Original Article

Newcastle Disease Vaccination Program in Broilers Using an Apathogenic Heat-Resistant Vaccine

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ABSTRACT

Newcastle disease (ND) is among the most common and deadliest poultry diseases worldwide. Thermostable Newcastle disease virus (NDV) vaccines have been widely used to protect village chickens against ND due to their decreased dependence on cold chains for transport and storage. The NDV4 Heat-Resistant (NDV4HR) vaccine is an apathogenic, heat-resistant, live vaccine that can induce immunity in chickens. In this study, 60 one-day-old Arain local hybrid broilers were divided into three groups of 20. Group A had the usual vaccination program in broiler flocks (seven days old: B1 type by eye drop and ND killed vaccine intramuscularly; 18 and 28 days old: LaSota strain orally). Group B did not receive any vaccine, and group C received the NDV4HR vaccine orally, six times from the first day to the 35th day of rearing. All groups were nasally challenged with acute Newcastle virus (genotype VIId) on the 35th rearing day. Regarding the hemagglutination inhibition serum antibody titer of the birds after the challenge, group B had the highest (7.12±1.05), and group C (6.37±1.31) had a higher titer than group A (6±1.22). After the challenge with the Newcastle virus, the necropsy findings and clinical symptoms of the disease were almost similar in groups A and C. Group B showed the most signs, with higher casualties than other groups. Regarding weight gain, group C had the highest weight at the end of the study (2204±106). On the second day after the challenge, groups A and B had virus shedding through the trachea and cloaca, while group B shed the virus only through the trachea. Furthermore, on the seventh day after the challenge, group A shed the virus through the cloaca, whereas group B did it through the trachea and the cloaca. On the other hand, there was no virus shedding in group C. This study showed that the NDV4HR vaccine provokes an antibody response that protects the birds against a virulent virus challenge.

Keywords: Broiler chickens, Heat-resistant vaccine, NDV4, Newcastle disease

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1. Introduction

Newcastle disease (ND) is a viral disease in domestic and wild birds that is characterized by gastrointestinal, respiratory, and neurological symptoms. Poultry infections with Newcastle disease virus (NDV) range from asymptomatic to highly fatal, depending on the pathotypes of the virus involved. The NDV is a paramyxovirus type 1 virus with apathogenic, lentogenic, mesogenic, and velogenic pathotypes. Clinical symptoms in viscerotropic velogenic forms are much more severe than those in neurotropic velogenic states. In viscerotropic velogenic forms, the bird suddenly loses its appetite and stops eating. However, the level of infection and clinical symptoms can differ according to the bird species and the isolate type (1). The head of the bird becomes swollen and has edema. The bird may have respiratory symptoms and distress. What is more specific is green excretion. Neurological symptoms include failure to respond to the perching test, neck twisting, stargazing, and paralysis. Autopsy symptoms include congestion and hemorrhage in the eyelid and conjunctival tissue, nasal discharge, petechiae between the tracheal rings, bleeding in the lungs, swelling and hemorrhagic points in the proventricular glands, and hemorrhage in the cecal tonsil and different parts of the gut, even the rectum. Horizontal transmission of the disease has been recorded repeatedly (2). Infected birds excrete the virus through feces and secretions of the upper respiratory tract (2). There is no etiologic treatment for this disease; only control and prevention can be achieved through vaccination and biosecurity (3). Many types of live and inactivated vaccines are used to protect poultry against ND. The lyophilized live vaccine of the NDV4HR strain (heat-resistant and apathogenic) has a higher shelf life at farm temperatures than conventional live vaccines. It provides increased protection of the respiratory and digestive systems against ND (4). Since this strain is resistant to heat, thermal shocks during transportation, storage, and vaccination procedures in commercial poultry have fewer undesirable effects on it than on usual vaccines (5). The source of the vaccine virus was taken from the NDV4 strain in Australia and transformed into a heat-resistant strain. Due to the apathogenicity of this strain, the use of this vaccine during production does not cause a drop in egg production, while some lentogenic strains have adverse effects on laying (6). Furthermore, different administration methods can be used for these vaccines, including drinking, spraying, and eye drops (7). The heat-resistant V4 (NDV4HR) vaccine against ND has brought encouraging results in backyard poultry in many countries, such as Africa and Southeast Asia (8, 9). The two strains of V4 and I-2 are used to produce NDV thermo-resistant vaccines; however, I-2 is more resistant than V4 (Aiders, 2014) (2020) investigated the molecular mechanism of thermostability in NDV vaccines (10). Chimeric viruses were constructed by exchanging fusion and hemagglutinin-neuraminidase (HN) genes between a heat-resistant and a thermolabile strain. The results showed that only chimeras with HN from the heat-resistant strain exhibited a thermostable phenotype. In addition, the molecular dynamics simulation revealed that specific amino acid substitutions affected viral thermostability, and mutant viruses with substitutions at positions 315 and 369 in the HN protein exhibited higher thermostability and activity. The study concluded that the HN gene is a significant determinant of thermostability in NDV vaccines, and specific amino acid residues have essential effects on viral thermostability. Another study by Zhao et al. (2018) investigated the mechanism of thermostability in NDV. The researchers focused on the role of the phosphoprotein (P) in NDV thermostability, as previous studies had not investigated this aspect. Using a reverse genetics system, they generated chimeric viruses by exchanging the P protein between a thermostable NDV strain and a thermolabile strain. The chimeric viruses were found to have similar growth properties, passage stability, and virulence compared to the parental strains. However, the thermostability of the chimeric virus with P derived from the thermolabile strain was reduced, while P from the thermostable strain enhanced the chimeric virus's thermostability. These findings indicated that P is an essential factor for NDV thermostability and suggested a theoretical basis for using the thermostable NDV4-C strain as a vaccine (11). Therefore, this study aims to investigate the immunogenic and protective effects of the NDV4HR strain vaccine compared to common Newcastle vaccines in broilers.

2. Materials and Methods

2.1. Study Design, Birds, and Grouping

A total of 60 one-day-old Arian local hybrid broilers (Iran) were transferred to the poultry house at the Faculty of Veterinary Medicine of Ferdowsi University of Mashhad, Mashhad, Iran, and reared under controlled environmental conditions. This facility has stringent biosecurity measures in place to prevent the transmission of diseases. Upon the chickens' arrival, the environmental temperature was set at 33°C and maintained at that level for the first 48 h to ensure the chickens' comfort and well-being. The humidity was also closely monitored and held at a level of at least 50%, which is optimal for the growth and development of broiler chickens. The chickens were provided with food and water as much as they desired throughout the experiment to ensure they received the necessary nutrients and water for their growth. At the end of the third week, the birds were transferred from the litter

to cages. The birds were randomly divided into three completely isolated groups of 20 (four replicates of five). Group A was the positive control group and received the usual vaccination program for broiler flocks. This group consumed the B1 ND vaccine (10^6-10^7TCID50; Avishield ND B1®, Dechra, UK) through an eye drop and the ND killed vaccine (Cevac®, UK) through an intramuscular injection on the seventh day after birth. On days 18 and 28, the birds of this group received the LaSota ND vaccine (Avishield ND®, Dechra, UK) orally. Group B did not receive any vaccines (the negative control group), while group C received the NDV4HR vaccine (MVP, Malaysia) weekly from the first day to the 35th day of rearing. All three groups were raised under similar conditions until the 35th day of breeding. On this day, all groups were challenged with a velogenic Newcastle virus (genotype VIId; 10^6 EID50/bird) through the nose. By carefully monitoring and controlling environmental conditions during the rearing period, the study's results were not affected by any external factors that could impact the chickens' health or susceptibility to disease.

2.3. Weighing, Feed Conversion Ratio, and Mortality

The birds were weighed weekly. The average weight of each group was then calculated. This weighing process continued until day 42 of rearing, which was the end of the experiment. The feed conversion ratio (FCR) was also measured and recorded weekly for each group. In addition to monitoring the growth and development of the broiler chickens, the mortality rate within each group was recorded. Any mortality within each group was examined and necropsied to determine the cause of death, and the number of dead birds was recorded for all groups. This information was used to assess the overall health and well-being of the chickens throughout the study period.

2.4. HI Test

The hemagglutination inhibition (HI) test is a commonly used method to measure the level of antibodies against NDV in poultry serum. In this study, the HI test was conducted on serum samples collected from the broiler chickens in each group every week by running the HI test to evaluate the effectiveness of different vaccination programs. Briefly, two-fold serial dilutions of sera were made, and eight HA NDV strains with an equal volume (25 µl) of diluted sera were used in each well of a 96-well microplate. After 45 min of incubation at room temperature, 25 µl of 1% chicken red blood cell was added, and after 30 min of incubation at room temperature, the last well, which had a complete inhibition, was considered the antibody titer (12).

2.5. RT-PCR Test

In this study, we used RT-PCR to detect the presence of field NDV in broiler chickens. Coacal swabs were collected from two birds per replication on a weekly basis from the beginning of the study until day 35. In addition, cloacal and tracheal samples were collected from all three groups on days 0, 2, 4, and 7 after challenging with the velogenic NDV. The RNA of the samples was extracted by the RNA extraction kit (CinnaGen Co., Iran), according to the manufacturer's manual. After that, the cDNA was synthesized by random hexamer primers and the cDNA Synthesis Kit (CinnaGen Co., Iran). The partial F gene was amplified by a pair of primers (forward: 5'-TTGATGGCAGGCCTCTTGC-3'; reverse: 5'-AGCGT(C/T)TCTGTCTCCT-3') (13). PCR was carried out in a 25 µl reaction volume consisting of 12.5 µl of Master Mix, 1 µl of each primer (10 pmol/µl), 2.5 µl of cDNA, and 8 µl of deionized water. The cDNA of a virulent Newcastle virus genotype (VII), which was kindly provided by the University of Tehran, was used as the positive control template. The reaction was programmed in the following conditions: 94°C for 3 min, followed by 35 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 60 sec, and a final extension at 72°C for 10 min. The PCR products were visualized by the electrophoresis of 1% agarose gel.

2.6. Statistical Analysis

The data obtained during this study were analyzed by SPSS software using the one-way analysis of variance (ANOVA) test with a significance level of $P<0.05$ to compare these data between the three groups.

3. Results

3.1. Weighing

All the chickens in each group were weighed on the first day and then randomly divided into study groups. Therefore, the initial weight recorded for all groups was the same. At the end of the study, the results showed that group C (test) had a higher weight than the other study groups, but this difference was not statistically significant ($P>0.05$) (Table 1).

3.2. Feed Conversion Ratio

The study results showed that groups A (the positive control) and C had a better FCR compared to group B (the negative control). However, it is necessary to note that the difference between groups A and C and group B was not statistically significant ($P>0.05$)(Table 2).

3.3. Serum Titer

Table 3 displays the mean and coefficient of variance of the birds' serum titers measured by the HI test in different study groups. The antibody titers of 42-day-old birds show that group B had the highest titer, followed by group C, and group A had the lowest.

Table 1: Mean body weight (grams) of birds during the experiment. There is no significant difference in body weight of different groups ($p<0.05$).

Group	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
A (positive control)	37	152	388	724	1057	1615	2190
B (Negative control)	37	156	397	771	1081	1618	1976
C (test)	37	160	405	735	1071	1606	2204

Table 2. FCR values of different groups during the experiment.

Group	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
A (positive control)	1.1	1.48	2.1	1.9	1.7	1.9
B (Negative control)	1.48	1.84	1.9	2.1	2.1	2.3
C (test)	1.14	1.5	1.9	1.8	1.8	1.9

Table 3. HI Mean titers and %CV in different groups during this experiment. Different superscripts indicates significant difference between groups at each day ($p<0.05$).

Group	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
A (positive control)	5.75^{a} (11.8)	3.25^{a} (15.2)	3.37^{a} (29.9)	4^{a} (17.4)	4.5^{a} (22.8)	4.87^{a} (39)	6^{a} (2.75)
B (Negative control)	5.37^{a} (11.8)	3.12^{a} (10.5)	2.37^{a} (23.6)	1.75^{b} (24.5)	1.12^{b} (55.9)	1^{b} (46.5)	7.12^{a} (20.2)
C (test)	5.62^{a} (11.8)	3.62^{a} (14)	3.87^{a} (15.2)	4.5^{a} (15.5)	4.62^{a} (35.4)	4.75^{a} (30.6)	6.37^{a} (20.4)

severe infection in birds with low antibody titers. Moreover, the findings of this study showed that on days 21, 28, and 35, the difference in serum titer between the vaccinated groups (A and C) and the unvaccinated group (B) was statistically significant ($P<0.05$)

3.4. Mortality

We recorded mortality in each study group. The results indicated that group B had the highest number of dead birds, with four dying post-ND challenge. One bird died on day 1 and another on day 5, and two birds died six days post-challenge. Group A had three dead birds on days 3, 5, and 6 post-challenge. Group C had two dead birds on days 3 and 5 post-challenge. Notably, necropsy findings in all birds revealed signs related to the ND challenge. Most clinical signs related to the ND challenge were resolved seven days post-challenge in groups A and C.

3.5. RT-PCR

Table 4 provides data on the status of virus excretion from the cloaca and trachea in the days following the challenge. The results show that groups A and B excreted the virus through the trachea and cloaca on the second day after the challenge. However, group C only passed the virus through the trachea. On the seventh day after the challenge, group A excreted the virus through the cloaca, while group B passed it through the trachea and cloaca. Meanwhile, no virus excretion was observed in group C at this time. These findings suggest that the NDV4HR vaccine may have been more effective in reducing virus shedding in group C than in groups A and B. The different patterns of virus excretion observed between the groups may also indicate differences in the immune response or susceptibility to the virus.

4. Discussion

Newcastle disease, a highly contagious and fatal disease caused by the paramyxovirus type 1, has plagued the poultry industry for decades (14). In the commercial poultry sector, there are many conventional vaccines to control ND (15). The disease can be controlled by strict biosecurity and through the administration of effective vaccines, which has dramatically reduced the incidence of ND on commercial poultry farms (16). Conventional vaccines are sensitive to heat (17).

Table 4. RT-PCR results in birds after challenge with virulent NDV. "+": positive; "-": negative.

Group	Day 0		Day 2		Day 4		Day 7	
	Trachea	Cloaca	Trachea	Cloaca	Trachea	Cloaca	Trachea	Cloaca
A (positive control)	-	-	+	+	+	+	-	+
B (Negative control)	-	-	+	+	+	+	+	+
C (test)	-	-	+	-	+	+	-	-

Almost all ND vaccines on the market require refrigeration and begin to be inactivated rapidly after 1-2 h if left at room temperature (about 25˚C). Consequently, since it may be challenging to maintain an adequate supply of refrigeration facilities in many countries that do not have reliable electricity sources, the development and large-scale production of an effective heat-resistant vaccine seem essential to supporting the poultry industry. Such vaccines should resist inactivation in hot environments, and thus there would be no concern about vaccine viability in response to temperature fluctuations. This study compared the usual vaccination program using B1, LaSota, and killed vaccines to a program using the NDV4HR vaccine. Interestingly, the mean body weight of unvaccinated birds at the ages of 21, 28, and 35 days was higher than the mean body weight of vaccinated birds. This growth retardation is linked to vaccination-induced stress (18). The decreased body weight of unvaccinated 42-day-old birds may be related to the stress linked to virulent ND challenge. There were differences between groups regarding HI antibody titer and protection against challenge. Although it is clear that vaccination of birds does not prevent ND infection, it reduces the shedding of the virus in challenged birds (19). Several researchers evaluated the efficacy of heat-resistant ND vaccines in poultry. A study by Najjari et al. compared the effectiveness of two vaccines, namely heat-resistant I-2 and commercial Hitchner B1, in protecting chickens from a virus. A total of 300 broilers were divided into four groups and vaccinated before being challenged with the virus. The results showed that both vaccines effectively protected the chickens from mortality and reduced virus shedding and spread. There was, however, no significant difference in serum titer between the two vaccines (20). Similar results were obtained in our study. There was no significant difference in ND antibody titers between V4HR and the usual vaccination program. Moreover, both vaccination programs were effective in reducing mortality and virus shedding. Conventional vaccines for ND are available in the commercial poultry sector. However, they are heat-labile and cannot be used in rural areas due to the lack of cold-chain facilities and the behavior of rural scavenging chickens. The oil-adjuvant inactivated vaccine has been used to control ND in rural chickens, but vaccination coverage has been low due to its high cost and the required skills for application. Therefore, heat-resistant live vaccines are made to protect birds against ND. Adwar and Lukesova (2008). evaluated a heat-resistant ND vaccine for its potential use in rural communities to protect free-range chickens against ND (4). In 2018, Mebrahtu et al. conducted a study to determine the effectiveness of the ND I-2 vaccine delivered through drinking water and spray in rural conditions. Twenty households were randomly divided into experimental and control groups, and blood samples were collected regularly for antibody assay from individual chickens vaccinated with the ND I-2 vaccine using different routes. The results showed that the vaccinated groups had significantly higher antibody titers than the non-vaccinated control group, and there was no statistically significant difference in antibody titers among the vaccinated groups. All vaccinated chickens survived after infection with the virulent NDV, while only 40% of the unvaccinated control group survived. Vaccination through different routes can protect chickens from NDV in rural conditions (21). Abdi et al. (2016) investigated the efficacy of using Ethiopian cereal grains as carriers for the orally administered ND I-2 vaccine in chickens in village chicken production settings where conventional ND vaccination strategies are impractical due to a shortage of cold chains, unsuitability of vaccine administration routes, and the need for trained personnel. The results showed that of the 15 treatment groups, drinking water, cracked maize, and parboiled barley induced significantly higher HI antibody titers than the other carrier grains and the naive control, resulting in a 100% survival rate. Chickens with higher HI antibody titers had a higher survival rate in the challenge experiment, indicating an inverse relationship between chicken mortality (%) and the mean HI titer. Booster vaccinations at ages 35 and 105 induced progressively higher HI antibody titers in all treatment groups (22). Promising results were also obtained in V4HR-vaccinated birds during this study. Therefore, heat-resistant ND vaccines, such as I-2 and V4, are suitable for vaccinating birds in rural conditions. In 2020, Habibi et al. evaluated the efficacy of using the thermostable NDV strain, I-2, in broiler chickens vaccinated via drinking water and coated on oiled wheat grain. They also assessed

its horizontal transmission and ability to protect unvaccinated chickens against a virulent strain of NDV. The results showed that both routes of administration provoked an adequate immune response, covered vaccinated chickens against NDV, and induced protective immunity in unvaccinated chickens against a local field isolate of virulent NDV. However, all unvaccinated and Newcastle-challenged broiler chickens died in the study, and the virus transmission from challenged chickens to susceptible ones was deficient (23). However, we did not assess horizontal transmission of the virus from vaccinated to non-vaccinated birds. Therefore, more studies are needed to ascertain the transmission of this virus from vaccinated to unvaccinated birds. In the present study, however, increased antibody titers and protection against virulent virus challenge were consequences of the V4 vaccination. The difference between vaccine genotype and field strain is one of the paramount causes of vaccine failures (24). Reverse genetic systems are employed by some researchers to develop new vaccines against ND. Ruan et al. (2020) created a thermostable, attenuated vaccine candidate strain, NDV/rHR09, using a heat-resistant virulent NDV strain, HR09, by the reverse genetics system (5). The results showed that NDV/rHR09 was lentogenic and stable after 15 serial passages in embryonated chicken eggs. The NDV/rHR09 strain exhibited hemagglutination activity and infectivity at 56˚C for 60 min. Compared to the commercially available LaSota and V4 vaccines, the NDV/rHR09 induced higher antibody titers and conferred complete protection against the virulent genotype VII NDV challenge and virus shedding from vaccinated chickens. A study by Cao et al. (2022) aimed to construct a thermostable and genotype VII-matched, live, attenuated vaccine against NDV, which has caused a pandemic in many countries and can have fatal consequences in infected chickens. The researchers used a thermostable genotype VIII virulent HR09 strain as a backbone. They replaced its F gene with that of the genotype VII DT-2014 strain while also mutating the cleavage site of the F gene to produce an avirulent class II F protein. The resulting chimeric viruses, rcHR09-CI and rcHR09-CII, were highly attenuated, showed similar growth kinetics and thermostability as the parental HR09 strain, induced a higher level of antibody response, and significantly reduced viral shedding, compared to the commercial LaSota vaccine strain, when tested on immunized chickens challenged with the virulent genotype VII ZJ1 strain. The study presents promising candidates for a thermostable and genotype VII-matched NDV vaccine (25). Genotype VII is the predominant NDV circulating in poultry flocks in Iran (26). Therefore, thermostable genotype VII ND vaccines are promising candidates for the vaccination of poultry flocks. However, more research is needed to evaluate the immunogenicity and protection of these vaccines. Mebrahtu et al. (2018) demonstrated that the ND I-2 vaccine can be delivered via drinking water, eye drops, and spray. All three delivery methods resulted in a significant increase in antibody titers compared to the unvaccinated control group. The survival rate of vaccinated chickens after infection with a virulent NDV was also significantly higher than that of the unvaccinated control group (20). These findings agree with the results of the present study, which show oral V4HR vaccination of birds stimulates high antibody titers and prevents the mortality rate after a challenge with the virulent NDV. The present study suggests that using the V4HR vaccine in the poultry vaccination program orally and weekly can be a suitable candidate for ND vaccination of poultry flocks. In this study, the vaccine was used orally and weekly. However, there was room for discussion and experimentation regarding the method of vaccine administration. Furthermore, the transmission of vaccine viruses to unvaccinated birds was not evaluated. In general, according to the obtained results and observing the performance of the tested chickens, it can be concluded that the use of the V4HR vaccine in meat broilers reduces the shedding of the virus during the conflict, reduces losses, and protects poultry when faced with the NDV. Concerning different dynamics and the process of production and breeding of backyard poultry in Iran, appropriate studies ought to be carried out on the appropriate vaccination dates and the number of vaccinations based on different conditions governing the backyard poultry breeding system in different regions of the country.

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Authors' Contribution

Study concept and design: S.A.G. and A.G.; Acquisition of data: S.A.G., A.G., M.M.; Analysis and interpretation of data: S.A.G., A.G., M.M.; Drafting of the manuscript: N.L. and A.G.; Critical revision of the manuscript: S.A.G. and A.G.; Statistical analysis: A.G. and N.L.

Ethics

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by all the authors.

Conflict of Interest

The authors confirm no conflicts of interest regarding financial, personal, or other relationships with other people or organizations related to this paper.

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Data Availability
The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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