are available in Iran. Therefore, present study was carried out to evaluate the avian influenza killed vaccine H9N2 on tissue distribution and shedding of the virus in ducklings.

**Materials and Methods**

**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 (EID50) was calculated for the second passage according to the method of Reed and Muench (Villegas et al., 2008).

**Experiment design.** The experiment was designed according to ethical permission
One hundred-day-old ducklings (hybrid strain) were purchased and after bleeding randomly from 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 groups 2 and 3 chicks were challenged with 0.1 ml allantoic liquid containing $10^5 \text{EID}_{50}$ A/chicken/Iran/Aid/2013(H9) virus intra-nasally at 30 days. Group 4 chicks were kept as uninfected unvaccinated control group. The duckling was observed two times daily. The AI virus used in this study was isolated from broiler flocks in Ahvaz city by Boroomand et al. (2016).

**Sampling**

**Serology.** Blood samples were collected from 20 hybrid ducklings at day-old via saphenous vein and at 31 and 41 days from 10 ducklings of each group via the saphenous vein to determine AIV antibodies using HI test (Villegas et al., 2008).

**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 and then euthanized by intravenous injection sodium pentobarbital (50.00 mg/kg) and trachea, lungs, bursa, spleen, intestine, liver, and brain samples were collected and stored at -70 °C up to molecular detection.

**Virus detection.** To detect the influenza virus, the RT-PCR test was performed on tissue samples and tracheal and cloaca swabs after challenge.
**RNA extraction.** For extraction of 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.

**Synthesis of cDNA.** For the c-DNA synthesis, random primer and cDNA Synthesis Kit (Yekta Tajhiz Azma, Iran) was used.

**cDNA amplification using PCR.** To conduct the process 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 GTT 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 microlitre, 6 microlitre water. The final volume of 20 µl was processed with a thermocycler gradient apparatus as follows: 35 cycles including: 95, 53 and 72 each for 1 min followed by 72 for 10 min.

**PCR product evaluation.** The 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.

**Hemagglutination inhibition test (HI).** The blood serum was separated and the HI (beta) test was performed (Villegas et al., 2008).

**Statistical method.** We ran a 2x2 ANOVA comparing Group and Time. There was a significant interaction between group and time (p<0.001). One-way ANOVAs were run at each point to determine how groups differed.

**Results**

**Clinical signs and autopsy**
Birds in all groups did not show any clinical signs, and gross lesions in post mortem of euthanized ducklings, were not observed.

**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 in the vaccinated-challenged group from day 3 to day 5. 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 and only in intestine of vaccine-challenged groups. The highest frequency of positive cases in different tissues was observed on days 3 and 5 (Fig. 1). Lymphoid tissue samples, Spleen and bursa, as well as brain and kidney tissues, were negative in all groups (table 1).

**Haemagglutination inhibition (HI) test results**

Days 31 and 41 had groups differences p<0.001. Post hoc comparisons with a Tukey adjustment was run to determine how groups differed on day 31 and 41. On the day 31th Vaccine-challenged and Vaccine-Control differed from both Non-vaccine groups (p<0.001) but did not differ from each other (p=1.000). Both non-vaccine groups did not differ from each other (p=1.000). On the day 41th Vaccine-Challenge and Vaccine-Control differed from both Non-vaccine groups (p<0.001) but did not differ from each other (p=0.152). The two non-vaccine groups differed (p<0.001) with the Challenge higher than control. The HI test results were shown Table 2. The control group had a decrease in HI antibody titer. In the vaccine group, after vaccination, the titer increased, and in the vaccine-challenge group, the titer of hemagglutination inhibition was higher than other groups. In the
challenge group, the raising of the titer shows that the virus, which originated in chickens, was able to stimulate the immune response in ducks, and although the level of antibodies in the challenge group increased, but the virus excretion was also detected in them. Therefore, the immunity created by the vaccine has been 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, 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 was 1.1 for the non-vaccine group. 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 ago, was able to provide protective protection against the influenza virus in ducks. The titer from the non-vaccine group indicates that the birds of these two groups have not been infected in these 14 days.

**Discussion**

The immune response depends on the type and age of the duck, the origin of the influenza virus, the tissues selected for sampling and the method of inoculation (Thayer et al., 2008). Ducks exhibit a weaker immune response than mammals and poultry to the H9N2 virus (Thayer et al., 2008). There is limited information on H9N2 infection in ducks (Thayer et al., 2008). All experiments with the LPAI virus have been performed in the laboratory and mostly on domesticated Mallard ducks. Almost all studies show no signs of disease or pathological lesions even when the virus spreads through the feces. In only one case, Cooley et al. (2007) 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 (Cooley et al., 2007). We find Avian influenza virus H9N2 chicken origin could not cause clinical and autopsy symptoms
in hybrid ducks and this finding correlated with other researcher; Kida et al. (1980) reported duck influenza lacking evidence of disease signs and immune response, Munster et al. (2007) following intra tracheal and intra pharyngeal inoculation of the LPAI virus, the virus did not cause clinical symptoms, but a marked reduction in weight gain was observed and Wang et al. (2019) inoculated H9N2 virus into duck via the intranasal route and did not observe any specific clinical signs. In challenged unvaccinated hybrid ducks, virus detected in lungs as well as intestinal tract and this finding correlated with some researcher reports; Kida et al. (1980) inoculated pecking duck with LPAI virus isolated from love bird and observed virus was replicated in the lower end of the gastrointestinal tract, while virus was isolated from respiratory tract of love bird, Munster et al. (2007) studied spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds and concluded influenza A viruses could be isolated from respiratory and cloacal swabs samples. Thayer et al., (2008) reported the main site for the virus replication in the duck and chicken respectively are the gastrointestinal and respiratory tract, Daoust et al. (2011) studied replication of low pathogenic avian influenza virus in naturally infected mallard ducks (Anas platyrhynchos) and concluded low pathogenic avian influenza A viruses isolated from oropharyngeal and cloacal swabs. Parmley et al. (2011) compare two sampling methods, cloacal swabs alone and combined oropharyngeal and cloacal samples to detect low pathogenic avian influenza viruses in wild ducks in Canada and reported combined samples improved virus detection. Daoust et al. (2012) studied replication of 2 subtypes of low-pathogenicity avian influenza virus of duck and gull origins in experimentally infected mallard ducks and concluded that the possible clinical significance of LPAI virus-associated pulmonary lesions and intestinal tract infection and Wang et al. (2014) reported up to day 14, the virus was detected in cloacal swabs. Present study showed in challenged vaccinated hybrid duck virus detected only in intestinal tract and vaccine prevent proliferation of low pathogenic influenza virus H9N2 in respiratory tract of hybrid ducks. We could not find any report in this regard and could not compare with other researcher. In our 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 (Kim et al., 2009). Some researcher showed that HPAI viruses multiply in the duck's respiratory tract, while LPAI viruses multiply in the gastrointestinal tract (Daoust et al. 2012). Wang et al. (2019) reported the virus excretion was higher in the cloacal swabs than in the tracheal swabs. In the present study, increased in antibody tiers in the vaccine group suggests that the influenza vaccine, which was injected intramuscularly in the ducklings of the vaccine group, was able to provide protective protection against the influenza virus in ducks. The challenged group that did not receive the vaccine had a higher tracheal virus shedding than the cloaca. Parmley et al. (2011) showed by Fluorescent antibody that the virus replicates more in the gastrointestinal and respiratory epithelial cells than in the lymphocyte cells. In this 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 tract, indicating the ability of the virus to replicate in intestinal and respiratory tracts, and killed vaccine prevent virus replication in the respiratory tract. Kim et al. (2009) inoculated LPAI virus in mallard and Muscovy duck embryos eggs and observed fewer deaths in mallard embryos than Muscovy embryos and viral antigen was detected in 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 response to influenza viruses is limited. Wang et al. (2019) by intranasal inoculation of H9N2 in Peking, Mallard and Muscovy showed that Muscovy ducks are susceptible to the H9N2 and Peking and Mallard ducks are resistant. In our study, the chicken-origin virus was able to stimulate the duck's immune response. The level of
antibody in the challenged group increased. Therefore, the vaccine immunity response was able to reduce virus excretion. Kida et al. (1980) isolated the influenza virus in pin teal wild ducks, but no immune response was observed in their blood, and their finding was not correlated with our findings.

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 (Kida et al., 1980). Kim et al. (2009) reported that White pelin ducks infected with the H7N2 virus, despite shedding virus 7 days after inoculation, through feces, produced poor hemagglutination levels response. 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 indicated that vaccination of ducks against AI H9N2 virus reduce shedding and tissue distribution of AI viruses in challenged ducks.

Acknowledgments

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Conflict of interest

The authors declare that there is no conflict of interest.

References

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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 bp DNA marker. M; marker, no.1: negative control, no.2: positive control, no.3 to 25 positive and negative samples.
Table 1: Detection of influenza virus by PCR in different tissues of experimental groups.

<table>
<thead>
<tr>
<th>tissue</th>
<th>Day 1 after challenge</th>
<th>Day 3 after challenge</th>
<th>Day 5 after challenge</th>
<th>Day 8 after challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ch</td>
<td>V-Ch</td>
<td>V</td>
<td>C</td>
</tr>
<tr>
<td>trachea</td>
<td>1/3</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>lung</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Intestine (Pieces of duodenum, jejunum, ileum)</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>spleen</td>
<td>-</td>
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<tr>
<td>brain</td>
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<tr>
<td>bursa</td>
<td>-</td>
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<tr>
<td>kidney</td>
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</tbody>
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V: vaccine  Ch: challenge  V-Ch: vaccine-challenge  C: control

Fraction = \frac{\text{positive samples}}{\text{Total samples}}
Table 2: HI titers, (Mean±std) of avian influenza virus based on logarithm 2

<table>
<thead>
<tr>
<th>Days</th>
<th>Day 8 (Before vaccination and challenge)</th>
<th>Day 31 (2 days before challenge and 21 days after vaccination)</th>
<th>Day 41 (8 days after challenge and 31 days after vaccination)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean±std</td>
<td>Mean±std</td>
<td>Mean±std</td>
</tr>
<tr>
<td>Vaccine Challenge</td>
<td>4.4±0.70</td>
<td>5.7±0.93&lt;sup&gt;bd&lt;/sup&gt;</td>
<td>7.1±8.8&lt;sup&gt;bd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Non-Vaccine Challenge</td>
<td>4.4±0.70</td>
<td>1.1±3.2&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>3.5±1.43&lt;sup&gt;ad&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vaccine control</td>
<td>4.4±0.70</td>
<td>5.7±9.5&lt;sup&gt;bd&lt;/sup&gt;</td>
<td>6.1±9.9&lt;sup&gt;bd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Non-Vaccine control</td>
<td>4.4±0.70</td>
<td>1.1±3.2&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>1±6.7&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

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