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Evaluation of inactivated vaccine's Antibody response to different H9N2 Vaccination programs with Hemagglutination Inhibition (HI) assay

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A B S T R A C T

Influenza is a significant poultry disease that can also affect humans, making it a zoonosis. The World Health Organization (WHO) and Contagious Diseases Organization (CDO) have increasingly focused on this disease in recent years. The isolation of influenza, particularly H5N1 and H9N2 subtypes, has dramatically increased, posing a global epidemic threat and causing deaths in various communities, as well as substantial losses in poultry. In response, using inactivated avian influenza vaccines has become common in controlling the disease. A comprehensive study was conducted in broiler farms in Iran to address the importance of influenza control through vaccination. The study involved 50,000 broiler chicks divided into seven groups with different vaccination programs. The groups were vaccinated at different ages and received varying vaccine doses. Serum samples were collected weekly and analyzed for antibody titers using hemagglutination inhibition (HI). The results showed that the groups vaccinated after seven days of age had a more consistent immune response and higher antibody titers than those vaccinated at a younger age or with only one dose. Early vaccination before seven days of age did not effectively stimulate the desired antibody response or achieve expected titers for the H9N2 influenza virus. Therefore, vaccinating older chicks improved immunity and flock protection more effectively. The study showed how vital it is to have good vaccination plans for managing flu in broiler chickens. The findings offer key details on when and how much vaccine to use for the best immune protection, especially against the H9N2 flu strain. By putting in place effective vaccination measures, the spread and severity of flu among poultry can be lessened, and the chance of it passing to humans can be lowered, helping to protect the health of both animals and people.

Keywords: *Broiler, Hemagglutination Inhibition, H9N2, LPAI, Vaccination.*

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1 Introduction

Avian influenza virus (AIV) is a Negative-Sense Single-Stranded RNA virus belonging to the family Orthomyxoviridae and genus (type) Influenzavirus A, and its genome consists of 8 segments. (1). According to the antigenic composition of surface glycoproteins among 18 types of hemagglutinin (HA) and 11 types of neuraminidase (NA), AIVs are classified into various subtypes (2). Genetic characteristics and pathogenicity tests (intravenous pathogenicity test) determine whether the virus is placed in the pathotypes of Highly pathogenic Avian Influenza virus (HPAIV) or Low pathogenic Avian Influenza virus (LPAIV). A wide range of subtypes exhibit the pathogenic features of LPAI, but HPAIs are associated only with H5 and H7 (2-5).

HA glycoprotein, as the main antigenic component, is the instrument of the virus for attachment to the sialic acid receptor of the host cell and fusion with its plasma membrane (1, 2). Most AIVs bind more closely to sialic acid α2,3 in birds' respiratory and digestive systems. A few mutations can increase the compatibility of the virus with mammalian α2-6 sialic acid, including human receptors, and this changes the host preference of the virus and causes efficient replication of the virus in the new host (1, 6). Influenza viruses are prone to viral polymerase errors and the exchange of gene parts between different viruses infecting the same host. Hence, they have a high mutation rate, and viruses with new genotypic and phenotypic characteristics appear rapidly in old and new hosts (1, 6, 7). Wild aquatic birds are the natural hosts of various AIV subtypes and reserve the virus. However, a wide variety of birds and mammals can be infected by the virus (8, 9).

Four high-mortality human influenza pandemics involving H1, H2, and H3-related subtypes have been recorded in the last century. Other subtypes, such as H5, H7, H9, and H10, have also been reported sporadically in human societies, which can sometimes be fatal (1, 3, 4, 9). Genetic evidence suggests that these strains are partially or completely related to influenza viruses circulating in birds. Therefore, avian influenza viruses should be considered an important zoonotic agent and a severe threat to public health (1, 7, 9). Considering the simultaneous and complex circulation of different subtypes of AIV in wild bird populations, industrial and backyard poultry, and, importantly, live bird markets, controlling the virus in bird populations is likely to reduce human exposure and provide a practical solution to prevent infections. Vaccination for humans cannot be considered the primary solution because the economic cost needs to be justified compared to the clinical disease that is currently observed (6, 10).

In poultry, extremely high mortality and severe production reduction can be caused by HPAIV in laboratory and field conditions, while LPAIV shows minimal clinical signs in experimental trials (11). In contrast, field conditions for these viruses can be very different. LPAIV infection can cause high casualties and severe economic losses when associated with other pathogens such as agents of infectious bronchitis, Newcastle disease, infectious bursal disease, mycoplasma, and Escherichia coli, or in stressful environmental conditions such as poor temperature and ventilation management, high levels of ammonia, and vaccination against other diseases (1, 2, 6, 12-14). Also, the immunosuppressive effect and increased susceptibility to other pathogens have been demonstrated in the case of AIV (2, 4).

HPAIs were first identified in China and have been circulating in Asia since then. Globally, new contamination streams with this agent in African, European, and American countries have challenged the poultry industry (15). Currently, LPAIV H9N2 is the most common strain in poultry worldwide. This virus was first detected in Wisconsin turkeys in 1996, and today, it is enzootic in many countries (including Middle Eastern countries such as Iran) (1, 3, 16). The simultaneous circulation of LPAIV and HPAIV in poultry flocks has been reported. In this situation, in addition to the individual threats of each, the role of the gene donor and the production of new strains that endanger the health of industrial poultry production and human society becomes important (3, 6).

Vaccination is suggested to control Avian influenza disease as part of an integrated strategy along with biosecurity principles and surveillance programs, and its positive effect in reducing the risk of infection and its complications has been proven (1, 4, 5). Vaccination against HPAI is currently not allowed in some countries but may be used in emergencies or when there is a reasonable prospect of eradicating HPAI in that area shortly (15). Vaccination against LPAI, primarily H9N2, is commonly performed in countries where the virus is enzootic. In these countries, when biosecurity measures are incompatible with dense breeding systems and testing and killing procedures are costly, vaccination is considered the first option for infection control (5, 6).

The bird's immune system is important in opposing the influenza virus. The stimulation of mucosal immune

responses, especially in the respiratory system, as the primary virus replication site, is significant. On the other hand, Cell-mediated immunity has an undeniable role in controlling AIV infection and primarily targets the internal components of the virus (4). In humoral immunity, most antibodies are against the HA glycoprotein, although anti-NA antibodies can also reduce disease severity (1, 4).

Inactivated whole virus vaccines are the most common influenza vaccines in poultry. These vaccines mainly stimulate humoral immunity, and the produced antibody is particularly effective in preventing the clinical appearance of disease and mortality. However, it cannot necessarily prevent virus shedding. The ease of production and non-return of these vaccines to pathogenic form are some advantages of these vaccines (4, 17). Inadequate stimulation of mucosal immunity reduces the effectiveness of these vaccines. In addition, their widespread and long-term usage has increased the immune selection pressure on AIV. Hence, gene exchange and antigenic drift in HA epitopes have progressed to the point where new viruses sometimes cause vaccination failure.

For this reason, infection and disease outbreaks are still observed in vaccinated flocks (6, 7, 10, 12, 18). Also another limitation of these vaccines is the inability to differentiate between vaccinated and infected animals with standard serological tests (19). Vector vaccines, live-attenuated vaccines, DNA and mRNA vaccines, virus-like particle vaccines, and recombinant protein vaccines are other options. However, many of them have not been used clinically (4).

Incompatibility of the vaccine seed with the field strain, emergence of new viruses, unregulated dose and interval, insufficiency of the bird's immune system, and inappropriate vaccination age can lead to vaccination failure (6, 20, 21). Therefore, evaluating the immune system's response to the vaccine becomes important. Antibody titers following influenza vaccination can be quantified by agglutination inhibition (HI) test (17). In the present study, induction of antibody response to killed H9N2 vaccine in broiler chickens following different vaccination regimens was assessed using the HI test.

2 Methods and Materials

2.1 Birds and breeding management

Fifty thousand one-day-old Ross308 broilers from the same mother flock and hatchery were purchased. Five houses with dimensions of 70 meters in length and 13 meters in width were prepared in Gorgan province, Iran, and 10 thousand chickens were kept in each for 42 days. All chickens' breeding management principles were the same during this period, except for the influenza vaccination regimen. Nutrition was based on a corn-soybean meal diet balanced with a primary energy of 3100 kcal and 22% crude protein.

2.2 Study Plan

Two spaces of 2 meters x 5 meters were considered, and 100 chickens in two groups were placed in each. The rest of the chickens were spread in the rest of the houses. Each house with a capacity of 1000 chickens was considered one of the groups A to E. Group A was inoculated subcutaneously with the killed H9N2 vaccine on days 7 and 17 of breeding. Groups B and C were vaccinated as a single dose on days 7 and 12, respectively. Group D received the vaccine once in 12 days but with a dose of 1.5 times higher than the other groups. Group E was vaccinated twice on days 1 and 12. Group F (100 chickens) was vaccinated only on the first day. Group G (100 chickens) did not receive a vaccine against AIV as the control group.

On the first day, 16 chickens were randomly selected, and their blood samples were taken. Then, on days 7, 14, 21, 28, 35, and 42, sixteen chickens from each group were randomly selected, and blood sampling was performed. On the last day of the breeding period, the groups were weighed, and the amount of feed consumed by each group was calculated.

2.3 Sample taking

1 ml of blood was taken from the jugular vein by sterile two cc syringes, and then air equal to the blood volume was drawn into the syringe. Then, the syringes were placed at an angle of 30 degrees until the serum was separated from the blood clot. The collected serum was referred to the laboratory for serology test.

2.4 Hemagglutination Inhibition assay

Each tested serum was serially diluted in 12 horizontal wells of the plate, and then 25 microliters of commercial antigen solution with 4 HA units were added to each well. After 30 minutes, 25 microliters of chicken RBC suspension were added to each well, and 30 minutes later, the antibody titers were recorded based on the logarithm of 2.

2.5 Statistical Analysis

Statistics were analyzed using SPSS software version 26. A chi-square test was also conducted to determine significance.

3 Results

Mean feed consumption, weight, and feed conversion rate (FCR) on the 42nd day for each group is revealed in Table 1. The final weights in groups B, C, D, F, and G were almost close but groups A and E's weights were in a lower range. The lowest final weight belonged to Group A, and the highest belonged to Group G (control). Group E consumed the lowest amount of feed, and Group G (control) consumed the highest. Calculation of FCR showed that group A had the highest conversion rate and group D had the lowest. The groups that received the vaccine once had lower mortality and higher weight at the end of the period than those vaccinated twice, or the vaccine dose was considered higher. Mortality rates in all groups were significantly increased on post-vaccination days compared to normal days.

Table 1. Record of production data and related indicators.

	Mean weight (g)	Feed Consumption (kg)	FCR	M42	MDM	MPV
Group A	1700	3014	1.77	430 (4.3%)	9	30
Group B	2200	3730	1.69	380 (3.8%)	7	28
Group C	2000	3010	1.50	390 (3.9%)	9	38
Group D	2050	3019	1.47	418 (4.18%)	10	18
Group E	1600	2998	1.87	420 (4.2%)	15	54
Group F	2250	3700	1.64	2 (2%)	-	-
Group G	2300	3794	1.64	2 (2%)	-	-

FCR (Feed Conversion Rate), M42 (Mortality during 42 days), MDM (Mean Daily mortality), MPV (Mean Mortality in days Post Vaccination), Group A: Two rounds of vaccination at days 7 and 17, Group B: One round of vaccination at day 7, Group C: One round of vaccination at day 12, Group D: One round of vaccination with 1.5 times dose at day 12, Group E: Two rounds of vaccination at days 1 and 12, Group F: One round of vaccination at day 1, Group G (control): Not vaccinated

During this study, 699 serum samples were collected, and antibody titers were assessed with the HI Test. Data related to this serological investigation are shown in Table 2. On the first day, the average antibody titer in 16 random chickens out of 50,000 chickens was 3.56. At seven days, a decreasing trend of antibody titer compared to the first day was observed in chickens. The difference between the titers of the groups was statistically significant (P>0.05). At the age of 14 days, this process continued, but the difference between the groups was not significant. At 21 days, titer was observed in all the groups that received the vaccine on days 1, 7, or 12, except for group D. All groups except group F showed an increase in titer on day 28. On the 35th day, all groups except group F had the highest titers compared to the previous days. At 42 days, group F showed an increase in titer, and group G showed a decrease in titer. In other groups, the titers were relatively close to the values of day 35. The titer difference between the groups was statistically significant on day 21 and after that. Serological examination showed that not only early vaccination against influenza before seven days of age did not lead to the induction of a favorable antibody titer, but also, at the age of 42 days, the mean titer obtained from this program was not significantly different from the group that did not receive the vaccine. The group that received only one dose of vaccine at seven days developed the highest antibody titers throughout the study.

Table 2. The results of the HI test in broiler chickens using different vaccination programs.

	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Group A	1.5_{a}	1	1.875_{a}	3.813_{a}	4.25_{a}	4.125_{a}
Group B	1.625_{a}	0.625	2_{a}	4.5_{a}	6.813_{b}	4.813_{a}
Group C	2.125_{b}	0.563	0.875_{b}	2.563_{b}	4.063_{ae}	4.688_{a}
Group D	1.375_{a}	0.938	0.563_{b}	4_{a}	4.938_{a}	4.688_{a}
Group E	2.563_{cd}	0.938	1.750_{a}	2.563_{b}	3_{ce}	2.563_{b}
Group F	2.375_{bd}	0.875	1.625_{a}	0.313_{c}	0.313_{d}	1.063_{c}
Group G	2.250_{bd}	1.063	0.5_{b}	1.25_{c}	1.938_{c}	0.438_{c}

Group A: Two rounds of vaccination at days 7 and 17, Group B: One round of vaccination at day 7, Group C: One round of vaccination at day 12, Group D: One round of vaccination with 1.5 times dose at day 12, Group E: Two rounds of vaccination at days 1 and 12, Group F: One round of vaccination at day 1, Group G (control): Not vaccinated, $_{a, b, c, d, e:}$ Different lower-case superscripts denote significant difference between treatments groups; Fisher's Exact test, P>0.05.

4 Discussion

This study concluded that Avian influenza vaccination with a killed vaccine before the appropriate time can produce a low antibody titer against the disease agent, and choosing the correct age, dose, and intervals between vaccines can positively affect the immune response following vaccination. However, we should not neglect the stressful effect of vaccine injection and its repetition in short intervals on the mortality rate and weight gain.

In order to discover the relationship between antigenic drift and persistence of the AIV H9N2 virus in vaccinated poultry flocks of China, the efficiency of a commercial AIV H9N2 vaccine against viruses that were in antigenic groups C, D, and E was investigated by Sun et al. The results showed that the effectiveness of vaccination is reduced when the vaccine virus belongs to the strains that are not antigenically compatible with Drifted viruses and can lead to the infection's stability in the specific region's poultry population (18).

In 2020, Talat et al. investigated the efficacy of two vaccination programs against the H9N2 avian influenza virus in broilers. Cobb-500 broiler chickens with maternal-derived immunity were treated with a killed vaccine based on the locally circulating virus and an imported vaccine in field and laboratory conditions at different ages. On day 28, chickens were challenged with the wild H9N2 virus. Examining the HI titer and virus shedding rate showed that vaccination on day 7 resulted in significantly higher immune response and less virus shedding than vaccination on the first day. In addition, the vaccine prepared from the local strain protected the flock more than the imported vaccine. As a result, it is recommended to use circulating virus seeds to produce vaccines and plan the H9N2 vaccination at 5 to 8 days of age (2).

Using a stochastic SIR model, Cui et al. (2021) showed that LPAI H9N2 vaccination, even in cases where high HI titers are obtained, may be unable to stop virus transmission after the challenge. They, therefore, stated that in addition to vaccination with killed vaccines, new strategies are needed to control avian influenza (21). Also, implementing the H9N2 vaccination program in Korea against the mentioned clade A subtype has dramatically reduced the prevalence of this clade. However, at the same time, the spread of clade B has increased in this country (7).

Evaluation of protection based on the severity of clinical symptoms, morbidity and mortality, and gross macroscopic lesions were considered important parameters for infection in a study by Khantour et al. HI monitored antibody titers against H9N2. Virus shedding was detected molecularly at 1, 3, 5, 7, 9, and 12 days after vaccination (14).

Another study showed that an HI titer of more than four can prevent Mortality and control virus shedding. It was demonstrated that shedding is further reduced when the vaccine and challenge strains are antigenically similar (22).

Kumar et al. (2007) stated that by vaccinating chickens at 25 days of age, HI titers less than 3.5 at week six did not confer immunity against clinical signs and mortality. The mortality stopped at titers between 3.5 and 4.5, but the shedding of the virus continued. Only titers above 4.5 were able to prevent mortality and shedding (17).

Amer et al.'s study (2011) showed that vaccination at seven days of age, compared to one day, can provide higher titers and a faster titer increase trend. Also, a decrease in antibody response was observed by halving the dose, and in this case, repeated vaccination failed to increase the antibody titer significantly (23).

De Vriese et al. stated that maternal-derived antibodies can remain in the progeny's serum until three weeks of age. Influenza vaccination on day one can cause much interference, but vaccination at ages between 7 and 10 days seems reasonable. They recommended that maternal antibody levels be measured on the first day and that vaccination may be repeated in high-risk situations to achieve adequate immunity levels (24). The results of Maas et al.'s study in 2011 are also significant. They stated that the antibody levels of the progeny at 14 days of age are eight times lower than that of their vaccinated mother flock and that this titer cannot provide reasonable protection against the challenge of HPAI. At the same time, it can interfere with vaccination and prevent obtaining the appropriate titers. In this study, chicks in the control group that lacked maternal antibodies had a higher HI titer 28 days after vaccination than the passively immunized group. It was also stated that several vaccination times can positively affect layer and mother flocks that live for extended periods (25).

Another study showed that the HI titer of maternal influenza antibodies is about 5.5 in the first week and decreases to less than 2 in the second week. Also, this study states that chicks vaccinated at five days of age show less protection against the HPAIV challenge than those vaccinated at ten days of age (26).

Abdelwahab et al. (2011) showed that the progeny of a vaccinated mother flock up to 10 days of age had protective antibody levels against the HPAI challenge; however, by day

14, maternal antibodies interfered with vaccination. Also, it is notable that chickens vaccinated on the 14th day could not resist the bird flu virus at 35 days (27).

Another critical factor for the effectiveness of vaccination is the bird reaching the right weight and the ability to produce sufficient antibodies. As stated by Swayne et al., two doses of vaccination in ducks and three doses in geese can provide long-term protection (52 and 32 weeks, respectively) (20).

Vaccines that simultaneously introduce two pathogens to the immune system may also be effective. Inactivated bivalent H9N2 and NDV vaccines were inoculated to White leghorn chickens at three weeks by Zhao et al. The highest antibody titer against H9N2 was achieved in the fourth week (mean titer 9.5), and this vaccine could completely stop virus shedding after challenge with the pathogenic virus (28). Also, In 2003, Swayne et al. evaluated the immune response following the use of recombinant influenza and Newcastle vaccines as eye drops in 2-week-old chicks. The serological response was compared by HI and ELISA tests. The results indicated that this vaccine partially protected against ultra-acute Newcastle strain and HPAIV pathotype challenges. In this study, chickens that received two vaccine doses produced higher antibody titers (29).

Lee et al. (2011) investigated the difference in immune response in different frequencies and adjuvants of H9N2 vaccination in 5 groups of SPF chickens. 4 groups received one or two doses of killed LPAI H9N2 vaccine with oil adjuvant or aluminum hydroxide gel adjuvant, and one group was not vaccinated as a control. The HI titer showed that the oil-adjuvanted vaccine produced a higher and faster antibody response in a single-dose regimen than the gel-adjuvanted vaccine. It was also observed that the Gel-primed + Oil-boosted regimen produced a slower but higher antibody titer than the Oil-Primed + Oil-boosted regimen. As a result, considering that the single-dose vaccination regimen may not be protective in field conditions and also the cost-effectiveness of aluminum hydroxide gel adjuvant, a two-dose regimen with an initial dose of Gel vaccine and a booster dose of Oil vaccine was suggested in this study (30). It also has been shown that in the production of inactivated vaccines, if we use beta-propiolactone and gamma irradiation, the humoral immune response increases compared to vaccines that were inactivated with formaldehyde (31).

It was stated that if the influenza vaccine is made from a seed with antigenic epitopes compatible with the challenge strain, and if the appropriate antibody titer is achieved, it can reduce the severity of symptoms and Mortality rate in case of infection. However, it is possible that, in some cases, it cannot stop virus shedding completely.

An adjusted vaccination program is needed to achieve the desired titer in the flock, which should consider several factors. It is necessary to pay attention to the type of vaccine and manufacturing technology, the coordination between the vaccine strain and the virus circulating in the region, the vaccine dose, the age and interval of vaccination, and the level of maternal immunity and its effect.

Conflict of Interest

The authors declared no conflicts of interest.

Author Contributions

All authors contributed to the original idea and study design.

Data Availability Statement

Data are available from the first author upon reasonable request.

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